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Research Article

Detection of Lysozyme in Foods: How to Identify Suitable Test for Official Laboratories

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Keywords

Lysozyme; Food allergens; ELISA test; Consumer's protection

Introduction

Lysozyme is an enzyme obtained from egg white and it is used as food additive for its lytic activity on the cell wall of gram-positive microorganisms. In particular, lysozyme is used as additive in hard cheese producing process to prevent the "late blowing" defect [1,2] as it results efficient in lysing the vegetative cells of Clostridia, specifically of *Clostridium tyrobutyricum*, gasogen bacteria responsible of the swelling during ripening. These bacteria can be present in high concentration in milk from bovines fed with the wide use of silage, and they are able to produce spores that survive the thermal treatment applied in making hard cheeses and can later germinate and produce gas causing the defect [3].

Hen egg is one of the main sources of lysozyme used in foods as additive. According to European law on food additives, lysozyme cab be added to food during producing processes as it is listed in part B annex II of EU Regulation 1333/2008 [4]. According to part E of the same regulation it can be used as food additive in hard cheese, in unpasteurized beers and some other drinks > 15% v/v at dose of *quantum satis*. In all these food categories, no maximum numerical level is specified and lysozyme shall be used in accordance with good manufacturing practice, at a level not higher than is necessary to achieve the intended purpose and provided the consumer is not misled. The E number of lysozyme is E1105.

Hen eggs are also one of the most frequent causes of food allergy. Lysozyme is a well-known major allergen from hen's egg named Gal d 4. It contains both conformational and sequential epitopes [5].

According to European Law (Regulation 1169/2011), foods ingredients must be declared in a list, in descending order of weight, as recorded at the time of their use in the manufacture of the food [6]. Substances or products causing allergies or intolerances must be emphasised through a typeset that clearly distinguishes it from the rest of the list of ingredients, for example by means of the font, style or background colour. Furthermore, they have to be indicated with a clear reference to the name of the substance or product causing allergies, as listed in Annex II, in order to guarantee clear information to allergic consumers. For this reason, when lysozyme is added as additives in food, its derivation from egg has to be clearly mentioned close to its name or E number.

Laboratories performing analyses on foods should have availability of test able to detect small amount of food allergens, because there is no correlation between its concentration in food and gravity of symptoms in consumers, a reliable lowest observed adverse effect levels for lysozyme is not recognized and because, to date, there is no regulation fixing criteria.

The aim of this paper is to describe the protocol followed in an official food control laboratory to identify the best test to be used to detect hidden lysozyme in food matrices. When a new screening method is needed in the laboratory Food Control Laboratory of Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta (Italy) a standardized protocol to identify the most suitable test is applied. The protocol includes a market survey, a theoretical score assignment, evaluation of the declared LOD and LOQ of the most suitable kits, a spiked samples test session and a reproducibility test session.

Materials and methods

Market survey

In the first phase of the project, a market survey was launched to verify how many commercial tests were available for detection of lysozyme in milk-based foods.

The survey was carried out by contacting companies producing commercial allergen detection kits, directly by email or through company websites. At the time of contact, companies were asked to provide the information requested in a questionnaire and related to:

Field of application, foods that can be analyzed with the kit

- Third part validation data available and certificates
- Known cross-reactivities with foods or other allergens
- Kind of reagents used
- Use of standard solutions provided by the kit
- Results elaboration and expression
- Execution time
- Need of with special filters/equipment for obtaining results

Theoretical score assignment

The information collected during the market survey was compared and for each of the characteristics under

investigation, scores were assigned based on a standardized grid. The score assignment criteria are summarized in Table 1. The table also describes the different scenarios that were supposed. Characteristics from 1 to 3 are referred to certification or validation documents; characteristics 4 and 5 are referred to reagents and solution used in the assay; characteristics from 6 to 8 are referred to results expression and time spending for execution.

	Characteristics	Score 0	Score 1	Score 2
1	**Field of application**	Dairy not included in the field of application	General reported as "foods"	Complete list of matrices including "dairy"
2	**Third part validation data**	Data are not available		Data are available
3	**Cross reactivity**	Cross reactivity with other allergen or other food proteins	No cross reactivity is reported	Absence declaration of cross reactivity is reported
4	**Reagents**	Other reagents are needed		All needed reagents are included
5	**Use of standard solutions**	To be prepared		Ready to use
6	**Results elaboration**	No info; Info are incomplete	Complete information on standard curve	Spreadsheet Software
7	**Execution time**	≥ 02:00 hours	≥ 01:30 hours	≤ 01:00 hour
8	**Need of special filters/instruments**	Filters or instruments not available in the lab	Filters or instruments not available in the laboratory but easily purchasable	Filters or instruments already available in the lab

Table 1: Score assignment criteria for commercial test for Lysozyme detection in dairy.

Investigation on declared LOD and LOQ

According to the results of the score assignment step, the five tests that obtained the highest score were selected and the producing companies were asked to give evidence of the declared LOD and LOQ of their ELISA tests and to communicate the lysozyme concentration of internal standards.

Spiked samples test-session

The five tests that obtained the highest score were used for a blind test session with spiked samples.

In particular 8 water and 8 milk samples were spiked with lysozyme at the final concentrations of 2, 5, 10, 20, 50, 100, 200, 400 ppb. Lysozyme from egg white L6876-1g, (Merck KGaA, Darmstadt, Germany) was used.

All samples were prepared in laboratory by operators not involved in any of subsequent analyses.

Analyses were performed in double by trained operators according to the different test producers' instructions.

Results and conclusions

During the market survey 20 emails and 27 information requests were sent to the email boxes or through company websites respectively. In total, the survey led to the evaluation of 16 commercial ELISA tests for the detection of lysozyme in foods and beverages.

For the subsequent step of the study, the tests were indicated with anonymity, with the numbers from 1 to 16. Table 2 reports for each test and each characteristic the assigned score.

After the evaluation of the first eight characteristics, a total of five tests obtained a score of 16, one kit obtained 13 points, one kit obtained 10 points, two tests obtained 1 point. At the kits having the six higher score, a special score related to the prize was assigned, from 0 to 5, from the more expensive to the cheapest respectively. At the end of evaluation, the maximum score was 21 (one kit) and the minimum 0 (7 kits); two tests had score 1 and finally 6 tests had a score ranging from 10 to 20. The five commercial tests having score from 17 to 21 were subjected to the spiked samples analyses session. They are named in Table 2 as test from 1 to 5.

During the preliminary analyse of spiked samples, test 5 and test 1 were the most responsive to the laboratories needs (Table 3). In particular, test 5 returned quantitative results close to the expected concentration, but did not identify lysozyme below the 20 ppb threshold; the test 1 was able to detect lower concentrations of lysozyme, but it seemed to underestimate the real concentrations of lysozyme added in the milk samples.

Based on these results, a test session was organized in order to check the reproducibility of tests 1 and 5: two different operators (operator A and operator B) were asked to test the same spiked milk samples in two different moments. Results are reported in Table 4.

Commercial	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Field of application	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0	2
Third part validation data	2	2	2	2	2	0	0	0	0	0	0	0	0	0	0	0
Cross reactivity	2	2	2	2	2	0	0	0	0	0	0	0	0	0	0	2
Reagents	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0	2
Use of standard solutions	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0	2
Results elaboration	2	2	2	2	2	0	0	0	0	0	0	0	0	0	0	1
Execution time	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0	2
Need of special filters/instruments	2	2	2	2	2	2	0	1	0	0	0	0	0	0	1	2
SUB-TOTAL	**16**	**16**	**16**	**16**	**16**	10	0	1	0	0	0	0	0	0	1	**13**
Special score for prize	4	2	1	5	3											0
TOTAL	**20**	**18**	**17**	**21**	**19**	10	0	1	0	0	0	0	0	0	1	13

Table 2: Score assigned to the tests. Test from 1 to 5 reached the higher scores and were subjected to the spiked sample analyses session.

Test						
Sample and Spike level (ppb)		**1**	**2**	**3**	**4**	**5**
Water	**2**	0,74	<LOD	<LOD	<LOD	<LOD
	5	1,08	<LOD	<LOD	<LOD	<LOD
	10	3,64	<LOD	0,685	<LOD	<LOD
	20	1,08	<LOD	0,662	<LOD	<LOQ
	50	1,21	<LOD	0,675	<LOD	<LOQ
	100	1,35	<LOD	0,601	<LOD	<LOQ
	200	1,42	<LOD	0,671	<LOD	<LOQ
	400	5,32	139,35	>2,00	<LOD	<LOQ
Milk	**2**	0,67	<LOD	<LOD	<LOD	<LOD
	5	1,21	<LOD	<LOD	<LOD	<LOD
	10	3,64	<LOD	0,588	<LOD	<LOD
	20	2,7	<LOD	0,563	<LOD	**<LOQ**
	50	6,87	<LOD	0,609	<LOD	39,59
	100	13,07	**<LOQ**	0,589	10,67	74,07
	200	25,61	126,12	0,754	23,25	165,03
	400	53,15	611,90	1,2	46,55	214,45

Table 3: Results of analyses of spikes water and milk samples.

Both of the test resulted to be characterized by high reproducibility: Both of them seemed to underestimate the concentration of lysozyme in particular test 1.

The protocol here described is a useful procedure helping official food laboratories to start decisional process for the expensive validation and accreditation documents.

When a new screening method is needed and dozen of commercial kits are available on the market, this protocol can help to select the most suitable tests to be fully validated and then accredited. In the study here reported out of 16 tests entering the survey, five were chosen for their characteristics and two resulted to be compliant with the laboratory needs.

	Spike (ppb)	Test 1 Operator A	Test 1 Operator B	Test 5 Operator A	Test 5 Operator B
Milk	**2**	0,67	0,57	<LOD	< LOD
	5	1,21	1,78	<LOD	< LOD
	10	3,64	4	<LOD	7,50
	20	2,7	6,73	<LOQ	11,17
	50	6,87	5,96	39,59	31,82
	100	13,07	12,44	74,07	68,91
	200	25,61	22,97	165,03	128,34

Table 4: Reproducibility test session by operator A and B using test 1 and 5.

In order to be used on official sample collected by competent authority the whole validation process must be completed. According to Article 12 of the Regulation (EC) 882/2004 indeed, competent authorities may only designate laboratories that operate and are assessed and accredited in accordance with some European standards and for example EN ISO/IEC 17025 on 'General requirements for the competence of testing and calibration laboratories. The study here reported, lead to select the commercial analytical test to be used in an official laboratory. The full validation process will allow to accredit the analyses and to start a monitoring plan to ensure health protection for allergic consumers. In North America and Western Europe egg allergy occurs in 0.5% to 2.5% of children under the age of five years and in one-third of cases, the allergy persists into adulthood [7]; Allergic reactions exclusively to lysozyme were reported by some authors [8]. Monitoring plans to detect hidden allergen not declared on the label are an in important instrument to protect allergic consumers and to recall incompliant foods from the market when they pose a risk for health.

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