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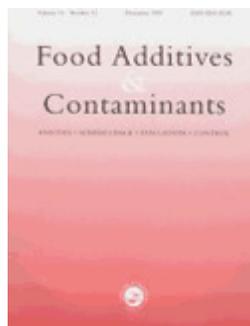
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Identification of hidden allergens - detection of pistachio traces in mortadella

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Abstract

An analytical method based on the detection of specific DNA was developed, and was applied to mortadella samples with and without pistachio (*Pistacia Vera*). The method is proposed for detection of traces of pistachio deriving from previous processes or from accidental contamination, since in predisposed individuals pistachios can cause allergic reactions leading to anaphylactic shock. Three pairs of primers were identified and tested by PCR on mortadella samples prepared with pistachio. Accidental contamination was also simulated. The optimised PCR was able to detect the presence of pistachio even at low concentrations. The primers pair PSTC 1-2 is suggested for unambiguous identification of pistachio in mortadella. The limit of detection for this primers pair was 100 mg/kg. No interference was observed from other spices or ingredients utilised in the formulation of the mortadella. The method enabled the identification of possible traces of pistachio remaining in the production plant after less than thorough washing.

Keywords:- allergens, pistachio, mortadella, PCR method

Introduction

Analysis of traces of food allergens is desirable to minimize the risk to consumers. Sensitive test systems are coming into use for some allergens but some analytical problems remain. The main problems are related to extraction, cross-reactivity, matrix effects and food processing. Enzyme-linked immunosorbent assay (ELISA) is the most frequently used analytical method to detect allergens. DNA- based methods are often controversial since they do not detect allergenic protein, but they have many advantages: good extraction of DNA from some problematic food matrix and its unchanging nature set against variability of protein composition (Poms et al. 2004).

An ingredient sometimes present in certain formulations of mortadella is pistachio (*Pistacia Vera*), a component that may cause serious allergic reactions in sensitive individuals. Pistachio belongs to the *anacardiaceae* family, together with cashew and mango, whereas the common sub-class of the *Rosidae* groups it with other allergens such as soybean, peanut, English walnut and hazelnut. Cross reactivity with cashew has often been observed in the past (Jansen et al. 1992; Fernandez et al. 1995) and has been confirmed in recent studies (Goetz et al. 2005; Roux et al. 2002); it is probable that the seed storage proteins belonging to 11S globulin and 2S albumins play a key role in clinical cross reactivity (Tawde, 2004).

Since processing of mortadella does not involve other types of nut other than pistachio, it constitutes the only real risk for consumers who are allergic to nuts. For this reason, it is important to be able to rule out its presence even in traces or, at any rate, to indicate its possible presence on the label, in accordance with the law (European Directive 2003/89/EC) Naturally, when it is a stated ingredient, its presence is clearly visible, since the whole nut is utilised in the mixture and can be seen in the slice. There may be pistachio dust even on products where its presence is not stated, owing to dust contamination in the environment in which the ingredients are kept, or to contamination from preceding processes if sufficient steps have not been taken to avoid it. This type of contamination may be difficult to identify and to quantify. A certificate testifying to the absence of pistachio, even in traces, is often required for the exporting of mortadella outside Italy.

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3 At present, no analytical methods are available for the specific detection of pistachio and
4 the only immunoenzymatic kits for nuts identification concern hazelnut and peanut
5 (Kiening et al. 2005). The aim of the work reported here was to develop a method of
6 identifying traces of pistachio in mortadella utilising PCR methodology. Analysis with the
7 PCR method for the detection of allergenic species, like all DNA tests is not as specific as
8 analysing for the presence of protein responsible. Nevertheless, with regard of pistachio in
9 mortadella, it constitutes a reasonable guarantee of absence of the relative allergen
10 protein.
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18 **Materials and methods.**

19 *Preparation of the samples*

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21 Pistachio were purchased, either fresh or toasted, from local markets, in order to obtain
22 pistachio DNA reference material. The mortadella, without pistachio (MP0) or with
23 pistachio (MP1), was produced in an industrial plant. The mortadella samples containing
24 pistachio were prepared by mixing in 5 g of whole pistachio nuts for every kg of mixture, as
25 is the custom for the traditional product.
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33 Contamination deriving from less than thorough cleaning of the plants is presumably very
34 slight and thus cannot be quantified. The same goes for contamination due to dust stirred
35 up by draughts of air inside a storage room. The sensitivity to these types of contamination
36 was simulated with two experimental strategies:
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- 40 a) A meat mixture was prepared with the intentional addition of a known and very low
41 quantity of pistachio dust M100 (100 mg/kg) and M40 (40 mg/kg).
- 42 b) A batch of mortadella (MCONT) without pistachio was prepared on production lines not
43 cleaned after a preceding processing with pistachio, simulating an involuntary
44 contamination of the first mortadella samples produced with the new mixture.
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51 A control test of the cleaning of the production line was carried out as follows: sterile paper
52 swabs lightly soaked in sterile water were rubbed onto the critical points of a mortadella
53 production plant (kneader screw, bagging machine bottom and outlet). DNA was
54 subsequently extracted from these.
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3 A further test of the effectiveness of the method was conducted, eliminating all the pieces
4 of pistachio from a slice of mortadella, including the product adjacent to the nut, and
5 mincing the rest, from which DNA was extracted and examined for traces of pistachio.
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8 Mortadella samples of around 12 kg in weight were produced.
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11 From mortadella samples of each batch of production, four slices were obtained in differing
12 positions: two slices from the ends, one from the middle and one three-quarters of the way
13 through the product. Each slice was minced, 100 g was homogenised with sterile water to
14 obtain a slurry, and a sample of around 200 mg was taken. The fresh and toasted
15 pistachios (PSTC) were milled in their turn, and 100 mg of these were utilised for the
16 analysis. The same protocol was used for the DNA extraction of ingredients, flavourings
17 and some types of nuts listed in Table 1 to check their interference with the pistachio test.
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26 *Extraction of the DNA and PCR*

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28 The samples were incubated in 2 mL test tubes at 37°C overnight, after the addition of
29 500 µL of extraction buffer (Tris-HCl 0.01 M pH 8.0, NaCl 0.15M, EDTA 2 mM), 50 µL of
30 10% Sodium dodecylsulphate and 5µL of proteinase K (20 mg/mL). After centrifugation for
31 15 min at 14 000 g, the supernatant was purified with 1 vol of CHCl₃/ isoamylic alcohol
32 24:1, and then with 1/3 vol of 10% ammonium acetate. From the supernatant obtained by
33 centrifugation (15 min at 14 000 g) the DNA was precipitated with 1 vol of 80%
34 isopropanol. The pellet was washed in 70% ethanol, air-dried, suspended in sterile water
35 solution, measured spectrophotometrically by absorption at 260 nm and diluted to a final
36 concentration of 100 ng/µL.
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46 In this study, we utilised the sequences of two genes codifying for a protein belonging to
47 the dehydrin family (Golan-Goldhirsh, 2001), and for a ribulose 1,5-bisphosphate
48 carboxylase/oxygenase (Savolainen et al. 2000). For the PCR on the DNA extracted, on
49 the basis of the sequences of two genes codifying for pistachio proteins obtained from the
50 literature, 3 pairs of primers were designed using the Primer3 software (Rozen and
51 Skaletsky, 2000). Two of these are planned on COR gene for dehydrin PSTC1 (5'-
52 GAATACGGGGAGCACAAAGA-3'), and PSTC2 (5'- AACCTCTCGCTAACCCATT-3') ,
53 PSTC 3 (5'- GGGACTGGTGGAGAAGATCA-3') and PSTC4 (5'-
54 TCTTTGTGCTCCCCGTATTC-3') (GenBank accession number Y07600.2). The last was
55 constructed on rbcL gene of ribulose 1,5-bisphosphate carboxylase/oxygenase PSTC 5
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3 (5'- GACAACGTGTGGACCGATG-3') and PSTC6(5'- CAGGGCTTTGAACCCAAATA-3')
4 (GenBank accession number AJ235786.1).
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9 The Master mix consisted of reaction Buffer 1X (Sigma), MgCl₂ 1.4 mM, dNTPs
10 (Eppendorf) 0.2 mM, Primer Forward and Reverse (Invitrogen) 0.25 μM (for PSTC 1-2 and
11 PSTC 3-4) or 0.5 μM (for PSTC 5-6), Taq polymerase (Sigma) 1U and 200 ng of DNA in a
12 final volume of 50 μL.
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17 Thermocycler conditions were as follows. PCR of the primers PSTC 1-2 and 3-4: 35 cycles
18 consisting of DNA denaturation at 94 °C for 10 s, primers annealing at 58 °C for 20 s, primer
19 extension at 72 °C for 15 s; with the primers PSTC 5-6, the annealing T° was lowered to
20 52 °C for 20 s and the extension time at 72 °C was prolonged to 30 s. Both protocols have a
21 final extension step at 72 °C for 5 min. PCR was carried out in PTC-100 thermocycler (MJ
22 Research). The amplification products were visualised with 0.5 μg/mL ethidium bromide
23 after an electrophoretic run on 2% agarose gel. The running condition were constant
24 voltage at 120 V for 1 h in Tris Borate EDTA 0.5X buffer.
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33 34 **Results and discussion**

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37 Figure 1 shows the electrophoretic pattern of the three PCRs with which we tested the
38 efficacy of the pairs of primers. These primers were designed to amplify sequences
39 characteristic of the pistachio species and any interference by various commercial flavours
40 (composition not declared by producer) used in the production of mortadella. Only the
41 pistachio samples gave a positive signal. No difference was observed for samples taken
42 from the differing position of the product (from the middle or the ends).
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50 The PSTC 1-2 pair yielded three amplicons, one of which was not justified by the PCR
51 product sequence, whereas the other bands correspond to the predicted amplicons (280
52 bp and 391 bp), as reported in figure 2A. The pair of primers 3-4 provided a 111 bp long
53 amplicon, although sometimes in the presence of high quantities of target DNA it also
54 yielded two extra amplicons (222 bp, 333 bp). In the tests, we utilised all three pairs of
55 primers, although the PCR carried out with PSTC 5-6 (figure 2B) resulted as being more
56 suitable on account of the univocal nature of the signal (193 bp), even though in some
57 cases the primers PSTC 1-2 demonstrated greater sensitivity.
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5 Specificity tests were carried out, as well as on three previously prepared commercial
6 flavours, with some natural ingredients normally utilised in mortadella flavourings. The
7 results are shown in table 1. None of them yielded a positive signal. The same table shows
8 the results of controls made on various types of nut with the three pairs of primers: it can
9 be observed that PSTC 1-2 and PSTC 3-4 are specific for pistachio, whereas there is an
10 interference with cashew and Brazil nut for the PCR conducted with PSTC 5-6. This fact,
11 however, is of only relative significance in the case of mortadella analysis, since these are
12 not ingredients utilised in the technology of this cold meat product.
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21 The PCR conducted with all pairs of primers on DNA extracted from the mortadella
22 samples always allowed for the unequivocal identification of the products with pistachio,
23 even those contaminated intentionally (MCONT and M100), and a positive result was also
24 yielded by a sample in which the whole pistachio nuts were manually removed from the
25 slice before the extraction of the DNA. The mere presence of the nut in contact with the
26 meat was sufficient to give a positive signal for the PCR analysis with primer PSTC 1-2.
27 However, the M40 sample yielded a positive signal only with PSTC 1-2, but was not
28 always detectable with the other pairs of primers, and was detected in only 3 samples out
29 of 12 (data not shown).
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39 The DNA extracted from the swabs taken at various places in the plant was sensitive to
40 the presence of pistachio. This yielded the amplicon of the pistachio with PSTC 1-2 and
41 PSTC 3-4 also after simple washing with water, disappearing only when rinses were done
42 first with soda and then with normal water.
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48 By carrying out a combined amplification of PSTC 1-2, PSTC 3-4 and PSTC 5-6, pistachio
49 can be detected in mortadella samples. This includes accidental contamination in the
50 case of neglect in cleaning the equipment (simulated by MCONT sample), or of cross-
51 contamination by dust from ingredients (simulated by M100 and M40 samples). A
52 contamination of 100 mg/kg of pistachio in mortadella was always detected. The limit of
53 detection is thus close to this value.
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Conclusions

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3 The presence of traces of pistachio due to accidental contamination can be unmistakably
4 detected using PCR using three pairs of primers (PSTC 1-2, PSTC 3-4, PSTC 5-6). The
5 first pair of primers is extremely sensitive although they yield co-amplified fragments. The
6 low detection limit (100 mg/kg of pistachio in mortadella) that these primers achieve
7 enables the detection in the ingredients of pistachio, even in traces, due to accidental
8 contamination. In addition, the two pairs of primers planned on the COR gene are very
9 specific, not reacting with other types of nut.
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17 The last pair of primers, however, are advantageous because of the presence of only one
18 amplicon in PCR, although the fact that they also give positive signals for cashew and
19 Brazil nut render them less useful beyond the analysis of mortadella. Common ingredients
20 used in the formulation of mortadella did not interfere with pistachio identification, as
21 evidenced by the negative tests conducted both on mortadella samples without pistachio
22 and on the potential ingredients analysed individually. Since no difference was observed
23 in the distribution of pistachio traces during cooking in the bulk of the mortadella, the
24 sample can be taken from any part of the meat. A further use for the analytical method
25 described could be to check on the cleanliness of mortadella production lines after the
26 processing of the product with pistachio and before passing to a subsequent processing
27 without pistachio. Samples taken at the critical points with swabs ensured that no traces of
28 the preceding mixtures remain.
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40 This method can be applied to various food products containing pistacchio (e.g
41 confectionary) or, albeit only in the case of the PSTC 5-6 primers, it could be directed
42 towards the detection of *anacardiaceae* in general. For the scope of this work, however,
43 the primers pair PSTC 1-2 is suggested to unambiguously identify pistachio in mortadella.
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Table 1. Commercial and natural flavours, and nuts tested by PCR PSTC 1-2 , 3-4, 5-6 to test interference with pistachio response.

SPECIFICITY TEST	PSTC 1-2 and PSTC 3-4	PSTC 5-6
Nuts		
Almond	negative	negative
Jungland nut	negative	negative
Cashew	negative	positive
Pecan nut	negative	negative
Hazelnut	negative	negative
Peanut	negative	negative
Brazil nut	negative	positive
Flavours	negative	negative
Commercial Mortadella Flavours A	negative	negative
Commercial Mortadella Flavours B	negative	negative
Commercial Mortadella Flavours C	negative	negative
Cloves	negative	negative
Mace	negative	negative
Cinnamon	negative	negative
Nutmeg	negative	negative
Coriander	negative	negative
Garlic	negative	negative
Pepper	negative	negative
Pimento	negative	negative

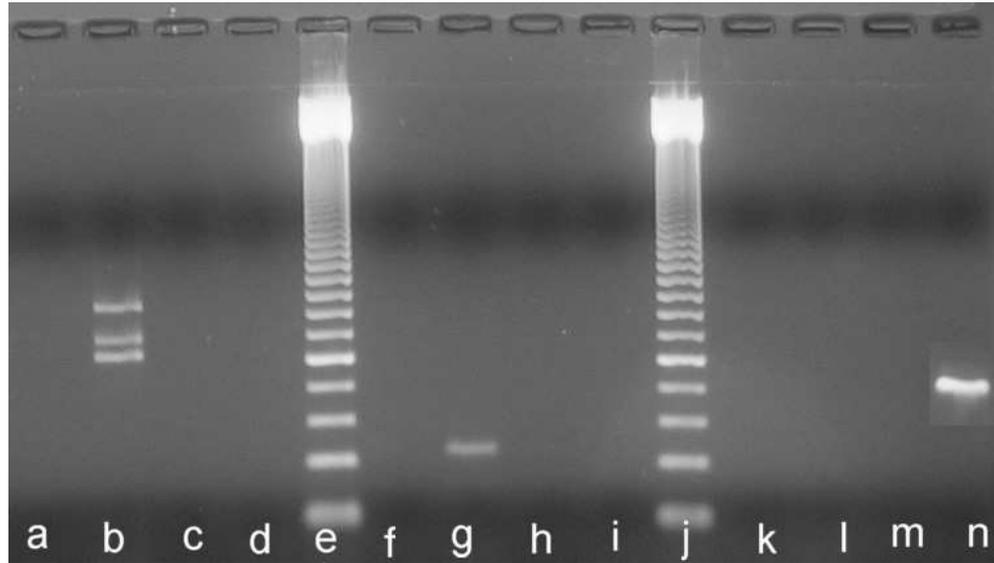


Figure 1. PCR carried out with three pairs of primers. a) - d): PSTC 1-2; f) - i): PSTC 3-4; k) - n): PSTC 5-6. b), g), n) are the pistachio samples; a), f), k) are commercial flavour A; c), h), l) are commercial flavour B; d), i), m) are commercial flavour C; e) and j) are marker (50 bp ladder).
96x54mm (200 x 200 DPI)

COR gene	GGGACTGGTGGAGAAGATCAAAGACAAGGTCCCTGGCCAAGGTGATCAACATGGACATGG
Primer	GGGACTGGTGGAGAAGATCA ----- PSTC3
COR gene	CATGCAGCAGCACCCGGCGGAATACAGGCCTGAATACGGGGAGCACAAAGAGGGACTGGT
Primer	----- GAATACGGGGAGCACAAAGAGGGACTGGT PSTC1 PSTC3
COR gene	GGAGAAGATCAAGGACAAGCTCCCTGGCCAAGGTGATCAACATGGACAGGGCGTGCAGCA
Primer	GGAGAAGATCA -----
COR gene	GCACCACGGCGAATACAGGCCTGAATACGGGGAGCACAAAGAGGGACTGGTGGAGAAGAT
Primer	----- GAATACGGGGAGCACAAAGAGGGACTGGTGGAGAAGAT PSTC1 PSTC3
COR gene	CAAGGACAAGCTCCCTGGCCAAGGTGATCAACATGGACAGGGCGTGCAGCAGCACCCGGG
Primer	CA -----
COR gene	CGAAAAACAGGCCTGAATACGGGGAGCACAAAGAGGGAATGGTGGAGCAGATCAAGGACAA
Primer	----- CTTATGCCCTCGTGTCT ----- PSTC4
COR gene	GGTCCATGGCCAAGGTGATCAATATGGATATGGCGTAGAGAAGAAGCACCCAGGATGGCCA
Primer	-----
COR gene	CATCAGCAGCCGTGACAAC TATTAAACATCCCAATATAGGTGAATGGGGTTAGCGAGAGG
Primer	----- TTACCCCAATCGCTCTC PSTC2
COR gene	TT
Primer	AA

Figure 2A. Nucleotide sequences of the primers PSTC 1-2 and PSTC 3-4 and target region on COR gene of pistachio.
177x145mm (96 x 96 DPI)

rbcl gene	GACAAC TGTGTGGACCGATGGCTTACCAGCCTTGATCGTTACAAAGGACGATGCTACA
Primer 5-6	GACAAC TGTGTGGACCGATG-----
rbcl gene	ACATTGAGCCCGTTGCTGGAGAANAAAATCAATATATATGTTATGTAGCTTACCCTTTAG
Primer 5-6	-----
rbcl gene	ACCTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCCATTGTGGGTAATGTATTG
Primer 5-6	-----ATAAAC
rbcl gene	GGTCAAAGCCCTG
Primer 5-6	CCAAGTTTCGGGAC

Figure 2B. Nucleotide sequences of the primers PSTC 5-6 and target region on rbcL gene of pistachio
177x145mm (96 x 96 DPI)