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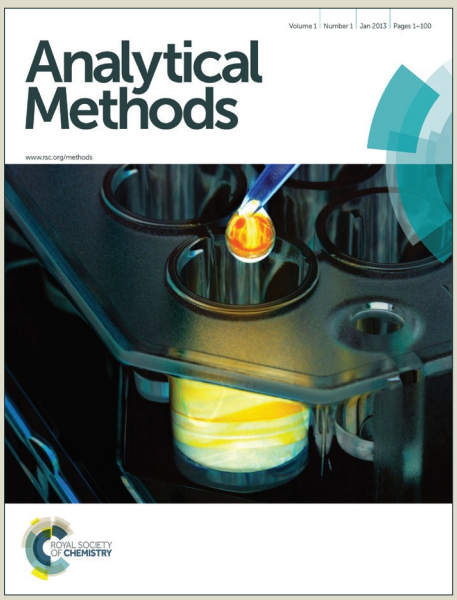

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FTIR microspectroscopy coupled with variable selection methods for the identification of flunitrazepan in necrophageous flies

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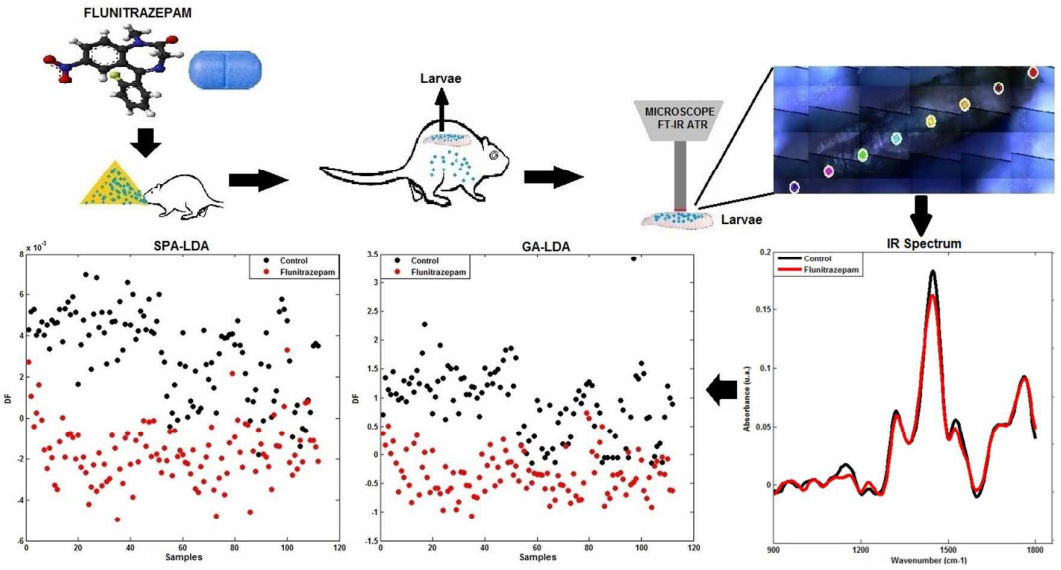

Abstract: The detection and identification of a drug in a corpse through the analysis of fly larvae feeding on the body by spectroscopic techniques promises to be of a great value, because of their sensitivity, promptness, low costs and simplicity. Therefore, the purpose of this study was to develop a method based on Fourier-transform infrared (FTIR) microscopy to identify and discriminate flunitrazepan in necrophagous flies (*Chrysomya megacephala, Chrysomya albicepse* and *Cochliomyia macellaria*) as a non-invasive and non-destructive technique. Thirty-two Winstar mice were divided into two groups of sixteen and supplemented in two categories: group 1 – ethanol, and group 2 – standard flunitrazepan at the dose of 2 mg kg^{-1}. Spectra from the larvae samples were analysed by principal component analysis–linear discriminant analysis (PCA–LDA), and variable selection techniques such as successive projection algorithm (SPA–LDA) and genetic algorithm (GA–LDA) to determine if control versus flunitrazepan could be segregated. In addition, the multivariate classification accuracy results were tested based on sensitivity, specificity, positive (or precision) and negative predictive values, Youden index, positive and negative likelihood ratios. For control vs. flunitrazepan category, the sensitivity and specificity levels, using 46 wavenumbers by SPA–LDA gave relatively good accuracy (up to 82.3% control vs. flunitrazepan). The resulting GA–LDA model also successfully classified both classes with respect to the main biochemical alterations induced by flunitrazepan using only 40 wavenumbers (up to 88.2% control vs. flunitrazepan). Compared to classical methods, this new approach could represent an alternative and an innovative tool for faster and cheaper evaluation in entomotoxicology.

Key-Words: Fourier-transform infrared microspectroscopy; flunitrazepan; Chrysomya megacephala, Chrysomya albicepse; Cochliomyia macellaria; Classification analysis

Introduction

Entomotoxicology ranks among the newest branch of the forensic entomology that deals with the identification and quantitation of drugs and other toxins in carrion-feeding arthropods in decomposing tissues, and the study of drug-induced changes in arthropod growth with respects to the estimate of the post-mortem interval (PMI) by entomological methods.[1,2] The use of necrophageous species as a matrix for qualitative compound (drugs[3,4], metals[5,6] and pesticides[7,8]) detection has been detected in insect tissues and generally accepted by forensic toxicologists. Although there has been recent progress in the detection of toxic substances in intact insects, there are some limitations such as insufficient knowledge of insect development and activity, proper use and validation of analytical procedures and lack of a general consensus concerning experimental set-up and sampling.

Several analytical drug detection/quantification procedures have been used for the analysis of insect tissues. These include radioimmunoassay (RIA)[9,10], gas chromatography/mass spectrometry (GC/MS)[11,12], and high performance liquid chromatography-mass spectrometry (HPLC-MS)[13,14], coupled with classic extraction techniques such as protein precipitation, liquid-liquid extraction (LLE) or solid phase extraction (SPE). Although these techniques carried out in entomotoxicological reports are expensive, invasive, destructive, involve numerical preparation steps and most of the time they require pools of specimens to detect any present drug. As a consequence, there has been increased interest in the use of alternative of new methods for detection and identification of a drug being present in a corpse.

The detection and identification of a drug present in a corpse through the analysis of fly larvae feeding on the body by spectroscopic techniques promises to be of great value, because of their sensitivity, promptness, low costs and simplicity. Recently,

we have used Near-infrared spectroscopy (NIRS) as a novel non-destructive method for the identification of flunitrazepan in *Chrysomya megacephala* (Fabricius) (Diptera: Calliphoridae) larvae, puparia and adults,[15] with the resulting study successfully detecting the biochemical alterations for the insect. NIRS is characterized by broad overlapping spectral peaks produced by the overtones and combination of infrared vibrational modes. While these results are encouraging, other databases of vibrational spectra for entomotoxicological results must be established. Fourier-transform infrared (FTIR) spectroscopy is one technique with potential applications in the field of entomotoxicology. FTIR has the ability to rapidly generate a "biochemical cell-fingerprint" of the material under analysis.[16] IR is also characterized by a minimum of sample handling, requiring no extractions and is non-destructive.[17] Unlike conventional techniques used in the analysis of insect tissues, IR yields a precise image of all the chemical bonds present in the sample and offers the opportunity to very quickly observe all metabolic modifications induced by a specific compound.[18] In this context, it could be interesting to develop a strategy based on infrared spectroscopy for detection and identification of a drug in a corpse.

On the other hand, the use of appropriate chemometric tools for multivariate calibration and classification is largely responsible for advancing spectroscopic techniques, for instance, IR and NIR. Computational approaches [e.g., principal component analysis (PCA)[19], linear discriminant analysis (LDA)[20], genetic algorithm[21] and successive projections algorithm (SPA)[22]] permit the processing of large amounts of spectroscopic data variables that subsequently require data reduction approaches in order to identify sources of variance across spectra and inter-class variation to be identified.

Not only is the choice and development of the computational approaches is important to ensure reliable drug detection and quantification using spectroscopic techniques; multivariate classification quality features such as sensitivity, specificity, positive (or precision) and negative predictive values, Youden index, and positive and negative likelihood ratios should be calculated to ensure the validity of the results in accordance with International guidelines.[23] When reviewing entomotoxicological publications, the methods tend to lack proper validation.

Herein, we have attempted to evaluate the potential of a novel non-destructive method based on attenuated total reflection-FTIR (ATR) microspectroscopy for identification of flunitrazepan in 32 larvae. Flunitrazepam is the N-methyl-2'-fluoro-analogue of nitrazepam and is available in a number of western European countries for use as a hypnotic (Rohypnol and Noriel® and anesthetic (Narcozep® agent). The detection of flunitrazepan, the most frequently abused pharmaceutical drug in the world, in necrophagous flies (*Chrysomya megacephala, Chrysomya albicep*s and *Cochliomyia macellaria*) as a non-invasive and non-destructive technique does not appear to be well-documented. In our study, sample preparation, spectroscopic measurement, data preprocessing, feature selection and analytical validation were addressed. To our knowledge, there is no reported use of FTIR microscopy for the detection and identification of a drug being present in a corpse.

Materials and Methods

Thirty-two Winstar mice (*Rattus norvegicus*) with an average weight of 300 g were divided into two groups of sixteen and supplemented in the following way: group 1 – ethanol, and group 2 – standard flunitrazepan at the dose of 2 mg kg^{-1}. One hour

after the supplementation, the mice were sacrificed, individually housed in fly traps and distributed at eight points along a track of a nearby forest. In the third and fourth days, 10 larvae were collected from each mouse and analyzed with ATR microspectroscopy. The average weight of one larva was estimated at 80 mg. All experiments were performed in compliance with the relevant laws and institutional guidelines, where the Ethics committee of the Federal University of Rio Grande do Norte (UFRN/Brazil) have approved the experiments [Research and Ethics Committee (REC) approval no.: 044/2013].

IR spectra [*n*= 264, 32 larvae (*Chrysomya megacephala, Chrysomya albiceps*e and *Cochliomyia macellaria*) and eight random points] were collected from larva (placing larva individually on their backs on the plate) using the Bruker Lumus FTIR spectrometer with motorized ATR crystal (Bruker Optics Ltd, Coventry, U.K.). Prior to analysing each specimen, the diamond crystal within the spectrometer was washed and a background spectrum was obtained to account for atmospheric composition.

The data import, pre-treatment and construction of chemometric classification models (PCA-LDA, SPA–LDA and GA–LDA) were implemented in MATLAB R2014a software (http://www.mathworks.com). Raw spectra were pre-processed by cutting between 1800 and 900 cm^{-1} (235 wavenumbers at 3.84 cm^{-1} spectral resolution) and baseline-corrected. For PCA-LDA, SPA–LDA and GA–LDA models, the samples were divided into training (70%), validation (15%) and prediction sets (15%) by applying the classic Kennard–Stone (KS) uniform sampling algorithm to the IR spectra. The KS algorithm was applied separately to each class for extract a representative set of objects from a given dataset by maximizing the minimal Euclidean distance between already selected objects and the remaining objects. The training samples were used in the modelling procedure (including variable selection for LDA), whereas the prediction

set was only used in the final evaluation of the classification. The optimum number of variables for SPA–LDA and GA–LDA was performed with an average risk G of LDA misclassification. Such a cost function is calculated in the validation set as:

$$G = \frac{1}{N_V}\sum_{n=1}^{N_V} g_{n,} \quad (1)$$

where g_n is defined as

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

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

In this definition, the numerator is the squared Mahalanobis distance between object x_n (of class index I_n) and the sample mean $m_{I(n)}$ of its true class. The denominator in Eq. (2) corresponds to the squared Mahalanobis distance between object x_n and the center of the closest wrong class.

The GA routine was carried out during 40 generations with 80 chromosomes each. Crossover and mutation probabilities were set to 60% and 10%, respectively. Moreover, the algorithm was repeated three times, starting from different random initial populations. The best solution (in terms of the fitness value) resulting from the three realizations of the GA was employed. For this study, LDA scores, loadings, and discriminant function (DF) values were obtained for the specimen.

Sensitivity (the confidence in a positive result for a sample of the label class is obtained), specificity (the confidence that a negative result for a sample of non-label class is obtained), Positive predictive value (PPV) (measures the proportion of correctly assigned positive examples and its value varies between 0 and 1), Negative predictive value (NPV) (measures the proportion of correctly assigned negative examples and its

value varies between 0 and 1), Youden's index (YOU) (evaluates the classifier's ability to avoid failure), The likelihood ratios (LR+) (represents the ratio between the probability to predict an example as positive when it truly is positive, and the probability to predict an example as positive when it actually is not positive) (LR-) (represents the ratio between the probabilities to predict an example as negative when it is actually positive, and the probability to predict an example as negative when it truly is negative) were calculated as important quality standards in test evaluation. The quality metrics used in this study for evaluating the classification results can be calculated following the equations:

$$\text{Sensibility (\%)} = \frac{TP}{TP + FN} x100 \quad (3)$$

$$\text{Specificity (\%)} = \frac{TN}{TN + FP} x100 \quad (4)$$

$$\text{PPV} = \frac{TP}{TP + FP} \quad (5)$$

$$\text{NPV} = \frac{TN}{TN + FN} \quad (6)$$

$$\text{YOU} = \text{SENS} - (1 - \text{SPEC}) \quad (7)$$

$$\text{LR}(+) = \frac{SENS}{1 - SPEC} \quad (8)$$

$$\text{LR}(-) = \frac{1 - SENS}{SPEC} \quad (9)$$

where FN is defined as false negative, FP as false positive, TP is defined as true positive and TN is defined as true negative. SENS is defined as sensibility and SPEC as specificity.

Results and discussion

In total, n= 264 spectra were acquired. The average IR spectra for each original class [control, black line; flunitrazepan, red line] in the range of 900–1800 cm^{-1} after baseline correction is shown in Fig. 1. As can be seen, discriminating between the two categories of specimens on the basis of IR measurements is not straightforward, owing to the complexity of the spectra. Although all spectra had a similar shape in the region of 1150–1190 cm^{-1}, 1470–1490 cm^{-1} and 1505-1520 cm^{-1}, the spectra were shifted downwards.

[Insert Fig. 1 here]

Distinguishing these categories only by spectral observation is difficult, so to identify markers, it is necessary to apply computation analysis [principal component analysis–linear discriminant analysis (PCA–LDA), and variable selection techniques such as successive projection algorithm (SPA–LDA) and genetic algorithm (GA–LDA)]. The optimum number of PCs for PCA and variables for SPA–LDA and GA–LDA was determined by power versus cost calculation using the minimum cost function G. These were adopted to systematically classify normal vs. flunitrazepan based on IR spectra. Further, comparisons were made between rates, interpretability and training times.

Fig. 2 is a 2-D PCA–LDA Fisher scores plot of the derived spectral points from each category. We can see that the Fisher scores do not show good segregation. The PCA–LDA model was built using five PC (93% variance in the data).

[Insert Figure 2 here]

Then, SPA–LDA and GA–LDA were applied to the dataset to obtain the optimum number of variables by the minimum cost function G. Fisher scores for both models (SPA and GA) were obtained and this improved segregation between categories when compared with PCA–LDA.

The SPA–LDA resulted in the selection of 46 variables (Table 1). Using solely 46 selected wavenumbers, the Fisher scores were calculated for both categories of the data set, as shown in Fig. 3. As can be seen in Fig. 3, there is a greater effect of homogeneity among classes, with a little misclassification being obtained. Examination of the selected wavenumbers following SPA–LDA showed that the main biochemical alterations discriminating control vs. flunitrazepan were lipids, proteins, nucleic acids, carbohydrates and, to a lesser extent, DNA vibrations. Several selected wavenumbers appear to be of particular interest, namely, the variables at 1315 cm^{-1}, 1389 cm^{-1}, 1505 cm^{-1} and 1550 cm^{-1}, associated with amide III of proteins, COO- symmetric stretch in fatty, amide II of proteins and C–O stretching predominantly a-sheet of Amide II, respectively.

[Insert Figure 3 here]

[Insert Table 1 here]

GA–LDA was applied to the dataset (control vs. flunitrazepan) and resulted in the selection of 40 variables (Table 1). Figure 4 is a scores plot that shows that GA–LDA generates best segregation between the two categories. Several selected wavenumbers (GA–LDA) appear to be of particular interest, namely, the variables at

1334 cm^{-1} and 1527 cm, representing the Amide III from proteins and CH bending and CH2 wagging, respectively. These findings suggest that FTIR microscopy is a very promising technique for the non-destructive identification of flunitrazepan in C. *Chrysomya megacephala, Chrysomya albicep*s*e* and *Cochliomyia macellaria* specimens. In addition, this finding is significant due to its potential for translation into entomotoxicology practice. Currently, no spectroscopic techniques are the gold standard in the detection procedures for the analysis of intact insects for toxicology decision-making.

Classification rates were carried out by using the best models. Table 2 presents the validation results for the optimized model (PCA–LDA, SPA–LDA and GA–LDA) of each category. According to results of sensitivity shown in Table 2, it is possible to see that the sensitivity rate from PCA–LDA, SPA–LDA and GA–LDA achieved scores of 64.7%, 70.5% and 64.7% for the control category, respectively, showing that the control category can be relatively classified by these methods. For the flunitrazepan category, the sensitivity values from PCA–LDA, SPA–LDA and GA–LDA models were 64.7%, 82.3% and 88.2%, respectively. Furthermore, the specificity as shown in Table 2 for both categories suggests that following SPA–LDA and GA–SPA, an improved accuracy in comparison with PCA–LDA was obtained. In general, distinguishing between the normal and flunitrazepan categories was more successful when using GA–LDA, demonstrating that ATR-FTIR microspectroscopy in conjunction with powerful chemometric approaches has the potential to detect and identify drugs present in a corpse.

[Insert Table 2 here]

Conclusions

The results of this study show that ATR–FTIR microspectroscopy coupled with variable selected techniques (SPA or GA) on necrophageous flies may be an alternative approach for the detection and identification of flunitrazepan. We report a fast, clean and non-destructive methodology involving minimal sample preparation to categorize the specimens. In a case study for flunitrazepan samples, the resulting GA–LDA model successfully detected the biochemical alteration based on 40 wavenumbers and produced 82.3% and 88.2% sensitivity and specificity accuracy. For this dataset investigated, these selected wavenumbers (SPA-LDA and GA-LDA) appear to be of particular interest for the detection and identification of flunitrazepan. This is required to robustly validate the classification and biomarker extraction models for this necrophageous flies and identification of flunitrazepan. Although the determination of abused drugs in insects is usually provided by gold standard (SPE-chromatography methods), the proposed methodology can be applied to new drugs or necrophageous where the processing time and reagent costs required are a major advantage. This method was thoroughly validated in accordance with international guidelines, being considered suitable for use as an official methodology for entomological methods. Further validation of these approaches exploiting other biospectroscopy techniques and using larger and architecturally more robust datasets is required.

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Captions for Figure

Fig. 1: Average spectrum for each original class (control, black line; flunitrazepan, red line).

Fig. 2: Discriminant function x samples calculated by using the PCA–LDA model from two categories (control and flunitrazepan).

Fig. 3: Discriminant function x samples calculated by SPA–LDA model from two categories (control and flunitrazepan) using 46 wavenumbers selected.

Fig. 4: Discriminant function x samples calculated by GA–LDA model from two categories (control and flunitrazepan) using 40 wavenumbers selected.

Legends for Tables

Table 1: Variables for SPA–LDA and GA–LDA determined from the minimum cost function G used to achieve classification of control and flunitrazepan for a given validation dataset.

Table 2: Values of quality performance features from three classification methods (PCA–LDA, SPA–LDA and GA–LDA) by FTIR microspectroscopy of each category.

Figures

Fig. 1

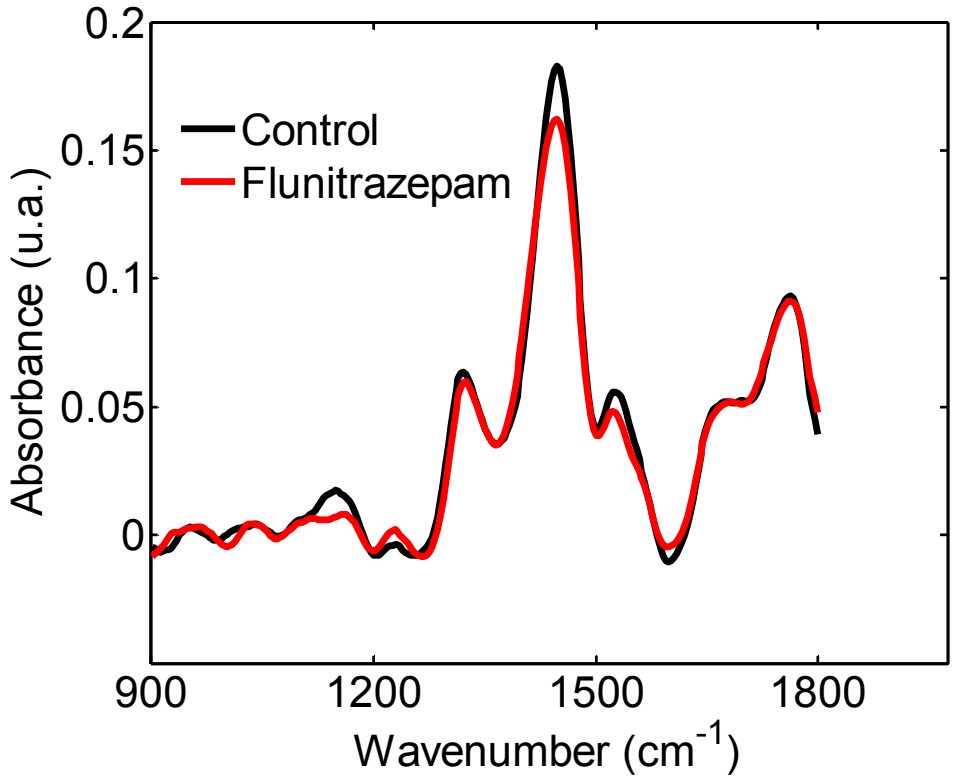


Fig. 2

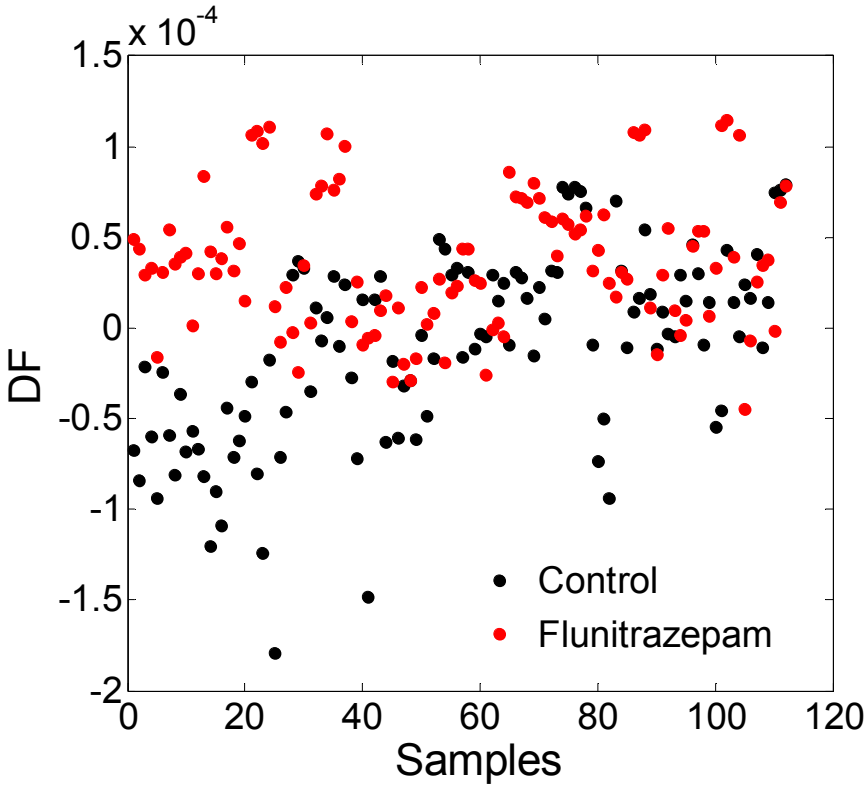


Fig. 3

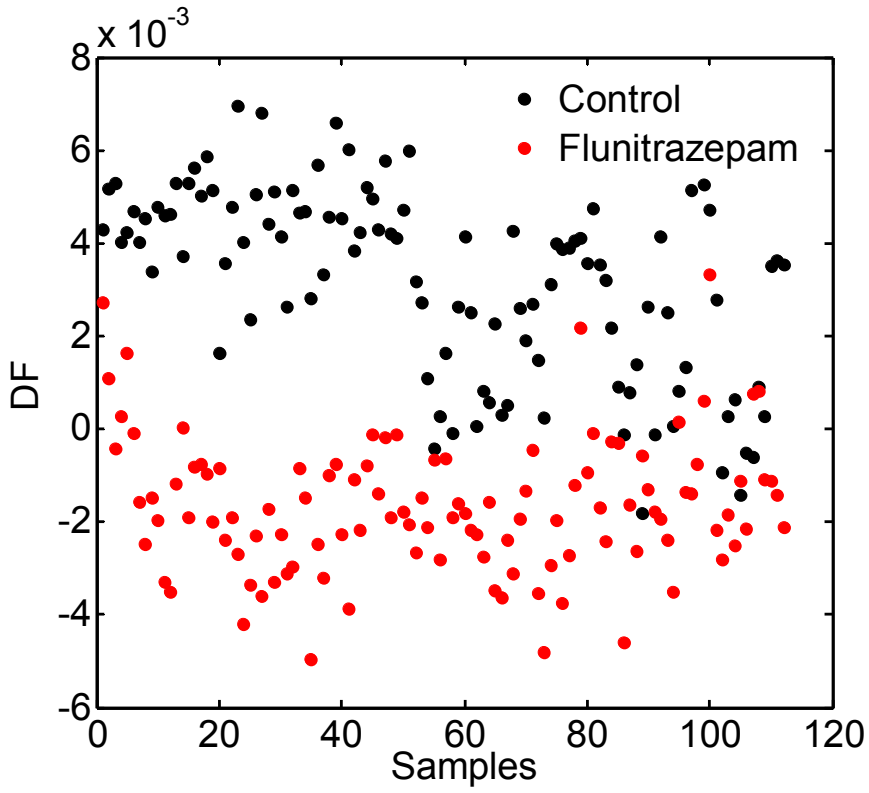
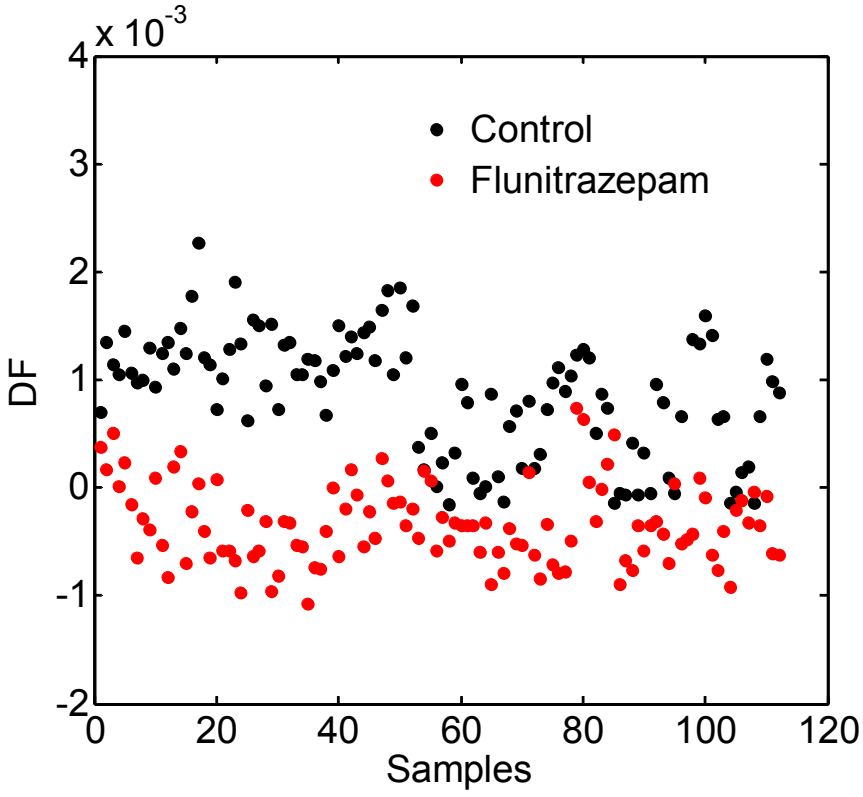


Fig. 4



Tables

Table 1

Chemometric analysis	Wavenumber (cm^{-1}) selected
SPA-LDA	1300 1304 1308 1315 1323 1327 1331 1339 1345 1350 1355 1361 1367 1373 1378 1382 1389 1396 1402 1409 1415 1420 1425 1430 1434 1441 1445 1449 1454 1458 1462 1467 1473 1478 1486 1492 1499 1505 1510 1517 1522 1527 1534 1540 1545 1550
GA-LDA	1304 1318 1319 1326 1327 1329 1331 1334 1335 1342 1345 1352 1356 1369 1371 1382 1386 1390 1391 1395 1403 1405 1421 1439 1441 1445 1447 1448 1450 1451 1471 1472 1477 1484 1486 1488 1500 1501 1505 1527

Table 2

Stage performance features	PCA-LDA	SPA-LDA	GA-LDA
Control			
Sensitivity (%)	64.7	70.5	64.7
Specificity (%)	76.4	70.5	64.7
Positive predictive values (PPV)	73.3	70.5	64.7
Negative predictive values (NPV)	68.4	70.5	64.7
Youden index (YOU)	41.1	41.1	29.4
Positive likelihood ratios (LR+)	2.7	2.4	1.8
Negative likelihood ratios (LR–)	0.4	0.4	0.5

Flunitrazepan			
Sensitivity (%)	64.7	82.3	88.2
Specificity (%)	64.7	82.3	88.2
Positive predictive values (PPV)	64.7	82.3	88.2
Negative predictive values (NPV)	64.7	82.3	88.2
Youden index (YOU)	29.4	64.7	76.4
Positive likelihood ratios (LR+)	1.8	4.6	7.5
Negative likelihood ratios (LR−)	0.5	0.2	0.1