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RESEARCH LETTER

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Low levels of gluten and major milk allergens *Bos d 5 and Bos d 11* identified in commercially available honey

To the Editor,

Despite allergy and allergic reactions to honey being widely regarded as rare, there have been documented systemic allergic reactions following ingestion of honey.[1] Current literature suggests that pollens and components derived from bees are the main cause of such reactions.[1,2] However, interestingly and perhaps unknown to many allergic patients and allergists, there are also reports of supplementary bee feeding with food allergen-loaded mixtures of soybean flour, dried brewer's yeast (containing high levels of residual gluten from brewing processes) and dry skimmed milk with sugar and water.[3] Furthermore, there have been reports of mould contamination within beehives.[4] Both factors suggest a potential for gluten, food and mould allergen protein presence in honey, which could account for some of the reported reactions following honey consumption. As such, the aim of this study was to determine if commercially available honey contained undeclared gluten and/or food or mould allergens, and at levels which could present a risk to individuals with hypersensitivities.

To investigate this, honey samples (n = 40) of UK, EU, Non-EU and blended Non-EU/EU origin were extracted and analysed for gluten using the Neogen Veratox Gliadin R5 Gluten ELISA, which is regarded as the gold standard for gluten measurements in the food industry. Major allergen content was measured using InBio MARIA and MARIA for Foods quantitative multiplex arrays for cow's milk, egg, peanut, soy, hazelnut, cashew and mould allergens. The MARIA immunoassay is based on xMAP® technology (Luminex Corp.) which uses polystyrene or magnetic microspheres that are internally labelled to create distinct sets of beads. Separate bead sets are covalently coupled with allergen-specific monoclonal antibodies, enabling the simultaneous capture and detection of multiple allergens in a single sample.[5]

Gluten (gliadin) assays were conducted according to manufacturer instruction. Honey samples were extracted by transferring 250 mg of honey to a 50-ml sterile centrifuge tube to which 2.5 ml of renaturing cocktail solution (Neogen 8515, 8515B, 8515S) was added. The resultant suspension was vortex mixed for 30 s and incubated in a water bath at 50°C for 40 min. Samples were then cooled for 10 min at room temperature (RT), and 80% v/v ETOH was added. Samples were mixed as previously described for 20 s and then rotated at 200 rpm for 60 min before 100 µl of the resultant solution was added to 1.25 ml of phosphate buffered saline (PBS). A negative honey control was employed during testing for the presence of Gliadin R 5, this was produced on the day of testing and consisted of; 28 g glucose, 14 g fructose and 8 g of Sterile distilled water. In all tests, the negative control did not produce a result above the assay limit of detection of 2.5 parts per million (ppm) of gliadin, or 5 ppm of gluten.

Analysis was repeated on two separate occasions and results are an average of these two measurements. Approximately, 50% of gluten is available as gliadin. Therefore, results for gliadin were multiplied by two to determine the levels of gluten.

Two MARIA arrays were used for sample analysis. A MARIA for foods multiplex array allowed the simultaneous quantification of Peanut (Ara h 3, Ara h 6), Cow's Milk (Bos d 5, Bos d 11), Egg (Gal d 1, Gal d 2), Cashew (Ana o 3), Hazelnut (Cor a 9) and Soy (Gly m 5) allergen. A second MARIA multiplex array allowed for the simultaneous quantification of mould (Asp f 1, Alt a 1) allergens. The arrays use highly purified allergen standards to quantify specific allergen proteins from samples. Assays were carried out as described by Filep and Chapman.[5]

Prior to analysis, samples (1 g) were extracted in 20 ml PBS 0.05% Tween-20, pH 7.4. Samples were briefly vortexed and incubated on a rocking platform for 120 min at RT. The resulting extracts were stored at −20°C prior to analysis. Positive results were confirmed by repeat extractions (n = 4–5 samples per honey) with results presented as an average of replicate extractions. Sample extracts were analysed in twofold dilution series, ranging from neat up to 1:80. Recovery of allergen proteins from honey was verified through production of an incurred honey sample (as described in online Open Science Framework repository: https://osf.io/vd28j/, 10.17605/OSF.IO/VD28J).

Of the 40 samples analysed, it was observed that 8 of the 40 (20%) samples contained gluten in the range of 5 ppm to 13.8 ppm. Positive honey samples categorized by origin are detailed in Table 1. Of the 21 non-EU honey samples analysed, six were positive for gluten. From the nine UK and nine EU/non-EU blend honey samples, each had one sample positive for gluten. This represents a gluten-positive sample rate of 28.6% and 11.1% for non-EU and both UK and EU/non-EU blends respectively.

Milk allergens Bos d 5 and Bos d 11 were detected in 7.5% of samples. The positive results ranged from 0.368 ppm (mg/kg), up to 0.567 ppm for Bos d 5, and from 0.030 ppm up to 0.182 ppm for

Bos d 11; with one sample being a blend of EU/Non-EU honey, and two being of UK origin. Two of the samples found to contain Bos d 5 and Bos d 11 were also positive for gluten. Results of the three positive samples are shown in Table 1. No detectable amounts of egg, peanut, soy, hazelnut, cashew or mould allergen was identified in the samples.

To our knowledge, this novel study is the first of its kind to identify the presence of milk allergen and gluten within honey. To determine whether the gluten and milk allergen detected in this study posed a risk to those with hypersensitivities, levels of allergen measured were compared to current consumption guidelines and thresholds. All honey samples analysed and measured here would be classified as "gluten free" under current EU legislation of <20 ppm,[6] offering some level of reassurance to those with gluten sensitivities. The levels of milk allergen detected were compared to the Voluntary Incidental Trace Allergen Labelling (VITAL) 3.0 reference doses. These reference doses suggest limits for the declaration of allergenic ingredients at a level that will prevent 99% of allergic individuals from reaction, termed "Eliciting Dose 01," or "ED01," which in the case of cow's milk is 0.2 mg of milk protein per serving.[7,8] Taking a worst-case scenario, the highest value of milk allergen detected in this study was for Bos d 5, measured at 0.567 ppm (mg/kg); which equates an estimated value of 5.67 µg of total milk protein / gram of food (honey) on the assumption that Bos d 5 comprises 10% of total cow's milk proteins.[9] Allergic individuals can take confidence that when considering a typical suggested serving size of 15 g of honey (data from six suppliers, 11 honey samples), they would consume a 0.085 mg dose of cow's milk protein per serving, less than half the ED01 reference dose. It may be argued that it is not uncommon for individuals to consume more than the suggested serving size of products. However, even when considering a consumption of 30 g; double the suggested serving size, an ED01 dose of milk protein is not reached and so the majority of the milk-allergic population should be well-guarded from adverse reaction.

TABLE 1 Measurement of gluten and milk allergens Bos d 5 and Bos d 11 in commercially available honey

Honey origin	Gluten	Bos d 5	Bos d 11
NON-EU *(6/21)*	6.10	–	–
	11.00	–	–
	12.40	–	–
	13.80	–	–
	10.60	–	–
	7.70	–	–
UK *(2/9)*	–	0.49	0.06
	5.50	0.57	0.18
EU/NON-EU *(1/9)*	10.70	0.37	0.03
EU *(0/1)*	–	–	–

Note: Commercially available honey samples ($n = 40$) were analysed for gluten and allergen content. Sample results are reported as parts per million (ppm), equivalent to milligram per kilogram (mg/kg). Samples are grouped by origin of production.

Key messages

- Gluten and milk allergen proteins were identified in commercially available honey samples
- Levels of gluten and milk allergen in honey samples were below current safeguarding levels
- Honey suppliers may wish to conduct analyses to ensure low levels in future batches

Although the levels of allergen detected in this study are low, one must still consider that the most sensitive individuals may react to these low levels and therefore supplementary bee feeding may offer an explanation for some of the rare cases of honey induced reactions. Additionally, it might be speculated that during the production of honey, enzymes produced by bees could hydrolyse or digest gluten and allergen proteins, thus rendering them indetectable by the immunoassay techniques used herein that are raised against native proteins. It is feasible that some peptides may present an unknown risk, but since allergen proteins typically must be in their intact form to elicit a response, such measurements may not be of clinical relevance.

The levels of gluten and allergens in honey identified in this study all fell below current safeguarding guidelines and recommendations, and so may not be a risk for the majority of allergic and gluten sensitive individuals. However, in light of these findings' suppliers may wish to undertake allergen testing of their products to offer greater safety to consumers and verify levels of gluten and allergen routinely fall below mentioned safeguarding levels, especially if honey is bottled in factories handling other milk and wheat products. The fact that no allergen protein contaminants other than milk were identified in the honey samples offers consumers further reassurances. Current literature does not give reason to suspect contamination from food sources other than milk, soy and gluten, so it is encouraging that the data show agreement.

AUTHOR CONTRIBUTIONS

All authors contributed to the concept and design of the study. MDB and KK ran the assays. MAO, MDB and JAB wrote the initial manuscript and all authors contributed to the final version.

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CONFLICT OF INTEREST

MDB and MAO are employees of InBio. InBio is in the business of manufacturing purified allergens, immunoassays for allergen quantification and subsequent use for analytical and contract research services. JB and KK declare no conflicts of interest.

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