

MUSTARD DETECTION IN FOOD SAMPLES

Ingrid Malmheden Yman, Johanna Almgren, Birgitta Kruse, Monica Ferm
National Food Administration, Box 622, SE 751 26 Uppsala, SWEDEN

Introduction



Table mustard is a mixture of yellow mustard (*Sinapis alba*) and black mustard (*Brassica nigra*). Outside Europe, oriental mustard (*Brassica juncea*) is more common. Mustard is present as an ingredient in various foods, sauces and spices.

Sinapis alba - Yellow mustard

Brassica nigra - Black mustard



The mustard plants belong to the *Brassicaceae* (*Cruciferae*) family. Other members of the family are oriental mustard (*Brassica juncea*), oil rape (*Brassica napus ssp. napus*), swede (*Brassica napus ssp. rapifera*), horse radish (*Armoracia rusticana*), radish (*Raphanus sativus*) and garden cress (*Lepidium sativum*).

The European labeling directives 2003/89/EC and 2006/142/EC state that certain 'allergens' and products derived thereof must always be declared. Mustard is one of the listed 'allergens'.

Allergic reactions to mustard, including severe anaphylaxis, have been reported. Reliable methods for the detection of mustard in food are thus needed.

- First case in 1980 caused by mustard on a pizza¹
 - Later there have been a number of reports on cases and patient series (France, Spain, Sweden, Finland and Italy)²
 - Provoking dose – high µg range
 - Case report – small amounts e.g. by contaminated cooking utensils
 - Cross-reactivity between mustard and other species of the *Brassicaceae* family has been reported in a few studies i.e. *Swede* and *radish*
 - A number of studies report cross-reactivity to *rape seeds*, Sin a 1 shows 94 % homology to Bra n 1³
- ¹ Panconesi E et al. *Contact Dermatitis* 1980;6:294-295
² The EFSA Journal 2004;32:120-128
³ González de la Peña MA et al. *Int Arch Appl Immunol* 1991;96:263-270

Mustard allergens have been isolated and characterized from yellow mustard (*Sinapis alba*) and from oriental mustard (*Brassica juncea*). Binding of specific IgE to proteins of 14 and 51 kDa in yellow mustard and to 16 kDa in black mustard have been shown by immunoblotting.

- Allergens isolated and characterized from yellow mustard are Sin a 1 and Sin a 2 and Bra j 1 from oriental mustard (*Brassica juncea*). The latter is closely related to black mustard
 - Sin a 1 is a 14 kDa protein and Bra j 1 is a 16 kDa protein
 - Sin a 1 and Bra j 1 are **2S albumins** consisting of two polypeptide chains with a molecular mass of about 9-10 kDa and 4-5 kDa, respectively, linked by four disulphide bonds
 - The 2S albumins are heat-resistant and resistant to enzymatic degradation, not affected by food processing
 - Sin a 2 has a molecular mass of 51 kDa
- Ref.
Menéndez-Arias L et al. *Int J Biochem* 1987;19:899-907
Monsalve RI et al. *Biochem J* 1993;293:625-632
Palomares O et al. *Ann Allergy Asthma Immunol* 2005;94:586-592

Protein isolation

Proteins from seeds of yellow (SIN) and black (BRA) mustard were purified. SDS-PAGE under reducing conditions revealed three main protein bands of about 20, 32 and 37 kDa in the yellow mustard protein fraction, in addition to minor bands of 5, 10 and 40 kDa. Black mustard proteins differed slightly in molecular mass with major bands at 20, 28 and 36 kDa, in addition to minor bands at 5 and 10 kDa.

Yellow mustard seeds

Black mustard seeds



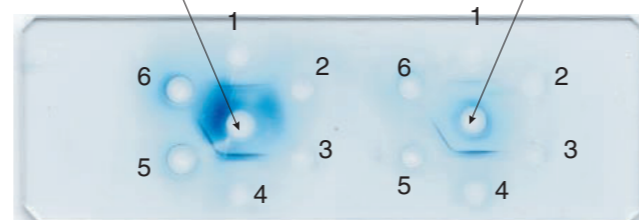
Antiserum

Rabbits were immunized with protein fractions from yellow and black mustard, respectively. Immune serum were collected at intervals.

Characterization of the antiserum to mustard Specificity

The specificity of the antisera to SIN and BRA were tested in immunodiffusion against other species of the *Brassicaceae* family like rape seed, cabbage, radish, horse radish, to selected nuts, seeds and, in addition, to milk and egg. Only mustard proteins and the extract of rape seeds reacted with the yellow as well as the black mustard specific antibodies.

Antiserum to yellow mustard Antiserum to black mustard



1. Purified protein from SIN and BRA respectively
2. Radish
3. Swede
4. Yellow or black mustard seed
5. Rape seed
6. Brazil nut

Specificity of the antiserum to SIN and the antiserum to BRA tested in immunodiffusion

Material	Reactivity (+/-)	Material	Reactivity (+/-)
Sesame seed	-	Garden cress	-
Sun flower seed	-	Radish	-
Brazil nut	-	Swede	-
Pecan nut	-	Rosé pepper	-
Marron	-	Egg yolk	-
Hazelnut	-	Egg white	-
Lima bean	-	Milk	-
Rape seed	+	Mustard seed	+
Horse radish	-	Mustard protein	+

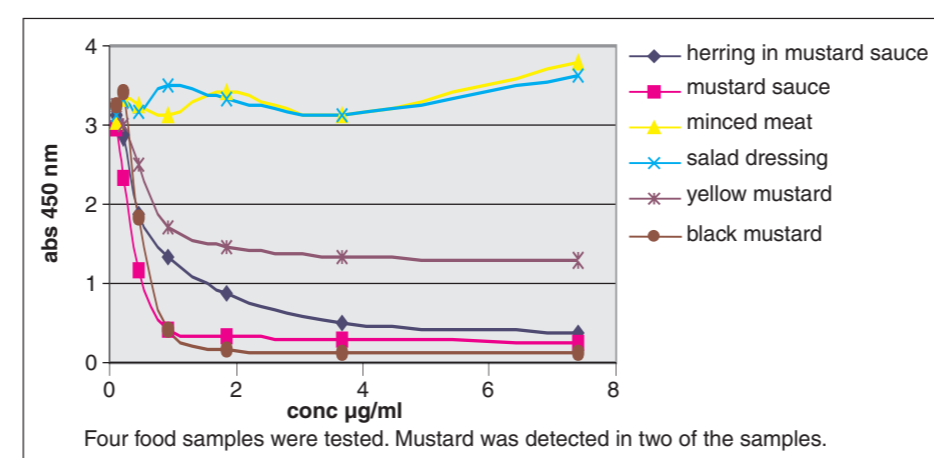
2 g or 5 g material was extracted with 10 ml buffer. For milk or egg equal volume was mixed with buffer.

Affinity purification of rabbit IgG

Purified protein from black mustard was covalently coupled to a NHS-activated HiTrap Sepharose HP column. The antiserum was passed through the column and specific antibodies eluted in glycine, pH 2.5. The IgG was pooled, neutralized and concentrated to 0.74 mg/ml.

Food sample analysis

Different foods, containing mustard in the ingredient lists, were purchased at a local store and analysed with immunodiffusion and the competitive ELISA. Results were compared to a commercial mustard ELISA.



Immunoblots

Immunoblotting with the two antisera showed a dominant reactivity towards proteins of 20 and 32 (28) kDa, respectively. Minor reactivity was seen against proteins of 5 and 10 kDa with the yellow mustard antiserum. Protein bands of 4, 10, 38 and 49 kDa were visible in immunoblots with the antiserum against black mustard.

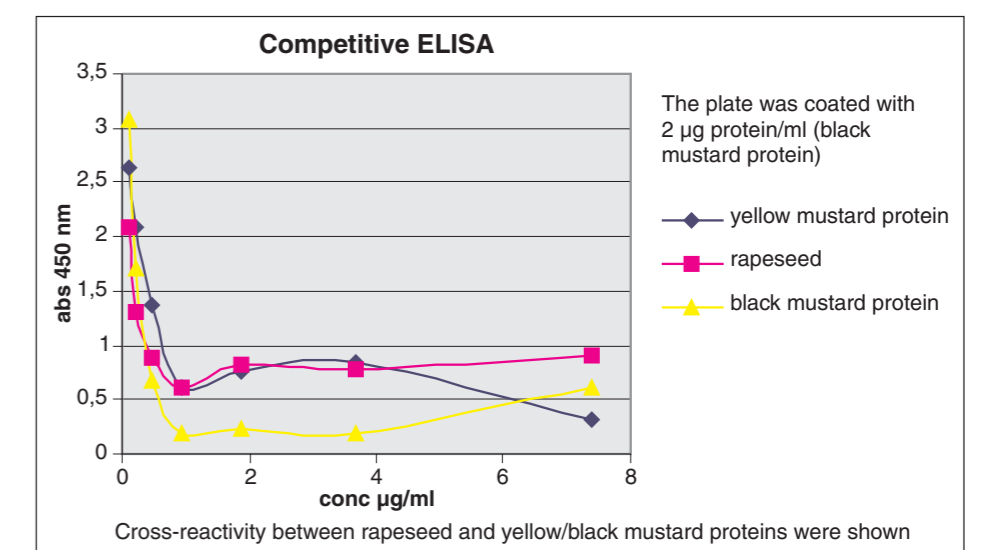
Immunoblotting with purified IgG against black mustard revealed that in extracts from seeds a concentration corresponding to 1.5 µg of mustard/ml could be detected. For the protein extract a dilution of 125 ng protein/ml was visible on the blot.

Immunoblot with IgG against black mustard



1-4. 1.5 µg/µl to 12.5 µg/µl 1-4. 125 ng/µl to 1.25 µg/µl

ELISA

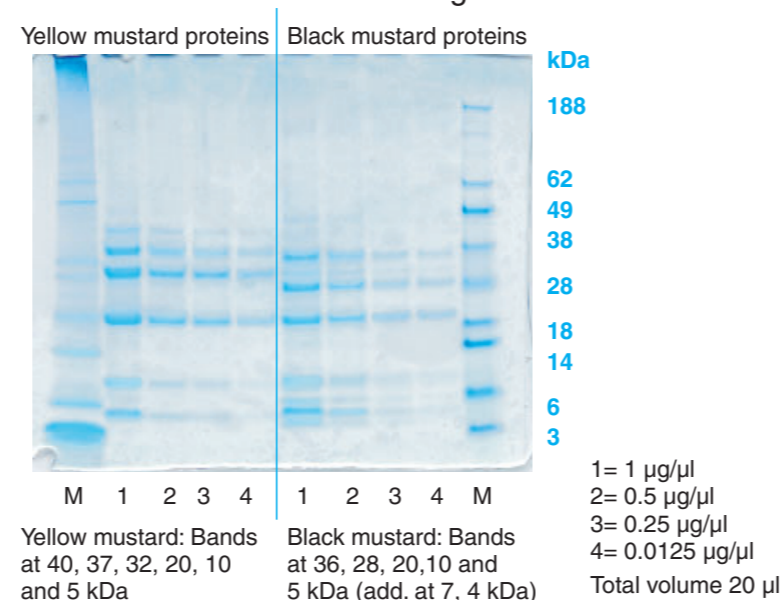


Competitive ELISAs to yellow mustard as well as black mustard were established. A concentration of 156 ng/ml of the purified mustard protein could inhibit 70% of the antigen/antibody reaction. This corresponds to a detection level below 1 ppm of mustard seeds.

Food	Immuno-diffusion	Competitive ELISA	ELISA Systems* ppm
Herring in mustard sauce	+	+	n.a.
Mustard sauce	+	+	2784
Mayonnaise	-	-	< 1 ppm
Sallad dressing	-	-	< 1 ppm
Cucumber mix	-	-	< 1 ppm
Chicken soup mix	+	+	728
Stroganoff sauce mix	-	-	< 1 ppm
Italian salad spice	+	+	27400
Curry spice	-	-	< 1 ppm
Bierwurst	+	+	1226
Salami	n.a.	n.a.	1.6
Minced meat burger	-	-	< 1 ppm
Sauce to the meat	-	-	< 1 ppm

* Mustard seed protein residue, prod code ESMUS -48 from Elisa Systems, Queensland, Australia.

SDS PAGE on 4-12 Bis/Tris gels



Conclusion

- Antibodies to yellow and black mustard were raised in rabbits
- The specificity of both antisera was excellent
- Cross-reacts only with extract from rape seed
- Establishment of a competitive assay revealed a detection level of 156 ng/ml of mustard protein
- This corresponds to a detection level below 1 ppm mustard seed
- Mustard in food products could be analyzed