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Measurement reliability in horseracing laboratory operation

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Abstract: Similar to many other testing laboratory activities, horseracing laboratory's operation requires formal recognition of measurement results, mainly to establish the presence of a wider range of prohibited substances (low concentrations) in biological matrices. The objective of this work is twofold: (i) the qualitative and semi-quantitative assessment of various substances detected among all (over 50,000) racehorses' urine analytical analysis conducted by the Antidoping Laboratory of the Brazilian Jockey Club (LAD/JCB) between 1996 and 2005 to identify the most frequent prohibited substances marked as 'forbidden' on the list of Brazilian National Horseracing Code (CNC) [1] and (ii) to develop and validate an specific mass spectrometry analytical method for the routine determination of traces of doping by caffeine. Caffeine confirmed to be the most common doping substance used in the Brazilian horseracing activity in 2002 when this investigation started. The tailored made analytical method developed (ALCAC-18), ensuring that false-negative' results are kept to a minimum, was then validated for the determination of caffeine.

Keywords: Horseracing doping. Metrology reliability. Caffeine. Mass spectrometry analytical method. Metrology in Chemistry.

1. INTRODUCTION

Due to the fact that horseracing (involving professional bookmakers and pure bred horses of high commercial value) is in fact a commercial activity, the Federation of Horseracing Authorities have motivated the International Laboratory Accreditation Cooperation (ILAC) to develop accreditation requirements and operating criteria for horseracing laboratories (ILAC-G7:1996) [2]. Besides disciplining the operation of this type of doping laboratories, accreditation introduces the formal recognition of technical competence. In recent years, the socio-economic impact of horseracing has grown very fast.

In many countries, the need for reliable measurements was anticipated for specific regulations in horseracing as well as for accreditation of the associated competent bodies [2]. Doping measurements were originally intended to assess the horses' health and to assure fair competition. More and more these assessments have become a field of study [3, 4, 5, 6] on its own involving metrological challenges, economic interests and ethic judgements.

2. FORBIDEN SUBSTANCES

Most of the substances used for doping are organic chemical compounds and present in biological fluids at very low concentration levels. As proposed in this work, a mass spectrometry analytical technique proved to be an adequate scheme applied for such analyses. In its routine practice, the LAD/JCB is able to detect up to 121 doping substances using eight different analytical procedures. As revealed by an internal survey carried out within the Antidoping Laboratory of the Brazilian Jockey Club, between 1996 and 2005, the LAD/JCB has performed over 50,000 analyses on racehorses' urine as summarized in Annex A. The analyses resulted in the qualitative and semi-quantitative assessment of various substances that are marked as 'forbidden' on the list of Brazilian National Horseracing Code (CNC) [1], based on international agreements. Caffeine and flunixin, in this order, were found to be the most frequent ones detected in these assays. This paper describes the development and validation processes associated with the determination of caffeine, the most frequently encountered forbidden substance up to 2002 [7]. It is well accepted nowadays that caffeine has a positive effect on the locomotor activity of horses [8]. In fact, in horseracing, there is no limit allowed; i.e. caffeine is not tolerated at all.

Following similar validation methodology, a new analytical method is in process of development to detect the presence of the forbidden substance *flunixin*, the new most frequent doping substance detected in the for after period 2002-2005.

3. THE METHOD

The method developed and validated for the detection of the forbidden substance caffeine originated from the multipurpose *Méthode Alcaline Sur C-18* originally proposed by the French *Laboratoire de Contrôle Antidopage*. This method applicable to the determination

of 39 substances proved not to be suitable for the LAD/JCB technical laboratory conditions, therefore the need for a new method which was based on gas chromatography-mass spectrometry (GC-MS). Upon implementation, validation of the adapted method for the determination of caffeine in race horse's urine was indispensable since the laboratory intends to have its quality system accredited for compliance with ISO/IEC 17025, a prerequisite for international laboratory accreditation.

Adherent to a basic metrological concept, one of the challenges is to assert traceability of the results of the critical analyses in a cost-effective way, avoiding unnecessary test duplication, waste of time, and hence, loss of credibility. Reliable measurements are equally needed to provide the technical base that corroborates to legal disputes of the recalcitrant in accordance with the Brazilian National Horseracing Code (CNC) [1].

The proposed method –now denoted as ALCAC-18– comprises two steps: (i) extraction of samples in alcaline medium and (ii) analysis of the extract obtained by gas chromatography coupled with mass spectrometry.

Implementation of the adapted method at LAD/JCB was as follows. A pool of negative urine samples collected from thoroughbred horses owned by the Brazilian Jockey Club is used as 'blank' reference material (absence of caffeine). A methanolic stock solution was prepared from certified reference material [9] "caffeine" at a final caffeine concentration of 0.01 mg/mL. Known quantities from this stock solution were spiked to the blank reference material, resulting in a series of urine samples with different caffeine levels for quality control.

To assure metrological reliability, the ALCAC-18 was monitored by the classical technique of internal standardization, using a methanolic diazepam solution (also prepared from a certified reference material at a final concentration of 10 μg/mL). The samples of urine (6 mL) were diluted with 3 mL of water (ultra-pure) to which 100 μL of the internal diazepam standard solution was added. The diluted samples were submitted to the *"salting out process"* by the addition of 500 mg of ammonia sulfate, kept still for 10 min. The pH was maintained in the 9.4 to 9.8 range by the addition of ammonia solution (10% m/v). The samples were then centrifuged for 20 min. at 4000 rpm. The solid phase extraction was carried out with C18HF extraction columns (high flux, 3 mL/500 mg).

The GC-MS analysis made use of a capillary column (0.25 mm diameter; 30m long; 0.25 μm thick) with phenylmethylpolysiloxane 35 % as stationary phase. Typical temperatures of the chromatographic programme were set at: 280 °C (injection temperature); 295 °C (interface temperature); 60 °C (initial oven temperature, adjusted for operation by the following programming rates: 22 °C/min up to 200 °C; 10 °C/min up to 270 °C and 30 °C/min up to 305 °C for 6min. The sample injections were of the *Splitless* type, using helium as a carrier gas (flux: 0.9 mL/min). The sample injection volume was 1 μL. The analyses were carried out at the electron impact mode, with ionization energy of 70 eV and scanning range from 40 to 550 amu (atomic mass units).

Tests conducted also suggest that specialized analytical methods applicable to the detection of single substances offers better overall performance than multipurpose methods applicable to the detection of many different doping substances. Suitable to the technical requirements of the existing doping laboratory, the validated method led to the reduction of the dilution and centrifugation times and reduction of the sample volume of the biological matrix.

4. VALIDATION OF THE ANALYTICAL METHOD

The metrological concept associated with the critical factors, the procedures for preparation of samples and the validation of ALCAC-18 applicable to the determination of caffeine are described in detail elsewhere [9].

The mass spectrum associated with ions 194 (mass-ion), 109 and 67, identified as the most characteristic ones present in caffeine, as given in Figure 1. As an additional criterion, the Association of Official Racing Chemists (AORC) recommends to verify the mass-charge ratios of these characteristic ions against their own agreed international reference standards for confirmation of positiveness (for caffeine).

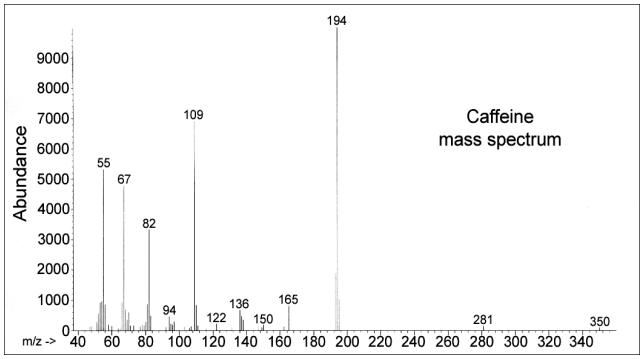


Figure 1. Caffeine mass spectrum.

Following international practices, the proposed method was then validated against classical critical factors:

Validation for selectivity, understood as the absence of interferences in the biological matrix studied was proven in all samples analyzed, cf Table 1.

Table 1. Validation for the critical factor selectivity

Sample	RT* (min)	Interferents
A	11,198	Absence
B	11,197	Absence
C	11,197	Absence
D	11,196	Absence
E	11,196	Absence

* Retention time

Validation for detection limit of caffeine in urine, found to be 6 ng/mL with a relative standard deviation ≤ 20 % in agreement with the criterion suggested by Chasin [10], cf Table 2. 6ng/mL was the concentration that produced a caffeine positive result for 30% of all replications of the 20 samples analyzed (100 samples in total) in five different concentrations (4; 6; 8; 10 and 12 ng/mL).

Table 2. Validation for detection limit and repeatability

Concentration ng/mL		**4**	**6**	**8**	**10**	**12**
RSD (≤ 20%) (AREA)	Íon 194	20	20	20	14	17
	Íon 194*	15	19	10	13	11
	R 194/109	7	8	12	9	8
	R 194/67	15	20	10	15	7
	R 109/67	17	12	10	13	8
RSD ≤ 2%	RT Íon 194	0,030	0,031	0,036	0,037	0,038

* with correction – R: Ratio – RT: Retention time

Validation for linearity, proved to be linear within $r^2 > 0.90$ as verified by linear regression ($r^2 > 0.90$ is the criterion recommended by INMETRO [11]).

Validation for the degree of accuracy, critical factor assessed by comparison of the actual amount of caffeine present in a pre-established reference solution with the one obtained by the proposed analytical procedure, cf Table 3.

Table 3. Validation results for the critical factor accuracy

Concentration (ng/mL)	**Ion 194 – obtained amount***	**Ion 194 – real amount***	**Accuracy %**
Blank Sample	109867	0	
6	487792	591870	82
8	676300	840451	80
12	1016304	1316557	77
18	1561100	1825573	85
24	2268996	2563160	88

* Chromatographic response

Validation for precision, assessed on basis of **repeatability** (adequate degree of agreement between the subsequent measurements, cf Table 2) and **reproducibility** (degree of agreement between measurements carried out by different technicians at different days, cf. Tables 4a and 4b). For the repeatability test, 100 samples in total (in the following five different concentrations, 4, 6, 8, 10 and 12 ng/mL) were analyzed.

Table 4a. Validation for the critical factor reproducibility

Concentration ng/mL		**6**	**8**	**12**	**18**	**24**
RSD (≤ 20%) (AREA)	Íon 194	12	10	19	11	7
	Íon 194*	4	5	4	5	16
	R 194/109	6	5	7	2	0
	R 194/67	17	11	5	6	5
	R 109/67	12	16	5	8	4
RSD ≤ 2%	RT Íon 194	0,045	0,033	0,018	0,005	0,024

* with correction – R: Ratio – RT: Retention time

Table 4b. Validation results for critical factor reproducibility

Concentration ng/mL		**6**	**8**	**12**	**18**	**24**
RSD (≤ 20%) (AREA)	Íon 194	19	11	12	17	9
	Íon 194*	5	7	4	11	5
	R 194/109	6	5	1	3	4
	R 194/67	13	8	10	1	3
	R 109/67	7	7	9	2	5
RSD ≤ 2%	RT Íon 194	0,010	0,017	0,035	0,031	0,017

* with correction – R: Ratio – RT: Retention time

Validation for recuperation, verified through the monitoring of two susequent reproductive analyses (88 ± 22% at the first day and 92 ± 25% at the second day).

Validation for robustness, evaluated by analyses of samples with pH varying in the 9.2-10 range (9.2 for the lowest concentration and 10.0 for the highest). Table 5, summarizes the results emphasizing that even for a wider pH range than the one specified by the proposed method (9.4-9.8) caffeine can be determined within a relative standard deviation smaller than 20%.

Table 5. Validation results for critical factor robustness

Concentration ng/mL		**6**	**8**	**12**	**18**	**24**
RSD (≤ 20%) (AREA)	Íon 194	11	16	7	17	2
	Íon 194*	5	1	5	15	8
	R 194/109	11	2	1	6	1
	R 194/67	16	9	16	13	2
	R 109/67	20	7	15	14	3
RSD ≤ 2%	RT Íon 194	0,090	0,076	0,048	0,049	0,003

* with correction – R: Ratio – RT: Retention time

5. INTERLABORATORY COMPARISON

Committed to formally demonstrate its technical competence, the LAD/JCB participates regularly in international proficiency testing schemes organized by non-profit associations dedicated to the improvement of measurement quality. Participation in such proficiency testing is certainly a reliable and a valuable continuous improvement tool to monitor and improve laboratory performance as it helps laboratories to gain insight into their measurement processes. It also provides to the laboratory' clients an unbiased analysis of measurement processes to enhance awareness of their technical competence and data integrity.

Association of Official Racing Chemists

This Document Certifies that

Anti-Doping Laboratory, Jockey Club Brasileiro

has participated in the *2005* Proficiency Testing Program,

and has successfully isolated and identified the required number of unknown specimens in accordance with the Association's requirements.

Signature
Chairman, Proficiency Testing Committee

Signature
President, AORC

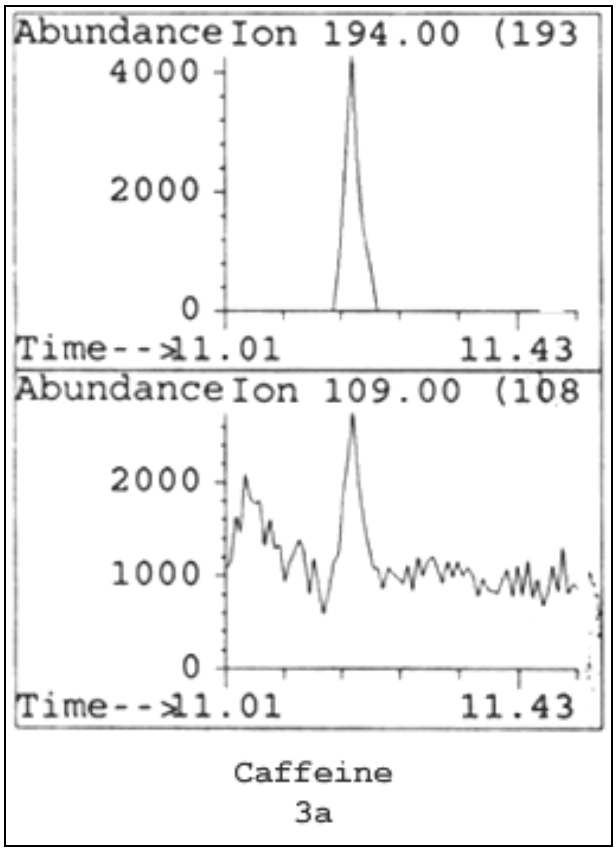
AORC

Figure 2. Certificate of participation in the 2005 round robin Proficiency Test conducted by the Association of Official Racing Chemists.

In the present case, after the mass-spectrometry analytical method ALCAC-18 was validated against the classical critical factors discussed in the previous session, the method was exposed to the international proficiency test program coordinated by the Association of Official Racing Chemists (AORC). An independent professionally administrated interlaboratory comparison recognized as being technically sound and in conformance with required standards. Typical from this type of interlaboratory qualitative comparisons the performance of the participant laboratory is expressed in a form of a certificate of participation. A certificate which instead of exhibiting comparative results reported by all participant laboratories it certifies the measurement performance of all participant laboratories. Figure 2 confirms the participation of the LAD/JCB in the qualitative 2005 AORC round robin which involved 47 laboratories from different countries. As documented in the full report accompanying the certificate, based upon an 80% approval criterion, LAD ranked among the top laboratories which have successfully identified the required number of specimens in accordance with the pre-established requirements within correctness of 100%.

6. RESULTS

Figure 3 depicts typical spectrograms used for validating the method by means of the analytical method taking into account positive and negative binary responses. The visual analysis of these spectra induces the analyst to issue a positive (3b in Figure 3) or negative (3a in Figure 3) binary response as a function of the abundance of the caffeine characteristic ions found in the samples.



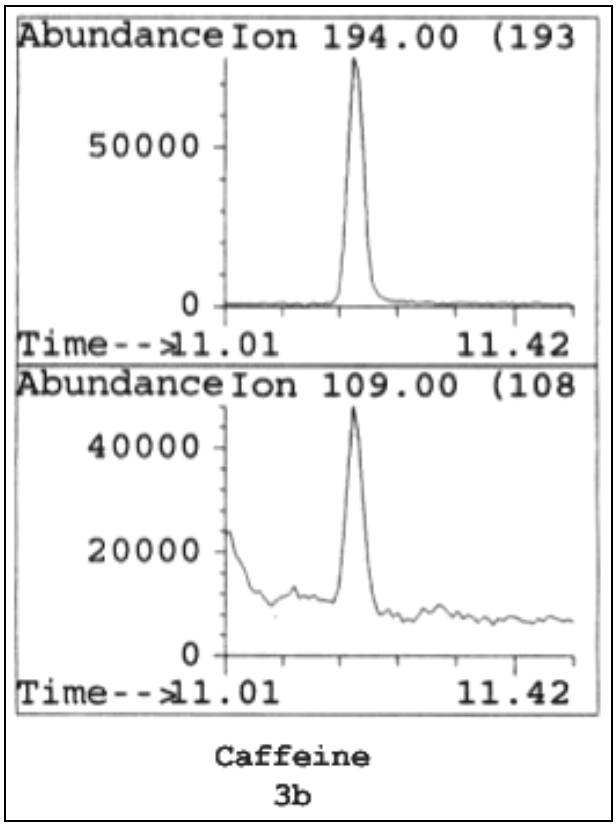


Figure 3. Typical spectrograms used in the validation of the analytical method (3-a: negative binary response. 3-b: positive binary response).

7. CONCLUSIONS

Horseracing activities are a promising market segment for mass-spectrometry laboratories offering services.

The implemented analytical method ALCAC-18 was in-house validated by traditional critical factors, and independently verified by its successfully participation in the 2005 round robin interlaboratory comparison proficiency testing programme organized by the International Association of Official Racing Chemists [12]. Consolidated results confirm the validation of the method based on the traditional critical factors: detection limit, selectivity, accuracy, linearity, precision (repeatability and reproducibility), recuperation and robustness.

Scientifically validated and internationally recognized, the method was then incorporated as a routine LAD/JCB method considered adequate and reliable for the determination of caffeine in the urine of (thoroughbred) racehorses. A reliable method to indicate the positiveness of the results found in the samples adding reliability to the measurement certificates issued by the LAD/JCB.

Following a unique approach, an evolution of the work is in progress to introduce an additional critical factor ("determination limit") intended to provide a convenient estimate of uncertainty associated with the qualitative determination of caffeine therefore contributing for this challenge process of expressing associated uncertainties.

Committed to become a reference horseracing antidoping laboratory for the region, LAD/JCB understands that accreditation and participation in international intercomparison laboratory programmes are pre-conditions for obtaining international mutual recognition of its doping tests performed.

8. ACKNOWLEDGEMENTS

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Annex A:

The most frequent forbidden substances in the horseracing activity (1996-2005)

Summary of a comprehensive internal survey carried out within the Antidoping Laboratory of the Brazilian Jockey Club. Between 1996 and 2005, the LAD/JCB has performed 50,394 analyses on racehorses' urine

Year >>	**1996**	**1997**	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**
Substances	Total number of testing: 2,643	Total number of testing: 4,891	Total number of testing: 4,649	Total number of testing: 5,492	Total number of testing: 5,709	Total number of testing: 5,151	Total number of testing: 5,490	Total number of testing: 5,746	Total number of testing: 5,452	Total number of testing: 5,171
Caffeine	**3**	**3**	**11**	**8**	**6**	**4**	**10**	**4**	**6**	**4**
Lidocaine	1	1	1	Abs	Abs	2	3	3	Abs	5
Procaine	Abs *	2	2	Abs	Abs	Abs	1	Abs	Abs	Abs
Mephentermine	Abs	1	Abs	Abs	Abs	Abs	Abs	Abs	Abs	Abs
Furosemide	Abs	1	1	Abs	1	1	Abs	Abs	Abs	Abs
Phenylbutazone	Abs	1	1	Abs	Abs	Abs	3	2	1	1
Isoxsuprine	Abs	1	Abs	1	1	Abs	Abs	Abs	Abs	1
Dexamethasone	Abs	Abs	Abs	Abs	1	Abs	Abs	Abs	Abs	Abs
Oxyphenbutazone	Abs	Abs	Abs	2	5	4	1	5	3	2
Dipyrone	Abs	Abs	Abs	Abs	1	1	2	Abs	1	2
Nimesulide	Abs	Abs	Abs	Abs	1	Abs	Abs	Abs	Abs	Abs
Clenbuterol	Abs	Abs	Abs	Abs	Abs	Abs	2	Abs	1	4
Diclofenac	Abs	Abs	Abs	Abs	Abs	Abs	1	2	1	1
Celecoxib	IM**	IM	IM	IM	Abs	Abs	1	Abs	Abs	Abs
Flunixin	Abs	Abs	Abs	Abs	Abs	Abs	12	10	17	2
Theobromine	Abs	Abs	Abs	Abs	Abs	Abs	2	Abs	1	Abs
Theophyilline	Abs	Abs	Abs	Abs	Abs	Abs	1	Abs	1	Abs
Positive cases	***4***	***10***	***16***	***11***	***16***	***12***	***39***	***26***	***32***	***22***

*****Abs: Absent – ******IM: Inexistent Method