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Comparative study of commercially available gluten ELISA kits using an incurred reference material

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Abstract

Handling major hypersensitivity reactions (e.g. celiac disease) triggered by proteins of wheat and other cereals is a challenging task for healthcare systems, legislative forces and the related fields of food analysis as well. In spite of the fact that there are available official threshold levels for labelling the absence of gluten, which is considered to be the toxic protein fraction of wheat, barley and rye, validation of the analytical methodology supporting regulatory requirements is currently problematic. The main limiting factors of method validation are the lack of reference methods and reference materials. The objective of this study was to provide a solution to this problem. An incurred reference material in a model food matrix was developed and studied by commercially available ELISA test kits as a part of the activity of the Food Allergen Working Group within the FP6 funded EU project MoniQA. After successful completion of the reference material development process, the incurred material was used as a basis of a comparative study examining the performance and applicability of seven commercially available ELISA kits designed for quantification of gluten/gliadin. In certain cases the obtained data showed discrepancies from the expected gliadin content that may carry both a food safety and an economic relevance. The evaluation of the effects of heat treatment on the analytical results is also presented, highlighting the fact that the food processing steps may have a considerable impact on the analytical data, thus should be carefully handled during method development and validation.

Keywords: data variability, effects of processing, method validation, quality assurance

1. Introduction

Celiac disease (gluten-sensitive enteropathy or nontropical sprue) is an autoimmune disorder appearing in genetically susceptible individuals triggered by the storage proteins of wheat (gliadins and glutenins), rye (secalins), barley (hordeins) and oats (avenins), although toxicity of the latter is a subject of debate. The immune response elicited by these proteins leads to a mucosal damage causing malabsorption and a wide range of other symptoms (e.g. chronic diarrhea, anemia, osteoporosis, infertility, etc.) depending on age and, according to certain studies, gender. Celiac disease is a significant public health issue having a prevalence of 1%. At present, the exclusive treatment of this disorder is undergoing a life-long diet avoiding the proteins of wheat, rye and barley (Briani *et al.*, 2008; Ciclitira *et al.*, 2005; Hischenhuber *et al.*, 2006). Wheat proteins responsible for the above-mentioned adverse effects belong to the storage proteins within which gluten and mainly its α-, γ- and ω-gliadin fractions show the highest toxic activity. Besides, the glutenin fraction of gluten was also proven to have toxic effect (Breitender and Radauer, 2004; Janssen, 2006; Petersen *et al.*, 2011; Silano *et al.*, 1999; Tye-Din *et al.*, 2010; Wieser 2001).

Helping celiac consumers to meet the requirements of their gluten-free diet is a concern of policy-makers too. In accordance with Codex Stan 118-1979 (revised 2008) of the Codex Alimentarius Commission, the EU Commission

Regulation 41/2009 (Commission of the European Communities, 2009) defines the following threshold levels for this protein type: (a) foods containing less than 20 mg/kg gluten (10 mg/kg gliadin) can be labelled as gluten-free; and (b) gluten concentrations between 20 mg/kg and 100 mg/kg (10 and 50 mg/kg gliadin, respectively) can be considered as low gluten-level. Since these values are defined in legislation, it is very important to have analytical tools capable of quantifying gluten content precisely and accurately. Reliability of results obtained by existing immunoanalytical methods like Enzyme-Linked Immunosorbent Assay (ELISA) and ELISA-based Lateral Flow Devices depends on different factors, e.g. complexity of the food matrix, effects of food processing on physical-chemical properties of the target proteins, the extraction method, type of the applied antibody, and the materials against which the methods were calibrated. These factors together with the fact that currently reference methods and incurred reference materials in this field are not available make the validation of these methods an important but equally challenging task (Janssen, 2006; Poms *et al.*, 2006; Van Eckert *et al.*, 2010; Werner and Wieser, 2003). They also highlight the need for harmonized method validation and reference materials (Abbott *et al.*, 2010; Allred and Ritter, 2010; Berger and Schmidt, 1996; Denery-Papini *et al.*, 1999; Geng *et al.*, 2008; Van Eckert *et al.*, 2010). Although there is no reference method at present, the ELISA method based on the R5 gliadin antibody is recommended by the Codex Alimentarius Commission and has been accepted by AOAC International as the 'Official First Action' method.

The objective of this study was to perform a comparative study of commercially available ELISA kits using a food matrix based incurred reference material that was developed previously in laboratory scale at the Budapest University of Technology and Economics as a part of the activity of the Food Allergen Working Group within the EU FP6 funded MoniQA Network of Excellence for supporting the validation process of gluten quantification (Bugyi *et al.*, 2012). The novel approach of this study is the application of an incurred reference matrix instead of spiked samples which allowed to evaluate analytical responses of the processed forms of the toxic target proteins and to compare presently used ELISA-based analytical methods under more realistic conditions.

2. Materials and methods

Materials for reference material production

The basis of the reference material production was a gluten-free flour (Schär Mix C, Dr. Schär Srl/GmbH, Burgstall, Italy). For producing the samples containing gliadin, a special gliadin isolate made of the mixture of 28 European wheat varieties by the Prolamin Working Group (PWG gliadin, Van Eckert *et al.*, 2006) was used. Further components of the applied recipe were as follows: margarine (Accento, Beluša Foods, Beluša, Slovakia), powdered sugar (Mester konyha, Budapest, Hungary; Salt-Image Kft., Kiskunhalas, Hungary), salt (Pro-team Kft., Nyíregyháza, Hungary) baking soda (Horváth Rozi, Gyál, Hungary; R-Coop 3 Kft., Szeged, Hungary) and water.

Production of the reference material

The reference material is made of a heat treated food matrix (cookie) based on a modified, previously published recipe (Bugyi *et al.*, 2012; Scaravelli *et al.*, 2008). The process of the model matrix production is shown in Figure 1.

Firstly, a blank (gliadin-free) powder mixture made of the dry components of the recipe was homogenized by mixing for 20 minutes in a valorigraph (FQA-205 METEFÉM, Budapest, Hungary). PWG gliadin dissolved in a 60% v/v ethanol solution was added to the blank powder mixture and was mixed for further 20 minutes to obtain the homogeneous distribution of gliadin in the blend. Following this step margarine and water was added to the powder mixture for dough formation that occurred also in the valorigraph (5 minutes). The dough was processed two ways: one part of it was freeze-dried, the other part was directly shaped into round shaped cookies (1 cm in height, 3.5 cm in diameter) and baked for 16 minutes at 180 °C (Bugyi *et al.*, 2012).

The cookies were incurred with gliadin at three concentration levels: blank, 10 and 50 mg/kg gliadin for modelling the legislative threshold levels of gluten-free and low gluten-level foods. The ethanol solution of PWG gliadin mentioned above was used at concentrations calculated to obtain a final gliadin content of the dough and the cookies of 10 and 50 mg/kg (dry matter basis). Three parallel batches (three cookies/batch) were prepared for each concentration level. For the comparative study and for the investigation of the effects of food processing steps (dough formation, heat treatment) on the measurable gliadin content, samples were taken from the major steps of the sample production process (indicated as grey boxes in Figure 1): powder mixture with gliadin, freeze-dried raw dough and cookies.

Moisture content of freeze-dried dough and cookie samples were also determined and compared by t-test showing no significant difference between the values (data not shown). Due to this reason moisture content was not taken into account during the evaluation of the data.

ELISA kits

During the comparative study, seven commercially available ELISA kits were examined. A list of the applied test kits and their main characteristics are shown in Table 1.

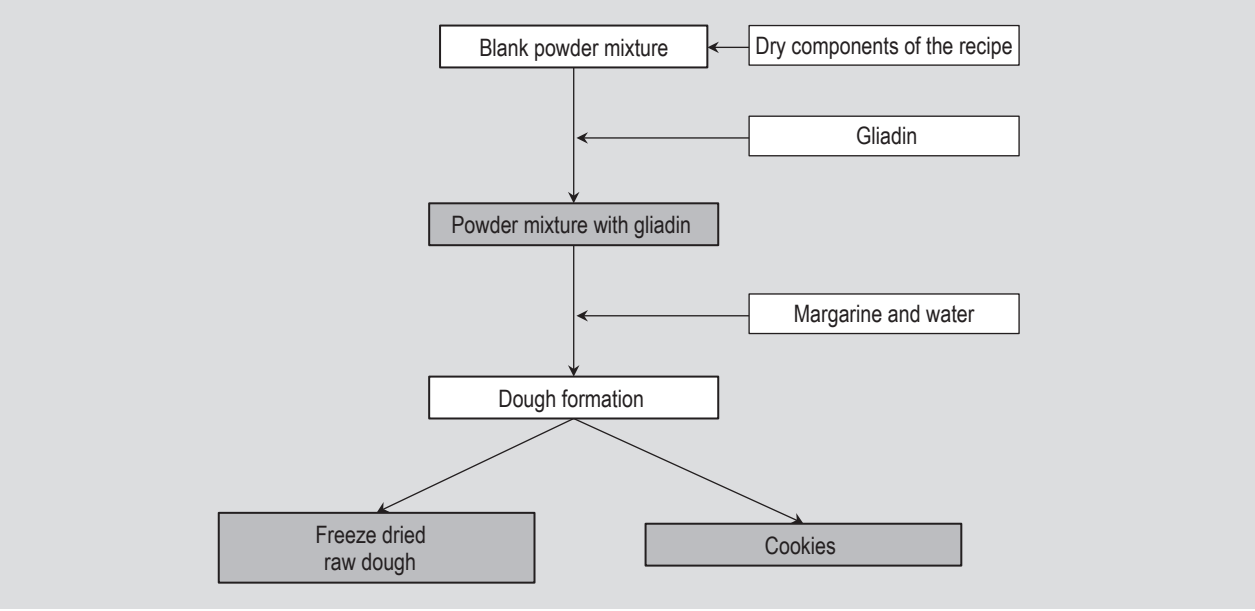

Figure 1. Flow chart of the reference material production. Samples were taken (grey boxes) to be used in a comparative study and to investigate the effect of certain food processing steps.

Table 1. Characteristics of the evaluated commercial ELISA kits according to the kit instructions.

Kit	Manufacturer	Test format	Antibody	Target protein	LoD	LoQ	Extraction	Calibrator
AgraQuant Gluten Assay	Romer Labs (Tulln, Austria)	sandwich	NA	gliadin	0.6 mg/kg gluten	4 mg/kg gluten	40% ethanol	NA
BIOKITS Gluten Assay Kit	Tepnel (Deeside, UK)	sandwich	monoclonal antibody (401/21)	glutenin and gliadin	1 mg/kg gluten	3 mg/kg gluten	extraction solution in 40% ethanol	Wheat gluten extract
Gliadin ELISA	ELISA Systems (Windsor, Australia)	sandwich	NA	gliadin	2.5 mg/kg gliadin	2.5 mg/kg gliadin	extraction solution in 40% ethanol	NA
HAVen Gluten-Check ELISA kit	Diagnostic Innovations (St. Asaph, UK)	sandwich	antibody developed by Skerritt & Hill (1991)	ω-gliadin	1 mg/kg gluten	7.5 mg/kg gluten	A6017 extraction kit[1]	NA
RIDASCREEN Gliadin	R-Biopharm (Darmstadt, Germany)	sandwich	R5 monoclonal	gliadin and corresponding prolamins of rye and barley	1.5 mg/kg gliadin (3 mg/kg gluten)	2.5 mg/kg gliadin (5 mg/kg gluten)	cocktail solution and 80% ethanol	PWG gliadin[2]
Veratox Quantitative Gliadin Test	Neogen (Lansing, United States)	sandwich	NA	gliadin	NA	5 mg/kg gliadin	cocktail solution and 55% ethanol	NA
Wheat protein ELISA kit	Morinaga (Yokohama, Japan)	sandwich	NA	gliadin	0.3 µg wheat protein/g food (sensitivity)	0.78 ng/ml wheat protein	extraction solution	NIST SRM-1567a wheat flour

Abbreviations used: NA = data not available in the instruction; LoD = limit of detection; LoQ = limit of quantification.

[1] Buffered ethanolic reagent with tannin binding additive, developed by Skerritt and Hill (1991).

[2] Gliadin isolate made of the mixture of 28 European wheat varieties by the Prolamin Working Group (Van Eckert *et al.*, 2006).

Sample preparation and implementation of the tests

As a first step of the analytical examination, freeze-dried raw dough and cookie samples were ground in a grinder (Retsch Grindmix 6M20, Retsch GmbH, Haan, Germany). Ground cookies of the same concentration level were mixed together, making a single batch for analysis. The same procedure took place with the dough samples. The extraction of gliadin and the measurements were carried out according to the instructions provided by the manufacturers. Five to eight replicates were taken for each sample depending on the number of available wells in the ELISA microtiter plate.

Data analysis

The analytical results were calculated using BioRad Microplate Manager 6 software. The applied calibration curves and gluten/wheat protein to gliadin conversion methods are summarized in Table 2. The data were evaluated by the examination of standard and relative deviations (SD and RSD, respectively) and two-sample t-tests.

For the presentation of the results kits were coded randomly and marked by capital letters from A to G, because the aim of this study was drawing attention to the present gaps of gluten analysis and not the actual ranking of the available kits.

3. Results and discussion

Evaluation of blank samples

As a first step of the experiment, blank samples (blank powder mixture, dough and cookie) were investigated to check their gluten-free status. For each kit the results were below the limit of quantification of the kits (data not shown) and the standard deviations were random. Therefore, the raw materials can be considered as gluten-free and it is also indicated that there was no cross-contamination during the cookie production. The obtained analytical values were taken into consideration as blank values.

Results of samples with defined gliadin content

Firstly, homogeneity of gliadin distribution in the model products was tested by R-Biopharm RIDASCREEN Gliadin ELISA (Darmstadt, Germany) by studying the SD and coefficient of variation (CV) values of three to five replicates. The applied homogenizing method is described in Section 2. Gliadin homogeneity was proven to be sufficient with CV values of 7.9% and 12.8% for cookies with 10 and 50 mg/kg gliadin content, respectively. The results of different incurred gliadin levels at 10 and 50 mg/kg concentration levels are shown in Figure 2. In case of raw dough, moisture content was not taken into account during the calculation of the gliadin concentration because it was proven that there is no significant difference between the moisture content of the dough and that of the cookies as described previously in Section 2.

The investigation of the recovery values of gliadin from these samples provides information on the performance of the kits as well as on the effects of the matrix and baking. It is well-observable that the results of the kits show a similar behaviour for the powder mixtures and for the dough. In several kits, significant discrepancies are observed between the actual and the expected concentrations. Recovery values of samples analysed by the ELISA kits are presented in Table 3. In the analysis of the incurred cookies, the most accurate results were provided by kits A, B and G, while kits C, D and E showed larger differences from the expected concentration values. The largest discrepancy in each case was found with kit F.

Table 2. Applied calibration curves and gliadin conversion methods for the evaluation of the ELISA results.

Manufacturer	Kit	Applied calibration curve	Kit reporting unit	Conversion to gliadin units
Romer Labs (Tulln, Austria)	AgraQuant Gluten Assay	quadratic	mg/kg gluten	/2 = mg/kg gliadin[a]
Tepnel (Deeside, UK)	BIOKITS Gluten Assay Kit	linear	mg/kg gluten	/2 = mg/kg gliadin[a]
ELISA Systems (Windsor, Australia)	Gliadin ELISA	linear	mg/kg gliadin	-
Diagnostic Innovations (St. Asaph, UK)	HAVen Gluten-Check ELISA kit	quadratic	ng/ml gliadin	x0.02 = mg/kg gliadin[a]
R-Biopharm (Darmstadt, Germany)	RIDASCREEN Gliadin	quadratic	µg/kg gliadin	x0.5 = mg/kg gliadin[a]
Neogen (Lansing, United States)	Veratox Quantitative Gliadin Test	quadratic	mg/kg gliadin	-
Morinaga (Yokohama, Japan)	Wheat protein ELISA kit (Gliadin) II	quadratic	ng/ml wheat protein	x0.16 = mg/kg gliadin[b]

[a] According to the users' manual.
[b] According to personal discussion with the manufacturer.

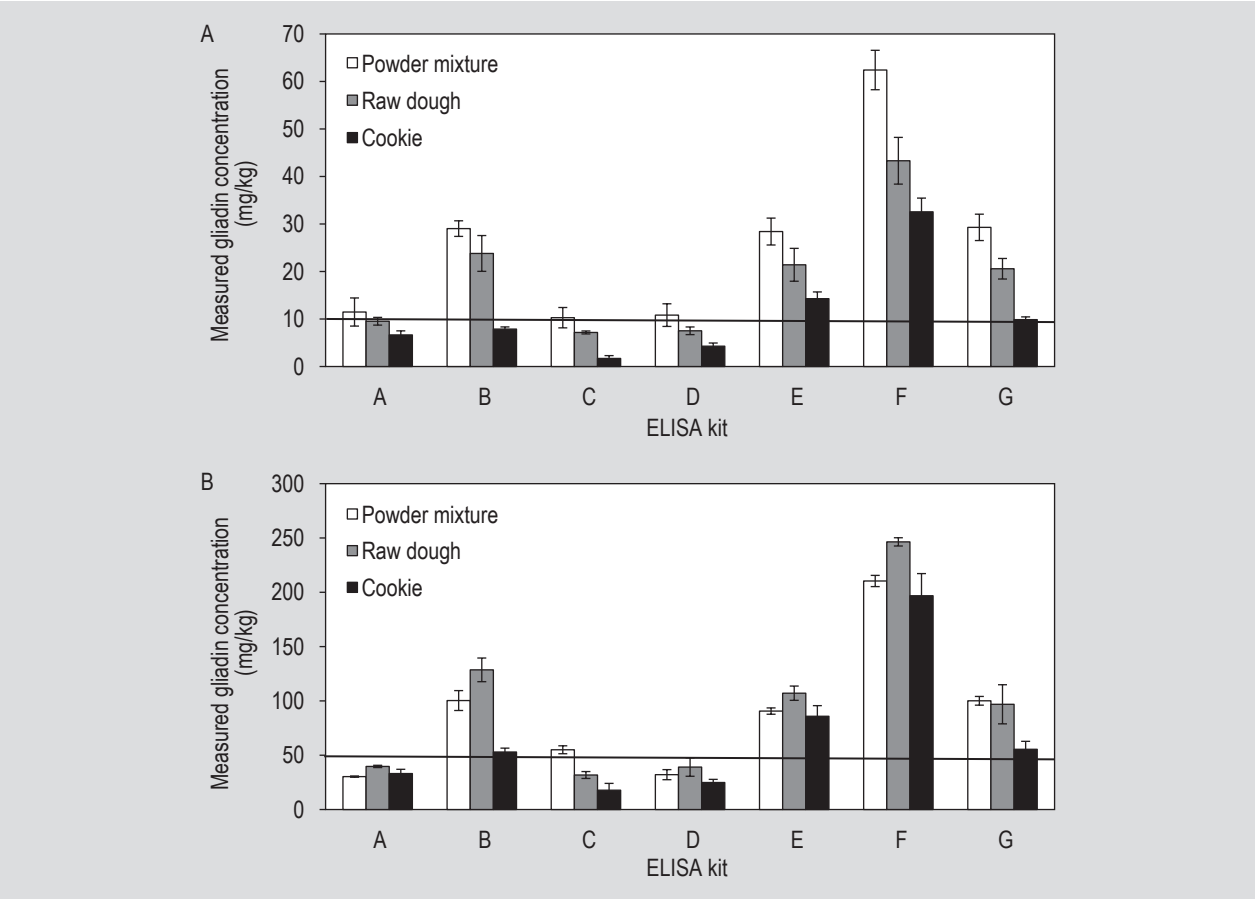

Figure 2. Average gliadin content of powder mixture, cookie dough and baked cookies incurred at (A) 10 mg/kg and (B) 50 mg/kg analysed by seven commercial ELISA test kits (error bars represent standard deviations).

Table 3. Recovery values of samples analysed by the commercial ELISA kits (A-G). Below the kit code the average recovery of gliadin in % is presented. The displayed order of the kits is based on the extent of the discrepancy of the recovery values from 100%.

Sample type-gliadin content	Kit ranking						
Powder mixture-10 mg/kg	C	D	A	E	B	G	F
	102.8	108.2	114.72	284.1	290.3	292.9	624.1
Powder mixture-50 mg/kg	C	D	A	E	G	B	F
	110.04	64.2	60.62	181.28	200.2	200.66	420.8
Raw dough-10 mg/kg	A	D	C	G	E	B	F
	95.28	75.2	71.7	205.8	214.1	238	433.1
Raw dough-50 mg/kg	A	D	C	G	E	B	F
	79.36	78.16	63.56	193.76	214.02	257.18	492.82
Cookie-10 mg/kg	G	B	A	E	D	C	F
	98.7	78.8	66.5	142.8	42.8	17.0	325.6
Cookie-50 mg/kg	B	G	A	D	C	E	F
	105.92	110.92	66.24	49.76	35.62	171.72	393.62

Furthermore, as for the results for cookies, the obtained concentrations are lower than those of the powder mixture and the dough, but in this case the results are likely affected by heat treatment that makes estimation of accuracy problematic (possible effects of heat treatment are discussed in the following section). However, as the baked model product is meant to be used as a reference material, detectability of gluten and performance of the

Table 4. RSD[1] values of the measured gliadin concentration values.

Gliadin content	Sample	ELISA kit						
		A	B	C	D	E	F	G
10 mg/kg	Powder mixture	0.26	0.06	0.21	0.22	0.10	0.07	0.09
	Raw dough	0.09	0.16	0.04	0.11	0.16	0.11	0.11
	Cookie	0.13	0.06	0.35	0.16	0.10	0.09	0.06
50 mg/kg	Powder mixture	0.02	0.09	0.07	0.14	0.03	0.02	0.04
	Raw dough	0.03	0.08	0.10	0.22	0.06	0.02	0.19
	Cookie	0.12	0.07	0.35	0.12	0.11	0.10	0.13

[1] RSD was calculated as: SD value of replicates/average value of replicates.

kits in this matrix and other heated products is extremely important. Another issue regarding cookies is that several kits underestimate the gliadin content, which may pose an increased food safety risk due to labelling 'gluten-free' on those products that may contain gluten at concentrations higher than the regulated threshold values. In a few cases overestimation can be observed as well, which may be important from economical point of view by hampering potentially proper products to be declared as gluten-free or low gluten-level.

For estimating the precision of the kits, the RSD of the data were calculated (Table 4.). These values were shown to be random, no tendencies were observable and none of them exceeded 0.35. As a conclusion, precision of the methods can be described as satisfying for this purpose.

Identification of the source of the experienced discrepancies of measured gliadin concentrations from the expected value and the variability of the results provided by different kits for the same samples needs a complex evaluation of several factors having an impact on the assay performance. Differences of individual kit performance are not unknown and were described previously by several studies. These studies state that the major factors influencing the analytical data provided by different ELISA kits are: (a) the analysed matrix; (b) the method development process (production and specificity of antibodies, extraction methods, target proteins/epitopes, type of detection, type of chromogen and substrate, calibrating materials); (c) prolamin polymorphism; and (d) genetic and environmental variability of storage proteins in cereal grains (Allred and Ritter, 2010; Berger and Schmidt, 1996; Denery-Papini *et al.*, 1999; Thompson and Mendez, 2008).

As for the assays used in this study, the major differences come from the application of different antibodies (target molecules), extraction solutions (simple ethanolic extractions vs. cocktail solutions with reducing agents) and calibrations. It was described earlier that all of these factors may have a significant effect on the analytical results. In case of the antibodies, e.g. R5 and Skerritt antibodies are both developed against prolamins, but their gluten-binding affinity is different. The Skerritt antibody shows higher affinity for glutenins than for gliadins while R5 antibody has lower affinity for glutenins than gliadins. This phenomenon also highlights the problem of calibration and the conversion of gliadin units into gluten units using a multiplying factor of two (Allred and Ritter, 2010; Geng *et al.*, 2008). As a conclusion, the discrepancies of the measured gliadin concentration can be originated from the different antibodies and extraction methods of the ELISA kits together with the fact that the gliadin material applied for the production of the reference material can be different from those used for calibrating the kits. The latter calls attention to the lack of standardized calibrating materials and the options of choosing a material for calibration, e.g. protein extracts (gliadin or glutenin) or more complex protein sources, like flours and the state of the proteins in the chosen material (native or processed). In case of this study another factor makes the picture even more complex, namely the detection of gliadin in a complex processed food matrix.

Effects of heat treatment on the analytical results

Since many food products are processed in some way, it is very important to understand the type of modification target proteins undergo during processing as well as the ability of the detection method to extract and detect these proteins. In addition, it would also be helpful for producing a proper reference material and for establishing method validation criteria. As we analyzed incurred samples from the unprocessed mixture of dry ingredients, from raw dough and from the baked cookies too, investigation of processing effects on assay performance was possible. A summary of the results concerning processing effects on measurable gliadin content measured by commercial ELISA kits is shown in Figure 2.

In case of samples containing 10 mg/kg gliadin, the measured protein concentration is lower in the dough than in the powder mixture, which may occur due to the effect of margarine and water added to the mixture. Besides, a remarkable decrease in the measurable gliadin content can be observed after baking. The degree of this decrease shows variability among the kits. Regarding dough samples incurred with 50 mg/kg gliadin, the estimation of gliadin content was higher than that found in the powder mixture in several cases which is a highly unexpected finding and will be studied further. As for cookies containing 50 mg/kg gliadin, the results were comparable to that of the 10 mg/kg incurred cookie samples, i.e. the measured gliadin content of the cookies was significantly lower than that of the dough.

Decrease of the measurable gliadin content during baking can be explained by several reasons. As a result of heat treatment proteins may suffer loss of tertiary structure (55-70 °C), cleavage of disulphide bonds (70-80 °C) and new inter- and intramolecular bonds can be formed (80-90 °C) such as aggregates (90-100 °C). Besides, these changes and other chemical modifications (e.g. Maillard reaction, crosslinking with oxidized lipid products) may occur in higher temperatures as well (100-125 °C and above). These changes may have a significant impact on the antibody-binding activity and solubility (thus extractability) of proteins depending on the severity of heat treatment, the matrix components and the properties of the protein (Kieffer *et al.*, 2007; Lagrain *et al.*, 2008; Monaci *et al.*, 2011; Takács *et al.*, 2010a,b; Thomas *et al.*, 2007; Wal, 2003). However wheat proteins are considered partially heat stable and resistant to heat denaturation (Mills *et al.*, 2009; Wal, 2003) it was also reported that wheat flour heated at 80-120 °C for 10-60 minutes showed a decreased IgE-binding activity (Poms and Anklam, 2004). The conditions of our experiments fits this latter range of time and temperature (16 minutes, 180 °C) so the decrease of measurable gliadin content may be explained by some of the above-mentioned effects of heat treatment and/or potential interactions of gliadin with other matrix components. This way the antibodies used in the kits may not be able to capture the modified proteins anymore, however the changes seem to be only partial as a part of the gliadin content was still detectable. It is also possible that the loss of measured gliadin concentration in cookies is caused by a change of its solubility that influenced the performance of the extraction methods. Another possibility is that changes of antibody-binding activity and solubility of gliadin both occurs, however the magnitude of these phenomena may vary among kits using different extraction methods and capturing antibodies.

The exact behaviour of gliadin during processing is not clear at present and it will be a subject of further research for better evaluating the negative impact and the degree of uncertainty it introduces in analytical results.

4. Conclusion

The results of this study highlight many factors regarding analytical methods, method development and validation that must be handled carefully for proper quantification of gluten. The main factors are choosing the target proteins for gluten determination and for the calibration of the ELISA assays which could be determinant for reference material development and method validation too. The analytical data indicates that the different protein sources and the denaturation effects could have a significant impact on the obtained results. To overcome this problem, which is probably one of the most challenging issues of current gluten analysis, consensus would be necessary on the following questions:

- What kind of material should be used as a calibrator and reference material (gluten, gliadin or glutenin; native proteins, wheat flour of a single wheat variety or a mixture of different varieties)?
- What kind of antibodies and target proteins are the most appropriate for the quantification of gluten (monoclonal or polyclonal, sandwich or indirect)?
- What is the best way to interpret the analytical data (how to convert gliadin or wheat protein units into gluten units)?
- How should the effects of processing steps be handled (what happens during processing on the molecular level; possible changes of antibody-binding affinity, solubility or both; matrix effects)?

The solution of these problems requires a complex approach. The questions above must be investigated on the level of methods and on the level of molecular studies too (e.g. monitoring protein changes during heat treatment) and the results should be handled together to develop such methods that provide reliable results independently of the matrix, the gluten source and the degree of processing.

In conclusion, harmonization is needed in method development and method validation too. This would be helpful for food manufacturers and policy makers as well for widening the range of gluten-free products with minimal food safety risk.

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