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The Use of Metabonomics to Uncover Differences between the Small Molecule Profiles of Eggs from Cage and Barn Housing Systems

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Abstract

Metabonomic techniques have been used to observe differences in the small molecule profiles of chicken eggs, to work towards the detection, and thus prevention, of fraud regarding the misrepresentation of egg farming systems. High Performance Liquid Chromatography-Quadrupole-Time-of-Flight-Mass Spectrometry (HPLC-Q-ToF-MS) was used to obtain profiles of the small molecules found in the yolks of eggs that were laid by hens in enriched cage systems, and in barn systems. Statistical analysis of these small molecule profiles, including the use of *XCMS Online* and multivariate statistics, was able to uncover differences between the yolks of cage and barn eggs. Several unidentified compounds were found to be present in significantly different abundances between cage and barn egg yolks and one of these compounds was tentatively identified, through the use of METLIN, as 1,2-dipalmitoyl-glycero-3-phosphocholine.

Keywords

Fraud, Eggs, Metabonomics, Chromatography, Mass Spectrometry

1. Introduction

Food fraud is the intentional deception of consumers regarding food products for purposes of financial gain (Spink & Moyer, 2011; Spink, Ortega, Chen, & Wu, 2017; Tähkäpää, Maijala, Korkeala, & Nevas, 2015), and it has been estimated that globally, food fraud costs approximately $US49billion a year (NSF, 2018). Misrepresentation is one of the main categories of food fraud; others being the adulteration and substitution of products (Spink et al., 2017; Tähkäpää et al., 2015). Food misrepresentation is the false advertisement or incorrect labelling of a food product (Spink et al., 2017), describing it to the consumer as something that it is not. An example of this would be labelling a box of

eggs as barn eggs, when they are in fact cage eggs, as the barn eggs could be sold at a higher price, resulting in an increased revenue.

Although food fraud is not a new problem, the globalization of food supply chains over more recent years has resulted in food fraud having a greater and more widespread impact (Manning & Soon, 2014; Spink & Moyer, 2011; Spink et al., 2017). As consumer awareness of food fraud is increasing (Hong et al., 2017; Spink & Moyer, 2011), particularly following the horsemeat scandal throughout Europe in 2013 (Fiorino et al., 2018; Spink et al., 2017), the trust that consumers have in Food Business Operators (FBOs), and their produce, is decreasing (Spink et al., 2017; Tähkäpää et al., 2015). This can result in a lack of sales, causing negative economic implications not only for other FBOs, but also for the government due to a loss of value added tax from sales (Tähkäpää et al., 2015). Economic issues can also arise from product recalls and authenticity testing of products following the discovery of fraudulence (Spink et al., 2017).

Metabonomics is the in-depth profiling of small molecules, typically below 1000 *m/z*, in organic tissues and biofluids, in order to observe changes in the small molecule profiles due to endogenous and exogenous factors (Tang & Wang, 2006; Wilson et al., 2005). It is a relatively modern technique, increasing in popularity over recent years, particularly in disease diagnostics (Rainville, Theodoridis, Plumb, & Wilson, 2014) as well as food authentication studies (Cubero-Leon, Peñalver, & Maquet, 2014). Non-targeted metabonomic studies aim to include as many small molecules as possible in the analysis. They are particularly useful when the differences between sample sets are very subtle, or when it is unknown which compounds or classes of compounds may be affected by the factor that is under investigation (Gika, Theodoridis, Plumb, & Wilson, 2014).

Legislation regarding food fraud and consumer protection exists in various forms. In the United Kingdom, Section 15 of the Food Safety Act of 1990 (“United Kingdom Food Safety Act,” 1990) bans falsely describing or presenting food, including any labels or advertisements that may mislead consumers. The Federal Food, Drug and Cosmetic Act (“Federal Food, Drug, and Cosmetic Act, 21 U.S.C. 9,” n.d.) in the United States of America prohibits the adulteration and alteration of food products, as well as the mislabelling of produce. For members of the European Union (EU), EU Regulation 178/2002 (“Regulation (EC) No 178/2002,” 2002) states that food law aims to protect consumers’ interests and allow them to make informed choices regarding the produce that they consume. It also states that food law aims to prevent “(a) fraudulent and deceptive practices; (b) the adulteration of food; and (c) any other practices which may mislead the consumer.” In addition to this, Directive 2000/13/EC (“Directive 2000/13/EC,” 2000) states that labelling of foodstuffs must not mislead the consumer as to the characteristics of the product, particularly its method of manufacture or production.

The EU Council Directive 1999/74 (“Council Directive 1999/74/EC,” 1999) outlines the minimum standards of living conditions for laying hens in enriched cage and barn (alternative) housing systems. One clear difference is that birds in enriched cages must have at least 750cm^2 area of space, of which 600cm^2 must be usable, whereas birds in barn housing systems have a maximum stocking density of nine laying hens per m^2 of usable area. Commission Regulation EC 589/2008 (“Commission Regulation (EC) No 589/2008,” 2008) states that eggs must be clearly labelled with the farming method. This, in conjunction with other food fraud legislation, makes it clear that eggs must be labelled with their farming system, where the farming system matches the guidelines in EU

Council Directive 1999/74. Clearly, any instances of eggs or packaging that are mislabelled with regard to their farming method are cases of food misrepresentation.

As there is no way for the consumer to determine the farming method behind the production of eggs that they purchase, other than trusting the label, it would be relatively straightforward for eggs or their packaging to be mislabelled. Mislabelling with false farming methods may make eggs more desirable and thus, more expensive, increasing the generated revenue on the products. There have been several cases of this type of fraud reported in the JRC Food Fraud Monthly Reports by the EU ("Monthly summary of articles on Food Fraud and Adulteration - European Commission," n.d.). However, the discovery of the fraud is usually due to inconsistencies in the paperwork, CCTV footage, or visual inspections of the farm, rather than through any scientific testing. Employing a robust scientific method to confirm the farming method of eggs would help to reliably detect cases of fraud, and deter those that may be tempted to mislabel eggs, preventing further occurrences.

Previous research has been carried out into the identification of egg housing system, including physical methods such as the observation of white, fluorescent, double parallel lines on the eggs to determine whether they were laid in a cage (Gregory, Gepp, & Babidge, 2005), the measurement of air cell height and Haugh unit, and eggshell thickness (Hidalgo, Rossi, Clerici, & Ratti, 2008). A study by van Ruth et al. developed a carotenoid fingerprint profile, which is capable of classifying eggs as either organic, or non-organic, but cannot categorize the origin of the egg any further (Van Ruth et al., 2011). A further study by van Ruth et al. developed a model for the identification of organic eggs using a fatty acid fingerprint however, again it cannot categorize the eggs any further (Tres, O'Neill, & van Ruth, 2011). The fatty acid composition of eggs was also studied by Samman et al., who found significant differences in the concentrations of fatty acids, and the percentage of saturated fats between organic and conventional eggs, and between cage eggs and barn and free-range eggs (Samman et al., 2009). Another study investigating fatty acids, by Torde et al., which used stable isotope labeling to relatively quantify fatty acids in egg yolk, found that omega-3 fatty acids are of a higher concentration in cage-free eggs compared to cage eggs (Torde, Therrien, Shortreed, Smith, & Lamos, 2013). A significant difference in cholesterol content was observed between battery cage, enriched cage, and free-range eggs by Radu-Rusu et al. (Radu-Rusu & Usturoi, 2014), and between organic and cage eggs by Minelli et al. (Minelli, Sirri, Folegatti, Meluzzi, & Franchini, 2007), however contradictory results were obtained by Anderson (Anderson, 2013).

There have been several studies examining the minerals and trace elements present in eggs from different housing systems. Radu-Rusu et al. (Radu-Rusu & Usturoi, 2014) found that the crude ash concentration of egg albumen is significantly different between eggs of different housing systems, and both Küçükyilmaz et al. (Küçükyilmaz et al., 2012) and Giannenas et al. (Giannenas, Nisianakis, Gavriil, Kontopidis, & Kyriazakis, 2009), using Inductively Coupled Plasma, coupled to either Optical Emission Spectroscopy or Mass Spectrometry, found significant differences in the concentrations of some elements between eggs from different housing systems. An investigation into whether the ratios of stable isotopes, such as ^{15}N and ^{13}C, differ between eggs from different housing systems was carried out by Rogers, who found that ^{15}N levels are higher in organic and free-range eggs, compared to cage and barn eggs (Rogers, 2009).

However, none of these studies utilise a non-targeted metabonomic approach to uncovering differences between eggs from different housing systems. A non-targeted

approach enables a wide range of compounds to be studied, increasing the potential of discovering a compound, or class of compounds, of interest, that could help discriminate between eggs originating from different housing systems.

This paper aims to show how a non-targeted metabonomic technique, using HPLC-Q-ToF-MS, can be used to uncover differences in the small molecule profiles of egg yolks from enriched cage and barn eggs. It will then show how this type of approach can lead to a tentative identification of a compound that has potential to be used as a biomarker of egg farming method in the future.

The general workflow that has been carried out in this research, from metabolite extraction to statistical analysis and compound identification, has been developed by the authors and used in a previous study (Johnson, Sidwick, Pirgozliev, Edge, & Thompson, 2018).

2. Materials and Methods

2.1. Materials

Methanol (HPLC, isocratic grade) and dichloromethane (stabilised with 0.002% 2-methyl-2-butene) were purchased from VWR (Radnor, PA). Ultra-pure water (18.2 MΩ/cm) was purified using a Milli-Q system from Elga (High Wycombe, U.K.). Formic acid (90%, laboratory reagent grade) and ammonium acetate, were purchased from Fisher Scientific (Loughborough, U.K). ESI-L low-concentration tuning mix and API-TOF reference-mass solution were purchased from Agilent Technologies (Santa Clara, CA).

2.2. Sample Collection

Fresh eggs, six from caged hens and six from barn hens, were collected from Oaklands Farm Eggs Ltd., Shrewsbury, U.K. Laying hens from both housing systems were of the Novogen breed, all fed the same diet, and were 50 weeks old at the point of lay. Eggs were stored at 23°C overnight and metabolite extraction was carried out the following day.

2.3. Metabolite Extraction

The metabolite extraction method was carried out as in previous work (Johnson et al., 2018). As egg yolk is more compound-rich than the albumen, which consists mainly of water (Li-Chan & Kim, 2008), analysis was focussed on the yolk. Egg yolk was separated from albumen using a stainless steel egg yolk separator, and approximately 50mg of each sample was weighed out into 1.5mL Eppendorf tubes. An organic extraction solvent mixture (3 dichloromethane: 1 methanol) was added (1mL per 50mg) and the samples were vortexed, then centrifuged for 20 minutes at 16,100rcf. From this, 0.75mL of supernatant was removed from each tube and allowed to evaporate overnight under ambient conditions. The dried extracts were then re-suspended in 0.75mL methanol, vortexed, and stored at -80°C prior to analysis.

2.4. Quality Control

Equal aliquots of all sample extracts were pooled together to create a Quality Control (QC) sample in line with published guidance (Sangster, Major, Plumb, Wilson, & Wilson, 2006). Ten injections of this QC sample were injected at the start of the analytical run in order to condition the column ready for analysis, and then one injection of QC sample was analyzed between every two samples throughout the analytical run in order to monitor instrumental

drift. The injection sequence of samples was randomized to ensure that any instrumental drift that affected the analysis, did not impact the final results.

2.5. Chromatographic Parameters

Separation was carried out at a flow rate of 0.3mL/min using a Thermo Scientific Accucore RP-MS column (100mm x 2.1mm, 2.6µm particle size) kept at 40°C, on an Agilent Technologies 1260 Infinity Binary HPLC system. The injection volume of sample was 5µL and a needle wash of methanol was carried out during each injection. Solvent (A) was 0.1% formic acid and 5mM ammonium acetate, and solvent (B) was methanol with 0.1% formic acid and 5mM ammonium acetate. The solvent gradient increased from 75% (B) to 81% (B) in the first 20 minutes, then up to 90% (B) in 1 minute, where it was held for 10 minutes, before it was increased to 100% (B) in 30 minutes, held for 20 minutes, then returned to starting conditions over 4 minutes. A post time of 5 minutes was included to allow the instrument to equilibrate prior to the next sample injection.

2.6. Mass Spectrometry (MS) Parameters

Samples were analyzed in positive ionization mode with a mass range of 100-1000 *m/z* using an Agilent Technologies 6530 Accurate-Mass Quadrupole-Time-of-Flight mass spectrometer with an electrospray ion source. This instrument has a mass resolution of >20,000 FWHM. The drying gas had a temperature of 300°C and a flow rate of 8 mL/min. Capillary, fragmentor and skimmer voltages were 3500 V, 175 V, and 65 V respectively, and the nebulizer pressure was 35 psi. The system was calibrated prior to analysis using the ESI-L low concentration tuning mix, in order to improve mass accuracy, and the API-TOF reference mass solution was used throughout the run to maintain this accuracy. Analysis was not carried out in negative ionization mode, as previous analysis of egg yolk by the authors revealed that very few molecules in egg yolk are ionized under negative ionization, compared to positive ionization, resulting in much smaller data sets.

2.7. Statistical Analysis

XCMS Online was used to pre-process data; feature detection, retention time correction, chromatogram alignment (Benton et al., 2015), and to produce a table consisting of molecular features (represented by their *m/z* values and retention times) and the peak areas for these features in each sample. Statistical analysis was then carried out based on this table, using *Microsoft Excel.*

Relative standard deviation percentages (RSD%) were calculated for each molecular feature, based on the peak intensities in the chromatograms of the QC samples that were analyzed throughout the analytical run. Any features with a RSD% greater than 30% were removed prior to further analysis, in accordance with recommended guidelines (Theodoridis, Gika, Want, & Wilson, 2012). Principal Component Analysis (PCA) was carried out, using an *Excel* Multivariate Analysis add-in, on all remaining compounds with a RSD% lower than 30%. The data was standardised, and the PCA included six principal components. Scores plots were then produced. The loadings from principal component 3 (PC3), the principal component which showed the greatest amount of variation between samples on the scores plots due to housing system, were used to rank the compounds from the highest to the lowest. The top 100 compounds, responsible for the most variation between samples due to housing system, were taken and any duplicates, isotopes, and adducts were removed. F-tests were carried out on the remaining compounds to test the equality of variances, and corresponding t-tests were then carried out, depending on the results of the F-tests.

Agilent Technologies' *MassHunter Qualitative Analysis* software was used to study the raw data and confirm the statistical significance of compounds that were found to show a significant difference from the t-tests. Extracted Ion Chromatograms (EICs) were produced for each statistically significant compound using this software, and the F-tests and t-tests were again carried out using the peak areas from the integrated EICs. This was done to ensure that all results were robust.

2.8. Identification

For those compounds that were still found to be statistically significant when confirmed using the raw data, attempts were made to identify them. EICs were produced for each of these compounds using *MassHunter Qualitative Analysis* and the software predicted potential molecular formulas for the compounds based on their mass spectra (Sana, Roark, Li, Waddell, & Fischer, 2008). Each predicted formula came with a likelihood score; those with a score of 95 or above were then searched against METLIN (Smith et al., 2005) (https://metlin.scripps.edu), a metabolite database, to see if there were any potential metabolite matches. For those formulas that did produce matches on METLIN, the mass spectra for the compounds from the analysis were compared with the mass spectra provided by METLIN, to see if they could be tentatively identified.

3. Results and Discussion

3.1. Metabolite Profiling

There is a subtle, but visible, difference in metabolite profile between cage and barn eggs, as can be seen in Figure 1. Although all peaks are present in both profiles, most chromatographic peaks appear to be of a slightly higher intensity for barn eggs compared to cage eggs.

3.2. Multivariate Statistics

Scores plots were produced following PCA and, as can be seen in Figure 2, they show that the QC samples are clustered tightly together, meaning that there was little to no instrumental drift throughout the analysis. This proves that the differences between samples and sample sets, as displayed on the scores plot, are due to true biological differences, rather than an instrumental effect. Although there is quite wide variation between samples within sample sets, a clear separation can be seen between barn and cage eggs. This separation is mostly across PC3, hence why the loadings of this principal component were then used to rank the compounds from highest to lowest, in order to choose the top 100 compounds responsible for the greatest amount of variation between the yolks of eggs from different housing systems. The variation within barn and cage egg sample sets can be explained by the fact that the eggs, even within one housing system, were laid by different birds, therefore the metabolite profiles will be different between the eggs due to differences in the birds themselves. This explains why scores plots using PC1 were not used to show the difference between barn and cage eggs, or to rank the compounds based on loadings; PC1 describes the highest amount of variation between samples (97.101%), however this variation is between random eggs, due to being laid by different birds, not between eggs produced by different farming methods. The scores plot in Figure 2 shows good, clear separation between barn and cage eggs however, the difference between the two sample sets is actually very subtle, with PC2 describing only 1.075% of the variation between samples, and PC3 describing only 0.764%. Although PC1 describes the largest amount of

variation between eggs, PC3 describes the variation that is due to differences between housing systems.

3.3. Potential Biomarkers

Following the removal of duplicates, adducts, and isotopes from the top 100 compounds based on PC3 loadings (as most separation was observed across PC3 on the scores plot in Figure 2), only 59 compounds remained. Of these 59 compounds, 29 were found to be significantly different in abundance between barn and cage eggs, with $P<0.05$, and only 23 compounds were still found to be statistically significant after analyzing the raw data. These final 23 compounds, identified by their *m/z* and retention time, can be seen in Table 1 in the appendix, along with their RSD% and the P-values resulting from t-tests. The abundances of all of these 23 compounds were found to be higher in barn eggs than in cage eggs, which supports what was observed in Figure 1, with most peaks showing higher intensity in the barn egg chromatogram compared to the cage egg chromatogram.

3.4. Tentative Compound Identification

The feature identification workflow was then applied to the 23 compounds in Table 1. Of these 23 compounds, 12 produced potential metabolite matches through METLIN, as can be seen in Table 2. The mass spectra of these 12 compounds produced by the analysis in this study were compared against the mass spectra for the potential matches provided by METLIN, and just one of these compounds resulted in a match; the compound with *m/z* 734.5699 and potential formula $C_{40}H_{80}NO_8P$ was tentatively identified as the phospholipid dipalmitoyl-glycero-3-phosphatidylcholine. Figure 3 shows the comparison of the mass spectra for this compound. If the identification of this compound was to be confirmed as dipalmitoyl-glycero-3-phosphatidylcholine using a chemical standard then, following further work, there would be potential for this compound to be used as a biomarker of egg housing system.

The remaining 11 compounds that produced potential metabolite matches through METLIN were all matched with various lipids; 3 potential diglycerides, 4 potential triglycerides, and 4 potential phospholipids. As all of these compounds were present in a higher abundance in barn eggs compared to cage eggs, this indicates that there is a higher lipid content in eggs from barn production systems compared to eggs from cage systems. These results are similar to those discovered by Pignoli et al., who found that there was a higher lipid content in free range eggs compared to cage eggs (Pignoli et al., 2009). In addition to the potential use of dipalmitoyl-glycero-3-phosphatidylcholine as a biomarker of egg housing system, it may be that a lipid profile, consisting of various lipids, could also be used as a method of distinguishing between eggs from different housing systems.

4. Conclusion

This research has shown that a metabonomic study is a viable approach to uncovering differences between eggs produced by different farming methods. It has shown that there is potential for the lipid profiles of egg yolks to be used as a method to distinguish between eggs from different housing systems, and has tentatively identified a compound that, with confirmation of its identity and further work, could have potential as a biomarker of egg housing system between cage and barn eggs.

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6. References

Anderson, K. E. (2013). Comparison of fatty acid, cholesterol, vitamin A and E composition, and trans fats in eggs from brown and white egg strains that were molted or nonmolted. *Poult Sci*, *92*(12), 3259–3265. https://doi.org/10.3382/ps.2013-03377

Benton, H. P., Ivanisevic, J., Mahieu, N. G., Kurczy, M. E., Johnson, C. H., Franco, L., … Siuzdak, G. (2015). Autonomous Metabolomics for Rapid Metabolite Identification in Global Profiling. *Analytical Chemistry*, *87*, 884–891. https://doi.org/10.1021/ac5025649

Commission Regulation (EC) No 589/2008. (2008). *Official Journal of the European Union*, (L 163), 6–23.

Council Directive 1999/74/EC. (1999). *Official Journal of the European Communities*, (L 203), 53–57.

Cubero-Leon, E., Peñalver, R., & Maquet, A. (2014). Review on metabolomics for food authentication. *Food Research International*, *60*, 95–107. https://doi.org/10.1016/j.foodres.2013.11.041

Directive 2000/13/EC. (2000). *Official Journal of the European Communities*, *L 109*, 29–42.

Federal Food, Drug, and Cosmetic Act, 21 U.S.C. 9. (n.d.).

Fiorino, G. M., Garino, C., Arlorio, M., Logrieco, A. F., Losito, I., & Monaci, L. (2018). Overview on Untargeted Methods to Combat Food Frauds: A Focus on Fishery Products. *Journal of Food Quality*.

Giannenas, I., Nisianakis, P., Gavriil, A., Kontopidis, G., & Kyriazakis, I. (2009). Trace mineral content of conventional, organic and courtyard eggs analysed by inductively coupled plasma mass spectrometry (ICP-MS). *Food Chemistry*, *114*(2), 706–711. https://doi.org/10.1016/j.foodchem.2008.09.079

Gika, H. G., Theodoridis, G. A., Plumb, R. S., & Wilson, I. D. (2014). Current practice of liquid chromatography-mass spectrometry in metabolomics and metabonomics. *Journal of Pharmaceutical and Biomedical Analysis*, *87*, 12–25. https://doi.org/10.1016/j.jpba.2013.06.032

Gregory, N. G., Gepp, M. J., & Babidge, P. J. (2005). Method for checking label accuracy in barn and free range eggs. *Journal of the Science of Food and Agriculture*, *85*, 1421–1426.

Hidalgo, A., Rossi, M., Clerici, F., & Ratti, S. (2008). A market study on the quality characteristics of eggs from different housing systems. *Food Chemistry*, *106*(3), 1031–1038. https://doi.org/10.1016/j.foodchem.2007.07.019

Hong, E., Lee, Y., Jeong, Y., Park, M., Kim, B. H., & Sook, H. (2017). Modern analytical methods for the detection of food fraud and adulteration by food category. *Journal of the Science of Food and Agriculture*, *97*, 3877–3896. https://doi.org/10.1002/jsfa.8364

Johnson, A. E., Sidwick, K. L., Pirgozliev, V. R., Edge, A., & Thompson, D. F. (2018). Metabonomic Profiling of Chicken Eggs during Storage Using High-Performance Liquid Chromatography–Quadrupole Time-of-Flight Mass Spectrometry. *Analytical Chemistry*, *90*, 7489–7494. https://doi.org/10.1021/acs.analchem.8b01031

Küçükyilmaz, K., Bozkurt, M., Yamaner, C., Inar, M., Atli, A. U., & Konak, R. (2012). Effect of an organic and conventional rearing system on the mineral content of hen eggs. *Food Chemistry*, *132*(2), 989–992. https://doi.org/10.1016/j.foodchem.2011.11.084

Li-Chan, E. C. Y., & Kim, H. O. (2008). Structure and Chemical Compositions of Eggs. In M. Y (Ed.), *Egg Bioscience and Biotechnology* (pp. 1–95). Hoboken, NJ USA: John Wiley & Sons, Inc.

Manning, L., & Soon, J. M. (2014). Developing systems to control food adulteration. *Food Policy*, *49*, 23–32. https://doi.org/10.1016/j.foodpol.2014.06.005

Minelli, G., Sirri, F., Folegatti, E., Meluzzi, A., & Franchini, A. (2007). Egg quality traits of laying hens reared in organic and conventional systems. *Italian Journal of Animal Science*, *6*(SUPPL. 1), 728–730. https://doi.org/10.4081/ijas.2007.1s.728

Monthly summary of articles on Food Fraud and Adulteration - European Commission. (n.d.). Retrieved from https://ec.europa.eu/jrc/en/food-fraud-and-quality/monthly-summary-articles

NSF. (2018). The Public Health and Safety Organization. Retrieved February 8, 2018, from http://www.nsf.org/services/by-industry/food-safety-quality/food-fraud

Pignoli, G., Rodriguez-Estrada, M. T., Mandrioli, M., Barbanti, L., Rizzi, L., & Lercker, G. (2009). Effects of Different Rearing and Feeding Systems on Lipid Oxidation and Antioxidant Capacity of Freeze-Dried Egg Yolks. *Journal of Agricultural and Food Chemistry*, *57*, 11517–11527. https://doi.org/10.1021/jf901537r

Radu-Rusu, R., & Usturoi, M. (2014). Chemical features, cholesterol and energy content of table hen eggs from conventional and alternative farming systems. *South African Journal …*, *44*(1). Retrieved from http://www.scielo.org.za/scielo.php?pid=S0375-15892014000100005&script=sci_arttext&tlng=pt

Rainville, P. D., Theodoridis, G., Plumb, R. S., & Wilson, I. D. (2014). Advances in liquid chromatography coupled to mass spectrometry for metabolic phenotyping. *Trends in Analytical Chemistry*, *61*, 181–191.

Regulation (EC) No 178/2002. (2002). *Official Journal of the European Communities*. https://doi.org/2004R0726 - v.7 of 05.06.2013

Rogers, K. M. (2009). Stable isotopes as a tool to differentiate eggs laid by caged, barn, free range, and organic hens. *Journal of Agricultural and Food Chemistry*, *57*(10), 4236–4242. https://doi.org/10.1021/jf803760s

Samman, S., Kung, F. P., Carter, L. M., Foster, M. J., Ahmad, Z. I., Phuyal, J. L., & Petocz, P. (2009). Fatty acid composition of certified organic, conventional and omega-3 eggs. *Food Chemistry*, *116*(4), 911–914. https://doi.org/10.1016/j.foodchem.2009.03.046

Sana, T. R., Roark, J. C., Li, X., Waddell, K., & Fischer, S. M. (2008). Molecular Formula and METLIN Personal Metabolite Database Matching Applied to the Identification of Compounds Generated by LC/TOF-MS. *Journal of Biomolecular Techniques*, *19*, 258–266.

Sangster, T., Major, H., Plumb, R., Wilson, A. J., & Wilson, I. D. (2006). A pragmatic and readily implemented quality control strategy for HPLC-MS and GC-MS-based metabonomic analysis. *The Analyst*, *131*(10), 1075–1078.

https://doi.org/10.1039/b604498k

Smith, C. A., Maille, G. O., Want, E. J., Qin, C., Trauger, S. A., Brandon, T. R., … Siuzdak, G. (2005). METLIN: A Metabolite Mass Spectral Database. *Therapeutic Drug Monitoring*, *27*(6), 747–751.

Spink, J., & Moyer, D. C. (2011). Defining the Public Health Threat of Food Fraud. *Journal of Food Science*, *76*, 157–163. https://doi.org/10.1111/j.1750-3841.2011.02417.x

Spink, J., Ortega, D. L., Chen, C., & Wu, F. (2017). Food fraud prevention shifts the food risk focus to vulnerability. *Trends in Food Science & Technology*, *62*, 215–220. https://doi.org/10.1016/j.tifs.2017.02.012

Tähkäpää, S., Maijala, R., Korkeala, H., & Nevas, M. (2015). Patterns of food frauds and adulterations reported in the EU rapid alarm system for food and feed and in Finland. *Food Control*, *47*, 175–184. https://doi.org/10.1016/j.foodcont.2014.07.007

Tang, H.-R., & Wang, Y.-L. (2006). Metabonomics: a Revolution in Progress. *Progress in Biochemistry and Biophysics*, *33*, 401–417.

Theodoridis, G. A., Gika, H. G., Want, E. J., & Wilson, I. D. (2012). Liquid chromatography-mass spectrometry based global metabolite profiling: A review. *Analytica Chimica Acta*, *711*, 7–16. https://doi.org/10.1016/j.aca.2011.09.042

Torde, R. G., Therrien, A. J., Shortreed, M. R., Smith, L. M., & Lamos, S. M. (2013). Multiplexed analysis of cage and cage free chicken egg fatty acids using stable isotope labeling and mass spectrometry. *Molecules*, *18*(12), 14977–14988. https://doi.org/10.3390/molecules181214977

Tres, A., O'Neill, R., & van Ruth, S. M. (2011). Fingerprinting of fatty acid composition for the verification of the identity of organic eggs. *Lipid Technology*, *23*(2), 40–42. https://doi.org/10.1002/lite.201100084

United Kingdom Food Safety Act. (1990).

Van Ruth, S., Alewijn, M., Rogers, K., Newton-Smith, E., Tena, N., Bollen, M., & Koot, A. (2011). Authentication of organic and conventional eggs by carotenoid profiling. *Food Chemistry*, *126*(3), 1299–1305. https://doi.org/10.1016/j.foodchem.2010.11.081

Wilson, I. D., Plumb, R., Granger, J., Major, H., Williams, R., & Lenz, E. M. (2005). HPLC-MS-based methods for the study of metabonomics. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *817*(1), 67–76. https://doi.org/10.1016/j.jchromb.2004.07.045

Figure Captions

Figure 1. Overlaid Total Ion Chromatograms for barn and cage eggs based on median values of retention time and detector response.

Figure 2. PCA scores plot of PC2 vs PC3 for compounds with RSD% <30% showing separation between barn and cage eggs

Figure 3. Comparison of METLIN mass spectrum (top) of dipalmitoyl-glycero-3-phosphatidylcholine and QC sample mass spectrum (bottom) of compound 734.5699 *m/z*

Table 1

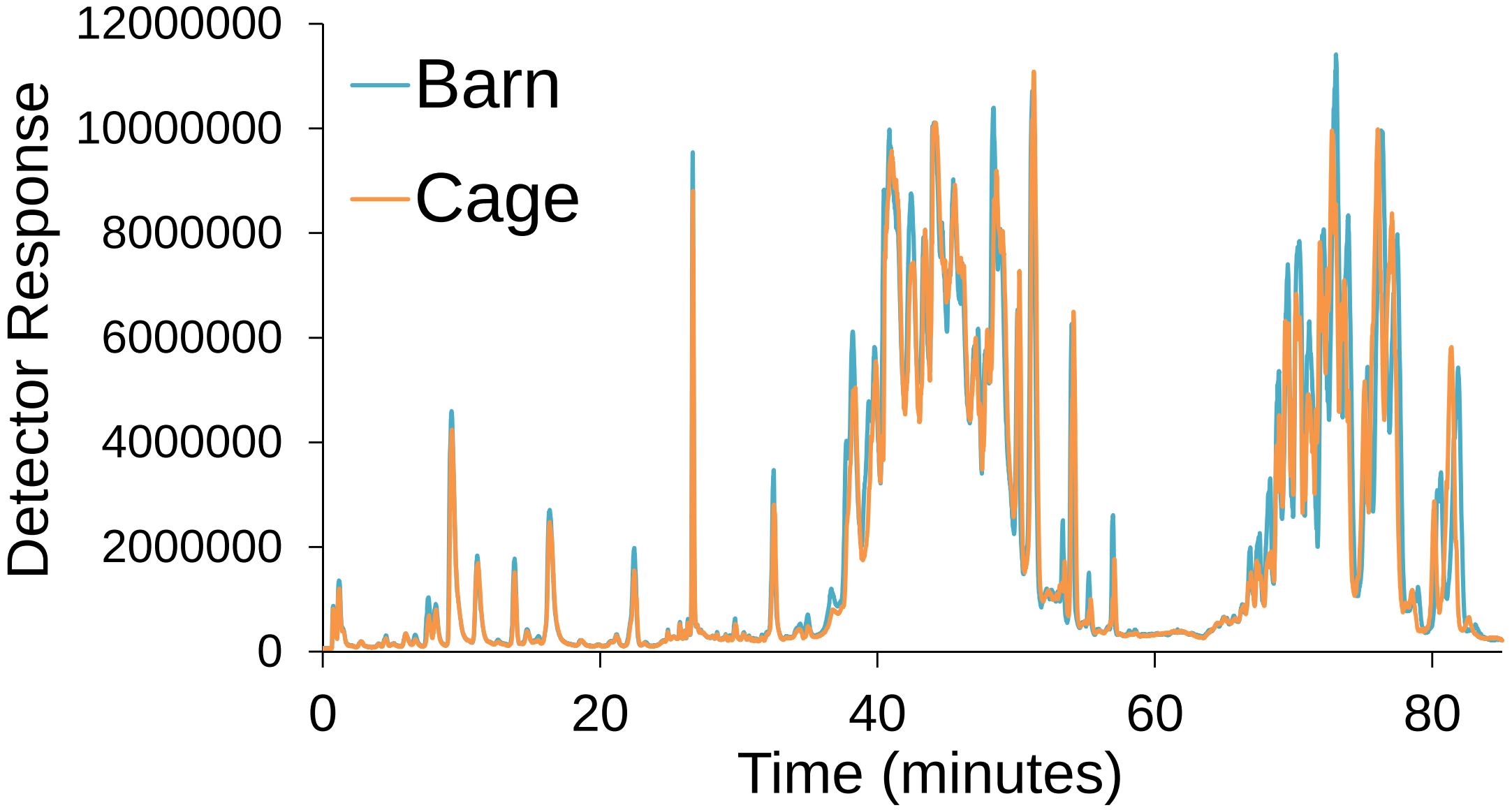
Table showing the final 23 compounds found to still be significantly different between barn and cage eggs in the raw data, and their RSD% and P-values from t-test

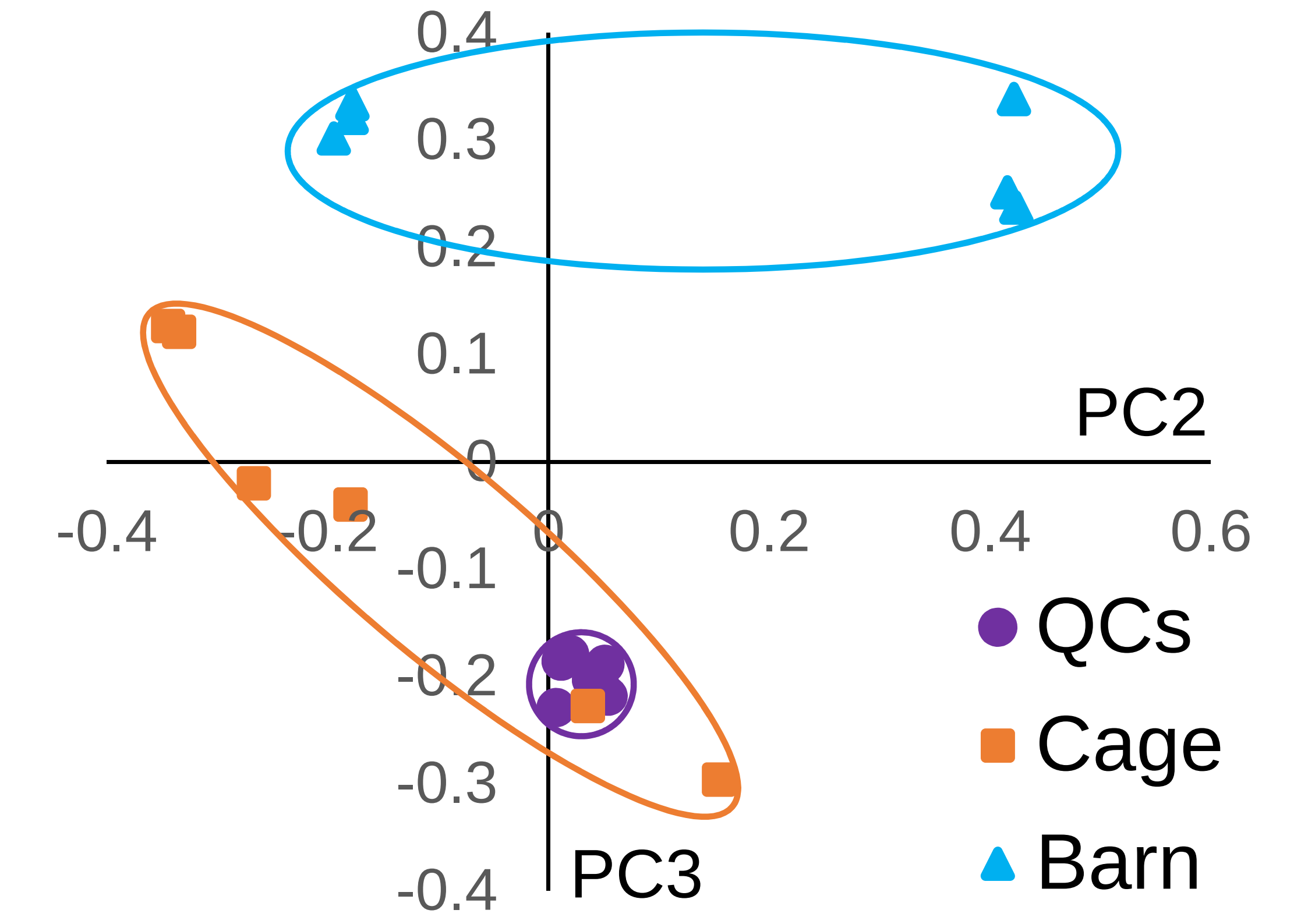
Feature *m/z* +/- *<1ppm*	Retention Time median (mins)	RSD%	T-test P-Value
520.3398	8.16	3.2	0.0041
601.5182	48.42	5.0	0.0015
608.5243	43.85	5.7	0.0467
634.5395	45.41	7.7	0.0005
636.5566	48.39	4.5	0.0004
700.5266	43.84	3.4	<0.0001
734.5699	43.39	3.1	0.0037
744.5543	46.16	1.8	0.0133
752.5215	34.90	3.1	0.0006
754.5371	37.36	6.7	0.0108
772.5851	43.83	3.4	0.0071
780.5529	39.39	2.4	0.0227
783.5737	39.56	2.3	0.0107
870.7605	68.12	8.9	0.0026
872.7705	70.11	1.6	0.0152
886.788	71.78	5.7	0.0078
888.8098	74.22	4.4	0.0331
896.7766	68.78	2.0	0.0001
898.7852	70.96	2.4	0.0002
898.7907	72.33	3.9	0.0078
901.8043	73.76	5.9	0.0104
926.8194	76.62	12.8	0.0064
928.8331	78.34	6.4	0.0360

Table 2

Table showing the 12 compounds that produced potential metabolite matches on METLIN. PC=phosphatidylcholine, PE=phosphatidylethanolamine, PA=phosphatidate

Feature *m/z* +/- <1ppm	Potential Formulas	Species	Score	Potential Identification
520.3398	C26 H50 N O7 P	(M+H)+	99.22	PCs, LysoPC, PE
608.5243	C37 H66 O5	(M+NH4)+	99.39	Diglycerides
634.5395	C39 H68 O5	(M+NH4)+	99.54	Diglycerides
636.5566	C39 H70 O5	(M+NH4)+	97.79	Diglycerides
700.5266	C39 H74 N O7 P	(M+H)+	97.37	PE
	C39 H71 O7 P	(M+NH4)+	97.37	PA
734.5699	C40 H80 N O8 P	(M+H)+	99.36	PE, PC
	C40 H77 O8 P	(M+NH4)+	99.36	PA
744.5543	C41 H78 N O8 P	(M+H)+	98.16	PE, PC
	C41 H75 O8 P	(M+NH4)+	98.16	PA
772.5851	C43 H82 N O8 P	(M+H)+	95	PE, PC
	C43 H79 O8 P	(M+NH4)+	95	PA
872.7705	C55 H98 O6	(M+NH4)+	99.53	Triglycerides
886.788	C56 H100 O6	(M+NH4)+	96.56	Triglycerides
898.7852	C57 H100 O6	(M+NH4)+	98.76	Triglycerides
928.8331	C59 H106 O6	(M+NH4)+	98.03	Triglycerides

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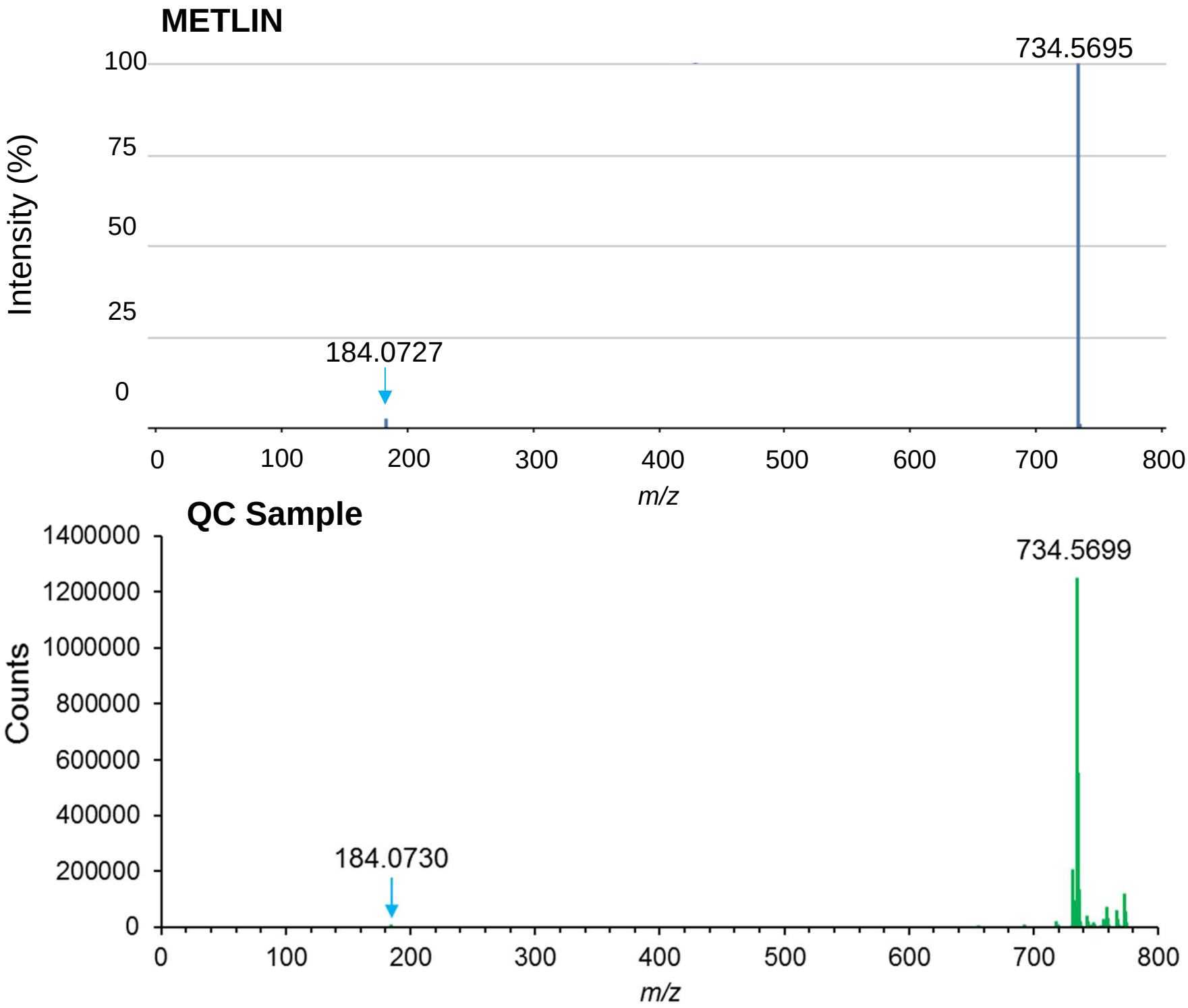
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.4
-0.2
0
0.2
0.4
0.6
PC2
PC3
QCs
Cage
Barn


METLIN
734.5695
184.0727
Intensity (%)
100
75
50
25
0
0
100
200
300
400
500
600
700
800
m/z
QC Sample
734.5699
184.0730
Counts
1400000
1200000
1000000
800000
600000
400000
200000
0
0
100
200
300
400
500
600
700
800
m/z

The Use of Metabonomic Profiling to Uncover Differences Between Cage and Barn Eggs

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Highlights

- Metabonomic studies can be used to observe differences between barn and cage eggs
- Lipid profiles of barn and cage eggs differ to each other
- Lipid content of barn eggs appears to be higher than that of cage eggs
- Dipalmitoyl-glycero-phosphatidylcholine tentatively identified as potential marker