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Journal Pre-proofs

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PII: S0963-9969(23)00477-5
DOI: https://doi.org/10.1016/j.foodres.2023.112932
Reference: FRIN 112932

To appear in: *Food Research International*

Received Date: 3 October 2022
Revised Date: 24 April 2023
Accepted Date: 27 April 2023

Please cite this article as: Beriziky, P., Cherkaoui, M., Linxe, L., Perrin, E., Rogniaux, H., Denery-Papini, S., Morisset, M., Larré, C., Dijk, W., Hemp seed: an allergen source with potential cross-reactivity to hazelnut, *Food Research International* (2023), doi: https://doi.org/10.1016/j.foodres.2023.112932

Hemp seed: an allergen source with potential cross-reactivity to hazelnut

Princy Beriziky[1,2&], Mehdi Cherkaoui[1,3], Laura Linxe[1], Emilie Perrin[1], Hélène Rogniaux[1,3], Sandra Denery-Papini[1], Martine Morisset[2], Colette Larré[1*], Wieneke Dijk[1*#]

[1] INRAE, UR BIA, F-44316, Nantes, France

[2] CHU d'Angers, unité d'allergologie, Angers, France

[3] INRAE, PROBE research infrastructure, BIBS Facility, F-44316 Nantes

[&] *current address* : CHU d'Angers, unité d'allergologie, Angers, France

* Co-senior authors

[#] corresponding author: Wieneke Dijk. Impasse Thérèse Bertrand-Fontaine, 44300, Nantes, France. Wieneke.dijk@inrae.fr

Abstract

The increasing exposure of the population to *Cannabis sativa* has revealed allergies to different parts of the plant, among which hemp seed. Nonetheless, the major hemp seed allergens remain to be identified. Several known families of allergens are present in hemp seed, including notably seed storage proteins. We therefore aimed to investigate the potential allergenicity of the hemp seed storage proteins and their potential cross-reactivity to different seeds and nuts. For this, we extracted hemp seed proteins sequentially using buffers with increasing levels of salinity (H_2O, T2 and T3) to yield extracts differentially enriched in storage proteins. We used these extracts to perform immunoblots and ELISAs using sera of patients either sensitized to hemp seeds or sensitized/allergic to other seeds and nuts. Immunoblots and proteomics analyses identified vicilins and edestins as potential hemp seed allergens. Moreover, ELISA analyses revealed a correlation between sensitization to hazelnut and the hemp seed T3 extract (enriched in storage proteins). The possible cross-reactivity between hazelnut and hemp seed proteins was further strengthened by the results from inhibition ELISAs: the incubation of sera from hazelnut-sensitized individuals with increasing concentrations of the T3 extract inhibited serum IgE binding to the hazelnut extract by about 25-30%. Our study thus identifies vicilins and edestins as potential hemp seed allergens and highlights a possible cross-reactivity with hazelnut. The clinical relevance of this cross-reactivity between hemp seed and hazelnut needs to be further investigated in hazelnut-allergic individuals.

Key words

Hemp seed, *Cannabis sativa*, food allergy, cross-reactivity, hazelnut, storage proteins

Abbreviations

Cs	Cannabis sativa
THC	Tetrahydrocannabinol
nsLTP	non-specific lipid transfer protein
PBS	Phosphate buffered saline
PBS-T	PBS-0.1% Tween20
PVP	Polyvinylpyrrolidone

1. Introduction

Cannabis sativa (*Cs*) is an annual herbaceous weed of the *Cannabinaceae* family that has been cultivated and consumed by mankind for several thousand years (Skypala *et al*., 2022). This herbaceous plant is well adapted to a temperate climate and is of particular interest in the current context of an agro-ecological transitioning towards a more plant-based diet. The main uses of *Cs* include the production of fibre, seeds for food and feed and psychoactive drugs. *Cs* varieties with a low tetrahydrocannabinol (THC) content (<0.3%) are grown for industrial and food purposes. To differentiate between these different uses, the term "hemp" has been dedicated to *Cs* plants used for industrial purposes, while the term "marijuana" is used for the flowers used for recreational purposes.

The increased exposure of the population to this plant has revealed allergies to different parts of the plant: pollen, leaves and seeds. *Cs* hypersensitivity can give different clinical symptoms: from isolated skin symptoms and mild respiratory manifestations to anaphylaxis following marijuana smoking or hemp seed ingestion (Bortolin *et al*., 2016; Cabrera-Freitag *et al*., 2019; Decuyper, Van Gasse, *et al*., 2019; Skypala *et al*., 2022; Stokes *et al*., 2000). Although hemp seed-induced anaphylaxis remains rare (Bortolin *et al*., 2016) multiple aspects encouraged us to explore the allergenic risk of this novel food. Hemp seed or hemp seed derivatives gain in popularity thanks to their nutritional properties and the trend towards an increased consumption of plant-based proteins. Hemp seed proteins are for example incorporated in high-fibre pasta, energy bars or hemp milk (House *et al*., 2010; Mamone *et al*., 2019). Despite this increasing exposure, the allergenic risk of these products remains poorly evaluated. Studies on allergies associated with *Cs* exposure have mostly focused on the allergens present in flowers, pollen or leaves, and not in seed. Indeed, the major *Cs* allergen Can s 3 is a non-specific lipid transfer protein (nsLTP) present in *Cs* flowers and leaves (Gamboa *et al*., 2007). Can s 3 is relatively conserved among plant species and responsible for allergic cross-reactions referred to as the "cannabis-fruit-vegetable" syndrome (Skypala *et al*., 2022). This allergen was not detected in a hemp seed concentrate using shot-gun proteomics (Mamone *et al*., 2019). In contrast, Can s RuBiSco, another allergen previously identified in *Cs* flowers (see **Supplemental Table 1**), was identified in hemp seed using proteomics (Aiello *et al*., 2016).

Nonetheless, many allergens implicated in severe anaphylaxis to nuts and seeds are storage proteins, which have not been identified as allergens for *Cs* (Costa *et al*., 2022). The major allergenic storage protein allergens comprise the 2S albumins (e.g. Ara h 2, Cor a 14 and Jug r 1, major allergens involved in severe allergy to peanut, hazelnut and walnut respectively), the

7S globulins (e.g. Ara h 1 (peanut) or Cor a 11 (hazelnut)) and the 11S globulins – also called edestins in hemp (e.g. Ara h 3 (peanut) or Cor a 9 (hazelnut)) (Costa *et al*., 2022). Furthermore, a preliminary study indicated that several patients with hemp seed sensitization were also sensitized to tree nuts (Alkhammash *et al*., 2019). This warranted us to investigate the potential allergenicity of hemp seed storage proteins and their potential cross-reactivity to nuts and other seeds. For this, we first characterized the protein composition of hemp seed using sequential protein extractions. The antigenicity of these extracts was then tested using sera of patients previously exposed to *Cs* and sensitized to hemp seed, or sera of patients sensitized or allergic to different seeds and nuts. These studies highlighted a potential cross-reactivity between hazelnut and hemp seed, which was further corroborated thanks to the identification of potential allergens in the hemp seed and an *in vitro* analysis of hazelnut-hemp seed cross-reactivity.

2. Materials & Methods

2.1 Hemp seeds & hazelnut powder

Organically grown hemp seeds (whole seeds and hulled seeds) were obtained from Sana Gaia (France). For hazelnut extractions, a powdered ground hazelnut flour was obtained from a factory that only handles hazelnut. Delipidated peanut extract was an in-house preparation produced using commercially available whole peanuts (supermarket, from Egypt or the United States) that were dehulled and mixed to obtain a paste. This paste was delipidated three times using petroleum ether and proteins were extracted using a 20mM phosphate buffer pH 7.4, NaCl 0.1M.

2.2 Hemp and food-allergic patients

Four separate patient groups were used for this study; 1) patients exposed to *Cs* and sensitized to hemp seeds, 2) patients allergic or sensitized to peanut, soy, tree nuts, and other seeds, 3) patients allergic to egg, 4) non-allergic individuals or individuals with isolated pollen allergies and reactive to Bet v 1 or profilin. Six sera from patients exposed to *Cs* and sensitized to hemp seed were selected on the basis of a positive skin prick test (SPT) to hemp seed (diameter > 3 mm) (see **Table 1**). These sera included sera from patients reporting contact urticaria to *Cs* (serum 2184) or suffering from allergic rhino conjunctivitis during professional exposure to *Cs* pollens (serum 2183). One patient also reported mild to moderate rhino conjunctivitis upon recreative exposure to *Cs* and episodes of oral pruritus and eyelid oedema possibly related to hemp seed ingestion (serum 2191). Other sera were selected among patients allergic or

sensitized to peanuts, soy, tree nuts and/or other seeds, but without known contact or sensitization to hemp seed (43 patients) (see **Supplemental Table 2**). No SPT to hemp seed were conducted in this patient population.

Sera from non-allergic individuals, individuals with isolated pollen allergies, and 10 egg-allergic patients with positive IgE to ovalbumin and without known allergies or sensitization towards peanut and/or tree nuts were also selected for this study as negative controls (see **Supplemental Table 2**). Sera from non-allergic controls and egg-allergic patients belong to a registered sera collection (DC-2008-809; of INRAE, UR1268 BIA Biopolymères, Interactions, Assemblages, Nantes, France). Sera from hemp seed-sensitized patients, patients sensitized or allergic to peanuts, soy, tree nuts or other seeds, and patients with isolated pollen allergies were obtained thanks to a collaboration with the Allergy unit and the laboratory of Immunology and Allergology of Angers University hospital, France (BB-0033-00038) (see **Supplemental Table 2**). All sera were obtained from patients who provided free and informed consent (or their legal representatives), and sera were stored at -80 °C until use.

2.3 Hemp seed and hazelnut extracts

Dehulled hemp seeds were frozen at -80 °C and crushed cold using a blender, and a pestle and mortar. After freeze-drying of the crushed material, a delipidation with cold hexane was performed (Devouge *et al*., 2007). Protein extraction of the freeze-dried, delipidated, dehulled seeds was carried out sequentially: first in distilled water (**H_2O**) (2h at room temperature), then in solutions of increasing salinity: **T2** (Tris 50mM + NaCl 50mM, pH 7.5, 2h at room temperature) and **T3** (Tris 50mM + NaCl 500mM, pH 7.5, 2h at room temperature) using 10 mL of solution per gram freeze-dried sample. Salt addition in buffers increases the extraction of globulins (7S, 11S). A direct extraction of dehulled, delipidated hemp seed powder in the Tris 50mM + NaCl 500mM, pH 7.5 solution (**TT**) was also made (2h at room temperature) using 10 mL of solution per gram freeze-dried sample. After each extraction step, the suspension was centrifuged for 30 min at 4200*g and the supernatant collected. For sequential extractions, the pellet was resuspended at each step in 10 mL of solution per gram of the initial freeze-dried material. Collected supernatants were desalted using dialysis (Cellu-Sep T1-19-30 ; T1 membrane, MCO 4000-6000, flat width 19mm, at 4°C), frozen and freeze-dried before analysis by SDS-PAGE. . A house-made hazelnut extract was made using the same method as for the hemp seed extract **TT**. A commercial hazelnut extract from the ALK company (Hørsholm, Denmark) was used for comparison.

2.4 Protein determination

Nitrogen determination of the dehulled seed and freeze-dried extracts was done according to the Dumas method (Rapid N exeed nitrogen analyser, Elementar). A conversion factor of 5.7 was used to evaluate the protein content. For SDS-PAGE and Western blot analyses, lyophilized protein extracts were reconstituted at a 2 mg/mL concentration in carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$) and protein concentration was determined using a BCA assay (Pierce).

2.5 SDS-PAGE & Western Blot

10 or 15 µg of proteins were separated by polyacrylamide gel electrophoresis, SDS-PAGE, under non-reduced or reduced conditions either using NuPAGE 4-12% Bis-Tris gels (#NP0335BOX, Life Technologies SAS, France) and a Bolt MES SDS running buffer (#B0002, Life Technologies), or using 8-16% Mini-Protean TGX Stain-Free gels (#4568104, Bio-Rad, France) and a Tris-Glycine-SDS running buffer (#161-0772, Bio-Rad). A 1x Laemmli sample buffer (#161-0747, Bio-Rad) was used for sample loading, with (reduced) or without (non-reduced) 5% 2-mercaptoethanol and heating at 95 °C for 5 min. A stained marker (#161-0394, Bio-Rad) was run in parallel. Gels were run at 100V and either used for immunoblotting or coloration with Coomassie Blue. For Coomassie Blue staining, the gels were fixed in 10% trichloroacetic acid (TCA) during 10 minutes and then stained overnight in a solution containing 2.4% Coomassie Blue R250, 10% glacial acetic acid, 30% ethanol in distilled water). Destaining was done using repetitive washes with a solution containing 50% ethanol and 10% glacial acetic acid (in distilled water).

For immunoblotting, the Trans-Blot® Turbo™ Blotting System (Bio-Rad) was used according to the machine's standard protocol. After transfer, the membrane (ImmunBlot PVDF membrane, #162-0174, Bio-Rad) was saturated with a solution of 4% Polyvinylpyrrolidone (PVP40, #P-0930, Sigma-Aldrich) in Phosphate buffered saline (PBS) and 0.1% Tween20 (PBS-T) for 4h at room temperature. This membrane was then incubated with sera from the different patients, diluted in a solution of PBS, 0.1% Tween20 and 2% PVP40 overnight with gentle agitation. The secondary antibodies used (anti-human IgE-HRP, #S9160-05 Southern Biotech) were diluted to $5x10^{-5}$ and incubated for 1.5 h at room temperature. After incubation with sera or secondary antibodies, the membrane was washed 3x 10 minutes using PBS-T. The revelation was performed with the Advansta Quantum Western Bright Kit (Advansta, #K-12042). Acquisition was done with a Fujifilm LAS 3000 camera.

2.6 Indirect Enzyme Linked ImmunoSorbent Assay (ELISA)

Protein extracts (hemp seed H_2O, hemp seed T2, hemp seed T3, hemp seed TT, walnut, hazelnut ALK, hazelnut TT, soy isolate, and delipidated peanut) were solubilised in a Na_2CO_3 (30 mM) + $NaHCO_3$ (70 mM) solution, pH 9.6, and diluted to a concentration of 8 µg/mL. A rabbit anti-human IgE (A0094, Agilent) in 1/1000 dilution was also coated to determine total IgE levels and for the IgE standard. 20 µL of the extracts were placed in each well (384-wells, NUNC Maxisorb #460372) in triplicates and allowed to stand for 2 hours at room temperature. The plates were washed 3x in PBS-0.1% Tween20 (PBS-T), followed by saturation with PBS-T with 0.5% porcine skin gelatin, 20 µL per well. Plates were incubated for 2 hours at 37 °C, before performing 3 washes in PBS-T. A range of standardized human IgE (NIBSC Code 11/234) was used as a standard at concentrations ranging from 0.07 to 160 ng/mL diluted in PBS-T with 0.5% gelatin. Patient sera were diluted 1:10 in PBS-T with 0.5% gelatin. 20 µL of diluted sera or IgE standard was added to each well and incubated overnight at 37°C, followed by 3 washes with PBS-T. An alkaline-phosphatase-conjugated anti-human IgE (A3525, Sigma, Saint-Quentin Fallavier, France), in PBS-T with gelatin (0.5%) was added at 1:500 dilution (20 µL) and incubated at 37 °C for 2 hours, followed by 3 washes with PBS-T. Revelation was done with 4-methylumbelliferyl phosphate (4-MUP, M-3168, Sigma-Aldrich, France) five times diluted in 1M Tris HCL pH 9.8. In each well, 30 µL of 4-MUP was placed and the plates were left to incubate for 90 minutes at room temperature in the dark. Fluorescence was measured by the SYNERGY HT microplate reader with excitation at 360 nm and emission at 440 nm. Total IgE and specific IgE were determined using the IgE standard curve (0.07 to 160 ng/mL) and regression analysis.

2.7 Inhibition ELISA

Hazelnut protein extracts or purified ovalbumin were solubilised in a Na_2CO_3 (30 mM) + $NaHCO_3$ (70 mM) solution, pH: 9.6, and diluted to a concentration of 4 µg/mL. 100 µL of the extracts were placed in each well (96 wells, NUNC Maxisorb #436110) in duplicates and incubated overnight at 4 °C. The plates were washed 3x in PBS-0.1% Tween20 (PBS-T), followed by saturation with PBS-T with 4% Polyvinylpyrrolidone (PVP) (#P0930, Sigma Aldrich), 200 µL per well. Plates were incubated for 1.5 hour at room temperature, before being washed 3x with PBS-T. Increasing dilutions of the hazelnut or hemp seed T3 protein extracts, or ovalbumin were incubated with 1/10 or 1/20 diluted patient sera (diluted in 1% PVP-PBS-T) for 2h at 37 °C. After washing, 100 µL per well of incubated sera and negative controls were added to the wells and incubated at room temperature for 2h. An HRP-conjugated anti-human

IgE (S9250-05, Southern Biotech) was added at a 1:5000 dilution (100 µL) and incubated at room temperature for 1 hour, followed by 3 washes with PBS-T. Revelation was done with o-Phenylenediamine dihydrochloride (OPD, Sigma-Aldrich, France), 10 mg diluted in 25 mL citrate buffer 50mM pH 5.5 and before usage 10 µl of H_2O_2 was added. In each well, 100 µL of OPD was placed and the plates were left for 30 minutes at room temperature in the dark. Reaction was stopped with 50 µl of 4N H_2SO_4. Absorbance was measured by the SYNERGY HT microplate reader at 490 nm and 630 nm.

2.8 Proteomics

Following identification in the hemp seed extracts by SDS-PAGE, bands of interest were selected based on a strong immunoblot signal and a signal in more than one patient, cut out and destained. These bands were reduced and alkylated before undergoing trypsinolysis, as described by Cherkaoui *et al*. (Cherkaoui *et al*., 2018). Two µL of the resulting peptides were then injected into a nanoLC-MS/MS system with a C18 column and eluted at a flow rate of 0.3 µL/min. The gradient elution solvents were (A) H_2O/0.1% formic acid (v/v) and (B) 90% ACN/ 0.08% formic acid (v/v). After a column equilibration step at 4% B from 0 to 15 minutes, the gradient consisted of a ramp from 4 to 50% B from 15 to 60 minutes, followed by a second ramp from 50 to 90% B from 60 to 63 minutes, a plateau at 90% B from 63 to 65 minutes and then a return to 4% B from 65 to 75 minutes. Mass spectrometry analysis was performed using a Q-Exactive HF mass spectrometer (Thermo Fischer Scientific). The Orbitrap analyser (m/z 400-2000) was used in DDA (Data Dependent Acquisition) mode to acquire MS scans at 60K resolution. HCD fragmentation spectra were recorded at 15k resolution for the 15 most intense MS ions. The raw LC-MS/MS files were processed in mgf files before interpretation against a database restricted to hemp proteins (Uniprot restricted to taxon 3483, version of 01/04/2021) using the X!Tandem pipeline software (version 0.4.27). Two modifications were set for the search: cysteine carboxymethylation and methionine oxidation. The search was done with tryptic hydrolysis with three allowed miss cleavages. Precursor mass and fragment mass tolerances were set at +/-5 ppm and 5 ppm respectively. The results obtained for peptide identification were validated for peptides with an e-value of less than 10^{-3}. Only the most abundant proteins in each SDS-PAGE band were retained for the presentation of the results. For mass spectrometry analysis of the global composition of the extracts, a similar approach was used as described above, except that extracts were subjected to SDS-PAGE for only 5 minutes before the migrated proteins were cut out from gel. For protein identification in extracts, only proteins with a minimum of 8 specific peptides were taken into consideration.

For their characterisation, proteins were identified with at least 2 peptides and a minimum e-value score of 10^{-4}. When protein function was not clearly annotated in the hemp database, a BLAST analysis on the UNIPROT platform (www.uniprot.org) was performed. For clarity, we chose to report the best homologous protein belonging to a seed used in human food. Sequence alignment was done using Clustal Omega ((Sievers *et al*., 2011) and visualized using mView ((Brown *et al*., 1998).

2.9 Statistics

A two-tailed Pearson correlation test was performed to show a relationship between IgE-mediated food sensitisations detected by ELISA and hemp seed sensitisation. Values outside the range of our ELISA were converted to unassigned values. A p-value <0.05 was defined as the threshold for statistical significance of the results. GraphPad Prism version 8 was used for statistical analyses.

3. Results

3.1 Sequential extractions of hemp seeds yield extracts differentially enriched in storage proteins

To obtain protein extracts differentially enriched in the diverse storage proteins present in the hemp seed (2S albumins, 7S and 11S globulins), a total extract (**TT**) and sequential extractions of dehulled, freeze-dried and delipidated hemp seed were made using buffers with an increasing level of salinity (**H_2O, T2**, and **T3**). SDS-PAGE analysis of the extracts suggested that the T2 extract was enriched in 2S albumins (migrating around 14 kDa), whereas the T3 extract was enriched in edestins (migrating around 50 kDa). The H_2O extract seemed to have a lower amount of storage proteins, whereas the TT extract, as expected, showed an intermediate profile between the T2 and T3 extracts (**Figure 1**). 7S vicilins were also identified in the different extracts by SDS-PAGE, as sample reduction does not impact their migration due to the absence of cysteines and thus disulphide bonds. No clear enrichment of vicilins was observed in any of the extracts (**Figure 1**). All extracts were significantly enriched in protein content compared to hemp seed, as demonstrated using nitrogen determination according to the Dumas total combustion method (see **Supplemental Table 3**).

Mass spectrometry analysis on the full extracts confirmed these visual observations, with a clear enrichment in edestin-specific peptides observed in the T3 extract, an enrichment of 2S-albumin

specific peptides in the T2 extract, and an intermediate peptide profile in the TT extract according to the number of spectra identified as specific peptides of these protein classes (**Supplemental Table 4**). Moreover, we observed a better average sequence coverage of edestin accessions in the T3 extract than in the T2 extract (81.4% vs 63.8%, respectively), and a better sequence coverage of albumin accessions in the T2 extract than in the T3 extract (33.1% vs 21.8%, respectively). An equivalent amount of 7S-like vicilins was detected in all extracts (**Supplemental Table 4**). Lastly, in all extracts, a small number of peptides identified as nsLTP and oleosins were also detected, the latter being known allergens notably in hazelnut and sesame (Leduc *et al*., 2006; Nebbia *et al*., 2021) (**Supplemental Table 4**).

3.2 Immunoblot of hemp extracts against sera of patients sensitized to hemp seed

In order to evaluate the possible allergenic risk of hemp seed consumption in a population exposed to *Cs*, sera from six patients presenting with a positive SPT to native hemp seed (**Table 1**) were tested *in vitro*. The reactivity of the sera was tested against the different hemp seed fractions in their reduced form (**Figure 2A**). Using immunoblotting, only two sera (2181, 2191) among six patients with a positive SPT to hemp seed showed IgE binding towards hemp seed proteins (**Figure 2B**). No notable IgE binding was detected using two control sera (2218, 2263), whereas the secondary antibody alone gave a very moderate or low binding signal around 8, 16 and 20 kDa (**Figure S1**). The two reactive patients showed IgE binding to a protein of around 55 kDa in the T3 extract, with and without reductive conditions (**Figure 2B**). An additional band at 30 kDa was recognised by the IgE of patient 2191 (**Figure 2B**). However, this band was only observed for patient 2191 and was therefore not selected for further MS identification. Mass spectrometry-based analyses of the IgE-binding band seen in both patients did not formally identify the protein of 55 kDa, but indicated a significant sequence homology with the 7S vicilins of *Corylus Avellana* (hazelnut) and *Juglans regia* (walnut) (**Figure 2C** *band 2.1/2.2***, Supplemental Table 5**). On top of this, conformational surface epitopes previously identified in different tree nut allergens (Barre *et al*., 2008) were found to be conserved in the *Cs* proteins (**Figure 2C**). A more minor sequence homology of around 25% was seen for Ara h 1, a well characterized peanut vicilin (**Supplemental Table 5**).

3.3 Potential cross-reactivity of hemp extracts with other food products

In order to study the potential cross-reactivity between hemp seeds and other nuts and seeds, we conducted ELISA with the sera from 43 patients sensitized or allergic to nuts and seeds and

using a variety of protein extracts (our house-made hemp seed extracts, peanut, soy, walnut and hazelnut) (**Supplemental Table 2**). Among these patients, 32 patients were sensitized or allergic to peanut, 21 sensitized or allergic to hazelnut and 16 sensitized or allergic to other tree nuts (**Table 2**). Patients were classified as 'allergic' when their food allergy was confirmed by an allergist, whereas patients with specific IgE or SPT but without a clear clinical history of food allergy were classified as 'sensitized'. A few patients were sensitized or allergic to other seeds: sesame, mustard and flax (**Supplemental Table 2**).

As expected from the clinical profile of the patients, peanut and hazelnut protein extracts were recognised by nearly half of the patients' sera. Among the different hemp seed extracts tested, seric IgE binding was mostly observed to the T3 hemp seed extract, which contains the highest concentration of edestins (**Supplemental Table 2, Tab 1a and 1b**). Interestingly, we observed that nearly all of the patients with specific IgE to the hazelnut extract also had specific IgE against the hemp seed T3 extract. We also observed that this was less the case for patients with specific IgE to the peanut or soy extract (**Supplemental Table 2, Tab 1a and Tab 1b**) For other seed and nut allergies, the number of included patients was too low to draw any firm conclusions. To determine to what extent the IgE binding to the hemp extracts and the hazelnut extract were correlated, Pearson correlations were performed using the data of specific IgE reactivity. When considering all patients with specific IgE to hazelnut based on the ELISA results, these analyses revealed a noticeable correlation between the hazelnut IgE reactivity and IgE reactivity towards the hemp T3 extract, with an R^2 of 0.3189 and a p-value <0.005 (**Figure 3, Figure S2**). The strength of this association between hazelnut and hemp seed IgE reactivity seemed to differ slightly between two patient groups (with R^2 of 0.7 and 0.8 when these groups were analysed separately), but no clear clinical differences could be identified between these two patient groups (**Figure 3, Figure S2**). A similar association was found when all patients with specific IgE to the hemp seed T3 extract (based on the ELISA results) were considered; a correlation was observed between their IgE reactivity to the T3 extract and their IgE reactivity towards the hazelnut T3 or a commercial hazelnut extract (**Figure S2**). In contrast, among patients with specific IgE to peanut, no correlation was observed between IgE reactivity of their sera to peanut and the IgE reactivity to hemp T3 extract (**Figure 3**). Taken together, these observations suggest a potential cross-reactivity between hemp seed and hazelnut proteins.

3.4 Immunoblot of hemp seed extracts using sera of patients sensitized or allergic to hazelnut

For immunoblotting, we next selected ten sera from hazelnut-allergic and hazelnut-sensitized patients that had a clear IgE reactivity to the hemp T3 extract in ELISA (**Table 2**, **Supplemental**

Table 2). Two sera from peanut-allergic individuals (2197, 2214) were used as negative controls. Immunoblotting showed significant IgE binding to proteins of the hemp seed T3 extract in six out of ten patients (sera 2203, 2219, 2248, 2258, 2216, 2201) under non-reduced and reduced conditions (**Figure 4B and 4C, Figure S3**). The reactivity of the sera was tested against hemp seed fractions in their non-reduced and reduced form in order to observe modifications of protein-IgE binding under these conditions and to obtain information on the proteins recognized by specific IgEs. In the non-reduced extract, sera 2258 and 2248 showed IgE binding to 4 bands of approximately 35, 45, 50 and 60 kDa, although the IgE binding of serum 2258 was rather diffuse (**Figure 4B**). Sera 2203 and 2216 show IgE binding to bands at approximately 35, 45 and 50 kDa, while serum 2201 only recognised bands at 45 and 50 kDa (**Figure 4B and 4C**). Serum 2219 showed a lower and more diffuse signal (**Figure 4B**). After reduction of the extract, most sera recognised proteins of 25 and 35 kDa (sera 2258, 2248, 2203, 2216, 2201), while two sera showed a persistent IgE binding to proteins of 20, 45 and 60 kDa (sera 2258 and 2248) (**Figure 4B and 4C**). The IgE binding by serum 2219 was again more diffuse, but binding to proteins at 50 and 60 kDa could be observed (**Figure 4B**). No noticeable IgE binding was detected using the sera of peanut-allergic individuals (**Figure 4D**).

To identify the nature of the hemp seed proteins that reacted with patient IgE, mass spectrometry analyses were performed. The IgE-binding bands of 35, 45 and 50 kDa in the non-reduced extract, as well the bands of 35, 25 and 20 kDa in the reduced extract were identified as 11S edestins (**Figure 4A**, *bands 4.1-4.6*, **Supplemental Table 5**). The main edestin of 50 kDa disappears upon reduction with appearance of a subunit located around 35 kDa, presumably the α-subunit of edestin, which was mostly recognised by IgE (**Figure 4B and 4C**). It should be noted that a band of 25 kDa was also identified as a 7S vicilin-like protein (**Figure 4A**, *band 4.5*, **Supplemental Table 5**).

Mass spectrometry analyses also showed that the IgE-binding proteins of 60 kDa in the non-reduced condition (*band 4.7*), and of 45 and 60 kDa in the reduced condition (*bands 4.8 & 4.9*) matched edestins (*band 4.7*), or were uncharacterized proteins with a high sequence homology to 7S vicilins (*band 4.8 & 4.9*) (~60%, *Corylus avellana*) (**Figure 4A**, **Supplemental Table 5**). The 60 kDa protein is likely similar to the proteins identified in bands 2.1 and 2.2, recognized by serum IgE of hemp seed-sensitized individuals and identified as 7S vicilin-like proteins (**Figure 2**). These data suggest that IgE of hazelnut allergic patients react towards the edestins and the 7S vicilins of hemp seed. Of interest, sequence homology analyses indicated a 55-60% sequence homology of *Cs* edestins to other tree nut allergens, such as the hazelnut allergen Cor

a 9, the walnut allergen Jug r 4 and the pecan nut allergen Car i 4 (**Figure 5**). Furthermore, several previously identified allergenic hot spots of tree nut legumins (Robotham *et al*., 2009) seem to be conserved in the *Cs* edestins (**Figure 5**). The identified 7S vicilin-like protein A0A7J6G321 was previously described in **Figure 2** and thus has a noticeable amino acid sequence homology with the allergenic vicilins of hazelnut (Cor a 11) and walnut (Jug r 6 and Jug r 2).

3.5 Potential cross-reactivity between hazelnut proteins and hemp seed proteins

To confirm the IgE cross-reactivity between the storage proteins of *Cs* and hazelnut, a competitive inhibition of IgE binding to hazelnut with T3 hemp seed extract was performed using an ELISA assay. We compared three different pools of sera. One pool consisted of 7 patients allergic or sensitized to hazelnut and reactive to the T3 extract by ELISA (sera 2201, 2216, 2253, 2241, 2248, 2254, 2258). A prior incubation of this pool of sera with increasing concentrations of hemp seed T3 extract revealed a reduction of their IgE binding capacity to the hazelnut extract with about 25% when 10 µg/mL hemp seed T3 extract was used (**Figure 6A**). To confirm these observations, we used three different individual sera pools : pool I, the serum of the hazelnut-sensitized patient 2216 (sera obtained at two different time points, used at 1:20 dilution); pool II, composed of the sera of two hazelnut-sensitized patients 2201 and 2254, which have a similar IgE reactivity profile to the hemp seed and hazelnut extracts (1:10 dilution); and pool III, corresponding to the sera of two hazelnut-allergic patients 2248 and 2258 (1:40 dilution). Again, a prior incubation of the sera with increasing concentrations of hemp seed T3 extract significantly reduced their IgE binding to the hazelnut extract by about 15-30% depending on the serum pool (**Figure 6B**). Importantly, these inhibitions appeared to be specific, as the IgE binding of egg-allergic patients to ovalbumin was not impaired when their sera were pre-treated with increasing concentrations of the hemp seed T3 extract (**Figure S4**). These results suggest that there is a potential cross-reactivity between hemp seed and hazelnut.

4. Discussion

Due to the increasing exposure to *Cs*, reports of allergic reactions to marijuana and anaphylactic reaction to cross-reacting food have dramatically increased (Skypala *et al*., 2022). Some allergic reactions to hemp seed have been reported, but no major allergens have so far been identified in hemp seed (Aiello *et al*., 2016; Alkhammash *et al*., 2019; Bortolin *et al*., 2016). In this work, we highlight the potential allergenicity of several proteins present in hemp seed. We observed

a moderate binding of IgE of hemp seed-sensitized patients to 7S vicilin-like proteins and a strong IgE binding to edestins and 7S vicilin-like proteins with the serum of hazelnut allergic patients.

Surprisingly, the seric IgE reactivity measured in ELISA was weak in patients mono-sensitized to *Cs* flower, whereas the strongest IgE reactivity in hemp seed-sensitized patients was observed in nuts- and seeds-allergic patients. Immunoblotting of hemp seed extracts confirmed this observation, as the IgE binding proteins vary according to the patient groups: an IgE binding to 7S vicilins was predominantly observed in hemp seed-sensitized patients, whereas a pronounced IgE binding to edestins was found in patients allergic or sensitized to nuts and seeds. This would imply a different reactivity profile that likely depends either on the sensitization route or on the food that caused primary sensitization (different parts of) *Cs* or another food. In a recent study of 15 patients sensitized to hemp seed (Alkhammash *et al*., 2019), 11/15 patients presented with allergic symptoms at the first ingestion of hemp seed. On the basis of SPT, they showed that five of the sensitized patients were also sensitized to pitted fruits or tree nuts and that 4/5 of sensitized individuals presented allergic symptoms following first-time hemp seed ingestion. Our results are consistent with this study and encourage further exploration of the relationship between hemp and tree nuts, in particular hazelnut and walnut, whose storage proteins have a significant sequence homology to those of hemp seed.

Our competitive inhibition ELISA confirmed a partial IgE-cross reactivity between some storage proteins (edestins) of hazelnut and hemp seed. Given that hazelnut allergy is the most common food allergy in Europe - especially in adults - the clinical relevance of cross-reactivity between hazelnut and hemp needs to be rapidly investigated (McWilliam *et al*., 2020). An extensive immunological cross-reactivity has been previously observed between the 11S globulins of hazelnut and walnut, which are the two tree nuts with the highest sequence homology with the *Cs* 11S edestins (55-60%) (Asero *et al*., 2004; Villalta *et al*., 2019). Although we do not provide clinical prove for the cross-reactivity between hazelnut and hemp seed, our results do warrant for vigilance amongst allergists. We identify hemp seed storage proteins as potential allergens with a possible cross-reactivity to hazelnut. Food allergies to storage proteins are usually severe, due to the high stability of these allergens during cooking and digestion, and extra vigilance is needed as hemp seed is a food without mandatory allergen labelling (Villalta *et al*., 2019).

Currently known cross-reactivities for *Cs* involve Can s 3, a ns-LTP, and dietary cross-reactions of Can s 3 with hazelnut and walnut ns-LTPs have been reported (Decuyper, Rihs, *et al*., 2019).

We hypothesized that Can s 3 would also be found in hemp seed, but we found only a minor quantity of a ns-LTP (accession: A0A7J6G7Y9) in our (dehulled) hemp seed extracts (**Supplemental Table 4**). Also, using sera of patients reactive to walnut ns-LTP, we did not observe a significant IgE binding to our different hemp seed extracts using ELISA (**Supplemental Table 2)**. It should be noted that a significant amount of ns-LTP might have been lost during the dehulling or delipidation steps, due to their high affinity to lipids.

In conclusion, this is one of the rare studies on the allergenic potential of hemp seed proteins. We identify the storage proteins of hemp seed, the vicilins and edestins, as potential allergens and highlight a possible cross-reactivity of hemp seed proteins with hazelnut proteins. Future studies are needed to formally confirm *Cs* edestins and vicilins as allergens through recombinant allergen production or allergen purification followed by immunoblotting. Also, the possible cross-reactivity of hemp seed with hazelnut needs to be further investigated in the clinic using basophil or mast cell activation assays with hemp seed extracts and conducting oral challenges with hemp seed in hazelnut-allergic individuals. Whether hemp seed proteins also cross-react with other tree nuts, such as walnut, is a question that remains open for future studies and that would require more sera of walnut-allergic individuals. Lastly, to what extent hemp seed ns-LTPs can clinically cross-react with other plant ns-LTPs should also be further explored, by assessing the presence of nsLTPs in the hull and the seed before delipidation and by immunoblotting of the hull and non-delipidated seed extracts.

Acknowledgements

The authors kindly thank the biological resource center (CRB) of Angers University Hospital (BB-0033-00038) for conditioning, storage and delivery of the sera we have studied. We would also like to express our gratitude to all the patients who offered their serum for the benefit of research. The authors also kindly thank Véronique Solé-Jamault (UR1268, BIA, Nantes) for excellent technical assistance. Finally, we thank the company Hemp-It for the generous supply of hemp pollen, flowers and leaves that were used in more exploratory stages of this study.

Author contributions

CL, MM, Conceptualization; PB, WD, Data curation; PB, WD, CL, Formal analysis; WD, CL, Funding acquisition; PB, MC, WD, LL, EP, Investigation; PB, MC, WD, LL, EP, Methodology; MM, HR, Resources; CL, SD, MM, HR, Supervision; PB, WD, Visualization; Roles/Writing - WD, PB, original draft; all authors, writing - review & editing.

Declaration of interest

None

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Table 1: Characteristics of patients sensitized to hemp seed. ND = not determined, SPT = skin prick test, Cs: cannabis sativa.

Patient code	**Age**	**IgE to hemp (kUA/L)**	**SPT to hemp seed (mm)**	**SPT to *Cs* flower**	**Exposition to Cannabis sativa (*Cs*)**	**Clinical reaction to *Cs***	**Other allergies**	**Other known IgE sensitization**
2181	33	ND	4	ND	*Cs* flower smoking in the past	None	Buckwheat anaphylaxis, Grass pollinosis	Pru p 3 (nsLTP), Alb 2S (Fag e 2)
2183	27	5.73	6	Positive	Professional exposure to hemp pollen	Rhino conjunctivitis upon respiratory exposure	Grass pollen, hemp seed	IgE Pru p 3 negative
2180	26	ND	5	Positive	Active smoker of *Cs*	None	Grass and Birch pollinosis	IgE Pru p 3 negative
2185	27	0.12	10	ND	Regular ingestion of hemp seeds	Unknown	Eosinophilic oesophagitis, (pollinosis)	Soy 7S and 11S
2184	41	0.59	8	Positive	*Cs* flower, active smoking	Contact urticaria, respiratory discomfort on inhalation	Birch pollinosis	IgE Pru p 3 negative
2191	31	ND	3	Positive	*Cs* flower, active smoking	Rhino conjunctivitis upon respiratory exposure, oropharyngeal oedema on inhalation	Birch pollinosis, Anaphylaxis to walnut, almond, peanut and peach	Pru p 3 (nsLTP)

*Table 2: Summary of clinical characteristics of patients allergic or sensitized to peanut, soy and/or tree nuts. Patients with a confirmed food allergy by an allergist are denoted under 'Allergic patients', whereas patients with IgE reactivity or skin prick sensitization but without a clear clinical history of food allergy are denoted under 'Sensitized individuals'. * sera from patients with a significant signal in immunoblot (IB) experiments (cf. not all patients were tested due to insufficient sera). # sera from patients used for inhibition ELISA.*

	Allergic patients			**Sensitized individuals**		
	Total	*Patient codes*	*Used for IB* or inhibition ELISA#*	*Total*	*Patient codes*	*Used for IB* or inhibition ELISA#*
Peanut	**23**	*2188, 2192, 2195, 2197, 2199, 2206, 2209, 2212, 2214, 2217, 2220, 2226, 2228, 2229, 2231, 2235, 2238, 2242, 2250, 2253, 2259, 2261, 2262*	*2253*#*	**9**	*2200, 2201, 2211, 2216, 2241, 2248, 2255, 2256, 2257*	*2201#*, 2216#*, 2241*#, 2248*#*
Soy	**3**	*2192, 2199, 2259*		**4**	*2200, 2201, 2245, 2265*	*2201*#*
Hazelnut	**8**	*2203, 2209, 2219, 2241, 2242, 2248, 2253, 2258*	*2203*, 2219*, 2241*#, 2248*#, 2253*#, 2258*#*	**13**	*2188, 2199, 2201, 2206, 2212, 2216, 2217, 2229, 2242, 2250, 2254, 2255, 2259*	*2201*#, 2216*#, 2254*#, 2259**
Other tree nuts	**8**	*2203, 2209, 2224, 2226, 2241, 2242, 2254, 2258*	*2241*#, 2241*#, 2254*#, 2258*#*	**8**	*2188, 2193, 2201, 2203, 2208, 2216, 2248, 2251*	*2201*#, 2216*#, 2248*#*

Figure legends

Figure 1. SDS-PAGE of different protein-enriched fractions from hemp seed. SDS-PAGE (4-12% Bis-Tris gel) with 15 µg of protein from sequential extractions of dehulled, freeze-dried and delipidated hemp seed with buffers with an increasing level of salinity (H2O, T2 (Tris 50mM + NaCl 50mM, pH 7.5) and T3 (Tris 50mM + NaCl 500mM, pH 7.5). A direct extraction in the Tris 50mM + NaCl 500mM, pH 7.5 solution was also done, yielding a total extract (TT). Protein-enriched fractions are shown under non-reduced and reduced conditions. Lanes with molecular weight markers are denoted as *M***.**

Figure 2. Western blot of hemp seed protein-enriched fractions using sera from hemp seed-sensitized individuals. A. SDS-PAGE (4-12% Bis-Tris gel) of 10 µg protein of *Cs* H2O extract, T2 extract and T3 extract under reduced conditions. B. Western blots (4-12% Bis-Tris gel) of 10 µg protein of H20 or T3 extract (serum 2181) or H20 extract, T2 extract and T3 extract (serum 2191) using sera from 2 different patients (2181 and 2191) under reduced conditions. Reactive bands (Figure 2A, in red) were cut from the corresponding Coomassie Blue gel and analyzed by mass spectrometry. The band surrounded by a red dashed line was only observed for patient 2191 and was therefore not selected for further MS identification. Lanes with molecular weight markers are denoted as *M*. C. Sequence alignment of two *Cs* 7S vicilin-like proteins identified by mass spectrometry ("*1"* and "*2"*) with the allergens Cor a 11 ("*3"*) of hazelnut and Jug r 6 ("*4"*) of walnut. Protein surface epitopes previously identified for tree nut vicilins (Barre *et al*., 2008) are boxed in black. Sequence alignment was performed with Clustal Omega.

Figure 3. A. Pearson correlation coefficient of determination of the IgE reactivity of hazelnut-sensitized individuals towards hazelnut and the hemp T3 extract, as measured using indirect ELISA. B. Pearson correlation coefficient of determination of the IgE reactivity of peanut-sensitized individuals towards a peanut extract and the hemp T3 extract, as measured using indirect ELISA.

Figure 4. Western blot of hemp seed protein-enriched fractions using sera from hazelnut-sensitised individuals. A. SDS-PAGE (4-12% Bis-Tris gel) of 10 µg protein of hemp seed T3 extract under non-reduced and reduced conditions. B-D. Western blots (4-12% Bis-Tris gels) of 10 µg protein of hemp seed T3 extract under reduced and non-reduced conditions, using sera from (B) 4 hazelnut-allergic (2248, 2258, 2203, 2219) or (C) 2 hazelnut-sensitized (2216, 2201) or (D) 2 peanut-allergic individuals (2197, 2214). Reactive bands (in red, blue and green)

identified in Figure 4B-D were cut from a corresponding Coomassie Blue gel (Figure 4A) and analyzed by mass spectrometry. Non-reduced conditions are denoted with « - », whereas reduced conditions are denoted with « + ». Lanes with molecular weight markers are denoted as *M*.

Figure 5. Sequence alignment of a legumin-type *Cs* protein identified by mass spectrometry ("*1*") with the allergens Cor a 9 ("*2*") of hazelnut, Jug r 4 ("*3*") of walnut and Car i 4 ("*4*") of pecan nut. Linear allergenic hot spots of different legumins as previously identified (Robotham *et al*., 2009) are boxed in black. Sequence alignment was performed with Clustal Omega.

Figure 6. Inhibition of IgE binding to hazelnut extracts following a pre-incubation of sera with the hemp seed T3 extract. A & B. Inhibition of IgE binding to hazelnut extracts using ELISA. The hazelnut T3 extract was used for coating (4 µg/mL), and increasing concentrations of the hemp seed T3 extract were used for inhibition of serum reactivity. (A) inhibition ELISA using a sera pool of 7 individuals sensitized or allergic to hazelnut that reacted to the hemp T3 extract (sera 2241, 2248, 2253, 2258, 2201, 2216, 2254), or (B) inhibition ELISA using 3 different sera pools. Pool I: sera from 1 hazelnut-sensitized individual (serum 2216, pool of 2 different serum samplings, 1/20 dilution; inhibition with hazelnut 10 µg/mL = 93%,), pool II: sera of 2 hazelnut-sensitized individuals (serum 2201 and 2254, 1/10 dilution, inhibition with hazelnut 10 µg/mL = 96%) or pool III: sera from 2 hazelnut-allergic individuals (serum 2248 and 2258, 1/40 dilution, inhibition with hazelnut 10 µg/mL = 93%). Percentage of inhibition is shown as a function of the protein quantity used for inhibition.

Author contributions

CL, MM, Conceptualization; PB, WD, Data curation; PB, WD, CL, Formal analysis; WD, CL, Funding acquisition; PB, MC, WD, LL, EP, Investigation; PB, MC, WD, LL, EP, Methodology; MM, HR, Resources; CL, SD, MM, HR, Supervision; PB, WD, Visualization; Roles/Writing - WD, PB, original draft; all authors, writing - review & editing.

Highlights

- The allergenic risk of exposure to hemp seed was assessed
- 7S vicilin-like proteins and edestins are potential hemp seed allergens
- A cross-reactivity exists between hazelnut and hemp seed proteins

Graphical Abstract

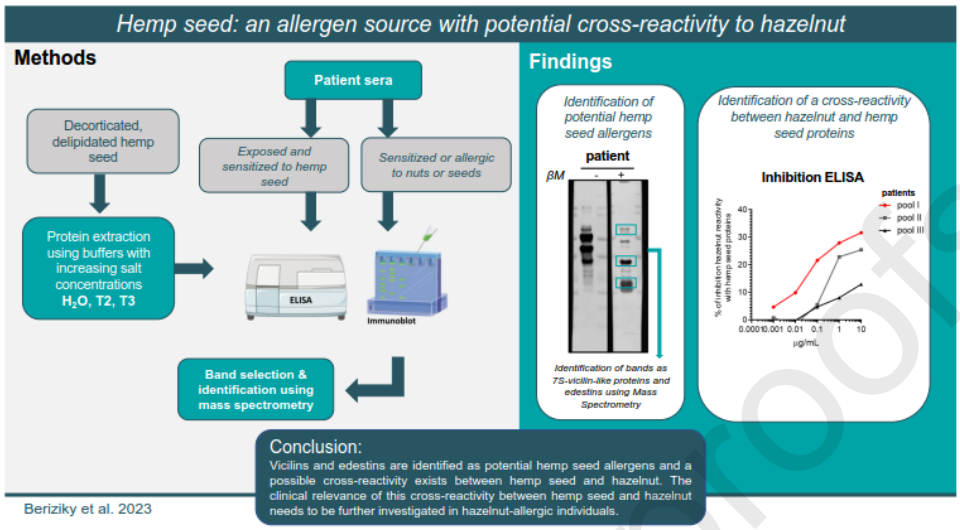

Figure 1

Click here to access/download;Figure;Figure1.pptx

Figure 1

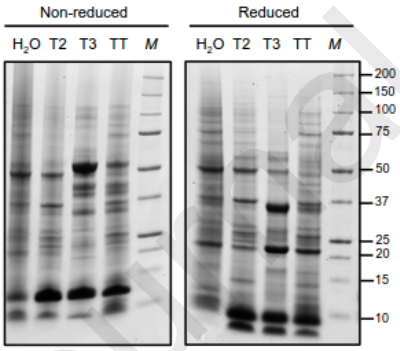

Figure 2

Figure 2

Click here to access/download;Figure;Figure2.pptx

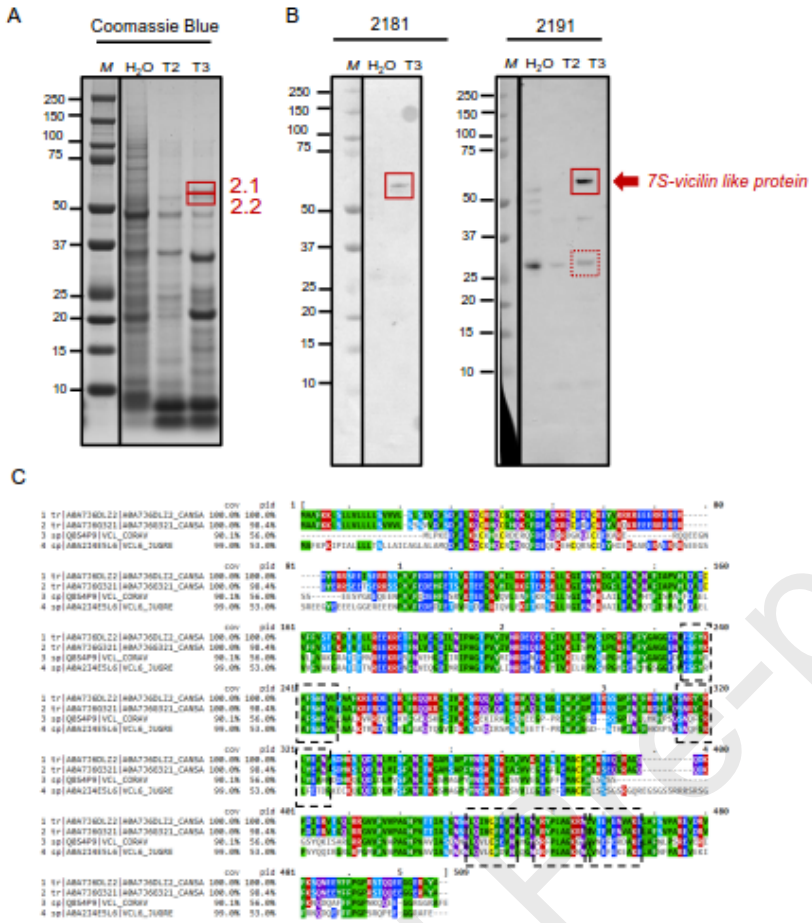

Figure 3

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Figure 3

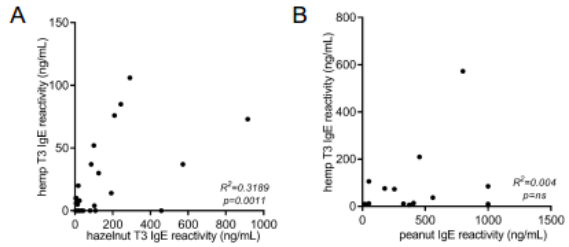

Figure 4

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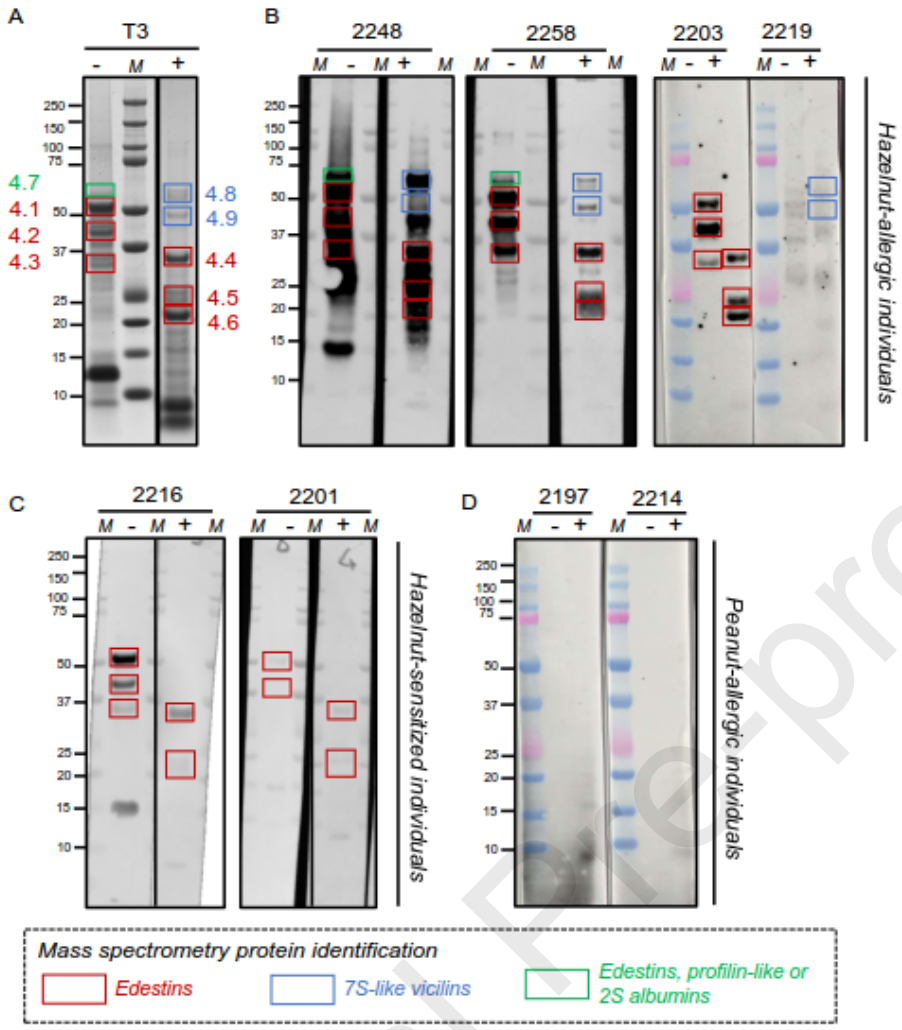

Figure 5

Figure 5

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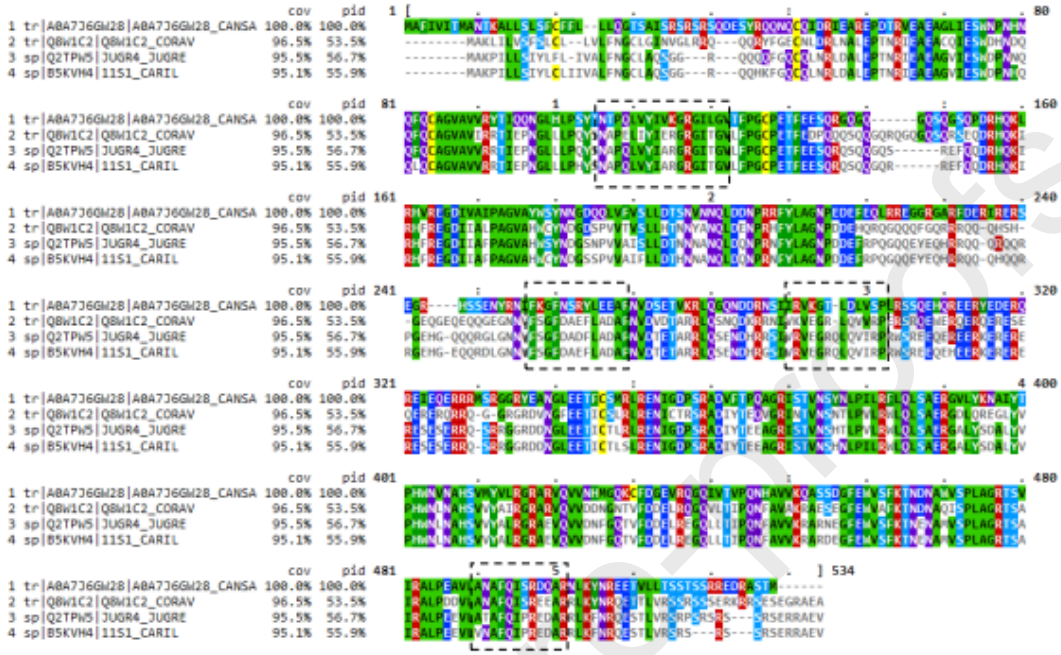

Figure 6

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Figure 6