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Comparison of commercial allergen ELISA kits for egg detection in food matrices

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ABSTRACT

Consumption of low levels of egg already can evoke harmful physiological responses in humans in those allergic to eggs. By detection of egg in food products, using Egg ELISA kits to determine its unintended presence, food producers can respond to avoid potential safety or quality risks of their products. Selection of an ELISA kit fit for the issue at hand is challenging due to, amongst others, lack of information on assay performances with specified matrices. In this study, performances of seven commercial egg ELISA kits are compared for nine different relevant matrices: cookie, chocolate, pasta, dressing, stock cube, wine, vegetable drink and milk, ice cream and meat/meat replacers. The presence of egg was unified for all ELISA kits to mg total egg protein kg^{-1} food product. In every matrix, kit performances for recovery, intra- and interassay were compared, and also processing is accounted for by determination of egg in incurred samples. All seven kits were able to detect egg qualitatively at the VITAL3 ED01 level of 0.2 mg total egg protein and the corresponding relevant portion size for each matrix. For quantitative results, each ELISA kit showed an increase in detected egg concentration with increased egg levels and performed within the set criteria for recovery for the cookie, chocolate, stock cube and wine. For pasta, vegetable drink and milk, ice cream, and salad dressing, recovery of egg was within the set criteria for at least 4 ELISA kits. Most challenging matrices were meat/meat replacers, showing high matrix effects which could not be explained by the possible egg presence in the cognate blank. Only one ELISA kit was able to recover egg within the set criteria for the meat/meat replacer matrix. Results enable food industry to choose for ELISA kits suitable for egg detection in the matrix of interest.

1. Introduction

Food allergies are a global health burden to the human populations, and their prevalence is rising. Currently, in Western countries, over 10% challenge-diagnosed food allergy has been reported in the population [1,2]. Allergy to egg contributes significantly to these allergenic reactions [3], and although regional differences in prevalence of food allergenic ingredients can be seen, egg allergy presents a global priority allergen [4]. For patients suffering from egg allergy, promising preliminary results with oral immunotherapy are

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obtained [5–7], however avoidance of egg in the diet still is the common treatment [8]. In Europe, it is mandatory for food business operators to label regulated allergen ingredients on the product in prepacked and unpacked foods, so consumers can make informed decisions about their diet [9]. In practice, however, unintended presence of allergens by, for instance, cross contamination during storage, transport or processing of food is possible [10]. To inform consumers about this possible risk, food producers can apply precautionary allergen labelling (PAL). The use of PAL, however, is controversial, as it reduces food choices for the allergic consumers without knowledge on the actual presence of the allergens [11]. In 74% of the food products with a PAL statement, the particular allergen in PAL does not necessary correlate with the undeclared allergens detected [12]. A proportion of allergic consumers are therefore known to ignore PAL, to broaden the range of products that they can consume. This however leads to incidences of reactions, in a Canadian survey of 1454 food-allergic consumers, up to 8% have reported allergic reactions after ingestion of PAL products [13]. First steps to ensure appropriate, consistent, risk-based application of PAL were taken by the Allergen Bureau of Australia and New Zealand, which started with an allergen management program named VITAL (Voluntary Incidental Trace Allergen Labeling). In VITAL, data on the eliciting dose (ED), i.e. the dose of the protein of the respective allergenic source that triggered an objective reaction in allergenic consumers, are collected from multiple sources and reviewed. For egg, the ED01, meaning the ED at which 1% of the egg allergic individuals are predicted to have an objective reaction, is set at 0.2 mg egg protein, and the ED05 at 2.3 mg egg protein [14]. Subsequently, the reference dose (RfD) set by the Joint FAO/WHO expert consultation is 2.0 mg egg protein [15]. These values are suggested to be used as risk management threshold values to determine if PAL is needed. Next steps to move towards a global harmonized risk-based approach to use of PAL are regulations at the European, or even the global level. For this an important step is harmonization on reference doses. At the moment discussions on these reference doses are ongoing, noting that there has been a recommendation from a Joint FAO/WHO expert consultation on reference doses for selected global priority allergens, which are based on ED05 values [16,17]. Next to this, specific, sensitive and reliable methods are needed for the detection of the presence of allergens in food items. Currently, for egg detection, ELISA is the most used technique [18], but also LC-MS/MS is developed and applied [19]. Next to ELISA, food business operators use lateral flow immunochromatography (LFIC) for detection of point of need in the factory environment (e.g. swabbing of surfaces) [20]. Although there is a broad choice of commercially available ELISA test kits, selection of an ELISA fit to the issue at hand is challenging [20]. By employing these ELISA test kits, difficulties on detection and quantification remain. For instance details on how the obtained results relate to total protein from the allergic source (in mg), or the lack of information on assay performances with specified matrices.

This study aimed to evaluate the performance of commercial available egg ELISA kits for relevant food matrix groups. Matrix groups were selected based on i) recommended matrixes to be included by AOAC [21], ii) challenging matrixes and iii) matrixes in which cross contaminations has been reported. Consequently, cookies, chocolate, pasta, wine, stock cubes, dressing, vegetable drink and milk, ice cream and meat/meat replaces were selected as matrix group. A comparison study was performed in these nine food matrixes using seven commercial available egg ELISA kits. Results were compared to LC-MS/MS analysis.

2. Experimental

2.1. Materials

Commonly used, commercially available ELISA test kits, for detection of whole egg or, if not available, egg white were purchased from the respective suppliers. These included: Spray-dried whole egg for allergen detection 8445 (NIST, MD, USA), 3 M™ Egg White protein Elisa kit (E96EGG, 3 M, MN, USA), Egg Elisa kit (EOM-EK-96, Biofront, FL, USA), Egg (Ovalbumin) Elisa kit II (M2111, Morinaga Japan), Veratox® for Egg (8450, Neogen UK), AgraQuant® plus egg (COKAL1848F), Romer, Austria), Ridascreen® egg (R6411, R-Biopharm, Germany), Enhanced egg residue kit (ESEGGPR-48, Elisasystems, Australia).

Matrix groups were selected on expected matrix effects and there relevance to egg being present and included the following nine groups: bakery products, chocolate, pasta, dressing, stock cube, vegetable drink and milk products, ice creams, wine and meat/meat-replacers. For every matrix group ten blank materials were selected. Blank materials were supplied by Unilever and Danone or selected from the local supermarket in the Netherlands. The criteria used to select the blank materials were: egg not present on the ingredient list of the product and, if possible, a precautionary allergen statement for egg being absent. Moreover, for every matrix group, when possible, also two incurred samples, i.e. samples with egg on their ingredient list were selected. The selected blank and incurred samples are presented in Supplementary information Table S1.

2.2. Expression egg content in food

A common reporting unit for allergens is encouraged, and most appropriate is to express allergen content of food products as mg total protein [22]. When applied to egg content expressions, these would be: mg total egg protein for the absolute dose and mg total egg protein per kg food product for concentrations [14,18,22,23]. Results from the Morinaga ELISA kit are already expressed in mg total egg protein per kg food product. For the other used ELISA kits, a conversion factor was needed. The conversion factors used were according to manufacturers' protocols and in line with described composition of egg [24]. The 3 M ELISA kit measures total egg white protein, and obtained results are, therefore, multiplied by a factor 2.0 to express the results in mg total egg protein per kg food product. For Biofront, Elisa Systems, Neogen, R-Biopharm and Romer results are expressed per whole dried egg or whole egg powder. Here, the obtained results are multiplied by a factor of 0.4805 to express the results in mg total egg protein per kg food product. Obtained results of each kit were converted to mg egg protein per kg product to allow comparison. Furthermore, in the course of this study a selection of samples were benchmarked against LC-MS/MS.

2.3. Spiking sample materials

2.3.1. Criteria used for selection of spike levels

For each matrix group, two different blank products were spiked. Three different levels were selected based on i) expected performances of the commercial ELISA kits used, and ii) relevant levels according to VITAL 3.0. To determine the lowest spiking level, the LODs as provided by the test kit manufacturers were transferred to mg egg protein/kg food product and compared as shown in Table 1.

Based on these results, 1 mg total egg protein per kg food product was chosen as lowest spike level. The chosen intermediate level was 3x this lowest level, i.e. 3 mg total egg protein per kg food product, which is in line with suggested spike levels in literature [25]. The highest level chosen was 10 mg total egg protein per kg food product. This level is still within the calibration curves of the commercial ELISA kits, with exception of the Elisa systems with an ULOQ of 5- and R-Biopharm kit with an ULOQ of 1 mg egg protein kg^{-1}. The selected spike levels in relation to the calibration curves of the commercial ELISA kits are shown in Supplemental Figure S1.

2.3.2. Preparation certified reference material for spiking purposes

To spike blank matrices, certified reference material sprayed dried whole egg was used. After acclimatization to room temperature, a stock solution of 100 mg mL^{-1} spray-dried whole egg powder (NIST 8445) in PBS was prepared. The stock solution was further diluted in PBS, to a final concentration enabling a blank sample material to diluted certified reference material ratio of 99.5 : 0.5 (m/v). Ensuring the same ratio for all spiked samples prepared.

Solid matrices. Solid matrices first were grounded to a fine powder. The diluted certified reference spray-dried whole egg material was distributed with a single-channel pipette over the grounded sample. Then, it was given a rest period for 30 min. Water was added, and a slurry was prepared using a blender. Samples were freeze dried and when fully dried, grinded again.

Emulsions. The diluted certified reference egg material was mixed in a mortar 1 : 1 (m/v) with the respective emulsion. To this mix, a similar amount of emulsion was added and again mixed in a mortar. This step was repeated until the total amount of emulsion was added. Finally, the emulsion was mixed, using a hand mixer for another 15 min.

Chocolate. Half of the total amount of chocolate (200g) was melted au-bain-marie in a water bath at 60 °C. The certified reference egg material was distributed over the chocolate and mixed for approximately 5 min. Then small pieces of the remaining chocolate were added to the chocolate mix, melted and mixed thoroughly with a hand mixer before adding a new piece. This was repeated until the remaining half was added in total. When totally mixed, the chocolate was frozen at −80 °C and grinded under liquid nitrogen using a blender.

Liquids. The total amount of liquid was poured into a beaker and stirred using a magnetic stirrer. When the liquid movement was stable, the certified reference egg material was slowly added to the matrix and mixed for another 10 min.

2.4. ELISA procedure

Each sample was analysed in duplicate (2 wells per sample) with each of the seven ELISA test kits, using the prescribed procedure following the respective manufacturer manual. No dilution steps were executed with exception of the samples that were analysed using the R-Biopharm kit, since for this kit it was clear beforehand that the sensitivity of the kit would not allow the measurement of the 3- and 10 mg egg protein per kg product spiked samples. Consequently, a 3-times dilution step was applied for the 3 mg egg protein per kg product spiked samples and a 10 times dilution for the 10 mg egg protein per kg product spiked samples.

2.5. LC-MS/MS analysis

Samples were analysed according to an in house method based on CEN17644:2022 [26] by Mérieux NutriSciences R&D (Resana, Italy). In brief, proteins were extracted, unfolded, denaturated, reduced, alkylated, enzymatic digested and subsequently solid phase extraction (SPE) purified. For food matrices with a fat content >10%, first a defatting step with hexane was executed. For protein extraction, samples were diluted and mixed by vortex and mechanical shaker using a neutral solution (Tris/urea/OGS). For subsequent unfolding, reduction and alkylation, samples were mixed with TCEP, and MMTS added and incubated. Samples then were trypsinated under mechanical shaking for 14–16 h at 37 °C. After trypsination samples were purified and concentrated by SPE with an C18-type stationary phase on a silica-supported column. For quantification a matrix matched calibration curve was prepared in the range of ~0,

Table 1
Expression of the limit of detection (LOD) of each egg ELISA kit in mg total egg protein/kg food product and the upper limit of quantification (ULOQ) based on the highest point in the calibration curve using the provided manufacturer information from each kit.

Kit brand	Measurand	LOD (ppm)	Conversion factor	LOD after conversion (mg egg protein kg^{-1})	ULOQ after conversion (mg egg protein kg^{-1})
3 M	Egg white	0.21	2.0	0.42	204
Biofront	Whole egg	0.3	0.4805	0.14	38
Elisasystems	NI[a]	NI[a]	0.4805	NI[a]	5
Morinaga	Egg protein	0.31	–	0.31	50
Neogen	Whole egg	0.6	0.4805	0.29	12
R-Biopharm	Whole egg	0.096	0.4805	0.046	1
Romer	Whole egg	0.5	0.4805	0.24	12

[a] Not indicated (NI).

4 - 4 μg/ml using the certified standard reference material whole egg powder from NIST (SRM 1845a). Analysis were performed using liquid chromatography (UHPLC, Infinity II 1290 Agilent Technologies) connected to a mass spectrometer (triple quadrupole analyzer, Sciex QTrap 6500+) with electrospray source (ESI) in positive mode and multiple reaction mode (MRM) acquisition. For egg, 3 MRM transitions of 4 peptides of ovalbumin were targetted, GGLEPINFQTAADQAR, ELINSWVESQTNGIIR, LYAEER and VYLPR respectively. An example of LC-MS/MS chromatogram obtained is shown in Supplemental Figure S2.

2.6. Statistics and calculations

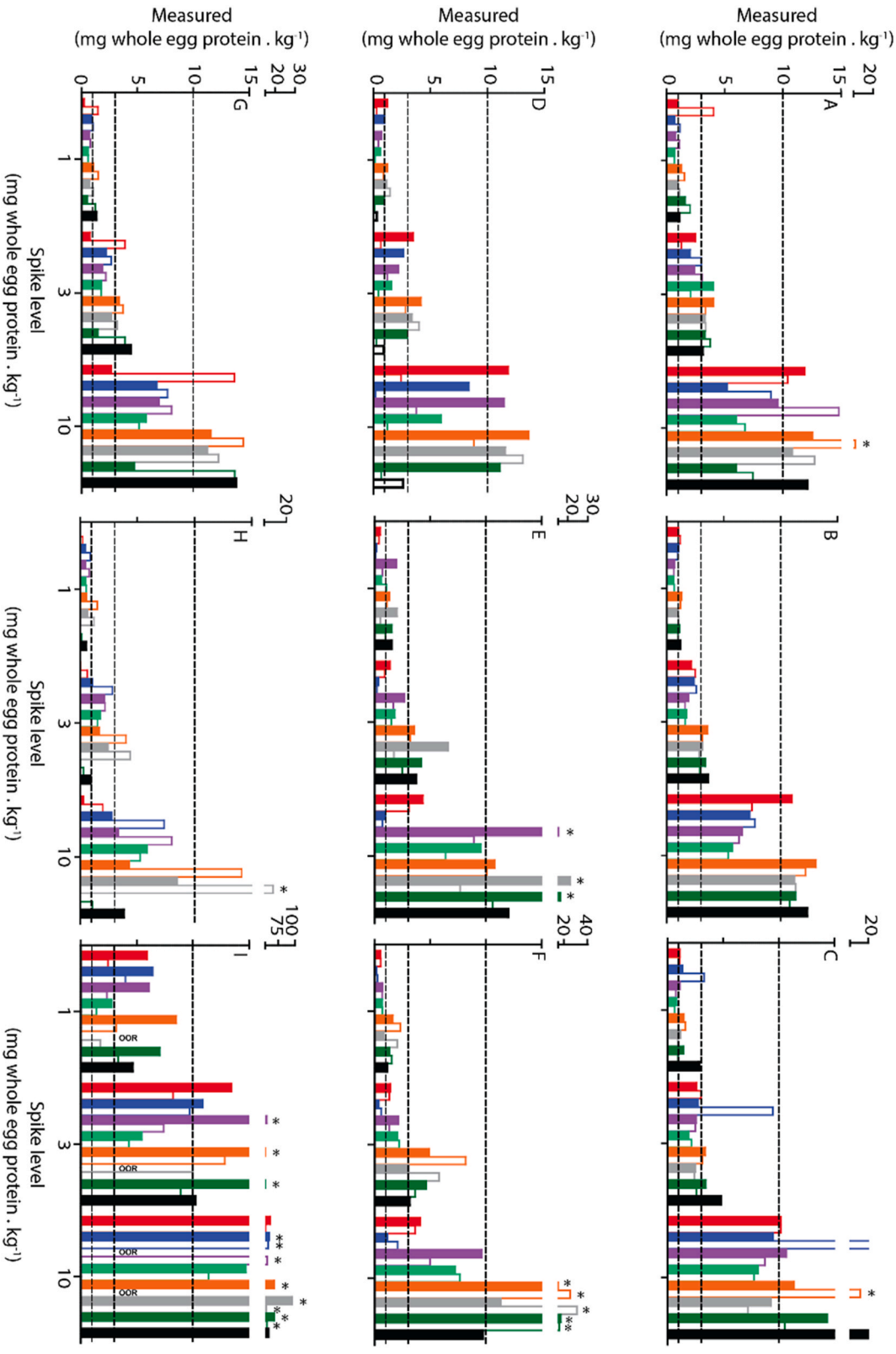
Sample homogeneity. Homogeneity was determined for the 10 mg total egg protein kg^{-1} food product spike level in both products of each matrix group (18 products in total). This level was chosen based on the high amount of protein, and the higher likeliness of protein-protein interactions or aggregation. The highest spike level would be the most difficult one to get homogeneous. The assumption was made that if we can reach homogeneity with the high spike level, homogeneity is likely to be reached also at the lower levels. Homogeneity was determined following The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [27] and ISO 13528 [28]. The between sample standard deviation (S_S) was compared with the standard deviation for proficiency assessment (σ_P), which is 25% of the grand mean of the homogeneity data [29]. The 10 mg total egg protein per kg food product spiked sample material was considered adequately homogeneous if $S_S < 0.3\ \sigma_P$. Ten samples were taken from each matrix, spiked with 10 mg total egg protein per kg food product and analysed in duplicate with a randomly chosen egg ELISA kit from the available kits. Which kit was used for which matrix is presented in Supplemental Table S2. For materials that were found not homogeneous, new materials were prepared until homogeneous, before start of the evaluation of the performance of the seven ELISA kits.

Performances commercial ELISA kits. Performance criteria were tested for all seven commercial ELISA kits with all matrices. Acceptance criteria described by Godefroy et al. were applied [30]. Determined method performance criteria were: i) recovery; ii) Limit of detection (LOD); iii) intra-assay variation, iv) inter-assay variation. Recovery is defined as: the percentage of mg total egg protein that is recovered after analysis of the spiked sample using the method of interest. The acceptance range is 50–150% [31]. LOD is defined as: The lowest concentration of total egg protein that can be distinguished by the respective test kit from a true blank sample. LOD determination was performed by analysing ten blank samples for each matrix group, followed by using a basic formula according to Abbot et al. [21]: $LOD = \overline{x} + 3\ SD$. For determining intra-assay variation, two randomly chosen sample materials, each spiked with 3 mg total egg protein kg^{-1} food product, assuming this spike level could be detected by all ELISA kits, were analysed in 8 fold on a single ELISA plate. Results were evaluated for their variability within the plate: $Intra-assay\ variation = \frac{SD}{\overline{x}} * 100\%$. For determining inter-assay variation, seven sample materials (1x cookie, 2x stock cube, 1x dressing, 2x wine and 1xmilk) spiked with 3 mg total egg protein kg^{-1} food product were analysed in duplo with each ELISA kit on seven different occasions. Results were evaluated for their variability between plates: $Inter-assay\ variation = \frac{SD}{\overline{x}} * 100\%$.

Determination of relevant total egg protein detection concentrations per *matrix.* As no harmonization on eliciting doses is set in current European legislation, the VITAL 3.0 ED01 level of 0.2 mg for total egg protein [14] was used for calculating the action levels per matrix. The portion sizes as described by Birot et al. [31] were used to calculate the action levels per matrix as shown in Table 2. These

Table 2
Action levels for detection of mg total egg protein kg^{-1} food product for different types of matrices using the VITAL3.0 ED01 reference dose of 0.2 mg total egg protein and portion sizes according to Birot et al. [31].

Food matrix group	Food matrix specification (no egg)	Portion size (g)	Food matrix group description Birot et al. [31]	Reference dose total egg protein (mg)	Action level (mg total egg protein kg^{-1} food product)
Cookie	Dough unprocessed Processed, Speculoos	42	Cookies (biscuits)	0.2	4.76
Chocolate	Creamy milk chocolate Dark chocoloate 70%	40	Chocolate and chocolate products	0.2	5.00
Pasta	Pasta (uncooked) macaroni Pasta (cooked) gnocchie	200	pasta	0.2	1.00
Wine	Red wine, Mesta tempranillo 2019 White wine, Pinot Grigio 2019	282.5	Alcoholic drinks, alcohol <15%	0.2	0.71
Vegetabledrink and milk products	Infant Formula Soy drink	317.5	Milk and milk products for drinking	0.2	0.63
Ice creams	Magnum Vegan Lemon sorbet	100	Ice cream	0.2	2.00
Stock cube	Bouillon powder vegetable – without yeast Concentrated bouillon, bone broth chicken	20	Herbs and spices mixes, bouillong cubes, yeast extracts	0.2	10.00
Salad dressing	Mayo vegan, Hellman Natural dressing salata	75	Sauces, savory, chutneys and pickles	0.2	2.67
Meat/meat replacers	Minced meat (vegan) Minced meat (beef)	113	Meal replacements and meat imitates	0.2	1.77



(caption on next page)

Fig. 1. Determined mg whole egg protein kg^{-1} food product in matrices: cookie (A), chocolate (B), pasta (C), wine (D), vegetable drink and milk I, ice cream (F), stock cube (G), dressing (H) and meat/meat replacers (I) for the tested Elisa kits 3 M (●), Biofront (●), Elisasystems (●), Morinaga (●), Neogen (●), R-Biopharm (●), Romer (●) and LC-MS/MS (●). Per matrix group, two products are spiked and tested (■, □), only with MS one of these matrix groups is tested. Obtained raw data are, if needed, transferred to whole egg protein to harmonize the expression of the results and enable comparison of the ELISA kits. (*) Interpolated concentrations above ULOQ for the respective ELISA kit, (OOR) out of range measurements (maximum absorbance in read out) for the respective ELISA kit.

matrix relevant action levels were used for spike level selection for presentation of recovery.

Incurred samples. For all matrices, with the exception of wine, products with egg on the ingredient list were selected as presented in the Supplementary information Table S3. Incurred samples were measured only to verify the ability of each kit to detect the presence of egg after possible alterations of the egg proteins due to processing.

3.

3.1. Sample total egg protein concentration

The determined total egg protein concentrations in the different food matrixes for all tested ELISA kits, in combination with the LC-MS/MS measurements, are shown in Fig. 1 (data are presented in Suplemental Table S4). For cookies, chocolate, stock cube, pasta, meat/meat replacers all ELISA kits were able to detect the presence of egg from the lowest spike level of 1 mg total egg protein kg^{-1} food product onwards. However, the matrix does affect the outcome of the detected concentration of egg, for instance represented by

Table 3
Average recovery of egg protein from fortified sample materials (n = 2) expressed in percentage egg protein weight. Data obtained without any sample dilutions with exception of the R-Biopharm ELISA kit. Green cells represent values that meet the recovery criteria according to Ref. [30]. For red cells these criteria are not met and blue cells represent values of samples which are diluted prior to measurement, as without dilution no results would have been obtained.

Matrix	Matrix relevant action level (mg egg protein . kg^{-1})	Nearest spike level (mg egg protein . kg^{-1})	3M (%)	Biofront (%)	Elisasystems (%)	Morinaga (%)	Neogen (%)	R-Biopharm (%)	Romer (%)
Cookie	4.76	3	109	84	90	101	123	111	118
Chocolate	5.0	3	77	83	58	56	112	101	104
Pasta	1.0	1	109	229	93	71	152	104	117
Wine	0.71	1	122	98	73	60	123	117	94
Veg drink and milk	0.63	1	43	13	132	81	121	124	126
Ice cream	2.0	3	45	15	57	70	217	148	137
Stock cube	10.0	10	82	72	75	55	130	118	92
Salad dressing	2.67	3	11	65	72	55	94	114	5
Meat/ meat replacers	1.77	1	242	399	233	140	319	175	333

egg concentrations analysed for spiked cookie and dressing (respectively A and H, Fig. 1), where for all ELISA kits analysed egg concentrations in cookie are higher than in dressing, although spiked with the same egg concentration. Even within the same matrix group, differences are found in egg concentration when analysing 2 different blank materials spiked with the same concentrations of egg as shown by the open and closed bars in Fig. 1. Analysis in the matrices dressing, vegetable drink and milk, wine and ice cream showed more often underestimation of the spiked egg concentration. For one dressing 2 ELISA kits showed not to be able to detect the presence of egg. On the other hand an overestimation of egg presence in meat/meat replacers is seen for all ELISA kits.

3.2. Performance of the test kits

Recoveries of the spiked levels, expressed in total egg protein kg^{-1} food product, are shown in Supplemental Table S5 for all matrixes in all tested kits. Given the relevant portion sizes (for each specific matrix group P75 is selected, which represents the portion size which is in 75% greater than the data obtained from all portion sizes) [31], and the VITAL3.0 ED01 for egg (0.2 mg), spiked levels which are nearest to these concentrations are presented in Table 3. For most kits, the recovery of egg protein per matrix was within the acceptable range of 50–150% [30], as shown by the green color in Table 3.

The Limit of Detection (LOD) and intra- and inter-assay variations are shown in Table 4. Godefroy et al. [30] describes performance criteria for the relative standard deviations to be ≤ 20 and $\leq 30\%$ for, respectively, intra- and inter-assay variation. For intra-assay variation this criteria was met for all kits with exception of Romer and R-Biopharm. It should, however, be mentioned that only 2 matrices were tested and for both kits one of the two matrices showed intra-assay results within the set criteria. For the inter-assay variation, the criteria was met for all kits, with exception of the 3 M and Biofront kit. The 3 M kit showed relative high inter-assay variation for 5 out of the 7 matrices, and for Biofront the criteria was not met for 2 out of the 7 matrices.

3.3. Incurred samples

With exception of wine, we were able to obtain and test incurred samples of all food matrices. Only for stock cubes, egg concentrations were very low or undetectable for all ELISA kits. LC-MS/MS results confirmed that egg was not detectable in one incurred stock cube, however, the second incurred stock cube sample resulted in 7.3 mg whole egg protein. kg^{-1} food. In this sample presence of egg was also detected by the Morinaga and R-Biopharm kits. For incurred cookie, chocolate, dressing, pasta, vegetable drink and milk, ice cream and meat/meat-replacers, concentrations of egg found were far above the upper limit of the detection range of the used ELISA kits, which was also confirmed by LC-MS/MS, all showing the ability to analyze processed egg (Table 5).

4. Discussion

Although ring trials for testing of ELISA kits for egg and milk in single matrices have been organized before [32], this study is the

Table 4
Limit of detection (LOD) based on measurements of blank samples expressed as mg whole egg protein kg^{-1} food product determined per matrix group and compared to the LOD specifications of the kit insert. The LOD is expressed as not detectable (nd) when results for the 10 blank samples of a matrix group analysed were below the calibration curve and result in 0 mg whole egg protein. kg^{-1} after interpolation of the data. Consequently this results in a LOD of 0 mg whole egg protein kg^{-1}.

Matrix	ELISA kit						
	3 M	Biofront	Elisasystems	Morinaga	Neogen	R-Biopharm	Romer
	LOD (mg whole egg protein. kg^{-1})						
Specifications kit	0.42	0.14	0.24	0.31	0.29	0.07	0.24
Cookie	0.06	nd	nd	nd	0.34	0.21	nd
Chocolate	0.18	0.34	nd	nd	0.16	0.19	nd
Pasta	0.10	0.05	0.40	0.28	0.65	0.07	0.40
Wine	0.21	0.1	0.05	0.55	0.08	0.08	0.03
Veg drink and milk	nd	nd	nd	0.32	1.23	0.03	0.03
Ice cream	0.12	0.06	6.43	0.48	0.64	0.02	0.01
Stock cube	0.04	0.07	0.05	nd	0.28	0.04	0.13
Salad dressing	2.20	0.07	0.61	0.04	0.05	0.11	0.02
Meat/meat replacers	5.04	2.56	0.70	0.79	1.05	1.27	4.58
Intra-assay variation (%)							
Wine	3	nd	7	16	6	5	29
Veg drink and milk	4	3	14	9	11	27	1
Inter-assay variation (%)							
Cookie	41	42	9	30	11	7	8
Stock cube 1	45	14	11	15	7	11	23
Stock cube 2	35	24	8	4	8	13	4
Dressing	64	11	6	17	7	11	29
Wine white	28	8	5	13	10	7	10
Wine red	39	40	9	25	19	5	28
Veg drink and milk	29	7	10	23	16	11	4

Table 5
Concentration of total egg protein. kg^{-1} food product in incurred samples tested with 7 selected ELISA kits and additional LC-MS/MS results. Matrices not measured with LC-MS/MS is presented as not applicable (N/A).

Incurred sample	ELISA kit							
	3 M	Biofront	Elisasystems	Morinaga	Neogen	R-Biopharm	Romer	LC-MS/MS
	Concentration (total egg protein. kg^{-1} product)							
Cookie 1	74	15	>5	>50	>12	>1	12	N/A
Cookie 2	28	35.8	>5	>50	>12	>1	9.1	>25
Chocolate 1	29	19.5	>5	>50	>12	>1	11	>25
Chocolate 2	175	32.6	>5	>50	>12	>1	>12	N/A
Stockcube 1	<LOD	<LOD	<LOD	<LOD	<LOD	0.9	<LOD	<LOD
Stockcube 2	0.7	<LOD	0.4	1.4	0.6	>1	<LOD	7.3
Dressing 1	111	>38	>5	>50	>12	>1	>12	>25
Dressing 2	71	38.2	>5	>50	>12	>1	>12	N/A
Pasta 1	3	20.1	>5	>50	>12	>1	4.3	N/A
Pasta 2	156	>38	>5	>50	>12	>1	>12	>25
Veg drink and milk 1	97	>38	>5	>50	>12	>1	>12	N/A
Veg drink and milk 2	>270	>38	>5	>50	>12	>1	>12	N/A
Ice cream 1	34	>38	>5	>50	>12	>1	>12	N/A
Ice cream 2	58	>38	>5	>50	>12	>1	>12	>25
Meat/meat replacers 1	67	>38	>5	>50	>12	>1	>12	>25
Meat/meat replacers 2	12	>38	>5	>50	>12	>1	>12	N/A

first one that compares a multitude of commercial available ELISA kits for the detection of egg in different food matrices. Suitability of detection of egg protein in divergent food matrixes is shown. Moreover, all kits could detect the presence of egg in the spiked and incurred samples. However, differences in sensitivity in different matrices are evident.

4.1. Expression egg content in food

In processed food not only eggs as total, but also solely egg yolk or egg whites are used depending on their properties for food preparation [33]. These egg yolks and whites are processed prior to use, for stabilization purposes and enhanced shelf-life [34]. In our experimental set-up, incurred samples are included to evaluate the performance of the ELISA test kits for processed egg. Calculations performed assume the use of total egg. Depending on the protein determined by the ELISA kit, this can lead to both under- and overestimation of total egg when parts of the eggs are used in food processing.

4.2. ELISA kits

The ability to determine the amount of egg using an ELISA kit is dependent on several factors like the extraction procedure applied and the antibodies used. The included ELISA kits showed to be able to detect egg in a broad concentration range (see Fig. 1), with one exception, the R-Biopharm kit. This kit was very sensitive and needed dilution steps prior to detection. As we knew the spiking levels beforehand, we were able to dilute the spiked samples accordingly. This however biases the comparison, as with unknown samples the present egg concentration is not known beforehand. Moreover, here we diluted the spiked materials to approximately 1 mg whole egg protein kg^{-1} product, always the same point in the calibration curve, whereas for the other kits concentrations on the lower and upper limits of the calibration curves were used for comparisons.

For the detection of whole egg using ELISA kits, ideally, extraction is efficient and a high sensitivity for all kinds of processing steps of the egg, without any cross reactivity, is reached. Moreover, ideally there are no matrix interferences. As shown in this study, all kits were able to detect the used NIST certified reference material. Moreover, all kits could detect the presence of egg in multiple incurred matrices as shown in Table 5. In incurred samples the egg ingredient is processed along with the matrix, showing the egg was still detectable after processing. But differences in sensitivity for egg detection in different matrices for the kits are evident (Fig. 1,Table 4), which can lead to underestimation of the egg present or false negative results. Future research therefore should further elaborate on the real effects of processing on the detection of egg in food matrices. The tested matrices have their own specifics and possible interfering substances in the egg ELISA kits. These interfering substances are further discussed for each matrix below.

4.3. Food matrices

4.3.1. Cookie

For the cookie matrix, all kits were able to qualitatively detect presence of egg from 1 mg egg protein kg^{-1} cookie onward (Fig. 1). Quantitative results for a cookie relevant concentration of 3 mg egg protein kg^{-1} product showed good recovery (Table 3). However, detection of egg in crisp cookies or rusk can be challenging, according to experienced kit users (personal communication Allergenen Consultancy). This is however not tested in this study. Cookies often are the matrix of choice for optimization and subsequent validation of egg ELISA kits, therefore most kits perform optimal for this matrix [35–37]. When looking at inter-assay variation (Table 4) however, 3 M, Biofront and Morinaga show a higher variability, as compared to the other tested ELISA kits.

4.3.2. Chocolate

All kits were able to detect egg in chocolate from the lowest spiking concentration onwards with recoveries in the set performances range (Table 3). For obtaining a homogeneously spiked chocolate a clear difference between dark and white chocolate was seen, where it was more difficult to homogeneously spike white chocolate. Probably contributing to the inhomogeneity, is the amount of fat. The addition of dairy ingredients influence the rheological properties of chocolate. For instance when adding whey to milk chocolate, an increase in particle size and crystallization of the chocolate is seen according to the amount whey added [38]. Another interfering compound for spiking chocolate can be the lecithin. The combination of the lecithin in the melted chocolate bar and the added protein can have an antagonistic effect as lecithin or the protein can be displaced from the interface. Interactions described are, for instance, change in net charge and incorporation of proteins into surfactant micelles and vesicles, which both can lead to inhomogeneity in the spiking experiment [39].

The chocolate matrix is a challenging matrix for ELISA kits, because the polyphenols can react with proteins during extraction in a rather non-specific manner which makes efficient extraction more difficult [40]. In addition, the presence of high fat content decreases protein epitope accessibility [41].

4.3.3. Pasta

All ELISA kits were able to detect egg in pasta matrix from the lowest spiking level onwards. All ELISA kits performed well in the recovery of the egg spiked into pasta matrix, with an overestimation at the action level of 1 ppm, determined with the Neogen kit and Biofront (Table 3). Moreover, processed egg was detected by all kits in the incurred samples (Table 5). However, for pasta it should be noticed that eggs are typically not used as a whole product. The egg yolk is added to color the pasta and to increase absorption of water during cooking. But it also inhibits the preferred formation of compact pasta protein network with gluten. Ovalbumin on the other hand enhances the compact pasta protein network with gluten. Therefore, food manufacturers will always optimize the ratio egg yolk/albumin for their pasta products, taken into account the regulatory amounts prescribed [42]. This should be taken into account when analysing egg in pasta especially when measuring cross contamination of egg-free pasta with egg. The kits often detect the egg white (Table 1), and for detection of whole egg, it is not clear from which part of the egg the detected protein originates. Cross contamination is a real concern, as shown by Marengo et al. [43], egg stays present for over an hour when, after production line cleaning of egg containing pasta, egg-free pasta is produced. In the Marengo et al. study [43] this correlated to 2000–4000 kg pasta.

4.3.4. Wine

All kits performed well in recovery of egg spiked to the wine matrix, although it should be mentioned that the spiking was executed freshly to prevent sedimentation of the egg and obtain a homogenous solution. Sedimentation of the eggs are a result of wine making, as eggs are used for their selective tannin adsorption, positively influencing the wine flavor, which results in sediments, clarifying the wine [44]. Detectability of egg in wine was described before. The signal obtained for the detection of egg reduced with >90% within 24 h [44]. This might be due to interaction between tannin and the fining proteins, as they can interfere with the detection of egg in the ELISA as proposed by Weber et al. [45] which might explain the decrease in detected egg protein. For the wine matrix, therefore, dilution of samples should be considered to decrease this matrix effect [45]. Similar to described with the pasta matrix, only the albumin fraction of the egg white, and not the total egg, is used as fining agent [46,47] which should be taken into account in the selection of the ELISA kit of choice and interpretation of results.

4.3.5. Vegetable drink and milk

As the portion size of milk is high, the action level for egg detection is low. All ELISA kits were able to detect egg in the spike concentration of 1 mg total egg protein per kg vegetable drink or milk, however recovery for the 3 M and Biofront kit were below the set performance criteria (Table 3). It should be noted that for all kits the recovered egg concentration in infant formula was higher than the recovered egg concentration in soy drink. As there is an increasing trend in replacing dairy milk by vegetable drink [48], it is often generally pointed to as milk. However the composition of vegetable drink is different from dairy milk, of which, in macronutrients, soy drink is the vegetable drink type that is most close to dairy milk [49]. This matrix difference possibly explains the difference in recovery for the vegetable drink and milk matrices.

4.3.6. Ice cream

Ice cream seems to be a difficult matrix for egg detection with ELISA. The recovery of using ELISA shows both overestimation and underestimation at the borders of the set performance criteria, with a range from 15% to 217% (Table 3). There is however not a difference seen in the 2 tested spiked ice creams, like with the vegetable drink and milk matrix for which a higher recovery was observed in the dairy matrix for every test kit. These results do not suggests specific possible interfering compounds for the detection of egg in the ice cream matrix.

4.3.7. Stock cube

Although stock cubes represents highly processed food products with a complex protein background [41], egg was detected in all ELISA kits from the lowest spiked level onwards (Fig. 1). Stock cubes are known to have a high salt content, and salts interfere in the binding of the antibody to the target [50,51]. Our spiked materials however were lower in salt content, 12% (m/m) and 0.3% (m/m) respectively. For all ELISA kits, recovery of egg was lower in the high salt containing matrix than the low salt containing matrix. This trend should be carefully taken into account when stock cubes higher in salt are tested. The two incurred samples contained respectively 14.9 and 22% salt, lower than the average salt concentrations found in stockcubes the Netherlands [52], but higher than

our spiked blank samples. The higher salt content might have decreased the detectability of the egg present. But in the incurred samples presence of egg was expected to be very low, or even not present as in the first sample, egg was only present as part of an aroma, and the second sample "may contain egg". These factors possibly explain why the incurred samples only showed non- and traces of egg present. Furthermore, the portion size of 20 g as described by Birot et al. [31] seems rather high, as packages state to dilute 10–20 g of stock cube in 1 L boiling water to prepare soup. Although people can use stock cubes differently, for instance as seasoning, portion size often will be lower, increasing the proposed action level.

4.3.8. Dressing

Detection of egg in dressing matrix is challenging for all ELISA kits. For the first spiked dressing matrix, the Hellman vegan mayo, an underestimation was observed for all ELISA kits. With some of the ELISA kits, the spikes were not detectable at all. For the second spiked material, the dressing salata, concentrations found were slightly higher, but also in this case some kits showed recoveries of approximately 10%. The combination of the high fat content with low pH of dressing ingredients, probably are causing the difficulties for detecting the egg present. Soares et al. [53] showed pH dependency of allergen extraction. Lower pH buffers showed lower protein extraction efficiency. As dressings are acidic [54], the buffer capacity of the used ELISA kits are challenged, and if the buffer capacity is unable to buffer the dressing enough this can lead to inefficient protein extraction. Moreover, also the antibody binding capacity is affected by incorrect buffering and consequently could give false-negative results or underestimation of results [55]. Furthermore, as described before for the chocolate matrix, presence of high fat content decreases protein epitope accessibility [41].

4.3.9. Meat/meat replacers

The detection of egg in the meat and meat replacers matrix was a challenge for all ELISA kits. All kits overestimated the presence of egg in both the meat replacer and beef minced meat. The beef minced meat itself gave blank results. However, for the minced meat replacer, egg was already detected in the blank material, which might be due to presence of egg in the blank material or influence of the matrix to the ELISA kits. For all 10 blank materials tested, this blank material was the only one giving positive results for egg presence with all 7 applied ELISA kits, which makes the presence of egg likely. This was also confirmed by the LC-MS/MS data which showed a concentration of 3.8 mg egg protein kg^{-1} meat replacer. If egg was present in the blank material, spiking this material should only give an increase in concentration in correlation with the added amount of egg. As a rough estimation of the egg content of the blank material by the used ELISA kits was 1 mg total egg protein per kg product, a concentration of 11 mg total egg protein per kg product was expected for the highest spike level. This however was not the case; for most kits the measured concentrations were above ULOQ, and if the obtained signal was within the kits range of the calibration curve, the egg protein concentration found was even 4x higher than expected. The matrix effect is therefore likely; meat replacers are protein rich, highly processed matrices [56], which challenges the egg ELISA kits for proper detection. As shown in this study egg content in both meat and meat/replacers are overestimated, with highest discrepancy for the meat replacers.

4.4. Performance criteria

4.4.1. Recovery

For commercial ELISA kits, the range of sample matrices which should be selected for determination of matrix effects is not harmonized [18]. This is, however, needed to enable comparison of recovery with relevant food matrices for the detection of egg. For practical reasons, in this study recovery was determined by spiking the already processed food products themselves, prior to extraction and analysis. This enabled comparison of matrix effects but not the effects of processing. It is good to be aware of the influence processing of food can have on recovery, as for instance described for sugared cookies [36,57]. Furthermore, we have chosen to use the recovery range of 50–150% as described by Godefroy et al. [30]. Although some studies advocating other ranges, like 60–120% as suggested by Paez et al. [58].

4.4.2. Limit of detection

In this study, the limit of detection of the test kits was calculated by using the background signal of blank materials for all matrix groups separately. Where the kit providers one LOD for their kit as shown in Table 1, the LOD is clearly matrix depended as can be seen in Table 4. Therefore, matrix depended LODs would be advisable. Furthermore, as clearly can be seen from the results, if the concentration measured in the blank materials is zero, the LOD cannot be determined. In Abbot et al. [21] this approach is pointed out, but also an alternative, more advanced method is proposed. The advanced method combines results of a blank and the blank spiked with 4 different concentrations of egg, measured in duplicate by 10 different laboratories. Since the aim of this study was not to investigate possible approaches for LOD determination, we did not apply this approach. Consequently, obtained data are not adequate for this purpose.

5. Conclusion

Performance of seven different commercial available ELISA kits for the detection of egg in nine different matrices was evaluated. All kits showed to be able to qualitatively detect egg at the VITAL3.0 ED01 level of 0.2 mg total egg protein and incurred egg protein in all matrices. For quantitative measurements, all seven ELISA kits showed to perform within the set criteria for recovery in cookies, chocolate, stock cube and wine. Quantifying egg in the matrices of vegetable drink and milk, pasta, ice cream, and salad dressing showed to be more challenging. However, at least four of the ELISA kits performed well within the set criteria. The most challenging

matrix showed to be the meat/meat replacers; this matrix showed to be of high influence on the general overestimation of the presence of egg by the kits. The results of this comparison study can be used by food industry and commercial and control laboratories to be well informed about the ELISA kits suitable for the matrix of interest.

Author contribution statement

Nathalie G.E. Smits: Conceived and designed the experiments; Performed the experiments; Analysed and interpreted the data; Wrote the paper.

Emiliano De Dominicis: Performed the experiments; Analysed and interpreted the data; Wrote the paper.

Andries J. Koops: Conceived and designed the experiments; Wrote the paper.

Rian Kraan: Performed the experiments; Analysed and interpreted the data.

Samim Saner: Contributed reagents, materials analysis tools and data; Wrote the paper.

H.J. Van Der Fels-Klerx: Analysed and interpreted the data; Wrote the paper.

Elise Hoek-van den Hil: Conceived and designed the experiments; Analysed and interpreted the data; Wrote the paper.

Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Nathalie G.E. Smits reports financial support and equipment, drugs, or supplies were provided by 3 M, Biofront technologies, Danone, Generon, Merieux NutriSciences, Morinaga, Neogen, Nutrilab, Progenus, Romer, Unilever.

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Rian Kraan reports financial support and equipment, drugs, or supplies were provided by 3 M, Biofront technologies, Danone, Generon, Merieux NutriSciences, Morinaga, Neogen, Nutrilab, Progenus, Romer, Unilever.

Elise Hoek-van den Hil reports financial support and equipment, drugs, or supplies were provided by 3 M, Biofront technologies, Danone, Generon, Merieux NutriSciences, Morinaga, Neogen, Nutrilab, Progenus, Romer, Unilever.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19687.

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