

# Factors influencing the quality of food extracts for *in vitro* and *in vivo* diagnosis

Vieths S, Hoffmann A, Holzhauser T, Müller U, Reindl J, Hausteil D. Factors influencing the quality of food extracts for *in vitro* and *in vivo* diagnosis.

Allergy 1998; 53 (Suppl 46): 65–71. © Munksgaard 1998.

Food extracts for diagnostic purposes often lack sufficient activity and consistency. Biologically standardized food extracts are not available on the market. Using extracts from plant-derived foods as examples, we investigated factors which may be important for the quality of such extracts. Divergent allergenic activities were found between strains of apples, but not within varieties of celery tuber (celeriac), hazelnut, and peanut, respectively. Heating of the food remarkably reduced the activity of apple, hazelnut, and celeriac, but had little effect on peanut. By contrast, heating of semipurified protein extracts from celery tuber and apple for 30 min at 100°C did not deplete the immunoreactivity of the major allergens, indicating that this is an inappropriate test for identifying labile food allergens. Due to their high endogenous enzyme activities, apples and other fruits require special extraction procedures applying either low temperature or enzyme inhibitors. Variation of extraction conditions had little effect on the composition and activity of extracts from hazelnut. The storage stability of skin test solutions from plant foods can be improved by avoiding phenol as an additive and by including 50% of glycerol. For model studies considering neoallergens, IgE was raised in mice against native and heated celery tuber, respectively. When extracts from nonthermally and thermally processed celeriac were subjected to an RBL-cell mediator release assay with these sera, an inverse ranking was obtained with anti-heated celeriac IgE and anti-native celeriac IgE, respectively. These data indicated that new epitopes had been formed by the heating process. Since all parameters were tested in model experiments with either human or murine IgE, their relevance has to be proven in further clinical investigations.

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Key words: allergen extract; food allergy; mediator release assay; murine IgE.

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Allergen extracts from plant-derived foods, particularly those from fruits and vegetables, often have poor biologic activity (1–4). This is partly caused by inherent enzyme activities leading to rapid allergen degradation during the preparation of extracts (5). However, there are also other ways in which the production of food extracts with high activity and consistency causes complex problems which do not occur with the majority of extracts from inhalants. Firstly, food proteins are either consumed in the native state or heated, or after various kinds of food technological treatments. Both native proteins and denatured “neoallergens” may be recognized by allergen-specific IgE. Secondly, sensitization can occur via ingestion in the case of classical food allergens such as peanut and fish, or after inhalation

or skin contact with cross-reacting primary immunogens such as birch pollen and latex. Thirdly, in contrast to major pollen allergens, a considerable fraction of proteins from specific foods such as cereals are poorly soluble in standard extraction buffers and require special extraction procedures (6,7). Apart from the observation that many allergens seem to be relatively resistant to digestive enzymes, many aspects of the impact of the gastrointestinal tract during sensitization still remain unknown. Coming back to allergen extracts, the possible importance of source materials, as in the case of different varieties from plant foods, has been neglected in many cases. Finally, biologically standardized extracts have not yet been licensed by the US Food and Drug Administration or the

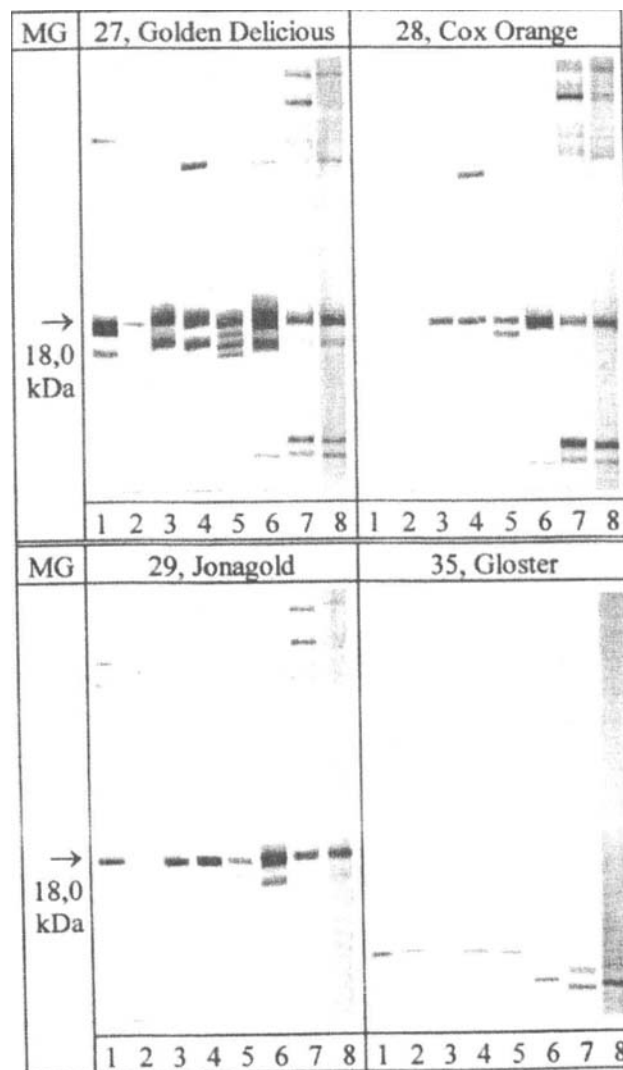


Fig. 1. Immunoblot analyses of four different apple strains: lanes 1, 2) mAbs against major birch-pollen allergen (Bet v 1) cross-reactive with major apple allergen (Mal d 1); 3, 4) rabbit sera raised against Bet v 1, also cross-reactive with Mal d 1; 5) cross-reactive rabbit antiserum against whole birch-pollen extract; 6) IgE from single apple-allergic patient with birch pollinosis; 7, 8) IgE from pooled sera of seven and 39 birch pollen-allergic patients, respectively. Dilutions: mAb, hybridoma supernatant 1:100; rabbit antisera, 1:5000; human sera, 1:6.7.

German Paul-Ehrlich-Institute. This paper briefly summarizes the results of our own model investigations touching various aspects of food extract quality.

### Material and methods

All apple extracts were prepared by the low-temperature acetone powder method (4, 8). Other foods were extracted with various buffers under different conditions as indicated in the text, dialyzed against distilled water, and stored freeze-dried until used. The allergenic activity of these preparations was assayed with IgE from patients allergic to the specific foods by the enzyme allergosorbent test (EAST) or by immunoblotting, as previously described (3, 9). Allergen-specific

rabbit antisera and monoclonal antibodies (mAb) were applied as indicated. A murine model of the type I reaction was applied, as reported earlier (10, 11). Pooled sera containing food-specific IgE from at least five sensitized mice were used for passive sensitization of rat basophil leukemia cells (subline RBL-2H3). After dose-related allergen challenge, mediator release was determined by assaying the  $\beta$ -hexosaminidase activity in the cell supernatants. For further conditions, see text.

### Results

#### Source material

From several to many different varieties exist of almost all kinds of food plants. The allergenicity of

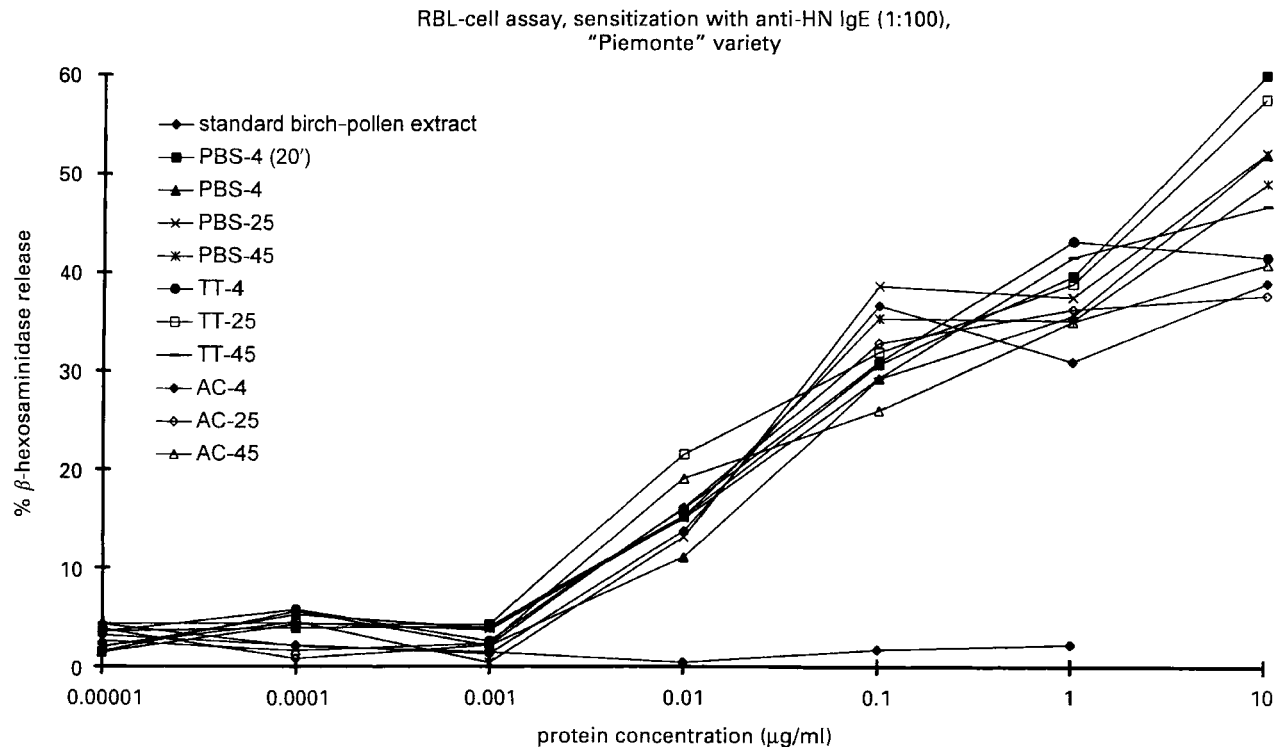


Fig. 2. Investigation of hazelnut extracts ("Piemonte" variety) prepared under different conditions, with extraction buffers PBS, tris/tricine (TT), and ammonium carbonate (AC). IgE cross-linking capacity was measured with RBL-cell mediator-release assay after passive sensitization with murine IgE raised against extract from native hazelnut. Samples: PBS-4 (20') PBS: extraction at 4°C for 20 min; PBS-4: extraction at 4°C for 4 h; PBS-25: extraction at 25°C for 4 h; PBS-45: extraction at 45°C for 4 h; further samples extracted with TT and AC buffers under conditions as described for PBS.

fruits from these plants can be related to storage proteins that are common in all varieties, or to proteins with biologic functions, such as enzymes, regulatory proteins, or pathogenesis-related proteins. With the last-named group of functional proteins, strain-dependent differences in the IgE-binding properties of the food may be suspected. Investigation of 16 different apple strains supported this hypothesis (3). As illustrated by the immunoblots shown in Fig. 1, the major allergen content varied over a wide range in the four varieties Golden Delicious, Cox Orange, Jonagold, and Gloster (12). Since an increase of the major allergen content during ripening and storage has also been reported (13, 14), the selection of source material can strongly influence the quality of apple extracts. By contrast, comparable levels of the major allergens were detected in four varieties of celeriac, four varieties of peanut, and six varieties of hazelnut, respectively (results not shown). Since allergen expression may be reduced by unknown factors or may also differ among various mutants of plant strains, pretesting of all new source material is required instead of selecting a variety that has been shown to be highly allergenic.

Since regional differences in sensitization may exist, the term "major allergen" has to be used with

caution. This is evident in the case of pollen-related food allergies where sensitization depends on the presence of particular kinds of pollen. In areas where birch trees are common, the major allergens of fruits such as apple, peach, and cherry are related to Bet v 1, the major birch-pollen allergen (4, 12), whereas, in the Mediterranean region, other allergens, such as profilin or a 13-kDa Prunoideae protein, may be the major IgE-binding proteins of these fruits (15, 16).

#### *Extraction and storage stability*

In the case of apples, the application of enzyme inhibitors or low temperature is required to produce active extracts, as commercially used standard procedures lead to highly degraded material (4, 5, 8). To optimize the allergen extraction from hazelnuts (HN), we applied three different buffers (0.05 M tris/tricine pH 8.6; 0.01 M PBS, pH 7.4; and 0.1 M  $(\text{NH}_4)_2\text{CO}_3$ , pH 8.0) at three different temperatures (4°C/25°C/45°C) for 4 h. In addition, a short time extraction (20 min) was tested with PBS. The maximal yield of soluble protein resulted from tris/tricine at 45°C. The mediator release capacity was determined with RBL-2H3 cells passively sensitized with murine

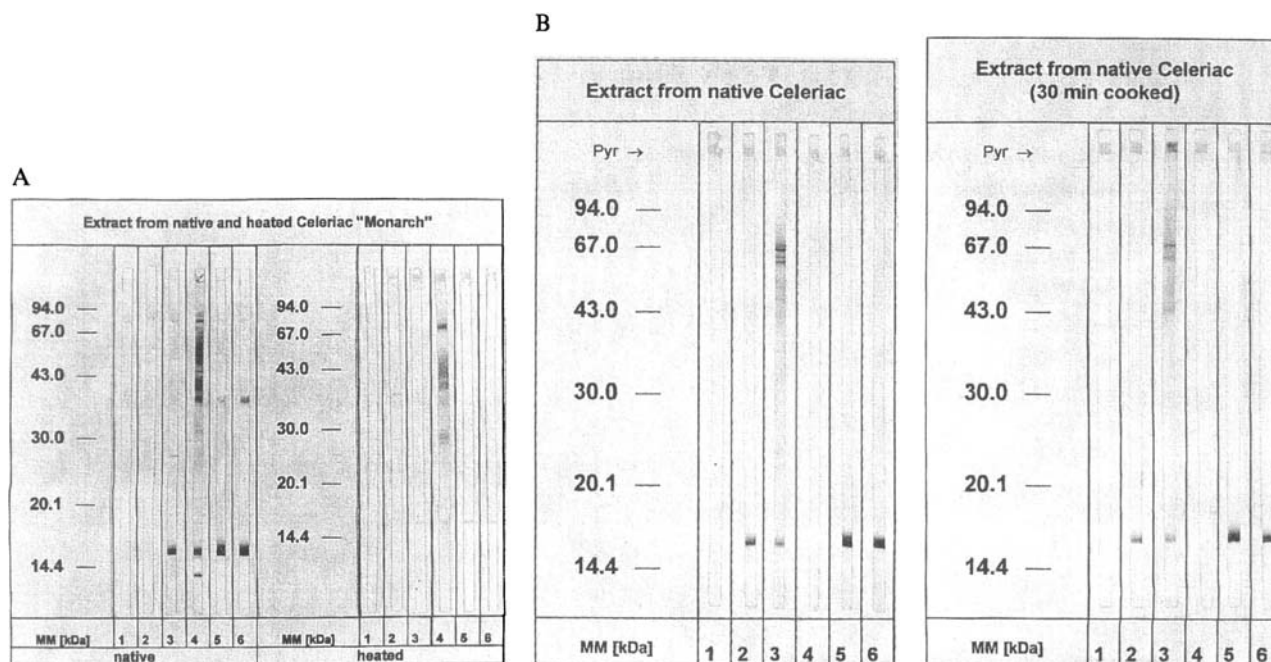


Fig. 3. A) Binding of IgE from celery-allergic patients and of mAbs raised against major allergen Api g 1 to blotted extracts from native and heated (30 min, 100°C) celeriac. Left side: native celery tuber, right side: microwaved celery tuber. Lane 1) buffer control; lane 2) nonallergic human control; lane 3) celery-allergic patient Bo211; lane 4) allergic patient M103; lanes 5, 6) mAbs against Api g 1, clones 12B2 and 12F2, respectively. Human sera were diluted 1:6.7; hybridoma supernatants with mAbs were applied at dilution of 1:5. Identical amounts of total protein from both preparations were subjected to electrophoresis. B) Immunoblotting with extract from native vegetable and after heating extract for 30 min at 100°C. Lane 1) nonallergic human control; lane 2) celery-allergic patient Bo211; lane 3) allergic patient M103; lane 4) mAb against Bet v 1 (clone PEI 16), not cross-reactive with Api g 1 (negative control); lanes 5, 6) mAbs against Api g 1, clones 12B2 and 12F2, respectively.

IgE against native HN (Fig. 2) and, due to the known cross-reactivity of HN with birch pollen (BP), with anti-BP IgE. The specific release activity of all preparations was very similar at protein concentrations up to 10 µg/ml. After sensitization of RBL cells with anti-BP IgE, all HN extracts caused a large cross-reactive mediator release at 10 and 100 µg/ml, respectively (not shown). To prove qualitative differences among the extracted allergens with human IgE, increasing amounts of protein (1.5–1500 µg/ml) from selected extracts were coupled to activated filter-paper disks and investigated by EAST with a serum pool from four HN-allergic patients who were exclusively sensitized to the Bet v 1-related major allergen. No differences in the IgE-binding capacity occurred between the extracts, thus confirming the results of the murine mediator-release assay. Saturation of the allergen disks was reached at 300 µg/ml HN protein in the coupling solution.

As mentioned earlier, extraction conditions may be even more important for allergen extracts from grains. In conclusion, as stated by Lehrer & Reese (6), conditions of extract preparation have to be chosen according to the properties of the proteins present in the source rather than by just employing established standard protocols. The same may be true for preservation of the allergenic activity.

Unfortunately, phenol is used as the standard preservative of prick test solutions. This contrasts with the observation that phenol rapidly deteriorates apple allergens (17) and also affects the activity of cereal grains (18). The latter were stable for many months at 4°C after simple use of solvents with 50% glycerol and 0.45% sodium chloride. Stabilization of apple prick test solutions with enzyme inhibitors such as phenylmethanesulfonyl-fluoride (PMSF) and diethylthiocarbamate (DIECA) (8) is not recommended for *in vivo* applications. However, after low-temperature extraction of apples without addition of such chemicals, the activity of prick test solutions was also preserved by replacing phenol with 0.12% cysteine and 0.7% sodium citrate in PBS/glycerol. Although the activity of the extracts decreased significantly during 6 months of storage at 4°C, the prick test remained positive in 9/12 apple-allergic patients (unpublished data).

#### *Influence of food processing*

Human nutrition is primarily based on heated and processed foods. Thus, the impact of processing on the biologic activity of food extracts has to be considered an important factor. In accordance with patients' reports, our data show that the major

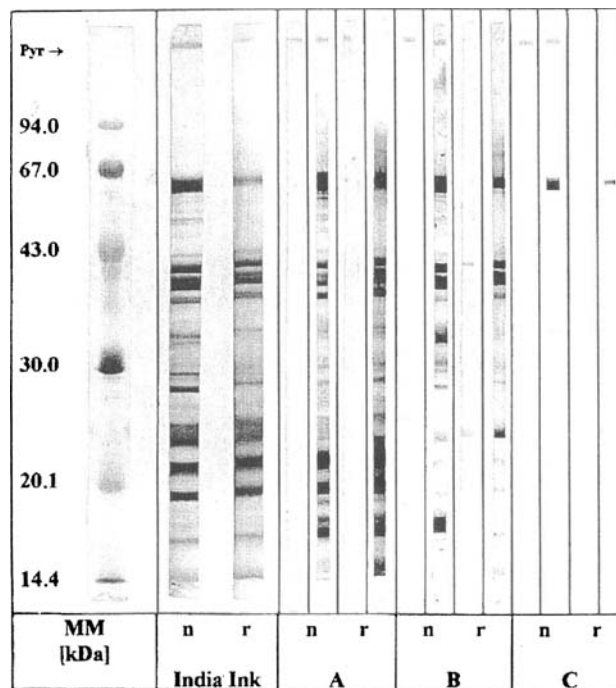


Fig. 4. Investigation of extract from native and roasted peanuts (45 min, 140°C) by immunoblotting. Lane n) native sample; r) roasted sample. A) IgE of serum pool from three peanut-allergic patients (1:10); B) rabbit antiserum against whole-peanut extract (1:7500); C) mAb PN-t specific for major allergen Ara h 1 (1 µg/ml). Left lanes show controls with nonallergic human serum (A), rabbit preimmune serum (B), and nonspecific mAb (C); right lanes show specific immunodetections.

apple allergen Mal d 1 becomes inactivated during all kind of processing or heating. A more complex situation exists with other pollen-related foods such as celeriac. Celery tubers are known to contain a thermostable and a heat-labile allergenic component (19–22). We have demonstrated the complete inactivation of the major allergen Api g 1 by even short heating procedures, but not by nonthermal treatments such as high-voltage impulse treatment or the preparation of celery powder (21). The lability of the 16-kDa Api g 1 is illustrated by Fig. 3A with extracts from native and 30-min microwaved celery tuber. Other IgE-binding structures in celeriac are more persistent; for example, multiple proteins of greater mass which have been shown to carry heat-stable glycan structures that form the IgE epitopes (illustrated by serum from patient M103, Fig. 3A, lane 4, with IgE against carbohydrate epitopes and Api g 1, respectively) (22). Fig. 3B demonstrates that it is important to perform heating experiments on the food itself, but not with extracts, as Api g 1 persists for 30 min at 100°C when heating is applied to semipurified protein fractions. Even the extremely labile Mal d 1 showed a residual IgE-binding potential after 30-min heating of apple extract (results not shown).

As determined by the RBL cell mediator-release assay, toasting of HN before extraction resulted in reduced immunoreactivity of the extracted allergens when the cells were sensitized by anti-birch

pollen IgE. After sensitization with anti-HN IgE, a considerable potential for specific mediator release persisted independently of toasting conditions (not shown). These data correspond closely to those obtained with apple (Mal d 1) and celery (Api g 1) and may further underline the heat lability of birch pollen-related major food allergens. The results of immunoblotting experiments with native and roasted peanuts are presented in Fig. 4. Most IgE-binding bands, including the major allergen Ara h 1 at 65 kDa, were recognized in both native and roasted peanuts (45 min, 140°C). Interestingly, a strong band at about 16 kDa was identified as being heat-resistant with IgE from peanut-allergic patients (Fig. 4A), but not with rabbit antibodies raised against peanut extract (Fig. 4B). In the RBL-cell assay, the mediator-release capacity of extracts from peanuts roasted at 140°C and 160°C for different periods showed only little differences (result not shown).

For study of the possible formation of neoallergens by heat treatment, celeriac was subjected to another series of model experiments (11). Specific IgE was raised in different groups of mice against native and heated celery tuber, respectively. Subsequently, mediator release tests were run with RBL-2H3 cells passively sensitized with the resulting sera (11, 21). With IgE against native celeriac, a strong release was measured with the native sample, with the major allergen Api g 1, and

with nonthermally treated samples such as irradiated celery tuber. By contrast, extracts prepared from heated products presented only little activity. Reversed allergenic properties were observed with murine IgE against heated celeriac. A strong release was triggered with all heated preparations including cooking water, whereas native celery showed the weakest activity of all tested preparations. Moreover, Api g 1 did not show significant activity in this system (results not shown). These data indicate that neoallergens of celery tuber are recognized by the murine immune system after sensitization to the native vegetable.

## Discussion

Food extracts for diagnostic purposes are far away from being standardized or even from being uniformly active. Moreover, it is evident that the extraction process itself, preparation of stable skin test solutions, selection of source material, and possibly technological treatment of the food before extraction are all important factors influencing the suitability of extracts. These factors have to be carefully adjusted with consideration of the particular food allergy under study. Despite the *in vitro* studies of IgE-binding properties, the relevance of extracts from heated foods for *in vitro* and *in vivo* diagnosis has to be further investigated. The database on sensitivity and specificity of diagnostic assays based on such preparations is poor. Extracts from native cereals have been shown to produce false-positive results in grass pollen-allergic patients (23). However, it is not clear whether improved results can be obtained with extracts from heated grains. Positive skin prick tests and IgE assays have been obtained with extracts from heated food in a subgroup of patients allergic to celeriac (19, 20). However, the correlation to clinical symptoms after ingestion of heat-processed celery tuber remains unclear. The application of extracts from heated material seems to be important in the diagnosis of allergy to mollusks (24, 25), a fact which, of course, is relevant only to a small number of patients. Finally, two important factors have to be borne in mind. On the one hand, sensitization to cross-reactive allergens and additional unknown factors may produce clinically insignificant skin tests and *in vitro* assays. On the other hand, false-negative results in food-allergic patients may result from inappropriate extracts; differences between skin sensitivity, humoral sensitization, and the reacting organ; and, again, from unknown reasons. Thus, apart from all efforts to improve the quality and consistency of food extracts, the double-blind, placebo-controlled

food challenge remains the reference standard for diagnosis of food allergy.

In conclusion, the model experiments described in this paper may be helpful to adjust and monitor the consistent biologic activity of food extracts. In the next step, these studies have to be supported by carefully designed clinical investigations.

## Acknowledgments

Parts of this work were supported by grants from the Landesregierung Baden-Württemberg (PUG P 94001), the Deutsche Forschungsgemeinschaft (DFG Vi 165/3), and the European Community (EC FAIR-CT97-3224). We are grateful to Dr G. Malgarini, Sorematec, Aarlon, Belgium, for kindly supplying samples of native and roasted hazelnuts and peanuts.

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