DOI 10.1002/mnfr.200600194 Mol. Nutr. Food Res. 2007, *51*, 360 – 366

Research Article

Optimized techniques for the extraction of grape allergens appropriate for *in vivo* and *in vitro* testing and diagnosis

Emilia V. Vassilopoulou[1], Laurian Zuidmeer[2], Jaap Akkerdaas[2], Neil Rigby[3], F. Javier Moreno[3], Nikolaos G. Papadopoulos[1], Photini Saxoni-Papageorgiou[1], Clare Mills[3] and Ronald van Ree[2]

[1] Allergy Research Centre of 2nd Paediatric Clinic, University of Athens, Athens, Greece
[2] Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
[3] Institute of Food Research (IFR), Norwich Research Park, Colney, Norwich NR4 7UA, UK

Standardized allergen extracts are needed for diagnosis and therapy purposes. For grapes, standardization is hampered by low protein and high tannin and pectin concentrations. The aim of the current study was to develop an optimized method for the extraction of grape proteins and possibly extend this to other fruits. Several existing or modified extraction methods were compared by means of protein concentration determination, SDS-PAGE, immunoblotting and radioallergosorbent test (RAST). An optimized extraction protocol was obtained in which we combined a high concentration of plant tissue, a concentrated, enriched and neutral buffer able to remove sugars and keep proteins soluble and a bivalent buffer for pectin removal. Both the quantitative (protein concentration) and qualitative parameters (SDS-PAGE protein patterns and IgE reactivity) were compared to standard protocols and commercial extracts used as diagnostic tools in the clinical practice. This method proved to be the most efficient mainly compared to the standard Björksten protocol in extracting the low molecular weight proteins, including the major grape allergen (lipid transfer protein, Vit v 1). It proved to be an easy, low cost and reproducible method proposed to prepare grape extracts that could replace the commercially available ones, used for diagnosis and possibly extend the method to other fruits especially in extracting LTPs.

Keywords: Allergen / Extract / Grape / Lipid transfer protein / Pectins

Received: October 5, 2006; revised: November 29, 2006; accepted: November 29, 2006

1 Introduction

Grapes belonging to the Vitaceae family are one of the oldest cultivated plants, dating back to more than 5000 years in Egypt, and were highly developed by the Greeks and Romans. Today there are nearly 200 cultivated varieties with modern cultivars derived from two main species, the European (Mediterranean) *Vitis vinifera* and the North American *Vitis labrusca*.

After a first report of sensitivity to grapes and wine from Brown in 1953 [1], a considerable number of reports have been published referring to allergic reactions after the consumption of grapes [1 – 8]. In 2003, three grape allergens were identified in a Mediterranean population, a 9 kDa lipid transfer protein (LTP) (Vit v 1), a thaumatin-like protein and an endochitinase 4A [4]. Recently, we have described a population of children, adolescents and young adults showing signs of severe allergic reactions after the consumption of grapes, and identified the allergens involved [9].

Grapes naturally contain a wide range of different proteins [10, 11], but their protein content is low compared to other fruits (~1%). Consequently, extracting proteins from grapes proves to be difficult, also due to their high content of tannins (polyphenols) that can complex and coprecipitate with proteins [10]. Additionally, grapes are relatively rich in pectic substances [10]. Standardization of grape and other fruit extracts is very important, as relevant reagents should be used for diagnosis in skin prick tests. Ideally, a

Correspondence: Dr. Emilia V. Vassilopoulou, Allergy Research Centre, 41 Fidippidou strasse, 11527 Goudi, Greece
E-mail: emi@allergy.gr
Fax: +30-210-7776964

Abbreviations: PVPP, polyvinylpolylirrodine; **RAST,** radioallergosorbent test

standardized extract must contain defined and consistent amounts of all the major and minor allergens and each allergen must be biologically active [12].

We focused on improving the current extraction protocols in order to further purify grape proteins responsible for allergic reactions in our population [13]. Several procedures were used to define a proper extraction method for further isolation of grape allergens.

We present an optimized method for extracting grape proteins, which combines features from already described standard methods. The new method is compared with the standard ones by means of different biochemical and immunochemical methods.

2 Materials and methods

2.1 Patients and sera

Sera were obtained from 15 patients (ten male) in the age group of 8 – 24 years (median, 14.6 years) that referred to the Allergy Unit of 2nd Paediatric Clinic, University of Athens, because of an allergic reaction after the consumption of grapes (12 anaphylaxis *vs.* 3 urticaria). Patients' clinical characteristics are described in detail elsewhere [9]. All the patients had a positive specific IgE CAP-FEIA (Pharmacia Sweden) (range 1.38 – 15.5 kU/L, median 4.521) and they were highly atopic, with 66% suffering from asthma, 73.3% from rhinitis and 46.7% from atopic dermatitis. Only one patient did not have any food allergy, whereas for the rest, 80% had a history of reaction to Rosacea fruit and 66.7% to nuts. All the patients were sensitized to various pollen allergens (100% to olive pollen, 86% to parietaria, 93% to grass pollen mix g × 2 and 6% to white pine). The study was approved by the local ethics committee, and informed consent was obtained from the patients.

2.2 Extracts

Starting from the standard Björksten protocol (method B) [14] that has already been used for the isolation of grape allergens [4], we have combined several features of previously described extraction buffers with slight modifications [14 – 17].

The resulting new method that proved to be the most efficient is described below in detail (method A):

White grapes (*V. vinifera*, cv. stafida) were peeled using a fruit juicer (Mulinex, Germany) and the skin (1/6 of total weight) was immediately frozen and ground to powder with liquid nitrogen in an analytical mill. Powder was homogenized with 200 mM potassium phosphate buffer (pH 7), containing 20 mM ethylenediamine tetraacetic acid (EDTA), 100 mM diethyldithiocarbamic acid (DIECA), 3 mM sodium azide (NaN_3) (3 : 1 w/v), 5% presoaked solid polyvinylpolylirrodine (PVPP) in potassium phosphate buffer and 0.5% Tween-20 reagent. During homogenization the temperature was checked not to exceed 15°C. Homogenate was stirred for 1 h at 1°C and then centrifuged at 1°C in 40 000 × *g* for 30 min. For pectin removal, the extract was treated with a bi-ionic buffer containing 50 mM calcium chloride ($CaCl_2$), 50 mM magnesium chloride ($MgCl_2$) and 50 mM manganese chloride ($MnCl_2$) as described by Jona and Fronda [12].

2.3 Source material

All the extracts were prepared from white grapes (*V. vinifera*, cultivar stafida). Results from comparison of the extracts ($n = 5$) prepared according to the optimized method A and the standard Björksten method B [14], as well as a commercial grape extract used for diagnosis are presented in detail:

Extract 1: Prepared from whole grapes using method B.
Extract 2: Prepared from grape skin using method A.
Extract 3: Prepared from grape skin using method A, without grinding in liquid nitrogen.
Extract 4: Prepared from whole grapes using method A, without grinding in liquid nitrogen.
Extract 5: A commercial grape extract used for diagnosis by skin prick testing (Stallergen).

For comparison we also extracted other fruits (apricot, pear, apple, peach and strawberries) purchased from local stores, according to the two protocols.

2.4 Reagents

Chemicals were obtained from Sigma (UK or Netherlands) and Merck (UK or Netherlands).

2.5 Protein determination

Protein concentrations were determined with the bicinchoninic acid assay (BCA) (Pierce, Rockford, IL) using serial dilutions of BSA as a standard [18].

2.6 SDS-PAGE/Immunoblotting

Protein separation, detection and immunoblotting were done as described previously [19]. Mr was calculated using prestained See-blue2 (Invitrogen, UK), unstained M-12 (Invitrogen) or unstained Precision standards (BioRad, UK).

2.7 Radioallergosorbent test (RAST)

RAST was performed for grape extracts 1 and 2 as previously described [20]. Results were expressed as international units IgE *per* mL (IU/mL). RAST values higher than 0.3 IU/mL, were regarded positive. Calculation was performed by means of a standard curve obtained with a dilution series of a chimeric monoclonal IgE antibody against

the major house dust mite allergen Der p 2 and Sepharose-coupled recombinant Der p 2 [21].

3 Results

3.1 Optimization of extraction method

After an extensive literature review on the extraction methods available, we selected and combined several methods and compared the results [14, 16, 17]. Different protocols used are briefly presented in Table 1.

Extracts prepared with the standard method B [14] resulted in a very low protein concentration (mean 0.15 mg/mL ± 0.06) (Fig. 1, lane 1). Trials to concentrate these extracts by ammonium sulphate precipitation [22], ultra filtration and lyophilization did not succeed. Additionally in some case proteins denatured and aggregated. Focusing on extraction of proteins from frozen grape skin as starting material as opposed to the whole fruit, indeed resulted in a higher yield.

Addition of either 0.5% Tween-20, as described in previous references for other plant tissues [16] or replacement of 5% PVPP with 5% soluble PVP-10, both had a significant effect on the solubilization and extraction of grape proteins and specifically the low Mr bands including grape LTP, identified as the major allergen for our population [9, 23]. On the other hand, the addition of 0.5% BSA (known to act as an antagonist to grape proteins on forming

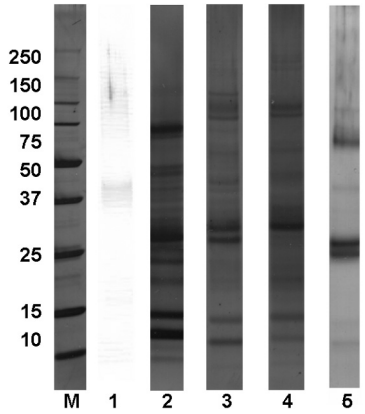

Figure 1. SDS-PAGE analysis of grape extracts. Lane 1: prepared according to the Björksten method B; lanes 2–4: extracts prepared according to the optimized method A; from grape skin with (lane 2) and without (lane 3) grinding, or from whole grapes (lane 4); lane 5: the commercial grape extract used for skin prick testing. M: molecular weight standard.

Table 1. Synoptic presentation of different extraction protocols which were applied aiming to isolate grape proteins. The numbered extracts (1–4) are presented in the figures

	Protocol	Ratio buffer : plant tissue v : v	Additional modification	Source material
1	Method B: Standard Björksten protocol [13]	2 : 1	Concentration with a, ammonium sulphate, b, ultrafiltration, c, lyophylization	Whole grapes
2	Method A: Björksten buffer (10 × concentrated) + 5% w/v PVPP + 0.5% Tween-20 + 0.5 mM $MgCl_2$+ 0.5 mM $MnCl_2$ + 0.5 mM $CaCl_2$+	1 : 3	–	Ground grape skin
3	As 2	1 : 3	–	Grape skin (not ground)
4	As 2	1 : 3	–	Whole grapes
a	Björksten buffer	1 : 25	Concentration with ammonium sulphate/ultrafiltration/lyophylization	Whole grapes
b	Björksten buffer with 20 mM Potassium Phosphate buffer (pH 7) + 3% w/v PVPP + 0.1% NaN_3	3 : 1	Concentration with ammonium sulphate/ultrafiltration/lyophylization	Ground grape skin
c	0.1 M Phosphate Buffer Saline (PBS) (pH 7) + 0.1% Tween-20	2 : 1	–	Whole grapes
d	Björksten buffer (10X) + 5% w/v PVPP	1 : 3	–	Grape skin (not ground)
e	Björksten buffer (10X) + 5% soluble PVP-10 [14]	1 : 3	–	Ground grape skin
f	(as d) + 0.5% BSA [15]	1 : 3	–	Ground grape skin
g	(as d) + 6.5% ascorbic acid [15]	1 : 3	–	Ground grape skin [16]
h	As d	1 : 3	–	Acetone treated grape skin [16]
i	(as d) + 5% Tween-20	1 : 3	–	Acetone treated grape skin [16]
j	As e	1 : 3	–	Acetone treated grape skin

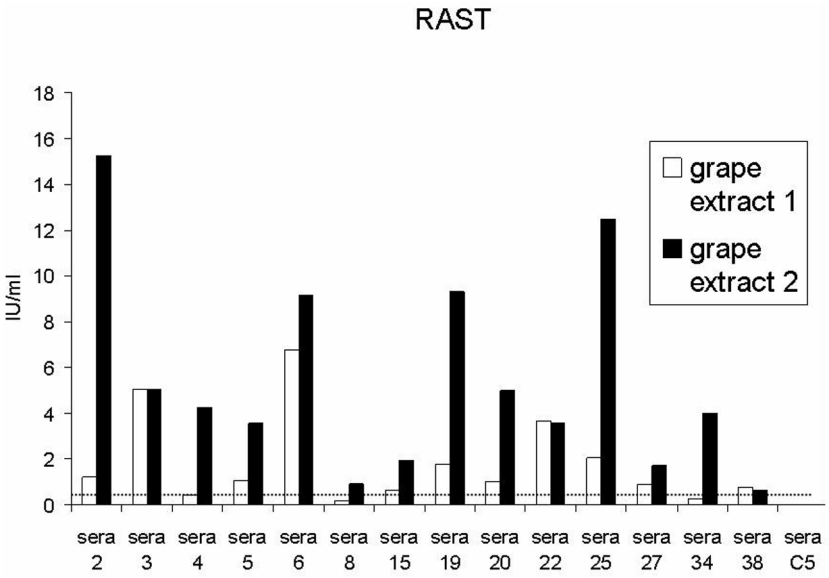

Figure 2. Comparison with RAST of the IgE reactivity of extracts 1 and 2 for a selected serum panel. C5: serum from a control donor. Values (> 0.3 IU/mL) above the dotted line are considered positive.

precipitates with tannins [24]) or 6.5% ascorbic acid, added to prevent oxidation of phenols (which when oxidized, bind proteins) [25] did not significantly improve the results (results not shown).

At the end, several factors were combined, which resulted in our optimized method A; *i.e.*, the use of frozen grape skin powder rather than the whole grape, reduction of the ratio buffer/plant material from 2:1 to 1:3, increase in the PVPP concentration from 2 to 5%, addition of Tween-20 reagent and increase in the pH from 5 to 7. This significantly improved solubilization and extraction of grape proteins resulting in an average increase in yield of 6.6 times compared to the Björksten method (B), as judged by BCA (seven extracts, mean protein concentration 0.88 ± 0.27 *vs.* 0.15 ± 0.06, respectively). This difference is also clearly visible on gel (Fig. 1, compare lanes 1 and 2). Grinding of grape skin in liquid nitrogen to a fine powder before extraction (Fig. 1, lane 2 *vs.* 3 and 4), as well as using grape skin instead of the whole seed (Fig. 1, lanes 3 and 4, respectively), further increases the protein yield as judged by SDS-PAGE. The commercial grape extract 5 (Stallergen) used for skin prick testing diagnosis (Fig. 1, lane 5) was found to contain less protein bands and, most importantly, lacked the low Mr protein identified as grape LTP.

Further comparison of both methods using other fruits (apple, strawberry, plum, pear and peach) indicated that method A is indeed more efficient, when compared with extracts prepared with method B (results not shown).

3.2 IgE reactivity of the extracts

By RAST, extract 2 (new method) shows an average increase of 56.5% in IgE binding compared to extract 1 (Björksten method) (Fig. 2). Also, sera 4, 8 and 34 gave false negative results with extract 1.

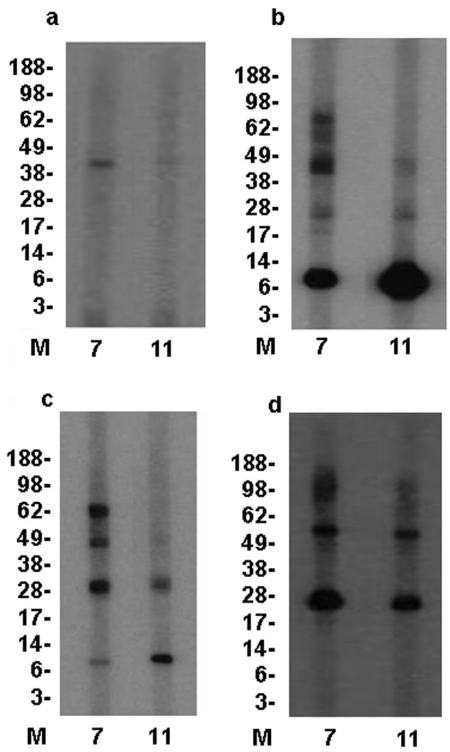

Figure 3. Immunoblotting with 2 selected sera (7, 11) using grape extract 1 (a), extract 2 (b), extract 3 (c) and extract 5 (d). M: molecular weight standard.

To compare both methods and determine the importance of grinding, immunoblotting was performed with several sera using extracts 1, 2, 3 and the commercial extract 5. A considerable variation between extraction methods was observed (Fig. 3). Extract 1 lacks some important IgE-reactive protein bands (Fig. 3a compared to b – d), both extracts

Table 2. Skin prick test results (mm^2) with different grape extracts of interest; our optimized extract 2, two different commercial extracts (Stallergen and Allergopharma) used for SPT in diagnosis compared with the wheal induced after prick-to-prick testing with fresh grapes

Pt number	Extract 2	Extract 5 (Stallergen)	Commercial grape extract (Allergo-pharma)	Prick-to-prick with fresh grapes
3	5	(−)	(−)	4
5	5	(−)	(−)	5
6	5	(−)	(−)	5
8	3.5	(−)	4	7.5
19	4	(−)	(−)	5.5
20	3	(−)	(−)	3
27	4	(−)	(−)	3
33	3	(−)	(−)	3
34	3	(−)	(−)	3.5
35	4	3.5	(−)	5.5
38	3	4	(−)	7
44	4	(−)	(−)	4

1 and 5 lack the low Mr IgE-binding protein identified as grape LTP, the major allergen for our population [9, 23] (Fig. 3a and d *vs.* b and c). Comparing extracts 2 and 3, the IgE-binding capacity of the ground material seems superior, especially for the 9 kDa band (Fig. 3b *vs.* c). Extract 5 was superior in identifying a 28 kDa IgE-binding protein (Fig. 3d *vs.* a–c).

Skin prick test results with extracts 2, 5 (Stallergen) and an additional commercial grape extract (Allergopharma), as well as prick-to-prick tests with fresh grapes, were in accordance with the *in vitro* testing results. Both commercial extracts used were positive only in 2/12 and 1/12 patients tested, respectively, whereas our proposed extract 2 gave similar wheal diameter (3.8 ± 0.8 mm) to the one revealed after prick-to-prick with the fresh fruit (4.6 ± 1.5 mm) (Table 2).

4 Discussion

Grapes have a water content of 80.6% [25] and are an important source of complex phenols, known as tannins, a group of astringents and bitter compounds found in the seeds and skin of grapes that slow down oxidation processes and promote ageing [26]. Tannins are implicated in grapes' pharmacological properties against cancer, inflammation and lipid oxidation [26]. In contrast to these beneficial characteristics, tannins can hamper protein isolation because, when oxidized, they bind proteins irreversibly and either precipitate or produce a gelatinous material [25]. Additionally, like most of the acidic fruits and vegetables, grapes also contain pectins. Pectins constitute a heterogeneous group of acidic structural polysaccharides which at low pH (<3.5) and in the presence of sufficient sugars rapidly form thermally irreversible gels, which are hard to separate [27]. Although the standard Björksten protocol [14], successfully implemented for the extraction of several plant allergens including grapes [4], takes some precautions against the processes described here, it did not lead to efficient extraction in our case. Grape content in various substances such as proteins, sugars and phenolic compounds is influenced by and dependent on their origin, climatic changes and conditions of cultivation (Aivaliotis, A., "*TABLE Soultanina Grapes OF ZEMENO*", http://www.soyltanina.gr/en/index 2006).

The insufficient extraction included low Mr proteins, such as the major allergen in our population [9], grape LTP, which in very low concentrations mainly appears in the skin of fruit [28–31].

Therefore, in this study we focused on generating a standardized extraction procedure providing a representative pattern of grape proteins [10–12] of allergenic interest [3, 4, 9] whilst protecting them against aggregation and precipitation and retaining antigen-binding capacity. By concentrating phosphate buffer and adding Tween-20 at neutral pH, the protein tannin hydrophobic interactions [15, 24] were minimized, resulting in a better extraction of grape proteins. A protective effect was obtained by increasing the concentration of reducing agent and proteolytic inhibitor (DIECA), combined with low extraction temperatures (below 15°C). Because grape skin had higher protein content compared to whole grapes and showed no remarkable differences in protein pattern on gel, grape skin was used as the optimal source material. Indeed, significant increase in the protein yield and IgE-reactivity was detected upon pulverization of frozen grape skin. Finally, the additional bi-ionic buffer, as described by Jona and Fronda [12], resulted in the removal of sugars (pectins, constituting almost 90% of grape's cell wall [32]), which reduced their interference on protein storage, testing and analyses.

The described protocol was implemented in five different cultivars more frequently consumed in Greece (roditis, kerino, koliniatis, asprouda and stafida) and no significant differences occurred in terms of their IgE-binding protein content. Therefore, cultivar stafida was used for our large-scale experimental procedures.

Satisfactory, qualitative grape protein extraction with content similar to the results of our optimized suggested protocol on gel, was in the case of extracts prepared using acetone-treated grape skin, as previously described by Lopez [15] and a concentrated phosphate buffer [14] containing 5% PVP-10. Further ionic fractionation followed by gel filtration gave less total protein yield. Therefore, we decided on the described optimal extract, because it is reproducible and less time consuming (in comparison with the cold acetone method), giving satisfactory results on isolating grape allergens for both small and large scale experiments.

The results of the comparison were in favour of our protocol not only for the self-prepared extracts according standard protocols, but also the two diagnostic commercial grape extracts we evaluated herein. Our optimized extract maintained allergenicity with respect to several IgE-binding proteins and especially the major allergen, grape LTP [9, 23]. This highlights the significance of good extracts used in the clinical practice due to the possibility of false negative results, added to the up-to-date reports describing difficulties in preparing allergen extracts from fruits, vegetables and other plant foods, mentioning wide variations between allergen range and content in extracts from different manufacturers [17, 33].

Furthermore, encouraging were the results from a first trial aiming to extend the protocol in other fruits. Extracts from apple, strawberry, plum, pear and peach prepared according to our optimized protocol seemed superior in terms of protein content and yield when parallelized with extracts prepared according to the standard Bjorksten protocol [14].

In conclusion, this study presents an easy and reproducible method to extract grape allergens suitable for diagnosis. The suggested protocol proved very efficient in comparison to several different standard extraction protocols and mainly the standard Bjorksten protocol and may in the future be implemented for the preparation of various commercial fruit extracts used in diagnosis as well.

E. Vassilopoulou was supported through the IFR Marie Curie Training Site Fellowship (QLK1-CT-2000-60030) and an Iraklitos fellowship for PhD students (Ministry of Greece for Education and Religions E.P.E.A.E.K II KA 70/3/7148). C. Mills and N. Rigby were funded by the BBSRC competitive strategic grant to IFR. L. Zuidmeer and R. van Ree were (partly) funded by a grant from the EC: SAFE QLK1-CT-2000-01394.

5 References

[1] Brown, E. A., Sensitivity to grape and grape products, including wine; case report, *Ann. Allergy* 1953, *11*, 590 – 593.

[2] Kalogeromitros, D. C., Makris, M. P., Gregoriou, S. G., Mousatou, V. G., *et al.*, Grape anaphylaxis: A study of 11 adult onset cases, *Allergy Asthma Proc.* 2005, *26*, 53 – 58.

[3] Rodriguez, A., Matheu, V., Trujillo, M. J., Martinez, M. I., *et al.*, Grape allergy in paediatric population, *Allergy* 2004, *59*, 364.

[4] Pastorello, E. A., Farioli, L., Pravettoni, V., Ortolani, C., *et al.*, Identification of grape and wine allergens as an endochitinase 4, a lipid-transfer protein, and a thaumatin, *J. Allergy Clin. Immunol.* 2003, *111*, 350 – 359.

[5] Caiaffa, M. F., Tursi, A., Macchia, L., Grape anaphylaxis, *J. Investig. Allergol. Clin. Immunol.* 2003, *13*, 211 – 212.

[6] Senna, G., Mistrello, G., Roncarolo, D., Crivellaro, M., *et al.*, Exercise-induced anaphylaxis to grape, *Allergy* 2001, *56*, 1235 – 1236.

[7] Rodriguez, A., Trujillo, M. J., Matheu, V., Baeza, M. L., *et al.*, Allergy to grape: A case report, *Pediatr. Allergy Immunol.* 2001, *12*, 289 – 290.

[8] Vaswani, S. K., Chang, B. W., Carey, R. N., Hamilton, R. G., Adult onset grape hypersensitivity causing life threatening anaphylaxis, *Ann. Allergy Asthma Immunol.* 1999, *83*, 25 – 26.

[9] Vassilopoulou, E., Zuidmeer, L., Akkerdaas, J., Tassios, I., *et al.*, Severe immediate allergic reactions to grape: Part of a lipid transfer protein-associated clinical syndrome, *Int. Arch. Allergy Immunol.* 2007, *143*, 92 – 102.

[10] Monteiro, S., Picarra-Pereira, M. A., Mesquita, P. R., Loureiro, V. B., *et al.*, The wide diversity of structurally similar wine proteins, *J. Agric. Food Chem.* 2001, *49*, 3999 – 4010.

[11] Monteiro, S., Picarra-Pereira, M. A., Teixeira, A. R., Loureiro, V. B., Ferreira, R. B., Environmental conditions during vegetative growth determine the major proteins that accumulate in mature grapes, *J. Agric. Food Chem.* 2003, *51*, 4046 – 4053.

[12] Jona, R., Fronda, A., Comparative histochemical analysis of cell wall polysaccharides by enzymatic and chemical extractions of two fruits, *Biotech. Histochem.* 1997, *72*, 22 – 28.

[13] Frankland, A. W., The characterization of allergens, *Clin. Exp. Allergy* 1996, *26*, 865 – 867.

[14] Bjorksten, F., Halmepuro, L., Hannuksela, M., Lahti, A., Extraction and properties of apple allergens, *Allergy* 1980, *35*, 671 – 677.

[15] López, J., Tejada, I., Vásquez, C., de Dios Garza, J., Shimada, A., Condensed tannins in tropical fodde crops and their in vitro biological activity: Part 2, *J. Sci. Food Agri.* 2004, *84*, 295 – 299.

[16] Nyman, B. F., Protein-proanthocyanidin interactions during extraction of scots pine needles, *Phytochemistry* 1985, *24*, 2939 – 2944.

[17] Vieths, S., Scheurer, S., Reindl, J., Luttkopf, D., *et al.*, Optimized allergen extracts and recombinant allergens in diagnostic applications, *Allergy* 2001, *56*, 78 – 82.

[18] Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., *et al.*, Measurement of protein using bicinchoninic acid, *Anal. Biochem.* 1985, *150*, 76 – 85.

[19] Bolhaar, S. T., Ree, R., Bruijnzeel-Koomen, C. A., Knulst, A. C., Zuidmeer, L., Allergy to jackfruit: A novel example of Bet v 1-related food allergy, *Allergy* 2004, *59*, 1187 – 1192.

[20] Aalberse, R. C., IgE-based radioimmunoassays for the quantitation of allergens, *Allergy* 1980, *35*, 236 – 238.

[21] Schuurman, J., Lourens, T. E., Perdok, G. J., Parren, P. W., Aalberse, R. C., Mouse/human chimeric IgE antibodies directed to the house dust mite allergen Der p 2, *Int. Arch. Allergy Immunol.* 1995, *107*, 465 – 466.

[22] Galland, A. V., Dory, D., Pons, L., Chopin, C., *et al.*, Purification of a 41 kDa cod-allergenic protein, *J. Chromatogr. B Biomed. Sci. Appl.* 1998, *706*, 63 – 71.

[23] Vassilopoulou, E., Rigby, N., Moreno, F. J., Zuidmeer, L., *et al.*, Effect of in vitro gastric and duodenal digestion on the allergenicity of grape lipid transfer protein, *J. Allergy Clin. Immunol.* 2006, *118*, 473 – 480.

[24] Kawamoto, H., Quantitative determination of tannin and protein in the precipitated by High-Performance Liquid Chromatography, *Phytochemistry* 1995, *40*, 1503 – 1505.

[25] Chaundhry, B. Y., Hunain, A., Riazuddin, T., Mini-scale Genomic DNA Extraction from Cotton, *Plant Mol. Biol. Rep.* 1999, *17*, 1 – 7.

[26] Palomino, O., Gomez-Serranillos, M. P., Slowing, K., Carretero, E., Villar, A., Study of polyphenols in grape berries by reversed-phase high-performance liquid chromatography, *J. Chromatogr. A* 2000, *870*, 449 – 451.

[27] Chaplin, M., Water structure and behaviour, *London South Bank Univ. Appl. Sci* 2000.

[28] Asero, R., Mistrello, G., Roncarolo, D., de Vries, S. C., *et al.*, Lipid transfer protein: A pan-allergen in plant-derived foods that is highly resistant to pepsin digestion, *Int. Arch. Allergy Immunol.* 2001, *124*, 67 – 69.

[29] Pastorello, E. A., Farioli, L., Pravettoni, V., Ortolani, C., *et al.*, The major allergen of peach (*Prunus persica*) is a lipid transfer protein, *J. Allergy Clin. Immunol.* 1999, *103*, 520 – 526.

[30] Pastorello, E. A., Pompei, C., Pravettoni, V., Brenna, O., *et al.*, Lipid transfer proteins and 2S albumins as allergens, *Allergy* 2001, *56*, 45 – 47.

[31] Sancho, A. I., Foxall, R., Rigby, N. M., Browne, T., *et al.*, Maturity and storage influence on the apple (*Malus domestica*) allergen Mal d 3, a nonspecific lipid transfer protein, *J. Agric. Food Chem.* 2006, *54*, 5098 – 5104.

[32] Barnavon, L., Doco, F., Terrier, N., Ageorges, A. *et al.*, Analysis of cell wall neutral sugar composition, β-galactosidase activity and a related cDNA clone throughout the development of *Vitis vinifera* grape berries, *Plant Phys. Biochem.* 2000, *38*, 289 – 300.

[33] Akkerdaas, J. H., Wensing, M., Knulst, A. C., Krebitz, M., *et al.*, How accurate and safe is the diagnosis of hazelnut allergy by means of commercial skin prick test reagents?, *Int. Arch. Allergy Immunol.* 2003, *132*, 132 – 140.

