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Detection of eggs of stored-product insects in flour with staining techniques

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Abstract

Chemical stains were investigated with the aim of developing a method for detecting eggs of stored-product insects in refined wheat flour (white flour), so that eggs and flour particles could be distinguished easily. Eggs of the rust-red flour beetle, *Tribolium castaneum*, the saw-toothed grain beetle, *Oryzaephilus surinamensis* and the rice moth, *Corcyra cephalonica* were detected by staining with 0.1% bromocresol green or 1:1 mixture of bromocresol green + 0.5% orange G solution after acid digestion of the flour particles. In contrast to the standard AACC method of staining with iodine solution where both eggs and flour particles were stained brownish-yellow or yellow, bromocresol green alone or in mixture with orange G, differentially stained the insect eggs and the flour particles distinct orange and green, respectively. Eggs in flour could also be detected using 0.05% acid fuchsin but both eggs and flour particles were stained uniformly pink.

Keywords: Wheat flour; *Tribolium castaneum*; *Oryzaephilus surinamensis*; *Corcyra cephalonica*; Eggs; Detection; Staining method

1. Introduction

In the milling industry, contamination of milled products of cereals by insect pests is cause for concern (Campbell, 2004). Infestations of the rust-red flour beetle, *Tribolium castaneum* (Herbst), the confused flour beetle, *T. confusum* du Val, the saw-toothed grain beetle, *Oryzaephilus surinamensis* (L.) and the rice moth, *Corcyra cephalonica* (Stainton) are observed generally in plan-sifters, purifiers, separators and conveyors. From these sources, insect infestation is likely to be passed on to finished products including refined wheat flour. Subsequently, the level of infestation in milled products increases according to the storage period in the retail market. Individual females of *T. castaneum* can lay up to 360 eggs (Howe, 1962) while those of *T. confusum* lay 970 eggs (Dick, 1937), *O. surinamensis* 375 eggs (Haines, 1991) and *C. cephalonica* about 150 eggs (Hodges, 1979) during their life. During storage, insect eggs in packaged wheat flour develop to the adult stage and the adults can proliferate resulting in high infestations in products. Infestation in packaged flour can lead to contamination of processed foods. Consumer complaints about the presence of insects in packaged products can affect the reputation of the brand or manufacturer. In this context it is important for millers to ensure that their products are free from contamination by any of the life stages of insect pests. Similarly, food industries buying wheat flour from millers for the preparation of different food products must ascertain that insect-free flour is supplied to them.

In infested flour, the larvae, pupae and adults are visible due to their larger size but the eggs are difficult to distinguish by naked eye from flour particles. In addition, adherence of flour particles to the eggs makes their identification even more difficult. Eggs of *T. castaneum* and *T. confusum* are 0.61 mm × 0.35 mm (Sokoloff, 1972), those of *O. surinamensis* 0.72 mm × 0.242 mm (Kučerová and Stejskal, 2002) and those of *C. cephalonica* 0.5 mm × 0.3 mm in size (Hodges, 1979). There are few reports about the detection of stored-product insect eggs in food commodities. Girish et al. (1972) showed that the eggs of *T. castaneum* and the *khapra* beetle, *Trogoderma granarium* Everts in wheat flour and grain dust could be

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detected by a centrifugation method. Abels and Ludescher (2003) reported that eggs of several stored-product insects including *T. castaneum*, *T. confusum*, *O. surinamensis*, *C. cephalonica*, *Lasioderma serricorne* (F.) (cigarette beetle), *Rhyzopertha dominica* (F.) (lesser grain borer), *Ephestia kuehniella* Zeller (Mediterranean flour moth) and *Plodia interpunctella* (Hübner) (Indian meal moth) were fluorescent under long-wave (365 nm) UV excitation. However, the authors could exploit the natural fluorescence only for detecting the eggs of the almond moth, *Ephestia cautella* (Walker) on cocoa beans. It was suspected that autofluorescence of some food commodities might limit the applicability of the fluorescence technique for detecting pre-adult stages including eggs.

There are staining methods to detect insect (*Sitophilus* spp.) egg plugs in whole grains using acid fuchsin (0.05%), gentian violet and berberine sulphate (20 ppm) (Rajendran, 2005). However, only limited studies have been carried out on detection of eggs of stored-product insects by staining milled products. In the recommended American Association of Cereal Chemists (AACC) (2000) method, white flour or refined wheat flour containing eggs is sifted using an 80–100 mesh sieve, and the flour residues digested with sulphuric acid. Subsequently, the eggs are stained with 0.1 N iodine solution and turn yellow. Blumberg and Ballard (1941) reported an acid–alkali method involving treatment with 5% nitric acid followed by 10% sodium hydroxide in which the eggs were orange-coloured even without the addition of any stain. It was of interest to investigate whether a detection method with a good colour contrast between eggs and flour particles could be developed. In this context, several chemical stains were examined to detect eggs of *T. castaneum*, *O. surinamensis* and *C. cephalonica* in refined wheat flour by differential staining.

2. Materials and methods

2.1. Collection of insect eggs

Tribolium castaneum and *C. cephalonica* cultures were maintained on whole wheat flour + 5% dried yeast, and *O. surinamensis* on 5:1:5 mixture of whole-wheat flour, dried yeast and rolled oats in the laboratory at 25 ± 1 °C and 65 ± 10% r.h. To collect *T. castaneum* or *O. surinamensis* eggs, whole-wheat flour was passed through an 85-mesh (pore size 180 μm) standard sieve. Into the sieved flour (200 g), 250 adults of each species from the cultures were released for oviposition. After 48 h, adults were removed from the flour using a standard 44-mesh sieve (pore size 355 μm). The flour was subsequently sieved through an 85-mesh sieve to separate the eggs. For eggs of *C. cephalonica*, about 50 newly emerged adults were placed in a metal cage with a mesh bottom (pore size 1200 μm) that allowed the eggs to pass through into a tray. The eggs were collected from the tray after 24 h for staining.

2.2. Staining

In preliminary tests, various stains and dyes (total 57) were evaluated for staining insect eggs (Table 1). Based on the results, bromocresol green (tetrabromo-*m*-cresolsulfonphthalein), orange G (Acid Orange 10) and acid fuchsin (Acid Violet 19) were chosen for the final tests. The AACC (2000) method with certain modifications was adopted for staining the eggs. Instead of the iodine solution, bromocresol green, a 1:1 mixture of bromocresol green + orange G and acid fuchsin were used for the staining and the duration of staining was 2–3 min. Also, for the final washing of flour particles and eggs, 10% sulphuric acid solution was used instead of 1% sulphuric acid. Bromocresol green (0.1%) in 100 ml of water containing 1.45 ml of 0.1 N sodium hydroxide and aqueous solutions of acid

Table 1
List of stains and dyes tested for detecting eggs of stored-product insects

1. *Arylmethane dyes*	6. *Nitro dyes*
Acid fuchshin[a]	Aurantia
Auramine O	7. *Anthroquinones*
Basic fuchsin	Alizarin red
Brilliant green	8. *Indigoid dyes*
Bromocresol green[a]	Indigo carmine
Bromophenol blue	9. *Quinone-Imine dyes*
Bromothymol blue	Amidazine
Chlorophenol red	Neutral red
Cresol red	Nile blue sulphate
Crystal violet	Safranin
Fast green	Thionin
Gentian violet	Toluidine blue
Malachite green	Wright's stain
Methyl violet	10. *Fluorescent dyes*
Naphthol benzein	Azur I
Phenol red	Azur II
Rosaniline hydrochloride	11. *Diazonium salts*
Thymol blue	Fast blue salt RR
Xylenol orange	12. *Natural dyes*
2. *Aminoxanthenes*	Carmine
Rhodamin B	13. *Others*
3. *Fluorones*	Ammonium purpurate
Eosin	Benzene azodiphenyl amine
Erythrosine	2,7 dichloro R. fluroscein
Phloxine	Itan yellow
4. *Acridines*	Neolan orange G
Acridine orange	Neolan yellow GR
5. *Azo dyes*	Prussian blue
Amido black 10B	Ruthenium red
Bismark brown	
Congo red	
Eriochrome black T	
Janus black	
Methyl orange	
Methyl red	
Oil red O	
Orange G	
Sudan IV	
Trypan blue	

[a]Stains showed good contrast between eggs and flour particles.

fuchsin (0.05%), orange G (0.5%), and iodine (0.1 N) were prepared.

Fifty grams lots of refined wheat flour were initially spiked with 20 eggs of *T. castaneum*, *O. surinamensis* or *C. cephalonica*. In subsequent tests, flour samples were spiked with only three eggs of individual species. The thoroughly mixed flour samples were sieved using an 85-mesh sieve to collect flour residues + eggs. Fifty grams of refined wheat flour samples normally contained 0.6–0.8 g of flour residues. The flour particles containing eggs were transferred to a 250 ml beaker and wet with 2–3 ml of alcohol. To this, 30 ml of diluted sulphuric acid (1:19) was added and the beaker was covered with a Petri plate and kept in a steam bath for 10 min. The prepared solution was then filtered through a Whatman No. 1 filter paper on a Buchner funnel with low suction. To each lot, 10–15 ml of stain was added to the filter paper without suction, i.e. either bromocresol green or 1:1 mixture of bromocresol green + orange G or acid fuchsin. After 2–3 min, mild suction was applied to allow the stain to pass through the filter paper, after which the filter paper was washed with 15–20 ml of 10% sulphuric acid followed by several small washes with water. Then, the filter paper was transferred to a Petri dish and examined under a binocular microscope. The staining method using bromocresol green alone, bromocresol green + orange G mixture and acid fuchsin alone was carried out four to five times with the eggs of *T. castaneum*, *O. surinamensis* and *C. cephalonica* giving consistent results.

3. Results and discussion

Bromocresol green stained eggs yellow-orange colour whereas the flour particles were green in colour (Fig. 1). In the tests either with 20 or 3 eggs, all the eggs in flour samples were detected and they were stained equally. The results of the tests with three eggs per 50 g flour sample did not vary when repeated four to five times. With 1:1 bromocresol green + orange G mixture, eggs were stained a distinct orange and the flour particles green. With *O. surinamensis* (Fig. 2) and *C. cephalonica* eggs, the colour was similar to that of *T. castaneum* but the intensity of staining was less. When acid fuchsin was used, both eggs and flour particles were stained pink, yet the eggs were distinct (Fig. 3). In the standard method (AACC, 2000) using iodine, eggs were yellow in colour and the flour particles were brownish yellow (Fig. 4).

In preliminary screening tests it was observed that some of the stains, Nile blue sulphate (blue), chlorophenol red (brick red), Wright's stain (blue), basic fuchsin (dark pink), bromocresol green (orange), toluidin blue (purple) and oil red O (red) stained the eggs. However, except for bromocresol green and acid fuchsin with all other dyes, when the eggs were washed with sulphuric acid and water, either the colour did not persist or both eggs and flour particles were indistinguishable. Bromocresol green either alone or in mixture with orange G and acid fuchsin alone proved useful for the detection of eggs. Bromocresol green in mixture with other dyes including methyl orange, orange G, dimethyl yellow, bromophenol blue, methyl green and iodine were also tested and it was noted that only the bromocresol green + orange G mixture gave desirable results and in other cases colour difference between eggs and flour particles was not distinct. Orange G alone imparted orange colour to both eggs and flour particles. Bromophenol blue (tetrabromophenolsulfonaphthalein) stained the eggs yellow and the flour particles were bluish green. However, the colour contrast between eggs and flour particles was not as distinct as with bromocresol green.

Blumberg and Ballard (1941) showed that in the acid–alkali method, eggs were yellow-orange even without the addition of any stain and flour residues were yellow or orange-red. They also reported that the contrast between eggs and flour particles can be improved by staining with either 1% aqueous iodine solution or with Ehrlich's triacid stain. In the acid–alkali method, the flour particles turned blue and green, respectively, when iodine and Ehrlich's triacid stain were used. In the present study using bromocresol green (or bromocresol green + orange G mixture), there was notable colour contrast between eggs (orange) and flour particles (green) (Figs. 1 and 2). Bromocresol green either alone or in mixture with orange G, and acid fuchsin alone when tested against *T. castaneum* eggs following the acid–alkali method (Blumberg and Ballard, 1941), did not provide a distinct contrast between eggs and flour residues.

In the present method, the staining period was 2–3 min. At shorter duration (1 or 1.5 min), staining was not sufficient and the colour difference between eggs and flour residues was inadequate. Staining for longer periods did not improve the colour difference between eggs and flour particles. Also, after staining the eggs, washing with 10% sulphuric acid solution is recommended since washing with $<10\%$ acid solution (1%, 5%, 8%) resulted in poor colour contrast between eggs and flour particles. Washing the eggs with $>10\%$ acid solution (e.g. 15%) did not improve the contrast and the treatment damaged the eggs so that they became shrunken. Eggs after staining with bromocresol green or bromocresol green + orange G mixture must be observed within 10 min, after which the flour particles around the eggs turned into an orange colour. Acid fuchsin alone stained the eggs as well as flour particles distinct pink (Fig. 3) and the colour was stable for more than 10 min.

In summary, the current study shows that with the method described, bromocresol green alone or in mixture with orange G, or acid fuchsin alone can be used to detect the eggs of *T. castaneum*, *O. surinamensis* and *C. cephalonica* that are encountered in refined wheat flour. Unlike the AACC (2000) method, the present method has the advantage of good colour contrast between insect eggs and the flour particles.

Fig. 1. Eggs of *Tribolium castaneum* stained with 0.1% bromocresol green.

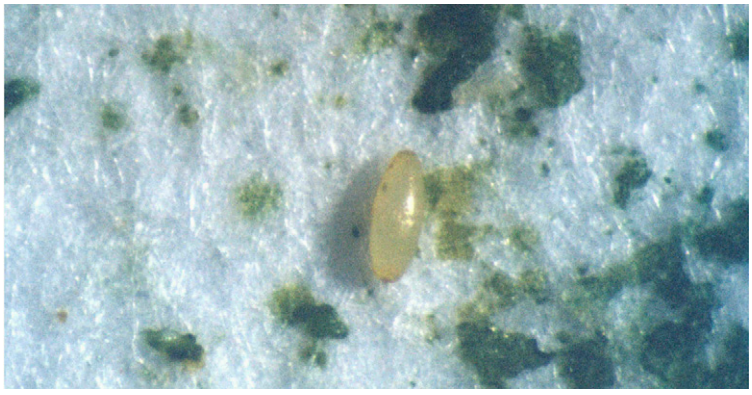

Fig. 2. Eggs of *Oryzaephilus surinamensis* stained with 0.1% bromocresol green.

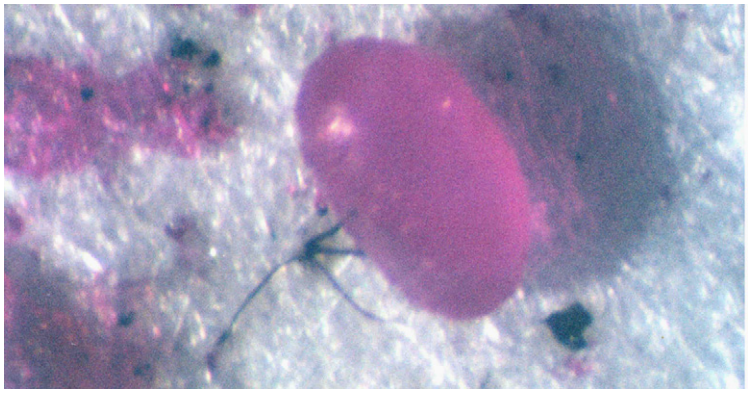

Fig. 3. Eggs of *Tribolium castaneum* stained with 0.05% acid fuchsin.

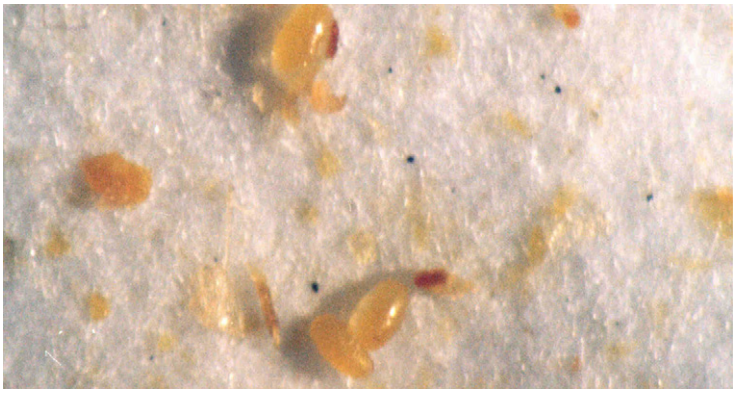

Fig. 4. Eggs of *Tribolium castaneum* stained with 0.1 N iodine solution (standard AACC method).

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