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Review

Hair analysis for veterinary drug monitoring in livestock production

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Abstract

This review summarizes the basic information and applications concerning the use of hair analysis for the detection of misuse of therapeutic and anabolic agents in livestock animals. Hair biology, hair-shaft structure and the mechanisms of drug incorporation are described, considering the different factors which can affect the deposition. Sampling and extraction methods are reviewed with special attention to the particularities of this matrix, while the use of different analytical techniques is discussed, taking into account the concentration and the sensitivity required for drug detection. Advantages, drawbacks, promising prospects and possible applications of this technique in the future are also discussed.

Keywords: Hair analysis; Veterinary drugs; Drug residues; Livestock

Contents

Abbreviations: APCI, atmospheric pressure chemical ionization; BSA, bovine serum albumin; CI, chemical ionization; DAD, diode array detection; DCM, dichloromethane; DDE, diphasic dialysis extraction; DTE, 1,4-dithioerythritol; DTT, 1,4-dithio-DL-trheitol; EC, electrochemical detector; EI, electron impact ionization; EIA, enzyme immunoassay; ELISA, enzyme linked immunosorbent assay; ESI, electrospray ionization; EtAc, ethyl acetate; EtES, ethinylestradiol; EtOH, ethanol; HPLC, high performance liquid chromatography; HRMS, high resolution mass spectrometry; GC, gas chromatography; LC, liquid chromatography; $LC_{prep.}$, preparative liquid chromatography; LLE, liquid–liquid extraction; λ_{max}, maximum absorption wavelength; MeOH, methanol; MeTS, methyltestosterone; MIC, multiple ion chromatogram; MPA, medroxyprogesterone; MRM, multiple reaction monitoring; MS, mass spectrometry; MS–MS, tandem mass spectrometry; SCAN, full scan *m/z* detection; SCX, strong cation exchanger; SDS, sodium dodecyl sulphate; SFE, supercritical fluid extraction; SIM, single ion monitoring; SIR, single ion recording; SMZ, sulfamethazine; SPE, solid phase extraction; T_{amb}, ambient temperature; TBME, tert-butyl methyl ether; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TFA, trifluoroacetic acid; USBath, ultrasonic bath

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1. Introduction

Food producing animals may be treated during their lifetime with veterinary drugs to prevent or control various diseases. The improper or illegal use of these compounds as growth promoters and repartitioning agents may cause the occurrence of drug residues in food products produced from these animals.

The European Community requires that all veterinary drugs be evaluated according to Council Regulation (EC) 2377/90 [1], which also describes the procedure for the establishment, if necessary, of the maximum residue limit (MRL) of a compound in a specific matrix. Moreover, the use of any drug to improve animal growth is banned within the European Union (Council Directive 96/22/EC amended by Directive 2003/74/EC) [2,3].

To guarantee a high level of consumer protection, each European Member state is obliged to draft a national residue monitoring plan, which evaluates the level of various residues in foods, including legal and illegal veterinary drugs and other substances such as pesticides and environmental contaminants [4].

Surveillance for the presence of residues of veterinary agents in food-producing animals and foods is regulated by the Council Directive 96/23/EC and Commission Decision 2002/657/EC [4,5] which contain specific guidelines for sampling procedures on farms and in slaughterhouses and establish criteria and procedures for the validation of analytical methods and for the interpretation of the results.

The correct implementation of these surveillance and monitoring programs involves the development of robust and sensitive analytical methods to provide the control authorities with effective tools.

The detection of veterinary drug residues is generally performed on urine and plasma samples taken from living animals as well as on tissue samples after slaughter (muscle, liver, kidney, vitreous humour, fat).

Veterinary drugs generally show high clearance rates in these biological samples, making the retroactive detection of improper or illicit uses for many active compounds difficult.

In the last decade various studies have investigated whether or not hair analysis could be considered a useful matrix to control this problem in food producing animals, as previously reported in the field of illicit drugs in human and forensic medicine [6,7].

Hair has been considered as a suitable sample for the determination of different organic drugs since Goldblum published in 1954 a report about detection of amphetamine in hair of Guinea pigs [8].

Since then many scientific papers have described the detection of doping agents, therapeutic compounds, drugs of abuse and tobacco residues in human hair samples [6,7,9,10]. At present, hair analysis is used in many different areas such as forensic toxicology, doping control, drugs of abuse studies and clinical human medicine [6,11,12].

In 1994 various authors reported the possible applications of hair analysis for the detection of fraudulent uses of anabolics and β-agonists in animals [13–15]. Since then, animal hair has been considered an interesting tissue in veterinary control, because if active compounds are incorporated in the strong, rigid hair structure, they can be retained for a longer time than in urine or blood, due to the low metabolic activity of this tissue [6,16,17].

In spite of these encouraging premises, the published studies on veterinary hair analysis are still scarce and only embrace a limited number of active compounds.

The general knowledge on veterinary drug deposition is poor for the greater part of active compounds and livestock species, and the effect of multiple endogenous and exogenous factors, which influence the accumulation of veterinary drugs in hair, is not thoroughly verified. For many compounds there is a need for reliable analytical methods to detect and confirm the presence of drug residues at ppb/ppt levels in this particular matrix.

The Society of Hair Testing developed inter-laboratory tests and established several statements concerning the examination of drugs in human hair, the legal aspects of hair analysis and a consensus opinion on hair testing for doping agents in humans (http://www.soht.org). These recommendations highlight the great difficulties to set up and harmonize the interpretation criteria for hair analysis and to establish an appropriate definition of "positive result" in human samples. These concerns are much more justified when livestock hairs have to be tested, due to the substantial novelty of this technique on animals.

So, the aim of this review is to summarize the relevant scientific literature on hair testing for veterinary drugs in farm animals.

Basic information on hair structure, factors which could affect the deposition of the veterinary drugs and procedures described in the scientific literature for the detection of therapeutic and anabolic agents in farm animal hair are described.

Advantages and drawbacks of this matrix are highlighted, as well as the analytical techniques in use for the evaluation of drug residues in hair, and some possible future development.

2. Hair structure and composition

In mammals, the hair shaft exhibits different protective functions against insects, parasites, electromagnetic radiations and temperature changes. Hairs also provide decoration, camouflage, information about the environment and support different social functions such as communication and spreading of pheromones [18].

The visible structure of hair is quite simple but its composition, biochemical mechanism of growth and structural organization are complex and only partially understood [7,19].

Hair is a composite tissue originated during the cyclical activity of hair follicle cells from the embryonic epidermis, which is located under the skin.

The whole hair follicle is surrounded by an intricate vascular system and muscular tissues. The hair follicle also incorporates various glandular structures whose secretions can soak hair (apocrine sweat and sebaceous glands), a dermal papilla, a root sheath and a bulge region [17,18,20].

The hair shaft consists of three different layers of cells, an inner medulla, a protective external cuticle and a central cortex. Hairs contain cross-linked and orientated fibrous proteins, basically α-keratins rich in cysteine (85–93%), melanins (0.3–1.5%) which are complex polymers derived from tyrosine

oxidation, water (3–5%), lipids (1–9%) and mineral compounds (0.25–0.95%) [17,20].

The high sulphur content of keratins enables the formation of multiple disulfide chemical bonds between cysteine molecules located in close keratin fibres. The highly stable arrangement of these chemical bonds and the tiled structure of the external cuticle protect the hair structure against environmental degradation factors, microbial agents and hydrolysis [17,19].

The cortex, which is the main component of the hair, contains longitudinally oriented keratinocytes and melanin granules while the medulla comprises randomly oriented and loosely packed rectangular cells. The keratinised region of the hair shaft is dehydrated and hygroscopic, whereas the hair cuticle shows hydrophobic properties [20].

3. Hair growth cycle

Hair does not grow constantly but undergoes defined periods of growth and quiescence, which are controlled by various factors only partially understood. Typically the cycle of hair growth encompasses the Anagen, a long period of active growth, the Catagen, a short transitional stage of slow growth, and the Telogen, a phase of no growth. When the hair shaft stops growing completely, it can be removed easily by pulling it out.

In humans and Guinea pigs the cycle of growth has been defined as "asynchronous" because each follicle possesses an independent behaviour. Other animal species show a different pattern of hair growth in which one hair follicle can influence the growth activity of the neighbouring follicles ("wave pattern") [18].

Different factors have been described as affecting the hair growth rate, such as the region of the body, the age, the gender, the seasonal and climatic factors, the photoperiod and the level of some hormones [17,18,21].

The growth rate of hair is approximately 6–7 mm per month in calves, whereas the thickness of the skin is between 2.5 and 3 mm for young animals [21,22].

All the factors cited should be considered when results of hair analysis are evaluated, because the time gap between the incorporation of a molecule in the follicle (during the drug administration) and the release of the corresponding hair portion at the animal's skin surface, could delay the detection of the drugs in hair samples [22].

4. Drug accumulation in the hair

Scientific literature gives evidence that different kinds of drugs and contaminants may accumulate in the hair and be detected [6,7,9,10,23].

The first mechanism proposed for the accumulation of drug molecules in the hair was a generic "passive diffusion" from the bloodstream into the growing cells at the base of the hair follicle. Consequently, drugs would be tightly bound in the interior of the hair shaft during the following keratogenesis. This mechanism is quite rational, since a dense network of capillaries surrounds the base of the hair follicle [19]. Following this model, drug accumulation would be strictly dependent on the drug concentration in the blood, which, in turn, depends on the dose of drug administered.

The "passive diffusion" model also draws on the scientific basis for "segmental hair analysis", which determines the time-course of the drug deposition analysing successive hair segments. Segmental analysis assumes that hair grows at a constant rate, so the position of drugs along the hair shaft can be correlated with the time the drug was present in the bloodstream. This has led to the claim that drug levels in hair reflect a "permanent record" of the drug content of the body during the growing phase of the hair [24]. Anyway, the precision of the estimation, calculated from the position of the drug along the hair shaft, may be strongly influenced by individual differences in hair growth rate and growing cycle synchronicity, as well as by the alignment of the hair fibres or by mechanisms of accumulation from sweat or skin exudates [24].

Various authors have studied the relationship between the drug level in plasma (or drug dosage) and its concentration in hair, indicating a good correlation under controlled conditions [25].

Notwithstanding, other experimental findings suggest the "passive diffusion" model may be oversimplified or may not be totally correct [24].

The first criticism is the marked individual variability sometimes observed in the quantities of drug accumulated in hair of different subjects receiving the same dose, which could only be partially explained by the individual pharmacokinetic and pharmacodynamic [7,26]. Furthermore, the ratios parent-drug/metabolites observed in hair seem to be considerably higher than those observed in blood, even when drug metabolites are the predominant form in the serum or plasma [27,28].

According to the passive diffusion model, which supposes a delay time between the drug accumulation in the follicle and the possibility of detecting the drug residues in the external hair shaft, Gaillard et al. [21] found a peak concentration of clenbuterol in calf hair on Days 26–36 after the treatment, followed by a decrease in the drug level, probably as a consequence of the perpetual replacement of the hair. However, other studies showed that the detection of drugs in hair was observed as early as 1 h after their administration [29], or the distribution of drug along the hair shaft was not always consistent with the predictions based on hair growth rate [24].

All these findings suggest that a more complex model might be necessary to explain how drugs get into hair. With this in mind, various authors have proposed a "multiple pool" model (Fig. 1) which takes into account that drugs exhibit complex equilibriums in the body compartments, and suggests several possible ways to explain their accumulation in hair.

This approach implies that drugs may be accumulated in the hair from the blood during the histogenesis ("endogenous pathway"), from sweat and sebum after formation ("endogenous–exogenous pathway"), from external environment after formation and/or after the hair has emerged from the skin ("exogenous pathway") [6,7,24].

A central point of the "multiple pool" model is that external contamination can be a potential route of entry for drugs, directly or by intradermal transfer. In the latter case skin could

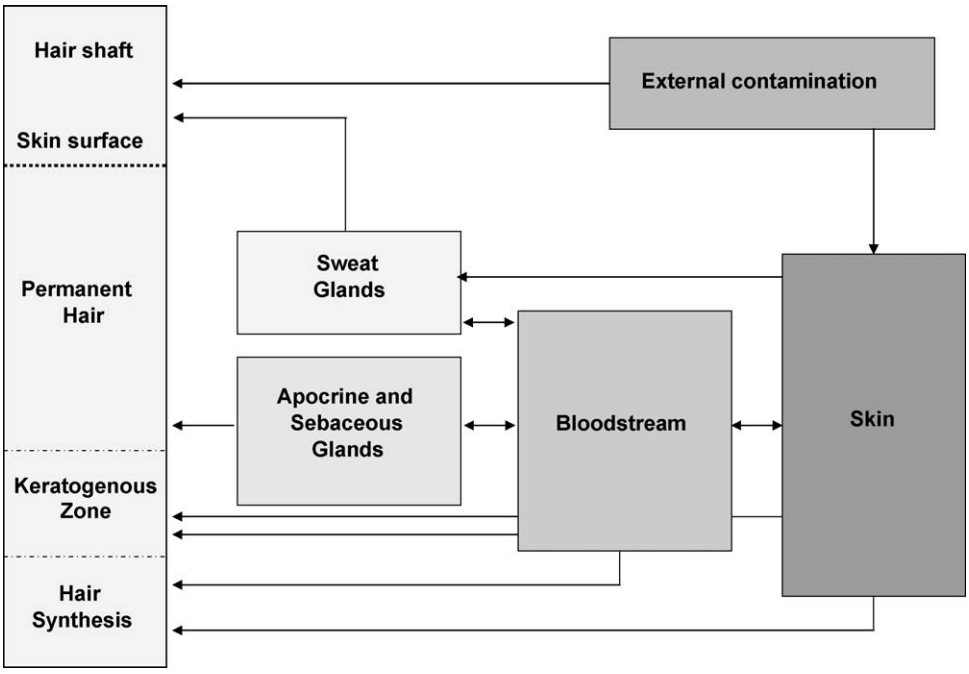

Fig. 1. Scheme for drugs incorporation in hair shaft. Adapted from [24].

act as a trap for some lipophilic drugs (which are retained in the subcutaneous layer) and then released slowly into the bloodstream and finally transferred to the hair follicle. It should be underlined that the pharmacokinetic of skin is quite complex and the potential drug–melanin binding sites in skin have not been completely evaluated yet [24,27].

In addition, drugs and metabolites may be transferred from several body compartments in the tissues that surround the hair follicle as well. For example sweat excretions may be transferred to hair after it has been formed; in this way drugs would be bound less tightly and consequently more easily removed during the sample pre-treatment steps (washes and rinses).

So, when glandular secretions are significant routes for drug accumulation in the hair shaft, the different procedures used by analysts to wash and extract drug residues could produce a great variability in the analytical results.

Several studies on the mechanism of the endogenous pathway suggest that passive diffusion from blood is strongly affected by the same parameters influencing the transport of drugs across biomembranes, such as molecular size and structure of drug, microenvironment (concentration gradient and pH gradient) nature of biomembranes, blood flow, plasma protein binding, lipid solubility of the drug and ratio of ionised to non-ionised drugs (pK_a, Henderson–Hasselbalch equation) [20,30].

Most of the veterinary drugs, after administration, are weak bases or acids that are present in blood bonded to plasma protein or in ionised form. In agreement with the proposed model, only the fraction of molecules which is non-bonded, non-ionised and shows a suitable liposolubility could diffuse across the cell membrane [6]. Then, depending upon the lipid solubility of the substance and the pH of the medium, the drug will be distributed in different cell compartments and will be retained in the keratinised fibre by interaction with other cell components such as melanin, proteins, etc.

Keratines are acidic proteins with an isoelectric point close to six and this could justify the easy accumulation of basic drugs observed in various studies [14,31].

It has also been suggested that the pigmentation could play an important role in the accumulation of certain drugs in hair by hydrophobic or electrostatic interactions with melanin. Various studies, evaluating the binding ability of different drugs to natural and synthetic melanins, have demonstrated that basic and hydrophobic drugs were bonded more strongly than the acid ones [31]. According to these premises, it would be logical to deduce that black hair accumulates basic drugs more efficiently than white or light hairs.

Actually, Gleixner and Meyer [32] found that clenbuterol (a basic drug) concentration in calf hair was higher in black samples than in white ones, but for methyltestosterone and ethynylestradiol no significant influence by pigmentation was observed [15].

On the other hand, Rambaud et al. [33] observed higher concentrations of 17α-methyltestosterone and 17β-estradiol-3-benzoate in black cattle hair than in white. Amphoteric drugs such as some potentiated sulphonamides and some quinolones, have also been reported to be accumulated predominantly in dark rather than in light hair [25,28].

However, hair pigmentation can only be reasonably considered as one of the multiple factors which seem to influence the deposition of drugs in hair [34]. If a basic compound has a better affinity to melanin than the acidic ones, the acidic drugs generally show a higher blood concentration, which can be a thousand times more than that of a basic molecule [6].

The method of administration of a drug (intravenous, oral) also seems to influence the rate and the possible manner of its

accumulation in the hair structure. It is well known that intravenous therapy quickly increases serum level of the parent drug while the oral administration enhances the presence of metabolites in the body fluids.

In accordance with these observations, Dunnet et al. [25] found higher relative levels of the parent drug (enrofloxacine) in horsehair after an intravenous administration than after an oral one.

In another study on the hair accumulation of a cationic compound (rhodamine), a rapid accumulation in cortex and medulla proteins was observed when the drug was injected, while it was gathered in the external cells when applied by soaking the hair in a rhodamine solution [27,35].

Regardless of the method of introduction, it is generally accepted that once drug residues are accumulated in the hair shaft they can persist for a long time in this protected microenvironment, lengthening the time window of its detection [26,28].

Despite there being limited data available on this subject, the stability of entrapped drugs is not absolute [36]. Hair shaft can be progressively damaged by various environmental factors, so drugs may gradually leak out from the injured hair by washing or might be decomposed over long periods, due to the effect of ultraviolet light or heating [7,36].

The close association of drugs with the hair components and the compact and solid nature of the hair may also create difficulties during hair analysis, as this could prevent a satisfactory release of the drugs and impede their detection. Consequently there are many reasonable doubts about whether the efficiency of the extraction, determined by spiking hair samples, simulates the behaviour of in-vivo incorporated drugs or not.

In this contest thorough validation procedures and the availability of reference material or certified samples (preferably obtained using labelled drugs) appear essential to extend the use of this matrix in official controls.

5. Sampling and extraction methods of veterinary drugs from hair

The basic concepts of hair analysis are similar to those included in monitoring plans for other biological matrices.

Hair samples, often taken from living animals, are generally washed before the analysis to reduce the external contamination and, after the extraction and purification steps, the presence of target analyte(s) is evaluated with suitable techniques of detection [6].

Despite the apparent simplicity, the practical execution of these operations could be complicated as a consequence of the lack of well established practices or official methods for hair analysis.

For example, the effects of different sample pre-treatments, such as washing and pulverising, on the extraction efficiencies of target compound(s) are really little known. Additionally, the hair extracts, generally obtained from small quantities of samples (50–200 mg), may be heavily loaded with interfering compounds. Both factors make the concentration of the extract difficult and necessitate the use of highly selective techniques for the clean-up and detection.

5.1. Specimen collection

The choice of the site and the technique for collecting the hair samples can influence the final results considerably, because differences in trace elements and drug concentrations have been reported for hair samples collected from different locations of the body [26]. So it is advisable to collect the hair samples from an area with a low variability in growth rate, which in turn may depend on anatomical region, age, gender, ethnicity or inter-individual variability [20].

It is necessary to reduce the possible external contaminations by taking the sample in the least dirty area of the body of the animal. It is also important to collect a uniform and adequate quantity of samples for the analysis, maintaining a constant distance from the scalp.

In a recent study Rambaud et al. [33] found higher accumulation of 17α-methyltestosterone and 17β-estradiol-3-benzoate near the treated zone when the drugs were administered intravenously.

In the same study the neck region was the cleaner zone, and in the samples taken there the authors found a major accumulation of some steroids as well as a higher stability and a longer detection window for these compounds.

If a segmental analysis is to be done, it is essential to keep the root ends aligned during the sampling, because in the next step the hair shaft must be cut into segments that will be analysed individually to correlate time of ingestion with location of the drug along the hair shaft [17,26].

5.2. Washing and pre-treatment protocols

The problem of external contamination is of great relevance for the detection of drugs of abuse in human samples. In this case the hair samples are routinely washed before analysis to remove lipids and other external contaminants. On the other hand, the establishment of "cut-off values" or "kinetic wash criteria" to discriminate between washing and extraction in forensic and doping investigations has been largely debated, but not yet resolved [6,26,37].

The same problem can often be experienced with veterinary drugs, because animals can be exposed occasionally to environmental particles, urine or faeces containing drug residues as a consequence of authorised veterinary therapies.

The washing procedures generally described in forensic hair analysis include the use of MeOH, EtOH, dichloromethane, methylene chloride, 0.1% sodium dodecylsulfate (SDS), aqueous buffers with/out detergents (Tween 20) and water as an additional rinse [7].

The use of extensive washing and/or low polarity solvents could partially remove incorporated drug residues from incurred samples [7,26]. As reported by Sauer, the concentration of 19-nortestosterone was significantly higher in unwashed hair than in hair washed three times with 0.2% aqueous SDS [38].

Whereas Hernández-Carrasquilla [39] showed that even a complete hair-washing procedure (with detergents, phosphate buffer or organic solvents) cannot completely eliminate the external contamination with β-agonist compounds in bovine hair.

Therefore, considering the many possible ways for drug accumulation, the presence of a certain degree of external contamination cannot be reasonably excluded in farm animals' hair. Notwithstanding, prohibited compounds and veterinary drugs not administered on the farm for therapeutic use should not be present in sampled hair at any detectable rate.

Once the hairs are washed and dried a pre-extraction treatment should be applied. Intact hair, fine cutting, powdering and homogenization have been reported as the form of the hair sample for extraction (Table 1).

Although some authors recognize that these pre-extraction treatments can influence the kinetic and the efficacy of the extraction, few experimental data have been published on the effects of these factors [35].

5.3. Extraction procedures

Various procedures for the extraction of drugs from hair have been described in forensic science, doping control and in veterinary drug detection [7], namely:

(a) *alkaline digestion*: generally involves incubation of the hair sample in 0.1–5 M NaOH at 45–100 °C for a variable length of time (10 min, overnight). The use of NH_4OH 0.2 M or KOH 0.5 M both at 60 °C was also reported [25,40]. After pH adjustment the aqueous solution is purified and analysed. The alkaline digestion seems to produce a complete break-up of the hair structure, but it is not applicable to drugs which are unstable under alkaline conditions [7];

(b) *enzymatic digestion*: several classes of enzymes have been used for the hair hydrolysis, such as β-glucuronidase/arylsulfatase (glusulase), proteinase K, protease VIII, Biopurase, pronase (with or without addition of dithiothreitol). The merit of these methods is that hair structure is dissolved at neutral pH, causing a limited degradation of unstable drugs, but are relatively expensive and have been typically applied only to human samples [7,41]. Recently, enzymatic digestion has been successfully applied for the detection of synthetic glucocorticoids in cattle hair [42].

(c) *acid digestion*: is generally carried out by the use of 0.1–0.6 M HCl or TFA 0.2 M at 47–70 °C for several hours (Table 1).

After the acid extraction the solution is neutralized and purified. In other studies mixtures between acids and MeOH or DTT have been applied to improve the extraction efficiency minimizing the hydrolysis [15,43];

d) *Others*: direct extractions of the hair samples with organic solvents (methanol, acetone) or aqueous buffers and with or without ultrasounds are also reported in literature [41,44–46]. Some authors proposed the use of aqueous TCEP (tris(2-carboxyethyl)phosphine hydrochloride) solutions to liberate anabolic molecules incorporated in hair matrix because this reactant appears to be more stable and effective than other reducing agents such as DTT [47,48]. The extracts obtained with organic solvent are generally clearer and more easily purified than those obtained with alkaline or enzymatic digestions, but there are serious doubts about the ability of organic solvents to adequately penetrate the keratinised region of the hair, which is very polar [33,49].

Supercritical fluid extraction (SFE) has also been used to extract drug residues from human hair samples without the need for lengthy preparation procedures. Another advantage of SFE over conventional methods is the direct extraction which reduces the risk of samples' cross-contamination [50,51].

Several authors recognized that alkaline and some kind of enzymatic digestions are the only ones which provoke a virtually complete breakdown of the hair structure, while other treatments cannot assure that the drug has been totally extracted from the keratised tissues [28,39,41,43].

During the development of a method for the detection of sulphonamides in calf hair, our research group studied the effect of various extraction media on the hair structure. By the use of electronic scanning microscopy it was possible to observe that the external hair arrangement was scarcely affected under our experimental conditions (acidified methanol, acetone and enzymatic treatment with papain) (Fig. 2). The chromatographic profiles of hair extracts obtained under different extraction conditions are shown in Fig. 3.

Acetone and methanol produced clear and clean extracts but poor extraction of the SMZ residues included in the hair structure. On the contrary, the NH_4OH solution gave satisfactory results in terms of apparent SMZ extraction, but the crude extract should be purified before HPLC analysis [52].

Recently Rambaud et al. [33] studied the extraction time-course of several anabolic steroidal hormones in incurred cattle hairs by sequentially washing or extracting samples with different solutions (DCM, MeOH and aqueous NaOH 1 M). Detectable levels of 17α-methyltestosterone were found in all fractions, supporting a time-course accumulation in accordance with the "multiple pool" model.

These observations reinforce the hypothesis that a great percentage of the drugs could be tightly retained in the biological structure of the hair and slowly released during washing and extraction [27].

5.4. Extract purification

Crude hair extracts are generally submitted to a clean-up step to reduce interfering compounds and to concentrate the target analyte(s) prior to analysis (Table 1).

Purification of hair extracts containing β-agonists has been carried out by LLE with TBME or hexane [32,53,54], by SPE cartridges (mixed-mode C_8 + SCX; C_{18}) [39] or by affinity chromatography [44].

LLE with organic solvents was also successfully applied to separate and purify diazepam and anabolic hormones in aqueous hair extracts [15,46,55].

Table 1
Screening procedure and published data for veterinary drug residues in livestock hair

Analyte(s)	Animal	Decontamination; sample weight	Extraction; clean-up	Chromatographic separation; detection conditions	Analytical results		Reference
					LD (ng/g)	(ng/g)[a]	
	Calves	Water SDS 1%; 100 mg	MeOH; Inmunoextraction, SPE (C_{18})	GC (HP5); EIA, SIM [clenbuterol, *m/z* 264:262:86]	3 pg/well	7.9–56.6	[44]
	Cattle	Water–BSA, 0.1%–Tween 80, 0.2%; 50 mg (powder)	50 mM DTT, NaOH 5 M, TBME, $T_{amb.}$, overnight; LLE with TBME	LC; EIA	0.03	1–100	[32]
	Bovine	300 mg	NaOH 1 M, 45 °C, overnight; LLE with *n*-hexane	LC (Novapack C_{18}) [150 mm × 3.9 mm]; EC; APCI (+), MS [clenbuterol, *m/z* 549:453:418]	NC[b]	8.3	[53]
β-Agonists	Calves	EtOH-buffer-DCM; 100 mg (powder)	HCl 0.1 M, 56 °C, 12 h; SPE(C_{18})	GC (CP SIL 8CB) [25 m × 0.25 mm]; MS, MIC [clenbuterol, *m/z* 285:243]	16	20–4372	[21]
	Cow	100 mg	NaOH 5 M, 95 °C, 10–30 min; LLE with TBME, SPE (Mixed mode C_8/SCX)	GC (CP SIL 5CB) [15 m × 0.25 mm]; ELISA; CI, MS–MS	1.1 (white); 2.9 (black)	9–590	[54]
	Bovine	Water and organic solvents; 500 mg	HCl 0.1 M, 60 °C, overnight; SPE (Mixed mode C_8/SCX)	GC (HP 5 ms) [30 m × 0.25 mm]; SIM [clenbuterol, *m/z* 262:243:212]	NC[b]	20–400	[39]
	Horse	50 mg	KOH 0.5 M, 60 °C, 3 h; SPE (HLB-OASIS)	GC; HRMS	0.2	0.5–23.25	[40]
EtES/MeTS and other anabolics	Bovine	Tween 20 (0.2%); 500 mg	Different extraction conditions; SPE(C_{18}), LLE with TBME	GC (HP 5 ms) [30 m × 0.25 mm]; EI, SIM [17α methyltestosterone, *m/z* 313:143:403]	40, 100, 200	ND[c]	[55]
Corticosteroids	Cow	100 mg	HCl 1 M:MeOH (3:2), 47 °C, 4 h; SPE(C_{18}), LLE with Na_2CO_3 10%, SPE(Si)	LC (Nucleosil C_{18}) [50 mm × 2 mm]; ESI (−) [dexamethasone, *m/z* 451:361:331]	2.9–93 pg/mg	ND[c]	[43]
Corticosteroids	Cattle	DCM; 100 mg (cut)	Proteinase, Tris–HCl 0.1 M (pH 9.6), 60 °C, 2 h; SPE(C_{18})	LC (Hypersil Hypercarb C_{18}) [100 mm × 2.1 mm]; ESI (−), MS–MS, MRM	–	6.1–348	[42]
EtES/MeTS	Calves	Water–EtOH 95%; 100 mg (powder)	100 mM DTT, HCl 1 M, TBME, $T_{amb.}$, overnight; LLE with TBME, SPE(C_{18}), LC_{prep}.	EIA	0.2	0.3–8.9	[15]
EtES	Cattle	Tween 80 (10% in water); 500 mg (cut)	NaOH 1 M, 100 °C, 10 min; DDE with DCM	GC (HP 5 ms) [30 m × 0.25 mm]; MS–MS, SIR	0.52	2.01–23.61	[22]
EtES	Cattle	Tween 80 (10% in water); 200 mg (cut)	NaOH 1 M, 100 °C, 10 min; DDE with DCM	GC (HP 5 ms) [30 m × 0.25 mm]; MS–MS, SIR	0.52	5.48	[16]
17βEstradiol-3-benzoate MPA, MeTS	Bovine	DCM–MeOH; 100 mg (powder)	NaOH 1 M, 85 °C, 25 min; LLE with Et.Ac., SPE (NH_2, Si)	LC (Nucleosil C_{18} AB); GC (OV-1); ESI (+), MS–MS, MRM; EI, MS–MS, MRM	0.2–4.1	0–17.9	[33]

Table 1 (*Continued*)

Analyte(s)	Animal	Decontamination; sample weight	Extraction; clean-up	Chromatographic separation; detection conditions	Analytical results		Reference
					LD (ng/g)	(ng/g)[a]	
19-Nortestosterone (Nandrolone)	Bovine	Unwashed, SDS 0.2%	–	LC, GC; APCI, ESI (+) MS Ion Trap; EI, MS, SIR	–	13.4 pmol/g (white); 28.9 pmol/g (black)	[38]
MeTS/nandrolone and other steroids	Cattle	MeOH–water–DCM; 100 mg (powder)	MeOH, USBath, 50 °C, 2 h NaOH 1 M, 80 °C, 15 min; LLE with Et.Ac., SPE (NH_2, Si)	GC (CP SIL 8CB) [30 m × 0.25 mm]; EI, MS	0.07–6.2	4.5–48.6	[45]
TS/nandrolone and precursors	Horse	MeOH:water (1:1) v/v; 100 mg (cut)	MeOH, USBath, 50 °C, 5 h; LLE with *n*-pentane:MeOH (25:1), HPLC	GC (HP 5 ms) [12.5 m]; MS–MS, HRMS	0.1–5.0	0–2	[61]
Estradiol benzoate	Cattle	500 mg (powder)	25 mM TCEP, 1 h; LLE with TBME., SPE(C_{18})	LC (Symmetry C_{18}) [150 mm × 3 mm]; ESI (+), MS–MS, MRM	5	8–19	[47]
TS esters/boldenone undecylenate	Bovine	200 mg (powder)	25 mMTCEP, 1 h; SPE(C_{18})	LC (Symmetry C_8) [150 mm × 2.1 mm]; ESI (+), MS–MS, MRM	2–50	ND[c]	[48]
Quinolones	Equine	Buffer–water–DCM; 50 mg (cut)	TFA 0.2 M, 70 °C, 24 h; SPE (Mixed ModeC_{18}/SCX)	LC (Genesis C_{18}) [150 mm × 2.1 mm]; DAD, λ_{max} = 450 nm	60–120	19–452 ng/mg	[25]
Stanozolol	Bovine	500 mg (powder)	NaOH 1 M, 65 °C, 2 h; LLE with isobutanol, SPE (mixed mode C_8/SCX)	LC (Symmetry C_{18}) [150 mm × 3 mm]; ESI (+), MS/MS, MRM [stanozolol, *m/z* 329-81:29-21]	0.25	0.8–25	[62]
Sulphonamides	Equine	SDS 2%–water–DCM; 100 mg (cut)	Ammonium hydroxide 0.2 M, 60 °C, 24 h; SPE (mixed modeC_{18}/SCX)	LC (Genesis C_{18}) [150 mm × 2.1 mm]; DAD, λ_{max} = 240, 270 nm	70–100	0.2–25 ng/mg	[28]
Sulphamethazine	Cattle/pig	Water; 50 mg (cut/powder)	Ammonium hydroxide 0.2 M, 60 °C/NaOH 0.1 M, 60 °C; tandem SPE (C_{18} + SCX)	LC (Luna RP C_{18}) [150 mm × 2 mm]; DAD, λ_{max} = 270 nm	46	7.5–59.2 ng/mg	[52]

[a] Range of concentration found in real samples.
[b] NC = not calculated.
[c] ND = not detected in real samples.

Dunnett et al. [25,28] proposed a purification procedure using mixed-mode (C_{18} + SCX) SPE cartridges for the detection of some potentiated sulphonamides and quinolones, which is based on the amphoteric properties of these compounds. A similar procedure, employing a tandem SPE (RP + SCX) was developed by Gratacós-Cubarsí et al. [52] to purify alkaline pig and calf hair.

Diphasic dialysis methods have also been applied for the extraction-purification of clenbuterol and ethinylestradiol in digested hair samples [16,22,56,57].

The complexity of hair extracts has sometimes obliged the development of combined clean-up procedures, involving both LLE and multiple SPE steps to reach a satisfactory degree of purification. For example, natural and synthetic steroids have been determined in hair extracts after a tandem LLE-SPE(NH_2/Si) [33,45].

Finally, some authors [58] proposed the use of headspace solid-phase microextraction (HS-SPME) for the control of volatile recreational drugs of abuse in human hair as a screening method. SPME is an excellent alternative to the above classical methods for volatile compounds, because the extracts appear cleaner, the procedure is fast and can be coupled to GC/MS easily [59].

6. Analytical techniques

The primary challenge in hair analysis frequently is the low concentration of the target analyte(s) and the interferences in the matrix.

Historically, radioimmunoassays (RIA) were first used for detecting drugs of abuse in human hair. Currently

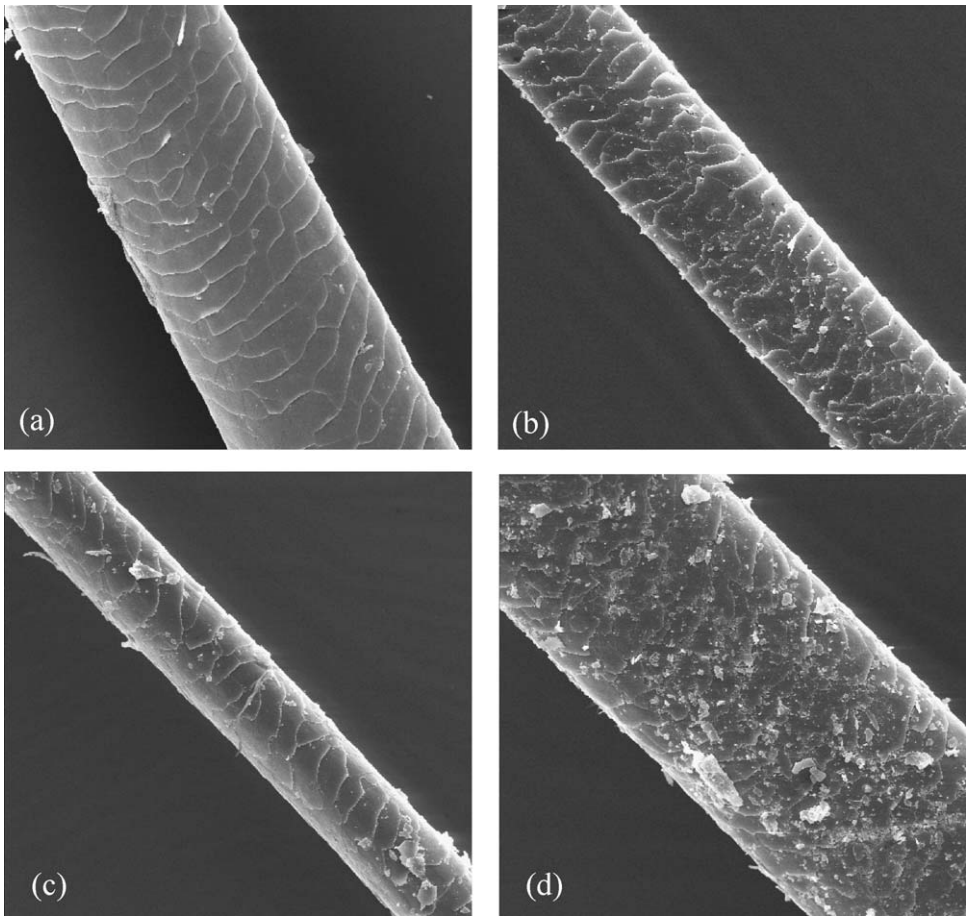

Fig. 2. Electronic scanning microscope images obtained from a calf hair sample treated with different extraction solutions: (a) not treated; (b) acetone; (c) enzymatic treatment (papain 1.7 μM, EDTA 3.8 mM in phosphate buffer 1 M pH 6.0); (d) acidified methanol (HCl 1 M:MeOH 60:40 v/v) (all treatments realized maintaining 200 mg of cut hair +5 ml of extraction solution at 40 °C for 4 h).

ELISAs and coated-plate 96-well microplate EIAs are generally employed for the detection of drug residues in hair extracts.

The immunoassays (IAs) used in hair analysis are usually the same commercial products designed to detect drug residues in other biological samples. They must not experience interference from the hair matrix and their antibodies must be suitable to work at the concentration of drug present in hair extracts [10]. Another requirement is that the extract be compatible with the antibody proteins of the immunoassay reagents. For this reason enzymatic digestions are better for IAs than chemical ones, because strongly acidic or alkaline extracts must be brought to a neutral pH before the IA.

Even if IAs represent a rapid, convenient and quite sensitive screening for the presence of drugs in hair [44,54,60], chromatography is undoubtedly the analytical technique of choice (Table 1). The great selectivity of LC or GC analytical columns allows the separation of the target analyte(s) from the interferences of the matrix as well as the DAD, or MS detectors may provide elements to confirm the identification [7].

Drugs with a relative degree of polarity, such as quinolones and sulfonamides, have been separated and quantified in animal hair extracts at ppm levels using RP-C_{18} analytical columns and DAD detectors [25,28,52].

Other substances, such as anabolics, β-agonist, benzodiazepines and corticosteroids, which accumulate in hair at lower concentrations (ppb, ppt), have been detected by LC or GC, usually coupled to MS detectors.

When these compounds have been separated on 100% dimethylpolysiloxane or 5% phenyl-95% dimethylpolysiloxane GC capillary columns, a derivatization step with MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide) was required to improve their volatility. The addition of TMIS, 2-mercaptoethanol, DTT, DTE or NH_4I has also been proposed to improve the efficiency of the derivatization reactions [16,22,33,40,45,54,55,61].

Hernández-Carrasquilla [55] proposed a double derivatization with MOHC (*O*-methylhydroxylamine monochloride) followed by MSTFA-TMIS (1-trimethylsilyl-imidazole) to enhance the detection of certain anabolics in hair extracts.

The use of trimethyl boroxime as derivatizing agent was also evaluated in a study on the detection of clenbuterol in calf hair. The authors stated that the formation of clenbuterol methylboronate derivatives increased the sensibility and the quality of the chromatograms [21].

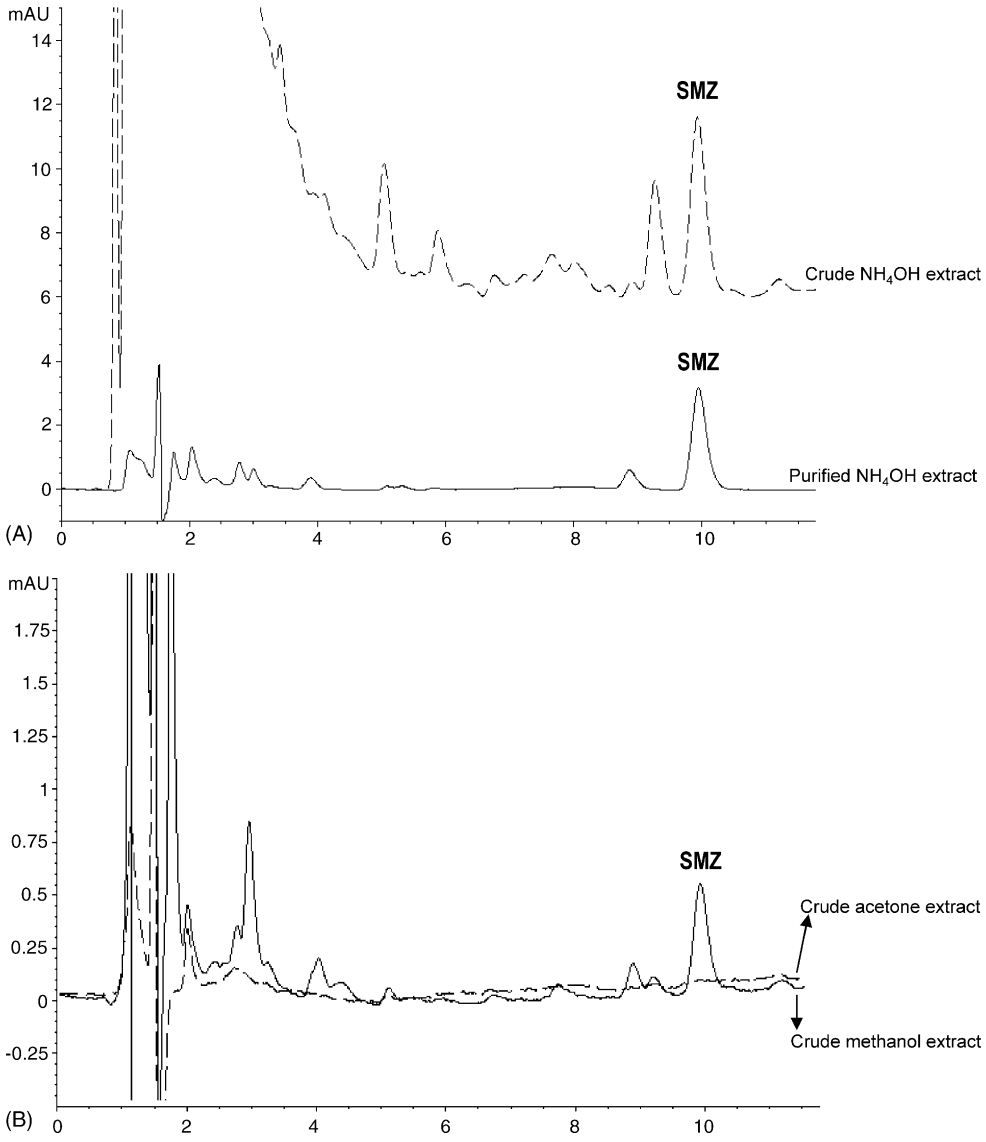

Fig. 3. Chromatographic profiles of different hair extracts obtained from the same pigmented hair samples taken from a calf which received a subcutaneous treatment with sulfamethazine (SMZ): (A) NH_4OH 0.2 M crude extract (dot line) and the same hair extract purified with tandem SPE (continuous line) as described in [52]; (B) acetone crude extract (dot line) and methanol crude extract (continuous line). All extractions realized with 50 mg of cut hair +4 ml of extraction solution at room temperature for 12 h. Chromatographic separation was carried out on a Luna RP-C_{18} (150 mm × 2.1 mm i.d.) column at a flow rate of 0.35 ml/min with a binary gradient elution between ammonium acetate 0.01 M (pH 4.5) and acetonitrile. Signal recorded at 270 nm, more details in [52].

More recently, high mass and relatively polar molecules, including some anabolics, corticosteroids and benzodiazepines, have been successfully detected in hair extracts by the use of LC–MS techniques [33,42,43,46,48,53,62]. Several methods for the detection of clenbuterol and diazepam in hair extracts use atmospheric pressure chemical ionisation (APCI) in positive mode [46,53].

Electrospray ionisation (ESI) has been the most extensively used LC-interface, either in positive (stanozolol and some steroids) or negative mode (corticosteroids) [33,42,43,47,48,62].

The possibility to use selective acquisition modes, as single ion monitoring or recording (SIM or SIR) [16,22,39,46,55], multiple ion chromatogram (MIC) [21] or multiple reaction monitoring (MRM) [42,47,48,62] has been generally applied to reduce matrix interferences and to reach the required sensitivity.

Other analytical techniques have been proposed for the analysis of different drug residues in hair extract, such as matrix assisted laser desorption ionisation-time of flight (MALDI-TOF), ion mobility spectrometry, immunocromatography, enzyme multiplied immunoassay technique (EMIT) and LC-coulometric detection [53,63].

7. Outcomes of hair analyses in livestock production

As previously mentioned, the detection of veterinary drug residues in animal hair started with the detection of clenbuterol – a molecule used exclusively in veterinary practice – in rat and in cattle hair [13,32]. Thereafter stazonolol was the first anabolic

steroid detected in farm animal hairs [64]. Since then, several molecules have been detected in livestock hair, including banned compounds or doping agents used in horse racing (Table 1).

The presence of anabolic agents such as β-agonist and steroidal compounds in hair has been extensively studied in farm animals [15,21,38,39,45,47,48,53,55,61,62].

These compounds are administered at low doses (few mg/animal), so the use of very sensitive techniques (GC–MS/MS or LC–MS/MS) is required for the detection of their ultra-trace residues in hair.

The efficacy of hair analysis to detect the illegal use of β-agonists has been largely demonstrated in cattle and horse (Table 1).

However, the suitability of hair analysis to detect hormonal steroids seems to depend greatly on the kind of compound considered in the studies.

Ethynilestradiol, estradiol benzoate, 17α-methyltestosterone, boldenone undecylenate, metandienone, several glucocorticoids, nandrolone, testosterone esters and 17β-estradiol-3-benzoate have been detected in animal hair samples. Surprisingly, no medroxyprogesterone acetate (MPA) and dexamethasone were detected in cattle hair after a single injection [33,42].

The accumulation of various antibiotics (sulphonamides and quinolones) has been clearly established in horse hair [25,28]. The authors observed a good correlation between administered dose and concentration of residues in hair, and a significant influence of hair pigmentation on the deposition. Accumulation of sulfamethazine was also verified in pig and calf hair after a therapeutic treatment [52].

The results of the residue monitoring plan in food of animal origin in the EU for 2003 shows that antimicrobials and hormones are the veterinary drugs with the highest percentage of non compliant results (75 and 5%, respectively) [65]. Furthermore, the complete banning of antibiotics as growth promoters is expected within a few years in the EU [66].

So, even if the presence of veterinary drug residues in hair does not imply a direct health risk for the consumers, and LMRs are not currently established for this tissue, hair analysis could gain relevance in the future as a complementary technique to detect their illegal uses.

Hypnotic benzodioazepines such as diazepam, alprazolam, estazolam and midazolam and their metabolites have been detected in rat and horse hair up to 38 days after the administration [46,67]. Other substances have been reported as being accumulated in equine hair such as procaine, benzilpenicilin, trimetoprim, sulfadiazine, metronidazol [17] or cocaine in sheep's wool [68].

From a quantitative point of view, the literature (Table 1) shows that drug residue can be accumulated within a considerable range of concentrations in animal hair, probably as a consequence of the specific conditions of each study. Furthermore, sometimes authors did not report the concentrations observed in incurred hair, even if they described the validation procedure using fortified samples, making the comparison of the results more difficult.

8. Drawback and future of hair analysis

Hair is a specimen that can be easily collected, transported, stored and extracted. The collection method is non-invasive and does not cause any damage or pain to the animal. The detection of veterinary drugs in hair could be interesting since in many cases it offers an enlarged retrospective detection when compared with other biological specimens, although some basic physiological and analytical problems have yet to be resolved satisfactorily.

A well-known weak point of hair analysis is the need for a comprehensive knowledge on the mechanisms of drug incorporation. Pharmacokinetic and pharmacodynamic models in hair are difficult to establish, due to the multiple factors influencing the drug accumulation in the hair shaft. Thus, it is quite impossible to identify general accumulation patterns or reliable relationships between the administered dose and quantity of drug detected in "blind" (unknown) hair samples.

Another central issue in hair testing is the quality control at all stages (pre-analytical and analytical phases). Basically, the development of inter-laboratory studies and/or proficiency tests will be fundamental to give increasing recognition to hair analysis. As mentioned before, it is very difficult to confirm if drugs are completely extracted from real hair samples, so the lack of reference materials, standardized procedures and reference intervals for the interpretation of the results, could make the quantitative findings particularly controversial, as well as the differentiation between medication and external contamination.

More studies are also needed to understand the stability of veterinary drugs once accumulated in hair fibres. The high stability reported for some drugs (over one year), means that therapeutic history could theoretically be studied during all the entire life of farm animals. This advantage can also imply some legal problems to establish where and when a drug has been administered, because often animals are born in one farm and are reared in a different farm (and possibly a different country).

A positive hair analysis result could be a useful indicator to increase or modify the sampling strategies in a specific group of animals. In practice, the results obtained from hair analysis may be very effective for improving the efficacy of control and surveillance plans when compared with those obtained from conventional and complementary biological samples (edible tissues, excreta, manure) [28].

Recent studies have shown that animal hair can accumulate sulphonamide metabolites or steroidal esters as a consequence of a pharmacological administration [28,33,47,48]. Thus, a promising field for future investigations could to be the identification of specific metabolites or derivates of the active compounds, which are accumulated in the hair structure only as a consequence of a veterinary treatment. As proved in humans, the detection in hair samples of these compounds could be a useful tool to exclude false positive results due to external contaminations or to discriminate between natural and administered hormones in farm animal studies.

Acknowledgments

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