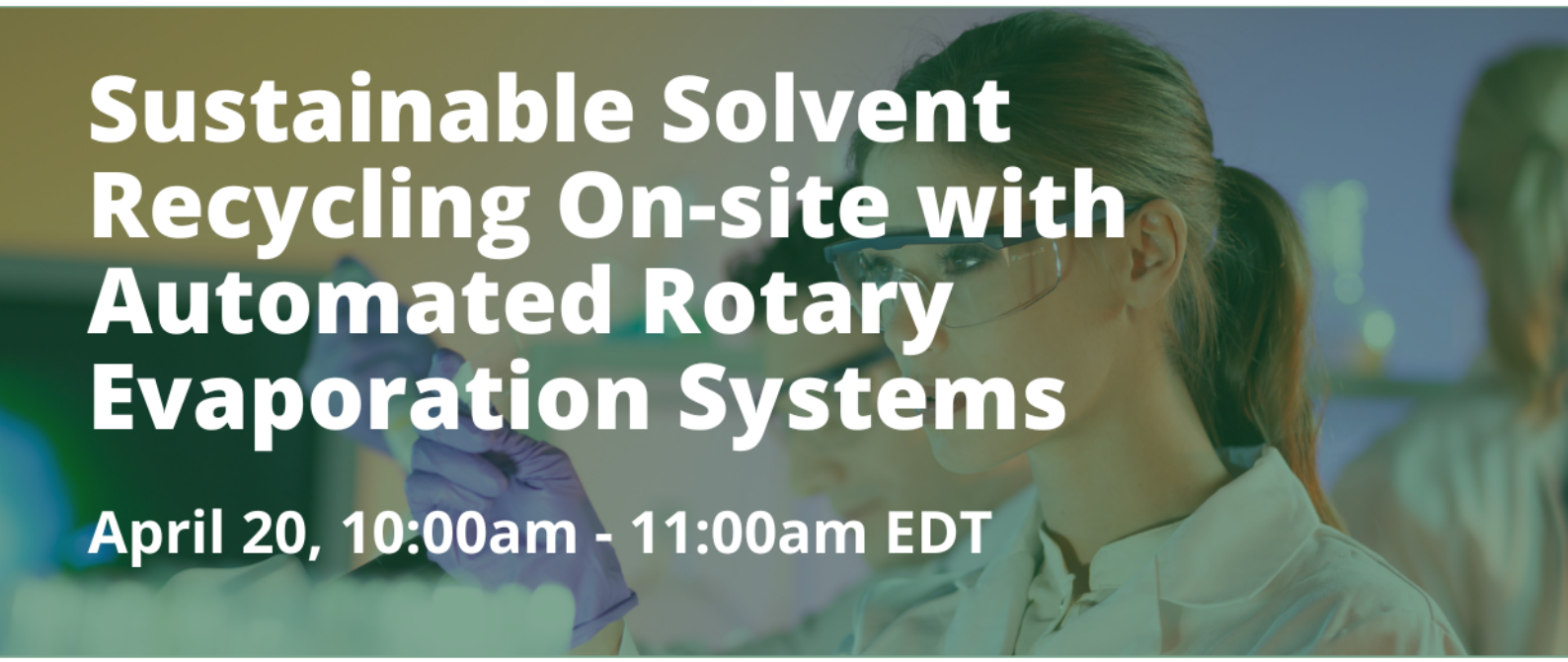
Wiley Analytical Science



Sustainable Solvent Recycling On-site with Automated Rotary Evaporation Systems

April 20, 10:00am - 11:00am EDT

With the steadily increasing costs of solvents and the huge CO2 emissions associated with the production of new solvents, on-site solvent recycling is not only a suitable way to reduce operating costs in the lab but also a sensible step towards more sustainable processes. An example is the life cycle of acetone: the production of 1 ton of acetone causes about 2000 kg of CO2 carbon footprint.

Watch this session during the WAS Virtual Conference:

Jürgen Heyder

Nate Kunzer

Lee Hibbett

Register Now

This talk is sponsored by heidolph research made easy

Report - Sport doping and medicine

Drug Testing and Analysis

Received: 31 July 2017 Revised: 10 October 2017 Accepted: 11 October 2017 Published online in Wiley Online Library: 16 November 2017

(www.drugtestinganalysis.com) DOI 10.1002/dta.2329

Doping control analysis at the Rio 2016 Olympic and Paralympic Games

Henrique Marcelo Gualberto Pereira,[a]* Vinicius Figueiredo Sardela,[a] Monica Costa Padilha,[a] Luciana Mirotti,[a] Alessandro Casilli,[a] Fabio Azamor de Oliveira,[a] Gustavo de Albuquerque Cavalcanti,[a] Lucas Martins Lisandro Rodrigues,[a] Amanda Lessa Dutra de Araujo,[a] Rachel Santos Levy,[a] Pedro Antonio Castelo Teixeira,[a] Felipe Alves Gomes de Oliveira,[a] Ana Carolina Giordani Duarte,[a] Ana Carolina Dudenhoeffer Carneiro,[a] Joseph Albert Medeiros Evaristo,[a] Gustavo Ramalho Cardoso dos Santos,[a] Giovanni Carlo Verissimo da Costa,[a] Fernando de Lima Castro,[a] Fabio Cesar Sousa Nogueira,[a] Fernanda Bertão Scalco,[a] Luciana Pizzatti[a,b] and Francisco Radler de Aquino Neto[a]

This paper summarises the results obtained from the doping control analyses performed during the Summer XXXI Olympic Games (August 3–21, 2016) and the XV Paralympic Games (September 7–18, 2016). The analyses of all doping control samples were performed at the Brazilian Doping Control Laboratory (LBCD), a World Anti-Doping Agency (WADA)-accredited laboratory located in Rio de Janeiro, Brazil. A new facility at Rio de Janeiro Federal University (UFRJ) was built and fully operated by over 700 professionals, including Brazilian and international scientists, administrative staff, and volunteers. For the Olympic Games, 4913 samples were analysed. In 29 specimens, the presence of a prohibited substance was confirmed, resulting in adverse analytical findings (AAFs). For the Paralympic Games, 1687 samples were analysed, 12 of which were reported as AAFs. For both events, 82.8% of the samples were urine, and 17.2% were blood samples. In total, more than 31 000 analytical procedures were conducted. New WADA technical documents were fully implemented; consequently, state-of-the-art analytical toxicology instrumentation and strategies were applied during the Games, including different types of mass spectrometry (MS) analysers, peptide, and protein detection strategies, endogenous steroid profile measurements, and blood analysis. This enormous investment yielded one of the largest Olympic legacies in Brazil and South America. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: anti-doping; Olympic and Paralympic Games; athlete biological passport; mass spectrometry; WADA-accredited laboratory

Introduction and facility description

The XXXI Summer Olympic Games (August 3–21, 2016) and the XV Paralympic Games (September 7–18, 2016) were held in Rio de Janeiro, marking the first time that these events were organised in South America. Doping control analyses have been conducted in Brazil since 1989, were incorporated into the International Olympic Committee (IOC) accreditation in 2002, and were absorbed by the World Anti-Doping Agency (WADA) accreditation system in 2005.[1,2] The Rio de Janeiro doping control team previously collaborated with the international anti-doping system, performing analyses for major events, such as the 2007 Pan-American Games,[3] the 2011 World Military Games, and the 2013 FIFA Confederations Cup. Nevertheless, the constant evolution of doping strategies and the increasing technical demand from WADA required a complete restructuring of the Brazilian anti-doping system. The milestone of this new era was the construction of a new laboratory facility on the campus of Rio de Janeiro Federal University (UFRJ) to support the Olympic and Paralympic Games.

* *Correspondence to: Henrique Marcelo Gualberto Pereira, Federal University of Rio de Janeiro – UFRJ, Brazilian Doping Control Laboratory – LBCD, LADETEC, Rio de Janeiro, Brazil.*
E-mail: henriquemarcelo@iq.ufrj.br; hmdoping@hotmail.com

a *Federal University of Rio de Janeiro – UFRJ, Brazilian Doping Control Laboratory – LBCD, LADETEC, Av. Horácio Macedo, 1281 - Polo de Química - Bloco C - Cidade Universitária - Ilha do Fundão - Rio de Janeiro, Brazil*

b *Federal University of Rio de Janeiro – UFRJ, Laboratório de Biologia Molecular e Proteômica do Sangue – LABMOPS, LADETEC, Av. Horácio Macedo, 1281 - Polo de Química - Bloco C - Cidade Universitária - Ilha do Fundão - Rio de Janeiro, Brazil*

The new facility, christened Laboratório Brasileiro de Controle de Dopagem (Brazilian Doping Control Laboratory – LBCD-LADETEC/IQ-UFRJ), has 5500 m^2 of usable area spread across 3 floors and 2 wings. One wing floor housed the sample reception area on the first floor, with 6 independent opening and splitting urine sample workstations. Blood samples are handled in a dedicated area near the cold-room storage areas with a storage capacity of 16 000 urine/blood samples. Security measurements were taken to hamper access by the sample delivery team to the sample opening area. The first floor also housed a chromatography/mass spectrometry (MS) hall (30 mass spectrometers) and a gas chromatography–combustion–carbon isotope ratio mass spectrometry (GC–C–IRMS) sample preparation laboratory. The second floor contained areas for the analyses of both DNA and protein and lab for B samples. Finally, the third floor housed the liquid chromatography (LC) and GC sample preparation areas; a dedicated laboratory for human growth hormone (hGH) – isoform and biomarker methods, including radioimmunoassay infrastructure; a reporting room, which was a management area for final reporting and connecting to the anti-doping administration and managing system (ADAMS); the technical manager's office; and dressing and resting rooms. The other wing housed offices, an information technology (IT) room, the main documentation storage room, a storage area, a canteen, a seminar room, a room for Internet access, a calibration room for volumetric materials, meeting rooms, backups of the main instruments (GCs, LCs, and mass spectrometers), preparation laboratories, and cold rooms. The complete blueprint of the facility is presented in Supporting Information Figure S1.

Special security measures

As expected, several security measures were implemented during the Games. These measures were taken particularly seriously in light of the recent doping scandals described in the McLaren Report.[4] The National Secretariat for Major Events Security (SESGE) from the Brazil Ministry of Justice coordinated the work, assembling all security-related institutions to ensure their harmonic operation. It also dispatched a group from the National Guard (*Força Nacional*), which is a special task force for national interventions. These efforts were supervised by the Brazilian Federal Police. In addition, a private security company was hired to guarantee lab security. All parties were placed in contact with the Games Crisis Centre (GCC) for our city region (the Maracanã region) and were in direct contact with the nearby military police battalion and the Rio Police Department. Specific authorisation was needed to pass through the gates, and a direct line between the gates and the LBCD's own 24/7 Crisis and Emergencies Group guaranteed the efficacy of this procedure. The LBCD site and its annexes had approximately 200 surveillance cameras with high-definition images stored for at least 12 months, according to WADA recommendations. The images taken from the cold chamber entrance, the sample reception, and the opening and aliquoting areas during the Games up to the shipment of the samples for long-term storage in Lausanne, Switzerland, were copied and delivered to the IOC and International Paralympic Committee (IPC). The entrance to the sample storage cold room was monitored by security guards 24 hours a day, and a mosaic of its cameras' images was displayed in the security control room for 24-hour surveillance. Entrance to the cold room was permitted only to at least 2 staff members together, and their stay was timed by the security guard. Any stay longer than 5 minutes was registered in a logbook, including details of the operation to justify the delay. The LBCD was also included under the umbrella of the Centre for Cybernetic Security (CD-Ciber) from the Ministry of Defence, which encompasses all the armed forces. The UFRJ has its own security detachment and cybersecurity unit from the IT superintendence, which are both directly in contact with the outfits mentioned. Despite the UFRJ being one of the most attacked sites in Brazil, there was no breach in cybersecurity during the Games. The connection from the LBCD to WADA's ADAMS data bank worked well for reporting results.

Additionally, all WADA requirements described in the World Anti-Doping Code International Standard for Laboratories[5] were fully heeded regarding Major Events accreditation requirements. Particular emphasis was placed on the physical layout of the space to ensure that adequate separation of various parts of the laboratory was maintained, and the number of individuals in controlled zones was limited based on work function using a combination of chip cards and fingerprint scanners. Moreover, all personnel had a thorough knowledge of their responsibilities, including the security of the laboratory, confidentiality of results, laboratory internal chain of custody protocols, and standard operating procedures (SOPs) for any method performed.

Accreditation, scope, and results management

The analytical strategies were defined according to the List of Prohibited Substances 2016 issued by WADA.[6] For the first time in the history of the Summer Olympic/Paralympic Games (O&PG), the analytical scope included erythropoiesis-stimulating agent (ESA) blood analysis. In addition, detection of growth-hormone-releasing peptides (GHRPs) and growth-hormone secretagogues (GHSs) were performed for all urine samples. All analytical and managerial procedures were accredited by the Brazilian National Metrological Institute (INMETRO) according to the ISO/IEC 17025 standard in conjunction with the WADA International Standard for Laboratories[6] (ISL v 9.0). The results were released electronically through ADAMS. The requirements of the new WADA Technical Documents/Guidelines were fulfilled a few months before the Games, including guidelines for IRMS, human chorionic gonadotrophin (hCG), luteinising hormone (LH), and 19-norandrosterone (19-NA) quantification and reporting, creating an additional challenge for the LBCD regarding proper implementation.

The Laboratory Information Management System (LIMS) was customised through the platform Sample Manager version 11.0 (Thermo Scientific, Manchester, UK). This software operates on a Windows server system based on an Oracle database in the c#. NET language. LIMS was accessible from any PC in the lab, operating with up to 80 simultaneous accesses. Access was restricted by login and password, which were personalised according to associated rights and functionalities. All user activity was logged and stored in the database for future reference. The data were added manually, and in several cases, bar coding was used to ensure the accuracy of the sample and aliquot numbers, except for the steroid profile, which was automatically updated from a csv file located in the LBCD database. To export the results, an xml file was generated per batch and per sample or group of samples and exported into ADAMS. Despite the complexity of the operation, which involved dozens of technical staff interacting with the system simultaneously, LIMS performed very well.

Personnel staff

In total, approximately 700 people were on staff during the O&PG in Rio. The technical team comprised 125 LBCD staff and 120

national and 79 international volunteers. As has become customary during the Games, the foremost doping control experts from around the world (international volunteers) were reunited in Rio, joining forces with the Brazilian team to attain the goal of reporting flawless results in due time. Notably, during the preparation of the Games, more than 50 training-purpose missions were accomplished, which counted as approximately 500 training days in WADA-accredited laboratories. The supporting team, including those charged with security, alimentation, transportation, medical assistance, external technical support, and cleaning, featured more than 300 people.

Experimental

Sample preparation

The main analytical protocols used as initial testing procedures (ITPs) and confirmation methods are described in Tables 1a and 1b.

Instrumental parameters

The instrumental parameters used during the Games are described in Table 3. Maintenance of all instruments followed the internal protocol established by the laboratory.

Quality control strategy

To ensure the quality of the analytical screening procedures, appropriate negative and positive quality control (QC) samples were included in all analyses. All QC charts followed the Westgard approach.[17] An SOP regulated the policy for control charts, which was established to monitor the performance of different methods. For the chromatography-MS-based methods, 2 positive controls were used: blank urine samples spiked with reference materials below the minimum required performance levels (MRPL) or threshold values (for threshold substances) and excretion urine samples from a reference collection. The QC strategy aimed to avoid unnecessary re-preparations/confirmations. A set of 13 control charts for each of the 12 GC tandem mass spectrometry (GC–MS/MS) instruments was established to follow the steroid profile parameters. For homologous blood transfusion (HBT), certified phenotyped red blood cells (ID-Diacell, Bio-Rad, São Paulo, Brazil) were used as controls. Immunoassay-based methods also adopted internal QCs in addition to those provided by the kit supplier. The performance of the hGH isoform method was controlled by 16 QC charts, including concentrations of recombinant (REC) and pituitary (PIT) hormone isoforms present in the kit controls and in-house predefined internal quality controls (iQCs), which were prepared by spiking different concentrations of recombinant somatropin standard (REC) (NIBSC 98/574) and pituitary human growth hormone standard (PIT) (NIBSC 80/505, UK) in sheep serum (Sigma 52263, St. Louis, MO, USA) to reach REC concentrations of 0.1, 0.5, 2.5, or 12.5 ng/mL and REC/PIT ratios between 1.5 and 2. For hGH biomarkers, high QCs were provided by WADA from an administration study, and human serum from Sigma (H4522, St. Louis, MO, USA) was used for low QCs. Figure 1 shows QC charts generated using data results obtained since the method validation for 2 iQCs of hGH isoforms (Figure 1). In total, the LBCD used 85 control charts to monitor the performance of the methods established for the Games.

Results and discussion

Test distribution plan: sample delivery, reception, and reporting

Because of the large number of samples, short reporting time, and need for an extensive analytical scope, the Summer Olympic Games is an unparalleled challenge in terms of logistics and workload. The commitment of the LBCD was to provide the same treatment to both Games; nevertheless, for the sake of clarity, information on the O&PG will be described separately whenever appropriate.

In agreement with the Testing Authorities (TA), the IOC, or the IPC, the reporting time for the urine screen analysis was established as 30 hours. This reporting time – somewhat longer than usual – was possible because 6 hours were conferred by using the LBCD reception area as a sample hub instead of an external hub to accumulate samples before they were delivered to the lab. Nevertheless, the LBCD's goal was to deliver results within 24 hours. The number of urine samples per batch was limited to 20 because of the number of controls necessary to guarantee accurate results in steroid profile measurements. This limited number of samples allowed a faster turnover of batches for the data evaluation team for both GC and LC screenings. This quick turnover enabled earlier decisions on batch reanalysis, suspicious sample evaluation, and confirmation procedure initiation. Elimination of the external hub provided the flexibility necessary for the staff to decide when to release sample batches. This strategy was shown to be efficient and should be considered for future Games or any major event. Figure 2 presents the number of urine and blood samples received per day at the LBCD during the O&PG, which were counted every day at 5 am. The busiest 14 days (August 7–20) had an average of 270 samples per day. The highest number of daily samples, ie, 350, occurred on August 11.

According to the internal procedure, all samples were inspected carefully as soon as they were received. A pre-established non-conformity (NC) list was created in agreement with the TA. All non-conformities were immediately reported to the TA, and it was agreed that the laboratory would conduct the analyses before obtaining the TA's directions whenever possible. The NC list is available in Supporting Information Table S1. For urine samples, pH and specific gravity were measured, and all pertinent information was added to LIMS. Blood samples were handled by a dedicated team trained to register, aliquot, and analyse the samples.

For the first time in Olympic history, doping control analyses were performed in respect of the in- and out-of-competition strategy (IC/OOC) regarding the WADA prohibited list.[6] The criterion was to flag as OOC all samples collected up to 24 hours before the competition; subsequently, the substances evaluated were restricted according to the WADA prohibited list.[6] The LBCD held the Olympic samples for a period of 32 days, from July 26 at 1:13 am to August 22 at 7:49 am. Paralympic samples were held for 26 days, from August 24 at 9:25 am to September 18 at 8:56 pm. Analyses were performed beyond that period, particularly for late and post-Games requests for IRMS analyses. Figure 3 presents the sample distribution observed during the O&PG regarding delivery time and classification between IC and OOC. As expected, the vast majority of samples were received by the laboratory between 6 pm and 6 am, consistent with the competition calendar. At night, OOC samples represented approximately 35% of the samples received. During the day, the fraction of OOC samples exceeded 70%. The large number of samples received between 2 am and 6 am is also noteworthy (Figure 3). This point

Table 1a. Summary of the ITP methods used during the Rio 2016 Olympic and Paralympic Games.

Class of substances	ITP methods
Anabolic agents, β-2 agonists, hormone and metabolic modulators, masking agents, stimulants, narcotics, carboxy-THC, β-blocker	To 2 mL of urine were added 40 μL of ISTD mix (Table 4), 750 μL of 0.8 M phosphate buffer, and 50 μL of β-glucuronidase obtained from *E. coli*. Incubated at 50°C for 1 hour, and then added 500 μL of aqueous buffer solution containing K_2CO_3/$KHCO_3$ (20% *w*/w) and 4 mL of TBME. After phase separation, the organic phase was evaporated under N_2 flow. Added 100 μL MSTFA/NH_4I/2-mercaptoethanol (1000:2:6), incubated at 60°C for 20 minutes and analysed by GC–MS–MS.[3]
hCG/LH	Homogenised urine (400 μL) was loaded into the automated immunoassay analyser.[3,7]
Anabolic agents, peptide hormones, growth factors, related substances and mimetics, β2 agonists, hormone and metabolic modulators, diuretics and masking agents, stimulants, narcotics, cannabinoids, glucocorticoids, β-blocker	Two urine aliquots were used. Aliquot I: 10 μL of urine was diluted in 50 μL of 2% acetic acid, centrifuged (10 G/5 minutes) and stored (10°C). Aliquot II: 10 μL of internal standard working solutions with 8 different ISTDs (Table 4) was added to 2.5 mL of sample. A 1.0 M acetate buffer solution (400 μL, pH 7.0) and 75 μL of β-glucuronidase obtained from *E. coli* were added and heated to 50°C for 1 hour. SPE with Strata-XC (30 mg) columns preconditioned with 2 mL methanol and 2 mL of ultrapure water. Sample was added and washed with 2 mL of ultrapure water and 1 mL of methanol:water (1:1). Elution with 3 mL of methanol:formic acid (95:5, *v*/v) followed by evaporation to dryness (N_2 flow at 40°C). The dried residue was diluted, and 8 μL of the resulting solution was injected on and analysed by LC–HRMS. Developed by the Rio 2016 Games team.
Plasma expanders	To 100 μL of urine was added 100 μL of $NaIO_4$ solution (5%) and 100 μL H_2SO_4 (2 N). Vortexing and incubation (6 minutes). Added 100 μL of $NaHSO_3$ (10%) and 50 μL of fuchsin reagent (0.3%) with 3% v/v ethanol. Incubated at 37°C for 15 minutes and at room temperature (15 minutes) and analysed by colourimetry.[8]
Erythropoiesis stimulating agents (ESAs)	For urine, 15 mL of sample was added to Tris–HCl buffer and protease inhibitor. Sample was concentrated by ultrafiltration and incubated in an anti-EPO ELISA plate. For blood, 0.25 mL of serum/plasma was analysed according to the kit (MAIIA Diagnostic, Sweden), and the immunoextract was concentrated by ultrafiltration. SAR-PAGE was loaded with 15 μL of sample (urine or blood). Running conditions: 125 V constant/3 hours, 10% Bis-Tris gel. Immunoblotting: 1.54 mA/cm^2, 45 minutes. Incubation with low-fat milk (5%) and dithiotreitol (DTT, 10 mM). BlotCycler incubations with antibodies at 4°C. Substrate was added, followed by chemiluminescence capture. Adapted from Reihlen et al.[9]
Haemoglobin-based oxygen carriers (HBOCs)	Fresh blood samples were centrifuged (1500 g for 15 minutes). Samples with altered plasma colour (> 0.6% Hemopure spiked in whole blood) had the haemoglobin concentration measured on a blood analyser instrument.[10,11]
Human growth hormone (hGH): Isoforms	Kits (CMZ-Assay GmbH, Germany) were used according to the manufacturer's instructions. Briefly, 50 μL of serum samples, controls, and calibrators were incubated with 150 μL of S buffer (2 hours, RT, 300 rpm), washed and incubated with 200 μL of tracer antibody. Washing procedure was repeated before luminometer reading.[12]
hGH: Biomarkers	IGF-I: Immunotech kit (Beckman-Coulter, Czech Republic) was used according to the manufacturer's instructions. Briefly, 25 μL of serum samples and controls were incubated with 500 μL of dissociation buffer (30–90 minutes, RT). 50 μL of calibrators and dissociated aliquots were incubated with 300 μL of tracer (1 hour, 280 rpm, RT). Washing procedure was performed before Gamma Counter reading.[13] P-III-NP: UniQ RIA kit (Orion Diagnostic, Finland) was used according to the manufacturer's instructions. Briefly, 100 μL of serum samples, calibrators, and controls were incubated with 200 μL of tracer and 200 μL of antiserum (2 hours, water bath, 37°C). Incubation with 500 μL of separation reagent (30 minutes, RT). Centrifuged (15 minutes, 2000 g, 4°C), supernatant discarded and Gamma Counter reading.[13]
Insulins, IGF-I analogues, GHRF (GH-RH), synacthen	Urine (5 mL) was subjected to SPE with an Oasis cartridge (60 mg, Waters Co., USA) after preconditioning. Elution with CH CN/H_2O and evaporation in a vacuum centrifuge. PBS reconstitution and immunopurification using magnetic beads adsorbed with mono- and polyclonal antibodies. Final elution performed with CH_3COOH. Analysed by LC–HRMS and nLC–HRMS/MS.[14]
Athlete biological passport (ABP)	Fresh blood samples in appropriate EDTA tubes were homogenised for at least 15 minutes and loaded in an automated blood analyser.[15]
Homologous blood transfusion (HBT)	Total blood (500 μL) was washed in CellStab (DiaMed, Brazil) and diluted to $10^{[6]}$ red blood cells (RBC)/25 mL. Samples (25 μL) and controls were labelled with RBC surface antibodies (BioRad, Switzerland) at optimal dilutions in a 96-well plate. Washed and incubated with PE-coupled IgM or IgG (Invitrogen, USA). Cell washing and flow cytometry acquisition. Unlabelled RBCs,

(Continues)

Table 1a. *(Continued)*

Class of substances	ITP methods
	glycophorin A and isotype control IgG1-FITC-labelled RBCs (Beckman-Coulter, France) were used as controls. Flow Check fluorospheres (Beckman, USA) were used for instrument check-up.[16]

Table 1b. Confirmation procedures applied to sample analyses during the Rio 2016 Olympic and Paralympic Games.

Class of substances	Confirmation procedure
Endogenous anabolic steroids, 19-NA, boldenone and its metabolite, formestane	Urine (10–25 mL) was purified with C_{18} SPE and drying. Phosphate buffer (1.5 mL, 0.2 M), 4 mL of TBME, and 100 μL of β-glucuronidase obtained from *E. coli* were added. Incubation was conducted for 1 hour at 50°C. K_2CO_3/$KHCO_3$ (500 μL, 20%, 1:1) buffer was added, and 2 instances of TBME LLE (2 × 4 mL) were performed. Solvent was dried under N_2 flow. For HPLC conditions, see Table 3. Analysed by IRMS.
Small peptides	SPE with STRATA X-CW cartridge (30 mg, Phenomenex, USA), elution with CH_3OH:CH_2O_2, and concentration by vacuum centrifuge. Final residue was reconstituted in CH_3COOH and analysed by LC–HRMS/MS or LC–MS/MS.
ESAs	Same procedure as the ITP (Table 1) but with double-blotting according Reichel et al.[8] Second transfer conditions: 1.0 mA/cm^2 for 10 minutes.
HBOCs	Native-PAGE followed by luminol exposure using 4%–15% native precast gels (Bio-Rad, São Paulo, Brazil).
HGH Biomarker (P-III-NP)	Immunoassay kit P-III-NP elf (Siemens, USA) was used according to the manufacturer's instructions. Briefly, after equipment maintenance washing, 100 μL of serum sample or QC was added to labelled tubes and read using an ADVIA Centaur system.
HGH Biomarker (IGF-I)	In 100 μL of human serum, it was added 400 μL of CH CN in acid medium. Evaporation in vacuum centrifuge until dryness. Fifty μL of NH_4HCO_3 was added, reduced with DTT, alkylated with C_2H_4INO, and digested with trypsin for 19 hours. Digestion step was stopped with H_2O_2, followed by analysis by LC–MS/MS.
Proteases	Urine (2 mL) supplemented with 10 μL of internal standard was subjected to SPE with a STRATA X-CW cartridge (30 mg) after preconditioning with 2 mL methanol and 2 mL of ultrapure water. Sample added and washed with 2 mL of ultrapure water and 1 mL of methanol. Sample was eluted with a CH_3OH:CH_2O_2 (95:5 *v*/v). Evaporation in a vacuum centrifuge, followed by residue reconstitution in CH_3COOH and analysis via LC–HRMS/MS or LC–MS/MS.
HBT	Same procedure as the ITP (Table 1), except that 2 additional concentrations of primary antibodies were used.

is critical for logistical preparation and should be considered when organising staff for future Games.

All classes of prohibited substances and methods were under surveillance, except for gene doping for specific genetic manipulation. Regarding gene-doping detection for ESAs, the first method developed with this goal is still undergoing final implementation in WADA-accredited laboratories, including the LBCD; if needed, analyses may be performed retrospectively.

During the Olympics, the LBCD received 4913 samples comprising 4071 urine and 842 blood samples (Figure 4) from 2781 male and 2132 female athletes. Among the urine samples, 905 were forwarded for ESA analysis, and 302 were requested for insulin and insulin-like growth factor-I (IGF-I) analogue analyses. Among the blood samples, 444 were analysed for the athlete biological passport (ABP) and 289 for hGH (isoforms and biomarkers analyses); additionally, among the same bottles, 154 were analysed for ESAs, 69 for HBT, and 805 for haemoglobin-based oxygen carriers (HBOCs). The total number of blood samples represents an increase of 5.8% compared with the number obtained during the 2012 Games in London. According to the preparation plan, the blood team also had proficiency in urine sample reception procedures; this flexibility was important in maintaining the flow of samples when a large number arrived outside of the Test Distribution Plan (TDP) agreed upon by the TA because of local organising committee logistical problems. In total, 2911 samples were flagged as IC and 2002 as OOC.

During the Paralympics, a total of 1687 samples were received, including 1396 urine and 291 blood samples. According to gender, 964 samples were from male athletes and 723 from female athletes. Among the urine samples, 205 were requested for ESA analysis, and no samples were forwarded for insulin or IGF-I analogue analysis. Among the blood samples, 2 were analysed for ESAs, 40 for HBT, 46 for ABP, 291 for HBOCs, and 244 for hGH, including isoform and biomarker analyses. Finally, 878 samples were flagged as IC and 809 as OOC.

ITPs and confirmation analyses

The screening approach was designed to use the lowest possible number of methods in order to meet the reporting time agreed. Hence, the methods employed needed to be as comprehensive as possible to cover the WADA prohibited list in a straightforward manner. For urine samples, 4 methods – GC–MS/MS, LC high resolution mass spectrometry (LC–HRMS), hCG, and LH – and colourimetry were used in the screening analysis. Blood sample analyses were performed as requested by the TA and included ESAs, HBOCs, hGH, ABP, and HBT.

Liquid chromatography mass spectrometry analysis

Orbitrap technology allowed a comprehensive approach, and the LC–HRMS method developed for the Games enabled the incorporation of the analyses of GHSs and GHRPs together with small molecules in 100% of the urine samples via a single injection after solid-phase extraction (SPE). This procedure was welcomed by the TA

Table 2. List of equipment available at the LBCD during the Rio 2016 Olympic and Paralympic Games. (N = quantity)

Key Instrument	Manufacturer (city, state and country)/model	N	Technical features
Procedure IV – Multi-analytes by GC–MS/MS			
GC–MS/MS	Thermo Scientific (Waltham, MA, USA)/ TSQ 8000	13	100% polydimethylsiloxane (Ultra-1®, 17 m × 0.2 mm × 0.11 μm; J&W Scientific, Agilent Technologies Inc.)
Procedure VI – hCG/LH			
Biochemical Analyser	Roche Basel, Switzerland/ Cobas e-411	1	ECL, solid-phase, 2-site chemiluminescent immunoassay
Biochemical Analyser	DPC Immulite (Munich, Germany)	1	Chemiluminescence, λ = 425–500 nm
Procedure VII – Anabolic agents by LC-HRMS			
LC–HRMS	Thermo Scientific (Waltham, MA, USA) / DionexLC - Q-Exactive Orbitrap	8	Thermo Scientific Syncronis, C18, 1.7 μm, 50 mm X 2.1 mm
LC–MS/MS	Thermo Scientific (Waltham, MA, USA) / DionexLC – TSQ Quantiva	4	Thermo Scientific Syncronis, C8, 1.7 μm, 50 mm X 2.1 mm
Procedure IX – Endogenous anabolic steroids, 19-NA, boldenone and its metabolite, formestane			
HPLC–DADTab	Thermo Scientific (Waltham, MA, USA) / Dionex UltiMate 3000	5	Waters XBridge™ Shield RP18 (250 × 4.6 mm × 5 μm) Waters XBridge™ C18 (150 × 4.6 mm × 5 μm)
GC–C–IRMS	Thermo Scientific (Waltham, MA, USA) / Trace 1310 – GC IsoLink II – Conflo IV – Delta V Plus	2	Agilent VF-17 ms (30 m × 0.25 mm × 0.25 μm)
GC–qMS–C–IRMS	Thermo Scientific (Waltham, MA, USA) / Trace 1310, ISQ LT, GC IsoLink II, Conflo IV, Delta V Plus	1	Agilent VF-17 ms (30 m × 0.25 mm × 0.25 μm)
Procedure X – ESAs			
Electrophoresis Cell	Life Technologies (Waltham, MA, USA)/ XCellSureLockMidiCell	4	Up to 4 midi-gels (8 × 13 cm)
		3	Up to 2 mini-gels (7 × 9 cm)
Power Supply	GE Healthcare Life Sciences (Chicago, IL, USA)/ EPS 3501 XL	2	3500 V, 400 mA, 200 W maximum 300 V, 500 mA, 150 W maximum
	Loccus Biotecnologia (Cotia, SP, Brasil)/ LPS 300 V	2	
Semi-dry Electrophoretic Blotting System	BioRad (Hercules, CA, USA)/Trans-Blot SD	1	Maximum gel size (W x L), 24 × 16 cm
	GE Healthcare Life Sciences (Chicago, IL, USA)/TE 70 PWR	1	Gels up to 14 × 16 cm
	CBS Scientific Company (Del Mar, CA, USA)/ EBU 6000	2	40 × 35 cm. Stainless steel cathode and platinum-coated titanium anode
BlotProcessor	Precision Biosystems (Mansfield, MA, USA)/ BlotCycler	3	Up to 2 midi membranes (60 samples) processing
Gel and Blot Image System	GE Healthcare Life Sciences (Chicago. IL, USA)/ImageQuant LAS 4000	1	Exposure Time: automatic, manual (1/100 seconds to 30 hours). Image Resolution Max. 3073 × 2048, 6.3 Mpixels
	Fujifilm (Minato, Japan)/LAS 3000	1	Image Resolution Max. 3072 2048
	GE Healthcare Life Sciences (Chicago, IL, USA)/Imager 600	1	Image Resolution Max. 3072 × 2048
Procedure XI – HBOCs			
Native-gels	BioRad (Hercules, CA, USA)/Mini Protean electrophoretic system	1	—
Procedure XII – hGH			
XIIa- hGH Isoforms Luminometer	Berthold Technologies (Baden-Württemberg, Germany) / AutoLumat Plus LB 953	2	Chemiluminescence: spectral range of 380 to 630 nm
XIIb- hGH Biomarkers	Perking-Elmer (Waltham, MA, USA)/ Gamma Counter Wizard 2470	1	Linear multichannel analyser of gamma radiation, within 15–1000 KeV range
	Siemens (Munich, Germany) / ADVIA Centaur CP	1	Direct chemiluminescence, acridinium ester technology
Procedure XV – ABP			

(Continues)

Table 2. *(Continued)*

Key Instrument	Manufacturer (city, state and country)/model	N	Technical features
Blood analyser	Sysmex (Kobe, Japan) XT-2000i	2	—
Procedure XVI – Proteins and peptides by MS			
XVIa	Thermo Scientific		
LC–MS QqQ	(Waltham, MA, USA)/	1	Syncronis® C8 (50 X 2.1 mm 1.7 μm)
LC–Orbitrap	TSQ Quantiva	1	
	Q-Exactive plus		
XVIb	Thermo Scientific		
LC–Orbitrap	(Waltham, MA, USA)/	2	Accucore® C18 (50 X 2.1 mm 2.6 μm)
nLC–Orbitrap	Q-Exactive plus	2	PicoChip® C18 (75 μm × 105 mm)
	Q-Exactive plus		
XVIc	Thermo Scientific (Waltham, MA, USA)/		
LC–MS QqQ	TSQ Quantiva	1	Syncronis® C8 (50 X 2.1 mm 1.7 μm)
XVId	Thermo Scientific (Waltham, MA, USA)/		
LC–Orbitrap	Q-Exactive plus	2	Syncronis® C8 (50 X 2.1 mm 1.7 μm)
LC–MS QqQ	TSQ Quantiva	1	
Screening XVIII – HBT			
Flow cytometer	Beckman-Coulter (Brea, CA, USA)/ FC-500 and Gallios	2	Automated 5-colour analysis with single or dual laser excitation

N = number of instruments

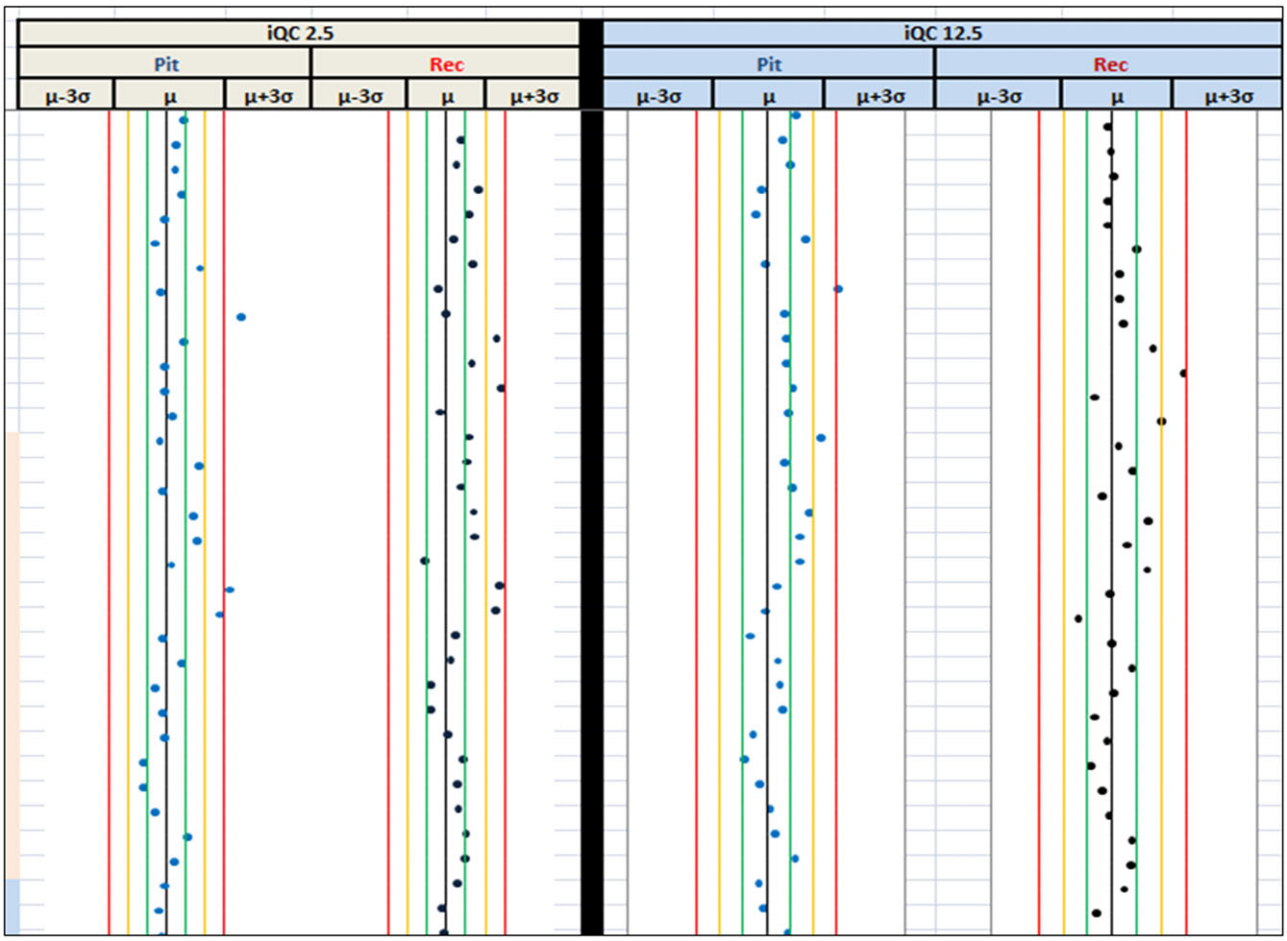


Figure 1. Control charts from in-house quality control samples (iQCs) containing 2.5 (iQC 2.5) or 12.5 ng/mL (iQC 12.5) recombinant hormone (REC) used for hGH isoform assays. Dots represent the concentrations of recombinant (REC) and pituitary (PIT) hormone in ng/mL. Averages (μ) and standard deviations (σ) were calculated after repeated analyses performed on different days and by different analysts.

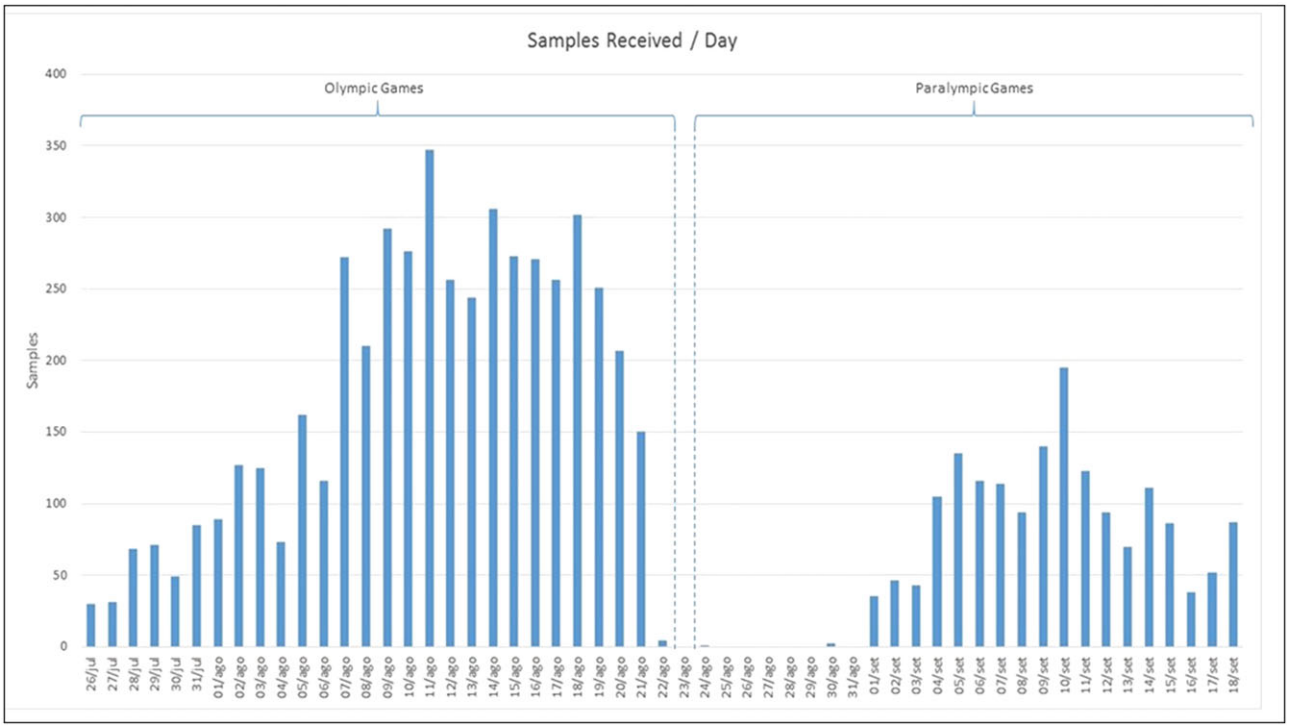


Figure 2. Olympic and Paralympic sample delivery profile, including urine and blood.

because it exceeded the requirements established by the Technical Document for Sport Specific Analysis (TDSSA version 2).[18] It also simplified the management of samples that otherwise would have needed to be selectively screened for different classes of substances.

Hence, the LC–HRMS screening analysis was performed for more than 400 target compounds, which is twice as many substances than the London 2012 LC–HRMS method.[8] Three QCs were employed (blank urine, reagent blank, and positive urine), and 9 internal standards (ISTDs) were spiked in all samples to

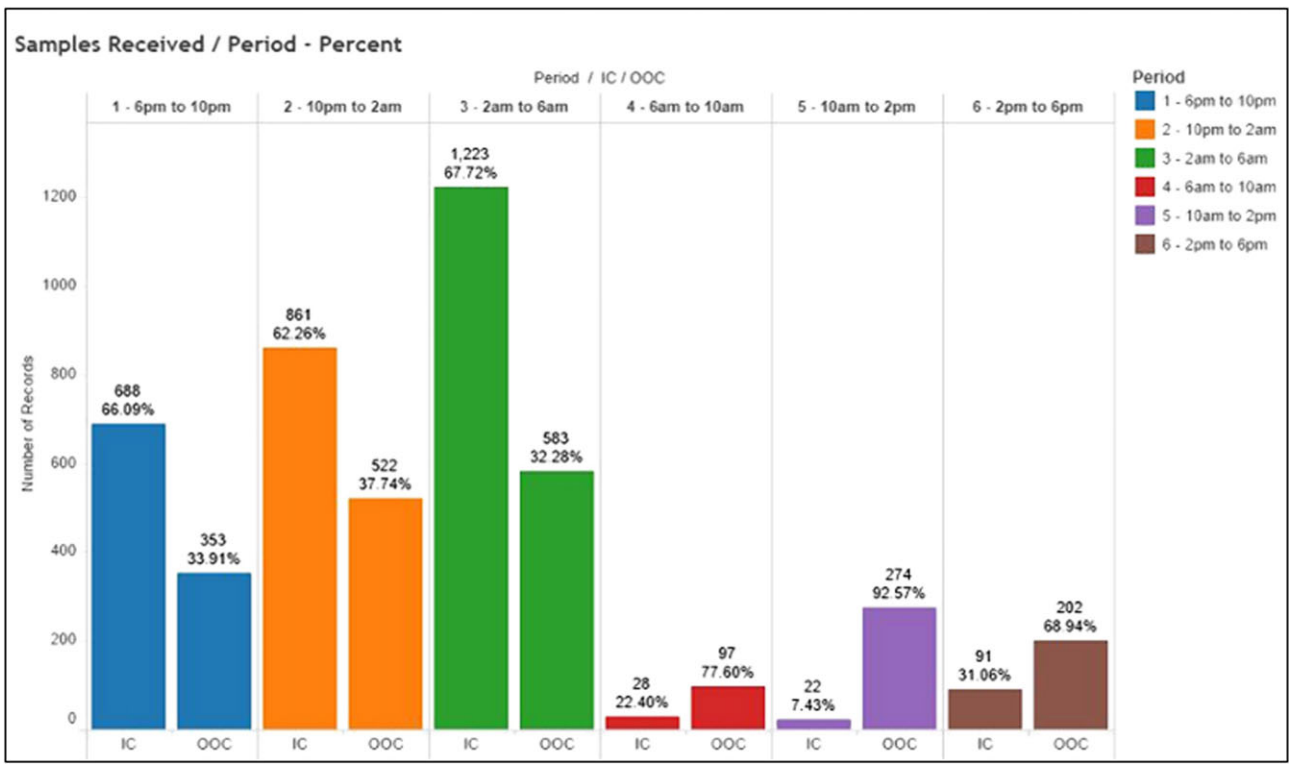


Figure 3. Sample distribution during the O&PG, separated by delivery time and IC/OOC classification.

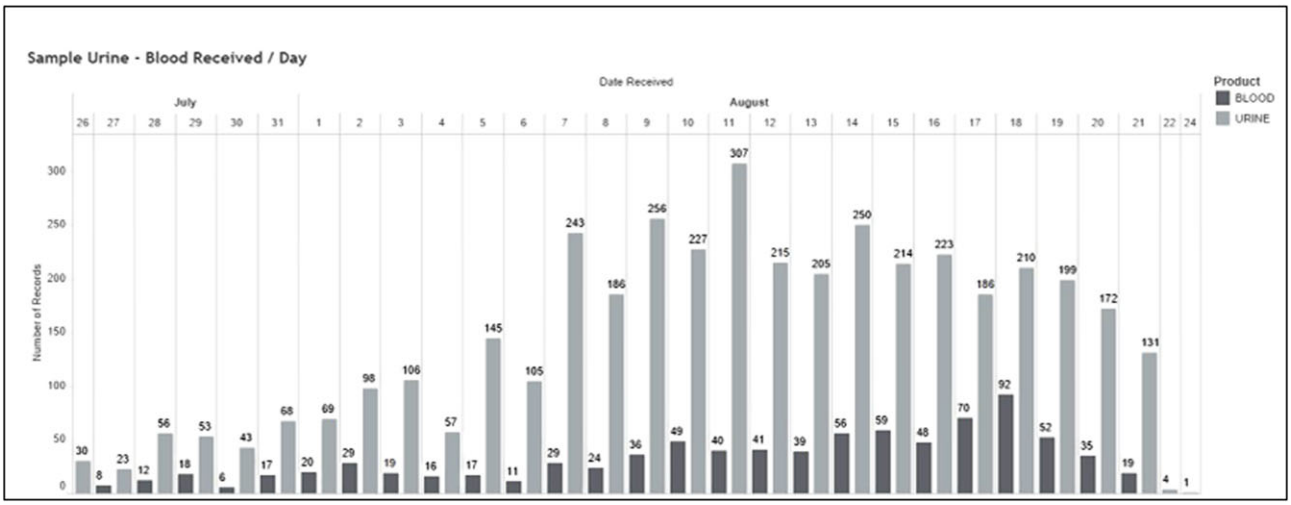


Figure 4. Urine and blood Olympic samples received in 24 hours.

Table 3. Internal standards applied to chromatography-based methods – low mass analytes.

LC–HRMS ITPs			
ISTD compounds	**ng/mL**	**Substance description**	**Evaluation aim**
7-Propylteophylline	40	Added in all validation procedures	Check whether the batch showed similar performance to validation
Buspirone	40	Used for Q-Exactive evaluation	Check whether the MS showed similar signal response to installation
Catine-D3	40	Ephedrine with the poorest response	Check the matrix effect on ephedrine detection
GHRP-4-D4	5	Peptide with the lowest recovery	Check sample preparation for peptide analysis
JWH-018-D11	4	Less polar cannabimimetic	Check the reconstitution for less polar compounds
Mefruside	100	Dual ionisation response	Check the ESI for negative and positive conditions
M-glicuronide-D3	20	Conjugated compound	Check the hydrolysis efficiency
Testosterone-D3	5	Steroid compound	Check the detection of steroid substances
A/Etio-glucuronide	–	Endogenous compound	Check whether the dilute-and-shoot fraction was added
GC–MS/MS ITPs			
ISTD compounds	**ng/mL**	**Substance description**	**Evaluation aim**
[16,16,17β-D3]-Epitestosterone [16,16,17α-D3]-Testosterone	15	Isotopically labelled internal standard	ISTD for single-point calibration approach to estimate E in "steroid profile"
[2,2,3β,4,4-D5]-Etiocholanolone [2,2,4,4-D4]-Androsterone	60	Isotopically labelled internal standard	ISTD for single-point calibration approach to estimate T in "steroid profile" and check the extraction efficiency of the ITP
β-glucuronic acid	500	Isotopically labelled internal standard	
[16,16,17-D3]- 5α-Androstanediol	500	Isotopically labelled glucuronide internal standard	ISTD for single-point calibration approach to estimate Etio and A in "steroid profile"
[2,2,3β,4,4-D5]-5β-Androstanediol	80	Isotopically labelled internal standard	Check the hydrolysis efficiency
	180	Isotopically labelled internal standard	ISTD for single-point calibration approach to estimate 5α-androstanediol in "steroid profile" ISTD for single-point calibration approach to estimate 5β-androstanediol in "steroid profile"

M, morphine; A, androsterone; Etio, etiocholanolone; ESI, electrospray ionisation.
E: Epitestosterone; A: Androsterone; T: Testosterone; Etio: Etiocholanolone

monitor different steps of the procedures (Table 3). Screening detection was achieved in 5 different MS experiments. Most of the substances were detected by a single signal based on $[M + H]^+$ or $[M-H]^-$ and the retention time determined in a chromatographic run of 11 minutes. Tetrahydrogestrinone, ethacrynic acid, modafinil, modafinil acid, *p*-hydroxyamphetamine, cathine, and acebutolol were detected by a specific fragment from all ion fragmentation (AIF) once all substances showed low specificity in the present approach. To improve sensitivity, a data independent analysis (DIA) was performed for methyldienolone, alexamorelin, aminoglutethimide, tetrahydrotestolactone, and oxilofrine. Two different reports were developed for data evaluation: one for all substances with reference materials available and the other for rare metabolites, scanning the entire chromatographic run based on only the theoretical exact mass. For both reports, the TraceFinder™ software from Thermo® was set to screening mode and used as a library for data evaluation. The LC–HRMS method in place during the Games was applied to 3500 samples before the Games. Three stress tests were performed, and the reports were shared by email with international scientists from fellow WADA labs for training purposes.

A few suspicious cases were observed; however, only 4% of the samples were reanalysed by an additional confirmation procedure. Before the start of a new aliquot analysis, a vial with a suspicious peak in its LC–HRMS spectrum was injected into an LC triple quadrupole mass spectrometer (LC–QqQ) in a different chromatographic column (C_8) and evaluated by 5 different transitions previously obtained for all monitored substances to accelerate the results. Approximately 1.3% of the samples remained suspicious even after reinjection of the same vial on the LC–QqQ; these samples had to be confirmed.

Polar substances were the most difficult for data analysis. Mildronate, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), and glycerol yielded numerous presumptive results in the ITPs and negative results upon confirmation analysis. An amide column was employed to confirm these substances, and early elution on the C_{18} column used during screening was observed to be the main problem, as these substances co-eluted with many other polar interferences. In addition, a target approach focusing on 19 protease autodigested peptides derived from trypsin, papain, subtilisin, and bacilysin was implemented in the LC–HRMS method for all urine samples. Hence, an inclusion list based on the m/z and retention time of each peptide was established to investigate protease misuse using the Q-Exactive plus mass spectrometer. Publications on these 2 tailormade methods developed for the Games are in preparation. One suspicious sample that indicated the presence of proteases was observed with this strategy. New aliquots were requested to perform the parallel reaction monitoring (PRM) or selected reaction monitoring (SRM) LC–MS confirmation method of analysis by HRMS or LC–QqQ, in which the detection of 3 transition ions should be obtained after fragmentation of a selected precursor ion to confirm an adverse analytical result. However, all analysed samples yielded negative results for protease manipulation.

Highly polar analytes such as glycerol and mannitol, which could be problematic under LC conditions, were screened by a previously described colourimetric method.[19] Approximately 1.2% of the samples were submitted to dedicated LC–MS methods developed for the confirmation of these analytes. All confirmations were negative.

Steroid profile

Changes in the endogenous profile of androgenic anabolic steroids (AASs) are markers of doping. In 2016, WADA's Technical Committee approved a new technical document on the interpretation of endogenous AASs in athletes[20] that introduced many other parameters for doping control in addition to those established in previous documents. During the ITP, the steroid profile was estimated using the single-point calibration approach instead of multipoint calibration because of the short Olympic timeframe. The performance of the single-point calibration approach was exhaustively assessed by WADA's external quality assessment scheme (EQAS), and the results were comparable to those of other WADA-accredited laboratories. The calibration samples were prepared by spiking synthetic urine with a reference material containing endogenous steroids in their free form. Nominal final concentrations of each compound in the calibration control are presented in Table 4.

Two QC samples, one of low concentration and one of high concentration, were prepared and analysed in every batch of samples. A set of 6 deuterated reference materials was used in the quantification procedure (Table 4). Among the materials, [2,2,4,4-D4]-androsterone β-glucuronic acid was used to monitor hydrolysis efficiency according to a ratio involving etiocholanolone-d5 spiked in its free form. A value of 1.00 corresponded to 100%, meaning that all androsterone-D4-glucuronide was hydrolysed.

The results were monitored by control charts. A derivatisation control was established by monitoring the androsterone-mono-OTMS/androsterone-bis-OTMS ratio. A value of 0.00 corresponded to 0%, indicating that no androsterone-mono-TMS derivative was formed and that derivatisation was complete. A ratio lower than 0.05 (5%) was adopted as a standard criterion. Microbial degradation control is performed by monitoring the 5β-androstanedione/etiocholanolone and 5α-androstanedione/androsterone ratios. Those values must always be lower than 0.10 (10%); otherwise, the steroid profile is reported as invalid.[20]

A direct channel was established between the Athlete Passport Management Units (APMUs – steroidal model) and the laboratory. Consequently, the LBCD was ready to start a new analytical batch

Table 4. Composition of endogenous steroids used for calibration control. Single-point calibration was adopted.

Endogenous steroid	ng/mL
11-ketoetiocholanolone (5β-androstan-3α-ol-11,17-dione)	800
Tetrahydrocortisol (5-pregnane-3α,11β,17α,21-tetrol-20-one)	1000
Pregnanediol (5β-pregnane-3α,20α-diol)	1000
Dihydrotestosterone (5α-androstan-17β-ol-3-one)	400
Dehydroepiandrosterone (5-androsten-3β-ol-17-one)	400
16-androstenol (5α-androst-16-en-3α-ol)	500
11β-hydroxy-androsterone (5α-androstan-3α,11β-diol-17-one)	400
11β-hydroxy-etiocholanolone (5β-androstane-3α,11β-diol-17-one)	200
5β-androstanediol (5β-androstane-3α,17β-diol)	80
5α-androstanediol (5α-androstane-3α,17β-diol)	180
Epitestosterone (androst-4-en-17α-ol-3-one)	40
Testosterone (androst-4-en-17β-ol-3-one)	40
Etiocholanolone (5β-androstan-3α-ol-17-one)	2000
Androsterone (5α-androstan-3α-ol-17-one)	2000

dedicated to steroid profile confirmation. IRMS confirmation batches were run in parallel with the steroid profile confirmation in view of the deadline for releasing results. Overall, 189 steroid profile confirmations were performed together with 177 GC–C–IRMS analyses, resulting in 2 adverse analytical findings (AAFs) and 2 atypical findings (ATFs). For details regarding the analytical methods, see Tables 1, 2, and 3.

Biological assays

ESAs in urine, plasma, and serum were screened and confirmed by sarcosyl-polyacrylamide gel electrophoresis (SAR-PAGE).[21]

For HBOCs, visual inspection of plasma and quantification of haemoglobin were used as screening methods. Samples with altered plasma colour (whole blood spiked with >0.6% Hemopure) had their haemoglobin concentration measured using a Sysmex XT-2000i instrument. Only 1.1% of the samples presented colouration above this threshold. The discrimination between haemoglobin from haemolysed samples and HBOCs in plasma was tested by native-polyacrylamide gel electrophoresis (native-PAGE) followed by luminol exposure using 4%–15% native precast gels. Native-PAGE separates plasma proteins by molecular weight and conformation. Endogenous haemoglobin and modified haemoglobin were identified on Native-PAGE gels by chemiluminescence after exposure to luminol. Twelve samples were submitted to the confirmation procedure, but they produced negative results.

Regarding the ABP (haematological module), samples were analysed twice, consecutively. Absolute differences between the 2 analyses were calculated, and the results were accepted according to WADA operating guidelines for ABP analysis.[22]

hGH detection was performed by an isoform differential immunoassay using kit 1 or kit 2 (CMZ-Assay GmbH, Berlin, Germany) for the ITP and both kits for the confirmation procedure.[23] Only 1 confirmation was performed due to extremely high concentrations of PIT, and the result was negative. hGH biomarkers, namely, IGF-I and N-terminal pro-peptide of type III collagen (P-III-NP), were detected by an assay pairing comprising an IGF-I assay and a P-III-NP assay for the ITPs and 2 different assay pairings for confirmation procedures, including immunoassays or MS-based approaches.[24] The radioimmunoassays Immunotech (IGF-I) and ORION (P-III-NP) were used for the ITP of 65% of the samples analysed, and the combination of Immunotech and ADVIA Centaur (immunoassay for P-III-NP detection) was used for the other 35% of the samples. Confirmation procedures were performed using 2 assay pairs: the pair used for the ITP and a second pair comprising 2 different methods, including LC–MS/MS for IGF-I detection. The analyses of 2 Paralympic samples had GH-2000 scores related to the positive criteria for pairing 1 (Immunotech IGF-I + ADVIA Centaur P-III-NP) and negative for pairing 2 [LC tandem mass spectrometry (LC–MS/MS) IGF-I - + Orion P-III-NP], resulting in 2 ATFs.

Flow cytometry analyses were performed to detect mixed populations of red blood cells (RBCs), a consequence of HBT. The tested blood groups antigens were C, c, E, JKa, JKb, K, Fya, Fyb, S, and s. Four samples were sent for confirmation due to unclear histogram patterns for one or more antigens, but the results were negative for all samples.

DNA analysis

WADA included gene-doping strategies in the list of banned substances in 2003. Since then, a few groups have focused on the subject, and only recently did an Australian group propose a molecular-biology-based method for the identification of non-viral vectors in plasma samples obtained from EDTA blood tubes to WADA. Nevertheless, gene-doping analysis methods still require validation using more current instrumentation, different reagents, other guidelines and, most importantly, interlaboratory analyses. The strategies for identifying erythropoietin (EPO) DNA sequences in plasma and mononuclear blood cell samples are based on the amplification of various exon-exon sequences of the EPO gene by real-time polymerase chain reaction (PCR).[25]

The EPO gene is expressed in renal cells, and only the EPO protein is secreted into the bloodstream; therefore, the identification of any concentration of EPO DNA sequences in blood is considered a positive result for gene doping. Considering the growing concern over gene doping, as well as the availability of new molecular biology tools, the LBCD implemented, improved, and validated 2 amplification assays for EPO cDNA using the real-time PCR instrument QuantStudio12K (Thermo Fisher, São Paulo, Brazil). For the analysis, TaqMan-MGB probes in a short cycle period (FAST chemistry reagents) and confirmation with the E-Gel electrophoresis system were used. The improvements were made to reduce the analysis time and costs, including those associated with additional alternative confirmation approaches, and required training staff. For this purpose, control samples established using a 3D digital PCR system (Thermo Fisher, São Paulo, Brazil) to confirm EPO were analysed, and DNA sequence analysis was performed to identify the transgenic sequences. All work was performed with WADA-certified reference material for EPO gene doping within a range of 1 to 4000 copies of reference material spikes and EPO gene-doping-positive samples.[26] However, in view of the absence of interlaboratory tests among the laboratories accredited by WADA, the analysis was not performed on the Olympic samples; it was only performed on samples selected exclusively for research.

Therapeutic use exemptions

The first strategy related to therapeutic use samples (TUEs) was established before the Games with the TAs. After a presumptive finding in the ITP, the laboratory contacted the TA using a specific form following item 5.2.4.3.1 from the ISL.[6] Nevertheless, because

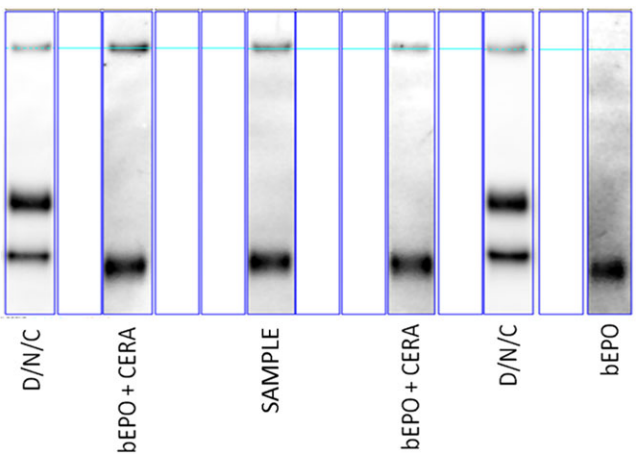


Figure 5. SAR-PAGE gel after double-blotting, demonstrating the presence of CERA in a road cycling athlete's blood. bEPO: blood EPO (negative control), D/N/C: Dynepo/NESP/CERA (reference material), bEPO + CERA (positive control).

of the high throughput of results, the strategy was amended by mutual agreement in the first days of the Olympic Games. Hence, all presumptive findings of β_2-agonists and glucocorticosteroids were confirmed without any previous contact and reported through ADAMS. This approach provided more time for the results management authority to address the cases without jeopardising laboratory activities.

Adverse analytical findings

Thirty-three samples were reported as AAFs during the Rio Olympic Games. The laboratory was notified that 4 of those samples were from WADA as part of the Games's EQAS double-blind programme. WADA also inserted 2 double-blind negative samples, which the LBCD reported as negative. Therefore, the LBCD displayed full compliance with WADA's external assessment scheme. Another 11 samples were covered by approved TUEs. Hence, 18 samples could be considered "real AAFs." Seven samples tested positive for continuous erythropoietin receptor activator (CERA). During the B sample procedures, it was clarified that these 7 findings were from 3 athletes, who were caught more than once by urine and blood analysis. First, a urine sample from a long-distance female athlete tested as a presumptive adverse analytical finding (PAAF) for CERA, but the result could not be confirmed because of CERA's well-known instability in urine.[27] As CERA is more stable in serum/plasma, a target collection was requested by the LBCD, and after analysis of a blood sample and a new urine sample, CERA abuse was confirmed. A second case involved a urine sample from a male road cyclist that tested as a PAAF for CERA. Another target collection was requested during the confirmation procedure. The

Table 5. Adverse and Atypical Findings reported during the Summer XXXI Olympic Games. (Classes: S1a. Exogenous AAS; S1b. Endogenous AAS; S2. Peptide hormones, growth factors, related substances, and mimetics; S3. β_2-agonists; Class S4. Hormone and metabolic modulators; S5. Diuretics and masking agents; S6. Stimulants; Class S7. Narcotics).

Adverse Analytical Findings			
Substance	**Class**	**Sport /Modality**	**Comments**
Mesterolone metabolite + Chlorothiazide	S1a; S5	N.A.	WADA EQAS sample
T, A, Etio, 5α-diol and 5β-diol	S1b	Weightlifting / Weightlifting	Confirmed by IRMS
T, A, Etio, 5α-diol and 5β-diol	S1b	Weightlifting / Weightlifting	Confirmed by IRMS
19-NA	S1b	N.A.	WADA EQAS sample
19-NA	S1b	Weightlifting / Weightlifting	Confirmed by IRMS
rEPO	S2	N.A.	WADA EQAS sample
CERA	S2	Cycling / Road	—
CERA	S2	Athletics / Long Distance 3000 m or greater	—
CERA	S2	Athletics / Long Distance 3000 m or greater	—
CERA	S2	Cycling / Road	—
CERA	S2	Cycling / Road	—
CERA	S2	Cycling / Road	—
CERA	S2	Athletics / Long Distance 3000 m or greater	—
Terbutaline	S3	Aquatics / Swimming	TUE granted
Terbutaline	S3	Aquatics / Swimming Sprint 100 m or less	TUE granted
Terbutaline	S3	Aquatics / Swimming Middle Distance 200–400 m	TUE granted
Clomiphene + pentazocine	S4; S7	N.A.	WADA EQAS sample
Hydrochlorothiazide	S5	Aquatics / Swimming Sprint 100 m or less	—
Hydrochlorothiazide	S5	Modern Pentathlon / Modern Pentathlon	—
Hydrochlorothiazide	S5	Modern Pentathlon / Modern Pentathlon	—
Strychnine	S6	Weightlifting / Weightlifting	—
Tuaminoheptane	S6	Boxing / Boxing	—
Methylphenidate + Ritalinic Acid	S6	Shooting / Rifle	TUE granted
Methylphenidate + Ritalinic Acid	S6	Gymnastic / Artistic	TUE granted
Methylphenidate + Ritalinic Acid	S6	Gymnastic / Artistic	TUE granted
Methylphenidate + Ritalinic Acid	S6	Gymnastic / Artistic	TUE granted
Methylphenidate + Ritalinic Acid	S6	Gymnastic / Artistic	TUE granted
Amphetamine	S6	Basketball / Basketball	—
Amphetamine	S6	Aquatics / Diving	—
Prednisone + Prednisolone	S9	Aquatics / Swimming	—
Betamethasone	S9	Field Hockey / Field Hockey	—
Dexamethasone + Triamcinolone acetonide	S9	Athletics / Throws	—
Prednisolone	S9	Triathlon / Triathlon	—
Atypical Findings			
19-NA with norethisterone	S1b	Aquatics / Swimming Long Distance 800 m or greater	Female sample
Testosterone	S1b	Weightlifting / Weightlifting	$\Delta\delta$ T-Pdiol $>$ 3. No other criteria reached
LH $>$ 50 mUI/mL	S2	Wrestling / Greco-Roman	Negative for LH-RH
LH $>$ 50 mUI/mL	S2	Taekwondo / Sparring	Negative for LH-RH
LH $>$ 50 mUI/mL	S2	Athletics / Throws	Negative for LH-RH

targeted blood (Figure 5) and urine samples were confirmed as AAFs for CERA, as was the first urine sample tested. Based on an APMU decision, the LBCD requested to analyse certain ABP blood samples for ESAs.

One of these samples, which belonged to the same athlete discussed in the previous case, tested positive for CERA. Finally, another sample obtained by an APMU request, an ABP sample from a male athlete (athletics, long distance), tested positive for CERA. Later, analysis of the urine from the same athlete did not detect CERA. However, a faint smear, representing recombinant EPO (rEPO), was observed, suggesting a degradation of CERA. These CERA cases demonstrated the importance of analytical flexibility regarding the matrix used (urine, plasma, and serum). In addition, the results provide remarkable proof of the success of the ABP (haematological module), as the altered results observed during ABP analyses led the lab team to contact the TA to request ESA analyses. At least 1 CERA case was identified based on this feedback.

The AAF for strychnine is also considered curious because this stimulant rarely appears in the annual test figures collected by WADA.[28] A very interesting case is associated with a sample from a weightlifting athlete reported as an AAF for 19-NA. Screening results estimated the concentration of 19-NA to be 4 ng/mL. Following the pre-established strategy defined for the Games, the quantification and the IRMS confirmation procedures were run in parallel. Because of the relatively high specific gravity of the urine containing 19-NA (1.025), the decision limit (DL) was adjusted to 3.1 ng/mL following the TD2016NA.[29] Because the quantification procedure resulted in a concentration of 3.0 ng/mL and the IRMS analysis confirmed an exogenous origin, the case represents a good example of the sensitivity of the IRMS approach in confirming 19-NA findings, even below the DL. Among the TUE-granted cases, the number of methylphenidate/ritalinic acid (5) and amphetamine [2] findings was surprising. In all cases, in addition to the TUE, the use of the relevant medications was declared in the doping control forms (DCFs). Substances with high incidence in doping control statistics, such as hydrochlorothiazide and glucocorticosteroids, also figured in the AAF results. LH analyses were conducted according to the guidelines for the reporting and management of urinary hCG and LH findings in male athletes. Samples with elevated LH concentration were required to be tested for gonadotrophin-releasing hormone (GnRH) analogues, anti-oestrogenic substances, or aromatase inhibitors. In this case, to implement a highly comprehensive LC–HRMS method, incorporating the GnRH analogues as a screening analysis yields a very convenient tool. No extra work was necessary to fulfil the guideline. Because no prohibited substances were detected in the elevated LH samples, they were reported as ATFs according to version 2.0 of the guideline.[30] A female sample with a low concentration of 19-NA was also reported as atypical because of the presence of norethisterone. Finally, a weightlifting sample was submitted to IRMS confirmation after a request from the APMU. Nevertheless, the sample showed alteration of the $\Delta\delta$ criteria for testosterone alone, disallowing a conclusion of exogenous administration. The sample was reported as atypical following the TD2016IRMS.[31] Excluding the EQAS sample and the TUE-granted samples, 0.37% of samples from the 2016 Olympic Games were reported as AAFs. Table 5 summarises the findings obtained from the Olympic Games sample analysis.

In the Paralympic Games, 12 samples were reported as AAFs. Interestingly, the majority of the substances found in the Paralympic samples were methadone, which has an extremely low incidence in annual statistics. Four samples from different athletes tested positive for the substance. Curiously, the 4 athletes declared the use of methadone in the DCFs. Additionally, as observed for the AAFs regarding 19-NA in the Olympic Games, an IPC sample was tested for the presence of the substance at a concentration

Table 6. Adverse and Atypical Findings reported during the Summer XV Paralympic Games (Classes: S1a. Exogenous AAS; S1b. Endogenous AAS; S1.2. Other anabolic agent; S2. Peptide hormones, growth factors, related substances, and mimetics; S4. Hormone and metabolic modulators; S5. Diuretics and masking agents; S7. Narcotics; S9. Glucocorticosteroid).

Adverse Analytical Findings			
Substance	**Class**	**Sport /Modality**	**Comments**
T, A, Etio, 5α-diol and 5β-diol	S1b	Aquatics / IPC Swimming Middle Distance 200–400 m	Confirmed by IRMS
19-NA	S1b	Athletics / IPC Throws	Confirmed by IRMS
Metenolone	S1a	Powerlifting / IPC Powerlifting	—
Tibolone	S1.2	Cycling / Para-cycling Road	—
Clomiphene	S4	Judo / Para-Judo	—
Hydrochlorothiazide	S5	Athletics / IPC Sprint 400 m or less	—
Hydrochlorothiazide	S5	Athletics / IPC Throws	—
Methadone	S7	Shooting / IPC Shooting	—
Methadone	S7	Rowing / Para-Rowing	—
Methadone	S7	Basketball / Wheelchair Basketball	—
Methadone	S7	Sailing / Para-Sailing	—
Triamcinolone acetonide	S9	Judo / Para-Judo	—
Atypical Findings			
Etiocholanolone	S1b	Powerlifting / IPC Powerlifting	$\Delta\gamma$ Etio-Pdiol >3. No other criteria reached
LH > 50 mUI/mL	S2	Powerlifting / IPC Powerlifting	Negative for LH-RH
LH > 50 mUI/mL	S2	Athletics / IPC Sprint 400 m or less	Negative for LH-RH
LH > 50 mUI/mL	S2	Aquatics / IPC Swimming Middle Distance 200–400 m	Negative for LH-RH
LH > 50 mUI/mL	S2	Powerlifting / IPC Powerlifting	Negative for LH-RH
hGH > DL	S2	Athletics / IPC Throws	Biomarkers approach
hGH > DL	S2	Athletics / IPC Throws	Biomarkers approach

of 4.2 ng/mL. Because the DL is corrected using a standard value of 1.020 for specific gravity and the sample had a specific gravity of exactly 1.020, the DL was 2.5 ng/mL. IRMS analysis was then performed, confirming the exogenous origin of 19-NA. Other anabolic substances were detected in 4 Paralympic samples: metenolone and tibolone, as confirmed by GC–MS/MS, and testosterone-related substances, as confirmed by IRMS. According to the LC–MS results, 2 athletes' samples tested positive for hydrochlorothiazide, and a judoka tested positive for clomiphene and its metabolite, desethyl-clomiphene. The presence of a glucocorticoid (triamcinolone acetonide) was confirmed, and the athlete had no TUE, thereby corroborating the AAF. The atypical results obtained during the Paralympic Games revealed 4 LH cases at concentrations above 50 IU/L with a negative result for GnRH analogues, anti-oestrogenic substances, or aromatase inhibitors. The criteria for an AAF for etiocholanolone is Etio $\Delta\delta > 4‰$ or Etio $\Delta\delta = 3$–$4‰$ with one of the Adiols $\Delta\delta > 3‰$. A powerlifting sample tested atypical for this substance, as Etio $\Delta\delta = 3.3‰$ (between 3‰ and 4‰) and both Adiols $\Delta\delta = 2.5‰$. The analyses of 2 samples by the hGH biomarkers test produced GH-2000 scores related to the positive criteria for pairing 1 (Immunotech IGF-I + ADVIA Centaur P-III-NP) and negative for pairing 2 (LC–MS/MS IGF-I + Orion P-III-NP). However, these results were deemed atypical for these blood samples. Table 6 summarises the findings obtained during the Paralympic Games sample analysis.

The continuous development of analytical strategies provides the potential to reveal new findings in the years to come. In the current paradigm, all negative samples were transferred to the Lausanne laboratory according to the long-term storage IOC programme[32] and could be retested whenever relevant.

Final remarks

As expected, the Rio 2016 Games involved considerable challenges regarding the technical, regulatory, and scientific aspects and the breadth of substance misuse screening compared with previous Games. All classes of prohibited substances were monitored. Therefore, state-of-the-art methods, technologies, and anti-doping methodologies were funnelled into Rio through typical scientific channels, mostly via a fruitful exchange among experts before the Games and the direct participation of 80 international experts during the Games, approximately a dozen of whom were directors of WADA-accredited laboratories.

From our perspective, the evaluation of laboratory work and performance was recognised by the community and the Olympic family. According to the Report by the Independent Observers, "LBCD made a tremendous effort to ensure it was fully prepared to meet these requirements. As a result, LBCD is an outstanding legacy from the Games for the anti-doping movement in South America."

Conclusions

The relevance, visibility. And precedence of the O&PG introduce a high possibility of doping abuse by athletes. Having the most effective doping control system in place to bring competitors to the same ethical levels of competition and sportsmanship is therefore of the utmost importance. The system has been improved throughout the years and reached its present apex at the Rio 2016 Summer Games. The development of indirect tools such as the ABP, as well as the introduction of proteomics and metabolomics approaches for the screening of forbidden substances, has enabled the full coverage of WADA's List of Prohibited Substances.[5] The mounting number of target compounds was effectively assessed by LC‰HRMS and non-target searches; by obtaining better data for endogenous steroid quantification with higher sensitivity and breadth of coverage for IRMS analysis of the exogenous application of endogenous steroids; by the effective use of the ABP (both haematological and steroidal modules) data in real time; and by ESA detection in the urine and blood, HBT, and HBOCs. Known small and large peptides and proteins were screened, including insulins, growth factors, hGH, hCG, LH, and proteases. Twenty procedures were established, and more than 30 000 tests were conducted with a relatively small number of AAFs, which is likely due to the effective and intelligent testing performed by almost all delegations before the Games.

The anti-doping community is still far from reaching 100% deterrence, but one may conclude that we are much closer to reaching this effectiveness than ever before.

Among the various "omics" fields, genomics is the next challenge. At the Rio Games, gene-doping testing was not performed but was deemed necessary in the future. Therefore, such testing will soon be implemented and could even be retrospectively applied to Rio 2016 samples, which will be stored by the IOC for at least 10 years.

Acknowledgments

It would be impossible to acknowledge all the sponsors, supporters, and contributors who were responsible for the success of this endeavour in so many diverse ways. A few representative ones include the Brazilian Federal Government, mainly through its Ministries of Sport and Education, which provided overall funding and political and logistical support, and WADA, WAADS, and WADA-accredited laboratories for their indefatigable support during our preparation and the training of the technical and managerial staff. Concerning the accredited laboratories, the release of so many experts to work during the Games is greatly appreciated. The full commitment of the UFRJ, the Chemistry Institute, and all LADETEC personnel to the Games's goals was essential to the outcome. Our family members and friends who stood by our side and accepted our continuous absence and lack of companionship during these years of extremely hard work can never be fully compensated for their altruism and understanding.

References

[1] F. R. Aquino Neto. O papel do atleta na sociedade e o controle de dopagem no esporte. *Rev Bras Med do Esporte*. **2001**, *7*, 138–148.
[2] H. M. G. Pereira, V. F. Sardela. Stimulant doping agents used in Brazil: prevalence, detectability, analytical implications, and challenges. *Subst Use Misuse*. **2014**, *49*, 1098–1114.
[3] H. M. G. Pereira, M. C. Padilha, R. M. A. Bento, T. P. Cunha, N. A. G. Lascas, F. R. Aquino Neto. Analytical and logistical improvements in doping-control analysis at the 2007 Pan-American Games. *TrAC – Trends Anal Chem*. **2008**, *27*, 648–656.
[4] McLaren RH. The Independent Person Report WADA Investigation of Sochi Allegations. https://www.wada-ama.org/en/resources/doping-control-process/mclaren-independent-investigations-report-into-sochi-allegations. [26 July 2017].
[5] WADA. The 2016 International Standard for Laboratories (ISL). https://www.wada-ama.org/en/resources/laboratories/international-standard-for-laboratories-isl. [26 July 2017].

[6] WADA. The 2016 Prohibited List. https://www.wada-ama.org/sites/default/files/resources/files/wada-2016-prohibited-list-en.pdf. [26 July 2017].
[7] P. Sardela, V. F. Sardela, H. M. Pereira, F. R. Aquino Neto. Urinary excretion profile of luteinizing hormone in Brazilian athletes. *J Braz Chem Soc*. **2015**, *26*, 1599–1607.
[8] A. Musenga, D. A. Cowan. Use of ultra-high pressure liquid chromatography coupled to high resolution mass spectrometry for fast screening in high throughput doping control. *J Chromatogr A*. **2013**, *1288*, 82–95.
[9] P. Reihlen, M. Blobel, R. Kempkes, et al. Optimizing SAR-PAGE. *Drug Test Anal*. **2015**, *7*, 1014–1016.
[10] C. Goebel, C. Alma, C. Howe, R. Kazlauskas, G. Trout. Methodologies for detection of hemoglobin-based oxygen carriers. *J Chromatogr Sci*. **2005**, *43*, 39–46.
[11] F. Lasne, N. Crepin, M. Ashenden, M. Audran, J. de Ceaurriz. Detection of hemoglobin-based oxygen carriers in human serum for doping analysis: screening by electrophoresis. *Clin Chem*. **2004**, *50*, 410–415.
[12] M. Bidlingmaier, J. Suhr, A. Ernst, et al. High-sensitivity chemiluminescence immunoassays for detection of growth hormone doping in sports. *Clin Chem*. **2009**, *55*, 445–453.
[13] I. Erotokritou-Mulligan, E. Eryl Bassett, D. A. Cowan, et al. The use of growth hormone (GH)-dependent markers in the detection of GH abuse in sport: Physiological intra-individual variation of IGF-I, type 3 pro-collagen (P-III-P) and the GH-2000 detection score. *Clin Endocrinol (Oxf)*. 21010. *72*, 520–526.
[14] M. Thevis, A. Thomas, P. Delahaut, A. Bosseloir, W. Schänzer. Qualitative determination of synthetic analogues of insulin in human plasma by immunoaffinity purification and liquid chromatography– tandem mass spectrometry for doping control purposes. *Anal Chem*. **2005**, *77*, 3579–3585.
[15] P. E. Sottas, N. Robinson, O. Rabin, M. Saugy. The athlete biological passport. *Clin Chem*. **2011**, *57*, 969–976.
[16] S. Giraud, N. Robinson, P. Mangin, M. Saugy. Scientific and forensic standards for homologous blood transfusion anti-doping analyses. *Forensic Sci Int*. **2008**, *179*, 23–33.
[17] J. O. Westgard, P. L. Barry, M. R. Hunt, T. Groth. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin Chem*. **1981**, *27*, 493–501.
[18] WADA. Technical Document for Sport Specific Analysis – TD2014SSA. https://www.wada-ama.org/sites/default/files/resources/files/wada-tdssa-v2.3_en.pdf. [26 July 2017].
[19] V. F. Sardela, F. B. Scalco, K. M. Cavalcante, et al. Systematic analysis of glycerol: Colourimetric screening and gas chromatography-mass spectrometric confirmation. *Drug Test Anal*. **2015**, *7*, 967–970.
[20] WADA. Technical Document - TD2016EAAS. https://www.wada-ama.org/sites/default/files/resources/files/wada-td2016eaas-eaas-measurement-and-reporting-en.pdf. [26 July 2017].
[21] C. Reichel, F. Abzieher, T. Geisendorfer. SARCOSYL-PAGE: a new method for the detection of MIRCERA- and EPO-doping in blood. *Drug Test Anal*. **2009**, *1*, 494–504.
[22] WADA. Athlete Biological Passport Operating Guidelines & Compilation of Required Elements. *V 5.0*, 63. https://www.wada-ama.org/sites/default/files/resources/files/wada-abp-operating-guidelines-v5.0-en.pdf. [26 July 2017].
[23] WADA Technical Document – TD2015GH Human Growth Hormone (hGH) Isoform Differential Immunoassays for Doping Control Analyses. https://www.wada-ama.org/sites/default/files/resources/files/wada_td2015gh_hgh_isoform_diff_immunoassays_en.pdf. [26 July 2017].
[24] World Anti-Doping Program GUIDELINES Human GROWTH HORMONE (hGH) BIOMARKERS TEST for Doping Control Analyses. https://www.wada-ama.org/sites/default/files/resources/files/wada-guidelines-for-hgh-biomarkers-test-v2.0-2016-en.pdf. [26 July 2017].
[25] A. Baoutina, T. Coldham, B. Fuller, K. R. Emslie. Improved detection of transgene and nonviral vectors in blood. *Hum Gene Ther Methods*. **2013**, *24*, 345–354.
[26] A. Baoutina, S. Bhat, M. Zheng, et al. Synthetic certified DNA reference material for analysis of human erythropoietin transgene and transcript in gene doping and gene therapy. *Gene Ther*. **2016**, *23*, 708–71747.
[27] Y. Dehnes, P. Hemmersbach. Effect of single doses of methoxypolyethylene glycol-epoetin beta (CERA, MircerаTM) and epoetin delta (DynepoTM) on isoelectric erythropoietin profiles and haematological parameters. *Drug Test Anal*. **2011**, *3*, 291–299.
[28] WADA. 2014 Anti-Doping Testing Figures Report. https://www.wada-ama.org/sites/default/files/wada_2014_anti-doping-testing-figures_full-report_en.pdf. [26 July 2017].
[29] WADA. Technical Document – TD2016NA. https://www.wada-ama.org/sites/default/files/resources/files/td2016na_eng.pdf. [26 July 2017].
[30] WADA. Guidelines Reporting & Management of Urinary Human Chorionic Gonadotrophin (hCG) and Luteinizing Hormone (LH) Findings in Male Athletes.
[31] WADA. Td2016Irms. https://www.wada-ama.org/sites/default/files/resources/files/wada-td2016irms-detection_synthetic_forms_eaas_by_irms-en.pdf. [26 July 2017].
[32] WADA. The International Olympic Committee Anti-Doping Rules Applicable to the Olympic Games in Rio **2016**. https://www.wada-ama.org/sites/default/files/resources/files/ioc_adr_rio_2016_-_revised_as_per_ioc_executive_board_decision_dated_1_march_2016_-_en.pdf. [26 July 2017].

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article.