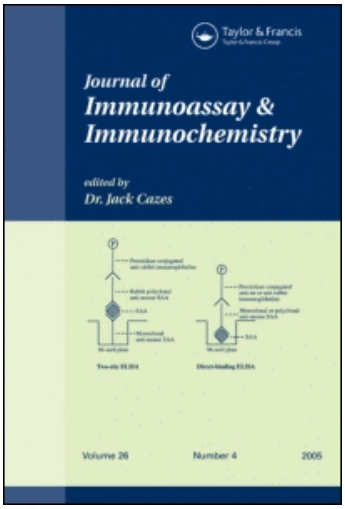


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# Serum IgG Responses to Food Antigens in the Italian Population Evaluated by Highly Sensitive and Specific ELISA Test

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**Abstract:** Using an optimized and validated ELISA method, we performed a serum test for assaying the binding capacity of serum IgG to proteins extracted from approx. 160 different foods to investigate the reactivity of specific IgG antibodies in the Italian population composed of 6,879 subjects (4,551 females and 2,328 males). 44 antigens showed an IgG response greater than 10% and only 14 aliments had an elevated reactivity greater than 20%, in particular, milk, from cow and goat, and several milk derivatives, along with egg albumen and yeasts. The IgG response to the high reactive food antigens depending on the age of the 6880 subjects was also analyzed. We demonstrated a high IgG response in a very large subject group to milk and milk derivatives, and egg albumin antigens, and we conclude that the validated ELISA test may be applied for the serum/plasma IgG antibody level determination as a useful indicator of adverse reactions to food and food hypersensitivity.

**Keywords:** ELISA, Food antigens, Food hypersensitivity, Food intolerance, IgG evaluation

## INTRODUCTION

A range of several adverse reactions to food have been identified in humans, including food allergy, defined as an immunologically-mediated

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excessive reaction to food substances in which cross-linking of food allergen-specific IgE molecules on the surface of mast cells and basophils occurs following ingestion of the food allergen to which the individual is sensitive.[1] Food allergy must be distinguished from food intolerance where non-immunological mechanisms are implicated.[2] In allergic diseases, IgE is regarded as the most important immunoglobin, but IgG antibodies have also been reported to be associated with allergic diseases.[3–6] Food sensitization is associated with an increased future risk of sensitization to inhalant allergens[5,6] and the results of several scientific reports[5,6–12] suggested that IgG might be useful in determining a child's risk of becoming allergic within a few years and, therefore, could be used as an early marker for the development of IgE-mediated allergy. Moreover, IgG antibodies may be responsible for several adverse reactions to food in humans due to other forms of immunological mechanisms as opposed to dietary allergy. In fact, such reactions could be mediated by IgG antibodies, which characteristically give a more delayed response following exposure to a particular antigen[13] and have been implicated in some cases of food hypersensitivity.[14–16] However, this mechanism is controversial and it is considered by some to be physiological[17–19] especially as IgG food antibodies can be present in apparently healthy individuals.[20–22] However, food allergy and intolerance may arise through abnormal responses on the part of the mucosal immune system to ingested food[23] with the gut being the largest immunological organ in the body able to normally generate local immune response, but systemic tolerance to, ingested antigen.[1] To date, the current understanding of the normal mucosal immune system is limited and proposed mechanisms of allergy and intolerance are generally speculative.[1]

Unconventional methods, such as cytotoxic food testing, the ALCAT test, bioresonance, electrodermal testing (electroacupuncture), reflexology, applied kinesiology, all of which lack scientific credibility and have been shown to have no clinical efficacy, are being increasingly used for diagnostic and therapeutic purposes in allergic diseases.[24–26] Some of these procedures, such as cytotoxic food testing and the ALCAT test, a new version of the previous leukocytotoxic food test, have the claim, not based on scientific evidence and credibility, to measure the serum concentrations of IgG and IgE antibodies specific for a variety of common food antigens.[24–26] Furthermore, food allergen extracts used in IgE- and IgG-mediated reactions are of paramount importance and they should be produced by means of suitable and standardized procedures controlled by sensitive, specific and reproducible quality control techniques.[27] ELISA is a convenient, sensitive, and reproducible analytical method used for determining antibody

production in serum generally applied for monitoring the occurrence of IgE and IgG antibodies specific for foods in animals[1,2] and humans.[28,29] Using an optimized and validated ELISA method according to the Guidance for Industry, Bioanalytical Method Validation from U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM) published in May 2001,[30] including specificity, linearity, detection (LOD), and quantitation (LOQ) limit, precision, accuracy, and robustness test, we performed a serum test for assaying the binding capacity of serum IgG to proteins extracted from approx. 160 different foods to investigate the reactivity of specific IgG antibodies in the Italian population composed of 6,879 subjects (4,551 females and 2,328 males). Proteins produced from the various aliments were purified according to a patent process and the quality of each extract was carefully determined quantitatively and qualitatively by sensitive and specific analytical procedures, such as spectrophotometric evaluation[31] and SDS-PAGE[32,33] coupled with densitometric acquisition.



## EXPERIMENTAL

### Materials

Maxisorp 96-well plates from Celbio were used. Blocking reagent, polyvinylpyrrolidone (PVP), goat anti-human IgG (Fab specific)-peroxidae (HRP) conjugated, 3,3′,5,5′-Tetramethylbenzidine (TMB), and IgG from human serum were from Sigma.

All the other reagents were analytical grade.

### Production of Food Antigens

Food antigens were produced by 164 aliments (Table 1) by a patent pending extraction and purification procedure. Briefly, aliments were diluted in a 50 mM pH 7.0 tris-Cl buffer and minced, when necessary, by an ultraturrax. Proteins were purified by means of filtration, gel-permeation, and ion-exchange chromatography, and freeze-dried. The extraction of food proteins was quantitatively evaluated by a specific spectrophotometric assay[31] (see Table 1) and qualitatively assured by SDS-PAGE and densitometric scanning to determine the relative amount of each electrophoretic band and the relative protein molecular mass (see, as example, Figure 1 and Table 2).

**Table 1.** The 160 various aliments from which food protein antigens were prepared. The protein content is also reported in mg/mL

| Food antigen | Proteins (mg/mL) | Food antigen | Proteins (mg/mL) |
|---|---|---|---|
| Albumen | 2.41 | Lemon | 0.99 |
| Almond | 2.63 | Lentil (*Lens esculenta*) | 1.44 |
| Amaranth (*Amaranthus*) | 0.50 | Lettuce (*Lactuca sativa*) | 0.93 |
| Pineapple (*Ananas sativus*) | 0.48 | Lime (*Tilia*) | 1.21 |
| Anchovy | 1.64 | Linseed | 3.74 |
| Aniseed (*Pimpinella anisum*) | 0.26 | Mackerel | 1.62 |
| Apple | 0.46 | Maize (*Zea mays*) | 0.34 |
| Apricot | 0.24 | Mallow (*Malva sylvestris*) | 0.72 |
| Artichoke (*Cynara cardunculus scolymus*) | 0.63 | Malt barley | 0.65 |
| Asparagus (*Asparagus officinalis*) | 1.28 | Mango (*Mangifera indica*) | 0.85 |
| Aubergine (*Solanum melongena*) | 1.29 | Marrow (*Cucurbita pepo*) | 0.75 |
| Avocado (*Persea gratissima*) | 1.93 | Melon (*Cucumis melo*) | 1.16 |
| Banana | 1.24 | Millet (*Panicum miliaceum*) | 0.73 |
| Barley (*Hordeum vulgare*) | 1.32 | Mint (*Mentha viridis*) | 0.24 |
| Basil (*Ocimum basilicum*) | 1.44 | Mozzarella | 1.34 |
| Bean (*Phaseolus vulgaris*) | 1.23 | Mushrooms | 1.49 |
| Beef | 2.22 | Mussels (*Mytilus edulis*) | 1.76 |
| Bilberry (*Vaccinium myrtillus*) | 0.14 | Mustard | 1.94 |
| Blackberry | 1.98 | Nettle (*Urtica dioica*) | 2.19 |
| Brazil nut | 2.24 | Nutmeg | 1.88 |
| Broccoli spear | 1.00 | Oats (*Avena sativa*) | 1.47 |
| Brussels sprout (*Brassica oleracea gemmifera*) | 0.71 | Olive | 1.37 |
| Buckwheat | 2.01 | Onion (*Allium cepa*) | 0.67 |
| Cacao (*Theobroma cacao*) | 0.75 | Orange | 0.27 |
| Calf | 2.57 | Oregano (*Origanum vulgare*) | 1.98 |
| Camembert cheese | 3.34 | Parmesan (cheese) | 2.81 |

(*Continued*)

**Table 1.** Continued

| Food antigen | Proteins (mg/mL) | Food antigen | Proteins (mg/mL) |
|---|---|---|---|
| Camomile (*Matricaria chamomilla*) | 0.66 | Parsley (*Petroselinum sativum*) | 1.29 |
| Carob | 0.10 | Pawpaw (*Carica papaya*) | 2.24 |
| Carrot | 0.97 | Pea (*Pisum sativum*) | 1.44 |
| Cashew (*Anacardium occidentale*) | 3.22 | Peach | 0.63 |
| Cauliflower (*Brassica oleracea botrytis*) | 0.36 | Peanut | 2.60 |
| Cayenne pepper | 2.52 | Pear | 0.55 |
| Celery (*Apium graveolens*) | 1.11 | Pepper (*Piper nigrum*) | 1.01 |
| Chard (*Beta vulgaris cicla*) | 0.99 | Pilchard | 2.34 |
| Cheese spread | 1.32 | Pistachio (*Pistacia vera*) | 1.91 |
| Cherry | 0.39 | Plaice | 0.98 |
| Chestnut | 1.42 | Plum | 0.40 |
| Chicken | 2.25 | Pork | 3.50 |
| Chickpea (*Cicer arietinum*) | 0.76 | Potato (*Solanum tuberosum*) | 1.19 |
| Chicory (*Cichorium intybus*) | 1.28 | Pumpkin (*Cucurbita maxima*) | 0.33 |
| Chive (*Allium schoenoprasum*) | 0.87 | Rabbit | 1.93 |
| Cinnamon (*Cinnamomum zeylanicum*) | 0.39 | Radish (*Raphanus sativus radicula*) | 1.69 |
| Cloves | 1.31 | Radish (*Raphanus sativus*) | 0.45 |
| Coconut | 1.25 | Raspberry (*Rubus idaeus*) | 0.83 |
| Codfish | 2.69 | Ribes | 1.23 |
| Coffee | 1.31 | Rice (*Oryza sativa*) | 1.14 |
| Coriander (*Coriandrum sativum*) | 1.17 | Ricotta | 2.84 |
| Cow's milk | 2.24 | Roebuck | 3.44 |
| Cucumber (*Cucumis sativus*) | 1.37 | Rosemary (*Rosmarinus officinalis*) | 1.06 |
| Cumin (*Cuminum cyminum*) | 1.03 | Rye (*Secale cereale*) | 3.38 |
| Curry | 3.02 | Safflower seed (*Carthamus tinctorius*) | 3.05 |

(*Continued*)

**Table 1.** Continued

| Food antigen | Proteins (mg/mL) | Food antigen | Proteins (mg/mL) |
|---|---|---|---|
| Date (*Phoenix dactylifera*) | 0.60 | Sage (*Salvia officinalis*) | 1.05 |
| Dill (*Anethum graveolens*) | 0.97 | Salmon | 1.52 |
| Duck | 3.60 | Savoy (*Brassica oleracea sabauda*) | 0.44 |
| Dutch cheese | 2.51 | Sea bass | 3.75 |
| Egg-yolk | 2.68 | Sesame (*Sesamum indicum*) | 1.37 |
| Emmenthal cheese | 3.22 | Sheep cheese | 1.62 |
| Emmer (*Triticum dicoccum*) | 2.52 | Sole | 1.36 |
| Endive | 1.98 | Soya bean (*Glycine max*) | 2.34 |
| Endive (*Cichorium endivia*) | 1.27 | Spinach (*Spinacia oleracea*) | 1.68 |
| European lobster | 1.05 | Strawberry (*Fragaria vesca*) | 0.77 |
| European lobster (*Homarus vulgaris*) | 2.51 | String bean | 2.03 |
| Fennel (*Foeniculum vulgare*) | 0.16 | Sunflower seed | 2.33 |
| Fig (*Ficus carica*) | 0.95 | Sweet marjoram (*Origanum majorana*) | 1.03 |
| Garden cress (*Lepidium sativum*) | 0.52 | Swordfish | 4.01 |
| Garlic (*Allium sativum*) | 2.14 | Tangerine (*Citrus nobilis*) | 1.08 |
| Ginger (*Zingiber officinale*) | 2.36 | Taraxacum (*Taraxacum officinale*) | 1.06 |
| Goat's milk | 1.83 | Tea-plant (*Thea sinensis*) | 1.03 |
| Gorgonzola cheese | 0.64 | Thyme (*Thymus vulgaris*) | 0.62 |
| Grapefruit (*Citrus paradisi*) | 0.37 | Tobacco (*Nicotiana tabacum*) | 1.75 |
| Grapes | 0.44 | Tomato (*Solanum lycopersicum*) | 0.57 |
| Guinea pepper (*Capsicum annuum*) | 1.36 | Trout | 1.23 |
| Hazelnut | 2.94 | Tuna | 2.34 |
| Herring | 3.16 | Turbot (*Psetta maxima*) | 2.73 |
| Honey | 0.75 | Turkey | 1.18 |

(*Continued*)

**Table 1.** Continued

| Food antigen | Proteins (mg/mL) | Food antigen | Proteins (mg/mL) |
|---|---|---|---|
| Hop (*Humulus lupulus*) | 0.87 | Turnip (*Brassica rapa*) | 0.83 |
| Hot pepper | 1.30 | Vanilla (*Vanilla planifolia*) | 0.47 |
| Kale (*Brassica oleracea acephala*) | 0.64 | Walnut-tree | 2.19 |
| Kiwi (*Apteryx australis*) | 1.48 | Watermelon (*Citrullus vulgaris*) | 0.76 |
| Lamb | 3.09 | Wheat (*Triticum vulgare*) | 1.53 |
| Laurel (*Laurus nobilis*) | 0.73 | Yeast | 0.83 |
| Leek (*Allium porrum*) | 0.54 | Yogurt | 2.29 |



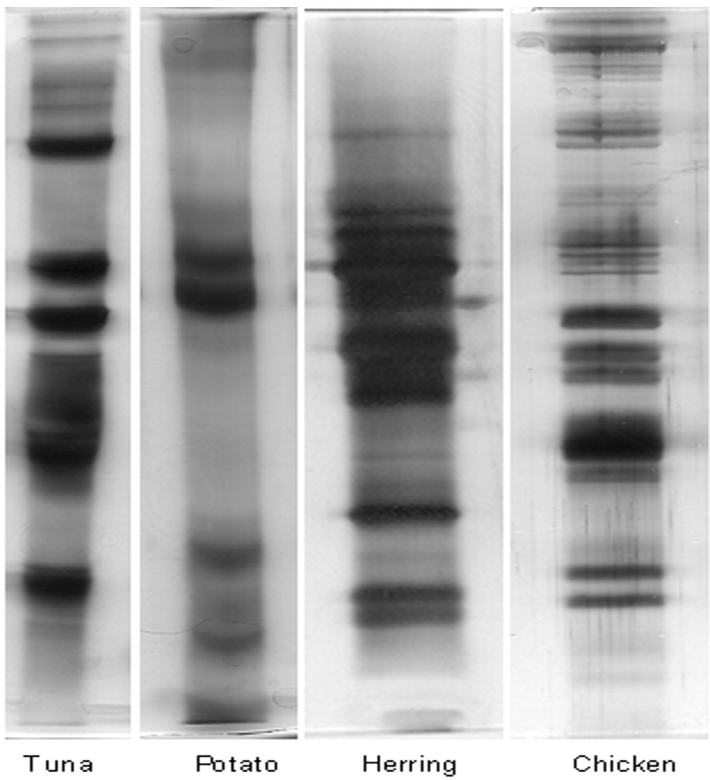


**Figure 1.** SDS-PAGE profiles of the proteins extracted and purified from several aliments reported as examples. See Table 2 for their molecular mass values and relative percentages.

**Table 2.** Molecular mass in kDa and the relative percentages of the main protein bands purified from several aliments reported as examples

| Tuna | | Potato | | Herring | | Chicken | |
|---|---|---|---|---|---|---|---|
| MW (kDa) | % | MW (kDa) | % | MW (kDa) | % | MW (kDa) | % |
| 109.1 | 3.3 | 53.9 | 31.0 | 69.6 | 4.5 | 155.9 | 3.6 |
| 71.0 | 44.8 | 49.6 | 32.8 | 52.1 | 11.3 | 119.7 | 3.1 |
| 59.4 | 43.3 | 27.8 | 19.7 | 49.6 | 11.6 | 65.5 | 7.8 |
| 36.7 | 4.9 | 19.1 | 16.5 | 43.9 | 11.8 | 58.1 | 7.4 |
| 23.3 | 3.7 | | | 33.6 | 20.8 | 52.8 | 7.4 |
| | | | | 27.7 | 11.0 | 41.5 | 55.4 |
| | | | | 18.4 | 10.5 | 38.6 | 5.1 |
| | | | | 14.4 | 9.6 | 27.5 | 5.3 |
| | | | | 13.4 | 8.8 | 25.0 | 4.8 |



## Human Subjects

The approx. 6,880 subjects participating in the study were selected by laboratories of Natrix S.r.L. (Reggio Emilia, Italy) and consisted of males (2,328) and females (4,551) from the entire Italian population aged between approx. 1 and 90 years (The mean age of males was $39 \pm 25.2$ years and $40 \pm 22.2$ years in the female group). The subjects were fully able to comprehend the nature and purpose of the study, to co-operate with the investigator, and to comply with the requirements of the study.

Venous blood samples (5–10 mL) were taken from a forearm vein and collected in tubes. The samples were stored at 4°C when necessary, then centrifuged at 4°C for 10 min to obtain serum and were immediately transferred into pre-labelled test tubes and stored frozen at −20°C until analysed in the laboratories of Natrix S.r.L. (Reggio Emilia, Italy).

## IgG ELISA

Plates were coated with food antigens diluted in carbonate/bicarbonate buffer. The quantity of each food antigen extract used to coat the ELISA plate was derived experimentally and represented the amount required to obtain maximum antibody binding. However, a large concentration range, from approx. 5 to 200 ng proteins, of the various allergens investigated was found to produce maximum response.

Plates were incubated at 4°C overnight with 100 μL of extract per well. Plates were subsequently blocked with 200 μL per well of blocking reagent at room temperature for 1 h. The blocking buffer was then decanted and the plates were left at 37°C overnight. Serum samples were

diluted in PBST/3% PVP 10 kD to 1/400 and 100 μL were added to each well for 30 min. After washing, goat anti-human IgG-peroxidae (HRP) conjugated, was diluted 1/2,000 in PBST/3% PVP 10 kD and 100 μL were applied to each well for 30 min with continuous shaking. 100 μL TMB substrate were applied to each well for 10 min. The reaction was stopped using 50 μL per well of 0.5 M sulphuric acid and the plates were read using a Bio-Rad ELISA plate reader at 450 nm.

The mean absorbance of each test specimen was compared to the absorbance of positive control serum samples for the group of food antigens applied to the ELISA plate. Positive food allergen ELISA control sera were identified from extensive preliminary screens and were defined as having a minimum absorbance greater than three times that of a control negative pool of human serum. Based on the control serum samples' responses, a calibration curve applicable to all the antigens was constructed by using a standardized and controlled amount of purified human IgG. As a consequence, the results were expressed in terms of percentage reaction calculated on the calibration curve and in comparison with 100% and 50% positive control serum samples.



## Validation of the ELISA Analytical Method

The ELISA test validation was established according to the Guidance for Industry, Bioanalytical Method Validation from U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM) published in May 2001.[30] including specificity, linearity, detection (LOD) and quantitation (LOQ) limit, precision, accuracy, and robustness test. The specificity of the IgG response to the food antigens was determined by immunoblotting against purified human IgG and human IgE, and no reaction with IgE was appreciated. Calibration curves were constructed for the group of food antigens by increasing the protein concentration. Linear regression analysis was used to calculate the slope, intercept and correlation coefficient ($r^2$) of each calibration curve. The precision of the ELISA method was assessed by determination of IgG response to five replicates ($n = 5$) of five different protein concentrations (from 5 to 200 ng). The intra- and inter-day precision, and accuracy of the method were estimated by percent of relative standard deviation (CV%) from the analysis of 10 different serum samples on 3 separate days. Robustness was assessed by analysis of several serum samples at different analytical conditions, food antigen protein amount, temperatures of antigens and blocking reagent incubation, times of treatment with serum, anti-human IgG and TMB, and various solution

concentrations buffer compositions, no greater than nor inferior to 10% of the adopted values.

### Statistical Analysis

The IgG responses to the various food antigens determined in the Italian population and the comparison according to age and sex are reported as means and standard deviation percentage (CV%). Differences between groups were compared by means of the $t$ -test with significance accepted at $p < 0.001$ and further confirmed using a multivariate approach, GLM, with additional post-hoc testing by SPSS software for Windows.

## RESULTS

### Food Antigens

Table 1 shows the food antigens produced in this study. As can be seen, antigens were produced from different kinds of aliments, such as meat, fish, crustaceans, milk products and eggs, vegetables, seeds, flowers and leaves, fruits and dried fruits, legumes and cereals, spices, yeast, and mushrooms. Proteins were extracted and purified by using a patent pending protocol based on filtration, gel-permeation, and ion-exchange chromatography. Final proteins were freeze-dried. Accurate quantitative analysis gives us the protein concentration depending on the kind of aliment and related to the extraction and purification protocol adopted and reported as mg protein diluted in 1 mL volume. This value for each extract was also used as quality control data for the purification process. SDS-PAGE and densitometric scanning to determine the relative amount of each electrophoretic band and the relative protein molecular mass (Figure 1 and Table 2) were adopted to evaluate the general protein composition of the extracts and also as a quality marker of the production process.

### IgG ELISA

The ELISA test is a highly specific analytical method suitable for IgG identification and response to different food antigens.[1,2,5,6] The intra- and inter-day variations (CV%), under the experimental conditions adopted, were between 3.6 and 18.9, and the calibration curves for the group of food antigens showed good linearity for the examined concentration range (5 to 200 ng) with an average correlation coefficient greater

than 0.990. The LOD and the LOQ of the method were 5 and 20 ng, respectively. Variations in food antigen protein amounts, temperatures of antigens and blocking reagent incubation, times of treatment with serum, anti-human IgG and TMB, and various solution concentration buffer compositions in comparison with adopted conditions below 10% do not modify the ELISA results.

## IgG Response to the Various Food Antigens

Figure 2 shows the IgG reactivity, expressed in percent, on the calibration curve constructed by using standardized and controlled amounts of purified human IgG, of the antigens produced from approx. 160 aliments used in this study. As appears evident, 44 antigens show an IgG response greater than 10% and only 14 aliments have a reactivity greater than 20%. 120 food extracts show a mean IgG reactivity lower than 10% evaluated in approx. 6,880 subjects. It is worth mentioning that these are mean values determined over a very large representative sample of Italian population and IgG response was found to range between 0 and 100% for each of the food antigens studied, depending on the human subject. By considering the variation (CV%) values of the ELISA test ranging



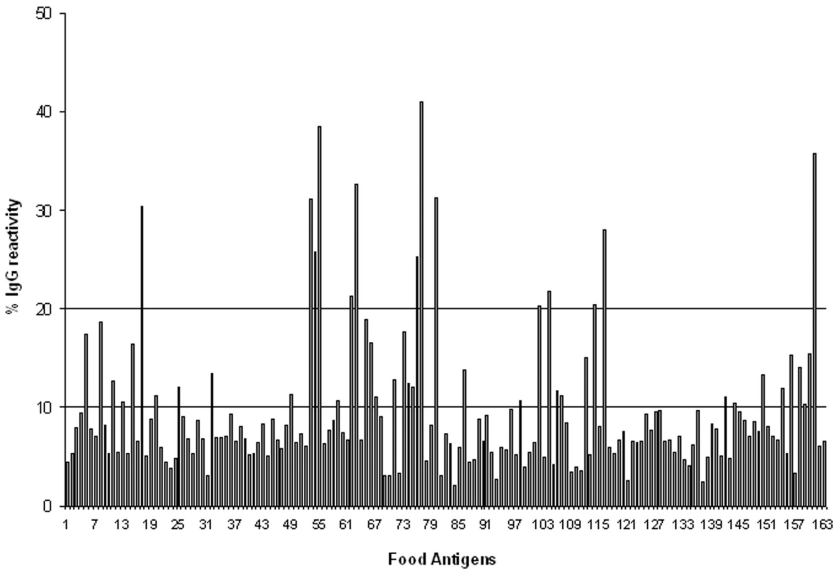


**Figure 2.** Percentages of IgG reactivity of the 160 food antigens evaluated by validated ELISA test.

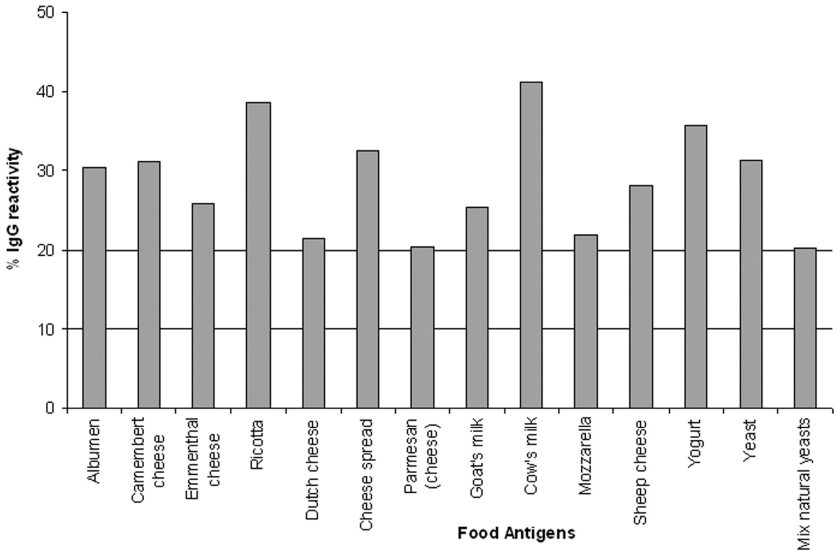


**Figure 3.** Percentages of IgG reactivity of the 14 very high reactive (>20%) food antigens evaluated by validated ELISA test. See also Figure 2.



between 3.6 and 18.9 (see above), IgG response greater than 20% was considered as a limit for high reactivity of food antigens. As a consequence, antigens extracted from 14 aliments produced a very strong IgG response in human subjects. Figure 3 illustrates in details the 14 highly reactive food antigens. In particular, milk from cow and goat, and several milk derivatives, like different kinds of cheese and yogurt, were found to produce a high IgG-response along with egg albumen and yeasts (Figure 3). Furthermore, 7 food antigens, e.g., pineapple, banana, kiwi, peanut, wheat, mushrooms, and egg-yolk (Figure 2), were found to give a moderate-high IgG-reactivity ranging between 15 and 20%.

We also analyzed the IgG response to the high reactive food antigens depending on the age of the 6,880 subjects (Figure 4). Two subgroups of subjects with a time span of 40 years were formed; one was composed of subjects born from 1904 to 1944, termed as the ''old group'', and the other of subjects born between 1967 and 2007, termed as the ''young group''. The data are illustrated in Table 3 in which the means of IgG response to the selected high reactive food antigens are reported for each subgroup, along with their standard errors and significance. As can be seen, albumin (and yolk, not shown) produced a significant ($p < 0.0001$) increase in the IgG response depending on age, approx. 90%. Milk, from cow and goat, and several milk derivatives, like different

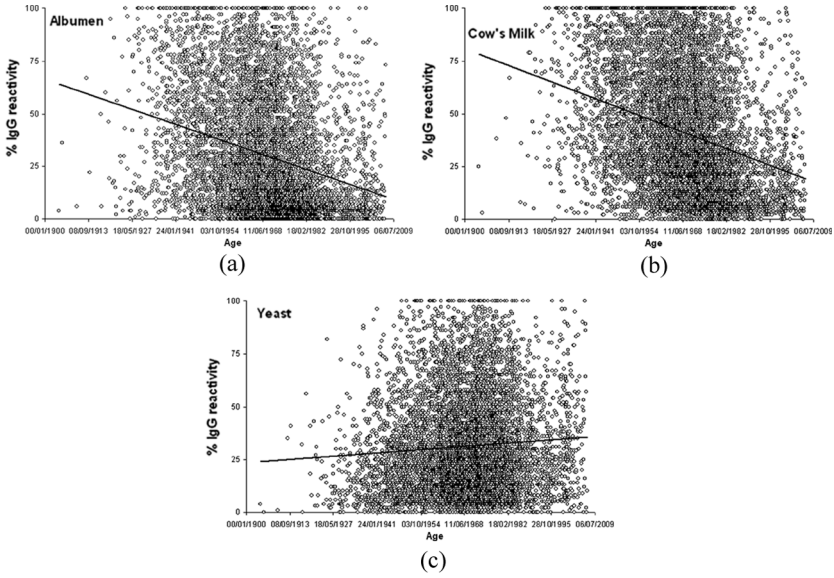


**Figure 4.** Regression line showing the distributions of percentages of IgG reactivity for several very high reactive (>20%) food antigens in relation to age of subjects.



kinds of cheese and yogurt, also showed a significant strong increase in the IgG reactivity in the group of "old" subjects, from about 33% up to 117% for mozzarella (Table 3). On the contrary, yeasts showed no significant modifications depending on age (Table 3).

No significant differences were found between female and male subjects.

## DISCUSSION

The ELISA test is the most accurate, specific, and sensitive analytical approach for the evaluation of serum IgE and IgG responses in normal and pathological subjects.[1,2] Other techniques, such as the cytotoxic test and ALCAT test (test for cellular responses to foreign substances), were found unproven and potentially dangerous, leading to misleading advice regarding treatments.[24–26] Furthermore, the preparation, processing, and stability of protein extracts for antigens was found to be a key point for an accurate IgE and IgG reactivity evaluation.[27,34] As a consequence, food antigens were accurately evaluated for protein content and quality by using a monodimensional SDS-PAGE separation to

**Table 3.** IgG response to the high reactive food antigens depending on the age of the 6880 subjects. Two subgroups of subjects with a time span of 40 years were formed; one composed of subjects born from 1904 to 1944, termed as the "old group", and the other of subjects born between 1967 and 2007 years, termed as the "young group". The data are reported as means of IgG response to the selected high reactive food antigens along with their standard errors and significance

| | IgG Response | St. Error | Difference (%) | Significance |
|---|---|---|---|---|
| Albumen 67–07 | 19 | 1.02 | | |
| Albumen 04–44 | 36 | 0.45 | 89.50 | $p < 0.0001$ |
| Cow milk 67–07 | 25 | 1.07 | | |
| Cow milk 04–44 | 47 | 0.46 | 88.00 | $p < 0.0001$ |
| Goat milk 67–07 | 21 | 0.96 | | |
| Goat milk 04–44 | 28 | 0.37 | 33.30 | $p < 0.002$ |
| Camembert 67–07 | 23 | 0.93 | | |
| Camembert 04–44 | 35 | 0.41 | 52.20 | $p < 0.0001$ |
| Emmenthal 67–07 | 16 | 0.86 | | |
| Emmenthal 04–44 | 30 | 0.42 | 87.50 | $p < 0.0001$ |
| Ricotta 67–07 | 26 | 1.13 | | |
| Ricotta 04–44 | 43 | 0.47 | 65.40 | $p < 0.0001$ |
| Dutch cheese 67–07 | 12 | 0.73 | | |
| Dutch cheese 04–44 | 25 | 0.38 | 108.30 | $p < 0.0001$ |
| Cheese spread 67–07 | 22 | 1.00 | | |
| Cheese spread 04–44 | 37 | 0.44 | 68.20 | $p < 0.0001$ |
| Parmesan 67–07 | 23 | 1.02 | | |
| Parmesan 04–44 | 39 | 0.45 | 69.60 | $p < 0.0001$ |
| Mozzarella 67–07 | 12 | 0.69 | | |
| Mozzarella 04–44 | 26 | 0.38 | 116.70 | $p < 0.0001$ |
| Sheep cheese 67–07 | 20 | 0.97 | | |
| Sheep cheese 04–44 | 32 | 0.43 | 60.00 | $p < 0.0001$ |
| Yogurt 67–07 | 22 | 1.02 | | |
| Yogurt 04–44 | 41 | 0.45 | 86.40 | $p < 0.0001$ |
| Yeast 67–07 | 32 | 1.06 | | |
| Yeast 04–44 | 30 | 0.36 | −6.25 | NS |
| Mix natural yeasts 67–07 | 21 | 0.88 | | |
| Mix natural yeasts 04–44 | 20 | 0.33 | −4.80% | NS |

assure reproducible production and quality control of the entire purification process. Furthermore, the capacity of each single extract to maintain its biological property was tested by using specific serum samples reactive towards each different food antigen to ensure biological quality control. Of course, monodimensional gel electrophoresis is unable to reach the resolution capacity of the bidimensional electrophoresis technique. However, the evaluation of the molecular mass value of the main extract

protein species and their relative percentage content determined by densitometric acquisition were found to represent quantitative and qualitative parameters able to give a reproducible and accurate food protein purification process. Finally, the ELISA method was validated and confirmed to be applicable in the evaluation and quantification of serum/plasma IgG reactivity to many food antigens.

120 food extracts were shown to have a mean IgG reactivity evaluated in approx. 6,880 subjects lower than 10% even if the IgG response was found to range between 0 and 100% for each of the food antigens evaluated, depending on the human subject. 44 antigens had an IgG response greater than 10% and only 14 aliments showed a reactivity greater than 20%. These very high reactive antigens were in particular from milk and milk derivatives, egg albumin (and yolk), and yeasts. As a consequence, the serum IgG evaluation produced a strong reactivity to proteins belonging to well-known allergen-food antigens.[28,11] Furthermore, the IgG-response to milk and milk derivative antigens, such as egg proteins, strongly increased in a subgroup of ''old'' subjects, born from 1904 to 1944, in comparison with a ''young'' group, born between 1967 and 2007. These data strongly support the hypothesis that increased antigen exposure, in particular for highly reactive proteins of very common aliments, produces an increased mucosal permeability[35] inducing a serum IgG production. Other very reactive antigens, e.g., yeasts, showed no significant modifications probably due to a lower capacity of these proteins to induce an increased mucosal permeability or due to a lower antigenic effect.

In allergic diseases, IgE is regarded as the most important immunoglobulin, but IgG antibodies have also been reported as being associated with allergic disorders.[34,36,37] In fact, it is well established that IgE to egg and milk are closely associated with the development of IgE to inhalant allergens.[38–41] However, also an increased IgG antibody level to foods, especially to milk and egg whites, but also to other aliments, was found to indicate an increased risk of having IgE to common allergens[10,11] in addition to a relation between increased IgG antibody level to foods and IgE antibodies to inhalant allergens, egg and milk, even in atopic subjects.[2] However, these studies were generally performed on low-number groups due to the necessity to have well controlled conditions, such as the age of the subjects or association with specific allergic sensitization and disease. In our study, we demonstrated a high IgG response in a very large subject group, approx. 6,900, to milk and milk derivatives, and egg albumin antigens. These data strongly support previous studies[2,5,10,11] showing that not only IgE-positive levels to milk and egg are useful indicators of atopic as well as topic allergic manifestations, but also IgG antibodies are equally good predictors. Furthermore, we showed a significant strong increase in the IgG levels to milk and egg

proteins with age confirming that allergic sensitization and disease increase with age[42,43] along with an increase in the serum IgG response.

In an ovalbumin-induced allergic rat model,[23] a chronic food allergy with morphological changes in the intestinal mucosa was established. Serum ovalbumin-specific IgG and IgE levels were increased in animals repeatedly exposed to this antigen, accompanied by increased infiltration of T lymphocytes in the small intestinal mucosa significantly accumulated in villus microvessels as well as in Peyer's patches via a MAdCAM-1-dependent process. Furthermore, according to Salvaggio et al.[35] the increased IgG response to milk and egg antigens in ''old'' subjects strongly suggests an increased permeability to macromolecules able to produce an increased antigen exposure.

## ABBREVIATIONS

CV%, Standard deviation percentage; ELISA, Enzyme-linked immunosorbent assay; GLM, General linear model; HRP, Horseradish peroxidase; LOD, Limit of detection; LOQ, Limit of quantitation; PVP, polyvinylpyrrolidone; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TMB, 3,3′,5,5′-Tetramethylbenzidine.



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