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Statistical discrimination of steroid profiles – introduction of steroidomics in doping analysis

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Introduction

In the last decade, a growing use of computer aided approaches has been used in fundamental research as well as in forensic science [1]. Particularly "omic"-technologies are being used in a broadening range of applications in health and life sciences [2]. Such data mining strategies rely upon advanced computer algorithms to establish high throughput processing of vast amounts of data and have already proven to be very helpful in biomarker development [2] or statistical classification of control and treated subjects [3, 4]. Few attempts to use such multivariate statistics in steroid profiling in doping analysis are limited to models discriminating doped from non-doped samples [3], classification of different phenotypes of steroid profiles with k-means clustering [5] or cluster analysis [6]. In the field of cattle testing to determine illicit use of growth promoters, multivariate differentiation has led to successful methodologies to screen full scan chromatographic profiles to detect the use of DHEA [7] or to predict the administration of nortestosterone in cattle [4].

Inspired by the success of recent cheminformatic-based classification models within the field of steroid analysis [4, 8], such methodology was extrapolated to the concept of extended steroid profile monitoring to facilitate the interpretation of multi-parametric steroid profiles and to detect different kinds of misuse with endogenous steroids. A recent and powerful machine learning technique called support vector machine (SVM) algorithms, also used in face recognition software and artificial intelligence were already successfully applied for the detection of blood doping in athletes [9] and growth promoters in cattle [4] based upon multiple registered parameters. Here, an SVM algorithm was proposed as steroidomic

approach for the global evaluation of the steroid profile and to improve the detection efficiency of testing methods for misuse with endogenous steroids.

Materials and Methods

Samples and analysis All samples were analysed with GC/MS according to the procedure described previously [10], which monitors 24 steroid metabolites. Six healthy male volunteers were administered with single small doses of T undecanoate (40mg), T-gel (100mg), DHT-gel (250mg) and DHEA (50mg) after provision of blank urine samples during one week before administration of any steroid. Between each steroid supplementation, one week of post-administration period was accounted for clearance from the body. Detailed information on the administration and urine collecting protocol can be found elsewhere [11].

With the same analytical procedure, reanalysis was performed of previously described excretion urines after administration of 100mg DHEA, 50mg Adion and 50mg 7-keto-DHEA was performed with the same analytical procedure. Detailed information on these administration protocols can be found elsewhere [12, 13]. These substances were taken by another volunteer than those who participated on the experiments with T undecanoate, T-gel, DHT-gel and DHEA.

All measured steroid concentrations were adjusted to a specific gravity of 1.020 according to the WADA 2004 technical document on endogenous steroids [14].

Bioinformatics We used an SVM for the optimal classification of 110 pre-administration and 565 post-administration steroid profiles into two classes of positive and negative samples. The SVM model considers each steroid profile as a mathematical vector in a 24-dimensional input hyperspace where a separation hyperplane is constructed based upon the input of the training sets with administration data. Based upon this hyperplane, the model generates a score for each steroid profile that indicates how it deviates from a normal profile. The value is referred to as the abnormal steroid profile score (ASPS).

Two versions of SVMs were modelled: one model was trained on the raw adjusted steroid concentrations (raw SVM model) while another SVM model was applied on the individually standardized data set after application of the adaptive model for longitudinal following (longitudinal SVM model). This longitudinal SVM model combines the individual results obtained with the Biological passport approach [15, 16] with the classification performance of a SVM algorithm.

All data were generated using a 'leave-one-subject-out' cross validation procedure. For each subject, the SVM was trained with the data set originating from the other 5 subjects, which each contained over 550 training instances. The samples from the considered subject were then applied as a test set to obtain their ASPS's. This sub-sampling technique ensures that no data are tested on a self-trained algorithm and hence guarantees the generalisation of the model. The models were tested on all administration data.

Classification accuracy was assessed with Receiver Operating Characteristics (ROC) analysis. In order to establish true and false positive rates, the samples collected in the pre-administration week were considered as negative (n=110) whereas all post-administration samples (n=565) were considered as positive. The area under the ROC curve (AUC) was associated with the accuracy of the SVM classifier.

The software used was the independent SVM-Kernel Methods Toolbox on Matlab [17], version 7.6.0. The adaptive Bayesian algorithm developed by Sottas *et al*. [18] was employed for the longitudinal evaluation of the datasets.

Results

Training and Classification results Both SVM methods resulted in an ASPS that quantifies how a given steroid profile deviates from a normal steroid profile. The mean ASPS's of six volunteers are presented in Figure 1A for the raw SVM model and in Figure 1B for the longitudinal SVM model. All blank pre-administration samples for both models presented low ASPS values, lying around or below one. As a consequence, thresholds values for which no false positives were recorded were assessed at 1.78 and 1.25 for the raw ASPS and the longitudinal ASPS, respectively. For both models, significant changes ($p<0.005$, unpaired T-test) could be noticed between the pre-administration samples and the post-administration samples per administrated steroid. Figure 1 illustrates the better detection performance of the raw SVM model since the differences between the ASPS's before and after administration are more pronounced. In the ASPS from the longitudinal SVM model, large increases of the ASPS for all volunteers could be observed within two days after intake of oral steroids. Hereafter, the ASPS slightly lowers towards suprabasal levels that still exceed the threshold levels for several days after administration.

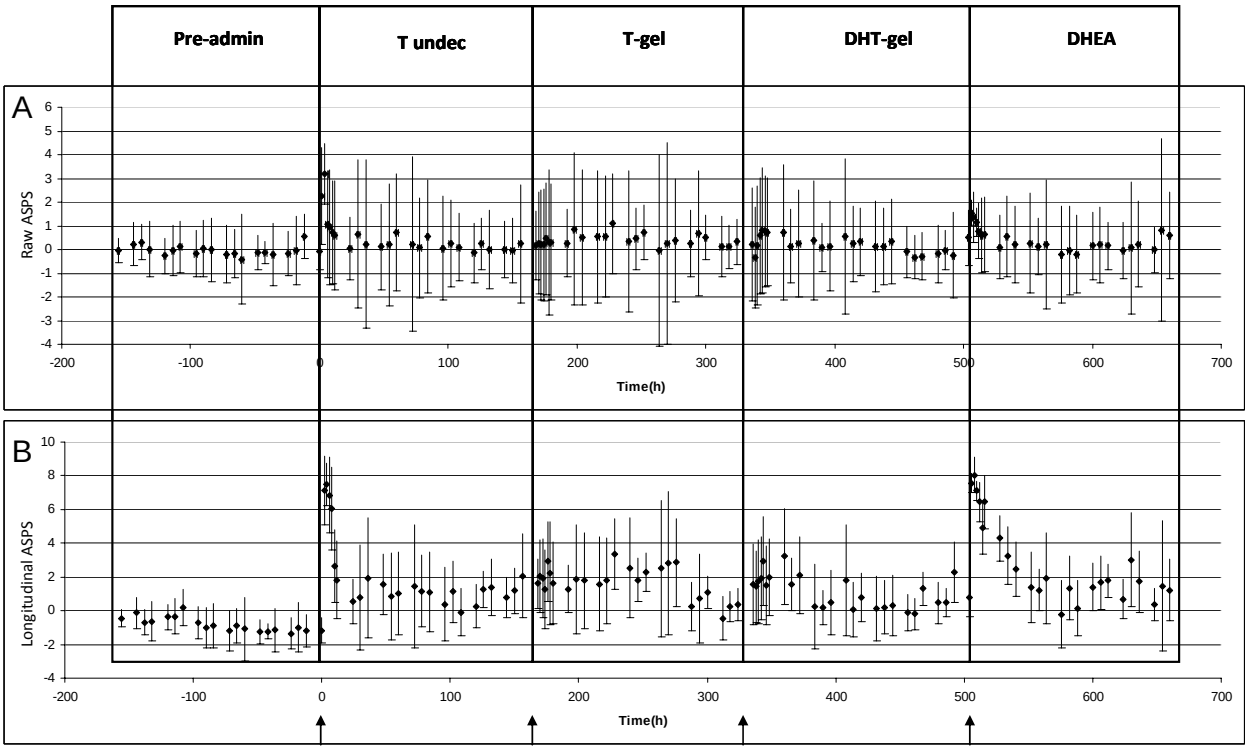

Figure 1: Mean ASPS-values resulting for the raw SVM (A) and longitudinal SVM model (B) during the pre-administration (negative time indication) and post-administration samples (positive time indication). The various steroid formulations were given at times points indicated with an arrow.

It is remarkable that the joint SVM model is capable to detect abnormal steroid profiles for a much longer time than the detection windows reported with any other strategy [11, 15, 16]. This is illustrated with the individual post-administration profiles in Figure 2. Small oral doses of testosterone undecanoate (40mg) and DHEA (50mg) resulted in abnormal steroid profiles which were detectable until 5 days after administration. Also detection of single doses of T-gel or DHT-gel could be established until 5 days after application.

Table 1 presents the sensitivities and overall diagnostic accuracies of both SVM models in relation with those of the longitudinal evaluation of the best biomarkers obtained previously [15, 16] per administered steroid. Table 1 also presents the number of post-administration samples per administered steroid and the corresponding number of positives according to a given marker.

With respect to the data obtained after administration of T undecanoate and T-gel, the raw SVM model shows a better sensitivity than all previously found biomarkers. After DHT and DHEA administration, the results from raw SVM model where similar as those from selected biomarkers. The longitudinal SVM, however, drastically improves sensitivities (up to 40%) and accuracies (up to 17%) in relation to the best biomarkers.

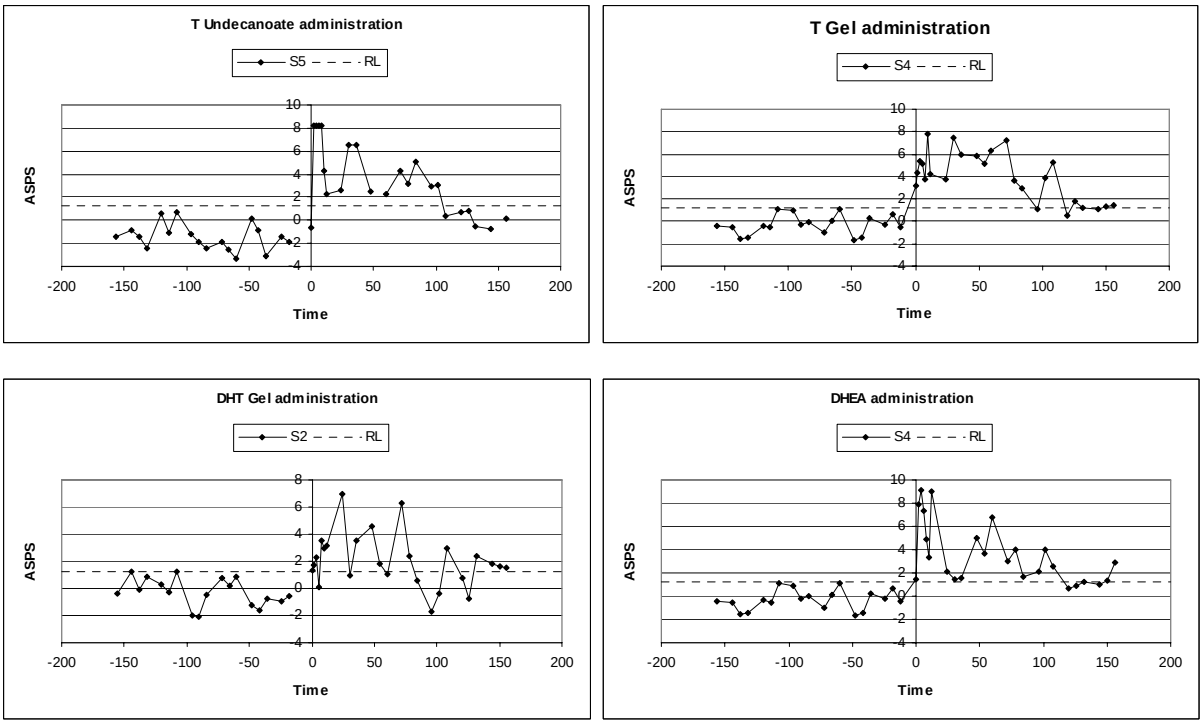

Figure 2: Individual ASPS profile over time calculated using the SVM model based upon individual information before and after administration of a single steroid. The 100% specificity threshold level is indicated by a dashed line.

Table 1: Parameter accuracy, sensitivities at 100% specificity level and corresponding number of detected samples are presented resulting from ROC analysis for the respective best biomarkers and both ASPS's from the raw and joint model. For each administered steroid, the pre- and post-administration period of one week was considered for ROC analysis. ROC analysis was also performed upon the complete clinical trial of 5 weeks on all biomarkers; all post-administration samples were considered for the estimation of the sensitivity.

Administered steroid	Parameter / Model	Sensitivity @ spec 100%	Accuracy	# positives	# post-administration samples
40mg T undecanoate	T/E	0.21	0.63	30	142
	4-OH-Adion /16α-OH-Adion	0.23	0.47	32	142
	ASPS	0.26	0.63	36	142
	ASPS+adaptive model	0.52	0.80	74	142
100mg T-gel	T/E	0.17	0.71	22	132
	ASPS	0.29	0.65	36	132
	ASPS+adaptive model	0.57	0.85	75	132
250mg DHT-gel	5ααβ-Adiol /5βαβ-Adiol	0.27	0.65	40	148
	ASPS	0.19	0.58	27	148
	ASPS+adaptive model	0.45	0.81	67	148
50mg DHEA	DHEA/E	0.31	0.68	45	143
	ASPS	0.20	0.62	28	143
	ASPS+adaptive model	0.66	0.84	95	143

The optimal SVM model even showed a twofold increase of sensitivity at the 100% specificity level. The obtained performance accuracies were established between 80% and 90%.

The capacities of the SVM are show in Figure 3 in relation to the well established T/E ratio. In Figure 3, the overall diagnostics were assessed for the both SVM models during the whole administration trial (n=675) by means their ROC curves as well as the ROC curves of the population statistic and longitudinal evaluation of the T/E ratio. In the ROC-region with higher specificity than 80%, both SVM models outperform even the longitudinal way of evaluating the T/E ratio. At a 100% specificity level, the joint SVM model reached a sensitivity of 55%. This means that 331 out of 565 post-administration samples could be identified as an abnormal steroid profile until one week after steroid intake. In contrast, the T/E ratio shows a maximal overall sensitivity of 6% and 13% if evaluated longitudinally. It must be mentioned that this set of post-administration data also includes excretion urines after DHT-gel application and DHEA administration, which normally do not alter the T/E ratio.

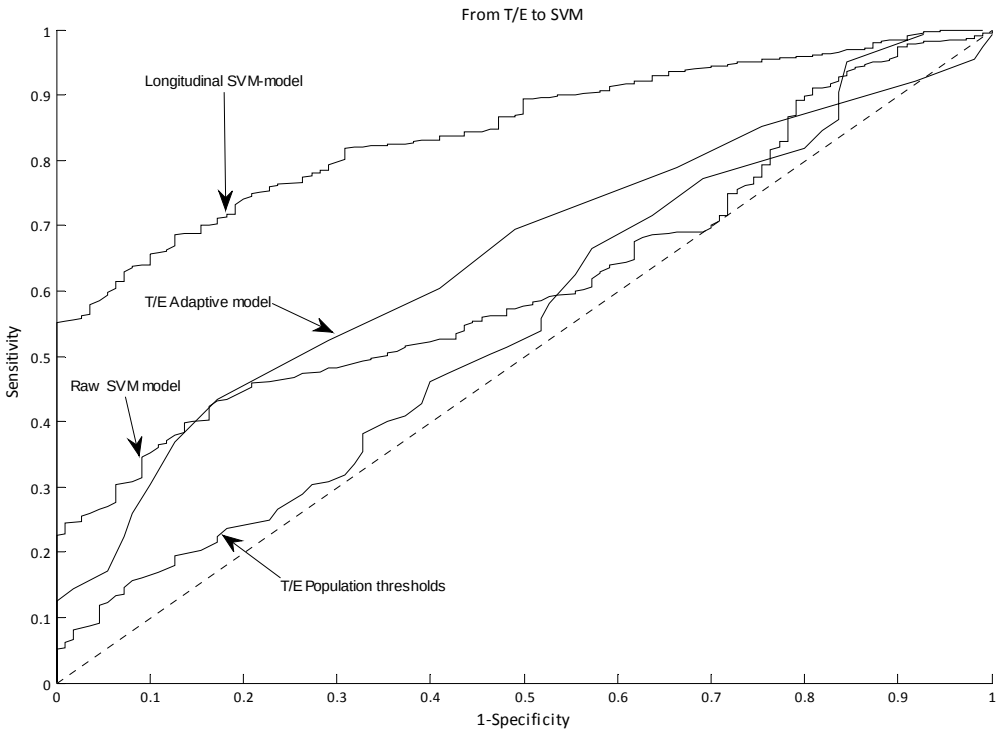

Figure 3: ROC plots of the T/E ratio evaluated with population statistics, the adaptive model and the SVM model as applied to the 24 steroid concentrations and 552 steroid ratios that were individually standardized using the data from the adaptive model

Tests on other administration samples The longitudinal SVM model was also applied to another set of steroid profiles, which was obtained after administration of Adion, 7-keto-DHEA and another dose of oral DHEA (100mg). The post-administration profiles of the corresponding ASPS are shown in Figure 4. The SVM model correctly classified 43 out of 48 profiles, yielding a sensitivity of 73% and an accuracy of 97%. The detection window acquired for DHEA and Adion were 2 days whereas administration with 7-keto-DHEA was detectable until 4 days post-administration.

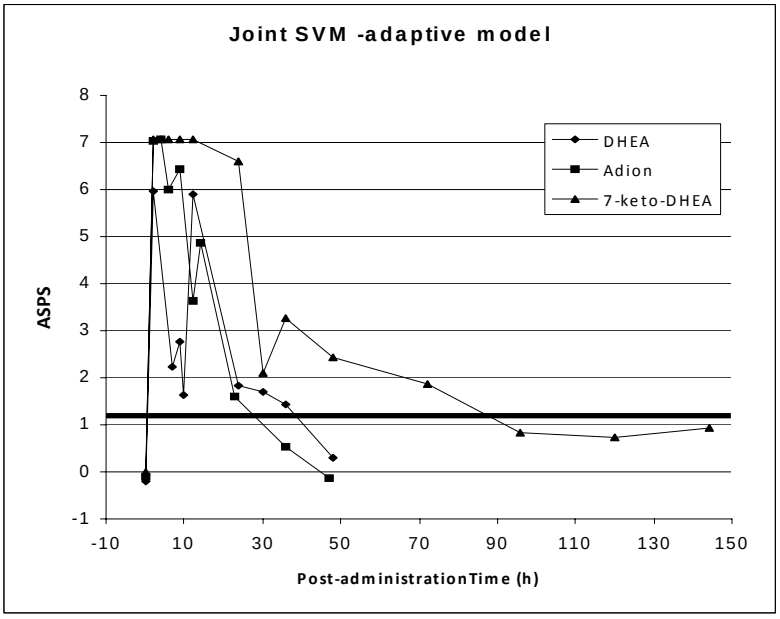

Figure 4: Excretion profiles of the ASPS resulting from both SVM model performed on samples obtained from past excretion studies with 100mg DHEA, 50mg Adion and 50mg 7-keto-DHEA involving another volunteer

Discussion and conclusion

A combination of the SVM algorithm with a comprehensive approach of steroid profiling resulted in a steroidomic model that enables to differentiate normal steroid profiles from abnormal ones. Theoretically, the SVM tool plots all monitored steroids in a multi-dimensional hyperspace which makes the use of steroid ratios redundant to obtain a strategy with optimal detection sensitivity. Hence, the whole set of steroid profile values can be evaluated at once, an analogous strategy as presented by Norli *et al*. [3]. In our model, however, the degree of abnormality was quantified by an ASPS (see Figure 1) for which values greater than 1.25 could be considered as deviating from normal. This raw model resulted in a SVM classifier that was at the same diagnostic level as longitudinal evaluation of the best biomarkers presented earlier [15, 16].

Since the introduction of the Athlete Biological Passport, the results of steroid profiling tests can be systematically stored in a central database enabling the estimation of the individual reference ranges [19]. From such databases, longitudinal steroid profiling data can be made readily available to elaborate longitudinal strategies, thereby omitting a large contribution of the inter-individual variance. Similarly, the raw SVM model was improved by standardizing the training set using individual mean and standard deviation obtained with the adaptive model. The combination of the adaptive model and the SVM enhances the general performance accuracy of the raw SVM model from 62% to 84%, disregarding the kind of endogenous steroid administered. The diagnostic sensitivity of the resulting ASPS was 55% in a post-administration period of 7 days. This is illustrated in Figure 2 indicating that altered steroid profiles can be found until 5 days after ingesting a small single doses of T or DHEA or after topical application of T or DHT in therapeutically recommended doses. This drastic increase in sensitivity can be explained by the ability of the model to sensitively distinguish a prolonged recovery state of the steroid metabolism which is restoring the homeostasis of steroid profile to known basal levels.

Since the model was trained on data obtained after T, DHT and DHEA administration, the model risked to be overfitted i.e. a specific detection tool for these steroids. This problem was addressed by leave-one-subject-out cross-validation and testing of the model on another volunteer, with another dose of DHEA and with other steroids. Testing of the excretion data from a 100mg dose of DHEA, 50mg Adion and 7-keto-DHEA ingested by another volunteer showed a clear response of the ASPSs in Figure 4. This indicates the polyvalent nature of the SVM model to detect any small disturbance of the steroid profile. Moreover, the high sensitivity of 97% obtained for this new test set illustrates the potential of the ASPS as a powerful biomarker for the general detection of misuse with endogenous steroids. Although, this single model shows excellent sensitivity for a wide range of administered steroids, it cannot specify which cause resulted in an aberrant steroid profile. For this information, specific metabolites should be evaluated separately.

Despite the excellent preliminary results on low dose administration studies conducted on a limited study population - including subjects with atypical T/E's that challenge the classification -, the applicability of this strategy will require further work and large scale validation procedure. In order to implement the ASPS in routine testing as a sensitive marker for of any misuse with endogenous steroids, the model should be tested on larger cohorts of data and external influences on the steroid profile that can alter the ASPS should be scrutinized in the future.

In conclusion, a new strategy was developed that returns a single value ASPS as a denotation of the degree of abnormality of a steroid profile containing 24 steroid metabolites. With this strategy, the alteration of the steroid profile, caused by a variety of endogenous steroids, can be detected very sensitively. The longitudinal SVM model was shown to be a general model which can result in long detection of small doses of oral and topical steroid formulations up to 5 days. The overall model performance was very good, particularly when coupled with the longitudinal results from the adaptive Bayesian model. The combination of computer aided techniques as the Bayesian adaptive model and SVM algorithm provide a valuable steroidomic strategy for the long term detection of misuse with endogenous steroids in complement with current steroid profiling methods.

References

1. Saks M.J., Koehler J.J. (2005) The Coming Paradigm Shift in Forensic Identification Science. *Science* **309**, p. 892-895.
2. Riedmaier I., Becker C., Pfaffl M.W., Meyer H.H.D. The use of omic technologies for biomarker development to trace functions of anabolic agents. *J. Chromatogr. A* **1216**, p. 8192-8199
3. Norli H.R., Esbensen K., Westad F., Birkeland K.I., Hemmersbach P. (1995) Chemometric evaluation of urinary steroid profiles in doping control. *J.Steroid Biochem. Mol. Biol.* **54**, p. 83-88.
4. Cunningham R.T., Mooney M.H., Xia X.L., Crooks S., Matthews D., O'Keeffe M., Li K., Elliott C.T. (2009) Feasibility of a Clinical Chemical Analysis Approach To Predict Misuse of Growth Promoting Hormones in Cattle. *Anal. Chem.* **81**, p. 977-983.
5. Rauth S., *Referenzbereiche von urinären Steroidkonzentrationen und Steroidquotienten*, in *Institut für Biochemie*. 1994, Sporthochschüle: Cologne.
6. Flenker U., Nolteernsting E., Schänzer W., Donike M. (1997) Detection and classification of different male steroid profiles by means of multivariate statistics. In:Schänzer, W., Geyer, H., Gotzmann, A., and Mareck-Engelke, U. (eds.) *Proceeding of the 15th Workshop on Dope Analysis* (4), Cologne. pp 203-210.
7. Rijk J.C.W., Lommen A., Essers M.L., Groot M.J., Van Hende J.M., Doeswijk T.G., Nielen M.W.F. (2009) Metabolomics Approach to Anabolic Steroid Urine Profiling of Bovines Treated with Prohormones. *Anal. Chem.*
8. Cannon E.O., Bender A., Palmer D.S., Mitchell J.B.O. (2006) Chemoinformatics-Based Classification of Prohibited Substances Employed for Doping in Sport. *J.Chem. Inf. Model.* **46**, p. 2369-2380.
9. Sottas P.-E., Robinson N., Giraud S., Taroni F., Kamber M., Mangin P., Saugy M. (2006) Statistical Classification of Abnormal Blood Profiles in Athletes. *Int. J. Biostat.* **2**, p. 1-21.
10. Van Renterghem P., Van Eenoo P., Van Thuyne W., Geyer H., Schänzer W., Delbeke F.T. (2008) Validation of an extended method for the detection of the misuse of endogenous steroids in sports, including new hydroxylated metabolites. *J. Chromatogr. B* **876**, p. 225-235.

11. Van Renterghem P., Van Eenoo P., Delbeke F.T. (2010) Population based evaluation of a multi-parametric steroid profiling on administered prohormones in single low dose *Steroids* **75**, p. 1047–1057.
12. Van Eenoo P., Delbeke F.T., Desmet N., De Backer P. (1998) Excretion studies with 4-androstene-3,17-dione. In:Schänzer, W., Geyer, H., Gotzmann, A., and Mareck-Engelke, U. (eds.) *Proceedings of the 16th Cologne Workshop on Dope Analysis* Cologne. pp 171-180.
13. Van Eenoo P., Delbeke F.T., Desmet N., De Backer P. (2001) Excretion studies with 7-keto-dehydroepiandrosterone. In:Schänzer, W., Geyer, H., Gotzmann, A., and Mareck-Engelke, U. (eds.) *Proceedings of the 19th Cologne Workshop on Dope Analysis* (9), Cologne. pp 91-98.
14. WADA, *Reporting and evaluation Guidance for testosterone, epitestosterone, T/E ratio and other endogenous steroids*, in *Technical document TD2004EAAS*. 2004.
15. Van Renterghem P., Van Eenoo P., Sottas P.E., Saugy M., Delbeke F.T. (2010) Subject-based steroid profiling and the determination of novel biomarkers for DHT and DHEA misuse in sports. *Drug Test. Anal.* **2**, p. 582–588.
16. Van Renterghem P., Van Eenoo P., Sottas P.-E., Saugy M., Delbeke F.T. (2011) A pilot study on subject-based comprehensive steroid profiling: novel biomarkers to detect testosterone misuse in sports. *Clin. Endocrinol.* **75**, p. 134–140.
17. Canu S., Grandvalet Y., Guigue V., Rakotomamonjy A., *SVM and Kernel Methods Matlab Toolbox*, in *Perception Systèmes et Information*. 2005, INSA de Rouen: Rouen, France.
18. Sottas P.-E., Baume N., Saudan C., Schweizer C., Kamber M., Saugy M. (2007) Bayesian detection of abnormal values in longitudinal biomarkers with an application to T/E ratio. *Biostat.* **8**, p. 285-296.
19. Sottas P.E., Saugy M., Saudan C. (2010) Endogenous Steroid Profiling in the Athlete Biological Passport. *Endocrinol. Metab. Clin. North Am.* **39**, p. 59-73.