

Comparison of Detection Methods for *Acarus siro* (Acari: Acaridida: Acarididae) Contamination in Grain

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ABSTRACT *Acarus siro* L. 1758 (Acari: Acaridida: Acarididae) is an important pest of stored grain because it contaminates the grain by allergens and transfers pathogenous microorganisms. Rapid detection of contamination enables to intercept an early grain infestation by the pest. In this study, we compared the usability and efficiency of various detection approaches. Under laboratory conditions, grain samples of various sizes were infested by different levels of the following contaminants: eggs, adults, and feces of *A. siro*. The samples were analyzed by enzyme-linked immunosorbent assay (ELISA) by using anti-*A. siro* polyclonal antibody (immunochemical method), extracted in Berlese-Tullgren funnels, sieved, and processed by filth-flotation (conventional methods). The adults or juveniles of *A. siro* could be detected by all the three tested conventional methods and ELISA with detection limits in the range from 221 to 1,157 mites/kg grain. Eggs were detected by filth-flotation only; the detection limit was 1,950 eggs/kg grain. The feces of *A. siro* were detectable by ELISA test, only. ELISA enabled the detection of the feces with the minimal threshold level of 1.04 µg feces/g grain; it means the assay allowed to trace less than one metabolically active mite per gram of grain. The study thus demonstrated that reliable *A. siro* detection in grain can only be achieved by combining different detection methods. European Union and U.S. administratives dictate zero or near-zero tolerance level for mite infestation in stored products. This demand is difficult to fulfill, because every detection method is limited by its detection limit; thus, it is hard to reliably detect infestation levels lower than obtained detection limits. This methodical limitation is discussed in context with the determined detection limits of the tested methods.

KEY WORDS extraction, ELISA, grain, mite, storage

Cereal crops are the main source of food for human consumption and animal feed. Not counting rice, *Oryza sativa* L., the cereal production in the European Union (EU) is ≈257.9 million tons, and the production of common wheat, *Triticum aestivum* L., amounts to 115.6 million tons (≈45%) (Ollier 2006). This makes common wheat the main cereal commodity in the EU. Common wheat is harvested from July to August in the climatic conditions of the Czech Republic. In 2005, the production amounted to 4.54 million tons (Ollier 2006). After harvest, wheat is stored in vertical or flat grain stores until its distribution (Stejskal et al. 2003). The storage period usually lasts 10 mo during which the stored wheat is attacked by pests, including stored-product mites and beetles. Unlike contamination with larger insects, stored-product mite contamination is usually hidden, because of their small body size and creamy white color.

Contamination of grain and food by stored-product mites is common in areas with humid climate. For example, contamination was found in 60% of grain samples in the Czech Republic (Stejskal et al. 2003),

in 21% of samples in the United Kingdom (Thind and Clarke 2001), and in 75% of samples in Ireland (Cusack et al. 1976). In addition, populations of mites in grain stores show metapopulation patterns (Hubert et al. 2006). All these findings indicate that stored-product mites are present in the majority of grain stores and farms and that they also occupy household storage facilities in rural areas, where mites were frequently found in low abundance (Eaton et al. 1985, Thind and Clarke 2001). This infestation could also reach dangerous levels if humidity and temperature conditions became favorable for the growth of the mite populations. Mites can multiply exponentially in suitable moisture and temperature conditions, with the development of the population very quick (Kawamoto et al. 1991, Aspally et al. 2007).

Mite infestation decreases the quality of grain by changing its proportions of nutrients. The mites' waste metabolites are smelly and change the odor and color of grain (Wilkin and Stables 1985). Moreover, mites transfer pathogenous microorganisms (White et al. 1979), and stored-product mites produce allergens causing health problems to store workers (Eaton et al. 1985, van Hage-Hamsten and Johansson 1992). Eating

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food contaminated by storage mites can cause anaphylactic reactions (Matsumoto et al. 1996, Sanchez-Borges et al. 2005). Transfer of mycotoxin-producing fungi was observed in stores contaminated by high levels of mites (Hubert et al. 2004). Mite infestation makes therefore grain unsuitable for milling and unpalatable to livestock. Detection techniques to find and quantify mite contamination are needed.

Sieving, extraction of mites in Tullgren-Berlese funnels, and filth-flotation are the most commonly used methods for the estimation of mite population densities. These conventional methods were introduced, reviewed, and commented by Solomon (1945). Except filth-flotation, these methods are widely used as the main detection/extraction techniques for stored-product mites in many laboratories. Filth-flotation has been improved and extended to the detection of the whole insect and their fragments and rodent hairs in storage commodities (Thind 2000). However, a comparison of the recovery efficiency of these conventional methods on stored-product mites is missing up to date.

Recently, a new grain screening tool, enzyme-linked immunosorbent assay (ELISA), has become available for insect detection (Quinn et al. 1992), and it has achieved reliable prediction of hidden contamination (Brader et al. 2002). These tests are based on monoclonal (mAbs), polyclonal antibodies (pAbs), or both. mAbs were developed for the detection of the allergens of house dust mites (*Dermatophagoides* spp.) in house dust (Lind 1986), and they became commercially available together with mAbs/pAbs against group 2 allergens (i.e., Lep d2, Tyr p2) (Parvaneh et al. 2002; <http://www.inbio.com>). Danielsen et al. (2004) applied a commercial mAb to detect the production and degradation of Lep d2 in grain contaminated by the stored-product mite *Lepidoglyphus destructor* (Schrank) under laboratory conditions. The levels of Lep d2 correlated with the abundance of the mites. mAb against a 39-kDa allergen (Härfast et al. 1992, 1996) was applied in combination with pAbs in the development of a detection technique for the *L. destructor* (Dunn et al. 2002, 2003). Recently, pAbs of high sensitivity to *Acarus siro* L. 1758 (Acari: Acarididae) and low cross-reactivity to other compounds and species in stored grain have been prepared, and they have demonstrated high potential for a rapid detection of mite contamination in grain (Kudlikova et al. 2004). mAbs/pAbs thus provide a tool for the detection of hidden mite contamination in food. However, a comparison with the conventional methods is needed to thoroughly evaluate the potential of mAb/pAbs in mite detection.

The main goal of this study was to compare immunochemical and more conventional methods for the detection of mite contamination in grain. In the current study, the recovery efficiency of the screening methods was tested on ≈ 200 grain samples artificially contaminated with mite individuals, eggs, and feces. *A. siro* was selected as the model contaminator based on its high frequency in grain and food samples and its cosmopolitan occurrence (Solomon 1962, Hughes

1976). We applied calibration statistical methods as a new approach to determine and compare the detection limits of all the methodical approaches tested.

Materials and Methods

Preparation of Contaminated Grain Samples. Common wheat grain (*T. aestivum* variety Nella) was placed into plastic containers (≈ 4 kg per container) and cleaned by bleach (5%) to eliminate possible background infestation with mites from previous storage, which may have confounded the highly sensitive immunochemical assay. The grain was washed in the bleach for 30 min, rinsed with water on sieves (2-mm mesh), and dried at 70°C for 6 h. Grain was remoistened to moisture contents from 14.8 to 15% and used for analyses.

The specimens of *A. siro* originated from laboratory cultures of the Research Institute of Crop Production, which were initiated in April 1996 with material collected in a grain store in Buštěhrad, Czech Republic (CZ). The mites were mass reared in frit-chambers (S0-frit, Kavalier Sázava, CZ) plugged by the rubber. The rubber was pierced by steal pipe (5 mm in diameter). Both ends of the steal pipe were covered by a muslin. The rearing diet consisted from oat flakes, wheat germ, and dried bakers yeast in the proportion 10:10:1 (wt:wt:wt), powdered, and sieved. Approximately 1.5 g of diet was used per each rearing chamber.

The chambers were placed into Secador desiccator boxes (P-Lab, Praha, CZ) at 85% RH and 25°C and kept in darkness. Adults and juveniles were collected by a brush from the surface of the rubber or the sides of the frit-chamber. After 1 mo of cultivation, the diets were sieved (Retsch 0.15-mm sieve, Retsch, Haan, Germany). The sieved fraction contained feces, small pieces of the diet, and mite eggs. The eggs were separated by a brush under a Stemi 2000-C dissection microscope (Carl Zeiss, Jena, Germany), and the rest was used as the feces fraction. These contaminants (adults/juveniles, eggs, and the feces fraction) were counted or weighted and directly transferred into grain samples.

The experimental design of the quantitative analyses (sample sizes and contamination densities) was developed based on the results of preliminary tests and ranged from 2.5 to 400 g of grain. The remoistened grain was weighted (0.01-g accuracy) on Navigator microbalance (Ohaus Inc., Pine Brook, NJ) and placed into plastic containers (Aliachem, Chropyně, CZ). The feces fraction was weighted (50- μ g accuracy) on AE240 Mettler microbalance (Mettler-Toledo, Inc., Columbus, OH) and mixed into the grain. The eggs and adults/juveniles were counted and added directly into grain samples under a dissection microscope. Five replicates were established per treatment (density $\times$ sample size $\times$ extraction method).

Extraction of Samples in Tullgren Berlese Funnels. The procedure is based on the creation of a temperature gradient in the samples and on active movement of mites from high to low temperature. Therefore, only samples containing adults/juveniles were analyzed.

Detailed descriptions of Tullgren-Berlese funnels and the extraction procedure can be found in Sinha (1964) and Golob et al. (1975). Brady (1969) described the physical changes in the extracted grain material.

The following combinations of sample size and adults/juveniles density were tested: 5, 25, 50, 100, 250, 500, and 10,000 specimens/50-g sample; 10, 50, 100, 500, and 1,000 specimens/100-g sample; and 20, 100, 200, 500, 1,000, and 2,000 specimens/200-g sample. The grain was put on the 2-mm sieves, and the sieves were placed into the funnel. The extraction temperature was 35°C, and the bulb heating was regulated by a voltage unit to keep stable temperature (Klen and Leskinen 1989). The extracted mites were collected in saturated solution of picric acid (cat no. 74069, Sigma-Aldrich, St. Louis, MO). The grain was extracted for 24 h, after extraction the mites in the picric acid were counted using a dissection microscope.

Extraction of Samples by a Sieve Shaker. This extraction is based on a separation of mites from the grain by shaking. The method was not tested for eggs and feces, because it is impossible to distinguish them in the sieved fraction. The following combinations of sample size and adults/juveniles density were tested: 10, 50, 100, 500, and 1,000 specimens/100-g sample; 20, 100, 200, 500, and 1,000 specimens/200-g sample; and 40, 200, 2,000, and 4,000 specimens/400-g sample. The grain samples were placed on the 2-mm sieves (Retsch) and shaken in sieve shaker AS 200 digit (Retsch) at 75 rpm for 3 min. The sieved fraction was removed into a petri dish with saturated picric acid solution, and the extracted mites were counted.

Filth-Flotation. We adopted Thind's filth-flotation apparatus (Thind and Griffiths 1979, Thind and Wallace 1984, Thind and Clarke 2001). Mites were separated from the grain by an overflow method in saturated aqueous salt solutions (Sasa et al. 1961) heated to 50°C. This method enables the separation and quantification of eggs and adults or juveniles. For eggs, the following densities were tested: 1, 5, and 25 eggs/2.5-g samples. For adults/juveniles, the following combinations of sample sizes and densities were analyzed: 3, 13, and 25 specimens/2.5-g sample; 5, 25, and 50 specimens/5-g sample; and 1, 5, 10, 50, and 100 specimens/10-g sample. The grain samples were extracted in filth-flotation apparatus, extracted fraction was deposited on the mesh of the apparatus, stained by phloxine B (cat. no. 28550, Sigma-Aldrich), and counted using dissection microscope (Thind 2000).

Immunochemical Detection. Samples were artificially contaminated with *A. siro* feces, eggs, and adults/juveniles in the following combinations: eggs: 1, 5, and 25 eggs/2.5-g sample; adults/juveniles: 1, 5, and 50 specimens/2.5-g sample; 1, 10, 50, and 100 specimens/25-g sample; feces: 1, 5, and 10 mg of feces/2.5-g sample; and 1, 5, 10, and 25 mg of feces/25-g sample. The contaminated and control grain samples were frozen at -32°C for at least 12 h. Then, they were homogenized in of phosphate-buffered saline (PBS). The PBS consisted of 0.137 M NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄·12H₂O, and 1 mM KH₂PO₄, pH 7.4. The volume of buffer was 2.5 ml for 2.5-g samples and 25

ml for 25-g samples. The homogenization takes 2 min at 17,500 rpm by using Ultra-Turrax T25 basic homogenizer (IKA Works, Inc., Wilmington, NC). After homogenization, the samples were extracted for 2 h at 4°C, and the crude extracts were drained off and centrifuged (2,500 rpm for 10 min at 0°C) on Mr23i Jouan centrifuge (Thermo Fisher, Inc., Waltham, MA). The resulting extracts were frozen (-45°C) until use. Extracts from eggs and adults/juveniles were applied in ELISA without dilution, whereas extracts from samples containing feces were diluted 1:9 in PBS.

For evaluating the reliability of the detection of feces in grain, samples of feces fraction were prepared and extracted without grain in the extraction buffer. Samples of 10 mg of feces and 10 mg of rearing diet (unspent growth medium) were extracted in 1 ml of PBS by agitation for 2 min on minishaker IKA at 200 rpm and left at 4°C for 2 h. The resulting crude extracts were centrifuged as described above, and the supernatant was diluted 1:100, 1:400, and 1:1000.

Plate-Trapped Antigen (PTA)-ELISA. Evaluation of the suitability of immunochemical methods for the detection of *A. siro* infestation was carried out by indirect PTA-ELISA in microtitration wells. The assay was accomplished as follows. Each well of the 96-wells assay plates was incubated with 100 µl of extract for 3 h at 25°C with agitation in heated microplate THERMOS star shaker (BMG Labtech, Offenburg, Germany). Plates were washed three times with 50% PBS containing 0.1% Tween 20 after incubation. Then, 200 µl of blocking solution (2% bovine serum albumin in PBS with 0.1% Tween 20) was added to block unoccupied binding sites in the wells. The solution was incubated for 30 min and washed as described above. The pAb anti-*A. siro* K53 (Kudlikova et al. 2004) was diluted to working concentration 0.1 µg/ml in blocking solution, filled in the wells (100 µl per well), and incubated for 3 h at 25°C. The wells were rinsed as described above, and alkaline phosphatase-conjugated goat anti-rabbit IgG (cat no. A3687, Sigma-Aldrich), diluted 1:2000 in the same buffer as the primary antibody, was added (100 µl per well) and incubated for 3 h at 25°C. After washing, the wells were filled with 100 µl of *p*-nitrophenylphosphate (cat no. N4645, Sigma-Aldrich) as substrate in substrate buffer (1 M diethanolamine and 0.5 mM MgCl₂·6H₂O, pH 9.8 adjusted with 1 M HCl). The absorbance of the plates was measured using ELISA Reader Multiscan Ascent (Thermo Fisher, Inc.) reader at 405 nm approximately after 60 min of incubation at 25°C in the dark (until the absorbance of the positive control reached value of 1.8–2.2). Every assay included both positive control (dilutions of the immunogen to a concentration of 0.1–1.0 µg/ml) and negative control (extract from control grain).

Features of pAb Used for Detection. For immunochemical assays, the previously prepared (Kudlikova et al. 2004) pAb anti-*A. siro* K53 was used. This pAb was characterized as specific and highly sensitive with the in vitro detection threshold reaching 100 ng of *A. siro* proteins per ml (at the working concentration of 1 µg/ml IgG). The pAb showed no cross-reactivity with other pests abundant and frequent in the grain

Table 1. Comparison of the recoveries and detection limits and optimal size of grain samples of the four extraction methods for *Acarus siro*

Detection method	Contaminant	Optimal sample size (g)	Recovery (%)	Detection limit
Antibodies	Adults/Juveniles	25	ND	574
	Eggs			
	Feces	2.5–25		1.04*
Filtth-flotation	Adults/Juveniles	2.5–25	61	1,157
	Eggs	ND	52	1,950
	Feces			
Sieving	Adults/juveniles	100	86	221
	Eggs			
	Feces			
Tullgren-Berlese funnel	Adults/juveniles	200	77	273
	Eggs			
	Feces			

Detection limits (LOD) are given as numbers of contaminants per kg of grain with the exception of faeces indicated by asterisk, which are given in mg/kg of grain.

ND, contaminants were not detected; *, mg of feces/kg of grain.

stores and food premises in central Europe (Stejskal et al. 2003, Hubert et al. 2006). The ability to detect *A. siro* adults and proteins present in their feces by this pAb was confirmed (Kudlikova et al. 2004).

Data Analyses. The analyses were done in QC Expert 2.5 Trilobite statistical software (Trilobite, Pardubice, CZ) and XLStat2007 (Addinsoft Inc., Brooklyn, NY). Two parameters were calculated for the conventional methods: 1) total and/or sample recovery and 2) detection limits. Detection limits only were calculated for the immunochemical method.

The sample recovery for each extraction was calculated by dividing the extracted (observed) numbers of *A. siro* adults/juveniles or eggs by the added numbers. The effects of the detection technique, sample size, and initial mite density on sample recoveries were compared by analysis of covariance (ANCOVA).

Calibration methods (Kupka 2002, Meloun and Miltky 2004) were used to calibrate the extraction procedures and to determine their detection limits. These methods work with linear or quadratic regression and include an absolute coefficient even if it is not significant. The initial numbers of the contaminants (added individuals or eggs) constituted the independent variable of the model, whereas the numbers of the recovered contaminants, the dependent variable. For the immunochemical method, absorbance constituted the dependent variable; the initial numbers of individuals or weights of feces and the sample sizes were the independent variables.

The calibration methods provided the back estimation of x values for the following limits: 1) instrument detection limit (IDL) is the threshold to which the measured value is noise, 2) limit of detection (LOD) is the lower bound from which positive samples are detected, and 3) limit of quantification (LOQ) is the lower bound from which reliable quantification of contamination is possible (Meloun and Miltky 2004). These limits were estimated using methods $K \times \text{Sigma}$ from regression on 5% probability level (Kupka 2002).

Results

On the qualitative level, *A. siro* adults and juveniles were detected by all the compared techniques. The feces of *A. siro* are detectable only by ELISA and eggs only by filtth-flotation. On the quantitative level, the adults or juveniles of *A. siro* can be detected by all tested methods with various recoveries. The recovery, detection limits, or both of *A. siro* mites extracted by shaking, in Tullgren-Berlese funnels and by filtth-flotation test, are summarized in Table 1.

When the sample recoveries of conventional methods was compared by ANCOVA, samples recoveries were significantly influenced by the detection methods ($F = 139.26$, $P < 0.0001$), sample size ($F = 10.71$, $P = 0.002$), and their interaction ($F = 8.384$, $P < 0.0001$). Although the effect of number of added individuals on the sample recovery was on the border of significance ($F = 4.074$, $P = 0.045$), the interaction between number of added individuals and methods was significant ($F = 3.563$, $P = 0.030$). Based on the sample recovery the most effective method was sieving, followed by extraction in Tullgren-Berlese funnels, and filtth test (Table 2). The detection limits (Table 3) based on calibration models corresponded with these findings. The sample size had no significant effect on sample recovery in filtth flotation extraction ($F = 0.476$, $P = 0.0493$), but it had significant effect on sieving ($F = 9.06$, $P = 0.003$) and extraction in Tullgren-Berlese funnels ($F = 15.96$, $P < 0.0001$). The sample recovery increased with increasing sample size in extraction by Tullgren-Berlese funnels, and it decreased with increasing sample size for sieving (Table 2).

The sample recovery of *A. siro* extraction in Tullgren-Berlese funnels ranged from 12 to 100%. The calibration models showed recovery from 52 to 77% (Table 3). The highest recovery and lowest detection limits were obtained for 200-g samples with detection limit LOD = 273 individuals per kg of grain (Fig. 1A). Extraction of *A. siro* from grain samples by sieving had sample recovery in the range from 34 to 100%. The calibration models yielded the highest recovery from

Table 2. *Acarus siro* extraction, the effect of various density and sample size on sample recovery of specimens extracted by the conventional methods

Method	Sample size (g)	Density (individuals/kg)				Total
		100–500	500–1,000	1,000–5,000	>6,000 and	
Filtration	2.5	100 ± 0		69 ± 27	53 ± 18	66 ± 25
	5			72 ± 11	46 ± 11	54 ± 17
	10	100 ± 0	35 ± 19	60 ± 20	61 ± 13	64 ± 24
Sieving	100	84 ± 15	80 ± 9	91 ± 10	85 ± 7	85 ± 10
	200	83 ± 10	79 ± 8	82 ± 7	84 ± 2	82 ± 7
	400	55 ± 18	60 ± 13	81 ± 12	87 ± 13	74 ± 19
Tullgren-Berlese funnel	50	73 ± 23	43 ± 30	51 ± 31	71 ± 10	58 ± 27
	100	78 ± 13	31 ± 12	86 ± 12	75 ± 18	72 ± 21
	200	52 ± 16	81 ± 13	76 ± 11	76 ± 9	74 ± 14

all extraction methods, which ranged from 81 to 86% (Table 3). The highest recovery was found in 100-g samples with detection limit LOD = 221 individuals per kg of grain (Fig. 1B). The filth-flotation test enabled detection of both the adults/juveniles and the eggs of *A. siro*. The ranges of sample recovery for eggs were from 0 to 100% and for adults/juveniles were from 20 to 100%. Because the sample size has no effect on sample recovery of adults/juveniles, the calibration model was constructed for all sample sizes. The yielded recoveries were between 61 and 72% (adults/juveniles) and 52% (eggs) (Table 3), and with detection limits they were LOD = 1,157 individuals per kg of grain and LOD = 3,900 eggs per kg of grain (Fig. 1C and D).

The detection of *A. siro* antigens from adults/juveniles and feces by ELISA gave positive results, but detection of eggs was not successful (Table 1). The assay in the range from 400 to 10,000 eggs per liter of the extraction buffer did not exceed the mean + 3 standard deviation of the absorbance of control uninfested grain samples. For adults/juveniles detection, ANCOVA model ($F = 51.86$, $P < 0.0001$) showed significant effect of sample size (2.5 and 25 g) on absorbance ($F = 11.62$, $P = 0.003$). The calibration model of the method showed different detection limits, LOD = 574 individuals per liter was in the 25-g grain sample, whereas LOD = 2,699 individuals/liter was in the smaller 2.5-g samples (Table 3; Fig. 2A and B). For detection of feces in grain samples, the best fit was obtained in ANCOVA model ($F = 21.62$, $P < 0.0001$) with logarithmically transformed weight of the feces, cf. $R^2 = 56$ and 35 for transformed and untransformed data, respectively. The effect of sample size (2.5 and 25 g) on absorbance was not significant ($F = 0.215$, $P = 0.646$). The calibration model showed detection limit LOD = 1.04 µg of feces per ml in the grain samples (Fig. 2C).

Discussion

Comparison of Recoveries of Conventional Detection Methods. It is obvious that a conventional detection method partly or seriously underestimates mite populations in the grain; consequently, the potential for an increase of mite infestation. But this could be corrected by calibrations of the techniques. In our study, both sieving and extraction of adults or juve-

niles of *A. siro* in Tullgren-Berlese funnels showed similar recoveries. The recoveries observed in Tullgren-Berlese funnels correspond with the ranges reviewed in Solomon (1945) and observed by Cusack et al. (1976). The recoveries of the extraction in these funnels are lower by ≈4 or 10% than in high gradient extractors (Klen and Leskinen 1989).

Surprisingly, sieving had a 2 or 3 times higher recovery of *A. siro* than previously reported. For example, Lynch and Thind (1985) obtained mean recoveries of *A. siro* in the range from 24 to 41% by sieving. The high sieving recovery in our experiments can be explained by the design. The dust fraction was removed from the grain before the addition of known numbers of *A. siro*. The dust in the extracted material begets problems with the counting of mites. This corresponds to the data obtained in the field analysis when sieving of the grain mass provides lower estimation than extraction in Tullgren-Berlese funnels. Furthermore, the used shaking Retsch device produced a three-dimensional throwing motion that moved the sieved sample equally over the whole sieving surface, whereas older shakers provided two-dimensional movements only. Therefore, our findings advocate sieving methods by using new generation shakers for the extraction of mites.

A low recovery (≈60%) was found in filth-flotation test in the comparison with modified methods by using mixtures of organic compounds substitute saline fluid. With this method, *A. siro* recoveries ranged from 84 to 93% (Thind and Wallace 1984). Although this modification provides the highest recoveries, its severe disadvantage consists in the handling and need for disposal of hazardous organic solvents (Brader et al. 2002). Nevertheless, this method seems to be unique for the detection of eggs, which was not possible by ELISA (Table 1). This method is rather limited for egg detection. The eggs small size complicates searching for eggs; we were not able to find one or two eggs per sample, indicating some limits. Another disadvantage is the impossibility of egg determination in practical application, because the keys for mite eggs are missing or scanning electron microscopic observation is necessary (Kucerova 2002, Kucerova and Stejskal 2002).

With the exception of filth-flotation test, the extraction efficiency was influenced by sample size. Our results indicate that larger (200-g) grain samples are more suitable for extraction in Tullgren-Berlese fun-

Table 3. The recoveries of *Acaris siro* extraction/detection for different sample sizes and types of contamination

Extraction method	Contaminat	Sample size (g)	Coef	Estimation	SD	Calibration model		Detection limits			Recovery (%)
						Lower limit	Upper limit	IDL	LOD	LOQ	
Tullgren-Berlese funnel	Adults/Juveniles	50	a	0.79	0.02	0.74	0.84	390	781	1171	48
			q	-310.58	109.34	-533.04	-88.12				
			a	0.72	0.03	0.66	0.79	338	676	1014	73
Sieving	Adults/juveniles	100	q	8.41	86.78	-167.41	184.24	136	273	409	77
			a	0.75	0.02	0.71	85.94				
		200	q	7.57	36.53	-67.47	82.63	110	221	331	86
			a	0.86	0.02	0.82	86.09				
			q	-2.71	33.91	-71.51	0.90	58	115	173	83
Filtration	Adults/juveniles	200	a	0.84	0.01	0.82	16.94	160	320	480	81
			q	-18.17	17.30	-53.27	0.86				
		400	a	0.88	0.04	0.80	30.28				
			q	-72.33	50.58	-174.94	0.97	578	1157	1735	61
			a	0.54	0.03	0.48	287.95				
Antibody	Faeces	2.5	q	63.30	112.58	-181.36	0.61	1950	3900	5851	62
			a	0.30	0.06	0.17	653.08				
		All sizes	q	222.49	199.32	-208.11	0.43	0.357	0.715	1.072	
			a	0.34736	0.01158	0.32420	0.37053	0.43	1.044	1.921	
Antibody	Adults/juveniles	2.5	q	0.12445	0.04423	0.03682	0.21309	1349	2699	4048	
			a	0.00003	0.00000	0.00002	0.00004				
		25	q	0.01362	0.01215	-0.01263	0.03987	287	574	860	
Antibody	Faeces	25	a	0.00008	0.00001	0.00006	0.00010				
			q	0.02543	0.00778	0.00908	0.04178				

The parameters of linear calibration models are $y = ax + q$. All slope parameters (a) were significant, while absolute parameters (q) were not significant at $P = 0.05$. The back estimation for the detection limits were calculated by K, sigma from calibration methods using linear regression and are following: IDL, interference limit; LOD, detection limit; LOQ, quantification limit. For faeces, logarithmically transformed data were analyzed, and *, recalculated limits are indicated by italic.

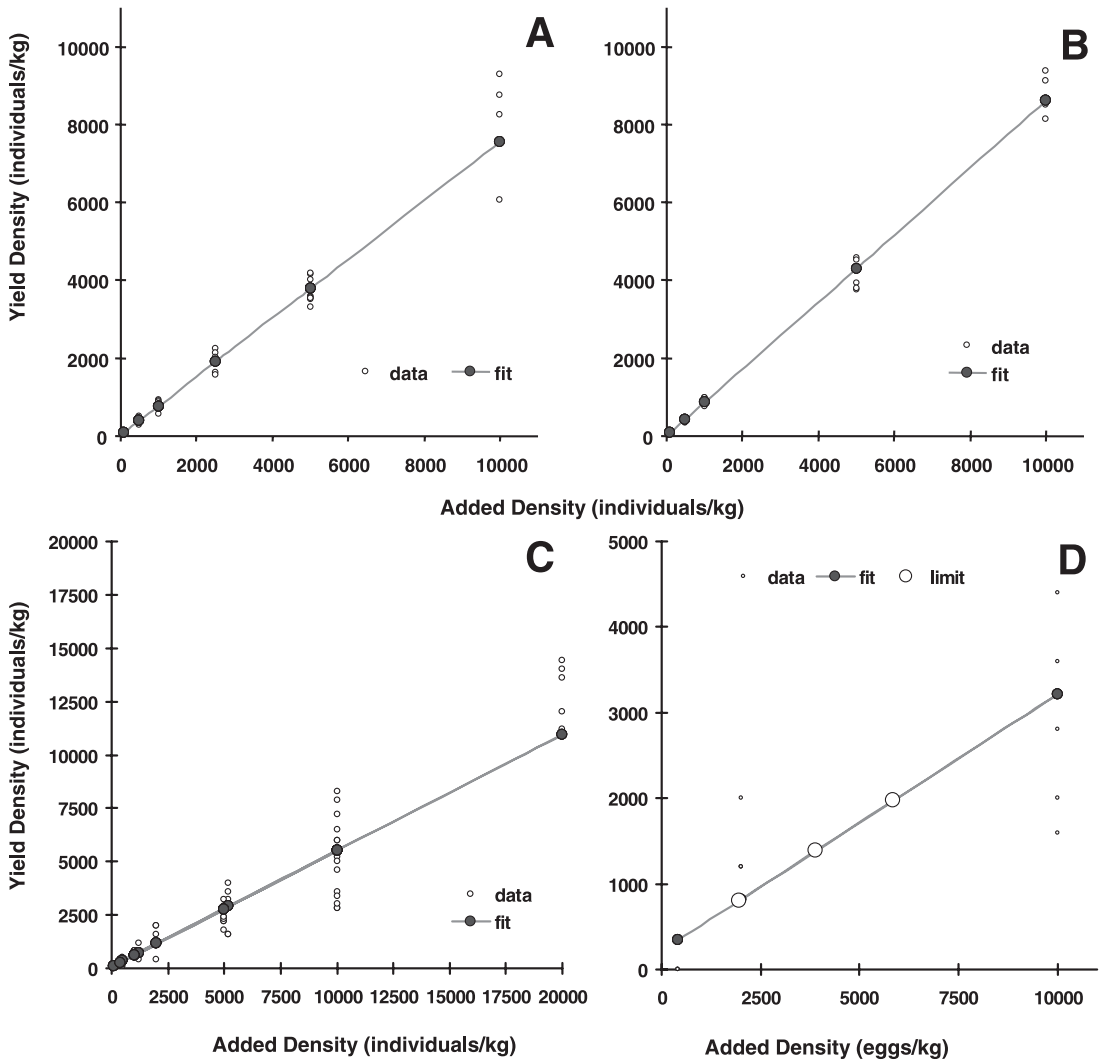


Fig. 1. The calibration method using linear regression model for detection of *A. siro* contamination by the conventional techniques. The parameters of linear regression models are shown in Table 3. (A) Calibration of adults/juveniles detection for 200-g grain sample extracted by Tullgren-Berlese funnel. (B) Calibration of adults/juveniles detection for 100-g grain samples extracted in sieve shaker AS 200 digit. (C) Calibration of adults/juveniles detection for filth-flotation. (D) Calibration of egg detection for filth-flotation, including the position of the detection limits in increasing range: IDL, LOD, and LOQ.

nels. The temperature/humidity gradient is wider in samples of larger volumes (Brady 1969), and it enables higher recovery. The opposite correlation was found for sieving methods, where samples of smaller size (100 g) had higher extraction efficiency. Shaking of smaller grain sample is more intensive than of larger ones. Generally, we recommend the application of calibration statistical procedures (Kupka 2002, Meloun and Militky 2004) for the conventional detection methods by using artificially contaminated samples as standards.

Immunochemical Detection Method. We achieved the detection limit of 1.04 μg of feces per ml, i.e., 1.04 mg of feces per kg of grain. The feces production is directly correlated with the metabolic activity of a mite. *A. siro* is metabolically active from $\approx 8^\circ\text{C}$ with a

wide peak of activity from 20 to 30°C at grain moisture content $>13.5\%$ (Solomon 1962). It is reasonable to expect that the weight of feces produced by an individual per day corresponds with the weight of consumed food. Calculation on food consumption expressed as weight losses of mite diet was the aim of several studies. Zdarkova and Reska (1976) determined the average weight of consumed food for exponentially growing populations of *A. siro* and *Tyrophagus putrescentiae* (Schränk) as 12 and 6 μg of diet per mite, respectively. This is in good agreement with Szlendak and Kraszpuski (1991), who calculated the consumption of rye germ for the 20 d of adult life of *A. siro* as 126 μg . According to this weight, we can speculate that our detection threshold allows safely detecting feces production by less than one adult mite

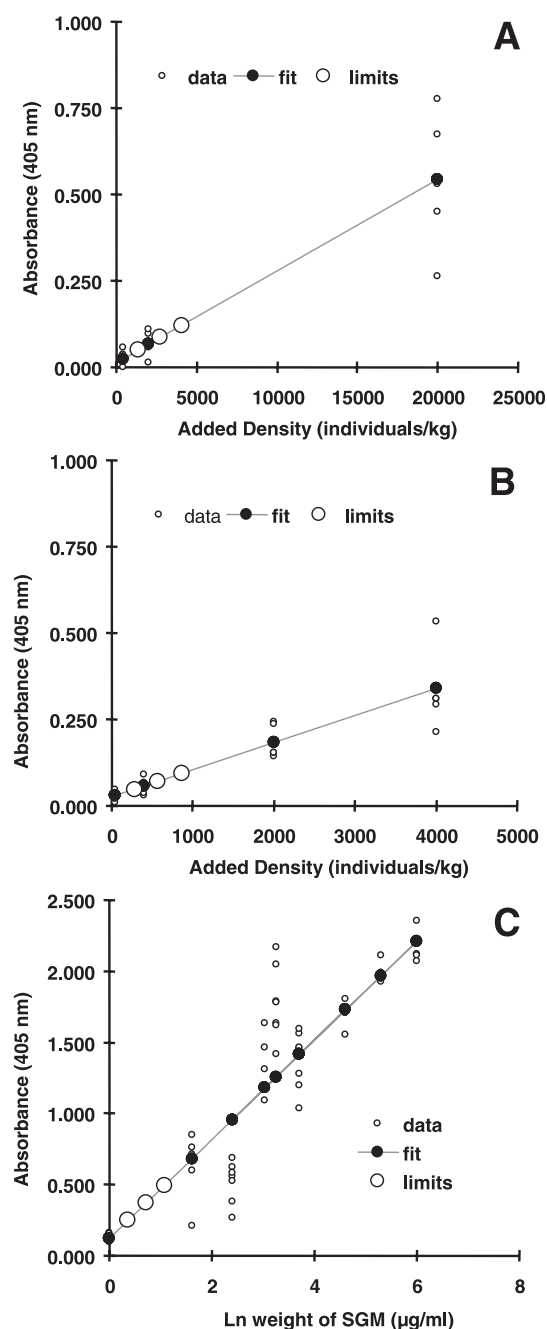


Fig. 2. The calibration of indirect ELISA test for detection of *A. siro* contamination by using linear regression models. The parameters of models are shown in Table 3. (A) Calibration models for the extraction of adults/individuals from 2.5 g of grain. (B) Calibration models for the extraction of adults/individuals from 25-g samples of grain, respectively, contaminated by *A. siro* adults/juveniles, including the position of the detection limits in increasing range: IDL, LOD, and LOQ. (C) Calibration model for *A. siro* feces, including the position of the limits in the same range as described above.

(approximately up to one sixth of specimen) per day in 1 g of grain.

Although the results of detecting *A. siro* adults in vitro were promising (Kudlikova et al. 2004), the ELISA procedure proved unsatisfactory for analyzing adults in contaminated grain on near-zero level (Table 3). Similar detection limit was achieved by Dunn et al. (2002) for *L. destructor* before they optimized their assay for *A. siro* to a sensitivity of 10 mites per kg of grain (Dunn et al. 2003). This decreasing of assay sensitivity was caused either by insufficient homogenization of the mites in the presence of grain and thus limited release of *A. siro* antigens or by a competition of *A. siro* antigens for surface of microwells with plant soluble material.

We contributed to the potentials of immunochemical detections by developing sensitive assay that can trace the history of the sample. Combining either conventional methods with the detection of feces or the detection of feces with the detection of adults/juveniles by immunochemical methods only (i.e., using homogenized and unhomogenized subsamples) allows evaluating whether a contamination is caused by a high-density population or by long-term infestation with a low-density population. Despite the high sensitivity of the assay, especially in terms of feces, we are still far from detecting very low infestation levels of grain samples as required by EU directives.

Detection Levels and Applied Techniques. Thind and Clarke (2001) reviewed the different administrative approaches giving zero tolerance level (any living mite) in the EU and 1,000 mite per kg of grain in the United States. Based on the previous finding, the EU legislative limit is hard to apply. The administrative limits are concerned with living mites, not with their feces. But the most hazardous compounds are mite allergens known to occur mainly in the feces (Tovey et al. 1981). Some allergens are stable in the feces for >1 yr (Sidenius et al. 2002). Therefore, allergen contamination or feces do not correspond to the actual population density of mites. Once the presence of mites is detected in grain, the infested loads are rejected and farmers suffer a loss. To reduce losses, the common practice is to mix infested with uninfested grain or to sieve the infested grain to remove the dust fraction and mites to obtain partially clean grain under the limits of living mite detection. The medical risk is not known, but this grain could still contain mite population under the detection limits or their feces.

The reasons for the need for a detection of mite contamination are therefore as follows: 1) identification of actual medical risk densities, when the density is higher than five mites per g; 2) identification of risk-potential densities, when the density is from 5 to 1 mite per g; and 3) identification of grain that was contaminated or still is contaminated by mites but under detection limits of conventional methods.

From this point of view, all detection methods, including conventional sieving, Tullgren-Berlese extraction, and filth-flotation test, are able to identify *A. siro* contaminations in stored grain (Table 3) on qualitative levels from 58 individuals per kg of grain and

quantify mites from 115 individuals per kg of grain. Despite the higher detection and quantification limits for ELISA than for sieving the possibility of feces detection is unique for the traceability of mite contamination of grain even after cleaning treatment. If the zero tolerance limits should be applied, the very low detection limit of ELISA for feces indicates high usability of this method.

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