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Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Analysis of the potential allergenicity of traditional apple cultivars by Multiplex Biochip-Based Immunoassay

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ARTICLE INFO

Article history:

Received 11 May 2011

Received in revised form 15 December 2011

Accepted 13 April 2012

Available online xxxx

Keywords:

Apple

Traditional cultivars

Pomological characterisation

Allergen pattern

Multiplex allergen microarray

SPHIAa test

ABSTRACT

Few apple cultivars are widespread in the markets worldwide. The characterisation of cultivars available only in local markets could reveal fruits with sufficiently low amounts of allergens to be tolerated by at least some allergic subjects. This study was focused on the characterisation of 10 ancient and 2 widespread apple cultivars cultivated in Southern Italy. Pomological characterisation showed good sensory features and high manipulation resistance for most of the selected ancient cultivars. Individual patterns of allergenic components were obtained when IgE, IgG and IgG4 inhibition tests were performed on a multiplexed allergen microarray, using sera from allergic subjects as probes. Results obtained allow the selection of potentially hypo- or hyper-allergenic apple cultivars. In addition, this study describes a possible use of allergen microarray technology to quickly investigate the potential allergenicity of specific foods that, after additional tests, can be considered for the inclusion in the diet of individual allergic subjects.

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1. Introduction

Apple (*Malus x domestica* Borkh.) is one of the most commonly eaten fruit worldwide. Several reports have described the healthy effects of apples due to the secondary metabolites contained in significant amounts in the freshly consumed fruit. For instance, many studies show that apples are a rich source of phenolic compounds with a high level of antioxidant activity (Chinnici, Bendini, Gaiani, & Riponi, 2004; Miene, Klenow, Veeriah, Richling, & Glei, 2009). Polyphenols help to neutralise free radicals, which are unstable molecules linked to the development of a number of pathologies including cancer, cardiovascular disease, cognitive impairment, immune dysfunction, cataracts and macular degeneration (Leborgne, Maziere, Maziere, & Andrejak, 2002; McCann et al., 2007). Unfortunately, some individuals have to avoid apple consumption because this fruit may also cause allergic reactions.

Four allergenic proteins have been identified in apple so far, and named Mal d 1, Mal d 2, Mal d 3 and Mal d 4 following the official WHO-IUIS nomenclature (www.allergome.org). Mal d 1 is a major

apple allergen, homologous to Bet v 1, the major birch pollen allergen. It is found in similar amounts in the pulp and in the peel of the fruit (Hoffmann-Sommergruber, 2005). It usually induces mild allergic symptoms that are often limited to the oral allergy syndrome, probably because the allergen is labile and sensitive to pepsin digestion and to heat (Andersen, Hall, & Dragsted, 2009; Bohle et al., 2006). It has been reported that in some geographical areas, such as Northern and Central Europe, apple allergy is associated to birch pollinosis (Andersen et al., 2009), probably resulting from a primary sensitisation to pollen and a subsequent cross-reaction of IgE antibodies synthesised against Bet v 1 and recognising also Mal d 1 epitopes. Conversely, in Southern Europe the allergic reaction to apple Mal d 3 seems to be related to a peach allergy via its homologous allergen Pru p 3 (Andersen et al., 2009; Fernandez-Rivas et al., 2006). Mal d 3 is a lipid transfer protein (LTP) especially abundant in the peel of the fruit. It may lead to severe symptoms probably connected to its high heat and proteolysis resistance (Fernandez-Rivas & Cuevas, 1999). The clinical relevance of the other known apple allergens, the thaumatin-like protein (TLP) Mal d 2 and the profilin Mal d 4 is still a matter of debate (Hoffmann-Sommergruber, 2005).

Some literature reports have described a significant variation of the allergen composition in different apple cultivars (Carnes,

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Ferrer, & Fernandez-Caldas, 2006; Sancho et al., 2008; Zuidmeer et al., 2006). In line with the results reported for other fruits, such as peach (Ahrazem et al., 2007) and kiwifruit (Ciardiello et al., 2009), it has been demonstrated that several factors, such as the cultivation method, the ripening stage and the storage conditions, may affect the allergen profile of the apple (Bolhaar et al., 2005; Matthes & Schmitz-Eiberger, 2009). Therefore, the characterisation of the available apple cultivars may provide important information on their potential allergenicity and allow the selection of hypoallergenic fruits the consumption of which can be suggested for the diet of subjects having mild symptoms following apple ingestion. Furthermore, these studies may have significant implications for clinical research, diagnostics and immunotherapy (Asero, Marzban, Martinelli, Zaccarini, & Machado, 2006; Son, Scheurer, Hoffmann, Haustein, & Vieths, 1999).

In general, the very few cultivars available on the market are those that have been better characterised. However, since more than hundred apple cultivars exist, it would be worth to investigate also those present only in local markets, because some of them might contain sufficiently low amounts of allergens to be tolerated by allergic subjects. The identification of hypoallergenic cultivars could induce their exploitation by apple growers in order to deal with the requests of allergic consumers.

Several ancient apple cultivars are grown in the Campania region, which is an area in Southern Italy having a great importance for apple production. Several cultivars, such as *Annurca*, *Limoncella*, *Mela Rosa* and *Zitella*, are traditionally cultivated in that region since ancient Roman times and are highly appreciated for their peculiar flavours and aromas (Guarino et al., 2007; Lo Scalzo, Testoni, & Genina, 2001). Apple cultivars such as *Annurca*, *Limoncella* and *Mela Rosa* have been reported to be rich in polyphenols (Cefarelli, D'Abrosca, Fiorentino, Izzo, & Monaco, 2005), mostly flavonoids whose activity and concentration vary with cultivar, ripening stage, environmental condition and fruit section (Aaby, Hvattum, & Skrede, 2004).

Taking into consideration the increasing interest in the revaluation of typical products and ancient flavours together with the demand of safe diets for allergic subjects, the aim of this study is the analysis of the pomological properties and of the protein and allergen contents in ten apple cultivars traditionally cultivated in the Campania region as well as in two widespread standard cultivars. Two different approaches have been used: a classical biochemical one, and a second using advanced immunochemical technologies. Using the former, the fruits were preliminary characterised by estimating the physical and chemical harvest indexes, and then used to measure the relative protein concentrations and to obtain the protein profiles on SDS–PAGE. The latter approach has been used to establish the allergen content, and therefore the potential allergenicity, by investigating the immunoglobulin (Ig; IgG, IgG4 and IgE isotypes) binding as performed by the Ig inhibition on a multiplex allergen microarray using the immobilised molecules as antigens, and the protein extracts of the twelve cultivars as inhibitors. Such approach has also been used to set a fast and approachable mean useful for similar purposes, not limiting the analysis to already known allergens.

2. Materials and methods

2.1. Apple samples

Twelve apple cultivars growing in the geographical area of the Campania region (Southern Italy) have been selected for this study and analysed immediately after harvesting. Ten of them are traditional cultivars available only in local markets: *Chianella di Volturara*, *Cassanese di Volturara*, *Bianca di Grottolella*, *Annurca Rossa del Sud*, *Suricillo di Volturara*, *Renetta di Serino*, *Fierro di Bucciano*, *Mela*

Rosa S. Nazzaro, *Sergente di Bagnoli Iripino*, and *Limoncella di Pater-nopoli*. Two of them are standard cultivars, available in worldwide markets: *Red Chief* and *Golden B*. Ten fruits of each cultivar were harvested at commercial ripening stage and used to analyse the main quality indexes. Pomological and qualitative traits were evaluated according to the *International Union for the Protection of New Varieties of Plants* (UPOV) descriptors. The physical and chemical harvest indexes were estimated as follows: (i) Total soluble solids content (TSS, °Brix) was determined on the flesh juice using a digital refractometer (Sinergica Soluzioni, DBR35, Pescara, Italy); (ii) Firmness (expressed as kg/cm²) was measured, after removing skin, on two opposite sides of five samples for each cultivar, by a digital penetrometer equipped with a 11 mm tip (TR snc, Forlì, Italy); (iii) Total acid content (TA), was determined by titrating 10 ml of flesh juice with 0.1 N NaOH (Wills, Scriven, & Greenfield, 1983). Results are expressed as g malic acid/L juice.

The fruits from all the twelve cultivars were stored in a refrigerator at 2 °C and 95% relative humidity for 150 days to evaluate the effect of cold storage. Physical and chemical harvest indexes were determined every 20 days to monitor their variation. Visual analysis were also performed to monitor any sign of rot and to evaluate the flesh decomposition degree. To define the maximum cold-storage time we evaluated the decrease of firmness, the physiological weight loss, and the number of rotted fruits due to pest attacks.

The firmness values were used to evaluate the manipulation resistance of each cultivar. The level of manipulation resistance was defined as follows: “discrete” when fruits have firmness values of 6–7 kg/cm², “good” when fruits have firmness of 8–9 kg/cm², and “high” for fruits with firmness values higher than 9 kg/cm².

For the protein extracts preparation, three samples of each cultivar, collected at the commercial ripening stage, were peeled and sliced, the seeds were removed, and the pulp samples were stored at –80 °C.

2.2. Protein extracts preparation

For each cultivar, the protein extracts from three fruits collected from the same tree were separately prepared and analysed. It is well known that an optimal extraction of different proteins would require different procedures. We chose a well-tested procedure showing a good efficiency in the extraction of several allergens. To protect proteins against oxidative damages, antioxidants were added. Briefly, 50 g of apple pulp were homogenised after addition of 25 ml of 2 M NaCl, 2 g of poly(vinylpyrrolidone) (Sigma, Steinheim, Germany), 0.820 ml of 0.12 M ethylenediaminetetraacetic acid (Sigma, St. Louis, USA), and 2.2 g of L-ascorbic acid (Panreac, Barcelona, Spain) dissolved in 25 ml of water and brought to pH 3.5 by the addition of NaOH. The final volume of the homogenate was 100 ml. After one hour on ice, the samples were centrifuged at 17,300g for 45 min, and about 50 ml of supernatant from each sample, representing the protein extract, were collected. To reduce the NaCl concentration from 0.5 to 0.1 M, all the extract samples were diluted five times by the addition of bidistilled water. Then, in order to obtain a solution with a protein concentration as close as possible to that present in the fruit, the extract volume was reduced ten times by ultrafiltration using Ultracel 3 K Amicon Ultra centrifugal filters (Millipore Corporation, Billerica, USA).

Protein concentrations were determined by the BIO-RAD Protein Assay (Biorad, Milan, Italy), using calibration curves made with bovine serum albumin, and statistical data analysis was carried out by SigmaPlot (Systat Software, Inc.).

2.3. Analysis of the protein components in SDS–PAGE

Protein samples (15 µg) of each extract were analysed by reducing 15% SDS–PAGE. Large volumes of protein extract solutions

were precipitated by the addition of trichloroacetic acid at a 1:10 ratio (vol:vol, final concentration 10%). After 30 min on ice, the samples were centrifuged at 12,000g for 10 min. The pellets were resuspended in SDS–PAGE loading buffer and then loaded on the gel. Following electrophoresis, proteins were stained with Coomassie Brilliant Blue.

2.4. IgE inhibition assay

Serum samples were obtained after informed written consent from allergic subjects affected by different allergic reactivity to several fresh fruits and vegetables, ranging from very mild oral allergy syndrome to severe anaphylactic reactions. Patients' sera were routinely screened for specific IgE using the ISAC 103 microarray system (PMD, Vienna, Austria) (Scala et al., 2010), thus their sensitisation profile was known in details and used for their selection. The study protocol has been approved by the Institutional Ethical Committee (IDI-CE 2005-106). In order to get the highest number of relevant allergens to be identified, one serum pool was obtained by mixing equal volumes of individual sera from seven subjects. Overall the following specificities were detected once the pooled sera were tested on the ISAC103: lipid transfer proteins, pathogenesis related-proteins 10, profilins, calcium binding proteins, cross reactive carbohydrates determinants, papain-like cysteine proteases, 11S globulins, vicilins, conglutins, 2S albumins, hevein-like proteins and alpha-amylase trypsin inhibitor. Detailed information about allergens listed in the present study can be obtained from the Allergome database (www.allergome.org).

IgE inhibition experiments were performed as previously reported (D'Avino et al., 2011). Briefly, 20 µl of the serum pool were incubated overnight with 20 µl of a solution containing, approximately, the amount of proteins deriving from 20 mg of apple pulp. After incubation, the IgE binding inhibition was evaluated by running the ISAC 103 microarray IgE assay (PMD), where recombinant and natural allergens of interest for the present study are immobilised in triplicate, as reported above. A control sample, where only the buffer solution was added, was used as reference value for no inhibition IgE values. The assay was run as a single point highest inhibition achievable assay (SPHIAa) (D'Avino et al., 2011). The testing protocol was adapted for IgG and IgG4 detection by mixing the additional two secondary antibodies (PMD) with the anti-IgE. To discriminate the different Ig determinations a different fluorochrome was used for both IgG4 and IgG, whereas the routine one was used for IgE detection. A two step procedure was set. The anti-IgE and the anti-IgG4 were mixed and used together. After incubation, washing, and first microarray image acquisition, the anti-IgG was added and incubated for 30 min. The same washing and image acquisition procedures were applied to the anti-IgG. All Ig values, either inhibited or not, were obtained by the triplicated spotting, thus simulating the usual replicated experiments. A mean value of the three raw values has been considered as the Ig inhibition result.

2.5. Data handling and processing

Pomological parameters were expressed as mean and standard deviation. The statistical differences among the twelve different cultivars were calculated with StatGraphic Software by LSD Test at 95% of confidence. This test applies a multiple comparison procedure to determine which means are significantly different from each others. The method used to discriminate among the means is Fisher's least significant difference (LSD) procedure.

All the clinical and routine diagnostic data related to each patient were saved in an allergy e-record as soon as they were made available from the patient (InterAll, Allergy Data Laboratories s.c., version 3.0, Latina, Italy). InterAll software is fully interfaced with

the ISAC system (PMD), allowing to store IgE, IgG4, and IgG data in real time. Data processing for the evaluation of SPHIAa results was performed by using the same InterAll software.

The statistical evaluation of each Ig inhibition difference, for each allergen between apples cultivars was calculated by comparing the 95% confidence intervals for each inhibition experiment. These intervals were obtained calculating the distribution probability for Ig measurement ratio, considering as uniform the distribution of a single Ig measure. Statistical significance level was set at a *p* value <0.05.

3. Results

3.1. Pomological characterisation of the fruits

The physical and chemical features of the fruit samples used in this study are summarised in Table 1. The harvesting time of the different cultivars ranged from late September to early November. The mean weight of the traditional apples fell in the range from 67.5 ± 3.06 to 123.8 ± 6.35 g. The highest values were recorded for standard varieties, namely *Golden B* and *Red Chief*, showing weights of about 190 and 195 g, respectively. Firmness, soluble solids (RSR) and total acidity (TA), are important parameters used to predict the consumer's perception of the fruit quality. The estimated soluble solids content was quite similar in the different cultivars, whereas some significant differences were recorded for the total acid content. In apple, it is generally established that the lowest value of accepted fruit firmness is 6/7 kg/cm². This is a value close to that obtained for *Renetta di Serino* and *Limoncella di Pater-nopoli*, whereas higher values were recorded for the other cultivars showing firmness ranging from 8 and 9.9 kg/cm². The firmness values are also used to predict the manipulation resistance of fruits. Results obtained indicate that only two traditional cultivars, *Renetta* and *Limoncella*, have a resistance to manipulation lower than that of the two standard ones, *Red Chief* and *Golden B*. The others have a resistance similar, and sometimes even higher (*Chianella*, *Suricillo*) than that of standard cultivars. This means that most of the tested cultivars have a good manipulation resistance, in terms of resistance to mechanical stresses or to normal physiological processes. A variability in the resistance to cold storage was observed. However, all the tested cultivars showed a good attitude to cold storage since it was possible to store most of them in the refrigerator for at least 120 days and some of them even for 150 days or more without significant alteration of chemical and physical parameters. In comparison with the standard ones, four traditional cultivars showed a similar resistance to cold storage, three a lower resistance and four an even higher resistance. In summary, the results obtained indicate an overall good inner quality of the traditional cultivars compared to the standard ones.

3.2. Extracts preparation

After salt dilution and sample concentration by ultrafiltration, the volume of each extract was adjusted to 1 ml/g of homogenised fruit pulp. Therefore, the experiments here described were performed using extract solutions having a protein concentration as close as possible to that present in the pulp of each fruit.

3.3. Analysis of the protein concentration in the extracts

The estimation of the protein concentration was carried out using the BIO-RAD Protein Assay. Fig. 1 shows the mean values and the standard deviations of the protein concentration determined in three different extracts each deriving from one of the three fruits collected from the same apple tree. The highest value

Table 1
Main features of the 12 apple cultivars at harvesting time and their storability.

	Cultivar	Harvesting time	Manipulation resistance	Fruit weight (g) ^A	Firmness (kg/cm ²) ^A	RSR (° Brix) ^A	Total acidity (TA) (g malic acid/L juice) ^A	Cold storage attitude (days)
1	Chianella di Volturara	Late October	Good	(114.8 ± 3.29) ^d	(9.1 ± 0.39) ^g	(14.64 ± 0.17) ^e	(7.37 ± 0.28) ^e	>150
2	Cassanese di Volturara	October/November	Good	(85.6 ± 3.10) ^b	(7.63 ± 0.24) ^c	(15.32 ± 0.50) ^f	(10.05 ± 0.41) ^h	>150
3	Bianca di Grottolella	Mid October	Good	(123.8 ± 6.35) ^e	(7.55 ± 0.23) ^c	(12.7 ± 0.60) ^a	(4.3 ± 0.28) ^b	<150
4	Annurca Rossa del Sud	Mid October	Good	(106.9 ± 3.10) ^c	(8.5 ± 0.43) ^f	(13 ± 0.69) ^{ab}	(9.0 ± 0.39) ^g	>150
5	Red Chief	Mid October	High	(195.5 ± 3.27) ^g	(9.91 ± 0.37) ^h	(13.1 ± 0.53) ^{ab}	(6.5 ± 0.44) ^d	<150
6	Suricillo di Volturara	Late October	Good	(67.5 ± 3.06) ^a	(8.46 ± 0.20) ^{ef}	(13.8 ± 1.10) ^{cd}	(6.36 ± 0.36) ^d	<120
7	Renetta di Serino	September/October	Discrete	(194 ± 4.27) ^g	(6.5 ± 0.44) ^a	(13.96 ± 0.76) ^{cd}	(5.02 ± 0.41) ^c	<150
8	Fierro di Bucciano	Late October	Good	(105.3 ± 3.16) ^c	(7.2 ± 0.53) ^b	(13.4 ± 0.86) ^{bc}	(3.35 ± 0.51) ^a	<120
9	Mela Rosa S. Nazzaro	Mid October	Good	(108 ± 5.57) ^c	(8.26 ± 0.26) ^e	(15.3 ± 0.51) ^f	(7.37 ± 0.42) ^e	<120
10	Sergente di Bagnoli Irpino	Early October	High	(105.2 ± 0.38) ⁱ	(11.1 ± 0.42) ^j	(13.48 ± 0.28) ^{bcd}	(7.87 ± 0.31) ^f	<150
11	Golden B di Pignataro	September/October	Good	(190 ± 4.67) ^f	(7.67 ± 0.31) ^c	(14 ± 0.54) ^d	(4.5 ± 0.20) ^c	<150
12	Limoncella di Paternopoli	September/October	Discrete	(106.5 ± 3.10) ^c	(7.0 ± 0.42) ^b	(14.7 ± 0.27) ^e	(7 ± 0.36) ^e	>150

^A Means with the same letter are not significantly different at $p = 0.05$ (LSD test).

(0.12 mg/g of fruit pulp) was measured for the cultivar *Fierro di Bucciano* (Fig. 1). A significant variation of the protein concentration can be observed. In fact, the highest protein content (sample 8) is 34-fold higher than the lowest one (sample 3).

3.4. Protein profiles

The protein pattern of the extracts was analysed by SDS-PAGE (Fig. 2). Variations in the number and relative abundance of the components were observed, and each protein profile was different from all the others. Some components appear to be characteristic of a specific cultivar, whereas other proteins seem to be shared among different cultivars. For instance, a variable amount of a 60 kDa protein band was found in all the apple extracts, whereas a 35 kDa protein, which seems to be absent in four cultivars (Fig. 2, samples 1, 3, 8, 10), represents the most abundant component in three of the others (Fig. 2, samples 4, 5, 11).

3.5. Immunoglobulin-binding inhibition experiments

Fig. 3 shows the IgE and IgG inhibition values only on already described allergen types, either as true apple allergens (Mal d 1) or as apple allergen homologs (TLP, LTP, profilin). Since more than one homolog of Mal d 3 and Mal d 4 were available on the microarray, only one of them, chosen among those displaying the highest inhibition values, were selected and included in the graph. As reported in Fig. 3, obtained Ig inhibition values were different for the 12 cultivars, even when considering a single protein as for TLP or Mal d 1.

Fig. 4 (panels A and B) shows the IgE, IgG and IgG4 inhibition data obtained with pollen profilins and the other plant food allergens available on the ISAC microarray, recognised by the serum pool used in these experiments. The list of the tested allergens (Table 2) includes five profilins (Bet v 2, Hev b 8, Mer a 1, Ole e 2, Phl p 12), seven Bet v 1-like molecules, belonging to the PR-10 protein family, (Api g 1, Ara h 8, Cor a 1, Dau c 1, Gly m 4, Mal d 1 and Pru p 1), two lipid transfer proteins (LTP) (Cor a 8 and Pru p 3), four 11S globulins (Ana o 2, Ara h 3, Cor a 9 and Gly m 6), two vicilins (Ara h 1 and Gly m 5), actinidin (Act d 1), a TLP (Act d 2), a conglutin (Ara h 2), a 2S albumin (Ses i 1), a hevein-like protein (Tri a 18), and an alpha-amylase/trypsin inhibitor (Tri a 30). The list of the components analysed in this study includes Ana c 2, that is not considered a real allergen, but is rather used as a marker of the presence of IgE to cross-reactive carbohydrate determinants in the sera (Mari, 2002). Eleven out of the twelve apple extracts used in these experiments totally inhibited the IgE binding to Ana c 2.

The data obtained revealed a high variability of the inhibition profiles as a function of the extract used to inhibit the Ig binding.

Extracts number 4 and 8 showed the highest and the lowest level of Ig-binding inhibition, respectively. Differences for inhibition capability were statistically evaluated and significance is reported in the Supplementary file.

A broad range of inhibition values were recorded for Mal d 1 and for the other Bet v 1-like allergens. Eleven extracts produced partial inhibition of IgE binding to Mal d 1, characterised by a high variability of the values, including an almost complete inhibition achieved by the sample 4. No inhibition on Mal d 1 was obtained with the extract number 2. However, the inhibition of other Bet v 1-like allergens strongly suggests the presence of Mal d 1 homologs also in the extract 2. For instance, 60% of IgE-binding inhibition on Gly m 4 and a complete inhibition of IgG4 on Dau c 1 were observed.

In general, a low level of IgE inhibition on Dau c 1 and Api g 1 was detected. Only the extract 4 showed a partial IgE inhibition on Dau c 1, whereas two extracts inhibited none of the tested antibodies on this allergen. Eight extracts displayed 100% inhibition of IgG4 on Dau c 1 in the absence of IgE inhibition. Again, only the extract 4 displayed a significant IgE inhibition (56%) on Api g 1, together with a complete inhibition of IgG4 binding. Eight extracts produced 0% inhibition on Api g 1 for each of the tested antibodies. Five extracts produced a partial inhibition of IgE binding on Gly m 4, whereas six extracts provided 0% inhibition of IgG, IgG4 and IgE. The inhibition of IgE binding to Cor a 1 was quite similar, and for some extracts even higher, than that observed on Mal d 1, Ara h 8 and Pru p 1. The extract number 4 provided a complete IgE inhibition on Cor a 1. Only the apple samples 3 and 4 produced IgE inhibition (41% and 44%, respectively) on the pollen allergen Bet v 1 (data not shown).

Three apple cultivars (samples 4, 5 and 6) produced a significant IgE inhibition, with values higher than 50%, on both the plant food LTPs immobilised on the ISAC microarray. The extract number 12 showed IgE inhibition on Cor a 8, significantly higher than that on Pru p 3. Some extracts partially inhibited the IgG and/or IgG4 binding on the two LTPs. The extract number 7 did not produce any Ig inhibition on both Pru p 3 and Cor a 8.

IgE-inhibition values ranging from 30% to 90% were obtained on all the five profilins available on the ISAC microarray for all the 12 cultivars. Complete inhibition of IgG, IgG4 and IgE on some profilins was detected for the cultivars 1, 3, 4 and 5.

Generally, a slight or absent IgE inhibition on the allergens of the 11S globulin protein group, Ana o 2, Ara h 3, Cor a 9 and Gly m 6, was observed. Only the extract number 4 provided an IgE-inhibition value higher than 50%. Nevertheless, some extracts showed a 100% IgG4 or IgG inhibition, sometimes in the absence of IgE inhibition.

Two extracts, samples 3 and 4, produced an IgE inhibition higher than 50% on the two vicilins, Ara h 1 and Gly m 5, whereas the

others showed low or absent inhibition. Only two apple cultivars provided a partial IgG4 or IgG inhibition on the vicilins.

None of the extracts provided IgE inhibition, even very low, on Act d 1, Act d 2 and Tri a 18. However, some extracts fully inhibited IgG and/or IgG4 binding on these allergens. Very low inhibition of all the three tested antibodies on the conglutin Ara h 2 was observed. Nine extracts did not produce any inhibition on Ses i 1, whereas the remaining three extracts fully inhibited the IgG binding. Just one extract produced a slight IgE inhibition on Tri a 30, whereas some other extracts provided IgG and/or IgG4 inhibition.

4. Discussion

The protection of biodiversity through the recovery of valuable indigenous germplasm plays an important role in the revival of agriculture in marginal areas. Recently it has been recorded an increasing interest in ancient fruits which leads to the revaluation of varieties which, compared to others more commercially known, have better nutritional and organoleptic characteristics, greater resistance to the most common biological adversities and a good cold storage attitude. In line with this observation, in the present study we selected ten traditional and two standard apple cultivars, growing in the geographic area of the Campania region (Southern Italy). The analysis of the major pheno-pomological parameters indicated that almost all the selected cultivars are characterised by good sensory features and high manipulation resistance giving them a good storability.

The analysis of the protein concentration, the profile on SDS-PAGE and the pattern of allergen inhibition on the multiplex microarray system (D'Avino et al., 2011; Scala et al., 2010) revealed that a unique combination of features characterises each of the twelve investigated apple cultivars. Individual patterns of IgG, IgG4 and IgE inhibitions were obtained for the cultivars tested on the available allergens. A significant variation of the protein concentration without a correlation with the potential allergenicity was observed in the protein extracts. For instance, the *Fierro di Bucciano* cultivar (sample 8) displayed the highest protein concentration associated to the lowest IgE inhibition, both in terms of percentage of inhibition and of number of inhibited allergens. Cultivars, such as *Fierro di Bucciano*, combining low IgE inhibition with good resistance to manipulation and storability of the fruit, could be selected as potentially hypoallergenic apple that after further appropriate testing and controlled challenge

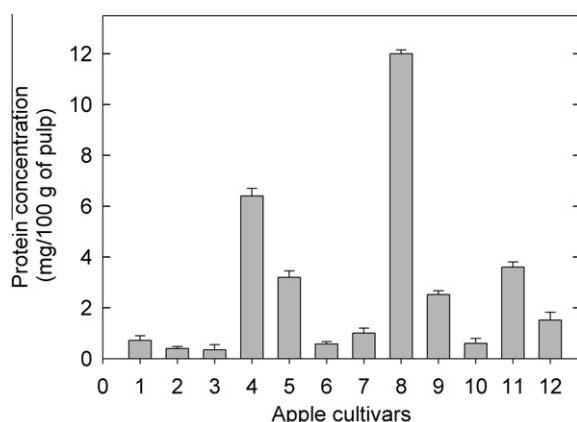


Fig. 1. Protein concentration, determined by the Bio-Rad Protein Assay, in the extracts obtained from the 12 apple cultivars. The cultivar numbering is that of Table 1. Bars indicate deviations from the mean values of measurements performed on the extracts from three different fruits for each cultivar.

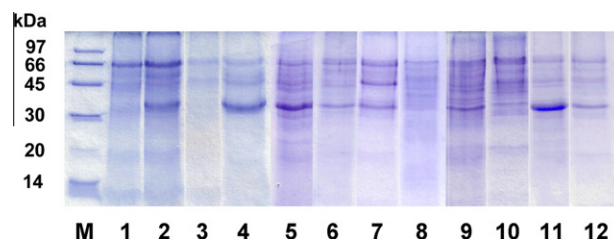


Fig. 2. SDS-PAGE of the extracts obtained from the 12 cultivars. The cultivar numbering is that of Table 1.

could be considered for the inclusion in the diet of selected allergic subjects. In contrast, the *Annurca Rossa del Sud* (sample 4) is characterised by a relatively high protein concentration and the highest potential allergenicity, as suggested by both the high IgE inhibition values obtained and the high number of allergens to which IgE binding was inhibited. The *Bianca di Grottolella* cultivar is characterised by the lowest protein concentration associated to a high level of IgE inhibitions, close to that of *Annurca Rossa del Sud*.

The IgE binding inhibition to some allergens, such as Act d 2, the homolog of Mal d 2, was never achieved by the pool of sera used in this study. However, the detection of IgG and/or IgG4 inhibitions on this allergen reveals the presence of protein molecules bearing one or more antigenic epitopes shared with Mal d 2, in all the twelve tested extracts. Nevertheless, the lack of IgE recognition suggests that Act d 2 and Mal d 2, or at least the Mal d 2 molecules/isoforms contained in the analysed cultivars, may bear unshared IgE-binding epitopes. In addition to Act d 2, none of the extracts inhibited IgE binding to Act d 1 and Tri a 18, although some extracts produced IgG and/or IgG4 inhibitions on these allergens. These results provide information on the possible presence in the apple fruits of protease (Act d 1) and hevein-like (Tri a 18) homologs, or at least protein structures bearing non-IgE antigenic epitopes shared with these allergens. Similarly, the possible presence of homologs of other allergenic proteins, such as 11S globulins, 2S albumins, vicilins, etc., could be inferred on the basis of the overall Ig-inhibition results.

Mal d 3 is an important apple allergen and, although it was not spotted on the ISAC103 microarray, it was possible to detect its presence in the extracts because of the availability on the microarray of two plant-derived food homologs, Pru p 3 and Cor a 8.

The presence of Mal d 4 in all the apple extracts was detected by the Ig inhibition on the five pollen profilins available on the microarray. The IgE-inhibition values, ranging from 30% to total inhibition, suggest a high degree of co-recognition between Mal d 4 and pollen profilins.

Mal d 1, as well as Bet v 1-like allergens, are usually described as labile proteins. Nevertheless, the results obtained in this study, displaying total IgE and/or IgG inhibition on Mal d 1 by at least some extracts, suggest that the used experimental conditions were suitable for the extraction and preservation of Bet v 1-like allergens. For instance, the extract of *Annurca Rossa del Sud*, sample 4, produced full IgG and IgE inhibition on Mal d 1 and about 40% of IgE inhibition on Bet v 1. Therefore, we argue that some IgE-binding epitopes of Bet v 1 are shared with isoforms of the major apple allergen Mal d 1 present in some apple cultivars, and probably may lead to co-recognition between these pollen and fruit homologous allergens. Furthermore, in line with many reports in the literature (Andersen et al., 2009; Ebo et al., 2010; Fernandez-Rivas et al., 2006), we cannot exclude the possibility that a primary sensitisation to pollen-derived Bet v 1-like molecules (Mari, Wallner, & Ferreira, 2003) might have induced the

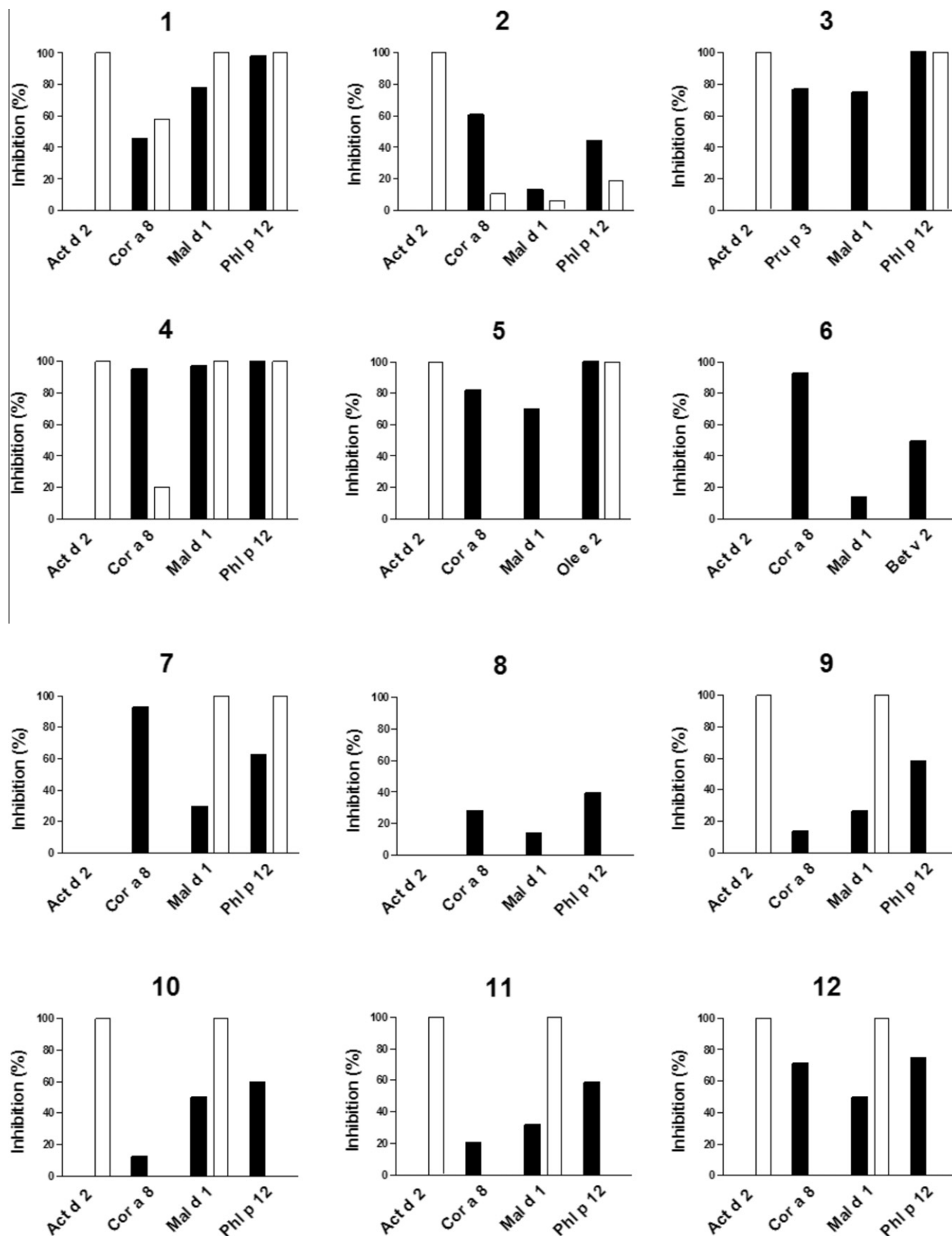


Fig. 3. Percentage of inhibition of IgG binding to TLP (Act d 2) (white bars) and of IgG (white bars) and IgE (black bars) binding to TLP (the TLP showing the highest IgE inhibition was selected), Bet v 1-like allergen (Mal d 1) and profilin (the profilin showing the highest IgE inhibition was selected). The cultivar numbering is that of Table 1.

synthesis of specific IgE capable of cross reactivity with Mal d 1 present in these cultivars. However, the observation that ten

cultivars did not inhibit Bet v 1 (data not shown), although they produced some inhibitions on Mal d 1 and/or other Bet v 1-like

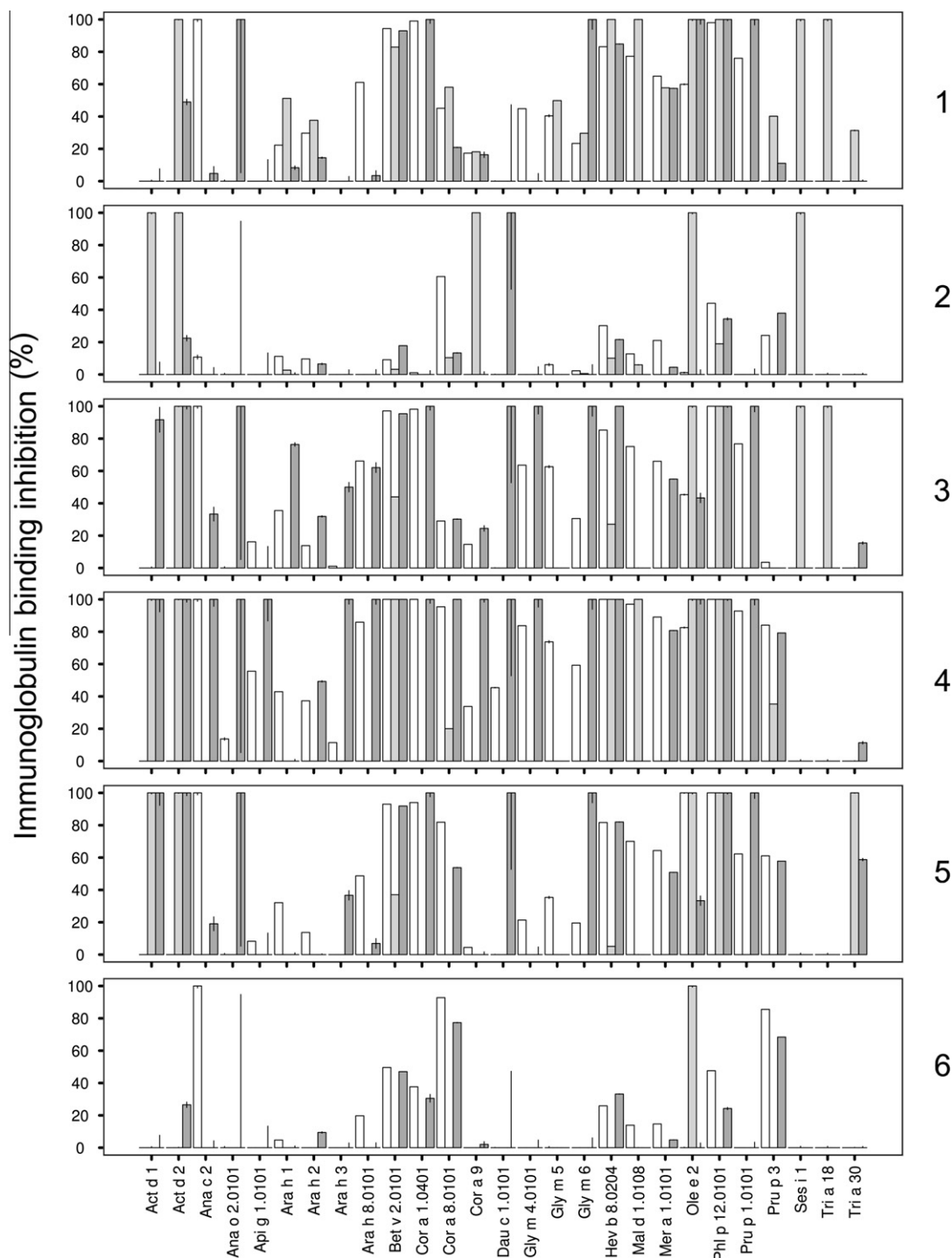


Fig. 4. (Panel A and B) Percentage of inhibition of IgE (white bars), IgG (light grey bars), and IgG4 (dark grey bars) binding to the allergens indicated on the X axis, obtained for the extracts from the different cultivars. The cultivar numbering is that of Table 1. Statistical evaluation has been carried out by comparing the 12 cultivars. Statistical significance is fully reported in the Supplementary file.

allergens, provides evidence of the presence in these samples of Mal d 1 isoforms bearing antigenic epitopes not shared with Bet v 1. Therefore, the recognition of these epitopes cannot be due to cross reactivity between Bet v 1 and Mal d 1. This is in line with literature reports showing that a different pattern of Mal d 1 isoforms, associated to variable allergenicities, can be found in different apple cultivars (Gao et al., 2008; Matthes and Schmitz-Eiberger, 2009).

In conclusion, in addition to the comparative characterisation of 12 apple cultivars, this study describes the potential use of an

allergenic molecule-based microarray to investigate the potential allergenicity of specific foods. It underlines that a significant collection of information related to the possible allergenicity of individual cultivars can be obtained by exploiting the multiplex inhibition method on ISAC system by performing Ig inhibitions in a single run with a very low amount of both patients' sera and extract preparations. Although this methodology does not provide information on the concentration of individual allergens in the extract, unless the molecule present in the sample is exactly the same spotted on the microarray, it anyhow allows the

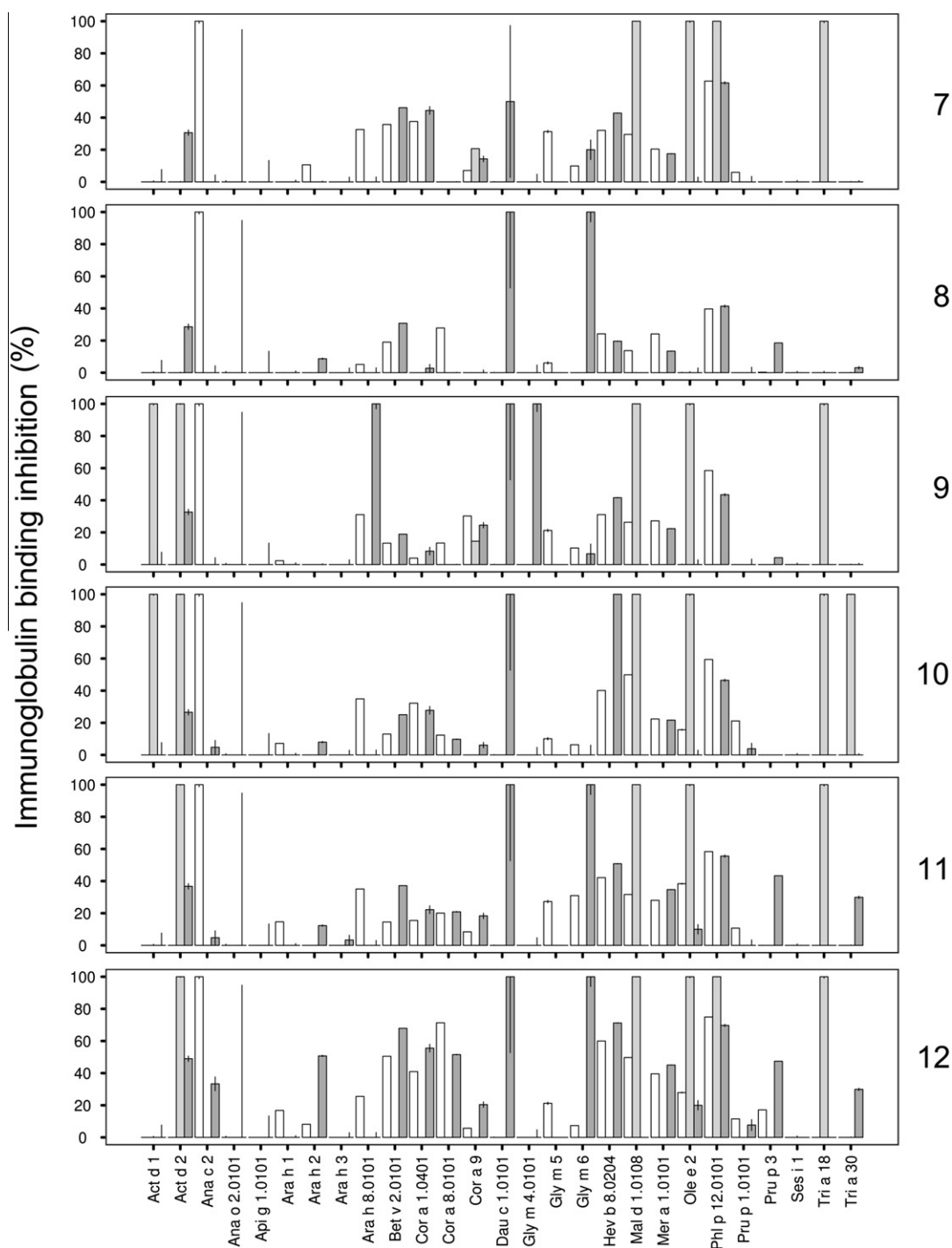


Fig. 4 (continued)

identification of allergenic molecules and their homologs in the sample. In fact, even a partial inhibition of the binding, to one or more spotted allergens, of any of the analysed antibody (IgG, IgG4 and IgE, in this study), represents an indication that molecules sharing one or more epitopes are present in the protein sample.

This high throughput methodology could provide general information on the relative hypo/hyper-allergenicity of specific foods and provides details on specific aspects such as the presence of already identified allergens and the presence of allergens/proteins not yet described in that food/cultivar. On the

basis of these data, the allergen pattern of individual cultivars can be obtained and used to select the foods most suitable for each allergic subject, taking into account that a designation as “low allergenic food” is not always true for each one of the allergens present in a cultivar. Hopefully, the inclusion of as many allergens as possible in the microarray system will allow an analysis of the allergen content even more accurate than that possible now (Mari, Ciardiello, Tamburrini, Rasi, & Palazzo, 2010). As reported recently by D’Avino et al. (2011), the creation of standard curves for allergen-binding inhibition could hopefully allow also allergen quantification in extracts.

Table 2

List of the allergens analysed in this study. Additional information can be obtained using the Allergome database (www.allergome.org).

Allergen	Protein family	Source
Act d 1	Papain-like cysteine protease	Kiwifruit
Act d 2	Thaumatin-like protein (TLP)	Kiwifruit
Ana c 2	Papain-like cysteine protease	Pineapple
Ana o 2.0101	11S globulin	Cashew
Api g 1.0101	PR-10 (Bet v 1-like)	Celery
Ara h 1	Vicilin	Peanut
Ara h 2	Conglutin	Peanut
Ara h 3	11S globulin	Peanut
Ara h 8.0101	PR-10 (Bet v 1-like)	Peanut
Bet v 2.0101	Profilin	Birch pollen
Cor a 1.0401	PR-10 (Bet v 1-like)	Hazelnut
Cor a 8.0101	Lipid transfer protein (LTP)	Hazelnut
Cor a 9	11S globulin	Hazelnut
Dau c 1.0101	PR-10 (Bet v 1-like)	Carrot
Gly m 4.0101	PR-10 (Bet v 1-like)	Soyabean
Gly m 5	Vicilin	Soyabean
Gly m 6	11S globulin	Soyabean
Hev b 8.0204	Profilin	Latex
Mal d 1.0108	PR-10 (Bet v 1-like)	Apple
Mer a 1.0101	Profilin	Annual mercury
Ole e 2	Profilin	Olive tree
Phl p 12.0101	Profilin	Timothy grass
Pru p 1.0101	PR-10 (Bet v 1-like)	Peach
Pru p 3	Lipid transfer protein (LTP)	Peach
Ses i 1	2S albumin	Sesame
Tri a 18	Hevein-like	Wheat
Tri a 30	alpha-Amylase/trypsin inhibitor	Wheat

Acknowledgements

We would like to thank Sara Zuzzi and Mario Santoro for performing the statistical evaluation of Ig inhibition values.

This study has been funded by the Italian Ministry of Health, Current Research Program 2008–2009, and by the program “Montagna viva – Progetto: Frutta antica d'Irpinia”, financed by Campania Region STAPA-Ce.P.I.C.A., Avellino, Italy (DR. D. No. 315, 21/07/2008).

The use of the InterAll allergy e-record has been kindly granted by Allergy Data Laboratories s.c. (Latina, Italy).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2012.04.075>.

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