Analysis of Hormones and Coccidiostats in Dust from Animal Husbandry

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A thesis submitted for the degree of
Doctor rerum naturalium (Dr. rer. nat.)
2026

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Meiner Oma
"Sei gerecht und scheue niemanden."

Acknowledgements

Obwohl auf dem Deckblatt einer Dissertation meist nur ein Name erscheint, ist eine solche Arbeit dann doch eher das Ergebnis einer Gruppenleistung und nur dank der Unterstützung und Zusammenarbeit vieler Personen möglich.

Daher gilt mein Dank zu allererst Prof. Dr. Gerd Hamscher. Nicht nur für die Bereitstellung dieses spannenden Themas und die Möglichkeit, diese Arbeit in seinem Arbeitskreis und mit seiner wissenschaftlichen Unterstützung anfertigen zu können, sondern auch für seine humorvollen Anekdoten und spannenden Vorträge über die Geschichte Gießens, beeindruckende Graffiti und Justus von Liebig, die ich mit Freuden, ob gefragt oder ungefragt, weitergebe.

Ebenso möchte ich mich herzlich bei Frau Prof. Dr. med. vet. Melanie Hamann für die Übernahme des Zweitgutachtens meiner Dissertation sowie bei Prof. Dr. Bernhard Spengler und Prof. Dr. Sylvia Schnell für den Beitritt zur Prüfungskommission bedanken.

PD Dr. Jochen Schulz, Prof. Dr. Isabel Hennig-Pauka und Dr. med. vet. Birgit Spindler von der Tierärztlichen Hochschule Hannover danke ich vielmals für die Versorgung mit neuen Staubproben.

Ebenfalls für die Bereitstellung von Staubproben bedanke ich mich bei allen Landwirten und Landwirtinnen, die mir erlaubt haben, in ihren Ställen frisches Probenmaterial zu sammeln.

Sophie Lennartz und Prof. Dr. Ines Mulder danke ich für die Bereitstellung ihrer Ergebnisse zum Thema QACs in Stallstäuben.

Bei Alexander Konrad aus dem Institut für Bodenkunde bedanke ich mich außerordentlich für die Rettung in der Not und der damit verbundenen Bereitstellung eines LC-MS für meine abschließenden Untersuchungen.

Für ihre enorme Unterstützung bei der ein oder anderen Sinnkrise und die richtigen Fragen zur richtigen Zeit, um das eigentliche Ziel nicht aus den Augen zu verlieren, gilt ein großer Dank Dr. Judith Delius.

Ein weiterer wichtiger Begleiter meiner Arbeit war Dr. Florian Kaltner. Du warst für mich nicht nur fachlich ein großartiger Kollege und Mentor, von dem

ich enorm viel lernen konnte, du hast auch mein Vokabular um viele bayrische Flüche und andere Begriffe erweitert.

Besonderer Dank gilt Dr. Panagiotis Steliopoulos für seine unermüdliche Unterstützung zum Thema DoE und seine ansteckende Begeisterung für Chemometrie. Ich bin dankbar für all die Zeit, die du dir genommen hast, um mir neue strukturierte Ansätze in verschiedensten wissenschaftlichen Fragestellungen beizubringen und mit mir meine Ergebnisse zu diskutieren.

Ein herzlicher Dank geht auch an alle Bachelor- und Masterandinnen, die ich betreuen durfte: Franka Gummert, Annika Haase, Paulina Michel und Saskia Lehr. Es hat mir viel Spaß gemacht, mit euch zusammenzuarbeiten und ihr wart eine wirklich tolle Unterstützung.

Ein großer Dank gilt auch Bianka Daubertshäuser und Peter Seum sowie Ivan und seinen Kollegen und Kolleginnen. Ohne euch wäre ein reibungsloser Laboralltag nicht möglich und nur halb so unterhaltsam.

Binglin Li gilt mein großer Dank für seine Unterstützung auf den kurzen „Side Quests“ meiner Doktorarbeit und die Erweiterung meines kulinarischen Horizonts, durch die ich feststellen durfte, dass Cola-Ingwertee nicht zu meinen neuen Favoriten gehören wird.

Doch nicht nur die fachliche Unterstützung ist es, die zum Erfolg einer solchen Arbeit beiträgt. Daher möchte ich mich an dieser Stelle vor allem bei meinen Freunden bedanken. Charlotte Schlieker danke ich für all ihre aufbauenden Worte, die Telefonate, die für genau die richtige Ablenkung gerade während des Schreibprozesses gesorgt haben, und dafür, dass sie immer für mich da ist. Alix Kaczmarek, Tobias Wagner und Linus Pohl danke ich für die Adoption in ihre Arbeitsgruppe und die entspannten Mittagspausen und Spieleabende, die sich daraus entwickelt haben. An dieser Stelle möchte ich mich auch nochmal bei Linus für sein Python-Skript bedanken, ohne welches ich wohl immer noch meine Daten sortieren würde.

Für die uneingeschränkte Unterstützung, nicht nur während meiner Doktorarbeit, sondern bereits mein ganzes Leben, danke ich meinen Eltern Agnes und Ralf. Ich weiß um euren Verzicht und eure Bemühungen, mir diesen Lebensweg und dieses Studium ermöglichen zu können. Dafür kann ich euch nicht genug danken. Ein ebensolcher Dank gilt auch meiner Oma, Agnes Pauelsen, und meinem Bruder, Sam, die in turbulenten Zeiten immer ein Anker für mich waren.

Zuletzt gilt mein größter Dank Joachim Reichelt. Du bist immer für mich da, ganz gleich, ob ich eine Schulter zum Anlehnen oder einen Motivationstritt benötige. Ich bin dir unendlich dankbar dafür, dass du mir in der ganzen Zeit den Rücken freigehalten, dir auch zum zehnten Mal denselben Vortrag angehört und dich auch einfach mal mit mir aufgeregt hast, wenn es mal nicht so lief, wie ich es mir vorgestellt hatte. Ich bin froh, dass du ein Teil meines Lebens bist und ich freue mich schon auf all die Abenteuer, die uns noch bevorstehen.

Abstract

The growth of the global population is accompanied by an increasing consumption of animal-derived food products. To meet this steadily growing demand, hormones, coccidiostats and other veterinary pharmaceuticals are employed to enhance the efficiency and profitability of breeding and fattening facilities while ensuring a certain level of animal welfare.

The use of various veterinary pharmaceuticals leads to environmental contamination via multiple exposure pathways. Whilst the primary routes of entry via manure and slurry are well established, stable dust has hitherto received little consideration as a potential exposure pathway. This dissertation systematically investigates the occurrence of steroid hormones (oestrogens, androgens, and progestogens) and coccidiostats (ionophores and synthetic compounds) in stable dust from various animal husbandry facilities using liquid chromatography–tandem mass spectrometry (LC-MS/MS).

A novel LC-MS/MS method was developed for the analysis of stable dust samples and fully validated according to Commission Implementing Regulation (EU) 2021/808, enabling the detection of 22 steroids and coccidiostats at trace analytical concentrations in complex matrices. Extraction parameters were optimised and validated through systematic optimisation experiments (Design of Experiments). For 17 of the 22 analytes, specificity, selectivity, and uncertainty related performance characteristics of the method comply with international requirements for residues in foodstuffs.

Analysis of 50 stable dust samples from laying hen facilities, broiler chicken operations and breeding sow facilities revealed coccidiostat concentrations in the mg/kg range (e.g. narasin up to 15.72 mg/kg, nicarbazin up to 11.50 mg/kg) and steroid hormone levels ranging from 0.30–2.62 mg/kg in some instances. Archive samples from the 1980s demonstrate the persistence of these substances over decades. A risk assessment using PEC/PNEC ratios for aquatic organisms indicates an elevated environmental risk.

The results provide further indications that stable dust represents a previously underestimated environmental and exposure compartment. Sub-therapeutic doses of coccidiostats in dust particles may promote the development of resistant parasite strains. The inhalation of dust contaminated with various pharmaceutical residues moreover may presents a previously underestimated occupational health risk. This work contributes substantially to the understanding of a novel entry route of veterinary pharmaceuticals and feed additives into the environment and provides a scientific foundation for future hygiene and occupational safety measures in intensive animal husbandry within the context of the One Health Approach.

Zusammenfassung

Mit einer wachsenden Weltbevölkerung geht ein zunehmender Konsum an tierischen Lebensmitteln einher. Um diesen stetig wachsenden Konsum zu decken, werden Hormone, Kokzidiostatika und andere Tierarzneimittel eingesetzt, um die Effizienz und Rentabilität von Zucht- und Mastanlagen zu steigern und ein gewisses Maß an Tierwohl zu gewährleisten.

Der Einsatz der verschiedenen Tierarzneimittel führt über verschiedene Eintragswege zu Kontaminationen in der Umwelt. Während die Haupteintragsrouten durch Gülle und Mist bekannt sind, wird Stallstaub als potenzieller Expositionspfad bisher nur wenig in Betracht gezogen. Diese Dissertation untersucht systematisch das Vorkommen von Steroidhormonen (Estrogene, Androgene und Gestagene) und Kokzidiostatika (Ionophore und synthetische Verbindungen) in Stallstaub aus verschiedenen Tierhaltungsanlagen mittels Flüssigchromatographie-Tandem-Massenspektrometrie (LC-MS/MS).

Zur Untersuchung von Stallstäuben wurde eine neue LC-MS/MS-Methode entwickelt und vollständig nach Durchführungsverordnung (EU) 2021/808 validiert, welche für den Nachweis von 22 Steroiden und Kokzidostatika im spurenanalytischen Konzentrationsbereich in einer komplexen Matrix verwendet werden kann. Mittels systematischer Optimierungsexperimente (Design of Experiments) wurden Extraktionsparameter optimiert und umfassende Validierungsstudien durchgeführt. Spezifität, Selektivität, Wiederholbarkeit, Reproduzierbarkeit und Wiederfindsungsrate der Methode erfüllen internationale Anforderungen für Rückstände in Lebensmitteln für 17 der 22 Analyten.

Die Analyse von 50 Stallstaubproben aus Legehennenbetrieben, Broilermasthühnerbeständen und Zuchtschweinebeständen zeigt Konzentrationen von Kokzidiostatika im mg/kg-Bereich (z.B. Narasin bis 15,72 mg/kg, Nicarbazin bis 11,50 mg/kg) sowie Steroidhormone teilweise im Bereich von 0,30–2,62 mg/kg. Archivproben aus den 1980er-Jahren demonstrieren die Persistenz dieser Substanzen über Jahrzehnte. Eine Risikoabschätzung unter Verwendung von PEC/PNEC-Quotienten für aquatische Organismen deutet auf ein erhöhtes Umweltrisiko hin.

Die Ergebnisse liefern weitere Anhaltspunkte dafür, dass Stallstaub ein bislang unterschätztes Umwelt- und Expositionskompartiment darstellt. Subtherapeutische Dosen von Kokzidiostatika in Staubpartikeln fördern möglicherweise die Entstehung resistenter Parasitenstämme. Die Inhalation von Staub, welcher mit diversen pharmazeutischen Rückständen belastet ist, stellt zudem ein bisher wenig beachtetes Berufsrisiko dar. Die Arbeit trägt wesentlich zum Verständnis einer neuen Eintragsroute von Veterinärpharmazeutika und Futtermittelzusatzstoffen in die Umwelt bei und liefert wissenschaftliche Grundlagen für zukünftige Hygiene- und Arbeitsschutzmaßnahmen in der intensiven Tierhaltung im Kontext des One Health Ansatzes.

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List of Abbreviations

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AC alternating current 26

ACN acetonitrile 43

ACTH adrenocorticotropic hormone 4

ADI acceptable daily intake 15

ALT altrenogest 33

ANOVA analysis of variance 117

API atmospheric pressure ionsation 23

ATMAC alkyltrimethylammonium compounds 65

BAC benzalkylammonium compounds 65

BC broiler chicken 52

CCD central composite design xxi

CE collision energy 41

CEM chain ejection model 24

CID collision-induced dissociation 27

COPD chronic obstructive pulmonary disease 19

CRM charged residue model 24

CUR curtain gas 34

CVMP Committee on Medicinal Products for Veterinary Use 15

CXP collision cell exit potential 41

DADMAC dialkyldimethylammonium compounds 65

DEC decoquinate 34

DC direct current 26

DIC diclazuril 73

DNC 4,4'-dinitrocarbanalide 99

DP declustering potential 41

E1 estrone 34

aE2 α-estradiole 34

bE2 β-estradiole 34

EE2 ethinylestradiole 34

E3 estriole 34

E3S estrone-3-sulphate 34

EFSA European Food Safety Authority 15

EMA European Medicines Agency 6

EP entrance potential 41

ESI electrospray ionisation 21

FEEDAP Panel on Additives and Products or Substances used in Animal Feed 15

FP fattening pigs 52

FWHM full width at half maximum 22

HAL halofuginone 34

HPLC high performance liquid chromatography 21

HRE hormone response elements 2

IEM ion evaporation model 24

JECFA Joint FAO/WHO Expert Committee on Food Additives 15

LAS lasalocid 34

LC liquid chromatography 33

LH leying hens 52

LSE liquid-solid extraction 56

MA megestrol acetate 34

MAPK mitogen-activated protein kinase 2

ME matrix effect 63

MeOH methanol 43

MON monensin 34

MPA medoxyprogesterone acetate 34

MRM multiple reaction monitoring 34

MS mass spectrometry 33

MS/MS tandem mass spectrometry 21

MT methyltestrosterone 62

MRL maximum residue limits 6

N nandrolone 34

NAR narasin 34

NIC nicarbazin 34

NOEL no observed effect level 15

NRCP national residue control plan 7

P progesterone 34

PA polyamid 74

PEC predicted environmental concentration 99

PET polyester 74

PI3K phosphoinositide 3-kinase 2

PM particulate matter 17

PNEC predicted no effect concentration 99

psi pounds per square inch 34

PVDF polyvinylidenfluorid 74

QqQ triple quadrupole 27

R resolving power 22

RASFF Rapid Alert System for Food and Feed 7

REC recovery 57

RF radio frequency 27

ROB robenidine 34

RP reversed phase 21

RSM response surface model 59

SAL salinomycin 34

SIM single ion monitoring 27

SPE solid phase extraction 66

STA stanozolol 34

T testosterone 34

Tb trenbolone 34

TIC total ion chromatogram 44

1 Introduction

Contents

1.1 Steroid Hormones

Hormones are endogenous messenger substances responsible for regulating various biochemical processes such as the adaptation of the organism to the environment, the regulation of sexual behaviour, and the coordination of growth. The multitude of hormones can be classified in various ways, whereby overlapping between different classifications is possible. Based on their biochemical properties, most hormones can be assigned to four groups:

- Amino acid-derivatives (e.g. insulin)
- Peptides (e.g. Catecholamines like adrenaline)
- Isoprenoid derivatives
  - Steroids (e.g. testosterone)
  - Lipid derivatives (e.g. retinoic acid)

All steroid hormones are formed from cholesterol, resulting in a common sterane core structure, classifying them as terpenes and thus isoprene derivatives. Cholesterol is primarily formed de novo in the liver from acetyl coenzyme A via a multi-step enzymatic reaction cascade. Bound to low-density lipoproteins (LDL), for example, cholesterol is transported to peripheral tissues and transferred to the target cell via receptor-mediated endocytosis using LDL receptors. Binding to steroidogenic acute regulatory protein (StAR) ultimately enables the transport of lipophilic cholesterol through the cytosol to the inner mitochondrial membrane.[1] The first step in synthesis, common to all steroid hormones, is the conversion of cholesterol to pregnenolone, catalysed by the cytochrome P-450 side chain cleavage enzyme (CYP450scc) CYP11a1. Depending on the cellular enzyme configuration, pregnenolone is converted into various steroid hormones via different reaction processes such as oxidation and reduction of hydroxy and keto groups or the introduction and hydrogenation of double bonds (see Figure 1.1).[2]

Primary formation of steroid hormones occurs in the mitochondria or endoplasmic reticulum in cells of the adrenal cortex, the Leydig cells of the testes (androgens) and the theca and granulosa cells of the ovaries (oestrogens and progestogens). To a lesser extent, steroids can also be produced in the placenta and the central nervous system. Due to their lipid solubility, steroid hormones are transported through the blood, bound to plasma proteins such as albumin or transcortin, to the corresponding intracellular steroid receptors. Due to their lipophilic nature, they enter the cells via passive diffusion through the cell membrane. Inside the cell, steroid hormones bind to specific intracellular nucleotide receptors, which can be located in the cytoplasm or in the nucleus. These nucleotide receptors are complexed within the cell by heat shock proteins (e.g. hsp 90, hsp 45 or hsp 70), which stabilise the receptors in a conformation suitable for hormone binding and protect them from degradation or untimely binding to DNA. Binding of a steroid hormone changes the conformation of the receptor, causing the heat shock proteins to dissociate. The active hormone-receptor complex can now dimerise, translocate to the nucleus and bind to hormone response elements (HRE) – specific DNA sequences in the promoter regions of hormone-responsive genes. The genomically mediated effects lead to modulation of the transcription rate of specific target genes. In addition to genomic mechanisms of action, steroid hormones can also bind to membrane-bound receptors and activate intracellular signalling cascades such as mitogen-activated protein kinase (MAPK) or phosphoinositide 3-kinase (PI3K) signalling pathways.

The endocrine system and its associated processes are based on a delicate equilibrium between the activation and inactivation of hormones. Within the

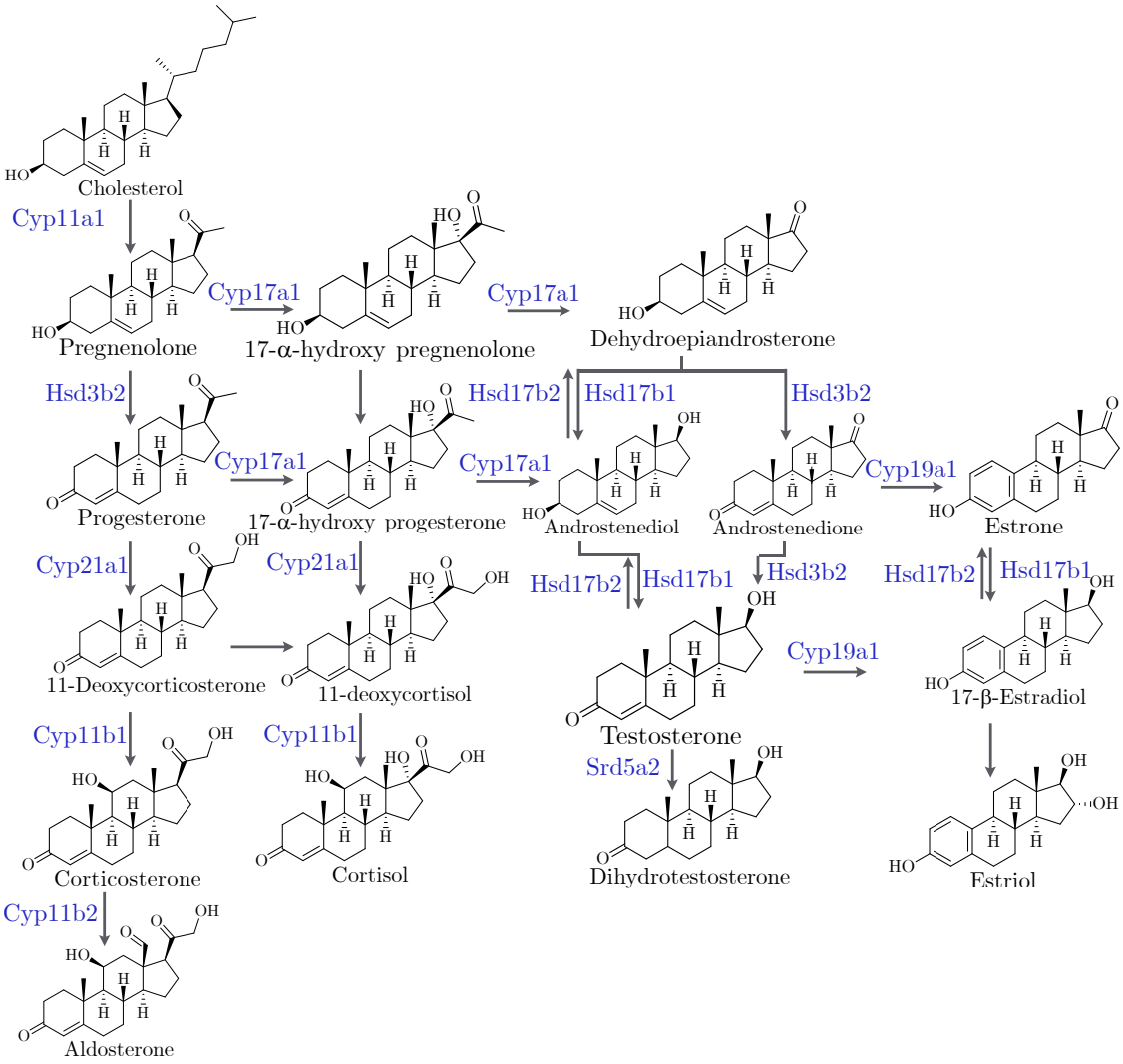

Figure 1.1: Steroid Synthesis adjusted from Chakraborty et al.[3]

peripheral target tissue, steroids are locally activated by phase I and II metabolism through the enzyme repertoire of the cell according to tissue-specific needs and subsequently inactivated. This mechanism is known as intracellular pre-and post-receptor metabolism or "intracrinology".[4]

When steroid hormones are no longer required and are destined for excretion, they are metabolised predominantly in the liver and in the tubular cells of the kidneys. This metabolism is essential, since steroids are inherently lipophilic compounds and their water solubility needs to be increased to enable efficient excretion in urine and bile. For conceptual understanding, metabolism is divided into phase I and phase II. In phase I, the biological activity of the steroid is modified and thereby functionally inactivated. This is achieved through the introduction, removal or modification of functional groups, which can also serve as binding sites for

subsequent phase II reactions. Examples of phase I reactions include 5α-reduction, catalysed by steroid 5α-reductase, and CYP3A4-catalysed β-hydroxylation. Typical phase II reactions are sulphation and glucuronidation. Further conjugation reactions include the methylation of oestrogens, conjugation with cysteine or glutathione, and esterification with fatty acids. Despite the theoretical sequentially differentiation between phase I and phase II metabolisation, depending on the structural moieties, some steroids can directly undergo phase II metabolism. With regard to subsequent environmental influences, it is important to note that steroid sulphation is reversible through the action of steroid sulphatases, which are ubiquitously expressed in virtually all tissues.[5]

1.1.1 Steroid Hormones in Veterinary Pharmaceuticals

The targeted use of both natural and synthetic hormones can interfere with the sensitive biochemical processes described above. This allows targeted control and influence of cycle disorders, oestrus synchronisation and even growth promotion. The use of hormones is divided into therapeutic and non-therapeutic approaches. Therapeutic applications include replacement therapies for endocrine dysfunction. For example, adrenocorticotropic hormone (ACTH) is used to stimulate the adrenal cortex to produce and release glucocorticoids in cats, dogs and dairy cows and as a therapeutic agent in primary bovine ketosis.

The administration of (steroid) hormones for non-therapeutic purposes is further subdivided into use in companion animals and food-producing animals. In companion animals, endocrine-disrupting pharmaceuticals are employed, for instance, to suppress oestrus in cats and dogs. Deslorelin acetate (GnRH agonist), for example, suppresses the production of testosterone and oestrogen and is therefore used to achieve temporary infertility in healthy, unneutered dogs.[6]

In food-producing animals, non-therapeutic uses of hormones include oestrus synchronisation and growth promotion. The hormones used for growth promotion include natural steroids (oestradiol, testosterone, progesterone), synthetic steroids (trenbolone acetate, melengestrol acetate, stanozolol, zeranol), thyreostatics and beta agonists (e.g. clenbuterol). Because of their superior efficacy compared with single-hormone preparations, combined implants containing both oestrogens and androgens are most commonly used.[7] Growth promotion is achieved through a shift in the balance from protein degradation towards protein synthesis, accompanied by an increase in satellite cell number and enhanced expression of IGF-1 mRNA in muscle tissue.[8]

In cattle, progesterone or synthetic gonadotropin-releasing hormone analogues such as buserelin are widely used for oestrus synchronisation, preparation for embryo transfer and the treatment of fertility disorders in individual animals.[9] In addition, the neuropeptide oxytocin is administered in both cattle and sheep for obstetrical purposes, and chorionic gonadotropin is used as a peptide hormone for oestrus synchronisation.[9] In veterinary practice, oestrus synchronisation in gilts in Germany relies predominantly on the synthetic progestagen altrenogest (Figure 1.2) administered at a dose of 20 mg/day over a period of 15–18 days.[10] Altrenogest inhibits the secretion of gonadotropin-releasing hormone in the hypothalamus and thereby also suppresses the release of luteinising hormone from the pituitary gland, resulting in a postponement of oestrus. Approximately five days after withdrawal of altrenogest, oestrus can be observed.[10] Proprietary products used for this indication include Regumate® and Suifertil®.

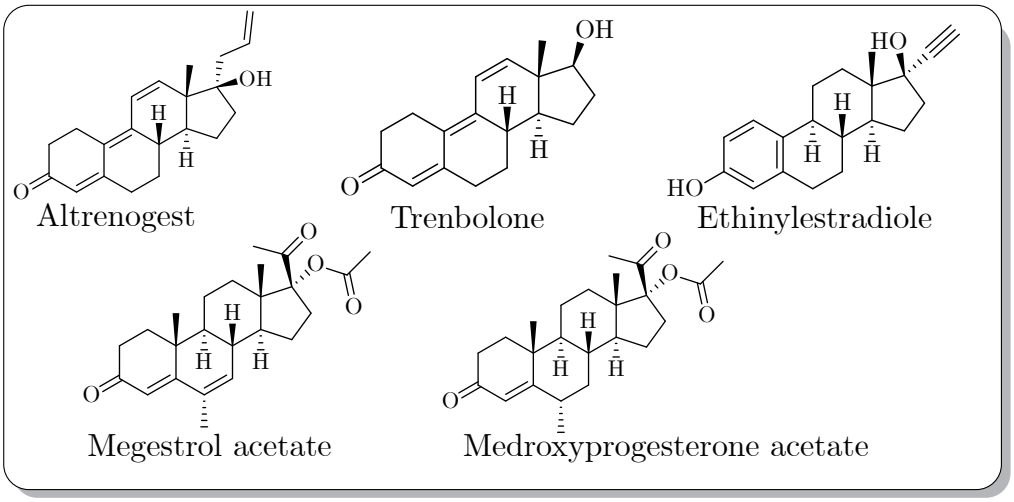

Figure 1.2: Structures of synthetic steroid hormones investigated in this study.

A systematic survey of the quantities of hormonally active substances dispensed in veterinary medicine does not exist in Germany. Nonetheless, in order to make a rough estimate of consumption volumes, it is first necessary to determine how many animals in Germany are treated with hormones at all. As an example, these considerations will hereafter be illustrated for breeding sows.

For food-producing animals, specific regulatory provisions apply depending on the production system. In accordance with the EU Organic Regulation (EU) No. 2018/848, the use of hormones without medical indication is prohibited in organic livestock production.[11] The proportion of holdings keeping pigs under organic management was, however, below 5 % in 2020. In the same year, there were approximately 1.7 million breeding sows in total, corresponding to about 7 % of

the German pig population.[12] Of these, around 22 000 breeding sows (1 %) were kept on organically managed farms, meaning that almost 99 % of all breeding sows were kept under conventional husbandry conditions.[12] On the basis of estimates from Lower Saxony, roughly one in three animals was treated with hormones.[13] A breeding sow produces on average 2.3 litters per year and would therefore be treated for approximately 35–41 days annually.[13], [14] Assuming, hypothetically, that only altrenogest were used, this would correspond to a total consumption of approximately 390–460 kg per year. However, since altrenogest is not routinely administered in every cycle but only in specific management situations (such as one-off synchronisation of gilts, in cases of return to oestrus, or for delaying parturition in the presence of pathological problems), the actual usage volumes are likely to be considerably lower. The total quantity of hormones used in veterinary medicine in Germany in 2020 has been estimated at approximately 232 kg.[15] Although the amount of hormones used is low compared to the amount of antibiotics used (> 200 tonnes), it is still relevant given the low effective concentration of hormones.

1.1.2 Legal Assessments Regarding the Use of Hormonally Active Pharmaceuticals

Due to the negative consequences for humans, animals and the environment caused by residues in food, the use of hormones in farm animals in Germany and the EU, unlike in the USA and Canada, for example, is restricted to therapeutic and zootechnological purposes and is stringently regulated. Since 1988, the use of steroid hormones for growth promotion has been prohibited in the EU. Furthermore, the application of substances with oestrogenic (except 17β-oestradiol), androgenic and gestagenic effects, as well as of altrenogest, is strictly regulated under Directive 96/22/EC and subject to specific conditions.[16] For instance, testosterone, progesterone or their derivatives may only be administered for the treatment of fertility disorders or for the termination of an unwanted pregnancy in individual animals (and thus only in sexually mature animals and not for fattening purposes) by means of injection or vaginal pessary and exclusively by a veterinarian.

Regulation (EC) No. 470/2009 establishes the framework for setting maximum residue limits (MRL)s of pharmacologically active substances (including hormonally active substances) in foodstuffs of animal origin.[17] This includes provisions on risk assessment, recommendations for risk management, as well as requirements concerning analytical methods and the marketing of foodstuffs. In this context, the European Medicines Agency (EMA) is responsible for risk assessment, recommendations on risk management and the establishment of MRLs. Based on Regulation

(EC) No. 470/2009 and EMA recommendations, Regulation (EU) No. 37/2010 lists all authorised substances that may be used, their permitted concentrations, the established MRLs and the prescribed withdrawal periods for food-producing animals.[18] Since regulations on the use of hormonally active substances are not uniformly applied worldwide – and hormones for growth promotion are still used, for example, in the USA and Canada – Regulation (EU) No. 2017/625 addresses import quotas (arising inter alia from Regulation (EU) No. 1308/2013 on a common organisation of the markets in agricultural products and various trade agreements) and the monitoring of import conditions through regular controls.[19], [20]

The effectiveness of these controls for both imported and EU-produced foodstuffs in recent years can be demonstrated by reports from the European Rapid Alert System for Food and Feed (RASFF). Since the year 2000, there have been only seven notifications related to hormonally active substances in food. Another control mechanism in the EU is the national residue control plan (NRCP). Based on Regulation (EU) No. 2017/625 in conjunction with Delegated Regulation (EU) No. 2022/1644 and Implementing Regulation (EU) No. 2022/1646, the NRCP serves to monitor residues in both living and slaughtered animals intended for food production, as well as their primary products (e.g. milk or eggs).[21], [22] The annual reports of the NRCP also recorded no residues of steroid hormones in the investigated planned samples from 2020 to 2022. Only in 2023, an elevated level of endogenously produced testosterone of 1.7 µg/L was detected in a sample from a heifer.

In summary, it can therefore be concluded that the consumption of animal-derived foodstuffs in Germany and the EU is safe. However, the environmental impacts of intensive livestock farming warrant more critical consideration.

1.1.3 Environmental Impact and Risk Assessment of Steroid Hormones

The reasons for the stringent regulations governing the use of natural and synthetic hormones are the potential effects on both humans and the environment. The manner in which these hormones are employed and their natural occurrence, particularly in the context of animal husbandry, lead to a variety of exposure pathways to humans and to the environment. According to estimates, around 360 tonnes of steroid hormones were excreted in animal husbandry in Europe in 2000 and 330 tonnes in the USA, with their release via animal manure and slurry representing a significant route for hormones and their metabolites to enter the environment.[23]. Measures such as modern wastewater and slurry management could markedly reduce the concentrations of individual hormones, but they cannot remove them completely.

Moreover, corresponding retrofits are not always feasible and can, in parts, be very expensive.[24]–[27] In the environment, further transport, degradation, and bioavailability of the hormones and their metabolites are influenced by a complex interplay of microbial activity within an environmental compartment, sorption to soil, and the chemical stability of the hormones.[28]–[30] Through microbial influences, hormones can not only be degraded but their metabolites can also be reconverted into their original hormonally active forms. Furthermore, an endocrine effect of the metabolites cannot be ruled out according to current knowledge.[31] To date, several studies have already demonstrated the occurrence of steroids and their metabolites in surface and groundwater at the low ng/L range, depending on the type and location of sampling [32]–[34]. In zones downstream of wastewater treatment plants or within the influence of intensive animal husbandry, these concentrations can rise to the µg/L range. Such concentrations may already induce intersexuality in aquatic organisms. These effects were observed in fish whose habitat was downstream of a pharmaceutical company.[35], [36] Due to substantial feminisation, there can be a decline in fish stocks. Moreover, hormones could potentially enter the drinking water at extremely low concentrations if it is sourced via bank filtration.[37] Research on hormone concentrations in drinking water is the subject of an ongoing project funded by the Federal Ministry of Health [38].

In contrast to the aquatic environment, there are few systematic investigations into the occurrence of steroid hormones and their metabolites or transformation products in soils.[13], [39] To estimate expected environmental concentrations in soil, Petri et al. developed a "Worst-Case Scenario." When considering typical agricultural practice and the condition that the applied slurry is incorporated into soil depths of 5–25 cm, concentrations fall below the threshold of 100 µg/kg.[40] This threshold generally applies to veterinary medicines with the exception of antiparasitics and is thus only cautiously transferable to hormones, because their activity, particularly for aquatic organisms, possesses substantially higher potency. Consequently, users need to be provided with sufficient information on appropriate manure management and environmentally friendly application. It is therefore evident that hormones, which are both produced endogenously and administered exogenously in animal breeding and husbandry, find their way into the environment, causing considerable damage.

1.2 Coccidiostats

1.2.1 Coccidiosis

Intensive livestock production requires not only optimised breeding strategies but also rigorous disease management. When animals are kept in large numbers and at high stocking densities, this promotes increased stress and enhances the risk of zoonotic transmission. Owing to low immunological barriers associated with limited genetic diversity, rapid dissemination of pathogenic agents can occur even in ostensibly closed systems through indirect contacts such as veterinary interventions, ventilation systems, and animal transport.[41], [42]

In intensive poultry production, coccidiosis, caused by protozoan parasites, represents one of the most prevalent and most difficult-to-control diseases.[43] Infection may lead to intestinal lesions, reduced egg production, impaired weight gain due to decreased feed conversion efficiency, and consequently elevated mortality rates.[43] The coccidia infecting chickens belong to the genus *Eimeria*. A key biological feature of *Eimeria* spp. is their strict host specificity. In the chicken, seven distinct species are recognised: *E. tenella*, *E. necatrix*, *E. brunetti*, *E. acervulina*, *E. maxima*, *E. mitis* and *E. praecox*.[44] The different *Eimeria* species colonise distinct segments of the intestinal tract and are associated with specific clinical manifestations and different prepatent periods ranging from 4 days (*E. mitis*) to 6 days (*E. necatrix*).[44]

The life cycle of *Eimeria* comprises an exogenous phase (sporogony) and an endogenous phase (schizogony and gametogony). It begins outside the host, where oocysts undergo sporulation and thereby become infective. The sporulated oocysts are ingested via feed or drinking water that has been contaminated, for example, with faeces.[45] Following ingestion, the mechanical action of the gizzard ruptures the oocyst walls, releasing sporocysts. Subsequently, in the duodenum, the combined effects of pancreatic secretions (trypsin), bile salts, and CO_2 trigger excystation, liberating the motile sporozoites. Following release, the sporozoites of the respective *Eimeria* species migrate to their specific intestinal sites and initiate invasion of the epithelial cells. Within these target cells, multiple rounds of asexual replication (schizogony) occur, giving rise to merozoites, which in turn can infect new host cells. Subsequently, sexual replication (gametogony) results in the formation of zygotes that develop into oocysts, which are finally excreted into the environment with the faeces.[45]

Young animals are particularly susceptible to infection owing to their still immature immune system. Moreover, as there is no complete cross-protective immunity between different *Eimeria* species in chickens, successive infections with heterologous species can occur.[46], [47]

1.2.2 Application and Modes of Action of Coccidiostats

The first agents employed for the prevention of coccidiosis were sulphonamides, such as sulphanilamide and sulphamerazine.[48] However, due to issues regarding toxicity and the emergence of resistance, the search for alternatives began rapidly. Currently, eleven coccidiostats are approved for use in the EU, including both ionophores and synthetic compounds.

Ionophores

Ionophoric coccidiostats are obtained via fermentation by various *Streptomyces* spp. and *Actinomadura* spp. They are composed of multiple tetrahydrofuran rings arranged in spiroketal moieties (see Figure 1.3). Due to this structure, ionophoric coccidiostats are capable of complexing specific cations: monovalent ionophores (e.g., monensin, narasin, salinomycin) primarily bind Na^+ and K^+, whereas divalent ionophores (e.g., lasalocid) bind Ca^{2+} and Mg^{2+}.[49] The resulting complex forms a pseudocyclic, electroneutral structure, stabilised by intramolecular hydrogen bonds between the carboxylate anion and two hydroxyl groups. Ionophores are classified into monovalent, monovalent glycosidic (maduramicin and semduramicin), and divalent ionophores.[43], [50]

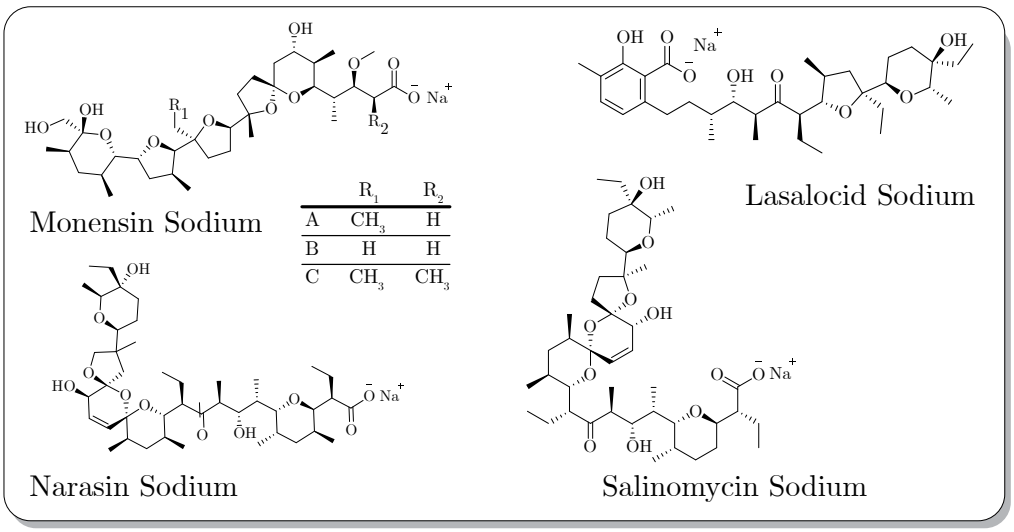

Figure 1.3: Molecular structures of ionophoric coccidiostats investigated in this study.

By complexing these ions, the lipophilic nature of the compound allows the transport of cations across the hydrophobic membrane of the parasite cell. This influx disrupts the physiological ionic gradients (e.g., Na^+/K^+ pump), forcing the parasite to expend excessive energy (ATP) to restore homeostasis. This results in osmotic and electrochemical imbalance, metabolic dysregulation, and finally,

vacuolisation and swelling of the parasite cell, leading to membrane lesions and necrosis. Ionophores act primarily on the initial asexual stages of development and are most effective against motile extracellular stages (sporozoites and merozoites). A major advantage of ionophores is that they do not completely suppress parasite development, thereby allowing the host to develop natural immunity.[43]

Synthetic Compounds

Synthetic coccidiostats comprise a heterogeneous group including quinolones, pyridones, alkaloids, guanidines, thiamine analogues, and triazine derivatives, each with distinct modes of action. Figure 1.4 displays the synthetic coccidiostats investigated in this study.

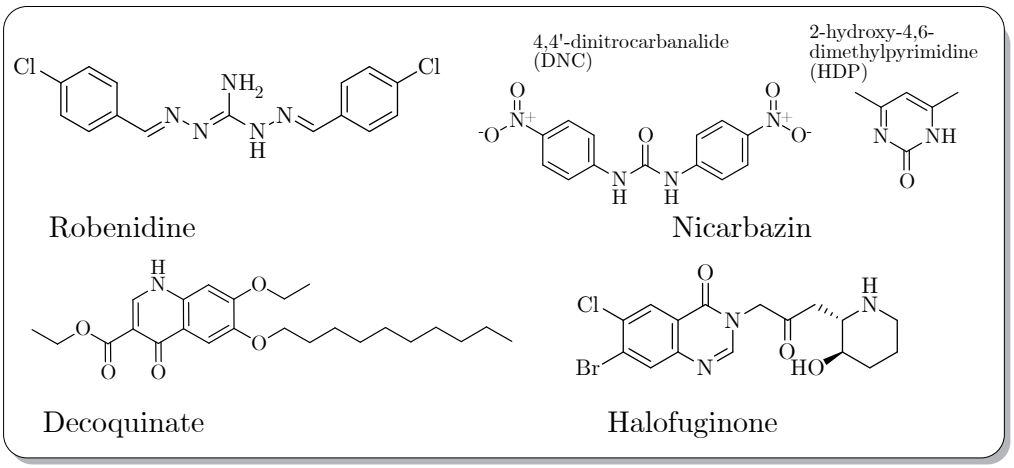

Figure 1.4: Molecular structures of ionophoric coccidiostats investigated in this study.

Decoquinate is a 4-hydroxyquinolone. Due to its significant structural lipophilicity, its absorption from the gastrointestinal tract is minimal, making it ideal for targeting intestinal coccidia. Decoquinate acts by inhibiting mitochondrial respiration in sporozoites; specifically, it blocks electron transport within the cytochrome system, thereby preventing ATP synthesis. Without sufficient energy, cell division is inhibited, and parasite proliferation is reduced.[51], [52] Upon ingestion, the complex of Nicarbazin dissociates into its components. HDP aids absorption but is rapidly excreted via urine, whereas DNC is absorbed and represents the active moiety. The mode of action of DNC is thought to involve uncoupling of oxidative phosphorylation and interference with the mitochondrial membrane potential; earlier hypotheses also suggested inhibition of transglutaminase activity.[53] Since nicarbazin acts intracellularly on the first and second schizont generations, it is frequently used in combination products with narasin or monensin. While

ionophores act coccidiostatically on early developmental stages, nicarbazin exerts a coccidiocidal effect, effectively killing schizonts.[54] Halofuginone is a synthetic analogue of febrifugine, an alkaloid extracted from the leaves and roots of *Dichroa febrifuga*. Although the precise mechanism is not fully elucidated, halofuginone is effective against multiple developmental stages of various *Eimeria* species. Current research indicates that halofuginone interferes with protein biosynthesis by inhibiting prolyl-tRNA synthetase, thereby preventing the incorporation of proline into proteins (e.g., collagen needed for oocyst walls).[55] Additionally, histological studies have demonstrated vacuolisation and degeneration of schizonts.[56] Robenidine belongs to the guanidine derivatives. Its mechanism of action is also not fully defined, but it is postulated that robenidine interferes with energy metabolism by inhibiting oxidative phosphorylation and the ATPase activity of mitochondria.[57]

Application Programs

To counteract the potential development of resistance to coccidiostats, two specific management strategies exist alongside single-drug application: "shuttle" and "rotation" programmes. The fundamental difference between these lies in their temporal application.

In a shuttle programme, the coccidiostat is changed within a single fattening cycle (e.g., starter vs. grower feed). This strategy leverages the different modes of action to target coccidia at various stages and to minimise the risk of toxicity or heat stress associated with certain compounds (e.g., nicarbazin) in older birds. Ideally, a chemical coccidiostat is used in the starter phase to reduce infection pressure, followed by an ionophore to allow immunity development.[58] In a rotation programme, the coccidiostat is changed between successive fattening cycles or flocks on a farm.

Fundamentally, and particularly when applying rotation programmes, strict hygiene management is crucial for the containment and prevention of coccidiosis. Beyond thorough cleaning of the housing, attention must be paid to contamination vectors such as veterinarians, personnel, and new stock. As sporulation of oocysts is favoured by high humidity and temperature, these environmental factors must be controlled. Additionally, infected animals should be quarantined and carcasses removed immediately.[59]

1.2.3 Legal Assessments of Coccidiostats

Regulation (EU) 1831/2003 establishes an authorisation procedure for coccidiostats and governs the placing on the market, use, and labelling of feed additives.[60] According to Article 3, a feed additive may only be placed on the market following specific authorisation by the European Commission and the European Food Safety Authority (EFSA). Authorisations for the various active substances are granted to manufacturers in the form of individual regulations for a period of ten years. Table 1.1 lists all coccidiostats investigated in this study that are currently authorised in the EU for broiler chickens and chickens reared for laying, along with their corresponding individual regulations.

Table 1.1: Authorised coccidiostats for poultry, including trade names, target animals, authorisation validity and regulations. * indicates that, in accordance with the provisions of Article 14 of Regulation (EC) No 1831/2003, an application for renewal of the authorisation was submitted. BC = broiler chicken, CRL = chicken reared for laying

Coccidiostat	**Trade name**	**Animal**	**Valid until**	**Regulation (EC)**
AMP HCl	COXAM	BC / CRL	14.12.2031	2021/2047
DEC	Deccox/ Avi-Deccox 60G	BC	20.12.2031	2021/2094
DIC	Coxiril	BC	04.02.2025	2015/46
	Clinacox 0.5%	BC	02.08.2023*	2019/138
		CRL (16 weeks)	23.12.2020*	1118/2010
LAS A Na	Avatec 150G	BC	06.07.2033	2023/1172
MON Na	Coxidin	BC	10.06.2021*	495/2011
		CRL (16 weeks)	09.03.2022*	140/2012
MON and NIC	Monimax	BC/ CRL (16 weeks)	30.07.2030	2020/994
NAR and NIC	Maxiban G160	BC	28.10.2020*	885/2010
ROB HCl	Robenz 66G	BC	25.02.2030	2020/148
SAL Na	Sacox120-/ 200 microGranulate	BC/ CRL (12 weeks)	09.11.2027	2017/1914

Within the respective individual regulations, specific "target species" are also defined. The use of various coccidiostats as feed additives is thereby restricted to broiler chickens, turkeys, chickens reared for laying (up to an age of 16 weeks), and rabbits. Animal species not explicitly listed for the application of coccidiostats are

classified as "non-target species" (e.g., laying hens). In addition to the prohibition of use in non-target species, Regulation (EU) 2018/848 bans the application of coccidiostats in animals from organic production.[61] As feed manufacturers frequently produce different types of feed within the same facility, unavoidable cross-contamination of coccidiostats into feed intended for non-target species may occur, despite the application of the ALARA principle (As Low As Reasonably Achievable) and adherence to all precautionary measures stipulated in Regulation (EC) No 183/2005.[62] For such instances, Commission Directive 2009/8/EC establishes maximum carry-over rates of 3 % for less sensitive species and 1 % of the authorised maximum content for more sensitive species and for withdrawal feed.[63] Maximum levels for coccidiostats in food resulting from this unavoidable carry-over into non-target feed are laid down in Regulation (EC) No 124/2009.[64]

Incidents involving the improper presence of coccidiostats are also recorded in the RASFF. Between 2020 and 2025, a total of 26 cases were associated with coccidiostats. These involved exceedances of MRLs in food (both from poultry and other animal species) and feed, as well as mislabelling of feed. Seven of the registered findings were declared as "potentially serious", "potential risk", or "serious". For five of the 26 cases, no risk decision has yet been made. Based on the previously introduced NRCP, 0.03 % (1 out of 3,086 control samples) of cases involving coccidiostats in food occurred in 2023. This specific case involved 128.25 µg/kg of decoquinate in muscle tissue of broiler chickens, a species for which decoquinate is not authorised in this context.[65] Consequently, the data indicates a generally high level of safety regarding coccidiostat residues in food.

1.2.4 Toxicology and Development of Resistance

Coccidiostats present a multifaceted toxicological dilemma: they are essential for the targeted control of coccidiosis in poultry, yet their usage inevitably leads to the exposure of non-target species and entry into the human food chain. Concurrently, their continuous prophylactic application contributes to the accelerated development of resistance in pathogenic *Eimeria* strains.

Mammals, particularly horses, are especially vulnerable to coccidiostats. This susceptibility is attributed to a relative deficiency in hepatic CYP450 demethylating enzyme capacity, resulting in significantly slower clearance rates.[66] Symptoms of monensin intoxication in horses, for instance, include depression, ataxia, paresis, and paralysis accompanied by partial anorexia.[67] In a controlled exposure study involving monensin, an LD50 of 1.38 mg/kg body weight was determined.[68] Depending on the specific ionophore, clinical manifestations vary in severity and may

include immobility, tachycardia, dyspnoea, cardiac insufficiency, and polyuria.[67]–[69] Symptoms of intoxication caused by synthetic coccidiostats depend heavily on the specific compound and the animal species affected; for example, signs may range from decreased respiratory rate, ataxia, tremors, and clonic convulsions to abomasitis (in ruminants).[70], [71]

However, caution is also required for "target species" due to the narrow margin of safety inherent to coccidiostats. In avian species, symptoms of intoxication resulting from overdosing with ionophoric compounds include locomotor disorders, severe dyspnoea, and moderate diarrhoea.[72]

Despite strict regulatory restrictions, notifications in the RASFF demonstrate that human exposure to coccidiostats cannot be entirely ruled out. Individuals involved in the production or direct handling of these substances are at a particularly elevated risk of exposure. The toxicological characterisation of coccidiostats for human risk assessment relies on experimental animal studies, particularly long-term studies in rats. From these data, no observed effect level (NOEL) values are derived. Scientific committees such as the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) Panel of the European Food Safety Authority (EFSA), the Committee on Medicinal Products for Veterinary Use (CVMP) of the EMA, and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) are responsible for evaluating these studies and, based on their findings, proposing toxicological reference values such as the acceptable daily intake (ADI). The ADI is calculated by applying a safety factor of 100 to account for inter- and intraspecies variability. Table 1.2 lists the corresponding ADI values for the coccidiostats investigated in this study.

Table 1.2: Acceptable daily intake (ADI) values of investigated coccidiostats expressed in µg/kg body weight (BW) per day.

Coccidiostat	**ADI [µg/kg BW/day]**
Lasalocid Sodium	5
Narasin	5
Salinomycin Sodium	5
Monensin	3
Robenidine	37.5
Decoquinate	75
Halofuginone	0.3
Nicarbazin	770

Regular intake of low doses of carboxylic ionophores has been observed to increase coronary blood flow through specific vasodilation. This pharmacological

effect may superimpose upon the body's autoregulatory dilation of partially occluded vessels, potentially diverting blood away from ischaemic regions of the heart—a mechanism that has been historically discussed in therapeutic contexts. In contrast, acute intoxication in humans manifests as headache, nausea, epistaxis (nosebleed), and skin irritation.[73]

A further critical issue regarding coccidiostats is the emergence of resistant strains. Although the development of resistance to coccidiostats, particularly ionophores, appears to be slower compared to sulphonamides, it is by no means precluded.[50] Arabkhazaeli et al. reported the development of resistance in six different *Eimeria* spp. across three separate inoculation trials against diclazuril, salinomycin, and amprolium.[74] Another study demonstrated cross-resistance between the monovalent ionophores monensin and narasin, but notably not simultaneous resistance to lasalocid.[75] A plausible explanation is that lasalocid, due to its ability to complex divalent cations, possesses a distinct mechanism of transport that may partially exclude it from cross-resistance patterns seen in monovalent ionophores. Beyond cross-resistance, multidrug resistance poses a significant challenge. Investigations from the UK revealed *E. tenella* isolates resistant to five coccidiostats with differing modes of action. Such resistance phenomena may stem from various causes: the sequential application of the same coccidiostats, as well as genetic recombination among already resistant strains, can drive the formation of multidrug resistance. Conversely, the rotation of coccidiostats sharing the same mode of action may promote cross-resistance.[50] Improper handling or inadequate hygiene management can lead to subtherapeutic dosing within a flock, exposing the parasites to the drug throughout their entire development cycle, thereby creating selection pressure for resistance.

In some instances, resistance may be temporary and can revert following a prolonged withdrawal of the respective substances. Nevertheless, resistance to pharmaceutical compounds entails severe health risks for both humans and animals. To reduce the reliance on coccidiostats and thus minimise resistance formation, vaccines against coccidia are already available, and alternative approaches such as probiotics are being explored. However, a complete cessation of coccidiostat use is currently not feasible.[76]

1.3 Dust as Vector of Toxic Contaminants and New Entry Pathway of Pharmaceuticals into the Environment

The application of veterinary medicinal products, such as coccidiostats or synthetic hormones, as well as the excretion of endogenously formed hormones, facilitate their entry into the environment via various pathways. Globally, manure, with an annual production volume of several billion tonnes, has been established as the primary entry route for pharmaceutical substances used in intensive livestock farming.[77], [78] Following excretion, administered pharmaceuticals accumulate in manure and are subsequently introduced into the environment through land application as organic fertiliser. However, the use of pharmaceuticals also leads to their incorporation into stable dust. Depending on the mode of administration, this entry occurs either passively via excretion in dried faeces and feed residues, or actively through application practices, such as the spray application of altrenogest.[79] This stable dust is ultimately released into the environment through ventilation systems or cleaning operations and subsequently dispersed. Additionally, humans are directly exposed to these contaminants via inhalation of stable dust.

Stable dust is characterised in the literature as a complex, heterogeneous, and biologically highly active mixture, the precise composition of which varies significantly depending on the animal species, housing system, feeding regime, and management practices. Consequently, its scientific definition typically relies on the description of its diverse constituents, physical properties, and biological effects.[80]

A critical parameter for characterising the physical properties of dust is particle size, expressed as particulate matter $(\mathrm{PM})_X$. Precise definitions regarding the size of particulate matter can be found in Directive 2008/50/EC.[81] The aerodynamic diameter of dust particles allows for an estimation of their penetration depth into the respiratory tract. Coarse particles ($\mathrm{PM} > 10\,\mu\mathrm{m}$) are retained by nasal hairs or the mucous membranes of the nasopharynx, whereas fine dust penetrates deep into the lungs, depositing at the level of the terminal bronchioles and alveoli.[82] Fine dust is further subdivided into PM_{10} and $\mathrm{PM}_{2.5}$. Particles smaller than $0.1\,\mu\mathrm{m}$ are even capable of translocating into the systemic circulation. The size distribution of stable dust particles varies according to animal species and housing type. For instance, a study by Lai et al. found the highest proportion of PM_{10} particles in poultry houses, whereas cattle barns exhibited the lowest.[83]

However, not only particle size but also the total dust concentration is influenced by the animal species and housing system. Generally, the highest concentrations

of inhalable dust are found in poultry housing (4.15 mg/m^3), followed by pig (2.32 mg/m^3), and cattle facilities (0.45 mg/m^3). Furthermore, aviary systems exhibit higher dust concentrations compared to cage systems. Regarding seasonal variations, concentrations are typically higher in winter than in summer due to reduced ventilation rates, although emissions may be higher in summer. Diurnal variations show higher concentrations during the day than at night, which correlates with increased animal activity generating and resuspending particles, as well as the availability of litter as a dust source.[84], [85] Yet, animal species and housing type are not the sole determinants of long-term dust concentration and composition. Optimised hygiene management systems, including advanced ventilation and air filtration, can significantly reduce dust loads.[86], [87]

Similar to dust concentration and particle size, the chemical composition—another vital characteristic of stable dust—is dependent on the animal species and housing form. The dust itself consists of organic and inorganic constituents, originating from the animals (hair, feathers, and skin) as well as from feed, litter, and building materials.[85], [88], [89] Figure 1.5 illustrates the distinct composition of stable dusts from pigs and laying hens.

Figure 1.5: Examples of dust samples originating from pig (left) and laying hen (right) facilities.

The stable dust samples presented originate from sedimentation. While pig stable dust consists predominantly of fine particles, chicken stable dust additionally contains components of down feathers. Resulting from differences in feed, the dusts also differ in colour and odour. A more detailed discussion on the chemical composition of dust based on Weender analysis can be found in Chapter 4.2.

Beyond constituents originating from the animal, feed, and housing materials, various studies have demonstrated that stable dust functions as a vector

for endotoxins, antibiotics, heavy metals, and bacteria.[90]–[94] Based on the composition of stable dusts, the concentrations present in livestock buildings, and the contaminants they carry, stable dusts pose a significant health risk to personnel, animals, and the environment. Chronic exposure to stable dust increases the risk for both personnel and animals of developing respiratory diseases, such as asthma-like syndrome, rhinosinusitis, hypersensitivity pneumonitis, organic dust toxic syndrome (ODTS), chronic obstructive pulmonary disease (COPD), and chronic bronchitis.[80], [95] Various mycotoxins present in stable dust can induce synergistic and additive toxic effects, including immune suppression, impaired reproduction, and organ damage. Residues of antibiotics, such as tetracyclines and fluoroquinolones, promote antibiotic resistance, thereby increasing infection risks for animals and their caretakers via inhalation.[93], [96]

2

Technical Background

Contents

2.1 Liquid Chromatography

In principle, chromatography describes the separation of analytes based on their distinct physico chemical properties due to adsorption and distribution of the analytes between a stationary and mobile phase. Many coccidiostats and steroid hormones, as well as their conjugates, which are formed by substitution with sulfates or β-glucuronates, are non-volatile and therefore unsuitable for gas chromatographic separation without prior hydrolysis and hazardous and time-consuming derivatization (e.g. silylation).[97] In combination with mass spectrometric analysis of hormones and coccidiostats, chromatographic separations are routinely carried out by high performance liquid chromatography (HPLC) predominantly operating under reversed phase (RP) conditions, implying a higher polarity of the mobile phase used for the elution as compared to the stationary phase.[98]–[100] The fundamental principles behind chromatography and HPLC are considered conversant and hence will not be explained in more detail.[101]

Despite the remarkable selectivity of modern mass spectrometric techniques, especially tandem mass spectrometry (MS/MS), it is not necessarily advisable to forgo coupling with HPLC. Using HPLC enables matrix components to be separated from analytes, thus reducing potential matrix effects. Especially with complex matrices such as stable dust and the use of electrospray ionisation (ESI),

the occurrence of matrix effects such as ion suppression is possible. Furthermore, analytes exhibiting identical m/z-transitions in MS/MS due to their structural composition can only be distinguished by prior separation using LC or other separation techniques. Thus, isomeric and therefore isobaric analytes such as α- and β-estradiol can only be distinguished by coupling with HPLC.[102]

2.2 Mass Spectrometry

2.2.1 Definitions of and within Mass Spectrometry

Mass spectrometer represent instruments capable of ionizing various atoms and molecules and separating and determining these ions according to their m/z ratio. As a result, they can be used for various applications, from structure elucidation to quantitative analysis or identification of unknowns by providing the unit mass or accurate mass.[103], [104] Depending on the purpose of application, many types of mass spectrometers have been developed, differing in physical principals and construction but all fulfil the above definition.

When it comes to definitions within mass spectrometry, there are two decisive characteristics: resolving power and accuracy.

Resolving Power

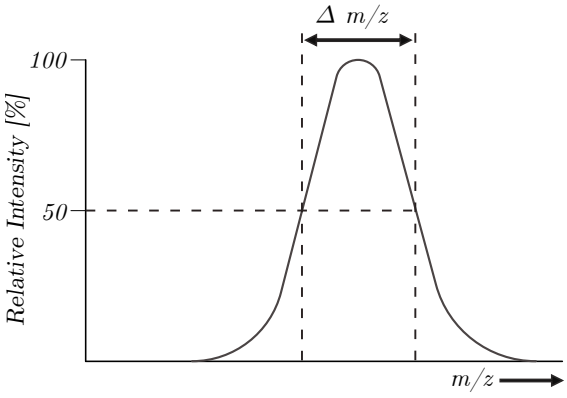

Figure 2.1: Definition of mass resolving power using the FWHM approach.

The resolving power (often used interchangeably with the term resolution) describes the ability (of a mass spectrometer) to separate two peaks in a mass spectrum. Current recommendations define the resolving power (R) according to the following equation [105], [106]:

$$R = \frac{m/z}{\Delta\ m/z} \tag{2.1}$$

Δ m/z is the width of a peak at a specific intercept of the peak height. Commonly used is half of the maximum peak height (full width at half maximum (FWHM)) as depicted in Figure 2.1.

(Mass) Accuracy

Mass accuracy describes the deviation from the experimentally determined to the theoretically calculated (exact mass) value (see Equation 2.2).

$$Mass\ error\,(ppm) = \frac{m/z_{experimental} - m/z_{theoretical}}{m/z_{theoretical}} \cdot 10^6 \tag{2.2}$$

The determination of the elemental composition of a molecule is dependent on a certain mass accuracy, according to the size of the molecule. With increasing m/z ratio, the number of possible formulas increases and thus complicates identification. If multiple charged molecules are present, a combination of high mass accuracy and resolving power is required to achieve unambiguous results.[107]

Another important characteristic is the duty cycle, especially when MS sensitivity is concerned, which indicates the percentage of target ions entering the MS that are effectively detected.

2.2.2 Ion Source

When coupling HPLC and mass spectrometers an atmospheric pressure ionsation (API) technique is required as interface, since the ion source region is usually not under vacuum. Its function is to ionise the analytes, transfer them into the gas phase and evaporate the solvent in order to maintain the vacuum in the mass spectrometer.

One of the most important and commonly used API techniques for the analysis of steroids and coccidiostats is ESI. [99], [100] The basis of ESI technology was laid by the work of Dole and co-workers [108], [109] and was further refined by the group of Fenn. [110]–[112] In ESI ionization occurs as a result of either charge separation, adduct formation, electrochemical or gas phase reactions (proton transfer). [113]

The analyte solution coming from the HPLC is passed through a capillary into an electric field, created by an electric voltage applied between the capillary and a counter electrode (see Figure 2.2). The ESI can be operated in both positive and negative mode. If positive polarity is used, the anions in solution are oxidised at the capillary (in this case the anode), which leads to a positive excess charge that can include H^+, NH_4^+, Na^+ and K^+ accumulating on the surface of the liquid.[114] As a result of the equilibrium between the surface tension of the liquid and the repulsive forces of the positive charges amplified by the counter electrode, a so-called Taylor cone is formed at the capillary outlet, pointing downfield. With increasing distance to the anode, the destabilization of the cone by the field strength prevails and a fine jet of positively charged droplets emits at the tip of the cone. [115] Often supported by

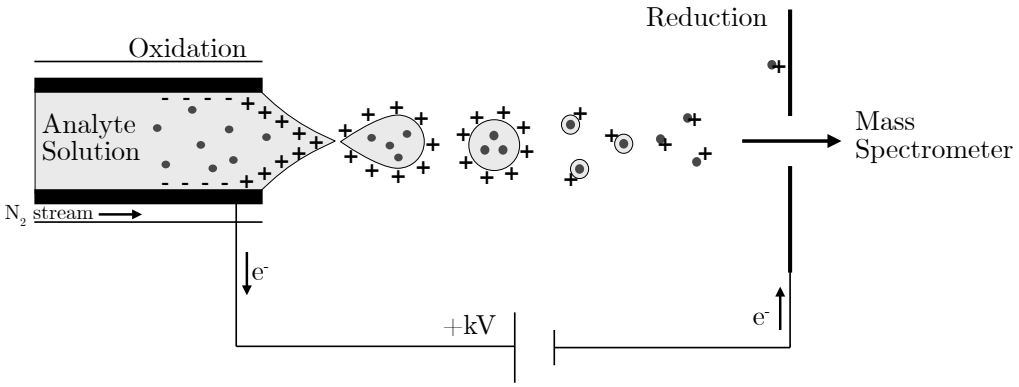

Figure 2.2: Schematic depiction of an ESI exemplarily operating in positive polarity.

a stream of nitrogen, the droplet undergoes rapid evaporation, which, depending on the flow rate and the solvent used, may be thermally assisted. When using a mixture of aqueous and organic solvents, the organic component evaporates more rapidly, causing the proportion of the aqueous phase to increase progressively.[116]–[118]

The location of the charge within a drop is described by two seemingly contradictory theories. According to Gauss's law, electrical charge in an isolated conductor (comparable to a drop of solvent in the gas phase) is located directly on its surface.[119] However, ions such as Na^+ tend to form a solvation shell and therefore remain on the inside of a droplet. Using a dipolar solvent seems to reconcile this conflict. Based on dipole-mediated polarisation effects, the solvent orientates itself around the ion and the charge is projected onto the surface of the droplet while maintaining the solvation shell around the ion. If the ion carries alkylated, and therefore organic, moieties the ion is presumably located on the surface of the droplet due to phase separation.

With continuous evaporation of the solvent and constant charge, the charge density on the surface of the droplet increases. If the electrostatic repulsion of the surface charges is equal to the surface tension, a so-called Rayleigh limit is reached. Below this limit, the droplet undergoes a Coulomb explosion to restore this equilibrium. Repeated evaporation and Coulomb explosions lead to droplets with a diameter of a few nm.

Based on the size and composition of the ions, there are three postulated models describing ionisation and the transfer to the gas phase: ion evaporation model (IEM), charged residue model (CRM), and chain ejection model (CEM).

According to the IEM, nanodroplets, which ultimately lead to the release of the gas phase ions, are formed from the outer layers of the μm droplets and are thus enriched with high surface activity species. These include primarily low molecular weight ions which possess hydrophobic moieties. Depending on a certain number of

charges and the radius of the droplet, an ejection of solvated ions occurs before the Rayleigh limit is reached. This ejection of solvated ions was described by Iribarne and Thompson using the transition state theory. Using this theory, the ejection rate constant k can be expressed as:[116], [120]

$$k = \frac{k_B \cdot T}{h} \cdot exp\left(\frac{-\Delta G^*}{k_B \cdot T}\right) \tag{2.3}$$

Here k_B corresponds to the Boltzmann constant, h to Planck's constant and T to the temperature. ΔG^* describes the barrier of the free activation energy, which is composed of the image charge, created by the solvent polarisation, which pulls the ion back into the droplet and the repulsive forces of the excess charges, pushing the ion further away from the droplet. Based on the solvent used and its aqueous proportions and the associated surface tension, the ion is initially connected to the droplet via a solvent bridge, which ruptures as the ion is released.[121]

The CRM describes the formation of gas-phase ions via Rayleigh-charged nanodroplets, containing a single analyte molecule. The analyte molecules for which the CRM applies are large, globular species such as natively folded proteins or water-soluble polymers. Extensive hydration of these polymers causes them to remain inside the droplet. Through continuous solvent evaporation and ejection of excess charges based on the IEM, the radius of the droplet decreases until remaining excess charge is transferred to the analyte and the analyte is present as a gas-phase ion.[108], [116] The charge state of the gas phase ion ultimately does not depend only on its charge state in solution but on the Rayleigh charge of the last solvation shell.[122]

Using acidic buffers in the solvent can lead to the denaturation and unfolding of proteins. As a result, the macromolecule is no longer able to shield the hydrophobic residues from the solvent and therefore no longer remains solvated inside the droplet. After migration to the surface of the droplet, one chain end is ejected from the droplet according to the IEM and sequentially pulls the rest of the molecule along, while surface charges are transferred to the macromolecule.[123] This model is described as the CEM.

2.2.3 Mass Analyser

The most commonly used mass analyser in hormone and coccidiostats analysis is the quadrupole.[99] A quadrupole consists of four cylindrical electrodes, of hyperbolical cross section, arranged radially opposed to each other (see Figure 2.3). Two opposing

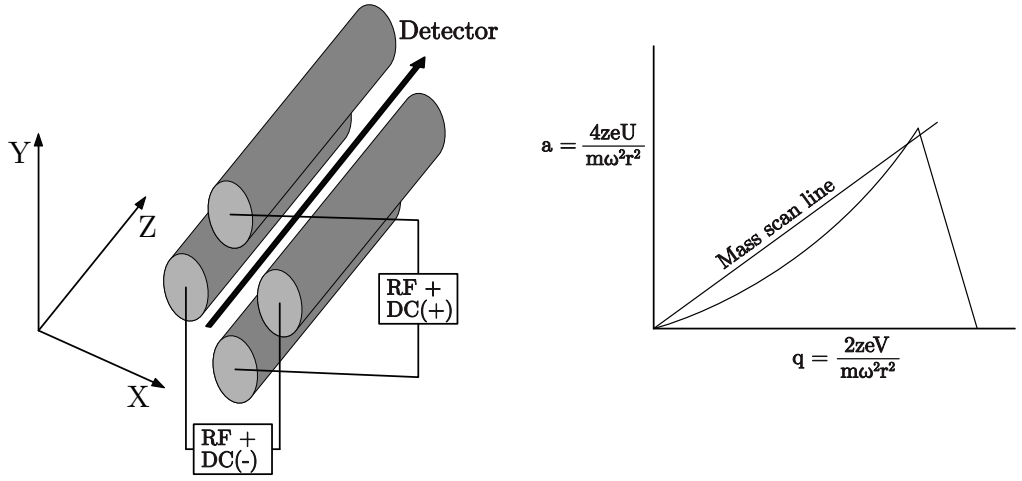

Figure 2.3: Schematic depiction of a Quadrupole (left) and a stability diagram (right).

electrodes form a pair, which is connected to both a time-dependent and a time-independent potential. A positive direct current (DC) is applied to one pair and a negative DC to the other. The applied alternating current (AC) potential has the same order of magnitude, but the amplitude is 180° out of phase between the pairs. The ions coming from the ion source pass through the quadrupole along the Z-axis (see Figure 2.3). Depending on the charge of the analyte ions and the applied DC potentials (positive or negative), the respective electrode pairs have a repulsive and thus stabilizing or attractive and thus destabilizing effect on the trajectory of the ions. By superimposing the AC and DC potentials, these effects can be varied by the amplitude and frequency of the AC potential allowing the quadrupole to be used as a mass filter. As a result of the influence of the average voltage of the superimposed AC and DC potentials on the ions, the electrode pairs operate as a high or low pass mass filter based on their DC potential. Electrode pairs with repulsive and thus stabilizing effects on the ions operate as high pass mass filters.

Based on Newton's second law of motion ($F = m \cdot a$), ions of higher mass are too inert for their trajectories to be destabilised by the momentary destabilising effects of the superimposed AC potential. In contrast, the trajectories of lighter ions are destabilised under the same settings of U (magnitude of the applied DC potential) and V (magnitude of the applied AC potential) of the same electrode pair to such an extent that they collide with the electrodes and neutralise. Consequently, the electrode pair to which a destabilising DC potential is applied acts as a low pass mass filter. Accordingly, the setting of the specific quadrupole parameters (U and V of the DC and AC potential respectively) determines whether an ion is heavy or light. By combining the two pairs of electrodes, a band pass filter with a specific bandwidth is obtained. This band pass filter represents the resolving

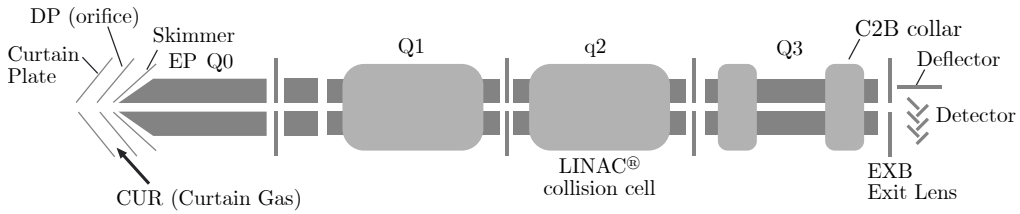

Figure 2.4: Schematic depiction of a triple quadrupole. Adopted and adjusted from [126]. With DP = declustering potential, EP = entrance potential.

power of the quadrupole. A quantitative approach to describe the motion of the ions through the quadrupole is provided by Mathieu's differential equations, the full understanding of which is beyond the scope of this thesis. A simplification of these equations leads to two terms, a and q, which describe the interrelation between the properties of the quadrupole and an ion. The solutions to these equations result in specific limits for U and V for certain m/z values of the ions, which can be depicted in a stability diagram (see Figure 2.3).

Based on different combinations of AC and DC potentials, the quadrupole can be used in three different modes. In single ion monitoring (SIM), U and V remain constant at a certain value throughout the entire measurement, allowing only one ion with a certain m/z ratio to pass through the quadrupole. Consequently, the duty cycle is almost 100 %. With continuous ramping of U and V while maintaining a certain ratio, the quadrupole operates in the so-called scan mode. Since various m/z ratios are successively stabilised in a predefined m/z range during the entire measurement, the respective duty cycle and therefore the sensitivity decrease compared to the SIM. Using only the AC potentials, the quadrupole operates in radio frequency (RF)-only mode (only radio frequency used), whereby the broadest possible m/z range is transmitted. This mode is used in collision cells or ion optics to focus the ion beam.[124], [125]

Today, almost all single quadrupole MS have been replaced by triple quadrupole (QqQ) instruments. QqQ instruments consist of two quadrupole mass analysers separated by a collision cell (see Figure 2.4).

The collision cell is basically a quadrupole (or hexa/octopole), which is filled with an inert gas (e.g. Nitrogen or Argon) and only operates in RF-only mode. Accordingly, no m/z selection is performed in the collision cell. Ions that enter the collision cell are accelerated by applied voltages, increasing their kinetic energy. During collisions with the inert gas, this energy is converted into internal energy. With sufficiently high internal energy, the bonds within the ion dissociate. This process is also called collision-induced dissociation (CID). Due to the ion species produced by the ESI, most fragmentations can be explained by σ-cleavage between

a carbon and a hetero atom or σ-cleavage with hydrogen shift. Since all ions are accelerated in the collision cell, sequential fragmentation can also occur, resulting in first- and higher-order product ions.

The two quadrupole mass analyser and their respective operating modes (SIM, Scan and RF-only) combined with the collision cell allow a versatile range of analytical questions to be answered. Table 2.1 lists the different QqQ modes and exemplary applications.

Table 2.1: Triple quadrupole operation modes and respective examples of application.

Mode	**Q1**	**q2 Collision Gas**	**Q3**	**Exemplary Application**
Q1 MS	Scan	off	RF-only	Complete mass spectrum
Product Ion Scan	SIM	on	Scan	Investigation of fragments/ Structure elucidation
Precursor Ion Scan	Scan	on	SIM	Detection of unknown compounds
Neutral Loss Scan	Scan	on	Scan (shifted by a fixed m/z difference to Q1)	Detection of unknown compounds
MRM	SIM	on	SIM	Target Analysis

Depending on the type of MS, detection is carried out either spatially separated (e.g. quadrupole instruments) or within the mass analyser (e.g. Orbitrap). In this study a Sciex 3200 QTrap was used, which is equipped with a channel electron multiplier detector. This detector consists of dynodes and generates an electronic signal through repetitive secondary emission, whereby the signal is intensified by cascade amplification of the colliding ions.

2.3 Applications of LC-MS/MS in Coccidiostat and Hormone Analysis

Validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been established for the detection of coccidiostats in food and feed matrices, and for hormones in aqueous environmental compartments. However, the simultaneous determination of both analyte classes in complex dust matrices has, to date, not been addressed in the published literature. This analytical gap represents a significant limitation in the comprehensive environmental and occupational risk assessment of intensive livestock facilities.

Coccidiostat analysis via LC-MS/MS in feed and food matrices is well-established through multiresidual methods. Chang et al. validated an LC-QTRAP-MS/MS

method for the simultaneous quantification of 20 coccidiostats in eight food-relevant matrices, achieving quantification limits of 0.5–2 ng/g.[127] Similarly, steroid hormone determination in aqueous matrices has been extensively developed, with methods routinely achieving detection limits in the ng/L range using solid-phase extraction and LC-MS/MS detection.[128] These analytical advances have been justified: class-specific methods inherently achieve superior selectivity and sensitivity, and regulatory control of coccidiostat residues mandates analytical performance that multiclass methods may compromise.

However, for the characterisation of stable dust as a complex environmental and occupational exposure compartment a multiclass method is favoured to first obtain a sophisticated overview over possible contaminants included in this matrix. The physical and chemical heterogeneity of stable dust, comprising feed residues, faecal matter, microbial components, feathers or hair, and organic contaminants, presents methodological challenges distinct from homogeneous food matrices or dissolved water samples. Furthermore, recent investigations have demonstrated that stable dust serves as a vector for antibiotics and mycotoxins, highlighting the matrix's significance as a multi phasic contaminant reservoir.[88], [89], [92], [96]

The development of a combined LC-MS/MS method capable of simultaneously detecting coccidiostats and steroid hormones in dust represents a development toward comprehensive characterisation. Such a method addresses structural and ionisation incompatibilities inherent to these analyte classes whilst accommodating the inherent variability and organic loading of dust matrices. This integrated approach adds up to the fundamentals needed to enable a holistic risk assessment addressing chronic inhalation exposure to farm workers and environmental pollution contributing to the one health approach.

3
Objectives

The presence of veterinary pharmaceuticals in the environment is a matter of growing scientific and regulatory concern. While the primary entry pathways, like manure, of these substances are well-documented, investigations of airborne particulate matter within livestock facilities remains scarce. Coccidiostats and steroid hormones, whether administered exogenously or produced endogenously, can partition into stable dust via dried faeces, feed residues, or direct aerosolisation during application. Once airborne, this dust serves as a vector, facilitating the dispersal of these compounds into the environment through ventilation systems and posing a direct inhalation risk to animals and their caretakers.

Consequently, the objective of this thesis is to establish stable dust as a significant, yet hitherto underestimated, environmental and occupational exposure compartment for steroid hormones and coccidiostats. To achieve this, an analytical framework was developed to detect, quantify, and give a first glance on the risk assessment of these residues in complex dust matrices. The specific research aims are defined as follows:

1. Development of a comprehensive LC-MS/MS method capable of the simultaneous detection and quantification of multi-class analytes, specifically steroid hormones (estrogens, androgens, progestogens) and coccidiostats (ionophores, synthetic compounds), at trace analytical concentrations within the complex matrix of stable dust.

2. Development and optimisation of extraction and clean-up methods to establish a rigorous sample preparation protocol that overcomes the heterogeneity of stable dust. This includes the characterisation of dust properties and the application of the Design of Experiments methodology to optimise liquid-solid extraction and dispersive solid phase extraction parameters, thereby maximising analyte recovery while minimising matrix effects.

3. Validation according to Commission Implementing Regulation (EU) 2021/808. This objective aims to demonstrate the method's reliability regarding specificity, selectivity, and to determine decision limits ($CC\alpha$), detection capabilities ($CC\beta$) and uncertainty related performance characteristics.

4. Application of the validated method to stable dust samples collected from diverse livestock systems (laying hens, broilers, breeding sows, finishing pigs). This includes assessing current contamination profiles, investigating the spatial distribution of residues within facilities, and analysing archived samples (1980s–2000s) to evaluate the long-term persistence of these compounds.

4

Results and Discussions

Contents

4.1 Detection-Method Development

Mass spectrometric methods employed for the detection of steroid hormones and coccidiostats at trace analytical concentrations in complex matrices, such as stable dust, require a high degree of sensitivity and selectivity. To fulfil these requirements, the variable parameters of the liquid chromatography (LC)-mass spectrometry (MS) method must be optimised according to the chemical and physical properties of the analytes.

Following successful optimisation of the method, the method should be capable of identifying and quantifying the following steroid hormones: altrenogest (ALT),

megestrol acetate (MA), medoxyprogesterone acetate (MPA), nandrolone (N), progesterone (P), stanozolol (STA), testosterone (T), trenbolone (Tb), α-estradiole (aE2), β-estradiole (bE2), estriole (E3), estrone (E1), estrone-3-sulphate (E3S), ethinylestradiole (EE2)

and coccidiostats: decoquinate (DEC), halofuginone (HAL), monensin (MON), narasin (NAR), robenidine (ROB), salinomycin (SAL), lasalocid (LAS), nicarbazin (NIC). Since the coccidiostats were integrated into the method at a later stage than the steroid hormones, parameters such as the curtain gas and source temperature were optimised solely for the steroids.

4.1.1 Ionisation

When using ESI, the efficiency of ionisation is essential. Hence, adaptation of adjustable ESI-parameter like curtain gas, source temperature, and mobile phase composition is crucial.

Curtain Gas

Depending on the instrument used, the curtain gas (CUR), generally Nitrogen, is directed either orthogonally or counter to the flow direction of the analytes between the curtain plate and the orifice. It primarily facilitates the evaporation of solvent droplets and prevents solvent residues, solvent clusters, and uncharged particles from entering the mass spectrometer. Optimal CUR settings, specified in pounds per square inch (psi), depend on the LC flow rate. Increased flow rates require higher CUR pressures to ensure efficient desolvation; however, excessive CUR can suppress ion transfer into the mass spectrometer and thus reduce signal intensities. To determine optimal CUR values for the method, settings of 25, 30, 40, and 50 psi were tested (see Figures 4.1 and 4.2). Determination of the optimal CUR was accomplished through the use of an analyte standard at 20 µg/L in H_2O:ACN:MeOH (70:15:15 v:v) and a preliminary multiple reaction monitoring (MRM) method.

Based on the data shown in Figures 4.1 and 4.2, a CUR of 40 psi was selected for the positive and 25 psi for the negative ionisation mode since using this settings lead to the highest area.

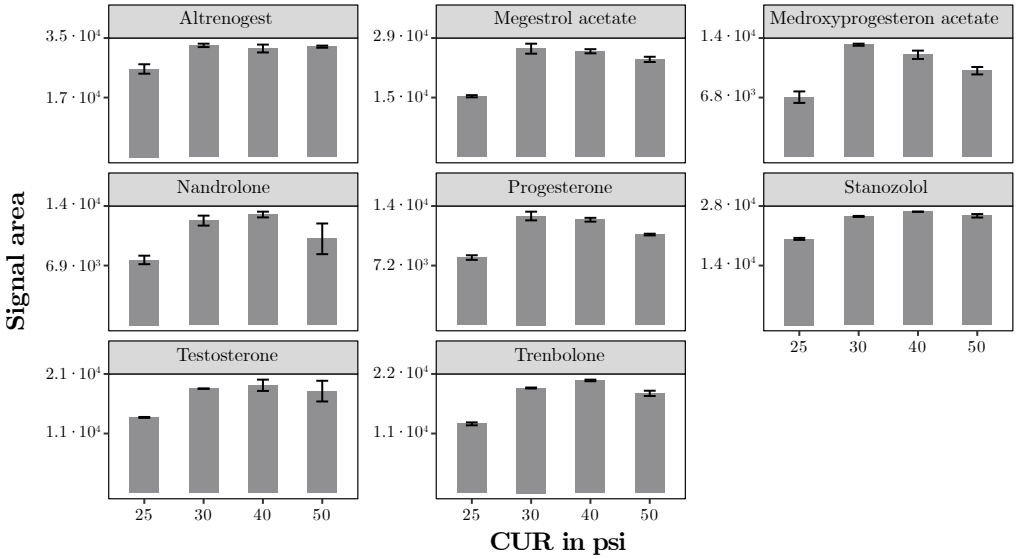

Figure 4.1: CUR optimisation for hormones using positive ionisation.

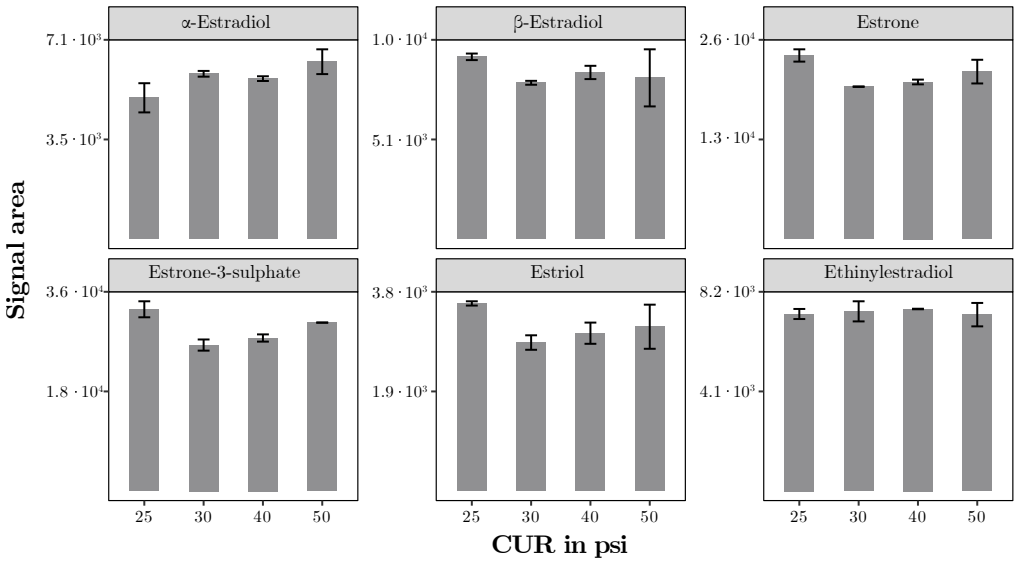

Figure 4.2: CUR optimisation for hormones using negative ionisation.

Source Temperature

The source temperature is not directly involved in the ionisation process itself, but can enhance ionisation efficiency depending on the analyte. The optimal source temperature depends on the solvent composition, the flow rate, and the thermal stability of the analytes. With increasing aqueous content of the eluent, the surface tension of the droplets increases correspondingly, leading to reduced ionisation efficiency due to a decreased evaporation rate. Increased temperature promotes faster solvent evaporation, facilitating the release of analytes into the gas phase and their subsequent ionisation. An elevated temperature produces the same enhancement

effect at increased flow rates. As the flow rate increases, the length of the jet increases, resulting in a larger diameter of the emitted droplets. Consequently, more Coulomb explosions are required until the solvent is completely removed.[129]–[131] However, depending on the thermal stability of the analyte, excessively high temperature can also produce negative effects, such as protein denaturation. The set source temperature does not act directly on the analytes because evaporative cooling occurs during the desolvation process. The effective temperature experienced by the analytes is consequently lower than the nominal instrument setting.

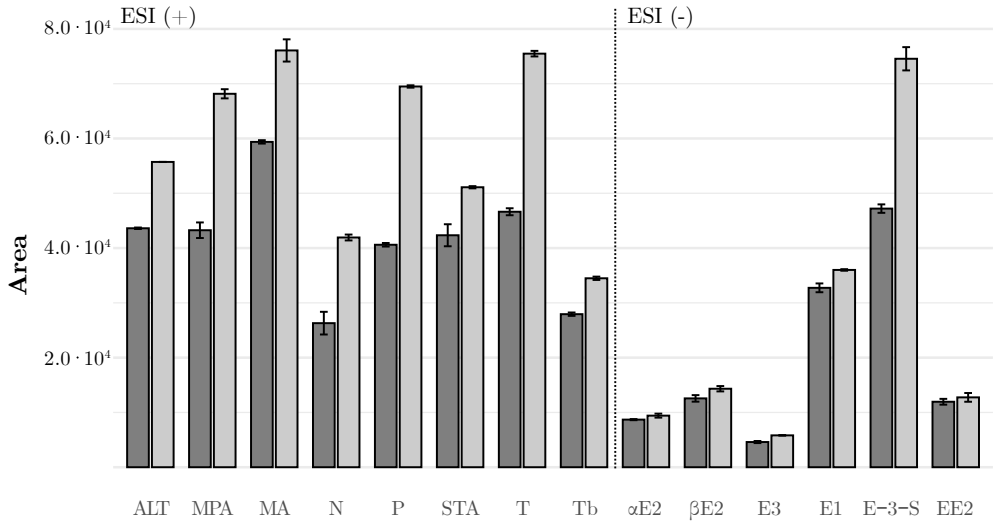

Figure 4.3: Optimisation of the ion source temperature. Dark grey indicates an ion source temperature of 400 °C and light grey of 600 °C. Abbreviations can be found under section 4.1.

The optimisation of the source temperature was carried out after optimising the LC gradient, but is already listed here as it is part of the ionisation optimisation. Figure 4.3 demonstrates that, with the eluents employed and the optimised gradient, including high water content and flow rates of 500 µL/min for positive ionisation mode and 300 µL/min for negative ionisation mode, a higher source temperature results in increased ionisation efficiency. Accordingly, a source temperature of 600 °C was selected for both the negative and positive ionisation methods. At this temperature, the manufacturer specifies gas flow rates of 55 and 60 for gas 1 and gas 2, respectively. Gas 1 is responsible for nebulising the liquid stream emerging from the source, while gas 2 transports thermal energy from the heating rods to the centre of the source.

Optimisation of Mobile Phase Composition and Ion Formation

The composition of the mobile phase used for chromatographic separation has a significant influence on both the formation of various ion species and the overall signal intensity of the detected ions. With respect to the ion species formed, the physicochemical properties of the analytes, such as the presence of functional groups, gas-phase basicity or acidity, and the overall atomic positive charge, are decisive factors.

Since ionophore coccidiostats such as lasalocid and monensin are particularly capable of complexing metal ions, and steroid hormones tend to form adducts as well, the initial focus was set on identifying the ion species showing the highest abundance.[132]–[134] For this purpose, the analytes were prepared in two different solvents: (1) MeOH:H_2O (80:20, v:v) with 2 % formic acid (for analysis in ESI^+ mode), and (2) MeOH:ACN:H_2O (15:15:70, v:v:v) with ammonia solution (pH 10) (for analyses in ESI^- mode). The measurements were performed by direct infusion using the *full scan* mode. Figure 4.4 exemplifies the mass spectrum of lasalocid and the detected ion species therein.

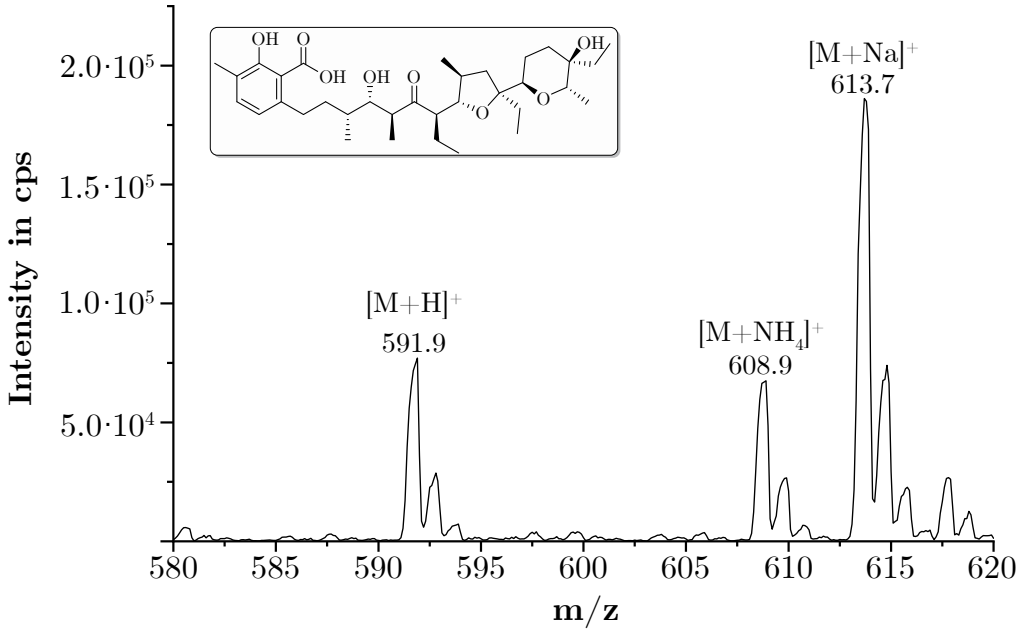

Figure 4.4: Different adducts for Lasalocid. Scan recorded using method 5.4.1 in positive ionisation mode.

According to the spectrum obtained (Figure 4.4), the $[M+Na]^+$-Ion shows the highest abundance. However, based on the pK_a values of lasalocid (see Table 4.2), resulting from the structural characteristics of the polyether, the formation of a molecular anion is favoured. Due to the inductive effect of the oxygen atoms of

the carboxylic acid group on the aromatic ring and their additional mesomeric stabilisation, it is deprotonated even at comparatively low pH values and is therefore preferentially present in ionic form. As described in Method 5.4.1, the preferred ion species and their total intensity were determined for each analyte and the results for the coccidiostats are shown in Table 4.1.

Table 4.1: Overview of detected coccidiostat ionspecies, including their respective charges, m/z-ratios, and peak areas.

Coccidiostat	**Charge**	**m/z**	**Area**
LAS	$[M+H]^+$	591.7	$4.62 \cdot 10^5$
	$[M+Na]^+$	613.7	$1.20 \cdot 10^6$
	$[M+NH_4]^+$	608.9	$4.17 \cdot 10^5$
	$[M–H]^-$	589.7	$1.31 \cdot 10^7$
MON	$[M+Na]^+$	693.8	$6.80 \cdot 10^6$
	$[M+NH_4]^+$	688.8	$7.30 \cdot 10^5$
NAR	$[M+Na]^+$	787.9	$2.95 \cdot 10^6$
	$[M+NH_4]^+$	783.0	$1.25 \cdot 10^6$
SAL	$[M+Na]^+$	773.9	$2.00 \cdot 10^6$
DEC	$[M+H]^+$	418.5	$2.75 \cdot 10^7$
	$[M+Na]^+$	440.5	$8.77 \cdot 10^5$
	$[M–H]^-$	416.4	$3.58 \cdot 10^6$
HAL	$[M+H]^+$	416.3	$1.10 \cdot 10^7$
	$[M+Na]^+$	437.6	$4.98 \cdot 10^5$
NIC	$[M+H]^+$ (HDP)	125.4	$1.87 \cdot 10^7$
	$[M–H]^-$ (DNC)	301.3	$8.13 \cdot 10^6$
ROB	$[M+H]^+$	334.0	$1.17 \cdot 10^7$

For the development of the final MRM methods, the molecular ions with the highest signal area were selected as *precursor ions*.

Considering the molecular structures of the steroid hormones analysed, it is noticeable that they generally do not contain functional groups that can be easily protonated or deprotonated under standard conditions (e.g. amines or carboxylic acids). Only oestrogens have one or more weakly alkaline hydroxy groups. Based on the pK_a values of the analytes (Table 4.2), it can be assumed that there are no significant ion concentrations in solution in the acidic pH range (approx. pH 2.7–3). Ionisation therefore occurs primarily in the gas phase within the ion source. Under basic conditions (pH 10), however, some of the estrogens are already deprotonated. These anions can be transferred to the gas phase in ESI^- mode by simple charge

separation. The deprotonation of the hydroxy groups is stabilised by mesomeric effects of the aromatic ring.

Table 4.2: pK_a and log P values at pH 8 of the analysed compounds.

Analyte	**pKa**	**log P at pH 8**
ALT	-0.18	3.19
MPA	n.A.	4.13
MA	n.A.	3.72
N	-0.88	3.07
P	n.A.	4.15
STA	3.36	3.81
T	-0.88	3.37
Tb	-0.89	2.25
α/β E2	10.33	3.74
E1	10.33	4.31
E3	10.33	2.67
E3S	1.75	2.50
EE2	10.33	3.90
DEC	10.76	6.87
HAL	14.59	0.41
MON	4.24	1.49
NAR	4.50	4.66
ROB	2.01	3.99
SAL	4.45	4.27
LAS	2.64	4.14
NIC	10.77	3.00

Investigations in ESI^+ mode showed only single-protonated molecular ions for androgens and progestogens without adduct formation. Since these compounds are not present in ionic form in solution at pH 2.7–3, their ionisation depends largely on gas-phase basicity or proton affinity.[135] With increasing proton affinity, the probability of the molecule reacting as a base in the gas phase and is thus protonated increases as well. For androgens and progestogens carbonyl compounds are suitable for this purpose since their oxygen atoms exhibit an increased proton affinity in the gas phase.

After selecting the most suitable precursor ions, the influence of various additives to the later mobile phase, used for the LC-method, on the ion yield was investigated. For this purpose, the following compositions were tested for ESI^+ mode: (1) 0,1 % formic acid, (2) 1 % formic acid, and (3) a 5 mM ammonium formate buffer adjusted to pH 3. The exact procedure is described in Method 5.4.2.

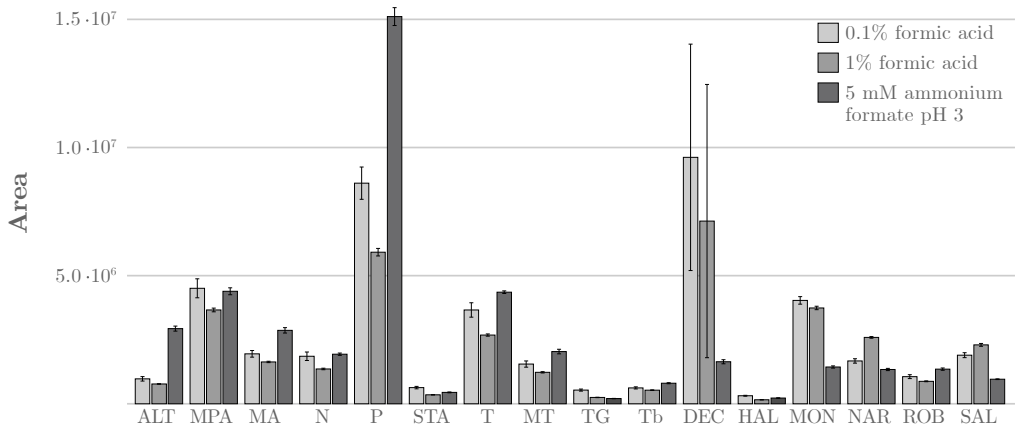

Figure 4.5: Optimisation of mobile phase composition for ESI^+ mode. The method is described in 5.4.2 with n = 4. Abbreviations can be found under section 4.1.

Figure 4.5 shows that for steroid hormones using an ammonium formate buffer led to the highest ion yields. However, even small amounts of ammonia in the matrix can suppress sodium adducts.[136] Accordingly, signal intensities for coccidiostats were higher with 0.1 % formic acid than with the ammonium formate buffer. Whether a potentially more intense $[M+NH_4]^+$ signal would surpass $[M+Na]^+$ signals when using formate buffer was not further evaluated. Since 0.1 % formic acid generated the highest yield, especially for analytes of overall low intensity, and was also less aggressive towards LC system components compared to 1 % formic acid, it was selected for the final method. Since a combined approach for simultaneous measurement of coccidiostats and steroid hormones was required, 0.1 % formic acid represented a practical compromise.

Two different mobile phase compositions were tested for ESI^- mode: (1) an aqueous ammonia solution at pH 10 and (2) a 5 mM ammonium acetate buffer (adjusted to pH 10). The addition of a buffer – and thus a salt – increases the number of possible ionic interactions in the mobile phase, which can cause dissolved ions to remain in the mobile phase rather than interacting with the stationary phase. While this effect was negligible in ESI^+ mode, ESI^- mode showed a significant impairment of chromatographic separation by the acetate buffer. Therefore, an ammonia solution was used as additive for the final ESI^- method.

4.1.2 MRM Transitions

The requirements demanded for a method aimed at detecting contaminants in the trace analytical concentration range include maximum sensitivity. This sensitivity can be achieved by employing the MRM method using triple quadrupole instruments.

For the development of an MRM method, individual standards of all analytes were prepared in the previously selected buffer (see subsection 4.1.1) and infused via direct infusion. Using the "Compound Optimization" function in *Analyst software* version 1.6.2., suitable transitions were initially selected for each analyte. A transition consists of the precursor ion and one fragment ion, where the fragment ions were selected at > m/z 90 so that they remained characteristic for the respective analyte.

For each transition, the parameters entrance potential (EP), declustering potential (DP), collision cell exit potential (CXP), and collision energy (CE) were optimised. The detailed results can be found for the ESI^+ mode in Table 4.3 and for the ESI^- mode in Table 4.4.

Table 4.3: m/z transitions and optimised mass spectrometry parameters for analytes analysed in ESI^+ mode including declustering potential (DP), collision cell exit potential (CXP), and entrance potential (EP) all expressed in volts and collision energy (CE) expressed in electron volt (eV).

ID	Q1 m/z	Q3 m/z	DP	EP	CE	CXP
Altrenogest	311.1	227.3	46	3.5	31	4
		115.1	46	3.5	103	4
Decoquinate	418.2	372.3	61	7	29	6
		204.2	61	7	53	4
Halofuginone	416.0	100.2	41	6.5	35	4
		120	41	6.5	27	4
Medroxyprog. acetate	387.2	327.4	36	6	19	6
		123.3	36	6	37	4
Megestrol acetate	385.2	325.3	31	6.5	19	6
		267.4	31	6.5	23	4
Methyltestosterone	303.2	109.1	46	3.5	35	4
		97.1	46	3.5	33	4
Monensine	693.6	479.5	106	9.5	67	6
		461.4	106	9.5	65	6
Nandrolone	275.1	109.1	46	3.5	37	4
		239.3	46	3.5	21	6
Narasin	787.5	431.4	101	9.5	65	4
		531.3	101	9.5	55	22
Progesterone	315.2	97.2	46	3.5	31	4
		109.2	46	3.5	31	4
Robenidine	334.0	155.1	46	4	27	4
		138.1	46	4	33	4
Salinomycin	773.5	431.4	101	10	63	4
		265.3	101	10	67	4

Continued on next page

Table 4.3 (Continuation)

ID	Q1 m/z	Q3 m/z	DP	EP	CE	CXP
Stanozolol	329.1	95.1	81	3.5	55	4
		121.2	81	3.5	49	4
Testosterone	289.2	97.1	41	3.5	29	4
		109.2	41	3.5	31	4
Trenbolone	271.1	115.2	56	3.5	85	4
		199.3	56	3.5	31	6

Table 4.4: m/z transitions and optimised mass spectrometry parameters for analytes analysed in ESI^- mode including declustering potential (DP), collision cell exit potential (CXP), and entrance potential (EP) all expressed in volts and collision energy (CE) expressed in electron volt (eV). The entrance potential (EP) was fixed at –10 V for all measurements.

ID	Q1 m/z	Q3 m/z	DP	CE	CXP
α-Estradiol	271.1	145.0	-95	-52	-7
		182.8	-95	-53	-5
β-Estradiol	271.1	183.0	-15	-54	-5
		144.9	-15	-50	-5
Estriol	287.1	170.9	-95	-48	-9
		144.9	-95	-54	-7
Estron	269.1	144.9	-90	-48	-9
		143.0	-90	-70	-7
Estron-3-Sulfate	349.0	269.0	-85	-44	-9
		144.9	-85	-68	-11
Ethinylestradiol	295.1	144.9	-105	-50	-7
		142.9	-105	-76	-11
Lasalocid	589.4	234.9	-75	-44	-7
		172.9	-75	-74	-5
Nicarbazin (DNC)	301.0	136.8	-50	-20	-13
		106.8	-49	-49	-4

For each transition, the fragment with the highest intensity was defined as the quantifier and the fragment with the second-highest intensity as the qualifier.

4.1.3 LC

Following determination of the quantifier and qualifier ions, further optimisation of the LC conditions were conducted, encompassing column selection, mobile phase composition, column oven temperature, and gradient parameters. Given the alkaline conditions (pH 10), a pH-stable stationary phase was essential. The XBridge C18

BEH column was selected because its bridged ethylene hybrid (BEH) particles provide stability across a pH range of 1–12.[137] This selection enabled the use of the same column for both ESI^+ and ESI^- modes, thereby simplifying the overall workflow. Alternative columns evaluated for ESI^+ mode - specifically Zorbax Eclipse XDB, Kinetex PFP, Accucore PhenylHexyl, and Ultracarb 5 µm ODS - exhibited reduced separation efficiencies and poor peak shapes.

Composition of Eluent B

As already described in the previous chapter 4.1.1, the organic component in the eluent contributes to both ionisation efficiency and chromatographic performance. Therefore, the composition of the organic phase was also investigated (Figure 4.6).

In ESI^- mode, sufficient signal intensities and chromatographic separation were already achieved using an eluent B composition of MeOH:ACN (50:50 v:v). However, the composition of eluent B for ESI^+ mode still required further optimisation. Various ratios of acetonitrile (ACN) and methanol (MeOH) containing 0.1 % formic acid were investigated for eluent B in ESI^+ mode. Figure 4.6 shows a representative section of the chromatogram ranging from t_R 8-12 min.

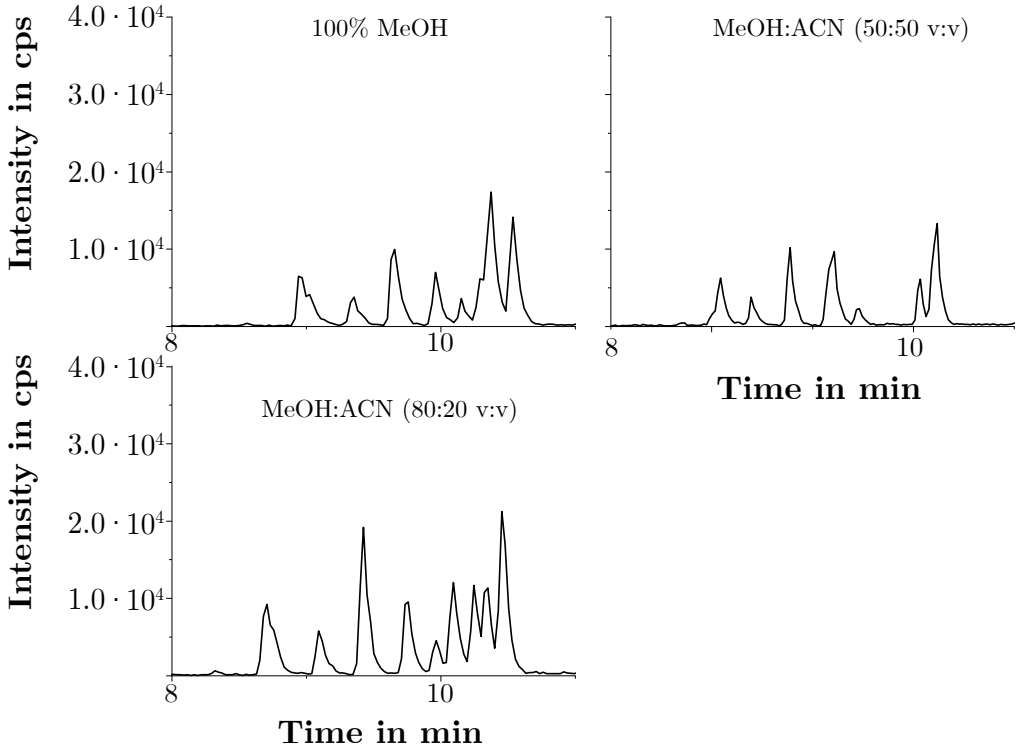

Figure 4.6: Comparison of different compositions of eluent B for the separation of components analysed in ESI^+ mode displayed as TIC. 0.1 % formic acid was added to each solvent. Eluent A used consisted of water + 0.1 % formic acid.

Considering the chromatographic separation, an increasing proportion of MeOH led to improved separation of the androgens and progestogens (Figure 4.6). ACN is less polar than MeOH and therefore exhibits a higher elution strength, resulting in earlier elution of the analytes under RP conditions. However, the specific properties of MeOH enhance the chromatographic selectivity for the compounds. MeOH is a protic solvent capable of forming hydrogen bonds. This increases the viscosity of the mobile phase and simultaneously reduces the efficiency of ion transfer into the gas phase. A small proportion of ACN, on the other hand, was found to increase the overall ESI response (see Figure 4.7). Accordingly, a mixture of MeOH:ACN (80:20 v:v) was used for the final method.

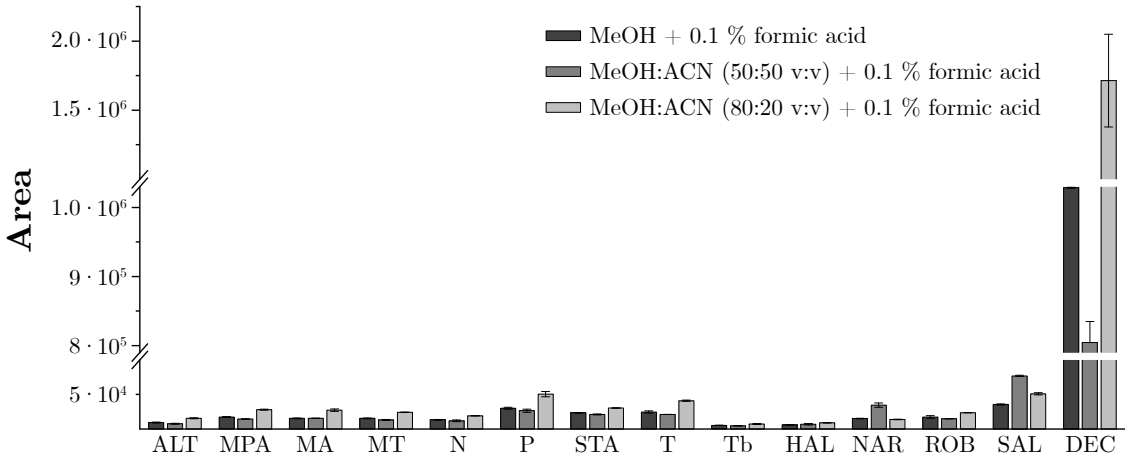

Figure 4.7: Comparison of different compositions of Eluent B regarding the ionisation efficiency of the components analysed in ESI^+ mode displayed as total ion chromatogram (TIC). 0.1 % formic acid was added to each solvent. Eluent A: H_2O with 0.1% formic acid. Abbreviations can be found under section 4.1.

Gradient and Flow Rate Optimisation

Optimally selected quantifiers and qualifiers provide reliable information for identifying different components. Therefore, baseline separation of the analytes is not mandatory. Nevertheless, the coelution of several substances can lead to matrix effects such as ion enhancement or ion suppression. This may result in reduced sensitivity, particularly when analysing dust samples.

In addition to separating analytes from matrix components structural isomers such as α- and β-estradiol, must be separated as well, as they share the same m/z transitions and retention time (t_R), which serves as an additional identification criterion. The starting point for gradient optimisation was based on preliminary work carried out by the working group. For the positive mode, a flow rate of

500 µL/min could be maintained from the preliminary work. After optimising the eluent and buffer compositions, the gradient required only minor adjustments to ensure sufficient separation of the hormones and coccidiostats. Figure 4.8 shows the final gradient and the resulting TIC of a solvent standard for ESI^+ mode.

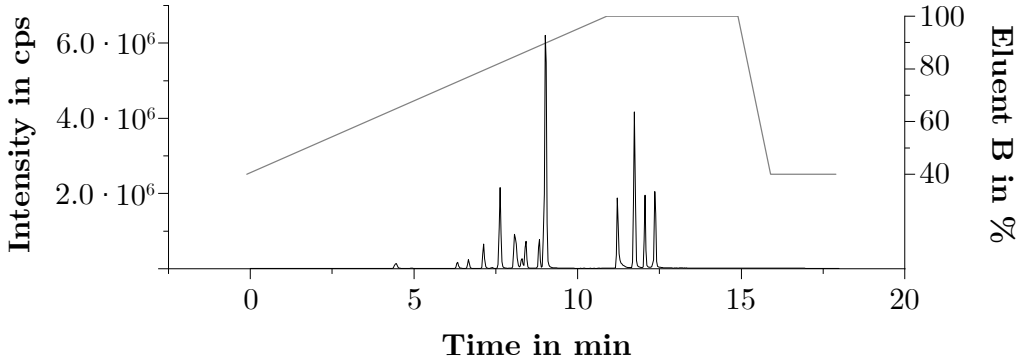

Figure 4.8: Optimised gradient and resulting TIC in ESI^+ mode. Eluent B: MeOH:ACN (80:20 v:v) with 0.1% formic acid; Eluent A: H_2O with 0.1% formic acid. Abbreviations can be found under section 4.1.

The starting conditions were set to 40 % of eluent B in order to dissolve the samples in the highest possible organic content after the extraction process, taking into account the solubility of the analytes. In addition, the first analyte (Halofuginone) only elutes above 40 % of eluent B, allowing for savings in both analysis time and solvent consumption.

For the ESI$^-$ mode, the eluents from the preliminary work could be retained (eluent A: H_2O with aqueous NH_3, pH 10; eluent B: MeOH:ACN (50:50 v:v)). Accordingly, a direct comparison can be made between the initial gradient from the preliminary work and the finalised method (see Figure 4.9).

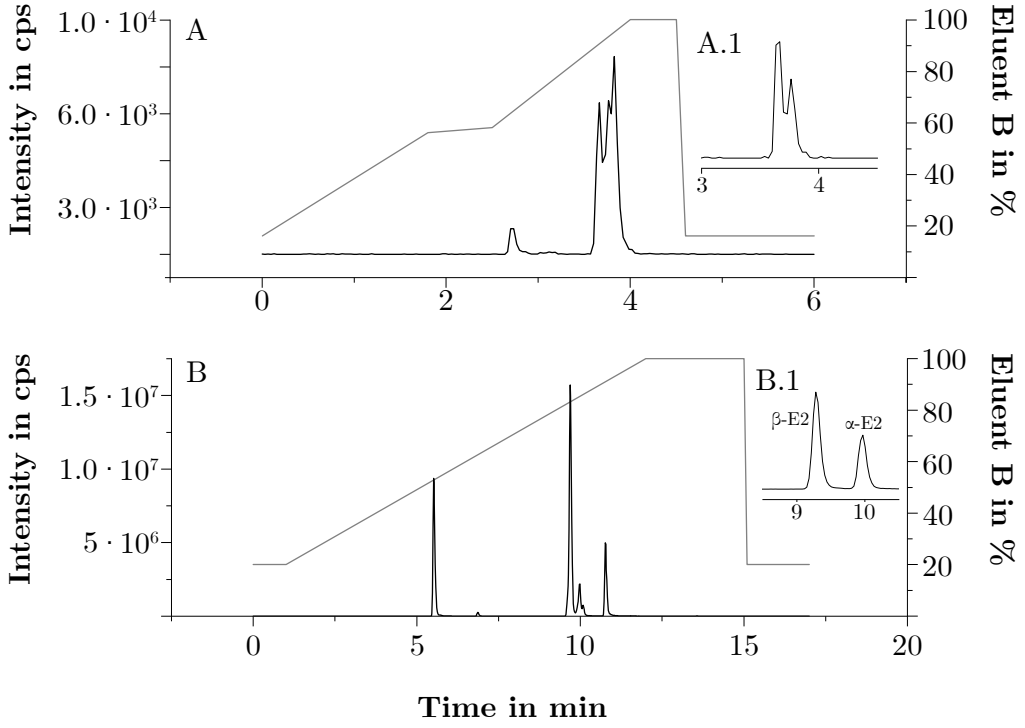

Figure 4.9: Optimisation of the gradient in ESI$^-$ mode. Chromatogram A shows the TIC obtained using the preliminary gradient. A.1 depicts the XIC only for α- and β-estradiol. Chromatograms B and B.1 show the corresponding TIC and XIC using the optimised gradient.

For the optimisation of the gradient in ESI$^-$ mode, the flow rate was initially reduced from 0.8 mL/min to 0.3 mL/min. The aim was to ensure that the flow rate was not too low, avoiding dominance of longitudinal diffusion, while remaining low enough to allow sufficient interaction time between the analytes and the stationary phase, thereby improving resolution.

In addition, the gradient was adjusted and a longer rinsing step was integrated to minimise potential carryover. Using the optimised gradient it was possible to achieve a baseline separation of α- and β-estradiol with the latter eluting first.

Column Oven Temperature

The use of a column oven not only improves the robustness of the method, but also influences both physical parameters, such as the viscosity of the mobile phase, and chemical parameters, such as reaction enthalpy. To investigate the effect of the oven temperature on the compounds analysed in this study, retention times and peak shapes were evaluated at three different temperatures (15 °C, 20 °C, and 30 °C). Using ESI^+ mode as an example, Figure 4.10 shows the corresponding TICs at 15 °C, 20 °C, and 30 °C.

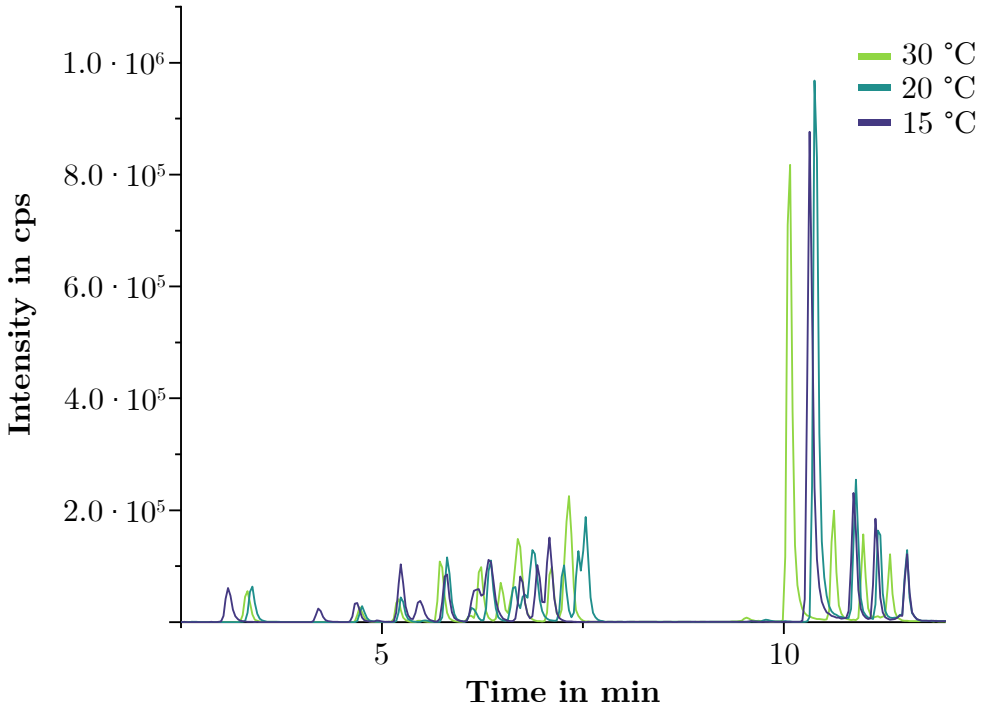

Figure 4.10: Optimisation of column oven temperature (at 15 °C, 20 °C, and 30 °C) exemplarily shown using the resulting TICs in ESI^+ mode.

Considering the peaks eluting after 10 minutes (from left to right: DEC, MON, SAL, and NAR), it becomes evident that increasing the column temperature leads to shorter retention times and narrower peak widths. This is the same for all compounds that are analysed using the ESI^- mode. A retention reversal is obtained for halofuginone (the first peak in the TIC), as well as for robenidine and the group of androgens and progestogens (with t_R between 4.7 and 7 minutes). This phenomenon can be explained by the van't Hoff equation, shown in Equation 4.1.

$$\ln k = \frac{\Delta H^\circ}{R \cdot T} + \frac{\Delta S^\circ}{R} \tag{4.1}$$

Where:

- $\ln k$ = natural logarithm of the retention factor
- ΔH° = standard enthalpy of transfer
- ΔS° = standard entropy of transfer
- T = absolute temperature in Kelvin (K)
- R = universal gas constant

If ln k is plotted against 1/T, information about the retention mechanism can be derived from the slope and the intercept. Figure 4.11 shows an example of this plot for altrenogest and monensin.

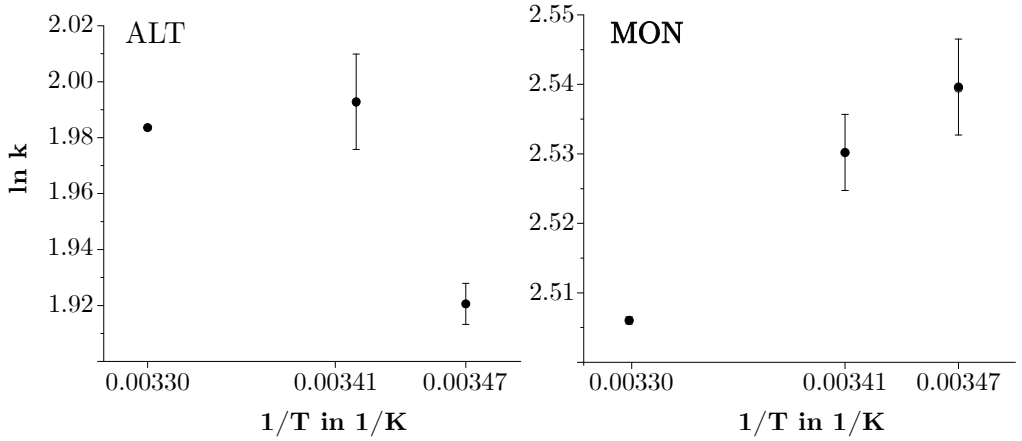

Figure 4.11: van't Hoff diagramm for altrenogest (ALT, left) and monensin (MON, right) with n = 2.

Since only three temperatures were investigated in this experiment, only an approximated trend can be estimated. Assuming that both curves shown in Figure 4.11 exhibit a linear relationship, the enthalpy of transfer (ΔH°) can be derived from the slope. In this case, ΔH° is positive for altrenogest and negative for monensin.

A negative ΔH° indicates that the transfer of the analyte to the stationary phase is exothermic and thus thermodynamically favoured. The more negative the value of ΔH°, the stronger the interaction between the analyte and the stationary phase, resulting in longer retention times. However, the relationship between ΔH° and the retention time is described by both terms of the van't Hoff equation – the

enthalpy and entropy term. Generally, a more negative ΔH° tends to result in longer retention times, provided that the entropy term ΔS° does not compensate this through positive contributions. The determined ΔH° values for each component are listed in table A.1 in the appendix. A linear van't Hoff plot also suggests that the retention mechanism remains constant over the examined temperature range.[138]

Analytes with negative ΔH° values (i.e. all compounds in ESI^- mode, as well as DEC, MON, NAR, and SAL) exhibited a clearer linear behaviour in the van't Hoff plot. In contrast, all other analytes displayed a pattern similar to altrenogest (see Figure 4.11). Non-linear curves indicate a shift in the retention mechanism. Possible causes include phase transitions within the stationary phase or changes in the properties of the mobile phase. This is particularly relevant when using gradient elution, as the mobile phase composition changes over time and the influence of temperature is therefore not constant throughout the run. As temperature increases, the hydrogen bonding network in water is altered, which in turn affects the entropy and ultimately the retention behaviour of the analytes.

Since the gradient ramps up to 100 % for eluent B starting at 11 minutes, this could partially explain the observed retention behaviour. However, additional temperatures should be investigated to enable a more accurate interpretation. Additionally, temperatures above 30 °C should be avoided, as they may reduce the lifespan of the column.

α- and β-Estradiol Differentiation

α- and β-estradiol are epimers. They differ only in the spatial orientation of the hydroxyl group at the C17 position (Figure 4.12).

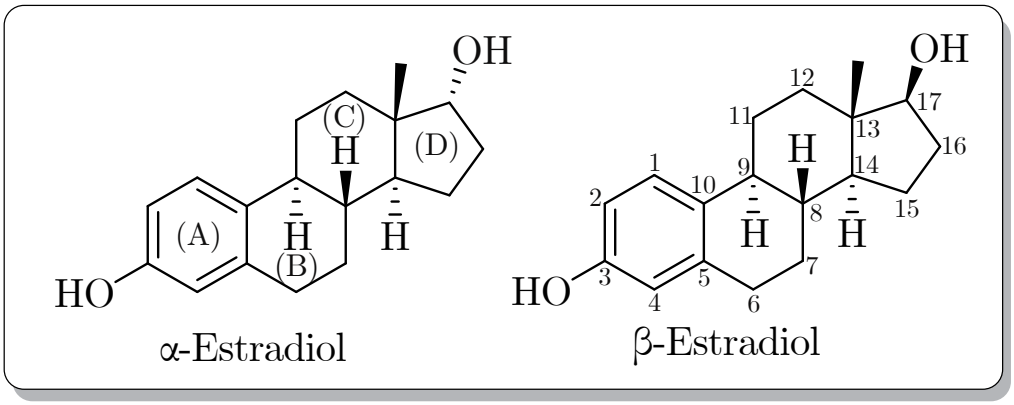

Figure 4.12: Structural formula of α (left)- and β (right)-Estradiol.

Using the optimised chromatographic gradient, α- and β-E2 could be baseline-separated. However, since they exhibit identical m/z transitions, it was desirable to integrate an additional identification criterion. Fragmentation pathways of α- and β-estradiol under CID conditions have already been described in detail in the literature and the proposed pathways are illustrated in Figure 4.13.[139]–[141]

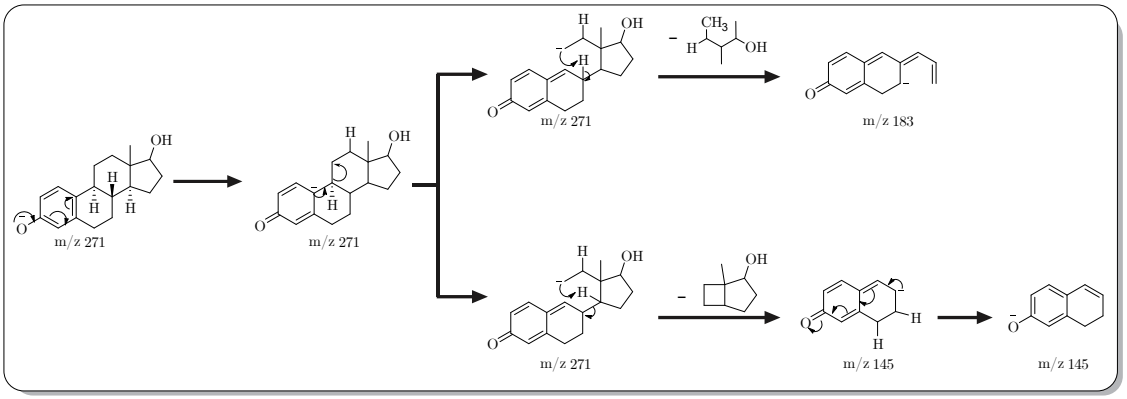

Figure 4.13: Fragmentation pathways of α- and β-estradiol according to the literature.[139]–[141]

Although α- and β-E2 exhibit the same m/z transitions, they differ in the relative intensities of the resulting fragment ions (see Figure 4.14).

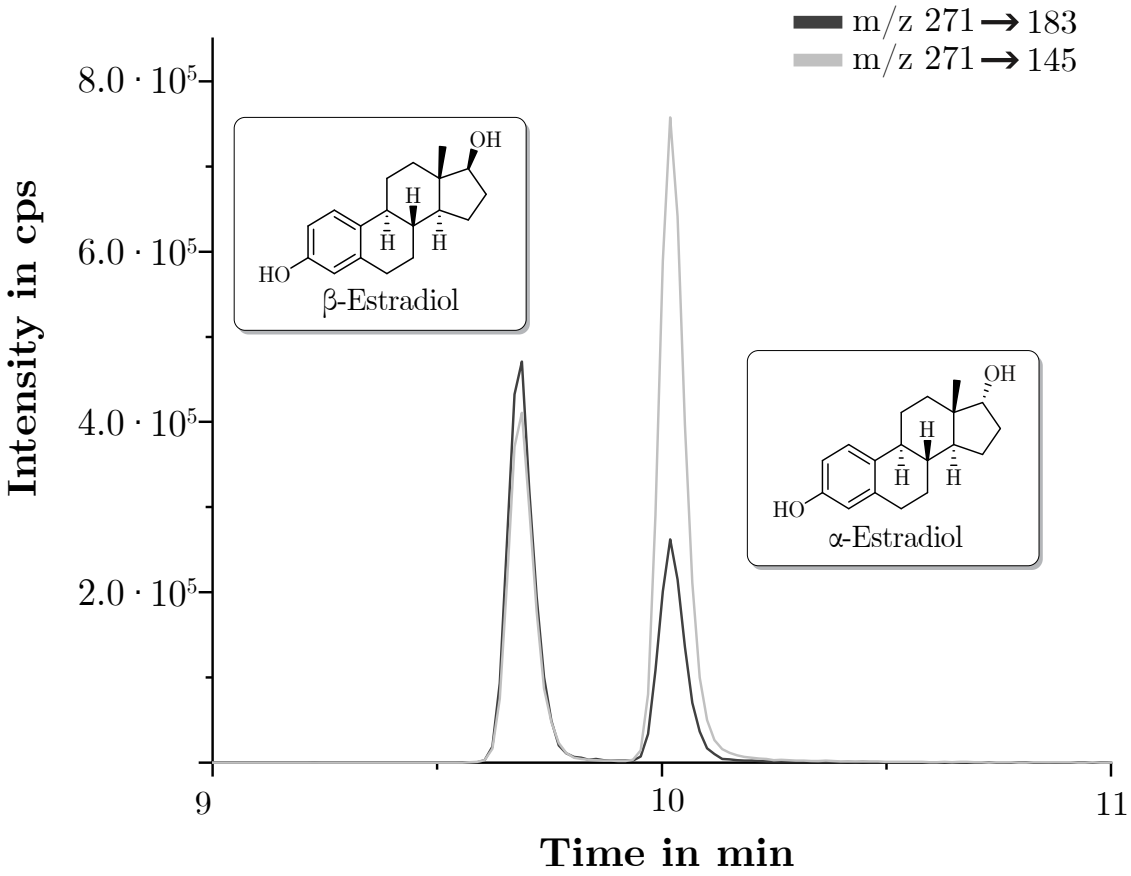

Figure 4.14: Chromatographic separation of α- and β-estradiol using he optimised method for the negative ionisation mode.

As can be seen in Figure 4.13, the formation of m/z 145 and 183 depends on whether the negative charge located at C11 targets the hydrogen at C14 or C8, respectively. Using a Python workflow based on RDKit for molecular structure processing and visualisation of atomic geometries, the spatial relationships between partial charges and hydrogen atoms involved in the fragmentation pathways were analysed. Partial atomic charges were calculated according to the AM1-BCC method using the Open Force Field Toolkit to support the mechanistic interpretation of the observed fragmentation patterns. The results suggested that the attack of the charge on the hydrogen located at C14 is less sterically impeded in α-estradiol resulting in preferred formation of the fragment m/z 145. For β-estradiol both sites appeared to be equally favoured. For more substantiated conclusions, the mechanisms and kinetics of these fragmentations must be investigated in more detail in further studies. For this work, however, it was important that the fragment ion ratios of 0.36 and 0.99 can be used as a further identification criterion for α- and β-estradiol, respectively.

4.2 Characterisation of Stable Dust

The following chapters address the optimisation of an appropriate extraction method for the steroid hormones and coccidiostats investigated herein. The objective is to remove as much matrix material as possible while simultaneously recovering the maximum analyte yield, resulting in reduced matrix effects and increased method sensitivity. To establish the requirements that the extraction method need to satisfy, the dust matrix will first be characterised in detail. The JLU institutional dust archive contains dust samples collected via a standardised sedimentation procedure (further information provided in Chapter 5.3.1). These samples span from the 1980s to the early 2000s and comprise dust from fattening pigs (FP)-, broiler chicken (BC)-, and leying hens (LH)- facilities. For some of these archived dust samples, results from Weender analysis are available obtained in the course of previous studies.[88] This conventional analysis is typically employed in feed analysis and serves for the quantitative determination of dry matter, crude protein, crude fat, crude fibre, crude ash, and Nitrogen-free extract. Dry matter encompasses all non-volatile constituents. It is important to note that volatile components consist not only of water, since dry matter is determined by simple drying of the sample, thereby removing all volatile constituents. Crude ash comprises all non-combustible components of the dust, such as minerals or sand. Crude protein content is typically determined via the Kjeldahl method.[142] However, this procedure quantifies not only protein Nitrogen but also Nitrogen from vitamins and nucleic acids and chitin from insects living in the stable.[143] Crude fat content can be determined using the Weibull–Stoldt method, which quantifies triglycerides and other similarly soluble substances such as phospholipids, waxes, and carotenoids.[144] The term crude fibre encompasses indigestible substances such as plant structural components, whilst Nitrogen-free extract primarily captures soluble carbohydrates such as starch.[143] Because no parameter measures a pure substance class, the designation "crude" is necessary. Using these results, it shall be determined how the dust samples are composed and whether significant differences in composition exist based on animal species, housing system, and farm location.

The null hypothesis (H_0) for the statistical analyses states that no difference exists in dust composition with respect to the Weender analysis parameters as a function of animal species, housing system, and farm location. To test this hypothesis, the residuals of the data used must first be evaluated for normal distribution. Figure 4.15 presents the Q–Q plots prepared for this purpose.

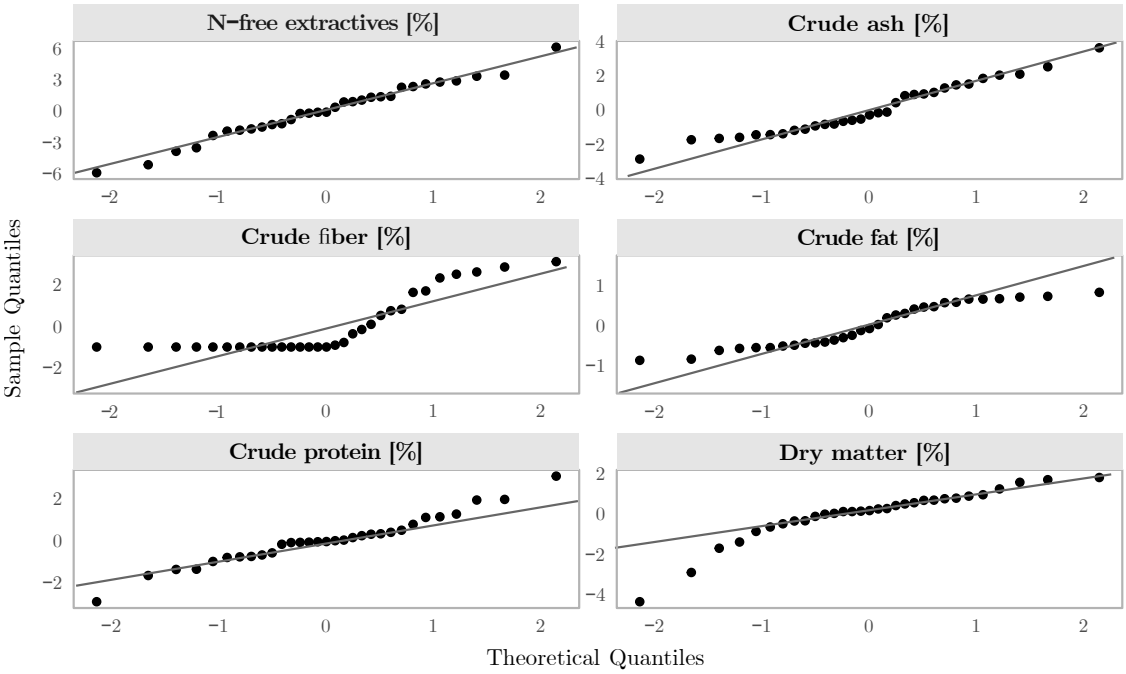

Figure 4.15: Normal distribution assessment of Weender analysis parameters via Q–Q plot analysis. Results exemplarily shown for group FP, housing system 1, location O1 with n = 31.

As shown in Figure 4.15, the crude fibre and crude fat parameters exhibited non-normal distributions, attributable to the fact that many samples yielded a value of zero for the respective content. Because the sample size was generally very small (eight to 90 samples), normal distribution was ultimately assumed for all parameters based on the central limit theorem.[145]

Since sample sizes differed between BC, LH, and FP groups, both fixed and random factors required consideration, and some results yielded a value of zero; a mixed model approach was selected to test the null hypothesis. It must be reiterated here that the overall sample size was very small and that mixed models possess only limited robustness under such conditions. The procedure for mixed model calculation and the R code employed are presented in section 5.3.2. Figure 4.16 shows the boxplots of the residual analysis from a mixed model that incorporated animal species as a fixed effect and location as a random effect.

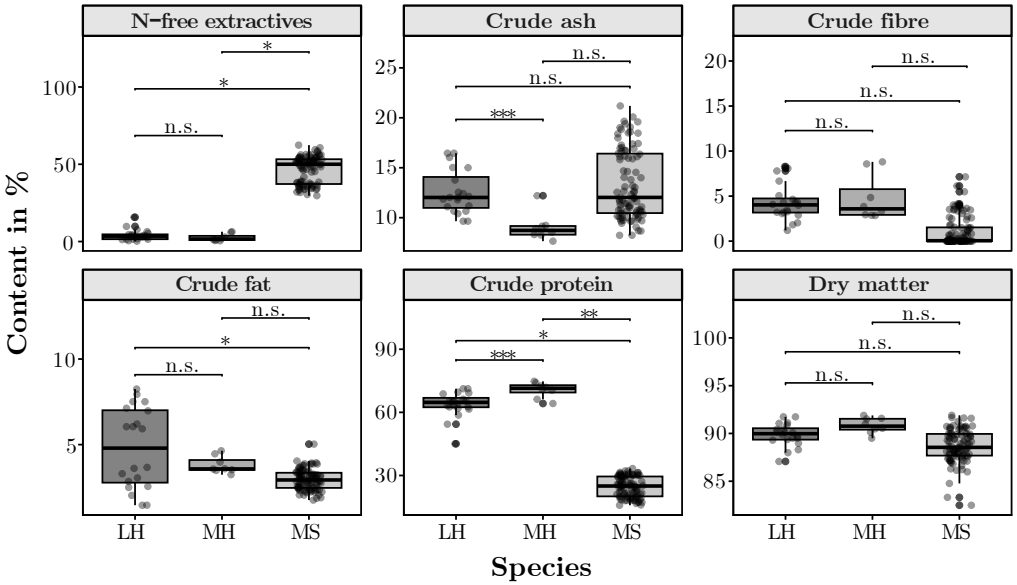

Figure 4.16: Comparison of Weender analysis parameter by animal species (broiler chicken (BC; n = 8), laying hens (LH, n = 20), and fattening pigs (FP, n = 90)) With * p = 0.05; ** p = 0.01; *** p = 0.001 and n.s. = not significant. For detailed information see section 5.3.2.

The results of Weender analysis indicate that dust samples from both poultry and fattening pig facilities comprise over 80 % organic material and do not differ significantly in this respect. This organic material consists primarily of feed components, litter, faeces, and animal constituents (such as feathers, skin scales, or hoof material), whilst the inorganic fraction additionally includes wear particles from walls and other stable surfaces.[89] Dust composition depends on temperature, humidity, movement of the animals, and ventilation, and thus varies with animal species, animal activity, animal age, housing system, and stocking density. Additional factors include farm hygiene management and the ventilation system, whereby particles from the environment can enter the stable.[88], [146], [147]

As shown in Figure 4.16, dust samples from poultry facilities (BC and LH) differ significantly in protein content from FP samples. While protein contents of poultry samples ranged from 60–70 %, FP dust samples exhibited protein contents of approximately 20–30 %. A possible explanation is the difference in feed protein content. Whilst poultry feed typically contains 20–25 % protein, fattening pig feed contains approximately 12–15 %.[148], [149] An additional explanation is the high proportion of feathers in the dust sample. Direct visual examination of dust samples reveals that fattening pig facility dust comprises an almost homogeneous particle distribution, whilst poultry facility dust exhibits an almost wool-like character. Because feathers consist predominantly of keratin, they contribute to an elevated

protein content. Nitrogen-free extract also showed significant differences. However, as these values are calculated taking into account the protein content, the results in this case represent a logical consequence.

In Figure 4.16, Weender analysis results for each animal species are aggregated across farm locations and housing systems. To more precisely evaluate whether differences in Weender analysis results depend not only on animal species but also on location, a significance test was performed using fattening pig samples alone. Figure 4.17 presents Weender analysis results for animal species FP alone, stratified by location (O1–O4). For statistical evaluation, a mixed model was again employed, with location as a fixed effect and sampling year as a random effect. Significant differences between locations were determined via a post-hoc test using Bonferroni correction and are indicated in the figure by asterisks. Further information can be obtained in section 5.3.2.

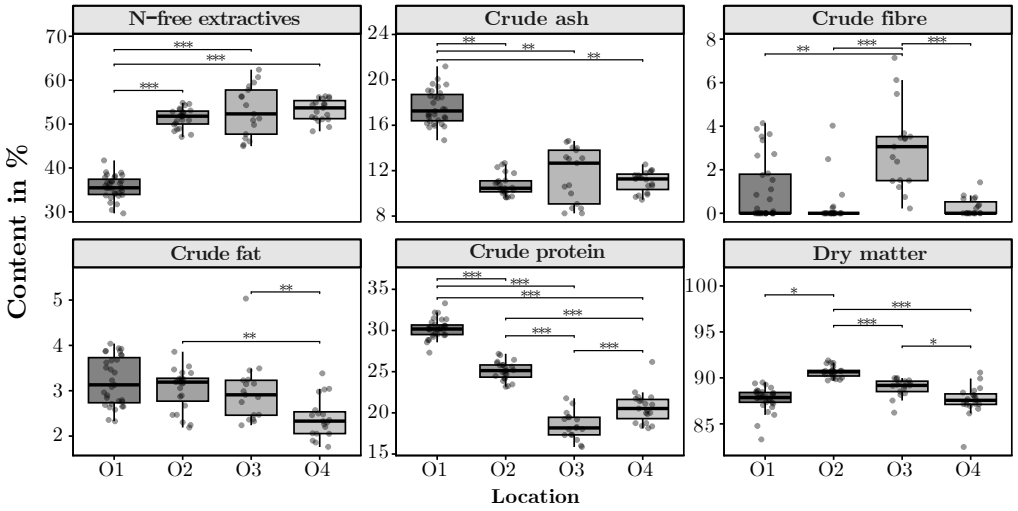

Figure 4.17: Comparison of Weender analysis parameter by farm locations (OX). With n = 31 (O1), n = 23 (O2), n = 17 (O3) and n = 19 (O4). * p = 0.05; ** p = 0.01; *** p = 0.001 and n.s. = not significant. Further information can be found in section 5.3.2

Regarding the results shown in Figure 4.17 it must be noted that animals at farm locations O1 and O2 were housed in the "finishing system", where a concrete floor is assumed whilst animals at locations O3 and O4 were housed "with straw bedding". Due to strong confounding, housing system was not included as a random factor in the model. Consequently, significant differences between O1 and O2 versus O3 and O4 cannot be unambiguously attributed to either location or housing system. Nevertheless, significant differences in dust sample composition are also evident within a single housing system across different locations.

In summary, dust matrix characterisation has demonstrated that the matrix "dust" is not only extremely complex but also highly variable in its individual

composition. Consequently, the requirements for an appropriate and, most importantly, robust extraction method are high and matrix effects should be considered during method validation.

4.3 Extraction

The results of the Weender analysis show the complexity and variability inherent in the composition of stable dust samples. To enable reliable quantitative determination of analytes in this matrix at trace analytical concentrations, an efficient extraction procedure is required. During method development, a liquid-solid extraction (LSE) approach was first developed and optimised to achieve satisfactory recoveries for the analytes. Subsequently, an additional clean-up step was established to further mitigate matrix effects while maintaining sufficiently high recoveries. For method development and optimisation, a pooled dust sample was used (see Section 5.3.3 for detailed information).

4.3.1 Significance of Extraction Parameters for Liquid-Solid Extraction

Numerous extraction methods exist for steroid hormones in matrices such as faeces, animal products, and tissues.[150]–[153] QuEChERS protocols, in particular, are widely used for biological matrices.[154]–[156] For stable dust, only a handful of publications are available, mostly concerning selected steroids or antibiotics.[157], [158] The situation for coccidiostats is similar.[159]–[161]

Based on the Weender analysis results, literature data, and preliminary experiments, a simple LSE was chosen as extraction method. The choice of extraction solvent was primarily based on the chemical properties of both the analytes and the stable dust. As shown in Figure 4.16, the stable dust samples exhibit a high crude protein content. Microbial and enzymatic activity in the dust can convert proteins to urea, then to ammonia, raising the pH of the dust. Therefore, a pH of approximately 7–9 was assumed for the dust.[88], [162] Steroids possess a nonpolar sterane backbone and, at pH 8, log P values greater than 3 (see Table 4.2). DEC, with a log P value of 6.87 at pH 8, is likewise strongly nonpolar. To achieve a quantitative extraction, n-hexane was selected as one of the extraction solvents. As the crude fat content of the samples was below 10 %, co-extraction was accepted at this stage and intended to be monitored and considered in the assessment and mitigation of matrix effects. Oestrogens such as E3S and E3 contain polar groups resulting in log P values less than 3. Coccidiostats are present in charged form

at pH 7–9, which increases their polarity (log P <3, see Table 4.2). Methanol was therefore chosen as a further extraction component to enable the extraction of more polar substances. Critical parameters of such a method are the solvent composition and volume, pH value, extraction time, and the number of extraction cycles. A statistical screening using a fractional factorial design (see Chapter 5.4.5 for practical implementation) of resolution V was performed to determine which of these parameters has a significant influence on extraction efficiency. The resolution characterises the degree to which estimated effects of different orders (main effects and factor interactions) are aliased with one another, and thus how clearly individual parameters can be distinguished. A high-resolution design (IV-VIII and full) ensures that a larger set of the investigated parameters and low-order interactions can be estimated independently, whereas low-resolution designs (I-III) imply increased confounding and hence limit the interpretability of parameter estimates. The response function was the (recovery (REC)) according to Equation 4.2.

$$REC = \frac{\bar{A}_{\text{pre}} - \bar{A}_{\text{blank}}}{\bar{A}_{\text{post}} - \bar{A}_{\text{blank}}} \cdot 100\% \tag{4.2}$$

Where:

- REC = Recovery in %
- $\bar{A}_{\text{pre}}$ = Peak area in the matrix spiked before extraction
- $\bar{A}_{\text{post}}$ = Peak area in the matrix spiked after extraction
- $\bar{A}_{\text{blank}}$ = Peak area in the matrix blank

Thus, for each experiment, three aliquots of the pooled sample were processed, with one spiked before extraction, one after extraction with 100 ng each, and one left unspiked. The spiked samples were homogenised, the solvent was evaporated, and the spiked samples were stored for 12 h at room temperature protected from light. The precise experimental plan is given in Section 5.4.5. Table 4.5 lists the natural variables at the two levels used.

The experimental design is designated as a 2^{5-1} design, meaning each of the five parameters was investigated at two levels. Furthermore, each main effect is confounded with a four-factor interaction and two-factor interactions are confounded with three-factor interactions. As a result, the number of experiments is reduced from 32 (full factorial design) to 16 (half fraction factorial design). Both the extraction experiments and LC–MS/MS measurements were conducted in randomised order to avoid systematic error. Significance was determined via ANOVA with a significance

Table 4.5: Coding of experimental conditions for the design of experiment(DoE) screening experiments representing the high (+1) and low levels(-1). The solvent composition consists of n-hexane and methanol. The given n-hexane content represents the respective methanol content inversely.

Factor	**+1**	**-1**
Solvent Composition (n-Hexane) [%]	100	20
Solvent Volume [mL]	5	10
Number of Cycles	1	5
Extraction Time [min]	10	60
Acid Concentration [%]	0	0.1

threshold of $p < 0.05$. Figure 4.18 shows which of the tested parameters have a significant effect on the recovery of individual analytes. The full experimental design, including natural variable settings, recoveries, and further information can be found in Section 5.4.5 and recoveries for each sreening experiment and analyte can be found in Section A.3.

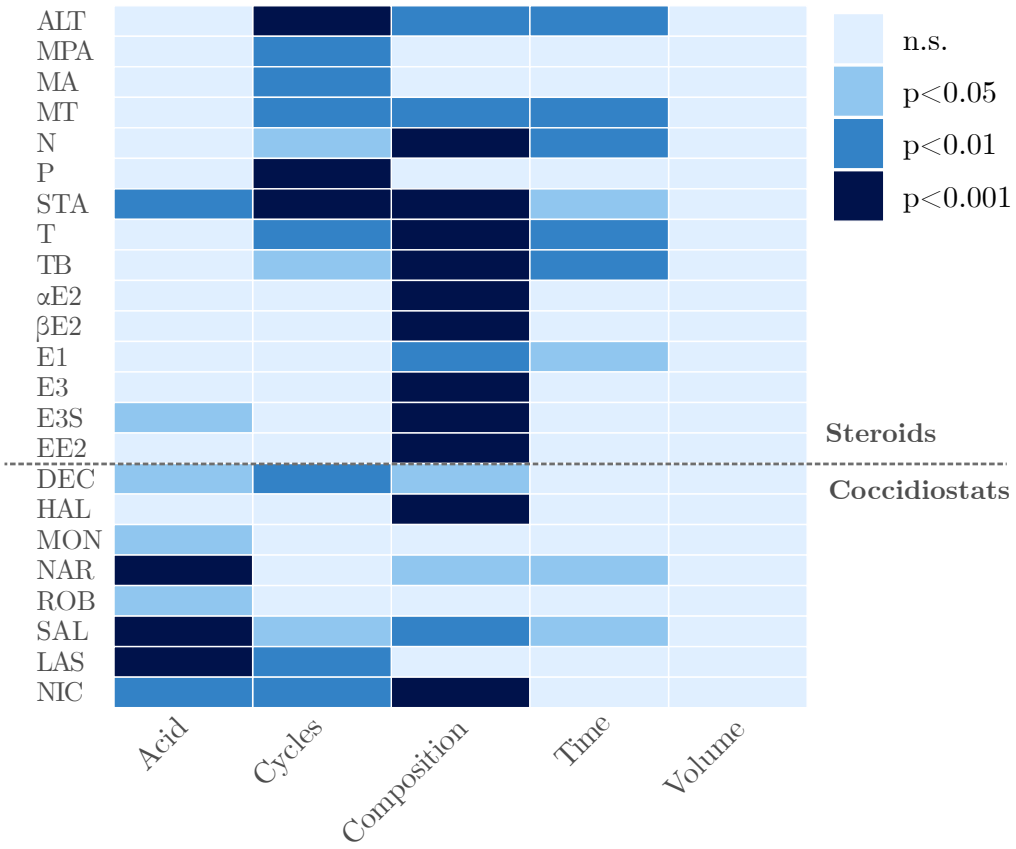

Figure 4.18: Screening of extraction parameter significance. With ns = not significant. Abbreviations can be found under section 4.1.

As shown in Figure 4.18, solvent composition is a significant extraction parameter for almost all analytes. In contrast, the extraction solvent volume does not have a significant influence for any analyte. Interestingly, formic acid has a significant impact only on the extraction of coccidiostats and two steroid hormones (STA and E3S). The reason is the pK_a values of these substances: as shown in Table 4.2, the pK_a values of those compounds not significantly affected by the acid are above 10. These therefore remain in neutral form with or without acid and their extraction behaviour remains unchanged.

The screening results were used to reduce the number of method parameters by excluding those with no significant effect, thereby reducing the number of experiments required for the full factorial design used in the subsequent optimisation. In the next phase of extraction method development, solvent volume will thus no longer be included as a variable and is fixed at 5 mL. The parameters extraction time, number of cycles, solvent composition, and acid concentration will be further optimised by response surface methodology and are discussed in the following sections.

4.3.2 Optimisation of Extraction Parameters Using a Central Composite Design

The central composite design (CCD) is used to optimise the factors (k) that have already shown a significant influence on the extraction of analytes from stable dust in the screening process. The CCD is based on a quadratic model function. Using this model, the dependence of the target variable (recovery) on the influencing factors (solvent composition, number of cycles, extraction time, and acid concentration, k = 4) can be described quantitatively and the optima can be calculated. The experimental design of the CCD used here, employing natural variables, is listed in Table 5.11. In principle, it consists of a full factorial design ($2^k = 16$ experiments) to estimate main effects and two-factor interactions, axial points ($2k = 8$ experiments) to estimate the quadratic terms, and eight central points to estimate the pure error, which in total results in 32 experiments. The axial points were set at $\alpha = \pm 2$. Table 4.6 shows the coding of the factors. The exact calculation of α is described in detail in Section 5.4.6. As before, both the extraction experiments and the LC–MS/MS measurements were carried out in a randomised order. Further details on the calculation of the statistical models are provided in Section 5.4.6.

Resulting recoveries and matrix effects of all analytes for each experiment is listed in Section A.4. First, a full response surface model (RSM) including all linear, quadratic, and two-factor interaction terms for all four factors was fitted. Evaluation of the quadratic RSM revealed an optimum for the factor acid concentration at

Table 4.6: Coding of experimental conditions for the CCD. The solvent composition consists of n-hexane and methanol. The given n-hexane content represents the respective methanol content inversely.

Factor	**+2**	**+1**	**0**	**-1**	**-2**
Solvent Composition (n-Hexane) [%]	100	80	60	40	20
Number of Cycles	5	4	3	2	1
Extraction Time [min]	60	47.5	35	22.5	10
Acid Concentration [%]	0.1	0.075	0.05	0.025	0

the boundary of the experimental domain. This indicates that the stationary point of the RSM for this factor lies outside the investigated factor space and that the function therefore exhibits a monotonic trend. Figure 4.19 shows the relationship between the natural variables used for acid concentration and recovery, exemplarily illustrated for altrenogest.

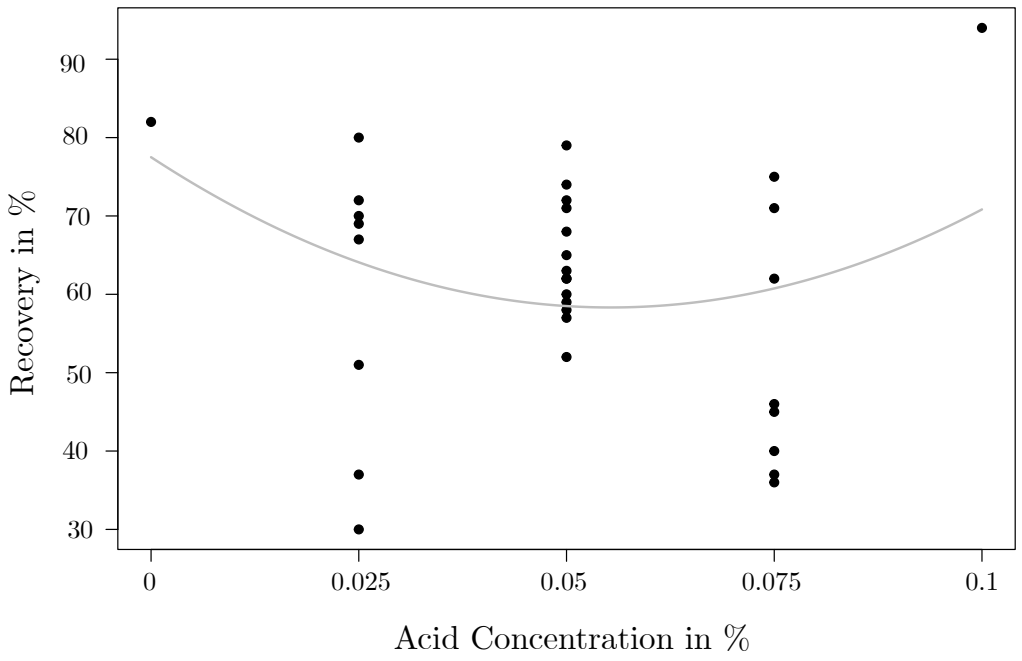

Figure 4.19: Acid concentration and obtained recovery for altrenogest resulting from the DoE optimisation experiments (see section 5.4.6 for further information).

Taking into account the pK_a values of the analytes (see Table 4.2), this trend appears plausible, since, particularly for steroid hormones, only strongly alkaline or strongly acidic conditions markedly affect protonation/deprotonation and thus alter extraction behaviour. As the linear effect of the factor dominated and the quadratic term was significant for only four analytes ($p > 0.05$), the factor was

fixed at the boundary value $-\alpha = 0\,\%$ for reasons of practicality and cost and was excluded from further modelling. Furthermore, none of the two-factor interactions showed a significant effect, suggesting that the factors act independently and that no synergistic or antagonistic effects occur. Consequently, the two-factor interaction terms were removed from the model to reduce its complexity. The reduced quadratic model was applied to the remaining factors to determine the optimum of each factor for every analyte. Figure 4.20 shows, exemplarily for altrenogest, the graphical representation of the response surface.

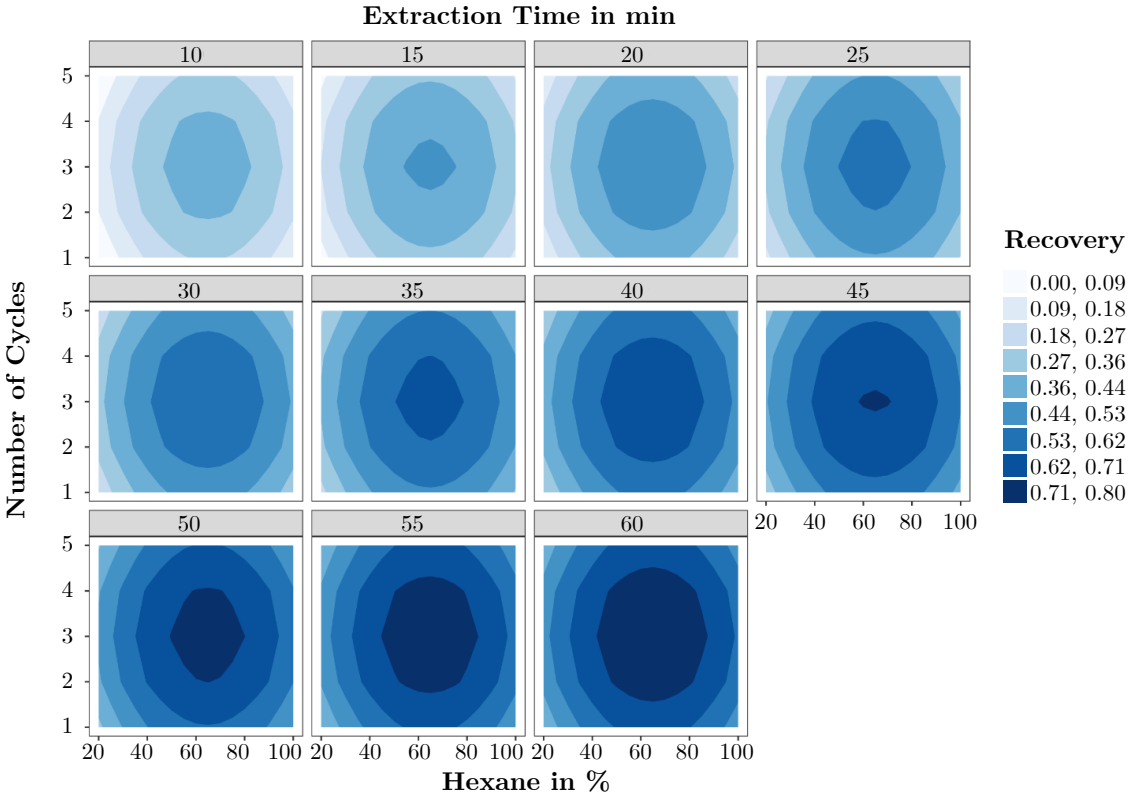

Figure 4.20: Response surface for altrenogest using varying factors: extraction time, number of cycles and n-hexane content. See section 5.4.6 for further information.

For the best possible recovery of altrenogest from dust samples, the RSM indicates optimal factor settings of three extraction cycles with 60 min extraction time each and a solvent composition of 64 % n-hexane and 36 % methanol. The optima of the RSM functions for the respective analytes were determined analogously and are summarised in Table 4.7.

Table 4.7: Optimised variables for LSE. methyltestrosterone (MT) was included as it was originally planed to be used as an internal standard.

Analyte	n-Hexane in %	Number of Cycles	Extraction Time in min
ALT	65	3	77
DEC	100	3	33
HAL	57	4	84
MPA	65	3	71
MA	78	4	41
MT	65	3	58
MON	76	3	34
N	64	3	87
NAR	82	3	30
P	66	2	100
ROB	62	3	36
SAL	100	2	29
STA	64	2	100
T	63	3	93
Tb	63	3	56
αE2	77	4	48
βE2	78	5	61
E3	85	1	52
E1	89	5	55
E3S	48	3	10
EE2	24	2	39
LAS	20	1	10
NIC (DNC)	53	5	68
Median	**65**	**3**	**56**

As the method developed here covers 23 analytes from different substance classes, the extraction parameters cannot be optimised individually for each analyte. To obtain conditions that are suitable for the majority of analytes, the median across all factor optima was used. For the final method, three extraction cycles of 60 min each (rounded up for practical reasons) with 65 % n-hexane and 35 % methanol were selected. At a mixing ratio of 65:35 (v:v) n-hexane:methanol, two phases are formed, which allows the simultaneous, selective extraction of both polar and nonpolar substances.[163] Using a rotary shaker at 8 rotations per minute generates an emulsion and local concentration maxima of each phase. Due to continuous agitation, dust compartments come into contact with both the nonpolar n-hexane phase and the polar methanol phase.[164], [165] A further advantage of the emulsion is the enlarged contact area between dust compartments and solvent, may increasing

extraction efficiency by providing a larger interaction surface. The optimised values were selected using recovery as the response. The aim was to maximise recovery and thus determine the local optimum of the RSM function. After exclusion of the factor acid concentration and all two-factor interactions, the model includes only the main effects of the factors extraction time, number of cycles and solvent composition, together with their quadratic terms (see Equation 4.3).

$$REC_{Analyte} = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_1^2 + \beta_5 x_2^2 + \beta_6 x_3^2 + \epsilon \qquad (4.3)$$

Where:

- $REC_{Analyte}$ = Recovery for the respective analyte
- β_0 = Intercept
- β_{1-6} = Regression coefficients
- x_{1-3} = Coded variables
- ϵ = Total error

By inserting the local optima in coded form into the response function, the maximum expected response, i.e. recovery, can be obtained for each analyte (see Figure 4.21). However, in addition to recovery, (matrix effect (ME)) are a crucial performance characteristic for the quality of an extraction method. Especially when using an ESI source, matrix effects are a decisive factor influencing method sensitivity. Therefore, matrix effects were also determined for each experiment of the full factorial design (see Equation 4.4).

$$ME = \frac{\bar{A}_{\text{post}} - \bar{A}_{\text{blank}}}{\bar{A}_{\text{Standard}}} \cdot 100\% \qquad (4.4)$$

Where:

- ME = Matrix effects in %
- $\bar{A}_{\text{post}}$ = Peak area in the matrix spiked after extraction
- $\bar{A}_{\text{blank}}$ = Peak area in the matrix blank
- $\bar{A}_{\text{Standard}}$ = Peak area in a solvent standard

Based on the ME results, regression coefficients for a function according to Equation 4.3 were likewise determined. The factor optima derived for recovery were then inserted into this model to estimate the matrix effects expected under the optimised extraction conditions. Recovery and the corresponding matrix effects are shown in Figure 4.21.

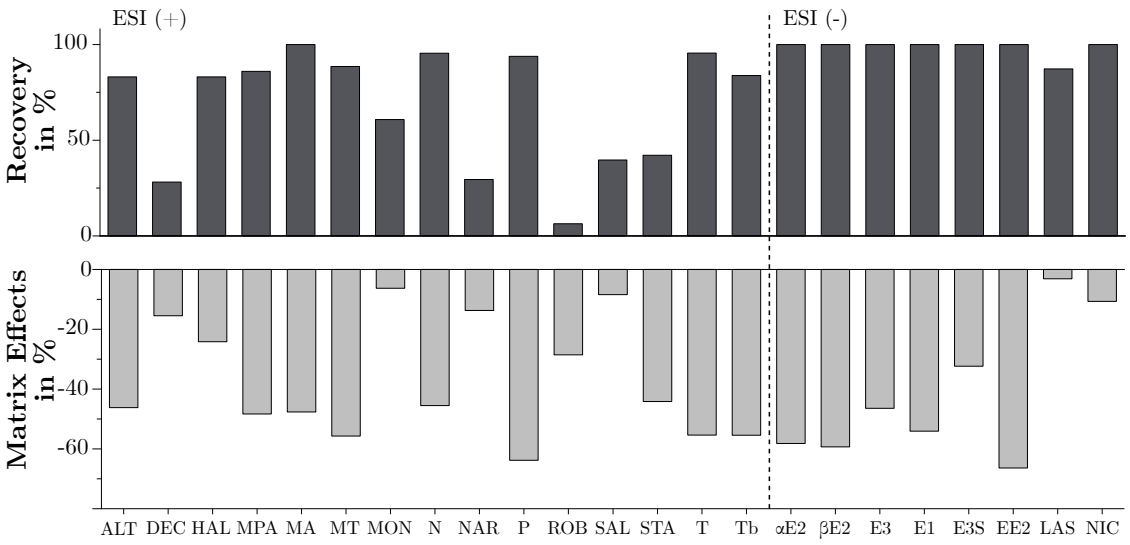

Figure 4.21: Recovery and matrix effects under optimised conditions. Recovery and matrix effects were calculated by inserting the optimised variables into the respective RSM functions. Abbreviations can be found under section 4.1.

As shown in Figure 4.21, using factor settings optimised solely with respect to maximum recovery would lead to ion suppression of up to 50 % for some analytes. Such pronounced matrix effects result in reduced sensitivity, measurement accuracy and precision, and may ultimately cause false-negative results in subsequent applications. Therefore, additional clean-up steps are required to reduce matrix effects.

4.3.3 Matrix Effects - Clean-up Methods

The term "matrix effect" encompasses all influences that cause a difference in analyte signal intensity between a solvent standard and the same analyte measured in a complex matrix such as a dust extract. Compounds originating from the sample matrices are referred to as endogenous, whereas contaminants such as polymers or salts, introduced via sample treatment or chromatographic separation, are considered exogenous.[166], [167] Therefore, it can be stated that the predominant reason for matrix effects is undesired compounds co-eluting with the target analyte during chromatographic separation, thereby altering the ionisation process. Matrix effects can generally be divided into ion suppression and ion enhancement. Ion enhancement is present when the analyte signal in the presence of matrix components is greater than in pure solvent. Possible causes include the presence of surfactants or other substances that decrease the surface tension of droplets formed during ESI, leading to more rapid evaporation and more efficient ion release.[168] In contrast, ion suppression describes the decrease in analyte signal due to matrix components compared with the solvent standard. Co-eluting compounds may compete with the

analyte for available charge and access to the droplet surface during the ionisation process, increase droplet viscosity and surface tension, or cause precipitation of analytes with non-volatile macromolecules. All these mechanisms reduce ionisation efficiency and result in lower analyte signal intensities.[167], [169], [170]

As revealed by the Weender analysis, stable dusts contain up to 70 % crude protein depending on animal species. Especially in ESI^+ mode, proteins, peptides, or amino acids are able to form positively charged ions and thus compete for charge, resulting in a higher degree of ion suppression.[171] Furthermore, 27 archived stable dust samples originating from pig fattening, broiler, and laying hen facilities were investigated at the Institute of Soil Science for quaternary alkylammonium compounds by Ines Mulder and Sophie Lennartz (Data not yet published). These compounds are used in disinfectants and cleaning agents for animal stables and thus have direct access to stable dusts.[172] In these studies, quaternary ammonium compounds were detected including alkyltrimethylammonium compounds (ATMAC)s, benzalkylammonium compounds (BAC)s, and dialkyldimethylammonium compounds (DADMAC)s (see Figure 4.22), with BACs C12-14 and DADMAC-C10 representing high production volume chemicals approved for the use in veterinary facilities.[173], [174]

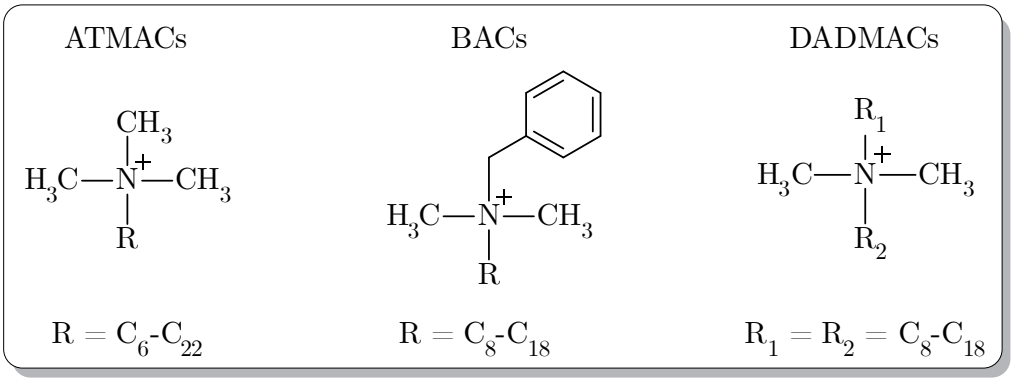

Figure 4.22: Structures of quaternary alkylammonium compounds.

Tang et al. describe the concentration dependence of analyte signals and charge competition effects. They refer to the ejection rate constant (k) from Equation 2.3 to describe the efficiency with which a specific ion enters the gas phase. Depending on the concentration, either surfactant (surface activity) effects or evaporation rate effects dominate.[169] Quaternary ammonium compounds have a high k value, as they are only weakly solvated and, due to their hydrophobic alkyl chains, surface-active. Consequently, they strongly compete for space at the droplet surface, suppressing the release of steroids and coccidiostats into the gas phase.

Thus, proteins, peptides, amino acids, quaternary ammonium compounds, and other matrix constituents synergistically lead to pronounced matrix effects (see Figure 4.21). To reduce these matrix effects and thus enhance method sensitivity, a clean-up step for stable dust extracts must be established.

Indications for the development of clean-up procedures for stable dust extracts were provided by studies of Blackwell et al., Moretti et al., as well as preliminary studies by F. Gummert, P. Michel, S. Lehr and recommendations from Hamm.[157], [175]–[179] Based on the preliminary studies, solid phase extraction (SPE) was selected for the clean-up of stable dust extracts. Taking into consideration both recovery and matrix effects, two normal phases (Florisil and SiOH), one reversed phase (HR-X), a combination of NP and RP (SiOH and HR-X), as well as a dispersive phase (combination of C18 and PSA), were compared. Detailed information on the methods and spiked concentrations can be found in Chapter 5.4.4. Each approach was analysed in duplicate. The resulting standard deviations were propagated according to Gaussian error propagation and used to calculate the error of recovery and matrix effects. Figure 4.23 depicts recoveries obtained from the various clean-up steps.

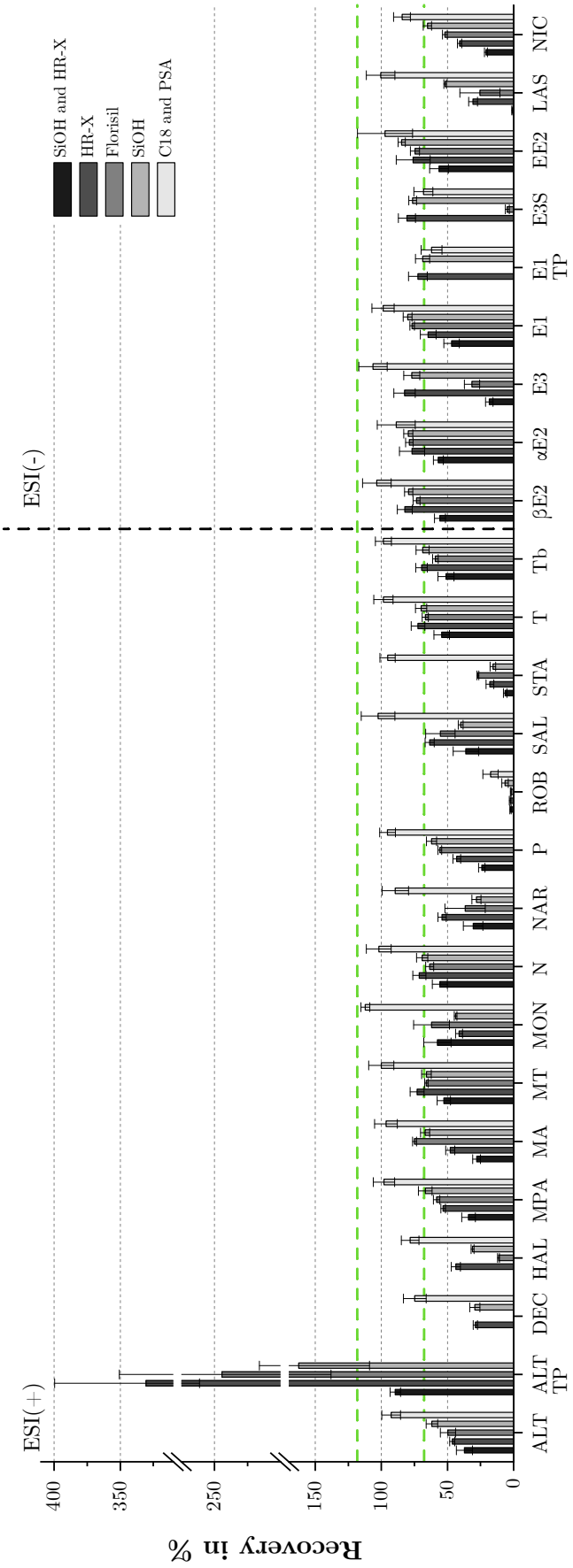

Figure 4.23: Comparison of recoveries for various SPE materials. (n = 2) Green lines indicate recoveries between 70 and 120 %. Abbreviations can be found under section 4.1.

SPE materials were utilised either as filters for the removal of matrix components (method using SiOH only) or for extraction of the analytes that were subsequently eluted from the material (all others). Since the analytes were dissolved in MeOH and n-hexane after LSE, the solvent needed to be evaporated for all methods except for the dSPE method, and the residue was reconstituted in a suitable solvent composition. This step was necessary to adjust the polarities for efficient extraction or filtration. As shown in Figure 4.23, the dSPE method yielded the highest recoveries (only ROB, E1 TP, and E3S REC $< 70\,\%$). The additional reconstitution step may be responsible, as it was not employed for dSPE. Depending on the solvent used for the reconstitution, analytes may not dissolve again, resulting in lower recoveries for other methods. For the combination of SiOH and HR-X materials, evaporation, and reconstitution were performed twice, which may lead to a loss of analyte due to incomplete dissolution. This resulted in the lowest recoveries and in case of HAL, DEC, E3S, and E1 TP even complete analyte loss. In this protocol both SiOH and HR-X were used for extraction and these analytes may not have been eluted from SiOH, due to their high polarity and strong interactions with silanol groups.[175] Additionally, these analytes (DEC, HAL, E3S and E1 TP) were only poorly extracted by HR-X. If SiOH was used as a filter for matrix separation only, recoveries were comparatively higher. Florisil (magnesium silicate), also a NP-SPE, was likewise used for analyte extraction, but yielded $>70\,\%$ recovery for only a few analytes.

Particularly notable were recoveries for ALT TP. This transformation product is formed by intramolecular [2+2] photocycloaddition from altrenogest (ALT CAP) under UV irradiation.[180] The longer ALT was exposed to UV, the more ALT TP was produced. As the SPE protocol exposed samples to UV longer than the dSPE protocol, the higher recoveries can be explained. E1 TP was a peak detected with the same m/z transitions as E1 but with an identical retention time as E3S. It was therefore assumed that this was E3S, which may had lost the sulphate group during the ionisation process. It was therefore additionally integrated into the method to enable correct quantification of E3S. The aim of the SPE/dSPE clean-up of stable dust extracts was to reduce matrix effects. The lowest median matrix effects were obtained using Florisil cartridges (see Figure 4.24).

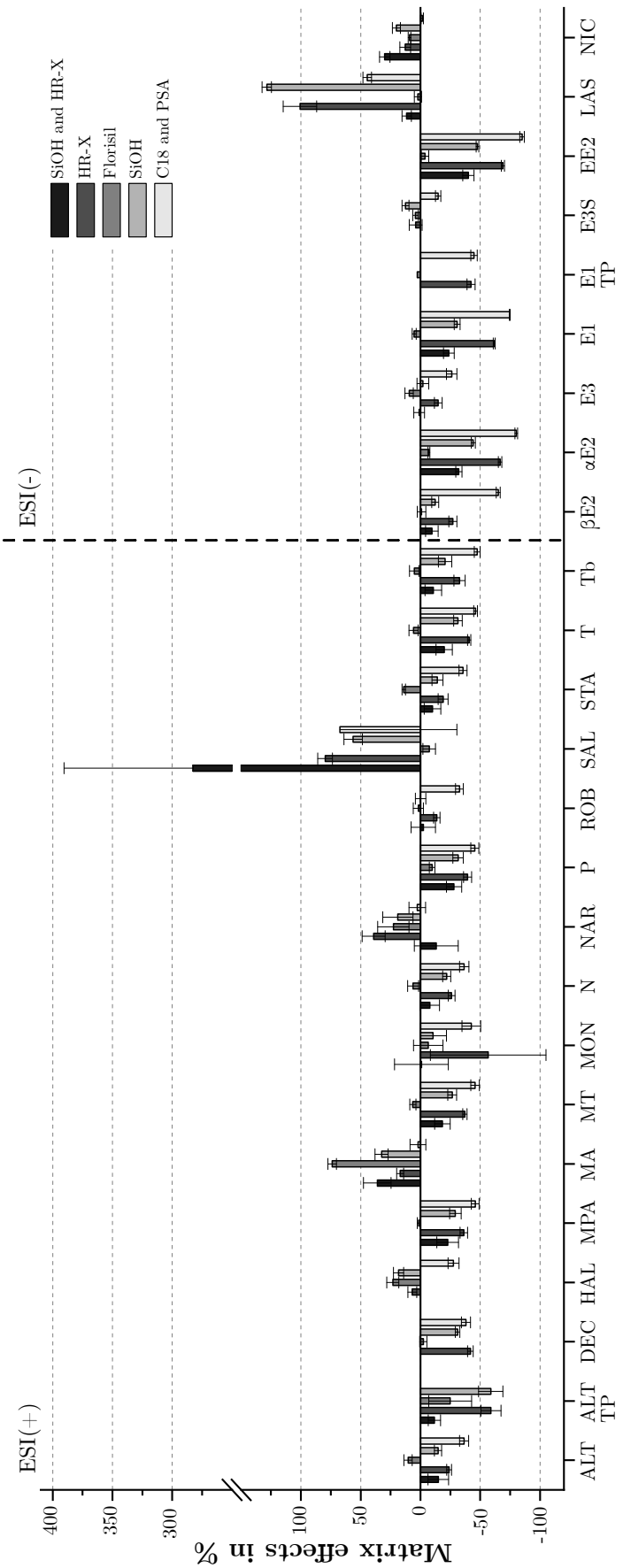

Figure 4.24: Comparison of matrix effects for various SPE materials. (n = 2) Abbreviations can be found under section 4.1.

Dissolution of the residue in n-hexane:DCM (95:5, v:v) ensures that proteins remain in their native and thus more polar form. Native proteins and quaternary ammonium compounds can interact with the silanol groups via hydrogen bonding and electrostatic interactions, resulting in their adsorption onto the SPE material. Elution was performed using a nonpolar solvent (ACN:n-hexane (60:40 v:v)), so that proteins and quaternary alkylammonium compounds were presumably not eluted. As a result, matrix effects were drastically reduced. However, proteins and quaternary alkylammonium compounds were not explicitly quantified.

Another important mechanism influencing matrix effects is the reconstitution of residue after LSE. For methods using SiOH, HR-X, and SiOH combined with HR-X, the residue was reconstituted in 100 % ethanol, ACN:water (10:90 v:v), or acetone:n-hexane (70:30 v:v), respectively, each causing different degrees of precipitation and aggregation of proteins.[181], [182] These aggregates are disrupted by vortexing and ultrasound but remain physically on the densely packed cartridge, resulting in significantly reduced matrix effects compared with LSE alone (see Figure 4.21). The dSPE method only marginally improved matrix effects, but yielded the highest recoveries and no complete loss of analytes. Matrix effects can generally be corrected by use of internal standards or standard addition. Higher recoveries lead to better signal-to-noise ratios, increasing method sensitivity and robustness.[183], [184] Accordingly, the dSPE method should be further optimised in the next step.

4.3.4 dSPE Optimisation

The experiments were conducted as part of Saskia Lehr's master's thesis.[179] For optimisation of the dSPE, a CCD was employed. The factors optimised were the initial n-hexane content at the beginning of the dSPE, the quantities of PSA and C18 as well as the extraction time. As the LSE was still in development at the time of dSPE optimisation and the final solvent composition had not yet been determined, potential effects of this composition on recovery and matrix effects were included in the evaluation. PSA, a polar sorbent, acts as a weak anion exchanger and removes fatty acids, polar organic acids, and, to some extent, sugars. C18 (octadecylsilane) is a lipophilic sorbent exhibiting strong affinity for hydrophobic substances such as fats, oils, and waxes. The contact time between the sorbents and the matrix was varied to establish the interaction duration required for efficient matrix removal. Given the four factors, a full factorial design was implemented without preliminary screening (see Section 4.3.2). Table 4.8 displays factor codings, and the full design matrix using natural variables is summarised in Table 5.4.6.

Table 4.8: Coding of experimental conditions for the CCD. The solvent composition consists of n-hexane and methanol. The given n-hexane content represents the respective methanol content inversely.

Factor	**+2**	**+1**	**0**	**-1**	**-2**
Solvent Composition (n-Hexane) [%]	70	57.5	45	32.5	20
PSA [mg]	90	70	50	30	10
C_{18}	90	70	50	30	10
Time [min]	12.5	10	7.5	5	2.5

Recovery and matrix effects, as defined in Equations 4.2 and 4.4, served as the responses for each experiment. Joint optimisation employed the desirability function according to Derringer and Suich.[185] Target value intervals were specified for both recovery and matrix effects. This ensured that, for matrix effects, unconstrained optimisation did not yield the greatest possible value (i.e. strong ion enhancement), but targeted a value of zero. For recovery, over-optimisation beyond 100 % was rare, except for ALT TP (see Figure 4.23). Target desirability was defined as shown in Equation 4.5:

$$d(y; L, T, U, r_1, r_2) = \begin{cases} 0, & y < L, \\ \left(\frac{y-L}{T-L}\right)^{r_1}, & L \leq y \leq T, \\ \left(\frac{U-y}{U-T}\right)^{r_2}, & T \leq y \leq U, \\ 0, & y > U. \end{cases} \tag{4.5}$$

Where:

- y = Response (REC or ME)
- L = Lower limit of the evaluation interval (0.4 for REC and -0.4 for ME)
- T = Target value (1 for REC and 0 for ME)
- U = Upper limit of the evaluation interval (1.2 for REC and 0.2 for ME)
- r_1 = Exponent controlling the slope between L and T (set to 1)
- r_2 = Exponent controlling the slope between T and U (set to 1)

The form parameters ($r_1 = r_2 = 1$) cause a linear scaling of the desirability between the limits and the target value.[186] The total desirability was calculated from the geometric mean of the desirabilities of the REC and ME (equation 4.6):

$$D_j = \sqrt{d(\mathrm{REC}_j) \cdot d(\mathrm{ME}_j)} \tag{4.6}$$

Where:

- D_j = Aggregated target value for observation j
- REC_j = Input value of the variable REC for observation j
- ME_j = Input value of the variable ME for observation j

If one of the two responses (REC or ME) exceeds the defined target range, the overall desirability declines to zero, regardless of the other response. This ensures that both responses must be optimised simultaneously. The experimental design and further details on the calculation of the optimised variables are described in Section 5.4.6. Evaluation of the RSM also showed that the two-factor interactions were not significant ($p < 0.05$). The extraction time factor was also not significant, which is why it was set to 5 minutes for the final method. The optimised variables and the corresponding desirabilities are listed in Table 4.9.

Table 4.9: Optimised variables of the dSPE and corresponding desirability values based on REC- and ME-Results. The given n-hexane content represents the respective methanol content inversely.

Analyte	**n-Hexane in %**	**PSA in mg**	**C18 in mg**	**Desirability**
ALT	70	10	55	0.74
DEC	20	90	55	0.69
HAL	46	90	55	0.83
MPA	20	10	55	0.71
MA	30	80	55	0.69
MT	41	90	55	0.73
MON	32	70	50	0.97
N	47	75	55	0.79
Nar	43	90	55	0.97
P	20	90	55	0.73
ROB	70	90	50	0.53
SAL	70	85	90	0.99
STA	32	80	55	0.78
T	41	70	55	0.72
Tb	20	10	55	0.74
αE2	21	10	10	0.40
βE2	38	60	55	0.33

Continued on next page

Table 4.9 (Continuation)

Analyte	n-Hexane in %	PSA in mg	C18 in mg	Desirability
DIC	20	10	50	0.73
E3	26	25	45	0.96
E1	40	60	10	0.31
E3S	30	10	30	0.84
EE2	70	10	60	0.25
LAS	42	50	10	0.94
NIC (DNC)	20	60	10	0.85
Median	**35**	**65**	**55**	

Table 4.9 shows results for diclazuril (DIC). When the method was optimised, this was still included, but it was later excluded due to detection difficulties. However, it is still listed in this table because its exclusion would lead to a change in the median PSA level (from 65 mg to 70 mg). However, this was not adjusted prior to validation, so the method was validated using the median values specified in Table 4.9. The optimised values of REC and ME resulting from extraction using LSE + dSPE can be seen in Figure 4.25.

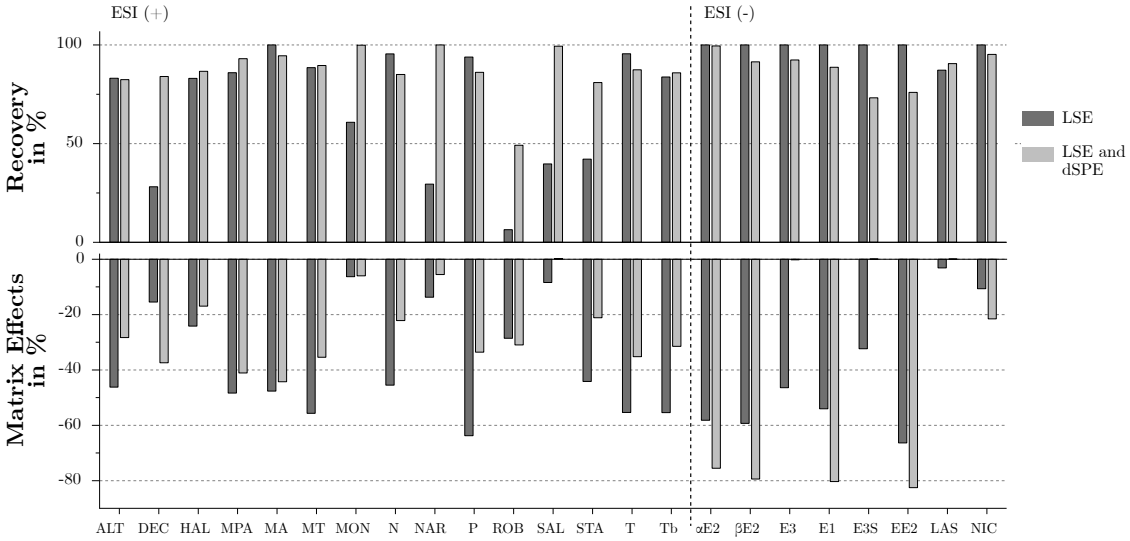

Figure 4.25: Comparison between Recovery and Matrix effects resulting from LSE and LSE + dSPE. REC from the LSE correspond to the optimised recoveries. The matrix effects of the LSE were calculated by inserting the optimised variables from the RSM for the REC (solvent composition, number of cycles and time) into the RSM of the ME. REC and MEs resulting from the combination of LSE and dSPE were calculated using the desirability function. Abbreviations can be found under section 4.1.

Only minor analyte losses resulted from the additional clean-up. For DEC, NAR, MON, ROB, SAL, and STA, recovery improvements between 39 % and 70 % were

observed. This effect is attributed to the change in solvent polarity preceding dSPE: after LSE (65,% n-hexane:35 % methanol), partial evaporation and replenishment with methanol (10 mL total) increased solvent polarity and may prevented analyte adsorption to polypropylene vials.[187]

Matrix effects were reduced by up to 30 % (as for P), and in some cases, such as E3 and E3S, eliminated. Stronger ion suppression of oestrogens (α-, β-estradiol, estrone, ethinylestradiol) occurred after including PSA and C18, likely due to sorbent-derived artefacts and the incomplete removal of phospholipids, which tend to concentrate during dSPE.[188], [189]

Improvement of matrix effects was achieved for 16 out of 23 analytes; therefore, the method optimisation was terminated. The remaining recoveries and matrix effects in quantification are corrected using the standard addition method. Alternatively, isotopically labelled standards may be employed.

4.3.5 Filter Test

To prevent instrumentation damage by minute particulates, extracts were passed through 0.2 µm syringe filters. For filter selection, polyvinylidenfluorid (PVDF), polyester (PET), and polyamid (PA) membranes were compared by filtering a solvent standard and relating signal intensities to unfiltered controls. Recoveries are shown in Figure 4.26.

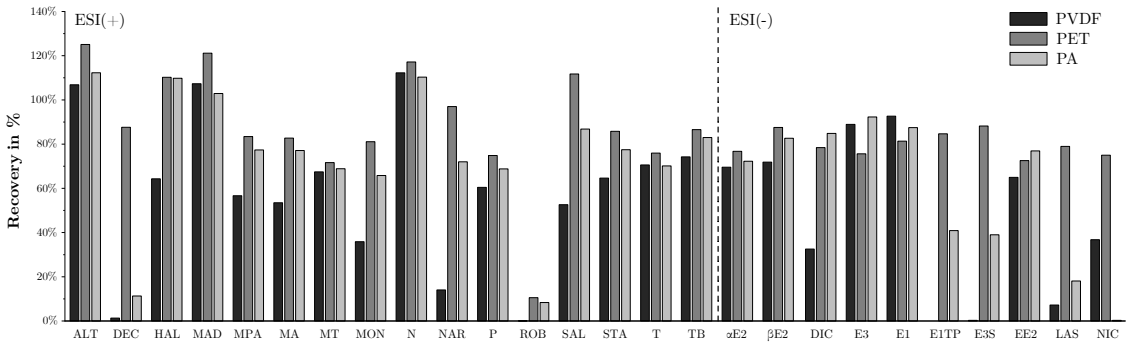

Figure 4.26: Comparison of recoveries for various filter materials. Abbreviations can be found under section 4.1.

The PET filter yielded the lowest analyte losses and was integrated into the extraction method.

4.3.6 Final Extraction Method

Figure 4.27 presents the final optimised extraction method. To achieve the optimised n-hexane fraction for dSPE, the extract resulting from LSE needs to be adjusted. Following LSE, 15 mL of extract consisting of 35 % MeOH and 65 % n-hexane is obtained. However, the optimal n-hexane fraction for dSPE is 35 %. Therefore, after LSE, the supernatant is evaporated to 5 mL and subsequently diluted to 10 mL with methanol. The evaporation was performed using a gentle N_2 stream at 25 °C at atmospheric pressure. Based on the vapour pressures of methanol and n-hexane under these conditions, the n-hexane fraction could be reduced to approximately 30 %.

LSE

1. Transfer a sample aliquot 0.1 g +/- 0.01 g to a 15 mL centrifuge tube.
2. Add 5 mL of 35 % methanol and 65 % n-hexane.
3. Extraction for 60 min on a rotary shaker.
4. Centrifuge for 5 min at 4500 rpm.
5. Quantitative transfer of the supernatant to a new 15 mL centrifuge tube.

dSPE

6. Reduce extraction solvent to 5 mL volume under a gentle stream of N_2.
7. Add 5 mL of methanol.
8. Add 65 mg PSA and 55 mg C18 sorbent.
9. Vortex for 3 min and centrifuge for 5 min at 4500 rpm.
10. Transfer supernatant to a new tube and evaporate to dryness under a gentle stream of N_2.
11. Ṙeconstitution in 1 mL H_2O:MeOH:ACN (60:20:20 v:v:v).
12. Syringe filtration using 2 µm PET filter.

LC-MS/MS Analysis

Figure 4.27: Final extraction method.

4.4 Validation

The objective of the method developed herein is the detection of steroid hormones and coccidiostats, expected at trace analytical concentration levels, in stable dust. Within the scope of validation, it is necessary to demonstrate that this method meets the requirements of its intended use. To ensure validation at the current regulatory and scientific standard, validation was conducted in accordance with Commission

Implementing Regulation (EU) 2021/808. Although Commission Implementing Regulation (EU) 2021/808 specifically addresses contaminants in food, there is no equivalent EU regulatory framework for environmental compartments. Given that the analytes under investigation are also relevant residues in food and that dust samples were collected from facilities for food-producing animals, application of Commission Implementing Regulation (EU) 2021/808 was deemed appropriate.

4.4.1 Stability of Analytes

In the course of the stability study, solvent standards (in H_2O:MeOH 1:1) at concentrations of 0.1, 1, and 10 mg/L were prepared. For 0.1 and 1 mg/L, two storage conditions were assessed: -20,°C and 4,°C, both protected from light. The 10 mg/L solutions were additionally stored at room temperature, with and without exposure to UV light. At specified time points, aliquots were obtained and analysed.

Figure 4.28 illustrates exemplary results for altrenogest and halofuginone. All further results are presented in Appendix A.5.1.

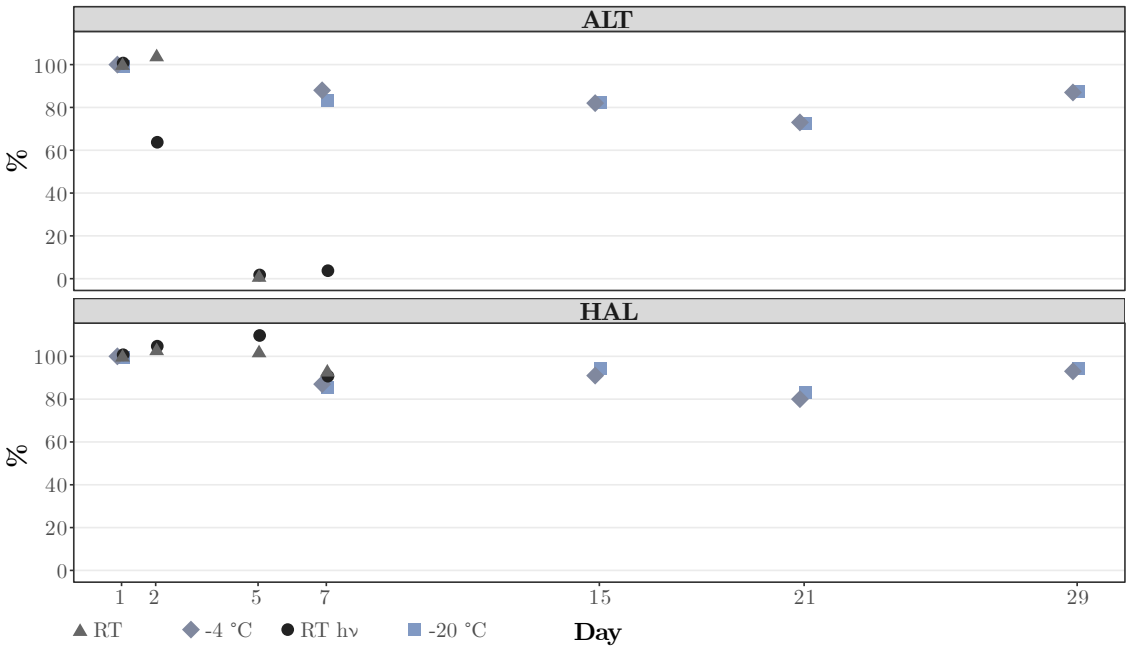

Figure 4.28: Stability of altrenogest and halofuginone under various storage conditions. RT = room temperature, hν = exposure to UV ligt.

As shown in Figure 4.28, altrenogest exhibited a drastic decrease, almost 100 % within five days, when exposed to light at room temperature. Simultaneously, the concentration of the photoisomer altrenogest-CAP increased, as already described in the literature.[180], [190] Under exclusion of light, the elimination of altrenogest proceeded considerably more slowly. Apart from this, only estrone-3-sulphate,

robenidine, and nicarbazin demonstrated a degradation of more than 30 % after seven days at room temperature under light exposure. In thermal degradation studies, nicarbazin (analysed by its complex component 4,4'-dinitrocarbanilide (DNC)) exhibited a marked reduction with increasing temperature during processes such as boiling, grilling, and microwave heating.[191] However, it could not definitively be established whether this was solely due to temperature or the sample matrix. After seven days of light exposure, robenidine exhibited a decrease of 32 %, while at the same temperature but under exclusion of light, only a 6 % loss was observed. This finding has also been previously reported for abiotic degradation of robenidine.[192] The degradation of estrone-3-sulphate has also been extensively investigated.[193] In the study by Scherr *et al.*, a temperature range below 25°C was examined, in which estrone-3-sulphate degradation was already apparent. Likewise, in the present work, temperatures of -20 °C and 4 °C resulted in substantial degradation (>30 %) across all concentrations after 15 days. Scherr et al. identified estrone as the main metabolite. In the present data, all oestrogens, including estrone, decreased by more than 40 % after 15 days. As estrone and estrone-3-sulphate were not independently quantified, a direct relationship cannot be established. All other androgens and progestogens exhibited concentration changes between +2 % and -30 % over 29 days, regardless of concentration, temperature, or light exposure. Of the coccidiostats investigated, only the synthetic compound halofuginone displayed less than 15 % degradation across all concentrations and temperatures. All other analytes demonstrated a pronounced decline, in some cases exceeding 90 % within 29 days.

For further validation and sample analyses, based on these results, it is recommended that extracted samples are analysed on the same day, to avoid crucial changes in concentration caused by degradation.

4.4.2 Specificity

Specificity entails detection and identification of the analyte without interference from matrix components. Specificity was evaluated by analysing matrix blanks for co-eluting peaks. Both progesterone and halofuginone showed the spectra depicted in Figure 4.29.

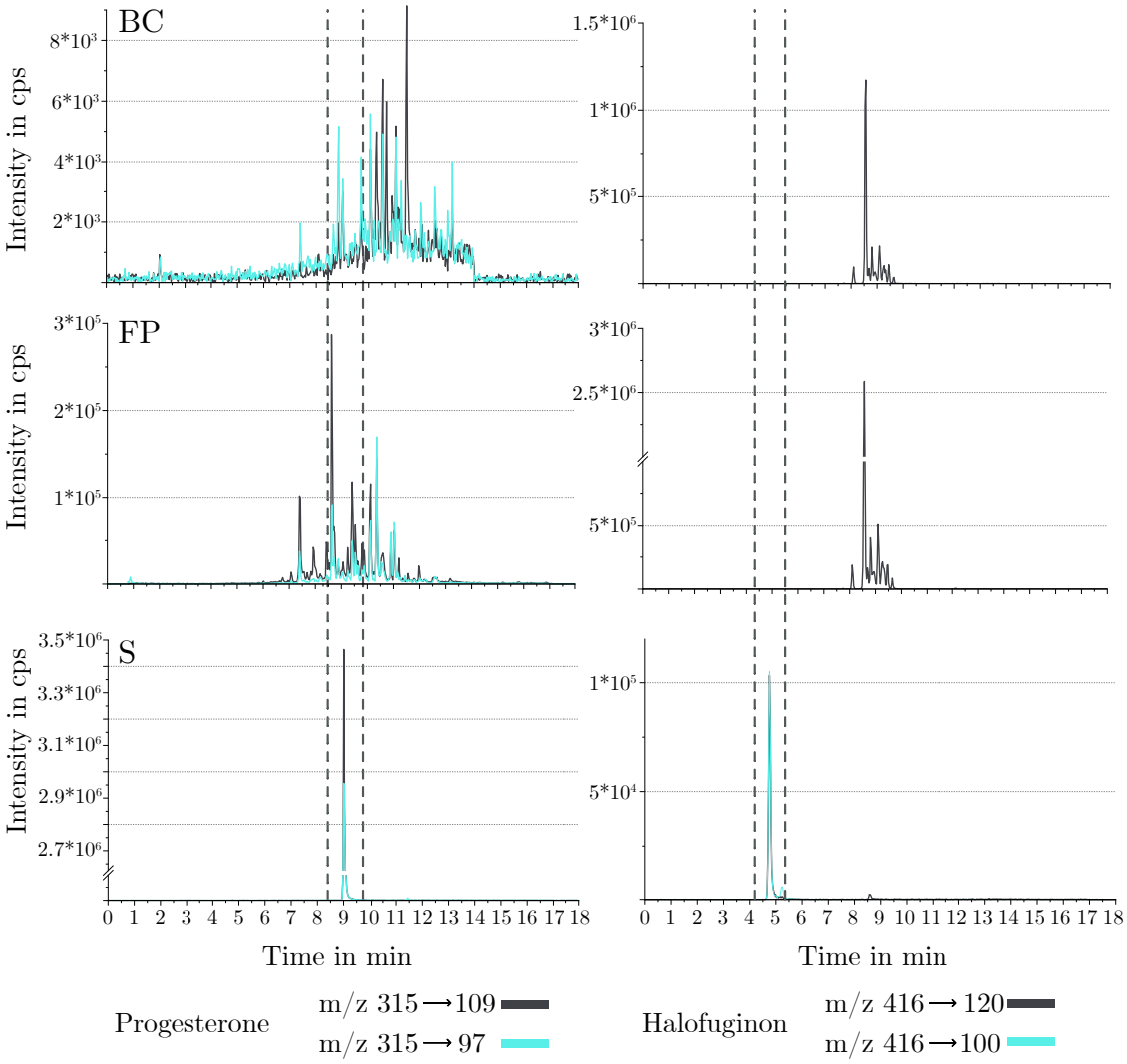

Figure 4.29: Ion chromatograms of m/z transitions of progesterone and halofuginon in comparison between different stable dust matrices (broiler chicken (BC) and fattening pigs (FP)) and a solvent standard (S).

Figure 4.29 presents the peaks of the respective m/z transitions of progesterone and halofuginone obtained from pig and broiler stable dust compared against a solvent standard. Investigations in the context of a Master's thesis (Paulina Michel) demonstrated that halofuginone signals (m/z 416.0 → 120.0) originate from exogenous matrix components. This was substantiated by processing a polypropylene

centrifuge tube without dust matrix or standard, following Method 4.3.6, which yielded an identical spectrum. For halofuginone specificity is supported by retention time and the second m/z transition (m/z 416.0 → 100.2). Halofuginone elutes at 4.7 min, while matrix-derived peaks with the transition m/z 416.0 → 120.0 elute between 8 and 10 min. The transition m/z 416.0 → 100.2 is absent in this retention interval. For progesterone, no corresponding signal could be detected. Variations in the progesterone signal profile between pig and broiler stable dust are presumed to result from endogenous matrix signals. Both quantifier and qualifier transitions m/z 315.19 → 109.2 and m/z 315.19 → 97.2, were detected resulting from matrix components eluting between 7 and 11 min, placing progesterone at 9.1 min in the centre of the retention time range of the matrix components. The progesterone spectra differ in intensity and signal profile between pig and broiler stable dust samples. Here, specificity is maintained by verifying appropriate relative abundances of diagnostic ions (quantifier and qualifier).

4.4.3 Selectivity

The example of progesterone in Figure 4.29 illustrates the importance of highly selective identification of analytes. Within a targeted LC-MS/MS method, selectivity refers to differentiating the target analyte from co-existing matrix constituents and other analytes. According to Commission Implementing Regulation (EU) 2021/808, each target analyte is assigned a confirmed retention time, two m/z transitions, and the ion ratio between transitions as identification criteria. The relative retention time of the analyte in the sample is permitted to deviate by ± 0.1 min from the standard. The deviation in ion ratio between sample and standard must not exceed ± 40 %. To assess retention times and m/z transitions established during method development, nine aliquots of a pig dust sample (Box 4 No. 170) and a laying hen dust sample (Box 16 No. 744) were spiked with increasing concentrations (0.05, 0.1, 0.15, 0.2, 0.5, 1, 1.5, 2, and 3 mg/kg) and processed according to Method 4.3.6. Solvent calibration was prepared at identical concentrations. Retention times and ion ratios across all concentrations were averaged, and standard deviations determined.

Table 4.10: Identification parameter retention time (mean($\overline{t_R}$) in min) and ion ratio (IR) for each component (Comp.), and their respective standard deviation (SD of n = 9) and relative standard deviation (RSD IR) and deviations compared between different stable dust matrices (M) (LH = laying hen, FP = fattening pigs) and a solvent standard (S) (Reft_R and Ref IR) SDt_R is displayed in min.

Comp.	Matrix	$\overline{t_R}$	SD t_R	Ref t_R	$\overline{IR}$	RSD IR	Ref IR
ALT	LH	8.4	0.1	0.0%	0.40	10.0%	2.8%
	FP	8.4	0.1	0.0%	0.41	6.7%	4.1%
	S	8.4	0.1		0.38	4.9%	
DEC	LH	11.2	0.0	0.0%	0.63	2.0%	0.1%
	FP	11.2	0.0	0.0%	0.62	2.3%	0.6%
	S	11.2	0.0		0.63	1.5%	
HAL	LH	4.4	0.3	0.1%	1.03	3.0%	0.5%
	FP	4.4	0.2	0.0%	1.06	3.0%	0.7%
	S	4.4	0.2		1.04	4.0%	
MPA	LH	9.0	0.1	0.0%	0.40	3.5%	0.5%
	FP	9.0	0.1	0.0%	0.40	2.2%	0.9%
	S	9.0	0.1		0.41	1.7%	
MA	LH	8.8	0.1	0.0%	0.56	2.7%	0.6%
	FP	8.8	0.1	0.0%	0.53	3.5%	1.6%
	S	8.8	0.1		0.55	1.6%	
MT	LH	8.0	0.1	0.0%	0.89	3.0%	0.1%
	FP	8.0	0.1	0.0%	0.88	2.6%	0.4%
	S	8.1	0.1		0.88	1.6%	
MON	LH	11.7	0.0	0.0%	0.84	1.0%	0.0%
	FP	11.7	0.0	0.0%	0.84	1.4%	0.0%
	S	11.7	0.0		0.84	2.0%	
N	LH	7.1	0.1	0.0%	0.54	3.6%	0.0%
	FP	7.1	0.1	0.0%	0.63	12.9%	7.3%
	S	7.1	0.1		0.54	1.3%	
NAR	LH	12.4	0.0	0.0%	0.48	1.7%	0.6%
	FP	12.4	0.0	0.0%	0.47	2.0%	1.2%
	S	12.4	0.0		0.49	1.4%	
P	LH	9.0	0.1	0.0%	0.91	2.4%	0.8%
	FP	9.0	0.7	0.1%	0.90	1.6%	1.3%
	S	9.0	0.0		0.93	2.8%	
ROB	LH	8.1	0.0	0.2%	0.93	4.8%	4.1%
	FP	8.1	0.0	0.2%	0.92	3.0%	3.1%
	S	8.1	0.1		0.86	20.5%	
SAL	LH	12.1	0.0	0.0%	0.33	2.3%	0.5%
	FP	12.1	0.0	0.0%	0.33	1.3%	0.6%
	S	12.1	0.0		0.33	2.6%	
STA	LH	8.3	0.1	0.0%	0.67	3.5%	0.3%

Continued on next page

Table 4.10 (Continuation)

Comp.	Matrix	$\overline{t_R}$	SD t_R	Ref t_R	$\overline{IR}$	RSD IR	Ref IR
	FP	8.3	0.2	0.1%	0.64	9.7%	3.0%
	S	8.3	0.1		0.67	5.0%	
T	LH	7.6	0.1	0.0%	0.90	1.6%	0.6%
	FP	7.6	0.1	0.0%	0.91	4.2%	0.1%
	S	7.6	0.1		0.91	1.0%	
Tb	LH	6.6	0.2	0.0%	0.67	8.3%	1.3%
	FP	6.6	0.1	0.0%	0.63	3.5%	1.8%
	S	6.7	0.1		0.65	6.9%	
αE2	LH	10.0	0.0	0.0%	0.34	2.1%	1.8%
	FP	10.0	0.0	0.0%	0.34	3.3%	2.8%
	S	10.0	0.0		0.36	0.9%	
βE2	LH	9.7	0.0	0.0%	1.00	1.6%	0.3%
	FP	9.7	0.0	0.0%	0.98	2.4%	0.4%
	S	9.7	0.0		0.99	1.6%	
E3	LH	6.9	0.1	0.0%	0.61	1.4%	0.2%
	FP	6.9	0.0	0.0%	0.60	2.4%	0.4%
	S	6.9	0.0		0.61	2.8%	
E1	LH	10.1	0.0	0.0%	0.42	2.5%	0.3%
	FP	10.1	0.0	0.0%	0.42	2.2%	0.5%
	S	10.1	0.0		0.42	1.5%	
E3S	LH	5.5	0.1	0.1%	0.28	2.7%	1.8%
	FP	5.5	0.1	0.0%	0.29	3.4%	0.0%
	S	5.5	0.2		0.29	4.4%	
EE2	LH	9.9	0.0	0.0%	0.80	3.6%	0.4%
	FP	9.9	0.0	0.0%	0.80	6.5%	0.7%
	S	9.9	0.0		0.79	4.1%	
LAS	LH	9.9	0.1	0.2%	0.23	1.3%	0.1%
	FP	10.0	0.2	0.3%	0.23	6.3%	1.9%
	S	9.9	0.1		0.23	2.1%	
NIC (DNC)	LH	10.8	0.0	0.0%	0.11	2.5%	0.2%
	FP	10.8	0.0	0.0%	0.11	5.0%	0.5%
	S	10.8	0.0		0.12	4.6%	

As evident from Table 4.10, retention times deviated by less than ±0.1 min in matrix calibration and relative to the solvent standard. Deviation in ion ratios of matrix calibration versus solvent standard calibration was within the permitted ± 40 %. These results substantiate sufficient selectivity across concentration ranges and matrix compositions. Only two matrices were investigated here. To ensure ongoing selectivity across diverse dust compositions, a solvent standard at 100 µg/L is measured alongside each run, and retention time and ion ratio of each sample are compared to those of the standard.

4.4.4 Uncertainty Related Performance Characteristics

The requirements to be validated for the method are the standard deviation of repeatability, the standard deviation of reproducibility, the decision limit (CCα), the detection capability (CCβ), and the recovery, which are defined according to Commission Implementing Regulation (EU) 2021/808 as:

Repeatability

"[...] precision under conditions, where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time"

Reproducibility

"[...] precision under conditions, where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment"

Here reproducibility was validated in house using identical test items in the same laboratory but different operators and different equipment.

Decision limit CCα

"[...] the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant [...]"

Detection capability CCβ

"[...] the smallest content of the analyte that may be detected or quantified in a sample with an error probability of β. in the case of prohibited or unauthorised pharmacologically active substances, the CCβ is the lowest concentration at which a method is able to detect or quantify, with a statistical certainty of1 - β, samples containing residues of prohibited or unauthorised substances. In the case of authorised substances, the CCβ is the concentration at which the method is able to detect concentrations below the permitted limit with a statistical certainty of 1 - β"

Recovery

"[...] the recovery corrected amount of an analyte divided by the fortified amount of the analyte in the matrix sample, expressed as a percentage"

The estimation of the performance characteristics is based on a variance components model, which represents the deviation between the measured value and the expected value as the sum of two variance terms. These variance components comprise the within-series variance and the between-series variance arising from systematic differences between measurement series. The measurement series were systematically varied according to a full factorial design in the factors matrix, operator, and date. Subsequent decomposition of the run-induced variance permits quantification of the contribution of each of these three factors to the total measurement variance. In total, four sample series were prepared, each consisting of a matrix blank and nine matrix aliquots fortified with increasing analyte levels, following the orthogonal, fully factorial design described in Section 5.4.7. Samples were processed according to Method 4.3.6 and analysed using Method 5.4.3.

The selection of the concentration range was guided by literature data on steroid levels in dust and by preliminary experiments estimating altrenogest and progesterone in stable dust. Blackwell *et al.* reported steroid hormones such as trenbolone, estrone, and melengestrol acetate in particulate matter at concentrations up to 20, 52, and 36 ng/g, respectively.[157], [194] Own preliminary investigations in dust from sow stables, where animals were actively treated with altrenogest, indicated concentrations of approximately 5 mg/kg for altrenogest and progesterone. Sun *et al.* reported monensin, salinomycin, and narasin in poultry litter at up to 0.3, 4, and 0.2 mg/kg respectively.[159] As coccidiostat concentrations in stable dust are expected to be lower than in poultry litter, a validation range of 0–3 mg/kg was selected (see Section 5.4.7). For Matrix variation, dust samples from FP (JLU dust archive box 4 no. 170) and LH (JLU dust archive box 16 no. 744) facilities were investigated. Figure 4.30 illustrates the linear relationship between the analyte content derived from the matrix-calibrated response and the true concentration, exemplified for progesterone and lasalocid.

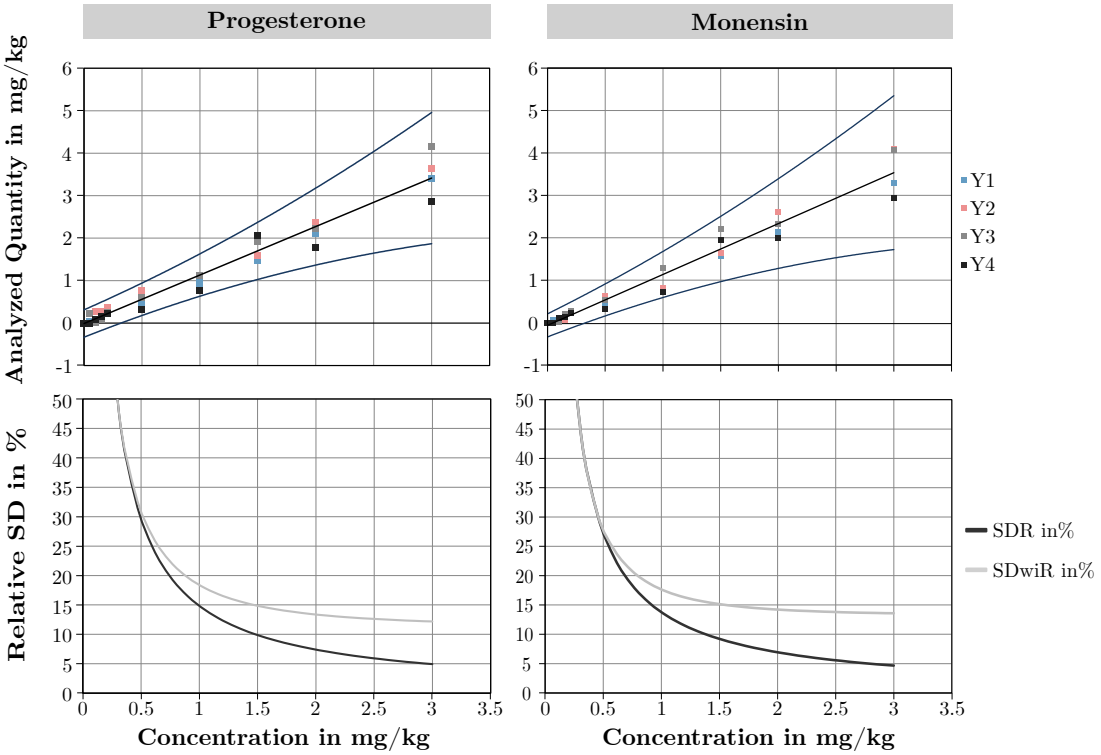

Figure 4.30: First row: Variance component model fitted to matrix calibration data obtained from four individual matrix calibrations (Response Y1-Y4 = concentration in mg/kg) with concentrations ranging from 0 to 3 mg/kg including 0.95-prediction bands. Second row: relative standard deviation of repeatability (SD_R in %) and relative standard deviation of within-laboratory reproducibility (SDwiR in %) as a function of concentration.

For concentrations above 120 µg/kg, the maximum admissible coefficient of variation according to Commission Implementing Regulation (EU) 2021/808 is calculated using the Horwitz equation (Equation 4.7):

$$CV = 2^{1-\frac{1}{2}\cdot\log(c)} \tag{4.7}$$

Where:

- CV = Variation coefficient in %
- c = Mass fraction expressed as a power of ten

For 0.1 mg/kg this yields an allowable reproducibility CV of 25 %; for 1 mg/kg, 16 %; and for 3 mg/kg, 14 %. Under repeatability conditions, the CV should not exceed two thirds of the corresponding reproducibility value, i.e. 17 %, 11 % and 9 %, respectively. Section A.5.2 in the Appendix lists the relative standard deviation of repeatability (SD_R) and the relative standard deviation of within-laboratory reproducibility (SDwiR) for all analytes. As shown in the lower row of Figure 4.30,

variances for progesterone and monensin from 1–1.5 mg/kg upwards lie within these specification limits. The upper row of Figure 4.30 illustrates that the prediction band width increases with concentration; this heteroscedastic variance is frequently observed in residue analysis and reflects the relationship between measurement variance under reproducibility conditions and analyte concentration.[195] Figure 4.31 shows recoveries, obtained by plotting the true concentration against the measured values determined via external calibration.

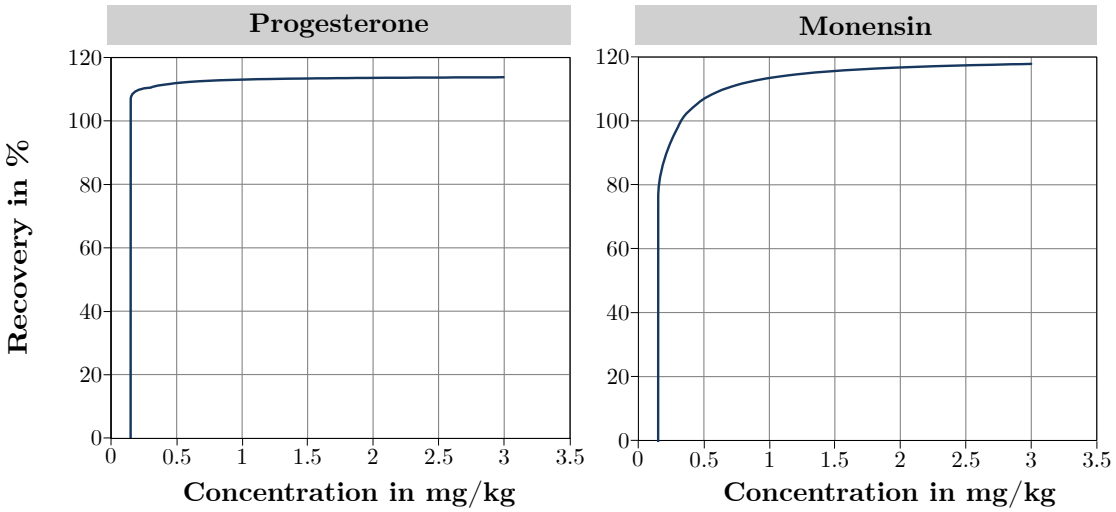

Figure 4.31: Recovery in % as a function of the concentration from the matrix calibration.

Recoveries comply with the acceptance criteria of Commission Implementing Regulation (EU) 2021/808 for the investigated concentration range (needs to be between 80 and 120 %). Table 4.11 summarises the recovery ranges for all analytes.

Table 4.11: Recovery values of all components. Abbreviations can be found under section 4.1.

Component	**Recovery** [%]
ALT	85–109
DEC	102–107
HAL	84–121
MPA	77–113
MA	79–112
MT	81–113
MON	76–118
N	75–104
NAR	92–116
P	107–114
ROB	100–104
SAL	66–120
STA	100–117
T	84–114
Tb	81–111
αE2	97–100
βE2	96–116
E3	95–107
E1	94–113
E3S	100–105
EE2	99–110
LAS	88–102
NIC (DNC)	95–106

As can be seen in Table 4.11 recoveries are compliant with Commission Implementing Regulation (EU) 2021/808 for almost all analytes. However, for MPA, MA, MON, N, and SAL, recoveries were above 80 % at respective CCα concentrations.

Decision limit (CCα) and detection capability (CCβ) were obtained under within-laboratory reproducibility conditions according to Commission Implementing Regulation 2021/808 as well. CCα values ranged from 0.2–0.7 mg/kg and CCβ values from 0.4–2.3 mg/kg across all analytes (Table 4.12).

Table 4.12: Decision limits (CCα) and detection capabilities (CCβ) in mg/kg stable dust obtained via variance components model for all analytes. Abbreviations can be found under section 4.1.

Component	**CCα at $X_0 = 0$**	**CCβ at $X_0 = 0$**
ALT	0.22	0.43
DEC	0.38	2.30
HAL	0.26	0.53
MPA	0.22	0.45
MA	0.22	0.44
MT	0.22	0.43
MON	0.24	0.47
N	0.21	0.62
NAR	0.33	0.68
P	0.26	0.53
ROB	0.71	–
SAL	0.32	0.63
STA	0.34	0.71
T	0.22	0.44
Tb	0.24	0.47
αE2	0.34	0.69
βE2	0.36	0.73
E3	0.27	0.55
E1	0.34	0.69
E3S	0.31	0.70
EE2	0.40	–
LAS	0.30	0.64
NIC (DNC)	0.44	1.12

In total, 5 (DEC, N, ROB, EE2, and NIC) of the 23 investigated analytes did not fully comply with the uncertainty-related performance characteristics specified in Commission Implementing Regulation (EU) 2021/808, resulting in comparatively higher CCα and CCβ values of e.g. 0.7 mg/kg (ROB) and 2.3 mg/kg (DEC) respectively. Variance decomposition showed that, depending on the analyte, either the matrix or the day (time factor) contributed the largest proportion to the total variance of these five analytes. Overall, the validation demonstrated that matrix-matched calibration yields accurate and precise measurement results and that the uncertainty-related performance characteristics are fit for purpose for the

analysis of stable dust samples to determine steroid hormones and coccidiostats at trace-level concentrations.

4.5 Investigation of Real Samples

To finally assess analyte concentrations in stable dust and to estimate whether the dust poses a certain risk potential for humans who come into daily contact with these dusts, and whether the dust represents a potential entry pathway into the environment, a total of 50 stable dust samples were analysed. These samples originate from different animal species (BC, LH, FP, and BC) and various housing systems (organic, conventional, aviary, cage housing). Within these stables, samples were sometimes taken from different locations, such as near feeding stations, lying areas, etc. (details are provided in method section 5.3.4). Since isotope-labeled standards for all analytes were not available, and no suitable analyte-free matrix existed for individual matrix calibration, quantification was initially intended to be performed via standard addition. However, during data evaluation, it became evident that the samples were not suitable for standard addition due to inhomogeneity in composition and analyte distribution, which prevented a linear regression in the standard addition method. Consequently, the samples were quantified using matrix calibration curves for LH and FP established during validation.

Collecting fresh stable dust samples also proved challenging, as stables are regularly cleaned, and thus, sample material was not always available in sufficient quantity. Due to the limited sample amount, some samples could only be analysed in duplicate or single determination. Accordingly, the results presented here represent initial indications and serve as orientation values, and should not be interpreted as overall average.

The samples investigated comprised samples from a farm that operates according to organic standards (O1-O4). This farm housed both laying hens in mobile stables and broiler chickens in a closed rearing stable. All animals were vaccinated with Paracox 8 and, thereafter, in accordance with Regulation (EU) 1831/2003 and Regulation (EC) 2018/848, received no further coccidiostats. Paracox 8 is a live coccidiosis vaccine administered to animals in the first days of life, either via drinking water or spray.

The laying hens (300 animals, age: 15 weeks) were kept in a mobile stable on pasture. Dust was collected from a shelf above the feeding station located outside the mobile laying hen stable. In the broiler rearing stable (500 animals, age: 10 days), samples were taken from heating lamps above the feeding station, from

wooden slats stored within the stable, and from a ledge above the entrance gate. No coccidiostats or steroid hormones were detected in any of these samples.

17 of the 50 samples originated from laying hen facilities. Table 4.13 lists the concentrations of the identified analytes. As shown in this table, monensin was detected in samples LH1 and LH3 at concentrations of 0.27 and 0.42 mg/kg, respectively, both below the CCβ threshold. These samples were collected in 1981 and 1983, respectively. Furthermore, nicarbazin was detected in sample LH2 (from 1982) at a concentration of 0.54 mg/kg. Coccidiostats such as monensin and nicarbazin were never and are currently not approved for use in laying hens. However, the use of coccidiostats for chickens reared for laying is permitted up to a maximum age of 16 weeks. As there is no information available regarding the age of the animals or the use of coccidiostats in these facilities, the detection of monensin and nicarbazin may indicate either that the hens were under 16 weeks old at the time of sampling or that the stable was also used for broiler chickens, with the detected coccidiostats representing residues. A further explanation could be related to cross contamination of the feed.

A further noteworthy finding was observed in sample LH8. This sample was analysed in duplicate. In one aliquot, nandrolone was detected at a concentration of 2.2 mg/kg, whereas it was not detected in the second aliquot. In the stable where this sample was collected, a total of 750 laying hens (age: 80 weeks) were kept. The sample was collected at the inlet fan of the stable. The laying hen stable was located on a farm that also kept dairy cows. The dust was found on the cover of the fan on the inside of the laying hen stable. Thus, it is possible that the dust components originated not only from the laying hen stable but also from surrounding animal facilities. Nandrolone is an endogenous anabolic steroid formed in small amounts during testosterone synthesis. It is therefore possible that the inlet fan acted as a kind of collection point for dust contaminated with nandrolone from the surrounding dairy cow and calf stables. This finding also clearly demonstrates the inhomogeneous distribution of analytes within the matrix.

Table 4.13: Concentrations in mg/kg of steroid hormones and and coccidiostats in stable dust samples from different laying hen housing systems (2025) and archived cage housing systems (1981–2004). # indicates concentrations below CCβ. Samples LH1 - LH6 originate from one facility, LH7 - LH11 from another facility, LH12 - LH15 from a third, and LH16 - LH17 from a fourth facility. Further information about dust sample origins can be found in section 5.3.4.

Dust Samples	Year	Housing System	MON	N	NAR	P	SAL	bE2	E1	E3	NIC
LH1	1981	Cage housing	0.27$^{\#}$					<CCα		<CCα	
LH2	1982	Cage housing	<CCα								0.54$^{\#}$
LH3	1983	Cage housing	0.42$^{\#}$					<CCα		<CCα	<CCα
LH4	2002	Cage housing									
LH5	2003	Cage housing	<CCα	<CCα	<CCα	<CCα		<CCα	<CCα		
LH6	2004	Aviary	<CCα		<CCα		<CCα	<CCα	<CCα		<CCα
LH7	2025	Floor housing			<CCα			<CCα	<CCα		
LH8	2025	Floor housing		2.21				<CCα			
LH9	2025	Floor housing		<CCα		<CCα		<CCα			
LH10	2025	Floor housing		<CCα				<CCα			
LH11	2025	Floor housing		<CCα		<CCα		<CCα			
LH12	2025	Mobile outdoor			<CCα						
LH13	2025	Mobile outdoor			<CCα	<CCα				<CCα	
LH14	2025	Mobile outdoor			<CCα						
LH15	2025	Mobile outdoor			<CCα						
LH16	2025	Mobile outdoor									
LH17	2025	Mobile outdoor			<CCα			<CCα			

9 of the 50 samples were collected from broiler chicken facilities (see Table 4.14). Samples BC1-BC3 were collected from inside the same stable (Lousiana stable housing system) in the years 1992, 1993, and 1994, respectively. No records regarding the use of coccidiostats were available for these samples. However, based on the results, monensin, salinomycin, and nicarbazin may have been used in these stables.

It is not possible to determine whether these coccidiostats were used simultaneously, as can be seen from samples BC6-BC11. These samples were collected in September 2025 in a broiler chicken stable (herd size: 20,500 animals (Hubbard 787), age: 32 days, housing level 3 according to current regulations.[196]) For these samples, it is known that the coccidiostats nicarbazin and narasin were applied at the time of sampling (via feed at 49 mg/kg). Samples BC4 and BC6-BC8 were collected from locations within the stable that are easily accessible during cleaning processes. In contrast, samples BC5 and BC9 were collected from less accessible locations, such as electrical installations or the outside of shutters, which are not fully cleaned during stable cleaning. In sample BC9, monensin (concentration below CCβ) was additionally detected alongside nicarbazin and narasin. This demonstrates that stable dust can serve as a medium in which coccidiostats persist for extended periods. To prevent resistance, coccidiostats are administered in a rotation or shuffle program.[197] However, if coccidiostats such as monensin remain in the stable at low concentrations, this could accelerate the development of resistant parasite strains.[161] As shown in Table 4.14, the concentrations are in parts significantly higher than in samples BC1-BC3. Considering the stability studies of coccidiostats, it can be assumed that these substances degrade over time to a rather low extend.[198]–[200] Nonetheless, since samples BC1-BC3 are already more than 30 years old, degradation of the analytes cannot be excluded, especially in complex matrices like stable dust. However, a reduced concentration is definitely also attributable to dilution effects caused by cleaning cycles.

Table 4.14: Concentrations of steroid hormones and coccidiostats in stable dust samples from different broiler chicken housing systems (2025) and archived broiler cage housing systems (1992–1994) in mg/kg. # indicates concentrations below CCβ. If standard deviations are given they rely on n = 2 otherwise, only single determination was possible. Further information can be found in section 5.3.4.

Dust Samples	Year	Housing System	MON	NAR	SAL	βE2	E1	E3	LAS	NIC
BC1	1992	Lousiana stable			0.52#	<CCα	<CCα	<CCα	<CCα	0.31#
BC2	1993	Louisiana stable	0.53							
BC3	1994	Lousiana stable	<CCα			<CCα			<CCα	
BC4	2025	Housing level 3		1.38 ± 0.24	<CCα					8.62 ± 0.15
BC5	2025	Housing level 3	<CCα	1.27	<CCα					6.75
BC6	2025	Housing level 3		1.59 ± 0.47		<CCα				8.73 ± 1.52
BC7	2025	Housing level 3		1.05 ± 0.04	<CCα	<CCα				7.61 ± 0.64
BC8	2025	Housing level 3	<CCα	13.60 ± 2.12	<CCα				<CCα	11.23 ± 0.27
BC9	2025	Housing level 3	0.41#	0.43 ± 0.07#	<CCα					4.48 ± 0.65

In total, 10 out of 50 samples originated from fattening pig stables (finishing pigs) from one farm (8 samples, years 1980–1999). In all these samples, only signals for decoquinate were detected, but only at concentrations below the CCα. Since decoquinate is not typically used in fattening pigs, these findings are likely due to carryover during sample injection. Different solvent compositions were tested for the cleaning solution of the injection needle. Using MeOH:ACN:H_2O:2-Propanol (1:1:1:1 v:v:v:v) could reduce the carry over, but failed to prevent it entirely. Resulting variations in the blank signal may lead to apparent signals in the evaluation even after blank signal subtraction (see section 5.4.3).

Additionally, a total of 10 samples were collected from breeding sow stables (see table 4.15). Nine of these samples were from stables where the sows were actively treated with altrenogest. One stable contained sows that were not treated with ALT (see section 5.3.4). Samples BS1–BS6 were collected from one stable but at six different locations within the stable (e.g. feeding stations, ceiling ducts, gestation crate bars). The detected concentrations of altrenogest in these samples were all below CCα. This could be due to the fact that the samples were already collected in 2023. Analyses shortly after sampling in 2023 revealed that altrenogest cycloaddition product concentrations of about 6 mg/kg (using a matrix matched calibration) were detected in only one of the six samples. Additionally, the fact that one sample (BS3) was collected from a gestation crate provides an explanation for the generally low altrenogest concentrations in these samples. Since sows are only placed in farrowing crates one week before farrowing, it can be assumed that altrenogest had not been used in this stable for at least about 15 weeks prior to sampling. Given the low stability of altrenogest and its transformation product in environmental matrices, it is likely that complete degradation of altrenogest had already occurred by the time of sampling.[180]

In contrast, progesterone showed different results. While approximately 1.8 mg/kg of progesterone was found in 2023, the concentration in the same sample in 2025 was still about 1.77 mg/kg. Progesterone thus appears to persist longer in stable dust than altrenogest. The sample analysed in 2023 was BS3, collected directly from the farrowing crate bars of the sows. In order to maintain pregnancy, the corpus luteum of sows continuously produces progesterone. Accordingly, progesterone levels are elevated during the approximately 115 days of pregnancy, which also leads to increased progesterone excretion.[201] This could be a possible explanation for the progesterone levels found in each of the BS samples.

Samples BS7 and BS8 were also collected from the same young sow breeding stable, with BS7 taken from a windowsill and BS8 from beside the sow feed dispenser.

These samples were collected in 2021. In sample BS7, neither altrenogest, its cycloaddition product, nor progesterone could be detected. In BS8, concentrations of about 1 mg/kg of altrenogest and progesterone were found. The distribution of analyte concentrations within the stable may correlate with dust fluctuation, so a decrease in analyte concentration with increasing distance from the application site cannot be ruled out.

BS9 and BS10 also originate from the same facility, where the sows were actively treated with altrenogest. These samples were colected in 2009. Altrenogest could be detected in sample BS10 (1.7 mg/kg determined in 2021) but not in BS9. Sample BS9 is a sample that was treated at 105 °C for 150 minutes as part of a bacterial count test. This treatment may have led to the complete degradation of altrenogest. New investgations of these samples showed no concentration of altrenogest in any of these samples but in both samples (BS9 and BS10), progesterone was detected at concentrations of about 1.7 and 1.8 mg/kg, respectively. Therefore, progesterone seems to be persistent over a long period of time and even under heat treatment conditions.

Table 4.15: Concentrations in mg/kg of steroid hormones and coccidiostats in stable dust samples from breeding sow (BS) facilities. # indicates concentrations below CCβ. If standard deviations are given they rely on n = 2 otherwise, only single determination was possible. Detailed information about the sample origins can be found in section 5.3.4.

Dust Samples	Year	ALT	ALT TP	P	N	SAL	STA	βE2	E1	NIC
BS1	2023	<CCα	<CCα	0.64 ± 0.04		1.11 ± 0.07		<CCα	<CCα	
BS2	2023	<CCα		<CCα					<CCα	<CCα
BS3	2023	<CCα	<CCα	1.77 ± 0.16			<CCα	<CCα		<CCα
BS4	2023	<CCα	<CCα	0.40 ± 0.07$^{\#}$			<CCα		<CCα	0.46
BS5	2023	<CCα	<CCα	0.44 ± 0.29$^{\#}$			<CCα	<CCα		<CCα
BS6	2023			0.37 ± 0.07$^{\#}$				<CCα		
BS7	2021	<CCα	<CCα	<CCα						
BS8	2021	0.95	0.27	1.14						
BS9	2009			1.69 ± 0.03				<CCα		<CCα
BS10	2009		<CCα	1.79 ± 0.83	1.48			<CCα		<CCα

Another important aspect, especially regarding altrenogest analytics, is sample storage. After arrival at the laboratory, samples collected after 2022 were stored in the dark at 4°C in a refrigerator. Archive samples, however, were stored at room temperature but in the dark in the basement. Whether the samples were exposed to light or cooled before arrival at the institute, or how long the dust and its contained analytes had already been in the stable, cannot be conclusively clarified.

A further notable finding can be seen in sample BS1 (Table 4.15). Here, salinomycin was unequivocally detected at a concentration of approximately 1.1 mg/kg. Sample BS1 was collected in 2023 from ceiling pipes inside the stable. There are studies demonstrating that the use of salinomycin improves sow and piglet health status and performance, thereby enabling more efficient piglet production.[202], [203] However, the use of salinomycin is not permitted in the EU under Regulation (EU) 1831/2003. Salinomycin was only found in 1/6 samples from this stable and also in a location that is presumably inaccessible during cleaning processes. It is therefore likely to be related to older dust. As the use of salinomycin in breeding sows is prohibited, a possible explanation is that the origin of the salinomycin is either contamination of the feed or unapproved use.

Literature concerning the occurrence of coccidiostats and hormones in stable dust remains scarce. Nevertheless, preliminary data on the presence of coccidiostats and hormones in dust originating from animal feed yards are already available. McEachran et al. detected monensin in dust collected from beef cattle feed yards at concentrations of 1.8 ± 0.37 mg/kg.[158] In the United States, monensin is administered as a feed additive in cattle to prevent coccidiosis and to enhance feed efficiency and the rate of weight gain. Although the concentrations used in feed for beef cattle (5 - 40 mg/kg) are lower than the permitted concentrations in feed for broilers in the European Union (100 - 125 mg/kg feed), the total quantities applied are considerably higher, which may result in substantially elevated monensin concentrations in dust. Due to wind dispersion, comparable concentrations to those found in the present study are ultimately observed. Wooten et al. reported monensin concentrations of up to 37 mg/kg in dust collected in close proximity to feed yards indicating a reduced dilution due to wind dispersion.[204]

Blackwell et al. identified hormonal residues in dust samples from beef cattle feed yards, detecting α-estradiol and estrone at concentrations of 0.02 and 0.01 mg/kg, respectively. Megestrol acetate and trenbolone were also measured, at concentrations of 1.3 and 1.9 ng/g, respectively.[194] Trenbolone and estradiol are, for instance, often administered in combination as implants to promote weight gain and to improve feed efficiency. The concentrations observed by Blackwell et al. were markedly

lower than those found in the present study. This may partly be attributed to the fact that, as implant-based applications, these hormones do not directly enter the dust matrix. Moreover, the authors emphasised the strong dependence of measured concentrations on wind direction and the orientation of the sampling sites. While the dusts analysed in the present work were collected inside stables with controlled ventilation systems, the samples analysed in the studies by McEachran, Wooten, and Blackwell originated from open feed yard environments. The lower concentrations reported in those investigations can therefore be ascribed to extensive dilution resulting from wind-driven dispersion.

As dust accumulates over time in the laboratory, this dust was also analysed for coccidiostats and steroid hormones to determine whether possible contamination of the laboratory air due to handling stable dust had occurred. But analyte concentrations (DEC, NAR, SAL, T, α- and β-E2, E1, E3, and E3S) detected in the laboratory dust sample were all far below the CCα.

4.6 Conclusion and Risk Assessment

The examination of various dust samples from different animal species and husbandry systems, and locations within individual facilities as well as across different facilities for steroid hormones and coccidiostats provides further insight into a matrix whose effects on the environment and organisms therein still contain numerous unknown variables.

Coccidiosis is a parasitic infectious disease of the intestinal tract caused by various protozoans such as Eimeria, Isospora, and Cryptosporidium of the phylum Apicomplexa. Due to its widespread prevalence, coccidiosis remains one of the major problems of the poultry industry, causing subclinical infections, intestinal lesions, and even death of infected animals. Additionally, coccidiosis facilitates epidemic diseases such as mycoplasmosis and colibacillosis, for example.[205] The economic damage is estimated at several billion euros annually.[43]

To control coccidiosis, the application of natural and synthetic coccidiostats such as monensin and decoquinate has been established. However, extensive use and misuse of coccidiostats have led to the development of drug-resistant strains, necessitating the implementation of rotation and shuttle programs.[205], [206] Investigations of dust samples from broiler chicken facilities have demonstrated that coccidiostats, which are administered via feed, accumulate at the highest concentrations in the dust near the feeding station but are distributed throughout the entire facility via air circulation, animal and human movement. In total,

coccidiostats could be detected in all investigated dust samples from broiler chicken facilities at concentrations in the mg/kg range. Results from dust samples from broiler chicken and laying hen facilities from the early 1980s and 1990s demonstrate that coccidiostats entering the dust are persistent and remain detectable in dust over decades at concentrations in the upper µg/kg range. Additionally, coccidiostats such as monensin could be detected despite their application likely dating back several cleaning cycles. Since large portions of dust originate from feces and thus most likely contain oocysts of coccidia, these come into contact with subtherapeutic doses of coccidiostats, promoting the development of resistant strains.

Hormones, particularly steroid hormones, are endogenous substances essential for regulating diverse biochemical processes in animals and humans. The targeted application of natural and synthetic hormones such as testosterone and altrenogest intervenes in these processes to increase muscle mass build-up (outside the EU), for example, or to synchronise estrus between multiple animals. These applications aim to optimise work processes resulting in increased production efficiency. Ultimately, both endogenously produced and exogenously administered steroid hormones are metabolised and excreted by the body, both in their original and metabolised forms, upon completion of their function. In this manner, steroid hormones consequently also can get into stable dust. The investigations conducted in the course of this work demonstrated that progesterone in particular can be present in stable dust at mg/kg concentrations and distributes throughout the entire facility. The synthetic steroid hormone altrenogest and its transformation product (ALT CAP) were also detectable in stable dust. However, due to its low stability, in investigations conducted on the same samples up to four years after sample collection, only concentrations <CCα - 1 mg/kg were found. Preliminary investigations, conducted shortly after sample collection, however, revealed significantly higher concentrations.

The background of this work was, on the one hand, the assessment of a possible health hazard posed by coccidiostats and hormones present in stable dust and, on the other hand, to determine whether dust represents a new entry route for these compounds into the environment. The qualitative detection of coccidiostats and steroid hormones in stable dust suggests that these compounds enter the environment via dust through ventilation or cleaning procedures and distribute therein. When ionophore coccidiostats such as salinomycin and narasin reach soil, their sorption is strongly dependent on the pH value and the resulting cations present. Nicarbazin, however, exhibits strong affinity to various soils. According to current studies summarised in various EFSA dossiers, the predicted environmental concentration (PEC) of the coccidiostats are only partially below the respective predicted no effect concentration (PNEC) for the respective animals in the corresponding environmental compartments (see Table 4.16).

Table 4.16: PEC/PNEC ratios for different coccidiostats in different taxa. Aquatic organisms include aquatic invertebrates, algae, and fish.

Compound	**Taxa**	**PEC/PNEC**	**Reference**
Monensin	Earthworm	0.02	[207]
	Plants	0.56	
	Aquatic organisms	0.86	
Lasalocid	Earthworm	0.05	[208]
	Plants	0.22	
	Aquatic organisms	0.09	
Narasin	Earthworm	0.20	[200]
	Plants	0.65	
	Aquatic organisms	0.30	
Salinomycin	Earthworm	0.06	[198]
	Plants	1.60	
	Aquatic organisms	0.06 – 0.23	
Nicarbazin (DNC)	Earthworm	0.02	[199]
	Plants	0.80	
	Aquatic organisms	4.70	

If the PEC exceeds the PNEC, their ratio is greater than 1 and a possible environmental risk caused by the corresponding substance can be assumed. For these substances, a risk assessment and appropriate measures must be established. On this basis, environmental hazard from coccidiostats cannot be excluded without exception, particularly with regard to 4,4'-dinitrocarbanalide (DNC) on aquatic invertebrates, algae, and fish.

The situation differs for steroid hormones. In particular, synthetic steroid hormones such as altrenogest, which possess a substantially lower effect concentration compared to their natural analogs such as progesterone.[209] The CVMP established PECs for altrenogest at 0.013 µg/kg in soil and 16 - 219 pg/L in surface water. For the synthetic steroid hormone altrenogest, a PNEC of 0.04 ng/L in surface water was estimated, resulting in PEC/PNEC ratios of >1.[210] Accordingly, environmental hazard from steroid hormones released into the environment must be assumed. That hormones released into the environment ultimately have negative effects on the environment and organisms therein has already been demonstrated in several studies using endogenously produced and exogenously administered steroid hormones from human pharmaceutical preparations.[211] In this context, the assessment of negative effects on the environment and organisms therein is complicated not only by the complexity of environmental compartments and the occurrence of synergistic and antagonistic effects (also with metabolites and transformation products of hormones),[33], [212], [213] but also often by the lack of clearly determinable toxicological endpoints to enable understanding of influences on organisms. In the case of estrogens the biomarker vitellogenin is used, which exhibits significant concentration changes in fish upon elevated estrogen exposure. For androgens, gestagens, glucocorticoids, and mineralocorticoids, however, these clear toxicological endpoints are still missing.[214]

To date, livestock manure (produced at over one billion tonns annualy worldwide [77], [78]) is considered the largest entry pathway for diverse veterinary pharmaceutical residues into the environment. However, the concentrations of coccidiostats and steroid hormones determined in this study suggest that stable dust also represents a relevant aspect in the discussion of environmental entry pathways.

Investigations by Takai et al. revealed a concentration of inhalable dust averaging 3.6 mg/m^3 in poultry buildings and 2.19 mg/m^3 in pig buildings across several European countries.[84] Using the 3.6 mg/m^3 dust concentration and considering the respiratory minute volume of a human under moderate physical activity, the amounts of coccidiostats and hormones inhaled following an 8-hour work day can be calculated (see Table 4.17).

Table 4.17: Maximum and minimum concentrations and corresponding inhaled analyte amounts in a 8 h workingday for different compounds. For the calculations, a respiratory minute volume during moderate physical activity of 4.75 (Max.) and 0.375 (Min.) m^3/h was used.[215] The maximum concentration of altrenogest in dust was derived from preliminary investigations.

Compound	**Concentration [mg/kg]**		**Inhaled analyte [µg/8 h]**	
	Max.	**Min.**	**Max.**	**Min.**
Narasin	15.72	0.36	2.2	0.004
Salinomycin	1.10	0.50	0.2	0.005
Nicarbazin	11.50	3.80	1.6	0.04
Monensin	0.41	0.00	0.1	0.0
Progesterone	2.62	0.30	0.4	0.003
Altrenogest	6.00	0.95	0.8	0.01
Nandrolone	2.21	1.48	0.3	0.02

Although the ADI-values listed in Table 1.2 in the introduction are not exceeded, preliminary investigations nevertheless demonstrated a persistence of coccidiostats exceeding 30 years in stable dust. Humans who accordingly work in pig and poultry confinement buildings over a similarly long time period are exposed daily over decades to subtherapeutic doses of various coccidiostats and hormones such as progesterone. In the case of coccidiostats, this can lead to histopathological changes such as peripheral neuropathies and focal muscle degenerations.[216] As the investigations presented here demonstrate, the analytes are not homogeneously distributed in the dust. Accordingly, "hot spots" can occur and it cannot be excluded that workers handling coccidiostats may exceed the ADI. In particular, direct contact with feed additives can result in inhalatory damage due to inhaled feed dust.[198]–[200], [207] Long-term exposure to steroid hormones at concentrations listed in Table 4.17 can alter physiological response and may result in reproductive disorders and depression of spermatogenesis.[217] Additionally, endotoxins, bacteria, and antibiotics have already been detected in stable dust. The results presented in this work thus demonstrate that coccidiostats and steroid hormones represent additional factors with regard to health hazards and may possibly act synergistically with the already demonstrated contaminants to burden the body.[92], [93], [96], [218]

The results of this study should therefore provide the impetus for further structured investigations of stable dusts, thereby establishing a foundation for subsequent discussions on appropriate measures in the areas of cleaning management and occupational safety contributing to the one health approach.

5 Material and Methods

Contents

5.1 Chemicals

Table 5.1: Standards of Coccidiostats used in this study. a.s = analytical standard, referring to the high purity Vetranal® line from Sigma-Aldrich

Compound	Purity	Manufacturer	Batch No.
Decoquinate	97.1 %	TargetMol Wellesley Hills, USA	113288
Diclazuril	>98 %	LKT Laboratories, Inc. St. Paul, USA	2599714
Halofuginone	>99 %	Selleck Chemicals Houston, USA	S8144
Lasalocid A sodium	>95 %	Sigma-Aldrich St. Louis, USA	73996-5MG

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Table 5.1 (Continuation)

Compound	Purity	Manufacturer	Batch No.
Maduramicin ammonium	a.s.	Sigma-Aldrich St. Louis, USA	BCBZ3261
Monensin sodium	>98 %	LKT Laboratories, Inc. St. Paul, USA	2597325
Narasin	98 %	Sigma-Aldrich St. Louis, USA	148476
Nicarbazin	a.s.	Sigma-Aldrich St. Louis, USA	10654285
Robenidine HCl	a.s.	Sigma-Aldrich St. Louis, USA	33979-100MG-R
Salinomycin	>90 %	Cayman Chemical Ann Arbor, USA	0615398-25

Table 5.2: Standards of steroid hormones used in the study. a.s = analytical standard, referring to the high purity Vetranal®line from Sigma-Aldrich

Compound	Purity	Manufacturer	Batch No.
Altrenogest	99.9%	Sigma-Aldrich St. Louis, USA	SZBD099XV
Estradiol	a.s.	Sigma-Aldrich St. Louis, USA	BCBT5161
Estradiol hemihydrate	99.7%	Sigma-Aldrich St. Louis, USA	SZBE203XV
Estriol	a.s.	Sigma-Aldrich St. Louis, USA	SZBE083XV
Estrone	a.s.	Sigma-Aldrich St. Louis, USA	SZBE205XV
Estrone-3-sulfate	>95 %	Toronto Research Chem. Toronto, Canada	10-NSR-1-1
Ethinylestradiol	>99 %	EDQM Strasbourg, France	3.1
Medroxyprogesterone acet.	a.s.	Sigma-Aldrich St. Louis, USA	BCBS8482V
Megestrol acetate	>97 %	MP Biomedicals Santa Ana, USA	SR01916
Methyltestosterone	>98.0%	Tokyo Chemical Ind. Tokyo, Japan	QX6ID-NN
Nandrolone	a.s.	Sigma-Aldrich St. Louis, USA	BCCC6108
Progesterone	99.5%	Sigma-Aldrich St. Louis, USA	SZBE164XV

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Table 5.2 (Continuation)

Compound	Purity	Manufacturer	Batch No.
Stanozolol	a.s.	Sigma-Aldrich St. Louis, USA	SLBN8458V
Testosterone	99.5%	Sigma-Aldrich St. Louis, USA	BCBS8823V
Trenbolone	>93 %	Sigma-Aldrich St. Louis, USA	SLCC7819

Table 5.3: Chemicals and reagents used in this study.

Chemical	Specifications	Manufacturer
2-Propanol	LC–MS grade	TH Geyer Renningen, Germany
Acetone(AnalaR NORMAPUR)	HPLC grade	VWR International Rosny-sous-Bois Cedex, France
Acetonitrile	LC–MS grade	TH Geyer Renningen, Germany
Formic acid	99%, LC–MS grade	SERVA Electrophoresis Heidelberg, Germany
Ammoniasolution (25%, LiChropur)	LC–MS grade	Merck Darmstadt, Germany
Ammonium acetate	–	Merck Darmstadt, Germany
Bondesil-C18	40 µm	Agilent Technologies Santa Clara, USA
Bondesil-PSA	40 µm	Agilent Technologies Santa Clara, USA
Ethanol(ROTISOLV®)	–	Roth Karlsruhe, Germany
Methanol	LC–MS grade	TH Geyer Renningen, Germany
n-Hexane	LC–MS grade	VWR International Rosny-sous-Bois Cedex, France

5.2 Equipment

Table 5.4: Instruments and materials used in this study.

Instrument / Material	Specifications	Manufacturer
Adsorbent	Supaclean™ LC-Florisil®	Sigma-Aldrich St. Louis, USA
Analytical balance	XA105 DualRange	Mettler Toledo Gießen, Germany
Balance	Atilon ATL-6292-I	Acculab Sartorius Group
Centrifuge	Universal 320 R	Hettich Zentrifugieren Tuttlingen, Germany
Centrifuge	Heraeus™ Megafuge™ 16R	Thermo Fisher Scientific Karlsruhe, Germany
Centrifuge tubes	Cellstar® 15 mL, PP, conical	TH Geyer Renningen, Germany
Disposable syringes	Omnifix® 3 mL	B. Braun Melsungen, Germany
Evaporation system	Techne™ Sample Concentrator SBHCONC/1	Thermo Fisher Scientific Karlsruhe, Germany
Evaporation system	Stuart™ Block Thermostat DB-3	Thermo Fisher Scientific Karlsruhe, Germany
HPLC column	XBridge™ C18 BEH, 2.5 µm (3.0 × 75 mm)	Waters Milford, USA
HPLC system	ExionLC™	AB Sciex Framingham, USA
HPLC system	UltiMate™ 3000 Rapid Separation (RS)	Thermo Fisher Scientific Sunnyvale, USA
Mass spectrometer	3200 QTRAP®	AB Sciex Framingham, USA
Mass spectrometer	4500 QTRAP®	AB Sciex Framingham, USA
Microliter syringes	Series 1000 TLL/TLLX, gastight (Type 1001 TLL)	Hamilton Bonaduz, Switzerland
Needles	0.80 × 120 mm	B. Braun Melsungen, Germany
Overhead shaker	Reax 2	Heidolph Schwalbach, Germany
pH electrode	SenTix® 41	Xylem Analytics Wertheim, Germany

Continued on next page

Table 5.4 (Continuation)

Instrument / Material	Specifications	Manufacturer
pH meter	InoLab® pH 730	Xylem Analytics Wertheim, Germany
Pipette tips	epT.I.P.S.® Standard (various sizes)	Eppendorf Hamburg, Germany
Pipettes	20, 100, 200 µL; 1 and 5 mL	Eppendorf Hamburg, Germany
Pipettes	1–10 mL	Brand Wertheim, Germany
Screw caps	N9 PP, hardness 55°, thickness 1 mm	–
Screw vials	1.5 mL, N9	TH Geyer Renningen, Germany
SPE cartridges	CHROMABOND® HR-X	MACHEREY-NAGEL Düren, Germany
SPE cartridges	CHROMABOND® SiOH	MACHEREY-NAGEL Düren, Germany
Syringe filters	Chromafil® PET-20/15 MS (0.20 µm)	MACHEREY-NAGEL Düren, Germany
Ultrasonic bath	USC300TH	VWR® Leuven, Belgium
Ultrapure water system	Milli-Q® / Q-Pod®	Merck Darmstadt, Germany
Vortex mixer	–	VWR® Darmstadt, Germany
Weighing boats	PS, black, 5 mL	TH Geyer Renningen, Germany

5.3 Stable dust

5.3.1 Sample Collection

For the experiments conducted, stable dust samples from the stable dust archive of the Justus Liebig University in Giessen were utilised. The dust collection was initiated during the period 1980–2009 at the Veterinary University Hanover by Professor Dr. Jörg Hartung and comprises over 900 stable dust samples from pig, cattle, and poultry holdings. Dust sampling was performed in the respective livestock facilities via sedimentation. A standardised metal frame with an effective sampling area of 3.002 cm^2, covered with aluminium foil, was employed. The sampling frame was positioned at approximately the mid-point of the stable at

a height of 1.5 m, corresponding to the typical inhalation height, for 14 to 30 days. Following this period, the sedimented dust sample was transferred from the aluminium foil into a glass container and sealed tightly; prior to sealing, residual dead insects, spiders, and coarse dust particles originating from the ceiling were removed.[93] Samples collected using these sampling technique are labelled as ST1.

LH samples, collected in 2025 were shoved into a polypropylene centrifuge tube using gloves, directly from surfaces inside the stables. This dust was also assumed to be sedimentation dust. These samples are labelled with ST2. For all other samples, exact sampling techniques are not available.

5.3.2 Statistical Investigation of Dust Compositions

Comparison of Weender Analysis Parameters by Species

Dust composition parameters from Weender analysis were compared across animal species using linear mixed effects models implemented in R (version 4.5.1) with the lme4 package.[219] Each parameter was modelled as:

```
Parameter ~ Animal Species + (1 | Farm Location)
```

where animal species was included as a fixed effect and farm location as a random intercept to account for site-specific variation. Pairwise comparisons between animal species were conducted using the emmeans package with Tukeys test. The significance level was set at $\alpha = 0.05$.

Assumptions of normality and homogeneity of variance were evaluated via Q–Q plots and visual inspection. Due to small sample sizes (n = 8–31 across groups) and the presence of zero values for certain parameters, normal distribution was assumed based on the central limit theorem. Results were visualised using boxplots with individual data points and pairwise significance indicators generated using ggplot2 and ggpubr packages.[220], [221]

Comparison of Weender Analysis Parameters by Location

To investigate whether significant differences existed in dust composition across farm locations, linear mixed effects models were employed as well. For each of the six Weender analysis parameters (dry matter, crude ash, crude protein, crude fat, crude fibre, and nitrogen-free extract) a separate linear mixed effects model was fitted according to the following specification:

```
model <- lmer(value ~ Location + (1 | Year), data = subdata)
anova_res <- anova(model)
emmeans_res <- emmeans(model, pairwise ~ Location, adjust = "bonferroni")
```

Pairwise comparisons between farm locations were conducted using the emmeans package with Bonferroni adjustment for multiple comparisons.[222] In total four different locations were analysed with n = 31 (O1), n = 23 (O2), n = 17 (O3) and n = 19 (O4)

5.3.3 Pooled Dust Sample

All experiments conducted for the development and optimisation of the extraction method were performed using a pooled dust sample. As the sample material from archived dusts was available in limited quantities, a composite sample was prepared from a variety of individual samples. In this manner, all optimisation experiments could be performed on a single matrix and were therefore comparable without depleting any individual sample. The composite sample consisted of varying proportions of both poultry house and pig house dust. Consequently, during method optimisation, all matrix components could be addressed simultaneously. Following composite preparation, the sample was homogenised using a mill. Table 5.5 lists the detailed composition of the composite dust sample:

Table 5.5: Pooled dust sample composition. R/V: Rethmer/Voigtländer.

Box	Sample No.	Species	Housing type / stable type	Sampling site	Date	Mass [g]
2	62	MS	n/a	R/V	21.03.83	2.10
2	115	MS	Stable I finishing	R/V	17.12.84	2.68
2	89	MS	Stable I finishing	R/V	27.12.83	2.88
2	73	MS	Stable III	R/V	30.06.83	3.63
2	97	MS	Stable I finishing	R/V	26.03.83	2.77
2	110	MS	Stable I finishing	R/V	16.10.84	1.01
2	114	MS	Stable I finishing	R/V	04.12.84	5.98
3	116	MS	Stable III finishing	R/V	04.12.84	5.98
3	129	MS	Stable I finishing	R/V	15.01.84	6.79
3	156	MS	Stable I finishing	R/V	08.01.85	10.28
3	132	MS	Stable I finishing	R/V	29.01.85	7.53
8	432	MS	Stable I finishing	R/V	25.10.93	2.60
8	423	MS	Stable I finishing	R/V	18.01.93	4.74

Continued on next page

Table 5.5 (Continuation)

Box	Sample No.	Species	Housing type / stable type	Sampling site	Date	Mass [g]
8	395	MS	Stable I finishing	R/V	11.05.92	5.50
4	172	MS	Stable III finishing	R/V	26.03.85	9.76
4	177	MS	Stable III finishing	R/V	23.04.85	10.65
4	200	MS	Stable III finishing	R/V	29.10.85	11.23
4	190	MS	Stable III finishing	R/V	06.08.85	11.42
4	173	MS	Stable III finishing	R/V	26.03.85	10.23
5	264	MS	Stable III finishing	R/V	14.04.86	10.14
5	242	MS	Stable III finishing	R/V	14.04.86	11.95
5	257	MS	Stable III finishing	R/V	17.11.86	9.97
14	691	MH	Lousiana barn	B/V	07.10.94	1.11
14	686	MH	Lousiana barn	B/V	17.08.94	1.02
14	669	LH	Stable II	Ruthe	16.05.83	2.23
14	663	LH	Stable II	Ruthe	05.04.83	1.85
14	668	LH	Stable II	Ruthe	16.05.83	1.01
14	673	LH	Stable II	Ruthe	13.06.83	1.15
14	665	LH	Stable I left	Ruthe	02.05.83	1.50
16	734	LH	Outdoor housing	3185	2001	1.75

5.3.4 Information about Archived and New Samples

Table 5.6: Overview of all Samples investigated in this study including sampling year, housing system, identification (ID), sampling technique (ST) and further informations about sampling location inside the stable or special sample treatment. ID represents the Name of the farm owner or the first two digits of the postal code. The number in brackets indicates that these samples were collected at the same farm. TiHo: University of Veterinary Medicine Hannover; R/V: Rethmer/Voigtländer *according to current regulations [196].

Name	Year	Farming system	ID	ST	Informations
LH1	1981	n.a.	Ruthe	ST1	
LH2	1982	n.a.	Ruthe	ST1	

Continued on next page

Table 5.6 (Continuation)

Name	Year	Farming system	ID	ST	Informations
LH3	1983	Stable I	Ruthe	ST1	
LH4	2002	Cage housing	Ruthe	ST1	
LH5	2003	Cage housing	Ruthe	ST1	
LH6	2004	Aviary	Ruthe	ST1	
LH7	2025	Floor housing	47 (3)	ST2	Exhaust fan
LH8	2025	Floor housing	47 (3)	ST2	Supply air fan
LH9	2025	Floor housing	47 (3)	ST2	Surface above perches
LH10	2025	Floor housing	47 (3)	ST2	Container for shell grid
LH11	2025	Floor housing	47 (3)	ST2	Feed supply tubes
LH12	2025	Mobile outdoor	53 (4.1)	ST2	Water pipe
LH13	2025	Mobile outdoor	53 (4.2)	ST2	Water pipe
LH14	2025	Mobile outdoor	53 (4.2)	ST2	Surface above feeding station
LH15	2025	Mobile outdoor	53 (4.1)	ST2	Surface above feeding station
LH16	2025	Mobile outdoor	47 (2)	ST2	Window sill
LH17	2025	Mobile outdoor	47 (2)	ST2	Surface above perches
BC1	1992	Lousiana	Verden	ST1	
BC2	1993	Lousiana	Verden	ST1	
BC3	1994	Lousiana	Verden	ST1	
BC4	2025	Housing level 3*	31 (5)	n.a.	Surface radiant heater
BC5	2025	Housing level 3*	31 (5)	n.a.	
BC6	2025	Housing level 3*	31 (5)	n.a.	
BC7	2025	Housing level 3*	31 (5)	n.a.	
BC8	2025	Housing level 3*	31 (5)	n.a.	
BC9	2025	Housing level 3*	31 (5)	n.a.	
BS1	2023	n.a.	TiHo	n.a.	Ceiling calbes
BS2	2023	n.a.	TiHo	n.a.	Feeding station
BS3	2023	n.a.	TiHo	n.a.	Gestation crate
BS4	2023	n.a.	TiHo	n.a.	Feeding station
BS5	2023	n.a.	TiHo	n.a.	Feed pipes
BS6	2023	n.a.	TiHo	n.a.	Feeding station
BS7	2021	n.a.	TiHo	n.a.	
BS8	2021	n.a.	TiHo	n.a.	
BS9	2009	n.a.	TiHo	n.a.	Treated: 105°C for 150 min
BS10	2009	n.a.	TiHo	n.a.	
MS1	1980	n.a.	R/V	ST1	
MS2	1981	n.a.	R/V	ST1	
MS3	1982	n.a.	R/V	ST1	
MS4	1983	n.a.	R/V	ST1	
MS5	1984	Stable III finishing	R/V	ST1	

Continued on next page

Table 5.6 (Continuation)

Name	Year	Farming system	ID	ST	Informations
MS6	1985	Stable III finishing	R/V	ST1	
MS7	1985	Stable III finishing	R/V	ST1	
MS8	1999	Stable I finishing	R/V	ST1	
MS9	2009	Finishing	n.a.	n.a.	Treated: 105°C for 150 min
MS10	2009	Finishing	n.a.	n.a.	
O1 (LH)	2025	Mobile outdoor org.	47 (1)	ST2	Surface outside the stable
O2 (BC)	2025	Mobile outdoor org.	47 (1)	ST2	Wooden planks inside the barn
O3 (BC)	2025	Mobile outdoor org.	47 (1)	ST2	Lamp surfaces
O4 (BC)	2025	Mobile outdoor org.	47 (1)	ST2	Surface on top of the door.

5.4 Methods

5.4.1 Ionspecies Detection

To select the appropriate ion species as precursor ions for the final MRM method, the analytes were prepared at a concentration of 2 µg/mL in the corresponding matrix. The matrix used was: (1) MeOH:H_2O (80:20, v:v) with 2 % formic acid for analysis in ESI^+ mode, and (2) MeOH:ACN:H_2O (15:15:70, v:v:v) with ammonia solution (pH 10) for analysis in ESI^- mode.

The samples were injected via direct infusion using a Harvard syringe with a diameter of 4.6 mm at a flow rate of 20 µL/min. The scan range was m/z 100–400 for steroid hormones and m/z 300–1000 for coccidiostats. The mass spectrometer used was a Sciex 3200 QTRAP.

5.4.2 Mobile Phase Composition Test

For investigations regarding the effect of the additives of the mobile Phase the following conditions were tested. For ESI^+ mode: (1) 0.1 % formic acid, (2) 1 % formic acid and (3) a 5 mM ammonium formate buffer adjusted to pH 3. And for ESI^- mode: (1) an aqueous ammonia solution at pH 10 and (2) a 5 mM ammonium acetate buffer (adjusted to pH 10). 10 µL of a solvent standard containing all analytes at a concentration of 100 µg/L was injected using a Sciex Exion LC connected to a QTRAP 4500 mass spectrometer from AB Sciex. The methods used were the final MRM-methods described in 5.4.3.

5.4.3 Final LC-MS Method

Positive Ionisation Mode Conditions

For ESI^+-mode, eluents were Eluent A: H_2O + 0.1 % formic acid and Eluent B: MeOH:ACN (80:20 v:v) 0.1 % formic acid. Flow rate was set to 500 µL/min. The Gradient is shown in Table 5.7.

Table 5.7: Gradient program ESI^+.

Time [min]	Eluent A [%]	Eluent B [%]
0.0	60	40
11.0	0	100
15.0	0	100
16.0	60	40
18.0	60	40

Negative Ionisation Mode Conditions

For ESI^--mode, eluents were Eluent A: H_2O + NH_3 pH 10 Eluent B: MeOH:ACN (50:50 v:v). Flow rate was set to 300 µL/min. The Gradient is shown in Table 5.8.

Table 5.8: Gradient program for ESI^+.

Time [min]	Eluent A [%]	Eluent B [%]
0.0	80	20
1.0	80	20
12.0	0	100
15.0	0	100
15.1	80	20
17.0	80	20

For both modes a XBridge™ C18 BEH, 2.5 µm (3.0 × 75 mm) was used for the separation. The sample plate temperature was set to 10 °C, the column oven temperature was set to 30 °C and the ion source temperature to 500 °C. Accordingly, gas 1 and gas2 were set to 55 and 60 respectively. The mass spectrometry conditions are summarised in Table 4.3 and 4.4. A dwell time of 50.0 milliseconds was selected for each analyte. Sample injection volume was set at 10 µL. The batch was structured to include a solvent blank (starting composition of the respective eluent) after every fourth real sample. The data was analysed using MultiQuant 3.0 software from sciex. Peaks of all samples and solvent blanks were integrated. After integration, areas of analytes, found in the blank samples were averaged and subtracted from the areas of the respective analyte found in the real samples.

5.4.4 SPE Methods

All experiments conducted to evaluate and select the most appropriate SPE material for matrix effect reduction were performed in duplicate. For each experiment, two aliquots of the homogenized pooled dust sample were spiked with the analyte mixture either prior to extraction or after completion of the extraction procedure and syringe filtration. In parallel, two additional aliquots were processed without spiking to serve as matrix blanks. Spiking prior to extraction was accomplished by adding 100 µL of a $1{\cdot}10^3$ µg/L methanolic standard solution containing all target analytes to the respective aliquots. The spiked samples were dried under a steam of nitrogen at temperatures 30 °C prior to further processing. Depending on the experimental procedure, the theoretical post-extraction analyte concentration was calculated to ensure accurate spiking of the final extracts. The final, dried extracts were dissolved in 1 mL of H_2O:ACN:MeOH (50:25:25,v:v:v) and vortexed for five minutes each and then subjected to ultrasonic treatment for five minutes. Subsequently, the extract solutions were filtered using a syringe filter (diameter of 15 mm) and transferred to an LC vial.

Liquid-Solid Extraction

The evaluation of different SPE materials was conducted prior to the full optimisation of the LSE protocol. At this stage, a modified version of the LSE procedure was employed: 0.10 ± 0.01 g of the pooled dust sample was weighed into a 15 mL polypropylene centrifuge tube and spiked with 100 µL of the methanolic standard. The samples were homogenized and dried overnight under a gentle stream of nitrogen in order to promote analyte adsorption onto the dust matrix. Subsequently, each sample was extracted with 4 mL methanol and 1 mL hexane, followed by overhead shaking for 60 min. After centrifugation (5 min, 4500 rpm), 4 mL of the supernatant was transferred to a further 15 mL centrifuge tube. With the exception of the experiments for the dSPE method, the extracts were dried under a stream of nitrogen at temperatures below 30 °C prior to further processing.

This LSE protocol was also used for the screening experiments, but taking into account the correspondingly varied parameters. Another deviation was the quantitative removal of the extraction solvent at the end of each cycle.

dSPE Method using PSA and C18

Four millilitres of the supernatant obtained after extraction were transferred to a 15 mL centrifuge tube preloaded with 40 ± 1.0 mg PSA and 60 ± 1.0 mg C18 sorbent. PSA is an alkylated amine sorbent that containing primary and secondary amines. The samples were vortexed for five minutes and centrifuged (5 min, 4500 rpm). Thereafter, 3.5 mL of the resulting supernatant were transferred into fresh 15 mL centrifuge tubes and evaporated to dryness under a gentle stream of nitrogen at temperatures below 30 °C.

SPE Method using Florisil

The samples were reconstituted in 5 mL of n-hexane:dichloromethane (95:5, v:v). Subsequently, the samples were vortexed for five minutes and then subjected to ultrasonic agitation for five minutes. The Florisil cartridges were conditioned twice with 5 mL of n-hexane:dichloromethane (95:5, v:v) and subsequently loaded with the sample solution. The sorbent material was dried both prior to and after washing of the Florisil cartridges with 5 mL of n-hexane:dichloromethane (60:40, v:v). Elution of the analytes was performed twice with 2.5 mL of ACN:n-hexane (60:40, v:v). The eluates were combined and evaporated to dryness overnight under a gentle stream of nitrogen at temperatures below 30 °C. The experimental procedure was adapted from the study by Blackwell et al.[157]

SPE Method using SiOH

The glass cartridge containing SiOH sorbent were initially dried in an oven at 100 °C for two hours and then cooled in a desiccator. This was necessary because of the hygroscopic nature of SiOH, whereby heating causes the evaporation of water molecules that had previously occupied some of the SiOH binding sites. After LSE, the samples were reconstituted in 10 mL of ethanol. The samples were subsequently vortexed for five minutes and subjected to ultrasonic treatment for an additional five minutes. In this method, the SiOH cartridge functioned as a filter. It was conditioned initially with 5 mL of ethanol prior to loading the sample solution. The eluate from the sample solution was collected directly. The SiOH cartridge was washed with 2 mL of ethanol and subsequently with a mixture of 6 mL of ethanol:H_2O (90:10, v:v) amended with 0.25 mL of a 25 % ammonia solution, with the eluates being collected as well. The combined eluates were evaporated to dryness under a gentle stream of nitrogen at temperatures below 30 °C. The experimental procedure was adapted from the study by Moretti et al.[175]

SPE Method using HR-X Cartridges

the samples were reconstituted in 4 mL of acetonitrile and 3.6mL of water. The samples were subsequently vortexed for 5min and subjected to ultrasonic treatment for an additional 5min. Conditioning of the HR-X cartridges was performed using 4 mL of ACN, followed by 4mL of water. The sample solutions were then loaded onto the HR-X cartridges, after which the cartridges were briefly dried under vacuum. The HR-X cartridges were subsequently washed with 4 mL of methanol:H_2O (1:20, v:v) and dried again. Elution of the analytes was accomplished using 5 mL of methanol. Finally, the eluate was evaporated to dryness under a stream of nitrogen at temperatures below 30 °C.

SPE Method using SiOH and HR-X Cartridges

Due to the limited number of silica gel glass cartridges, non spiked samples were not used when testing the combination of HR-X and SiOH. The SiOH containing glass cartridge was dried in an oven at 100 °C for 2 hours and subsequently cooled in a desiccator. The samples were reconstituted in 4 mL of n-hexane:acetone (3:7, v:v). The samples were subsequently vortexed for five minutes and subjected to ultrasonic treatment for an additional five minutes. In the meantime, the silica gel glass cartridge was conditioned with 4 mL of n-hexane/acetone (3:7, v:v) and subsequently loaded with the sample solution. Elution of the analytes was performed using 4 mL of n-hexane:acetone (7:3, v:v). The eluate was then evaporated to dryness under a stream of nitrogen at temperatures below 30 °C and stored overnight in a refrigerator.

The sample residue was reconstituted in 0.4 mL of ACN and vortexed for 5 minutes. Subsequently, 3.6 mL of water was added to the sample solution, and the mixture was vortexed for five minutes and subjected to ultrasonic treatment for an additional five minutes. The HR-X cartridge was conditioned with 4 mL of ACN, followed by 4 mL of water. The HR-X column was subsequently loaded with the sample solution and dried under vacuum for approximately 5 minutes. The HR-X cartridge was washed with 4 mL of methanol/water (1/20, v/v) and dried again under vacuum for approximately 5 minutes. Elution was accomplished using 5 mL of methanol. The eluate was subsequently evaporated to dryness under a stream of nitrogen at temperatures below 30 °C overnight.

5.4.5 DoE Screening

The design matrix for the screening experiments was a designated one-half fraction design with

$$2^{k-p} = 2^{5-1} = 16 \ \ Experiments \tag{5.1}$$

Where:

- k = Number of factors
- p = Number of times the design was divided in half

Each set of experiments consisted of three aliquots of 0.1 g ± 0.01 g of the pooled dust sample (see Section 5.3.3). One aliquot was spiked with 100 µL of a 1 mg/L stock solution containing all target analytes dissolved in methanol. The methanol was subsequently removed under a gentle stream of nitrogen. All three aliquots were then processed identically according to the protocol outlined in section 5.4.4. Following syringe filtration, one aliquot was additionally spiked with 100 µL of the 1 mg/L stock solution. The third aliquot remained unspiked and served as the matrix blank. Using a one-half fraction design results in a design resolution of V meaning no main effects or two-factor interaction are confounded with other main effects or two-factor interactions. All factors listed in Table 4.5 were coded to standardized units (-1, +1) to render effects directly comparable and to ensure the validity of the analysis of variance (ANOVA) procedure. The complete experimental design matrix is listed in Table 5.9.

Table 5.9: Coded design matrix for the screening experiments.

Nr.	Solvent composition	Volume	Cycles	Time	Acid
1	1	-1	1	-1	-1
2	-1	-1	-1	1	-1
3	1	1	-1	1	1
4	-1	1	1	-1	1
5	1	-1	-1	-1	1
6	-1	-1	1	1	1
7	1	1	1	1	-1
8	-1	1	-1	-1	-1
9	1	-1	1	-1	-1
10	-1	-1	1	1	1
11	1	1	1	1	-1
12	-1	1	-1	-1	-1
13	1	-1	-1	-1	1
14	-1	-1	-1	1	-1
15	1	1	-1	1	1
16	-1	1	1	-1	1

The experiments were carried out in a randomised order to avoid systematic errors. Statistical analysis was performed fitting linear regression models for each analyte indivdually. ANOVA was performed to assess the statistical significance of each factor. Significance levels were set at $\alpha = 0.05$. All statistical analyses were conducted using R (version 4.5.1) with packages tidyverse, broom, car, FrF2, and DoE.base.[220], [223]–[226]

5.4.6 DoE Optimisation

Optimisation of the LSE and dSPE was carried out using the response surface methodology. The design matrix consisted of a full 2^4 factorial design, 8 central points, and 8 axial points. All optimisation experiments were conducted using the pooled sample and three aliquotes of 0.1 g ± 0.01 g treated as described in the previous chapter 5.4.5. The coded design matrix for LSE and dSPE optimisation can be seen in table 5.10.

Table 5.10: Coded design matrix for optimisation.

Nr.	Solvent composition	Cycles	Time	Acid concentration
1	1	1	1	1
2	1	1	1	-1
3	1	1	-1	1
4	1	1	-1	-1
5	1	-1	1	1
6	1	-1	1	-1
7	1	-1	-1	1
8	1	-1	-1	-1
9	-1	1	1	1
10	-1	1	1	-1
11	-1	1	-1	1
12	-1	1	-1	-1
13	-1	-1	1	1
14	-1	-1	1	-1
15	-1	-1	-1	1
16	-1	-1	-1	-1
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0
21	0	0	0	0
22	0	0	0	0
23	0	0	0	0
24	0	0	0	0
25	2	0	0	0
26	-2	0	0	0
27	0	2	0	0
28	0	-2	0	0
29	0	0	2	0
30	0	0	-2	0
31	0	0	0	2

Continued on next page

Table 5.10 (Continuation)

Nr.	Solvent composition	Cycles	Time	Acid concentration
32	0	0	0	-2

To maintain rotatability of the design, axial points (α) were calculated as:

$$\alpha = \left(2^k\right)^{\frac{1}{4}} \tag{5.2}$$

Where:

- k = Number of factors = 4

With $\pm\alpha = \pm 2$, natural variables could be calculated using:

$$X = X_{center} + \alpha \cdot \frac{X_{+1} - X_{-1}}{2} \tag{5.3}$$

Where:

- X = Natural variable
- X_{center} = Mean value of natural variables
- α = Coded axial value (2)
- $\frac{X_{+1}-X_{-1}}{2}$ = Range

Design Matrix for LSE Optimisation:

Table 5.11: Design matrix for the LSE optimisation with the natural variables of hexane content [%], extraction cycles, extraction time [min], and acid concentration [%].

Nr.	Hexane Content [%]	Cycles	Time [min]	Acid Conc. [%]
1	80	4	47.5	0.075
2	80	4	47.5	0.025
3	80	4	22.5	0.075
4	80	4	22.5	0.025
5	80	2	47.5	0.075
6	80	2	47.5	0.025
7	80	2	22.5	0.075
8	80	2	22.5	0.025
9	40	4	47.5	0.075
10	40	4	47.5	0.025
11	40	4	22.5	0.075
12	40	4	22.5	0.025
13	40	2	47.5	0.075
14	40	2	47.5	0.025
15	40	2	22.5	0.075
16	40	2	22.5	0.025
17	60	3	35.0	0.050
18	60	3	35.0	0.050
19	60	3	35.0	0.050
20	60	3	35.0	0.050
21	60	3	35.0	0.050
22	60	3	35.0	0.050
23	60	3	35.0	0.050
24	60	3	35.0	0.050
25	100	3	35.0	0.050
26	20	3	35.0	0.050
27	60	5	35.0	0.050
28	60	1	35.0	0.050
29	60	3	60.0	0.050
30	60	3	10.0	0.050
31	60	3	35.0	0.100
32	60	3	35.0	0.000

Design Matrix for dSPE Optimisation:

Table 5.12: Design matrix for the dSPE optimisation with the natural variables of hexane content [%], PSA [mg], C18 [mg], and extraction time [min].

Nr.	Hexane content [%]	PSA [mg]	C18 [mg]	Time [min]
1	57.5	70	70	10
2	57.5	70	70	5
3	57.5	70	30	10
4	57.5	70	30	5
5	57.5	30	70	10
6	57.5	30	70	5
7	57.5	30	30	10
8	57.5	30	30	5
9	32.5	70	70	10
10	32.5	70	70	5
11	32.5	70	30	10
12	32.5	70	30	5
13	32.5	30	70	10
14	32.5	30	70	5
15	32.5	30	30	10
16	32.5	30	30	5
17	45.0	50	50	7.5
18	45.0	50	50	7.5
19	45.0	50	50	7.5
20	45.0	50	50	7.5
21	45.0	50	50	7.5
22	45.0	50	50	7.5
23	45.0	50	50	7.5
24	45.0	50	50	7.5
25	70.0	50	50	7.5
26	20.0	50	50	7.5
27	45.0	90	50	7.5
28	45.0	10	50	7.5
29	45.0	50	90	7.5
30	45.0	50	10	7.5
31	45.0	50	50	12.5
32	45.0	50	50	2.5

Statistical analyses were conducted using R (version 4.5.1) and the rsm package.[222] The optimal LSE parameters were determined by means of systematic optimisation over a regular grid with the following resolution:

For LSE optimisation:

- Solvent composition: 20 % - 100 % hexane, intervals of 5 percentage points
- Number of cycles: 1 - 5, intervals of 1
- Extraction time: 1 min - 60 min, intervals of 5 min

For dSPE optimisation:

- Solvent composition: 20 % - 70 % hexane, intervals of 1 percentage points
- PSA: 10 mg - 90 mg, intervals of 5 mg
- C_{18}: 10 mg - 90 mg, intervals of 5 mg

5.4.7 Validation

Eight series of samples, each consisting of one blank and several blanks spiked with increasing amounts of analyte, were prepared, processed, and analysed according to an orthogonal fractional factorial design. The fortified concentrations were 0.05, 0.1, 0.15, 0.2, 0.5, 1, 1.5, 2, and 3 mg/kg. As matrices, two archived barn dust samples were used: No. 170 (fattening pigs, final fattening phase, location: Rethmer/Voigtländer, 1985) as matrix 1 and No. 744 (laying hens, aviary housing, location: Ruthe, 2003) as matrix 2. Experiments were carried out by an experienced operator (1) and an untrained operator (2) in two consecutive weeks (date 1 and date 2, respectively). The complete experimental design is shown in Table 5.13.

Table 5.13: Coded, experimental matrix for validation. With samples No. 170 (fattening pigs, final fattening phase, location: Rethmer/Voigtländer, 1985) as matrix 1 and No. 744 (laying hens, aviary housing, location: Ruthe, 2003) as matrix 2, a trained (1) and untrained (2) operator and two consecutive weeks (date 1 and date 2, respectively).

	Matrix	**Operator**	**Date**
y_{i1}	1	1	1
y_{i2}	1	2	2
y_{i3}	2	1	2
y_{i4}	2	2	1

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Appendices

A Appendix

A.1 Column Oven Temperature Data

Table A.1: Retention time, mean $\ln k$, and standard deviation of $\ln k$ for each analyte at three different temperatures.

Temp. [°C]	**Comp.**	**Retention Time [min]**	**Mean** lnk	**Std. Dev.** lnk	**1/T** [1/K]
15	ALT	6.3	1.920565505	0.007326138	0.003470415
20	ALT	6.57	1.992853055	0.017037423	0.003411223
30	ALT	6.61	1.983583968	0.000859845	0.003298697
15	DEC	10.49	2.485913123	0.008324854	0.003470415
20	DEC	10.26	2.477040187	0.006824253	0.003411223
30	DEC	10.05	2.448847017	0.001079914	0.003298697
15	HAL	3.09	1.051695369	0.000000000	0.003470415
20	HAL	3.31	1.157179627	0.013753323	0.003411223
30	HAL	3.31	1.147394579	0.003968275	0.003298697
15	MPA	7.07	2.048444100	0.010475806	0.003470415
20	MPA	7.24	2.101716249	0.016044157	0.003411223
30	MPA	7.27	2.091091861	0.000772201	0.003298697
15	MA	6.85	2.013184794	0.010017030	0.003470415
20	MA	7.06	2.072273709	0.014949973	0.003411223
30	MA	7.08	2.062103358	0.001589826	0.003298697
15	MT	5.85	1.837556629	0.004975165	0.003470415
20	MT	6.16	1.918622789	0.016515263	0.003411223
30	MT	6.20	1.911390936	0.001848431	0.003298697
15	MON	11.01	2.539607721	0.006903463	0.003470415
20	MON	10.79	2.530203560	0.005475416	0.003411223
30	MON	10.60	2.506035881	0.000509944	0.003298697
15	N	4.71	1.582829815	0.003851110	0.003470415
20	N	5.07	1.695402513	0.020645134	0.003411223
30	N	5.18	1.702470184	0.002277908	0.003298697
15	NAR	11.64	2.601286828	0.005099720	0.003470415
20	NAR	11.44	2.591509371	0.003745336	0.003411223
30	NAR	11.30	2.575470284	0.000951475	0.003298697

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Temp. [°C]	**Comp.**	**Retention Time [min]**	**Mean** lnk	**Std. Dev.** lnk	**1/T [1/K]**
15	P	7.20	2.069977537	0.009464005	0.003470415
20	P	7.34	2.116142097	0.015061380	0.003411223
30	P	7.31	2.098781862	0.002298855	0.003298697
15	ROB	6.42	1.942306634	0.007168582	0.003470415
20	ROB	6.68	2.011424280	0.016723967	0.003411223
30	ROB	6.68	1.996398108	0.001697795	0.003298697
15	SAL	11.27	2.565410498	0.006247078	0.003470415
20	SAL	11.09	2.558670265	0.004354164	0.003411223
30	SAL	10.97	2.543568080	0.000982319	0.003298697
15	STA	6.21	1.905816378	0.005576266	0.003470415
20	STA	6.46	1.972220896	0.015653452	0.003411223
30	STA	6.46	1.959210621	0.002643178	0.003298697
15	T	5.28	1.718282263	0.004484335	0.003470415
20	T	5.63	1.817281752	0.019291733	0.003411223
30	T	5.71	1.816450016	0.002032523	0.003298697
15	Tb	4.24	1.455699563	0.002915460	0.003470415
20	Tb	4.59	1.576184350	0.020674780	0.003411223
30	Tb	4.70	1.587951541	0.003831436	0.003298697
15	αE2	9.53	2.389908921	0.000000000	0.003470415
20	αE2	9.38	2.371994374	0.000583090	0.003411223
30	αE2	9.06	2.334568139	0.000000000	0.003298697
15	βE2	9.22	2.353159202	0.000594177	0.003470415
20	βE2	9.06	2.334568139	0.000000000	0.003411223
30	βE2	8.73	2.293796587	0.000000000	0.003298697
15	E3	6.46	1.958331114	0.001763670	0.003470415
20	E3	6.36	1.938741660	0.000000000	0.003411223
30	E3	6.10	1.890850372	0.000000000	0.003298697
15	E1	9.69	2.408070601	0.000000000	0.003470415
20	E1	9.52	2.388762789	0.000000000	0.003411223
30	E1	9.17	2.348394451	0.000597015	0.003298697
15	E3S	4.98	1.658216739	0.004761941	0.003470415
20	E3S	5.05	1.654529624	0.015532910	0.003411223
30	E3S	4.54	1.547548362	0.005319199	0.003298697
15	EE2	9.46	2.382435308	0.000577034	0.003470415
20	EE2	9.32	2.364972693	0.000587199	0.003411223
30	EE2	9.00	2.327277706	0.000000000	0.003298697
15	LAS	10.02	2.448300347	0.003781758	0.003470415
20	LAS	10.04	2.429062467	0.017622970	0.003411223
30	LAS	9.22	2.354346851	0.000593472	0.003298697
15	NIC (DNC)	10.42	2.487507297	0.000519481	0.003470415
20	NIC (DNC)	10.19	2.462788845	0.000000000	0.003411223

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Temp. [°C]	**Comp.**	**Retention Time [min]**	**Mean** lnk	**Std. Dev.** lnk	**1/T** [1/K]
30	NIC (DNC)	9.72	2.411439498	0.000000000	0.003298697

A.2 Dust Characterisation

Table A.2: Shapiro-Wilk test for normal distribution of the individual parameters of the Weender analysis for the MS group, housing type 1, location O1. Listed are the test statistic value W and the corresponding p-value.

Nr.	**Parameter**	**W**	**p-Value**
1	Dry matter [%]	0.8641	0.0010
2	Crude ash [%]	0.9630	0.3485
3	Crude protein [%]	0.9701	0.5207
4	Crude fat [%]	0.9134	0.0158
5	Crude fibre [%]	0.7327	<0.0001
6	N-free extractives [%]	0.9849	0.9297

A.3 Screening Experiment LSE

Table A.3: Recoveries of steroid hormones resulting from screening experiments for LSE optimisation.

Experiment	ALT	MPA	MA	MT	N	P	STA	T	TB	αE2	βE2	E3	E1	E3S	EE2
1	0.37	0.46	0.43	0.41	0.35	0.44	0.16	0.37	0.28	0.14	0.13	0.00	0.32	0.02	0.12
2	0.81	0.70	0.64	0.83	0.79	0.76	0.69	0.91	0.77	1.47	0.94	0.81	1.40	0.82	1.02
3	0.83	0.85	0.79	0.83	0.72	0.76	0.44	0.77	0.59	0.24	0.33	0.00	0.65	0.00	0.24
4	0.60	0.60	0.63	0.65	0.64	0.57	0.31	0.67	0.62	0.60	0.65	0.62	0.69	0.78	0.71
5	0.60	0.62	0.60	0.62	0.55	0.68	0.29	0.62	0.42	0.20	0.22	0.00	0.45	0.00	0.20
6	0.75	0.72	0.67	0.81	0.89	0.66	0.36	0.88	0.83	0.65	0.83	0.62	0.91	0.85	0.84
7	0.63	0.66	0.64	0.72	0.69	0.62	0.29	0.73	0.57	0.34	0.39	0.00	0.68	0.01	0.37
8	0.80	0.76	0.67	0.77	0.85	0.76	0.69	0.84	0.81	1.02	1.10	0.87	0.61	0.84	1.17
9	0.48	0.59	0.57	0.53	0.41	0.57	0.16	0.47	0.34	0.13	0.13	0.00	0.34	0.01	0.12
10	0.75	0.73	0.72	0.82	0.86	0.76	0.37	0.91	0.88	0.83	0.98	0.91	0.94	0.72	1.17
11	0.46	0.54	0.53	0.51	0.45	0.52	0.17	0.46	0.38	0.19	0.19	0.00	0.47	0.03	0.19
12	0.81	0.88	0.81	0.90	0.82	0.87	0.69	0.85	0.77	0.65	0.73	0.75	0.96	0.90	0.69
13	0.69	0.79	0.72	0.72	0.64	0.76	0.37	0.68	0.46	0.23	0.16	0.00	0.57	0.01	0.11
14	0.95	0.95	0.90	0.97	0.88	0.87	0.83	1.02	0.85	0.65	0.72	0.95	0.83	1.05	0.75
15	0.76	0.81	0.80	0.77	0.71	0.78	0.34	0.74	0.54	0.28	0.30	0.00	0.65	0.00	0.25
16	0.46	0.50	0.55	0.59	0.55	0.51	0.09	0.64	0.54	0.75	1.01	0.75	0.78	0.71	0.74

Table A.4: Recoveries of coccidiostats resulting from screening experiments for LSE optimisation.

Experiment	DEC	HAL	MON	NAR	ROB	SAL	LAS	NIC
1	0.33	0.02	0.58	0.54	0.08	0.59	0.58	0.03
2	0.23	0.56	0.50	0.27	0.09	0.37	0.71	0.68
3	0.37	0.00	0.47	0.27	0.03	0.36	0.46	0.01
4	0.17	0.71	0.48	0.17	0.06	0.24	0.23	0.61
5	0.38	0.00	0.49	0.44	0.03	0.58	0.55	0.01
6	0.17	0.78	0.45	0.17	0.02	0.24	0.28	0.59
7	0.32	0.03	0.49	0.32	0.02	0.46	0.82	0.02
8	0.37	0.56	0.71	0.49	0.06	0.59	0.87	0.79
9	0.36	0.00	0.94	0.50	0.03	0.67	0.46	0.01
10	0.21	0.75	0.40	0.27	0.00	0.32	0.44	0.63
11	0.34	0.02	0.66	0.48	0.03	0.50	0.50	0.02
12	0.43	0.65	1.05	0.44	0.21	0.60	0.78	0.72
13	0.40	0.00	0.75	0.40	0.02	0.55	0.59	0.01
14	0.49	0.70	1.19	0.45	0.30	0.59	0.90	0.79
15	0.38	0.00	0.65	0.30	0.03	0.41	0.49	0.01
16	0.15	0.59	0.28	0.18	0.00	0.23	0.33	0.56

A.4 Optimisation LSE and dSPE

Table A.5: Recoveries of steroid hormones resulting from optimisation experiments for LSE method

Experiment	ALT	MPA	MA	MT	N	P	STA	T	TB	αE2	βE2	E3	E1	E-3-S	EE2
1	0.75	0.82	0.85	0.92	0.88	0.85	0.22	0.91	0.93	1.30	1.17	1.03	1.05	0.89	1.03
2	0.67	0.76	0.77	0.82	0.88	0.69	0.19	0.80	0.79	1.10	1.12	0.99	1.05	0.98	1.06
3	0.46	0.61	1.43	0.76	0.67	0.60	0.17	0.74	0.58	1.00	1.00	1.09	1.06	0.95	1.20
4	0.30	0.41	1.24	0.57	0.48	0.42	0.19	0.53	0.47	0.96	0.92	1.06	1.08	0.95	1.19
5	0.71	0.80	0.80	0.86	0.91	0.76	0.34	0.89	0.82	1.03	1.14	1.04	1.18	0.86	1.39
6	0.80	0.78	0.85	0.89	0.90	0.80	0.33	0.93	0.80	1.35	1.24	1.63	1.63	0.92	1.46
7	0.37	0.48	1.39	0.69	0.64	0.56	0.14	0.68	0.51	1.57	1.21	1.32	1.51	0.70	1.23
8	0.72	0.83	1.85	0.91	0.91	0.81	0.27	0.93	0.82	1.44	1.63	1.33	1.18	0.85	1.45
9	0.62	0.60	0.61	0.59	0.64	0.60	0.23	0.60	0.60	0.99	0.90	0.90	1.13	0.84	1.10
10	0.70	0.71	0.75	0.73	0.70	0.66	0.19	0.75	0.71	1.25	1.26	1.35	1.49	0.86	1.40
11	0.45	0.56	1.44	0.69	0.68	0.58	0.18	0.71	0.56	0.97	0.90	0.84	1.03	0.92	1.04
12	0.37	0.53	1.34	0.65	0.58	0.51	0.19	0.65	0.53	1.13	1.17	0.97	1.07	1.00	1.22
13	0.36	0.54	0.61	0.57	0.63	0.50	0.12	0.58	0.51	1.32	1.25	1.28	1.64	0.79	1.46
14	0.69	0.77	0.80	0.85	0.77	0.86	0.23	0.86	0.76	1.27	1.39	0.90	1.20	0.82	1.52
15	0.40	0.55	1.42	0.70	0.61	0.53	0.19	0.70	0.58	0.80	0.83	0.89	0.91	0.89	1.25
16	0.51	0.61	1.54	0.77	0.66	0.60	0.33	0.77	0.57	1.29	0.98	0.92	1.17	0.88	1.08
17	0.71	0.86	1.62	0.89	0.88	0.74	0.23	0.92	0.87	0.86	0.91	0.78	0.88	0.74	0.79
18	0.62	0.59	1.27	0.74	0.83	0.62	0.22	0.77	0.67	1.28	1.29	1.28	1.56	0.82	1.80
19	0.59	0.62	1.30	0.71	0.75	0.60	0.20	0.75	0.61	0.99	1.09	0.76	0.94	0.76	0.81
20	0.74	0.77	1.48	0.93	0.86	0.74	0.22	0.95	0.91	0.86	0.91	0.82	0.99	0.80	1.16
21	0.68	0.84	1.63	1.01	0.97	0.85	0.25	1.01	0.88	1.08	1.17	0.97	1.05	0.84	1.14
22	0.60	0.68	1.25	0.75	0.72	0.62	0.17	0.78	0.69	0.91	0.98	0.72	1.00	0.77	1.07
23	0.63	0.77	1.44	0.82	0.74	0.65	0.21	0.80	0.65	1.47	1.32	1.36	1.79	0.90	1.79

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Table A.5 (Continuation)

Experiment	ALT	MPA	MA	MT	N	P	STA	T	TB	αE2	βE2	E3	E1	E-3-S	EE2
24	0.65	0.74	1.38	0.84	0.86	0.68	0.22	0.82	0.72	1.07	1.00	0.82	0.94	0.74	1.07
25	0.52	0.58	0.57	0.61	0.51	0.55	0.24	0.55	0.42	0.11	0.18	0.00	0.27	0.01	0.16
26	0.58	0.60	0.65	0.72	0.71	0.62	0.26	0.76	0.62	1.13	1.26	1.31	1.29	0.76	1.09
27	0.72	0.73	0.78	0.78	0.80	0.70	0.32	0.77	0.77	0.97	0.88	0.82	1.00	0.82	0.96
28	0.57	0.70	0.76	0.74	0.69	0.69	0.26	0.75	0.69	1.03	0.76	0.66	1.46	0.93	0.69
29	0.79	0.86	0.87	0.94	0.90	0.80	0.36	0.96	0.83	1.02	1.07	1.04	1.06	0.98	1.15
30	0.62	0.65	0.67	0.70	0.77	0.65	0.31	0.75	0.57	1.48	1.51	1.36	1.61	0.83	2.02
31	0.94	0.99	0.97	0.95	0.96	0.91	0.47	0.98	0.95	0.88	0.75	0.76	0.96	0.89	0.82
32	0.82	0.80	0.84	0.83	0.85	0.78	0.56	0.82	0.79	1.27	1.84	1.36	1.31	0.94	1.11

Table A.6: Matrix effects of steroid hormones resulting from optimisation experiments for LSE method

Experiment	ALT	MPA	MA	MT	N	P	STA	T	TB	αE2	βE2	E3	E1	E-3-S	EE2
1	-0.53	-0.55	-0.19	-0.64	-0.58	-0.63	-0.49	-0.67	-0.62	-0.67	-0.64	-0.56	-0.54	-0.36	-0.65
2	-0.47	-0.45	-0.04	-0.56	-0.53	-0.54	-0.42	-0.61	-0.55	-0.61	-0.65	-0.51	-0.53	-0.38	-0.65
3	-0.52	-0.52	-0.57	-0.61	-0.55	-0.59	-0.51	-0.64	-0.57	-0.56	-0.59	-0.60	-0.57	-0.39	-0.69
4	-0.33	-0.35	-0.42	-0.43	-0.38	-0.39	-0.31	-0.44	-0.42	-0.58	-0.57	-0.54	-0.55	-0.36	-0.66
5	-0.46	-0.49	-0.08	-0.58	-0.52	-0.55	-0.42	-0.62	-0.56	-0.54	-0.60	-0.50	-0.52	-0.31	-0.67
6	-0.52	-0.47	-0.15	-0.58	-0.51	-0.57	-0.46	-0.61	-0.56	-0.59	-0.66	-0.54	-0.59	-0.39	-0.67
7	-0.47	-0.46	-0.52	-0.56	-0.53	-0.52	-0.44	-0.59	-0.56	-0.64	-0.61	-0.52	-0.55	-0.33	-0.65
8	-0.47	-0.47	-0.53	-0.54	-0.47	-0.50	-0.46	-0.58	-0.52	-0.63	-0.64	-0.46	-0.53	-0.26	-0.69
9	-0.41	-0.41	0.04	-0.48	-0.43	-0.49	-0.33	-0.53	-0.48	-0.59	-0.57	-0.49	-0.55	-0.31	-0.65
10	-0.50	-0.47	-0.08	-0.55	-0.49	-0.54	-0.41	-0.60	-0.54	-0.54	-0.59	-0.52	-0.50	-0.34	-0.63
11	-0.48	-0.48	-0.55	-0.58	-0.55	-0.55	-0.47	-0.61	-0.53	-0.49	-0.39	-0.37	-0.49	-0.33	-0.58
12	-0.48	-0.49	-0.55	-0.57	-0.50	-0.54	-0.45	-0.60	-0.55	-0.51	-0.60	-0.28	-0.35	-0.43	-0.60
13	-0.40	-0.39	0.07	-0.47	-0.43	-0.46	-0.32	-0.53	-0.44	-0.56	-0.60	-0.48	-0.55	-0.32	-0.65
14	-0.47	-0.45	-0.02	-0.55	-0.48	-0.55	-0.37	-0.58	-0.53	-0.66	-0.74	-0.46	-0.61	-0.27	-0.80
15	-0.46	-0.46	-0.52	-0.56	-0.49	-0.52	-0.44	-0.59	-0.52	-0.56	-0.59	-0.46	-0.52	-0.31	-0.71
16	-0.54	-0.54	-0.58	-0.60	-0.50	-0.58	-0.53	-0.63	-0.56	-0.73	-0.79	-0.32	-0.70	-0.48	-0.78
17	-0.55	-0.55	-0.60	-0.61	-0.57	-0.58	-0.53	-0.66	-0.62	-0.51	-0.56	-0.46	-0.45	-0.25	-0.59
18	-0.47	-0.45	-0.53	-0.56	-0.56	-0.53	-0.45	-0.61	-0.54	-0.55	-0.59	-0.52	-0.55	-0.35	-0.70
19	-0.49	-0.48	-0.54	-0.55	-0.53	-0.53	-0.47	-0.60	-0.53	-0.39	-0.43	-0.23	-0.24	-0.31	-0.42
20	-0.50	-0.48	-0.54	-0.58	-0.53	-0.54	-0.46	-0.61	-0.56	-0.52	-0.56	-0.49	-0.51	-0.30	-0.67
21	-0.43	-0.48	-0.53	-0.57	-0.51	-0.55	-0.44	-0.60	-0.52	-0.63	-0.63	-0.54	-0.52	-0.31	-0.67
22	-0.44	-0.46	-0.51	-0.54	-0.49	-0.52	-0.42	-0.59	-0.50	-0.53	-0.57	-0.48	-0.53	-0.30	-0.67
23	-0.44	-0.48	-0.52	-0.56	-0.48	-0.52	-0.43	-0.59	-0.48	-0.60	-0.59	-0.55	-0.57	-0.34	-0.67

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Table A.6 (Continuation)

Experiment	ALT	MPA	MA	MT	N	P	STA	T	TB	αE2	βE2	E3	E1	E-3-S	EE2
24	-0.47	-0.45	-0.52	-0.56	-0.52	-0.51	-0.46	-0.59	-0.53	-0.59	-0.60	-0.49	-0.50	-0.24	-0.64
25	-0.26	-0.21	-0.29	-0.24	-0.20	-0.23	-0.21	-0.21	-0.21	-0.28	-0.24	0.06	-0.34	-0.08	-0.37
26	-0.48	-0.44	-0.53	-0.55	-0.50	-0.49	-0.47	-0.58	-0.51	-0.49	-0.55	-0.52	-0.50	-0.30	-0.65
27	-0.48	-0.46	-0.51	-0.54	-0.52	-0.51	-0.46	-0.57	-0.53	-0.48	-0.38	-0.25	-0.41	-0.29	-0.49
28	-0.42	-0.42	-0.48	-0.50	-0.41	-0.46	-0.40	-0.54	-0.48	-0.67	-0.54	-0.24	-0.70	-0.41	-0.61
29	-0.44	-0.45	-0.51	-0.55	-0.48	-0.50	-0.43	-0.60	-0.50	-0.53	-0.58	-0.53	-0.52	-0.33	-0.65
30	-0.44	-0.40	-0.45	-0.50	-0.45	-0.46	-0.38	-0.55	-0.45	-0.48	-0.55	-0.41	-0.46	-0.20	-0.69
31	-0.48	-0.48	-0.53	-0.56	-0.54	-0.52	-0.47	-0.61	-0.53	-0.47	-0.40	-0.35	-0.48	-0.32	-0.58
32	-0.43	-0.42	-0.48	-0.50	-0.44	-0.45	-0.40	-0.54	-0.46	-0.80	-0.82	-0.52	-0.76	-0.37	-0.85

Table A.7: Recoveries and matrix effects of coccidiostats resulting from optimisation experiments for LSE method.

Exp.	DEC REC	ME	HAL REC	ME	MON REC	ME	NAR REC	ME	ROB REC	ME	SAL REC	ME	LAS REC	ME	NIC REC	ME
1	0.16	-0.22	0.85	-0.28	0.57	-0.15	0.17	-0.38	0.04	-0.35	0.23	-0.08	0.13	-0.03	0.71	-0.10
2	0.13	-0.10	0.65	-0.16	0.50	-0.17	0.14	-0.19	0.00	-0.36	0.22	0.32	0.20	-0.06	0.73	-0.15
3	0.17	-0.16	0.57	-0.26	0.53	0.05	0.21	-0.10	0.02	-0.30	0.34	0.15	0.18	0.08	0.78	-0.19
4	0.14	0.00	0.44	-0.11	0.56	0.14	0.13	0.40	0.00	-0.14	0.27	0.63	0.28	0.13	0.75	-0.13
5	0.20	-0.09	0.73	-0.18	0.48	-0.05	0.19	-0.17	0.04	-0.28	0.25	0.22	0.20	-0.08	0.73	-0.07
6	0.23	-0.26	0.67	-0.23	0.50	0.07	0.24	0.05	0.00	-0.73	0.30	0.50	0.41	0.12	0.80	-0.21
7	0.23	-0.12	0.49	-0.16	0.46	-0.01	0.45	-0.32	0.02	-0.25	0.44	0.21	0.24	0.14	0.57	-0.11
8	0.23	-0.13	0.77	-0.22	0.60	-0.10	0.23	0.08	0.00	-0.33	0.37	0.52	0.51	0.14	0.72	-0.05
9	0.17	-0.03	0.63	-0.09	0.44	0.11	0.12	0.31	0.05	-0.22	0.20	0.61	0.13	0.12	0.72	-0.06
10	0.17	-0.16	0.65	-0.20	0.46	0.14	0.16	0.10	0.00	-0.49	0.23	0.60	0.17	0.19	0.69	-0.12
11	0.17	-0.08	0.62	-0.22	0.47	-0.20	0.19	-0.09	0.02	-0.25	0.26	0.36	0.26	0.05	0.71	-0.10
12	0.16	-0.14	0.51	-0.23	0.55	-0.12	0.20	0.08	0.00	-0.26	0.28	0.47	0.40	-0.01	0.80	-0.27
13	0.11	-0.01	0.46	-0.06	0.48	0.18	0.12	0.40	0.00	-0.27	0.19	0.66	0.19	0.16	0.58	-0.06
14	0.14	-0.07	0.59	-0.13	0.50	0.28	0.15	0.11	0.00	-0.35	0.22	0.73	0.43	0.18	0.70	-0.04
15	0.17	-0.11	0.55	-0.21	0.44	-0.01	0.18	0.01	0.00	-0.24	0.26	0.49	0.39	0.12	0.70	-0.07
16	0.16	-0.28	0.45	-0.29	0.72	-0.23	0.25	0.22	0.00	-0.63	0.34	0.47	0.68	-0.08	0.72	-0.33
17	0.23	-0.26	0.82	-0.34	0.65	-0.22	0.21	-0.23	0.04	-0.37	0.38	0.02	0.13	0.04	0.65	-0.01
18	0.20	-0.13	0.70	-0.20	0.58	0.04	0.26	-0.13	0.04	-0.23	0.39	0.29	0.11	-0.03	0.67	-0.11
19	0.22	-0.19	0.68	-0.23	0.50	-0.13	0.33	-0.09	0.05	-0.31	0.43	0.21	0.12	-0.06	0.65	-0.13
20	0.17	-0.17	0.74	-0.22	0.51	0.01	0.25	-0.17	0.03	-0.27	0.29	0.20	0.10	0.02	0.63	-0.07
21	0.21	-0.14	0.79	-0.18	0.59	-0.09	0.37	-0.20	0.04	-0.22	0.32	0.30	0.12	0.02	0.70	-0.10
22	0.13	-0.12	0.66	-0.16	0.54	0.02	0.16	-0.16	0.02	-0.21	0.21	0.29	0.11	0.00	0.60	-0.09
23	0.24	-0.13	0.72	-0.17	0.50	0.18	0.23	0.19	0.06	-0.27	0.34	0.51	0.12	0.03	0.75	-0.12
24	0.15	-0.18	0.78	-0.20	0.64	-0.22	0.22	-0.24	0.03	-0.25	0.23	0.17	0.08	0.11	0.53	-0.03
25	0.34	-0.06	0.00	0.16	0.68	-0.12	0.31	-0.15	0.01	-0.20	0.43	0.27	0.46	0.30	0.01	0.06
26	0.20	-0.27	0.66	-0.25	0.36	0.04	0.18	-0.06	0.00	-0.58	0.27	0.33	0.26	0.23	0.68	-0.11
27	0.26	-0.20	0.79	-0.22	0.62	-0.06	0.24	-0.11	0.07	-0.24	0.35	0.23	0.13	-0.04	0.69	-0.06
28	0.15	-0.22	0.50	-0.14	0.51	0.22	0.17	0.28	0.02	-0.54	0.31	0.79	0.71	0.01	0.72	-0.13
29	0.14	-0.17	0.77	-0.20	0.47	0.19	0.13	0.10	0.01	-0.33	0.20	0.51	0.22	0.13	0.80	-0.15
30	0.15	-0.12	0.69	-0.15	0.38	0.16	0.19	-0.04	0.02	-0.15	0.24	0.49	0.09	0.21	0.58	0.04
31	0.23	-0.18	0.94	-0.19	0.78	-0.30	0.14	-0.15	0.11	-0.22	0.22	0.13	0.22	-0.02	0.83	-0.09
32	0.40	-0.22	0.61	-0.18	0.67	0.22	0.60	0.38	0.26	-0.38	0.59	1.01	0.79	0.01	0.79	-0.17

Table A.8: Matrix effects of steroid hormones resulting from optimisation experiments for dSPE method.

Exp.	ALT	MPA	MA	MT	N	P	STA	T	TB	αE2	βE2	E3	E1	E3S	EE2
1	-15.82	-22.81	-32.46	-26.40	-11.97	-19.86	-4.50	-23.24	-24.57	-85.22	-76.69	8.22	-83.72	5.09	-82.43
2	-30.76	-38.81	-42.67	-34.41	-23.88	-29.60	-20.58	-35.81	-45.08	-86.45	-80.04	14.86	-84.39	-6.19	-84.39
3	-31.27	-37.73	-44.40	-39.55	-23.16	-33.91	-24.27	-38.18	-42.90	-87.14	-79.50	1.23	-84.41	0.21	-84.80
4	-36.43	-43.86	-48.13	-39.11	-32.71	-37.80	-30.88	-41.26	-48.51	-72.72	-83.26	-31.16	-79.23	-22.09	-87.51
5	-48.19	-52.07	-57.64	-55.46	-46.76	-49.79	-35.21	-56.21	-54.79	-85.61	-81.32	4.99	-88.48	3.17	-83.75
6	-38.47	-38.27	-45.03	-37.74	-22.10	-34.62	-22.47	-39.88	-45.55	-87.09	-81.72	-22.37	-84.41	-18.12	-86.49
7	-27.22	-35.86	-42.88	-35.19	-24.57	-28.71	-19.18	-35.46	-29.31	-86.86	-81.33	-3.28	-86.45	-1.85	-86.05
8	-20.73	-32.91	-40.66	-34.50	-27.97	-27.00	-23.75	-36.43	-27.39	-79.79	-83.04	21.84	-89.43	11.18	-81.39
9	-29.08	-36.93	-43.31	-37.35	-25.28	-33.24	-20.55	-36.92	-43.04	-85.79	-77.40	-6.78	-80.86	-7.61	-83.97
10	-47.36	-54.15	-57.42	-52.71	-41.80	-49.03	-40.79	-51.09	-60.79	-87.13	-82.16	-2.37	-85.55	-19.47	-86.52
11	-29.75	-35.55	-40.49	-34.15	-22.88	-29.46	-18.45	-36.95	-45.28	-85.83	-83.16	-27.27	-88.36	-39.34	-85.93
12	-25.68	-34.51	-40.87	-33.36	-24.77	-31.56	-25.47	-34.81	-44.32	-85.65	-78.11	-16.56	-78.12	0.41	-85.82
13	-16.71	-32.34	-39.13	-30.13	-28.15	-23.78	-21.35	-29.70	-26.76	-83.45	-84.80	18.09	-90.04	11.61	-84.35
14	-20.00	-30.38	-35.29	-26.23	-15.43	-23.40	-13.30	-30.04	-39.98	-80.38	-76.21	-14.12	-73.13	4.80	-86.14
15	-32.11	-34.05	-39.78	-30.09	-15.50	-28.39	-13.75	-34.85	-36.20	-86.22	-81.05	-17.75	-83.48	-13.37	-87.26
16	-42.84	-49.56	-55.79	-52.20	-42.65	-46.50	-33.67	-54.48	-44.83	-85.93	-83.95	-5.51	-89.94	-3.34	-86.16
17	-30.35	-39.51	-43.85	-36.80	-23.24	-35.40	-16.84	-36.24	-39.61	-87.93	-80.38	-22.48	-80.60	-2.38	-87.02
18	-31.49	-38.55	-41.03	-36.90	-23.38	-31.31	-27.53	-37.26	-44.21	-83.69	-77.92	-25.44	-77.79	-10.13	-85.55
19	-29.22	-30.77	-38.39	-30.36	-15.49	-28.43	-14.91	-33.26	-38.13	-85.97	-81.12	-15.96	-83.81	-15.90	-85.54
20	-48.49	-55.58	-57.47	-52.44	-46.28	-48.81	-46.84	-52.75	-62.99	-80.78	-75.59	-21.37	-74.47	-0.99	-84.76
21	-29.04	-32.46	-37.59	-27.41	-17.41	-27.25	-15.27	-33.44	-42.07	-87.44	-82.10	-13.70	-85.21	-13.54	-86.57
22	-37.71	-47.30	-48.54	-41.69	-32.35	-40.65	-24.12	-42.84	-49.41	-85.32	-78.44	-18.29	-77.36	3.92	-86.85
23	-41.50	-42.78	-50.43	-40.63	-31.92	-39.29	-31.57	-44.98	-48.90	-87.37	-82.88	-23.96	-86.85	-26.86	-87.44

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Table A.8 (Continuation)

Exp.	ALT	MPA	MA	MT	N	P	STA	T	TB	αE2	βE2	E3	E1	E3S	EE2
24	-24.12	-33.71	-38.68	-31.73	-17.32	-27.82	-12.05	-32.21	-36.79	-84.31	-76.64	2.50	-81.54	-0.82	-82.36
25	-22.95	-31.59	-38.30	-31.39	-21.28	-25.70	-13.14	-31.11	-31.35	-85.35	-78.17	3.55	-85.02	0.13	-82.12
26	-37.14	-44.20	-50.58	-47.32	-36.92	-40.69	-19.54	-51.16	-42.40	-88.14	-82.13	1.92	-87.54	5.15	-86.45
27	-34.37	-32.67	-42.66	-30.63	-20.15	-28.17	-19.16	-29.18	-40.47	-83.35	-79.58	-18.81	-83.59	-22.06	-84.43
28	-38.32	-43.68	-49.59	-46.74	-38.10	-41.74	-27.95	-48.83	-39.34	-84.63	-83.80	-2.18	-89.83	-2.00	-84.94
29	-41.27	-41.25	-48.41	-36.67	-31.80	-38.61	-29.93	-40.61	-48.29	-86.18	-81.17	-16.05	-85.03	-15.91	-84.53
30	-39.16	-45.16	-47.86	-42.06	-33.41	-38.42	-31.93	-43.79	-52.69	-67.90	-82.55	-29.68	-76.93	-24.61	-85.63
31	-25.90	-33.45	-41.30	-33.42	-21.69	-30.55	-12.65	-33.34	-39.28	-86.04	-77.71	-1.53	-81.69	-6.16	-83.41
32	-36.63	-42.08	-46.36	-40.30	-29.30	-34.16	-27.98	-39.90	-49.57	-84.13	-79.77	6.05	-84.33	-10.07	-83.16

Table A.9: Matrix effects of coccidiostats resulting from optimisation experiments for dSPE method.

Experiment	DEC	HAL	MON	NAR	ROB	SAL	LAS	NIC
1	-27.34	2.64	-1.72	11.58	-17.60	48.82	36.70	-15.09
2	-42.05	-13.06	-7.12	5.61	-41.69	42.60	11.68	-16.74
3	-40.07	-19.57	-17.14	-8.39	-34.74	21.42	30.91	-19.84
4	-47.62	-21.80	-18.88	-5.64	-48.76	17.90	-6.01	-4.87
5	-44.83	-31.53	-22.64	-19.52	-50.19	8.26	38.08	-30.79
6	-44.67	-9.03	-3.68	4.63	-41.31	43.58	14.91	-35.88
7	-39.94	-2.84	-8.16	-0.67	-36.56	34.98	38.16	-33.29
8	-32.90	-15.09	-20.12	-10.25	-31.27	12.12	43.17	-23.40
9	-38.10	-17.92	-15.71	-6.77	-34.91	31.26	24.18	-17.54
10	-59.74	-38.79	-29.19	-25.92	-65.60	-4.06	1.22	-36.25
11	-40.51	-8.38	-1.24	11.17	-45.82	54.74	-25.36	-51.51
12	-43.20	-18.82	-13.77	-7.48	-49.55	21.45	11.45	-6.98
13	-31.95	-6.28	-17.28	-8.49	-29.77	15.59	43.94	-17.92
14	-35.05	-9.06	-6.68	-2.29	-42.33	39.83	28.64	-0.08
15	-37.48	7.01	12.63	31.57	-37.30	76.74	23.68	-31.99
16	-44.91	-30.93	-16.57	-8.43	-51.79	18.20	27.83	-37.70
17	-39.18	-7.22	7.15	23.55	-36.49	69.01	12.00	-18.30
18	-43.65	-20.58	-16.09	-12.11	-49.39	21.53	8.83	-17.95
19	-37.93	-4.53	10.38	15.34	-34.77	68.42	18.90	-24.88
20	-60.49	-43.93	-43.33	-44.92	-60.82	-32.23	15.60	-8.99
21	-38.50	-0.54	3.40	18.75	-32.98	63.90	18.96	-26.41
22	-45.67	-20.55	-8.01	9.52	-43.07	40.72	14.38	-8.48
23	-49.42	-21.17	-12.08	3.80	-54.85	35.09	3.26	-37.93

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Table A.9 (Continuation)

Experiment	DEC	HAL	MON	NAR	ROB	SAL	LAS	NIC
24	-32.84	-8.53	-6.52	-2.17	-27.35	37.25	33.71	-19.06
25	-34.78	-5.64	-12.98	0.46	-26.44	34.99	33.82	-27.47
26	-35.01	-18.62	5.39	4.56	-40.59	54.26	39.15	-27.39
27	-40.74	-10.81	-11.02	1.25	-39.16	41.80	8.49	-18.06
28	-39.34	-20.80	-17.18	-7.20	-46.18	19.07	35.15	-30.75
29	-46.12	-19.84	-13.17	9.32	-48.27	35.82	13.61	-28.17
30	-48.03	-21.50	-17.75	-10.29	-48.15	9.06	-0.34	-4.46
31	-33.61	-7.63	-0.35	5.43	-27.97	48.08	33.59	-20.63
32	-47.32	-18.45	-15.40	-9.17	-44.64	19.73	10.45	-34.59

Table A.10: Recoveries of steroid hormones resulting from optimisation experiments for dSPE method.

Exp.	ALT	MPA	MA	MT	N	P	STA	T	TB	αE2	βE2	E3	E1	E3S	EE2
1	64.13	62.17	73.61	71.91	70.73	64.67	63.53	69.44	67.69	72.89	67.10	67.75	65.65	17.82	68.76
2	88.99	86.28	93.03	88.57	89.87	83.41	81.06	86.78	87.36	101.50	87.59	82.71	80.67	27.91	91.00
3	74.07	77.94	89.93	83.62	76.87	79.82	78.60	88.16	86.07	81.79	87.68	91.97	86.12	22.85	90.12
4	81.60	80.55	90.69	83.51	87.82	78.70	80.66	89.24	78.10	86.71	83.15	91.99	86.91	29.01	88.38
5	84.49	83.27	87.68	87.69	88.33	82.74	69.93	91.21	94.62	88.00	84.97	88.57	91.40	40.97	83.01
6	92.34	90.31	93.48	96.00	91.84	88.02	76.84	94.35	93.77	88.77	86.18	99.84	88.87	50.60	90.60
7	78.94	81.95	89.78	84.08	87.68	79.62	65.15	85.76	79.48	94.33	93.35	89.28	93.05	47.42	96.36
8	72.32	75.71	76.16	78.60	80.00	77.46	67.23	79.41	77.11	59.67	73.32	76.32	80.07	44.16	66.76
9	71.16	75.98	86.94	80.43	80.41	74.64	76.16	87.57	81.52	82.35	81.38	84.68	81.21	25.83	87.26
10	88.89	100.96	103.82	105.55	94.91	100.02	89.21	98.80	100.90	89.56	94.37	92.06	97.70	36.19	94.09
11	84.46	82.36	90.35	93.95	88.26	85.77	80.74	90.81	94.15	105.26	89.71	92.40	135.74	30.37	76.63
12	80.60	86.85	95.74	91.00	88.69	86.04	82.20	92.50	90.08	113.03	103.84	95.64	102.33	39.09	111.82
13	75.96	83.21	91.40	85.36	77.35	75.86	69.50	84.75	90.61	84.25	76.28	86.96	78.74	44.07	86.65
14	89.16	91.48	99.11	89.20	93.22	89.96	72.54	95.83	95.36	91.58	101.89	95.39	93.00	55.76	107.71
15	65.36	67.00	67.22	67.04	65.64	65.26	48.95	70.41	68.02	89.94	84.93	76.08	82.85	48.15	91.38
16	83.60	89.12	95.34	93.46	89.75	87.75	73.66	95.37	84.39	87.84	87.36	94.79	92.42	53.92	101.13
17	86.37	89.52	101.00	97.29	91.98	91.55	84.73	96.44	94.78	109.57	102.53	98.03	99.24	41.88	107.40
18	90.75	91.43	97.54	96.99	90.50	90.18	87.45	99.84	91.87	104.99	103.49	103.37	103.27	41.26	102.19
19	70.55	68.79	77.35	77.17	74.20	72.86	63.39	76.92	74.14	92.22	92.61	83.99	87.92	35.09	98.14
20	113.18	120.35	125.97	123.61	123.38	112.18	106.03	123.93	125.83	87.37	91.25	85.80	87.73	34.18	100.12
21	72.88	70.25	75.40	74.32	79.10	71.32	67.24	77.98	79.93	74.28	80.46	77.31	79.25	33.78	82.84
22	89.87	96.57	102.50	96.06	98.65	87.58	85.85	98.45	100.12	83.81	91.15	100.14	84.29	36.06	98.36
23	83.97	80.31	92.52	87.80	88.40	83.17	80.49	90.17	87.21	82.59	90.01	90.69	95.16	37.35	98.44
24	65.90	69.64	71.97	70.29	67.35	64.49	59.86	73.40	71.87	69.49	75.14	83.72	74.46	32.85	78.41
25	72.44	71.68	74.84	74.44	76.86	68.28	65.22	72.14	73.70	72.23	71.04	80.48	74.41	27.51	65.12
26	79.37	87.22	95.17	90.06	87.60	87.39	73.57	96.03	83.74	108.86	98.10	93.88	87.98	37.23	106.44
27	77.03	74.07	85.57	82.28	82.70	73.36	77.32	72.99	77.78	62.25	76.00	80.48	75.50	26.33	69.57
28	87.95	87.83	89.31	89.26	86.61	87.81	63.60	92.64	82.57	98.24	92.50	95.36	94.06	72.94	97.83
29	73.83	76.13	82.40	77.86	83.84	79.52	72.44	78.63	80.18	77.55	80.46	89.70	83.22	32.27	78.32
30	72.94	72.54	78.66	77.78	75.85	71.88	69.09	80.39	75.39	75.51	74.09	91.30	70.27	32.20	70.44
31	73.05	73.90	81.56	78.14	76.25	74.11	66.82	81.53	80.65	80.03	79.66	81.69	80.60	23.26	79.89
32	85.93	88.55	93.22	95.40	92.08	85.94	80.27	93.82	93.29	77.97	93.57	95.77	92.01	43.71	85.42

Table A.11: Recoveries of coccidiostats resulting from optimisation experiments for dSPE method.

Experiment	DEC	HAL	MON	NAR	ROB	SAL	LAS	NIC
1	60.33	62.79	80.68	72.32	38.33	77.17	67.23	60.88
2	72.71	83.91	97.42	90.50	37.27	92.45	77.02	64.86
3	75.82	79.72	96.68	87.80	56.12	93.14	85.12	78.51
4	75.36	73.67	99.40	90.96	42.71	93.76	85.90	76.07
5	61.13	67.60	88.41	76.12	31.40	81.94	86.38	80.03
6	69.25	74.47	97.26	79.81	39.41	92.03	91.40	75.31
7	74.50	66.63	90.42	76.46	54.81	84.68	86.10	81.69
8	56.96	64.33	97.30	70.77	28.01	83.19	80.41	68.43
9	74.56	80.38	100.35	88.85	43.93	93.60	81.38	68.93
10	85.67	89.48	114.03	98.69	49.15	111.04	86.35	76.05
11	77.20	81.93	113.62	96.67	44.83	95.22	95.97	112.39
12	84.98	84.58	96.77	85.03	48.09	94.10	91.30	84.66
13	62.74	67.32	83.45	71.51	29.14	77.89	83.11	80.58
14	76.02	80.84	98.14	86.88	45.72	92.86	91.05	85.53
15	43.99	46.06	63.28	40.48	23.15	48.39	73.61	65.34
16	68.18	69.72	91.26	68.86	30.98	80.76	87.50	86.07
17	73.25	78.13	105.72	86.67	39.74	97.10	93.55	88.46
18	80.98	85.04	103.65	93.81	48.83	97.13	94.82	91.15
19	54.96	61.66	71.04	62.61	24.95	63.88	79.78	62.98
20	107.21	103.48	138.44	131.64	61.24	155.28	82.47	75.12
21	53.54	63.86	95.92	73.52	21.04	83.15	77.09	62.66
22	79.04	86.29	113.70	95.57	44.30	106.73	93.50	83.92
23	68.02	74.27	94.97	66.62	40.28	78.88	90.38	72.00

Continued on next page

Table A.11 (Continuation)

Experiment	DEC	HAL	MON	NAR	ROB	SAL	LAS	NIC
24	57.49	61.02	77.10	68.71	34.22	70.60	82.24	76.95
25	63.36	65.11	88.33	73.91	38.05	79.02	79.74	77.64
26	75.08	70.40	83.83	77.80	30.40	80.91	78.99	81.73
27	68.19	80.05	112.30	97.60	38.81	97.13	77.29	63.65
28	56.49	66.40	95.18	62.63	32.30	74.77	87.68	66.44
29	63.40	68.14	98.18	75.91	33.27	87.57	83.13	68.80
30	61.39	63.21	86.17	74.00	32.97	79.97	90.60	78.83
31	64.49	70.43	85.03	75.35	38.42	78.14	78.57	68.95
32	78.02	81.06	102.17	92.69	45.37	99.66	88.94	82.78

A.5 Validation

A.5.1 Stability Data

Table A.12: Results of the stability study for steroid hormones: concentrations and conditions, and recovery in %.

Analyte	**Day**	**0.1 mg/L** -20 °C	**0.1 mg/L** 4 °C	**1 mg/L** -20 °C	**1 mg/L** 4 °C	**10 mg/L** -20 °C	**10 mg/L** 4 °C	**10 mg/L** RT +UV	**10 mg/L** RT -UV
ALT	1	100	100	100	100	100	100	100	100
ALT	2							63	104
ALT	5							1	1
ALT	7	74	81	80	84	84	88	3	
ALT	15	74	90	79	78	83	82		
ALT	21	63	68	67	67	73	73		
ALT	29	73	96	88	87	88	87		
αE2	1	100	100	100	100	100	100	100	100
αE2	2							111	106
αE2	5							89	80
αE2	7	95	84	97	101	108	109	116	119
αE2	15	43	42	52	49	58	56		
αE2	21	37	37	45	41	50	50		
αE2	29	46	48	49	52	59	62		
βE2	1	100	100	100	100	100	100	100	100
βE2	2							113	111
βE2	5							95	84
βE2	7	98	81	94	105	118	117	125	127
βE2	15	46	47	51	56	65	60		
βE2	21	37	36	43	39	54	53		
βE2	29	53	46	53	56	66	65		
E1	1	100	100	100	100	100	100	100	100

Analyte	**Day**	**0.1 mg/L** -20 °C	**0.1 mg/L** 4 °C	**1 mg/L** -20 °C	**1 mg/L** 4 °C	**10 mg/L** -20 °C	**10 mg/L** 4 °C	**10 mg/L** RT +UV	**10 mg/L** RT -UV
E1	2							98	96
E1	5							83	77
E1	7	102	80	96	106	100	98	99	102
E1	15	47	43	48	44	55	54		
E1	21	37	35	41	38	47	48		
E1	29	46	43	45	48	58	60		
E3	1	100	100	100	100	100	100	100	100
E3	2							87	85
E3	5							71	68
E3	7	103	78	104	99	105	112	108	108
E3	15	35	30	34	41	41	43		
E3	21	25	26	33	30	33	34		
E3	29	34	28	38	33	41	43		
E3S	1	100	100	100	100	100	100	100	100
E3S	2							75	81
E3S	5							56	59
E3S	7	99	81	97	101	91	107	98	101
E3S	15	71	47	53	55	44	49		
E3S	21	59	49	52	48	40	43		
E3S	29	62	43	49	52	45	51		
EE2	1	100	100	100	100	100	100	100	100
EE2	2							114	108
EE2	5							93	83
EE2	7	112	99	109	106	106	120	115	115
EE2	15	57	57	57	51	61	63		

Analyte	**Day**	**0.1 mg/L** -20 °C	**0.1 mg/L** 4 °C	**1 mg/L** -20 °C	**1 mg/L** 4 °C	**10 mg/L** -20 °C	**10 mg/L** 4 °C	**10 mg/L** RT +UV	**10 mg/L** RT -UV
EE2	21	42	42	45	40	51	58		
EE2	29	54	52	57	51	62	68		
MA	1	100	100	100	100	100	100	100	100
MA	2							101	99
MA	5							107	102
MA	7	83	82	82	84	82	87	88	90
MA	15	84	87	93	87	92	93		
MA	21	74	76	75	74	77	80		
MA	29	83	94	92	91	90	91		
MPA	1	100	100	100	100	100	100	100	100
MPA	2							113	96
MPA	5							117	98
MPA	7	84	80	83	81	81	86	105	93
MPA	15	83	83	89	88	91	91		
MPA	21	72	70	74	72	78	79		
MPA	29	84	79	89	90	87	92		
MT	1	100	100	100	100	100	100	100	100
MT	2							127	122
MT	5							142	128
MT	7	83	89	91	89	94	97	95	97
MT	15	80	94	93	86	103	104		
MT	21	79	82	83	79	95	94		
MT	29	92	112	107	95	107	112		
N	1	100	100	100	100	100	100	100	100
N	2							102	98

Analyte	**Day**	**0.1 mg/L** -20 °C	**0.1 mg/L** 4 °C	**1 mg/L** -20 °C	**1 mg/L** 4 °C	**10 mg/L** -20 °C	**10 mg/L** 4 °C	**10 mg/L** RT +UV	**10 mg/L** RT -UV
N	5							116	107
N	7	90	89	89	83	83	87	86	91
N	15	79	90	85	78	85	83		
N	21	81	82	78	72	80	77		
N	29	89	107	98	84	93	88		
P	1	100	100	100	100	100	100	100	100
P	2							105	103
P	5							113	108
P	7	90	89	90	90	87	88	89	92
P	15	92	102	96	92	95	96		
P	21	80	74	79	75	79	80		
P	29	95	105	102	93	95	98		
STA	1	100	100	100	100	100	100	100	100
STA	2							95	99
STA	5							113	110
STA	7	90	98	97	99	95	95	93	100
STA	15	80	86	86	83	95	91		
STA	21	74	75	76	76	85	78		
STA	29	86	104	103	94	102	99		
T	1	100	100	100	100	100	100	100	100
T	2							99	105
T	5							109	98
T	7	85	90	84	85	83	84	78	89
T	15	81	91	86	81	87	86		
T	21	75	76	77	72	80	76		

Analyte	**Day**	**0.1 mg/L** -20 °C	**0.1 mg/L** 4 °C	**1 mg/L** -20 °C	**1 mg/L** 4 °C	**10 mg/L** -20 °C	**10 mg/L** 4 °C	**10 mg/L** RT +UV	**10 mg/L** RT -UV
T	29	84	105	99	89	93	92		
Tb	1	100	100	100	100	100	100	100	100
Tb	2							108	108
Tb	5							111	101
Tb	7	70	78	82	84	81	84	79	90
Tb	15	73	87	88	82	86	85		
Tb	21	59	72	71	70	77	73		
Tb	29	70	89	85	80	87	85		

Table A.13: Results of the stability study for coccidiostats: concentrations and conditions, and recovery in %.

Analyte	**Day**	**0.1 mg/L** -20 °C	**0.1 mg/L** 4 °C	**1 mg/L** -20 °C	**1 mg/L** 4 °C	**10 mg/L** -20 °C	**10 mg/L** 4 °C	**10 mg/L** RT +UV	**10 mg/L** RT -UV
DEC	1	100	100	100	100	100	100	100	100
DEC	2	0	0	0	0	0	0	35	38
DEC	5	0	0	0	0	0	0	37	4
DEC	7	0	0	0	0	0	0	0	0
DEC	15	63	802	320	46	18	19		
DEC	21	166	2066	69	19	2	27		
DEC	29	48	438	170	31	24	39		
HAL	1	100	100	100	100	100	100	100	100
HAL	2	0	0	0	0	0	0	104	103
HAL	5	0	0	0	0	0	0	109	102
HAL	7	86	87	87	83	86	87	89	93
HAL	15	91	107	97	89	95	91		
HAL	21	79	80	79	83	84	80		
HAL	29	87	98	91	89	95	93		
LAS	1	100	100	100	100	100	100	100	100
LAS	2	0	0	0	0	0	0	117	113
LAS	5	0	0	0	0	0	0	1.5	73
LAS	7	91	83	100	115	66	108	109	111
LAS	15	103	82	74	76	41	69		
LAS	21	119	101	87	85	35	68		
LAS	29	108	82	80	90	29	76		
MON	1	100	100	100	100	100	100	100	100

Analyte	**Day**	**0.1 mg/L** -20 °C	**0.1 mg/L** 4 °C	**1 mg/L** -20 °C	**1 mg/L** 4 °C	**10 mg/L** -20 °C	**10 mg/L** 4 °C	**10 mg/L** RT +UV	**10 mg/L** RT -UV
MON	2	0	0	0	0	0	0	101	88
MON	5	0	0	0	0	0	0	81	71
MON	7	80	71	78	75	81	78	101	83
MON	15	82	57	75	75	83	75		
MON	21	81	63	78	75	97	91		
MON	29	75	48	68	71	93	89		
NAR	1	100	100	100	100	100	100	100	100
NAR	2	0	0	0	0	0	0	101	86
NAR	5	0	0	0	0	0	0	67	60
NAR	7	81	65	71	78	73	86	115	93
NAR	15	32	20	29	29	37	39		
NAR	21	56	41	49	49	62	68		
NAR	29	44	24	38	40	44	55		
NIC	1	100	100	100	100	100	100	100	100
NIC	2	0	0	0	0	0	0	103	93
NIC	5	0	0	0	0	0	0	47	46
NIC	7	83	82	66	110	46	63	62	57
NIC	15	69	59	44	69	33	39		
NIC	21	93	66	50	76	32	35		
NIC	29	69	72	49	68	25	34		
ROB	1	100	100	100	100	100	100	100	100
ROB	2	0	0	0	0	0	0	104	103
ROB	5	0	0	0	0	0	0	99	89
ROB	7	93	95	98	95	53	95	78	94
ROB	15	83	94	95	95	66	107		

Analyte	**Day**	**0.1 mg/L** -20 °C	**0.1 mg/L** 4 °C	**1 mg/L** -20 °C	**1 mg/L** 4 °C	**10 mg/L** -20 °C	**10 mg/L** 4 °C	**10 mg/L** RT +UV	**10 mg/L** RT -UV
ROB	21	73	78	93	81	46	87		
ROB	29	23	5	30	4	7	2		
SAL	1	100	100	100	100	100	100	100	100
SAL	2	0	0	0	0	0	0	105	92
SAL	5	0	0	0	0	0	0	76	69
SAL	7	85	76	78	85	87	91	123	100
SAL	15	47	33	45	44	57	54		
SAL	21	63	46	61	61	83	81		
SAL	29	58	32	52	55	68	70		

A.5.2 SD_R and SD_{wiR} Data

Table A.14: SD_R and SD_{wiR} of androgens and gestagens expressed in percent at different concentration levels.

Conc. [mg/kg]	ALT [%]		MA [%]		MPA [%]		MT [%]		N [%]		P [%]		STA [%]		T [%]		Tb [%]	
	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}
0.15	83.89	83.89	85.37	85.37	86.11	86.11	83.59	83.59	81.15	81.15	98.98	98.98	131.85	131.85	85.83	85.83	90.65	90.65
0.30	41.95	41.95	42.69	42.69	43.06	43.06	41.80	41.80	40.57	45.65	49.49	49.49	65.93	65.93	42.91	42.91	45.32	45.32
0.45	27.96	27.96	28.46	28.46	28.70	28.70	27.86	27.86	27.05	35.88	32.99	33.92	43.95	43.95	28.61	28.61	30.22	30.22
0.60	20.97	22.09	21.34	21.34	21.53	21.93	20.90	20.90	20.29	31.66	24.74	26.58	32.96	33.37	21.46	21.70	22.66	22.66
0.75	16.78	18.95	17.07	17.59	17.22	18.46	16.72	17.39	16.23	29.46	19.80	22.35	26.37	28.39	17.17	18.18	18.13	18.63
0.90	13.98	17.02	14.23	15.26	14.35	16.28	13.93	15.17	13.52	28.16	16.50	19.66	21.98	25.42	14.30	15.95	15.11	16.26
1.05	11.98	15.75	12.20	13.65	12.30	14.82	11.94	13.66	11.59	27.33	14.14	17.84	18.84	23.54	12.26	14.43	12.95	14.67
1.20	10.49	14.88	10.67	12.49	10.76	13.79	10.45	12.59	10.14	26.76	12.37	16.54	16.48	22.30	10.73	13.35	11.33	13.54
1.35	9.32	14.26	9.49	11.62	9.57	13.04	9.29	11.80	9.02	26.36	11.00	15.58	14.65	21.46	9.54	12.56	10.07	12.72
1.50	8.39	13.80	8.54	10.96	8.61	12.47	8.36	11.20	8.11	26.06	9.90	14.85	13.19	20.87	8.58	11.96	9.06	12.11
1.65	7.63	13.45	7.76	10.43	7.83	12.04	7.60	10.73	7.38	25.84	9.00	14.29	11.99	20.44	7.80	11.49	8.24	11.64
1.80	6.99	13.19	7.11	10.02	7.18	11.70	6.97	10.36	6.76	25.66	8.25	13.84	10.99	20.13	7.15	11.13	7.55	11.27
1.95	6.45	12.98	6.57	9.68	6.62	11.43	6.43	10.07	6.24	25.52	7.61	13.48	10.14	19.91	6.60	10.83	6.97	10.98
2.10	5.99	12.81	6.10	9.40	6.15	11.22	5.97	9.82	5.80	25.41	7.07	13.18	9.42	19.74	6.13	10.59	6.47	10.74
2.25	5.59	12.68	5.69	9.16	5.74	11.04	5.57	9.63	5.41	25.31	6.60	12.94	8.79	19.61	5.72	10.40	6.04	10.55
2.40	5.24	12.57	5.34	8.97	5.38	10.89	5.22	9.46	5.07	25.23	6.19	12.74	8.24	19.51	5.36	10.23	5.67	10.39
2.55	4.93	12.48	5.02	8.80	5.07	10.77	4.92	9.32	4.77	25.17	5.82	12.56	7.76	19.44	5.05	10.09	5.33	10.26
2.70	4.66	12.40	4.74	8.66	4.78	10.66	4.64	9.20	4.51	25.11	5.50	12.42	7.33	19.38	4.77	9.97	5.04	10.15
2.85	4.42	12.34	4.49	8.53	4.53	10.57	4.40	9.10	4.27	25.06	5.21	12.29	6.94	19.34	4.52	9.87	4.77	10.06
3.00	4.19	12.29	4.27	8.43	4.31	10.50	4.18	9.01	4.06	25.02	4.95	12.18	6.59	19.30	4.29	9.79	4.53	9.98

Table A.15: SD_R and SD_{wiR} of estrogens expressed in percent at different concentration levels.

Conc. [mg/kg]	αE2 [%]		βE2 [%]		E1 [%]		E3 [%]		E3S [%]		EE2 [%]	
	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}
0.15	129.56	130.53	139.45	139.45	114.26	119.71	100.94	102.28	119.34	119.34	130.30	135.94
0.30	64.78	65.91	69.72	69.72	57.13	58.97	50.47	51.37	59.67	59.82	65.15	67.68
0.45	43.19	44.32	46.48	46.48	38.09	39.89	33.65	34.90	39.78	41.88	43.43	47.89
0.60	32.39	33.47	34.86	34.97	28.56	31.18	25.24	27.03	29.84	33.57	32.58	39.88
0.75	25.91	26.94	27.89	28.51	22.85	26.54	20.19	22.56	23.87	29.02	26.06	36.18
0.90	21.59	22.55	23.24	24.27	19.04	23.86	16.82	19.77	19.89	26.27	21.72	34.35
1.05	18.51	19.40	19.92	21.28	16.32	22.23	14.42	17.92	17.05	24.50	18.61	33.41
1.20	16.19	17.01	17.43	19.07	14.28	21.20	12.62	16.64	14.92	23.30	16.29	32.93
1.35	14.40	15.14	15.49	17.38	12.70	20.52	11.22	15.72	13.26	22.46	14.48	32.70
1.50	12.96	13.62	13.94	16.06	11.43	20.08	10.09	15.04	11.93	21.85	13.03	32.60
1.65	11.78	12.37	12.68	15.00	10.39	19.78	9.18	14.53	10.85	21.40	11.85	32.58
1.80	10.80	11.31	11.62	14.13	9.52	19.58	8.41	14.14	9.95	21.06	10.86	32.60
1.95	9.97	10.40	10.73	13.41	8.79	19.44	7.76	13.83	9.18	20.79	10.02	32.65
2.10	9.25	9.62	9.96	12.80	8.16	19.35	7.21	13.59	8.52	20.58	9.31	32.71
2.25	8.64	8.92	9.30	12.29	7.62	19.29	6.73	13.40	7.96	20.42	8.69	32.78
2.40	8.10	8.30	8.72	11.85	7.14	19.25	6.31	13.24	7.46	20.29	8.14	32.85
2.55	7.62	7.75	8.20	11.47	6.72	19.23	5.94	13.11	7.02	20.18	7.66	32.93
2.70	7.20	7.24	7.75	11.15	6.35	19.22	5.61	13.00	6.63	20.09	7.24	33.00
2.85	6.82	6.82	7.34	10.86	6.01	19.21	5.31	12.92	6.28	20.01	6.86	33.07
3.00	6.48	6.48	6.97	10.60	5.71	19.22	5.05	12.84	5.97	19.95	6.52	33.14

Table A.16: SD_R and $SDSD_{wiR}$ of coccidiostats expressed in percent at different concentration levels.

Conc. [mg/kg]	DEC [%]		HAL [%]		LAS		NAR [%]		NIC [%]		ROB [%]		SAL [%]	
	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}	SD_R	SD_{wiR}R
0.15	146.18	146.18	101.04	101.04	114.07	115.05	126.04	126.04	169.24	174.29	273.71	273.71	115.97	115.97
0.30	73.09	73.09	50.52	50.52	57.04	58.59	63.02	63.02	84.62	90.06	136.86	136.86	57.99	57.99
0.45	48.73	50.67	33.68	33.68	38.02	40.25	42.01	42.01	56.41	62.35	91.24	94.44	38.66	38.66
0.60	36.54	42.14	25.26	26.42	28.52	31.42	31.51	32.47	42.31	48.74	68.43	77.21	28.99	29.87
0.75	29.24	37.89	20.21	22.62	22.81	26.36	25.21	27.60	33.85	40.75	54.74	68.09	23.19	25.15
0.90	24.36	35.56	16.84	20.29	19.01	23.15	21.01	24.60	28.21	35.55	45.62	62.75	19.33	22.34
1.05	20.88	34.20	14.43	18.77	16.30	20.99	18.01	22.65	24.18	31.93	39.10	59.42	16.57	20.58
1.20	18.27	33.37	12.63	17.72	14.26	19.46	15.75	21.32	21.16	29.28	34.21	57.24	14.50	19.43
1.35	16.24	32.84	11.23	16.97	12.67	18.34	14.00	20.37	18.80	27.28	30.41	55.74	12.89	18.66
1.50	14.62	32.50	10.10	16.43	11.41	17.49	12.60	19.68	16.92	25.73	27.37	54.69	11.60	18.12
1.65	13.29	32.28	9.19	16.02	10.37	16.84	11.46	19.17	15.39	24.49	24.88	53.93	10.54	17.75
1.80	12.18	32.13	8.42	15.70	9.51	16.33	10.50	18.78	14.10	23.48	22.81	53.36	9.66	17.48
1.95	11.24	32.03	7.77	15.45	8.77	15.92	9.70	18.47	13.02	22.65	21.05	52.94	8.92	17.29
2.10	10.44	31.97	7.22	15.25	8.15	15.59	9.00	18.23	12.09	21.96	19.55	52.61	8.28	17.15
2.25	9.75	31.93	6.74	15.10	7.60	15.32	8.40	18.04	11.28	21.38	18.25	52.36	7.73	17.05
2.40	9.14	31.91	6.32	14.97	7.13	15.09	7.88	17.89	10.58	20.88	17.11	52.16	7.25	16.98
2.55	8.60	31.90	5.94	14.86	6.71	14.91	7.41	17.76	9.96	20.45	16.10	52.00	6.82	16.93
2.70	8.12	31.90	5.61	14.77	6.34	14.75	7.00	17.66	9.40	20.07	15.21	51.88	6.44	16.89
2.85	7.69	31.90	5.32	14.70	6.00	14.61	6.63	17.57	8.91	19.74	14.41	51.78	6.10	16.87
3.00	7.31	31.91	5.05	14.64	5.70	14.49	6.30	17.50	8.46	19.46	13.69	51.70	5.80	16.85

A.6 R-Codes

A.6.1 Screening

```
#  1. Pakete laden
library(tidyverse)
library(broom)
library(car)
library(FrF2)
library(DoE.base)
library(ggplot2)

#  2. Faktoren definieren und Response-Namen festlegen
factor_names <- c("H", "V", "Z", "T", "S")
factor_labels <- c("Hexan_MeOH", "Volume", "Cycles", "Time",
   "Acid")
response_names <- names(ScreeningREC)[!names(ScreeningREC) %
   in% factor_names]
response_names <- response_names[response_names != "T.1"]  #
   falls doppelte T-Spalte

# 3. Faktoren kodieren
ScreeningREC$H_coded <- ifelse(ScreeningREC$H == 20, -1, 1)
ScreeningREC$V_coded <- ifelse(ScreeningREC$V == 5, -1, 1)
ScreeningREC$Z_coded <- ifelse(ScreeningREC$Z == 1, -1, 1)
ScreeningREC$T_coded <- ifelse(ScreeningREC$T == 10, -1, 1)
ScreeningREC$S_coded <- ifelse(ScreeningREC$S == 0, -1, 1)
coded_factors <- c("H_coded", "V_coded", "Z_coded", "T_coded"
   , "S_coded")

# 4. Analyse-Funktion
analyze_analyte <- function(analyte_name, data) {
        cat("\n", strrep("=", 60), "\n")
        cat("ANALYSE für:", analyte_name, "\n")
        cat(strrep("=", 60), "\n")
        analyte_escaped <- paste0("`", analyte_name, "`")
        formula_str <- paste(analyte_escaped, "~", paste(
           coded_factors, collapse = " + "))
        model <- lm(as.formula(formula_str), data = data)
        summary_model <- summary(model)
        anova_model <- anova(model)
        coefs <- summary_model$coefficients
        rownames(coefs)[1] <- "Intercept"
        for(i in seq_along(coded_factors)) {
                rownames(coefs)[i+1] <- factor_labels[i]
        }
        effects_df <- data.frame(
        Factor = rownames(coefs),
```

```
40          Estimate = coefs[, "Estimate"],
41          Std_Error = coefs[, "Std. Error"],
42          t_value = coefs[, "t value"],
43          p_value = coefs[, "Pr(>|t|)"],
44          Significance = ifelse(coefs[, "Pr(>|t|)"] < 0.001, "*
               **",
45          ifelse(coefs[, "Pr(>|t|)"] < 0.01, "**",
46          ifelse(coefs[, "Pr(>|t|)"] < 0.05, "*", "")))
47          )
48          main_effects <- list()
49          response <- data[[analyte_name]]
50          for(i in seq_along(coded_factors)) {
51                  low_mean <- mean(response[data[[coded_factors
                       [i]]] == -1])
52                  high_mean <- mean(response[data[[coded_
                       factors[i]]] == 1])
53                  main_effects[[factor_labels[i]]] <- c(Low =
                       low_mean, High = high_mean,
54                  Effect = high_mean - low_mean)
55          }
56          return(list(model = model, effects = effects_df, main
               _effects = main_effects,
57          anova = anova_model, summary = summary_model))
58  }
59
60  # 5. Analyse durchführen
61  results_list <- list()
62  for(analyte in response_names){
63          results_list[[analyte]] <- analyze_analyte(analyte,
               ScreeningREC)
64  }
65  cat("\nAnalyse abgeschlossen!\n")
66
67  # 6. Signifikanz-Matrix erstellen
68  sigmat <- matrix(NA, nrow=length(response_names), ncol=length
       (factor_labels))
69  rownames(sigmat) <- response_names
70  colnames(sigmat) <- factor_labels
71  for(i in seq_along(response_names)){
72          eff <- results_list[[response_names[i]]]$effects
73          for(j in seq_along(factor_labels)){
74                  idx <- which(eff$Factor == factor_labels[j])
75                  if(length(idx) == 1) {
76                          p <- eff$p_value[idx]
77                          sigmat[i, j] <- ifelse(p < 0.001, 3,
78                          ifelse(p < 0.01, 2,
79                          ifelse(p < 0.05, 1, 0)))
80                  } else {
```

```
                        sigmat[i, j] <- NA
                }
        }
}

#7. Dataframe für ggplot erzeugen
library(tidyr)
heat_df <- as.data.frame(sigmat)
heat_df$Analyt <- rownames(heat_df)
heat_df <- pivot_longer(heat_df,
cols = -Analyt,
names_to = "Parameter",
values_to = "Signifikanz")
heat_df$Signifikanz <- factor(heat_df$Signifikanz,
levels=c(0,1,2,3),
labels=c('ns','p<0.05','p<0.01','p<0.001'))

# 8. HEATMAP erstellen
ggplot(heat_df, aes(x=Parameter, y=Analyt, fill=Signifikanz)) +
geom_tile(color='white') +
scale_fill_manual(values=c('#e0efff','#90c6ef','#3382c6','#00124b')) +
labs(title='Heatmap der Faktorsignifikanz',
fill='Signifikanz') +
theme_minimal(base_size=12) +
theme(axis.text.x = element_text(angle=45,hjust=1),
axis.text.y = element_text(size=9),
panel.grid=element_blank())


# Definierte Reihenfolge der Analyten
analyte_order <- c("ALT", "MPA", "MA", "MT", "N", "P", "STA", "Te", "TB",
"aE2", "bE2", "E1", "E3", "E3S", "EE2", "DEC", "HAL",
"MON", "NAR", "ROB", "SAL", "LAS", "NIC")

# Im Schritt 5 (Analysen) diese Reihenfolge verwenden:
results_list <- list()
for(analyte in response_names_ordered){
        results_list[[analyte]] <- analyze_analyte(analyte, ScreeningREC)
}
cat("\nAnalyse abgeschlossen!\n")

# Und im Schritt 6 Matrix und Heatmap mit dieser Reihenfolge erzeugen:
```

```
sigmat <- matrix(NA, nrow=length(response_names_ordered),
   ncol=length(factor_labels))
rownames(sigmat) <- response_names_ordered
colnames(sigmat) <- factor_labels

for(i in seq_along(response_names_ordered)){
        eff <- results_list[[response_names_ordered[i]]]$
           effects
        for(j in seq_along(factor_labels)){
                idx <- which(eff$Factor == factor_labels[j])
                if(length(idx) == 1) {
                        p <- eff$p_value[idx]
                        sigmat[i, j] <- ifelse(p < 0.001, 3,
                        ifelse(p < 0.01, 2,
                        ifelse(p < 0.05, 1, 0)))
                } else {
                        sigmat[i, j] <- NA
                }
        }
}

# Heatmap Dataframe generieren (wie im vorherigen Code)
heat_df$Analyt <- factor(heat_df$Analyt, levels = rev(
   response_names_ordered))

ggplot(heat_df, aes(x=Parameter, y=Analyt, fill=Signifikanz))
    +
geom_tile(color='white') +
scale_fill_manual(values=c('#e0efff','#90c6ef','#3382c6','
   #00124b')) +
labs(title='Heatmap der Faktorsignifikanz',
fill='Signifikanz') +
theme_minimal(base_size=12) +
theme(axis.text.x = element_text(angle=45,hjust=1),
axis.text.y = element_text(size=9),
panel.grid=element_blank())

colnames(ScreeningREC)
```

A.6.2 DoE LSE

```
        library(rsm)


        Results$x_H <- (Results$H - 60)/20
        Results$x_Z <- (Results$Z - 3)/1
        Results$x_T <- (Results$T - 35)/12.5
        Results$x_S <- (Results$S - 0.05)/0.025
```

```
8
9
10          # setting the realtionship between the coded and the
               natural variables
11
12          Results <- as.coded.data(Results,
13          x_H ~ (H - 60)/20,
14          x_Z ~ (Z - 3)/1,
15          x_T ~ (T - 35)/12.5,
16          x_S ~ (S-0.05)/0.025)
17          str(Results)
18          optimized_params <- list()
19          # Functions for desirability
20          # (Derringer and Suich, 1980)
21
22          # y = response
23          # L = unacceptability boundary
24          # T = target acceptability boundary T
25          # U = upper unacceptability boundary
26          # r, r1, r2 = exponent 1 for L and U
27          # d = desirability function
28
29          # Maximum
30          maxD <- function(y, L, U, T, r) {
31                  if (y < L){d <- 0}
32                  else if (y > T){d <- 0}
33                  else{d <- ((y - L) / (T - L))^r}
34                  return(d) }
35
36
37          # Minimum
38          minD <- function(y,L, U, T, r) {
39                  if (y < T){d <- 1}
40                  else if (y > U){d <- 0}
41                  else{d <- ((U - y) / (U - T))^r}
42                  return(d) }
43
44          # Target
45          targetD <- function(y, L, T, U, r1, r2) {
46                  if (y < L){d <- 0}
47                  else if ( L<=y & y<=T ){
48                          d <- ((y - L) / (T - L))^r1}
49                  else if( T<=y & y<=U ){
50                          d <- ((U - y) / (U - T))^r2}
51                  else {
52                          d <- 0}
53                  return(d)}
54
```

```
55          # Schleife für die Responsepaare REC1 bis REC24
56          for (i in 1:24) {
57
58                  REC_response <- paste0("REC", i)
59
60                  # Modell für REC für das aktuelle
                       Responsepaar erstellen (mit H, Z, T)
61                  model.REC <- rsm(as.formula(paste(REC_
                       response, "~ FO(x_H, x_Z, x_T) + PQ(x_H, x
                       _Z, x_T)")), data = Results)
62
63                  # Gitter für die Desirability-Berechnung
                       erstellen (nur H, Z und T)
64                  dataD <- expand.grid(seq(20, 100, by = 5),  #
                        Hexangehalt (H)
65                  seq(10, 60, by = 5),  # T (neuer Parameter)
66                  seq(1, 5, by = 1))    # Z (neuer Parameter)
67                  colnames(dataD) <- c("H", "T", "Z")
68
69                  # Natural-/Coded-Variables für H, T und Z
70                  dataD$x_H <- (dataD$H - 60) / 20
71                  dataD$x_T <- (dataD$T - 35) / 12.5
72                  dataD$x_Z <- (dataD$Z - 3) / 1  # Angenommene
                        Skalierung für Z
73
74                  # Vorhergesagte Antwort für REC
75                  dataD$REC <- predict(model.REC, newdata =
                       dataD)
76
77                  # Desirability berechnen für jedes Datenpaar
                       basierend auf REC
78                  for (j in 1:nrow(dataD)) {
79                          # Desirability für REC
80                          d1 <- targetD(dataD$REC[j], L = 0.10,
                                T = 1, U = 1.20, r1 = 1, r2 = 1)
81                          D <- d1  # Desirability nur von REC
82
83
84                          dataD[j, c("d1", "D")] <- c(d1, D)
85                  }
86
87                  # Optimierung: Finden der optimalen Parameter
                        H, Z, T
88                  optimal_row <- dataD[which.max(dataD$D), ]
89                  optimized_params[[i]] <- list(
90                  H = optimal_row$H,
91                  Z = optimal_row$Z,
92                  T = optimal_row$T,
```

```
93                  Desirability = optimal_row$D,
94                  REC_value = optimal_row$REC  # Berechneter
                       Wert für REC
95                  )
96
97                  # Plot der Desirability-Ergebnisse für das
                       aktuelle Responsepaar
98                  ggplot(data = dataD, aes(x = H, y = Z, z = D)
                       ) +
99                  geom_contour_filled(bins = 9) +
100                 scale_fill_brewer() +
101                 theme_bw() +
102                 theme(
103                 panel.grid.major = element_blank(),
104                 panel.grid.minor = element_blank(),
105                 legend.position = "right",  # Legende rechts
                       neben dem Diagramm
106                 legend.title = element_text(size = 12),
107                 legend.text = element_text(size = 10)
108                 ) +
109                 labs(x = "Hexan (%)", y = "Z", fill = "
                       Desirability") +
110                 ggtitle(paste("Desirability für ", REC_
                       response)) +
111                 facet_wrap(~T)
112
113                 ggsave(paste0("desirability_HZT_", i, ".png")
                       , width = 10, height = 7, dpi = 1000)
114         }
115
116
117         optimized_df <- do.call(rbind, lapply(optimized_
               params, function(x) data.frame(x)))
118         write.csv(optimized_df, "optimized_parameters_HZT.csv
               ", row.names = FALSE)
119
120         optimized_df
121
122         ####################ME aus optimierten REC Parametern
                berechnen###################
123
124
125         optimized_df <- read.csv("optimized_parameters_HZT.
               csv")
126
127         # Erstelle einen leeren Vektor, um die berechneten ME
               -Werte zu speichern
128         optimized_df$ME_value <- NA
```

```
129
130         # Schleife durch jedes Compound (1-24), um die zugehö
               rigen ME-Werte zu berechnen
131         for (i in 1:nrow(optimized_df)) {
132                 # Extrahiere die Parameter für jedes Compound
                        (H, Z, T)
133                 H <- optimized_df$H[i]
134                 Z <- optimized_df$Z[i]
135                 T <- optimized_df$T[i]
136
137                 # Umrechnung in kodierte Variablen (skaliert)
138                 x_H <- (H - 60) / 20
139                 x_T <- (T - 35) / 12.5
140                 x_Z <- (Z - 3) / 1
141
142
143                 dataME <- data.frame(x_H = x_H, x_T = x_T, x_
                       Z = x_Z)
144                 optimized_df$ME_value[i] <- predict(model.ME,
                        newdata = dataME)
145         }
146
147         # Ausgabe der berechneten ME-Werte
148         optimized_df
149
150         # Optional: Speichern der erweiterten Tabelle mit ME-
               Werten
151         write.csv(optimized_df, "optimized_parameters_with_ME
               .csv", row.names = FALSE)
```

A.6.3 DoE dSPE

```
1
2  library(rsm)
3
4  # calculating the coded variables
5
6  Results$x_H <- (Results$H - 45)/12.5
7  Results$x_PSA <- (Results$PSA - 50)/20
8  Results$x_C18 <- (Results$C18 - 50)/20
9  Results$x_T <- (Results$T - 7.5)/2.5
10
11
12 # setting the realtionship between the coded and the natural
      variables
13
14 Results <- as.coded.data(Results,
15 x_H ~ (H - 45)/12.5,
```

```
x_PSA ~ (PSA - 50)/20,
x_C18 ~ (C18 - 50)/20,
x_T ~ (T - 7.5)/2.5)
str(Results)


model_summaries <- list()


# Schleife über alle 24 REC-Datenreihen
for (i in 1:24) {
        # Dynamisch den Namen der Response-Variable für jedes Modell erstellen
        response_name <- paste0("REC", i)

        # Formel für das RSM-Modell erstellen
        formula <- as.formula(paste(response_name, "~ FO(x_H, x_PSA, x_C18, x_T) + TWI(x_H, x_PSA, x_C18, x_T) + PQ(x_H, x_PSA, x_C18, x_T)"))

        # Modell erstellen
        model <- rsm(formula, data = Results)

        # Zusammenfassung des Modells speichern
        model_summaries[[response_name]] <- summary(model)

        # Ausgabe der Zusammenfassung für jedes Modell
        cat("\nZusammenfassung für Modell", response_name, ":\n")
        print(summary(model))
        sink(paste0("model_summary_", response_name, ".txt"))
        print(summary(model))
        sink()
}

#####################################
model_summaries <- list()

# Eine Schleife für 1 bis 24
for (i in 1:24) {
        # Dynamisch den Namen der Response-Variable für jedes Modell erstellen
        response_name <- paste0("REC", i)

        # Formel für das RSM-Modell erstellen
        formula <- as.formula(paste(response_name, "~ FO(x_H, x_PSA, x_C18, x_T) + TWI(x_H, x_PSA, x_C18, x_T) + PQ(x_H, x_PSA, x_C18, x_T)"))
```

```
57
58
59          model <- rsm(formula, data = Results)
60
61
62          model_summary <- summary(model)
63
64
65          summary_data <- data.frame(
66          Term = rownames(model_summary$coefficients),
67          Estimate = model_summary$coefficients[, "Estimate"],
68          Std.Error = model_summary$coefficients[, "Std. Error"
               ],
69          t.Value = model_summary$coefficients[, "t value"],
70          Pr = model_summary$coefficients[, "Pr(>|t|)"]
71          )
72
73
74          model_summaries[[response_name]] <- summary_data
75  }
76
77  # Alle Zusammenfassungen in eine Excel-Datei schreiben
78  write_xlsx(model_summaries, "model_summaries.xlsx")
79
80  View(model_summaries)
81
82
83  model.REC1 <- rsm(REC1 ~ FO(x_H, x_PSA, x_T, x_C18) + TWI(x_H
       , x_PSA, x_T, x_C18) + PQ(x_H, x_PSA, x_T, x_C18), data =
       Results)#nicht signifikante Parameter entfernt außer Hexan
        Gehalt H
84  summary(model.REC1)
85
86  # contour plots of the responses
87
88  par(mfrow=c(2,3))
89
90  contour(model.REC1, ~x_H+x_C18,
91  image = TRUE,
92  zlim = c(0,1),
93  xlabs=c("Hexan (%)","C18"))
94  points(Results$H, Results$C18)
95  title(main = list("(a) Recovery", cex = 1, font = 1))
96
97  contour(model.REC1, ~x_H+x_PSA,
98  image = TRUE,
99  zlim = c(0,1),
100 xlabs=c("Hexan (%)","PSA"))
```

```
101 points(Results$H, Results$PSA)
102 title(main = list("(a) Recovery", cex = 1, font = 1))
103
104 contour(model.REC1, ~x_C18+x_PSA,
105 image = TRUE,
106 zlim = c(0,1),
107 xlabs=c("C18", "PSA"))
108 points(Results$C18, Results$PSA)
109 title(main = list("(c) Recovery", cex = 1, font = 1))
110
111 contour(model.ME, ~x_H+x_C18,
112 image = TRUE,
113 xlabs=c("Hexan (%)", "C18"))
114 points(Results$H, Results$C18)
115 title(main = list("(d) Matrix Effect", cex = 1, font = 1))
116
117 contour(model.ME, ~x_H+x_PSA,
118 image = TRUE,
119 xlabs=c("Hexan (%)", "PSA"))
120 points(Results$H, Results$PSA)
121 title(main = list("(d) Matrix Effect", cex = 1, font = 1))
122
123 contour(model.ME, ~x_C18+x_PSA,
124 image = TRUE,
125 xlabs=c("C18", "PSA"))
126 points(Results$C18, Results$PSA)
127 text(main = list("(d) Matrix Effect", cex = 1, font = 1))
128
129
130
131 # Functions for desirability
132 # (Derringer and Suich, 1980)
133
134 # y = response
135 # L = unacceptability boundary
136 # T = target acceptability boundary T
137 # U = upper unacceptability boundary
138 # r, r1, r2 = exponent 1 for L and U
139 # d = desirability function
140
141 # Maximum
142 maxD <- function(y, L, U, T, r) {
143         if (y < L){d <- 0}
144         else if (y > T){d <- 0}
145         else{d <- ((y - L) / (T - L))^r}
146         return(d) }
147
148
```

```
149 # Minimum
150 minD <- function(y,L, U, T, r) {
151         if (y < T){d <- 1}
152         else if (y > U){d <- 0}
153         else{d <- ((U - y) / (U - T))^r}
154         return(d) }
155
156 # Target
157 targetD <- function(y, L, T, U, r1, r2) {
158         if (y < L){d <- 0}
159         else if ( L<=y & y<=T ){
160                 d <- ((y - L) / (T - L))^r1}
161         else if( T<=y & y<=U ){
162                 d <- ((U - y) / (U - T))^r2}
163         else {
164                 d <- 0}
165         return(d)}
166
167 # models for the responses
168
169 model.REC <- rsm(REC7 ~ FO(x_H, x_C18, x_PSA) + PQ(x_H, x_C18
      , x_PSA), data = Results)
170 model.ME <- rsm(ME7 ~ FO(x_H, x_C18, x_PSA) + PQ(x_H, x_C18,
      x_PSA), data = Results)
171
172 dataD <- expand.grid(seq(20, 70, by = 5),  # Hexangehalt
173 seq(10, 90, by = 5),   #C18
174 seq(10, 90, by = 5))   # PSA
175 colnames(dataD) <- c("H", "C18", "PSA")
176 str(dataD)
177 #View(dataD)
178
179 # add the natural/coded variables
180
181 dataD$x_H <- (dataD$H - 45)/12.5
182 dataD$x_C18 <- (dataD$C18 - 50)/20
183 dataD$x_PSA <- (dataD$PSA - 50)/20
184
185 dataD$REC <- predict(model.REC, newdata = dataD)
186 dataD$ME <- predict(model.ME, newdata = dataD)
187
188 for (i in 1:nrow(dataD)) {
189         d1 <- targetD(dataD$REC[i], L = 50, T = 100, U = 120,
              r1 = 1, r2 = 1)
190         d2 <- targetD(dataD$ME[i], L = -40, T = 0, U = 20, r1
              = 1, r2 = 1)
191         D <- (d1 * d2)^(1/2)
192         dataD[i, c("d1", "d2", "D")] <- c(d1, d2, D)
```

```
193 }
194
195 # plot desirability results
196 library(ggplot2)
197 ggplot(data = dataD, aes(x=H, y=C18, z=D)) +
198 geom_contour_filled(bins =9) +
199 scale_fill_brewer() +
200 theme_bw() +
201 theme(panel.grid.major = element_blank(), panel.grid.minor =
       element_blank()) +
202 labs(x = "Hexan (%)", y = "C18") +
203 facet_wrap(~PSA) +
204 theme(legend.position.inside = c(0.8, 0.2))
205 ggsave("desirability_ALT.png", width = 10, height = 7, dpi =
       1000)
206
207 summary(predict(model.REC, newdata = dataD))
208
209 ################################################
210
211 optimized_params <- list()
212
213 # Schleife für alle 24 Responsepaare (REC1 - REC24, ME1 -
       ME24)
214 for (i in 1:24) {
215
216         REC_response <- paste0("REC", i)
217         ME_response <- paste0("ME", i)
218
219         # Modell für REC und ME für das aktuelle Responsepaar
              erstellen (nur C18 und H)
220         model.REC <- rsm(as.formula(paste(REC_response, "~ FO
              (x_H, x_C18) + PQ(x_H, x_C18)")), data = Results)
221         model.ME <- rsm(as.formula(paste(ME_response, "~ FO(x
              _H, x_C18) + PQ(x_H, x_C18)")), data = Results)
222
223         # Gitter für die Desirability-Berechnung erstellen (
              nur C18 und H)
224         dataD <- expand.grid(seq(20, 70, by = 5),  #
              Hexangehalt (H)
225         seq(10, 90, by = 5))   # C18
226         colnames(dataD) <- c("H", "C18")
227
228         # Natural-/Coded-Variablen  (nur C18 und H)
229         dataD$x_H <- (dataD$H - 45) / 12.5
230         dataD$x_C18 <- (dataD$C18 - 50) / 20
231
232
```

```
233         dataD$REC <- predict(model.REC, newdata = dataD)
234         dataD$ME <- predict(model.ME, newdata = dataD)
235 
236         # Für jedes Datenpaar Desirability berechnen
237         for (j in 1:nrow(dataD)) {
238                 d1 <- targetD(dataD$REC[j], L = 40, T = 100,
                       U = 120, r1 = 1, r2 = 1)
239                 d2 <- targetD(dataD$ME[j], L = -40, T = 0, U
                       = 20, r1 = 1, r2 = 1)
240                 D <- (d1 * d2)^(1/2)
241                 dataD[j, c("d1", "d2", "D")] <- c(d1, d2, D)
242         }
243 
244         # Optimierung: Finden der optimalen Parameter H, C18
245         optimal_row <- dataD[which.max(dataD$D), ]
246         optimized_params[[i]] <- list(
247         H = optimal_row$H,
248         C18 = optimal_row$C18,
249         Desirability = optimal_row$D,
250         REC_value = optimal_row$REC,
251         ME_value = optimal_row$ME
252         )
253 
254         # Plot der Desirability-Ergebnisse für das aktuelle
              Responsepaar
255         ggplot(data = dataD, aes(x = H, y = C18, z = D)) +
256         geom_contour_filled(bins = 9) +
257         scale_fill_brewer() +
258         theme_bw() +
259         theme(
260         panel.grid.major = element_blank(),
261         panel.grid.minor = element_blank(),
262         legend.position = "right",  # Legende rechts neben
              dem Diagramm
263         legend.title = element_text(size = 12),
264         legend.text = element_text(size = 10)
265         ) +
266         labs(x = "Hexan (%)", y = "C18", fill = "Desirability
              ") +  # Legende bezeichnen
267         ggtitle(paste("Desirability für ", REC_response, "
              und ", ME_response))
268 
269 
270         ggsave(paste0("desirability_2F_", i, ".png"), width =
               10, height = 7, dpi = 1000)
271 }
272 
273 optimized_df <- do.call(rbind, lapply(optimized_params,
```

```
    function(x) data.frame(x)))
write.csv(optimized_df, "optimized_parameters_2Faktoren.csv",
    row.names = FALSE)

optimized_df
```

Scientific Contributions

Scientific Paper and Further Articles

F. Pauelsen, M. Haman and G. Hamscher, "Einsatz von Hormonen in der Tierhaltung und deren potentiellen Auswirkungen auf Mensch und Umwelt," *Deutsche Lebensmittel-Rundschau*, vol. 121, no. 2, Feb. 2025

F. Pauelsen, S. Huppertsberg, T. P. Knepper and D. Zahn, "Narrowing the analytical gap for water-soluble polymers: A novel trace-analytical method and first quantitative occurrence data for polyethylene oxide in surface and wastewater," *Science of The Total Environment*, vol. 882, April. 2023

S. Huppertsberg, D. Zahn, F. Pauelsen, T. Reemtsma and T. P. Knepper, "Making waves: Water-soluble polymers in the aquatic environment: An overlooked class of synthetic polymers?," *Water Research*, vol. 181, no. 1, May 2020

Conference Abstracts

F. Pauelsen, S. Lehr, P. Steliopoulos and G. Hamscher, "Steroid Hormones and Coccidiostats in Dust Samples Originating from Animal Husbandry," Oral presentation presentation at TRACES, Ghent, Belgium, Jun. 2025

F. Pauelsen, P. Steliopoulos and G. Hamscher, "Design of Experiments (DoE) approach for the development and optimisation of an extraction method for steroid hormones and coccidiostats from stable dust," Poster presentation at 52. Deutschen Lebensmittelchemietagen, Freisingen, Germany, Sep. 2024

F. Pauelsen, I. Hennig-Pauka, J. Hartung and G. Hamscher, "LC-MS/MS Determination of Altrenogest and further Steroid Hormones in Dust from Animal Husbandry" Poster presentation at 51. Deutschen Lebensmittelchemietagen, Bonn, Germany, Aug. 2023

F. Pauelsen and G. Hamscher, "Development of Targeted and non-Targeted LC-MS Approaches for Determining Hormones and their Transformation Products in Stable Dusts" Poster presentation at 50. Deutschen Lebensmittelchemietagen, Hamburg, Germany, Sep. 2022

F. Pauelsen, S. Huppertsberg, T. P. Knepper and D. Zahn, "Polyethyleneoxide in the Aquatic Environment – Development and Optimisation of a Quantitative Trace-Analytical Method and First Occurrence Data," Oral presentation at 24. IMSC, Maastricht, Netherlands, Aug. 2022

Declaration in Lieu of an Oath

Ich erkläre: Ich habe die vorgelegte Dissertation selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Ich stimme einer evtl. Überprüfung meiner Dissertation durch eine Antiplagiat-Software zu. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten. Angaben zu auf künstlicher Intelligenz (KI) basierender Hilfen wie ChatGPT oder SchulKI von OpenAI oder Gemini von Google zur Erstellung meiner Dissertation (Zutreffendes angekreuzt):

- ☐ Ich habe bei der Erstellung dieses Textes kein KI-Tool verwendet.
- ☒ Ich habe ein KI-Tool in den folgenden Bereichen eingesetzt (Mehrfachnennungen möglich):
  - ☐ Ideen finden, meine Kreativität anregen
  - ☐ Verstehen von Konzepten, Recherche von Fakten und Definitionen
  - ☒ Optimierung eines von mir verfassten Textes
  - ☐ Erstellen ganzer Textpassagen nach meinen Vorgaben

Folgende KI-Tools habe ich verwendet, damit aufgeführte Teile meines Textes von dem Tool wie folgt profitiert haben:

Ich habe neben online Wörterbüchern deepL.com zur Übersetzung verwendet. Mit Hilfe von ChatGPT habe ich die Tabellen in dieser Arbeit in ein LaTeX-Format konvertiert und R-Codes korrigiert. Perplexity.ai habe ich zur Korrektur von Rechtschreibung und Grammatik meiner Texte verwendet.