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Research Article

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The presence of prednisolone in complementary feedstuffs for bovine husbandry

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Abstract

BACKGROUND: According to European Union legislation, prednisolone, a steroid that belongs to the glucocorticosteroid group, is banned as a growth promoter in cattle husbandry and therefore should not be present in bovine feedstuffs. As our preliminary investigations detected prednisolone in this matrix, we performed a study on different commercially available complementary feedstuffs, stored at the farm and/or in the laboratory, in order to verify whether its presence was due to neo-formation during storage.

RESULTS: Prednisolone was detected in almost all (95%) feedstuffs collected at the farm. When the feedstuffs were stored at the laboratory, the frequency (31%) and the concentration of prednisolone-positives were lower. This difference, which is likely due to different environmental conditions, implies the possibility of its neo-formation.

CONCLUSION: Our data indicate that the neo-formation of prednisolone can occur in feedstuff, and that the frequency and concentration could be related to the storage conditions. The individuation of an objective parameter that is useful for the identification of the compliance of feed is therefore essential.

Keywords: prednisolone; bovine feedstuff; neo-formation; storage

INTRODUCTION

The intensive production of food animals has triggered the development of minutely elaborated diets and has induced increased utilisation of veterinary drugs for therapeutic or preventive purposes.

The ban of any growth-promoter in the European Union (EU), was accomplished on 1 January 2006 with the last four antimicrobial agents – monensin sodium, salinomycin sodium, avilamycin and flavophospholipol[1] – set very precise limits upon the use of drugs or medicated feeds in animal husbandry, with the aim of ensuring 'a high level of consumer protection with regard to food and feed safety', and 'animal health and animal welfare'[2] as well as limiting antimicrobial resistance.[3] The concern of the EU legislator was the control of the use of veterinary drugs in food producing animals,[4–6] the enactment of regulation on feedstuff hygiene,[2] the use of additives in animal nutrition,[1] and the presence of undesirable substances – such as inorganic contaminants, nitrogenous compounds, dioxins and polychlorobiphenyls – in animal feed, as stated by Directive 2002/32/EC and its subsequent amendments.[7,8] The monitoring of residues in feed and food of banned or undesirable substances requires great effort by official control organisations, whose investigations are regulated by the National Animal Feed Plan and the National Residues Plan in each EU Member State. The work of these organisations is made more difficult, however, by the possible presence of active principles of drugs, which may be included in the category of pseudo-endogenous substances, i.e. synthetically produced hormones that are also known to be endogenous under certain conditions, due to their dual synthetic/endogenous nature.[9] This is the case for thiouracil, a thyreostatic drug that was banned in the EU in 1981 for use in livestock for fattening purposes. This drug, and other naturally goitrogen substances, may originate from the ingestion of *Brassicaceae*, glucosinolate-rich plants. Myrosinase, an endogenous enzyme of these plants freed from the cell vacuoles after disruption, or by myrosinase-like intestinal bacterial activity during digestion, which causes glucosinolate hydrolysis, can induce the presence of thiouracil in the urine of livestock.[10]

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Also, the anabolic steroid boldenone has been extensively studied since Arts *et al.*[11] showed its possible endogenous origin in calves. Some authors hypothesised an *ex vivo* neo-formation in contaminated urine.[12] A study on human athletes who tested positive for boldenone showed, by using gas chromatography–combustion–isotope ratio mass spectrometry (GC/C/IRMS), its endogenous presence in urine, and suggested its formation in the gut, defined as an 'endocrine active side organ'.[13] The role of phytosterols in the diet was studied on veal calves:[14] it was shown that these sterols do not significantly increase the urinary level of 17α-boldenone, nor induce the formation of 17β-boldenone, both in their conjugate forms. The EU regulations require the presence of the total conjugate fraction in bovine urine as an unambiguous demonstration of boldenone administration[15] and, to demonstrate the difficulties experienced by control laboratories, more recent studies have shown that the detection of only the sulfo-conjugate fraction of 17β-boldenone should unequivocally demonstrate treatment with the anabolic steroid ester.[16,17]

In these pseudo-endogenous substances, prednisolone must be mentioned. This corticosteroid was demonstrated to be produced by cattle under stress conditions;[18] additionally, it was found in 612 out of 780 racehorse urine samples at concentrations around 1 ng mL^{-1},[19] in all urine samples of 34 untreated human volunteers of both genders[20] and, finally, possible *ex vivo* neo-formation in human urine[21] and in bovine urine and faeces[22,23] was demonstrated. Besides its endogenous origin, it was recently suggested that exogenous prednisolone administrated in bovines, could influence the metabolism of some natural corticosteroids.[24]

Currently, studies of the natural presence of prednisolone in feed are not available in the literature: although the possibility of endogenous production or of *ex vivo* formation in urine cannot be excluded, the involuntary administration of prednisolone with complementary feed should be accounted for. The term 'complementary feed' is precisely described in Article 3, Paragraph 1 of the Regulation (EC) No 767/2009[25] as: 'compound feed which has a high content of certain substances but which, by reason of its composition, is sufficient for a daily ration only if used in combination with other feed'. Therefore, specific, 'dense' composition of complementary plant feedstuffs can serve as a good basis to start with the examination of the presence of corticosteroids in this milieu. Bearing this in mind, we undertook an investigation of the presence and origin of prednisolone in complementary plant feedstuff samples.

EXPERIMENTAL

Reagents and chemicals

Cortisol and prednisolone were purchased from Sigma–Aldrich (St Louis, MO, USA). The internal standard prednisolone-d6 was from CDN Isotopes (Pointe-Claire, Quebec, Canada). All other chemicals were from Fluka Chemie GmbH (Buchs, Switzerland). Standard stock solutions were prepared in ethanol (1 mg mL^{-1}) and stored at −18 °C. Working solutions were prepared daily by diluting the stock solutions with methanol/water (50:50, v/v).

Sample selection

The experiment was designed according to available feed samples. Initially, feeds were collected at the farms (FARM group) and included into two samples sets. The first set included five feed samples that were randomly collected in farms during hot summer months. After collection, the samples were stored in the laboratory, at room temperature. In the late autumn, the samples were analysed. The second set consisted of 15 samples of cattle feed of four different compositions. These samples were stored at the farm in the summer and autumn, collected in the late autumn, taken to the laboratory and, unlike the first set, immediately analysed. A second analysis was carried out after a month of storage at room temperature.

On the basis of preliminary results obtained for the FARM group, a new experimental group was formed, which included feeds stored in the laboratory (LAB group). The LAB group included 18 samples of cattle feed of different compositions, which were collected in the spring. These samples were taken to the laboratory before their delivery to the farm. Upon their arrival at the laboratory, these samples were immediately analysed. A storage period of 5 weeks at room temperature followed, with sampling on every seventh day.

Complementary feed composition

We used commercially available, vegetable complementary feeds. All of the information about the feedstuff compositions came from the manufacturer's certificates. A total of 38 feeds were considered in the experiment. There were 16 types of feed, named with the letters of the English alphabet from A to P, as some samples came from different batches of the same feed type. The feeds were:

- Feed A was for veal calves weaning
- Feed B was for veal calves weaning and for young beef
- Feeds C to G were for young beef
- Feeds H to O for adult beef
- Feed P for dairy cows.

Feeds A, B, C, D and F came from different farms; the remaining feeds were obtained directly from the manufacturer.

All feeds contained calcium carbonate, sodium chloride, sodium bicarbonate, magnesium oxide and calcium salts of fatty acids. Feeds G and M also contained dicalcium phosphate, and feed H contained calcium sulfate.

All feeds, except K and N, contained wheat as flour middling (B, D, E, O, P), bran (A–E, I, J, L, M, P) or middling (H).

Corn was present in all feeds except J, K, M and N; in B, C, E–G this was present as gluten feed, in E–G as germ, too; in I and L it was as bran, and in A and E as corncob. In the remaining feeds, the presence of corn was generically indicated. In O, corn was genetically modified (GM).

All feeds except M contained soy as dehulled soybean flour (A–D, F–L, N–P), soybeans (E, P), soybean oil (G), and soybean hulls (A). In O, soy was GM.

Sunflower meal was present in all feeds except D. Feeds K and O contained barley flour; GM canola flour (O) and rice bran were also present. Sugarcane or beet molasses were in A–E, I, J, M–O; sugar beet pulp was in A, E, F, H, L and N.

Saccharomyces cerevisae was in I and L–N; wheat distillers in I; sulfur bloom and saponified vegetable oil in L; *Yucca schidigera*, brewers grain, linseeds and carob in M.

The analytical constituents were: proteins from 14.5% (O) to 35.0% (K); lipids from 1% (K) to 9% (M); cellulose from 5.10% (D) to 12.0% (L); ash from 6.20% (A) to 35.0% (K); calcium from 0.9% (A, D, E) to 3.5% (M); phosphorus from 0.40% (E) to 0.80% (G); sodium from 0.30% (E) to 4.8% (K); magnesium from 0.30% (A) to 0.90% (M); methionine from 0.20% (A) to 0.60% (P). Feed H was supplemented with selenomethionine (22.75 mg kg^{-1}).

Vitamins A, D_3 and E were present as additives in all complementary feeds (from 6500 to 125 000 UI kg^{-1}, from 750 to 25 000 UI kg^{-1} and from 25 to 1400 mg kg^{-1}, respectively). B vitamins were present at different concentrations in feeds H, I, K–N and P. Choline was present in feeds H, L and M. In L, vitamin K was also reported. Feeds H, J and K contained urea (from 18 000 to 40 000 mg kg^{-1}). Selenium, zinc, manganese, iron, copper, and iodine were present. Feed M contained sorbent and binding materials, while flavourings were present in feed O.

Sample extraction

A 2 g portion of cattle feed (pellets or flour), transferred to a 50 mL polypropylene tube, was spiked with 40 µL of a 100 ng mL^{-1} internal standard solution. After the addition of 20 mL water, the sample was shaken for 1 min until complete dispersion was achieved. A solution (4 mL) of 80/20 *tert*-buthylmethylether/ethyl acetate (v/v) was added, and the resulting mixture was shaken in a vertical rotary shaker for 20 min and centrifuged for 15 min at 3000 × *g*. The tube was kept at −18 °C for about 1 h, until the aqueous phase froze and the lipids solidified. The organic liquid supernatant was then transferred to a glass 10 mL tube. The sample was dried under vacuum in a centrifugal evaporator. The residue was dissolved in 200 µL of a mixture of methanol/aqueous formic acid 0.1% (50:50 v/v), 800 µL of petroleum ether was added, and then the solution was vortexed for 30 s and centrifuged for 2 min at 3000 × *g*. The lower aqueous phase was collected with a disposable 1 mL syringe and transferred to the autosampler vial.

LC-MS^3 analysis

Analysis conditions have been previously described elsewhere.[18] Briefly, the HPLC system comprised a quaternary pump equipped with a degasser and a Surveyor AS autosampler (Thermo Electron, San Jose, CA, USA). The chromatographic separation was performed using a HPLC column (100 mm × 2.1 mm i.d., 3 µm particle size Allure Biphenyl) (Restek Corporation, Bellefonte, PA, USA) in an oven set at 30 °C with an isocratic elution (40% aqueous formic acid (0.1%) and 60% methanol at a flow rate of 0.2 mL min^{-1}). An LCQDecaXpMax ion trap mass spectrometer (Thermo Electron) was operated in negative electrospray ionisation (ESI−) mode with the following conditions: sheath and auxiliary gas (nitrogen) flow rates of 40 and 18 arbitrary units, respectively; a spray voltage of 5.50 kV, an ion transfer capillary temperature of 245 °C, a capillary voltage of −23 V, and a tube lens offset of −77 V. Helium was used for collision-induced dissociation. All of the investigated compounds showed, in full scan MS, very abundant formiate adducts, $[M + HCOO]^-$. Consequently, these ions were used as precursor ions for the MS^2 fragmentation: for each analyte; the most abundant ion detected after collision was then used as a precursor for the MS^3 fragmentation. The analysis was performed in consecutive reaction monitoring. The precursor ions were the formiate adducts of the studied compounds ($[M + HCOO]^-$), and are shown in Table 1 together with the product ions and collision energies. The quantifications were made on one ion. Representative chromatograms and mass spectra of a spiked feed sample are reported in Fig. 1.

LC-HRMS analysis

The presence of prednisolone was qualitatively confirmed by high-resolution mass spectrometry (HRMS) in four samples in full MS scan mode. All data were processed with a mass tolerance of 5 ppm. The exact mass of the prednisolone formiate adduct is 405.19187 Da. The chromatographic separation was performed on a reversed-phase SunfireW column (~~150 2.1 mm, 3.5 mm~~; •Waters, Milford, MA, USA), with a mobile phase consisting of a mixture of 75% water with 0.1% formic acid and 25% acetonitrile at a flow rate of 0.3 mL min^{-1}. The HRMS instrumentation was an Exactive™ Benchtop high-resolution mass spectrometer equipped with an HESI-II source (Thermo Fisher, San José, CA, USA) operating in negative mode. The method is thoroughly described elsewhere.[20]

LC-MS^3 method validation

The presence of the studied corticosteroids in feed samples was checked by the analytical method described above. A calibration curve was thus prepared with blank samples, which were spiked to give known concentrations of prednisolone and cortisol (0.10, 0.2, 0.3, 0.4, 0.5, 1.0, 2.0, 5.0 and 10 ng g^{-1} feed). Three replicates were measured on three different days after liquid–liquid extraction. The following parameters were calculated: (1) precision, expressed as intra-day and inter-day coefficients of variation (CV%), on four blank feed samples, spiked with 0.6 ng g^{-1} feed, roughly corresponding to twice the detection capability (CCβ); (2) recovery (%), on the same four samples, expressed as the percentage of measured concentration to a fortified concentration ratio; (3) the decision limit (CCα) and detection capability (CCβ); and (4) between-run accuracy, on three different days using four different samples spiked with 0.6 ng g^{-1} feed (twice the CCβ).

The calculation of CCα and CCβ was made following the method proposed by Galarini *et al.*,[26] starting with the determination of the 'minimum required performance level', which indicates the concentration above which the curves must be built.

Statistical analysis

Means, medians and standard deviations were calculated for every set/group of feeds. In order to determine if a difference

Table 1. Ions for prednisolone, cortisol and the internal standard prednisolone-d6 detected by LC-MS^3 in consecutive reaction monitoring mode

	MS^2		MS^3		
Compound	$[M + HCOO]^-$ precursor ion (*m/z*)	Collision energy (%)	Precursor ion (*m/z*)	Collision energy (%)	Product ions (*m/z*)
Prednisolone	405	25	329	26	313, 295, 280, **187**
Cortisol	407	35	331	25	315, **297**, 189
Prednisolone-d6	411	25	333	26	317, 299, 284, **191**

Results in bold type are the ions for quantification.

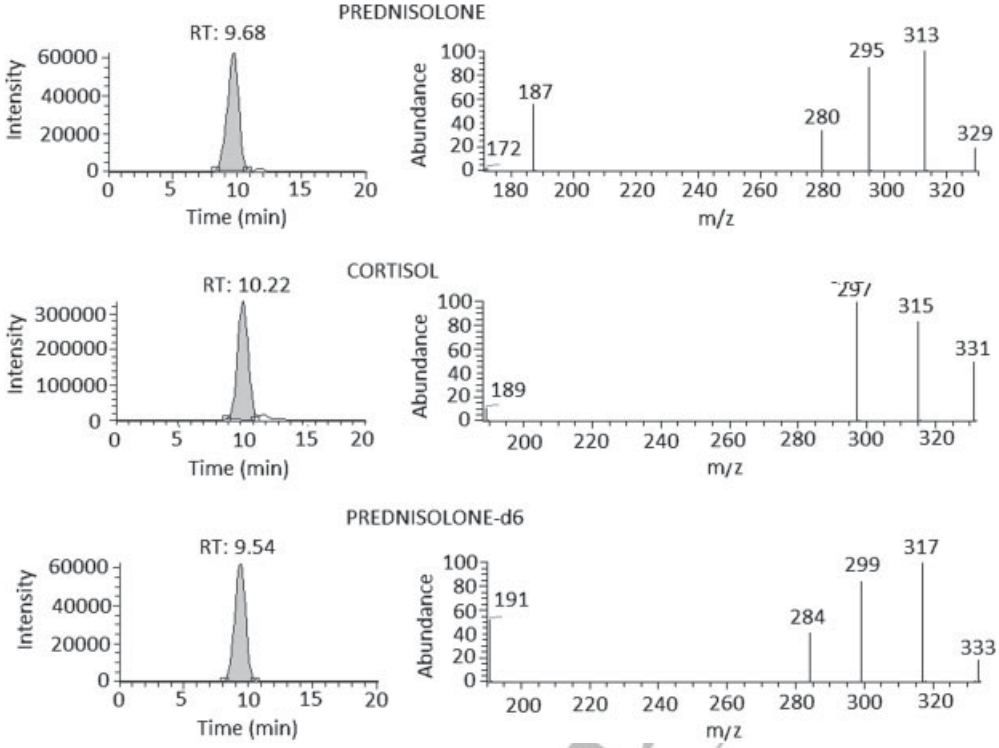

Figure 1. Reconstructed chromatogram and consecutive reaction monitoring (CRM) mass spectra of a blank feed sample spiked with 2 ng g^{-1} prednisolone and cortisol. The concentration of the internal standard prednisolone-d6 is 2 ng g^{-1}.

existed in prednisolone concentrations, we compared the different sets/groups of feeds. The Kolmogorov–Smirnov method was used to verify the normality of the value distribution. When a comparison was made between two sets/groups, we always used the Mann–Whitney test as at least one of the populations did not pass the normality test. To compare three sets of values, we performed the ordinary analysis of variance (ANOVA) if the normality test was passed by all sets, or the Kruskal–Wallis test (non-parametric ANOVA) in all other cases. The software used was GraphPad InStat™ version 3.00 (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

Table 2. Validation performance characteristics of prednisolone and cortisol

Characteristic	Prednisolone	Cortisol
Linearity R^2	0.98	0.97
Intra-day CV (%)	7.4	9.5
Inter-day CV (%)	12.7	14.2
Recovery (%)	91	85
CCα (ng g^{-1})	0.22	0.22
CCβ (ng g^{-1})	0.29	0.29

CCα, decision limit; CCβ, detection capability; CV, coefficient of variation.

RESULTS AND DISCUSSION

Although there is a need for sensitive, accurate and quick analytical methods to monitor the abuse of corticosteroids, only a limited number of analytical methods have been published for feedstuff. Animal feed is a very complex matrix; not only does the composition differ for each type but starting materials also differ for each production batch, leading to each sample of feed having its own characteristics. This means that the interfering compounds differ from sample to sample, which makes method development challenging. Therefore, we paid special attention to the sample handling and extraction procedure. The parameters calculated for method validation are reported in Table 2. All validation data for prednisolone and cortisol determination in feedstuff were adequate and indicated good performance of the developed analytical procedure. The level of cortisol was below the decision limit in all of the analysed samples.

Prednisolone was detected in all samples from the preliminary study (first set, FARM group) and could be quantified in four. The mean ± SD value was 1.6 ± 1.5 ng g^{-1} (Table 3). The unexpected presence of prednisolone in these samples strongly suggested the possibility of its neo-formation, similarly to the faecal matter as already observed.[23] The samples were randomly collected from farms, and then transferred to the laboratory. The time and temperature of their storage at the farm were neither uniform nor exactly monitored; the period was from 1 to 2 months. The storage period in the laboratory was 2 months, also without any caution with regard to the storage temperature. Therefore, neo-formation could occur during both of the indicated intervals.

In order to gain a clearer picture of where and when prednisolone was formed, a new approach was designed; the results are given as a second set of the FARM group. As the values obtained for this set were not normally distributed, the Mann–Whitney test was used to compare them to the first set. The 15 samples showed a prednisolone concentration value of 1.6 ± 1.3 ng g^{-1} (mean ± SD), which did not differ significantly from the first set (Table 3). The second set of the FARM group seemed to confirm the initial

Table 3. Concentrations of prednisolone detected in the feed samples of the FARM group: first set (1 to 5) and of the second set (6 to 20), after a storage period at the farm and at the laboratory •

Sample	Feed	Storage (farm)	Storage (laboratory)	First analysis Month	First analysis Prednisolone (ng g^{-1})	Second analysis Month	Second analysis Prednisolone (ng g^{-1})
1	B	June to September	September to November	November	0.97	–	NP
2	C				1.0		NP
3	F				1.7		NP
4	A				0.22		NP
5	D				4.0		NP
6	C	August to December	~~	~~	3.9	~~	ND
7	D				0.88		ND
8	D				0.35		ND
9	D				0.73		ND
10	F				2.4		ND
11	B	October to December	~~December to January~~	~~December~~	0.98	~~January~~	ND
12	B				2.1		ND
13	C				0.51		ND
14	D				0.82		ND
15	D				3.9		290[a]
16	F				0.88		ND
17	F				3.6		ND
18	B	November to December	–	–	ND	–	ND
19	B				1.7		ND
20	B				1.0		ND

[a] Estimated value; out of the calibration range.
ND, not detected; NP, not performed.

hypothesis. Prednisolone was, in fact, detected in 14 out of 15 samples independent of the variable environmental conditions (temperature, humidity, etc.). It has to be noted that the samples of this set had been stored only at the farm when the first analysis was undertaken. The second analysis on the presence of prednisolone was performed after a storage period of 1 month in the laboratory at room temperature: all samples were negative except no. 15 (Table 3). The extremely high concentration found in this feed specimen could not be interpreted by the simple addition of the corticosteroid to the feed, as its concentration in the first analysis was about 74-fold lower. A possible explanation for this could be a high level of precursors or more presumably high microbiological activity due to the particular conditions in the jar. More profound studies should be conducted to clarify why other samples of the same composition did not behave in the same manner (Table 3).

In order to compare feed samples according to their stay in farm, the samples were merged (samples 1–5, 6–10, 11–17 and 18–20, respectively) and ANOVA test was performed; no significant difference was observed ($P = 0.81$). On the other hand, the Kruskal–Wallis test was performed to evaluate the prednisolone concentration in the feed samples merged according to their composition. When the mean prednisolone concentrations of feedstuffs B to F were compared, no significant difference was shown. Feed A could not be considered due to the presence of only one sample.

Because of the lack of a significant difference between the prednisolone concentrations in feedstuffs studied in the FARM group, a second experiment was undertaken. Commercially available vegetable feedstuffs ($n = 18$) were randomly chosen, regardless of their composition. The results obtained for this group are shown in Table 4. Only one sample showed the presence of prednisolone upon arrival at the laboratory. A total of 108 analyses were performed and prednisolone was found on 34 occasions. Only one sample (no. 22) was always negative. In the other samples, no relationship was found between the collection time and the presence of prednisolone: the corticosteroid was in fact detected between one and four times in each sample. The concentration was either roughly constant, increasing, decreasing or with a bell-shaped profile. The mean ± SD prednisolone concentrations ranged from 0.74 ± 0.26 ng g^{-1} (day 28) to 1.13 ± 1.07 ng g^{-1} (day 14), with no difference shown between the collection days. The positives were: one upon arrival, two on the seventh day and, even if the distribution was random, seven at any further collection time (Fig. 2).

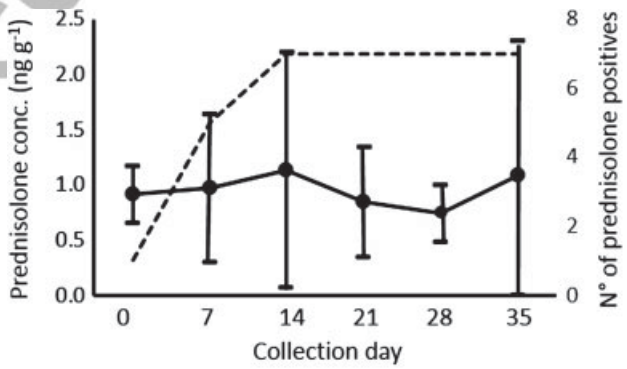

Figure 2. Mean ± SD concentrations (●) and number of positives (_ _ _) to prednisolone in samples of laboratory (LAB) group, related to the collection day.

Table 4. Concentrations (ng g^{-1}) of prednisolone detected at different collection times in the feed samples of the LAB group during the storage period at the laboratory

Sample	Feed	On arrival	Day 7	Day 14	Day 21	Day 28	Day 35
21	E	ND	ND	0.29	0.45	ND	ND
22	P	ND	ND	ND	ND	ND	ND
23	D	ND	ND	ND	ND	ND	0.40
24	B	0.73	ND	ND	0.57	ND	ND
25	B	ND	1.6	1.8	ND	ND	ND
26	B	ND	ND	ND	ND	0.99	1.2
27	G	ND	ND	0.53	1.2	1.1	0.64
28	G	ND	1.8	0.70	0.35	ND	ND
29	G	ND	ND	ND	ND	0.63	1.2
30	H	ND	ND	ND	ND	0.79	3.7
31	I	ND	ND	ND	0.69	0.54	ND
32	J	ND	0.65	ND	ND	ND	ND
33	K	ND	ND	3.0	1.8	0.34	ND
34	L	ND	ND	0.43	ND	ND	ND
35	M	ND	0.43	ND	ND	ND	ND
36	N	ND	0.38	ND	0.86	0.75	0.36
37	O	ND	ND	0.31	ND	ND	ND
38	O	ND	ND	ND	ND	ND	0.22

ND, not detected.

The Kruskal–Wallis test was performed to compare prednisolone-positive samples, merged by collection day, but no significant statistical relationship was found again.

Beyond this, the integrated data from positive samples of the FARM group were compared to the corresponding data from the LAB group. The mean ± SD values were 1.66 ± 1.28 and 0.95 ± 0.76 ng g^{-1}, respectively, and the Mann–Whitney test ($P = 0.024$) demonstrated a difference in prednisolone concentration between the samples stored at the farm and in the laboratory. Nevertheless, apart from this statistical significance, one fact remains: prednisolone is formed either at the farm or in the laboratory. In the LAB group, in contrast to the FARM group, the sample storage after production was performed only in the laboratory: the neo-formation of prednisolone occurred in this environment as well. However, the frequency was lower, as only 31% of analyses were positive for prednisolone, versus 95% of samples stored at the farm, at least for the short term. These data suggest that different storage conditions differently evoke prednisolone neo-formation. Also, the variability observed did not exclude the possibility of its degradation. In the second set of the FARM group, 14 samples out of 15 were found to be negative after 1 month of storage in the laboratory. In the LAB group, the higher frequency of prednisolone detection was seen in seven out of 18 samples, observed from day 14 to day 35. Hence, most of the samples (about 60%) were negative for these collection days and when prednisolone was observed early, it generally disappeared. The poor stability of the corticosteroids has recently been shown by De Clercq *et al.*,[27] who, to preserve glucocorticoids in bovine urine for a long period (20 weeks), recommended filter sterilising and storage under acidic conditions, preferentially at pH 3 and at a temperature of −80 °C (or at least −20 °C). This last observation, made on a different matrix, shows the real possibility of microbiological degradation of corticosteroids. Currently, the only explanation for the higher frequency of prednisone-positive

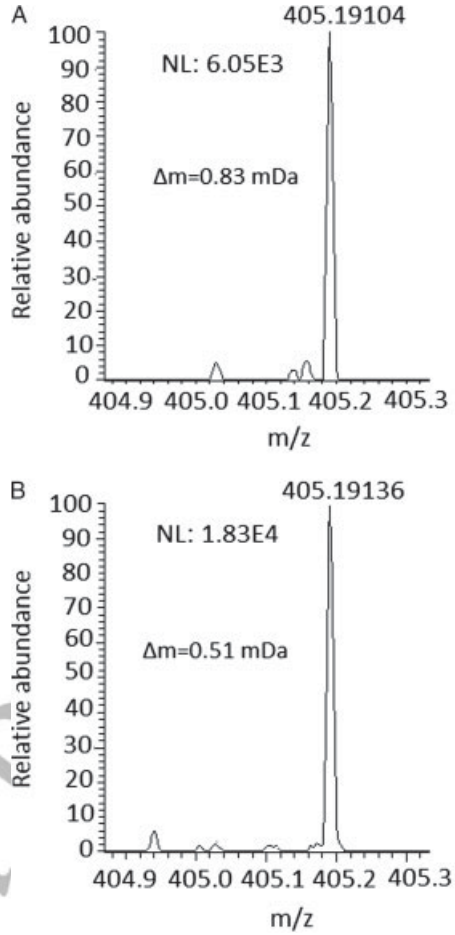

Figure 3. Total ion spectra of the prednisolone peak acquired by HRMS. (A) Standard solution (1 ng mL^{-1}), (B) a positive feed sample. The exact mass of prednisolone formiate ($[M + HCOO]^-$) is 405.19187 Da.

samples in the FARM group with respect to the LAB group could be found in the different sanitary hygienic storage conditions. Conservation in closed jars, which is performed in the laboratory, preserves the possibility of contamination; while, on the farm, the hygienic conditions are objectively different and obviously more favourable for prednisolone neo-formation. The appearance of prednisolone in a very high concentration in sample no. 15, collected after 1 month of storage in the laboratory, could represent indirect, although controversial, evidence of this observation; in fact, it took place in a closed container where the conditions could have been different compared to all other samples that were stored in closed jars.

Finally, the identification of prednisolone with a low mass resolution spectrometer was fully confirmed in four randomly selected samples, through the accuracy of the measured mass of the formiate precursor $[M + HCOO]^-$ in HRMS analysis, as shown in Fig. 3.

CONCLUSIONS

Based on the results obtained, we hypothesise that feedstuffs without the addition of drugs may be non-compliant for prednisolone presence upon inspection by the health authorities. Due to the low possibility of affecting the storage conditions at the farms, the studies that would indicate objective parameters, e.g. a cut-off

level or metabolite markers, are essential. To this aim, special attention must be paid to the definition of the prednisolone metabolic precursors in the feedstuffs and the nature of their origin. All of this would allow the official control organisations to make the most accurate decisions that are possible about the cause and importance of the presence of prednisolone in complementary feedstuff.

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AQ1. Please clarify the dimensions here. For example, was the column 150 × 2.1 mm, 3.5 μm coating ?

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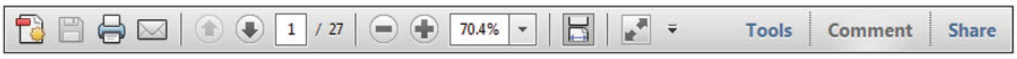
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/uk/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



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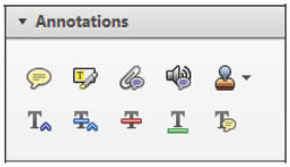

1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
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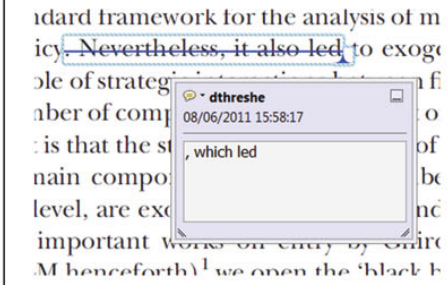
- Type the replacement text into the blue box that appears.

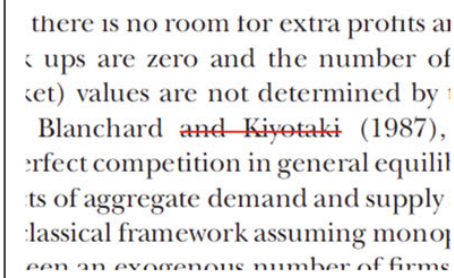


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.



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- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
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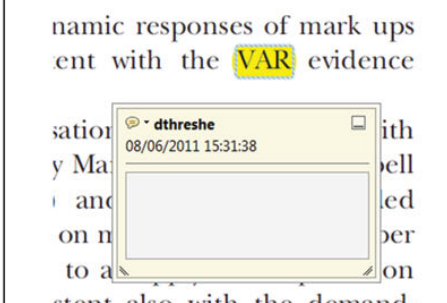

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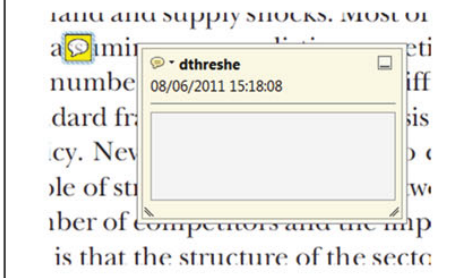
How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

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- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
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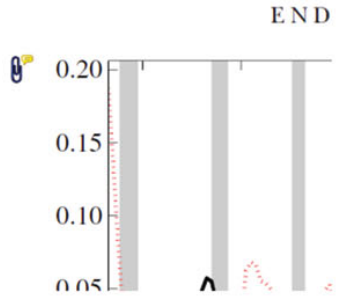

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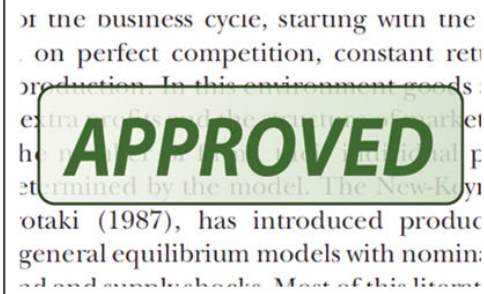

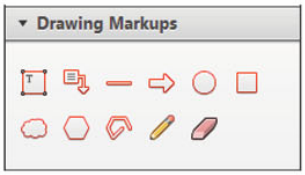

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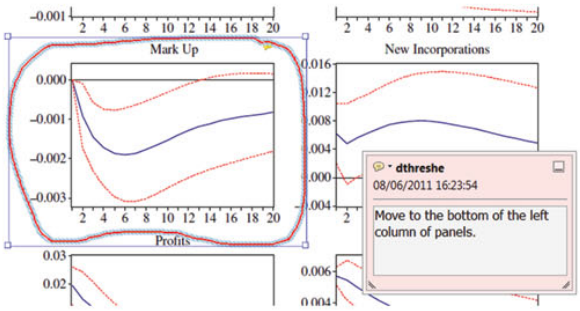

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