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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 |

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| | approach. As such the method is suitable for a more systematic study on differences between conventionally and organically produced food. |
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3 **Application of untargeted metabolomics approach for identification of**
4 **compounds that may be responsible for observed differential effects in**
5 **chickens fed an organic and a conventional diet**
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27 **Abstract**
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30 The aim of this study was to apply an untargeted NMR and LC-MS based metabolomics
31 approach to detect potential differences between an organically and a conventionally
32 produced feed, that caused statistically significant differences in growth, in the response
33 to an immunological challenge and in the gene expression profiles in the small intestine
34 of laying hens. A fractionation procedure was set-up to create multiple fractions of the
35 feed, which were subsequently analyzed by NMR and UPLC-TOF/MS operating in
36 positive mode. Comparison of the profiles revealed that the most apparent differences
37 came from the isoflavones in the soy as well as a compound with a molecular mass of
38 441.202 (M+1)⁺, which was identified as N,N'-diferuloylputrescine (DFP) and came
39 from the corn. Whether the observed differences in effects are due to the higher levels
40 of isoflavones and DFP is unclear, as is the fact whether the observed differences are
41 typical for organic or conventional produced corn and soy. However, this study shows
42 that this metabolomics approach is suitable for detecting potential differences between
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3 products, even in levels of compounds that would have been overlooked with a more
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5 targeted approach. As such the method is suitable for a more systematic study on
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7 differences between conventionally and organically produced food.
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10 **Keywords:** feed, organic farming, metabolomics, health, secondary metabolites
11

12 13 **Introduction**

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16 There is an increasing interest in products that are organically produced. Major reasons
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18 are the care for animals, the environment, but also the potential benefits for the health of
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20 the consumer (Finamore et al., 2004; Rembialkowska, 2007; Givens et al., 2008). A
21
22 large number of studies (Rembialkowska, 2007; Baker et al., 2002; Jestoi et al., 2004;
23
24 Hoogenboom et al., 2008) have addressed the latter issue, focusing e.g. on differences in
25
26 the residues of compounds used during production, like pesticides and veterinary drugs,
27
28 but also on mycotoxins and environmental contaminants. Other studies addressed the
29
30 potential contamination with micro-organisms or the resistance of micro-organisms
31
32 against antibiotics (Philips et al., 2004; Pol and Ruegg, 2007, Hoogenboom et al.,
33
34 2008). However, also potential differences in primary and secondary plant metabolites
35
36 are of interest since some of these compounds may affect human health in a positive or
37
38 negative way (Caris-Veyrat et al., 2004; Asami et al., 2003; Schulzová et al., 2007).
39
40 Differences in the fertilization and the use of pesticides may result in different levels of
41
42 plant metabolites (Lydon et al., 1989; Daniel et al., 1999; Carbonaro et al., 2002). A
43
44 dilemma in all these studies is how to relate the composition of the agricultural product
45
46 to health aspects. Furthermore, most of the studies performed in this regard focus on a
47
48 limited number of compounds, which are assumed to be of interest or could have an
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50 effect (Bourn and Prescott, 2002; Magkos et al., 2003; Roose et al., 2009). An
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52 additional factor is that many environmental and genetic factors can influence
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3 composition as well (Bourn and Prescott, 2002). So, in any case large numbers of
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5 samples and broad chemical analyses should be considered. An approach based on the
6
7 profiling of extracts may be a good way for searching for and locating potential
8
9 differences, and for correlating differences to health claims. Various examples of
10
11 metabolomics studies on plants are available in the literature (Le Gall et al., 2003;
12
13 Noteborn et al., 1998; 2000; Tikunov et al., 2005), showing the opportunities of this
14
15 approach.
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19 Recently a large study was performed on potential differences in the health of
20
21 laying hens raised on feeds prepared from either organically or conventionally produced
22
23 ingredients like soy, corn, barley, wheat, peas and triticale (Huber et al., 2010). Animals
24
25 raised on the organic feed showed a stronger immune response after a challenge with
26
27 keyhole limpet hemocyanin (KLH), but also a slower growth than the animals on
28
29 conventional feed. Since other factors like housing were identical for all animals, the
30
31 effects had to be somehow related to differences in the composition of the feed and thus
32
33 the ingredients. In order to address these questions, a broad conventional analysis of
34
35 both ingredients and feeds was performed, focusing on amino acids, vitamins, minerals
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37 and a selected number of secondary plant metabolites. However, such an approach is
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39 laborious and expensive and important differences may be overlooked.
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45 In this context, the objective of this work was to develop an alternative approach
46
47 to compare the different feeds in an untargeted way. Fingerprinting methodologies, such
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49 as Near and Mid-Infrared Spectroscopy (NIR, MIR) or FT- RAMAN Spectroscopy,
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51 have been used with this aim. These techniques have shown to be useful to confirm, for
52
53 example, the geographical origin of food products. However, fingerprinting techniques
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55 are less suitable to identify or quantify individual compounds. Other techniques, such as
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57 the ambient ionization technique Direct Analysis in Real Time (DART), have also been
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3 successfully used in food fingerprinting and profiling (Hajslova et al., 2011). However,
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5 with this method high temperatures are sometimes required to volatilize metabolites. In
6
7 the present study it was decided to apply an untargeted metabolomics approach, based
8
9 on the use of both NMR and UPLC-TOF/MS, that is able to identify potential
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11 differences between products and is suitable for broader studies on the potential
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13 differences between organic and conventional food. The main advantage is that the
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15 approach is untargeted and as such may also detect novel compounds.
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20 **Materials and Methods**

21 *Samples from the chicken study*

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24 Chicken feeds, previously used in the animal study, were prepared with ingredients
25
26 from organic or conventional production systems with an essentially identical
27
28 composition. As primary ingredients for the feeds, wheat, barley, triticale, corn, peas
29
30 and soy were used (Huber et al., 2010). Ideally, ingredients were to be obtained from
31
32 controlled cultivation systems, but this was only achieved for barley. For the other
33
34 ingredients, neighbouring certified organic and conventional farms, with the same soil
35
36 and climate, and following 'best practices' were chosen as the second best.
37
38 Furthermore, using the same variety of the product in theory would have been the best
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40 option. However, only for soy was this achieved. For the other ingredients, this was not
41
42 realistic because of cultivation requirements for both types of production, so in those
43
44 cases the varieties used were typical for the production system (see Table 1).
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50 The chicken feed was adapted to the age of the chicken. Therefore, three types
51
52 of chicken feed with different composition were prepared (see Table 2). Starter feed
53
54 (ST) was given to the chicken from 0 till 6 weeks, grower feed (GR) was used to feed
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56 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
4
5 conventionally (c) produced ingredients, so in total there were 6 different chicken feeds:
6
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
14
15 (KH_2PO_4), ethylenediaminetetraacetic acid (EDTA) and deuterated chloroform
16
17 ($CDCl_3$), water (D_2O) and methanol (CD_3OD) were purchased from Merck (Darmstadt,
18
19 Germany), perchloric acid from Acros Organics (Geel, Belgium), daidzin, genistin,
20
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
39
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
43
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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3 Fraction A was also prepared from the individual ingredients. Each chicken feed, as
4 well as each individual ingredient, was fractionated in 6-fold to account for possible
5 variations in the extraction.
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9 Fraction A (low molecular weight polar components) was obtained by extracting
10 300 mg of the powdered sample with 3 ml of CD₃OD/D₂O (60/40, v/v) by thorough
11 shaking for 45 min. After centrifuging, 400 µl of the supernatant was separated for a
12 further analysis by UPLC-TOF/MS and the rest was dried and stored at -20°C for
13 analysis by NMR.
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20 Fraction B (low molecular weight apolar components) was obtained by using
21 240 mg of sample mixed in 3 ml of 200 mM NH₄Ac. After vortexing the mixture for 30
22 seconds, it was centrifuged for 20 min and the supernatant was removed. This procedure
23 was repeated 5 times in total, in order to remove the low molecular weight polar
24 components present in the sample. The pellet was freeze-dried and then ground to get a
25 very fine powder. This powder was then extracted 5 times with 2 ml of chloroform. The
26 chloroform extracts were then pooled and dried under nitrogen at room temperature.
27 The resulting sample was stored at -20°C for analysis by NMR.
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38 The extracted leftover pellet (high molecular weight insolubles) resulting from
39 the preparation of Fraction B was dried under nitrogen at room temperature and
40 subjected to methanolysis as a first step in obtaining Fractions C and D. For this, 20 mg
41 of dried pellet was put in the Teflon container of a Parr bomb (Illinois, USA) and 0.5 ml
42 of perchloric acid was added. After 40 min of careful stirring, 11.5 ml of methanol was
43 added and the mixture was bubbled through for 2 min with argon, in order to remove
44 oxygen. Then the teflon container was put into the Parr bomb and the latter was placed
45 in a preheated oven at 100°C for 9 h. The content was transferred into a 60 ml glass tube
46 to which 4 ml of chloroform was added. The sample was then further diluted with 40 ml
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3 of Millipore water. The tube was placed under argon for 2 min and thoroughly mixed
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5 for 20 min, after which it was centrifuged at 500 rpm at 4°C for 25 min. The two phases
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7 obtained constitute Fractions C (polar phase) and D (apolar phase). The chloroform
8
9 phase (Fraction D) was transferred to another tube to which 2 ml of ascorbic acid (1 M)
10
11 was added. After extensive shaking, it was centrifuged in order to separate the two
12
13 phases. The chloroform fraction was then transferred to a vial, dried at room
14
15 temperature and kept at -20°C for analysis by NMR.
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19 The polar phase (Fraction C) had to be deproteinized prior to analysis. This
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21 fraction was passed through a cationic exchange AG50 W-X2 column previously
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23 washed with methanol. The non-retained fluid was then transferred to an anionic
24
25 exchange AG 4x4 column also previously washed with methanol. This deproteinized
26
27 fraction was freeze-dried and kept at -20°C for analysis by NMR.
28

29 *NMR analysis*

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31 Fraction A was dissolved in 1 ml of CD₃OD/D₂O (60:40, v/v), containing 200 mM
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33 K₂HPO₄/KH₂PO₄, 2.5 mM TMSP and 2.5 mM EDTA; Fraction C was dissolved in 1 ml
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35 of D₂O containing 400 mM K₂HPO₄/KH₂PO₄, 2.5 mM TMSP and 2.5 mM EDTA;
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37 Fraction B and D were dissolved in 1 ml of CDCl₃ containing 0.03% of
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39 tetramethylsilane (TMS) as internal standard. From all samples 0.600 (± 0.010) ml of
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41 sample in high quality NMR sample tubes (5.0 mm, 535-PP-7, Wilmad, Buena, USA)
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43 were used to ensure optimal field homogeneity and optimal reproducibility of the
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45 magnetic field. The ¹H NMR spectra were recorded at 400.13 MHz at 300.0 (± 0.05) K
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47 on a Bruker Avance 400 narrow bore using a 5.0-mm probe. The spectrometer settings
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49 were: 2 s relaxation delay, number of scans 128 (4 dummy scans) (for Fraction C,
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51 1024), spectral width of 5000 Hz, a 60 degree pulse and 16 K data points. Prior to data
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3 analysis the raw NMR data were subjected to a squared sine bell filter (shifted $\frac{1}{2}$ pi),
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5 zero-filling to 128 K data points, Fourier Transformation and phase correction.
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8 9 *UPLC-TOF/MS analysis*

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11 The study of Fraction A of the chicken feed and of the individual ingredients was
12 carried out by diluting the NMR samples 10 times in CD₃OD/D₂O (60:40, v/v) and
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14 injecting 5 μ l of each sample on the system described below. The injection sequence
15
16 was randomized according to Vos et al. (2007). The analyses were performed on a LCT
17
18 Premier LC-TOF-MS system (Waters, Milford, MA, USA) equipped with a dual spray
19
20 electrospray source. The lock mass calibrant (leucine/enkephaline) was measured every
21
22 10 scans. The gradient was provided by an UPLC system (model Acquity, Waters) with
23
24 a 150 mm x 2.1 mm UPLC BEH-C8 with 1.7 μ m particles (Waters). The mobile phase
25
26 consisted of water, acetonitrile and formic acid (A:100/0/0.2 and B: 0/100/0.2).
27
28 Gradient elution was performed at 0.4 ml min⁻¹. The initial eluent composition, 100%
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30 A, was kept for 2 min after which the composition was changed to 70% A in 13 min.
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32 This composition was maintained for 5 min and afterwards, the eluent composition was
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34 increased till 100% B in 0.20 min and was kept this way for 20 min more. The effluent
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36 of the LC system was interfaced directly with the TOF-MS. The instrument was
37
38 operated in positive mode polarity. A stable spray was obtained with a capillary voltage
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40 of 2.8 kV, a source temperature of 120°C and desolvation temperature of 350°C. The
41
42 desolvation and cone gas flow were 500 and 50 L h⁻¹ respectively. The cone voltage
43
44 was 25 V. Spectra were collected in centroid mode from m/z 100 to 1450 with a scan
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46 time of 0.2 s. Accurate masses were obtained after lock mass correction. The mass
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48 spectrometer was operated in W mode Dynamic Range Enhancement turned on and the
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50 resolution was 10,000 (FWHM).
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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.

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3 Besides this, the aligned fingerprint data $^1\text{H-NMR}$ as well as UPLC-TOF/MS- in the
4 form of generated spreadsheets were subjected to multivariate analysis using Genemaths
5 XT (<http://www.applied-maths.com/genemaths/genemaths.htm>). An investigation of
6 differences in metabolic profiles was done for the chicken feed prepared with
7 organically and conventionally produced ingredients, as well as for the organically and
8 conventionally cultivated soybean and corn samples.
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18 **Results and Discussion**

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

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3 example for two different chicken feeds (ST and GR) with regard to the aromatic region
4
5 of the $^1\text{H-NMR}$ spectrum typical of isoflavone resonances.
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8 Complementary and analogous to the $^1\text{H-NMR}$ experiments, semi-polar
9
10 secondary metabolites in Fraction A were separated and analyzed on a C8-column in an
11
12 UPLC system coupled to a TOF/MS. The UPLC-TOF/MS raw data sets were processed
13
14 and aligned with the metAlign software package as in previous studies (Tikunov et al.,
15
16 2005; De Vos et al., 2007; Lommen, 2009; Keurentjes et al., 2006). An example of
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18 chromatograms pre-processed with MetAlign of organic GR, ST and L feed given in
19
20 additional file figure S1. The output data matrix of the exact masses, obtained after the
21
22 alignment, was subjected to multivariate analysis. The PCA plot of this fraction was
23
24 very similar to that obtained for Fraction A measured by $^1\text{H NMR}$ (see additional file
25
26 figure S2). Again, masses related to isoflavone derivatives (GR > L > ST) showed to
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28 contribute heavily to the separation in the PCA through analysis of the peak loadings.
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30 Commercial standards were used for confirmation of the identity of some of the
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32 isoflavones present in the chicken feed samples. This was done for $^1\text{H-NMR}$ as well as
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34 UPLC-TOF/MS by spiking. The main isoflavones identified were daidzin, genistin and
35
36 the acetyl and malonyl derivatives of daidzin and genistin. A study of the polar fraction
37
38 of the individual ingredients by $^1\text{H NMR}$ and UPLC-TOF/MS showed that isoflavones
39
40 arose exclusively from soybean (data not shown).
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46 In Fraction B, which contained the low molecular weight apolar components,
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48 also differences between the various types of feeds were found (see Figure 2B). These
49
50 were related to the acyl group (i.e. fatty acid) composition. It was observed that the ST
51
52 feeds had a higher proportion of saturated and mono-unsaturated acyl groups, whereas
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54 the L and GR feeds had a higher proportion of polyunsaturated groups.
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3 Among the fractions of high molecular weight components, the largest
4 differences between the types of feed were detected in the polar fraction (see Figure
5 2C). After examination of the peak loadings it could be noted that Fraction C showed
6 higher amounts of monomerized sugars in the ST feeds than in the L and GR feeds. The
7 differences in the apolar fraction were shown to be minimal and therefore not further
8 examined (see Figure 2D).
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19 *Differences between organic and conventional feeds*

20 As shown in Figure 2A (see clustering of samples in PCA), there were also differences
21 between feeds prepared with organically and conventionally produced ingredients,
22 although these differences were much smaller in comparison to the differences between
23 the types of feed (ST, GR and L) (see also Table 3 and additional file figure S3, which
24 shows the MetAlign pre-processed chromatograms of organic and conventional grower
25 feed). A complicating factor in analyzing the effects of organically and conventionally
26 produced feed in the reference study (Huber et al., 2010) was that the feed composition
27 of ST, GR and L (used in different phases of growth, Table 2) was different. These
28 differences in composition also complicated the search for feed factors that might have
29 caused the differences in effects. A way to look for consistent feed-independent
30 differences between organically and conventionally produced feeds was by regrouping
31 the ST, GR and L data in 2 groups, respectively organically and conventionally
32 produced feed. The results of this approach are given in Figure 4A and 4B for fraction
33 A analyzed by NMR and UPLC-TOF/MS respectively, as well as in Table 3 part B.
34 This table shows that there is still a significant number of peaks surviving after ANOVA
35 ($p < 0.05$). The peak loadings resulting in Figure 4A (Fraction A analyzed by ^1H NMR)
36 suggested that there might be a trend in different isoflavone contents. This trend, which
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3 was also observed in the mass loadings resulting in Figure 4B (Fraction A analyzed by
4 UPLC-TOF/MS), could be explained from the analysis of Fraction A (by ^1H NMR and
5 UPLC-TOF/MS, in 6 fold) of the organic and conventional soybean samples. This
6 analysis showed that the organic sample contained between 20 and 30% more
7 isoflavones than the conventional counterpart, as was also observed in a classical
8 targeted analysis (Huber, 2007).
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16 Apart from these isoflavone signals, a distinct mass of 441.202 Da ($\text{M}+\text{H}$)⁺ was
17 observed as a difference in the UPLC-TOF/MS data. This mass came out of the
18 multivariate analysis in Figure 4B and was a “survivor” mass peak in Table 3 part B.
19
20 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
21 the corn (data not shown). This structure could correspond to N,N'-dyferuloylputrescine
22 (DFP), a polyamine conjugate already described as an ingredient of corn. Unfortunately
23 no standard could be purchased. Therefore, in order to confirm the identity of this mass,
24 MS/MS analysis on Fraction A of the organic corn sample was performed, since the
25 organic feed and corn showed higher concentration of the mass of interest. The main
26 fragments obtained from the MS/MS analysis were the following: 177.054, 248.128 and
27 291.134 Da, which were consistent with those already described for DFP by other
28 authors (LeClere et al., 2007) and with those predicted by Mass Frontier 6.0
29 (<http://www.highchem.com/downloads/mass-frontier/>). Furthermore, the ^1H NMR
30 spectra of Fraction A of the organically and conventionally produced corn samples
31 exhibited the characteristic proton signals of DFP in the aromatic region of the spectra
32 (Miller et al., 1996). This analysis, which was also done in 6 fold, showed that the
33 organic sample contained more than two fold higher levels of DFP.
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54 This finding could be of interest because DFP has been found to have antibiotic
55 activities against certain pests in corn (Arnason et al., 1992), to prevent aflatoxin
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3 production by *Aspergillus flavus* (Mellon and Moreau, 2004) and also to possess
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5 antimelanogenic, antioxidant and radical scavenger activities (Choi et al., 2007).
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8 9 ***Relation between feed composition and observed health effects***

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

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47 This paper has shown that the chemical fingerprinting of the ingredients and chicken
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49 feed is a powerful tool for detecting potential differences between samples, not only of
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51 different composition (ST, L & GR) but also of different origin (in this case organic vs
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53 conventional). The extraction method, together with the analytical techniques used in
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55 this study, provide very reproducible and reliable results. It has also been shown that the
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57 fractions containing low molecular weight metabolites (fractions A and B) provide the
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3 most valuable information. Therefore, the analysis of these fractions, either by ^1H NMR
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5 or by UPLC-MS or both, could be used as a straightforward method to profile feeds .
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8 One of the biggest advantages of this metabolomics approach, compared to classical
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10 analysis, is that within this approach, only when differences are found, there is a need
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12 for identification of the responsible compounds. Furthermore, using an approach as the
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14 one described, a wide range of both known and potentially unknown metabolites can be
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16 covered. The untargeted metabolomics approach described in this paper has allowed us
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18 to find differences in the composition of the various chicken feeds prepared from
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20 organic and conventionally produced ingredients which were not detected in the broad
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22 classical analysis. The observed differences were due to the higher content of a
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24 compound with mass 441.202, identified as DPF, in the organic feed. In addition higher
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26 levels of isoflavones were detected, as also observed in the targeted analysis.
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30 Even though the feeds were found to be different, this study could not answer the
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32 question whether the differences were typical for the organic and conventional
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34 production systems. Although efforts were made to include samples from controlled
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36 trials, this was not always possible. Therefore, it cannot be excluded that some of the
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38 differences observed were due to varietal and/or production differences, not related to
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40 organic or conventional practices. For this question to be answered many more products
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42 from each production type should be analysed to have an idea what the average situation
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44 is in practice. In this respect, application of untargeted, broad analytical techniques may
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46 allow a better examination of differences between products from different production
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48 types and hence allow a better selection of representative samples used for animal
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50 studies.
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53 54 55 56 **Acknowledgements** 57 58 59 60

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2
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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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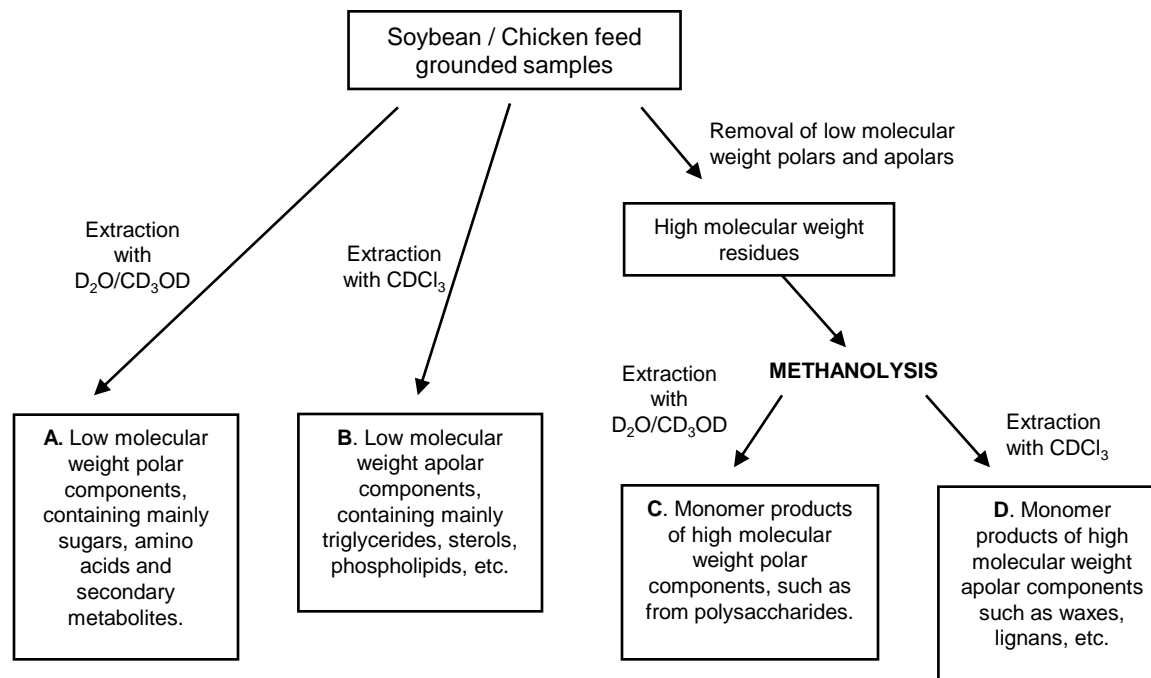


Figure 1

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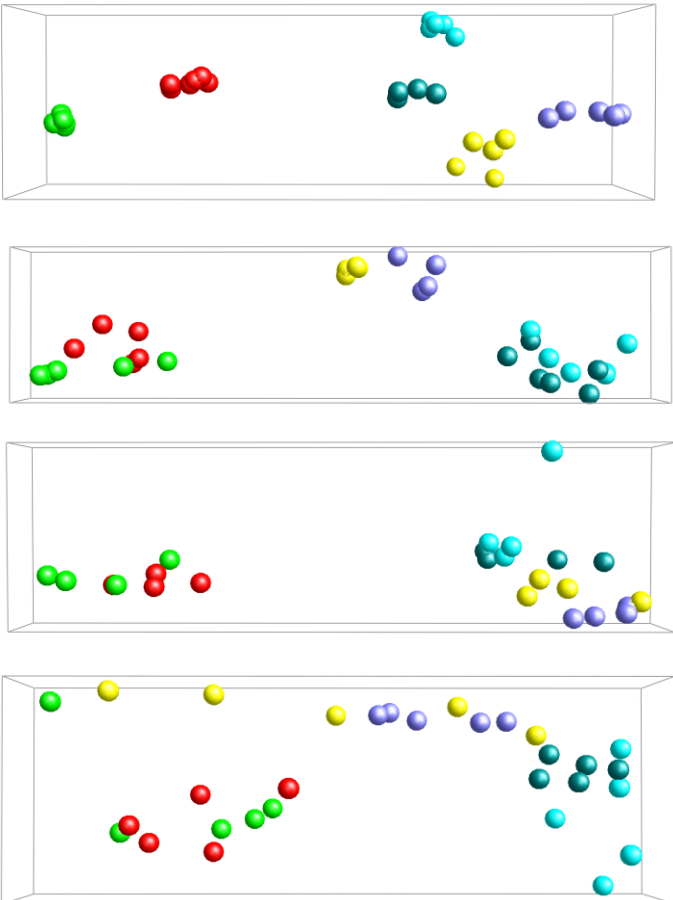


Figure 2

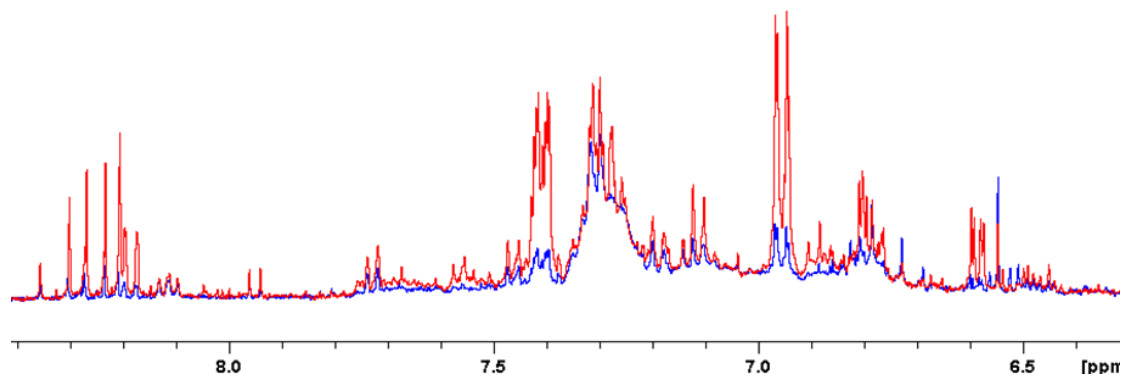


Figure 3

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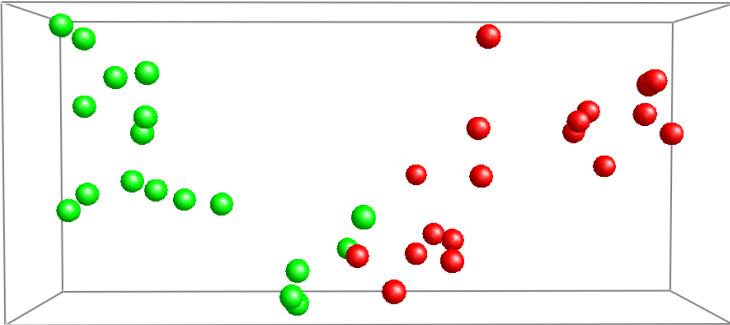
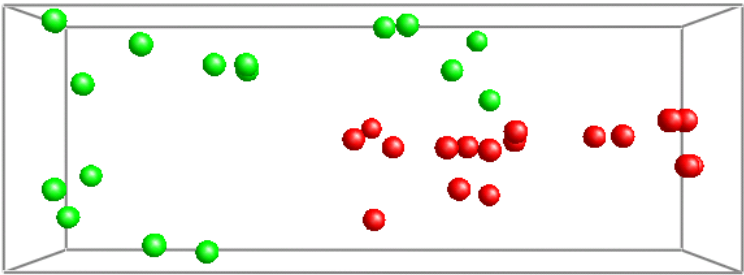


Figure 4

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

| Ingredients | Starter feed | Grower feed | Layer feed |
|---------------------|--------------|-------------|-------------|
| | (STo & STc) | (GFo & GFc) | (LFo & LFc) |
| | % | % | % |
| 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) |

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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**

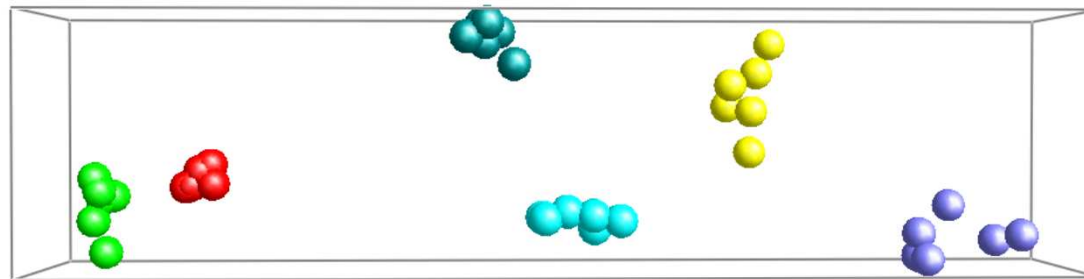
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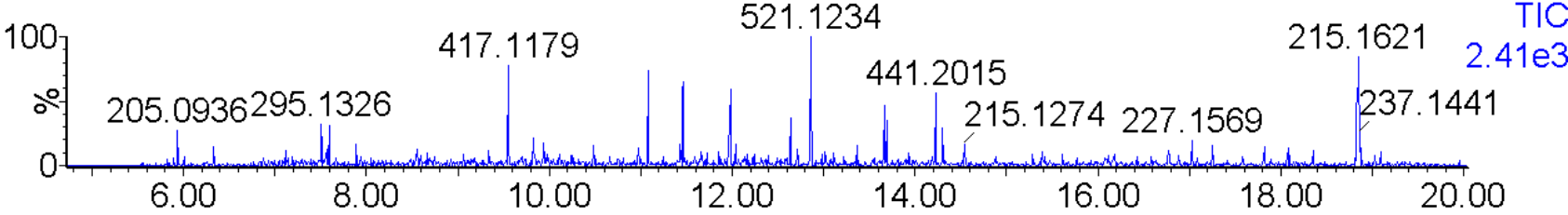
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SUPPLEMENTARY DATA

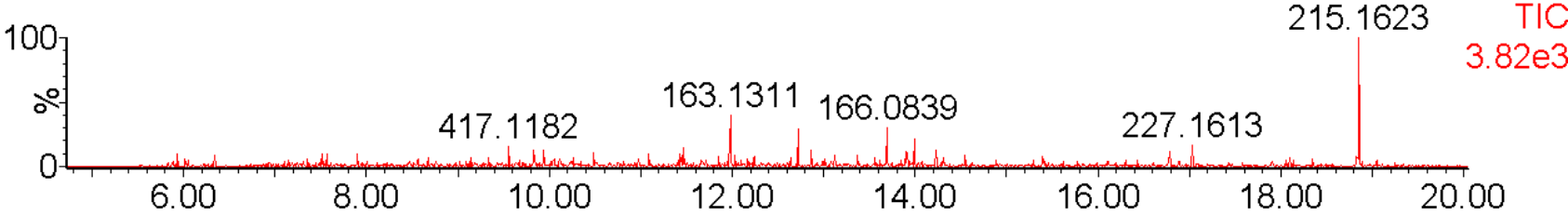


S1. PCA plot (after ANOVA $p < 0.05$) of Fraction A of chicken feed analyzed by UPLC-TOF/MS and pre-processed and aligned using MetAlign. Spheres with the same color are technical replicates of the same sample. STo: green; STc: red; GRo: lila; GRc: yellow; Lo: light blue; Lc: dark blue.

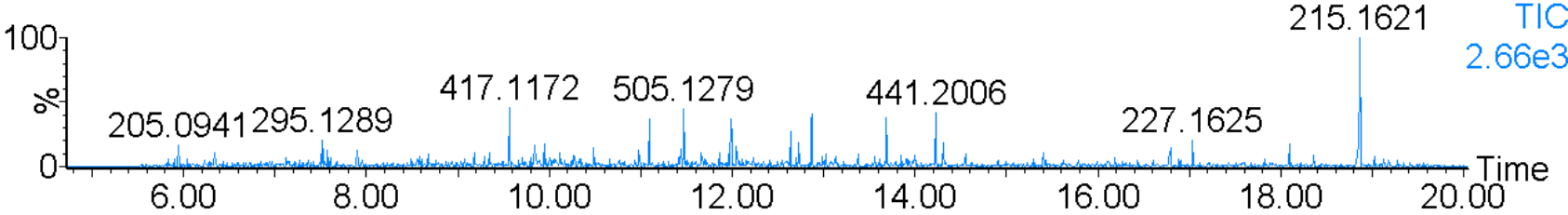
GR organic



ST organic

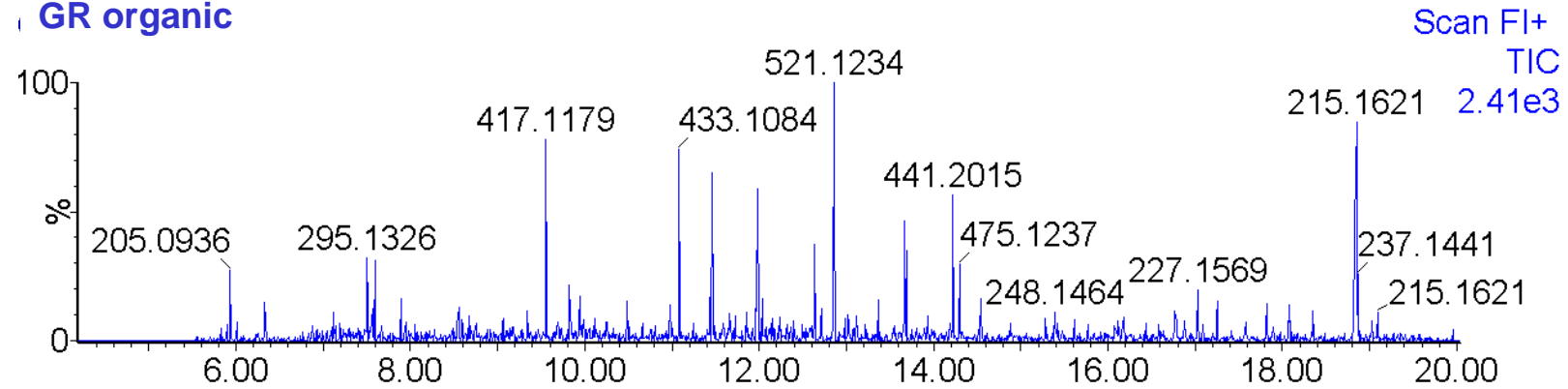


L organic

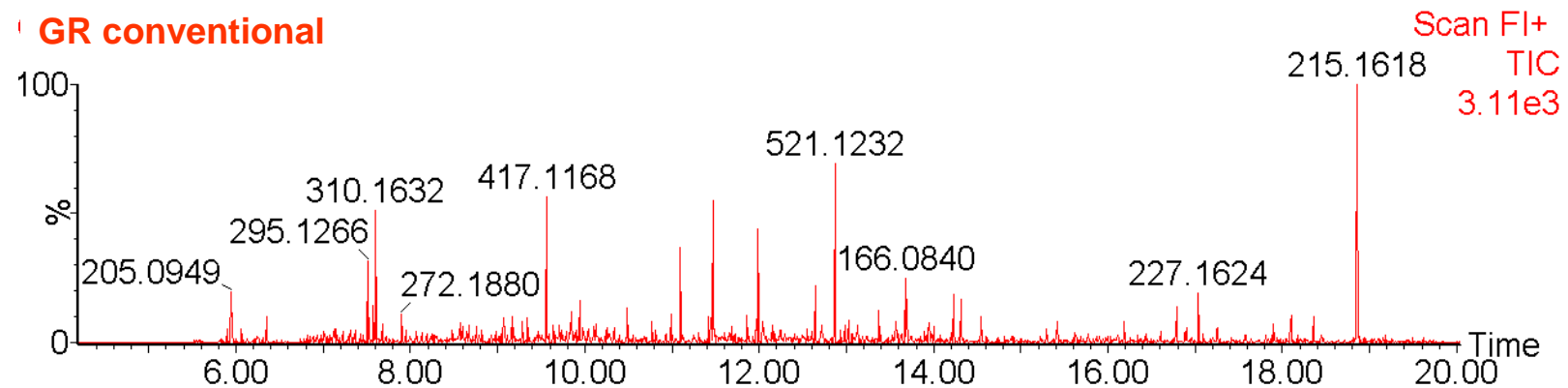


S2. Chromatograms of the organic grower (GR), started (ST) and layer (L) feeds pre-processed using MetAlign (Lommen, 2009)

GR organic



GR conventional



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