Chromatography Today Help Desk

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A question of taste?

It is a sad indictment of today's civilisation that in the pursuit of money there are an assortment of fake items that are readily available. Whether this is products at the local market or at a site on the world wide web, the availability of the range of fraudulent goods is immense. These types of items are particularly prevalent when dealing with high cost goods, and for example the number of Gucci or Louis Vuitton own labelled handbags that are available at a fraction of the price on the official websites is quite startling. Where there is a clear price differential it is obvious that there is something not quite correct, however, where the seller purports to be selling the genuine article at the genuine price then the high profit margins the seller is obtaining is at the expense of the consumer. It is not just the fashion industry that has suffered with food and other consumer products industries also being affected by the lure of large profits.

Food fraud has been a problem for thousands of years [1] but due to globalisation and the expansion of food chains, the prevalence and impact of these activities has increased substantially over recent years [2]. This can sometimes lead to tragic conclusions as with the melamine substitution of protein in baby formula milk, which resulted in the hospitalisation of over 54,000 babies in China [3]. In other cases, the impact is not as severe but whatever the situation the role of the analytical chemist is clear; to ensure the quality of the food for public consumption.

In this edition of the helpdesk, the problem is not of an analytical making, but more of a fraudulent labelling one. The helpdesk was involved in solving this problem using separation science as the core technology and so in a twist to a conventional problem page, in this issue the problem is resolved by separation science in a rather neat manner. The research was performed at Keele University [4] and the Helpdesk is grateful to the team there for allowing the data to be shared.

A problem often encountered by supermarkets is how to determine the quality and authenticity of a food product, and one such example presented itself relatively recently, where a local supermarket had cause to question the sell by dates of eggs that were being supplied. Customers had complained substantially regarding the quality of the product but the sell by date had not been exceeded, and so enter a team of analytical scientists who were tasked with identifying how old the eggs were, and had they been mislabelled.

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 [5]. They also may only be labelled as 'fresh' or 'extra fresh' within the first 9 days after lay [6]. However, other than trusting the labelling 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 and go unnoticed and for eggs or packaging to be mislabelled with incorrect dates, falsely giving the eggs an extended period of time before reaching their sell-by date. In this

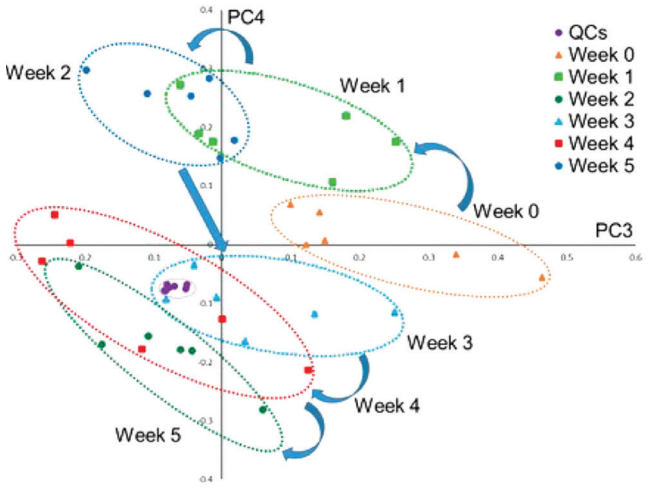

Figure 1
Principal component analysis plot obtained from eggs of different ages, showing that the age difference can be detected.
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case it was thought that this was not due to an error in labelling, but more on individuals looking to make a profit from out of date eggs.

It is known that if an egg is left long enough then there will be a build-up of hydrogen sulphide which will result in the eggs floating in water, this is because H2S is lighter than air. Over a period of time the build-up of hydrogen sulphide will cause the egg to gradually stand on its pointed end, before eventually floating to the surface of the water, however it can take up to 5 weeks for an egg to 'go off', and it would be much better to identify the age of a batch of eggs on arrival at the store, which avoids the issue of selling customers eggs that are past their sell by date.

The Keele team looked initially to identify a chemical marker that would enable the egg age to be determined. The approach employed was taken from the field of metabolomics, where different sample types are analysed samples analysed using LC-HRMS and an authentomic approach applied to discover the differences in the small molecule profiles of eggs of different ages. Once suitable markers have been identified it would be possible to develop a targeted analytical methodology that would allow for determination of the age of the egg.

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

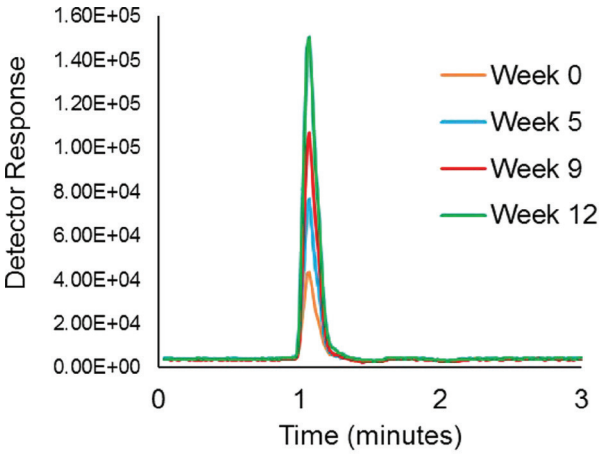

Figure 2
The variation of choline from week 0 to week 12
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dissolution, and then stored at -80°C prior to analysis. The intention with the extraction procedure is to reduce the complexity of the sample without removing any potential biomarkers from the system.

Very early in the development of the field of metabolomics it was identified that during the mathematical analysis, drift could occur in the results interpretation, and so the use of a pooled quality control (QC) sample is important to ensure data integrity. The drift can be associated with a range of variables that are not within the control of the analytical scientist, and so using a pooled QC sample which is injected throughout the run will ensure that any drift is seen [7]. If drift is seen in the pooled QC experiments, it would suggest that the experimental data is invalid.

QC samples were produced in the nontargeted study by pooling equal aliquots of each individual sample in the analytical run together, in accordance with published guidelines [8]. A series of ten QC injections were analysed immediately prior to the analytical run to condition the column for analysis. A QC sample was injected and analysed with every six samples. 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 on the basis of their peak areas, and those with an RSD% greater than 30% were discarded in accordance with suggested guidelines [9].

The analysis was performed on a UHPLC system coupled to a Quadrupole Time of Flight Mass Spectrometer (QTof), using a 100 x 2.1 mm superficially porous RP column. The mobile phase had a flow rate of 0.3 mL/min and consisted of solvents A, 0.1% formic acid and 5 mM ammonium acetate, and B, methanol with 0.1% formic acid and 5 mM ammonium acetate. The solvent gradient started at 75% B and was increased to 81% B in 20 min, increased to 90% B in 1 min, held for 10 min, increased to 100% B in 30 min, held for 20 min, and then returned to starting conditions in the final 4 min. There was then a post analysis time of 5 min to allow the instrument to equilibrate prior to the next injection.

The data was processed using XCMS Online [10] which produced a molecular feature table, showing the peak areas for each compound in all samples. This table was then transferred to Microsoft Excel, and after removal of the statistically irrelevant data, principal-component analysis was then performed. The features were then ranked in order of the PC1 loadings, highest to lowest. After further sifting of the data to ensure that there was statistical relevance between the different sample sets, the raw data was analysed in the MS software to confirm the initial findings, thereby identifying the retention time and mass spectra of a potential biomarker.

Figure 1 shows the resulting PCA plot, and it can be readily seen that it is possible to differentiate between the different aged eggs using this approach. The QCs are very close together which demonstrates that the experiment has been performed correctly and that there is minimal drift. It also demonstrates that the differences that are observed between the different sample sets are due to the biological differences within the system, i.e. it is an age effect, and that the PCA is identifying potential biomarkers from the analytical measurements.

With further analysis of the raw analytical data it was possible to identify a potential biomarker. In this case a m/z of 104.1 was detected, which corresponds to choline. Choline is a precursor to the neurotransmitter acetylcholine as well as to various choline-containing lipids [7,11]. It exists in its free form mainly as a result of the catabolism of one of these choline-containing lipids, phosphatidylcholine [12,13], which is highly abundant in egg yolk. It has been observed that the phosphatidylcholine content of egg yolk decreases with increasing storage times as the phospholipases in the egg yolk hydrolyse the phosphatidylcholine, resulting in the release of choline. This catabolism of phosphatidylcholine explains the observed increase in the abundance of choline over the egg-storage period.

Once the team had identified a suitable biomarker, in this case choline, it was possible to develop a targeted approach to the analysis of this key biomarker. The sample preparation approach taken was the same as that employed for the untargeted analysis of the eggs, and with the final analysis, the chromatography and mass spectrometric methodologies were altered to allow optimal chromatographic performance and also detection. Figure 2 shows the resulting chromatograms obtained by monitoring the egg for choline from week 0 to week 12. It is very evident that the Keele team have developed a very sensitive and specific methodology that can be readily applied to the primary issue that the supermarket had with a case of potential food fraud.

This approach to determining food authenticity would require the destructive sampling of a single egg within a batch from the supplier, which is a similar approach taken by the regulatory authorities to determine the presence of growth hormones in eggs. Clearly using this approach, it is not feasible to test every egg, however as with the other approaches employed by the regulatory authorities, the mere knowledge that it is possible to determine the age of the egg, acts as an effective deterrent in most cases, and obviously it is feasible to test any suspected product.

The approach taken by the Keele team highlights the importance of analytical science within our community, and in particular for ensuring the general wellbeing of the general public. In these most uncertain of times the importance of analytical scientists has been brought to the fore. Previously scientists would reside in quiet, producing data that supports the wellbeing of society without any notoriety, however with the world in crisis the role of the scientific community has never before had such a profile and indeed such significance. The approach taken by the group in Keele is very applicable to not just the determining the age of eggs but also for identifying key biomarkers within any biological system.

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