

Almond or Mahaleb? Orthogonal Allergen Analysis During a Live Incident Investigation by ELISA, Molecular Biology, and Protein Mass Spectrometry

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It is now well known that an incident investigated in the United Kingdom in 2015 of cumin alleged to be contaminated with almond, a risk for people with almond allergy, was caused by the *Prunus* species, *Prunus mahaleb*. In the United Kingdom, the Government Chemist offers a route of technical appeal from official findings in the food control system. Findings of almond in two official samples, cumin and paprika, which had prompted action to exclude the consignments from the food chain, were so referred. Herein are described the approaches deployed to resolve the analytical issues during the investigation of the incidents. The cross-reactivity of ELISA to *Prunus* species was confirmed, and although this is useful in screening for the genus, orthogonal techniques are required to identify the species and confirm its presence. Two novel PCR assays were developed: one specific for *P. mahaleb* and the other a screening method capable of identifying common *Prunus* DNA. Peptides unique to almond and mahaleb were identified, permitting LC-tandem MS and criteria were developed for peptide identification to forensic standards. This work enables a staged approach to be taken to any future incident thought to involve *Prunus* species and provides a template for the investigation of similar incidents.

It is now well known that an incident investigated in the United Kingdom in 2015 of cumin alleged to be contaminated with almond, a risk for people with almond allergy, was caused by the *Prunus* species, *Prunus mahaleb*. Also now well known is the potential for ELISA cross-reactivity to closely related *Prunus* species.

The integrity of the food supply chain has recently been notably compromised, e.g., in the horse meat scandal (1), but also by allergens fraudulently introduced into the supply chain,

with a risk of acute ill health or fatality (2). In October 2014, Canadian authorities reported undeclared peanut and almond protein in products containing cumin, *Cuminum cyminum*, an umbelliferous fruit of the Apiaceae (celery, carrot, or parsley) family. Hence, it was feared that a further, potentially life-threatening, breach of supply chain integrity had occurred (3). Suspicions in North America initially fell on adulteration (bulking out) of spices with cheap almond shells, or in parallel findings of peanut in spices, adulteration with cheaper peanut hulls or peanut meal (animal feed). Cross-contamination in harvesting, transport, storage, or milling was also a possibility, and this was considered the more likely scenario in the U.K. incidents. In the United Kingdom, the Government Chemist is mandated to offer a route of technical appeal from official findings in the food control system (4, 5). Two official samples, cumin and a paprika (*Capsicum annum*), both of which had prompted official action to exclude their consignments from the food chain, were so referred. Figure 1 shows the timeline in which these U.K. technical appeals were set. Herein are described the complementary approaches deployed to resolve the analytical issues during the live investigation of the incidents in 2015.

Almond and the *Prunus* Family

Almond *P. dulcis* (sweet almond), is part of the genus *Prunus* that contains over 400 species including apricot (*P. armeniaca*), cherry (*P. avium*), sour cherry (*P. cerasus*), peach (*P. persica*), plum (*P. domestica* and *P. salicina*), and mahaleb (*P. mahaleb*), all of which have multiple cultivars developed for commercial fruit production. It is also apparently common for almond to be grafted to peach rootstock (Berryman, P., personal communication, 2016). Mahaleb (also mahlab, white mahlab, mahleb, English cherry, Rock cherry, or St. Lucie cherry; *P. mahaleb* L. of the Rosaceae family, subfamily Prunoideae) is grown where the flesh and seed are important, e.g., in Turkey. Mahaleb puree, flour, and oil are produced from mahaleb fruit. Mahaleb seeds are an important source of protein and oil. The stones are cracked to extract the seed kernel, which is about 5 mm in diameter (6–8); however, little was known about *P. mahaleb* in the United Kingdom until 2015.

Analytical Strategy

Table 1 outlines the typical steps in a Government Chemist technical appeal (i.e., “referee”) case. The default analytical strategy is multiple replicate analyses on multiple days by

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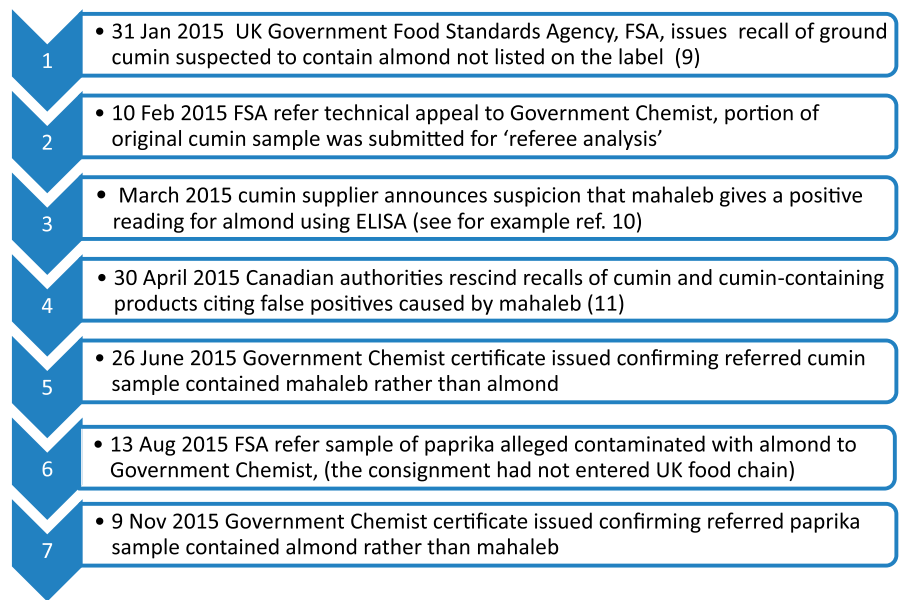


Figure 1. Timeline of cumin and paprika recall episodes in relation to UK technical appeals (9–11).

more than one technique, if possible. In the United Kingdom, food law falls under criminal law, and confirmation of the presence of an allergen to the forensic burden of proof (beyond reasonable doubt) requires orthogonal approaches. Initially, five approaches were considered: protein ELISA, bioinformatics, DNA PCR, protein MS, and GC-MS of volatile compounds.

Materials and Methods

Control materials were sourced as shown in Table 2. Unless preground, materials were ground to a fine powder using an IKA Tube Mill control with single-use milling vessels (IKA-Werke GmbH & Co. KG, Germany). Their species identity was subsequently confirmed using PCR sequencing primers annealing to the internal transcribed spacer 2 (ITS2) and

ribulose biphosphate carboxylase long chain (rbcL) regions of the nuclear and chloroplast genomes, respectively, followed by Sanger DNA sequencing of the resultant PCR products and sequence confirmation using the Barcode of Life Database (BOLD; 12) and GenBank database (13; data not shown).

ELISA

Three commercial ELISAs were applied: R-Biopharm RIDASCREEN FAST Almond kit, Romer Agraquant Almond kit, and ELISA Systems Mandel/Almond Residue kit (batch numbers on file). Kit instructions were followed and three replicates of the controls and the samples were plated in duplicate. Absorbances were read on a Tecan Sunrise plate reader (Männedorf, Switzerland). Quantification was done by

Table 1. Typical steps in a Government Chemist technical appeal (“referee”) case

Step	Description
1	Decision to accept and appropriate funding contribution
2	Scientific and administrative case meeting to identify appropriate techniques and methods (more than one technique when possible), allocate tasks and responsibilities, and schedule work
3	Appropriate legislation identified and checked
4	Method(s) investigated, and, if necessary, modified
5	Experimental work, multiple replicates on multiple days, reference materials and certified reference materials when possible, spikes, and all key steps witnessed by a second scientist
6	Data compiled and all data transcriptions checked
7	Results reviewed against quality criteria
8	Batches repeated or new analytical runs if required
9	Statisticians review datasets for outliers and batch effects and case-specific measurement uncertainties are calculated as appropriate
10	Results interpretation
11	Certificate drafted and reviewed and data independently checked
12	Formal case review meeting with Government Chemist for examination of the case file, queries addressed, appropriate steps repeated if required, and rereview if necessary
13	Certificate (official report typically 1500–3000 words) released to the immediate parties concerned

Table 2. Control materials

Sample	Brand/purchased from	Variety/cultivar	Country of origin	Batch No.
Organic cumin seeds	Just Ingredients (via Amazon)	Unknown	Unknown	DO-04-2014-H
Ground mahaleb	Steenbergs (via Amazon)	Unknown	Lebanon	17023
Mahaleb seeds	Supplied by the Food Standards Agency	<i>P. mahaleb</i>	Turkey	L131217
Almonds in shell	Buy Whole Foods Online (via Amazon)	Unknown	Australia/United States	Best before July 13, 2015
Whole almonds	Neal's Yard Wholefoods, Holland and Barrett	Unknown	United States	15071 09:15 2 90209
Apricots	Asda	Bebeco	South Africa	716975
Peach	Asda	Ohenry	Chile	728360
Peach (2)	Tesco	Florida Prince	Egypt	DPT4394

using the calibration curves of the standards supplied with each kit, with both kits specifying curve-drawing instructions, and an in-house quadratic equation.

Molecular Biology

A commercial DNA PCR assay (14) was applied to almond, apricot, and mahaleb kernels, all with positive responses. An in-house assay (15) was assessed, but owing to the evident sequence homologies, it was clear that no differentiation between *Prunus* species would be possible. A real-time PCR screening assay targeting the gene encoding for the Pru du 5 allergen in almond, which uses a fluorescent intercalating dye and high-resolution melt curve analysis (16), was applied, but we found that it was not appropriate. National Center for Biotechnology Information (NCBI; 17), GenBank, and BOLD databases were accessed to evaluate the available DNA and amino acid sequence information to assess the feasibility of designing bespoke assays specific for almond not cross-reactive with other members of the *Prunus* genus and/or applying a DNA-sequencing approach. These focused on differentiating between *P. armeniaca* (apricot), *P. dulcis* (sweet almond), and *P. mahaleb* (mahaleb). At the time the searches were conducted (March 24, 2015), 904 individual records of *Prunus* in the BOLD database were found, representing 188 different species within this genus and originating from 19 different countries. A search of the GenBank database revealed 236 565 nucleotide sequences for *Prunus*, 99 491 of which were derived from *P. armeniaca* and 1747 from *P. dulcis*, but only 49 from *P. mahaleb*. Searches of the NCBI nucleotide and BOLD Systems DNA databases were also conducted to identify all available sequences for the background matrix of *C. cyminum* (cumin) and, later, *C. annuum* (paprika). Table 3 descriptively summarizes the extent of bioinformatics available. Four common molecular phylogenetic markers—rbcL, maturase K, 5.8s ribosomal RNA, and ITS2—were examined for their potential to differentiate between the three queried *Prunus* species. An initial analysis of these markers revealed the existence of a very high degree of sequence similarity. However, a candidate ITS region was identified and a representative *P. mahaleb* sequence selected for subsequent assay design. DNA from the referred cumin sample and control samples was extracted, cleaned up, and precipitated. DNA pellets were washed and resuspended in nuclease-free water and the DNA quantified. All control materials were represented by two independent DNA extractions, whereas the referred cumin

sample was represented by four independent DNA extractions. Full details and the experimental design of the real-time PCR for the specific detection of *P. mahaleb* are described elsewhere (18).

In addition, for the paprika investigation, a general *Prunus* species real-time PCR-based assay was adapted from the above *P. mahaleb*-specific assay. Real-time PCR was performed under two-step thermal cycling conditions incorporating a melt analysis step using a 7900HT Fast Real-Time PCR System set to monitor fluorescence within the FAM™ dye spectral region (Life Technologies, Paisley, United Kingdom). The mp (dissociation of double-stranded DNA) of PCR product is a function of its constituent nucleotides and is instrumentally exhibited as an inflection in the fluorescence–temperature curve, in which the first derivative thereof generates peaks at the melting temperature. Full details of the developed novel rapid differentiation of common *Prunus* allergen species by PCR product melt analysis are described elsewhere (16).

Protein MS

MS following extraction—enzymatic (trypsin) digestion of protein in a reproducible manner to yield chromatographed peptides characteristic of and unique (20) to the protein of interest—was investigated. The method described by Heick et al. (21) was applied to kernel (apricot, almond, and mahaleb) and shell (apricot and almond) specimens. Three peptides resulting from the tryptic digests from the protein prunin were monitored by LC-tandem MS (MS/MS) by performing selective-reaction monitoring experiments. In order to improve the recovery of the peptides from the food matrixes and therefore increase sensitivity, an in-house

Table 3. Summary of the bioinformatics data available as of March 2015

Food	Quality and quantity of bioinformatics available
Cumin	Limited
Almond	Extensive, but fragmented
Apricot kernel	References linked to apricot in general rather than the kernel
Mahaleb	Very limited
Almond shell	None
Apricot shell	None

developed extraction method was applied to the samples and resulted in significantly improved extraction efficiency and sensitivity (19). The amino acid sequence of the protein prunin from almonds (*P. dulcis*) was also searched against sequences reported on open access databases, and strong similarities were observed when compared with the sequence of an uncharacterized protein from peaches (*P. persica*). In order to identify potential peptide targets not reported in the literature, which could differentiate almonds from other members of the *Prunus* genus, full-scan analysis of the above specimens was performed on a LC quadrupole time-of-flight instrument. Full experimental details of the applied method are described elsewhere (22).

GC-MS Volatile Compounds Profile

Spices impart aroma through volatile oils and taste through oleoresins; hence, the volatile compound profiles of cumin, almond, and mahaleb were explored by a combination of simultaneous distillation and extraction and were semiquantified based on a deuterated chlorobenzene internal standard. Several compounds were identified (acetone cyanohydrin, 2-hydroxypropanenitrile, and benzaldehyde) that may be characteristic for *P. mahaleb*. However, this approach was not taken forward in favor of the DNA and MS approaches, which were deemed to be more capable of yielding definitive outcomes.

Results

The referred cumin sample had the appearance of ground cumin and weighed 21.5 g. The referee paprika sample had the appearance of ground paprika and weighed approximately 160 g.

ELISA

Figure 2 shows the ELISA response to control apricot and mahaleb kernels relative to the response to almond kernel (set at 100%). The responses of each platform to milled almond and apricot shell were negligible. These data demonstrated that the then available ELISA kits were not a viable approach other than

for screening *Prunus* species in general, although the impact of the cross-reactivity of the items tested on kit C (see Figure 2) was less than on the other two kits examined. As expected, milled almond and apricot shell did not exhibit strong binding to the kit antibodies. ELISA findings in the referred samples of cumin and paprika are shown in Table 4 and confirm both samples contained a *Prunus* protein. In the case of the referred cumin sample, our findings were well below those of the official control laboratory (i.e., almond 306 mg·kg⁻¹) that triggered the recall. However, the *Prunus* protein was detected at concentrations above the LOQs of three ELISA platforms with statistical significance. The data do not include replication over different days; hence, the day-to-day variability of the different platforms has not been taken into consideration.

Real-Time PCR

Referred cumin sample.—Extracted DNA was assessed for quality and quantity, and the in-house real-time mahaleb-specific PCR assay (15) was applied to four individual extractions of the laboratory sample (at 25 ng and a 1:10 dilution; 2.5 ng total DNA per PCR) and to DNA extracts of control material with the results exhibited in Table 5. All samples were represented by a triplicate level of PCR replication. The instrumental response to positive and negative controls was completely satisfactory. DNA extracted from the laboratory sample generated a real-time PCR measurement response consistent with mahaleb being detected. From the quantification cycle values obtained, it is possible to infer that in relation to the control mahaleb and cumin materials examined, the amount of mahaleb present in the referred cumin sample was <0.01% (<100 mg·kg⁻¹) and could be <0.001% (<10 mg·kg⁻¹) based on the assumption that no other *Prunus* species were present in the sample that would compete as targets for the assay.

Referred paprika sample.—Extracted DNA was assessed for quality and quantity, and the in-house real time mahaleb-specific PCR assay (15) applied to six individual extractions of the referee sample (50 ng per PCR reaction) to 10% mahaleb and 10% control materials spiked into a background of paprika and to DNA extracts of control material. All samples were represented

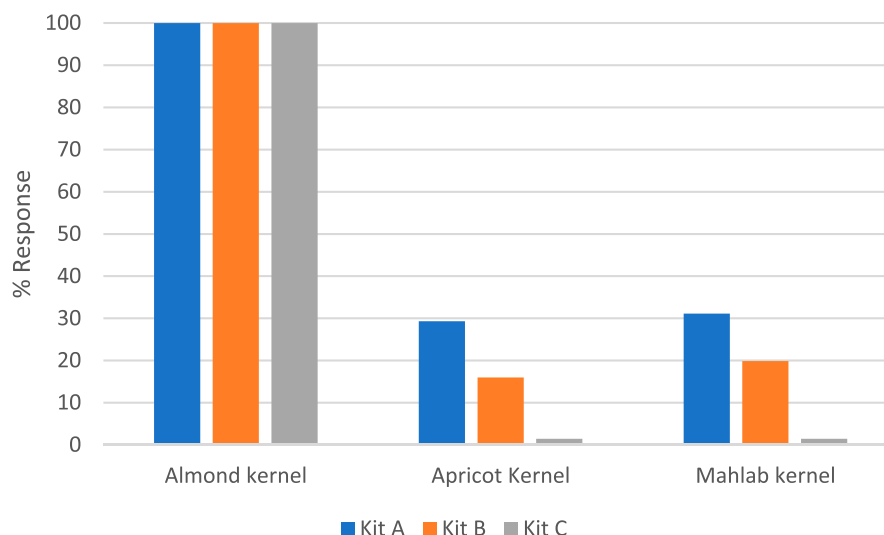


Figure 2. Relative responses of three ELISA platforms to almond, apricot and mahaleb kernels.

Table 4. Summary of ELISA results on the referred samples of cumin and paprika

Cumin	ELISA platform results for referred sample		
	A	B	C
Presumptive <i>Prunus</i> protein mean result mg·kg ⁻¹ (<i>n</i> = 3) expressed as almond kernel ±U ^a	3.3 ± 0.4	11.1 ± 1.1	1.4 ± 0.1
<i>Prunus</i> protein not less than, mg·kg ⁻¹ expressed as almond kernel	2.9	10.0	1.3
LoQ, mg·kg ⁻¹ as almond kernel	2.5	0.4	0.5
<i>P</i> ^b	0.012	<0.001	<0.001
Cumin control assumed free from <i>Prunus</i> species estimate expressed as mg·kg ⁻¹ almond kernel	0.4	0.4	0.5
Paprika			
Presumptive <i>Prunus</i> protein mean result mg·kg ⁻¹ expressed as almond kernel ±U	590 ± 110	390 ^c	Not performed

^a U = Expanded measurement uncertainty, as at least a 95% confidence interval, with 2 degrees of freedom and a coverage factor of 4.3.

^b *P* = Significance of the difference between mean result and LOQ at 95% confidence level.

^c *n* = 1.

by at least a triplicate level of PCR replication. The instrumental response to positive and negative controls was completely satisfactory. Mahaleb was detected in all the spiked referred paprika samples, indicating that a background matrix does not inhibit amplification of mahaleb. Six independent DNA extractions from the referred paprika sample alone generated no amplification for mahaleb in any of the six PCR replicates assayed. Hence, mahaleb was reported as not detected. These findings were supported by the *Prunus* PCR screening assay with species-specific capabilities, which detected two PCR products: one characteristic of almond and the other of an unknown PCR product that was not consistent with almond, mahaleb, apricot, or peach DNA (Table 6, (19)).

LC-MS/MS

The referred samples and control material were analyzed in triplicate with simultaneous extraction and trypsin digestion,

followed by solid-phase extraction cleanup and LC-MS/MS. In the referred cumin sample, no peptides solely attributable to almond were detected. All three transitions for one of the three peptides shown to be unique for mahaleb were detected. In the referred paprika sample, no peptides solely attributable to mahaleb were detected. Two peptides were detected for which the chromatographic retention times and response ratios between the three SRM transitions acquired for each peptide were in agreement with those observed in the sample of almond kernels. In addition, four other peptides indicative of the presence of *Prunus* were also detected. Further data are presented elsewhere (22).

Discussion

The response by the U.K. Food Business Owner (FBO) to the initial alleged finding of almond in cumin is instructive. It is important to bear in mind that on the industry side, the response

Table 5. Real-time PCR DNA results control materials and referred cumin sample

Specimen	Amplification	C _q ± SD ^a
Almond kernel 1	No amplification	NA ^b
Apricot kernel 1	No amplification	NA
Peach kernel 1	No amplification	NA
Mahaleb kernel powder	Strong detection exponential amplification plot	17.87 ± 0.06
2× independent cumin extractions	No amplification	NA
Referred cumin sample (1) ^c	Strong detection exponential amplification plot	30.39 ± 0.10
Referred cumin sample (1), 1:10	Strong detection exponential amplification plot	33.49 ± 0.34
Referred cumin sample (2)	Strong detection exponential amplification plot	30.14 ± 0.07
Referred cumin sample (2), 1:10	Strong detection exponential amplification plot	33.58 ± 0.14
Referred cumin sample (3)	Strong detection exponential amplification plot	30.40 ± 0.04
Referred cumin sample (3), 1:10	Strong detection exponential amplification plot	34.04 ± 0.22
Referred cumin sample (4)	Strong detection exponential amplification plot	30.12 ± 0.15
Referred cumin sample (4), 1:10	Strong detection exponential amplification plot	33.65 ± 0.25
1% DNA:DNA mahaleb:cumin ^d	Strong detection exponential amplification plot	23.10 ± 0.14
Extraction blanks, <i>n</i> = 4	No amplification	NA

^a C_q = Quantification cycle.

^b NA = Not applicable.

^c (*n*) where *n* is each of four successive extractions of the sample; 1:10 is a one in ten dilution.

^d 1% DNA:DNA mahaleb:cumin sample: DNA was extracted from mahaleb powder and cumin (1) independently, normalized to 5 ng/μL, and then combined on a volumetric basis to make 1% DNA:DNA mahaleb in the cumin sample based on 25 ng template DNA per PCR well.

Table 6. Melt peak (T_m) data quantitative PCR DNA referred paprika sample

Sample	Mean Peak(s) (T_m) ^a
Ground mahaleb	84.90
Apricot stone	85.33
Peach stone	89.20
Almond	88.37
Peanut	74.50 ^b
Paprika	74.93 ^b
Smoked paprika	74.90 ^b
Cumin seeds	85.37
Paprika-based referred sample 1	82.60
Paprika-based referred sample 2	82.80
Paprika-based referred sample 3	82.60
10% w/w mahaleb in paprika referred sample DNA 1	85.23
10% w/w mahaleb in paprika referred sample DNA 2	85.10
10% w/w mahaleb in paprika referred sample DNA 3	85.07
10% mahaleb & 10% almond in paprika DNA	84.83 and 88.07
Extraction control	75.03 ^b
No Template Control	74.63 ^b

^a Mean T_m results (expressed in °C) from triplicate technical replicates. The predominant melt peaks (repeatable across all replicates) associated with each sample analysis are listed. Standard deviation associated with each mean value was always less than 1% of the mean value.

^b Probable primer artefact when no specific PCR product is amplified with a T_m of approximately 75°C.

to an incident such as this is handled by well-informed managers with food technical experience who are generally not scientists, let alone forensic specialists. After the initial adverse findings, unless an immediate cause is self-evident, there are many possibilities that must be considered under time pressure, against pressing knowledge gaps, including the extent to which other product lines are implicated and often in the glare of publicity. Among other lines of inquiry, the FBO requested a list of all materials milled in the same premises in Turkey as the cumin. The FBO worked through the list, investigating any unfamiliar species, in due course, focusing on mahaleb, then largely unknown in the United Kingdom. Work commissioned by the FBO suggested that almond ELISAs cross-reacted with mahaleb (Corbishley, G., The Barts Ingredients Co., Ltd, personal communications, 2016 and 2017). The investigation described herein led to the confirmation of industry suspicions that *P. mahaleb* and not *P. dulcis* (almond, a Regulation 1169/2011 Annex II-listed allergen) was the contaminant in the referred sample of cumin. Based on our findings, the recall that had been issued by the Food Standards Agency (FSA) was rescinded. We were also able to confirm that a sample from a consignment of paprika that had been refused access to the U.K. food chain did indeed contain almond. Our investigation was conducted according to established forensic standards, although additional MS criteria for the confirmation of peptides had to be developed. The investigation of the cumin incident was hampered somewhat by the small mass of sample available; nevertheless, it was brought to a successful conclusion. As a result of the incidents, an industry best practice for assessing and protecting supply chains for culinary dried herbs and spices has been published (23).

Throughout the analytical investigations, regular written and verbal updates were provided by a designated scientist (the corresponding author of the present paper) to the multidisciplinary team and to the parties involved via the FSA. This communication proved valuable, but was challenging in the face of the asynchronous emerging tentative findings until final orthogonal resolution and parallel competing demand-led work including other technical appeals.

ELISA is the workhorse technique for the detection of food allergens and is likely to remain so for the medium-term future. It has many advantages. However, drawbacks include variable- and manufacturer-specific sensitivities, cross-reactivity, and a potential for poor protein recovery: These contribute to difficulties in obtaining accurate quantification (24–26). Almond ELISAs are well known (27), but in 2015 were known to exhibit cross-reactivities with apricot, which have been acknowledged both by ELISA manufacturers (28) and the literature (29). Application of PCR amplification of extracted DNA is expected to be more species-specific than ELISA. Nonexhaustively, PCR methods for almond have been reported; some with reported negligible cross-reactivity for a very limited number of phylogenetically closely related Prunoideae foods (30–32), but others with higher (1.2% signal) cross-reactivity to apricot (29). MS of allergen proteins is a developing area, promising a number of advantages over ELISA and PCR as MS can be made to be specific for the protein to be quantified, provides protein identity information, permits a wider linear dynamic range, is less prone to be affected by food processing and, if appropriately applied, can be used as a reference method or for the production of certified reference materials. However, LC-MS methods remain as vulnerable as other approaches with regard to poor extraction efficiency and a difficulty in determining recovery owing to a lack of reference methods and reference materials and a lack of metrological traceability to secure standardization and harmonization of results (33). Hence, during the live investigation of the incidents, complementary approaches were necessary, none of which would have alone satisfied the criminal burden of proof (Table 7). It is interesting that the semiquantitative data from both the ELISA and PCR were in broad agreement.

Although, to our knowledge, there are no clinical records of allergy to mahaleb, there is very probable risk to almond-allergic individuals from mahaleb cross-contamination owing to amino acid homology, which appears to have been recently confirmed by the demonstration of mahaleb reactivity with almond-allergic patient IgE (34). Thus, it was definitively important to identify by MS detection peptides characteristic to mahaleb in the cumin and almond in the paprika; detection of multiple *Prunus* peptides in both confirmed the allergy risk.

Although members of the animal kingdom, particularly meat and fish species, are well represented in publicly available DNA databases, there is much less information available regarding fruits and spices. What information is available regarding these species requires very careful examination in order to identify and capitalize on areas of sequence homology or polymorphism on which to potentially design generic and specific PCR and MS assays. It is often prudent to reinforce *in silico* data through experimental evaluation and sequencing within the laboratory.

The assays described in this paper depend on validation with randomly sourced commercial samples as control materials. Although these were checked as to species and purity and

Table 7. Summary of complementary approaches to resolve the disputed identity of *Prunus* allergen in the referred samples

Technique	Almond kernel	Mahaleb kernel
Commercial ELISAs	Capable of screening for <i>Prunus</i> species protein and ascribing presumptive quantitative data albeit expressed as 'almond' In the referred paprika sample two platforms produced data in the range 390 – 700 mg kg ⁻¹ expressed as almond	In the referred cumin sample three platforms produced data in the range 1.3 – 12.2 mg kg ⁻¹ expressed as almond
Real time PCR assay demonstrated to be species-specific	No almond-specific assay available	The developed mahaleb-specific assay detected mahaleb DNA in the referred cumin sample in the approximate range <10 - <100 mg kg ⁻¹ expressed as mahaleb. Mahaleb was not detected in the referred paprika sample
Real time screening PCR <i>Prunus</i> assay (This assay was not developed until after the cumin case)	Almond DNA detected in the referred paprika sample	Mahaleb DNA not detected in the referred paprika sample
LC-MS/MS Referred cumin sample	No peptides uniquely characteristic of almond were detected	Of 3 peptides known to be present in mahaleb 1 was detected
LC-MS/MS Referred paprika sample	Two peptides uniquely characteristic of almond were detected	No peptides uniquely characteristic of mahaleb were detected

every effort was taken to ensure they were representative of the species in question (Table 2), there is a small likelihood that different cultivars within the same species may exhibit small differences in base pair and amino acid sequences. The mahaleb-specific DNA assay, the *Prunus* PCR screening assay with species-specific capabilities, and the LC-MS/MS investigation have not been tested on all cultivars and species of almond, apricot, plum, cherry, mahaleb, or peach. It is assumed that the DNA and LC-MS/MS assays will not respond with false-positives to untested *Prunus* species, but unless experimental work is conducted to verify this, it remains a possibility, albeit, we believe, of low probability. Indeed, the differing pattern of ELISA platform cross-reactivity between the control materials and the referred samples and the presence of an additional unidentified peak in the melt curve of the referred paprika sample, presumed to be a separate mahaleb cultivar, attest to the complexity of the analysis of species in the *Prunus* genus.

We also note our cumin result was much lower than the original official finding by ELISA of some 300 mg·kg⁻¹ as almond, suggesting that even in powdered spice matrices, inhomogenous distribution of contaminants is likely.

Conclusions

In the opinion of the parties involved (FBO, regulators, and other interested parties), the U.K. cumin incident was handled about as well as it could have been throughout, based on the information and knowledge available at each stage, as the investigation proceeded. The FSA was correct to advise a product recall at the outset because the initial result was not irrefutably incorrect and the putative allergen was one of possibly significant impact. Subsequently there was open and honest communication that allowed the true cause of the incident to be uncovered and confirmed. Transparency throughout as to the progress of the investigation, avoidance of apportioning blame, or seeking damages for the cost of the incident were lauded as helpful in achieving a true understanding of cause and thereby assisting in the avoidance of a similar problem in the future (Corbishley, G., The Barts Ingredients Co., Ltd, personal communications, 2016 and 2017).

The utility of ELISA solely as a screening tool for the *Prunus* family and extent of cross-reactivity has been confirmed. Two novel PCR assays were developed: one specific for *P. mahaleb* and the other a screening method capable of identifying common *Prunus* DNA. Peptides unique to almond and mahaleb have been identified, along with the conditions required for their extraction, tryptic digestion, chromatography and MS, and criteria for peptide identification to forensic standards. The publication of this work enables a staged approach to be applied to any future incident thought to involve *Prunus* species and provides a template for the investigation of similar incidents. Successful resolution of the incidents described was aided by access to a multidisciplinary team of forensically aware analytical chemists, molecular biologists, protein mass spectrometrists, statisticians, and food law specialists. A similar investigation of peanut and almond contamination of cumin in North America bears out the complex nature of the spice supply chain and the need for orthogonal confirmation of ELISA screening results (35).

The suspicions of bulking out of expensive spices with much cheaper almond or apricot shells were not born out by our investigation, but remain live possibilities. ELISA, as a screening tool, would be unlikely to pick up such adulteration, although the described novel PCR screening assay may be capable of doing so and MS confirmation may be a possibility. However, this is an area of potential fraud in which other techniques, such as multispectral imaging (36), may be of more immediate utility.

The use of control materials deserves some thought and we recommend multiple sources, knowledge of the supply chain (if possible), recording and publication of label details (see Table 1), and independent species confirmation (DNA sequencing).

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References

- (1) Walker, M.J., Burns, M., & Thorburn Burns, D. (2013) *J. Assoc. Public Analysts (Online)* **41**, 67–106
- (2) Gowland, M.H., & Walker, M.J. (2014) *J. Sci. Food Agric.* **95**, 1979–1990. doi:10.1002/jsfa.6988
- (3) Seasoning and Spice Association (2015) The Seasoning and Spice Association Q&A on Undeclared Peanut/Almond Protein, <http://www.seasoningandspice.org.uk/publicgeneral/Q&A.pdf> (accessed on June 29, 2017)
- (4) Walker, M.J., & Gray, K. (2013) *J. Assoc. Public Analysts (Online)* **41**, 1–27
- (5) Boley, N. (2016) Government Chemist Annual Statement of Statutory Scope, <https://www.gov.uk/government/publications/government-chemist-annual-statement-of-statutory-scope-2015-2> (accessed on June 30, 2017)
- (6) Özbey, A., Öncül, N., Yıldırım, Z., & Yıldırım, M. (2011) *J. Agri. Faculty Gaziosmanpaşa Univ.* **28**, 153–158 [Abstract; full text in Turkish]
- (7) Ercisli, S. (2004) *Genet. Resour. Crop Evol.* **51**, 419–435. doi:10.1023/B:GRES.0000023458.60138.79
- (8) Ieri, F., Pinelli, P., & Romani, A. (2012) *Food Chem.* **135**, 2157–2162. doi:10.1016/j.foodchem.2012.07.083
- (9) Food Standards Agency (2015) Bart Ground Cumin Recalled, <https://www.food.gov.uk/news-updates/news/2015/13512/bart-ground-cumin-recalled> (accessed on June 29, 2017)
- (10) Bawden, T. (2015) Food Firm in “Nuts-for-Spices” Row Blames False Test Results, The Independent, <http://www.independent.co.uk/life-style/food-and-drink/news/food-firm-in-nuts-for-spices-row-blames-false-test-results-10089275.html> (accessed on June 29, 2017)
- (11) Canadian Food Inspection Agency Agence Canadienne d’Inspection des Aliments (2015) Correction - Food Recall Warning - Rescindment of Food Recall Warnings (Allergen) Cumin and Cumin-Containing Products Recalled Due to Undeclared Almond, <http://www.inspection.gc.ca/about-the-cfia/newsroom/food-recall-warnings/complete-listing/2015-04-30/eng/1430432363404/1430432364107> (accessed on June 29, 2017)
- (12) Ratnasingham, S., & Hebert, P.D. (2007) *Mol. Ecol. Notes* **7**, 355–364. doi:10.1111/j.1471-8286.2007.01678.x
- (13) Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., & Wheeler, D.L. (2005) *Nucleic Acids Res.* **33**, D34–D38. doi:10.1093/nar/gki063
- (14) Polymerase Chain Reaction, PCR, Almond kit PATH-SS-P. Dulcis, Primer Design Ltd., Chandler’s Ford, Hampshire, U.K
- (15) Valdivia, H., & Burns, M. (2010) *J. Assoc. Public Analysts (Online)* **38**, 1–12
- (16) Costa, J., & Mafra, I. (2012) *Food Chem.* **133**, 1062–1069. doi:10.1016/j.foodchem.2012.01.077
- (17) NCBI database, National Center for Biotechnology Information, <https://www.ncbi.nlm.nih.gov>
- (18) Burns, M., Walker, M., Wilkes, T., Hall, L., Gray, K., & Nixon, G. (2016) *Food Nutr. Sci.* **7**, 703–710. doi:10.4236/fns.2016.78071
- (19) Nixon, G., Hall, L., Wilkes, T., Walker, M., & Burns, M. (2016) *Food Nutr. Sci.* **7**, 920–926. doi:10.4236/fns.2016.710091
- (20) Johnson, P.E., Baumgartner, S., Aldick, T., Bessant, C., Giosafatto, V., Heick, J., Mamone, G., O’connor, G., Poms, R., Popping, B., Reuter, A., Ulberth, F., Watson, A., Monaci, L., & Mills, E.N. (2011) *J. AOAC Int.* **94**, 1026–1033
- (21) Heick, J., Fischer, M., & Pöpping, B. (2011) *J. Chromatogr. A* **1218**, 938–943. doi:10.1016/j.chroma.2010.12.067
- (22) Inman, S. E., Groves, K., McCullough, B., Quaglia, M., & Hopley, C. (2017), Food Chemistry, <https://doi.org/10.1016/j.foodchem.2017.10.101>
- (23) British Retail Consortium, Food and Drink Federation, and Seasoning and Spice Association, Guidance on Authenticity of Herbs and Spices: Industry Best Practice on Assessing and Protecting Culinary Dried herbs and Spices, https://www.fdf.org.uk/corporate_pubs/guidance-herbsandspices.pdf (accessed on June 30, 2017)
- (24) Cryar, A., Pritchard, C., Burkitt, W., Walker, M., O’Connor, G., & Quaglia, M. (2013) *J. AOAC Int.* **96**, 1350–1361. doi:10.5740/jaoacint.12-438
- (25) Johnson, P.E., Rigby, N.M., Dainty, J.R., Mackie, A.R., Immer, U.U., Rogers, A., Titchener, P., Shoji, M., Ryan, A., Mata, L., Brown, H., Holzhauser, T., Dumont, V., Wykes, J.A., Walker, M., Griffin, J., White, J., Taylor, G., Popping, B., Crevel, R., Miguel, S., Lutter, P., Gaskin, F., Koerner, T.B., Clarke, D., Sherlock, R., Flanagan, A., Chan, C.H., & Mills, E.N. (2014) *Food Chem.* **148**, 30–36. doi:10.1016/j.foodchem.2013.09.115
- (26) Garber, E.A., & Perry, J. (2010) *Anal. Bioanal. Chem.* **396**, 1939–1945. doi:10.1007/s00216-009-3424-2
- (27) Ben Rejeb, S., Abbott, M., Davies, D., Cléroux, C., & Delahaut, P. (2005) *Food Addit. Contam.* **22**, 709–715. doi:10.1080/02652030500158450
- (28) R-Biopharm AG, RIDASCREEN FAST Mandel/Almond, <http://www.r-biopharm.com/products/food-feed-analysis/allergens/nuts/item/ridascreenfast-mandel-almond> (accessed on May 31, 2015)
- (29) Köppel, R., Dvorak, V., Zimmerli, F., Breitenmoser, A., Eugster, A., & Waiblinger, H.-U. (2010) *Eur. Food Res. Technol.* **230**, 367–374. doi:10.1007/s00217-009-1164-3
- (30) Röder, M., Vieths, S., & Holzhauser, T. (2011) *Anal. Chim. Acta* **685**, 74–83. doi:10.1016/j.aca.2010.11.019
- (31) López-Calleja, I.M., de la Cruz, S., Pegels, N., González, I., Martín, R., & García, T. (2014) *Lebensm. Wiss. Technol.* **56**, 31–39. doi:10.1016/j.lwt.2013.10.039
- (32) Haase, I., Brüning, P., Matissek, R., & Fischer, M. (2013) *J. Agric. Food Chem.* **61**, 3414–3418. doi:10.1021/jf3052175
- (33) Groves, K., Cryar, A., Walker, M., & Quaglia, M. (2017) *J. AOAC Int.* in press
- (34) Noble, K.A., Liu, C., Sathe, S.K., & Roux, K.H. (2017) *J. Food Sci.* **82**, 1786–1791. doi:10.1111/1750-3841.13757
- (35) Garber, E.A., Parker, C.H., Handy, S.M., Cho, C.Y., Panda, R., Samadpour, M., Reynaud, D.H., & Ziobro, G.C. (2016) *J. Agric. Food Chem.* **64**, 1202–1211. doi:10.1021/acs.jafc.5b05497
- (36) Wilkes, T., Nixon, G., Bushell, C., Waltho, A., Alroichdi, A., & Burns, M. (2016) *Food Nutr. Sci.* **7**, 355–361. doi:10.4236/fns.2016.75037