



Analytical Methods

Detection of hen's egg white lysozyme in food: Comparison between a sensitive HPLC and a commercial ELISA method

Barbara Kerkaert, Frédéric Mestdagh, Bruno De Meulenaer*

NutriFOODchem Unit, Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

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ABSTRACT

Recently some cases described allergic reactions to undeclared lysozyme present in foods. In order to protect the allergic consumer, a better control is needed based on reliable and specific detection and quantification methods. The two currently used methods, a commercially available enzyme-linked immunosorbent assay (ELISA) for egg white allergens and a liquid chromatographic method, were compared. Despite the technical specifications of the ELISA kit, ensuring quantitative analysis of lysozyme in absence of other egg white proteins, no reliable quantitative and even qualitative data could be obtained which contrasted with the capabilities of the instrumental technique. A more elaborated evaluation of the methods on spiked samples identified the causes of the observed discrepancies. It was shown that next to a poor sensitivity of the ELISA kit also an insufficient lysozyme extraction and a co-extraction of interfering substances were the causes of the anomalies observed during application of the immunochemical method.

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1. Introduction

Hen's egg white (HEW) lysozyme is a food preservative, because of its lytic activity on the cell wall of Gram-positive bacteria. Cunningham, Proctor, and Goetsch (1991) described its use in a variety of foods such as fresh fruits, vegetables, meats and seafoods. The most known and studied application is however, as an alternative to nitrate, to prevent late blowing of semi-hard and hard cheeses by inhibiting growth of *Clostridium tyrobutyricum* (Pellegrino & Tirelli, 2000; Proctor & Cunningham, 1988). Besides, lysozyme is used as a preservative in wine, in order to control malolactic fermentation (Proctor & Cunningham, 1988; Tirelli & De Noni, 2007).

In the last decade several case studies revealed severe allergic reactions due to the presence of lysozyme. In Sweden, five of 21 case studies of allergic reactions to eggs were attributed to the presence of lysozyme as an additive in cheese (Malmheden, 2004). The percentage of lysozyme-sensitised patients in the egg-allergic population is however under discussion. While Frémont, Kanny, Nicolas, and Moneretvastrin (1997) noted that 35% of egg-allergic patients had anti-lysozyme IgE, Poulsen et al. (2001) concluded that the frequencies of reactivity to lysozyme amounted 15% in persons with egg sensitisation.

Given this relatively high incidence of lysozyme sensitisation and according to the recently changed EC legislation (2003/89/EC), the use of lysozyme as an additive has to be declared on the

ingredient label. An alert notification for insufficient labelling of deep frozen meals in April 2006, after an allergic reaction to lysozyme, proves the need for labelling and moreover the need for reliable analytical methods (European Food Safety Authority, 2006). Such method should be highly specific, sensitive and robust (Besler, 2001; van Hengel, 2007).

The first detection methods for lysozyme were based on the lytic activity of lysozyme on *Micrococcus lysodeikticus* cells (Liao, Brown, & Martin, 2001; Proctor & Cunningham, 1988). These microbiological methods are simple, rapid and reproducible with a low detection limit of 1.94 µg/mL (Liao et al., 2001). However they are not feasible for routine analyses and inaccurate in quantification because of the sensitivity to interfering substances and to lysozyme denaturation (Pellegrino & Tirelli, 2000; Vidal, Gautron, & Nys, 2005). Lysozyme determination can also be based on the quantification of the protein itself using chromatographic and immunological methods. The enzyme-linked immunosorbent assays (ELISA) are currently the most used commercial available methods to determine allergens in food (Besler, 2001; Poms, Klein, & Anklam, 2004). The immunoassays are promising because of their low detection limit, their high specificity and sensitivity (Vidal et al., 2005). Yet, questions arise about this specificity since it is known that immunological methods suffer from unexpected cross reactivities (van Hengel, 2007). Yoshida, Takagaki, and Nishimune (1991) who developed a specific sandwich ELISA for hen egg white HEW lysozyme, noted moreover a low recovery for lysozyme in more complex matrices due to interfering components. These interfering substances were also observed in the ELISA of Rauch,

* Corresponding author. Tel.: +32 9 264 61 66; fax: +32 9 264 62 15.
E-mail address: Bruno.DeMeulenaer@UGent.be (B. De Meulenaer).

Hochel, and Kas (1990), who determined lysozyme in cheese and noted a low accuracy and poor reproducibility. In conclusion, the developed specific HEW lysozyme ELISA assays is not fully optimised for several food matrices and thus not commercially available. Therefore commercial HEW ELISA's, which ensure the quantitative analysis of lysozyme, are currently used in order to detect the allergen. Although questions arise about the specificity of the HEW assays towards lysozyme, the kits are still used in the industry to detect this allergen.

In order to have a more robust and reliable method, Pellegrino and Tirelli (2000) developed a sensitive HPLC method for the detection of HEW lysozyme in milk and dairy products. They concluded that the optimised HPLC method was more accurate and reliable than the microbiological assay, however a comparison with the available immunochemical method was not made. Therefore in the current study, a commercially available ELISA was compared with a chromatographic method for the quantification of HEW lysozyme in cheese and cheese containing prepared meals.

2. Materials and methods

2.1. Chemicals

A freeze-dried egg white lysozyme standard (p.a.), Fluka 62971, was obtained from Sigma Aldrich (Belgium). NaCl (p.a.) and HPLC grade water was supplied by Chemlab (Belgium). HPLC grade acetonitrile and trifluoroacetic acid (p.a.) were from VWR International (Belgium) and Sigma Aldrich (Belgium), respectively.

2.2. Test samples

Different cheeses were used for the validation and comparison studies. Pamesello bulk and Gouda were provided by IDB (Belgium). Grana Padano was produced by Galbani (Italy) and a Gouda Cheese "Oud Brugge" by Belgomilk (Belgium). Besides cheese, also ready-to-eat meals were analysed which contained only lysozyme as egg white protein. The meals were frozen prepared pasta dish with spinach and ham, delivered by Crops (Belgium). This pasta meal contained 38% pasta, 22% vegetables (spinach, paprika, tomato), 6% cooked ham, 34% sauce (milk, grana padano, tomatoes, rapeseed oil and sunflower oil, rice and potato starch, soy protein, emulsifier (E322), stabilizer (E412, E415)).

2.3. Liquid chromatographic procedure

The chromatographic method of Pellegrino and Tirelli (2000) was slightly adapted. The final extraction procedure and HPLC conditions are given below.

2.3.1. Extraction of egg white lysozyme

About 3 g of homogenised cheese or 10 g of homogenised meal was mixed with 35 mL of 1 M NaCl. The pH of these solutions was adjusted to 6.0. The samples were heated (40 °C) during 10 min, mixed with the Ultra Turrax (CAT, UAF-25R) for 2 min at 25 000 rpm and placed on a shaker for 1 h. Afterwards, the extract was acidified to a pH lower than 4.3 in order to precipitate the caseins. Finally, the extracts were diluted to 50 mL with 1 M NaCl and filtered over a paper filter (Schleicher & Schuell, Germany) and a 0.20 µm HPLC filter (FP30-0.2CA, Novolab, Belgium).

2.3.2. HPLC conditions

The HPLC (1100 system, Agilent Technologies, Switzerland) was equipped with a reversed-phase polymeric column (PLRP-S 250 × 4.6 mm, 300 Å pore size, 5 µm particle size) from Varian Inc. (Belgium). The mobile phase consisted of a gradient of water

and acetonitrile both containing 0.1% trifluoroacetic acid (v/v). Eluting conditions expressed as proportion of water with 0.1% TFA: 0–10 min: 69%, 19 min: 49.3%, 20 min: 25%, 20–21 min: 25%, 22 min: 69%, 22–30 min: 69%. The flow rate was 1 mL min⁻¹, the column temperature 45 °C and the injected volume 50 µL. Detection was carried out with a fluorescence detector (FLD, G1321, Agilent Technologies, Switzerland) set at 280 nm ex. and 340 nm em. An emission spectrum was taken between 300 and 500 nm in order to confirm the identity of the peak and as such guarantee the specificity of the analysis.

2.3.3. Validation

The linearity was tested with standard solutions of lysozyme in a concentration range between 0.2 and 1 µg/mL and from 2 to 10 µg/mL, injected in duplicate. The low and high range standard curves were both linear with correlation coefficients of respectively 0.990 and 0.996. The limit of detection (LOD) and quantification (LOQ) were determined by analysing a spiked cheese, initially not containing lysozyme, in duplicate on five days.

The repeatability of the HPLC analysis was determined with standard solutions which were injected in duplicate on five days. To assess the intra-reproducibility and the repeatability of the whole procedure, a blank matrix was spiked with 3, 15, 30, 45, 60 µg/g and analysed together with a sample Grana Padano containing 200 µg/g lysozyme, in duplicate during five days. The duplicates of the same day were used to calculate the repeatability, the duplicates between days were used to assess the reproducibility. To calculate the reproducibility and repeatability, the following formula was used $r = t_{\infty} \cdot \sqrt{2} \cdot SD$ with t_{∞} , the Student's two tailed value for $v = \infty$ for a given confidence (for a normal confidence state of 95%, $t_{\infty} = 1.96$) and SD the standard deviation measured under repeatability and reproducibility conditions (Eurachem Group, 1998). For the recovery, a sample was spiked within the following concentration range: 15, 30, 45, 60 µg/g of lysozyme. The specific amounts of lysozyme were added at the beginning of the extraction procedure. The analytical procedure was performed in duplicate on five days.

2.4. Enzyme-linked immunosorbent assay

The Ridascreen FAST egg ELISA (Biopharm AG, Germany) was used to detect lysozyme. It is a commercially available enzyme-linked immunosorbent assay for the rapid detection of egg white proteins in several food matrices. Egg white protein consisted of 80% allergenic proteins amongst which ovalbumin (54%), ovomucoid (12%), ovomucoid (11%) and lysozyme (3.5%). In contrast to other available egg white protein assays, this assay is the only one which contains antibodies recognising all four HEW allergens. Most commercial egg ELISA kits only react to ovalbumin or ovomucoid (Veratox, Neogen; BioKits Egg Assay, Tepnel; Egg, ElisaSystems). Biopharm ensured that lysozyme quantification is possible with the egg FAST assay if lysozyme is used directly for calibration instead of the protein standard mixture included in the assay. In addition, no other HEW should be present in the sample as well (Biopharm, personal communication).

The extraction and test procedure were followed exactly according to the manufacturers specifications (Biopharm). Briefly, about 1 g of the homogenised sample was dissolved in 20 mL of extraction buffer. This extract was incubated for 10 min at 60 °C and centrifuged at 2500 g during 10 min. The sandwich ELISA started with the addition of 100 µL of standards and filtered extraction solutions to the wells coated with antibodies against HEW. After 10 min incubation, the wells were washed and 100 µL of enzyme conjugated antibody was added followed by an incubation period of 10 min at 37 °C. After a last washing step, substrate solution was added. Finally the colour reaction was stopped

with 2 M H₂SO₄ and absorbance was measured at 450 nm with a microplate spectrophotometer (Benchmark Plus, BioRad). The assay was each time calibrated using the protein standard mixture included in the assay. For quantitative lysozyme analyses however, a supplementary series of standard solutions of lysozyme in extraction buffer was used.

2.5. Comparison of the liquid chromatographic method and the ELISA method using spiked samples

Two spiking procedures, illustrated in Fig. 1, were applied. First of all, the samples were spiked with 5000 µg/g 24 h before the ELISA extraction. A second extract was spiked at the start of the extraction with 5000 µg/g. The ELISA extracts spiked before and during extraction were analysed with the ELISA assay in order to check the recovery of the ELISA procedure (extraction + assay). Besides, the extracts were also injected on HPLC in order to check the recovery of the ELISA extraction.

3. Results and discussion

3.1. Validation and results of the chromatographic method

The described HPLC method was completely validated for cheese. A LOD of 2.07 µg/g and a LOQ of 4.13 µg/g were obtained. Yet, Pellegrino and Tirelli (2000) noted a lower limit of detection of 0.8 µg/g. Since there is no threshold level known to which lysozyme allergic patients react there is no imposed detection limit for quantification methods, although a reasonable LOD for allergens is in a range of 1–100 µg/g (Poms et al., 2004). The repeatability of the HPLC analysis, assessed on lysozyme standard solutions, was 1.79% for higher concentrations (33–167 µg/g) and 9.99% for the lower concentration range (3.3–16.7 µg/g). The repeatability of the whole procedure, including the extraction, was 4.82% for cheese containing lysozyme concentrations between 20 and 200 µg/g. The reproducibility was assessed by taking the day-to-day variation into account, yielding 8.32% for a concentration range between 20 and 200 µg/g and comparable with a reproducibility of 7.2% determined by Tirelli and De Noni (2007) who analysed lysozyme in wine. Pellegrino and Tirelli (2000) noted however a reproducibility of 1.47% for lysozyme analysis in hard cheese. The specificity of the analysis was guaranteed by an elaborated extraction procedure, specific fluorescence detection and a good chromatographic separation without interfering peaks at the retention time of lysozyme. The accuracy was assessed by a recovery study which resulted in a final recovery of 90.2% for cheese. The

same recovery was determined by Pellegrino and Tirelli (2000) in milk. In ready-to-eat meals, however, a recovery of 127% was determined in a less extended validation in which 9 and 18 µg/g lysozyme were added to meals containing around 15 µg/g lysozyme.

Cheese and ready-to-eat meals containing various amounts of lysozyme were analysed on three different days. The results, corrected for the determined recovery, are given in Table 1. Both Grana Padano cheeses contain lysozyme with concentrations varying between roughly 100–200 µg/g. The low lysozyme concentration in the second ready-to-eat meal originates from the parmesan cheese which is processed in the meal as mentioned on the label. Most of these meals contain approximately 3–7% hard cheese (200 µg/g) or between 6 and 14 µg/g lysozyme. Based on the results of Table 1, it could be concluded that this optimised chromatographic procedure is a reliable, reproducible technique to quantify lysozyme not only in wine, cheese and milk but also in ready-to-eat meals.

3.2. Enzyme linked immuno assay

Since the Ridascreen assay was primarily designed to quantify a mixture of egg white proteins in foods, the kit was initially used in a qualitative way to detect presence of lysozyme in the samples. Consequently samples were considered to be positive for lysozyme if an absorbance value was obtained which was significantly higher than the one obtained for the standard not containing egg white protein, provided in the kit. If absorbance values were significant lower than this reference value, samples were considered not containing lysozyme. The experiment, including the calibration, was repeated on several days, in order to have an idea about the reproducibility of the method. Results are shown in Table 2 together with the qualitative appreciation of the HPLC-analysis reported in Table 1. Only for two samples analysed reproducible qualitative results were obtained, which proved however not to be accurate on basis of the HPLC analysis. For the other samples analysed, a variable qualitative result was obtained from day to day, which consequently was not always in correspondence with the previously obtained HPLC results. Thus both false positive and false negative qualitative results were obtained using the immunological procedure. In order to evaluate the qualitative lysozyme analysis, the ELISA assay was calibrated using an additional pure lysozyme stan-

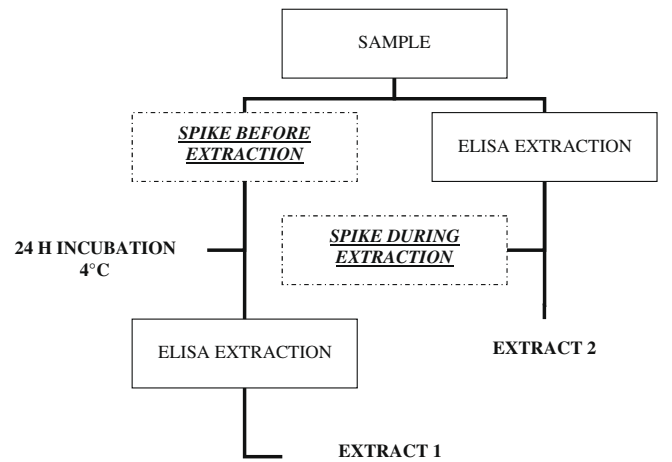


Fig. 1. Comparison study of the ELISA assay and the HPLC procedure.

Table 1
Lysozyme concentration of different samples analysed with the HPLC procedure.

Samples	Concentration lysozyme (µg/g) – (n = 3)
Ready-to-eat meal 1	<LOD
Ready-to-eat meal 2	15.2 ± 1.2
Grana Padano 1	94.5 ± 4.2
Grana Padano 2	202.7 ± 7.9
Gouda 1	<LOD
Gouda 2	180.5 ± 29.6

Table 2
Qualitative results of the ELISA and HPLC.

Samples	ELISA				HPLC
	Day 1	Day 2	Day 3	Day 4	
Ready-to-eat 1	+	–	–	–	–
Ready-to-eat 2	+	–	+	+	+
Grana Padano 1	–	–	–	–	+
Grana Padano 2	–	–	+	+	+
Gouda 1	+	–	–	+	–
Gouda 2	–	–	–	–	+

dard. Surprisingly, it was shown that the sensitivity of the kit towards pure lysozyme was about three orders of magnitude lower compared to the standard mixture of proteins provided in the kit (Fig. 2). Since lysozyme concentrations, as determined via HPLC analysis, in the analysed samples proved to be much lower than the actual working range of the kit, if calibrated with pure lysozyme, it was moreover quite surprising that in the qualitative analysis (Table 2), several positive samples were identified.

3.3. Comparison of the liquid chromatographic method and the ELISA method using spiked samples

In order to further elaborate the quantitative capabilities of the ELISA kit, samples spiked with lysozyme at a 5000 ppm level were analysed using both the ELISA and the HPLC method. The applied spiking levels were chosen in view of the calibration data represented in Fig. 2. In food however, it should be noted that such high lysozyme concentrations are unrealistically high. Spiking was performed in two different ways as explained in Fig. 1. All extracts were obtained using the extraction buffer used in the ELISA kit. Extract 1 was obtained from a sample which was spiked 24 h before extraction. Extract 2 was obtained by spiking lysozyme during the extraction of an unspiked sample. All ELISA extracts obtained were analysed using the HPLC method and the ELISA assay. These spiked samples were moreover also extracted according to the HPLC procedure, diluted in NaCl (1 M) and injected on HPLC to confirm the results of the earlier discussed validation study. Results are expressed as a recovery on basis of the total theoretical lysozyme concentration present in the extract which was calculated from the initial lysozyme amount present in the sample, as obtained in the earlier HPLC analysis (Table 1) and from the spiked lysozyme concentration.

The recovery of the spiked samples extracted by the HPLC method and injected on HPLC varied between 75% and 110% with an average recovery of 93% which is comparable with the recovery

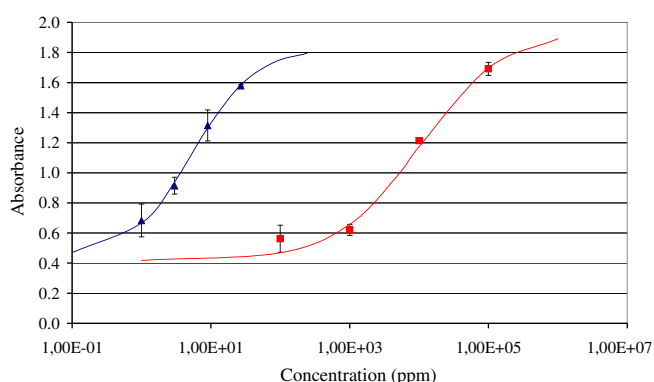


Fig. 2. The ELISA standard curves of egg white protein (▲) and lysozyme (■) (confidence interval; $n = 3$).

Table 3

Recovery of the lysozyme from the spiked samples determined with HPLC analysis after ELISA extraction.

	Recovery of lysozyme	
	Extract 1 (%)	Extract 2 (%)
Ready-to-eat meal (1)	52.9 ± 7.6	57.8 ± 7.1
Ready-to-eat meal (2)	41.9 ± 1.5	64.0 ± 10.0
Grana Padano (1)	45.3 ± 6.8	63.6 ± 0.3
Grana Padano (2)	59.2 ± 11.3	59.3 ± 1.4
Gouda (1)	34.0 ± 5.1	65.8 ± 2.7
Gouda (2)	50.8 ± 11.2	81.1 ± 3.7

Table 4

Recovery of the lysozyme from the spiked samples determined with ELISA after ELISA extraction.

	Recovery of lysozyme	
	Extract 1 (%)	Extract 2 (%)
Ready-to-eat meal (1)	54.5 ± 4.4	63.6 ± 5.1
Ready-to-eat meal (2)	48.0 ± 3.8	41.9 ± 3.4
Grana Padano (1)	18.5 ± 1.5	32.0 ± 2.6
Grana Padano (2)	34.5 ± 2.8	29.2 ± 2.3
Gouda (1)	28.3 ± 2.3	28.8 ± 2.3
Gouda (2)	16.3 ± 1.3	34.9 ± 2.8

of the validation study. Results of the ELISA extracts analysed by HPLC and ELISA are shown in Tables 3 and 4.

Comparing the HPLC results obtained for the various ELISA extracts (Table 3), it can be concluded that an incomplete extraction of lysozyme is achieved using the extraction procedure of the assay. Considering the cheese samples, it seems that, when spiking is performed in presence of a significant concentration of caseins, a substantial amount of lysozyme is not extracted with recoveries varying between 34.0% and 81.1%. In view of the strong interaction between caseins and lysozyme, this is not surprising. It seems however that an interaction with other food components may also negatively influence the extraction efficiency, since only 42–64% of the lysozyme was extracted from the spiked ready-to-eat meals, which contained only a limited amount of cheese and thus also of caseins.

By comparing the lysozyme concentration of extract 1 with extract 2 for the same samples, it seems moreover that the longer the interaction between lysozyme and the matrix, the lower the recovery obtained during extraction. Probably, better extraction efficiency can be obtained if a strong saline solution (1 M NaCl) is used as was used in the chromatographic procedure. The brine solution is necessary in order to release lysozyme which is associated with other proteins by electrostatic and hydrophobic interactions (de Roos, Walstra, & Geurts, 1998; Howell & LiChan, 1996). High salt concentrations are as such necessary for the extraction but have however a negative effect on the immunological reaction since De Meulenaer, De la Court, Acke, De Meyere, and Van de Keere (2005) for instance noted that an increase in ionic strength related to an increase in sodium chloride, resulted in a decrease of the interaction between antibody and peanut protein. More research needs to be done in order to have an extraction buffer which extracts all the lysozyme without negatively influencing the immunological reaction. A recent study moreover noted big discrepancies in extraction procedures and buffers of different egg white protein assays giving rise to very different and even contradictory results (Faeste, Lovberg, Lindvik, & Egaas, 2007). Another study compared different extraction buffers to extract hen egg proteins and concluded that phosphate buffered saline gave the best quantifiable results, followed by physiological saline, water, acetate buffer and tween 20 (Hildebrandt, Steinhart, & Paschke, 2008). Phosphate buffer saline may be a good extraction buffer for HEW in general, it is however doubtful if the salt concentration of 0.15 M will be sufficiently high to recover lysozyme in view of its strong interaction with other proteins.

After analysing the extractability, the different spiked extracts were analysed with the ELISA assay as well. Comparing the results as reported in Table 4 with those obtained from the HPLC analysis of the same extracts (Table 3), it can be observed that for the cheese samples an additional underestimation of the lysozyme concentration actually present in the extract is obtained for both lysozyme extracts. This can be illustrated with the data of the Gouda 2 – extract 1 – samples for which only 51% of the spiked lysozyme could be extracted with the ELISA extraction procedure.

Moreover after analysing this extract with the immunoassay, only 16% of the total lysozyme amount spiked could be detected, which indicates that only 30% of the extracted lysozyme could be quantified by the assay. Probably matrix components interfere during the immunological reactions, thus giving rise to more dramatic underestimation of the spiked lysozyme concentration. This trend was however not observed for the ready-to-eat meals.

Possible interfering substances include cross reactive proteins but also enzymes and even lipids, as summarised by [Pesce and Michael \(1992\)](#). Since the interfering compounds differ from matrix to matrix, care should be taken if a validated ELISA assay is applied in a new matrix not included in the validation studies. [van Hengel \(2007\)](#) mentioned the importance of a common experimental design for validation studies since the outcome of the validation depends strongly on the type of matrix which is considered and the used spiking procedure. The present study confirms these findings for the case of lysozyme detection in cheese and prepared meals.

4. Conclusions

An ELISA to quantify egg white allergen proved to be unsuitable for lysozyme analysis in foods. In contrast to the HPLC method, the ELISA was prone to incomplete lysozyme extraction and matrix interferences during the immunological reactions. The latter disadvantage could be resolved by using more specific antibodies. Even in this case, it should be kept into account that due to the strong interaction between lysozyme and other food proteins the addition of chemicals lowering protein–protein interactions during extraction remains indispensable, which undoubtedly would also affect the antibody-analyte interaction in an ELISA.

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