REPORT

Proficiency testing

Egg analysis in sweet and sour sauce and in cookies by ELISA

November 2014 – March 2015

CER Groupe, Département Santé

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Table of Contents

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Objective

A proficiency test was organized by CER to evaluate the ability of service labs to detect egg residues in food matrices. Testing materials were prepared and characterized during the 2014 egg validation study requested by FASFC then sent to the participating labs. It consisted of three different sample types (sweet and sour sauce, spiked cookies and incurred cookies) contaminated with either one of four different concentrations of egg: 0 ppm, 50 ppm, 100 ppm or 250 ppm of dry whole egg powder. Each participant received 7 blinded samples (15 g) consisting of:

- sweet and sour sauce, 0 ppm
- sweet and sour sauce, 50 ppm
- sweet and sour sauce, 100 ppm
- blank cookies, 0 ppm
- spiked cookies, 50 ppm
- spiked cookies, 100 ppm
- incurred cookies, 250 ppm

Twelve labs providing routine allergen detection services (Belgium: 6; Netherlands: 2; France: 2; Switzerland: 2) were invited to participate to the study (see Appendix 1 for invitation form). Six labs answered positively to the request. Registration form (Appendix 2) and instructions (Appendix 3) were provided by email to the participating labs prior to sending the samples. The list of participating labs and their coordinates are detailed in Appendix 4. The samples were sent at room temperature on November 24th and the labs were invited to use their standard operating procedure for egg detection. Deadline for reporting results was December 15th.

Material

Standardized egg powder (RM 8415, dry whole egg powder, National Institute of Standards and Technology, USA) was used as contaminating ingredient. Two types of food matrices were investigated: cookies (manufactured at CER) and sweet and sour sauce (purchased locally). Contamination of the cookies with egg powder was achieved in two ways, either the egg powder was added after the baking (spiked cookies) or added to the dough before the baking (incurred cookies). Details on the preparation of the test materials are given in Appendix 5. Tests were conducted to confirm the homogenous distribution of the egg contamination in the different food matrices (Appendix 6) and the stability of the samples (Appendix 7).

Samples with decreasing egg concentrations (100 ppm or 50 ppm) were analyzed to evaluate the sensitivity of the methods used by the participating labs. Higher egg concentration (250 ppm) was used for the incurred cookies to compensate for the degradation of epitopes that occur during the baking process. Equal portions of 15 g were weighted for the 7 tests samples and sent to the participating labs.

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Results

Qualitative results ("detected" or "not detected") were requested from the participating labs for the 7 provided samples. Quantitative results could also be submitted on a voluntary basis. An Excel file was sent by email to the labs to standardize the reporting of the results (Appendix 8).

The results from all participating labs were returned by January 15th. One lab provided results for the sweet and sour sauce only but none for the cookies samples. Two labs provided quantitative results for all samples and one lab provided quantitative results for the sweet and sour sauce samples only.

Six data sets were obtained for the sweet and sour sauce samples and 5 for the cookies. The kit commercialized by R-Biopharm (RidaScreen Fast Ei/Eigg Protein, Germany) was used by 4 labs, one lab used the kit from ELISA System (Enhanced Egg Residue, Australia) and one lab used an in-house developed ELISA method.

Detailed results of the analysis performed are shown in **Table 1**.

Table 1. Results of the detection by ELISA of different concentrations of egg powder in 7 test samples

Sample (ppm)	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6
Sweet and sour sauce (0)	-	-	-	-	-	-
Sweet and sour sauce (50)	+	+	+	+	+	+
Sweet and sour sauce (100)	+	+	+	+	+	+
Cookies (0)	-	-	-	-	no results	-
Spiked cookies (50)	+	+	+	+	no results	+
Spiked cookies (100)	+	+	+	+	no results	+
Incurred cookies (250)	+	-	+	+	no results	-
Commercial kit used	R-Biopharm	R-Biopharm	ELISA System	R-Biopharm	R-Biopharm	in-house developed
Reporting units	whole egg powder	whole egg powder	whole egg powder	whole egg powder	whole egg powder	whole egg proteins
LOD (ppm)*	0.1	0.13	0.5	0.5	0.25	1.3
LOQ (ppm)*	0.5	0.3	1	0.5	0.5	2
Calibration curve (ppm)	0 - 0.5 - 1.5 - 4.5 - 13.5	0 - 0.5 - 1.5 - 4.5 - 13.5	0 - 1 - 2.5 - 5 - 10	0 - 0.5 - 1.5 - 4.5 - 13.5	0 - 0.5 - 1.5 - 4.5 - 13.5	0 - 2 - 5 - 10 - 20 - 50
Starting material (g)	1	1	2	1	1	2

*: as reported by the participating lab, not necessarily as documented by the kit manufacturer.

No labs reported false positive results in sweet and sour sauce or in cookies. Lab 5 didn't fill any results for the cookies matrix.

All labs detected the presence of traces of egg in the sweet and sour sauce samples for the highest (100 ppm) and lowest (50 ppm) contamination levels.

Labs that reported results for the cookies matrices (all labs except Lab 5) detected the presence of traces of egg in the cookies spiked with the highest (100 ppm) and lowest (50 ppm) egg concentrations.

Discrepancies between the labs appeared for the reporting of the results of the incurred cookies containing 250 ppm of egg powder. Three labs correctly detected the presence of egg in the samples (using R-Biopharm or ELISA System kits) while 2 labs failed to detect it (using R-Biopharm or an internally developed test).

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Four labs reported complete quantitative data sets and a fifth one reported quantitative results on the sweet and sour sauce only. The results are displayed in **Table 2**.

Table 2. Quantitative results reported for the presence of egg residues in 7 test samples

Sample (ppm)	Lab 1 (ppm)	Lab 2 (ppm)	Lab 4 (ppm)	Lab 5 (ppm)	Lab 6 (ppm)
Sweet and sour sauce (0)	<0.5	<0.3	<0.5	<0.5	<2
Sweet and sour sauce (50)	1.6	0.54	2.8	2.2	2.2
Sweet and sour sauce (100)	4.3	0.95	4.6	3.8	4.2
Cookies (0)	<0,5	<0.3	<0.5	no results	<0.2
Spiked cookies (50)	3.8	0.61	3.2	no results	2.4
Spiked cookies (100)	6.5	1.52	7.9	no results	6.4
Incurred cookies (250)	1.0	<0.3	1.2	no results	<2

All the labs reporting quantitative results used R-Biopharm kit except one (in-house developed method). The linearity of the test for the concentration analyzed is good for both the spiked cookies and the sweet and sour sauce. The values calculated for samples spiked with 100 ppm are roughly twice the value calculated at 50 ppm (maximum difference between values at 100 ppm and 2x values at 50 ppm = 20%). Lab 2 reported lower values than Lab 4, Lab 5 and Lab 6.

Importantly, the calculated egg concentration is lower than expected in regards to the quantity of egg powder used for the contamination. A possible explanation would be that the manufacturing process of the powder (drying + treatment by γ-radiations for long term storage) modified the epitopes of the allergenic proteins inducing a drop in the signal. To test this hypothesis, blank cookies samples were spiked with either 100 ppm of NIST 8415 egg powder or 100 ppm of freshly lyophilized egg and processed according to kit protocol. The results confirm that for an equivalent contamination, the NIST 8415 powder gives a lower concentration (3.3 ppm vs 27.7 ppm for freshly lyophilized egg). Contamination levels (50 ppm, 100 ppm and 250 ppm) should be considered with caution as the above results indicate that they are strongly overvalued.

Conclusion

Ridascreen Fast Ei/Egg kit (R-Biopharm) is the method used by the majority of Belgian labs (3 out of 5). The two commercial kits evaluated (Ridascreen Fast Ei/Egg from R-biopharm and Enhanced Egg Protein from ELISA System) were able to detect egg at the lowest concentration (50 ppm) in both spiked cookies and sweet and sour sauce as well as in incurred cookies (250 ppm). Two labs did not detect egg residues in incurred cookies, one was using the R-Biopharm kit and the second was using an in-house developed kit. The latest has a higher limit of quantification than the other kits (2 ppm vs 0.5 ppm for R-Biopharm and 1 ppm for ELISA System).

Traces of egg are more difficult to detect in processed food as it is indicated by the lower concentrations found in incurred cookies (250 ppm) compared with the one of spiked cookies (50 ppm and 100 ppm). These results confirm observations made during the egg validation study.

As a conclusion, R-Biopharm Kit and ELISA System kit display similar analytical sensitivity and are suitable to detect traces of raw and cooked egg in food. The internally developed method is less sensitive for

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cooked egg but display similar analytical sensitivity to the commercial kits for raw egg contamination. Additionally, differences in the procedures followed by labs using identical kits may lead to significant variations for quantitative results. This is exemplified by quantitative results obtained by Lab2 and Lab4 which are using the same kit but report a constant difference of 5-fold for similar samples.

Appendix 1. Invitation form

Marloie, November 06th, 2014.

Dear Colleagues,

Regarding : Proficiency test
Detection of egg residues in cookies powder and sauce.
Invitation

We are pleased to invite you in participating to the proficiency test '**Detection of egg residues in cookies powder and sauce.**' organized by the Belgian NRL for Allergens.

The PT materials are 4 cookies powder and 3 sauce samples which could contain all targeted compounds.

For the official Control Laboratories approved for Allergens, egg, by the Belgian Federal Agency for the Safety of the Food Chain (FASFC), participation is mandatory.

Time schedule :

- deadline for registration (see attached Registration Form) : **November 17th, 2014**
- shipping of samples : November 24th, 2014
- deadline for submission of results : December 15th, 2014

Participation charges :
none

Please send all replies and/or messages related to this PT to the following e-mail address : infolnr@cergroupe.be.

Looking forward to hearing about your participation,
Best regards,

Dr P. DELAHAUT
CER Groupe, Département Santé

Enclosure : (1) Registration Form

Appendix 2. Registration form

+++ REGISTRATION FORM +++

Proficiency test
Detection of egg residues in cookies powder and sauce.

Contact person

Name and address of the laboratory

Phone

E-mail

VAT number

Shipping address (if different from the above mentioned address)

I hereby accept the terms of participation as specified in the invitation letter.

Date/signature :

Please return your signed registration form by e-mail to infolnr@cergroupe.be or by fax to +32 (0)84 31 61 08 **before November 17th, 2014**.

Appendix 3. Instructions

Proficiency test

Detection of egg residues in cookies powder and sauce.

Instructions

Shipment content

- 4 identified samples of cookies powder
- 3 identified samples of sauce.

Instructions

A) acknowledgement of receipt

Please fill in the attached "Acknowledgement of Receipt" form and return it immediately upon receipt of the samples :

- by email to : **infolnr@cergroupe.be**

or

- by fax to : **+32 (0)84 31 61 08**

B) samples storage and reconstitution

- **Upon arrival**, the samples must be stored **at -20 °C** until start of analysis.
- Each vial contains 15 g of sample.
- Prior analysis, each sealed vial must be placed at room temperature.
- The mixture must be homogenized before the extraction.

C) samples analysis

Each sample may contain egg.
Please perform a single analysis for each sample by using your routine method.

D) reporting of results

Please report analyses results **before December 15th, 2014** by using the attached "Reporting of Results" form.
Please report qualitative results for egg.

Appendix 4. List of participating labs

Country	Laboratory name	Address
Belgium	AFSCA, LFSAL	495, Rue de Visée 4020 Wandre
Belgium	CER Groupe, Département Santé	8, Rue du point du jour 6900 Marloie
Belgium	Instituut voor Landbouw en Visserijonderzoek (ILVO)	115, Burgemeester Van Gansberghelaan 9820 Merelbeke
Belgium	Laboratorium ECCA NV	3, Ambachtsweg 9820 Merelbeke
Belgium	Laboratorium voor Onderzoek Van levensmiddelen en Aanverwante Produkten (LOVAP)	11, Klaus-Michael Kuehnelaan 2440 Geel
The Netherlands	NutriLab B.V.	12, Burgstraat 4283 GG Giessen

Appendix 5. Test material preparation

Each starting material for both sauce and cookies were tested with CER kit to confirm the absence of detectable egg residues (data not shown).

Dry whole egg powder (catalogue reference: RM 8415) from the National Institute of Standards and Technology (NIST, USA) was used as contamination source for the different tested matrices. According to the product datasheet, the total protein content of the reference material is 37.8% ± 1.2%.

For each tested matrix, 4 contamination levels were prepared: 0 ppm, 50 ppm, 100 ppm and 250 ppm of dry whole egg powder. The lowest contamination level (50 ppm) is above the quantification limit of all commercial kits, it is hence expected that all samples except the negative controls should be classified as positive for egg residues. The test samples were aliquoted (15 g) and stored at -20°C until analysis.

Sweet and sour sauce

An off-the-shelf sweet and sour sauce was bought in a local supermarket and spiked with 250 ppm dry whole egg powder then thoroughly homogenized using an industrial grinder (Blixer 4.V.V., Robot Coupe, France). Recipe as stated on the jar is as follows: onions, carrots, red pepper, green pepper, bamboo, celery, water, sugar, pineapple, tomatoes, vinegar, starch, chicken bouillon, calf bouillon and spices (proportions not specified).

The homogenized sweet and sour sauce spiked with 250 ppm egg powder was diluted and further homogenized as described below by adding uncontaminated sauce with the following ratios:

- 100 ppm sauce: 250 ppm sauce (40%) mixed with uncontaminated sauce (60%)
- 50 ppm sauce: 250 ppm sauce (20%) mixed with uncontaminated sauce (80%)

Spiked cookies (egg powder added after baking)

The recipe used to produce the cookies was based on the one published by Dumont[1] *et al.* (2010) as described in Table 3.

Table 3. Cookies composition

Ingredients	Proportion (%)
Olive Oil	16
Wheat Flour	59
Dust Sugar	18
Water	6,5
Sodium Chloride	0,3
Sodium Hydrogen Carbonate	0,1
Amonium Bisulphate	0,1

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The thickness of the cookies was 8 mm and the diameter of the punch was 60 mm. The cookies were baked for 17 min at 200°C. The weight of each cookie before baking was 22 g ± 2 g and 20 g ± 2 g (n = 18) after baking, indicating a water loss of 10%.

The cookies were thoroughly homogenized as described above, spiked with 250 ppm egg powder and homogenized again. The 250 ppm cookies sample was afterwards diluted with uncontaminated cookie powder with the following ratios:

- 100 ppm spiked cookies: 250 ppm cookies powder (40%) mixed with uncontaminated cookie powder (60%)
- 50 ppm spiked cookies: 250 ppm cookies powder (20%) mixed with uncontaminated cookie powder (80%)

Incurred cookies (egg powder added before baking)

The same recipe as described above was followed with the exception that egg powder was added in the wheat flour. The wheat flour was then homogenized before being used to prepare the dough. The dough was then baked as described for spiked cookies.

A summary of the analysed samples is given in Table 4.

Table 4. Summary of the samples tested for the validation study

sample type	contamination level (dry whole egg powder)			
	0 ppm	50 ppm	100 ppm	250 ppm
sweet sour sauce	blank sauce	320 g of the 250 ppm + 1280 g of the blank matrix	640 g of the 250 ppm + 960 g of blank matrix	400 mg egg powder in 1600 g sauce
spiked cookies	blank cookie powder	400 g of the 250 ppm + 1600 g of blank matrix	800 g of the 250 ppm + 1200 g of blank matrix	500 mg egg powder in 2000 g blank cookie powder
incurred cookies	blank cookie powder	400 g of the 250 ppm + 1600 g of blank matrix	800 g of the 250 ppm + 1200 g of blank matrix	500 mg egg powder in 1180 g wheat flour used for the dough

1. Dumont V, Kerbach S, Poms R, Johnson P, Mills C, Popping B, Tömösközi S and Delahaut P. Development of milk and egg incurred reference materials for the validation of food allergen detection methods. Quality Assurance and Safety of Crops & Foods. 2010 Dec; 2(4): 208-15.

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Appendix 6. Homogeneity study

To ascertain that potential variations in the measurement are not due to a lack of samples homogeneity, 10 samples from each sample batch (matrix type and contamination level) were randomly collected and analyzed for their egg content using the CER kit (only six samples were tested for the sauce). The samples are considered homogenous if the calculated standard deviation (SD) for the measured optical densities (OD) is below 16% (16% = 2 x SD calculated for the first point of the standard curve (2 ppm) during the validation study of the CER kit for egg).

1. Sweet and sour sauce

Table 5. Homogeneity testing for sauce samples

Sample	Sweet and sour sauce batch			
	0 ppm	50 ppm	100 ppm	250 ppm
Sample 1	0,055	0,501	0,576	0,978
Sample 2	0,053	0,526	0,648	0,993
Sample 3	0,053	0,516	0,698	0,837
Sample 4	0,039	0,543	0,730	0,806
Sample 5	0,052	0,511	0,722	0,961
Sample 6	0,054	0,517	0,732	1,016
Mean value (OD)	0,051	0,519	0,684	0,932
Standard deviation (OD)	0,006	0,014	0,062	0,088
Standard deviation (%)	12%	3%	9%	9%

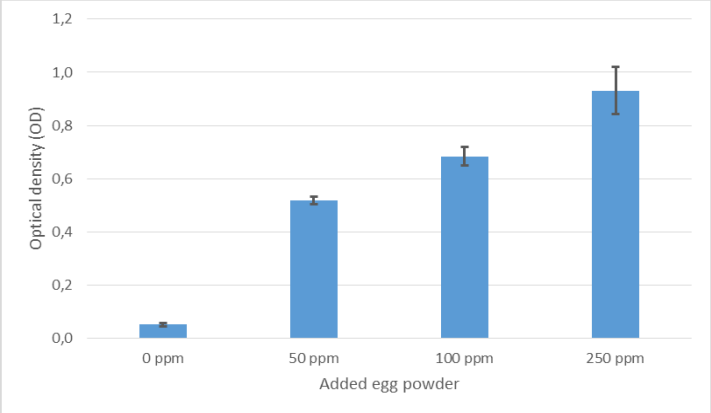

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Fig.1 : Distribution of OD for sauce with different concentrations of egg powder.

2. Spiked cookies

Table 6. Homogeneity testing for spiked cookies samples

Sample	Spiked cookies batch			
	0 ppm	50 ppm	100 ppm	250 ppm
Sample 1	0,056	0,380	0,486	0,965
Sample 2	0,056	0,336	0,533	0,713
Sample 3	0,059	0,367	0,469	0,713
Sample 4	0,055	0,346	0,511	0,763
Sample 5	0,058	0,347	0,514	0,779
Sample 6	0,060	0,334	0,499	0,758
Sample 7	0,059	0,352	0,541	0,719
Sample 8	0,057	0,379	0,530	0,741
Sample 9	0,056	0,377	0,528	0,701
Sample 10	0,054	0,374	0,755	0,743
Mean value (OD)	0,057	0,359	0,536	0,759
Standard deviation (OD)	0,002	0,017	0,0759	0,0724
Standard deviation (%)	3%	5%	14%	10%

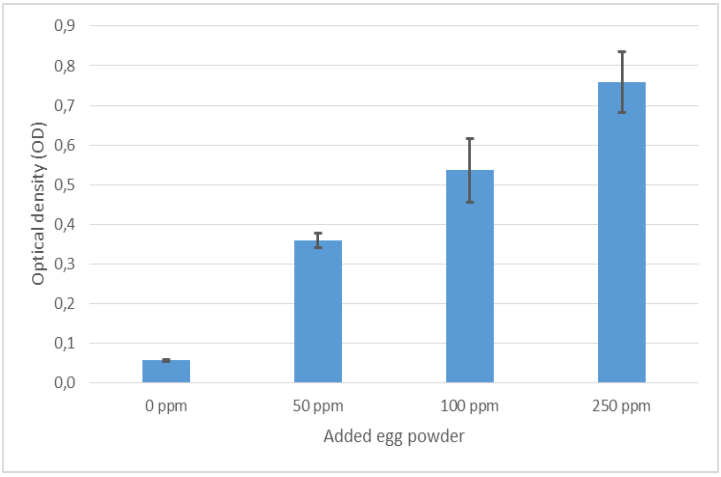

Fig.2 : Distribution of OD for spiked cookies with different concentrations of egg powder.

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3. Incurred cookies

Table 7. Homogeneity testing for incurred cookies samples

Sample	Incurred cookies batch			
	0 ppm	50 ppm	100 ppm	250 ppm
Sample 1	0,052	0,118	0,169	0,320
Sample 2	0,054	0,116	0,162	0,308
Sample 3	0,052	0,119	0,175	0,320
Sample 4	0,052	0,111	0,183	0,309
Sample 5	0,052	0,122	0,173	0,327
Sample 6	0,054	0,125	0,183	0,302
Sample 7	0,056	0,112	0,185	0,314
Sample 8	0,054	0,138	0,185	0,307
Sample 9	0,053	0,120	0,169	0,320
Sample 10	0,052	0,120	0,163	0,325
Mean value (OD)	0,053	0,120	0,174	0,315
Standard deviation (OD)	0,001	0,007	0,0085	0,008
Standard deviation (%)	3%	6%	5%	3%

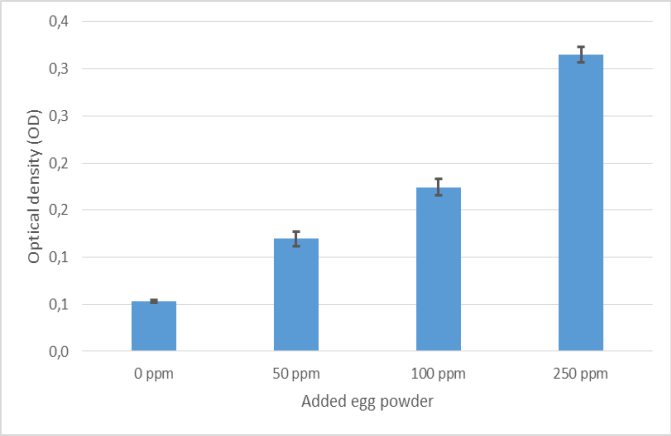

Fig.3 : Distribution of OD for incurred cookies with different concentrations of egg powder.

As shown in Tables 5, 6 and 7, all prepared sample batches (sauce and cookies) displayed good homogeneity with a global variation from sample to sample no more than 14%.

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Appendix 7. Stability study

The stability of egg was tested in each sample batch. The aim was to prove that egg is stable during shipment and storage in the validation conditions. The impact of freeze/thaw cycles on the detection of egg residues was evaluated by comparing values obtained after a single or after 3 freeze/thaw cycles (-20°C for 1h followed by 1h at room temperature). Two randomly chosen samples were tested with the CER kit for each batch (matrix type and contamination level). The samples are considered stable if the difference between 1 and 3 freeze/thaw cycles is below 16%.

Table 8. Stability testing after repeated freeze/thaw cycles

Matrix type	Contamination level	Sample ID	1 Freeze/thaw cycle (OD values)	3 Freeze/thaw cycle (OD values)
Sauce	0 ppm	sample 1	0,052	0,052
		sample2	0,053	0,080
	50 ppm	sample 1	0,384	0,378
		sample2	0,388	0,378
	100 ppm	sample 1	0,479	0,521
		sample2	0,530	0,526
	250 ppm	sample 1	0,680	0,677
		sample2	0,692	0,686
Spiked cookies	0 ppm	sample 1	0,067	0,064
		sample2	0,083	0,063
	50 ppm	sample 1	0,425	0,429
		sample2	0,410	0,432
	100 ppm	sample 1	0,606	0,655
		sample2	0,615	0,640
	250 ppm	sample 1	0,813	0,831
		sample2	0,822	0,903
Incurred cookies	0 ppm	sample 1	0,064	0,064
		sample2	0,062	0,060
	50 ppm	sample 1	0,124	0,135
		sample2	0,130	0,153
	100 ppm	sample 1	0,206	0,213
		sample2	0,204	0,195
	250 ppm	sample 1	0,349	0,351
		sample2	0,353	0,355

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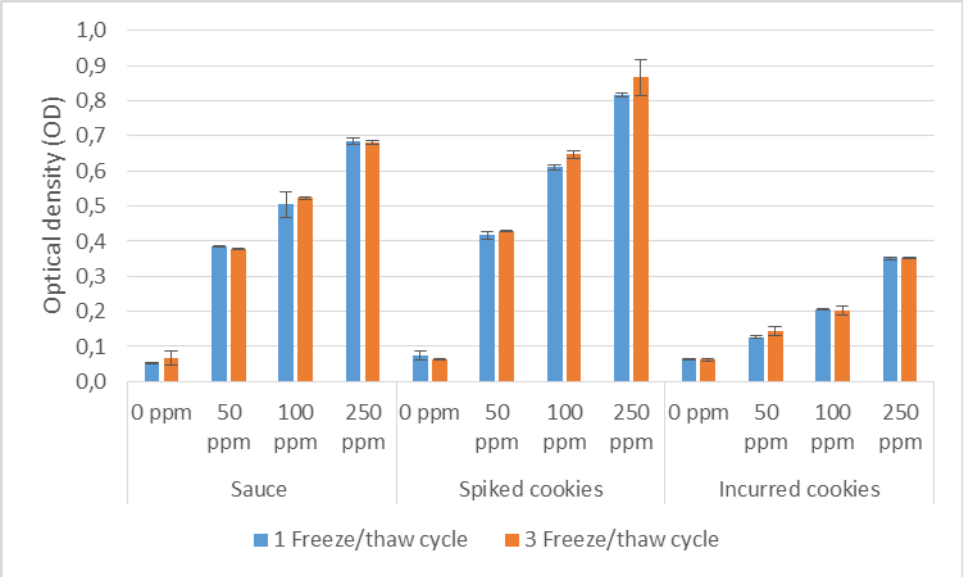

Fig.4 : Stability study, comparison of samples OD after several freeze/thaw cycles.

No significant differences were observed between the samples analyzed after one or three freeze/thaw cycles. The highest disparity is recorded for samples of spiked cookies (250 ppm) but the difference between the two samples is lower than 10% and has no statistical significance. This is indicating that the storage conditions had no impact on the samples.

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Appendix 8. Reporting template

Proficiency test : Detection of egg residues in cookies powder and sauce.

Reporting of Results +++ Deadline: December 15th, 2014 +++

Participating laboratory(*)	
Detection kit used	

(*) : to be completed

Qualitative results for cookies powder

Allergens	LOD (ng/g)	LOQ (ng/g)	identification (yes/no)			
			Sample ID .. - .. - …	Sample ID .. - .. - …	Sample ID .. - .. - …	Sample ID .. - .. - …
egg						

Qualitative results for sauce

Allergens	LOD (ng/g)	LOQ (ng/g)	identification (yes/no)			
			Sample ID .. - .. - …	Sample ID .. - .. - …	Sample ID .. - .. - …	
egg						

Date : ___/___/___
Name & Signature : _____

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