Set-up of a multivariate approach based on serum biomarkers as an alternative strategy for the screening evaluation of the potential abuse of growth promoters in veal calves

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Set up of a multivariate approach based on serum biomarkers as an alternative strategy for screening evaluation of potential growth-promoters abuse in veal calves

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Abstract

A chemometric class modeling strategy (unequal dispersed classes, UNEQ) is applied for the first time as possible screening method to monitor growth-promoters abuse in veal calves. Five serum biomarkers, known to reflect the exposure to classes of compounds illegally used as growth-promoters, were determined from 50 untreated animals in order to design a model of controls, representing veal calves reared under good, safe, and highly standardized breeding conditions. The class modeling was applied to 421 commercially bred veal calves, to separate them in "compliant" and "non-compliant" in respect to the modeled controls. Part of the "non-compliant" animals underwent further histological and chemical examinations to confirm the presence of either alterations in target tissues or traces of illegal substances commonly administered for growth-promoting purposes. Overall, the congruence between the histological or chemical methods and the UNEQ "non-compliant" outcomes was approximately 58%, likely underestimated due to the blindness nature of this examination. Further research is needed to confirm the validity of the UNEQ model in terms of sensitivity in recognizing untreated animals as "compliant" to the controls, and specificity in revealing deviations from ideal breeding conditions, for example due to growth-promoters abuse.

Introduction

The Council Directive 96/22/EC, as amended by Directives 2003/74/EC and 2008/97/EC, stipulates that all use of steroids, β-agonists or other substances for the chemical manipulation of animal growth is strictly banned in the EU. Accordingly, a National Residue Control Plan (NRCP) is drawn up each year by the EU Member States (MS) to monitor the abuse of growth-promoters, involving samplings both on live animals at farm level and on carcasses at slaughterhouse and the subsequent chemical analysis performed by recognized laboratories. Based on the official results of samples taken for analysis by MS during the period 2005–2010, a low incidence (around 0.2 %) of non-compliances for illegal growth-promoters has been reported (EFSA 2013). However, the wide availability of anabolic substances on the black market, the seizures of growth-promoting preparations by veterinary officers or the police, and the results of histological screenings of target organs would point to a more widespread use of anabolic agents in meat cattle production than that emerging from the official figures (Cacciatore et al. 2009, Courtheyn et al. 2002, Imbimbo et al. 2012, Stephany 2010). Such a discrepancy may be explained by the use of cocktails of different active principles, each at very low dosage, or of drugs retaining the growth-promoting effects of a given class (e.g. steroids) but not included in the list of the molecules subjected to chemical monitoring in the frame of NRCPs (Mooney et al. 2009). The issue of designing a battery of screening tests based on the countless biological effects of growth-promoters – mainly gonadal steroids, corticosteroids, and β-agonists – to target the chemical analyses has been raised by several researchers (for a review, see Nebbia et al. 2011) and also by EFSA in a recent opinion on meat inspection of bovine animals (EFSA 2013).

Aside from the histological screening, which is routinely applied in the frame of the Italian NRCP, a number of assays have been developed at slaughterhouse level. Changes in the expression of specific genes (Cannizzo et al. 2013, Carraro et al. 2009, Divari et al. 2011) or proteins (Gardini et al. 2006) in different target tissues (e.g. muscles, thymus, liver, gonads) have been proposed as indirect biological markers that may be suggestive of the exposure to a number of anabolic

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substances. A parallel approach involves the investigation of blood-based biomarkers, which have the advantage of being less invasive and theoretically more fit-for-purpose than the tissue-based ones in that they may be applied over the entire animal breeding cycle and are potentially suitable to high throughput screening (Mooney et al. 2008).

Several serum/plasma components have been proposed as indicators of the exposure to a number of anabolic agents in veal calves, either used alone or in combination. The application of a growth-promoting protocol comprising 17β-oestradiol, clenbuterol, and dexamethasone (DEX) resulted in a marked lowering of the serum antioxidant capacity (SAC) in sampling corresponding to the administration of the oestrogen and the β-agonist (Nebbia et al. 2003); a superimposing SAC response was observed in veal calves exhibiting serum 17β-oestradiol concentrations above the legal limits (Brambilla et al. 2003). By contrast, DEX administration to veal calves according to a growth-promoting schedule caused SAC to slightly increase over both the controls and the pre-treatment values (Carletti et al. 2007). A decrease in the circulating levels of cortisol, resulting from the known interference of glucocorticoids (GCs) with the hypothalamic-pituitary, has been reported as one of the most reliable indicators of the prolonged exposure to "anabolic" dosages of such hormones, especially in conjunction with other biological tests like the histological screening of thymuses (Vascellari et al. 2008, Vascellari et al. 2012,). Osteocalcin, a small protein preferentially expressed in bone matrix, closely reflects osteoblast activity (for a review, see Neve et al. 2013). Consequently, the detrimental effects of GCs on bone formation and strength are associated with a notable decrease in circulating osteocalcin in humans (O'Brien et al. 2004) or in DEX-treated veal calves (Cacciatore et al. 2009). Gonadal peptide hormones called inhibins, belonging to the transforming growth factor-β superfamily that regulates the pituitary follicle stimulating hormone (FSH) secretion, have been proposed as further biomarkers, based on their involvement in both gonadal function and in the regulation of bone mass (Suresh et al. 2011). A consistent fall in the circulating levels of ir-inhibin, representing the pool of different inhibins reacting with specific antibodies, has been detected in bulls subjected to either oestrogen or androgen treatments (Godfrey

et al. 1992) or administered with oestrogens and androgens followed by DEX (Cacciatore et al. 2009). Finally, decreased serum urea levels are indicative of the exposure to anabolic agents in cattle (Preston et al. 1995), in line with their positive effects on body nitrogen balance.

In view of a possible application under field conditions for screening purposes, however, it should be noted that the reliability of all the indirect serum biomarkers mentioned above has been proven under experimental conditions by comparison with matched controls. To provide reference profiles, a database should therefore be established and based on surely untreated veal calves raised under controlled conditions reproducing the current European animal management and breeding standards. In addition, there is increasing evidence that, rather than looking at deviations from the "normal" range values of a single biomarker, the combination of multiple biomarkers and the application of multivariate class modelling or discriminant classification techniques may improve the diagnostic potential of screening assays. Either approach has been successfully applied not only in humans to doping detection (Pottgiesser and Schumacher 2013), alcohol abuse (Oliveri and Downey 2012, Pirro et al. 2013), or food origin control (Marini, et al. 2006a, Marini et al. 2006b), but also in cattle to predict misuse of growth-promoting hormones (Cunningham et al. 2009). Among the supervised pattern recognition methods, multivariate class modeling represents a suitable mean for data analysis, whenever a class of interest (i.e., untreated veal calves) has to be mathematically described e.g. on the basis of several biomarkers' values and with no bias from any other classes in the computation of the model. Compliance of other unknown samples (i.e., objects) to the modelled class can be investigated in decision-making processes (Oliveri and Downey 2012, Pirro et al. 2013). These techniques differ to the more widely used discriminant classification methods. A detailed description of their respective features in a decision-making process is beyond the aim of this article, and can be found elsewhere (Oliveri et al. 2010, Oliveri and Downey 2012, Marini 2013, Pirro et al. 2013).

A wide portfolio of class modelling methods is available as tools for data analysis (Forina et al. 2008, Oliveri and Downey 2012, Marini 2013). Among them, the UNEQ (Unequal dispersed

classes) class model offers the advantage to work directly on the original variables (i.e. serum biomarkers) and build simple models (Oliveri and Downey 2012, Pirro et al. 2013). Its simplicity and straightforwardness in the interpretation of the outcomes directly translate in robustness (Seasholtz and Kowalski 1993) and ease of introduction in complex inter-disciplinary contexts, such as that of food safety.

The present study describes the application of the UNEQ class model to define the class of untreated veal calves on the basis of five serum biomarkers (SAC, osteocalcin, urea, cortisol, ir-inhibin) that could be used as an alternative, biologically based, screening strategy for the evaluation of potential growth-promoter abuse.

Materials and methods

Animals and study protocol

The study was performed on two groups of 6/7 month-old male Holstein-Friesian calves. The first group of untreated animals (controls, C) was composed by 50 animals enrolled in the study at 1/2 months of age and reared according to standard programs of veal production following EC Directive 91/629, under controlled conditions, for five months. Calves were housed in groups of 5 and bucket-fed with a milk replacer for 10 weeks; then, the diet was gradually modified with the addition of a concentrated feed (composed by milk serum and fat), and the introduction of a fibrous quota (a mixture of cereals in form of flakes). The mean values ± SD of animal weight were 51 ± 10.9 kg, and 208 ± 24.5 kg, at the beginning and at the end of the study, respectively. Animals were vaccinated against the main viral diseases (IBR, Parainfluenza 3, BRSV and BVDV) (CATTLEMASTER® Pfizer Animal Health, New York, USA). Blood sampling was performed from each animal after 22 weeks of controlled rearing, corresponding to the age of 6/7 months. A second group (unknown, U) was included in the study, consisting of 421 veal calves of the same sex and breed, coming from commercial UE breeding units. Blood samples were taken at the same age intervals as for the controls.

Blood and urine collection

Blood and urine sampling was performed 3 to 4 weeks before slaughtering, which took place in EU certified abattoirs. Blood was collected in the morning (9-11 a.m.) using 10 ml tubes (Venosafe®, TERUMO) from the jugular vein. After clotting, serum was separated by centrifugation at 1272 g for 15 min at room temperature, divided into aliquots, immediately frozen in liquid nitrogen, and then stored at -80°C until analysis. When appropriate, urine samples were concurrently collected after spontaneous micturition, divided into aliquots, immediately frozen in liquid nitrogen, and stored at -20 °C pending analysis. Particular care was taken in avoiding faecal contamination. Sampling was conducted in the frame of routine controls by licensed veterinarians, avoiding any possible physical pain or stress condition, in accordance to the EU current legislation on animal welfare.

Biomarker assays

Serum levels of osteocalcin, cortisol, ir-inhibin, and urea, and SAC were evaluated using commercially available kits, adapted for bovine serum, and namely N-MID® Osteocalcin Elisa (IDS), Cortisol EIA kit® (Arbor Assays), Inhibin Alpha-Subunit (1-32) (Porcine) EIA kit® (Phoenix Pharmaceuticals, Inc.), respectively. All analyses were performed as recommended by the manufacturer's protocols. Osteocalcin, cortisol, and ir-inhibin levels were measured by interpolation of the standard curve of log standard concentrations versus normalized absorbance values. Serum samples were appropriately diluted with kit buffers for cortisol and ir-inhibin assays, to fall within the concentration range of the standard curve. The sensitivity of the osteocalcin assay was 0.5 ng ml^{-1}, whereas that of inhibin and cortisol assays were 0.04 ng ml^{-1}, and 45.4 pg ml^{-1}, respectively.

Urea concentrations were measured by an enzymatic ultraviolet (UV) method validated for bovine serum (IL Test™ Urea®) using an ILAB Aries spectrophotometer (Instrumentation Laboratory). Briefly, urea is hydrolyzed enzymatically by urease to yield ammonia and carbon

dioxide. The ammonia and α-oxoglutarate are converted to glutamate in a reaction catalyzed by L-glutamate dehydrogenase. The rate of NADH disappearance at 340 nm is proportional to the urea concentration in the sample. The sensitivity of the test is 1.7 mg urea dl^{-1} .

The OXY-Adsorbent test was employed to evaluate SAC. The test measures the ability of a serum sample to prevent the oxidative insult induced by a solution of hypochlorous acid. The sensitivity of the OXY-Adsorbent Test amounts to 25 µmol HClO ml^{-1}.

UNEQ class modeling technique

UNEQ is a probabilistic class modelling technique, which originated in the work of Hotelling in 1947 and was introduced into Chemometrics by Derde and Massart (1986). UNEQ is based on the hypothesis of a multivariate normal distribution in each category and on the use of the Hotelling's T^2 statistics to define a class space. Given a real matrix, the class model is defined as the centroid (i.e., the row vector containing the mean values of each variable for the samples of the category being modelled). A closed class space is calculated around the model. The class space is an ellipse in the case of two variables, an ellipsoid in the case of three variables and a hyper-ellipsoid in the case of more than three variables. The eccentricity and the orientation of the ellipse depend on the correlation between the variables and on their dispersion (Oliveri and Downey 2012). The equation of the boundary represents a formulation of the squared Mahalanobis distance (MD), which takes into account the correlation among variables for its computation (De Maesschalck et al. 2000). The space boundaries - used for the decision-making strategy on unknown samples - are defined by the critical value of T^2 statistics, at a predetermined confidence level (p = 1–2α) (Forina et al. 1995). Whenever the squared MD of a specific sample is smaller than the critical value (sample that falls inside the closed boundary), the sample is recognized as "compliant" with the class model, at a selected confidence probability. Conversely, if its squared MD is larger than the critical value, the sample is rejected by the model (samples falling outside the boundary).

The UNEQ class model of the present study was developed by in-house Matlab (The MathWorks, Inc., MA, USA) routines.

Model preliminary confirmation analyses

Specimens of thymus, prostate and bulbourethral glands were collected at the abattoir from a representative number of group U animals, classified as "non-compliant" based on the UNEQ class modelling set up in this study. Samples were immediately fixed in 10% neutral buffered formalin and stored at room temperature until paraffin-embedding according to standard protocols. Representative sections (4 µm) were stained with haematoxylin and eosin for histological examination, which was performed by certified pathologists. Tissues were classified as "negative", "uncertain", or "suspect" based on the absence or presence of specific histological lesions, in accordance with the NRCP recommendations (PNR 2013). In particular, the morphology of the thymus parenchyma was evaluated for light or severe adipose tissue infiltration and consequent cortical atrophy. As for prostate and bulbourethral glands, hyperplasia, and light or severe metaplasia were considered.

Urine and serum samples from a representative number of group U animals, classified as "non-compliant" on the basis of the UNEQ class modelling, were submitted for chemical analysis to an ISO 17025 accredited laboratory using methods validated according to 2002/657/EC Regulation. Serum samples were analyzed for the presence of natural sexual steroids by gas-chromatography/high-resolution mass spectrometry (GC-HRMS), while the presence of synthetic steroids and GCs in urine samples was detected through liquid chromatography/tandem mass spectrometry (LC-MS/MS). A list of the searched analytes and their respective LOD and LOQ values is reported in Table 1.

Results and discussion

The descriptive statistical data for the five examined biomarkers (SAC, osteocalcin, urea, cortisol, ir-inhibin) of group C (n=50) are reported in Table 2. As far as SAC, urea, cortisol, and ir-inhibin are concerned, average values from the present study comply with those already documented for the bovine species (Carletti et al. 2007, Matsuzaki et al. 2001, Mooney et al. 2008, Mosher et al. 2013). The range of serum osteocalcin levels in calves has been reported as relatively wide (Cacciatore et al. 2009, Matsuo et al. 2014). Such heterogeneity, reported also for humans, is attributable both to biological variability (e.g. age, diurnal and seasonal variation) and, principally, to the specificity of the commercial assays (Lee et al. 2000). Indeed, osteocalcin exhibits a quite short half-life due to the proteolysis in fragments of various molecular weight and stability that gives reason for the divergent results from assays characterized by different immunorecognition. Nevertheless, the average values found in the control calves are in line with those obtained in bull calves using a commercial kit detecting the same N-terminal mid fragment of osteocalcin as in our study (Matsuo et al. 2014). Table 3 depicts the descriptive statistical data for the examined biomarkers of veal calves from Group U (n= 421).

The UNEQ class modelling technique has been used to study the class of controls (untreated veal calves, C, n=50) and to define them according to the values of the serum biomarkers mentioned above. Then, the UNEQ model has been utilized to verify the compatibility of the U samples with the features of the C calves. By this class modelling approach, the decision regarding sample conformity is completely independent from the nature of "non-compliant" samples (e.g. possibly growth-promoter treated veal calves), and therefore, suitable to be used for applications where the representativeness of the class of "non-compliant" samples is hardly definable. Indeed, the class of "treated calves" is inherently inhomogeneous, because of the various illegal treatments that can be perpetrated, involving different classes of compounds (e.g. sexual steroids, GCs, β-agonists, etc.) and administration conditions (e.g. dosages, duration, schedules, combination of active principles) (Courtheyn et al. 2002). On the contrary, the class of controls can be defined clearly as veal calves

reared under good, safe, and highly standardized breeding conditions following European guidelines (EC Directive 91/629), and this is the only class of samples affecting the decision-making process according to the adopted approach.

Figure 1 shows the Mahalanobis distance (Log scale) of all the controls (C, blue symbols) and the unknowns (U group). The horizontal red line in the graph defines the critical distance (based on the T^2 statistics) used to discriminate between "compliant" and "non-compliant" samples, at a fixed confidence level for the class space (95% of sensitivity). The samples above the line are rejected from the model and identified as different from the controls (182 samples, 43.2% of the total unknown), while the samples with a smaller distance (below the line) are accepted and recognized as "compliant" (239 samples, 56.8% of the total unknowns). In Figure 1, the order of the unknowns has been arranged so that all the "compliant" cases are grouped together and colour-labelled in green, while all "non-compliant" cases are grouped sideways and colour-labelled in red.

Whenever models are built for decision-making processes, even if confined at a screening level as in our case, validation of the prediction performances of those models is of great importance, in order to provide information about their actual validity and usefulness in relation to the studied problem (Forina et al. 2008). In this specific study, no further controlled trials have been performed, in that no surely untreated and treated veal calves - outside the training sample set (control calves, Group C) - were available to estimate model sensitivity and specificity, respectively (Forina et al. 2008, Oliveri and Downey 2012). Nevertheless, a rough estimation of model specificity was indirectly and tentatively estimated by further independent techniques (i.e., chemical and histological examinations) described in the Methods section. More in details, 52 out of the 182 calves classified as "non-compliant" by the model based on the examined serum biomarkers were selected randomly from farms showing percentages of non-compliance higher than 60% and the corresponding tissue samples submitted to histological examination. Furthermore, 24 serum and urine samples taken randomly from the same calf group (n=52) were submitted to chemical analysis (Figure 2). The overall results of both the target tissue histological evaluation and the analytical

investigations are depicted in Table 4. In veal calves, thymic lesions are typically associated with the prolonged exposure to synthetic halogenated GCs, such as dexamethasone, even at low dosages (Biolatti et al. 2005, Cannizzo et al. 2008). By constrast, the fraudulent administration of estrogens alone or associated with other sexual steroids entails the onset of a various degree of epithelial metaplasia in the accessory sex glands (Groot et al. 1990, Groot et al. 1998, Schilt et al. 1998). In our study, 24 samples out of the 52 (46 %) investigated calves exhibited lesions in at least one of the examined tissues, and were therefore classified as "uncertain" or "suspect". The large majority of the histological alterations (n=21) occurred in the thymus, while the prostate (n=7) and the bulbourethral glands (n= 1) were affected to a lesser extent. Lesions simultaneously involving different target tissues (i.e. thymus and prostate or bulbourethral glands) were observed in 5 animals. A similar quali-quantitative pattern was reported in two different histological screening surveys conducted in northern and southern Italy, respectively (Regione Piemonte 2009, Imbimbo et al. 2012). As regards the results of the chemical analyses, none of the 24 serum and urine samples were certified as legally "non-compliant" with respect to any of the investigated analytes. However, amounts of 17β-boldenone and/or prednisolone higher than the respective LODs but lower than the respective LOQs were detected in urine samples from 6 calves, which did not exhibit histological changes in the examined target tissues. Although scientific evidences suggest that 17β-boldenone and prednisolone may have an endogenous origin in cattle (Ferranti et al. 2013, Scarth et al. 2009), it should be noted that a relative high incidence of non-compliances for either compound in bovines has been reported in the results from NRCPs performed by EU Member States in the 2005-2010 period (EFSA 2013). The trace urinary levels found in our study might be consistent with the use of very low doses of hormonal active principles for growth-promoting purposes, which is known to be the strategy currently adopted by dishonest breeders (Nebbia et al. 2011). Moreover, 17β-boldenone has a rapid excretion kinetics, meaning that concentrations below the established LOQ value can be reached in about 36 hours, after a single oral administration (Draisci et al. 2007). On the other hand, prednisolone undergoes to an extensive biotransformation, resulting in most cases in urinary GC

levels below the LOD even during the treatment with a growth-promoting schedule (Cannizzo et al. 2011). Interestingly, none of the 6 calves in which trace levels of either analyte (both in one case) could be demonstrated displayed appreciable histological changes in target tissues. Aside from the kinetic characteristics of 17β-boldenone and prednisolone detailed above and the likely use of very low dosages, the lack of correspondence between the histological and the analytical results in such calves might be explained by the peculiar effects of either active principle on target tissues. Furthermore, in calves treated with 17β-boldenone, the epithelial cells of sex accessory glands were characterized by a picture of hypersecretion, rather than by epithelial hyperplasia or metaplasia (Groot and Biolatti 2004). On the other hand, the administration of prednisolone to beef cattle for growth-promoting purposes did not elicit the lymphocyte depletion and thymus atrophy (Cannizzo et al. 2013), as typically observed upon the prolonged exposure to other synthetic GCs such as dexamethasone (Vascellari et al. 2008).

Conclusions

In the present study, we have elaborated a multivariate UNEQ analysis on 50 veal calves bred under standard conditions, based on the levels of five serum biomarkers known to reflect the exposure to the main classes of compounds illegally used as growth-promoters in veal calves. In the official screening methods, the relatively low sensitivity and specificity of the applied techniques are critical factors in determining the compliance of a given sample. Our model could represent a biologically-based alternative screening test, in which the unknown samples are rejected (i.e. are considered "non compliant") based on their distance from a centroid (i.e. the Mahalanobis distance) which takes into account both the absolute biomarkers' values and their inter-correlations. The application of our statistical approach to 421 commercially bred veal calves resulted in the identification of 182 "non-compliant" individuals, part of which (n=52) were selected for further histological (n=52/52) and chemical (n=24/52) investigations. Our findings indicate that 24 out of the selected 52 calves exhibited histological changes in target tissues (thymus, prostate or

bulbourethral glands), and hence classified as uncertain or suspect cases. In addition, trace amounts (< LOQ) of 17β-boldenone or prednisolone were found in urine samples from 6 calves, which did not show any histological lesions. Overall, the congruence between the histological and chemical methods and the proposed multivariate UNEQ analysis based on serum levels of five selected biomarkers was approximately 58% (30/52). Such a percentage of congruence is likely to be underestimated. Indeed, in our study, blood samplings for biomarker assays were collected three to four weeks before slaughtering and the subsequent tissue collection, a period that closely approaches the duration of the histological signature in cattle thymus (Cannizzo et al. 2011) or in bovine male accessory sex glands (Duffour and Grandmontagne 1990). As a further line of evidence, routine histological screening tests alone are unsuitable to detect the exposure to another class of widely used illicit growth-promoters like β-agonists, and only partially able to detect lesions in target tissues from low dosages sexual steroid-treated calves, unless they are integrated with specific immunohistochemical assays (Cannizzo et al. 2007, Pezzolato et al. 2013). More to the point, as already outlined in the previous sections, the rapid excretion kinetics of many anabolic compounds in the bovine species as well as the use of different active principle combinations, often at low dosages and/or of unknown structure, greatly limit the general reliability of the chemical analysis. Finally, the list of the analytes used for analytical investigations in the present study did not include β-agonists, which are still used as illegal growth promoters and are able to affect the serum levels of the measured biomarkers (Nebbia et al. 2011).

In this study, only a preliminary estimation of method validation is presented. Further experiments are warranted to verify both experimental sensitivity, by analyzing other certainly untreated animals, and experimental specificity, by analyzing animals that will be purposely treated with growth-promoting substances. Over time, the application of the model on a higher number of samples analyzed under diverse conditions will provide a broaden overview of the general applicability of this methodology. The possibility to understand to which extent an illicit treatment can be reflected into a significant variation of the examined serum biomarkers is of interest, as well

as the role played by potential confounding factors, e.g., stress conditions, diseases or medications, that might alter the panel of biomarkers. Indeed, there is scant information about the effects of physio-pathological events (e.g. stress or diseases) or allowed therapeutic treatments (that should be officially declared) on the levels of the selected biomarkers. However, it should be noted that any of such conditions that could alter the biomarker profile and be identified by our model would potentially represent a deviation from a picture representing good, safe and highly standardized breeding conditions. Therefore, the multivariate approach elaborated in this study could be potentially used as a part of quality control programmes in veal calf industry.

References

Biolatti B, Bollo E, Cannizzo FT, Zancanaro G, Tarantola M, Dacasto M, Cantiello M, Carletti M, Biolatti PG, Barbarino G. 2005. Effects of low-dose dexamethasone on thymus morphology and immunological parameters in veal calves. J Vet Med A Physiol Pathol Clin Med. 52:202-208.

Brambilla G, Ballerini A, Civitareale C, Fiori M, Neri B, Cavallina R, Nardoni A, Giannetti L. 2003. Oxidative stress as a bio-marker of estrogen exposure in healthy veal calves. Anal Chim Acta. 483:281-288.

Cacciatore G, Eisenberg SWF, Situ C, Mooney MH, Delahaut P, Klarenbeek S, Huet AC, Bergwerff AA, Elliott CT. 2009. Effect of growth-promoting 17 beta-estradiol, 19-nortestosterone and dexamethasone on circulating levels of nine potential biomarker candidates in veal calves Anal Chim Acta. 637:351-359.

Cannizzo FT, Zancanaro G, Spada F, Mulasso C, Biolatti B. 2007. Pathology of the testicle and sex accessory glands following the administration of boldenone and boldione as growth promoters in veal calves. J Vet Med Sci. 69:1109-1116.

Cannizzo FT, Miniscalco B, Riondato F, Bollo E, Barbarino G, Giorgi P, Mazzini C, Biolatti B. 2008. Effects of anabolic and therapeutic doses of dexamethasone on thymus morphology and apoptosis in veal calves. Vet Rec. 163:448-452.

Cannizzo FT, Capra P, Divari S, Ciccotelli V, Biolatti B, Vincenti M. 2011. Effects of low-dose dexamethasone and prednisolone long term administration in beef calf: Chemical and morphological investigation. Anal Chim Acta. 700:95-104.

Cannizzo FT, Pegolo S, Cucuzza LS, Bargelloni L, Divari S, Franch R, Castagnaro M, Biolatti B. 2013. Gene expression profiling of thymus in beef cattle treated with prednisolone. Res Vet Sci. 95:540-547.

Carletti M, Cantiello M, Giantin M, Nebbia C, Cannizzo FT, Bollo E, Dacasto M. 2007. Serum antioxidant enzyme activities and oxidative stress parameters as possible biomarkers of exposure in veal calves illegally treated with dexamethasone. Toxicol In Vitro. 21:277-283.

Carraro L, Ferraresso S, Cardazzo B, Romualdi C, Montesissa C, Gottardo F, Patarnello T, Castagnaro M, Bargelloni L. 2009. Expression profiling of skeletal muscle in young bulls treated with steroidal growth promoters. Physiol Genomics. 38:138-148.

Courtheyn D, Le Bizec B, Brambilla G, De Brabander HF, Cobbaert E, de Wiele AV, Vercammen J, De Wasch K. 2002. Recent developments in the use and abuse of growth promoters. Anal Chim Acta. 473:71-82.

Cunningham RT, Mooney MH, Xia X-L, Crooks S, Matthews D, O'Keeffe M, Li K, Elliott CT. 2009. Feasibility of a Clinical Chemical Analysis Approach To Predict Misuse of Growth Promoting Hormones in Cattle. Anal Chem. 81:977-983.

De Maesschalck R, Jouan-Rimbaud D, Massart DL. 2000. The Mahalanobis distance. Chemometr Intell Lab Syst. 50:1-18.

Derde MP, Massart DL. 1986. UNEQ - A class modeling supervised pattern-recognition technique. Mikrochim Acta. 2:139-152.

Divari S, Mulasso C, Uslenghi F, Cannizzo FT, Spada F, De Maria R, Brina N, Biolatti B. 2011. Progesterone receptor up-regulation: a diagnostic tool for the illicit use of oestrogens in adult beef cattle. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 28:1677-1686.

Draisci R, Montesissa C, Santarnaria B, Dambrosio C, Ferretti G, Merlanti R, Ferranti C, De Liguoro M, Cartoni C, Pistarino E, et al. 2007. Integrated analytical approach in veal calves administered the anabolic androgenic steroids boldenone and boldione: urine and plasma kinetic profile and changes in plasma protein expression. Proteomics. 7:3184-3193.

Duffour CC, Grandmontagne C. 1990. Evolution of prostate histological pictures after use of new molecules in calf breeding. Rev Med Vet. 141:187-194.

EFSA. 2013. Scientific Opinion on the public health hazards to be covered by inspection of meat (bovine animals). EFSA J.11:261.

Ferranti C, Famele M, Palleschi L, Bozzetta E, Pezzolato M, Draisci R. 2013. Excretion profile of corticosteroids in bovine urine compared with tissue residues after therapeutic and growth-promoting administration of dexamethasone. Steroids. 78:803-812.

Forina M, Lanteri S, Sarabia L. 1995. Distance and class space in the uneq class-modeling technique. J Chemom. 9:69-89.

Forina M, Oliveri P, Lanteri S, Casale M. 2008. Class-modeling techniques, classic and new, for old and new problems. Chemometr Intell Lab Syst. 93:132-148.

Gardini G, Del Boccio P, Colombatto S, Testore G, Corpillo D, Di Ilio C, Urbani A, Nebbia C. 2006. Proteomic investigation in the detection of the illicit treatment of calves with growth-promoting agents. Proteomics. 6:2813-2822.

Godfrey RW, Lunstra DD, Schanbacher BD. 1992. Effect of implanting bull calves with testosterone propionate, dihydrotestosterone propionate or estradiol-17-beta prepubertally on the pituitary testicular axis and on postpubertal social and sexual-behavior. J Reprod Fertil. 94:57-69.

Groot MJ, Denhartog JMP, Arts CJM, Ossenkoppele JS, Gruys E. 1990. Histological-changes in the prostate of veal calves after the administration of natural hormones. Arch Lebensmittelhyg. 41:37-42.

Groot MJ, Schilt R, Ossenkoppele JS, Berende PLM, Haasnoot W. 1998. Combinations of growth promoters in veal calves: Consequences for screening and confirmation methods. J Vet Med A Physiol Pathol Clin Med. 45:425-440.

Groot MJ, Biolatti B. 2004. Histopathological effects of boldenone in cattle J Vet Med A Physiol Pathol Clin Med. 51:58-63.

Hotelling H. 1947. Multivariate quality control. In: Eisenhart C, Hastay MW, Wallis WA, eds. Techniques of Statistical Analysis. New York (NY): McGraw-Hill; p. 111-184.

Imbimbo P, Castigliego L, Armani A, Biolatti B, Cannizzo FT, Gianfaldoni D, Guidi A. 2012. A Histologic Study on Growth Promoter Target Organs of Slaughtered Beef in Molise Region (Italy). J Vet Med Sci. 74:1253-1259.

Lee AJ, Hodges S, Eastell R. 2000. Measurement of osteocalcin. Ann Clin Biochem. 37:432-446.

Marini F, Magri AL, Bucci R, Balestrieri F, Marini D. 2006a. Class-modeling techniques in the authentication of Italian oils from Sicily with a Protected Denomination of Origin (PDO). Chemometr Intell Lab Syst. 80:140-148.

Marini F, Bucci R, Magri AL, Magri AD. 2006b. Authentication of Italian CDO wines by class-modeling techniques. Chemometr Intell Lab Syst. 84:164-171.

Marini F. 2013. Chemometrics in food chemistry. In: Marini F, editor. Data Handling in Science and Technology. Elsevier; p. 1-512.

Matsuo A, Togashi A, Sasaki K, Devkota B, Hirata TI, Yamagishi N. 2014. Diurnal Variation of Plasma Bone Markers in Japanese Black Calves. J Vet Med Sci. DOI: http://dx.doi.org/10.1292/jvms.14-0021.

Matsuzaki S, Uenoyama Y, Okuda K, Watanabe G, Kitamura N, Taya K, Cruzana MBC, Yamada J. 2001. Prepubertal changes in immunoreactive inhibin concentration in blood serum and testicular tissue in Holstein bull calves. J Vet Med Sci. 63:1303-1307.

Mooney MH, Situ C, Cacciatore G, Hutchinson T, Elliott C, Bergwerff AA. 2008. Plasma biomarker profiling in the detection of growth promoter use in calves. Biomarkers. 13:246-256.

Mooney MH, Le Bizec B, Elliott CT. 2009. Combining biomarker screening and mass-spectrometric analysis to detect hormone abuse in cattle. Trends Analyt Chem. 28:665-675.

Mosher RA, Wang C, Allen PS, Coetzee JF. 2013. Comparative effects of castration and dehorning in series or concurrent castration and dehorning procedures on stress responses and production in Holstein calves. J Anim Sci. 91:4133-4145.

Nebbia C, Cantiello M, Giuliano Albo A, Gusson F, Dacasto M, Bellino C, Gardini G. 2003. Serum antioxidant status and oxidative stress parameters as biomarkers for illicit treatments with growth promoting agents in veal calves. J Vet Pharmacol Ther. 26:1.

Nebbia C, Urbani A, Carletti M, Gardini G, Balbo A, Bertarelli D, Girolami F. 2011. Novel strategies for tracing the exposure of meat cattle to illegal growth-promoters. Vet J. 189:34-42.

Neve A, Corrado A, Cantatore FP. 2013. Osteocalcin: Skeletal and extra-skeletal effects. J Cell Physiol. 228:1149-1153.

O'Brien CA, Jia D, Plotkin LI, Bellido T, Powers CC, Stewart SA, Manolagas SC, Weinstein RS. 2004. Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. Endocrinology. 145:1835-1841.

Oliveri P, Casolino MC, Forina M. 2010. Chemometric Brains for Artificial Tongues. Adv Food Nutr Res. 61:57-117.

Oliveri P, Downey G. 2012. Multivariate class modeling for the verification of food-authenticity claims. Trends Analyt Chem. 35:74-86.

Pezzolato M, Richelmi GB, Maurella C, Pitardi D, Varello K, Caramelli M, Bozzetta E. 2013. Histopathology as a simple and reliable method to detect 17 beta-oestradiol illegal treatment in male calves. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 30:1096-1099.

Pirro V, Oliveri P, Sciutteri B, Salvo R, Salomone A, Lanteri S, Vincenti M. 2013. Multivariate strategies for screening evaluation of harmful drinking. Bioanalysis. 5:687-699.

PNR. 2013. [Internet]. [cited 2014 Aug 11]. Available from: http://www.regione.calabria.it/sanita/allegati/dpgr_2013/dpgr_2013/all._dpgr_n._5_-_pnr_2013.pdf.

Pottgiesser T, Schumacher YO. 2013. Current strategies of blood doping detectionAnal Bioanal Chem. 405:9625-9639.

Preston RL, Bartle SJ, Kasser TR, Day JW, Veenhuizen JJ, Baile CA. 1995. Comparative effectiveness of somatotropin and anabolic-steroids in feedlot steers. J Anim Sci. 73:1038-1047.

Regione Piemonte. 2009. [cited 2014 September 16] Available from: http://www.regione.piemonte.it/sanita/cms2/documentazione/category/97-2010?download=978:area-c-piano-regionale-di-controllo-dei-residui-indesiderati-nelle-carni-e-negli-animali-allevati-pdf-180.49-kb

Scarth J, Akre C, van Ginkel L, Le Bizec B, De Brabander H, Korth W, Points J, Teale P, Kay J. 2009. Presence and metabolism of endogenous androgenic-anabolic steroid hormones in

meat-producing animals: a review. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 26:640-671.

Schilt R, Groot MJ, Berende PLM, Ramazza V, Ossenkoppele JS, Haasnoot W, Van Bennekom EO, Brouwer L, Hooijerink H. 1998. Pour on application of growth promoters in veal calves: analytical and histological results. Analyst. 123:2665-2670.

Seasholtz MB, Kowalski B. 1993. The parsimony principle applied to multivariate calibration. Anal Chim Acta. 277:165-177.

Stephany RW. 2010. Hormonal growth promoting agents in food producing animals. Handb Exp Pharmacol. 2010:355-367.

Suresh PS, Rajan T, Tsutsumi R. 2011. New targets for old hormones: inhibins clinical role revisited. Endocr J. 58:223-235.

Vascellari M, Pozza G, Poppi L, Capello K, Angeletti R, Ravarotto L, Andrighetto I, Mutinelli F. 2008. Evaluation of indirect biomarkers for detecting corticosteroids used as illegal growth promoters in beef cattle. Vet Rec. 163:147-152.

Vascellari M, Capello K, Stefani A, Biancotto G, Moro L, Stella R, Pozza G, Mutinelli F. 2012. Evaluation of thymus morphology and serum cortisol concentration as indirect biomarkers to detect low-dose dexamethasone illegal treatment in beef cattle. BMC Vet Res. 8:129.

Table 1. List of investigated analytes in serum and urines from veal calves (n=24) of the "unknown" group

Substance	Matrix	LOD ($\mu g\ l^{-1}$)	LOQ ($\mu g\ l^{-1}$)
Sexual steroids			
17β-oestradiol	Serum	0.03	0.03
17β-Testosterone	Serum	1	2
Progesterone	Serum	0.06	0.15
Ethinyloestradiol	Urine	0.30	1.5
Fluossimesterone	Urine	0.55	1.5
16β-Hydroxystanozolol	Urine	0.15	0.55
Methylboldenone	Urine	0.30	1.5
Methyltestosterone	Urine	0.30	1.5
17α-Nortestosterone	Urine	0.30	1
17β-Nortestosterone	Urine	0.30	1
Stanozolol	Urine	0.30	0.55
17α-Trenbolone	Urine	0.25	1.5
17β-Trenbolone	Urine	0.55	1.5
17α-Boldenone	Urine	0.30	0.55
17β-Boldenone	Urine	0.30	0.55
Corticosteroids			
Beclomethasone	Urine	0.2	1.5
Betamethasone	Urine	0.2	1.5
Dexamethasone	Urine	0.2	1.5
Flumethasone	Urine	0.2	1.5
Fluorometholone	Urine	0.2	1.5
Methylprednisolone	Urine	0.2	1.5
Prednisolone	Urine	0.2	1.5
Prednisone	Urine	0.2	1.5
Triamcinolone acetonide	Urine	0.2	1.5

Table 2. Serum biomarker values from veal calves of the control group (n=50)

Biomarker	Mean ± SD	Minimum	Median	Maximum
Osteocalcin (ng ml^{-1})	13.73±3.29	6.70	14.15	21.60
Cortisol (ng ml^{-1})	22.82±7.1	8.81	22.03	48.53
Inhibin (ng ml^{-1})	3.32±1.86	0.60	3.03	10.24
Urea (mg dl^{-1})	10.28±3.03	5.00	10.00	20.00
SAC μmol HClO ml^{-1}	352.63±60.01	250.27	354.61	488.66

Note: SAC, Serum antioxidant capacity

Table 3. Serum biomarker values from veal calves of the unknown group (n=421)

Biomarker	Mean ± SD	Minimum	Median	Maximum
Osteocalcin (ng ml^{-1})	9.62±2.17	0.10	9.42	9.83
Cortisol (ng ml^{-1})	24.83±17.01	3.13	19.45	106.41
Inhibin (ng ml^{-1})	1.13±0.63	0.04	1.04	6.36
Urea (mg dl^{-1})	14.21±4.89	0.23	13.75	14.67
SAC μmol HClO ml^{-1}	348.01±72.12	3.47	342.21	661.04

Note: SAC, Serum antioxidant capacity

Table 4. Results of the histological evaluation (n=52) and of the chemical analysis (n=24) of selected samples from the "unknown veal calves" group classified as "non compliant" with respect to the UNEQ class model based on the levels of five serum biomarkers

Sample	Histological evaluation			Chemical analysis	
	Thymus	Prostate	Bulbourethral glands	Serum	Urine*
V01	SUSPECT	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V02	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	17β-BOLD Detected <LOQ
V03	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V04	UNCERTAIN	COMPLIANT	COMPLIANT	n.a.	n.a.
V05	UNCERTAIN	COMPLIANT	UNCERTAIN	n.a.	n.a.
V06	UNCERTAIN	COMPLIANT	COMPLIANT	n.a.	n.a.
V07	UNCERTAIN	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V08	UNCERTAIN	UNCERTAIN	COMPLIANT	n.a.	n.a.
V09	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V10	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V11	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	17β-BOLD Detected <LOQ PRED Detected <LOQ
V12	UNCERTAIN	UNCERTAIN	COMPLIANT	COMPLIANT	COMPLIANT
V13	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V14	UNCERTAIN	COMPLIANT	COMPLIANT	n.a.	n.a.
V15	SUSPECT	UNCERTAIN	COMPLIANT	n.a.	n.a.
V16	COMPLIANT	UNCERTAIN	COMPLIANT	COMPLIANT	COMPLIANT
V17	UNCERTAIN	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V18	UNCERTAIN	COMPLIANT	COMPLIANT	n.a.	n.a.
V19	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V20	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	17β-BOLD Detected <LOQ
V21	UNCERTAIN	COMPLIANT	COMPLIANT	n.a.	n.a.
V22	UNCERTAIN	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V23	UNCERTAIN	COMPLIANT	COMPLIANT	n.a.	n.a.
V24	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V25	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V26	UNCERTAIN	UNCERTAIN	COMPLIANT	n.a.	n.a.
V27	COMPLIANT	UNCERTAIN	COMPLIANT	n.a.	n.a.
V28	UNCERTAIN	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V29	UNCERTAIN	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V30	SUSPECT	COMPLIANT	COMPLIANT	n.a.	n.a.
V31	UNCERTAIN	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V32	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V33	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V34	COMPLIANT	UNCERTAIN	COMPLIANT	COMPLIANT	COMPLIANT
V35	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V36	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V37	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V38	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT

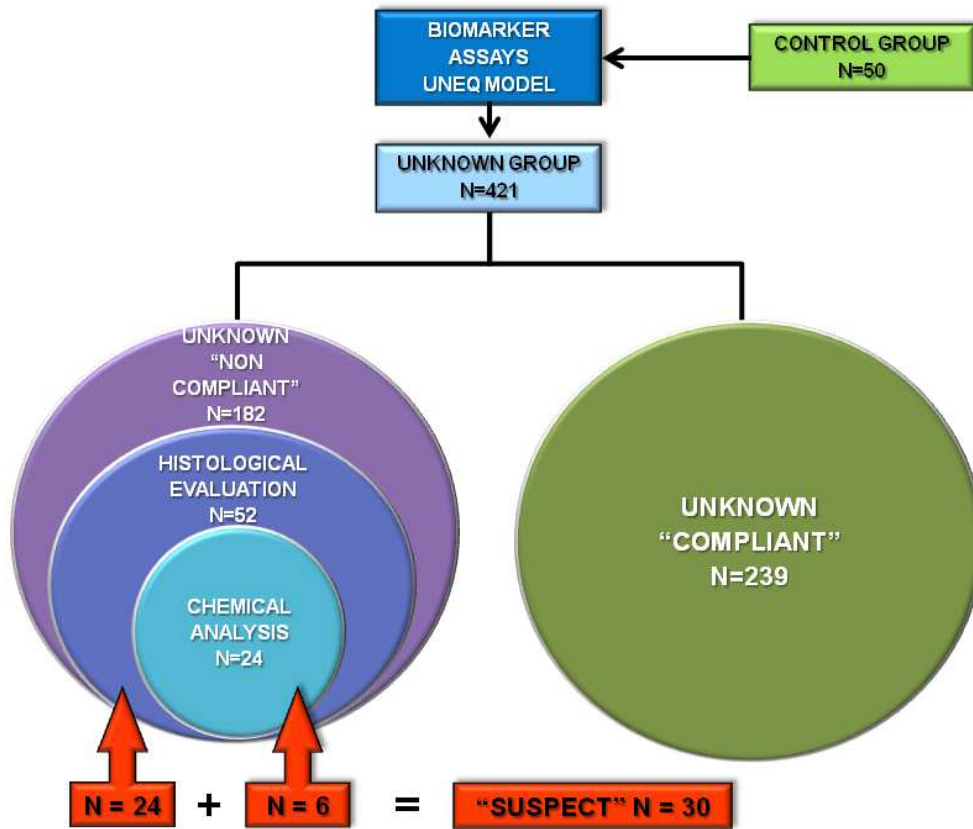|					
V39	UNCERTAIN	COMPLIANT	COMPLIANT	n.a.	n.a.
V40	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V41	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V42	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	17β-BOLD Detected <LOQ
V43	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	17β-BOLD Detected <LOQ
V44	SUSPECT	COMPLIANT	COMPLIANT	n.a.	n.a.
V45	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V46	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V47	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V48	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	PRED Detected <LOQ
V49	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT	COMPLIANT
V50	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V51	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.
V52	COMPLIANT	COMPLIANT	COMPLIANT	n.a.	n.a.

Note : Serum samples were assayed for the presence of natural sexual steroids; urine specimens were analyzed for the presence of synthetic sexual steroids and of corticosteroids. The full list of the searched analytes and the corresponding LOD and LOQ values is depicted in Table 1.

17β-BOLD, 17β-Boldenone; PRED, Prednisolone; n.a., not analysed.

The concentration of the identified analytes was in all cases higher than LOD but lower than LOQ
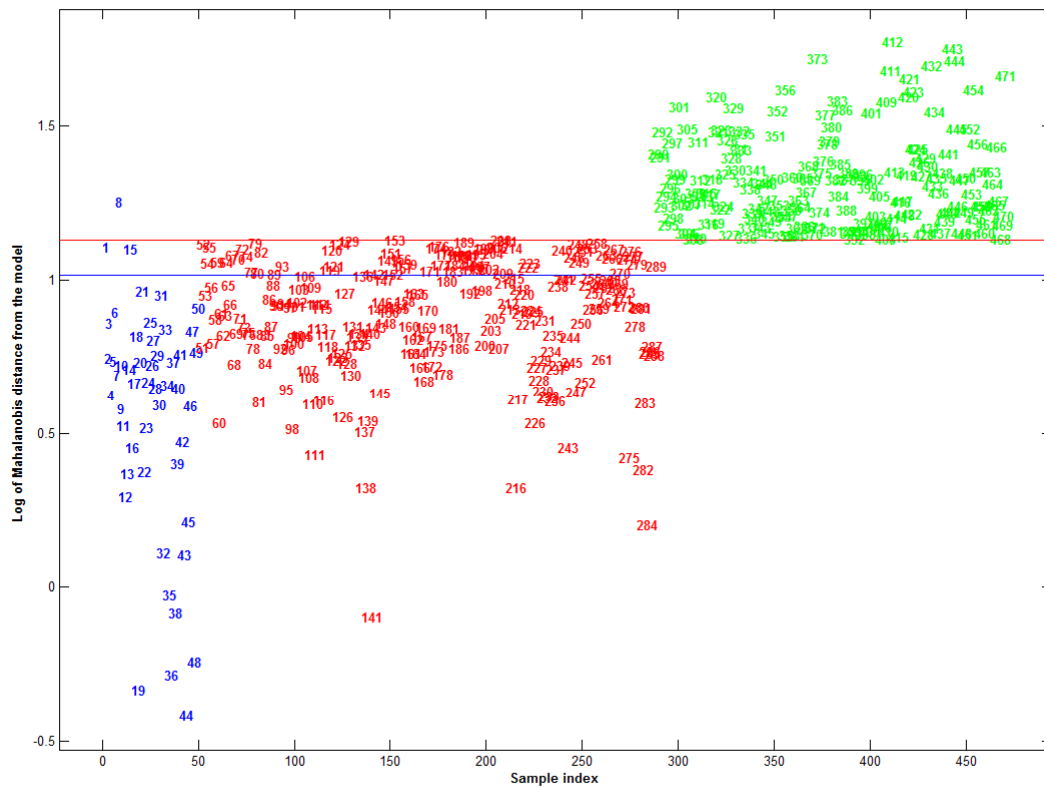
Figure 1 Distribution of Mahalanobis Distance (log values) for Group C and Group U animals.

Blue figures = Group C; green figures = Group U, compliant animals; red figures = Group U, non-compliant animals.

Figure 2 Flow chart of the study