

Optimization, Standardization and Control of Food Allergens

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Introduction

Today, commercial food allergen extracts as used for in vitro and in vivo diagnosis often lack good quality [1-3]. Some food allergens, such as peanut [4] and codfish [5, 6], often show a good predictive value, whereas other allergens (e.g. fruit, vegetables, milk and egg) [2, 7, 8] have shown a much lower predictive value, when compared to the double-blind, placebo-controlled food challenge (DBPCFC).

The basis of a reliable diagnostic test for IgE-mediated food allergy is a standardized food allergen extract with a documented predictive value in comparison with the DBPCFC and a documented stability of the finished and intermediate product. In a good quality extract all potential allergens must be present and the extract must have a defined level of potency. Moreover, for in vivo testing the potency of the extract should be expressed in biological units, no adverse or false positive reactions through irritants should occur.

The fresh food skin prick test (SPT) might for some allergens (e.g. fruit and vegetables) give more reliable results [2, 3] as compared to the current available commercial extracts. However, fresh foods are not standardized, may contain irritants that interfere with the SPT and the used raw material may vary in allergen content and composition from batch to batch.

The aim of this study was to standardize the seven most abundant food allergens: apple, hazelnut, peanut, milk, egg, codfish and wheat flour in order to obtain reliable allergen extracts both for in vivo and in vitro diagnosis of IgE-mediated food allergy. Further, the stability of the SPT formulation was determined.

Selection of Raw Materials

Of most food allergens a wide variety of raw materials can be obtained. The pretreatment of the material is important as this can influence the content of allergens. Nearly all peanuts consumed by humans are roasted during processing, however it was reported that the number and quantities of allergens are higher in not-roasted peanut extracts [4, 9, 10]. Cooking of foods can degrade allergens and it was also reported that by cooking new allergens can be formed [3]. In sterilized milk, thermolabile allergens are completely absent.

As food allergens are biological materials, batch-to-batch variation of raw materials may exist, especially between harvesting seasons. The cultivation conditions in the country of origin is likely to influence the allergen composition. Some raw materials may contain contaminants, such as pesticides. Therefore, we prefer to use 'ecological' cultivated raw materials.

Because of these great variations in source materials it is very important to screen for the best source material and chose one or two suppliers. Once the raw material and suppliers are selected these should not be changed. An example is given for peanut. We have studied the influence of the sort of peanut, the pretreatment and the country of origin. Nine varieties of peanut, available from local stores, were selected. After grinding and petroleum ether extraction the peanuts were extracted for 2 h in phosphate-buffered saline (PBS) under exactly the same conditions. In SDS-PAGE all protein bands of extracts 5, 7 and 9 have the highest intensity. Bands could be detected at 64 kD (probably Ara h I) and 20–30 kD and at 20, 18 (probably Ara h II) and 13 kD. The band at 64 kD is relatively stronger in the not-roasted peanuts, indicating that this protein is relatively sensitive to roasting. The protein content and relative potency (LIA inhibition) of the same extracts was determined. It can be seen in figure 1 that the not-roasted peanut extracts gave the highest protein content and relative potency. The not-roasted peanut extract No. 9 has an equal protein content, but a lower relative potency as compared to the other not-roasted peanuts. It can be concluded that peanuts 5 and 7 are the optimal source material and that protein content and SDS-PAGE alone are not sufficient for the selection of raw materials. Further studies are necessary to determine differences in specific allergens.

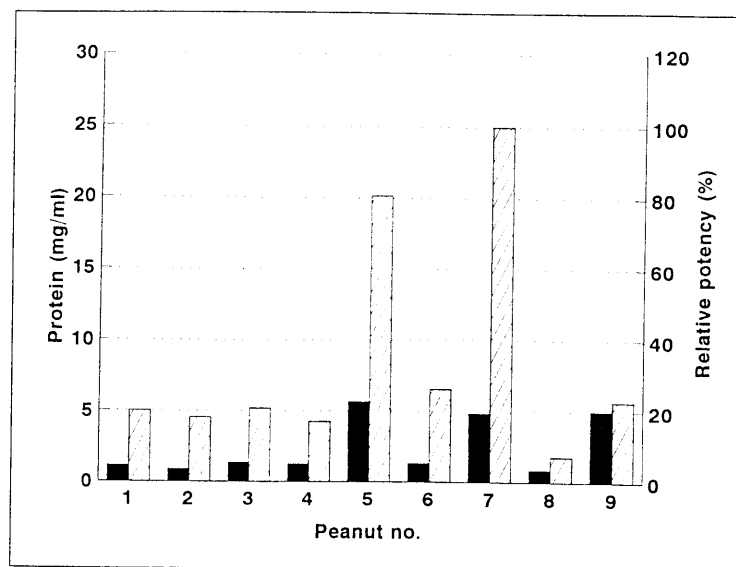


Fig. 1. Relative potency (%) (LIA inhibition) (▨) and protein contents (mg/ml) (■) of extracts of nine sorts of peanut. Peanut 1: roasted in shell, USA, Valencia; peanut 2: roasted in shell, China; peanut 3: roasted, without skin, salted, Valencia; peanut 4: roasted, without skin, USA, Runner; peanut 5: not roasted, without skin, USA, Runner; peanut 6: roasted, without skin, king size, USA, Virginia; peanut 7: not roasted, without skin, USA, Virginia; peanut 8: roasted with skin, Natal; peanut 9: not roasted with skin, Natal.

Selection of the Extraction Method

The extraction circumstances should preferably resemble the physiological conditions in the tractus digestivus. The pH could be around 7, resembling extraction in the mouth; a low pH, resembling the condition in the stomach or a high pH resembling conditions in the gut. The optimal extraction pH is dependent of the charge of a protein at a given pH. Different extraction procedures have been used for food allergens. Most authors use a PBS buffer at pH 7.0 [11–17]. Some have added PVPP, EDTA and sodium azide for hazelnut [12], egg [15] and apple [18] in order to prevent degradation during extraction. Others have used buffers with another composition and pH for peanut [19] and wheat flour [20]. Most authors have used overnight or 24-hour extractions [11, 12, 14, 15, 19], others have used 1- to 4-hour extractions [13, 16, 18].

The extraction time should be as short as possible to prevent possible degradation of extracted allergens. The extraction buffer should contain as little additives as possible and phenol should be omitted, because phenol has been shown

to have a deleterious effect on allergens. In addition, the circumstances should be such that microbiological contamination is as low as possible; cooled extraction and removal of bacteria by filtration. Additives should only be used when needed for the preservation of the allergenic activity during extraction. Defatting is necessary for nuts and other fat- or oil-containing foods. Grinding is necessary for nuts. It is important that no heat is generated by the grinding procedure, which could cause degradation of allergens.

Again an example is given for peanut. Peanut 7 was selected and 10 g was extracted in 100 ml in a buffer with or without PVPP and EDTA at pH 5.4, 7.4 and 9.4. The extracts were directly used for SDS-PAGE under exactly the same conditions. The extraction time tested was 15 min, 2 h and 18 h. From the SDS-PAGE results it could be concluded that the extraction time and buffer composition have no influence on the extraction of proteins, whereas the pH has a significant influence. The highest quantities of proteins are extracted at pH 9.4. In the immunoblot bands at 75, 58, 47, 34 and 18 kD could be detected. No differences between different buffers and extraction times could be seen.

In figure 2 the protein content and relative potency (LIA inhibition) of the above-mentioned extracts are shown. There is a relatively large increase in the protein content and relative potency when the pH of the extraction buffer is increased from 7.4 to 9.4, which corresponds well with the SDS-PAGE results. The conclusion of these experiments is that peanut is best extracted at pH 9.4 during 15 min.

Standardization and Control

In order to standardize the allergen extract it is necessary to select and characterize an in-house reference (IHR) preparation. This IHR is prepared in exactly the same way as the normal freeze-dried intermediate product and is characterized by an electrophoresis profile, an allergogram with at least 20 sera of DBPCFC-positive patients. The biological potency should also be determined in patients having true IgE-mediated food allergy confirmed by DBPCFC. All analytical methods and production steps should be validated.

The batch-to-batch consistency of freeze-dried intermediate product (IMP) batches is determined relative to the IHR. The qualitative composition is determined with electrophoretic techniques, ensuring that all potential allergens are present. The quantitative composition should be determined using a total potency determination and a determination of at least two major allergens. Further, the protein content and the water content is determined, as a too high water content could facilitate degradation. An example of the batch-to-batch consistency of three produced batches is given in table 1 for egg. The results of the water,

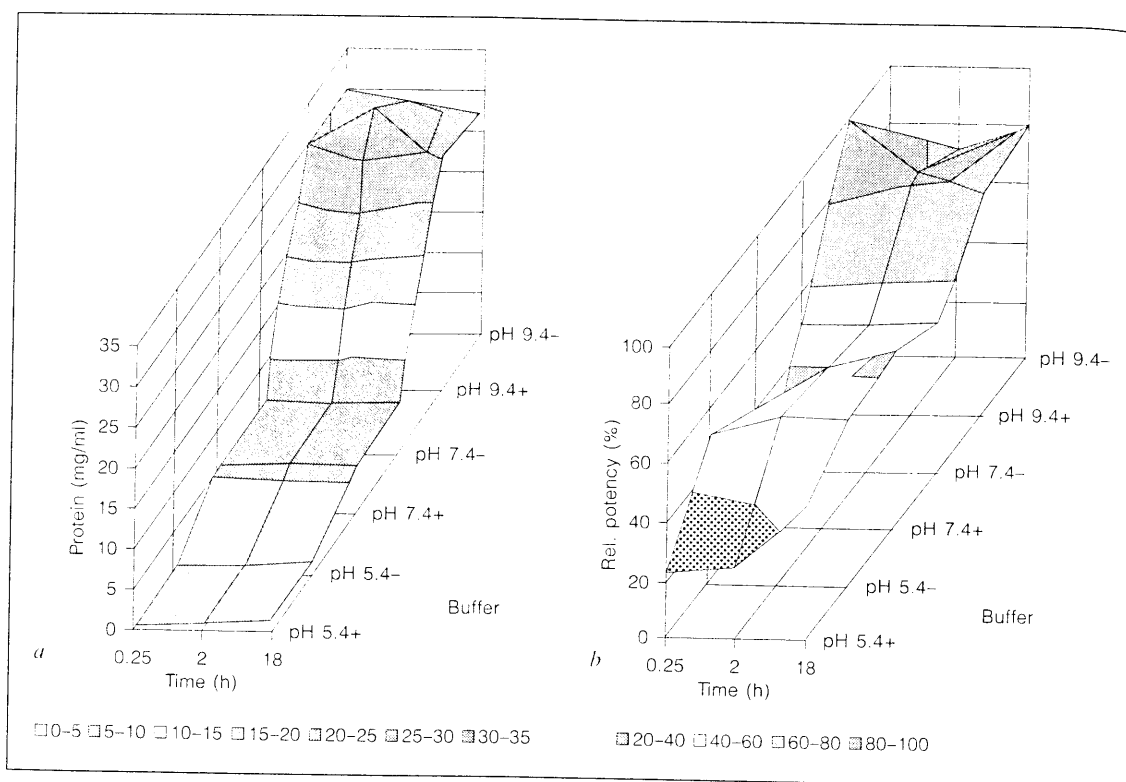


Fig. 2. Protein content (mg/ml) (a) and relative potency (%) (b) of peanut 7, extracted at pH 5.4, 7.4 and 9.4 with or without PVPP and EDTA during 15 min, 2 h and 18 h.

Table 1. Batch-to-batch consistency of three batches of freeze-dried intermediate product of egg as determined with LIA inhibition, rocket immunoelectrophoresis and SDS-PAGE

Batch	Water %	Protein %	Relative potency, %	Ovalbumin %	Ovomucoid %	SDS-PAGE
1	9.80	19.2	93	103	128	passed
2	10.0	16.7	125	103	131	passed
3	10.0	19.4	119	95	116	passed

protein, the ovalbumin (RIE), ovomucoid content (RIE), relative potency (LIA inhibition) and SDS-PAGE are shown. It can be concluded that all batches are within specifications and a good batch-to-batch consistency is obtained.

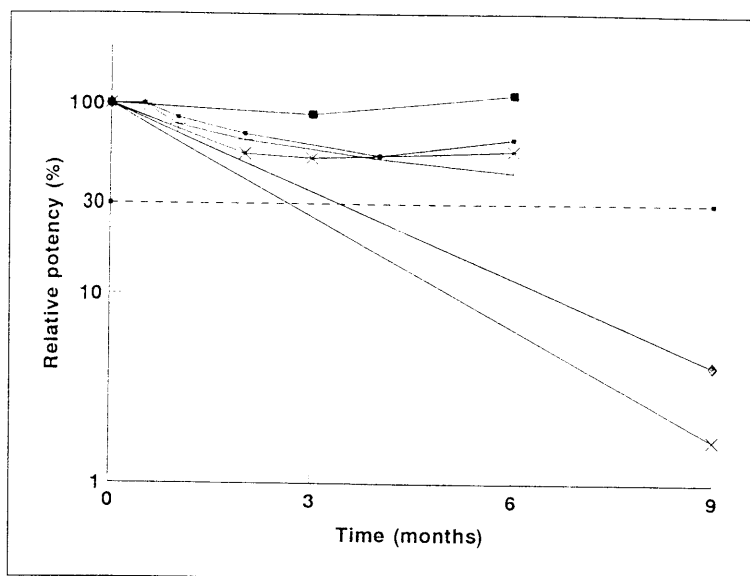


Fig. 3. Results of a stability study of the SPT formulation for apple extract at 6 (■) and 25°C (+), hazelnut extract at 6 (■) and 25°C (*) and milk extract at 6 (◆) and 25°C (×), as measured with RAST inhibition.

Stability Skin Prick Test Formulation

The stability of the SPT formulation (PBS with 50% glycerol) was determined for apple, hazelnut and milk during storage for up to 6 or 9 months at 6 and 25°C (fig. 3). Milk extract is stable for only approximately 3 months at 6°C, apple for at least 6 months and hazelnut shows no decline at 6°C, but a degradation at 25°C. As aqueous SPT formulations of food allergens extracts seem not to be stable for some allergens, a freeze-dried SPT preparation may be stored without fast degradation.

Conclusions

It is important to select the optimal raw material and extraction method for each allergen. A freeze-dried SPT preparation can be stored without fast degradation. Using this procedure a good batch-to-batch consistency can be obtained and the diagnostic capacity of the in vivo (SPT) and in vitro tests (Magic Lite) can probably be improved. Further clinical and in vitro studies are necessary to confirm this.

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