***"Improved Steroids Analysis Employing the Novel Approach of Regioselective Anion Attachment Mass Spectrometry"***

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## Project Summary

This proposal describes a detailed plan to improve the ability to analyze prohibited doping substances by the development of brand new, original methodologies in Analytical Chemistry. The proposal teams up an advanced mass spectrometry laboratory at the University of Paris (VI) with France's leading organization in the battle to ensure the absence of illegal performance enhancing substances in athletes, the "Agence Française de Lutte contre le Dopage" (AFLD).

Using the original approach of regioselective anion attachment mass spectrometry that has been recently developed by the Cole research group, we seek to improve the limits of detection of prohibited steroids, especially those of limited polarity that exhibit poor responses by conventional mass spectrometric ionization approaches. In addition, we seek to expand the ability to unequivocally identify novel doping substances (those not on the current Prohibited list), while also enabling the direct analysis of conjugated steroids. The expected outcomes of this work are a substantial improvement in the ability to detect prohibited steroids at ultra-trace levels, and an enhancement of the specificity in structural analysis that is obtainable by tandem mass spectrometry.

## Results and Conclusions

Neutral anabolic steroids are doping agents that do not provide strong signals by electrospray ionization-mass spectrometry (ESI-MS), thus making their detection challenging when using this technique. While some steroids may be analyzed adequately with older more labor-intensive anti-doping analysis methods (such as GC-MS employing electron ionization (EI), often preceded by derivatization), there remains a need for the development of a new method that is capable of efficiently analyzing more difficult compounds, especially those that are less susceptible to ionization. We have investigated the addition of anions, in ammonium salt form, to anabolic steroid samples as ionization enhancers and confirmed that better signals and lower limits of detection are obtained using Anion Attachment Mass Spectrometry as compared to both positive and negative ESI in the absence of additives. Overall, the developed method allows for substantially improved detection of neutral steroids. For the neutral steroids targeted in this study, results show that the instrumental limit of detection (LOD) obtained using Anion

Attachment Mass Spectrometry is always equal to, or better than, that obtained in either positive or negative ion ESI-MS in the absence of any additive. In the case of the most difficult compounds (calusterone-M, norbolethone-M), no signal was obtained in negative ion ESI-MS, but upon addition of $NH_4F$, it becomes possible to detect these compounds at concentrations as low as 25 ng/mL, which represents a 100-fold improvement compared to the LODs obtained in positive ion ESI-MS.

Atmospheric Pressure Photo-Ionization (APPI) Mass Spectrometry was also tested for its ability to produce mass spectrometric signals from trace level samples, and obtained results were compared to results acquired using Anion Attachment Mass Spectrometry. As a test case, the fluoxymesterone metabolite produces the cation radical ion $M^{+\cdot}$ when analyzed using APPI, but a 50-fold loss in signal is observed from APPI when compared to the Anion Attachment approach. In summary, none of the tested "difficult to ionize" steroids exhibited better signal responses by APPI as compared to those obtained by Anion Attachment Mass Spectrometry. On the other hand, the formation of anion adducts does not appear to improve the sensitivity of conjugated steroids containing a carboxylic acid that is already susceptible to deprotonation and $[M-H]^-$ formation in conventional ESI-MS.

Looking ahead to the implementation of this new approach to "real-world" antidoping samples, we have obtained preliminary Liquid Chromatography-Multiple Reaction Monitoring (LC-MRM) results that show trace level detection of the problematic fluoxymesterone metabolite down to the 5 ng/mL level. This improved sensitivity, relative to existing methods, will serve to reduce the number of false negatives in real sports doping analyses.