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Development of a novel non-destructive method based on spectral fingerprint for determination of abused drug in insects: An alternative entomotoxicology approach

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ABSTRACT

Near-infrared spectroscopy (NIRS) is emerging as the tool of choice for non-destructive analysis and the detection of different compounds in biological systems. We evaluate the potential of a novel non-destructive method for the identification of flunitrazepam in *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) larvae, puparia and adult. Necrophagous insects in particular the larvae found in cadavers have been applied as an alternative at the time of death and can also contribute to the qualitative identification of abused drugs present in the corpse. Using this strategy, we have combined a portable NIR spectrometer with variable selection techniques, such as, genetic algorithm–linear discriminant analysis (GA–LDA) and successive projection algorithm (SPA–LDA) for the identification of this drug in the insects based on the unique spectral "fingerprints" of their biochemical composition. Larva, puparium and adult calibrators containing flunitrazepam at concentrations ranging from 0, 4, 8, 16 to 32 pg g^{-1} were prepared and analyzed. The resulting GA–LDA model successfully classified adult female with respect to their concentration using only 9 wavenumbers. This alternative approach for entomotoxicology requires further testing, but the obtained results suggest that NIR spectra could be used for abused drug identification in insects.

1. Introduction

Investigations about the use of arthropods (specifically, arachnids, mites, ticks, scorpions, and spiders) as an alternative toxicological matrix and the effects of many compounds (drugs, metals and pesticides) on insect physiology are associated on forensic entomotoxicology. Investigations on the use of carrion-feeding arthropods as alternative toxicological specimens, and on the impact the tissue toxins and drugs have on the development of immature insects feeding on these substances, currently comprise the major avenues of exploration in the emerging field of entomotoxicology [1].

The major interest of entomotoxicology is the determination of these compounds just before death, mainly in skeletonized remains where no tissue or fluids are left [2]. Alcohol [3], drug antidepressants [4–7], barbiturates [8,9], benzodiazepines [10–12], opioids [13–15], metals [16,17], and pesticides [18,19] are commonly involved in cases where entomotoxicology is investigated. Basically these studies have detailed the detection of toxic substances in different developmental stages of insects, a comparison of sample preparation and analytical procedures for each toxic substance. In these reports, the recovered arthropods have also been generally homogenized and subsequently processed in a manner similar to that for other, more traditional tissues and fluids, or subjected to extraction techniques developed for the analysis of rigorous tissues, such as hair and nails.

The determination of abused drugs in insects is usually performed by gas chromatography–mass spectrometry (GC/MS) [3,12] and liquid chromatography–mass spectrometry (LC/MS) [10,14], coupled to classic extraction techniques such as protein precipitation, liquid–liquid extraction (LLE) or solid phase extraction (SPE). The choice of analytical drug detection/quantification procedures for the analysis of insect tissues depends on the physicochemical properties of the drugs of interest and the required selectivity and sensibility. Although the SPE-chromatography methods present high sensitivity, they present several inherent drawbacks for the analysis of insect tissues, such as invasive and destructive technique, besides the use of bulky instrumentation that impairs in-field monitoring.

Alternatively, the near-infrared spectroscopy (NIRS) can be utilized to determine the insect metabolic fingerprint (lipids, proteins, cellular processes) [20], emerging as an interesting alternative for a rapid and cost-effective identification of living specimens [21–25]. NIRS is also characterized by a minimum of sample handling. It requires no extractions and is non-destructive. The NIR absorption is affected by the

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internal and external biochemical composition of the organism. For example, the composition of the insect cuticle may be different for different species and may change as the insect ages. Rodríguez-Fernández et al. [20] used NIRS to identify nine species of flies in the genus *Neodexiopsis* Malloch (Muscidae, Diptera). The authors concluded that NIRS may provide a new source of data to test and organize hypotheses of species delimitation. Fischnaller et al. [21] tested the usefulness of cuticular chemical profiles collected via the NIRS for discriminating live individuals of *Drosophila obscura* and *Drosophila subobscura* (Diptera). The authors found a classification success for wild-caught specimens of 85%. Ntamatungiro et al. [22] described a series of experiments using laboratory-reared *Anopheles arabiensis* of known chronological age and physiological status, to determine how physiological status impacts the NIR absorption and age prediction. The authors concluded that NIRS cannot be used to determine physiological age and further research is necessary to better understand the factors that influence changes in NIR absorption of the mosquito along their lifetime. In other similar work, Mayagaya et al. [23] evaluated the use of NIRS to determine vector species and age of *A. arabiensis*. The relative age of young or old females was predicted with approximately 80% accuracy. Accordingly, authors described that NIRS offers a valuable alternative to traditional methods such as polymerase chain reaction (PCR) methods. Sikulu et al. [24] evaluated the accuracy of the NIRS as a complementary age grading and species (*A. arabiensis* and *Anopheles gambiae*) identification tool for African malaria vectors. NIRS classified female *A. arabiensis* and *A. gambiae* with 89% ($n = 377$) and 78% ($n = 327$) accuracy, respectively, and differentiated them with 89% ($n = 704$) accuracy. Lastly, Dowell et al. [25] used the NIRS to rapidly and nondestructively determine the age of fresh mosquitoes (*A. gambiae*) stored in RNA*later*. The results from this study show that age can be predicted from mosquitoes preserved in RNA*later* with confidence intervals <1.4 days. However, the metabolic fingerprint generated by NIRS reflects the balance of some factors such as compositional and quantitative differences of biochemical compounds in living specimens.

On the other hand, the use of appropriate chemometric tools for multivariate calibration and classification is largely responsible for the advancement of the NIR technique. These include partial least squares (PLS) [26], principal component regression (PCR) [27], artificial neural networks (ANN) [28] and least squares-vector support machine (LS-SVM) [29]. Moreover, we can include principal component analysis (PCA) for the initial data reduction [30], hierarchical cluster analysis (HCA) for the analysis of groups in a set of data on the basis of spectral similarities [31], and linear discriminant analysis (LDA) for the classification of unknown samples into predetermined groups [32]. However, when employing full spectrum in the construction of these mathematical models, many variables are redundant and/or non-informative, and their inclusion may affect the performance of the final model. A well-succeeded approach to overcome this drawback is the successive projection algorithm (SPA) [33] in conjunction with the linear discriminant analysis (LDA) and genetic algorithm (GA) [34].

Although these studies have shown that calibrations using NIR spectra could be used for wavelength method selection, little research has been directed toward the use of NIRS and variable selection to be used in entomotoxicology. Herein, we have attempted to evaluate the potential of a novel non-destructive method for the identification of flunitrazepam in *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) larvae, puparia and adult. For this, the present paper proposes the NIR spectral region, or the combination of variables, that reflects a specific biochemical feature of flunitrazepam in *C. megacephala* samples. We employed SPA and GA to select an appropriate subset of wavenumbers for LDA. Other goals were the elucidation of the altered variables using different concentrations of flunitrazepam into insects and the identification of the altered biochemical-insect fingerprint. This novel approach can lead to a more selective and specific drug detection for entomotoxicology field. Nevertheless, flunitrazepam was never calibrated by NIRS using wavelength selection to detect in *C. megacephala* (Fabricius) (Diptera: Calliphoridae).

2. Experimental

2.1. Laboratory-reared insects

Larvae were from a stock colony of *C. megacephala*, maintained in an insectarium at 24 ± 2 °C with $70 \pm 5\%$ relative humidity (RH) and a cyclical artificial lighting simulating 16 h daylight and 8 h darkness. About 200 flies were kept in gauze cages (35 cm × 35 cm × 35 cm) and fed ad libitum with water, sugar, powdered milk and brewer's yeast. Larvae were reared on either drug-free artificial food (beef heart) or drug-laden beef heart spiked with flunitrazepam at a concentration of 1 μg/g. Post-feeding larvae were harvested at day 5. Larvae were rinsed thoroughly with deionized water and then dried on absorbent filter paper prior to killing. Larvae were killed by freezing to −20 °C and then stored at this temperature until further analysis. After completion of metamorphosis and emergence of the adult fly, empty puparium cases were harvested for toxicological analyses. These were stored at −20 °C until NIR analysis.

2.2. Calibrators and quality control samples

2.2.1. Larvae

The average weight of one larva was estimated at 80 mg. Distilled water was added to a pool of 50 drug-free larvae to give a final volume of 25 mL. Following homogenization, 0.5-mL aliquots (thus, containing 1 larva) were prepared and either used immediately or stored at −20 °C until use. A series of larva calibrators (0, 4, 8, 16, and 32 pg/g larva) were prepared by spiking the drug-free larva aliquots with flunitrazepam standards.

2.2.2. Puparia and adult

Before pulverization, the average weight of a single puparium was determined to be 4 mg. A pool of 15 puparia was pulverized and then methanol was added to give a final volume of 15 mL. Aliquots of 1 mL (thus, containing one puparium) were prepared and used immediately. A series of puparium calibrators (0, 4, 8, 16, and 32 pg/g puparium) and adult (male and female) calibrators (0, 4, 8, 16, and 32 pg/g adult) were prepared by spiking the pulverized drug-free puparium/adult aliquots with flunitrazepam standards.

2.3. NIR spectroscopy

Each NIR spectrum (8 cm^{-1} spectral resolution, co-added for 32 scans and in triplicate) was directly acquired, in reflectance mode on a miniature scanning Fourier-transform spectrometer from ARCspectro ANIR (Neuchâtel, Switzerland), which is based on a lamellar grating interferometer (35 mm × 35 mm × 65 mm) as used in a micromechanical actuator. The portable NIR device uses an InGaAs photodiode (900 nm to 2500 nm) and the reflected light was directed to the spectrometer via a bundle of optical fiber (model R600-7-VIS-125F, Ocean Optics, USA) which is linked to the probe end and governed via the software ARCspectro ANIR 1.64. Individual animal (larvae, puparia and adult) was manually positioned under the transflectance probe (less than 1 cm and 90° from the surface), and the animal was scanned at a time from two different positions (head and thorax). The average value from two different locations of each sample was properly stored, and the mean spectrum was then calculated for each sample. Sample positioning, data collection, and storage took less than 1 min per animal. The transflectance probe was washed with ethanol (70% v/v) and dried using tissue paper after each sample. Cleanliness of the transflectance probe was verified by collecting an absorbance spectrum of the probe using the most recently collected background as a reference. Spectral measurements were done in an acclimatized room under controlled