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Application of untargeted metabolomics approach for identification of compounds that may be responsible for observed differential effects in chickens fed an organic and a conventional diet

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Abstract:	<p>The aim of this study was to apply an untargeted NMR and LC-MS based metabolomics approach to detect potential differences between an organically and a conventionally produced feed, that caused statistically significant differences in growth, in the response to an immunological challenge and in the gene expression profiles in the small intestine of laying hens. A fractionation procedure was set-up to create multiple fractions of the feed, which were subsequently analyzed by NMR and UPLC-TOF/MS operating in positive mode. Comparison of the profiles revealed that the most apparent differences came from the isoflavones in the soy as well as a compound with a molecular mass of 441.202 (M+1)⁺, which was identified as N,N'-diferuloylputrescine (DFP) and came from the corn. Whether the observed differences in effects are due to the higher levels of isoflavones and DFP is unclear, as is the fact whether the observed differences are typical for organic or conventional produced corn and soy. However, this study shows that this metabolomics approach is suitable for detecting potential differences between products, even in levels of compounds that would have been overlooked with a more targeted</p>

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Application of untargeted metabolomics approach for identification of compounds that may be responsible for observed differential effects in chickens fed an organic and a conventional diet

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Abstract

The aim of this study was to apply an untargeted NMR and LC-MS based metabolomics approach to detect potential differences between an organically and a conventionally produced feed, that caused statistically significant differences in growth, in the response to an immunological challenge and in the gene expression profiles in the small intestine of laying hens. A fractionation procedure was set-up to create multiple fractions of the feed, which were subsequently analyzed by NMR and UPLC-TOF/MS operating in positive mode. Comparison of the profiles revealed that the most apparent differences came from the isoflavones in the soy as well as a compound with a molecular mass of 441.202 (M+1)⁺, which was identified as N,N'-diferuloylputrescine (DFP) and came from the corn. Whether the observed differences in effects are due to the higher levels of isoflavones and DFP is unclear, as is the fact whether the observed differences are typical for organic or conventional produced corn and soy. However, this study shows that this metabolomics approach is suitable for detecting potential differences between

products, even in levels of compounds that would have been overlooked with a more targeted approach. As such the method is suitable for a more systematic study on differences between conventionally and organically produced food.

Keywords: feed, organic farming, metabolomics, health, secondary metabolites

Introduction

There is an increasing interest in products that are organically produced. Major reasons are the care for animals, the environment, but also the potential benefits for the health of the consumer (Finamore et al., 2004; Rembialkowska, 2007; Givens et al., 2008). A large number of studies (Rembialkowska, 2007; Baker et al., 2002; Jestoi et al., 2004; Hoogenboom et al., 2008) have addressed the latter issue, focusing e.g. on differences in the residues of compounds used during production, like pesticides and veterinary drugs, but also on mycotoxins and environmental contaminants. Other studies addressed the potential contamination with micro-organisms or the resistance of micro-organisms against antibiotics (Philips et al., 2004; Pol and Ruegg, 2007, Hoogenboom et al., 2008). However, also potential differences in primary and secondary plant metabolites are of interest since some of these compounds may affect human health in a positive or negative way (Caris-Veyrat et al., 2004; Asami et al., 2003; Schulzová et al., 2007). Differences in the fertilization and the use of pesticides may result in different levels of plant metabolites (Lydon et al., 1989; Daniel et al., 1999; Carbonaro et al., 2002). A dilemma in all these studies is how to relate the composition of the agricultural product to health aspects. Furthermore, most of the studies performed in this regard focus on a limited number of compounds, which are assumed to be of interest or could have an effect (Bourn and Prescott, 2002; Magkos et al., 2003; Roose et al., 2009). An additional factor is that many environmental and genetic factors can influence

composition as well (Bourn and Prescott, 2002). So, in any case large numbers of samples and broad chemical analyses should be considered. An approach based on the profiling of extracts may be a good way for searching for and locating potential differences, and for correlating differences to health claims. Various examples of metabolomics studies on plants are available in the literature (Le Gall et al., 2003; Noteborn et al., 1998; 2000; Tikunov et al., 2005), showing the opportunities of this approach.

Recently a large study was performed on potential differences in the health of laying hens raised on feeds prepared from either organically or conventionally produced ingredients like soy, corn, barley, wheat, peas and triticale (Huber et al., 2010). Animals raised on the organic feed showed a stronger immune response after a challenge with keyhole limpet hemocyanin (KLH), but also a slower growth than the animals on conventional feed. Since other factors like housing were identical for all animals, the effects had to be somehow related to differences in the composition of the feed and thus the ingredients. In order to address these questions, a broad conventional analysis of both ingredients and feeds was performed, focusing on amino acids, vitamins, minerals and a selected number of secondary plant metabolites. However, such an approach is laborious and expensive and important differences may be overlooked.

In this context, the objective of this work was to develop an alternative approach to compare the different feeds in an untargeted way. Fingerprinting methodologies, such as Near and Mid-Infrared Spectroscopy (NIR, MIR) or FT- RAMAN Spectroscopy, have been used with this aim. These techniques have shown to be useful to confirm, for example, the geographical origin of food products. However, fingerprinting techniques are less suitable to identify or quantify individual compounds. Other techniques, such as the ambient ionization technique Direct Analysis in Real Time (DART), have also been

successfully used in food fingerprinting and profiling (Hajslova et al., 2011). However, with this method high temperatures are sometimes required to volatilize metabolites. In the present study it was decided to apply an untargeted metabolomics approach, based on the use of both NMR and UPLC-TOF/MS, that is able to identify potential differences between products and is suitable for broader studies on the potential differences between organic and conventional food. The main advantage is that the approach is untargeted and as such may also detect novel compounds.

Materials and Methods

Samples from the chicken study

Chicken feeds, previously used in the animal study, were prepared with ingredients from organic or conventional production systems with an essentially identical composition. As primary ingredients for the feeds, wheat, barley, triticale, corn, peas and soy were used (Huber et al., 2010). Ideally, ingredients were to be obtained from controlled cultivation systems, but this was only achieved for barley. For the other ingredients, neighbouring certified organic and conventional farms, with the same soil and climate, and following ‘best practices’ were chosen as the second best. Furthermore, using the same variety of the product in theory would have been the best option. However, only for soy was this achieved. For the other ingredients, this was not realistic because of cultivation requirements for both types of production, so in those cases the varieties used were typical for the production system (see Table 1).

The chicken feed was adapted to the age of the chicken. Therefore, three types of chicken feed with different composition were prepared (see Table 2). Starter feed (ST) was given to the chicken from 0 till 6 weeks, grower feed (GR) was used to feed the chicken from week 7 till 17, and layer feed (L) was given to the chicken from 18

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3 weeks on. The three chicken feeds were prepared with organically (o) and
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5 conventionally (c) produced ingredients, so in total there were 6 different chicken feeds:
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7 STo and STc; GRo and GRc; and Lo and Lc.
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10 11 *Chemicals*

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13 Ammonium acetate, dipotassium phosphate (K_2HPO_4), monopotassium phosphate
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15 (KH_2PO_4), ethylenediaminetetraacetic acid (EDTA) and deuterated chloroform
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17 ($CDCl_3$), water (D_2O) and methanol (CD_3OD) were purchased from Merck (Darmstadt,
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19 Germany), perchloric acid from Acros Organics (Geel, Belgium), daidzin, genistin,
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21 methanol and chloroform from Fluka Chemical Co. (Buchs, Switzerland),
22
23 Trimethylsilyl propanoic acid (TMSP) from Sigma-Aldrich (St. Louis, MO, USA), the
24
25 cationic exchange resin (AG50 W-X2) and the anionic exchange resin (AG 4x4) from
26
27 Biorad Laboratories (Hercules, CA, USA), acetyl genistin, acetyl daidzin, malonyl
28
29 genistin and malonyl daidzin from Nalacai Tesque Inc. (Kyoto, Japan).
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36 *Sample extraction and fractionation*

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38 The extraction was carried out following a modified protocol of that used in previous
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40 studies (Noteborn et al., 1998; 2000). Four fractions were prepared from each chicken
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42 feed sample (Figure 1). Fraction A: low molecular weight polar components, containing
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44 mainly sugars, amino acids and secondary metabolites; Fraction B: low molecular
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46 weight apolar components, containing basically triglycerides, but also some sterols,
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48 phospholipids, etc.; Fraction C: monomer methanolysis products of high molecular
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50 weight polar components, such as C1-methylated monomers units from
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52 polysaccharides; and Fraction D: monomer methanolysis products of high molecular
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54 weight apolar components, such as monomers of waxes, lignans, etc. Furthermore,
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Fraction A was also prepared from the individual ingredients. Each chicken feed, as well as each individual ingredient, was fractionated in 6-fold to account for possible variations in the extraction.

Fraction A (low molecular weight polar components) was obtained by extracting 300 mg of the powdered sample with 3 ml of CD₃OD/D₂O (60/40, v/v) by thorough shaking for 45 min. After centrifuging, 400 µl of the supernatant was separated for a further analysis by UPLC-TOF/MS and the rest was dried and stored at -20°C for analysis by NMR.

Fraction B (low molecular weight apolar components) was obtained by using 240 mg of sample mixed in 3 ml of 200 mM NH₄Ac. After vortexing the mixture for 30 seconds, it was centrifuged for 20 min and the supernatant was removed. This procedure was repeated 5 times in total, in order to remove the low molecular weight polar components present in the sample. The pellet was freeze-dried and then ground to get a very fine powder. This powder was then extracted 5 times with 2 ml of chloroform. The chloroform extracts were then pooled and dried under nitrogen at room temperature. The resulting sample was stored at -20°C for analysis by NMR.

The extracted leftover pellet (high molecular weight insolubles) resulting from the preparation of Fraction B was dried under nitrogen at room temperature and subjected to methanolysis as a first step in obtaining Fractions C and D. For this, 20 mg of dried pellet was put in the Teflon container of a Parr bomb (Illinois, USA) and 0.5 ml of perchloric acid was added. After 40 min of careful stirring, 11.5 ml of methanol was added and the mixture was bubbled through for 2 min with argon, in order to remove oxygen. Then the teflon container was put into the Parr bomb and the latter was placed in a preheated oven at 100°C for 9 h. The content was transferred into a 60 ml glass tube to which 4 ml of chloroform was added. The sample was then further diluted with 40 ml

of Millipore water. The tube was placed under argon for 2 min and thoroughly mixed for 20 min, after which it was centrifuged at 500 rpm at 4°C for 25 min. The two phases obtained constitute Fractions C (polar phase) and D (apolar phase). The chloroform phase (Fraction D) was transferred to another tube to which 2 ml of ascorbic acid (1 M) was added. After extensive shaking, it was centrifuged in order to separate the two phases. The chloroform fraction was then transferred to a vial, dried at room temperature and kept at -20°C for analysis by NMR.

The polar phase (Fraction C) had to be deproteinized prior to analysis. This fraction was passed through a cationic exchange AG50 W-X2 column previously washed with methanol. The non-retained fluid was then transferred to an anionic exchange AG 4x4 column also previously washed with methanol. This deproteinized fraction was freeze-dried and kept at -20°C for analysis by NMR.

NMR analysis

Fraction A was dissolved in 1 ml of CD₃OD/D₂O (60:40, v/v), containing 200 mM K₂HPO₄/KH₂PO₄, 2.5 mM TMSP and 2.5 mM EDTA; Fraction C was dissolved in 1 ml of D₂O containing 400 mM K₂HPO₄/KH₂PO₄, 2.5 mM TMSP and 2.5 mM EDTA; Fraction B and D were dissolved in 1 ml of CDCl₃ containing 0.03% of tetramethylsilane (TMS) as internal standard. From all samples 0.600 (± 0.010) ml of sample in high quality NMR sample tubes (5.0 mm, 535-PP-7, Wilmad, Buena, USA) were used to ensure optimal field homogeneity and optimal reproducibility of the magnetic field. The ¹H NMR spectra were recorded at 400.13 MHz at 300.0 (± 0.05) K on a Bruker Avance 400 narrow bore using a 5.0-mm probe. The spectrometer settings were: 2 s relaxation delay, number of scans 128 (4 dummy scans) (for Fraction C, 1024), spectral width of 5000 Hz, a 60 degree pulse and 16 K data points. Prior to data

analysis the raw NMR data were subjected to a squared sine bell filter (shifted $\frac{1}{2}$ pi), zero-filling to 128 K data points, Fourier Transformation and phase correction.

UPLC-TOF/MS analysis

The study of Fraction A of the chicken feed and of the individual ingredients was carried out by diluting the NMR samples 10 times in CD₃OD/D₂O (60:40, v/v) and injecting 5 µl of each sample on the system described below. The injection sequence was randomized according to Vos et al. (2007). The analyses were performed on a LCT Premier LC-TOF-MS system (Waters, Milford, MA, USA) equipped with a dual spray electrospray source. The lock mass calibrant (leucine/enkephaline) was measured every 10 scans. The gradient was provided by an UPLC system (model Acquity, Waters) with a 150 mm x 2.1 mm UPLC BEH-C8 with 1.7 µm particles (Waters). The mobile phase consisted of water, acetonitrile and formic acid (A:100/0/0.2 and B: 0/100/0.2). Gradient elution was performed at 0.4 ml min⁻¹. The initial eluent composition, 100% A, was kept for 2 min after which the composition was changed to 70% A in 13 min. This composition was maintained for 5 min and afterwards, the eluent composition was increased till 100% B in 0.20 min and was kept this way for 20 min more. The effluent of the LC system was interfaced directly with the TOF-MS. The instrument was operated in positive mode polarity. A stable spray was obtained with a capillary voltage of 2.8 kV, a source temperature of 120°C and desolvation temperature of 350°C. The desolvation and cone gas flow were 500 and 50 L h⁻¹ respectively. The cone voltage was 25 V. Spectra were collected in centroid mode from m/z 100 to 1450 with a scan time of 0.2 s. Accurate masses were obtained after lock mass correction. The mass spectrometer was operated in W mode Dynamic Range Enhancement turned on and the resolution was 10,000 (FWHM).

UPLC-QTOF-MS/MS analysis

The measurements were performed on an Acquity UPLC (Waters, Milford, MA, USA) coupled via ESI interface to a Bruker microTOF-Q (Bruker Daltonics, Bremen, Germany) system operating in positive mode. 20 μl of sample were injected and separated on a 150 mm x 2.1 mm UPLC BEH-C8 column with 1.7 μm particles (Waters). The flow rate was set at 0.4 ml min^{-1} . Since the retention time of the compound of interest was known, the gradient used was similar to that described above but adapted to run in a shorter time. Briefly, the initial eluent composition, 95% A, was kept for 1 min and was changed to 70% A in 5 min. This composition was maintained for 2 min and then, the eluent composition of B was increased again till 70% B in 4 min. Afterwards, B was increased to 100% in 0.5 min and kept this way for 2 min more. For MS/MS analysis, the capillary voltage was set to 3500 V, the nebulizer gas to 2 bar, the dry gas to 8 L/min and the temperature at 200°C. The transfer time of the source was 75 μs . For MS/MS analysis of the compound of interest, a precursor ion at m/z 441.2 was selected. The collision energy was 15 eV and nitrogen was used as the collision gas. Full spectra were collected at m/z 50–700. Instrument calibration was performed externally prior to each sequence with a sodium formate/acetate solution.

Data Analysis

The NMR data were pre-processed and aligned using an in-house developed program (Noteborn et al., 2000; Lommen et al., 1998). The LC-MS data sets were pre-processed and aligned using the metAlignTM software package (Lommen, 2009) (a free download is available at <http://www.metalign.nl/UK/>). Both software programs were used to generate differential data sets (using univariate statistics) where required in this study.

Besides this, the aligned fingerprint data –¹H-NMR as well as UPLC-TOF/MS- in the form of generated spreadsheets were subjected to multivariate analysis using Genemaths XT (<http://www.applied-maths.com/genemaths/genemaths.htm>). An investigation of differences in metabolic profiles was done for the chicken feed prepared with organically and conventionally produced ingredients, as well as for the organically and conventionally cultivated soybean and corn samples.

Results and Discussion

The four fractions of the chicken feed, prepared as described above and depicted in Figure 1, were first measured by ¹H NMR. Visual inspection of replicates of all fractions through overlaying spectra showed a high level of reproducibility. The spectra were baseline corrected and aligned using an in-house developed program (Noteborn et al., 2000; De Vos et al., 2007). The output data were subsequently put through multivariate analysis. Figures 2A, 2B, 2C and 2D show the resulting PCA plots of the four different fractions. The high reproducibility level was also reflected in the PCA plots through good clustering of the six different replicas of each sample, which is enhanced after performing an ANOVA with a p<0.05. From the clustering of the samples in Figures 2A, 2B and 2C it can be clearly observed that differences between the three different types of feed (starter (ST), grower (GR), layer (L)) were much larger than the differences between the six technical replicates and also than the differences between the two production types, organic or conventional. The differences between feed samples, as deduced from the apolar components of the monomer methanolysis products of high molecular weight (Figure 2D) were less clear and smaller. The differences between the various types of feed on the one hand, and feeds produced from ingredients from the two different production types will be further discussed below.

Differences between starter, grower and layer feeds

The clear differences observed between starter (ST), grower (GR) and layer feed (L) were expected due to their different composition with respect to the ingredients (see Table 2). At the same time, this result confirms the potential of the approach to detect quantitative differences in the levels of different components in the feeds.

A different way of examining the differences between the various types of feed is given in Table 3A, showing an ANOVA performed for $p < 0.05$, either with or without a Bonferroni correction; the latter is used to take into account the effects of multiple testing on increasing the chance to find significant differences. Using this technique, the fraction of identified peaks that show a significant difference in terms of peak area was calculated. It is clear that many NMR peaks survived this selection, indicating the existence of significant differences. This is especially true when the comparison is based on the different types of feed, which can be explained by the different composition. However, also when looking at the production type, a number of significant differences can be observed, especially in the low molecular weight polar fraction (A) (see below).

From the peak loadings giving rise to the separation of samples in the PCA in Figure 2, it was possible to create a list of resonance positions, which contributed most to the observed separation between the types of feed. Using such a list and the original ^1H -NMR data, positions of interest in the spectra could be localized for identification. This constitutes an untargeted way of searching for differences in samples. The first principle component (x-axis) in the PCA in Figure 2A was primarily arising from resonance peaks of aromatic protons of isoflavone derivatives. Figure 3, shows an

example for two different chicken feeds (ST and GR) with regard to the aromatic region of the ^1H -NMR spectrum typical of isoflavone resonances.

Complementary and analogous to the ^1H -NMR experiments, semi-polar secondary metabolites in Fraction A were separated and analyzed on a C8-column in an UPLC system coupled to a TOF/MS. The UPLC-TOF/MS raw data sets were processed and aligned with the metAlign software package as in previous studies (Tikunov et al., 2005; De Vos et al., 2007; Lommen, 2009; Keurentjes et al., 2006). An example of chromatograms pre-processed with MetAlign of organic GR, ST and L feed given in additional file figure S1. The output data matrix of the exact masses, obtained after the alignment, was subjected to multivariate analysis. The PCA plot of this fraction was very similar to that obtained for Fraction A measured by ^1H NMR (see additional file figure S2). Again, masses related to isoflavone derivatives ($\text{GR} > \text{L} > \text{ST}$) showed to contribute heavily to the separation in the PCA through analysis of the peak loadings. Commercial standards were used for confirmation of the identity of some of the isoflavones present in the chicken feed samples. This was done for ^1H -NMR as well as UPLC-TOF/MS by spiking. The main isoflavones identified were daidzin, genistin and the acetyl and malonyl derivatives of daidzin and genistin. A study of the polar fraction of the individual ingredients by ^1H NMR and UPLC-TOF/MS showed that isoflavones arose exclusively from soybean (data not shown).

In Fraction B, which contained the low molecular weight apolar components, also differences between the various types of feeds were found (see Figure 2B). These were related to the acyl group (i.e. fatty acid) composition. It was observed that the ST feeds had a higher proportion of saturated and mono-unsaturated acyl groups, whereas the L and GR feeds had a higher proportion of polyunsaturated groups.

Among the fractions of high molecular weight components, the largest differences between the types of feed were detected in the polar fraction (see Figure 2C). After examination of the peak loadings it could be noted that Fraction C showed higher amounts of monomerized sugars in the ST feeds than in the L and GR feeds. The differences in the apolar fraction were shown to be minimal and therefore not further examined (see Figure 2D).

Differences between organic and conventional feeds

As shown in Figure 2A (see clustering of samples in PCA), there were also differences between feeds prepared with organically and conventionally produced ingredients, although these differences were much smaller in comparison to the differences between the types of feed (ST, GR and L) (see also Table 3 and additional file figure S3, which shows the MetAlign pre-processed chromatograms of organic and conventional grower feed). A complicating factor in analyzing the effects of organically and conventionally produced feed in the reference study (Huber et al., 2010) was that the feed composition of ST, GR and L (used in different phases of growth, Table 2) was different. These differences in composition also complicated the search for feed factors that might have caused the differences in effects. A way to look for consistent feed-independent differences between organically and conventionally produced feeds was by regrouping the ST, GR and L data in 2 groups, respectively organically and conventionally produced feed. The results of this approach are given in Figure 4A and 4B for fraction A analyzed by NMR and UPLC-TOF/MS respectively, as well as in Table 3 part B. This table shows that there is still a significant number of peaks surviving after ANOVA ($p < 0.05$). The peak loadings resulting in Figure 4A (Fraction A analyzed by ^1H NMR) suggested that there might be a trend in different isoflavone contents. This trend, which

was also observed in the mass loadings resulting in Figure 4B (Fraction A analyzed by UPLC-TOF/MS), could be explained from the analysis of Fraction A (by ^1H NMR and UPLC-TOF/MS, in 6 fold) of the organic and conventional soybean samples. This analysis showed that the organic sample contained between 20 and 30% more isoflavones than the conventional counterpart, as was also observed in a classical targeted analysis (Huber, 2007).

Apart from these isoflavone signals, a distinct mass of 441.202 Da ($\text{M}+\text{H}^+$) was observed as a difference in the UPLC-TOF/MS data. This mass came out of the multivariate analysis in Figure 4B and was a “survivor” mass peak in Table 3 part B. Mass 441.202 Da, which matched the structure $\text{C}_{24}\text{H}_{29}\text{N}_2\text{O}_6$, was shown to arise from the corn (data not shown). This structure could correspond to N,N'-dyferuloylputrescine (DFP), a polyamine conjugate already described as an ingredient of corn. Unfortunately no standard could be purchased. Therefore, in order to confirm the identity of this mass, MS/MS analysis on Fraction A of the organic corn sample was performed, since the organic feed and corn showed higher concentration of the mass of interest. The main fragments obtained from the MS/MS analysis were the following: 177.054, 248.128 and 291.134 Da, which were consistent with those already described for DFP by other authors (LeClere et al., 2007) and with those predicted by Mass Frontier 6.0 (<http://www.highchem.com/downloads/mass-frontier/>). Furthermore, the ^1H NMR spectra of Fraction A of the organically and conventionally produced corn samples exhibited the characteristic proton signals of DFP in the aromatic region of the spectra (Miller et al., 1996). This analysis, which was also done in 6 fold, showed that the organic sample contained more than two fold higher levels of DFP.

This finding could be of interest because DFP has been found to have antibiotic activities against certain pests in corn (Arnason et al., 1992), to prevent aflatoxin

production by *Aspergillus flavus* (Mellon and Moreau, 2004) and also to possess antimelanogenic, antioxidant and radical scavenger activities (Choi et al., 2007).

Relation between feed composition and observed health effects

Although at this point it is not possible to ascribe the effects observed in the chicken study (Huber et al., 2010) to the differences in the composition found between the feeds of different origin, it is noteworthy that using the untargeted approach herein described, differences in the content of several bioactive compounds such as isoflavones (Sakai and Kogiso, 2008) and DFP (Choi et al., 2007) have been found. Additional classical analyses, in which individual feeds (i.e. ST, GR and L) of both production types (i.e. organic and conventional) were compared, also showed results on the isoflavones compatible to the above (Huber, 2007). However, differences in the content of DFP between both types of feed were overlooked. At the same time it should be clear that even an untargeted metabolomics approach will not cover the full range of possible compounds; for instance, compounds that do not ionize in positive mode will not be detected in the UPLC-MS approach used in this study. Also proteins and peptides are not determined and trace contaminants will not be within the dynamic range of the applied techniques.

Conclusions

This paper has shown that the chemical fingerprinting of the ingredients and chicken feed is a powerful tool for detecting potential differences between samples, not only of different composition (ST, L & GR) but also of different origin (in this case organic vs conventional). The extraction method, together with the analytical techniques used in this study, provide very reproducible and reliable results. It has also been shown that the fractions containing low molecular weight metabolites (fractions A and B) provide the

most valuable information. Therefore, the analysis of these fractions, either by ^1H NMR or by UPLC-MS or both, could be used as a straightforward method to profile feeds .

One of the biggest advantages of this metabolomics approach, compared to classical analysis, is that within this approach, only when differences are found, there is a need for identification of the responsible compounds. Furthermore, using an approach as the one described, a wide range of both known and potentially unknown metabolites can be covered. The untargeted metabolomics approach described in this paper has allowed us to find differences in the composition of the various chicken feeds prepared from organic and conventionally produced ingredients which were not detected in the broad classical analysis. The observed differences were due to the higher content of a compound with mass 441.202, identified as DPF, in the organic feed. In addition higher levels of isoflavones were detected, as also observed in the targeted analysis.

Even though the feeds were found to be different, this study could not answer the question whether the differences were typical for the organic and conventional production systems. Although efforts were made to include samples from controlled trials, this was not always possible. Therefore, it cannot be excluded that some of the differences observed were due to varietal and/or production differences, not related to organic or conventional practices. For this question to be answered many more products from each production type should be analysed to have an idea what the average situation is in practice. In this respect, application of untargeted, broad analytical techniques may allow a better examination of differences between products from different production types and hence allow a better selection of representative samples used for animal studies.

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Figure Captions

Figure 1. Scheme of the extraction protocol of the fractions A, B, C and D.

Figure 2. Multivariate statistical analysis of pre-processed and aligned ^1H NMR data from chicken feed samples. A, B, C, D correspond to PCA plots (after ANOVA $p < 0.05$) for resp. fractions A, B, C, and D. Spheres with the same color are technical replicates of the same sample. STo: green; STc: red; GRo: lila; GRc: yellow; Lo: light blue; Lc: dark blue.

Figure 3. Expanded region of a ^1H NMR spectrum of the chicken feed STo (blue) and GRo (red) illustrating differences due to isoflavone resonances.

Figure 4. A: PCA plot (after ANOVA $p < 0.05$) of Fraction A of chicken feed analyzed by ^1H NMR and pre-processed and aligned using an in-house developed program. Green: ORG; red: CONV. **B.** PCA plot (after ANOVA $p < 0.05$) of Fraction A of chicken feed analyzed by UPLC-TOF/MS and pre-processed and aligned using MetAlign. Green: ORG; red: CONV.

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Additional files

PDF file containing Figures S1, S2 and S3.

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Figure 1

Figure 2

Figure 3

Figure 4

Table 1. Background and varieties of the ingredients used to prepare the chicken feeds

Ingredient	Origin		Variety	
	Conv.	Org.	Conv.	Org.
Barley	Netherlands	Netherlands	Class	Prestige
Wheat	Netherlands	Netherlands	Bristol	Lavett
Triticale	Netherlands	Netherlands	Talentro	Cairo
Pea	Denmark	Denmark	Unknown	Unknown
Corn	Austria	Austria	Mix of 8	Mix of 5
Soy	Austria	Austria	Essor	Essor

Table 2. Composition of the different chicken feeds

	Starter feed	Grower feed	Layer feed
	(STo & STc)	(GFo & GFc)	(LFo & LFc)
Ingredients	%	%	%
Corn	20.00	20.00	25.00
Wheat	30.00	26.42	25.23
Barley	5.00	10.00	5.00
Triticale	12.05	0.00	0.00
Soybeans heated	0.00	10.17	19.87
Soy flakes	10.16	20.00	0.00
Peas	10.00	10.00	10.00
Potato proteins*	7.00	0.00	2.50
MonoCalFos*	1.13	0.73	1.01
FX Layers premix*	1.00	1.00	1.00
Fat of plan origin*	1.50	0.00	0.52
Salt*	0.07	0.09	0.06
Chalk*	1.64	1.16	7.65
Shells broken*	0.00	0.00	2.00
NaCO ₃ *	0.09	0.08	0.00
Methionine*	0.11	0.04	0.15

*Additional feed component

Table 3: Numbers of NMR selected peaks after anova ($p < 0.05$) with and without Bonferroni correction). Comparisons are made for either: A- type of feed, or B- type of production. For the ANOVA selection, replicates of each sample were grouped together.

			ANOVA $p < 0.05$	ANOVA $p < 0.05$ & bonferroni
A: Types of feed (ST, GR, L)	NMR	Low polar	83.7% (478/578)	59.5% (344/578)
		Low apolar	82.8% (217/262)	61.1% (160/262)
		High polar	50.0% (144/288)	21.2% (61/288)
		High apolar	57.5% (61/106)	27.4% (29/106)
	LC-MS	Low polar	11.5% (541/4719)	3.3% (157/4719)
B: Type of Cultivation (Org vs Conv)	NMR	Low polar	19.6% (113/578)	1.6% (9/578)
		Low apolar	12.2% (32/262)	-----
		High polar	1.0% (3/288)	-----
		High apolar	2.8% (3/106)	-----
	LC-MS	Low polar	5.0% (238/4719)	0.1% (6/4719)

**Application of untargeted metabolomics approach for
identification of compounds that may be responsible for
observed differential effects in chickens fed an organic and a
conventional diet**

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SUPPLEMENTARY DATA

S1. PCA plot (after ANOVA $p < 0.05$) of Fraction A of chicken feed analyzed by UPLC-TOF/MS and pre-processed and aligned using MetAlign. Spheres with the same color are technical replicates of the same sample. STo: green; STc: red; GRo: lila; GRc: yellow; Lo: light blue; Lc: dark blue.

S2. Chromatograms of the organic grower (GR), started (ST) and layer (L) feeds pre-processed using MetAlign (Lommen, 2009)

GR organic **GR conventional**

S3. Chromatograms of the organic and conventional grower feeds (GR organic & GR conventional) pre-processed using MetAlign (Lommen, 2009)