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# A RAPID TEST FOR SPECIES IDENTIFICATION OF YELLOW MEALWORM BASED ON MTDNA

Małgorzata Natonek-Wiśniewska[1], Piotr Krzyścin[1], Mariusz Pietras[2], Monika Bugno-Poniewierska[1,3]

[1]National Research Institute of Animal Production, Department of Animal Molecular Biology, Balice
[2]National Research Institute of Animal Production, Department of Animal Nutrition and Feed Science, Balice
[3]University of Agriculture, Department of Animal Reproduction, Anatomy and Genomics, al. Mickiewicza 24/28, Kraków
e-mail: malgorzata.natonek@izoo.krakow.pl
Małgorzata Natonek-Wiśniewska ORCID: 0000-0003-2132-2829
Piotr Krzyścin ORCID:
Mariusz Pietras ORCID: 0000-0003-4683-9840
Monika Bugno-Poniewierska ORCID: 0000-0001-7537-6641

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## Abstract

*The increasing interest in insects as an alternative source of food for humans and animals derives from their high content of easily available protein, vitamins, minerals and fat. Regulations increasingly allow for the commercial use of insects (e.g. EU 2017/893). Considering food safety and the increasing public awareness of the ingredients, production process and origin of foods, the availability of insects as food requires the development of tests for reliable identification of their DNA. Yellow mealworm is one of the species most frequently used as a food ingredient. The method proposed in this paper enables determining the potential presence of biological material from this species.*

*The method is based on real-time PCR analysis of an mtDNA fragment (cytochrome c oxidase subunit I) and involves a simple test for determination of mealworm DNA. The method is effective for a very wide range (0.1% to 100%) of its content in a product. The standard curve parameters show species specificity and linearity across the entire range of the method, which is essential due to a possible quantitative use of the test. The test is effective for analysis of mealworm in raw form and in processed products that contain it, regardless of the stage of insect development (larva, adult form), and can be used to monitor foods containing insects.*

*The application of the test in commercial products detected mealworm in all the products in which mealworm presence was declared by the manufacturer. In positive samples, the rate of the reaction depended on mealworm content – the higher the content, the more quickly the reaction product was formed.*

*Key words: species identification of yellow mealworm, COI, identification of insect DNA*

## Introduction

The use of insects as a source of food is as ancient as humanity, although for the last dozen or so centuries it has been slightly forgotten and reserved only for the developing countries. Only recent years have seen a renewed interest in insects as food ingredient for both animals and humans. According to a report of The Food and Agriculture Organization of the United Nations (FAO), insects are a promising source of food for biological, environmental and economic reasons (Tomotake et al., 2010; Van Huis et al., 2013). Due to the high content of easily assimilable protein, vitamins, minerals and fat (Makkar et al., 2014; Józefiak et al.,

2016), as well as low ammonia emissions and rearing costs, many countries have investigated their potential use as a source of food. These studies led to authorization of the use of insects in the food industry and in animal feeds. For example, the Belgian Federal Agency for the Safety of the Food Chain (AFSCA) approved 10 species of edible insects, while the Korean Ministry of Food and Drug Safety (MFDS) legalized seven species of edible insects as food ingredients (Ghosh et al., 2017). Likewise, in 2017 the European Commission approved the use of insects to feed farmed fish (UE, 2017). In addition, in the same document the European Commission states that farmed insects could represent an alternative and sustainable solution to conventional sources of animal proteins destined for feed for non-ruminant farmed animals. Several worm species, including the yellow mealworm, are considered. The fact that it differs from other insect species may cause problems due to its similar morphological appearance (e.g. mealworm larvae are similar to superworm larvae) or change of external appearance as a result of thermal processing.

For the last two decades, species composition of food products has been mainly authenticated by molecular analysis because DNA is identical in all somatic cells of an organism and remains unchanged regardless of the source of origin (blood, muscles, etc.). Furthermore, because researchers use degradation-resistant DNA fragments, the analyses are effective for highly processed food products, and also for trace contaminants. The methods are highly sensitive because the amount of required material may be just a few cells (Sun et al., 2014). The most common techniques used to analyse food include PCR (conventional and real-time), single-strand conformation polymorphism (PCR-SSCP), random amplified polymorphic DNA (RAPD) (Sforza et al., 2011).

The methods reported in the literature for determination of yellow mealworm are based on real-time PCR analysis of cytochrome c oxidase subunit I (COI). The method should be sensitive and specific regardless of the insect's biological form and processing degree.

The aim of the study was:

– determination of a universal DNA isolation method suitable for each biological form of mealworm and independent of their degree of thermal and baric processing

– development of a mealworm species identification test for raw and processed samples and for each stage of biological development based on detection of the final fragment of cytochrome I oxidase (mtDNA) specific for this species

– test validation, i.e. determination of its parameters

– verification test for analysis of commercial samples available on the Polish market

## Materials and methods

The material of the study consisted of:

– adult insects of the following species (12 each): field cricket, Dubia cockroach, Madagascar hissing cockroach, banded cricket, migratory locust, yellow mealworm, superworm

– house fly (2 insects), lacewing (2 insects)

– yellow mealworm and superworm larvae (around 100 each)

– reptile food samples containing dried yellow mealworm larvae with different content, dried crickets, crustaceans, dipterans

– plant samples: lemon, banana, tomato, wheat grain, oat grain

– meat samples from cattle, pig, turkey, chicken, fish

The insects were purchased from breeders of feeder insects, which were reared under Veterinary Inspectorate supervision to ensure that the material conforms to the declaration. Furthermore, the species of test material was confirmed using an insect identification key and comparison with the photographs (Gwinner et al., 1990, http://www.medianauka.pl). Meat samples and reptile food samples were purchased at a food store and at a pet shop, respectively.

For each insect species 3 test samples, each weighing 0.1 g, were prepared.

### DNA extraction

The isolation method was adjusted to the type of tissue material – insect DNA was extracted using the Sherlock kit (A&A Biotechnology), and DNA from meat and plants was obtained using the AxFood kit (A&A Biotechnology). Insect isolations were improved by using supplemental DTT, extended heating time (4 h) and intense vortexing. The content of DNA obtained was determined with Nanodrop 2000. The purity of DNA isolates was determined from absorbance ratio 260/280.

### qPCR reaction

The obtained DNA was subjected to Real-Time PCR with StepOne Plus Thermal Cycler Software v2.3 (Applied Biosystems), using primers (0.34 μM concentration in the reaction mixture) flanking the species specific region for the mealworm, Tamra probe compatible with their DNA (0.54 μM concentration in the reaction mixture) and TaqMan Master Mix

(Thermofisher). The primers and the probe were taken from the literature (Debode et al. 2017).

| | |
|---|---|
| TM-WING-Forward | 5'- CAGGGTTGAACGGGTTCAGT |
| TM-WING-Reverse | 5'- ATACTATTTCGGGCAACAGCATC |
| TM-WING-Sonda | 5'- AAGCCGTACTTGTGTTACGGCGGTTCAC |

The thermal program was as follows:

| | | | |
|---|---|---|---|
| initial denaturation | 15 minutes | 95ºC | 1 cycle |
| denaturation | 15 seconds | 95ºC | 50 cycles |
| annealing/extension | 1 minute | 60ºC | |

All analyses were performed in triplicate. For all the samples, the cycle at which the amplification threshold crosses the plot of fluorescence versus reaction cycle, was determined. This value, known as the threshold cycle ($C_T$), is correlated with the presence and original amount of biological material whose DNA is compatible to the test primers and probe. For the threshold cycle also the absolute standard deviation (SD) and the relative standard deviation (RSD%) were determined to check the repeatability of the result obtained for independent DNA isolations.

**Molecular specificity of the method**

Molecular specificity of the method was determined by in silico analysis s of primers using Basic Local Alignment Search Tool (BLAST). Next, specificity of the primers was experimentally tested using DNA extracted from insects (adult and larval forms), plants and meat. PCR cross-reactions with other species than mealworm confirm the lack of species-nonspecific PCR products. The presence of PCR product for mealworm DNA and the concurrent lack of the product for the DNA of other species are indicative of the biological specificity of the applied test. Each DNA isolate was analysed at a concentration of 25 ng/µl. Water served as negative control for DNA isolation (KN) and PCR (NTC).

**Validation of the test: linearity and sensitivity: limit of detection (LOD) and limit of quantification (LOQ)**

As part of the test validation, parameters such as linearity and sensitivity were determined. Method sensitivity was tested by determining the limit of detection and the limit of

quantification. To this end, mealworm DNA dilution analysis was performed at concentrations of 25, 10, 1, 0.1 and 0.025 ng per qPCR.

To determine the linearity of the test, we constructed a standard curve of the solutions and determined its slope, coefficient of correlation ($R^2$) and PCR yield (%) based on the formula: E = [10 (-1 / slope) - 1]. Information about the linearity of the test is necessary to determine the potential application of the test for quantitative determinations.

**Application of the method for commercial samples**

To provide a more complete presentation of the method, analysis was made of the commercial reptile food samples which could possibly contain mealworm and other insects.

## Results

**DNA extraction**

DNA concentration and quality ranged from 10.369 to 757.629 ng/µl with A260/280 absorbance between 1.27 and 1.88. Detailed parameters of DNA isolation for all the analysed insects are shown in Table 1.

**Qualitative analysis. Validation. Method specificity and sensitivity, limit of detection and quantification**

In silico analysis demonstrated that the proposed primers are only species specific for mealworm. The experimentally tested specificity showed positive reactions for mealworm DNA, no reactions for all the other species (field cricket, Argentinian cockroach, banded cricket, superworm larvae) or late stage products after the 44th cycle (Madagascar cockroach, migratory locust). The results are shown as mean $C_T$ from three independent isolations of the samples as well as standard deviation (SD) between them (Table 2)

The analysis of sensitivity showed positive reactions for all the dilutions between 0.025 and 25 ng (Table 3). Standard deviation was 1.16-4.92%, which resulted in relative uncertainty of the measurement below 12.46%.

The standard curve generated from the qPCR results obtained for mealworm DNA dilutions had the following formula:

$C_T = -3.374 \log c + 33.407$

with the correlation coefficient $R^2=1$ and efficiency E=97.888,

indicating that the test is linear across the entire range. The limit of detection (the smallest amount of product that can be determined quantitatively) was set at 0.025 ng, corresponding to a concentration of 0.1%. The limit of quantification was set at the same level because no tests for the lower concentrations were performed. Graphical presentation of the result for some samples is shown on Figure 1.

Table 1. Insect DNA concentration and quality

| Type of sample | Sample composition | Concentration [ng/µl] | $A_{260/280}$ |
|---|---|---|---|
| S | field cricket | 590.77 | 1.87 |
| S | superworm | 757.629 | 1.88 |
| S | Dubia cockroach | 559.754 | 1.88 |
| S | Argentinian cockroach | 475.096 | 1.87 |
| S | yellow mealworm | 18.35 | 1.39 |
| S | migratory locust | 475.766 | 1.86 |
| S | banded cricket | 700.614 | 1.90 |
| S | mealworm larvae | 26.31 | 1.33 |
| S | superworm larvae | 19.834 | 1.36 |
| P | dried mealworm larvae | 687.304 | 1.86 |
| P | mealworm larvae 20%, dried | 217.946 | 1.78 |
| P | mealworm 10% | 95.211 | 1.71 |
| P | crickets 0.3%, mealworm 0.3% | 94.788 | 1.71 |
| P | mealworm larvae 4% | 92.101 | 1.73 |
| P | dried mealworm | 10.369 | 1.27 |
| P | crustaceans | 297.007 | 1.80 |
| P | dipterans/crustaceans | 538.833 | 1.87 |

Insect DNA quantity and quality. S – fresh sample, P – processed sample

**Application of the method for commercial samples**

For the samples containing mealworm, positive reactions were obtained between cycles 26 and 37, and no reaction product was obtained for the samples containing no mealworm. In the positive samples, the rate of the reaction depended on mealworm content – the higher the content, the more quickly the reaction product was formed. Detailed results for DNA determination of mealworm in the commercial samples, together with the interpretation of the

result, are presented in Table 4. Graphical presentation of the result for some samples is shown on Figure 1.

Table 2. Results for species specificity of the yellow mealworm

| Type of sample | Sample composition | Mean $C_T$ | SD $C_T$ | RSD % | Results |
|---|---|---|---|---|---|
| S | field cricket | NR | | | - |
| S | superworm | 46.386 | 0.748 | 0.016 | - |
| S | Dubia cockroach | NR | | | - |
| S | Madagascar cockroach | 44.855 | | 0.026 | - |
| S | mealworm | 30.940 | 1.533 | 0.040 | + |
| S | migratory locust | 45.315 | 0.323 | 0.008 | - |
| S | banded cricket | NR | | | - |
| S | mealworm larvae | 31.821 | 3.999 | 0.126 | + |
| S | superworm larvae | NR | | | - |
| DNA | cattle/pig | NR | | | - |
| DNA | lemon/banana | NR | | | - |
| DNA | turkey/chicken | NR | | | - |
| DNA | fly/lacewing | NR | | | - |
| DNA | tomato | NR | | | - |
| DNA | fish | NR | | | - |
| DNA | grain | NR | | | - |
| DNA | KN | NR | | | - |

NR – no reaction, $C_T$ – amplification threshold cycle, SD $C_T$ – standard deviation of the amplification threshold cycle, RSD – relative standard deviation of the amplification threshold cycle.

Table 3. Analysis of the sensitivity test for species determination of the yellow mealworm

| Tested species/Amount of DNA used | $C_T$ | Mean $C_T$ | SD $C_T$ | RSD % |
|---|---|---|---|---|
| mealworm 25 ng | 31.38 | 31.46 | 3.55 | 11.27% |
| mealworm 25 ng | 27.96 | 31.46 | 3.55 | |
| mealworm 25 ng | 35.05 | 31.46 | 3.55 | |
| mealworm 10 ng | 32.89 | 32.64 | 3.32 | 10.16% |
| mealworm 10 ng | 29.21 | 32.64 | 3.32 | |
| mealworm 10 ng | 35.83 | 32.64 | 3.32 | |
| mealworm 1 ng | 38.76 | 35.95 | 3.16 | 8.80% |
| mealworm 1 ng | 36.56 | 35.95 | 3.16 | |
| mealworm 1 ng | 32.52 | 35.95 | 3.16 | |
| mealworm 0.1 ng | NR | 38.81 | 4.92 | 12.46% |
| mealworm 0.1 ng | 36.00 | 38.81 | 4.92 | |
| mealworm 0.1 ng | 42.96 | 38.81 | 4.92 | |
| mealworm 0.025 ng | 39.63 | 39.48 | 1.16 | 2.98% |
| mealworm 0.025 ng | 37.99 | 39.48 | 1.16 | |
| mealworm 0.025 ng | NR | 39.48 | 1.16 | |

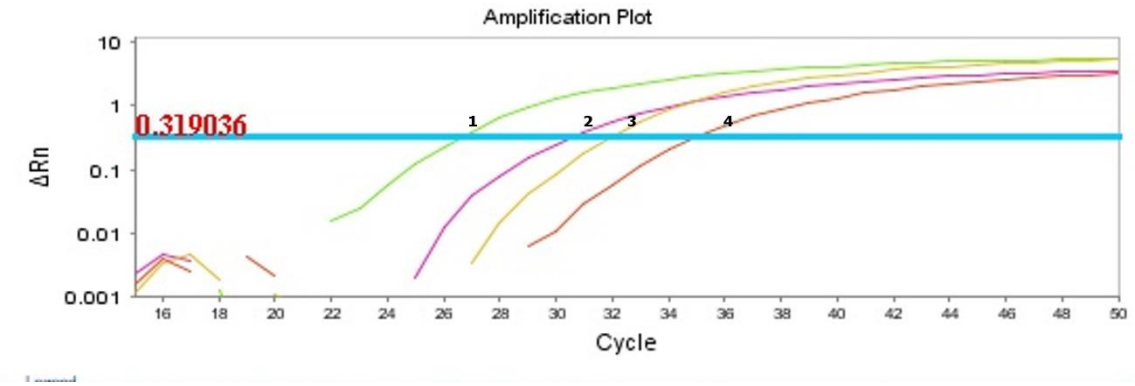
NR – no reaction, $C_T$ – amplification threshold cycle, SD $C_T$ – standard deviation of the amplification threshold cycle, RSD – relative standard deviation of the amplification threshold cycle.

Table 4. Detailed results for determination of mealworm DNA in commercial samples

| Type of sample | Sample composition | Mean $C_T$ | SD $C_T$ | RSD % | Results |
|---|---|---|---|---|---|
| P | dried mealworm larvae | 26.316 | 0.428 | 0.016 | + |
| P | mealworm larvae 20%, dried crickets 10% | 28.362 | 0.638 | 0.022 | + |
| P | mealworm 10% | 32.116 | | 0.031 | + |
| P | crickets 0.3%, mealworm 0.2% | 37.120 | 0.758 | 0.020 | + |
| P | mealworm larvae 4% | 32.922 | 0.038 | 0.001 | + |
| P | dried mealworm larvae | 25.778 | 1.299 | 0.050 | + |
| P | crustaceans | NR | | | - |
| P | dipterans/crustaceans | NR | | | - |

NR – no reaction, CT – amplification threshold cycle, SD $C_T$ – standard deviation of the amplification threshold cycle, RSD – relative standard deviation of the amplification threshold cycle .

Figure 1. Amplification plot of selected samples




1-dried mealworm larvae (P)

2-mealworm (S)

3-mealworm larvae 4% (P)

4-mealworm 1 ng (S)

KN – no reaction product

## Discussion

Insects as an alternative source of food for humans and animals enjoy continuing interest. The commercial availability of insects as food requires reliable identification of the product, which is essential with regard to food safety and building consumer confidence. It is therefore necessary to develop methods for species identification of insects. The more that in the process of producing food products, different components, in this case insects, can be subjected to thermal, mechanical or baric treatment, which can very often make it difficult or impossible to make a determination based on morphological assessment. In this case, DNA-based species identification may be the only way to perform species verification independent of how the sample is processed. In recent years, the species identification of insects in feed and food products has emerged as a developing field of research allowing the detection of insects with just a few methods (Zagon et al., 2018, Marien et al., 2018, Debode et al., 2017).

Most of them are based on analysis of cytochrome oxidase I using Sanger sequencing (Tembe et al., 2014; Siozios et al., 2020; Park et al., 2011). Much of the credit for this belongs to Kim et al. (2019), who elaborated verification methods comparing the obtained insect sequence with NCBI data, which allows distinguishing six insect species. Although scientifically interesting, such methods are less practical when a single species (such as the yellow mealworm) needs to be identified.

The test developed as part of the present study has no such limitations and enables specific identification of this species. Where the potential presence of biological material from mealworm needs to be quickly established, the species specific, sensitive and straightforward test has an advantage over more complex methods.

DNA was isolated using the Sherlock kit with our own modifications, which was a new approach in the extraction of DNA from insects. In the available literature the authors used other kits (Siozios et al., 2020). DNA isolation from insects is generally troublesome due to the presence of chitin shells. Our use of DTT and long heating coupled with prolonged vortexing improved the quality of DNA isolates enough to enable PCR reaction. As shown in the publication, the processed samples showed more favourable parameters but their raw counterparts, mainly mealworm larvae, had a high protein content. Despite these inconveniences, the obtained DNA isolates allowed us to obtain the PCR reaction product for all the samples. Moreover, for repeated DNA isolations we obtained PCR products with similar cycle, which suggests that the applied DNA isolation method is repeatable.

The presented method for identification of insect DNA is effective over a very wide range of DNA content, for both 0.025 ng and 25 ng, i.e. for a sample from 0.1% to 100%. The standard curve parameters further point to species specificity and linearity across the entire range of the method, which is crucial because it enables both qualitative and quantitative determination. Given that falsely positive reactions appear sporadically after the 42nd reaction cycle, the limit of detection can be lowered to 0.01%, which is an attractive result because the existing methods can detect 1% adulterations (Veys et al., 2018).

Our test is effective for processed products and can be used to monitor feeds. Its use for commercial products revealed that mealworm was detected in all the products in which they were declared by the manufacturer, so the qualitative composition of the tested feeds conformed to the declaration.

Development of methods for identification of mealworm DNA is crucial for monitoring feeds that contain mealworm and for detecting potential contaminants in food products with representatives of this species. Our experiment showed that the applied method can be successfully used to identify insects regardless of their biological form and processing degree. The parameters of this method as well its high sensitivity and specificity allow it to detect trace amounts of this species, while the linearity holds promise for quantitative determination of the reaction product. The results are repeatable and the relative standard deviation in all the samples is below 13%.

## Conclusions

- the method of DNA extraction using the Sherlock kit with DTT and extended heating/vortexing time is applicable to a wide range of samples containing insects, regardless of their biological form and processing method
- the presented test allows for quick and effective determination of the species of mealworm on the basis of mtDNA.
- the parameters of the method indicate the high sensitivity of the test, the operating range for 0.01-100% mealworm content, biological specificity and linearity
- the linearity of the test promises to be used for quantification
- the test is effective for processed products and can be used for feed monitoring

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