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Optimizing the cost/benefit ratio of the laboratory procedures: A way to match the WADA requirements (with the help of microwaves)

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INTRODUCTION

The period embracing the last three years has been perhaps the most dense of changes for the activity of the antidoping laboratories. Apart from the increase of the overall number of samples to be tested (representative data from the Antidoping Laboratory of Rome: 6500 samples received in 2002; almost 8000 samples in 2004), the workload has increased also from a "qualitative" point of view, as a consequence of the progressive upgrade of the list of doping substances and methods released by the World AntiDoping Agency (WADA) [1]. The inclusion of new substances/classes of substances, the approval of other biological matrices (blood) to be used for doping analysis, the revision of the technical guidelines for the accredited laboratory, including the criteria for confirmation (also with respect to the existence of therapeutic use exemptions), has forced the laboratories to deeply reorganize their internal activity, such a reorganization being more and more drastic as the availability of further financial resources did not keep the pace with the new requirements.

From a very general point of view, the progressive upgrades of the list, of the International Standard for Laboratories and of the related technical documents, forced the labs to deal with the following constraints:

1. an increased number of screening procedures (including new matrices, e.g. blood);
2. an increased number of target compounds to be screened for in each screening procedure;
3. an increase of the overall number of confirmation analysis;
4. different criteria for the in-laboratory storage of biological samples.

This contribution outlines the approach followed by the antidoping laboratory of Rome to deal with the new WADA requirements, including the development of a deeply reorganized pool of screening and confirmation procedures, also in view of the activity of the laboratory on the occasion of the 2006 Winter Olympic and Paralympic Games.

LABORATORIES "FIT TO PURPOSE": THE EVOLUTION OF THE "PURPOSE"

The most striking changes consequent to the 2003-2005 upgrade of the WADA list are outlined below:

1. the analysis of blood samples;
2. the screening for all the synthetic glucocorticoids in all "in competition" samples;
3. the screening for THG, gestrinone and other steroids not detectable by GC-MS methods;
4. the screening for the new class of anti-oestrogen agents;
5. the screening of class 3 (above) and of hCG also in females athletes;
6. the reduction of the threshold value of the T/E ratio from 6 to 4;
7. the need of confirming all beta-agonists and glucocorticoid samples, regardless of the existence of a therapeutic use exemption (TUE);

More specifically, blood has been approved as an additional biological matrix, to perform additional self-consistent screening and confirmation procedures, and not only in combined blood-urine tests. Although publication of the relevant methods (mandatory for their official application) is still in progress, they were already and will again be applied on the occasion of major international events. This imposed an "extra-instrumental" upgrade of the labs, to comply with the new requirements for blood manipulation that, in some Countries (including Italy), are regulated by strict, specific guidelines.

Figure 1. The "physical" upgrade of the antidoping laboratory of Rome. The brand new section (hosting the areas for EPO/NESP and blood analyses and the -20 °C walk-in freezers and the +4 °C cold-room for the storage of samples) is indicated by the white oval.

This imposed the most evident upgrade of the laboratory in Rome: the addition of a new, physical extension of the lab (Figure 1). Apart from architectural aspects, all points 1-7 required a drastic reorganization of the available human and instrumental resources. For indeed, the "lot" of the target compounds (not including blood analysis) whose screening is mandatory for all WADA labs, progressively increased in the last 4 years (see Table 1).

Table 1 Number of the substances potentially included in the reaccreditation/proficiency test control samples

2002 (IOC-MC reaccreditation test)	***2005 (WADA PT programme)***
Anabolic Agents: Anabolic Androgenic Steroids (AAS): exogenous	
18	**34**
Anabolic Agents: Anabolic Androgenic Steroids (AAS): endogenous	
3	**5**
Other Anabolic Agents	
1	**2**
Peptide hormones	
1	**3**
Beta-2-agonists	
3	**6**
Diuretics & Masking Agents	
11	**14**
Stimulants	
34	**45**
Narcotics + Cannabinoids	
6	**6**
Beta-blockers	
14	**17**
Anti-oestrogens	
---	**12**
Glucocorticoids	
---	**13**
Alpha-reductase inhibitors	
---	**2**
Total	
91	**159**

"ANALYTICAL DARWINISM": HOW TO SURVIVE WITH A RAPIDLY CHANGING LIST

Considering that the available resources do not keep the pace with the evolving picture of WADA requirements, the labs experienced a sort of "analytical Darwinism", critically balancing auto-organization and evolution to adapt themselves to the (rapidly) changing "environment". In this process, some components went towards extinction (in our case: the equipment/methods dedicated exclusively to the screening for beta-blockers and phenolalkylamines), some others arose and become predominant. The "logical" evolutionary process (i.e. the development of new, dedicated methods for the screening of any newly added substances [2]) would have brought the "lab organisms" to the extinction, due to the "energetic" (mainly financial) non sustainability of such an approach. The adapting organism (in Rome) survived evolving towards the system structured as shown in Figure 2, where a comparison to its precursor (in the period 2002-2004) is also given.

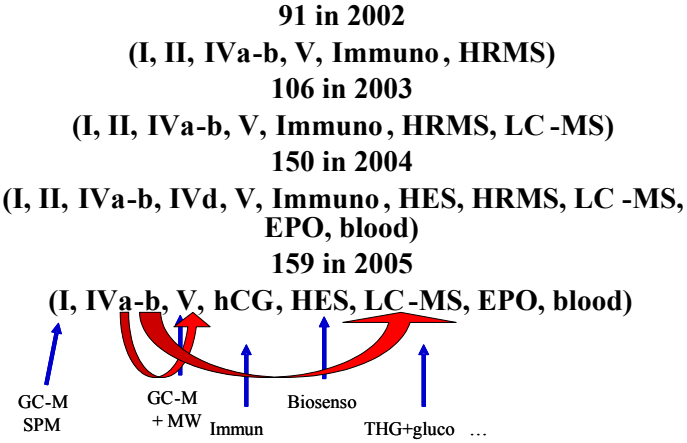

Figure 2. The evolution, in the period 2002-2005, of the screening procedures carried out at the antidoping laboratory of Rome, as a function of the total number of substances to be screened for (see again Table 1). Bend arrows indicate that the pre-treatment process is partially in common between two procedures. Blood analyses, GC-HRMS, GC(C)-IRMS and other confirmation procedures (e.g. GC-MS in negative chemical ionization mode for beta-blockers and phenolalkylamines) are not considered in the scheme.

The key components of this (forced) evolutionary process, whose basic goal was the recovery of any reusable human, instrumental and financial resource, were (i) the reduction of the costly consumables (i.e. C18 SPE cartridges), (ii) the automation of the pre-instrumental process, being now partially in common among different screening procedures, (iii) the reduction of the time required for each analysis (mostly at the pre-instrumental stage, i.e. by microwave irradiation). An example on the reorganization of screening procedures, planned to optimize the instrumental and human resources needed for every analytical line and, at the same time, to reduce the time and cost of the screening procedures, is outlined in Figure 3.

Free fraction,. Methyl derivatives		**Free fraction, TMS-derivatives**
• Urine volume	**6 mL**	
• Internal standard	Indomethacine/mefruside/methyltestosterone	
• 1st Extraction	**No cartridge!**	
	pH corrected to > 10 (300 µl carbonate/bicarbonate buffer)	
	extraction by 12 ml $CHCl_3$:isopropanol:TBME (80:10:10)	
• Evaporation	N_2 stream, 70 °C	N_2 stream, 70 °C
• 2nd Extraction		
– pH corrected to < 4 (100 µl formate/formic acid 5M)		
– extraction by 5 ml $CHCl_3$:isopropanol:TBME (60:10:30)		
• Evaporation	N2 stream, 40 °C	
• *Derivatization*	*Acetone/CH_3I= 9/1, 200 µL*	50 µL derivatizing reagent
	Anhydrous K_2CO_3, 50 mg	(MSTFA:NH_4I:DTET 1000:2:4)
	6 min MW 1200W or 10 min 110 °C	***30 min 70 °C (or 5 min MW 600 W)***
• Injection volume	1 µL (split 1:10)	1 µL (split 1:10)
• Analysis	GC-MS EI SIM	GC-MS EI SIM

Figure 3. The newly proposed common urine pretreatment process for the detection of TMS-derivatives and of methyl-derivatives (both excreted free in the urine) by two independent GC-MS-EI procedures. Extracts are splitted before the final derivatization stage.

A further aspect of the research activity of the lab in Rome is considering the potential application of electrochemical methods to the antidoping field [3-4]. In this context, we have evaluated the possibility of employing electrochemical enzymatic biosensors for the analysis of the plasma volume expanders (PVE) HES and dextran, the final aim of the project being the screening of all polysaccharide-based PVE at the pre-instrumental stage (Figures 4-5).

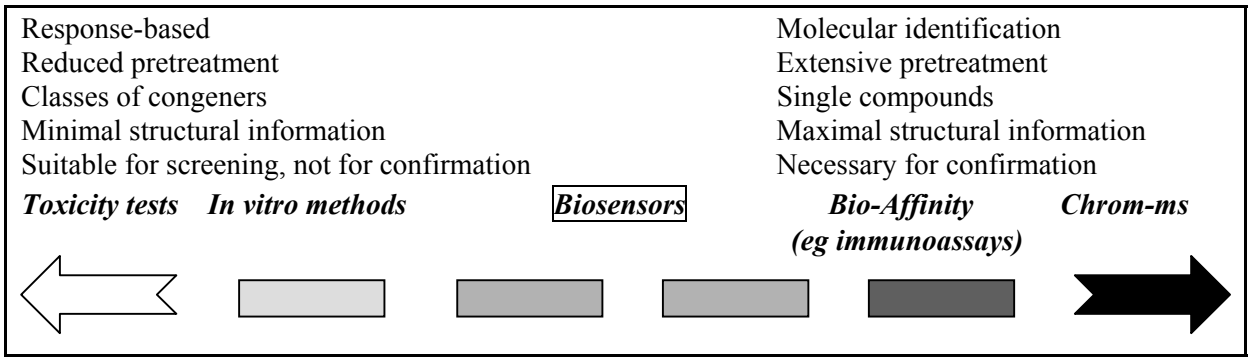

Figure 4. The position of biosensors, and especially of enzymatic electrochemical sensors, in the scale from response-based to physico-chemical methods of analysis

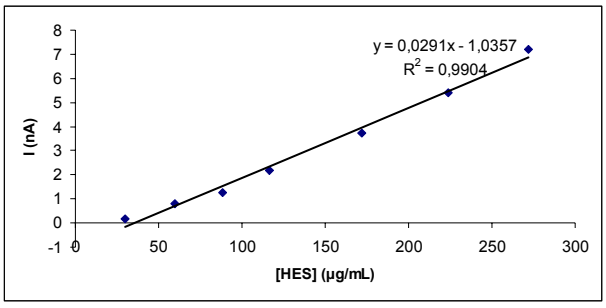

Figure 5. A typical calibration curve obtained by the maltose bioelectrode

SPEEDING UP THE DERIVATIZATION STEP: THE ROLE OF MICROWAVE IRRADIATION

Whenever the time factor is a constraint, a further help can come from the use of microwave ovens at the derivatization stage. The laboratory of Rome is presently involved in a series of research programmes aimed to both optimize the conditions for the derivatization reaction under microwave irradiation and clarify some aspects of the mechanisms driving the derivatization processes. Most generally, the effect of the microwave irradiation, if compared to the "traditional" thermal incubation, can affect either the rate and/or the yield of the derivatization process (Figure 6).

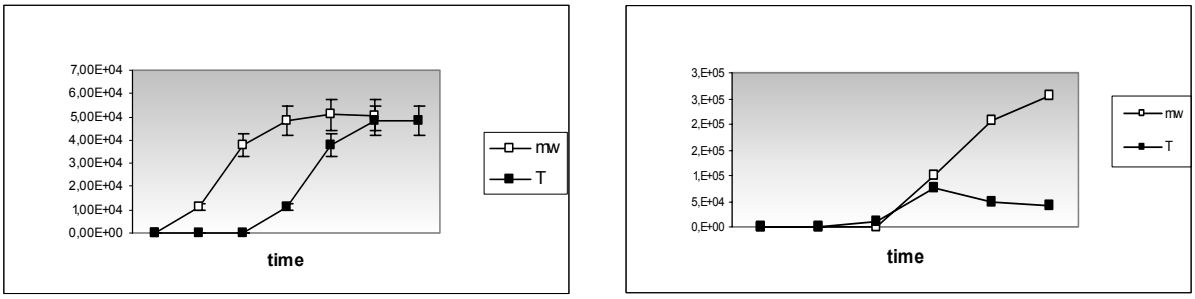

Figure 6. Two possible effects of microwave irradiation with respect to thermal incubation, affecting either the rate (left) or the yield (right) of derivatization reactions. Data on the y axis refer, in general, to the normalized intensity of the diagnostic ion fragment monitored to follow the derivatization reaction of the target compound.

The situation depicted in the left part of the figure above is typical of the methylation reaction of diuretics, stimulants, and, in general, of all those target substances screened for by the procedure summarized in figure 3 [5]; while a pattern similar to that shown on the right plot was detected in the case of the GC-MS analysis of glucocorticoids (whose screening and confirmation analysis are now performed in Rome by LC-MS-MS). Apparently, data obtained on the GC-MS characterization of synthetic glucocorticoids suggested that microwave irradiation could led to a significant increase of the rate of the derivatization process with respect to thermal incubation [6]. It is however self evident that, to compare data obtained by the two procedures, a reliable thermostatic system has to be set up; in our case, this was ensured, in both the thermal incubation and the microwave irradiation, by an outer jacket filled with (boiling) water. A deep re-evaluation of all experimental data led us to reconsider our results in the light of the role played by the microwave irradiation on the external water bath itself. Measurements carried out by temperature probes have shown that the temperature reached by the boiling water under irradiation in the microwave oven is not the same of that of the thermal incubation. A "superheating effect" is indeed observed under microwave

irradiation, and, while the temperature recorded inside the "thermostating" jacket containing boiling water is (obviously) 100.0 °C, the temperature of the same jacket, again containing boiling water, measured under microwave irradiation gave (less obviously) the value of 103.5 °C. This effect can be attributed to retardation of nucleation during microwave heating [7]. The effect is minimal using low microwave power (< 800 W) and it can be totally eliminated in well stirred reaction chambers; but, nonetheless, it is still detectable under our experimental conditions (microwaves irradiated at 1200 W). In theory, a sufficiently strong magnetic field could cause the solvent to freeze. This "boiling ice" ("ghiaccio bollente" in Italian, see also figure 7) effect is responsible for the apparent increase of the derivatization yields by microwave irradiation with respect to direct thermal incubation.

Figure 7. A symbolic representation of a biochemical interaction, involving steroid hormones, occurring in a water bath. The shot is taken from Federico Fellini's "La dolce vita", the movie that did not win the Academy Award Oscar in 1961. The two "species", binding under the effect of non-thermal irradiation in a water bath, are Marcello Mastroianni and Anita Ekberg, the nickname of the latter being the most famous oxymoron of Italian language ("ghiaccio bollente", boiling ice).

It might be interesting to note that, once the microwave irradiation is carried out on a reaction chamber without the external water jacket, the effect on the derivatization yields is generally much more pronounced. Data reported in Table 2 compare the derivatization yields obtained in the derivatization reaction of synthetic glucocorticoids (to form the corresponding poly-TMS derivatives) by the procedure described in [8].

Table 1 Comparison among the yield of the derivatization process, with formation of poly-TMS derivatives, of some representative synthetic glucocorticoids, carried out in different experimental conditions. Data refer to the percent of the concentration of the most substituted derivative with respect of the total concentration of all derivatives.

Time (min)	Thermal	Microwave irradiation (water bath)	Microwave irradiation (dry)
Fluocortolone-3TMS			
15	24	18	**95**
30	32	18	**81**
45	34	35	**97**
60	40	43	**99**
Budesonide-3TMS			
15	11	9	**75**
30	15	9	**72**
45	18	14	**87**
60	26	12	**88**
Methylprednisolone-4TMS			
15	15	6	**81**
30	13	8	**80**
45	15	8	**86**
60	18	6	**76**
Prednisone-3TMS			
15	17	9	**91**
30	15	9	**92**
45	19	28	**100**
60	36	10	**100**
Prednisolone-4TMS			
15	5	3	**62**
30	5	3	**65**
45	12	4	**90**
60	6	2	**89**

On the basis of the experimental work carried out so far, the following consideration can be drawn:

1. the yields obtained by thermal and microwave assisted derivatization are comparable if the incubation is carried out at the same temperature (the water bath is a good thermostatic system, but one has to consider that comparing data obtained by microwave irradiation and by thermal incubation has no significance if the temperature is not the same in both cases);
2. if the microwave irradiation is carried out on a reaction chamber with an outer water bath jacket, it is indeed the water to preferentially absorb most of the irradiated energy: in this "boiling ice" configuration the derivatization reaction only benefits of the higher constant temperature of the outer (boiling) water bath;
3. the real potential and usefulness of microwaves, especially if higher reaction yields are to

be achieved, particularly on poorly reactive residues, are pushed by incubating without the outer water bath;

4. nonetheless, the absence of a polar medium absorbing the microwaves imposes the maximum of precautions: the oven must be perfectly clean, with no traces of humidity, powders or other "pinpoint targets" that could cause a local overheating, with severe risk for the oven and for the operators;
5. apart from theoretical issues, and in the routine activity of an antidoping laboratory, microwave irradiation can be a powerful strategy to minimize the duration of the derivatization process.

CONCLUSIONS

This brief overview outlines some of the most evident changes in the organization of the activity of the WADA laboratories, supporting the idea that human and instrumental resources have to be thoughtfully optimized inside an antidoping laboratory, keeping their "useless fraction" to a minimum. Should the future upgrades of the list keep the same pace of the last three years, the WADA laboratories will have to deal with the lack of sufficient human, instrumental and environmental (in terms of space available in the lab) resources. In this context, the laboratory of Rome is continuously trying to reduce as much as possible the unnecessary screening and confirmation procedures, helping the lab itself to evolve, again, towards a new "fit to purpose" state that can still be sustained by the available resources. This is most true now that the laboratory tools used for the fight against doping in sport are progressively shifting from the field of chromatographic-spectrometric techniques to that of a more integrated approach [9]. It is self evident that, for such a complex system, no extra-laboratory speculation can justify a team of professional experts wasting human and instrumental resources for procedures that will finally appear useless.

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