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Hair analysis for screening horses for exposure to dietary toxic residues

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Summary

Hair analysis has been proposed and evaluated as a potential diagnostic technique to retrospectively monitor exposure to a range of environmental, dietary and other toxins including drugs, pesticides, mycotoxins, plant toxins, heavy metals and other toxic elements, such as selenium and arsenic, in humans and animals. The purpose of this review is to report and discuss, where data exists, the development and use of this technique to identify such toxic residues in equine hair. In the absence of research specific to the horse, this review draws upon comparative data in humans and other species, on hair analysis and potential dietary toxins of particular relevance in equine nutrition and feedstuffs production.

Keywords: Hair analysis, residues, exposure, drugs, metabolites, mycotoxins, pesticides, heavy metals, selenium, plant toxins, screening

Die Haaranalye zum Nachweis unerwünschter oder toxischer Stoffe beim Pferd nach oraler Applikation

Die Haaranalyse gilt als Instrument der retrospektiven Diagnostik. Prinzipiell können Rückstände aus der Umwelt, nutritive Einflüsse oder Toxinbelastungen inklusive Pharmaka, Pestizide, Mykotoxine, Pflanzengifte, Schwermetalle oder weitere toxische Elemente wie Selen oder Arsen beim Menschen oder Tieren detektiert werden. Dieses Übersichtsreferat geht auf die morphologischen und chemischen Eigenschaften des Pferdehaares ein (Wasser <15 %, Protein 80-85 %, Fette 1-9 %, Melanin 0,3-1,5 %, Mineralien 0,25-1%) und skizziert die Perspektiven, oben genannte toxische Substanzen im Pferdehaar zu identifizieren. Zum Teil fehlen fundierte Ergebnisse vom Pferd, so dass auf Angaben zum Rückstandsverhalten von Substanzen bei anderen Tierarten einschließlich Mensch zurückgegriffen werden muss. Das Haarwachstum von rd. 22-25 mm/Monat unterscheidet die anagene (Formung des Haarschaftes durch aktive Follikel), catagene (Übergangsphase) und telogene (Ruhephase) Phase. Die Inkorporation xenobiotischer Stoffe erfolgt besonders im ersten Wachstumsabschnitt. Salicylate und DMSO zeigen prinzipielle Aspekte der Haaranalyse auf. Diese Stoffe werden oral als Pflanzeninhaltsstoffe aufgenommen, können aber auch aus gezielter Verabreichung entstammen und im forensischen Zusammenhang Beachtung verdienen. Morphin, Codein, Coffein, Theobromin sind ebenfalls im Haar des Pferdes nachweisbar, was bezüglich Hordenin noch der Überprüfung bedarf. Experimentelle Arbeiten zur Applikation von Steroiden und ihrem Verteilungsverhalten zeigen, dass diese rechtlich bedeutsame Substanzgruppe über Haaranalysen detektierbar ist. Dies ist besonders wegen der langfristigen Ablagerung im Haar von Interesse. Für Toxine aus dem Bereich der Pestizide ist sind nur unzulänglich Daten vom Pferd verfügbar. Bei Schwermetallen und weiteren Elementen wie Selen und Arsen sind z.T. gezielte Untersuchungen vorhanden, die auch den Stellenwert einer Harranalyse im Vergiftungsfall belegen. Ob auch bezüglich einer Belastung des Pferdes mit Mykotoxinen sowie Giftpflanzen diagnostisch relevante Perspektiven bestehen, bedarf noch der Abklärung.

Schlüsselwörter: Haaranalyse, Rückstände, Belastung, Medikamente, Stoffwechselprodukte, Mykotoxine, Pestizide, Schwermetalle, Selen, Pflanzentoxine, Screening

Introduction

Hair analysis is a diagnostic technique utilised in humans and animals that can identify and assess exposure of individuals, groups and populations to various harmful/toxic substances that can be present in the environment. Many xenobiotics including drugs, metals, pesticides and biological toxins accumulate and are retained in hair for the lifetime of the hair shaft.

Hair has not been generally regarded as a major excretory route for endogenous or exogenous substances, as quantitatively, amounts eliminated in hair, expressed as a percentage of the total acquired dose or exposure, are small. Hair however, compared with other body tissues and fluids provides an extremely stable analytical matrix. It is a dehydrated, metabolically inert and mechanically tough tissue that is highly resistant to environmental change or damage. Its nature ensures that analytical residues degrade only very slowly and remain detectable for prolonged periods without the need for refrigerated sample storage. Thus, hair analysis can provide an historical record of exposure, with retrospective detection possible for weeks, months or even years after systemic challenge. Contrastingly, hair root analysis offers the possibility of identification of recent acute exposure. *Gygi* et al. (1995) detected codeine in the follicles of plucked hair samples only one hour after administration. Other attractions for hair as an analytical matrix include ease of sample collection, transport and storage. Furthermore, a single hair sample, unlike a one-off blood or urine sample, is able to discriminate between a single acute toxin challenge, repeated dosing or chronic exposure.

The efficacy and breadth of this screening approach continues to be an active area of research and this review will identify some of the potential applications of the technique and its inherent limitations, specifically in relation to horses and their diet.

Hair structure, composition and growth

Hair is a living tissue comprising two gross structural features; the follicle and the hair shaft. The follicle can be considered a miniature organ, as it is associated with vascular, muscular and glandular components, and enzyme systems that determine the biochemical composition of the hair shaft. The hair shaft derives from follicle growth, but in itself is metabolically inert and once formed undergoes no further biogenic turnover.

Three principal hair types are recognised within equine skin; the temporary hair of the coat, the permanent hair of the mane and tail, and the tactile hairs around the muzzle, eyes and ears. They have in common, three distinct structural components: an outer cuticle, a medial cortex, and a central medulla. The thin cuticle is a protective scale-like structure, the overlapping cells of which also anchor the hair shaft within the inner root sheath. The cortex makes up the bulk of the hair shaft, comprising longitudinally orientated keratinocytes constructed from macrofibrils, or fibrous keratin (protein) bundles. These have a high cysteine content and cross-link through disulphide bridges to give the hair shaft mechanical strength.

Hair is a cross-linked orientated polymeric structure that is approximately 80 – 85 % protein. Other significant components of the hair shaft are; water (< 15%), lipids (1 – 9%), melanins (0.3 – 1.5%) and minerals (0.25 – 1 %). Although the hair shaft is essentially a dehydrated structure it can contain variable amounts of water, derived from sweat and atmospheric moisture. Hair lipids include triglycerides and free fatty acids, that are intrinsic structural components (cell membrane complexes) and surface deposits of sebaceous or apocrine origin. Melanins (eumelanins, black-brown; pheomelanins, red-yellow) are endogenous pigments, formed from tyrosine oxidation within melanocytes in the follicle and distributed as melanin granules within the cortex. The mineral content comprises macro-elements, such as calcium and phosphorus, trace elements and heavy metals.

Equine hair has a cyclical growth pattern, beginning with an extended period of growth (anagen) during which the follicle actively forms new hair shaft. This is followed by a short transitional phase (catagen) when growth ceases and shrinking of the follicle occurs, and subsequently, a quiescent period (telogen) during which an inactive club hair is formed. Finally, shedding of the hair shaft (exogen) is precipitated by the underlying formation of a new hair shaft in the new anagen phase. The rate of growth during anagen in horses has been variously studied (*Beresford* et al. 1998, *Popot* et al. 2000, *Dunnett* and *Lees* 2003). Measurements in 29 horses of different breeds showed continual hair growth and a linear growth-rate (r^2=0.994-0.998) in the permanent hairs of the mane and tail over a 12 month period. This varied with anatomical location and breed, but not with gender. Average growth-rate was greatest in the tail and cranial region of the mane (24.8 mm/month) and least in the caudal region of the mane (21.3 mm/month). Mane growth was fastest in UK native pony breeds including Shetland and Welsh Mountain ponies, (24.8 mm/month) and slowest in Thoroughbreds (21.6 mm/month). Unlike the coat, there was no evidence of a statistically significant seasonal influence on equine mane and tail growth (*Dunnett* and *Lees* 2003).

Potentially toxic feed residues and deposition in hair

A number of potential routes exist for the uptake of xenobiotics into hair, many of which are interrelated and may be synergistic. The broadly accepted multi-compartment model proposed by *Henderson* (1993), identifies uptake from the follicle vascular supply, lymphatic system, sebaceous and apocrine secretions, eccrine sweat, transfer from surrounding tissues, including skin, and surface deposition from the environment. The biochemical concept of xenobiotic incorporation and deposition in hair is based on, principles of molecular transport across cell membranes, principles of metabolism/biotransformation, and xenobiotic-melanin binding (*Potsch* et al. 1997). A number of physico-chemical factors, such as blood flow, plasma protein binding, lipid solubility, degree of ionisation, molecular size and geometry, and pH and concentration gradients influence xenobiotic transport into hair. Once deposited within the hair milieu, the exact mechanisms and/or structures involved in xenobiotic binding remain speculative, but include lipids of the cell membrane complex, structural proteins and melanins (*Potsch* et al. 1997).

Numerous harmful or toxic substances can potentially contaminate the equine diet, whether manufactured feeds and supplements, or grazing and preserved forages. Such dietary contaminants can be divided into six categories: drugs (pharmacologically active substances), heavy metals, non-metallic toxic elements, pesticides, mycotoxins, and plant toxins. Self-evidently there is some blurring between the definitions of plant toxins and pharmacologically active substances (phytochemicals). Examples within these categories are given in Table 1.

Table 1 Potential equine dietary toxins and their sources. *Potentielle Futtertoxine beim Pferd und deren Herkunft.*

Category	Sources	Examples
Drugs	Weed contamination of crops Manufacturing contaminants Medicated feeds cross-contamination	Atropine, morphine Methylxanthines Ionophore antibiotics
Heavy metals	Mining and metal smelting, contaminated soil, grazing	Lead, cadmium
Other toxic elements	Soils, grazing and water	Selenium, arsenic
Pesticides	Rodent control Pasture management	Coumarins
Mycotoxins	Grains, mixed feeds, forage	Fumonisins, zearelanone
Plant toxins	Grazing, preserved forage, ornamental plants	Alkaloids

Drugs and metabolites

The most commonly encountered pharmacologically active 'drug' residues in equine feedstuffs include salicylates, dimethylsulphoxide (DMSO), the methylxanthines caffeine and theobromine, morphine, hyoscine, atropine and hordenine. However, there are a considerable number of pharmacologically active substances present in manufactured feeds, grazing and preserved forages that can be considered as 'drugs' and certainly as prohibited substances, in terms of competition rules. Their presence in equine feedstuffs can be categorised as, natural feed constituents, feed crop contaminants, or manufacturing or shipping contaminants. Examples of these together are given in Table 2, however the list is indicative rather than exhaustive.

Table 2 Indicative examples of pharmacologically active contaminants and components in equine feedstuffs and grazing.
Beispielhaft pharmakologisch wirksame Substanzen (Kontaminationen oder originäre Inhaltsstoffe) in Futtermitteln für Pferde

Pharmacologically active substance (drug)	Source
Salicylic acid	Alfalfa, willow
Dimethylsulphoxide (DMSO)	Alfalfa, others
Caffeine	Coffee
Theobromine	Cocoa
Theophylline	Coffee, Cocoa
Morphine	Papaver ssp.
Codeine	Papaver ssp.
Hordenine	Germinating barley
Hyoscine	Datura spp.
Atropine	Datura spp.
Lupanine	Lupin seed
Bufotenine	Phalaris grasses
Valerenic acid	Valerian
Dicoumarol	Spoiled sweet clover
Borneol	Carrots, wood savings

Natural feed constituents

Salicylates and dimethylsulphoxide

Salicylates (*Beaumier* et al. 1987) and dimethylsulphoxide (DMSO) (*Law* 1990) are present in numerous equine feedstuffs and many pasture species. Salicylates are found at particularly high concentrations in grazing and forage legumes, such as clover and alfalfa (*Beaumier* et al. 1987), respectively, and willow-based herbal remedies. Phytogenic salicylates are metabolised in vivo to salicylic acid, an analgesic anti-inflammatory. DMSO also occurs at high levels in alfalfa (< 32 mg/kg) and is a weak analgesic anti-inflammatory (*Law* 1990). Owing to their widespread occurrence and pharmacological properties, racing jurisdictions have established thresholds for their presence in post-competition urine and blood samples. Although, in itself it is unlikely that feed-related salicylate load will cause such testing thresholds to be exceeded, it can be used as an example of how hair analysis can identify exposure to dietary phytopharmaceuticals. Salicylic acid is readily detected in mane hair of horses fed comparatively high alfalfa diets; 3 x 2 kg per day (Fig. 1).

Hair analysis can also discriminate between horses on high and low salicylate-containing diets (Fig. 2), and furthermore, when the diet of an individual horse changes from a high to low salicylate content (Fig. 3). Neither of these substances appears to be associated with acute or chronic toxicoses in horses.

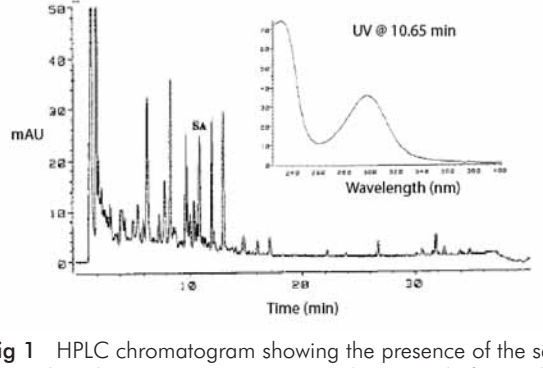

Fig 1 HPLC chromatogram showing the presence of the salicylic acid peak at 10.65 min in a mane hair sample from a horse on a high alfalfa (salicylate) diet, and inset the UV absorption spectrum in confirmation of the identity of the salicylic acid peak.
Signal für Salicylsäure in einem HPLC-Chromatogramm bei einer Retentionszeit von 10:65 Minuten für eine Probe vom Mähnenhaar eines Pferdes, das eine luzernereiche Ration erhielt; verkleinert eingefügt: UV-Absorptionssprektrum zur Absicherung der Identität des Salicylsäurepeaks

Fig 2 Comparison of mane hair salicylic acid concentrations in horses on high and low alfalfa (salicylate) diets. Mean (± SD) hair salicylic acid concentration was significantly higher ($p < 0.001$) in horses on high alfalfa (salicylate) diets.
Vergleich der Konzentrationen von Salicylsäure im Mähnenhaar von Pferden mit hohem und niedrigem Luzerneanteil in der Ration. Im Haar von Pferden mit hoher Luzerneaufnahme wurden auch signifikant höhere Konzentrationen an Salicylsäure gefunden als im Haar luzernearm versorgter Pferde.

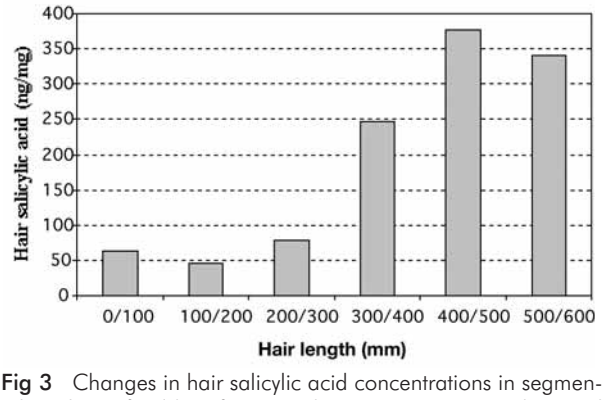

Fig 3 Changes in hair salicylic acid concentrations in segmental analysis of tail hair from one horse over a 24 month period during which the diet was changed from a high to low alfalfa (salicylate) content.
Verlaufsuntersuchungen zu Veränderungen in der Konzentration von Salicylsäure im Schweifhaar während einer 24monatigen Periode mit einem Wechsel von hohem zu geringem Luzerneverzehr.

Hordenine and bufotenine

Hordenine (a sympathomimetic) and bufotenine (a hallucinogen) are recognised as occasional contaminants of equine feedstuffs. Both substances are present in Phalaris grass species (Canary grass), and hordenine also occurs in germinating barley and other Gramineae species. Although neither bufotenine nor hordenine are specifically associated with toxic syndromes in horses, excessive consumption of Phalaris grass species is associated with a number of syndromes that are collectively known as Phalaris staggers (*Bourke* 1994, *Colegate* 1999). As both substances affect the CNS of horses they are regarded as prohibited substances under competition rules, and they have both been detected in post-race urine samples in Europe and Australia (*Schubert* et al. 1990, *McCaffrey* et al. 2002). There are no reports in the literature describing the

detection of bufotenine or hordenine in animal or human hair, however, given that these substances are weak organic bases, they should be amenable to detection in this tissue.

Feed crop contaminants

Morphine and codeine

Morphine and codeine present a less common but significant feed contamination issue. Firstly, their presence in post-race samples is a breach of equine competition prohibited substance rules. Secondly, morphine is legally classified as a controlled drug. Finally, opiate drugs can have significant adverse behavioural (CNS stimulatory) effects on horses even at low doses (*Suann* et al. 1990). Feed contamination with material from opium poppies (Papaver somniferum), wild poppies (P. somniferum ssp setigerum) or ornamental poppies (P. Orientale), resulting in post-race urine samples testing positive for opiates occurred in Australia in the 1990s and the UK and Ireland in 2002 (*Vine* et al. 2002, *Scott* et al. 2004). Morphine has been detected in mane hair of horses following experimental morphine administration at doses of 0.1 and 0.7 mg/kg body weight (bwt) up to three months post-administration (Fig. 4).

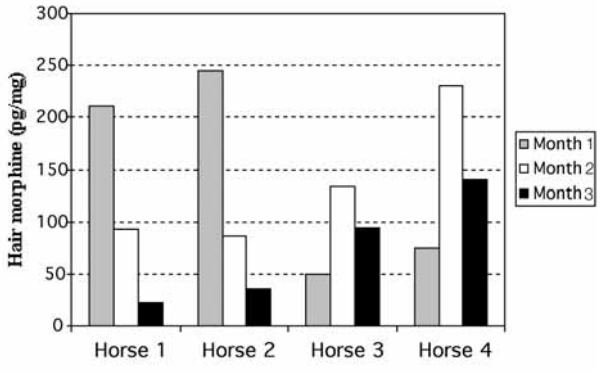

Fig 4 Distribution of morphine residues in sequential hair sections from 4 horses after morphine administration at doses of 0.1 and 0.7 mg/kg body weight to each horse. Samples were collected 2 months after the second administration in each case. The sequence of administrations was randomised between animals. Adapted from tabulated data in *Beresford* et al. (1998).
Morphinrückstände in aufeinanderfolgenden Untersuchungen von Haarproben (4 Pferde) nach Morphinapplikation (2mal im Abstand von einem Monat jeweils 0,1 und 0,7 mg/kg Körpermasse in randomisierter Reihenfolge). Entnahme der Proben erfolgte 2 Monate nach der 2. Morphinapplikation. Die Haarproben wurden entsprechend dem Haarwachstum Zeitabschnitten zugeordnet.

Concentrations of morphine residues in hair were correlated with dosing levels (*Beresford* et al. 1998, *Whittem* et al. 1998). No reports appear in the literature on the detection of codeine in equine hair, although this drug has been successfully detected in human and rat hair in studies either under experimental conditions (*Scheidweiler* et al. 2005) or following drug abuse (*Charles* et al. 2005).

Hyoscine and atropine

Tropane alkaloids, hyoscine (scopolamine) and atropine, are found in belladonna-type Solanaceous plants including Datura, Scopolia and Duboisia species. Atropa belladonna (deadly nightshade) contains predominantly atropine, whereas Hyoscyamous niger (henbane) contains primarily hyoscine. These pharmacologically active alkaloids competitively block muscarinic acetylcholine receptors, and induce a range of pathological effects in horses, such as CNS dysfunction, tachycardia and gastrointestinal atonia. *Schulman* and *Bolton* (1998) reported the euthanasia of two horses following the ingestion of feed contaminated with seeds from Datura species, where the pathology was consistent with tropane alkaloid intoxication. Owing to their potent pharmacological effects upon the CNS and cardiovascular system, the presence of hyoscine or atropine in post-competition urine samples is regarded as a breach prohibited substances rules. Hyoscine and atropine are detected in urine after experimental administration of Datura and in post-race samples, presumably arising from accidental ingestion of Datura sp. (*Galey* et al. 1996). either atropine nor hyoscine appears to have been detected in hair from any species.

Manufacturing or shipping contaminants

Caffeine and theobromine

The methylxanthines, caffeine and theobromine, are recognised equine feed contaminants. Historically, cocoa husk (Theobroma cacao) was used as a feed bulking agent. In contemporary equine feedstuffs, their presence presumably arises from contamination from other feed residues, such as biscuit meal. Methylxanthines are stimulants that possess degrees of CNS, cardiac and respiratory activity. Theophylline, a metabolite of caffeine and a potent respiratory stimulant, is also used clinically in the treatment of respiratory disease, such as recurrent airway obstruction (RAO) in horses, formerly known as chronic obstructive pulmonary disease (COPD). Given the

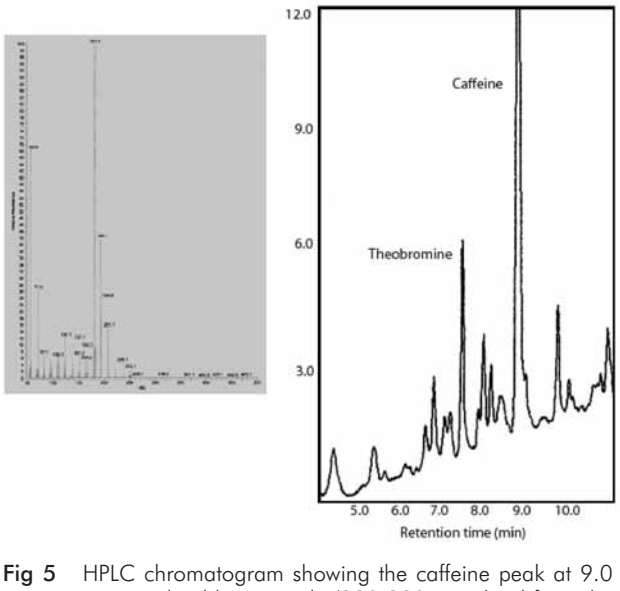

Fig 5 HPLC chromatogram showing the caffeine peak at 9.0 min in a sectional tail hair sample (220-280 mm distal from the follicle) collected 13 months after oral administration of caffeine, and inset mass spectrum in confirmation of the identity of the caffeine peak.
HPLC chromatogram mit einem Signal für Koffein bei einer Retentionszeit von 9 Minuten für eine Schweifhaarprobe (220-280 mm distal der Haarfollike) 13 Monate nach einer oralen Gabe von Koffein; verkleinert eingefügt: massenspektrometrische Bestätigung der Identität des Peaks für Koffein.

prevalence of methylxanthines in the feedstuffs production chain and the extreme difficulty in removing such inadvertent contamination, racing's regulatory authorities implemented a threshold for post-race urine theobromine concentration of 2 µg/mL (*Haywood* et al. 1990). Caffeine and its metabolites theobromine and theophylline were detected in tail hair samples from two horses, at concentrations of <0.1 ng/mg, 13 months after low-dose oral caffeine administration (*Dunnett* et al. 2002, Fig. 5).

Supplements

There is increasing manufacture, marketing and use of dietary supplements in horses, not only so-called 'ergogenic aids', but also those purportedly offering health benefits. Such complementary feedstuffs are also potentially at risk of contamination. Although there has been no comprehensive survey of contamination in equine feed supplements, three such surveys have been conducted on human sports supplements. Results indicated that approximately 20% of supplements tested contained prohibited substances (under IOC rules), principally anabolic steroids including nandrolone (19-nortestosterone) and testosterone, and related pro-hormones (*Maughan* 2004). Contamination levels were generally low and highly variable, so it was assumed their presence arose through inadvertent manufacturing contamination, either by the supplement manufacturer or the ingredient supplier(s). However, methanedienone has recently been detected in one supplementary product at a level sufficient to produce an anabolic effect, but also to potentially cause deleterious health effects. Unlabelled stimulants, such as caffeine and ephedrines have also been detected in human sports supplements (*Maughan* 2004). Such analytical findings are suggestive of deliberate adulteration to improve efficacy. A recent doping case suggests that equine supplement contamination may become an issue for the feed industry and regulatory authorities. However, this positive post-competition urine test, for the presence of nandrolone and estranediol, seems to have arisen through the use of a human sports supplement in the horse (*Russell* et al. 2004).

Under experimental conditions, hair analysis was used successfully to detect anabolic steroid administration in horses. Boldenone was detected in mane and tail hair up to twelve months after a single administration of the drug, and stanozolol was identified in mane, tail and coat hair up to 70 days post-administration (*Popot* et al. 2002). Testosterone was also detectable in mane and tail hair after a single intra-muscular injection, but residues of endogenous testosterone and nandrolone were not identifiable in hair samples (*Popot* et al. 2004). Numerous other synthetic anabolic steroids including methenolone and testosterone esters, have been detected in human hair under experimental and real conditions, as reviewed by (*Kintz* 2003). Various stimulants have been identified in human and laboratory animal hair, collected under experimental conditions and in forensic investigations. Ephedrine and pseudoephedrine are of particular interest as they can be of plant origin (Ephedra ssp). High levels of ephedrine, up to 10 ng/mg, have been found in hair of bodybuilders (*Dumestre-Toulet* et al. 2002).

Pesticides

Dietary exposure to fungicides, herbicides, insecticides, rodenticides and their degradation products in horses can arise via ingestion in contaminated cereals, pasture, and preserved forages. Numerous pesticides are utilised during feedstuffs production, from crop management to finished product storage. Pesticide residues can pose risks to health through acute exposure, such as incorrectly judged application rates and chronic exposure from environmentally persistent organochlorine pollutants (POPs). These include polychlorinated biphenyls (PCBs), such as DDT, DDE, and other organochlorine pesticides including HCH and lindane, and degradation products polychlorinated dibenzodioxins (dioxins). Chronic exposure can occur through environmentally persistent residues due to use in developed countries prior to their prohibition and from continued use in emerging agricultural economies. The properties of organochlorines that make them effective insecticides, include high lipid-solubility, chemical stability and resistance to biotransformation, ultimately led to their demise. Bioconcentration in the food chain and resultant cumulative body burdens lead to demonstrable interference with fertility and reproduction.

Several studies on POP deposition in hair have been conducted in humans and animals. *Dauberschmidt* and *Wennig* (1998) demonstrated the potential of hair analysis to identify human exposure to DDE and other PCB pesticides, with concentrations ranging from 0.5-4.9 ng/mg. Lindane and DDT residues have been found in the hair of children (*Neuber* et al. 1999) and dogs (*Liu* and *Pleil* 2002). *Covaci* et al. (2002) observed greater residues of PCBs, HCH and DDT in hair from subjects with occupational exposure to such pesticides compared with non-exposed controls. Dioxin residues have also been detected in human hair (*Schramm* et al. 1992). Residue levels were reported to be 2.5 times greater in occupationally exposed workers (incineration plants) than in the general population (*Nakao* et al. 2002; *Nakao* et al. 2005).

Exposure to pesticides other than POPs is also identifiable through hair analysis. The literature includes examples of hair analysis being used to demonstrate exposure in experimental situations to pesticides, such as the organophosphate diazinon (*Tutudaki* et al. 2003), the carbamate methomyl, and pyrethroids (*Tsatsakis* and *Tutudaki* 2004). Overall however, there appears to be little research that attempts to link hair pesticide residues to dietary exposure. *Covaci* et al. (2002) suggested PCB deposition in hair to be attributable to dietary intake but offered no supportive evidence. *Paton* and *Petterson* (1997) reported that dieldrin accumulates in wool of sheep that have ingested dieldrin contaminated soil.

Pesticide toxicosis in horses is comparatively rare, but there are reports of metaldehyde (molluscicide) and zinc phosphide (rodenticide) poisoning (*Plumlee* 2001), brodifacoum toxicosis (second-generation anticoagulant rodenticide) (*McConnico* et al. 1997), and methyl bromide poisoning (*Knight* and *Costner* 1977). Horses may also be exposed to dietary pesticides at levels causing no acute clinical signs. Fenitrothion, a grain storage protection pesticide, is metabolised to aminofenitrothion by caecal microflora in the equine gut, and both the parent pesticide and its metabolite have been detected in post-competition urine samples (*Wynne* et al. 1994). Despite

the potential of hair analysis to identify such exposure, there have been no publications on the deposition and accumulation of pesticides in equine hair.

Heavy metals

Heavy metals, most commonly associated with toxicoses are lead (Pb), cadmium (Cd), mercury (Hg), nickel (Ni) and molybdenum (Mo). These toxins pose a health risk to horses primarily through ingestion of fresh forage and water, but through manufactured cereal based feedstuffs and preserved forages grown on contaminated soil. Usual sources of such contamination are ore processing and metal manufacturing, rather than natural environmental origins. Hair analysis to establish human exposure history to certain toxic heavy metals is well established. *Shamberger* (2002) has reported relationships between body burden, dosage, and exposure or toxicity, and residue levels of a number of potentially toxic elements in hair including arsenic, Cd, Pb, Hg and Ni. There has been little evaluation or use of hair analysis for this purpose in horses, however where such research has been conducted, hair analysis so far appears to be suggestive of toxicosis rather than indicative, except in gross population measurements.

Cadmium and lead

Hair analysis can potentially identify lead toxicosis. *Ward* and *Savage* (1994) employed hair analysis to monitor toxic element residues in horses including Pb, Cd, chromium, Ni and bromine arising from vehicle emissions. Elevated Pb and Cd levels were identified in hair and blood, and were significantly correlated ($r = +0.69$). *Anke* et al. (1989) observed significantly increased ($p < 0.01$) hair Cd residues in horses grazing near metal smelters in contrast to those grazing 'unpolluted' pasture, with the effect being greater in geldings than mares. Pb and Cd poisoning due to environmental heavy metal contamination in horses grazing near non-ferrous metal smelters have been diagnosed by hair analysis. Residues of Pb and Cd were significantly higher than control animals and reference values ($p < 0.01$). Estimates of Pb and Cd intake from forage were 6.0 and 1.1 mg/kg bwt/d, respectively; compared with quoted fatal doses of 1.7 and 1.0 mg/kg bwt/d (*Lui* 2003). Cd may be a cumulative toxin in horses, as residues in mane hair were positively correlated with age ($r = 0.546$, $p < 0.01$), however, there appear to be significant positive and negative effects of hair colour on residue levels for some toxic elements (*Asano* et al. 2005).

Molybdenum and nickel

Although evidence is limited, hair Mo concentrations appear to be indicative of excess dietary intake of this heavy metal in horses. Increased hair Mo concentrations were strongly associated with excess dietary Mo (*Cape* and *Hintz* 1982). Mean hair Mo concentrations between horses on low and high dietary Mo intakes (3 and 50 mg Mo/kg feed, respectively) were 1.0 ± 0.7 and 3.0 ± 2.5 ng/mg, respectively ($p < 0.02$). Furthermore, levels of Mo residues in hair were not affected by age, season, or other minerals (*Cape* and *Hintz* 1982).

(*Wells* et al. 1987) reported that hair Ni concentrations were indicative of dietary Ni intake and that there was a significant direct correlation between hair Ni concentrations of mares and their foals in samples collected on the day of foalin ($p < 0.02$).

Mercury

Hair analysis as an effective indicator of environmental and dietary exposure to inorganic and organic Hg species, such as methylmercury, in humans is well recognised. Indeed, hair analysis is the method of choice to identify exposure to methylmercury (*Wilhelm* and *Idel* 1996). Numerous publications have reported the use of this technique in human subjects in a variety of circumstances over the past 40 years. *Katz* and *Katz* (1992) have extensively reviewed this application and have stated hair Hg concentrations >5ng/mg as being indicative of Hg toxicosis. Although Hg poisoning in horses occasionally occurs from the use of Hg-containing blistering agents (*Casteel* 2001), there appear to be no reports in the literature regarding the use of hair analysis to identify such toxicoses.

Other toxic elements

Selenium

Selenium performs a number of significant roles pertaining to cellular function and is an essential component of the equine diet. Indeed, given its importance to normal physiological processes and the naturally low levels present in many soils within many EU member countries, Se is added to manufactured feeds and supplements in the from of inorganic sodium selenite or the more bioavailable organic forms selenomethionine, selenocysteine, or Se enriched yeasts. However, organic forms of Se and enriched yeasts are not authorised additives for use in horses under European feedstuffs legislation. Care needs to be taken with dietary Se supplementation as chronic toxicity can evolve when dietary levels exceed 5 mg/kg, and acute toxicity occurs when levels reach 25-50 mg/kg. Toxic selenosis is a serious health threat to horses (*Crinion* and *O'Connor* 1978, *McLaughlin* and *Cullen* 1986; *Dewes* and *Lowe* 1987, *Witte* et al. 1993). In such circumstances it is assumed that in regions where soils are rich in Se containing minerals, then Se substitutes for sulphur in sulphur-containing amino acids leading to the production of selenomethionine and selenocysteine in grazing flora. Subsequent ingestion leads to the incorporation of absorbed preformed seleno-amino acids during keratogenesis and results in an impaired ability to form disulphide cross-links and subsequent reduced structural integrity and mechanical strength. Clinical signs of selenosis in horses are usually the development of brittle hair and its progressive loss from the mane and tail, and in extreme cases a generalised alopecia and hoof sloughing. It has been suggested that changes to the integrity of hoof structures, presumably via a similar mechanism, may occur at much lower intakes of selenium .

Selenium is readily detected in equine hair and hair selenium concentrations are partially cumulative and reflect historical exposure to this element. *Witte* et al. (1993) observed Se con-

centrations ranging from 0.3 to 7.1 ng/mg in hair samples from horses with varying chronic exposure to Se containing alfalfa forage. Hair Se concentrations above 5 ng/mg are considered to be suggestive of selenosis and values greater than 10 ng/mg to be indicative (*Salbe* and *Levander* 1990). Potentially toxic levels of Se in blood and serum have been strongly correlated (r = 0.76-0.94) with Se concentrations in equine coat, mane and tail hair (*Witte* et al. 1993). *Dewes* and *Lowe* (1987) also observed a strong correlation between blood and hair Se concentrations (r=0.96). Hair selenium concentrations have also been significantly correlated with dietary Se intake in pigs (*Kim* and *Mahan* 2001) and cattle (*Christodoulopoulos* et al. 2003). Organic, as opposed to inorganic, forms of Se are more bioavailable and thus lead to higher Se concentrations in hair and other tissues of rats, cattle and pigs (*Salbe* and *Levander* 1990; *Christodoulopoulos* et al. 2003).

Arsenic

Arsenic is a prohibited substance under equine competition rules, but as it is a ubiquitous environmental substance, threshold levels have been established for its presence in post-competition samples. Additionally, As levels in the environment can be elevated by contamination from the use of pesticidal As compounds, the most commonly encountered being CCA (chromated copper arsenate) wood preservative. Arsenic is a systemic toxin and in cases of severe poisoning the major effect is haemorrhagic gastro-enteritis that may lead to severe dehydration, collapse, shock and death. Reports of As toxicosis in horses are rare (*Pace* et al. 1997) and the application of hair analysis to investigate such poisoning has not been reported. However, hair analysis has been employed in the pathological examination of As toxicosis in cattle. *Riviere* et al. (1981) reported hair As residue levels of 0.80-3.40 ng/mg in affected cattle in contrast to values of 0.09-0.10 ng/mg in randomly selected controls. Despite the current lack of application, hair analysis could be used for As residue determination in horses. In humans it useful for identifying chronic exposure, given appropriate washing procedures to exclude exogenous contamination (*Hindmarsh* 2002, *Shamberger* 2002), although *Wilhelm* and *Idel* (1996) suggests that such measurements are only applicable in population comparisons. Furthermore, hair analysis can identify inorganic As and its organic metabolites (dimethylarsinic acid and monomethylarsonic acid), after acute or chronic exposure, and up to 30 days after residues are no longer detectable in other tissues (*Lin* et al. 2004).

Mycotoxins

Mycotoxins are secondary fungal metabolites of diverse chemical structures that mostly derive from Aspergillus, Fusarium and Penicillium spp. For example, fumonisins, deoxynivalenol, T-2 toxin and zearalenone produced by Fusarium spp., and aflatoxins and ochratoxin A by Aspergillus spp. pose a significant threat to equine health. Mycotoxicoses that most commonly affect horses are leucoencephalomalacia, caused by Fusarium monliforme and fescue toxicosis, caused by Acremonium coenophialum. The causative agents are fumonisin B1 and ergot alkaloids, respectively. Less commonly, horses may be affected with aflatoxicosis, and grass staggers and ergotism (Acremonium and Claviceps spp).

The literature contains no reports on the detection of mycotoxins in equine hair, however two studies describe the detection of fumonisins in human hair. *Sewram* et al. (2003) detected accumulated residues of fumonisins B1 and B2 (FB1 and FB2) in hair samples from subjects in South Africa having an environmental exposure to fumonisin contaminated maize. Liquid chromatography-mass spectrometry identified FB1 mean concentrations of 23.5-33.0 pg/kg and FB2 mean concentrations of 5.7-11.1 pg/mg, but failed to detect fumonisin B3. In earlier experiments (*Sewram* et al. 2001) where non-human primates and rats were fed a control diet or diets having low or high fumonisin contents, levels of fumonisins B1, B2 and B3 and their hydrolysis products, the aminopolyols AP1 and AP2, in hair were correlated with exposure levels. Furthermore, in addition to chronic exposure, acute exposure was also identifiable by hair analysis in rats, four weeks after gavage administration of FB1 at doses of 1 and 10 mg/kg bwt.

The chemical structures of fumonisins are similar to that of sphingosine and thus their toxicity has been attributed to the blocking of enzymes involved in sphingosine biosynthesis. *Morgan* et al. (1997) proposed using hair sphinganine/sphingosine ratio as a non-invasive biomarker of fumonisin exposure, however under experimental conditions there was no change observed in this ratio in mink fed diets adulterated with fumonisins B1, B2 and B3.

Although mycotoxins have a range of chemical structures that reflect the genetic diversity of the fungi that produce them, most will be amenable to detection in hair given the development and use of appropriate methodologies. For example, in fescue toxicosis and ergotism the fungi Claviceps purpurea produces the causative ergot alkaloids including ergotamine, ergonovine and lysergic acid amide. Exposure to these would be readily detectable given modification of existing techniques for the detection of LSD, lysergic acid diethylamide and metabolites, in rat and human hair (*Nakahara* et al. 1996, *Rohrich* et al. 1999).

Plant toxins

Hair analysis for the detection and monitoring of plant toxin residues is a potentially valuable application that has not begun to be studied until very recently. Plant poisoning in horses is an ongoing issue in many countries, but its economic impact is unclear given that comparatively few poisoning cases are confirmed and thus the full extent of the problem is not known. Plant toxicoses can arise from ingestion of poisonous plants during grazing when normal pasture is starved or when behavioural quirks cause horses to graze toxic ornamental plants.

Within the UK, the greatest problem relates to exposure to common (or tansy) ragwort (Senecio jacobaea), although other Senecio spp, such as groundsel (S. plattensis) and common groundsel (S. vulgaris) also pose a potential risk. Although horses may graze on the fresh plant if pasture is star-

ved of grass species, the green plant is bitter tasting, and this is a less common cause of Ragwort ingestion. The greatest risk of exposure comes from ingestion as contamination in preserved forage, as drying or ensiling renders the plant more palatable. Ragwort is a pernicious weed, ingestion of which has been estimated to cause fatal hepatic disease in 500 horses per annum. The causative agents present in S. jacobaea are pyrrolizidine alkaloids (PAs), principally seneciphylline and senecionine and lesser amounts of jacobine, jacozine and jaconine. In groundsels the toxic principle is retrorsine N-oxide. Comfrey (Symphytum officinale) is an oral herbal remedy marketed for use in horses. It is purported to promote fracture healing ostensibly through the action of one of its components allantoin. However, comfrey also contains high levels of PAs, symphytine and echimidine (*Stickel* and *Seitz* 2000). PAs have been shown to cause veno-occlusive disease in the liver and lungs of humans and rats, and are also known to be potent carcinogens, teratogens and abortifacients (*Prakash* et al. 1999).

Given that the greatest health risk from PAs arises from chronic ingestion, a retrospective test, such as hair analysis, would be an appropriate approach to monitor exposure. The chemical nature of PAs, as lipid-soluble weak organic bases, makes them amenable to deposition, accumulation and therefore detection in hair. In a recent pilot study we attempted to detect the presence of seneciphylline, senecionine and retrorsine N-oxide in equine mane and tail hair samples from horses with suspected exposure to Senecio spp (on the basis of clinical blood biochemistry) in contrast to those with no known exposure. PA residues were absent in hair from horses with exposure history and from most with suspected exposure. However, we tentatively identified the presence of seneciphylline and senecionine residues at concentrations up to 180 and 250 pg/mg, respectively, in sequential hair sections analysed from one horse. Peak concentrations were suggestive of ragwort ingestion 20 months prior to hair sampling. These results were consistent with an observed chronic liver pathology. Serum gamma-GT and bile acids were significantly elevated at 12 months and 6 months prior to hair collection. Retrorsine N-oxide was not detected in this horse, thus apparently ruling out groundsel ingestion. These findings are far from conclusive and further work is ongoing, but they seem to support the hypothesis that dietary ragwort exposure could be identified by hair analysis for PA residues.

Discussion and conclusions

In humans, hair analysis has become an established and commonly employed analytical procedure for the retrospective identification of drug, and to a lesser extent heavy metal, exposure in various forensic situations. The development of this technique in horses is much more recent and ongoing, and to date application is sparse. Despite this, the horse is an excellent species in which hair analysis could effectively be used. This review has illustrated a number of toxins where hair analysis has been used, or through ongoing research, may be effective in monitoring exposure. In addition to these, hair analysis can potentially identify exposure to less common dietary contaminants, including medicated feeds containing ionophore growth-promoting antibiotics such as monensin, lasalocid and salinomycin (*Whitlock* 1990), and industrial solvents, such as alcohols. Alcohol exposure has been identified in human hair samples through levels of ethyl glucuronide or fatty acid ethyl esters, such as ethyl oleate and ethyl stearate (*Yegles* et al. 2004).

Hair analysis should not necessarily be considered as a substitute for other forms of analysis such as blood, urine or tissue, but rather as a complementary diagnostic approach. Hair is a unique biological matrix for the retrospective determination of exposure to various ingested toxins. The continuous growth of mane and tail hair produces a permanent long-term historical record of exposure. The duration of the anagen phase is uncertain in the horse, however it evidently lasts for many months, and possibly as long as 3 years as potentiated sulphonamide residues have been detected in tail hair from a horse 2 years after intra-venous administration of these drugs (*Dunnett* and *Lees* 2004). This retrospective analytical potential is the principal advantage in utilising hair, as opposed to more common samples types, such as blood and urine. If drugs are used as an example, the detection time window for equine hair is many months in contrast to the typically short-term windows of 24-48 h and 5-10 days for blood and urine, respectively. The dehydrated keratinaceous nature of hair makes it a chemically stable protective medium for most analytes and obviates the need for refrigerated storage. These two characteristics, continuous growth and matrix stability, enable repeatability in sampling and analysis that are not achievable with samples from the highly variable and dynamic circulatory and urinary systems. Furthermore, given its linear growth, sectional analysis of the hair shaft can be performed. In this, consecutive longitudinal sections of equal length (each section typically representing one month's growth) from the follicle to the distal region are analysed individually. This enables discrimination between a single acute challenge, intermittent multiple toxic events, or chronic exposure.

Table 3 Examples of other drugs detected in equine hair. *Beispiele für Arzneimittel, die im equinen Haar nachgewiesen werden* können.

Drug	Metabolites	Drug class	Reference
Metronidazole	No	Antibacterial	(Dunnett and Lees 2003)
Sulphonamides	No	Antibacterial	(Dunnett and Lees 2004a)
Trimethoprim	No	Antibacterial	(Dunnett and Lees 2004a)
Enrofloxacin	Ciprofloxacin	Antibacterial	(Dunnett *et al.* 2004)
Procaine	No	Local anaesthetic	(Dunnett and Lees 2002)
Etamiphylline	Desethyletamiphylline	Bronchodilator	(Dunnett *et al.* 2002)
Pentoxifylline	Lysophylline	Vasodilator	(Dunnett *et al.* 2002)
Clenbuterol	No	Bronchodilator	(Schlupp *et al.* 2004)
Diazepam	No	Tranquilliser	(Jouvel *et al.* 2000)
Hydroxyzine	No	Antihistamine	(Dunnett *et al.* 2004a)

In equestrian sports prohibited substance testing, hair analysis may able to identify the source of drug or phytochemical residues. In instances of a positive post-competition drug test, feed contamination or ingestion of unusual grazing species may be the cause, rather than deliberate drug administration, for example morphine positive urine tests arising from feed contamination with poppy material (*Vine* et al. 2002, *Scott* et al. 2004). Unlike urine, which mainly contains drug metabolites, parent drugs are predominantly detected in hair (Table 3). This opens up the possibility of botanical marker analysis, where for example, in addition to morphine the presence of other opiate congeners of poppy origin including papaverine, codeine, thebaine, protopine and cryptopine, would be indicative of feed contamination. This same approach could also discriminate between ingestion of Phalaris species and bufo-

tenine or hordenine administration if numerous other tryptamine-type alkaloids, such as horsfiline and coerulescine, were are present.

Despite the benefits offered, considerable care needs to be taken in the appropriate application of hair analysis and in the interpretation of the resultant data. The physiological basis of hair analysis and the methodologies employed are complex and several important factors can significantly influence the outcome of such analysis (*Pragst* 2005). There can be no generalised method for hair analysis, as even within a specific toxin group the diverse chemical nature of its various constituents may necessitate the need for a single analyte-specific methodology. This may include not only a targeted analytical method, but also tailored sampling, decontamination and extraction procedures. Discrimination between endogenous exposure and exogenous deposition through surface contamination from dust and aerosols is vital. This is a particular difficulty during investigation of exposure to toxic metals or pesticides with low-level, broad environmental distribution. Appropriate and rigorous sample decontamination procedures need to be applied, often with concurrent analysis of the wash fractions. For similar reasons, mane hair is preferred over tail hair, particularly in drug and plant toxin residue analysis where an accurate exposure-time profile from sectional analysis is important. Tail hair, especially in more for more distal portions, is at risk of surface contamination from urinary and faecal components. These can be analytes and/or their metabolites that may distort the profile, or other substances that could cause analytical interference.

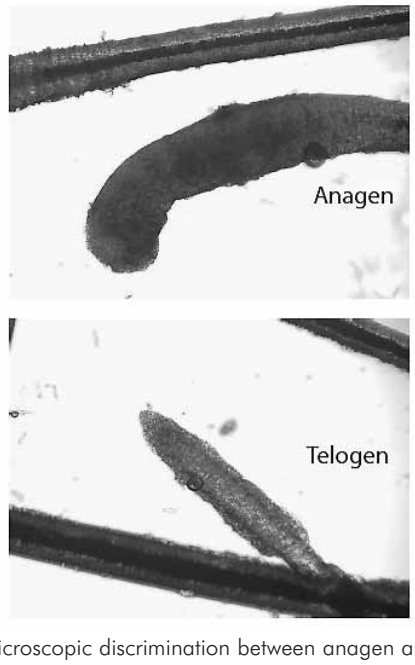

Fig 6 Microscopic discrimination between anagen and telogen hairs.
Mikroskopische Abgrenzung von anagenem und telogenem Haar.

For the most accurate determination of exposure in terms of magnitude and time of occurrence, it is vital to analyse only anagen hairs. By the very nature of the hair growth cycle telogen hair is considerably older than anagen hair. Furthermore, as telogen hair has no vascular supply there cannot be any immediate residue incorporation from blood, but only subsequently from sebum, sweat and tissue deposits. This will confer a time lag on deposition and where relevant may also alter parent compound-metabolite ratios. The potential error from this is relatively small as at any time only approximately 8 % of equine mane hair is in telogen (*Dunnett* et al. 2004b). This contrasts with up to 20 % for human scalp hair (*Pragst* 2005). This error can be further reduced by selectively sampling anagen hairs for analysis. Anagen and telogen hairs are easily discriminated by low-power microscopy (Fig. 6).

It is recognised that melanin binding is a prominent factor in determining the extent toxin residue deposition in hair, certainly for those substances for which there exists a strong affinity, such as multivalent cationic species and weak organic bases. Thus hair colour can have a significant effect on the levels of toxin residues present. This effect appears to be greatest for organic bases. When drug residues are measured in hair from horses of different colours after administrations at equal doses on a body weight basis, darker coloured hair invariably contains higher concentrations. Furthermore, when black and white mane and tail hairs from a grey horse were analysed separately, enrofloxacin and ciprofloxacin residues were forty-fold greater in black hair compared with white (*Dunnett* et al. 2004). Such a situation is also likely to exist for plant alkaloids as they are weak bases, but this has not been demonstrated experimentally. A significant effect is also seen with heavy metals and other metallic elements (*Asano* et al. 2005), however the relationship between residue level and colour is pronounced.

In conclusion, hair analysis in horses has the potential to be an effective means of identifying past exposure to a range of dietary toxins. Given the relationship between nutrition and equine performance sport, the most extensive research has focussed on drug residues. However, there is increasing awareness and concern over the effects of feed and grazing quality on equine health, particularly where these effects are long-term with no apparent acute clinical signs. Toxins implicated include dioxins, mycotoxins, and hepatotoxic and teratogenic plant alkaloids. Hair analysis may be especially useful in diagnosing exposure to these.

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