SAFEFOODERA

date

Project no.: 08125
Project acronym:
Project title: Detection of traces of allergens in foods

SAFEFOODERA Call 2008 topic:

Periodic activity report (1)

Period covered: December, 31. 2009 –June, 2011

Date of preparation:

Start date of project: March, 1st 2009 (to march, 1st 2011)
Duration: 24 months

Project coordinator name:
Jorge Martínez-Quesada. Dept. Immunology, Microbiology and Parasitology. Faculty of Pharmacy. University of Basque Country. Vitoria. Basque Country. Spain.

Project coordinator organisation name:
University of Basque Country. Basque Country. Spain.

Project participants

(1) Jorge Martínez-Quesada. Dept. Immunology, Microbiology and Parasitology.

Fac. of Pharmacy. University of Basque Country (UPV/EHU). Vitoria. Basque Country. Spain.

(2) Miguel Angel Pardo AZTI – Tecnalia / Food Research Division (AZTI).

Derio. Vizcaya. Basque Country. Sapin

(3) Dana Gabrovska Food Research Institute Prague (FRIP). Prague. Czech Republic.

(4) Kristiina Takkinen : VTT Technical Research Centre of Finland (VTT). Espoo. Finland

1 Report on deliverables and milestones

In Table 1 and Table 2 the Milestones and Deliverables, as formulated in the Description of Work, are listed. The final reports follow the “Guideline for final Reporting”.
Those Deliverables and Milestones not included in the original description of Work should be clearly marked as NEW.
Progress to be marked as:

- “Completed” for completed task. Details should be given on publications or other presentation of results in the column for Comments if applicable
- ”On track” for tasks still going on.
- “Delayed” for tasks that are not past their due date but are delayed. The reasons/corrective measures should be given in the column for Comments.
- “Not achieved” for tasks exciding their due date. The reasons/corrective measures should be given in the column for Comments.

Table 1: Milestone List

Item	Acitivity	Date due	Partner responsible	Progress	Comments
1	Project start-up	July, 1999	UPV/EHU, AZTI, FRIP, VTT	Completed	
2	Project start-up meeting	May, 1999	UPV/EHU	Completed	
3	Project Kick off meeting	April, 1999 (Amsterdam)	UPV/EHU	Completed	
4.1	Communication plan (1)	July 2010 (International Innovation Report)	UPV/EHU, AZTI, FRIP, VTT	Completed	Research Media Ltd. Economical Journal “Cinco Dias”. Publications
4.2	Communication plan (2)	July 2010 First published article	UPV/EHU, AZTI, FRIP, VTT	On going	Ring trial. Publication first paper (accepted two papers).
5	1st 6-month activities/results meeting	November, 1999	FRIP	Completed	
6	1st Status report (1-6 months)		UPV/EHU	Completed	
7	2nd 24-month activities/results meeting		ND		
8	Safefoodera midterm meeting	Juny 2010	UPV/EHU	Completed	
9	2nd Status report (6-12 months)	Juny, 2010	UPV/EHU	Completed	
10	3rd 18-month activities/results meeting		Tele-conference	Completed	
11	3rd Status report (12-18) months)	December, 2010	UPV/EHU	Completed	
12	Project finalization meeting	February, 2011	UPV/EHU		February, 2011 Publication

date

					second paper
13	Final report	March, 2011	UPV/EHU		
14	Final Communication Plan	March, 2011	AZTI, FRIP, VTT		June, 2011
15	Project end date	March, 2011	UPV/EHU, AZTI, FRIP, VTT		June, 2011

Table 1: Deliverable List

Delive-rable number	Deliverable name	Date due	Partner responsible	Progress	Comments
WP 1.	Establishment of homogeneous reference systems (allergens and detection methods)	01.01.10	VTT/AZTI/UPV-EHU/FRIP	Completed	
Task 1	Preparation IHR	01.09.09	UPV-EHU	Completed	
Task 2	Establishment validated commercial ELISA kits pool	31.12.09	FRIP	Completed	
WP 2.	Evaluation of food processing on allergen detectability	28.02.11	AZTI	Completed	
WP 3	Development of innovative methodologies for allergen traces detection		VTT/AZTI/UPV-EHU	Completed	
Task 1	Generation recombinant antibodies	28.02.11	VTT	Activity in progress	
Task 2	Optimization of sensitive immune detection systems	28.02.11	AZTI	Completed	
.Task 3	Detection of food allergen by allergen microarrays	28.02.11	UPV-EHU	Completed	
WP 4.	Validation of detection methods with a collaborative studies	28.02.11	FRIP	Completed	
WP 5.	Diffusion and exploitation of results	28.02.11	FRIP, VTT, AZTI, UPV-EHU	Activity in progress	

Key: ✓ - completed; Δ - reformulated; → - activity in progress;
≈ - activity ‘frozen’ (waiting to get started after request/activity from other WP)
fa – future activity

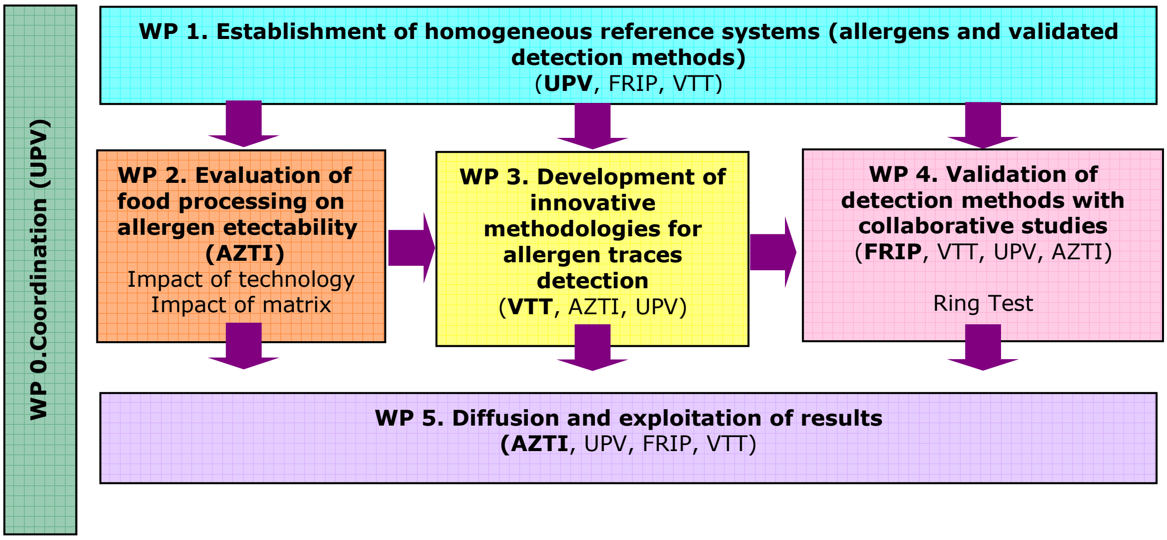
2 Problems/challenges/deviances

List changes within the participant group, unanticipated expenses/earnings etc and any other issues pertinent to progress not covered in the tables above.

3 Indications, preliminary conclusions and results

Account for indications, preliminary conclusions and results that have been revealed thus far in the project.

Organization chart of the project



WP1: **Establishment of homogeneous reference systems (allergens and validated detection methods)** Researcher Groups: UPV/AZTI.

<u>In-house references obtained form milk and *Anisakis simplex* row materials</u>:

Samples were standardized according to the rules proposed by: Larsen and Dreborg. (Standardization of allergen extracts. Methods Mol Med. 2008;138:133-45); Larenas-Linnemann, et al.(European allergen extract units and potency: review of available information. Ann Allergy Asthma Immunol. 2008 Feb;100(2):137-45.); van Ree et al. (The CREATE project: development of certified reference materials for allergenic products and validation of methods for their quantification. Allergy. 2008; 63(3):310-26.); Poulsen et al (Immunochemical and biological quantification of peanut extract. Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M. 2003;(94):97-105.); Akkerdaas et al. (In vitro and in vivo characterization of hazelnut skin prick test extracts. Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M. 2003;(94):87-95.) van Ree (The CREATE project: a new beginning of allergen standardization based on mass units of major allergens. Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M. 2003;(94):70-3.), Lowensteinn (Overview: biological standardization in Europe and the USA. Arb Paul Ehrlich Inst Bundesamt Sera Impfstoffe Frankf A M. 2003;(94):67-8.).

According to the above mentioned rules and procedures, native tropomiosin from shrimp, have been produced as in-house reference. The purification of this protein was carried out by immunoadsortion using polyclonal specific rabbit antiserum by immunization with cockroach recombinant tropomiosin (JA Asturias et al. Molecular characterization of American cockroach tropomiosin (*Periplaneta americana* Allergen 7), a cross-reactive allergen. Journal of Immunology. 1999; 162: 4342-4348). 2D electrophoresis and ELISA were used to characterize and quantify the tropomiosin respectively.

***WP1, WP4*: Validation of detection methods with collaborative studies.** All partners (FRIP, AZTI, VTT, UPV/EHU)

RING TRIAL

A) ELISA kit for determination of egg white proteins – Collaborative study.

The results of this ring trial have been published at Journal of AOAC International 2010, 93(6): 1923-1929

Gabrovská D[1]., Rysová J[1]., Hanák P.[1], Štumr F. [2],Plicka J[3]., Tomková K. [2], Dvorská P.[2], Cuhra P. [4], Kubík M. [4], Baršová S. [4], Karšulínová L. [4], Bulawová H. [5], Brychta J. [5], Iametti S.[6], Guisantes J.A.[7], Martínez J.[7], Suñen E.[7], Postigo I.[7], Takkinen K., [8], Laukkanen M.L. [8], Pardo M.A. [9], Baranda A.[9], Martínez de Marañon I.[9] Jimenez E. [9], Piknová L.[10], Langerholz T.[11], Čenčič A.[11]

1 – Food Research Institute Prague, Radiová 7, 102 31 Praha 10, Czech Republic
2 – SEDIUM RD, Ltd., Železničního pluku 1361, 530 02 Pardubice, Czech Republic
3 – Immunotech a Beckman Coulter Company, Radiová 1, 102 27 Praha 10, Czech Republic
4 – Czech Agriculture and Food Inspection Authority, Za Opravnou 300/6, 150 06 Praha 5, Czech Republic
5 – State Veterinary Institute, Rantířovská 93, 586 05 Jihlava, Czech Republic
6– Universitâ degli studi di Milano, DISMA, Via Celoria2, Milano, Italy
7 – University of the Basque Country, Faculty of Pharmacy, Paseo de la Universidad 7, Vitoria-Gasteiz, Basque Country, Spain
8 –VTT Technical Research Centre of Finland, P.O.Box 1000, Espoo, Finland
9 – AZTI Tecnalia, Technological Park of Biskay, Astondo bidea, Edif. 609, Derio, Basque Country, Spain
10 – Food Research Institute, Priemyselna 4, Bratislava, Slovakia
11- University of Maribor, Pivola 10, 2311 Hoče, Slovenia

Abstract

A collaborative study in 11 laboratories was performed to prove the validation of ELISA method developed for quantitative egg white proteins (EWP) determination in foods. The ELISA kit used for this study is based on sheep polyclonal antibody. This kit does not produce any false positive results or cross-reactivity with broad food matrix range with zero egg white proteins content. All participants obtained Egg ELISA kit-native kit with standard operational procedure, the list of samples, samples and a protocol for test results recording.

The study included 10 food samples (rice, mixture for yoghurt cake, farmer soup, egg pasta, 2 samples of red wine, mixture for potato dumplings, mixture for bread roll dumplings, mixture for pancake, pizza mixture and 6 flour mixtures.

Four samples of food matrix with zero EWP content showed EWP content lower than the first standard (EWP content 0.5 mg kg^{-1}). One sample of food matrix with zero EWP content revealed EWP content higher than standard 3 (1.5 mg EWP kg^{-1}). Five food samples containing EWP as an ingredient were tested as positive and one as negative. The statistical tests (Cochran, Dixon and Mandel) and analysis of variance (ANOVA) were used for the evaluation of the collaborative study results. Repeatability and reproducibility limits as well as a limit of quantification (LOQ, 0.28 mg EWP kg^{-1}) and a limit of detection (LOD, 0.08 mg EWP kg^{-1}) for the kit were calculated.

B) ELISA kit for mustard protein determination – Collaborative study.

The results of this ring trial have been published at Journal of AOAC International 2011, 94 (2): 605-610.

Gabrovská D[1]., Rysová J[1]., Hanák P.[1], Štumr F. [2],Plicka J[3]., Tomková K. [2], Dvorská P.[2], Cuhra P. [4], Kubík M. [4], Baršová S. [4], Karšulínová L. [4], Bulawová H. [5], Brychta J. [5], Malmheden Yman I.[6], Iametti S.[7], Guisantes J.A.[8], Martínez J.[8], Suñen E.[8], Postigo I.[8], Takkinen K., [9], Laukkanen M.L. [9], Pardo M.A. [10], Baranda A.[10], Martínez de Marañon I.[10],Jimenez E. [10], Piknová L.[11] Langerholz T.[12], Čenčič A.[12]

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9 –VTT Technical Research Centre of Finland, P.O.Box 1000, Espoo, Finland
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11 – Food Research Institute, Priemyselna 4, Bratislava, Slovakia
12- University of Maribor, Pivola 10, 2311 Hoče, Slovenia

Abstract

A collaborative study in 12 laboratories was performed to prove the validation of the ELISA method developed for quantitative mustard protein determination in foods. The ELISA kit used for this study is based on rabbit polyclonal antibody. This kit does not produce any false positive results or cross-reactivity with a broad food matrix range with zero mustard protein content. All participants obtained the Mustard ELISA kit with standard operational procedures, the list of samples, samples and a protocol for recording test results.
The study included 15 food samples and 2 spiked samples. Seven food matrix samples of zero mustard content and four samples with mustard declared as an ingredient showed mustard protein content lower than the first standard (mustard protein content of 0.42 mg kg^{-1}). Four samples with mustard declared as an ingredient revealed mustard protein content above 12.5 mg kg^{-1} (the highest standard). The statistical tests (Cochran, Dixon and Mandel) and analysis of variance (ANOVA) were used for the evaluation of the collaborative study results. Repeatability and reproducibility limits as well as a limit of quantification (LOQ, 0.15 mg mustard proteins/kg) and a limit of detection (LOD, 0.06 mg mustard proteins/kg) for the kit were calculated.

C) ELISA kit for gliadin protein determination – Collaborative study.

An interlaboratory study with 5 participants was performed to obtain validation and performance data for an enzyme-linked immunosorbent assay (ELISA) kit developed for quantitative gluten determination in foods. The ELISA kit used for this study is based on 2 monoclonal and 1 polyclonal antibody developed by Immunotech, a Beckman Coulter Co. This kit did not show any false positive results or cross-reactivity with oat, rice, maize, and buckwheat. The gliadin standard from the Working Group on Prolamin Analysis and Toxicity was included in the kit as reference material for calibration. All participants obtained a gliadin ELISA kit with Standard Operational Procedures. The study included 13 samples labeled as "gluten-free" and 2 samples spiked by wheat flour.

List of the samples

1. Gluten-free rolls, 2. Cereal mini bread, 3. Gingerbread in chocolate, 4. Croissant with chocolate cream, 5. Gluten-free pretzels, 6. Gluten-free mixture dark, 7. Gluten-free mix for cakes, fruit pie, 8. Gluten-free mix, 9. Gluten-free mix for sunflower bread, 10. Gluten-free mix for bread, 11. Gluten-free mix for muffins, 12. Spiked sample, 13. Spiked sample, 757. Buckwheat, 758. Millet

Gliadin ELISA kit

Standard

Gliadin standard.—Prepared and characterized by the Working Group on Prolamin Analysis and Toxicity used for preparation of calibration solutions.

Chemicals

Gliadin ELISA kit.—Ref. IM3717. The format of the newly developed ELISA kit for gluten determination in food products and raw materials is a 2-step sandwich assay based on 2 monoclonal antibody clones, 7C6 and 8D4, for detecting peptide motifs from α-gliadin, γ-gliadin, and ω-secalin (Immunotech, a Beckman Coulter Co., Fullerton, CA) used for solid-phase coating and signal conjugate of polyclonal antibody with horse radish peroxidase from Sigma (Part No.A 1052, St. Louis, MO). This polyclonal antibody forms a suitable pair with developed monoclonal antibodies. The protein applied for mice immunization was crude gliadin from wheat gluten (Sigma Part No. G3375).

The kit contains:

(**a**) *Calibration solutions*.—Six 2 mL vials (lyophilized). After reconstitution, the vials contain concentrations of gliadin in a range from 0 to 300 ng/mL in buffer with bovine serum albumin; the exact concentration is indicated on each vial label. Add 2 mL of 10% ethanol into the vial containing lyophilized calibration substance and mix carefully. After reconstitution, solutions must be stored frozen.

(**b**) *Dilution buffer*.—Two 125 mL vials (concentrate); the solution must be diluted before use by addition of 14 mL of 96% ethanol directly into the original vial.

(**c**) *Wash solution* (*20x*).—One 50 mL vial (concentrate). The solution must be diluted before use: pour 25 mL of
concentrate into 475 mL distilled water and mix; the diluted wash solution can be stored at 2–8°C.

(**d**) *Conjugate concentrate* (*49x*).—One 0.6 mL vial (concentrate). For instance, the amount sufficient for 6 strips will be prepared by addition of 250 μL of concentrate into the vial for the diluted conjugate and then by addition of 2 mL of conjugate diluents; the conjugate working solution must be used on the day of analysis.

(**e**) *Conjugate diluent*.—One 24 mL vial (ready-to-use).

(**f**) *Tetramethylbenzidine (TMB) substrate*.—Two 12 mL vials (ready-to-use).

(**g**) *Stop solution*.—One 0.6 mL vial (ready-to-use); 2 M hydrochloric acid.

Chemicals not included in the kit:

(**a**) *Ethanol*.—96% p.a. or ethanol denatured with methanol, up to 10% (v/v); the use of industrial spirit or
ethanol denatured with other additives (petrol, pyridine) is not suitable.

(**b**) *Distilled or deionized water, or equivalent*.—18.2 Megohm·cm resistivity.

Sample Treatment and Extraction Procedure

The sample (1.00 g) was weighted into a suitable vial with a stopper, and 10 mL of 40% ethanol was added. The sample was shaken using a Vortex mixer for 1 min and immediately centrifuged at 1800 *g* for 15 min. The supernatants were diluted 100 x with dilution buffer before the assay.

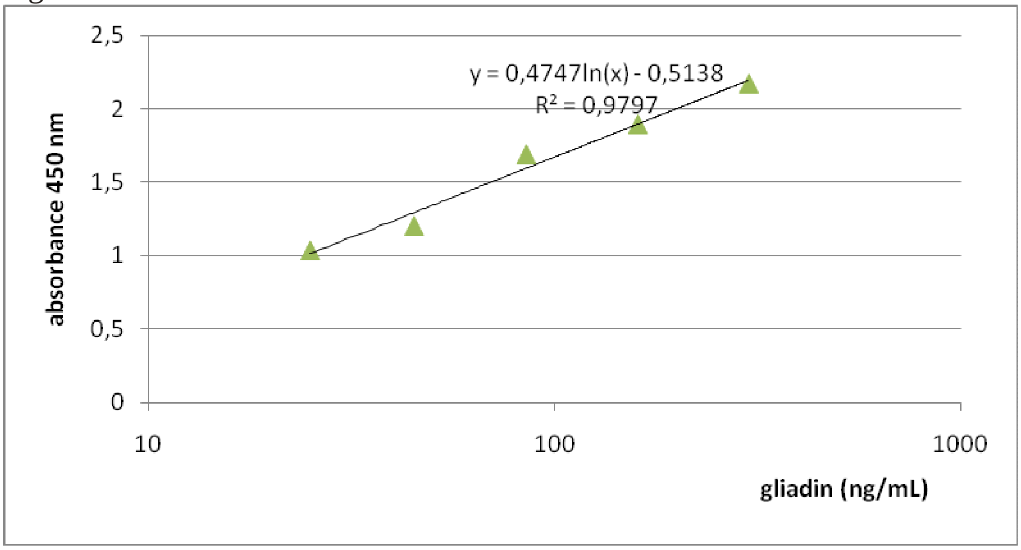
Determination

The calibration solution or diluted sample (200 mL) was added into appropriate wells. The strip holder was covered and incubated for 1 h at room temperature without shaking. The contents of all wells were washed with 350 mL of wash solution 4 times using the washer or repeating dispenser. Conjugate working solution (200 mL) was immediately dispensed into all wells. Dispensing was finished in 2 min. The strip holder was covered and incubated for 2 h at room temperature without shaking. The contents of all wells were washed with 350 mL of wash solution 4 times using the washer or repeating dispenser. TMB substrate (200 mL) was immediately dispensed into all the wells. Dispensing was finished in 2 min. The plate was incubated for 10 min in the dark without shaking; 50 mL stop solution was added and shaken briefly, and within 20 min the reading was made at 450 nm.

Calculations

The results were calculated using Excel software.

Figure 1 Calibration curve



Results and discussion

The results are showed in Tables 1 (a, b) and 2 (a, b). They show a very good agreement at the samples from 1 to 10 and sample 757. A few discrepancies were found at the samples 11, 12, 13 and 758 probably due to a sample exchange.

Table 1a Absorbencies received from participants

Participant	sample No.	1	2	3	4	5	6	7
FRIP	1st day	0,245	0,212	0,181	0,230	0,314	0,183	0,663
		0,219	0,201	0,226	0,196	0,324	0,186	0,668
	2nd day	0,335	0,315	0,275	0,264	0,380	0,285	0,648
		0,413	0,346	0,261	0,282	0,590	0,288	0,688
VTT Finland	1st day	0,275	0,307	0,535	0,188	0,236	0,173	0,360
		0,318	0,471	0,479	0,216	0,253	0,207	0,374
	2nd day							
Participant	sample No.	1	2	3	4	5	6	7
UBC Vitoria	1st day	0,216	0,163	0,477	0,127	0,199	0,126	0,432
		0,156	0,122	0,773	0,158	0,225	0,134	0,382
	2nd day	0,140	0,163	0,157	0,117	0,177	0,120	0,313
		0,150	0,144	0,165	0,116	0,171	0,119	0,303
AZTI	1st day	0,231	0,264	0,411	0,309	0,240	0,178	1,056
		0,244	0,277	0,402	0,323	0,221	0,192	1,158
University	1st day	0,148	0,274	0,770	0,335	0,196	0,159	1,855
Maribor		0,254	0,138	0,414	0,194	0,221	0,136	2,686
	2nd day	0,182	0,220	0,248	0,290	0,218	0,358	1,217
		0,164	0,145	0,128	0,467	0,226	0,136	1,064

Table 1b Absorbencies received from the participants

Participant	Sample No.	8	9	10	11	12	13	757	758
FRIP	1st day	0,234	0,359	1,716	0,243	3,068	3,056	0,981	0,987
		0,184	0,351	1,567	0,202	3,012	2,963	0,937	1,233
	2nd day	0,280	0,293	1,470	0,314	3,072	2,670	0,983	0,963
		0,300	0,388	1,504	0,319	3,009	2,867	0,944	1,102
VTT Finland	1st day	0,202	0,321	1,044	0,360	3,112	2,319	0,215	0,311
		0,343	0,540	1,028	0,316	3,230	2,613	0,382	0,421
	2nd day								
UBC Vitoria	1st day	0,150	0,203	0,817	0,229	2,607	2,569	0,144	0,172
		0,148	0,208	0,940	0,344	2,130	2,871	0,240	0,257
	2nd day	0,127	0,178	0,890	0,252	2,526	2,391	0,249	0,120
		0,119	0,164	0,874	0,353	2,703	2,346	0,264	0,222
AZTI	1st day	0,169	0,226	0,436	0,208	0,227	0,242	0,183	3,270
		0,179	0,233	0,425	0,269	0,279	0,351	0,254	3,240
University Maribor	1st day	1,072	0,177	0,660	2,605	1,975	1,616	0,293	0,474
		0,639	0,269	0,672	2,312	1,966	1,402	0,665	0,622
	2nd day	0,682	0,182	1,020	2,308	2,137	1,818	0,160	0,252
		0,490	0,247	0,802	2,723	2,011	2,014	0,156	0,318

Table 2a. Results of gluten content from participating laboratories

Participant	Sample No.	1	2	3	4	5	6	7
FRIP	1st day	3,2	3,0	3,1	3,1	3,6	2,9	6,3
		<st	<st	<st	<st	<st	<st	<st
	2nd day	3,2	2,9	2,6	2,5	3,1	2,6	5,8
		<st	<st	<st	<st	<st	<st	<st
VTT Finland	1st day	17,90	21,65	21,86	15,78	16,52	15,60	19,19
		<st	<st	<st	<st	<st	<st	<st
	2nd day							
UBC Vitoria	1st day	10,8	10,9	11,0	10,3	11,3	10,3	14,1
		<st	<st	<st	<st	<st	<st	<st
	2nd day	14,0	13,3	24,5	13,3	14,5	13,1	18,6
		<st	<st	<st	<st	<st	<st	<st
AZTI	1st day	15,4	16,1	19,0	17,0	15,3	14,5	45,2
		<st	<st	<st	<st	<st	<st	<st
	2nd							
University Maribor	1st day	15,1	15,2	27,7	16,7	15,3	13,9	371,5
		<st	<st	<st	<st	<st	<st	<st
	2nd day	11,4	11,6	11,7	16,5	12,5	13,0	65,0
		<st	<st	<st	<st	<st	<st	<st

Table 2b Results of gluten content from participating laboratories

participant	sample No.	8	9	10	11	12	13	757	758
FRIP	1st day	3,0	3,8	30,2	3,2	288,4	269,5	10,9	12,0
		<st	<st		<st	>STD	>STD	<st	<st
	2nd day	2,6	2,9	32,6	2,9	871,3	482,2	5,5	4,3
		<st	<st		<st	>STD	>STD	<st	<st
VTT Finland	1st day	18,47	23,58	43,19	17,86	663,18	308,50	19,38	20,34
		<st	<st	<st	<st	>STD		<st	<st
	2nd day								
UBC Vitoria	1st day	10,4	11,2	36,6	14,0	650,1	432,0	12,9	11,2
		<st	<st	<st	<st	>STD	>STD	<st	<st
	2nd day	13,4	14,4	33,8	16,0	224,5	350,7	14,2	14,6
		<st	<st	<st	<st			<st	<st
AZTI	1st day	14,3	15,3	19,6	15,4	15,7	16,6	15,1	641,7
		<st	<st	<st	<st	<st	<st	<st	>STD
Participant	sample No.	8	9	10	11	12	13	757	758
University Maribor	1st day	41,6	15,7	31,1	496,9	233,5	114,4	23,3	25,9
		<st	<st	<st				<st	<st
	2nd day	24,0	12,3	43,0	768,4	347,7	261,7	11,1	14,0
		<st	<st	<st	>STD			<st	<st

***WP2*: Evaluation of Food Processing on Allergen Detectability** (AZTI)

Extract preparation and treatments:

Allergen extracts were prepared mixing a determined and constant concentration of β-lactoglobulin (2.5 mg/mL, isolated and provided by the University of the Basque Country) with other ingredients such as, lactose, glucose, sucrose, casein, ovomucoid, starch, and gelatine, in buffers at two different pH (4.6 and 6.8) to study interactions of the components during the treatments.

Heat treatments were performed in a water bath in hermetically sealed glass capillars (20 µL samples) to assure good heat transfer. Treatments were carried out at 75 and 90 ºC for 30, 60 and 90 seconds respectively.

For High Pressure, polystyrene bags with 300 µL of the correspondent sample were treated at 450 and 600 MPa for 100 and 300 seconds at 25 ºC (under pressure).

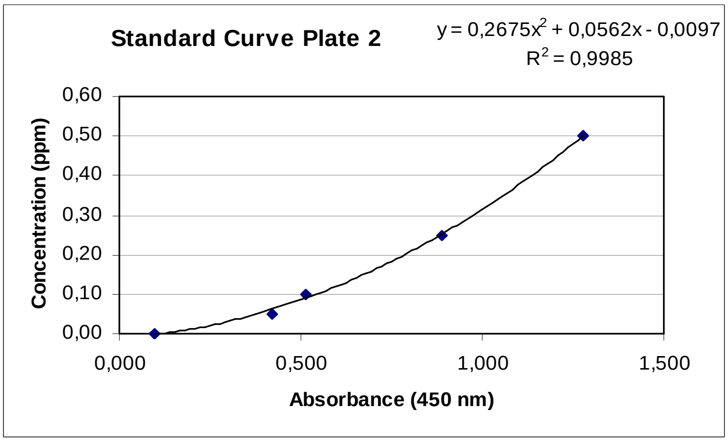
Allergen Detection:

For detection and quantification of β-lactoglobulin, commercial ELISA kits (ZEU-Inmunotec, Zaragoza, Spain), containing coated plates, extraction and washing buffers, standard solutions, antibody-enzyme conjugates, enzyme substrate and stopping solution were used.

All the treatments were carried out by duplicate, to test repeatability of the results, and each sample was analysed by triplicate.

A volume of 100 µl per well of standard or diluted sample were added to an ELISA plate coated with antibodies against β-lactoglobulin, and incubated for 30 min at room temperature. The plate was washed five times with 300 µl/well of washing buffer composed by PBS containing 0.05% Tween (PBST) and then, 100 µl/ well of a solution of peroxidase labelled antibodies was added and incubated for 30 min at room temperature. The plate was washed again and 100 µl/well of a solution of 3, 3', 5, 5'-tetramethylbenzidine (TMB) was added and incubated for 30 min at room temperature. The reaction was stopped by adding 50 µl/ well of stopping solution containing 1M sulphuric acid and the absorbance of wells was measured at 450 nm.

The calibration standard solutions were assayed simultaneously with the food extracts. The average absorbance of triplicate wells was used for the calculation. For β-lactoglobulin sandwich test, calibration curves were obtained using the relationship between the value of absorbance and the concentration of standard solutions. The concentration of β-lactoglobulin detected in the test samples was calculated using the correspondent calibration curves:



Results and Discussion:

As every sample contained originally the same concentration of β-lactoglobulin, after treatment, the detected concentration for each sample was represented in relation to the concentration of untreated sample (100% of detection). In this way, values of detection over 100% indicated that treatment enhances the detection, while values under 100% indicated that the treatment has a negative effect on the detection of the protein.

The codifications for the samples in the figures are the following:

1.- 2.5 mg/mLβ-lactoglobulin, pH 6.8
2.- β-lactoglobulin + Lactose, pH 6.8
3.- β-lactoglobulin + Sucrose, pH 6.8
4.- β-lactoglobulin + Glucose, pH 6.8
5.- β-lactoglobulin + Fructose, pH 6.8
6.- β-lactoglobulin + Starch, pH 6.8
7.- β-lactoglobulin + Gelatine, pH 6.8
8.- β-lactoglobulin + Ovomucoid, pH 6.8
9.- β-lactoglobulin + Casein, pH 6.8
10.- 2.5 mg/mLβ-lactoglobulin, pH 4.6
11.- β -lactoglobulin + Lactose, pH 4.6
12.- β-lactoglobulin + Sucrose, pH 4.6
13.- β-lactoglobulin + Glucose, pH 4.6
14.- β-lactoglobulin + Fructose, pH 4.6
15.- β-lactoglobulin + Starch, pH 4.6
16.- β-lactoglobulin + Gelatine, pH 4.6

As we can see in the figures 1 and 2, results obtained in this work indicate that the quantitative determination of β-lactoglobulin, in processed foods depends on the intensity of heat processing and pressure treatment applied and food matrix (components and pH) in the conditions studied in this work.

Figure 1. Effect of Thermal Treatment (temperature and time) on the detection of β-LG by ELISA Sandwich.

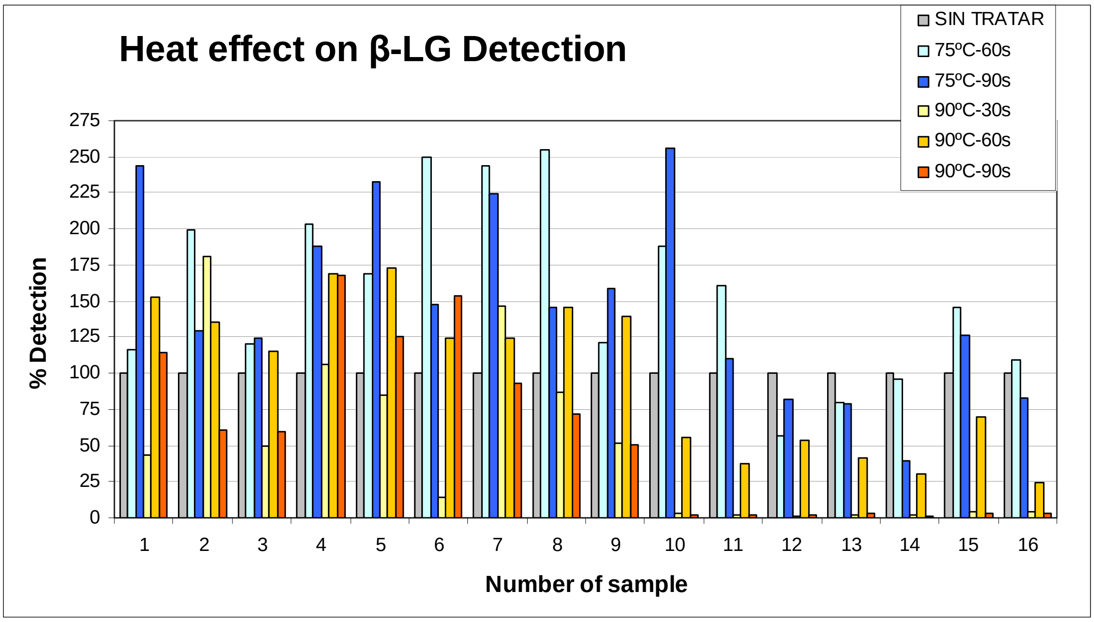
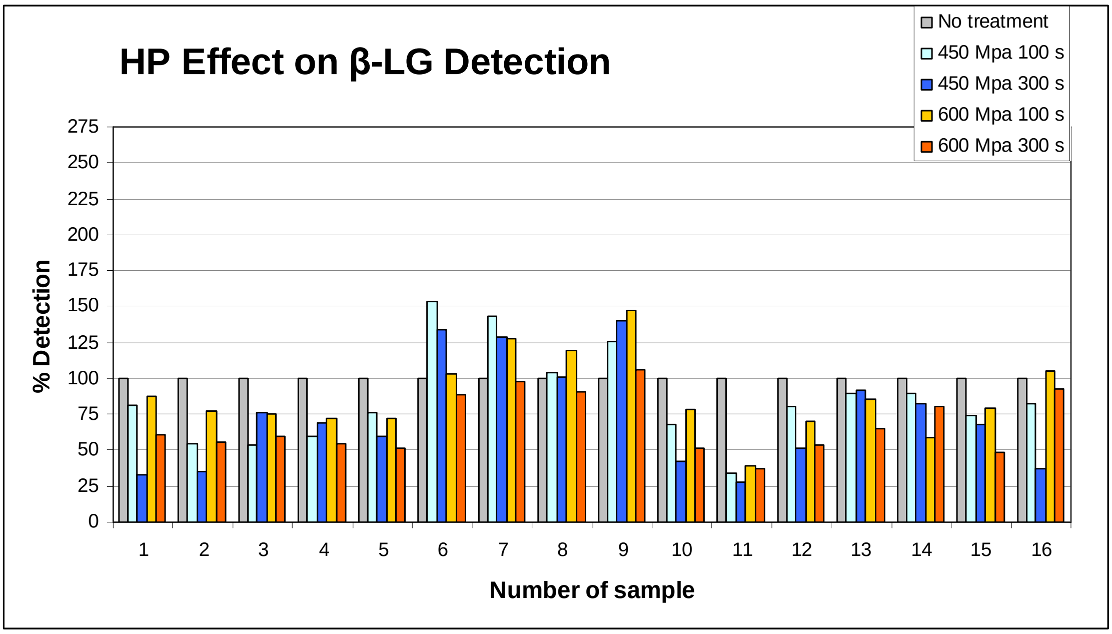


Figure 2. Effect of High Pressure Treatment (Pressure and time) on the detection of β-LG by ELISA sandwich



In general, at pH 6.8, β-LG is more resistant to heat than to high pressure, but macromolecules, as starch, gelatine or other proteins, protect β-LG against high pressure effect (Figure 1, samples 6, 7, 8 and 9). As reported in previous studies, β-LG is very pressure sensitive in proportion to its small size and globular structure. Upon pressure treatment,

conformational changes occur resulting in solvent exposure of tryptophan residues and increased activity of the free thiol group on Cys121, which can react easily with other groups. This reactivity could enhance the formation of complexes with other proteins or polysaccharides, and could explain the protective effect when these macromolecules are present in treatment medium. At pH 4.6 this reaction is not as favourable as at pH 6.8, and we cannot see the cited effect (Figure 1, samples 15 and 16). At pH 4.6 however, we can see more reactivity in samples added with monosaccharides (glucose and fructose, Figure 1, samples 13 and 14), than at pH 6.8 (samples 4 and 5). This fact has already been observed by other authors, who indicated that a high pressure treatment can enhance the formation of Maillad's reaction products which may have more allergenicity that the β-LG by itself.
Heat treatments studied enhances β-lactoglobulin detection at pH 6.8 (Figure 2). This fact may be due to the release of epitopes when the structure of the protein starts the denaturation process. However, at 90ºC and pH 4.6 denaturation of β-LG is more intense.

Conclusions:

These findings confirm our hypothesis, and underline the fact that the determination of allergenic proteins in food products is greatly influenced by the particularities of ELISA format used as well as by processing conditions applied to food products. These considerations should be taken into account for a correct interpretation of results obtained when using different immunoassays to detect allergens in foods.

As in this study, detection of allergens by ELISA is frequently performed by IgG, which recognise conformational epitopes. This kind of epitopes are more easily destroyed by treatments, but linear epitopes (recognised by IgE, responsible of the allergenic response) may remain undistorted. Thus, our results indicates that this method of detection may lead to a great variability on obtained results, thus, its important to develop more reliable and optimised methods of detection (WP3, in this project). This fact becomes more important since the high-pressure technology has recently become of increased commercial interest, and consumers can find in the market more and more products treated by this technology. However, detection test are usually validated against heat treatment and, as demonstrated, β-LG is more sensitive to pressure treatment, thus methods used currently could lead to false negative test in some food samples, and represent a risk for sensitive consumers.

WP 3. Development of innovative methodologies for allergen traces detection (AZTI).

During this term we have immunized a number of rabbits with *Anisakis simplex* extracts and recombinant AniS3 allergen. After a period of time of 3-4 months the rabbits were bled and their spleens were removed. The total RNA was isolated and checked their integrity by electrophoresis. This RNA has been used as template for cDNA synthesis. To obtain this cDNA we have successfully optimised the PCR conditions with the final aim of obtaining a representative population of VL and VH fragments. In fact, the first-strand cDNA has been used as template for PCR amplification of V gene sequences (VH and VL). The PCR products has been restricted and ligated into the appropriate sites in a phage display vector pSD3 (provided by Kevin C. Gough). The recombinant phagemid has been electroporated into methylase deficient cells (JM110 *E. coli)* to create and scFv library.

WP 3. Development of innovative methodologies for allergen traces detection (VTT Technical Research Centre of Finland). Task 1.

Generation of recombinant antibodies

In the SAFEFOODERA project VTT Research Centre of Finland has focused on the milk and egg allergies to develop recombinant IgE antibodies and allergens as well as optimize and validate immunoassay formats for specific and sensitive detection of trace amounts of allergens in food.

A human IgE scFv library from a clinically validated, voluntary egg-allergic patient was constructed. The blood donor for the construction of IgE library had very high allergen-specific IgE levels for several different allergens including Gal d 1 (ovomucoid) and Gal d 2 (ovalbumin) as determined by Phadia ISAC. The scFv-κ and scFv-λ library with the sizes of 9.62×10^6 (κ) and 4.05×10^5 (λ) clones and background level of 0.76 and 5.9 %, respectively, were constructed.

Recombinant IgE antibodies specific to the major egg allergen, ovalbumin and ovomucoid, were selected by panning procedures. The library selections were carried out in solution using biotinylated allergens. The enrichment of the ovalbumin and ovomucoid specific phages was already seen after first and second selection round as studied by phage ELISA. However, the isolated clones, regardless of which allergens were used in the selection, bind to ovomucoid. It is known that the commercial ovalbumin contains ovomucoid as an impurity. Interestingly, this fact might have lead to an overestimation of dominance of ovalbumin as the major egg allergen in literature. To overcome the ovomucoid contamination problem we have modified the phage display selection protocol as well as the recombinant Gal d 2 will be used as a selection antigen.

Selected, ovomucoid-specific IgE scFv fragments were converted to Fab fragments with the human IgG heavy and lambda light chain constant domains to avoid potential aggregation problems of the scFv fragment and, thus, to achieve more efficient purification yields. The IgE Fab fragments were produced in *E.coli* expression system and chromatographically purified in a large scale. The binding properties such as affinity and specificity of the purified IgE Fab fragments were characterised in detail. The binding kinetics of the Fab fragments has been analyzed by BIAcore. The preliminary results indicate a strong binding to ovomucoid ($K_D \sim 10^{-9}$M) which is typical to IgE antibodies. Sensitivity of the ovomucoid detection in a sandwich-type assay with the resulting IgE antibodies will be evaluated.

In addition to the development of recombinant IgE antibodies also IgG antibodies will be isolated from phage display libraries constructed from mice immunized with ovalbumin or beta-lactoglobulin, one of the major allergens of cow’s milk. The performance of the IgG antibodies in combination with the IgE antibodies in the optimization of a sandwich immunoassay for the sensitive detection of ovalbumin or beta-lactoglobulin allergens will be evaluated.

WP 3. Development of innovative methodologies for allergen traces detection (Dept. Immunology, Microbiology and Parasitology. University of the Basque Country). Task 3.

***Detection of food allergen by allergen microarrays. Validation of allergen detection by ISAC-inhibition*.**

Standardisation of the inhibition-enzymoimmunoassay by microarray for the quantification of allergens from plant sources.

The individualization of allergenic components has led to the development of new diagnostic tools based on the component-resolved diagnosis. Microarrays are biotechnological devices based on molecular biology and used in the diagnostics of the IgE mediated hypersensitivity. This multiplex platform allows the identification of the allergenic molecule implicated in the allergenic reaction in an easy way. The use of competitive inhibition and microarrays technology enables the detection of trace of allergens in foods. This technology have been used for quantification of allergens from plant sources in samples of food. For the standardization of the microarray-inhibition assay the most critical points to consider in establishing the inhibition test were the sample processing and the level of IgE present in the reference serum used in the assay. The best results were obtained by non-aggressive extraction processes (aqueous extractions) of the allergenic source for a period not exceeding two hours. When the level of the specific IgE in the serum was more than 5 ISU, the inhibition assays were able to detect concentrations of inhibitor allergens ranging 1-5 ng/ml. The development of this technology allows the detection of trace of allergens belonging to different allergenic source as egg, milk or vegetables in one unique multiparametric step.

Figure 1. Influence of the titer serum in the inhibition assay results

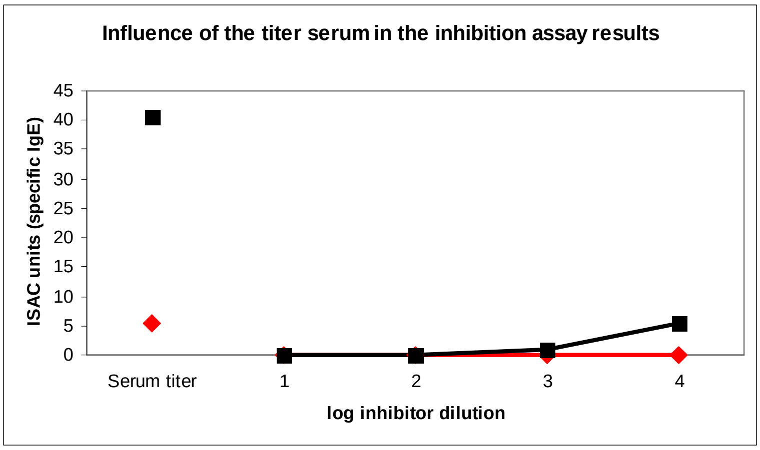
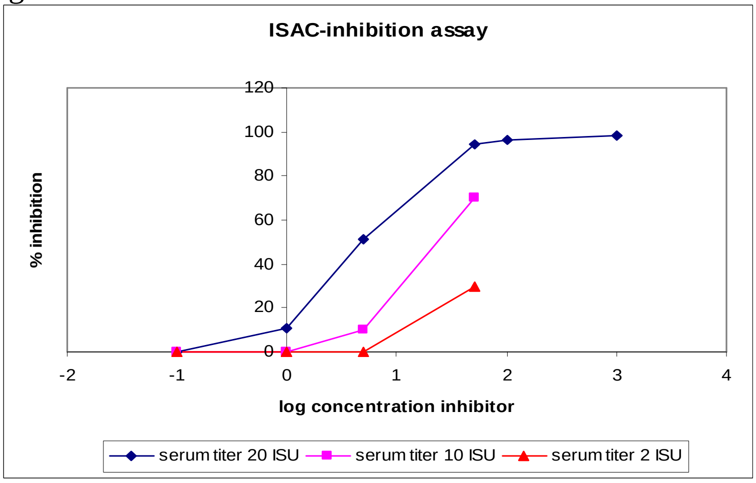


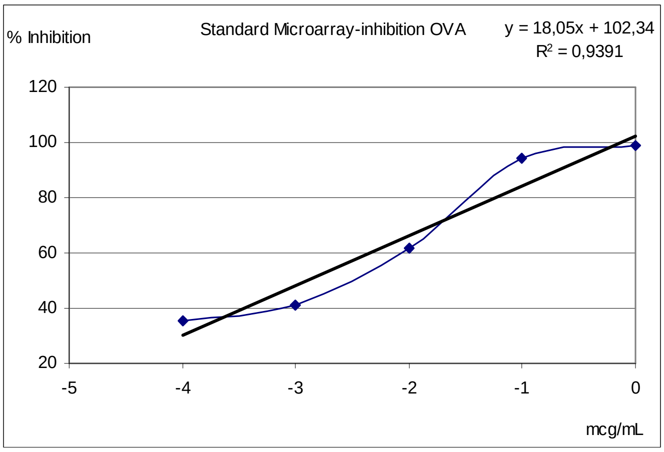
Figure 2. ISAC-inhibition results



Detection of Gal d 1 (ovoalbumin) in food samples by microarray-inhibition: Validation of the assay and comparison with ELISA results.

For the validation of the assay a standard microarray-inhibition curve was made with pure ovoalbumin (SIGMA) at different inhibitory concentrations ranging from 100 ng/mL to 0.1 ng/mL (Figure 1), as was standardised before.

Figure 1. Standard microarray-inhibition curve for ovoalbumin (OVA)



To measure the levels of ovoalbumin in the food samples (Table 1) they were extracted in PBS during 2 h, then centrifugated and incubated with the reference sera (25ISU to Gal d 1). The IgE was measured by microarray following the microarray manufacture's guidelines (ISAC-ImmunoCap). The results were compared with the results obtained by the ELISA validated in the ring trial. The analysed samples were the same ones that were used in the ELISA (golden standard). The results are showed at Table 1.

Table 1. ELISA-Microarray-inhibition comparative data

Sample list	ELISA mg EWP/kg sample	Microarray ng OVA/g sample	Agreement with ELISA
Pancake mixture	2,180	>Standard	Yes
Pizza mixture	>Standard	>Standard	Yes
Potato dumpling mixture	<Standard	<Standard	Yes
Rice	<Standard	<Standard	Yes
Bread roll mixture	42,300	<Standard	No
Red wine	<Standard	<Standard	Yes
Red wine	<Standard	<Standard	Yes
Yoghurt cake mixture	<Standard	<Standard	Yes
Flour mixture 1	6,290	0,15	Yes
Flour mixture 2	1,820	0,15	Yes
Flour mixture 3	7,980	<Standard	No
Flour mixture 4	0,800	0,15	Yes
Flour mixture 5	<Standard	<Standard	Yes
Flour mixture 6	<Standard	<Standard	Yes

The lowest concentration of OVA that could be detected by microarray-inhibition was 1 ng/g of sample and the highest was 100 ng/g of sample (Figure 1). The results showed in Table 1 demonstrate that there was a good correlation (based on agreement with detection/no detection of the allergen) between microarray-inhibition assay and the gold standard (ELISA). Only two false negative results were obtained with the microarray-inhibition assay.

Detection of Gal d 1 (ovoalbumin)and Gal d 2 (ovomucoid) in food samples by Fluoroenzimoimmunoassay-inhibition (FEIA-inhibition): Validation of the assay and comparison with ELISA results

For the validation of the assay standard FEIA-inhibition (ImmunoCap) curves were made with pure ovoalbumin (SIGMA) and pure ovomucoid at different inhibitory concentrations ranging from 100 mcg/mL to 0.1 mcg/mL (Figure 1 and 2), as was standardised before.

Figure 1. Standard FEIA-inhibition curve for ovoalbumin (OVA)

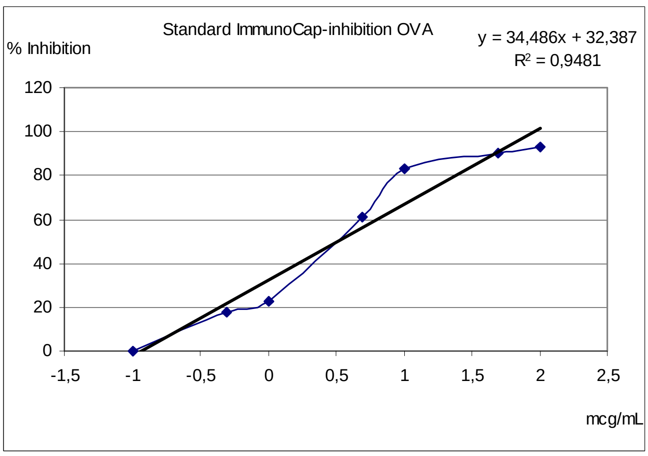
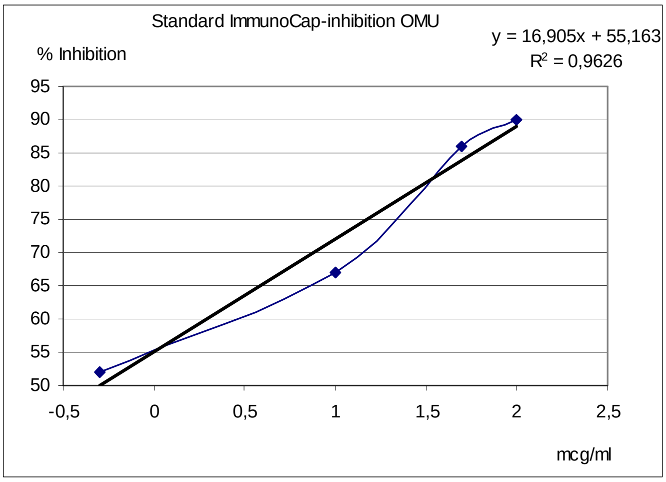


Figure 2. Standard FEIA-inhibition curve for ovomucoid (OMU)



To measure the levels of ovoalbumin and ovomucoid in the food samples (Table 1) they were extracted in PBS during 2 h, then centrifugated and incubated with the reference sera (100 kU/L of specific IgE to Gal d 1 and to Gald 2). The IgE was measured by the authomatic system ImmunoCap 100 following the manufacture's guidelines. The results were compared with the results obtained by the ELISA validated in the ring trial. The analysed samples were the same ones that were used in the ELISA (golden standard). The results are showed at Table 1.

Sample list	ELISA mg EWP/kg sample	FEIA-inhibition mcg OVA/g of sample	FEIA-inhibition mcg OMU/g of sample	Agreement with ELISA
Pancake mixture	2,180	<0,5	<0,5	Yes
Pizza mixture	>Standard	>100	>100	Yes
Potato dumpling mixture	<Standard	<0,5	<0,5	Yes
Rice	<Standard	<0,5	<0,5	Yes
Bread roll mixture	42,300	52,00	35,62	Yes
Red wine	<Standard	<0,5	<0,5	Yes
Red wine	<Standard	<0,5	<0,5	Yes
Yoghurt cake mixture	<Standard	<0,5	<0,5	Yes
Flour mixture 1	6,290	9,4	40,2	Yes
Flour mixture 2	1,820	0,11	0,15	Yes
Flour mixture 3	7,980	9,95	13,8	Yes
Flour mixture 4	0,800	<0,5	<0,5	Yes
Flour mixture 5	<Standard	<0,5	<0,5	Yes
Flour mixture 6	<Standard	<0,5	<0,5	Yes

The lowest concentration of OVA and OMU that could be detected by FEIA-inhibition was 0.5 mcg/g of sample and the highest was 100 mcg/g of sample (Figure 1). The results showed in Table 1 demonstrate that there was a full correlation (based on agreement with detection/no detection of the allergen) between FEIA-inhibition assay and the gold standard (ELISA).

These two methods provide an advantage over the ELISA because through the microarray and FEIA-inhibition we can measure in foods the presence in only one assay of the allergenic molecules Gal d 1 (OVA) or Gal d 2 (OMU) independently.

***WP5*:** Activity in progress

Communications to the RAFA Congress in Prague (Nov, 2009) of the two following works:

1. ELISA kit for mustard protein determination – Collaborative study
Petr Cuhra[a], Dana Gabrovská[b], Jana Rysová[b], Petr Hanák[b], František Štumr F[c], Jan Plicka[d], Květa Tomková[c], Pavla Dvorská[c], Martin Kubík[a], Soňa Baršová[a], Lenka Karšulínová[a], Hana Bulawová [e], Josef Brychta[e], Ingrid Malmheden Yman[f], Stefania Iametti[g], Jorge A.Guisantes Del Barco[h], Jorge Martínez Quesada[h], Esther Suñen Pardo [h], Idoia Postigo Resa[h], Kristiina Takkinen[i], Marja-Leena Laukkanen[i], Miguel A.Pardo[j], Ana Baranda[j], Iñigo Martínez de Marañon [j], Elisa Jimenez[j], Lubica Piknová[k] Tomaz Langerholc[l], Avrelija Čenčič[l]

2. ELISA kit for determination of egg white proteins – Collaborative study
Gabrovská D[1]., Rysová J[1]., Hanák P.[1], Štumr F. [2],Plicka J[3]., Tomková K. [2], Dvorská P.[2], , Cuhra P. [4], Kubík M. [4], Baršová S. [4], Karšulínová L. [4], Bulawová H. [5], Brychta J. [5], Iametti S.[6], Guisantes J.A.[7], Martínez J.[7], Suñen E.[7], Postigo I.[7], Takkinen K., [8], Laukkanen M.L. [8], Pardo M.A. [9], Baranda A.[9], Martínez de Marañon I.[9] Jimenez E. [9], Piknová L.[10], Langerholz T.[11], Čenčič A.[11]

Both were sent to the Journal of AOAC international, to be submitted for its publication and the two papers has been published:

3. Tomková el al. ELISA kit for detection of egg white proteins: Interlaboratory Study. Journal of AOAC International 2010, 93(6): 1923-1929.

4. Cuhra et al. ELISA Kit for Mustard Protein Determination: Interlaboratory Study. Journal of AOAC International 2011, 94(2): 605-610

5*. I.* Postigo et al .Assessment of inhibition assays in microarrays platforms to measure individual allergenic components in foods. Euro-Mediterranean Symposium “Fruit &Veg Processing”. Avignon, France. April, 2011.

4. Other comments/suggestions

The reports appeared in International Innovation edited by Research Media Ltd and in Cinco Dias (A Journal for the Economy and Business areas) were sent previously.

5. Person months used.

Partner organisation	Contact person	Person month so far
UPV/EHU	Jorge Martinez	2
AZTI	Miguel Angel Pardo	2
FRIP	Dana Gabrovska	2
VTT	Kristiina Takkinen	2