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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A B S T R A C T

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 insect in the 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.

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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
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 NIRS 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 NIRM 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 RNAlater. The results from this study show that age can be predicted from mosquitoes preserved in RNAlater 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 ± 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 larval 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/puparium) and adult (male and female) calibrators (0, 4, 8, 16, and 32 pg/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 micro-mechanical 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 transfectance 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 transfectance probe was washed with ethanol (70% v/v) and dried with tissue paper after each sample. Cleanliness of the transfectance 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
temperature of 22 °C, and 60% relative air humidity, and samples were allowed to equilibrate to this temperature before analysis. Fig. 1 shows the experimental arrangement for sampling identification of flunitrazepam in C. megacephala (Fabricius) (Diptera: Calliphoridae) using NIR spectroscopy.

2.4. Chemometrics methods: LDA, SPA–LDA and GA–LDA

LDA is a supervised linear transformation that projects the variables (wavenumbers, for example) into a variable-reduced space which is optimal for discrimination between treatment classes. An LDA seeks for a projection matrix such that Fisher criterion (i.e. the ratio of the between-variance scatter to the within-class variance) is maximized after the projection. The variables created through LDA (factors) are linear combinations of the wavenumber–absorbance intensity values [35]. Thus, the use of LDA for the identification or classification of spectral data generally requires appropriate variable selection procedures [33,36]. In the present study, the SPA and GA were adopted for this function. In the SPA–LDA and GA–LDA models, the validation set was used to guide the variable selection, a strategy to avoid overfitting. The optimum number of variables for SPA–LDA and GA–LDA was determined from the minimum of the cost function $G$ calculated for a given validation data set as:

$$G = \frac{1}{N_v} \sum_{n=1}^{N_v} g_n,$$

(1)

where $g_n$ is defined as

$$g_n = \frac{r^2(x_n, m_{l(n)})}{\min_{l(n) \neq l(n)} r^2(x_n, m_{l(n)})},$$

(2)

where $l(n)$ is the index of the true class for the $n$th validation object $x_n$.

In the GA–LDA model, the mutation and reproduction probabilities were kept constant, 10 and 80%, respectively. The initial population was 120 individuals, with 60 generations. The best solution resulting from the three realizations of the GA was kept.

For this study, LDA scores, loading, and discriminant function (DF) values were derived for the biochemical-insect fingerprint region. The first LDA factor (LD1) was used to visualize the alterations of the insect sample in 1-dimensional (D) score plots that represented the main chemical alterations. SPA–LDA and GA–LDA were used to detect the biochemical alterations relative to the corresponding vehicle control (uncontaminated insect).

2.5. Software

The data import, pre-treatment, and the construction of chemometric classification models (LDA, SPA–LDA and GA–LDA) were implemented in the MATLAB version 6.5 (Math-Works, Natick, USA). Different preprocessing methods were used, including the baseline correction, derivative, smoothing Savitzky–Golay methods by using a first and second-order polynomial, and varying the number of window points (3, 7, 15 and 31). For the SPA–LDA and GA–LDA models, each class was treated separately. The samples were divided into training, validation and test sets by applying the classic Kennard–Stone (KS) uniform sampling algorithm [37] to the NIR spectra. The mean centering was applied to all spectra before performing variable subset selection and calibration. The number of samples in each set is presented in Table 1.

3. Results and discussion

We show that NIRS combined with variable selection techniques (GA–LDA and SPA–LDA) can be employed to the identification of different levels of flunitrazepam in C. megacephala (larva, puparium and adult stages) based on the spectral “fingerprints” of biochemical composition. Although this method has been successfully applied in entomology...
science, this is the first time in which its utility for evaluation on entomotoximology has been demonstrated.

3.1. Larva data set

The raw NIR spectra (larva data set, 25 samples) are shown in Fig. 2a. The averages of duplicate measurements for each sample (head and thorax) were recorded in the region from 900 to 2500 nm. The spectra are highly overlapping and noisy, and solely a visual inspection is difficult to distinguish similar features between the control and treated flies. As larva cuticular layers contain complex mixtures of hydrocarbons, lipids and water content, the interpretation of NIR spectrum is much more complicated. Nevertheless, it was observed that the offset showed some correlation with the amount of water present in the samples; the higher the sample’s water content, the higher its spectral offset. In addition, it is possible to assign some overtones and combination bands evidenced in the spectrum. The peaks at 1000, 1400, 1450, and 1800 nm correspond to water absorption [38]. The peaks at 980, 1050, 1220, 1450, and 1765 nm correspond to molecules assigned of C–H function which are likely caused by absorption of cuticular lipids. Insect cuticular lipids are composed mainly of fatty acids, alcohol, esters, glycerides, sterols, aldehydes, ketones and hydrocarbons [39]. Moreover, flunitrazepam, in turn, is characterized by the second overtone C–H stretching band at 1142 and 1160 nm, first overtone C–H stretching band at 1652, 1664, 1674, 1690, 1718, 1760, 1776 and 1793 nm and combination C–H stretching band at 2216, 2255, 2287, 2327, 2329, 2354, 2362, 2402, 2431, 2458, 2468 and 2488 nm [40].

In addition, consistent baseline offsets and bias are present, as can be seen in Fig. 2a. Noise and systematic behavior are undesirable features in the spectra. These are quite common features in NIR spectra acquired by diffuse reflectance techniques. In our study, as each individual animal was manually positioned under the transfectance probe, the baseline offsets couldn’t have avoided. To solve this, some pre-treatments were applied, such as smoothing (first-order), multiplicative scattering correction (MSC) and first- and second-order derivatives (Savitzky–Golay). The best window for average-smoothing was determined to be that consisting of 31 points, as it gave models with better predictive abilities than those built using other windows. In all cases, the MSC was applied effectively to reduce the offset originally present in the spectra and to remove baseline features. These effects are shown in Fig. 2b.

GA was applied to the larva data set and resulted in the selection of 7 variables, namely 995, 1022, 1066, 1069, 1087, 1265 and 1284 nm, as shown in Fig. 2c. Using the 7 selected wavelengths, the Fisher scores were calculated for all the samples of the data set. Fig. 2d presents the first two (non-standardized) discriminant functions (DF1, DF2) for the overall data set. The coefficients of these functions were calculated by using the training-set statistics (class means and pooled covariance matrix) for the 8 selected variables. As can be seen in Fig. 2d, DF1 × DF2 does not discriminate well among all larva concentration samples, especially for low concentrations (4, 8 and 16 pg g⁻¹). Perhaps low doses of the drug change relatively little inside the larva cuticle when compared with higher doses or perhaps metabolic changes associated with blood feeding are creating larger difference than would be seen in low doses. However, there was a clear segregation between the control and flunitrazepam for 32 pg g⁻¹. These last results that are corroborated by the main biochemical alterations induced by flunitrazepam were on cuticular lipids and water vibrations. Several selected wavenumbers appear to be of particular interest, namely, the variables at 995, 1066

![Fig. 2](image-url)

Fig. 2. (a) Raw absorbance spectra of the biochemical-larva fingerprint regions for NIR spectroscopy; (b) NIR spectra with smoothing Savitzky–Golay using a window of 31 points and multiplicative scattering correction (MSC); (c) NIR spectrum (smoothing Savitzky–Golay using a window of 31 points and multiplicative scattering correction (MSC)). The closed circle indicates the position in the spectra of the 7 wavenumber variables selected by the GA–LDA; (d) DF1 × DF2 discriminant function values calculated by using the variables selected by GA–LDA of the larva data set (control: ●, 4; ▲, 8; ▼, 16; △, 32; ●, pg g⁻¹).
and 1265 nm, representing the C–H groups from insect cuticular and internal lipids. In the SPA–LDA solution some of the wavenumbers selected by SPA are located in regions where no information is apparent. Hence, the inclusion of such variables has an adverse effect on the sensitivity of the LDA model to instrumental noise.

3.2. Puparium data set

The raw NIR spectra (puparium data set, 25 samples) are shown in Fig. 3a. Initially, the best pre-processing strategies were chosen for the spectra to develop the NIR model non-destructively, and the original spectra were transformed by a smoothing (first-order, 31 points) and multiplicative scattering correction (MSC), as can be seen in Fig. 3b.

The spectra also are highly overlapping and noisy, and a visual inspection is difficult to distinguish similar features between the control and treated flies. Fig. 3c and d presents the wavelength selected by using GA–LDA with pre-processing spectra and the first two (non-standardized) discriminant functions (DF1, DF2) for the overall data set. GA was applied to the puparium data set and resulted in the selection of 9 variables, namely 930, 1108, 1319, 1387, 1455, 2232, 2276, 2314 and 2349 nm. As can be seen in Fig. 4c, DF1 × DF2 discriminates some overtones and combination bands evidenced in the spectrum, including the following water bands (1455 nm and 2100–2230 nm). In the SPA–LDA solution some of the wavenumbers selected by SPA are located in regions where no information is apparent when compared by GA–LDA.

3.3. Adult data set

The raw NIR spectra (adult male data set, 25 samples) are shown in Fig. 4a. As performed in the previous models (larvae and puparia), the original spectra were transformed by a smoothing (first-order, 31 points) and multiplicative scattering correction (MSC), as can be seen in Fig. 4b.

The spectra also are highly overlapping and noisy, and a visual inspection is making it difficult to distinguish similar features between the control and treated flies. Fig. 4c and d presents the wavelength selected by using GA–SPA with pre-processing spectra and the first two (non-standardized) discriminant functions (DF1, DF2) for the overall data set. GA was applied to the adult male data set and resulted in the selection of 9 variables, namely 930, 1108, 1319, 1387, 1455, 2232, 2276, 2314 and 2349 nm. As can be seen in Fig. 4d, DF1 × DF2 does not discriminate well among all larva concentration samples, especially for low concentrations (4 and 8 pg g⁻¹). Perhaps male cuticle changes relatively little when compared with females, and thus there was not a good segregation between the control and flunitrazepam for adult male models. However, several selected wavenumbers appear to be of particular interest, namely, the variables at 930 and 1108 nm, representing the C–H groups from insect cuticular and internal lipids, and 2314 and 2349 nm are related to the combination C–H stretching flunitrazepam band. In addition, it is possible to assign some overtones and combination bands evidenced in the spectrum, including the following water bands (1455 nm and 2100–2230 nm). In the SPA–LDA solution some of the wavenumbers selected by SPA are also located in regions where no information is apparent when compared by GA–LDA.

On the other hand, the raw NIR spectra (adult female data set, 25 samples) are shown in Fig. 5a. As performed in the adult male, the original spectra were transformed by a smoothing (first-order, 31 points) and multiplicative scattering correction (MSC), as can be seen in Fig. 5b.
Fig. 4. (a) Raw absorbance spectra of the biochemical-adult male fingerprint regions for NIR spectroscopy; (b) NIR spectra with smoothing Savitzky–Golay using a window of 31 points and multiplicative scattering correction (MSC); (c) NIR spectrum (smoothing Savitzky–Golay using a window of 31 points and multiplicative scattering correction (MSC)). The closed circle indicates the position in the spectra of the 9 wavenumber variables selected by the GA–LDA; (d) DF1 × DF2 discriminant function values calculated by using the variables selected by GA–LDA of the larva data set (control: ●, 4: ▲, 8: , 16: ►, 32: ▽, pg g⁻¹).

Fig. 5. (a) Raw absorbance spectra of the biochemical-adult female fingerprint regions for NIR spectroscopy; (b) NIR spectra with smoothing Savitzky–Golay using a window of 31 points and multiplicative scattering correction (MSC); (c) NIR spectrum (smoothing Savitzky–Golay using a window of 31 points and multiplicative scattering correction (MSC)). The closed circle indicates the position in the spectra of the 9 wavenumber variables selected by the GA–LDA; (d) DF1 × DF2 discriminant function values calculated by using the variables selected by GA–LDA of the larva data set (control: ●, 4: ▲, 8: , 16: ►, 32: ▽, pg g⁻¹).
GA and SPA were applied to the adult female data set. The GA resulted in the selection of 9 variables, namely 901, 916, 965, 1369, 1451, 1931, 2332, 2427 and 2494 nm, as shown in Fig. 5c. Using solely 9 selected wavelengths, the Fisher scores were calculated for all the samples of the data set, as shown in Fig. 5d. As can be seen in Fig. 5d, there is a greater effect of homogeneity among classes, being obtained no misclassification, using only the 9 wavenumbers selected by GA in the LDA modeling. Examination of the selected wavenumbers following GA–LDA (Fig. 5d) indicated that the main biochemical alterations induced by *C. megacephala* were on cuticular lipids (901, 916 and 965 nm). Several selected wavenumbers appear to be of particular interest, namely, the variables at 1451, 2332, 2427 and 2494 nm, representing the combination C–H stretching flunitrazepam band (fingerprint region). These findings suggest that NIR is a very promising technique for the non-destructive identification of flunitrazepam in *C. megacephala*.

4. Conclusions

In conclusion, the joint use of NIRs and discrimination analysis is a powerful means for the identification of flunitrazepam in *C. megacephala* (Fabricius) (Diptera: Calliphoridae) larva, puparium and adult stages. This study proposed to evaluate the potential of a novel non-destructive method for the identification of flunitrazepam as an alternative approach for entomotoxicology field, employing portable NIR spectrometry and LDA analysis coupled with the GA for wavenumber selection. Variable selection techniques (SPA and GA) using LDA were subsequently performed in an attempt to gain more information regarding potential differences among the flunitrazepam concentrations from the NIR spectra. In a case study involving 4 different concentrations and one control insect sample, the resulting GA–LDA model successfully detected the biochemical alterations for the insect (larvae, puparia and adult) using 9 wavenumbers as maximum. Although the determination of abused drugs in insects is usually provided by gold standard (SPE–HPLC) methods, the processing time and reagents costs required further alternative approaches in entomologic surveys. In contrast, the NIR technique described can handle a much larger volume of samples, is nondestructive and becomes increasingly cost-effective as the number of samples for analysis increases. While these results are encouraging, much larger databases of vibrational spectra of a wider range of samples, is nondestructive and becomes increasingly cost-effective as the number of samples for analysis increases. While these results are encouraging, much larger databases of vibrational spectra of a wider range of insects by immunohistochemistry in dipteran larvae of forensic importance, J. Forensic Med. Pathol. 21 (2000) 59–61.


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