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Occurrence of chloramphenicol in cereal straw in north-western Europe

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ABSTRACT

Two surveys are presented of straw analysed for naturally occurring chloramphenicol (CAP), a drug banned for use in food-producing animals. In the first study, CAP was analysed by LC-MS/MS and detected in 37 out of 105 straw samples originating from the Netherlands, France, the UK, Germany and Denmark. The highest level found was 6.3 μg kg^{-1}, the average 0.6 μg kg^{-1} and the median 0.2 μg kg^{-1}. The second study included a method comparison between ELISA and LC-MS/MS and a survey of CAP in cereal straw sampled at farms in all areas of Sweden. A total of 215 samples were screened by ELISA and a subset of 26 samples was also analysed by LC-MS/MS. Fifty-four of the samples contained more than 1 μg kg^{-1} CAP and the highest level found was 32 μg kg^{-1} (confirmed by LC-MS/MS). The highest contents of CAP in this study were allocated to the Baltic sea coast in the south-eastern part of Sweden (the county of Skåne and the Baltic Sea isle of Gotland). These results indicate a high incidence of CAP in straw in north-west Europe and have a severe impact on the enforcement of European Union legislation.

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Introduction

Chloramphenicol (CAP) is a broad-spectrum antibiotic with a historic use in all major food-producing animal species. The drug has been evaluated by a number of organisations (USFDA 1985; IARC 1990; EMEA 1996), most recently in 2005 by the Joint Expert Committee on Food Additives at its 62nd meeting (Wongtavatchai et al. 2004). CAP is a suspected carcinogen and due to its linkage with the development of non-dose-related aplastic anaemia in humans (Wongtavatchai et al. 2004) the drug is banned for use in food-producing animals in the European Union (EU/37/2010 2010) and in many other countries, including the United States, Canada, Australia, Japan and China. A minimum required performance limit (MRPL) of 0.3 μg kg^{-1} was set by the European Commission for analytical methods to be used in testing for CAP in products of animal origin (2003/181/EC 2003), which is nowadays considered as a reference point of action (RPA) (EC/34/2005 2005).

In addition, CAP, as all antibiotics, is prohibited as a feed additive according to EC/1831/2003 (1831/2003/EC 2003). Traditionally, it is produced for commercial use by chemical synthesis (Wongtavatchai et al. 2004), but it is biosynthesised by the soil organism *Streptomyces venezuelae* and several other Actinomycetes (Aouiche et al. 2012).

In 2010, the detection of CAP in plants and soil of mainly Mongolian origin (Berendsen et al. 2010) was reported and a first monitoring of CAP in European straw (n = 21) resulted in 57% positive samples with concentrations mainly below 1 μg kg^{-1}, but the maximum level was as high as 11 μg kg^{-1} (Stolker et al. 2012). More recent studies (Berendsen et al. 2013) suggest that CAP can be produced in the soil by soil organisms and subsequently be transferred to crops. These findings make it a much more realistic prospect that products of animal origin can contain CAP residues that are not due to (illegal) use of the drug, but rather to its natural occurrence. Because straw is fed to animals to prevent ruminal acidosis (De Campeneere et al. 2004) and is used as stall bedding for animal welfare, straw is a significant part of the livestock diet. All together the risk arises that animals ingest CAP-contaminated straw that might, depending on the level of intake, result in residues in animal products. Transfer studies in pigs showed that CAP is found in urine and plasma, and to a lower extent in kidney and muscle after oral administration (data not yet published). So far, transfer studies in calves have

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focused on the excretion though urine and yield contradictory results depending on the mode of administration of the drug (no data published). No data on the transfer of CAP to edible animal products, including milk and milk products, after low level intake have been published. Nevertheless, it is realistic that farmers could unjustifiably be convicted for illegal use of the drug and CAP residues might end up in human food products. For example, within the Swedish programme for official control of drug residues in food of animal origin, CAP was detected in pig urine and muscle in late 2012. During the work of tracing the contamination to feed and feed materials no illegal use of CAP was shown. On the contrary, CAP could be detected only in bedding straw. Three samples of wheat straw contained 2–4.5 μg kg^{-1} CAP, which might have been the origin of the CAP in the animal matrices. Clearly, information on the processes that influence the natural occurrence of CAP and additional transfer studies are urgently needed to address the CAP issue better.

CAP monitoring is regularly carried out within European Union member states in various products of animal origin, among which are urine, muscle and milk. However, only very limited monitoring is carried out in straw and, therefore, only little information is available on the extent of the CAP contamination regarding the fraction of positive straw samples and the CAP levels that are to be expected in naturally contaminated straw. Furthermore, no data are available on possible correlations between a positive CAP finding and the type of straw or its geographical origin. Because CAP contaminations are hypothesised to be related to natural processes, such data might give further insight into the factors influencing the natural occurrence of CAP.

This paper presents the results of two surveys focused on CAP in straw. First, a survey including 105 straw samples of various types and origin (all in north-west Europe) carried out in 2012; and second, a survey including 215 straw samples of various types, all taken from Sweden in 2013. The latter study was initiated after the above-mentioned findings of CAP in pig urine and muscle within the Swedish official control of drug residues in animal and food of animal origin. In this survey the exact geographical origin of the samples was registered, resulting in a unique and highly valuable dataset.

Experimental

Sample collection and preparation

In the first survey, 105 straw samples were collected from north-western Europe, including the Netherlands, Germany, the UK, France and Denmark. These samples were sent to RIKILT (Wageningen UR, Wageningen, the Netherlands) for analysis. Samples were homogenised by cryogenic mincing.

In the second survey, 215 samples of cereal straw were collected by official district veterinarians at animal-producing farms all over Sweden. Samples of about 1 kg were sent to the National Veterinary Institute (SVA) in Uppsala, Sweden, and dried at < 60°C under weight control overnight in a ventilated cupboard. All samples were ground on a hammer mill to pass a 1 mm screen prior to analysis. All results are presented on an as-is basis corrected for water losses during sample preparation.

Reagents

Ethyl acetate CAS no. [141-78-6], p.a.
Methanol CAS no. [67-56-1].
n-Hexane CAS no. [110-54-3], p.a.
Chloramphenicol CAS no. [56-75-7].
ELISA-kit (Ridascreen Chloramphenicol, R Biopharm AG, Darmstadt, Germany).

Methods

In the first survey, CAP was analysed by LC-MS/MS according to a previously published method (Berendsen et al. 2011), with some minor adaptations. Cryogenically minced straw (2.5 g) was extracted using 20 ml Milli-Q water. After shaking using a rotary tumbles (15 min) and centrifugation (3000*g*, 15 min) the extract was applied onto SPE as described.

In the second survey, CAP was analysed with an ELISA kit designed for the analysis of CAP in food of animal origin. The analysis was adapted by adding 20 ml deionised water to 2 g ground straw in a 50 ml polyethylene centrifuge tube and was left for 30 min at RT. A total of 10 ml ethyl acetate were added and CAP was extracted on a rotary shaker for 20 min followed by centrifugation at 3700*g* for 20 min. A total of 2 ml of the supernatant was evaporated to dryness by a stream of dry nitrogen. The dry extract was reconstituted in 1 ml hexane and re-extracted into the buffer solution from the ELISA kit by liquid–liquid partition on a vortex mixer for 1 min followed by centrifugation for 1 min at 1000*g*. A total of 50 μl of the lower phase were transferred to a well in the ELISA plate and the content of CAP was then determined as described for the ELISA kit.

In addition, 26 samples were analysed by LC-MS/MS for confirmation (see above). These included all samples with ELISA results of CAP > 8 μg kg^{-1} (n = 7)

as well as a selection of samples with lower ELISA results.

Results and discussion

In the first survey, CAP was analysed using LC-MS/MS. As SRM was chosen as the technique to use, in order to detect CAP at levels as low as reasonably possible, this concerns a targeted analysis. Also, in the present study the focus was on freely extractable CAP only; neither CAP metabolites nor bound CAP, if any, were included in the analyses. In order to confirm the relevance of this assumption, a few positive straw samples were analysed using an additional hydrolysis step using ß-glucuronidase/arylsulphatase (VWR, Radnor, PA, USA) during extraction aiming to hydrolyse CAP glucuronides. No significantly different quantitative results were obtained and, therefore, it was concluded that CAP was present in its free form and that no hydrolysis is needed to obtain accurate quantitative results. It remains questionable if freely extractable CAP (after cryogenic mincing) remains the only relevant form in general, but it is beyond any doubt that the analysis of only free CAP yields useful surveillance data.

In the first study, CAP was detected in 36 straw samples, of which 20 were > 0.1 μg kg^{-1}. The highest level was 6.3 μg kg^{-1}, the average 0.6 μg kg^{-1} and the median 0.2 μg kg^{-1}, demonstrating the uneven distribution of the drug among the samples. In Table 1 the samples and the results are grouped according to the country of origin. No relation between CAP content and the region, nor the type of straw was observed.

In the Swedish survey ELISA was used for screening purposes. This was done by adapting a kit designed for the analysis of food of animal origin for use with straw. The sensitivity of the ELISA assay is fit for purpose for analysis of CAP in food of animal origin; limit of detection is ≤ 0.3 μg kg^{-1} for meat and other major foods. Preliminary data (unpublished) indicated a somewhat lower sensitivity when analysing straw, so it was decided to set a cut-off value of 1 μg kg^{-1} for the detection of CAP in straw in this study. To verify the ELISA results, 26 samples (including all containing CAP > 8 μg kg^{-1} by ELISA) were selected for confirmatory analysis by LC-MS/MS. The results from this method comparison are presented in Figures 1 and 2. It can be observed that the ELISA method overestimated the low values (minimum detected level was 0.6 μg kg^{-1}) giving rise to a number of 'false-positives' compared with LC-MS/MS where samples apparently free from CAP were present (LOD = 0.05 μg kg^{-1}). This might be explained by cross-reactivity of trace amounts of structurally similar compounds, as is

Table 1. Summary of results of content of chloramphenicol in straw sampled in Northwest Europe grouped per country of origin.

Country	Number of samples	Number of CAP containing samples (> 0.1 μg kg^{-1})	Highest content of CAP (μg kg^{-1})	Average content of CAP (μg kg^{-1})	Median content (μg kg^{-1})
The Netherlands	75	25	6.3	0.7	0.3
Germany	13	4	4	0.4	0.2
France	14	4	0.2	0.2	0.2
UK	2	2	0.2	0.2	0.2
Denmark	1	1	1	< 0.1	< 0.1

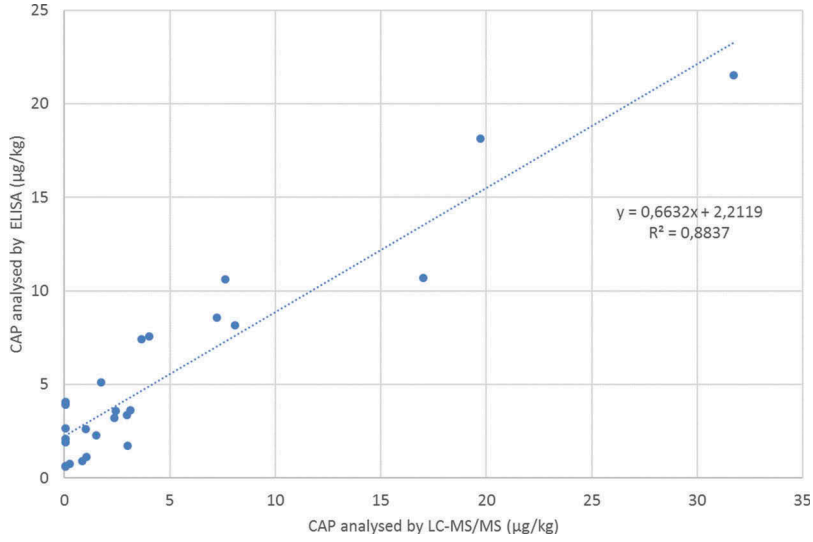

Figure 1. ELISA data plotted against LC-MS/MS data with linear regression statistics.

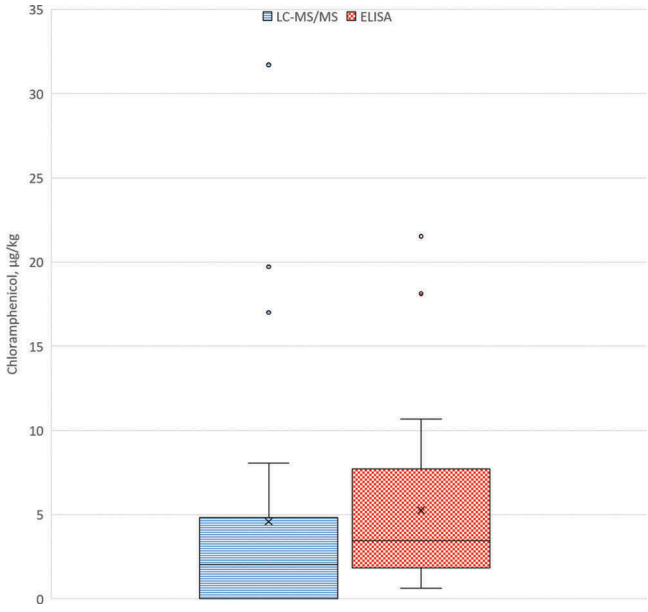

Figure 2. (colour online) Box–Whisker plot of the results of analysis of CAP by LC-MS/MS (LOQ = 0.1, LOD = 0.05) and ELISA (minimum observed level = 0.6) respectively. All values are in µg kg^{-1}. Boxes represent second and third quartiles with median (horizontal line) and mean (×) indicated.

also sometimes observed when applying this test in animal feed testing (Hsieh et al. 2013). Of the 'validation' set consisting of 26 samples, five were erroneously classified as having a CAP content of more than 1 µg kg^{-1} by ELISA analysis. Considering the high throughput and relatively inexpensiveness of the ELISA method, the drawback of a number of false-positives may be accepted when the method is used for screening purpose. More important, from the small validation study, it was concluded that the ELISA analysis did not produce any 'false-negatives' indicating that applying the combination of ELISA and LC-MS/MS yields trustworthy results.

The geographical distribution of the sampling points is presented in Figure 3. The density of sampling points also represents the general distribution of grain producing areas in Sweden. CAP was detected in 54 of the samples representing all types of straw. The highest level of CAP detected was 32 µg kg^{-1}, which is high compared with earlier findings by Stolker et al. (2012) who found a maximum of 11 µg kg^{-1} and compared with the Western European study reported in the present paper where the highest level was 6.3 µg kg^{-1}. The distribution of CAP throughout the different samples was very uneven, with 161 samples below the cut-off of 1.0 µg kg^{-1} as defined for the ELISA test and an average of 3.3 µg kg^{-1} among the 'positive' samples whereas the corresponding median value was 1.8 µg kg^{-1}.

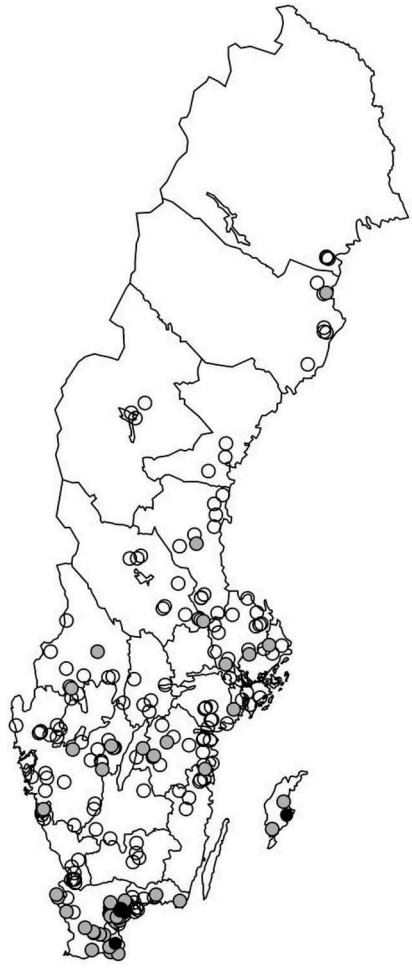

Figure 3. Geographical distribution of samples with indication of CAP content. Open circles: < 1 µg kg^{-1}; grey circles 1–10 µg kg^{-1}; black circles > 10 µg kg^{-1}.

Table 2. Summary of results of analyses of chloramphenicol in cereal straw sampled at Swedish farms.

Species	Number of samples	Number of samples with CAP > 1 µg kg^{-1}	Highest content of CAP (µg kg^{-1})	Average content of CAP (µg kg^{-1})	Median content (µg kg^{-1})
Barley	106	30	21.5 (31.7[a])	1.5	< 1
Wheat	46	12	10.7 (18.0[a])	1.1	< 1
Oats	29	2	4.1 (0[a])	< 1	< 1
Triticale	8	1	1.3 (–)	< 1	< 1
Unspecified bedding straw	20	9	3.2 (2.4[a])	1.1	< 1

Note: [a]Figures in parentheses represent confirmed contents quantified by LC-MS/MS.

In Table 2 the samples and the results are grouped according to botanical species of straw; in Table 3 the results are grouped according to the sample's origin (county). Barley straw was the most common species representing about half of all samples, which also reflects the fact that much of Swedish grain is grown for the purpose of feeding animals. In fact, only for

Table 3. Regional distribution in Sweden of straw samples containing more than 1 µg/kg CAP.

County where CAP was detected	Number of samples	Number of Samples with CAP > 1 µg kg^{-1}	Highest content of CAP (µg kg^{-1})
Skåne	50	32	20
Gotland	3	3	32
Västra Götaland	24	4	2
Uppland	16	2	2[a]
Hälsingland	7	1	4[a]
Östergötland	16	3	3
Halland	11	1	2[a]
Västerbotten	7	1	2[a]
Södermanland	13	1	1[a]
Värmland	12	2	1[a]
Blekinge	10	2	1[a]
Kalmar	8	1	1[a]
Dalarna	11	1	1[a]

Note: [a]Not positively identified and quantified by LC-MS/MS.

straw of barley (N = 106) and of wheat (N = 46) were there enough samples to be systematically evaluated. The degree of contamination, i.e. CAP > 1 µg kg^{-1} by ELISA, was 28% for the barley straw samples and 26% of the wheat straw samples. Thus, in accordance with the first survey, no clear relation was observed between CAP content and type of straw. On the contrary, when considering the relation between CAP content and region, some interesting observations may be made. Most CAP positives were detected in straw from the county of Skåne in the southern part of Sweden, which is also the major pig production region of Sweden. Clearly this area is at the highest risk for CAP-containing straw. In general, most CAP positives were detected near the east coast and on the isle of Gotland, which might suggest a relation with climate, but definite conclusions cannot be drawn from this survey only.

Conclusions

A commercially available ELISA kit for the analysis of CAP was demonstrated to be a high-throughput and efficient screening tool. The tendency of slight overestimation of low contents giving rise to a number of 'false-positives', may be accepted since the absence of 'false-negatives' still ensures safety regarding the possible presence of CAP in animal feed.

This study furthermore shows that CAP may occur naturally in widespread areas of Northern and Western Europe, and at levels that may be of significance for production of food of animal origin. The highest level of contamination, 32 µg kg^{-1} is more than 100 times the RPA set in the legislation of European Union. The biology of the formation of CAP in arable soil is largely not understood and thus more research is needed in order to predict and possibly control the presence of CAP in cereal straw and other plant material.

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Disclosure statement

No potential conflict of interest was reported by the authors.

References

1831/2003/EC. 2003. Commission Regulation 1831/2003/EC on additives for use in animal nutrition. Off J Europ Commun. L268:29–43.

2003/181/EC. 2003. Commission Decision 2003/181/EC amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin. Off J. L71:17–18.

Aouiche A, Sabaou N, Meklat A, Zitouni A, Bijani C, Mathieu F, Lebrihi A. 2012. Saccharothrix sp. PAL54, a new chloramphenicol-producing strain isolated from a Saharan soil. World J Microb Biotechnol. 28:943–951.

Berendsen B, Pikkemaat M, Römkens P, Wegh R, Van Sisseren M, Stolker L, Nielen M. 2013. Occurrence of chloramphenicol in crops through natural production by bacteria in soil. J Agr Food Chem. 61:4004–4010.

Berendsen BJA, Essers ML, Stolker AAN, Nielen MWF. 2011. Quantitative trace analysis of eight chloramphenicol isomers in urine by chiral liquid chromatography coupled to tandem mass spectrometry. J Chromatogr A. 1218:7331–7340.

Berendsen BJA, Stolker L, Jong J, Nielen MWF, Tserendorj E, Sodnomdarjaa R, Cannavan A, Elliott RC. 2010. Evidence of natural occurrence of the banned antibiotic chloramphenicol in herbs and grass. Anal Bioanal Chem. 397:1955–1963.

De Campeneere S, Van Herck A, Fiems LO, De Brabander DL. 2004. Effect of double-muscling and of removing straw, fed besides concentrates, on the chewing behaviour of bulls. J Anim Feed Sci. 13:627–630.

EC/34/2005. 2005. Commission Decision 2005/34/EC laying down harmonised standards for the testing for certain residues in products of animal origin imported from third countries. Off J Eur Commun. L16:61–63.

EU/37/2010. 2010. Commission Regulation on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Off J Eur Commun. L15:1–72.

[EMEA] European Agency for the Evaluation of Medicinal Products. 1996. European committee for veterinary medicinal products. Chloramphenicol summary report. [cited 2013 Jan 21]. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500012060.pdf

Hsieh KH, Lin CP, Cheng CC, Lai CP, Sheu SY, Yao CH, Lei YC, Chang TH, Kuo TF. 2013. Screening procedures for

chloramphenicol residue determination in serum, milk, and feedstuff using immunochromatographic assay. Food Biotechnol. 27:4,328–341.

[IARC] International Agency for Research on Cancer. 1990. Summaries & evaluations - chloramphenicol. Lyon (France): IARC; Vol. 50.

Stolker AAM, Jehoel G, van Baak M, Wegh RS, Berendsen BJA. 2012. Natural occurrence of chloramphenicol in straw. Poster on EuroResidue VII conference; 2012 May 14–16. The Netherlands: Egmond aan Zee.

[USFDA] US Food and Drug Administration. 1985. Chloramphenicol oral solution; opportunity for hearing. 50 Fed. Reg. 27059. Washington (DC): U.S. Government Printing Office.

Wongtavatchai J, McLean L, Ramos F, Arnold D 2004. World health organization: joint FAO/WHO expert committee on food additives (JECFA), chloramphenicol. IPCS (international programme on chemical safety) INCHEM WHO Food Additives series: 53. Authored by WHO Food Additives series. 53: 7–85.