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Investigation on the uptake of ciprofloxacin, chloramphenicol and praziquantel by button mushrooms

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ABSTRACT

Button mushrooms are widely produced edible basidiomycetes. Commercially, they are cultivated on substrates containing fermented horse manure and chicken feces. Since pharmacologically active substances (PAS) might be introduced into the food chain via animal treatment, their residues may be present in manure used for mushroom growth. Previous studies in plants have demonstrated an uptake of PAS from the agricultural environment. The present study was performed to investigate the presence of PAS in button mushrooms. For analysis, a multi-analyte method for the detection of 21 selected PAS using liquid chromatography tandem-mass spectrometry was developed, successfully validated and applied to commercially available button mushrooms. Traces of chloramphenicol were detected in two of 20 samples. Additionally, in a mushroom cultivation experiment an uptake of ciprofloxacin, chloramphenicol and praziquantel was conducted. Throughout the whole experiment, praziquantel was present in quantifiable amounts in mushrooms and in high quantities in soil.

1. Introduction

Pharmacologically active substances (PAS) are, among others, used for animal treatment. In 2017, about 6,703 tonnes of veterinary PAS with antimicrobial effect were sold in the EU, predominantly for use in livestock (European Medicines Agency, 2019). Since residues of PAS may be present in food and might have an impact on the health of consumers, the European Commission has set maximum residue limits (MRL) for PAS in food of animal origin, as laid down in Regulation (EU) No 37/2010 (European Commission, 2010). The control of the presence of PAS in food is indispensable to verify the compliance with the requirements of the Regulation and to guarantee a high level of consumer health protection. PAS are directly detectable as residues of treatments of animals in food of animal origin, but also other pathways of the import of PAS into the food chain are possible. For example, PAS can be introduced into soil by application of manure used as fertilizer in the agricultural practice. In experimental studies up to 216 mg/L PAS were found in manure (Kumar, Gupta, Chander, & Singh, 2005). Furthermore, several studies demonstrated an uptake of PAS from soil by plants (Boxall et al., 2006; Kumar, Gupta, Baidoo, Chander, & Rosen, 2005; Panja, Sarkar, Li, & Datta, 2019). Nevertheless, the potential risk for human health caused by PAS via the route of manure, soil and plants is considered to be low, especially when the acceptable daily intake (ADI) of the PAS is high and exposure does not occur via a number of different routes simultaneously (Boxall et al., 2006).

A route not yet been investigated thoroughly is the transition of veterinary PAS from contaminated soil to button mushrooms (*Agaricus bisporus*). Button mushrooms are among the most extensively and commercially cultivated basidiomycetes which are consumed worldwide. For large-scale mushroom production, a well-defined fermented mixture of manure from domestic or food-producing horses, chicken manure, straw, gypsum and water is used. PAS administered to horses or

Abbreviations: *Epi*-CTC, 4-Epichlortetracycline; *Epi*-OTC, 4-epioxytetracycline; *Epi*-TC, 4-epitetracycline; ADI, acceptable daily intake; Anhydro-ERY-A, anhydroerythromycin A; CAP, chloramphenicol; CAP-d5, chloramphenicol-d5; CTC, chlorotetracycline; CIP, ciprofloxacin; CIP-d8, ciprofloxacin-d8; DEX, dexamethason; DIX, difloxacin; ESI, electrospray ion source; ENR, enrofloxacin; ENR-d5, enrofloxacin-d5 hydrochlorid; ERY-A, erythromycin A; ERY-C13,d3, erythromycin-C13,d3; FLX-d3, flunixin-d3; IP, identification point; LC-MS/MS, liquid chromatography tandem-mass spectrometry; MRL, maximum residue limit; MTBE, methyl *tert*-butyl ether; MRPL, minimum required performance limit; MRM, multiple reaction monitoring; NOR, norfloxacin; NOR-d5, norfloxacin-d5; OTC, oxytetracycline; PAS, pharmacologically active substances; PRA, praziquantel; SARA, sarafloxacin; SARA-d8, sarafloxacin-d8; SPI, spiramycin; SD, sulfadiazine; SME, sulfamethazine; SMX, sulfamethoxazole; TC, tetracycline; TRI, trimethoprim; TRI-d9, trimethoprim-d9.

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chicken for therapeutic reasons can potentially be excreted unchanged or metabolized and occur in the fermented substrate, which is deeply pervaded by the mushroom mycelium. An uptake of antimicrobial PAS by mushrooms is possible as was recently reported for doxycycline (Gbylik, Gajda, Nowacka-Kozak, & Posyniak, 2020).

For our study on a potential transition of PAS into button mushrooms we chose PAS according to the following criteria: i) frequent administration to horses or chicken and ii) excretion mainly unchanged or as active metabolites. PAS fulfilling these criteria were found for the groups of anthelmintics, fenicols, makrolides, quinolones, sulfonamides and tetracyclines. Among those PAS are ciprofloxacin (CIP), which is the active metabolite of the broad-spectrum antibiotic enrofloxacin (ENR), and the anthelmintic praziquantel (PRA). The fluoroquinolone ENR is frequently administered to horses and chicken and shows a high chemical stability. In general, ENR is *N*-deethylated into CIP in animals, except poultry. In chicken, 74% of ENR is excreted as unchanged parent compound while only 25% is metabolized to form the chemically stable and more potent CIP (Asambe, Babashani, & Salisu, 2018; Pasquali & Manfreda, 2007; Slana, Pahor, Cvitkovič Maričič, & Sollner-Dolenc, 2014). In chicken manure, CIP was detected in amounts up to 2.1 mg/kg (Martínez-Carballo, González-Barreiro, Scharf, & Gans, 2007) and 45.59 mg/kg (Zhao, Dong, & Wang, 2010). Due to the use of CIP in human medicine, a spread of antibiotic resistance or the triggering of adverse immunological reactions through CIP sources other than human treatment may be possible (Witte, 1998). Therefore, CIP is discussed as an emerging environmental pollutant (Wang et al., 2020). PRA is frequently used for therapy of internal parasites in domestic horses and chicken. It was excreted in amounts of 31% via urine and 24% via feces within 24 h after application of a radiolabeled compound (European Agency for the evaluation of Medicinal Products, 1998). It is a thermally stable (Horvat et al., 2012) and environmentally persistent PAS, found in waste water in concentrations of up to 0.4 μg/L (Babić et al., 2010). Another thermally stable PAS investigated in our study is the broad-spectrum antibiotic chloramphenicol (CAP) being active against Gram-positive and some Gram-negative bacteria. In the EU, it is not authorized for food-producing animals but approved for use in non-food producing animals. Natural contamination of plant-based feed, especially straw, by CAP-producing soil bacteria like *Streptomyces venezuelae* is described (Berendsen et al., 2013). In chicken, CAP is rapidly metabolized to form dehydro-CAP, nitrophenylaminopropanedione-CAP and nitroso-CAP (Anadón, Bringas, Martinez-Larrañaga, & Diaz, 1994). While in general the principal pathway for CAP is its inactivation via glucuronidation, about 5–15% of CAP is still excreted unchanged via urine (Wongtavatchail, McLean, Ramos, & Arnold, 2004). Due to its carcinogenic potential for humans and the lack of data for a dose–response relationship for aplastic anemia, it is banned for use in food-producing animals. The EU has set a minimum required performance limit (MRPL) of 0.3 μg/kg for food (European Commission, 2003). Taking into account a substance-specific risk assessment by the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain in 2014 (European Food Safety Authority, 2014) a reference point for action (RPA) of 0.15 μg/kg for food of animal origin shall apply from November 2022 (European Commission, 2019).

The aim of the present study was to evaluate a possible transfer of frequently used veterinary PAS from soil into button mushrooms. Initially, 21 PAS were identified for a potential transition into mushrooms according to the above mentioned criteria (frequent administration to chicken or horses, excretion mainly unmetabolized or as active metabolite). These PAS exhibit a broad range of activities, including antibiotic, anthelmintic and anti-inflammatory activity. A multi-analyte method using liquid chromatography tandem-mass spectrometry (LC-MS/MS) for these 21 PAS was developed, validated and applied to commercially available mushrooms. In a mushroom cultivation experiment, the transition of CIP and PRA was monitored from soil into button mushrooms. Due to two findings of the broad-spectrum antibiotic CAP in mushrooms at concentrations between limit of detection (LOD) and limit of quantitation (LOQ), CAP was also selected for the mushroom cultivation experiment. The three PAS were added to the top layer, which was deeply pervaded by the mushroom mycelium. At defined intervals, mushrooms as well as the top layer were analyzed for CIP, CAP and PRA.

2. Material and methods

2.1. Chemicals and reagents

Anhydroerythromycin A (anhydro-ERY-A), chloramphenicol (CAP), chloramphenicol-d5 (CAP-d5), chlorotetracycline (CTC), ciprofloxacin (CIP), ciprofloxacin-d8 (CIP-d8), dexamethason (DEX), difloxacin (DIX), enrofloxacin (ENR), enrofloxacin-d5 hydrochlorid (ENR-d5), 4-epioxytetracycline (*Epi*-OTC), 4-epitetracycline (*Epi*-TC), erythromycin A (ERY-A), flunixin-d3 (FLX-d3), NaCl, norfloxacin (NOR), oxytetracycline (OTC), praziquantel (PRA), spiramycin (SPI), sulfamethazine (SME), tetracycline (TC), trimethoprim (TRI) and trimethoprim-d9 (TRI-d9) were purchased from Merck (Darmstadt, Germany). Sarafloxacin-d8 (SARA-d8) and sulfamethoxazole (SMX) were purchased from Altmann Analytik (München, Germany), sarafloxacin (SARA) and sulfadiazine (SD) from LGC (Teddington, Middlesex, United Kingdom), 4-epichlortetracycline (*Epi*-CTC) from Acros Organics (Thermo Fisher Scientific, Waltham, Massachusetts), norfloxacin-d5 (NOR-d5) from Fluka (Thermo Fisher Scientific, Waltham, Massachusetts), erythromycin-C13, d3 (ERY-C13,d3) from Santa Cruz Biotechnology (Heidelberg, Germany), acetonitrile, methanol, formic acid and methyl *tert*-butyl ether (MTBE) from Merck (Darmstadt, Germany) and isopropanol and ammonium formate from VWR (Darmstadt, Germany). All solvents and additives were of LC-MS grade. Purified water was prepared by a Milli-Q system (Millipore, Eschborn, Germany).

2.2. Apparatus

For freeze-drying of samples, a Martin Christ Gefriertrocknungsanlage (Osterode am Harz, Germany) was used. Solid-phase extraction using OASIS HLB, 200 mg sorbent per Cartridge (hydrophilic-lipophilic-balanced, Waters, Milford, MA) for sample clean-up was performed on a Visiprep DL solid-phase extraction vacuum manifold from Merck (Darmstadt, Germany). Analytes were separated and quantified using an Agilent 1260 Infinity LC coupled with an Agilent 6500 Triple quadrupole tandem-mass spectrometer (Agilent, Santa Clara, CA) interfaced with an electrospray ion source (ESI).

2.3. Preparation of standard solutions for liquid chromatography-tandem mass spectrometry

A standard mix solution containing DEX, SME, TRI, PRA, NOR, DIX, ENR, OTC, SD, TC, CTC, ERY-A, anhydro-ERY-A, CIP, *Epi*-TC, *Epi*-CTC, SPI, *Epi*-TC, SMX and SARA (at a concentration of 10 μg/mL each) was prepared from stock solutions of DEX, SME, TRI, PRA, DIX, ENR, OTC, TC, CTC, ERY-A, CIP, *Epi*-TC, *Epi*-CTC, SPI, *Epi*-TC, SMX (1 mg/mL in MeOH, each) and NOR, SD, anhydro-ERY-A and SARA (100 μg/mL in MeOH, each) with MeOH. A further standard solution containing 10 μg/mL of CAP was prepared by dilution of a stock solution (1 mg/mL in MeOH) with MeOH. Stock solutions were stored at –20 °C for six months. Standard mix solutions were stable for three months at –20 °C.

An internal standard mix solution (IS-Mix) containing TRI-d9, CIP-d8, ENR-d5, SARA-d8, ERY-C13,d3 and NOR-d5 (at a concentration of 5 μg/mL, each) was prepared from stock solutions of TRI-d9 and CIP-d8 (110 μg/mL in MeOH), ENR-d5, SARA-d8 and ERY-A-C13,d3 (100 μg/mL in MeOH) and NOR-d5 (40 μg/mL in MeOH) with MeOH. A 10 μg/mL internal standard solution of CAP-d5 (IS-CAP) was produced by dilution of a stock solution (1 mg/mL in MeOH) in MeOH. Stock and internal standard solutions were stored at −20 °C for six months and three months, respectively. For calibration, a 0.5 μg/mL working solution of IS-Mix and a 100 ng/mL working solution of CAP-d5 were

prepared freshly on the day of use by dilution in MeOH. TRI-d9, CIP-d8, ENR-d5, SARA-d8, ERY-C13,d3, NOR-d5 and CAP-d5 were used as internal standards for the quantification of TRI, CIP, ENR, SARA, ERY, NOR and CAP. The remaining analytes were analyzed without internal standard correction. For analysis of the top layer, FLX-d3 was used for the quantification of PRA. For this purpose, a 5 μg/mL solution of FLX-d3 was prepared in MeOH. CIP was analyzed without internal standard.

2.4. Mushroom cultivation procedure and sample collection

For the cultivation experiment, 12 white mushroom breeding boxes from Pilzhof & Edelpilzzucht Breck GbR (Malschwitz, Germany) were used consisting of a mycelium-containing straw material and about 1 kg moist top layer each. The top layer of all mushroom breeding boxes were merged and homogenized by mixing. The water-holding capacity of the merged top layers determined from the loss of water of 5 g top layer after drying to constant mass at 105 °C was 79.2%. For pH determination, 5 g dried top layer were mixed with 25 mL water, allowed to swell overnight and determined using a pH meter from Mettler Toledo (Gießen, Germany). The pH of the merged top layer was 7.3. Subsequently, the remaining moist top layer was divided into four parts of 3 kg each. Three parts of moist top layer were separately treated with 4 mg substance (CIP, CAP or PRA) dissolved in 500 mL water (1,333 μg/kg dry weight), mixed with an electrical concrete mixer for 30 min and dried at 35 °C to the initial moisture content. The concentration of PAS was chosen as a low-concentration scenario compared to the use of manure containing 216 mg/L PAS (Kumar et al., 2005). One part was treated with the same amount of water for control. Finally, each treated top layer was spread evenly on three mushroom breeding boxes. Homogeneity of the analytes in the top layer was determined by taking three samples of top layer from different parts of each box and analyzing the samples as described (see Section 2.6, a total of 9 samples per treatment). Every two weeks several samples of top layer were taken for homogeneity testing and analyzed for CAP, CIP and PRA. The boxes were kept in the dark at 20 °C. After two weeks, the top layer was deeply pervaded by the mushroom mycelium. To induce fungal growth, the temperature in the room was reduced to 16 °C. The mushrooms were harvested four and six weeks after addition of the treated top layer and directly freeze-dried. Subsequently they were ground and stored under exclusion of light until extraction.

2.5. Extraction and clean-up of mushrooms

Freeze-dried mushrooms corresponding to 10 g fresh mushroom were weighed into a 50 mL falcon tube. After addition of 80 μL IS-Mix working solution and 30 μL working solution of CAP-d5, the sample was allowed to incubate for 10 min at room temperature before addition of 4 mL of 86% acetonitrile in water. The sample was intensively vortexed for 1 min, sonicated for 10 min in an ultrasonic bath and vortexed again for 1 min. The mixture was cooled for 10 min in ice-cold water, mixed with 40 μL formic acid, vortexed briefly and subsequently centrifuged for 10 min at 5 °C and 2.000 × *g*. The supernatant was filtered through a pleated filter. The residue was redissolved in 10 mL water, vortexed for 1 min, sonicated for 10 min and centrifuged as described before. The supernatants were combined and adjusted with water to 20 mL. Prior to LC-MS/MS analysis of CIP and PRA (as part of the multi-analyte method), the extracts were transferred to vials via a 0.45 μm PTFE-syringe filter and kept at 4 °C until analysis.

For CAP in food, a minimum required performance limit (MRPL) of 0.3 μg/kg was set by the EU (European Commission, 2003). To meet this requirement, additional extraction steps for CAP were established. For this purpose, 10 mL of the extract for multi-analyte detection were vigorously vortexed with 4.5 g NaCl and 5 mL of MTBE for 15 min. The mixture was centrifuged for 10 min at 2.000 × *g* and the organic phase transferred to a 15 mL falcon tube. The procedure was repeated with another 5 mL of MTBE. The combined organic phases were evaporated to dryness at 40 °C in a stream of nitrogen. The dry residue was redissolved in 2 mL of 10% acetonitrile / 90% 10 mM ammonium formate in water + 0.2% formic acid. The redissolved residue was intensively vortexed for 1 min, sonicated in a water bath for 10 min and filtered via a 0.45 μm PTFE-syringe filter prior to LC-MS/MS analysis.

2.6. Extraction and clean-up of top layer

The extraction procedure for the analysis of top layer is based on the method for the extraction of mushrooms. For sample preparation, about 50 g of top layer was dried at 35 °C for about 29 h until constant weight was reached. For analysis of CIP, CAP and PRA 1 g of dried top layer was used and processed as described above (Section 2.5) with the exception of omitting the second extraction step for analysis of CAP.

2.7. Liquid chromatography tandem-mass spectrometry operating conditions

Mushrooms were analyzed using a validated in-house method. However, for the analysis of CAP some settings needed to be optimized for chromatographic separation, parameters of ion source and MS/MS detection. In the following, the LC-MS/MS configurations for analysis of 20 PAS (multi-analyte method) in mushrooms are referred to as “system 1”, those applied for detection of CAP are referred to as “system 2”.

System 1: Chromatographic separation was carried out using a Thermo Fisher Hypersil Gold column (150 × 2.1 mm, 3 μm particle size) with corresponding guard cartridge (4 × 2 mm, C18 phase) and a solution of 10 mM ammonium formate in water (eluent A) and acetonitrile (eluent B), both containing 0.2% formic acid. A volume of 20 μL of the sample extract of mushrooms or soil was injected into an initial mobile phase consisting of 10% eluent B. At a flow rate of 0.3 mL/min, analyte separation was achieved with a 15.4-min linear gradient and a 3 min subsequent isocratic elution ending at 80% eluent B. The total run time for one analysis including re-equilibration of the HPLC was 27.0 min. The column temperature was 30 °C and the autosampler temperature was maintained at 4 °C.

System 2: Analyte separation was carried out using an Agilent Poroshell 120 EC-C18 analytical column (50 × 4.6 mm, 2.7 μm particle size) with guard cartridge (4 × 2 mm, C18 phase) and a solution of 10 mM ammonium formate in water (eluent A) and acetonitrile (eluent B), both containing 0.2% formic acid. A volume of 20 μL of the sample extract of mushrooms or soil was injected into an initial mobile phase consisting of 30% eluent B. At a flow rate of 0.5 mL/min, analyte separation was achieved with a 4-min linear gradient ending at 58% eluent B. The total run time for one analysis including re-equilibration of the HPLC was 10 min. The column temperature was 30 °C and the autosampler temperature was maintained at 4 °C.

The following ion source parameters were used for multi-analyte analysis and CAP: polarity = positive (*system 1*), negative (*system 2*); curtain gas = 55 psi; collision gas = MED; ion spray voltage = 5500 V (*system 1*), −2000 V (*system 2*); temperature = 400 °C; gas sources 1 = 60 psi; gas sources 2 = 50 psi and entrance potential = 10 V (*system 1*), −7 V (*system 2*). For quantification, the multiple reaction monitoring (MRM) approach was used. The retention time, optimized declustering potential, collision cell exit potential and collision energy for each MRM transition are summarized in Supplemental Table 1.

Analytes were distinguished by retention time and MRM transitions against standard solutions. At least two mass transitions for each analyte were monitored, whereby the mass transition of highest intensity was used for quantification. Only peaks in the range of retention time ± 2.5% and an ion ratio in compliance with standard solutions were used for calculation. Results were calculated against a matrix-matched calibration in the range of 2.5 to 50 μg/kg for multi-analyte analysis and of 0.125 to 1.5 μg/kg for CAP and only if positively checked for linearity using Mandel’s test. All data were corrected for recovery. Sample preparation and measurements were performed on the same day.

CIP, PRA and CAP in top layer were analyzed using the same LC-MS/MS settings as described for mushrooms. For quantification, an external calibration curve in the range of 5.0 to 75 μg/mL CIP, PRA and CAP, without matrix was applied. All data were corrected for recovery. Sample preparation and measurements were performed on the same day.

2.8. Method validation

For validation of the method used for the analysis of 21 PAS in mushrooms, a factorial design according to Commission Decision 2002/657/EC (European Commission, 2002) was applied. Calculation was done using the software InterVAL (Quodata, Dresden/München, Germany). Relevant factors that may influence the analysis were varied on two levels: experienced and unexperienced operator, different batches of LC column and mushroom color (white and brown button mushrooms). The experimental design is shown in Supplemental Table 2.

Test samples for eight runs were prepared by spiking freeze-dried blank mushroom samples with 0, 5, 10, 20, 30 and 40 μg/kg analyte which, referring to a fresh weight basis of mushrooms had been calculated against a matrix-match calibration of 2.5, 5, 10, 20, 30, 40 and 50 μg/kg for multi-analyte analysis using *system 1*. Blank freeze-dried mushroom samples were spiked with 0, 0.1, 0.2, 0.3, 0.4 and 0.5 μg/kg of CAP. With reference to fresh mushroom weight, the amounts had been calculated against a matrix-matched calibration of 0, 0.125, 0.25, 0.5, 0.75, 1, 1.25 and 1.5 μg/kg using *system 2*. One matrix-matched calibration was used for two runs, prepared within one day. Assessed were: the decision limit CCα (α set to 1%), defined as lowest concentration level at which a method can discriminate with a statistical certainty of 1–α whether the particular analyte is present; the detection capability CCβ (β set to 5%), defined as lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1-β; the repeatabilites (RSD_r); the in-house reproducibilities (RSD_{wR}); recovery; and recovery-corrected measurement uncertainty. Additionally, LOD and LOQ were calculated according to DIN 32645 (Deutsches Institut für Normung, 2008).

3. Results and discussion

3.1. Method development

An accurate and robust analytical method for the quantification of 21 PAS and metabolites of the groups of tetracyclines, sulfonamides, quinolones, makrolides, fenicols and anthelmintics in button mushrooms using LC-MS/MS was developed. Methods for detection of antimicrobial PAS are already published (Gbylik, Gajda, Nowacka-Kozak, & Posyniak, 2019, 2020). For the first time, using our method also anthelmintics, anti-inflammatory drugs and CAP can be detected in mushrooms simultaneously. For sample preparation, commercially available mushrooms were freeze-dried. Mushrooms typically contain about 90% of water. Freeze-drying allowed preparation of a finely ground homogenate and long-term storage. During the process of freeze drying, no analyte loss was observed. The subsequent extraction procedure of the freeze-dried mushroom samples included a large dilution step. This step was effective for extracting the analytes and diluting the matrix components sufficiently. It constitutes a reliable, low-cost and time-saving alternative to other extraction procedures such as solid-phase extraction. The whole procedure allows the simultaneous analysis of 20 PAS and metabolites in mushrooms using the positive ion mode of the electrospray ion source (ESI) within 18.4 min (multi-analyte analysis) with a total run time for one analysis including re-equilibration of 27 min. For CAP an additional extraction step was performed. It was separately quantified within a 4 min run using the same eluents as for the multi-analyte method, but with a shorter column and in the negative ion mode. The methods were successfully validated using an alternative validation approach and applied to commercially available mushrooms.

3.2. Method validation and application for mushrooms

Full method validation was performed according to the guidelines of Commission Decision No. 2002/657/EC using the software InterVal. For each analyte, the method performance was assessed using the qualitative parameters analyte specificity as well as molecular identification in term of retention time (Rt) and transition ion ratios. The quantitative parameters tested were linearity, repeatability, within-laboratory reproducibility, recovery and analytical limits CCα and CCβ. These parameters provide information regarding linear range, detection capability, precision, trueness and decision limits. Validation was done using a set of eight series (runs) of analyses with five concentration levels per run, resulting in a total number of 40 experiments. The experimental design is based on the factors experience of the operator, batches of LC column and mushroom color, which varied on two levels each. This experimental design was also used for calculation of the analytical limits LOD and LOQ according to DIN 32645:2008. In detail, LOD was calculated as three times the standard deviation of the lowest concentration sample to the slope of the calibration curve. LOQ was expressed as 3.3 times LOD.

3.2.1. Specificity

Specificity was determined for each analyte directly in the chromatograms obtained from standard solutions, different blank and fortified mushroom samples. Any extra-peak in the retention time window of the analyte for the MRM transitions of interest was checked in comparison to the blank matrix chromatograms. No interferences were observed at the retention times of the 21 PAS in mushrooms and of the internal standards (Supplemental Fig. 1). Also, retention times of analytes when determined in spiked samples and standard solutions, were comparable with the required tolerance of ± 2.5% (Commission Decision No. 2002/657/EC).

3.2.2. Identification

All selected PAS except CAP belong to group B of Annex 1 of Directive 96/23/EC. The minimum number of identification points (IP) according to Commission Decision No. 2002/657/EC for liquid chromatography coupled with tandem-mass spectrometry is set to three. CAP belongs to group A6 of Annex 1 of Directive 96/23/EC. For CAP, the minimum number of IPs is set to four. Since at least two relevant MRM transitions were monitored for each analyte, four IPs were obtained. Stability of the ion ratios between two transitions was tested for each analyte by first calculating the mean ion ratio (least intense signal against the most intense one) for all calibrating standards. Subsequently, the relative deviation (%) of the ion ratios was determined with spiked mushroom samples. The mean ratio of calibration standards and spiked mushroom samples was in accordance with the tolerances recommended by the Commission Decision No. 2002/657/EC (Annex I, Section 2.3.3.2) for all analytes.

3.2.3. Linearity

One matrix-matched calibration curve was built each day with 7 calibration levels. Calibration levels for multi-analyte determination (system 1) ranged from 2.5 to 50 μg/kg and from 0.125 to 1.5 μg/kg for CAP. One matrix-matched calibration curve was used for analysis of two runs. Linearity of the matrix-matched calibration curves was determined using the Grubbs' test, Mandel's test and calculation of the coefficient of determination (R^2). The linearity of the analytical response across the studied range of all matrix-matched calibrations over all 21 analytes was satisfactory with coefficients of determinations higher than 0.95.

3.2.4. Accuracy in terms of trueness

Trueness was assessed by calculating the recovery of the spiked blank freeze-dried mushroom samples against the matrix-matched calibration curve. The mean recoveries are shown in Table 1. Some recoveries were outside the range of 80 to 110% recommended by Commission Decision

Table 1
Results of the method validation for the studied compounds in button mushrooms; decision limit (CCα), detection capability (CCβ), limit of detection (LOD), limit of quantitation (LOQ), repeatability (RSDr), in-house reproducibility (RSDwR), recovery and measurement uncertainty.

	CCα (μg/kg)	CCβ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	RSD_r (%)	RSD_{wR} (%)	Recovery (%)
Tetracyclines							
TC	8.7	12.4	4.6	15.1	9.0	16.1	68.2
Epi-TC	10.0	15.3	4.3	14.2	6.9	19.2	60.1
OTC	17.9	26.5	5.1	16.9	2.4	15.3	60.4
Epi-OTC	8.9	12.4	4.4	14.6	8.5	15.8	56.1
CTC	7.7	10.2	2.2	7.3	9.5	12.8	59.4
Epi-CTC	12.3	20.9	6.4	21.2	14.8	22.6	64.6
Sulfonamides							
SD	8.8	13.3	3.7	12.2	7.1	19.4	86.6
SME	9.4	14.1	4.8	15.8	7.0	18.8	76.8
SMX	8.3	11.2	3.2	10.6	7.5	12.9	78.8
Diaminopyrimidine							
TRI_a	7.1	9.1	3.9	12.8	7.2	12.3	90.3
Quinolones							
DIX	11.0	17.4	6.5	21.5	15.1	20.3	77.9
CIP_a	8.5	11.3	4.4	14.6	8.0	11.9	89.5
ENR_a	8.6	11.1	3.6	12.0	6.1	9.5	81.6
NOR_a	9.4	13.1	3.7	12.3	7.9	13.4	89.0
$SARA_a$	9.2	12.7	3.7	12.1	8.4	12.6	84.4
Makrolides							
ERY-A_a	9.4	14.5	2.1	6.9	8.4	20.2	98.3
Anhydro-ERY-A	7.9	10.4	2.9	9.5	7.2	11.8	81.5
SPI	8.6	11.7	3.5	11.4	7.9	12.7	76.0
Anti-inflammatory drugs							
DEX	8.3	10.9	3.0	9.9	6.8	10.2	75.8
Anthelmintics							
PRA	8.1	10.5	3.3	10.8	6.6	10.5	64.7
Fenicols							
CAP_a	0.18	0.23	0.06	0.19	6.3	9.6	96.6

No. 2002/657/EC. For example, the group of tetracyclines showed recoveries between 56.1 and 68.2%, only. The recoveries for the sulfonamides SME (76.8%) and SMX (78.8%), the quinolone DIX (77.9%), the macrolide SPI (76.0%), the anti-inflammatory DEX (75.8%) and the anthelmintic PRA (64.7%) were not within the default range. Optimal extraction and detection of all analytes are not always achievable when several compounds are targeted simultaneously. The use of isotopically labeled internal standards would improve the recovery of these analytes. Unfortunately, such compounds were not commercially available at the time the experiments were conducted. Since this method was developed for research purposes, and not for official food and feed control, the validation results obtained were considered acceptable for this multi-method. To mitigate this limitation, it is always recommended to include spiked control samples to evaluate the recovery of the series of analyses.

3.2.5. Precision

The precision of the method was evaluated by determining the repeatability and within-laboratory reproducibility. For all analytes, the repeatability was in the range of 2.4 (OTC) to 15.1% (DIX) and the within-laboratory reproducibility was in the range of 9.5 (ENR) to 22.6% (*Epi*-CTC). These results show a satisfactory performance of the method in accordance with Commission Decision No. 2002/657/EC, listing a repeatability not exceeding 15.3% and a within-laboratory reproducibility as low as possible for concentrations lower than 100 μg/kg. Results are shown in Table 1.

3.2.6. Analytical limits

For the selected PAS, no MRLs are set for the matrix mushroom. Therefore, the level of interest is set at 0.00 μg/kg. Consequently, the two critical limits CCα and CCβ were determined to be in the range of 7.1 (TRI) to 17.9 μg/kg (OTC) and 9.1 (TRI) to 26.5 μg/kg (OTC), respectively. For CAP in food, a MRPL of 0.3 μg/kg has been set. The CCβ value for CAP is below the MRPL, confirming that the method achieves the MRPL established for CAP measurement in food. LOD and LOQ are in the range of 2.1 (ERY-A) to 6.5 (DIX) and 6.9 (ERY-A) to 21.5 (DIX). The lowest level of CAP that can be reliably detected according to DIN 32645 is 0.06 μg/kg. The LOQ for CAP was determined to be 0.19 μg/kg. The results obtained for the critical limits CCα and CCβ as well as LOD and LOQ during this validation are summarized in Table 1.

3.2.7. Robustness

Robustness of the method was tested by varying the factors experience of the operator, batches of LC column and mushroom color on two levels each. The factorial effects contribute considerably to the total uncertainty of the method. However, the critical concentrations were acceptable. Separate calibration and/or separate validation were not required.

3.3. Method application to commercially available mushrooms

The validated method was applied to 20 commercially available mushroom samples obtained in the period from 2015 to 2018. In one brown and one white mushroom sample, CAP was found in concentrations between LOD and LOQ. In the EU, CAP is not authorized for use in food-producing animals and an MRPL of 0.3 μg/kg for food has been set. Nevertheless, for mushroom production, horse and chicken manure from non-food-producing animals can be used. Another explanation for the finding might be the presence of CAP-producing soil bacteria such as *Streptomyces venezuelae*. Since top layer has not to be sterilized for mushroom cultivation and may contain CAP-producing soil bacteria, CAP may occur as a contaminant in top layer and be taken up by the mushroom mycelium. A formation of over 100 μg/kg CAP in non-sterile top soil within one day as well as an uptake of CAP from soil by plants was reported (Berendsen et al., 2013). Moreover, CAP is often used to keep the spawn (pure culture of mycelium) free from bacterial contamination. The availability of spawn free from contamination is one of the most critical factors in mushroom production. None of the other tested PAS was found in any sample investigated, not even below the CCα.

3.4. Mushroom cultivation experiment

In a mushroom cultivation experiment a possible transition of the PAS CIP, CAP and PRA from top layer into button mushrooms were determined. PRA and CIP were chosen for their frequent administration to horses or chicken and a mainly unchanged excretion or excretion as active metabolite. In our investigations, CAP was found in two of 20 commercially available mushroom samples at concentrations between LOD and LOQ. After addition of 1,333 μg/kg PAS to top layer (dry matter) and starting the mushroom cultivation experiment, all treated and untreated mushroom boxes showed adequate mushroom growth. There was no obvious difference in shape, color, size and water content between mushrooms of control and treated boxes. About 870 g mushrooms per box were collected in two harvests. Between day 24 and 28, after addition of the top layer, mushrooms were mature and harvested for the first time with a mean mass of 800 g per box. In the second harvest, only 77.8 ± 52.7 g mushrooms per box were collected. In one box (PRA-treated top layer) no mushrooms were collectable at the second harvest. Interestingly, the growth of mushrooms in the CAP-treated boxes was significantly increased at the first harvest (Fig. 1). Taking into account the second harvest of mushrooms from CAP-treated top layer on day 42, a comparable total amount of mushrooms was found for the two harvests. CAP might have a growth-enhancing effect but no influence on total mushroom mass. Further investigations are needed to prove this finding.

All harvested mushrooms were analyzed for CIP, CAP and PRA. In the mushrooms of control boxes without PAS treatment, no analyte was detected. However, after the first harvest, traces of CAP, CIP and PRA below CCα were found in all mushrooms of the treated boxes (Table 2). An uptake of PAS by the mushroom mycelium might explain these findings. Considering that the levels of PAS added to top layer were chosen to reflect realistic and not artificial worst-case scenarios, amounts of PAS in the mushroom substrate used in commercial farming might be too low for appropriate detection in mushrooms. Moreover, it might be possible that the PAS were taken up by mushrooms but subsequently metabolized by the mycelium. It was for example shown that basidiomycetes are able to metabolize CIP (Wetzstein, Stadler, Tichy, Dalhoff, & Karl, 1999). Our method is suitable for the detection of CIP, CAP and PRA but not their metabolites. Finally, the findings could also be a result of remaining top layer particles on the mushroom surface. For sample preparation, mushrooms were carefully cleaned from soil particles after the harvest, but not treated with water. Thus, an uptake of CIP, CAP and PRA by button mushrooms after the first harvest (24–28 days) could not be sufficiently confirmed. After the second harvest on day 42, however, PRA was found in mushrooms with mean amounts of

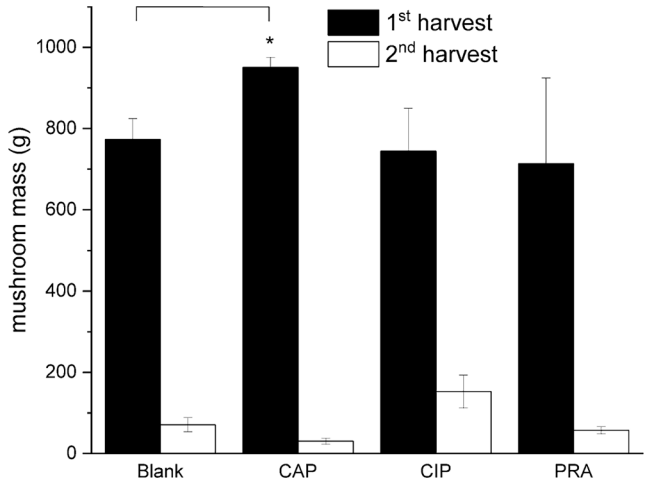

Fig. 1. Mean total harvested mass of white button mushrooms per box. Data are means ± SEM (n = 3, PRA 2nd harvest n = 2). *p < 0.05 (Student's *t*-test).

Table 2
Results of mushroom analysis during the cultivation experiment. Data are means ± SD (n = 3).

	Harvest 1			Harvest 2		
Top layer spiked with	box 1	box 2	box 3	box 1	box 2	box 3
CAP	<CCα	<CCα	<CCα	–	–	–
CIP	<CCα	<CCα	<CCα	–	–	–
PRA	<CCα	<CCα	<CCα	98.9 ± 6.3 μg/kg	25.9 μg/kg*	no mushrooms

* (n = 1).

98.9 μg/kg (box 1, n = 3) and 25.9 μg/kg (box 2, only one out of three samples with positive result), while in the third box (box 3) no mushroom growth was observed. This indicates an inconsistent uptake of PRA by mushroom mycelium after long-time incubation (42 days). CIP and CAP were not detected in mushrooms after 42 days of treatment, not even below the CCα.

The Committee for Veterinary Medicinal Products established an ADI of 0.17 mg/kg for PRA (European Agency for the evaluation of Medicinal Products, 1998). The potential exposure to PRA due to contaminated mushrooms and the risk to human health were estimated using the European Food Safety Authority Pesticide Residue Intake Model (European Food Safety Authority, 2019). Irish adults were identified consuming the highest daily amount of mushrooms with approximately 0.2367 g/kg body weight. If all consumed mushrooms are contaminated with 0.0989 mg/kg PRA (the highest amount found in the mushroom cultivation experiment), a theoretical daily intake of 0.023 μg/kg body weight can be calculated. This represents only around 0.01% of the ADI so that the risk for human health is considered to be low.

3.5. Analysis of CIP, CAP and PRA in top layer

During mushroom cultivation, the top layer was also tested for CAP, CIP and PRA. The validated method for mushroom analysis was used, however, the additional extraction step for CAP was omitted. A short validation demonstrated the suitability of the method for soil and allowed to determine the analytical limits LOD and LOQ: untreated top layer from the control box was spiked with 0.5, 1, 2.5, 5, 10 μg/kg CAP, CIP, PRA and analyzed as described before (section 2.6). This procedure was conducted in duplicate on three different days. LOD and LOQ were between 0.80 μg/kg (CAP) and 2.01 μg/kg (PRA) and 3.04 μg/kg (CAP) and 7.80 μg/kg (PRA), respectively (Supplemental Fig. 2, Supplemental Table 3). A high inter-day precision (1.1 – 2.7%) and an acceptable recovery (69.3 – 88.2%) were found for all analytes. The high sensitivity and high precision demonstrate the suitability of the method for the difficult matrix soil. The detection limits are comparable to other published methods analyzing antibiotics in soil (Berendsen et al., 2013; Huang et al., 2013).

Using this method for the analysis of CAP, CIP and PRA in top layer, a decrease of CAP and CIP within 14 days was observed (Fig. 2). In general, PAS in soil can be subject to sorption, photohydrolysis, oxidation and biodegradation. They interact with soil minerals, organic matter and microorganism so that they are no longer bioavailable. A fast decrease of CAP in soil is already known. Depending on the soil composition, a half-life of approximately one day for CAP in non-sterile top soil due to bacterial activity was reported (Berendsen et al., 2013). For CIP, a strong binding to soil is described making CIP recalcitrant to biodegradation. After 28 days (first mushroom harvest), CAP was detectable in levels close to the LOQ while CIP was detectable in levels above the LOQ. Neither CAP nor CIP were detectable on day 42 (second mushroom harvest). The fast decrease of CAP and CIP in top layer might be responsible for the fact that both substances could not be detected in mushrooms after 42 days. Nevertheless, metabolites of CIP and CAP may

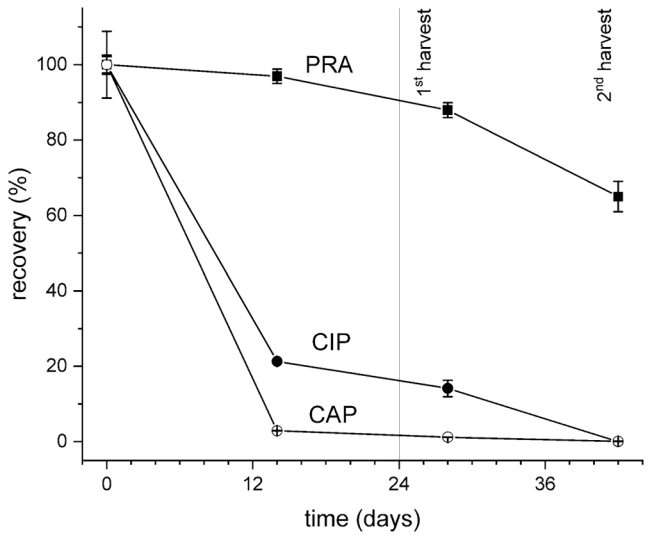

Fig. 2. Time course of CAP, CIP and PRA added to top layer on day "0" (1,333 μg/kg, set to 100%) of the cultivation experiment. White button mushrooms were harvested on days 24–28 (1st harvest) and day 42 (2nd harvest). Data are means ± SD (n = 18).

be present in soil and mushrooms. These metabolites could not be determined using our extraction procedure. In contrast to the results with CIP and CAP, PRA was found in high amounts in top layer throughout the whole experiment. Even after 42 days, about 70% of the initial PRA levels were still detectable. This indicates a high stability of PRA in soil and might explain the uptake of PRA by button mushrooms. Furthermore, this finding indicates that PRA could be discussed as an emerging environmental pollutant.

4. Conclusion

A sensitive and robust LC-MS/MS method for the analysis of 21 selected PAS in mushrooms was developed and validated using an alternative validation approach. The method can simply be implemented by any laboratory operator using any batch of LC column and applied to any kind of button mushrooms. Using our method not only antibiotics but also anthelmintics, anti-inflammatory drugs and CAP can be detected in mushrooms simultaneously. Now it is possible to determine the fate of different antibiotics not only in animals, manure, soil, water and plants but also in button mushrooms. By applying the method to 20 commercially available mushrooms, CAP was found to be present at trace levels between LOD and LOQ in two samples. Furthermore, a possible transition of CAP as well as CIP and PRA from soil by button mushrooms during a mushroom cultivation experiment was conducted. While no uptake of CAP and CIP from top layers treated with 1,333 μg/kg each was observed, PRA was found at levels of up to 98.9 μg/kg in mushrooms after 42 days of cultivation. Analysis of top layer during mushroom cultivation using LC-MS/MS revealed a fast decrease of CAP and CIP, while PRA was detectable in high amounts even after 42 days of cultivation. Our data confirm, that residues of veterinary drugs can be detected not only in animal-based foods but also in plant-based foods. This should be subject to further investigation.

CRediT authorship contribution statement

Julia Schildt: Methodology, Validation, Project administration, Funding acquisition. **Max Rüdiger:** Methodology, Validation, Investigation. **Annemarie Richter:** Methodology, Validation, Investigation. **David M. Schumacher:** Writing - original draft, Writing - review & editing. **Corinna Kürbis:** Conceptualization, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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