Use of NMR metabolomic plasma profiling methodologies to identifyillicit growth-promoting administrations.

Use of NMR metabolomic plasma profiling methodologies to identify illicit growth-promoting administrations

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Abstract Detection of growth-promoter use in animal pro-duction systems still proves to be an analytical challenge despite years of activity in the field. This study reports on the capability of NMR metabolomic profiling techniques to discriminate between plasma samples obtained from cattle treated with different groups of growth-promoting hormones (dexamethasone, prednisolone, oestradiol) based on recorded metabolite profiles. Two methods of NMR analysis were investigated—a Carr-Purcell-Meiboom-Gill (CPMG)-pulse sequence technique and a conventional ^{1}H NMR method using pre-extracted plasma. Using the CPMG method, 17 distinct metabolites could be identified from the spectra. ^{1}H NMR analysis of extracted plasma facilitated identification of 23 metabolites—six more than the alternative method and all within the aromatic region. Multivariate statistical analysis of acquired data from both forms of NMR analysis separated the plasma metabolite profiles into distinct sample cluster sets representative of the different ani-mal study groups. Samples from both sets of corticosteroid-treated animals—dexamethasone and prednisolone—were found to be clustered relatively closely and had similar alterations to identified metabolite panels. Distinctive metabolite profiles, different from those observed within plasma from corticosteroid-treated animal plasma, were observed in oestradiol-treated animals and samples from these animals formed a cluster spatially isolated from control animal plasma samples. These findings suggest the potential use of NMR methodologies of plasma metabolite analysis as a high-throughput screening technique to aid detection of growth promoter use.

Keywords Growth promoters . Plasma . Metabolite profiling . NMR . CPMG

Introduction

The administration of chemical agents to livestock to promote animal growth for financial gain is still practised within the European Union despite the banning of their use by European Directive 88/146/EC [1]. To police and combat these illegal practices, statutory monitoring for growth-promoter abuse is performed whereby samples of animal matrices are screened using relatively simple and rapid techniques including immunoassays, inhibitory sub-stance testing and chromatographic techniques, to initially clear compliant samples and identify potential non-compliant samples for further testing. A relatively small proportion of cattle (approximately 0.05%) are subjected to any form of screening to detect growth-promoter presence prior to entry into the food supply chain. Subsequent con-firmatory testing (post-screening) of non-compliant samples is typically based on hyphenated gas chromatography or liquid chromatography (LC)-based mass spectrometry (MS) analysis [2]. Although MS-based techniques used to monitor for multiple growth-promoting agents have and continue to advance consistently in terms of sensitivity and sample throughput, such analysis, as with existing screening approaches for abuse detection, is typically dependent on the targeted analysis and detection of specific known growth-promoting agents.

In the intervening years since the introduction of the ban on the use of growth promoters, there have been clear advances too in the practises of those who use these illicit administrations and developments on this front have centred primarily on aiding avoidance of detection by monitoring agencies [3]. This evolution in growth-promoting activities relates principally to the type and class of growth-promoting agents used and in the manner by which they are administered. There has been an increasing trend towards the use of naturally occurring hormones which are impossible to distinguish from endogenous hormones and a tendency to use repeated low concentration dosing regimes that consist of formulations of multiple growth-promoting agents including corticosteroids at levels which once administered to animals are difficult to detect analytically. To overcome these practises which, as evident from the current low rates of abuse detection, have proven themselves to be analytically chal-lenging to existing testing methodology, attempts have been made by various research groups to develop novel approaches both to improve overall detection rates and increase sample throughput in screening analyses [4, 5]. Direct approaches have included the use of isotope ratio MS to distinguish between metabolites of administered syn-thetically derived analogues from endogenous hormones [6] and the detection of steroid esters incorporation into animal hair [7]. Other proposed techniques include identification analysis of histological anomalies in target animal organs [8], measurement of alterations to transcriptomic [9] and proteomic profiles [10] within tissue. Such methods all aim to facilitate higher sample throughput to target confir-matory residue analysis towards highly suspect samples and thus significantly improve detection rates through a more robust monitoring of banned agent use.

Newly emerging attempts to develop improved screening techniques have been made possible through technological and bioinformatical advances in sample and data analysis and have focused on the application of metabolomic methodologies to analyse changes to metabolite profiles in animals [11]. Metabolomic approaches have found widespread application in diverse fields of research and recent activities have predominantly focused on using LC-MS-based techniques to perform targeted or untargeted analysis of alterations to urinary metabolite profiles in response to exogenously administered compounds [12-15]. An alternative to MS-based metabolomic analysis techniques utilises nuclear magnetic resonance (NMR) instrumentation [16] and NMR-based approaches have been widely used in a range of metabolite profiling studies of various matrices including wheat [17] and meat [18]. Whilst NMR metabolomic approaches are not as sensitive as MS techniques, this non-destructive form of analysis offers the opportunity to readily identify the metabolites of interest a current limitation of MS-based metabolomic approaches [19]. A metabolomic method to identify growth-promoter-treated animals utilising NMR analysis was previously described by Dumas et al. [20]. Urine samples from cattle treated with a range of anabolic hormone compounds were profiled using H-13C-HMBC NMR analysis, and based on the NMR spectra obtained from sample analysis, distinct urinary metabolite signatures were used to identify and classify samples derived from treated or untreated animal groupings.

The aim of the present study was to investigate the potential of applying NMR analytical approaches to differentially distinguish between non-treated livestock and animals treated with various growth-promoting agents through combining metabolomic NMR profiling of plasma from these animals with multi variate statistical analysis. Two alternative NMR methodologies of plasma sample analysis are investigated with the ultimate aim of developing a high-throughput screening technique. The first method examined employs a conventional NMR technique requiring a solvent extraction sample clean-up step whilst the second utilises a rapid NMR analysis method involving altered (optimised Bruker Carr-Purcell-Meiboom-Gill (CPMG) pulse program) ^{1}H CPMG pulse sequences. Plasma samples from animals treated with oestra-diol, dexamethasone and prednisolone derivatives will be analysed alongside plasma from non-treated animals by these two analytical approaches. The performance of these two techniques will be assessed through the analysis of identical plasma samples and compared based on their individual performance in distinguishing between samples from non-treated and growth-promoter-treated animals based on identified metabolite profiles.

Materials and methods

Growth-promoter animal treatment study

Twenty-four male 17-22-month-old Charolais beef cattle, with an average bodyweight of 600 kg, were randomly allocated into four groups and treated as indicated: group A (n06)— untreated control animals; group B (n06)—received 0.7 mg/ day per os dexamethasone-21-sodium phosphate (Desashock, Fort Dodge Animal Health, Bologna, Italy) for 40 days begin-ning at study day 1; group C (n06)—received 15 mg/day per os prednisolone acetate (Novosterol, Ceva Vetem SpA, Milan, Italy) for 30 days beginning at day 8 and group D (n06)— received intramuscularly in the neck 0.01 mg/kg bodyweight oestradiol benzoate (Sigma-Aldrich, Milan, Italy) dissolved in benzylic alcohol and ethyl oleate weekly on days 12,19,26,33 and 40. All animals treated orally were given one capsule containing compound per application using a drenching gun.

Administration dosages used in the study were chosen accord-ing to known practices from the literature [3, 21, 22] and information derived from observations of local veterinary au-thorities through inspections on suspect farms. Control group animals were not administered matching administration exci-pients so that reported metabolic findings would be fully representative of the situation in on-site farm animals receiving no treatments at all. All groups of experimental animals were kept in separate boxes and fed with a diet consisting of corn silage, corn, hay and a commercial protein supplement, and water was supplied ad libitum. The experiment was authorized by the Italian Ministry of Health and the Ethics Committee of the University of Turin. All animals were sacrificed after a 6-day drug withdrawal period and carcasses of treated animals destroyed appropriately. Blood samples were collected from all animal groups throughout the study period and plasma stored frozen at -20 °C until further use. Plasma samples analysed by NMR in this study were obtained from individual animals at day 35 of the study.

Preparation of animal study plasma samples for NMR analysis

Carr—Pur cell—Meiboom—Gill NMR sample preparation

Plasma samples were allowed to come to room temperature, and 100 \A was mixed with 550 \A of saline (0.9% NaCl in 10% D_2O). Following centrifugation at 16,000xg- for 15 min, a 600-(j.l aliquot was pipetted into a 5-mm NMR tube prior to analysis by CPMG pulse sequence NMR.

Conventional ^{1}H NMR sample preparation

To a 10-ml glass tube containing 1 ml of plasma, 4 ml of ice cold acetone was added and mixed for 30 s. The solution was subsequently kept on ice for 12 min followed by centrifugation (l,200xg) at 4 °C for 15 min. The supernatant was collected

and dried under nitrogen before being re-suspended in 1.2 ml of D_2O and transferred to 2 ml plastic tube. The sample was then centrifuged at 4 °C for 15 min at 13,000xg\ The upper lipid layer was removed, and to the remaining mixture 500 *A* of $CDCL_3$ was added and then mixed for 30 s. The resulting mixture was then centrifuged at 13,000xg- at 4 °C for 15 min and the aqueous phase collected. This step was repeated to remove any remaining lipids and the supernatant collected and placed in a clean vial until analysis. To 540 *ii* of the sample, 60 *ii* of 1 M phosphate buffer in 99.95% D_2O, containing 2.5 mM of the internal standard trimethylsilyl-2,2,3,3,-tetradeuteroproprionate (TSP; Sigma Aldrich, UK), was added and used for NMR analysis. The pH of prepared samples (pH 7) was checked after the addition of phosphate buffer solution [23].
NMR analysis

CPMG NMR spectral acquisition

All 1D 1H CPMG NMR experiments were recorded at 300.0 (±0.05) K on a Bruker AVIII 400-MHz spectrometer (Bruker-Bopspin, UK) operating at 400.11 MHz. *^1H* NMR spectra were acquired using a Bruker CPMG pulse sequence (cpmgpr1d; RD-90°-{T-180°-T}$_n$-acquire) where ris the spin echo delay (τ=400 *xs*, overall CPMG length080 ms). The CPMG sequence generates spectra edited by T_2 relaxation times, reducing broad resonances from high molecular weight compounds facilitating the observation of low molecular weight metabolites. For all the 1H spectra 64 free induction decays (FIDs) were collected into 32 K data points, using a spectral width of 8,223.69 Hz (20 ppm), with a 4-s relaxation delay between pulses (17 (a.s pulse length) and a rf-field strength (1/(4 x 90 pulse)) of 29.4 kHz. A water pre-saturation delay was applied between pulses. Replication of results was insured by running a single sample throughout the experiment and direct comparison of the acquired spectra was used as a measure of reproducibility and quality control. As with standard 1D spectra, an exponential line-broadening func-tion of 0.3 Hz was applied to the FID prior to Fourier transfor-mation. All the sample spectra were phased and manually baseline corrected using ACDlabs (version 11.0, Toronto, ON, Canada). The sample spectra were referenced to lactate (CH_3, *SI.33* ppm) [24, 25]. Data reduction was carried out by manually binning the spectra and measuring the integral for each bin/bucket between 0.70 and 6.0 ppm—a total of 41 buckets were recorded. The region from 54.60 to 55.00 ppm, which contained the residual water signal, was excluded.

Conventional ^1H NMR spectral acquisition

The *^1H* NMR spectra were recorded at 400.13 MHz at 300.0 (±0.05) K on a Bruker Avance 400 narrow bore. For all the spectra, 512 FIDs were collected into 32 K data points using a spectral width of 5,000 Hz (12 ppm), with a 2-s relaxation delay between pulses. Four dummy scans were used in all cases. An exponential line-broadening function of 0.5 Hz was applied to the FID prior to Fourier transformation. All spectra were automatically phase and baseline-corrected, and the sample spectra were referenced to TSP (0.00 ppm). One dimensional 1H NMR data were pre-processed and aligned using an in-house developed program [26].

Metabolite identification

Metabolites were assigned based on chemical shift and identified from a library of in-house pure compounds, NMR databases (Madison Metabolomics Consortium Database) and from the literature [27]. After initial designation, metabolite identifications were confirmed through spiking and 2D NMR (data not presented).

Data processing and multivariate statistical analysis

Data obtained from plasma sample NMR analysis was ana-lysed using orthogonal partial-least-squares discriminant analysis (OPLS-DA). Scores plots facilitated visualisation of the different sample sets, whilst loadings plots revealed the char-acteristic underlying metabolic differences by showing the influence of the spectral integrals on the principal components. OPLS-DA was performed using SIMCA-P+ 12.0.1 (Umetrics AB, Umeå, Sweden). The spectral integrals were normalised to the sum of the total spectral intensity and scaled using the Range scaling technique as outlined by Xia et al. [28, 29] (mean centred and divided by the range of each variable). Consequently, samples were mean centred and analysed by OPLS-DA. Three correlated components and five orthogonal components were calculated for CPMG data. Three correlated and two orthogonal components were calculated for the con-ventional data. Constructed models were validated using Sim-ca's leave data out cross-validation technique.

Results and discussion

NMR spectroscopic analysis of bovine plasma samples

Figure 1 displays a representative spectrum obtained follow-ing CPMG ^1H NMR analysis of a bovine plasma sample. In essence, the 1D ^1H CPMG pulse program aids removal of the broad resonances associated with high molecular weight macromolecules and motionally constrained compounds, thus facilitating the observation of low molecular weight

metabolites. Using this methodology, a range of metabolites was positively identified within plasma between 50.00 and 6.00 ppm with some spectral resonances remaining uniden-tified. Despite CPMG ^ NMR being a high-throughput method for analysis (requiring no sample preparation of plasma samples), it is worth noting that the acquisition time required is slightly longer relative to that needed to obtain a conventional 1D ^{1}H NMR sequence (3.28 and 4.36 s per scan, respectively). Although we do expect T_2 losses in the CPMG experiment, the main reason for the longer acquisition times was due to the larger dilution factor of samples (CPMG samples were diluted by 85%; conventional ^{1}H NMR experiments were diluted by ~30%). Using pre-saturation will affect the intensities of any exchangeable protons in the spectra because of saturation transfer and, in addition, will affect the chemical shifts and intensities of all protons near the saturation frequency. Pre-saturation can also cause the sample to heat up if the power is too high, but this is not the case here whereby a 50-Hz pre-saturation power and variable temperature techniques were used to minimise any effects due to temperature increase. In contrast to the CPMG pulse sequence technique, a sample preparation step to extract polar metabolites is required prior to the analysis of plasma by the conventional NMR techniques. This step is necessary as a substantial component of all plasma samples consists of albumin, and using conventional ^{1}H NMR analysis, this protein negative-ly impacts on the observation of other low level components within the sample. Figure 2 illustrates a typical 1D ^{1}H spectrum obtained from an extracted plasma sample using this form of conventional NMR analysis with prior sample clean-up. As can be observed, the resulting spectra obtained are cleaner compared to CPMG obtained spectra, with over 23 identified metabolites and some resonances remaining unidentified. The most obvious addition to the metabolite profile obtained from this form of analysis is the identifica-tion of aromatic group compounds including tyrosine, phe-nylalanine, 3-methylhistidine, histidine, 1-methylhistidine and formate, between 56.50 and 59.00 ppm (see insert Fig. 2). A distinct advantage of this form of NMR analysis over the CPMG pulse sequence technique is that identified metabolites can potentially be quantified if required through the addition of an internal standard (TSP) provided the proper relaxation delay between pulses is applied (5x the longest T1 in the sample). For example, acetate methyl T1s are on the order of 6 s and TSP methyls 3 to 4 s. Therefore, a minimum 30-s relaxation delay would be needed to insure that their relative peak areas reflect the proton concentra-tions of each species. As a semi-quantitative analysis is used (i.e. high versus low) in this manuscript, the lack of an appropriately long relaxation delay between pulses (2 s is used for the extracted plasma samples) is suffice. However, if the T1's are significantly different, this could affect the high versus low classification.

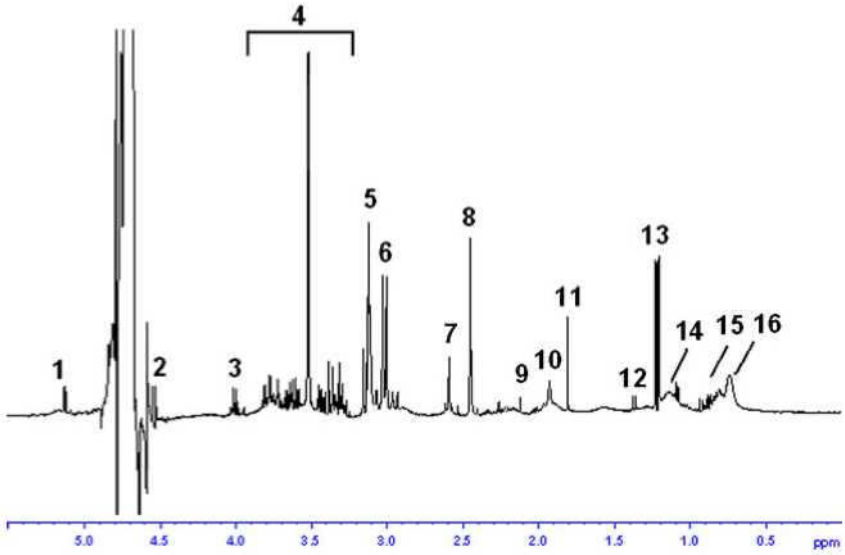

Fig. 1 ^{1}H CPMG NMR spectrum following analysis of bovine plasma sample. All identified peaks are referenced to the lactate peak at 51.33 ppm (d, 3, *J* 0 6 Hz, CH3). Major peak assignments: 1, a-glucose (<55.23 ppm; d, 1, *J0* 3 Hz, CH); *2*, |3-glucose ((54.64 ppm; d, *1* , *J 0* 6, CH); *3*, lactate ((54.11 ppm; q, *1* , *J 0 7* , CH); *4*, glucose (large carbohydrate region); 5, choline/phosphocholine *(83.21* ppm; s, , CH_3); *6*, unidentified ((52.69 ppm; s); 7, dimethylamine ((53.15 ppm; s, 3, CH_3); *8*, methylamine *(82.55* ppm; s, 3, CH_3); *9*, acetoacetate ((52.23 ppm; s, 3, CH3); *10*, glycoprotein (acetyls); *11*, unidentified ((51.91 ppm, s); *12*, alanine *(81.47* ppm; d, *3,J07*, CH_3); *13*, lactate ((51.33 ppm; d, 3, *J 0* 6 Hz, CH_3); *14*, lipid (CH_3); *15*, isoleucine/ leucine/valine (overlapped); *16*, lipoprotein (CH_3)

Multivariate analysis of metabolomic profiles of bovine plasma samples

CPMG pulse sequence NMR plasma sample data interpretation

Plasma samples *(n 0* 24) obtained on the same study day (day 35) from animals within the various growth-promoter groups were individually analysed by both forms of NMR analysis and metabolomic profiles representative of each sample recorded. OPLS-DA was utilised to analyse the reported spectral data to determine if it was possible to discriminate between those samples from non-treated control and those from growth-promoter-treated animal groups [20]. Figure 3 illustrates the scores plot obtained following OPLS-DA interpretation of the spectral data obtained following sample analysis via the CPMG pulse sequence. From the scores plot, it is visually possible to clearly identify four distinct animal study groups. The four different animal study groups are differen-tially distinguished along three latent components—latent component (LC) 1 explaining 17.6% of the variation, LC2 10.6% and LC3 5% *(R2* 0 0.93; *Q2* 0 0.66).

It is evident that animals from all treatment groups can be distinguished from control animals due to differences occurring in the relative levels of various identified metabolites present with analysed plasma. Table 1 highlights the relative levels of major metabolites, identified through the analysis of the spec-tral data using loadings plots and s-plots (plots not presented). It is notable that plasma from dexamethasone- and prednisolone-treated animals has similar high and low concentrations of particular metabolites relative to levels within control animals.

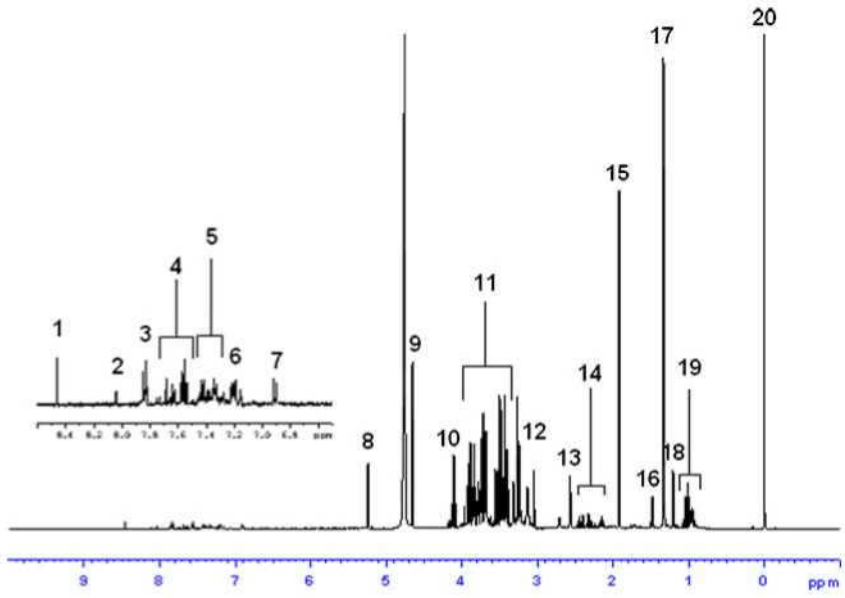

Fig. 2 ^{1}H spectrum obtained following conventional NMR analysis of an extracted plasma sample. All peaks are referenced to the internal standard TSP at 60.00 ppm. Insert -magnified view of the aromatic region of the spectrum. Major peak assignments: 1, unidentified, (68.46 ppm; s); 2, 1/3 methylamine (68.14; s, H2-ring); 3, hippurate (67.82 ppm; d, *J* = 7.40, o-ring); 4, hippurate (ring structure); 5, phenylalanine (ring structure); 6, tyrosine (67.20 ppm; d, *J* = 8.3, H2/H6-ring); 7, tyrosine (66.90 ppm; d, *J*= 8.3, H3/H5-ring); 8, a-glucose (65.23 ppm; d, 1, *J*= 3Hz, CH); 9, |3-glucose (64.64 ppm; d, 1, *J*= 6, CH); 10, lactate (64.11 ppm; q, 1, *J* = 7, CH); 11, glucose (Large carbohydrate region); 12, choline/phosphocholine (63.21 ppm; s, 3, CH_3); 13, unidentified (62.57 ppm, s); 14, region corresponding to glutamine and glutamate; 15 unidentified (61.91 ppm, s); 16, alanine (61.47 ppm; d, 3, *J*= 7, CH_3); 17, lactate (61.33 ppm; d, 3, *J* = 6 Hz, CH_3); 18, isoleucine/leucine/valine (Overlapped); 19, |3-hydroxybutyricacid(61.2ppm, d, 3,J= 6.4,yCH_3); 20, TSP (60.00 ppm, s, 9)

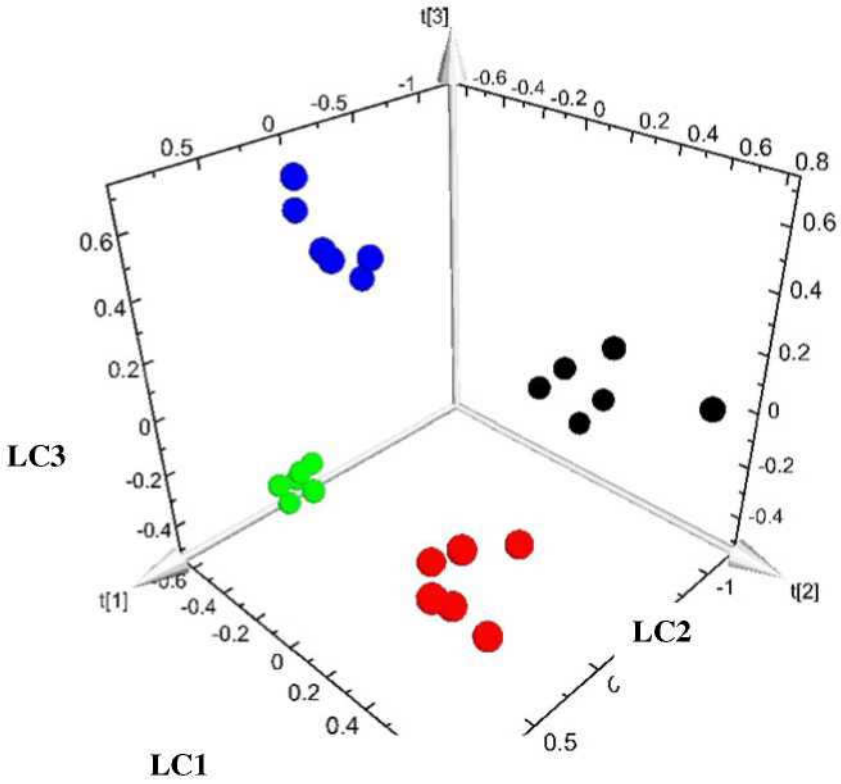

Fig. 3 3D OPLS-DA scores plot of the first three components from the analysis of ^{1}H CPMG NMR spectra obtained from plasma samples of non-treated control and growth-promoter-treated animal groups *(n* 0 6): dexamethasone *(red)*, prednisolone *(blue)*, oestradiol benzoate *(black)* and control *(green). R2 0* 0.93; *Q2 0* 0.66

This observation would indeed be anticipated as these two compounds are corticosteroid agents and are therefore likely to exert effects through similar mechanisms in vivo. However, plasma from animals treated with prednisolone differs slightly from controls (compared to dexamethasone -treated animals) in that they have higher levels of an unknown metabolite (a singlet at 52.69 ppm). From an extensive review of the literature and web libraries, we were unable to implicitly identify this particular compound. We suspect that it maybe 3-methyl-nicotinic aid. Animals treated with oestradiol, an agent with oestrogenic activity, were found to have altered plasma levels of a panel of metabolites relative to control animals which are totally dis-tinctive from those observed within samples from corticosteroid-treated animals.

Direct comparison of metabolite panels identified in this study with previous metabolomic studies investigating bio-logical responses to growth promoters [12, 15, 20, 30] is complicated by variations in utilised treatment regimes, in sample analysis methods, and in the type of matrices ana-lysed, i.e. plasma versus urine. Plasma metabolites distin-guishing between growth-promoter-treated and non-treated animals identified in this study include markers of nitrogen flux and metabolic balance. These metabolites reflect the impact of administered agents on the processes of gluconeo-genesis, glycogen deposition and protein metabolism. Glu-tamine and alanine are major inter-organ nitrogen carriers and are released by muscle tissue under corticosteroid influ-ence to accelerate nitrogen transfer to the liver. Plasma glutamate also plays a central role in mammalian nitrogen flow and protein synthesis acting as a substrate for gluta-mine production in peripheral tissues such as skeletal muscle. Observed alterations to creatinine and creatine levels also signify effects on protein accretion and nitrogen reten-tion and have previously been shown to be perturbed in other studies also investigating blood [30] and urine [15, 20] parameters affected by growth promoters.

Table 1 Levels of metabolite concentrations in plasma samples from various growth-promoter-treated animal groups relative to the matching control non-treated animal group as de-termined through data obtained by the CPMG pulse sequence NMR analysis method

Growth-promoter study animal treatment group					
Prednisolone		Dexamethasone		Oestradiol	
High	Low	High	Low	High	Low
Choline	Creatine	Alanine	Creatine	Alanine	Acetoacetate
Creatinine	Glucose	Choline	Glucose	Choline	Creatine
Glutamine		Creatinine		Glutamine	Isobutyric acid
Glutamate		Glutamine		Glutamate	Glucose
Unidentified		Glutamate		Isoleucine	
Lactate		Isobutyric acid		Lactate	
		Lactate		Leucine	
		Methylamine		Valine	
		Valine			
		Methionine			

Conventional NMR analysis of extracted plasma samples

The scores plot obtained following OPLS-DA interrogation of data from spectra derived through conventional NMR analysis of plasma samples from various growth-promoter-treated and control animal samples is visualised in Fig. 4 and illustrates a very similar pattern of study group sample separation to that obtained following analysis of data from CPMG sequence analyses. The four animal groups form separate distinct clusters with a single outlier observed for the dexamethasone treatment group which, following closer inspection of the quality of the obtained spectrum, was removed. From the OPLS-DA analysis, LC1 was found to account for 11% of the variation amongst the samples whereas LC2 explained 8.2% and LC3 68.5% *(R2* 0 0.78; *Q2* 0 0.33). Although the OPLS-DA scores plots obtained from data from both types of NMR analyses are similar, the information obtained from the conventional NMR analysis of extracted samples was found to pro vide less compact and defined clusters in comparison to the CPMG sequence data. Furthermore, it does not explain the same amount of variation as evident from CPMG analysis (LC1-LC3 accounting for 33.2% of the variation). In addition, it can be determined from the validation of the models that the CPMG analysis was more efficient as evident from the obtained *R2* and *Q2* values. This suggests that the less time-consuming CPMG sequence plasma analysis method could provide analogous results to that achieved by conventional NMR analysis but more rapidly, potentially making it more amenable for adop-tion in high-throughput screening approaches.

Table 2 displays the relative levels of key metabolites observed through conventional NMR analysis and identified through loadings plots and s-plots as being responsible for accounting for the variation between plasma extracts from growth-promoter-treated animals and plasma from control animals. Similar to observations from high-throughput CPMG analysis, plasma extracts from prednisolone- and dexamethasone-treated animals were revealed to contain altered levels of a similar range of identified metabolites. Plasma from oestradiol-treated animals was, also as before, found to display metabolite profiles distinct from that within corticosteroid-treated animal plasma samples. There are some variations in the metabolites identified through the different methods of NMR analysis as having increased or decreased levels relative to control animals. This may be as an artefact of analysing intact versus extracted plasma samples resulting in some metabolites been weighted more heavily in one form of analysis than another. This is evident when comparing the two types of analyses with regards to the small differences recorded between prednisolone and control samples for the separate NMR methodologies. Conventional NMR acquisition allows for identification of increased numbers of metabolites, and this has a direct impact on subsequent model building. For the samples measured by the CPMG pulse program, the plasma proteins and lipids remain in the sample whilst being analysed by NMR. As a result, many aromatic compounds present in the samples bind to albumin. Whilst bound, these small molecules take on the motional properties of the protein and are also atten-uated by the CPMG pulse train. For the samples analysed by the conventional proton pulse sequence, the plasma proteins were precipitated using acetone. In this process, many bound small molecules can be released and consequently are detected in the resulting NMR spectrum. As a result, specific metabolites are more heavily weighted and have a greater variable importance in the OPLS-DA analysis lead-ing to the small differences between sample acquisition methods. Additionally, as the CPMG pulse program removes broad resonances and other motionally constrained molecules, it may suppress larger signals within the plasma samples resulting in other identified metabolites not being as heavily weighted in the OPLS-DA analysis.

Conclusion

The present work has examined the potential to detect the illicit use of growth-promoting agents through the metabolomic profiling of bovine plasma by applying NMR methodologies and utilising multivariate data analysis to differentially distinguish between non-treated and treated animals based on reported metabolite profiles. Oestradiol administration, either alone or in combination with other agents, has long been recognised for its growth-promoting activity in animal production processes, whilst the synthetic corticosteroids dexamethasone and prednisolone, typically associated with veterinary health applications, have come into more recent focus as continuing evidence of their illegal use arises [3]. Due to their rapid excretion in urine irrespective of the administration route [31], glucocorticoids are typically administered orally in growth-promoting regimes at repeated low doses over sustained periods of time. Administration of such agents has been shown to increase animal weight gain, water retention and fat content, whilst improving overall feed efficiency [32]. These effects of growth promoting agents are reflected in changes at the biological level and effects on circulating blood components [33-36], and tissues [37, 38] have been clearly demonstrated. These effects have led to attempts to use the detection of changes to biological path-ways in response to exogenously administered agents as a means of identifying illegally treated animals [4, 5]. Metabolomic techniques examining changes in specific panels of metabolites within various biological matrices in response to administration of growth-promoting compounds are currently being investigated with the aim of developing easy-to-use high-throughput sample screening techniques. The analytical capabilities of two types of NMR analysis to analyse plasma samples have been compared in this study and their ability to distinguish between samples from hormone-treated and control non-treated animals assessed. Despite different and dis-tinct means of plasma analysis, both techniques produced similar metabolite profiling results with subsequent defined clustering of plasma samples from animals of different study groups upon multivariate analysis of obtained data. The CPMG pulse sequence analysis, requiring little or no sample preparation prior to

sample analysis, was found to be a rapid form of NMR analysis providing good metabolite profiling of samples and separation of animal study groups through data analysis by PLS-DA. Conventional one dimensional ^{1}H NMR analysis, although requiring a prior plasma sample extraction step and thereby proving to be more time-consuming, also provided a good degree of sample clustering and differentia-tion. This latter method resulted in cleaner NMR spectra requiring less processing time and facilitated the identification of a wider range of metabolites particularly those within the aromatic region of the spectra not observed via analysis by the CPMG pulse sequence method. This study has demonstrated the potential of NMR metabolomic techniques to differentiate between plasma samples within animal groups based on the metabolite profile accruing from different forms of growth-promoter administrations. Future work will investigate the effectiveness of this approach in larger animal cohorts and at different phases of treatment regimes and following growth-promoter withdrawal.

The implementation of this or other emerging forms of indirect techniques based on sample profiling to identify growth-promoter abuse will require modification of existing EU regulatory legislation—targeted MS-based residue analysis is the only approach currently accepted. The status quo of continuing illegal growth-promoter abuse accompanied by ineffectual monitoring is not only a regulatory deficiency but also a potential consumer confidence issue. The EU ban on growth-promoter use was introduced essentially as a precautionary human health measure and prior to ban intro-duction the treatment of animals with growth promoters relied principally on regulated compounds and associated administration regimes. However, post-ban treatment prac-tices have evolved to avoid detection by residue-based analysis and have developed to such an extent that existing EU monitoring programmes for growth-promoter abuse have arguably been rendered practically ineffectual. As a conse-quence, consumer health may be jeopardised by a reluctance to examine, and if deemed appropriate implement, promis-ing new methodologies as screening techniques for growth-promoter abuse in food producing animals. As argued by others [4, 6, 11], updating of EU legislation to bring growth-promoter monitoring regulations up to date with major advances in analytical methodology and more in line with anti-doping enforcement in human and equine sport would be a major advance towards the effective policing of the current ban, acting as a greater deterrent to growth-promoter users and helping to maintain consumer confidence.

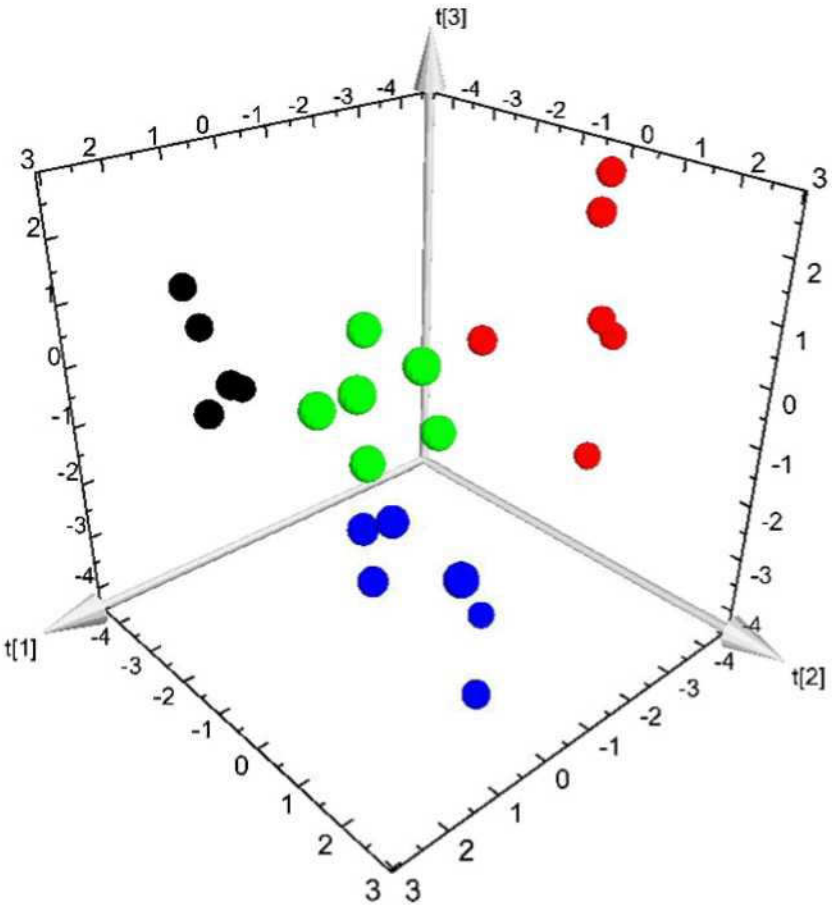

Fig. 4 3D OPLS-DA scores plot of the first three latent components of an analysis of 1D ^{1}H NMR spectra obtained from extract plasma samples of non-treated control and growth-promoter-treated animal groups *(n* 0 6): dexamethasone *(red)*, prednisolone *(blue)*, oestradiol benzoate *(black)* and control *(green)*. *R2* 0 0.88; *Q2* 0 0.44

Table 2 Relative levels of metabolite concentrations in plasma samples from various growth-promoter-treated animal groups compared to control non-treated animals as determined from data obtained by conventional NMR analysis of extracted plasma samples

Growth-promoter study animal treatment group					
Prednisolone		Dexamethasone		Oestradiol	
High	Low	High	Low	High	Low
2-Oxoglutarate	Creatine	2-Oxoglutarate	Creatine	Leucine	2-Oxoglutarate
Acetoacetate	Isoleucine	Glutamine	*Myo*-inositol	Lysine	Creatine
Glutamine	Lysine	Glutamate	Phenylalanine		Glutamine
Glutamate	*Myo*-inositol	Glucose			Glutamate
Glucose	Phenylalanine	Histidine			Glucose
Histidine		Isoleucine			Histidine
Leucine		Leucine			Isoleucine
Unsaturated fatty acids		Lysine Unsaturated fatty acids Valine			*Myo*-inositol Unsaturated fatty acids Valine

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