PRELIMINARY STUDY FOR SIMULTANEOUS DETECTION AND QUANTIFICATION OF ANABOLIC ANDROGENIC STEROIDS USING ELISA AND PATTERN RECOGNITION TECHNIQUES

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Abstract

A first step towards the multidetection, identification and quantification of anabolic androgenic steroids by Enzyme-linked immunosorbent assays (ELISA) has been performed in this study. This proposal combines multiple competitive ELISA assays with different cross-reactivities profiles and multivariate data analysis techniques. Data has been analyzed by principal component analysis in conjunction with a novel k-nearest line classifier. This proposal allows to detect simultaneously up to four different steroids in the range of concentration from 0.1 to 316.2 nM with a total rate of 90.6% of correct detection, even in the presence of cross-reactivities. A methodology for concentration prediction is also presented with satisfactory results.

Keywords: ELISA, anabolic androgenic steroid, multidetection, quantification, pattern recognition, k-nearlest line

Introduction

Anabolic androgenic steroids (AAS) are illegally used as growth promoters in cattle, to increase the performance in athletes and in horse racing[1]. The use of substances having a hormonal action for growth promotion in farm animals was prohibited in 1981 by the European Union (Directive 81/602/EEC). With regard to human sports, every year, the World Antidoping Agency (WADA) publishes a list of prohibited drugs[2], where anabolic steroids are included in the S1 group.

As consequence of the illegal use of the AAS the development of new methodologies for detecting these compounds becomes indispensable in our society. The immunochemical methods provide an accurate measurement with fast results and they can measure simultaneously a large number of samples[3, 4], in consequence, there is a decrease in the price per analysis. One of the most widely used immunochemical techniques is the enzyme-linked immunosorbent assay (ELISA). ELISAs are based on the specific recognition of antigens by specific antibodies; when the antigen is a small molecular weight molecule, like AAS, a competitive format is used. Work with immunoassays helps to reduce the sample preparation time, because matrix effects which usually appear with other type of sensors can be avoided by high dilutions of the sample[5]. In contrast, the classical techniques based on chromatography coupled to mass spectrometry, GC-MS or LC-MS[6-10], used as confirmatory methods for the detection and identification of this compounds, are more time consuming, because a more complex sample treatment is needed, and are more expensive.

However, the ELISA technique has few disadvantages. Its main problem is the cross-reactivity that appears in the determination of compounds with a very similar structure, like AAS. The fact that one assay can detect several compounds makes difficult to discriminate which is the analyte present in the sample. However, since different assays

may show different sensitivities to different compounds the use of an array of assays in conjunction with multivariate data analysis open new doors for the determination and identification of compounds of the same family. In this point, multivariate data analysis can help us to improve the system selectivity and also to perform the multidetection approach. A multianalyte assay brings us advantages such as simplicity, faster measurements, and lower costs compared to perform each assay individually. There are some classical approximations to perform the multidetection using immunoassays techniques[11], but in general these methods avoid the cross-reactivity recognition with a previous optimization of the immunoreagents concentrations. Other types of multidetection strategies are forced to use very specific immunosensors to perform the multidetection with a sequential analysis, a spatially separation of immunosensors[12] or using multiple labels. But in any case, these classical methods of multidetection use a single calibration curve for each analyte.

The multivariate data analysis techniques are usually used for solving problems in the selectivity (due to cross-recognition effects)[13], as well as, to perform multidetection using high dimensionality data[14, 15] (for instance multiple ELISAs). This strategy, that is well-known in artificial olfaction[16] and electronic tongues[17], uses an unique mathematical model to detect and/or quantify various analytes simultaneously. This approach usually improves the possibilities of single calibrations[13], because the effect of the cross-reactivity recognition is counteracted in the final mathematical model. In fact, it has been already reported for analysing mixtures of two pesticides using immunosensors[18, 19].

The development and cross-reactivity characterization of different ELISAs for 24 different AAS has been performed to achieve the final aim of this work, which is to

combine some ELISA assays and multivariate data analysis techniques to perform AAS multidetection and quantification.

Regarding data evaluation, here we use a novel pattern classifier. Most of the classical pattern recognition methods assume Gaussian clusters, where sample scatter is due to perturbances and experimental errors. In the current scenario, every class correspond to an analyte, and the data processing intends to recognise the correct class for a large range of concentrations; in these cases, and in particular for non-linear sensors, the pattern dispersion due to potential variations in concentration of the analyte in the sample under analysis dominates other sources of dispersion and the clusters become elongated and in many occasions curvilinear. If the clusters remain linear, every class can be represented by a different subspace. This is the strategy behind SIMCA classifiers[20]. However, for curvilinear clusters this strategy fails. This problem has been already considered by A. Ortega et al.[21]. The authors proposed here the use of Self-Organizing Maps followed by a pruning sterp to build a Minimum Spaning Tree. Here in this work, and following the same ideas, a simplified version is presented. It is an extension of the well-known K-Nearest Neighbour where the prototypes are now lines. This method can be called as K-Nearest Line (K-NL) classifier in analogy.

Experimental

Materials

(a) ELISA: Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Washing steps were carried out using a SLY96 PW microplate washer (SLT Labinstruments GmbH, Salzburg, Austria). Absorbances were read using a SpectramaxPlus (Molecular Devices, Sunnyvale, CA) at a single wavelength mode of 450 nm. The competitive curves were analyzed with a four-parameter equation using

the software SoftmaxPro v2.7 (Molecular Devices) and GraphPad Prism v4 (GraphPad Software Inc., San Diego, CA). Unless otherwise indicated, data presented correspond to the average of at least two well replicates. Immunochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). The immunoreagents for AAS were produced in the laboratory, and were described before. For the detection of St (**8**BSA, **5**BSA and As147)[22, 23], for THG (hG-BSA and As170)[24] and the immunoreagents employed in this work for the detection of B (**13**BSA and As138) and MB (**15**BSA, **12**BSA, **14**BSA and As140, As142, 143) will be described elsewhere.

(b) Buffers: PBS is 10 mM phosphate buffer 0.8% saline solution, and unless otherwise indicated the pH is 7.5. Coating buffer is 50 mM carbonate-bicarbonate buffer pH 9.6. PBST is PBS with 0.05% Tween 20. Citrate buffer is a 40 mM solution of sodium citrate pH 5.5. The substrate solution contains 0.01% TMB (tetramethylbenzidine) and 0.004% H_2O_2 in citrate buffer.

Analytes

Stanozolol (St), Boldenone (B), a-Boldenone (a-B), Methylboldenone (MB), Androstandiendione (ADD), Methyltestosterone (MT), 19-nortestosterone (NT), Progesterone (P), Testosterone (T), Androstandione (A1), Pregnenolone (Preg), Cholesterol (Ch), Dihydrotestosterone (DHT), Estrone (E1), Dexamethasone (D21P), Norstanozolol (Norst), 16b-hydroxystanozolol (16OH), 3'hydroxystanozolol (3OH), Trenbolone (Tr), Estradiol (E2), Ethynylestradiol (EES), Tetrahydrogestrinone (THG), Gestrinone (G) and Norethandrolone (NEth).

Stanozolol, Boldenone, Methylboldenone, Testosterone and Gestrinone were purchased from Sequoia Research Products, Ltd. (Oxford, UK). Other chemical reagents were

purchased from Aldrich Chemical Co. (Milwaukee, WI). Tetrahydrogestrinone was synthesized in the laboratory[24].

ELISA assay

The ELISAs employed in this work have been optimized, demonstrated and published its applicability before, for St[22, 23] and THG[24]. In the case of the assays for the determination of B and MB will be published elsewhere.

(a) General procedure: Microtiter plates were coated with the coating antigens, BSA conjugates, (in coating buffer; 100 μL/well in all cases) overnight at 4 °C. The following day, the plates were washed four times with PBST, and the standard curves prepared (0.064 nM-1000 nM different AAS) were added to the microtiter plates (in PBST; 50 μL/well) followed by the corresponding dilution of antiserum, previously optimized in the laboratory (in PBST; 50 μL/well) and incubated for 30 min at RT [22-24]. The plates were washed again as before, and a solution of anti-IgG-HRP (1/6000 in PBST) was added (100 μL/well) and incubated for 30 min more at RT. After a new washing step, the substrate solution was added (100 μL/well) and the enzymatic reaction was stopped after 30 min at RT with 4N H_2SO_4 (50 μL/well). The absorbances were read at 450 nm. The standard curves were fitted to a four parameter equation according to the following formula:

$$Y = \left[\frac{(A - B)}{1} - \left(\frac{x}{C} \right)^{D} \right] + B \qquad (1)$$

where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximum absorbance (or IC_{50}), and D is the slope at the inflection point of the sigmoid curve. The limit of detection (LOD) for a single

ELISA is defined as the concentration that produces 90% of the maximum absorbance (IC_{90}).

(b) Cross-reactivity characterization: Stock solutions of different steroidal compounds were prepared (10 mM in dimethyl sulfoxide) and stored at 4ºC. Standard calibration curves for each one were prepared by serial dilutions in PBST and measured with the different ELISAs (same procedure as above). The IC_{50} were determined in the competitive experiments as described above and the cross-reactivity values were calculated according to the following equation:

$$CR = \left[\frac{IC_{50(\mathrm{Re}\,ference\_assay)}}{IC_{50(AAS\_tested)}} \right] \cdot 100 \qquad (2)$$

Dataset description and statistical methods

The pattern recognition proposed in this work has basically three steps: (i) preprocessing, (ii) dimensionality reduction, (iii) classifier. This section gives a short introduction to the techniques used. In this work, a modified version of k-NN[25] is presented, specially designed to operate with cluster elongation due to concentration scatter. A description of the procedure is given in this section.

(a) Description of data set: Two cases with different number of ELISAs and analytes were selected in order to study the possibilities of multidetection. The first case consists on use all eight previously characterized ELISA assays. Four analytes (DHT, MB, P and St) at 8 concentrations from 0.03 to 1000nM were measured twice with these immunoassays. Thus, there were 64 assays in the first case (4 analytes x 8 ELISAs x 2 replicates). In the second case, only 4 ELISAs (As138/**13**BSA, As140/**15**BSA, As142/**15**BSA and As147/**8**BSA) were used for measuring up to 7 AAS, also twice at 8

concentrations from 0.03 to 1000nM. In this case there were 56 assays (7 analytes x 4 ELISAs x 2 replicates).

(b) Data preprocessing: First, the mean of each pair of replicates in the training set was performed. In order to build lines that follow the expected evolution of the patterns due to concentration changes, synthetic samples were obtained by linear interpolation within the available training data. The interpolated patterns correspond to concentrations from 0.03 to 1000 nM with increments of 0.1 (in logarithm units of nM). Thus, there were 46 synthetic samples per each ELISA assay (that included the original concentrations).

Next, the Logit transformation was applied to linearize the response[26]:

$$x = \log\left(\frac{y}{1-y}\right) \tag{3}$$

After that, the data was autoscaled before Principal Component Analysis is carried out. Finally, principal components analysis was used to reduce the dimensionality and to visualize data. Thanks to PCA, original data set is transformed from a high number of variables (up to eight ELISAs) to few numbers of new variables (three principal components). The final number of principal components was selected according to the minimal number of principal components that achieve to 95% of the total variance.

(c) Classification and quantification: Next, the k-nearest line classifier is applied in the principal components space. The novel k-nearest line is a supervised method to classify unknown samples and it can also be used for concentration estimation. There is one line per class/analyte that is made up for a dense sequence of samples with increasing concentrations. In fact, no actual line is built, however due to the density of the interpolated data, one assume that looking for the nearest neighbour is the same than looking for the nearest line.

The main principle of k-NL is to assign the class of one sample by the majority vote of its k nearest lines. Using k=1, the classifier k-NN was considered as a nearest line

approach. The distance used for evaluating the nearest neighbours was the Euclidean distance in the principal component space.

Once the detection of the compound is done, the concentration of the nearest neighbour (it can be a synthetic or real sample) was used to assign the concentration of the test sample. In the interpolation scheme every synthetic sample has an associated concentration value.

(d) Validation and interpretation of results: In order to evaluate the predictive ability of the classifier, a leave-one-pair-out validation was used. This scheme is due to the use of replicates. If a measurement replicate is let in the training set, the results are always almost perfect for very simple classifiers. Leaving both replicates out of the training set, means that the measurement conditions in the validation set are not present in the training set.

This methodology uses all real sample pairs, excluding one pair, as a training set and the excluded sample pair is used as a test set. After that, the model is constructed again using all samples without the next test sample. This process is repeated N times, where N is the number of test pair samples. In this work, the original samples were used as test samples.

The classification results are presented in the confusion matrix that contains the classification rate per analyte, and the confusions between analytes. If the model predicts perfectly, the confusion matrix will show a diagonal matrix with 100% in its diagonal. To facilitate the interpretation of the results, the rate of the correct classification for each concentration was also calculated.

As mentioned before, this scheme also permits to estimate concentrations. The evaluation of the quantification was performed by plotting the obtained concentrations versus the expected concentrations. When the quantification is done correctly, the linear

regression of this comparison has values of slope, intercept and correlation near to 1, 0 and 1 respectively.

Data organization, mathematical transformations and graphics were performed using Matlab® R2007a from The MathworksTM. PCA was carried out using the special functions provided by PLS_Toolbox 5.0 from Eigenvector Research Inc. for Matlab use.

Results and Discussion

ELISA assay

Different ELISA assays have been developed[22-24] for the determination of AAS. The assays employed in this study are summarized in the table 1. The concentrations of the immunoreagents were previously optimized in the laboratory. The assay As138/**13**BSA achieved a detectability value (IC_{50}) of 23.77 nM for the determination of Boldenone. Regarding the determination of Methylboldenone, there are different assays with different behaviour: As140/**15**BSA, As142/**15**BSA, As143/**12**BSA and As143/14BSA, with a detectability of 4.33, 0.79, 10.51 and 3.98 nM, respectively. In the case of the analysis of Stanozolol, we characterized that the assays As147/5BSA and As147/**8**BSA had an IC_{50} value of 2.60 and 0.38 nM, respectively. Finally, the assay employed for the determination of Tetrahydrogestrinone was As170/hG-BSA with an IC_{50} of 1.39 nM.

Cross-reactivity study

Regarding the cross-reactivity studies, as it can be observed in the table 2, each assay has a different profile of recognition of the different analytes of interest. Remarkable is, for instance, that the assay As138/**13**BSA can recognize equally B and ADD. For the assays developed for MB, it can be observed that the recognition of MT is very similar

than MB. Within these assays, the recognition of St is also important, as in the assay of As143/**12**BSA. In the case of the St ELISAs, a different profile was obtained only changing the coating antigen. For As147/**5**BSA were obtained cross-reactivity values of 100, 21, 45, 18 and 51% for St, MB, NorSt, 16OH and 3OH respectively. And, on the other hand, for As147/8BSA were obtained 100, 21, 2.5, 1.5 and 2% for the same analytes. In the case of THG detection, a specific assay was obtained which can only partially recognize G and NEth with a 20 and 62%, respectively.

With a general perspective, one can observe that all assays have cross-reactivity for at least 5 different compounds, which makes difficult the individual detection and quantification of AAS using the classical approach of the single ELISA.

Multianalyte ELISA assay

All ELISAs were performed independently ones from others. However, the pattern of response is built by grouping the responses of the different ELISAs for the same analyte concentration.

Case 1: Four analytes and eight ELISAs procedure

In this case, four analytes (DHT, MB, P and St) and the data from the 8 ELISAs were selected. After data pre-processing, PCA captured 80.04% of the total variance in the first principal component, the second one captured 11.95% and the third captured 5.27%. These three PCs with an accumulated variance of 97.26% were used to perform the k-nearest line classifier. The Figure 1 shows the first principal component (PC1) vs the second principal component (PC2) (also called scores plot) for all synthetic and real samples. It can be clearly observed that PC1 evolution basically reflects the common mode response the assay to the increase of concentration of the different analytes. In the

scores plot, there are two main tendencies: the first with values PC1<1.5, where different analytes are overlapped (at low concentrations all assays converge to the same point on the principal component space since assays do not show response) and the second with higher values of PC1, where we can distinguish visually the analytes. One can see in the scores plot that the samples were distributed as a line per analytes, as expected.

After applying the 1-NL classifier, the total rate of correct classification using leave-one-pair-out validation was 90.6%. As shown in the confusion matrix (table 3), the samples that had MB and P were correctly classified. The St had a rate of 87.5%, and its confusions are with DHT. The worst classified analyte was DHT that had a 75% of correct classification. DHT is sometimes confused with MB or P.

The combined rate of correct classification for each concentration shows that the classifier fails only at lowest and highest concentrations, with 62% and 75% at values of 0.03 and 1000 nM respectively. The fault at lowest concentrations can be caused by the overlapping in the scores plot, and at highest concentrations the fail is produced because an extrapolation is done using the leave-one-pair-out strategy (sample falls out of the domain covered by the calibration points). In conjunction, all analytes were well recognised between 0.1 to 316.2 nM with more than 85% of correct answers at each concentration. According to this concentration range, 0.1 nM can be assigned as the limit of identification using this methodology.

The comparison plots of quantification are presented in the figure 2, where the concentrations obtained versus expected values and its linear regression are presented for all analytes. One can observe that all regressions have good behaviour, near to the ideal. The correlations are higher than 0.97 and the slopes and intercepts are near to 1

and 0, respectively. That confirms that our 1-NL model to perform the quantification works correctly.

An important fact is that, the best cross-reactivities values achieved for P and DHT using the immunoassays are 8 and 26%, respectively (see Table 2). Although, using this methodology, P and DHT can be detected correctly even without a specific ELISA for them.

Case 2: Seven analytes and four ELISAs procedure

The second selection of data included seven analytes (A1, B, DHT, MB, P, St and T) and four assays (As138/**13**BSA, As140/**15**BSA, As142/**15**BSA and As147/**8**BSA). The scores plot, where first principal component vs second principal component are plotted for all samples, is shown in the figure 3. The 99.21% of the variance of the data set is captured in the three first components, with the 82.62, 9.72 and 6.87% in the PC1, PC2 and PC3, respectively. We can observe a similar behaviour as in the previous case. At low values of PC1, which corresponds to low concentrations of compounds, an overlapping effect exists for all analytes. However, now there are some overlapped lines at higher concentrations. DHT, MB, P and T lines are quite close to each other in the PC1 vs PC2 projection

Once a 1-NL classifier is applied, some of these overlapping lines cause multiple confusions in the detection. The confusion matrix is shown in the table 4, where one can observe that only 4 analytes are well detected (with more than 75% of correct answers): A1, MB, P and St. These compounds are sometimes confused: A1 with DHT; MB with T; P with DHT and St; St with P. The analyte B has obtained 68.8% of correct answers and presents confusions with St and T. The detection of T was performed with 50% of correct answers, and it has quite confusions with B, DHT and MB. Finally, the DHT

has a rate of 31.3% of correct detections and presents high confusions with A1, B, P and T.

When the rate of correct classification in function of the concentration was studied, it is showed that the A1, MB, P and St have more than 90% of correct detection from 3.16 nM to the highest concentration, at each value of concentration. Although the results are a bit worse than in the previous case, it is important to note that in this case there are more analytes than sensors.

The results of quantification only for the analytes with more than 75% of correct classification were presented, because when a sample is misclassified, this sample is not taken into account for the quantification evaluation. Analytes with the best classification rate have been selected, in order to have enough samples to build relevant statistical comparison regressions. The comparison results are presented in the figure 4. The worst behaviour corresponds to the regression of A1 that has a correlation of 0.92 but the parameters for the three other analytes are really near to the ideal.

As well as the previous case, this methodology is able to detect and quantify two AAS that have no specific ELISA assay. These compounds are A1 and P, and their maximum values of cross-reactivity are only 8 and 10% for the ELISAs used (see Table 2).

Conclusions

ELISA technique had been used in this work to detect and quantify various anabolic androgenic steroids simultaneously. The different recognition profile of eight ELISAs has been evaluated for a variety of steroids. After data preprocessing using statistical tools, the PCA showed the data structure. The structure visualization gave us an idea about if multidetection of AAS was possible and how we could perform it.

The k-Nearest Line procedure was implemented. Line formation was accomplished by producing a dense line representation by adding synthetic samples between consecutive training samples (once ordered in analyte concentration).

Finally, the multianalyte ELISA approach was performed using 1-NL classifier and using the leave-one-pair-out validation. Our proposal carried out multidetection and quantification of four different compounds using eight assays in the range from 0.1 to 316.2 nM. The proposed methodology also can be used with few assays, obtaining correct multidetection and quantification of four different compounds in the range from 3.16 to 1000 nM using only three assays.

We can conclude that multidetection and quantification of anabolic androgenic steroids using ELISA data is possible in buffer, even when no specific ELISA is available. Just, ELISAs with a wide cross-reactivity profile and multivariate data analysis is needed to be able to perform an identification and quantification of AAS. In a future this method could be used to analyze these substances in real samples, like human sera, because it has been already demonstrated in a previous work[22] that with a simple pre-treatment consisted on precipitate the proteins presents in the serum and then dilute the sample in the assay buffer, the matrix effect disappear.

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Figure 1. 2D data set representation consisting of eight different ELISAs and four analytes (case 1). In this graph, the two first principal components are plotted, that explain a total variance of 91.99%.

Figure 2. Quantification results for case 1 (eight different ELISAs and four analytes). Correlations between the obtained and the expected concentrations of DHT (a), MB (b), P (c) and St (d). The dashed line represents the ideal values, the solid one the regression of the obtained vs expected values.

Figure 3. 2D data set representation consisting of four different ELISAs and seven analytes (case 2). In this graph, the two first principal components are plotted, that explain a total variance of 92.34%.

Figure 4. Quantification results for case 2 (four different ELISAs and seven analytes). Correlations between the obtained and the expected concentrations of A1 (a), MB (b), P (c) and St (d). The dashed line represents the ideal values, the solid one the regression of the obtained vs expected values.

Table 1. Details of the ELISA assays employed and their value of IC_{50} for its target analyte

Assay	Target analyte	As dilution	Coating Antigen (μg/mL)	IC_{50} (nM)
As138/**13**BSA	B	1/64000	0.0625	23.77
As140/**15**BSA	MB	1/16000	0.0625	4.33
As142/**15**BSA	MB	1/32000	0.125	0.79
As143/**12**BSA	MB	1/16000	0.125	10.51
As143/**14**BSA	MB	1/16000	0.5	3.98
As147/**5**BSA	St	1/2000	0.625	2.60
As147/**8**BSA	St	1/32000	0.039	0.38
As170/hG-BSA	THG	1/16000	0.125	1.39

Table 2. Recognition of anabolic androgenic steroids, expressed by their percentage of cross-reactivity of each ELISA assay

	Cross-reactivity (%)							
Compound	**As138/ 13BSA**	**As140/ 15BSA**	**As142/ 15BSA**	**As143/ 12BSA**	**As143/ 14BSA**	**As147/ 5BSA**	**As147/ 8BSA**	**As170/ hG-BSA**
St	<2	33	11	104	57	**100**	**100**	4
B	**100**	8	15	<1	<0.4	0.5	0.3	0.5
MB	14	**100**	**100**	**100**	**100**	21	21	2
a-B	12	<0.4	<0.1	<1	<0.4	-	-	-
ADD	96	2	<0.1	-	-	-	-	<0.1
MT	6	93	75	372	123	55	-	0.5
NT	3	-		3	<0.4	<0.2	-	0.7
P	10	<0.4	<0.1	8	<0.4	4	1	3
T	5	3	4	-	19	2	<0.1	<0.1
A1	8	<0.4	<0.1	-	-	-	<0.1	<0.1
Preg	<2	<0.4	4	-	<0.4	-	-	<0.1
Ch	<2	<0.4	<0.1	<1	2	1	-	<0.1
DHT	4	9	2	26	12	4	<0.1	<0.1
E1	<2	15	9	2	<0.4	1	<0.1	<0.1
D21P	-	-	-	<1	<0.4	-	-	-
NorSt	-	-	-	-	-	45	2.5	-
16OH	-	-	-	-	-	18	1.5	-
3OH	-	-	-	-	-	51	2	-
Tr	-	-	-	-	-	1	-	0.5
E2	-	-	-	-	-	1	-	<0.1
EES	-	-	-	-	-	4	-	0.3
THG	-	-	-	-	-	-	-	**100**
G	-	-	-	-	-	-	-	20
NEth		-	-	-	-	-	-	62

- Not tested

Table 3. Classification results for case 1 (eight different ELISAs and four analytes). A confusion matrix is presented, where the rates of correct answers for each analyte are plotted and one can see how the misclassification happens.

	Obtained DHT	Obtained MB	Obtained P	Obtained St
Expected DHT	75.0%	12.5%	12.5%	0.0%
Expected MB	0.0%	100.0%	0.0%	0.0%
Expected P	0.0%	0.0%	100.0%	0.0%
Expected St	12.5%	0.0%	0.0%	87.5%

Table 4. Classification results for case 2 (four different ELISAs and seven analytes). A confusion matrix is presented, where the rates of correct answers for each analyte are plotted and one can see how the misclassification happens.

	Obtained A1	Obtained B	Obtained DHT	Obtained MB	Obtained P	Obtained St	Obtained T
Expected A1	87.5%	0.0%	12.5%	0.0%	0.0%	0.0%	0.0%
Expected B	0.0%	68.7%	0.0%	0.0%	0.0%	6.3%	25.0%
Expected DHT	18.7%	6.3%	31.2%	0.0%	6.25%	0.0%	37.5%
Expected MB	0.0%	0.0%	0.0%	87.5%	0.0%	0.0%	12.5%
Expected P	0.0%	0.0%	12.5%	0.0%	81.2%	6.3%	0.0%
Expected St	0.0%	6.3%	0.0%	0.0%	12.5%	81.2%	0.0%
Expected T	0.0%	18.7%	18.8%	12.5%	0.0%	0.0%	50.0%

Figure 1

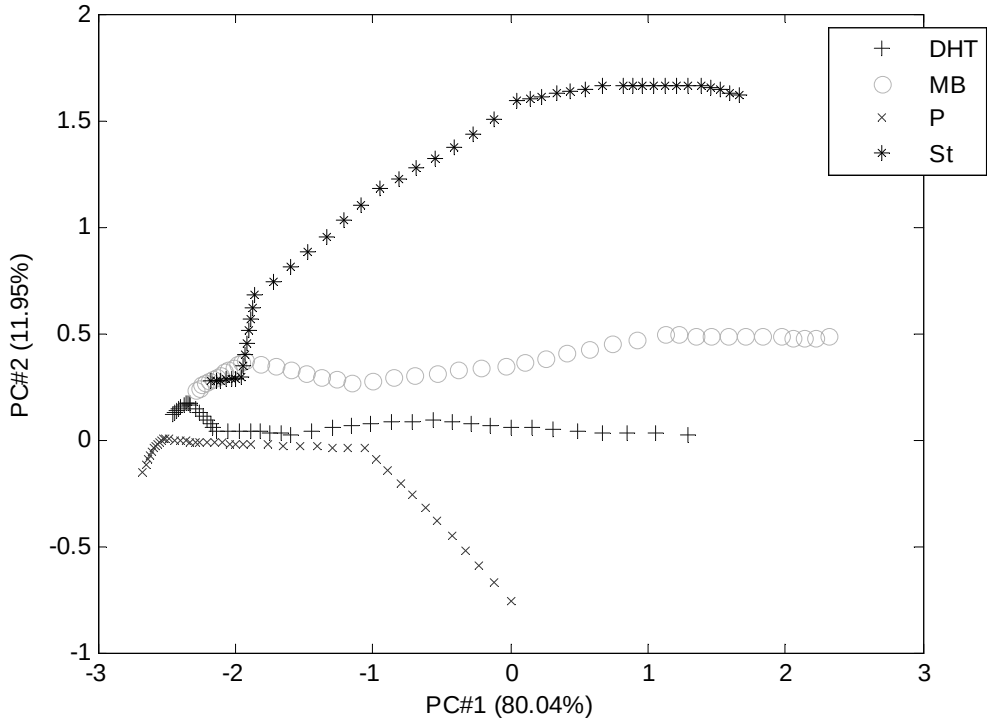


Figure 2

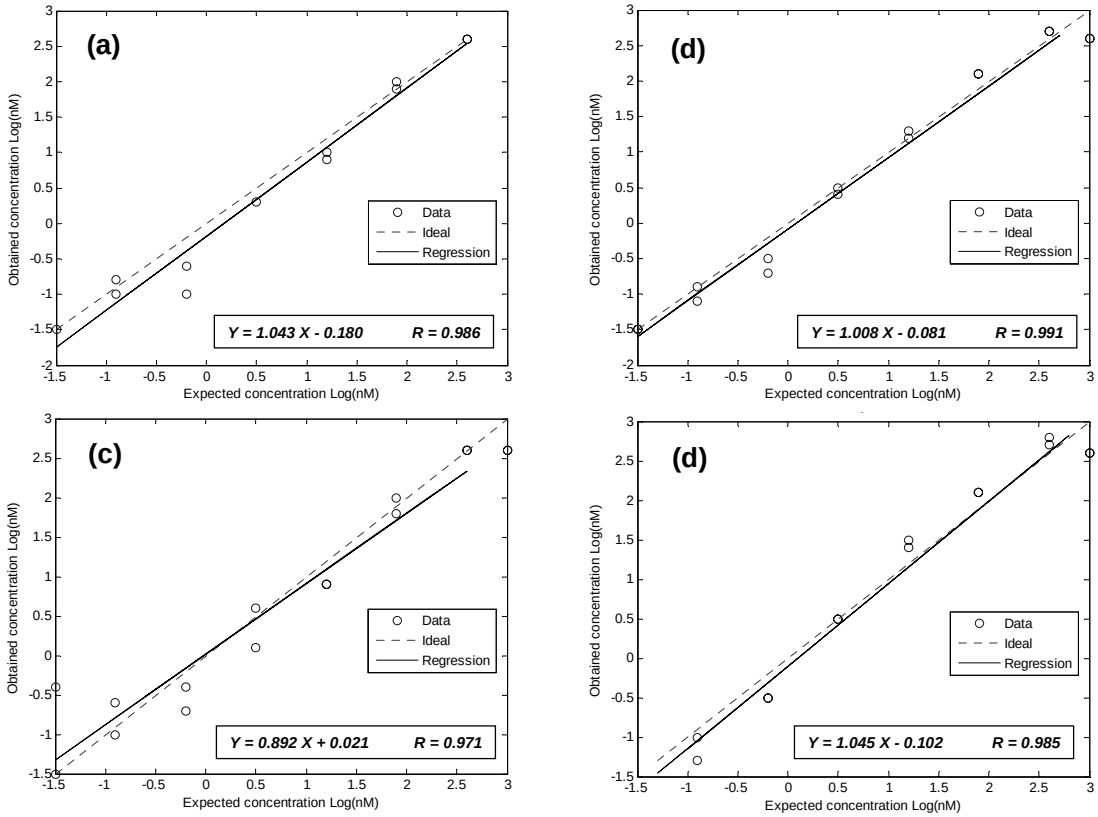

(a)
Obtained concentration Log(nM)
Expected concentration Log(nM)
Data
Ideal
Regression
Y = 1.043 X - 0.180
R = 0.986
(d)
Obtained concentration Log(nM)
Expected concentration Log(nM)
Data
Ideal
Regression
Y = 1.008 X - 0.081
R = 0.991
(c)
Obtained concentration Log(nM)
Expected concentration Log(nM)
Data
Ideal
Regression
Y = 0.892 X + 0.021
R = 0.971
(d)
Obtained concentration Log(nM)
Expected concentration Log(nM)
Data
Ideal
Regression
Y = 1.045 X - 0.102
R = 0.985

Figure 3

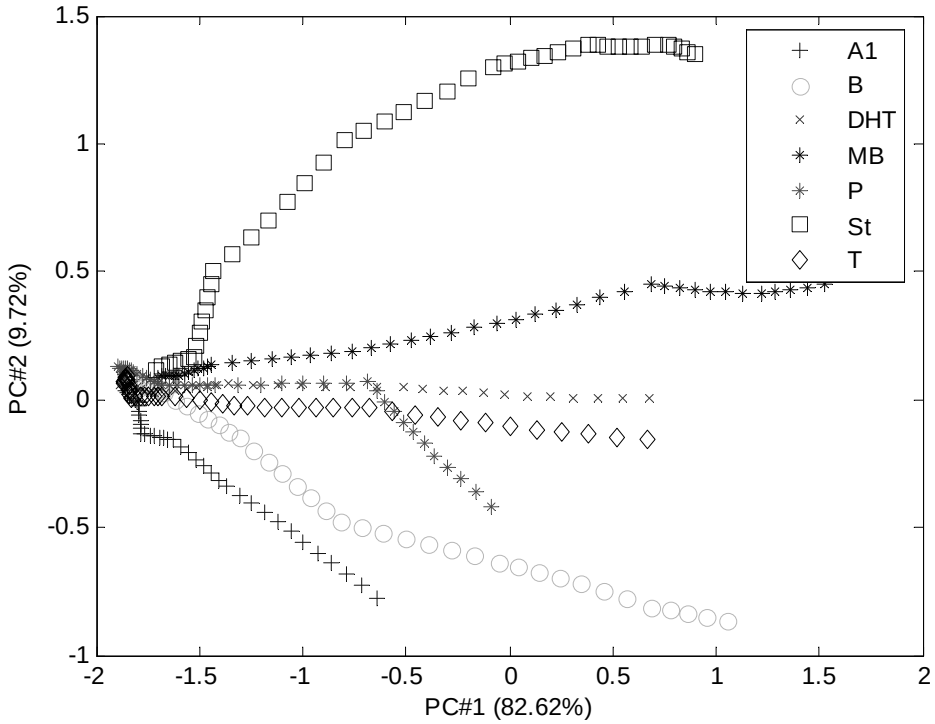
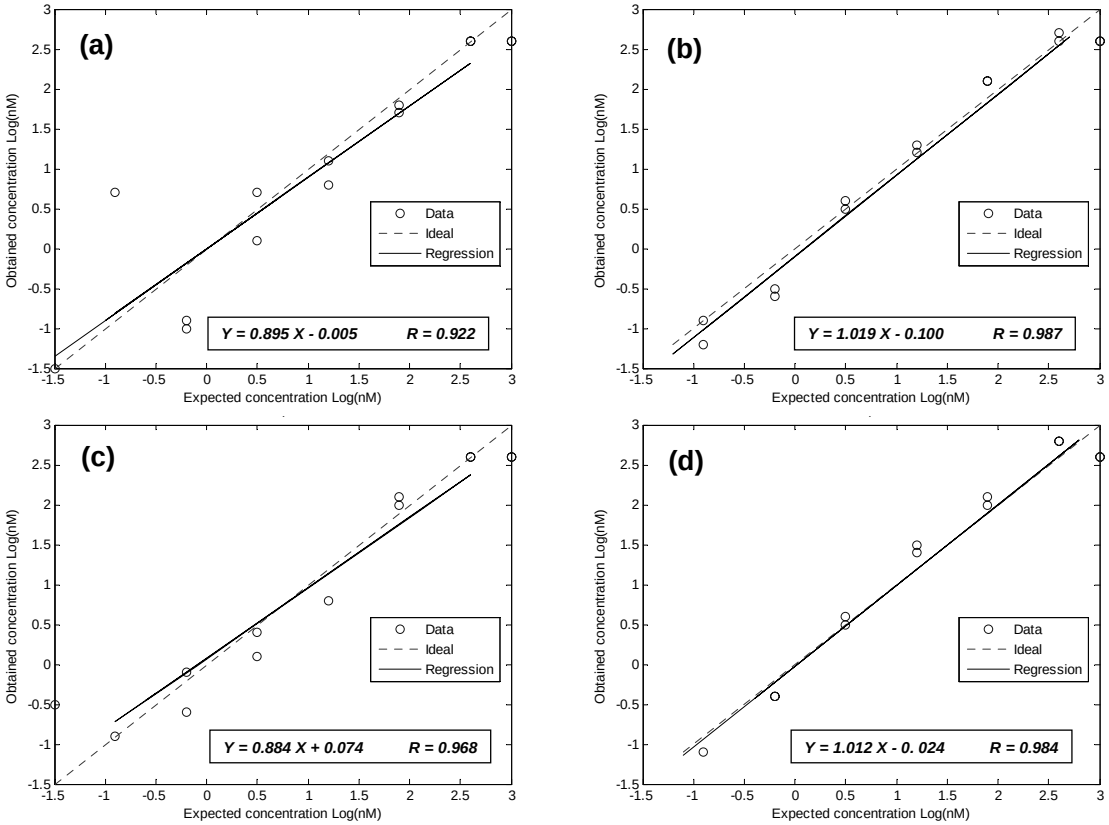


Figure 4


(a)
Obtained concentration Log(nM)
Expected concentration Log(nM)
Data
Ideal
Regression
Y = 0.895 X - 0.005 R = 0.922
(b)
Obtained concentration Log(nM)
Expected concentration Log(nM)
Data
Ideal
Regression
Y = 1.019 X - 0.100 R = 0.987
(c)
Obtained concentration Log(nM)
Expected concentration Log(nM)
Data
Ideal
Regression
Y = 0.884 X + 0.074 R = 0.968
(d)
Obtained concentration Log(nM)
Expected concentration Log(nM)
Data
Ideal
Regression
Y = 1.012 X - 0. 024 R = 0.984