The Metabonomic Profiling of Chicken Eggs During Storage Using High Performance Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry

Amy E. Johnson[a], Kate L. Sidwick[a], Vasil R. Pirgozliev[b], Anthony Edge[c], and David F. Thompson*[a]

[a] School of Chemical and Physical Sciences, Keele University, Keele, Staffordshire, ST5 5BG, United Kingdom

[b] The National Institute of Poultry Husbandry, Harper Adams University, Newport, Shropshire, TF10 8NB, United Kingdom

[c] Department of Chemistry, Liverpool University, Crown Street, Liverpool, L69 7ZD, United Kingdom

ABSTRACT: Metabonomic techniques have been used to discover subtle differences in the small molecule profiles of chicken eggs, which could help to combat fraud within the egg industry. High Performance Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (HPLC-Q-ToF-MS) was used to obtain profiles of the small molecules present in the yolks of chicken eggs stored for different lengths of time. Statistical analysis, including the use of *XCMS Online* and further exploratory statistics, was able to uncover differences in the abundances of several of the small molecules found in these egg yolks. One of these small molecules was identified, through the use of METLIN and MS/MS analysis, as choline. A targeted study was then carried out over a longer storage period, using the same instrumentation and analytical techniques, in order to observe how the concentration of choline in egg yolk changes over a longer period of time.

Food fraud is a problem that has existed for thousands of years[1], but due to the globalization and expansion of food supply chains, the prevalence and impact of these fraudulent activities have increased over recent years[2]. This growing impact of food fraud has resulted in consumers becoming increasingly wary of the products that they purchase[3], and their trust in the food industry is decreasing significantly. Although several techniques have been developed and used to detect cases of food fraud[4], there is still much to be done regarding the detection of more subtle instances of food fraud, particularly the misrepresentation of food products due to mislabeling.

Metabonomics is the comprehensive profiling of low molecular weight species in organic tissues and biofluids, and the observation of how these profiles are affected by both endogenous and exogenous factors[5,6]. It is a relatively recent discipline that is becoming increasingly popular, particularly within areas of research such as: disease diagnostics, toxicology, environmental research, as well as food authentication studies[6–9]. However, there is opportunity for metabonomics to be utilized further in the field of authentomics, in exploring the more subtle differences in food products in order to detect instances of fraudulent activity.

There are two broad categories of metabonomic studies; targeted and non-targeted[7]. Targeted studies can be useful when particular biological pathways are known to be affected by the factor that is being investigated, as the experimental methods can be tailored to particular compounds or compound classes that are found in that pathway. Non-targeted metabonomic studies aim to profile as many metabolites, or small compounds (up to 1000Da), as possible, in order to get a more holistic view of the metabolome[7]. This is particularly useful in authentomic studies when looking for very subtle differences between products, as it is often unknown what biological pathways may be affected, and so a more global approach is preferred. Non-targeted studies generate large amounts of data, providing information on thousands of compounds found in the samples. From this data it is possible, through the use of statistical workflows, to discover compounds that show a significant trend between different sample sets[7]. With the identification of just one of these compounds, following further work and larger studies it could act as a marker to aid in the authentication of the food product.

According to legislation from the European Union, shell eggs that have been laid by chickens must reach the consumer within 21 days of being laid, and have a use-by date of 28 days after lay[10]. They also may only be labeled as "fresh" or "extra fresh" within the first nine days after lay[11]. However, other than trusting the labeling on the eggs and the packaging, there is no way to confirm whether eggs that are for sale fall within these time frames or not. This makes it easy for errors to be introduced unnoticed, and eggs or packaging to be mislabeled with incorrect dates, falsely giving the eggs a longer period of time before reaching their sell-by date.

This paper aims to show how a non-targeted metabonomic technique using HPLC-Q-ToF-MS can be applied to an authentomic study in order to discover differences in the small molecule profiles of eggs stored for different lengths of time. It will then show how this type of study can lead to the identification of a compound that, with further work and larger studies, has the potential to be used as a biomarker, in this case to detect cases of food fraud.

MATERIALS AND METHODS

Materials Methanol (HPLC, isocratic grade) and dichloromethane (stabilized with 0.002% 2-methyl-2-butene) were purchased from VWR (Pennsylvania, USA). Formic acid (90%, laboratory reagent grade) and ammonium acetate were purchased from Fisher Scientific (Loughborough, UK). Ultra pure water (at 18.2MΩ-cm) was purified in-house using a Milli-Q system from Elga (High Wycombe, UK). ESI-L low concentration tuning mix and API-TOF reference mass solution were purchased from Agilent Technologies (California, USA). Chemical standard choline chloride ≥99% was purchased from Sigma-Aldrich (Missouri, USA).

Sample collection non-targeted study Eggs were collected from The National Institute of Poultry Husbandry, Harper Adams University. Laying hens were of the Hy-line brown breed, and were 21 weeks old at the point of lay. They were kept in enriched cages, with 8 birds per cage.

Sample collection targeted study A separate set of eggs was collected from Oaklands Farm Eggs Ltd. Laying hens were of the Hy-line brown breed and were 46 weeks old at the point of lay. They were kept in enriched cages with 80 birds per cage.

Storage of eggs For both studies, eggs were kept at 23°C. Six eggs were taken at each time point (every 7 days, including day 0 – no storage time) and the yolk metabolites were extracted. The non-targeted study took place over five weeks (six time points), and the targeted study took place over twelve weeks (thirteen time points).

Metabolite extraction Egg yolk and albumen were separated using a stainless steel egg separator, which was cleaned with ultra-pure water and methanol between uses. Approximately 50mg of yolk was weighed out into 1.5mL Eppendorf tubes and an organic extraction solvent mixture (dichloromethane/methanol, 3:1) was added (1mL solvent per 50mg yolk, or part thereof). The sample was vortexed and then centrifuged for 20 minutes at 16,100rcf. From this, 0.75mL of supernatant was removed and allowed to evaporate in a fume cupboard under ambient conditions overnight. The dried extracts were then reconstituted in 0.75mL methanol, vortexed to ensure thorough dissolution, and then stored at -80°C prior to analysis.

Quality control Quality control (QC) samples were produced in the non-targeted study by pooling equal aliquots of each individual sample in the analytical run together, in accordance with published guidelines[12]. A series of ten QC injections were analyzed immediately prior to the analytical run to condition the column for analysis. For the targeted study the QC sample used was a chemical standard of 1.4µg/mL choline in methanol, from the middle of the calibration range used. A QC sample was injected and analyzed at regular intervals of every six samples in the non-targeted study and every six or seven samples in the targeted study. These QC samples throughout the analytical run were used to monitor the stability and reproducibility of the analytes found in the samples during the analysis. The relative standard deviation (RSD%) was calculated for all analytes found in these QC samples in the non-targeted study, based on their peak areas, and those with a RSD% greater than 30% were discarded in accordance with suggested guidelines[13].

Chromatographic parameters All samples were analyzed using an Agilent Technologies 1260 Infinity Binary HPLC system and were randomized prior to analysis. The HPLC column used was a Thermo Scientific Accucore RP-MS column (100mm x 2.1mm, 2.6µm particle size).

Non-targeted chromatographic parameters The column was kept at a temperature of 40°C and the injection volume of sample was 5µL. A needle wash of methanol was carried out during each sample injection. The mobile phase had a flow rate of 0.3mL/min and consisted of solvents (A) 0.1% formic acid and 5mM ammonium acetate, and (B) methanol with 0.1% formic acid and 5mM ammonium acetate. The solvent gradient started at 75% (B), increased to 81% (B) in 20 minutes, increased to 90% (B) in 1 minute, held for 10 minutes, increased to 100% (B) in 30 minutes, held for 20 minutes, then returned to starting conditions in the final 4 minutes. There was then a post time of 5 minutes to allow the instrument to equilibrate prior to the next injection.

Targeted chromatographic parameters The column was kept at a temperature of 40°C and the injection volume of sample was 1.5µL. A needle wash of methanol was carried out during each sample injection. The mobile phase had a flow rate of 0.2mL/min and consisted of solvents (A) and (B), as previously described. The solvent gradient started at 5% (B) and held for 1 minute, increased to 10% (B) in 0.1 minutes, then returned to starting conditions in 1.4 minutes and held for 0.5 minutes. External standard solutions of choline chloride in methanol were analyzed both before and after samples at a range of concentrations: 0.2, 0.6, 1, 1.4, 1.8, 2.2, 2.6µg/mL.

Mass Spectrometry (MS) parameters All samples were analyzed using an Agilent Technologies 6530 Accurate-Mass Quadrupole-Time-of-Flight mass spectrometer in positive ionization mode using an electrospray ion source. The parameters were: drying gas temperature of 300°C, drying gas flow rate of 8L/min, capillary voltage of 3500V, nebulizer pressure of 35psi, fragmentor voltage of 175V, skimmer voltage of 65V, and a mass range of 100-1000 *m/z*. The ESI-L low concentration tuning mix was used to calibrate the system prior to analysis to improve mass accuracy, and the API-TOF reference mass solution, consisting of purine (121.0509 *m/z*) and hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine (922.0098 *m/z*), was used throughout the run to maintain this mass accuracy. MS parameters were all kept the same for targeted analysis, except for the mass range which was lowered to 50-150 *m/z*.

MS/MS parameters These were kept the same as previously outlined, but the mass range was lowered to 25-200 *m/z*. Spectra were collected at three different collision energies: 10V, 20V and 40V, for the precursor ion 104.1 *m/z*.

Non-targeted statistical analysis Data was processed using *XCMS Online* which detected features, corrected retention times, and aligned chromatograms[14]. A feature table, with each molecular feature represented by its *m/*z and retention time, was produced by this program, showing the peak areas for each compound in all samples. This table was then transferred to *Microsoft Excel* for statistical analysis. As mentioned previously, RSD% was calculated for all features, and those that had a RSD% >30% were removed prior to further analysis. An initial one-way ANOVA was carried out on all remaining features and those with a significance

of P>0.01 were removed. Principal Component Analysis was then carried out on the remaining features and scores plots were produced. The features were then ranked in order of the PC1 loadings, highest to lowest. The top 100 of the remaining features based on PC1 loadings were taken and any duplicates, isotopic equivalents, and adducts were removed. A Levene's test was then carried out on the remaining features to test for equality of variances; one-way ANOVA was carried out on those with equal variances, and a Welch's t-test was carried out on those with unequal variances. Compounds that were found to show significant differences between sample sets then had their significance confirmed by examining the raw data in Agilent Technologies' *MassHunter Qualitative Analysis* software. Extracted Ion Chromatograms (EICs) were produced for each compound in all samples, and the same statistical tests that were carried out previously were repeated using the peak areas from the integrated EICs. This ensured that these features were robust in their significance between sample sets.

Targeted statistical analysis EICs of choline in the external standards of choline chloride were produced from the raw data using *MassHunter Qualitative Analysis*, and the peak areas were used to construct standard and drift calibration curves with R^2=0.99. Accuracy and precision of the data were measured and were found to be within acceptable limits according to published Food and Drug Administration (FDA) guidelines[15]. Relative Errors (REs) were calculated for the choline chloride standards to measure accuracy and all were found to be less than 8% for the standard curve, and less than 14% for the drift curve. To measure precision, the RSD% of all choline chloride standard solutions were calculated and found to be less than 6% for the standard curve and less than 8% for the drift curve, and the RSD% of a QC sample analyzed throughout the analytical run was 1.6%. EICs of the targeted compound were produced for all samples, and concentrations were then calculated based on the equation of the standard curve. The same statistical tests that were used in the non-targeted study were carried out based on the concentrations of the compound in all samples, with t-tests then being carried out between individual sample sets of eggs stored for different lengths of time.

RESULTS AND DISCUSSION

Non-targeted study

Metabolite profiling The profiles of yolk organic extracts are visibly different between fresh eggs (week 0) and those that were stored for five weeks (week 5), as shown in Figure 1. Most peaks have a higher abundance in the Total Ion Chromatogram (TIC) for week 0 egg yolk extracts than for the extracts from week 5 egg yolks.

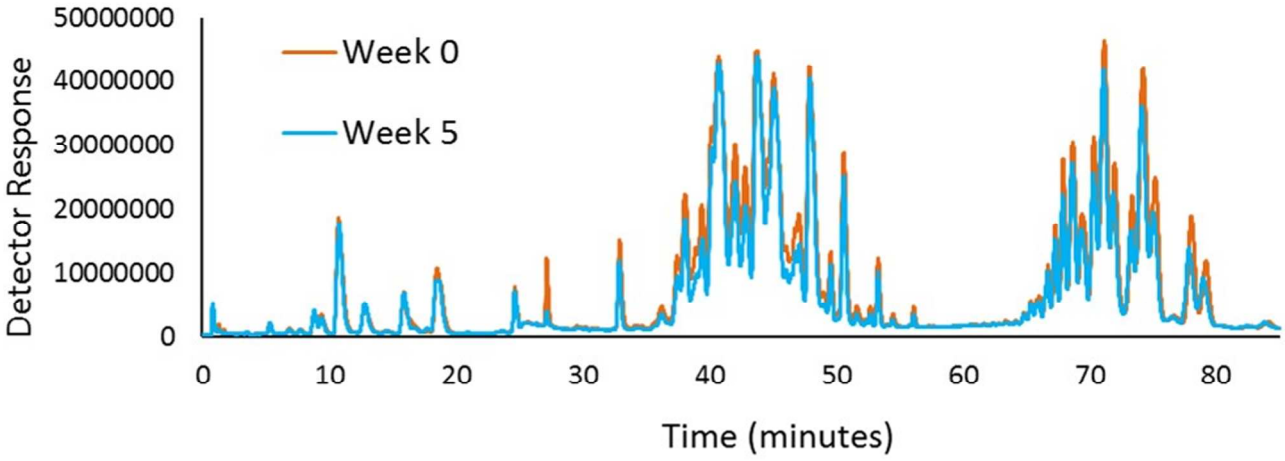

Figure 1. Overlaid Total Ion Chromatograms of yolk organic extracts from fresh eggs (Week 0) and eggs stored for five weeks (Week 5).

Multivariate statistics PCA scores plots, as seen in Figure 2, display a tight clustering of QC samples which shows that there was minimal instrumental drift throughout the analysis. There is some degree of separation between sample sets of eggs stored for different lengths of time. The distance between the samples and the separated sample sets is greater than the spread of the QC samples which shows that these are true biological differences between the samples, rather than instrumental drift. Fresh eggs form a group separate to any other sets of eggs, which seems to indicate that there is a large change in the metabolic profiles of egg yolks within the first week of egg storage. There is also some separation between other sets of eggs stored for different lengths of time. Although the separation between the different sample sets on the scores plot is very subtle, it is important to realize that the differences between the sets of egg samples were also very subtle; the eggs were all laid by the same batch of birds of the same age and breed, on the same day, the only difference was the length of time that the eggs were stored for prior to extraction. However, the metabonomic profiling and statistical analysis employed in this study were still able to observe these differences. The subtlety of these differences is highlighted by the very small percentage variance explained by PC3 and PC4, which is 0.41% and 0.19% respectively.

Potential biomarkers Table S-1 gives the *m/z,* retention time, RSD% and P-value (from either ANOVA or Welch's t-test) of the top 41 compounds remaining following the removal of duplicates, isotopic equivalents and adducts from the top 100 compounds based on PC1 loadings. These compounds were all found to be significantly different between eggs stored for different lengths of time, based on the information in the feature table produced by *XCMS Online*. Those in bold were also found to show significant differences when using information from the raw data via *MassHunter Qualitative Analysis.*

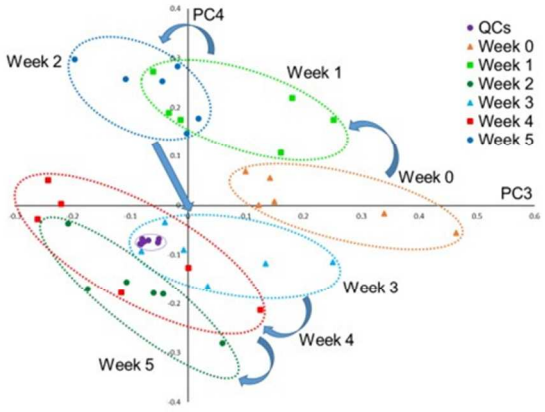

Figure 2. PCA scores plot of PC3 vs PC4 showing groupings of samples of eggs stored for different lengths of time.

Feature identification For those features that also showed significant differences between eggs stored for different lengths of time based on the raw data, (those in bold in Table S-1), attempts were made to identify them. EICs were produced in *MassHunter Qualitative Analysis* and potential molecular formulae were predicted for the compounds by the software, based on the monoisotopic mass, isotope abundance and isotopic peak spacing in the mass spectra[16]. All formulae with a likelihood score of 95 and above were searched on METLIN, an online metabolite database, to find metabolite matches. For those that produced matches, the sample mass spectra were compared with the mass spectra on METLIN to see if they could be putatively identified[17]. One of these compounds, that with an *m/z* of 104.1 in Table S-1, was predicted the formula $C_5H_{13}NO$ with a score of 98.85, and was putatively identified via METLIN as choline. This identification was then confirmed when a chemical standard of choline chloride was analyzed using HPLC-MS/MS alongside a QC sample, and the compound *m/z* 104.1 was found to match choline in both retention time and mass spectrum, with all fragment peaks matching at three different collision energies. Figure S-1 shows the matching retention times of the choline standard and compound *m/z* 104.1 and Figure S-2 shows the mass spectra for both the choline standard and the compound *m/z* 104.1 at three different collision energies.

Choline The trend of choline abundance in egg yolk was found to increase steadily over the five week period, as can be seen in Figure 3.

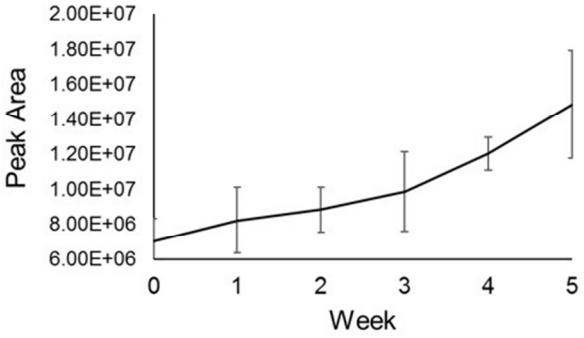

Figure 3. Trend of choline abundance in egg yolk over a five week period, measured as mean values of peak area at each time point, with error bars of +/-1 standard deviation.

Choline is a precursor to the neurotransmitter acetylcholine[18,19] as well as various choline-containing lipids[19,20]. It exists in its free form mainly due to the catabolism of one of these choline-containing lipids, phosphatidylcholine[18,21], which is found in all cell membranes[22] and is highly abundant in egg yolk[23]. Phospholipases (PLs) are responsible for the catabolism of phosphatidylcholine as they hydrolyze certain bonds in the molecule: PLA1 and PLA2 hydrolyze the fatty acyl bonds between the fatty acids and the glycerol backbone, PLC hydrolyzes the glycerophosphate bond, and PLD hydrolyzes the choline phosphate ester bond[18].

It has been observed that the phosphatidylcholine content of egg yolk decreases over an increasing storage time of eggs as the phospholipases, which are endogenous to egg yolk, hydrolyze the phosphatidylcholine, resulting in the release of choline[24]. This catabolism of phosphatidylcholine explains the observed increase in abundance of choline over the egg storage period; the decrease of phosphatidylcholine abundance results in an increase of choline abundance.

These results have also been observed in this study. Several compounds in Table S-1 have been recognized, through the use of *Lipid Maps* and METLIN, as lipids belonging to the phosphatidylcholine-choline metabolic pathway. Some of these compounds have been classified as potential phosphatidylcholines and show a general trend of decreasing abundance over an increasing egg storage time, supporting the observed results. Other compounds have been classified as potential monoacylglycerophosphocholines, which result from the hydrolysis of one of the fatty acyl bonds in phosphatidylcholine by PLA1 or PLA2 phospholipases. The abundances of these compounds were found to generally increase over increasing egg storage time, due to the catabolism of phosphatidylcholine by phospholipases, again supporting the results found in this study.

Targeted study The EICs of choline in the yolk organic extracts show a clear difference in abundance between weeks 0, 5, 9, and 12 as can be seen in Figure 4, with eggs that were stored for longer periods of time having a higher abundance of choline.

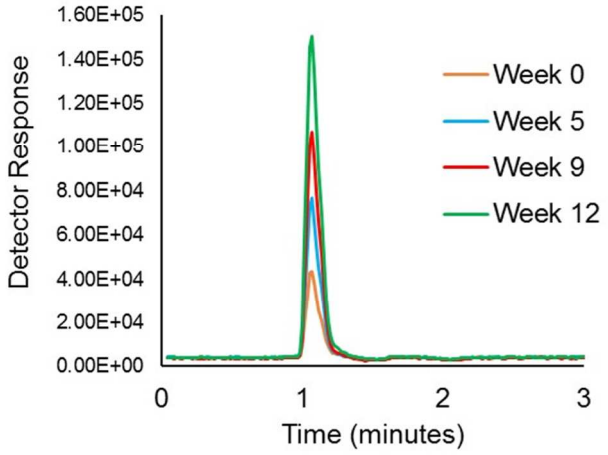

Figure 4. Extracted Ion Chromatograms (EICs) for choline in yolk organic extracts for samples from four different lengths of storage time.

Table S-2 gives the concentration of choline in egg yolk for each sample, and a mean yolk choline concentration for each time point. Across the first five weeks of egg storage, there is a statistical significance of P=0.001, and over the full twelve week storage period there is a significance of P<0.001 for the difference in choline abundance in the yolks of eggs stored for different lengths of time. The trend of choline abundance over the first five weeks is similar to that of the non-targeted study, with the abundance gradually increasing over the five week period. The trend then continues in a similar manner over the remaining weeks up to week 12, but is slightly more erratic, as can be seen in Figure 5. There is also a large increase in abundance of choline at week 8, which then decreases again in week 9. Two of the samples that were stored for eight weeks had a much higher concentration of choline than the other four samples from this time point, as can be seen in Table S-2, which explains this sudden increase in the average concentration at this point. It would be expected that the effect of biological variation within groups of eggs stored for the same length of time would be reduced with larger sample sizes.

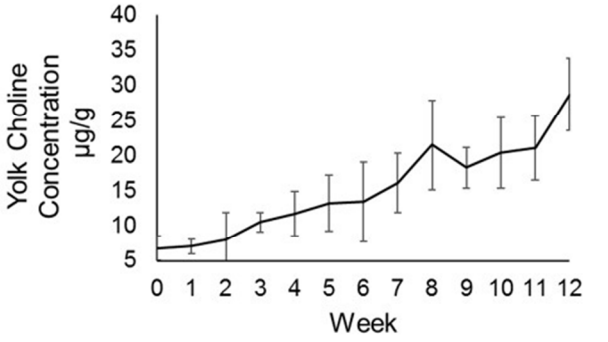

Figure 5. Trend of choline concentration in egg yolk over a 12 week period, measured as mean values of peak area at each time point, with error bars of +/-1 standard deviation.

The average concentration of choline in a fresh egg yolk was found to be approximately 6.8µg/g as can be seen in Table S-2. There was a significant difference in yolk choline concentration of P=0.002 between fresh eggs and eggs stored for three weeks, i.e. at their sell-by date, with an increase in choline concentration of approximately 3.8µg/g up to 10.6µg/g. The concentration of choline in the yolk at four weeks of egg storage, i.e. the eggs' use-by date, had increased to approximately 11.7µg/g. There was a significant difference in yolk choline concentration of P=0.012 between eggs at their sell-by date of three weeks and eggs stored for seven weeks which had an average yolk choline concentration of 16.1µg/g; and a significant difference of P=0.011 between eggs at their use-by date of four weeks and eggs stored for eight weeks, at which point the choline concentration in the yolks had increased to 21.5µg/g. The overall increase of average choline concentration in egg yolk between fresh eggs and eggs that were stored for twelve weeks was 22.0µg/g, up to a final concentration of 28.7µg/g.

CONCLUSION

This study has shown that it is possible, using a non-targeted metabonomic workflow, to uncover differences in the small molecule profiles of chicken eggs stored for different lengths of time. It has also shown that this technique is capable of resulting in the identification of a particular biomarker that is significantly different in abundance between these eggs of different ages. The follow-up targeted study shows that choline is reproducibly of statistical significance in egg aging studies, and the trend of its abundance is also reproducible between different sets of eggs. Significant differences can be seen in the concentration of choline in egg yolk between eggs at their sell-by and use-by dates, and eggs that have been stored for just four weeks longer.

Although it is clear that further work and larger studies would be required to validate the use of choline as a biomarker of egg age, this study has shown both the potential of this metabonomic technique in discovering potential biomarkers to uncover food fraud, and the potential use of choline as a biomarker of egg age.

ASSOCIATED CONTENT

Supporting Information

Table of features found to be significantly different between eggs of different ages; image showing comparable retention times of choline and compound 104.1 *m/z*; image showing matching MS/MS spectra of choline and compound 104.1 *m/z*; table of choline concentrations in sample extracts and yolks (PDF)

AUTHOR INFORMATION

Corresponding Author

* E-mail: d.f.thompson@keele.ac.uk

ORCID

David F. Thompson: 0000-0002-2834-0109

Author Contributions

All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

This work was supported by Keele University, with help and advice contributed by Harper Adams University. Thanks to both The National Institute of Poultry Husbandry and Oaklands Farm Eggs Ltd for providing eggs for this study. Thanks

also to Dr Vladimir Zholobenko for his help and support with this research.

REFERENCES

(1) Shears, P. *Br. Food J.* **2010**, *112* (2), 198–213.

(2) Spink, J.; Moyer, D. C. *J. Food Sci.* **2011**, *76*, 157–163.

(3) Capuano, E.; Boerrigter-Eenling, R.; van der Veer, G.; van Ruth, S. M. *J. Sci. Food Agric.* **2013**, *93* (1), 12–28.

(4) Reid, L. M.; O'Donnell, C. P.; Downey, G. *Trends Food Sci. Technol.* **2006**, *17* (7), 344–353.

(5) Wilson, I. D.; Plumb, R.; Granger, J.; Major, H.; Williams, R.; Lenz, E. M. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2005**, *817* (1), 67–76.

(6) Tang, H.-R.; Wang, Y.-L. *Prog. Biochem. Biophys.* **2006**, *33*, 401–417.

(7) Gika, H. G.; Theodoridis, G. A.; Plumb, R. S.; Wilson, I. D. *J. Pharm. Biomed. Anal.* **2014**, *87*, 12–25.

(8) Moco, S.; Bino, R. J.; De Vos, R. C. H.; Vervoort, J. *Trends Anal. Chem.* **2007**, *26* (9), 855–866.

(9) Cubero-Leon, E.; Peñalver, R.; Maquet, A. *Food Res. Int.* **2014**, *60*, 95–107.

(10) *Off. J. Eur. Union* **2004**, L139/55.

(11) Commission Regulation. *Off. J. Eur. Union* **2008**, *51* (L 163), 6–23.

(12) Sangster, T.; Major, H.; Plumb, R.; Wilson, A. J.; Wilson, I. D. *Analyst* **2006**, *131* (10), 1075.

(13) Theodoridis, G. A.; Gika, H. G.; Want, E. J.; Wilson, I. D. *Anal. Chim. Acta* **2012**, *711*, 7–16.

(14) Benton, H. P.; Ivanisevic, J.; Mahieu, N. G.; Kurczy, M. E.; Johnson, C. H.; Franco, L.; Rinehart, D.; Valentine, E.; Gowda, H.; Ubhi, B. K.; Tautenhahn, R.; Gieschen, A.; Fields, M. W.; Patti, G. J.; Siuzdak, G. *Anal. Chem.* **2015**, *87*, 884–891.

(15) FDA. **2001**. Guidance for Industry: Bioanlytical Method Validation.

(16) Sana, T. R.; Roark, J. C.; Li, X.; Waddell, K.; Fischer, S. M. *J. Biomol. Tech.* **2008**, *19*, 258–266.

(17) Dunn, W. B.; Erban, A.; Weber, R. J. M.; Creek, D. J.; Neumann, S.; Kopka, J.; Viant, M. R. *Metabolomics* **2013**, *9*, 44–66.

(18) Lockman, P. R.; Allen, D. D. *Drug Dev. Ind. Pharm.* **2002**, *28* (7), 749–771.

(19) Ueland, P. M. *J. Inherit. Metab. Dis.* **2011**, *34* (1), 3–15.

(20) Simon, J. *Worlds Poult. Sci. J.* **1999**, *55*, 353–374.

(21) Zeisel, S. H.; Da Costa, K. A. *Nutr. Rev.* **2009**, *67* (11), 615–623.

(22) Küllenberg, D.; Taylor, L. A.; Schneider, M.; Massing, U. *Lipids Health Dis.* **2012**, *11*, 1–16.

(23) Anton, M.; Nau, F.; Nys, Y. *Worlds. Poult. Sci. J.* **2006**, *62*, 429–438.

(24) Wang, Q.; Jin, G.; Wang, N.; Guo, X.; Jin, Y.; Ma, M. *Czech J. Food Sci.* **2017**, *35*, 229–235.

Table of Contents Graphic – For TOC only