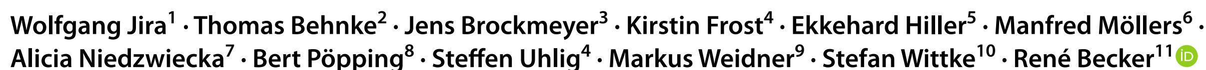
Food Analytical Methods
https://doi.org/10.1007/s12161-022-02289-0

Inter-laboratory Validation of an HPLC–MS/MS Method for the Detection of Microbial Transglutaminase in Meat and Meat Products

Wolfgang Jira[1] · Thomas Behnke[2] · Jens Brockmeyer[3] · Kirstin Frost[4] · Ekkehard Hiller[5] · Manfred Möllers[6] · Alicia Niedzwiecka[7] · Bert Pöpping[8] · Steffen Uhlig[4] · Markus Weidner[9] · Stefan Wittke[10] · René Becker[11]



Received: 26 May 2021 / Accepted: 3 April 2022
© The Author(s) 2022

Abstract

Microbial transglutaminase (TG) is an enzyme isolated on an industrial scale from *Streptomyces mobaraensis*. Technical TG, a formulated powder, is primarily used to restructure meat in the meat-processing industry, typically at a 1% concentration and is often referred to as "meat glue." In the European Union, meat restructured with TG requires the indication "formed meat" on the label according to Regulation (EU) No 1169/2011. In order to detect food fraud like the undeclared TG usage in meat and meat products, a qualitative mass spectrometric method using specific tryptic marker peptides has been published in 2017. Here the successful inter-laboratory validation and first-time standardization of a proteomics method for food control is described, which was subsequently included into the Official Collection of Analysis Methods according to the German Food and Feed Code (§ 64 LFGB). Thirteen laboratories from governmental, academic, and private institutions participated in the study, whereas four laboratories did not meet the minimal quality criteria and therefore their results had to be excluded. Three different test materials containing between 0.2 and 2% technical TG as well as blank samples were produced and tested. The laboratories used triple-quadrupole mass spectrometers from several vendors as well as quadrupole time-of-flight instruments. The detection of TG was considered to be positive, if three mass transitions for the marker peptides VTPPAEPLDR (TG-1) and SPFYSALR (TG-2), each, showed a signal-to-noise ratio of at least 3. The level of detection $LOD_{95\%}$ for the median laboratory with intermediate performance was 0.31%, the false-positive rate was 0% and the false-negative rate was 2.1%.

Keywords Formed meat · Food authenticity · Proteomics · Collaborative study · Method validation · Probability of detection

✉ René Becker
rene.becker@bvl.bund.de

[1] Max Rubner-Institut (MRI), Department of Safety and Quality of Meat, E.-C.-Baumann-Straße 20, 95326 Kulmbach, Germany

[2] Institut Kirchhoff Berlin GmbH, Oudenarder Straße 16, 13347 Berlin, Germany

[3] Analytical Food Chemistry, University of Stuttgart, Allmandring 5 B, 70569 Stuttgart, Germany

[4] QuoData GmbH, Prellerstraße 14, 01309 Dresden, Germany

[5] Chemisches und Veterinäruntersuchungsamt Stuttgart, Schaflandstr. 3/2, 70736 Fellbach, Germany

[6] Chemisches und Veterinäruntersuchungsamt Karlsruhe, Weißenburger Straße 3, 76187 Karlsruhe, Germany

[7] Bundesinstitut für Risikobewertung (BfR), Max-Dohrn-Straße 8-10, 10589 Berlin, Germany

[8] FOCOS Food Consulting Strategically, Zum Kälterhaus 6b, 63755 Alzenau, Germany

[9] Landeslabor Schleswig-Holstein, Max-Eyth-Straße 5, 24537 Neumünster, Germany

[10] University of Applied Sciences Bremerhaven, An der Karlstadt 8, 27568 Bremerhaven, Germany

[11] Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), Diedersdorfer Weg 1, 12277 Berlin, Germany

Introduction

In the European Union, food enzymes that are applied to perform a technological function are covered in Regulation (EC) No 1332/2008. This legislation will harmonize the national provisions relating to the use of food enzymes in the European Union. In the future, food enzymes must be permitted for their use within the Community. Therefore, food enzymes must appear in a Community list that should clearly describe the enzymes and specify any conditions governing their use, including, where necessary, information on their function in the final food and, where appropriate, specific labelling requirements for foodstuffs produced with these enzymes. The consumption of an enzyme must be harmless to health, its use must be technologically necessary, and must not mislead the consumer.

To establish this Community list, a Register (European Commission 2020a) was drawn up by the Commission. Interested parties could submit applications for food enzymes to be included in this list until 11 March 2015. The Commission then draws up the Community list based on the documents submitted, with the European Food Safety Authority (EFSA) carrying out a safety assessment. As soon as the assessments are completed, the Community list will be published. From this point on, only the enzymes on the Community list are permitted. Until that time, the use of food enzymes and food produced with food enzymes is subject to the legislation of the EU Member States.

An enzyme intensively used in the food industry (Lerner et al. 2020) is protein-glutamine γ-glutamyltransferase (transglutaminase (TG); Enzyme Commission (EC) number 2.3.2.13), which catalyzes the formation of isopeptide bonds between an ε-amino group of a lysine residue and a γ-carboxamide group of a glutamine residue (Kanaji et al. 1993; Yokoyama et al. 2004) leading to highly cross-linked protein polymers. The enzyme is isolated from the microorganism *Streptomyces mobaraensis* to produce microbial TG on an industrial scale (Kieliszek and Misiewicz 2014). The enzyme is a monomeric 38-kDa protein containing 331 amino acids and maintains its total enzymatic activity even at temperatures close to 0 °C (Yokoyama et al. 2004). Therefore, TG can be used for restructuring of meat (Jira et al. 2017; Lennon et al. 2010; Sadeghi-Mehr et al. 2016).

In the list of applications (European Commission 2020b), the following applications were submitted for TG: production of bakery products and other cereal-based products, protein processing, dairy processing, fruit and vegetable processing, and grain processing. The technological effects achieved also include consistency and texture improvements. The most well-known application, the production of restructured meat, keyword “glue ham” or “glue meat,” is obviously indicated here with the term “protein processing.”

According to Article 7 of Regulation (EU) No 1169/2011, food information shall not be misleading the consumer, particularly as to the characteristics of the food, composition, and method of manufacture or production. In the same sense, Article 6 c) of Regulation (EC) 1332/2008 requires that the use of food enzymes does not mislead the consumer. Misleading the consumer includes, but is not limited to, issues related to the nature, freshness and quality of the ingredients used, the naturalness of a product or of the production process, or the nutritional quality of the product.

To prevent misleading the consumer, meat, meat products, and meat preparations, which may give the impression, that they are made of a whole piece of meat, but actually consist of different pieces combined together shall bear the indication: “formed meat” (Annex VI, Part A No. 7 Regulation (EU) No 1169/2011).

In order to verify the labelling of meat products restructured with TG and to protect consumers from misleading and deception, a sensitive high-performance liquid chromatography-tandem mass spectrometry (HPLC–MS/MS) method for the detection of TG from *Streptomyces mobaraensis* in restructured meat using six tryptic marker peptides was developed recently (Jira and Schwägele 2017). Out of these six TG marker peptides, two marker peptides (VTPPAEPLDR and SPFYSALR) are suitable for the detection of all three TGs (Commission IDs 2015/169, 2015/188 and 2015/10) registered in the European Union (European Commission 2020a).

In Germany, the Federal Office of Consumer Protection and Food Safety (BVL) publishes an Official Collection of Analysis Methods in accordance to the German Food and Feed Act (LFGB), which contains validated methods for the official food control laboratories of the German federal states. In addition, the validated official methods are also preferably used by private laboratories as well as official laboratories of other German speaking countries (namely Austria and Switzerland). With the aim of including a qualitative HPLC–MS/MS method for the detection of the registered TGs into the Official Collection, the method with two marker peptides was validated by means of an inter-laboratory validation study. The inter-laboratory validation was planned and organized by the working group “Mass Spectrometric Protein Analysis” (Stoyke et al. 2019). A total of thirteen laboratories, comprising seven authorities, four companies, and two academic institutions, participated in the study.

Materials and Methods

Preparation of Spiked Meat and Meat Products

Determination of the Protein Content in Technical TG

The protein content of technical TG (Activa WM, Ajinomoto, Hamburg, Germany) was determined in accordance with the respective method in the German Food and Feed Code (Official Collection of Analysis Methods 2014).

Thermal Treatment of Suspensions of the Mixtures of Technical TG and Maltodextrin

The required amounts of mixtures of technical TG (Activa WM, Ajinomoto, Hamburg, Germany) and maltodextrin (total weight: 80–200 g) and the 2.5-fold (for materials 1 and 2) or the 1.25-fold (for material 3) amount of water were filled in a beaker. The suspensions were cooked on a magnet stirrer with a magnetic stir bar for 30 min and subsequently cooled to room temperature. Afterwards, the amounts of evaporated water were refilled and the suspensions were placed in an ultrasound bath and left for 15 min. After cooling to room temperature, the suspensions were stored at 2 °C.

Preparation of Emulsion-Type Sausages (Materials 1 and 2)

The basic formulation of a 10-kg batch applied to a 13-L bowl chopper (Müller Food Machines, Saarbrücken, Germany) was 56% turkey meat, 20% sunflower oil, 20% ice, 1.8% salt (containing sodium nitrite; 0.4%), 0.2% dipotassium hydrogen phosphate, 2% maltodextrin-technical TG mixture for material 1 and 55.5% turkey meat, 20% sunflower oil, 20% ice, 1.8% salt (containing sodium nitrite; 0.4%), 0.5% spice mixture, 0.2% dipotassium hydrogen phosphate, and 2% maltodextrin-technical TG mixture for material 2. For material 1, five batches (0 (negative control), 0.2, 0.5 (positive control), 1.0, and 2.0% technical TG) and for material 2, four batches (0 (negative control), 0.2, 0.5 (positive control), and 1.0% technical TG) were produced (Table 1). The maximum temperature of the sausage meat was 12 °C. The sausage meat was filled into 200 g tinplate cans (type 99/36 mm; Dosen-Zentrale Züchner GmbH, Cologne, Germany) and heated to full preserves to obtain F values of 6.3 (material 1) and 8.5 (material 2), respectively, and cooled subsequently to room temperature and stored at 2 °C.

Preparation of Hamburger Patties (Material 3)

The basic formulation of the hamburger patties was 92% beef (minced meat; 3 mm), 5% water, 1% salt, and 2% maltodextrin-technical TG mixture. The minced meat and a suspension of the maltodextrin-technical TG mixture were

Table 1 Materials used in the inter-laboratory study

Material		Sample	Concentration of transglutaminase (%)	
			Technical mixture in maltodextrin	Pure enzyme
1	Emulsion-type sausage with turkey and sunflower oil	Matrix sample	1.0	0.0067
		P01 (negative control)	0.0	0.0000
		P02	0.2	0.0013
		P03 (positive control)	0.5	0.0034
		P04	1.0	0.0067
		P05	1.0	0.0067
		P06	2.0	0.0134
2	Emulsion-type sausage with turkey, sunflower oil, and spice mixture	P07 (negative control)	0.0	0.0000
		P08	0.2	0.0013
		P09 (positive control)	0.5	0.0034
		P10	1.0	0.0067
		P11	1.0	0.0067
3	Raw hamburger patties (beef)	P12 (negative control)	0.0	0.0000
		P13	0.2	0.0013
		P14 (positive control)	0.5	0.0034
		P15	1.0	0.0067
		P16	1.0	0.0067

mixed in a stirring machine for 1 min. After the addition of salt, the samples were mixed once again by hand for about 1 min and filled in metal forms (diameter: 9 cm) to obtain patties of about 90 g. The patties were lightly frozen for 1 h at − 20 °C and afterwards filled in vacuum bags and stored at − 20 °C.

Standard Operating Procedure

A standard operating procedure (SOP), consisting of the method to be validated (Official Collection of Analysis Methods 2021) and a protocol with further instructions, was dispatched to the participating laboratories with detailed information concerning (a) the identification of the seven most intense mass transitions for each peptide and for the optimization of the MS/MS parameters (identification of the most intense mass transitions and optimization of the MS/MS parameters section), (b) the sample preparation (Sample preparation of test materials section), and (c) the sequence of the HPLC–MS/MS measurements (sample preparation and HPLC–MS/MS measurements schedule section). The participants could freely choose the vendor for the required chemicals, provided that they were of appropriate quality.

Sample Preparation of Test Materials

An amount of 3 g homogenized meat or meat product was suspended with 20 mL acetone using a homogenizer (90 s at 25,000 rpm). After a pause of 5 min, the supernatant was carefully decanted. Afterwards, the sample was suspended in a further 20 mL acetone and filtered. The filtration residue was dried in a desiccator overnight. The defatted and dehydrated sample was homogenized in a ball mill (at least for 1 min) or in a homogenizer (at least 2 × 15 s) to obtain a fine powder. The further sample treatment (protein extraction, tryptic digestion, and solid phase extraction (SPE)) has been described elsewhere (Jira and Schwägele 2017). As a slight modification, the elution from the SPE columns was performed with acetonitrile (ACN)/water/formic acid, 90/9/1, v/v/v.

Sample Preparation and HPLC–MS/MS Measurement Schedule

The sample preparations should be performed on two independent days: 10 of the 16 randomized samples on day 1 and the remaining 6 samples on day 2. It was determined by the organizer of the inter-laboratory study, which samples had to be prepared on which day. The separation of peptides should be performed on a reversed-phase column and a water/ACN/formic acid-gradient. The detection of the tryptic marker peptides should be carried out by MS/MS in the positive electrospray ionization (ESI) mode. The HPLC–MS/MS measurement of the samples should also be carried out on two independent days following a predefined scheme (see Table 2), whereas the order of samples was randomized. However, the specific requirements were made concerning which sample had to be measured on which day. Blanks had to be measured between the single samples. A marker peptide mixture had to be measured at the beginning and the end of each measuring day.

HPLC–MS/MS Method

Synthetic Peptide Standards and Test Material for Optimization

In addition to the test materials (P01–P16), the participating laboratories were provided with the synthetic standard peptides VTPPAEPLDR (TG-1) and SPFYSALR (TG-2) (Peptide Specialty Laboratories GmbH, purity based on 216 nm HPLC–UV signal > 95%) dissolved in ACN/water/formic acid 96.9/3.0/0.1 (v/v/v) to a final concentration of 10 ng/µL and a matrix test sample (material 1) of which the participants knew that it contained TG.

HPLC Optimization

The optimization of the HPLC conditions was carried out with the standard peptide mixture and the matrix test sample (synthetic peptide standards and test material for optimization section) by each laboratory individually, as each laboratory used different HPLC systems (Table 3).

Identification of the Most Intense Mass Transitions and Optimization of the MS/MS Parameters

Since each laboratory used different mass spectrometers, the participating laboratories should select the seven most abundant theoretically explainable mass transitions for each marker peptide and optimize the MS/MS parameters of mass spectrometers (Table 4) by syringe pump injection (direct infusion) of the standard peptides (synthetic peptide standards and test material for optimization section). For orientation, the participants were provided with a list of the mass transitions identified during method development.

Statistical Analysis

The statistical evaluation was performed with the inter-laboratory study software PROLab™ Plus (Version 2020.12.3.0).

The detection of TG is evaluated by the rate of detection (ROD), which describes the relative frequency of detection in independent replicates. To be distinguished from ROD is the probability of detection (POD), which can be

Table 2 Schematic procedure of the analysis of samples P01 to P16, whereas the sequence of samples within a measuring day varied between the laboratories. Blank samples were measured between each matrix sample

Measuring day	Sample	Material	Concentration of technical mixture of transglutaminase (%)
1	Marker peptide mix	Marker peptide mix	
	P01	1	0.0
	P02		0.2
	P03		0.5
	P04		1.0
	P06		2.0
	P07	2	0.0
	P08		0.2
	P09		0.5
	P10		1.0
	P15	3	1.0
	Marker peptide mix	Marker peptide mix	
2	Marker peptide mix	Marker peptide mix	
	P05	1	1.0
	P11	2	1.0
	P12	3	0.0
	P13		0.2
	P14		0.5
	P16		1.0
	Marker peptide mix		

Table 3 HPLC instruments and columns of participating laboratories

Lab	HPLC instrument	HPLC column	length (mm)	ID (mm)	particle size (µm)
A	Agilent 1290 Infinity UHPLC	Zorbax Eclipse Plus C18	150	2.1	1.8
B	Agilent 1290 Infinity II UHPLC	Macherey Nagel Nucleosil 100–3 C18 HD	125	2.0	3.0
C	Waters Acquity UPLC	Acquity UPLC HSS T3	100	2.1	1.8
D	AB Sciex Eksigent MicroLC 200	HALO Fused-Core C18	50	0.5	2.7
E	Shimadzu UHPLC-20ADXR	Phenomenex Aeris Peptide XB-C18 100 A	100	2.1	1.7
F	AB Sciex M3 MicroLC	YMC Tirart C18	150	0.3	3.0
G	AB Sciex ExionLC	Phenomenex Aeris Peptide XB-C18 100 A	150	2.1	1.7
H	Agilent 1200 Series LC	Raptor ARC-18	100	2.1	2.7
I	Shimadzu Nexera X2	Phenomenex Aqua C18	150	2.0	3.0
J	Agilent 1290 Infinity UHPLC	Phenomenex Luna C18	150	2.0	5.0
K	Agilent 1100 Series LC	HALO Fused-Core C18	150	2.1	2.7
L	Agilent 1260 Infinity LC	Phenomenex Kinetex C18	100	2.1	2.6
M	Shimadzu SCL 10	Phenomenex Kinetex C18	100	2.1	2.6

regarded as the theoretical mean value of the ROD (Uhlig et al. 2011; Wehling et al. 2011).

The POD curve describes the probability of detection as a function of the TG concentration. The main features of this POD curve are the TG concentrations at which the POD curve reaches a value of 0.5 ($LOD_{50\%}$) and 0.95 ($LOD_{95\%}$), respectively. The LOD (ISO 2021) characterizes the used methodology (e.g., if the value to be measured exceeds the $LOD_{95\%}$, the probability of detection is higher than 95%).

The calculation of the POD curve and thus the $LOD_{50\%}$ and $LOD_{95\%}$ across laboratories is based on a generalized mixed effects model (GLMM) together with a four-parameter sigmoid curve as given in Sect. 6.2 of ISO/DTS 27878 (2021). The resulting $LOD_{50\%}$ and $LOD_{95\%}$ are given for a laboratory with median performance together with its upper

Table 4 Mass spectrometers and MS/MS parameters of participating laboratories

Lab	Mass spectrometer	Ion source (+)	Collision gas	Source temperature (°C)	Desolvation temperature (°C)
A	Agilent 6460 Triple Quad	Jet Stream ESI	Nitrogen	330	400
B	Agilent 6495 Triple Quad	ESI	Nitrogen	250	150
C	AB Sciex QTrap 6500	ESI	Nitrogen	430	–
D	AB Sciex TripleTOF 4600	Duo Spray ESI	Nitrogen	420	–
E	AB Sciex QTrap 5500	TurboIonSpray ESI	Nitrogen	450	–
F	AB Sciex TripleTOF 6600	Duo Spray ESI	Nitrogen	300	–
G	AB Sciex QTrap 6500+	Turbo Spray ESI	Nitrogen	430	–
H	AB Sciex API 4000	TurboIonSpray ESI	Nitrogen	500	–
I	Shimadzu Triple Quad 8060	ESI	Argon	300	250
J	Agilent 6460 Triple Quad	ESI	Nitrogen	–	300
K	Thermo Scientific TSQ Vantage	ESI	Argon	400	350
L	AB Sciex QTrap 5500	ESI	Nitrogen	600	–
M	AB Sciex API 4000	TurboIonSpray ESI	Nitrogen	450	–

95% confidence limit. The standard deviation of the laboratory-specific values for $\ln(LOD_{50\%})$ and $\ln(LOD_{95\%})$ for a randomly selected laboratory is referred to as laboratory standard deviationσ_L (Sect. 6.2 of ISO/DTS 27878 2021). For σ_L, the upper 95% confidence limit is additionally calculated. All parameters and confidence/prediction intervals were estimated using an extension of the approach described by Uhlig et al. (2013).

Results and Discussion

Principal considerations

The suitability of the qualitative method was successfully evaluated in a preliminary ring trial with 10 participating laboratories (A–J) analyzing seven randomized sausages (3 or 4 samples without TG, and 4 or 3 samples with 1% TG, each). For the production of the three different test materials included in the main validation study, materials 1 and 2 were produced as emulsion-type sausages due to their high homogeneity. Material 3 was produced as raw hamburger patties, representing a compromise between the homogeneity of the sample material and the matrix similarity to raw restructured meat. A technical TG mixture with maltodextrin as the sole further ingredient (and no further protein additives such as caseinate or pork protein) was selected for the preparation of the test materials to ensure the highest possible matrix similarity of samples with and without TG. In total, 16 test samples (Table 1) with and without TG and one matrix sample (1% TG; for optimization) were produced for each of the 13 participating laboratories, whereas the sample numbers of the test samples were randomized. The concentration of technical TG added ranged between 0.2%, a concentration which has proven to be too low for meat binding (Jira and Schwägele 2017), and 2%, a concentration twice as high as recommended by the manufacturer. The samples with a TG concentration just sufficient for meat binding (0.5%) were used as positive control samples. This was necessary, since a relevant number of laboratories participating in this study had no or only limited experience in targeted proteomics. Therefore, minimal quality criteria for the overall analytical performance were announced to identify and exclude systematic analytical errors that are not method based. A determination of the protein content in technical TG (no additional protein ingredients; determination of the protein content in technical TG section) revealed protein contents of 0.67%, confirming the TG concentrations in this product found by Kütemeyer (2007). Therefore, the concentration of TG (pure enzyme) in the test materials ranged from 0.0013 to 0.0134% (Table 1). The technical TG was deactivated by cooking (Zhang et al. 2012) before the production of the test materials to avoid that the participants could identify the presence or absence of TG in the test samples based on different sample consistencies. From each matrix, a sample with 1% TG should be analyzed in duplicate determination.

The original published method (Jira and Schwägele 2017) identified six marker peptides for the detection of the predominantly distributed type of microbial TG in the EU (Kanaji et al. 1993; Kashiwagi et al. 2002). Further investigations into other commercially available TGs, in part from other strains of *S. mobaraensis* (e.g., NCBI accession AAV31068.1), revealed that only two of the marker peptides were common for all available TGs. Therefore, only the marker peptides VTPPAEPLDR (TG-1) and SPFYSALR (TG-2) were used in the inter-laboratory validation study.

In the schematic procedure of the analysis of samples (Table 2), blank samples were measured between each

matrix sample to investigate a possible carry-over of marker peptides due to the fact that some peptides show a non-specific adsorption that can occur at every part of the HPLC–MS/MS system (Maes et al. 2014). However, in the inter-laboratory validation study, only one laboratory detected traces of TG-2 in blank samples demonstrating that both peptides do not show a relevant carry-over.

The detection of TG in a meat sample was considered to be positive, if (a) at least three mass transitions for TG-1 and TG-2, each, showed a signal-to-noise ratio (SNR) ≥ 3, (b) the results of the negative control samples were negative, and (c) the results of the positive control samples (sample with 0.5% TG) were positive.

Identification of the Most Intense Mass Transitions for Each Marker Peptide

According to the SOP, each participating laboratory had to identify the seven most intense theoretically explainable mass transitions for the two marker peptides, each, by direct infusion of the peptide standard solutions (synthetic peptide standards and test material for optimization section and identification of the most intense mass transitions and optimization of the MS/MS parameters section). However, three participating laboratories determined only a lower number of mass transitions (laboratory G: 3; laboratory H: 5; laboratory K: 4 for TG-1 and 3 for TG-2). For each of the marker peptides TG-1 and TG-2, three mass transitions were identified in all thirteen participating laboratories (see Table 5). These most prevalent fragment ions (TG-1: *m/z* 447.7 ($y8^{2+}$), 500.3 (y4), 797.4 (y7); TG-2: *m/z* 446.3 (y4), 609.3 (y5), 756.4 (y6)) were all y-ions with *m/z* > 400. They were determined among the most intense mass transitions also in the laboratories I and K using argon (instead of nitrogen) as collision gas. Furthermore, four mass transitions, each, were identified in at least seven laboratories. The most prevalent fragment ions were usually among the most intense fragment ions.

After MS/MS parameter optimization, the meat samples (Table 1) were analyzed for all previously identified mass transitions according to the SOP. Three samples (P03, P09, and P14) were further evaluated to determine whether the most abundant fragment ions also showed the highest SNR. Overall, the most abundant fragment ions also showed high SNR values in the sample materials.

Intra-laboratory Deviations of the Retention Times of the Marker Peptides

Deviations in retention times of the matrix-adjusted positive control samples between individual laboratories were analyzed to examine the general robustness and whether additional quality controls can be applied. For this purpose, the deviations of retention times between the (up to) three matrices were calculated for each laboratory and both marker peptides. The distributions of these deviations are displayed by means of kernel density estimation (KDE) in Fig. 1. Based on robust statistics (Q/Hampel), the 95% tolerance intervals for the respective deviations were calculated (vertical red lines in Fig. 1). For both marker peptides, the tolerance limits were frequently exceeding ± 0.1 min, but were always within ± 0.2 min. It can also be stated that the deviations of retention times for marker peptide TG-1, which elutes earlier and therefore has less interactions with the stationary phase, vary more than for marker peptide TG-2. This tentatively can also be ascribed to variations of the DMSO volume which was added to the sample after SPE before solvent removal and which is more pronounced for the less interacting peptide. The results obtained led to the quality requirement in the official method (Official Collection of Analysis Methods 2021) specification that the retention times of TG-1 and TG-2 in a sample must not deviate more than ± 0.2 min from the retention times of the marker peptides in the matrix-adjusted positive control sample. Considering also the variety of applied HPLC instruments and columns (see Table 3), this confirms the stability of the retention times of the marker peptides.

Statistical Analysis of the Detection of TG

The data basis for the statistical analysis of the detection of TG is given in Table 6. Corresponding ROD values and false-negative rates across laboratories are displayed in Table 7.

Applying the identification criterion that results are only to be considered as verified, if TG was detected in the 0.5% TG sample (positive control sample), the complete results of laboratories E, I, and M as well as the results for hamburger patties (material 3: P13–P16) of laboratory J must be excluded from the calculations of the false-positive and the false-negative rates and for the POD curve. The laboratories I and J had no experience in targeted proteomics. Laboratory I reported about a low sensitivity of the mass spectrometer and laboratory M had problems with the SPE cleanup and assumed as a consequence a noticeable suppression of the ESI ionization. The incorrect detection of TG in the positive control samples of laboratory E (despite having experience in targeted proteomics and having no problems in the preliminary ring trial) referred exclusively to the missing detection of TG-2. Laboratory J did not detect both TG marker peptides in any samples of the hamburger patties. Therefore, a systematic error during sample preparation of this matrix is probable, which could not be further specified. Nevertheless, even after exclusion of the 3 or 4 laboratories mentioned above, the remaining number of laboratories was still sufficient to fulfil the requirements for collaborative

Table 5 Most abundant fragment ions of the TG marker peptides determined by the participating laboratories. Gray marked cells: mass transitions identified in all laboratories

		Laboratory													Number of labs
Marker (*m/z*)	Fragment ion	A	B	C	D	E	F	G	H	I	J	K	L	M	detected
TG-1	175.1 (y1)	+													1
(547.8)	201.1 (b2)		+	+	+		+		+	+	+		+	+	9
	298.2 (b3)		+	+	+					+	+		+	+	7
	399.2 ($y7^{2+}$)	+	+	+	+		+		+	+	+	+	+	+	11
	447.7 ($y8^{2+}$)	+	+	+	+	+	+	+	+	+	+	+	+	+	13
	498.3 ($y9^{2+}$)	+				+									2
	500.3 (y4)	+	+	+	+	+	+	+	+	+	+	+	+	+	13
	700.4 (y6)		+	+	+	+	+			+	+		+	+	9
	797.4 (y7)	+	+	+	+	+	+	+	+	+	+	+	+	+	13
	894.5 (y8)	+				+	+								3
TG-2	175.1 (y1)	+													1
(470.7)	185.1 (b2)						+								1
	304.2 (a3)		+	+	+					+	+		+	+	7
	332.2 (b3)		+	+	+		+			+	+		+	+	8
	359.2 (y3)	+													1
	378.7 ($y6^{2+}$)	+	+	+	+					+	+		+	+	8
	427.2 ($y7^{2+}$)	+	+	+	+	+	+		+	+	+		+	+	11
	446.3 (y4)	+	+	+	+	+	+	+	+	+	+	+	+	+	13
	467.2 (a4)					+									1
	495.2 (b4)					+									1
	609.3 (y5)	+	+	+	+	+	+	+	+	+	+	+	+	+	13
	756.4 (y6)	+	+	+	+	+	+	+	+	+	+	+	+	+	13
	853.5 (y7)						+								1

study procedures to validate an analytical method (Appendix D of AOAC Official Methods of Analysis 2005; ISO 2020).

The false-positive rate was calculated based on the three blank samples P01, P07, and P12 (Table 1). Regardless of the sample matrix, there was no misclassification, so that a false-positive rate of 0% could be achieved. The false-negative rate was calculated based on the samples containing a TG concentration of at least 0.5%. In total, two false-negative results were reported: one for material 2 and one for material 3 which both contain 1% TG. This resulted in an overall false-negative rate of 2.0% and in false-negative rates of the specific TG concentrations as shown in Table 7.

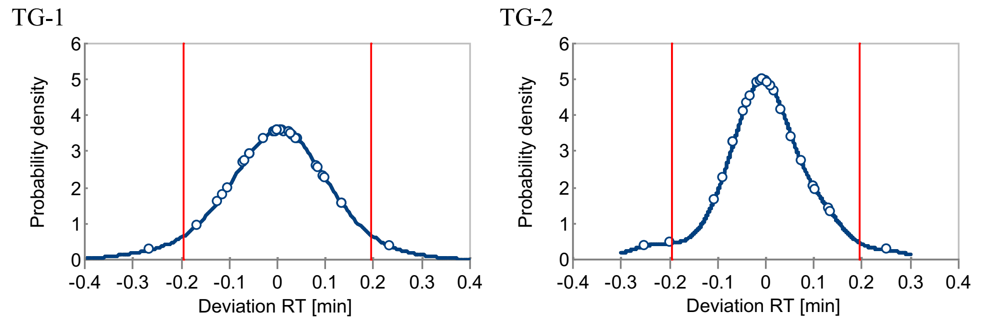


Fig. 1 KDE of lab-specific deviations of retention times of the matrix-adjusted positive control sample between the (up to) three matrices for both marker peptides TG-1 and TG-2; vertical red lines: 95% tolerance limits of retention time deviations

Table 6 Sample-specific results of laboratories; + = TG detected, $-^{1}$ = TG-1 negative, $-^{2}$ = TG-2 negative, $-^{12}$ = TG-1 and TG-2 negative; gray marked cells = excluded from statistical analysis

	Added technical transglutaminase												
	0.2 %			0.5 %			1.0 %						2.0 %
Lab	P02	P08	P13	P03	P09	P14	P04	P05	P10	P11	P15	P16	P06
A	+	+	$-^{12}$	+	+	+	+	+	+	+	+	+	+
B	$-^{12}$	+	+	+	+	+	+	+	$-^{12}$	+	+	$-^{12}$	+
C	+	+	$-^{1}$	+	+	+	+	+	+	+	+	+	+
D	+	+	+	+	+	+	+	+	+	+	+	+	+
E	$-^{2}$	$-^{2}$	$-^{2}$	$-^{2}$	$-^{2}$	$-^{2}$	$-^{2}$	$-^{2}$	$-^{2}$	$-^{2}$	+	+	$-^{2}$
F	+	+	+	+	+	+	+	+	+	+	+	+	+
G	+	+	+	+	+	+	+	+	+	+	+	+	+
H	+	+	+	+	+	+	+	+	+	+	+	+	+
I	$-^{12}$	$-^{2}$	$-^{12}$	$-^{2}$	$-^{2}$	$-^{12}$	$-^{2}$	$-^{2}$	$-^{2}$	$-^{2}$	$-^{12}$	$-^{12}$	$-^{2}$
J	+	+	$-^{12}$	+	+	$-^{12}$	+	+	+	+	$-^{12}$	$-^{12}$	+
K	+	+	$-^{1}$	+	+	+	+	+	+	+	+	+	+
L	+	+	+	+	+	+	+	+	+	+	+	+	+
M	$-^{1}$	$-^{1}$	$-^{12}$	$-^{12}$	+	$-^{12}$	$-^{1}$	+	+	+	$-^{1}$	$-^{1}$	+

Table 7 Summary of the results across laboratories separately for each TG concentration

TG concentration (%)	Number of samples	Number of positive tests /total number of tests	ROD	False-negative rate (%)
0.0	3	0/30	0.00	–
0.2	3	25/30	0.83	17
0.5	3	30/30	1.00	0
1.0	6	58/60	0.97	3
2.0	1	10/10	1.00	0

Probability of detection and $LOD_{95\%}$

Since the conventional statistical approach for the validation of quantitative methods (according to ISO 2019) is not feasible for the validation of a binary qualitative method, the statistical analysis was based on the POD approach, which is currently being discussed to become an international validation standard (ISO 2021).

The probability of detection (POD) across laboratories as a function of the TG concentration could be modeled

independently of the matrix, since no significant effects between the three matrices in terms of detection probability were detected. The resulting POD curve across laboratories with associated 95% confidence interval as well as the 95% prediction range and the laboratory-specific ROD values are shown in Fig. 2.

The evaluation range of the POD curve is 0.2–2.0% TG. Outside this range, extrapolation is necessary, so that the value of 0.03% TG for the $LOD_{50\%}$ (upper confidence limit 0.12% TG) can only be regarded as an estimation value. The $LOD_{95\%}$ for the median laboratory is 0.31% TG (upper confidence limit 1.16% TG). However, it should be noted that the qualitative method in most cases yields a positive result at all investigated concentration levels (above 0%) provided that laboratories with systematic problems are disregarded. The determination of method performance parameters is therefore difficult and associated with considerable uncertainties.

For the laboratory standard deviation σ_L, a high value of 0.81 (upper confidence limit 3.26) is obtained, which corresponds to a relative laboratory standard deviation of 81% according to the standards of a quantitative method (ISO 2019). There are several reasons: (1) for laboratory B, the laboratory-specific $LOD_{95\%}$ is outside the measured range; and (2) the determination of the threshold for the detection of TG is not fully standardized and both systematic and random differences between laboratories are to be expected. Therefore, for the practical implementation of the method, it is recommended to regularly use suitable control samples with a TG concentration of 0.5% as a positive control. In addition, upon introduction of the method into a laboratory, it is recommended to perform a verification study in which six independent measurements of a sample containing 0.5% TG are performed. If all six results of this verification are positive, it can be concluded that the laboratory is capable of determining a level of 1% TG with a probability of at least 0.8.

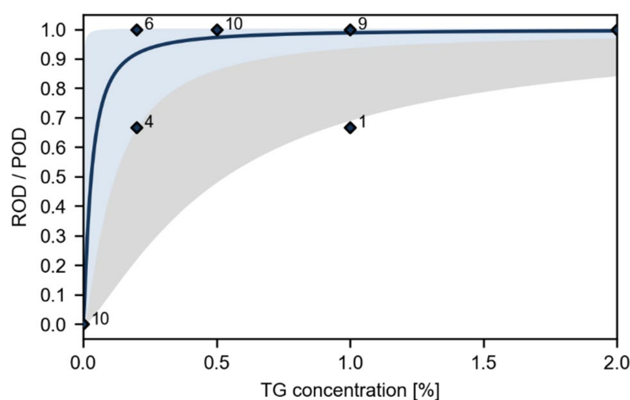


Fig. 2 POD curve across laboratories (blue line) with associated 95% confidence interval (light blue) and 95% prediction interval (light blue + light gray) as well as laboratory-specific ROD values (blue squares – numerical values = number of laboratories with corresponding ROD)

Robustness of the Method

In some laboratories, sample processing and protein extraction deviated slightly from the SOP. Three laboratories used blending devices with lower maximal processing speeds (24,000 rpm at D, 15,000 rpm at F, 9500 rpm at E) than the 25,000 rpm specified in the method. Laboratories E, F, and M did not use a ball mill, whereas laboratories C and K only used a ball mill for some samples. Laboratory H used a mortar and pestle on the defatted and dehydrated samples. Despite the differences in sample preparation, each laboratory obtained a fine powder. Laboratory D recovered the finished protein extracts with centrifugation at 5000 rpm instead of 8000 rpm. In addition to the deviations from the SOP, the method itself provided the laboratories with some liberties in processing steps and especially in the analysis. Therefore, the tryptic digestion was conducted using trypsin from five different manufacturers and for SPE, two different systems were used. In terms of analysis, the laboratories could use any suitable HPLC–MS/MS system after optimization. Despite all these variations, no negative impact on the overall characterization of the samples was observed in the validation study.

Comparison of the Results of Triple-Quadrupole (MRM) and Quadrupole Time-of-Flight Instruments (Pseudo-MRM)

An important aspect for the transfer of mass spectrometric methods to routine applicability is their platform independence. Although quantitative analyses in routine applications are still mainly established on triple-quadrupole instruments, the benefits of high-resolution MS (HRMS) are increasingly exploited (Domon and Gallien 2015; Faktor et al. 2017; Higgs et al. 2013). It is important to note that the mechanisms of targeted approaches are different on triple-quadrupole and quadrupole time-of-flight (Q-ToF) instruments. Triple-quadrupole instruments are mainly run in multiple reaction monitoring (MRM) mode. In Q-ToF HRMS, pseudo-MRM transitions (also referred to as MRM-HR or PRM (parallel reaction monitoring)) are generated by the instrument as it cycles through predefined sets of precursor ions and collects full scan fragment ion spectra of each precursor. MRM transitions are reconstructed by software and are rather extracted ion chromatograms in high resolution. The major advantages are enhanced specificity due to high-resolution and the availability of full scan fragment spectra.

To demonstrate the platform independence of the developed method, the pseudo-MRM approach using two Q-ToF HRMS instruments (Sciex TT4600 and Sciex TT6600, respectively) was included in the inter-laboratory study for the detection of TG. All samples in the analyzed concentration range from 0.2 to 2% TG were classified correctly in both laboratories using pseudo-MRM. Furthermore, it was shown that the SNRs for TG-1 and TG-2 were comparable for the triple-quadrupole and Q-ToF instruments in all analyzed matrices (TG concentration 0.5%). These convincing results are also reflected in the fact that the $LOD_{95\%}$ for the hybrid MS and the triple-quadrupoles were at least comparably good. Both laboratories that employed Q-ToF instruments in the inter-laboratory study achieved ROD values of 1 (Fig. 2). Overall, these results demonstrate that the Q-ToF mass spectrometers used in the inter-laboratory comparison proved to be completely suitable for the method application.

Conclusions

The method for the detection of TG in meat products is the first successful inter-laboratory validation and standardization of a proteomics method for food control, which was subsequently included into the Official Collection of Analysis Methods according to the German Food and Feed Code (§ 64 LFGB). Two TG-specific peptides suitable for the detection of all three TGs registered in the EU were selected out of six candidate peptides. The high discriminative power of the targeted HPLC–MS/MS method allows the reliable detection of TG-1 and TG-2 in a complex mixture for most laboratories despite differences in proteomics expertise and facilities, even if the pure enzyme is present in low concentrations down to 0.0013% in the test samples. Due to the observed problems of one laboratory with SPE preparation in the inter-laboratory study, adjustments in the final official method were made (e.g., drop speed) to avoid such errors in the future. Furthermore, the inter-laboratory study revealed the suitability of different HPLC–MS/MS platforms to detect microbial TG in meat products in concentrations commonly used by the industry for restructuring. For calculating the POD curve and the $LOD_{95\%}$, a non-linear mixed effects model was successfully applied in combination with a 4-parameter sigmoid curve to describe the variability of the POD curves across laboratories. Although the calculated laboratory standard deviations close to 1 may appear high, they are considered acceptable for a qualitative method. In order to assure the results according to the requirements of a method of the German Official Collection of Analysis Methods, also considering the difficulties of some laboratories, it is advisable to use appropriate control samples with a TG concentration of 0.5%. On the one hand, these controls should be carried along with the measurements as a positive control, and on the other hand, they should be measured independently several times in a laboratory as part of a verification when the method is first implemented. Based on the outcome of the validation with the current set of matrices, it can be concluded that the application of this method could also be extended to other matrices (e.g., fish) in the future as listed in the application documents.

Acknowledgements We kindly thank Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (Oberschleißheim, Germany), Chemisches- und Veterinäruntersuchungsamt Rhein Ruhr Wupper (Krefeld, Germany), Coop (Basel, Switzerland), Chemisches und Veterinäruntersuchungsamt Münsterland-Emscher-Lippe (Münster, Germany), IGV GmbH testlab (Nuthetal, Germany), and Sciex (Darmstadt, Germany) for participation in the validation study.

Funding Open Access funding enabled and organized by Projekt DEAL.

Data Availability The raw data of this study was generated according to a statutory mandate (German Food and Feed Code). It is therefore not available for publication.

Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest Wolfgang Jira declares that he has no conflict of interest. Thomas Behnke declares that he has no conflict of interest. Jens Brockmeyer declares that he has no conflict of interest. Kirstin Frost declares that she has no conflict of interest. Ekkehard Hiller declares that he has no conflict of interest. Manfred Möllers declares that he has no conflict of interest. Alicia Niedzwiecka declares that she has no conflict of interest. Bert Pöpping declares that he has no conflict of interest. Steffen Uhlig declares that he has no conflict of interest. Markus Weidner declares that he has no conflict of interest. Stefan Wittke declares that he has no conflict of interest. René Becker declares that he has no conflict of interest.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

Appendix D of AOAC Official Methods of Analysis (2005) Guidelines for collaborative study procedures to validate characteristics of a method of analysis. https://www.eoma.aoac.org/app_d.pdf

Domon B, Gallien S (2015) Recent advances in targeted proteomics for clinical applications. Proteom Clin Appl 9:423–431. https://doi.org/10.1002/prca.201400136

European Commission (2020a). Register of food enzymes to be considered for inclusion in the Union List. Retrieved on April 28, 2021 from: https://ec.europa.eu/food/sites/food/files/safety/docs/fs_food-improvement-agents_enzymes_register.pdf.

European Commission (2020b). Food enzyme applications submitted to the Commission within the legal deadline (from 11 September 2011 to 11 March 2015). Retrieved on April 28, 2021 from: https://ec.europa.eu/food/sites/food/files/safety/docs/fs_food-improvement-agents_enzymes-applications.pdf.

European Union (2008). Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on food enzymes. Official Journal of the European Union. 31.12.2008. L 354/7–15.

European Union (2011). Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers. Official Journal of the European Union. 22.11.2011. L 304/18–63.

Faktor J, Sucha R, Paralova V, Liu YS, Bouchal P (2017) Comparison of targeted proteomics approaches for detecting and quantifying proteins derived from human cancer tissues. Proteomics 17:1600323. https://doi.org/10.1002/pmic.201600323

Higgs RE, Butler JP, Han B, Knierman MD (2013) Quantitative Proteomics via High Resolution MS Quantification: capabilities and limitations. Int J Proteomics 2013:674282. https://doi.org/10.1155/2013/674282

ISO 5725–2:2019–12, Accuracy (trueness and precision) of measurement methods and results - Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method

ISO 16140–4:2020–07, Microbiology of the food chain - method validation - Part 4: Protocol for method validation in a single laboratory

ISO/DTS 27878:2021, Reproducibility of the LOD of binary methods in collaborative and in-house validation studies

Jira W, Schwägele F (2017) A sensitive high performance liquid chromatography-tandem mass spectrometry method for the detection of microbial transglutaminase in different types of restructured meat. Food Chem 221:1970–1978. https://doi.org/10.1016/j.foodchem.2016.11.148

Jira W, Sadeghi-Mehr A, Brueggemann DA, Schwägele F (2017) Production of dry-cured formed ham with different concentrations of microbial transglutaminase: mass spectrometric analysis and sensory evaluation. Meat Sci 129:81–87. https://doi.org/10.1016/j.meatsci.2017.02.021

Kanaji T, Ozaki H, Takao T, Kawajiri H, Ide H, Motoki M, Shimonishi Y (1993) Primary structure of microbial transglutaminase from streptoverticillium sp strain-S-8112. J Biol Chem 268:11565–11572

Kashiwagi T, Yokoyama K, Ishikawa K, Ono K, Ejima D, Matsui H, Suzuki E (2002) Crystal structure of microbial transglutaminase from streptoverticillium mobaraense. J Bio Chem 277:44252–44260

Kieliszek M, Misiewicz A (2014) Microbial transglutaminase and its application in the food industry. A Review Folia Microbiol 59:241–250. https://doi.org/10.1007/s12223-013-0287-x

Kütemeyer C (2007) Beiträge zur Qualitätssicherung von Fischprodukten am Beispiel des Schwarzen Heilbutts (Reinhardtius hippoglossoides). Dissertation Universität Hannover. http://d-nb.info/987041967/34

Lennon AM, McDonald K, Moon SS, Ward P, Kenny TA (2010) Performance of cold-set binding agents in re-formed beef steaks. Meat Sci 85:620–624. https://doi.org/10.1016/j.meatsci.2010.03.014

Lerner A, Ramesh A, Matthias T (2020) The temperature and pH repertoire of the transglutaminase family is expanding. FEBS Open Bio 10:492–494. https://doi.org/10.1002/2211-5463.12839

Maes K, Smolders I, Michotte Y, Van Eeckhaut A (2014) Strategies to reduce aspecific adsorption of peptides and proteins in liquid chromatography-mass spectrometry based bioanalyses: An overview. J Chromatogr A 1358:1–13. https://doi.org/10.1016/j.chroma.2014.06.072

Official Collection of Analysis Methods according to § 64 of the German Food and Feed Code (LFGB), L 06.00–7: Bestimmung des Rohproteingehaltes in Fleisch und Fleischerzeugnissen; Titrimetrisches Verfahren nach Kjeldahl; Referenzverfahren: 08–2014.

Official Collection of Analysis Methods according to § 64 of the German Food and Feed Code (LFGB), L 06.00–70: Nachweis mikrobieller Transglutaminase aus Streptomyces mobaraensis in Fleisch und Fleischerzeugnissen mittels Flüssigkeitschromatographie und Tandem-Massenspektrometrie (LC-ESI-MS/MS): 03–2021.

Sadeghi-Mehr A, Lautenschlaeger R, Drusch S (2016) Sensory, physicochemical and microbiological properties of dry-cured formed ham: comparison of four different binding systems. Eur Food Res Technol 242:1379–1391. https://doi.org/10.1007/s00217-016-2641-0

Stoyke M, Becker R, Brockmeyer J, Jira W, Popping B, Uhlig S, Wittke S (2019) German Government Official Methods Board Points the Way Forward: Launch of a New Working Group for Mass Spectrometry for Protein Analysis to Detect Food Fraud and Food Allergens. J AOAC Int 102:1280–1285. https://doi.org/10.1093/jaoac/102.5.1280

Uhlig S, Niewoehner L, Gowik P (2011) Can the usual validation standard series for quantitative methods, ISO 5725, be also applied for qualitative methods? Accredit Qual Assur 16:533–537. https://doi.org/10.1007/s00769-011-0811-0

Uhlig S, Kruegener S, Gowik P (2013) A new profile likelihood confidence interval for the mean probability of detection in collaborative studies of binary test methods. Accredit Qual Assur 18:367–372. https://doi.org/10.1007/s00769-013-0993-8

Wehling P, LaBudde RA, Brunelle SL, Nelson MT (2011) Probability of detection (POD) as a Statistical Model for the Validation of Qualitative Methods. J AOAC Int 94:335–347

Yokoyama K, Nio N, Kikuchi Y (2004) Properties and applications of microbial transglutaminase. Appl Microbiol Biot 64:447–454. https://doi.org/10.1007/s00253-003-1539-5

Zhang L et al (2012) Enzymatic characterization of transglutaminase from Streptomyces mobaraensis DSM 40587 in high salt and effect of enzymatic cross-linking of yak milk proteins on functional properties of stirred yogurt. J Dairy Sci 95:3559–3568. https://doi.org/10.3168/jds.2011-5125

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.