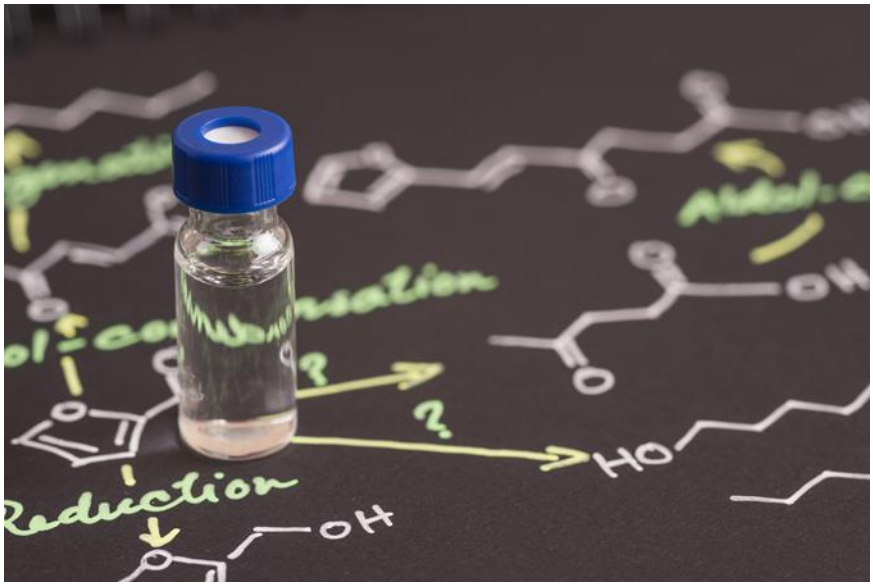
Exploration of novel technologies for counteracting food fraud

A technology viability study



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Foreword & Acknowledgments

The fraudulent exploitation of food chain vulnerabilities has come into focus in recent years, particularly after the horsemeat scandal of 2013 in the EU. Food fraud is now recognised as a serious and widespread whole food chain phenomenon that not only leads to consumer deception, but can undermine the protection of consumer health as well. There is now an urgency to develop cost-effective, rapid and reliable analytical methodologies that can be used for assuring the authenticity of food. This report details an investigation into the viability of a number of analytical techniques to be applied to food authenticity testing.

***safe*food** would like to thank the Principal Researchers on this project, Prof Chris Elliott and Dr Simon Haughey of the Institute for Global Food Security, Queen's University Belfast. Thanks are also due to Dr Haughey's research colleagues at Queen's University Belfast including Dr Olivier Chevallier, Dr Tassos Koidis, Mr Daniel McDowell, Miss Claire McVey and Mr Connor Black. We would also like to acknowledge the participation of Prof Tom Buckley, Mrs Claire Egan and Mr Mark Sherry of the Irish Equine Centre, Naas, Co. Kildare who collaborated on this research. Finally, we would like to thank all those food businesses and processors throughout the island of Ireland and abroad without whose participation this research would not have been possible.

Executive Summary

The incidence of food fraud continues to increase globally with negative impacts on consumer confidence and potentially their health and well-being. It is estimated to cost the global food industry anything from US$30 to $40 billion (€28-37 billion) annually. To try and reduce the prevalence of food fraud, we need to develop methods that will rapidly indicate whether a foodstuff is authentic or not. With this in mind, the team at the Institute for Global Food Security (IGFS) at Queen's University Belfast, in collaboration with the Irish Equine Centre in Naas, Co. Kildare, investigated the application of modern analytical techniques for the detection of food fraud by applying these techniques to a range of foods including cheese, meat, fish and rapeseed oil.

A number of analytical techniques and associated equipment were identified as showing promise in the development of methods for food authenticity testing. These were (1) benchtop Nuclear Magnetic Resonance (60MHz); (2) the loop-mediated isothermal amplification (LAMP) assay, and (3) Rapid Evaporative Ionisation Mass Spectrometry (REIMS). Although the 60MHz NMR showed potential, there were also drawbacks associated with this technique, so other spectroscopy techniques were explored including mid-infrared (FT-IR), Raman and high field NMR.

The key outputs from the research were:

1. Sample libraries of food commodities were built for use during the project which was achieved with the assistance of the red meat, fish, rapeseed oil and artisan cheese industries.
2. The LAMP assay was initially used for cheese and fish speciation but in addition to this (and as an extra outcome), meat speciation was also included. The post-validation sample survey revealed the most suitable use of LAMP for species-specific assays, namely for single species identification in small sample volumes. The use of LAMP assays for multiple speciation identification in red meat and fish samples is unattainable. Even a solitary sample cannot be screened for multiple targets in a single run. This is due to the single target nature of each assay and the limited capacity of the Optigene Genie II LAMP machine.
3. The feasibility of low field 60MHz NMR for the determination of rapeseed authenticity was investigated. As an extra outcome, other spectroscopic techniques were also trialled.
4. REIMS was used to determine speciation of meat and fish as well as the method of “catch” (trawler vs line caught). The determination of the geographic origin of beef showed some tentative discrimination but would need further work. Initial results indicate that the method could not be used to determine the differences in meat tissues between drug-treated and control animals. The authors recommend that the latter two pieces of work should be continued with perhaps different experimental conditions used for analysis. It is worth

noting that this is the first time REIMS has been used in the area of food authenticity and integrity.

5. Following retail surveys on meat and fish, indications of fraud or unintentional contamination were found for both meat and fish products.

Key Recommendations

- REIMS coupled with advanced data processing and chemometrics could potentially be used in many areas related to food integrity. It is a key recommendation of the authors that the full potential of this equipment be explored.
- The LAMP assay could be used for speciation of meat, fish and cheese and was found to be quicker than PCR. However the authors recommend that further work is necessary to make this a high throughput technique.
- NMR (60MHz) showed some potential for detected rapeseed oil adulteration but 400MHz NMR, FT-IR and Raman spectroscopies would appear to work just as well or even better.

Glossary of terms

AMS	Accelerator mass spectrometry
CPRSO	Cold pressed rapeseed oil
DESI	Desorption Electrospray Ionization
DNA	Deoxyribonucleic acid
EU	European Union
FT-IR	Fourier Transformed-Infrared Spectroscopy
IoI	Island of Ireland
IPC	Internal positive control
IRMS	Isotope ratio MS
LAMP	Loop-mediated isothermal amplification
LDA	Linear discriminant analysis
LOD	Limit of Detection
MS	Mass Spectrometry
NI	Northern Ireland
NMR	Nuclear Magnetic Resonance
OPLS-DA	Orthogonal Partial Least Square-Discriminant Analysis
PCA	Principal Component Analysis
PESI	Probe Electrospray Ionization
PLS-DA	Partial Least Squares Discriminant Analysis
REIMS	Rapid Evaporative Ionization Mass Spectrometry
RoI	Republic of Ireland
RRSO	Refined rapeseed oil
SFO	Sunflower oil
SIMCA	Soft Independent Modelling by Class Analogy

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1 Research aims and objectives

This project investigated three different analytical techniques and developed procedures for foods known to be very prone to fraud: (1) Cheese (2) Fish (3) Red Meat (4) Rapeseed oil, which are economically very important commodities produced and sold on the island of Ireland (IoI). The first procedure is based on a molecular technique called Loop Mediated Isothermal Amplification (LAMP) which is an isothermal nucleic acid amplification technique. Unlike PCR, where the reaction is carried out with a series of alternating temperature steps or cycles, LAMP is carried out at a constant temperature and doesn't require a thermal cycler. This makes the procedure much faster, cheaper and simpler to undertake than PCR. As with all the analytical techniques investigated in this research, a partnership with LAMP manufacturer (Optigene) was established and the technology was provided at no cost to the project. Optigene have developed LAMP assays for speciation tests (cheese, fish) and a rigorous validation across a range of fish species and cheese types (goat's, cow's) was undertaken. Large sample numbers were provided free of charge to the project by the cheese industry but also by the red meat, fish and artisan rapeseed oil industries as well.

The second analytical technique investigated was benchtop NMR (60MHz) which was used to profile cold-pressed rapeseed oil, a high value artisan product produced on the IoI and prone to adulteration with cheaper oils. This method was assessed to determine what level of substitution with these cheap oils can be detected.

The third analytical technique investigated was Rapid Evaporative Ionization Mass Spectrometry (REIMS). This is an emerging technique that allows near real-time characterization of tissue by metabolite analysis of the aerosol released during dissection. The coupling of REIMS technology with dissection for tissue identification is known as Intelligent Knife (iKnife). The metabolite fingerprint generated shows a unique pattern for the type of tissue. For the first time, REIMS was investigated for its ability to (a) differentiate between different types of meat and fish species, (b) detect chemical contamination of meat, and (c) provide information on the geographical origin of meat simultaneously. A successful outcome to this research had the potential to bring about a paradigm shift in food fraud detection.

REIMS technology was currently only available at one other research institute in the world (Imperial College, London) where they were pioneering the technique to be used for real-time detection of cancerous tissue during surgery. Imperial College and Waters Corporation (USA) have supported the project in terms of supplying expertise and the REIMS technology itself. The project was also strongly supported by the ABP Food Group. As one of the companies most effected by the 2013 Horsemeat

Scandal, they have a strong desire to work with research groups to deliver better ways of protecting their supply chains from fraud. ABP supplied hundreds of meat samples together with full traceability details to the project free of charge. Young's Seafood, Grimsby, United Kingdom did the same for the various fish species analysed.

Main objectives of the project

1. Building a sample library
    a. Meat speciation library: Samples of beef, pork, lamb, horsemeat, goat meat were obtained, coded and stored at -80°C.
    b. Fish speciation library: Samples of fish were obtained from various contacts in the fish industry and libraries of raw, cooked and processed samples were prepared.
    c. Cheese speciation library: Samples of goat's cheese had already been collected from producers as part of a previous ***safe***food-funded project. These were tested to ensure they all register as having no evidence of cow's material present.
    d. Rapeseed oil library: Samples of rapeseed oil had already been collected from artisan producers on the IoI and were blended with a cheap vegetable oils.
    e. Geographic origin library: Meat samples from across the IoI were collected and transported to Queen's University Belfast. Details of location of slaughter, geographic region, etc. were provided. Further samples of meat were obtained from various global meat trade contacts.
    f. Antibiotic treated library: Samples of meat taken from animals known to be treated with a range of nitroimidazoles were used. Control samples (i.e. untreated) were obtained from the same sources.
2. LAMP Assay Validation

    A full validation of LAMP assays was undertaken for cheese and fish speciation. As a bonus to the project, meat speciation was also included. The commercial LAMP kits were evaluated for sensitivity, specificity, false positive rates, false negative rates and user robustness using the cheese and fish speciation libraries.

3. Building the NMR model for rapeseed oil determination and detection of adulteration

    Samples of pure rapeseed oil from local suppliers were used to build the model. During the NMR data analysis, the calibration models were validated using authentic rapeseed oil samples and relevant admixtures (n=30) to evaluate predictability and reliability.

4. Building REIMS databases

Meat samples from the IoI were analysed using REIMS and Quadrupole Time-of-flight (QToF) instrumentation with data used to build a 'country of origin model'. Further samples collected from the IoI and other locations were assayed to determine the capability of REIMS to correctly identify IoI from non- IoI beef. Meat from the meat speciation library was also analysed by REIMS to determine the capability of this procedure to identify species contamination and country of origin of the beef simultaneously. Samples of antibiotic-treated beef were subject to REIMS profiling with a database developed to identify illegal treatment of meat with hormones.

2 Authenticity of cold pressed rapeseed oil using 60Hz pulsar NMR

Introduction

Cold pressed rapeseed oil (CPRSO) is a relatively new culinary oil on the UK and Irish markets that is locally cultivated, harvested and pressed. The seeds from the oil seed rape crop are mechanically crushed at a low temperature and the oil is collected. The oil is filtered and bottled and this concludes the processing. According to the Codex Standard for named vegetable oils "all cold-pressed vegetable oils must be obtained without altering the oil, by mechanical procedures only e.g. expelling or pressing, without the application of heat. They may have been purified by washing with water, settling, filtration and centrifuging only." The final product is a deep yellow, nutty flavoured oil which is used for dressings and high temperature cooking. It commands a premium price at retail.

High quality edible oils such as this are particularly susceptible to adulteration due to the ease with which cheap oils can be mixed in to increase profit. Although no incidences of adulteration of CPRSO have been recorded, it is still important to have measures in place which have the ability to detect fraud. This will go further to strengthening the already robust CPRSO industry.

Refined rapeseed oil (RRSO), sometimes branded as "vegetable oil" in supermarkets, is a cheap oil which has been popular for decades. The seeds from the oil seed rape plant undergo a much more complex processing pathway than the previously mention cold pressed sequence. The seeds are mechanically crushed and solvent extracted to ensure maximum levels of oil are harvested from the seeds. This oil then undergoes a range of intensive processing techniques to removed chemicals associated with colour, flavour and odour. The resulting oil is low cost and used exclusively for high temperature cooking. RRSO is a low cost oil and, in terms of chemical composition, is very similar to cold-pressed rapeseed oil. Therefore, it would be a good candidate for adulteration. In this event, it can be expected that the fatty acid composition of the two oils would be very similar and the main difference would be in minor compound constitution which makes up around 2-4% of cold-pressed rapeseed oil. Therefore, detecting RRSO in CPRSO would be challenging.

Generally speaking, refined sunflower oil (SFO) can be a low cost oil usually used in high temperature cooking and considered healthy due to its favourable fatty acid composition. Refined SFO was selected as another possible oil of adulteration due to its cheap price and wide spread availability

(Figure 2.1). Refined SFO is produced from sunflower seeds by undergoing a similar intensive processing pathway as described in RRSO. However, the differences are more significant as it originates from a different seed to rapeseed oil. Therefore, SFO should be easier to detect in cold-pressed rapeseed oil.

Figure 2.1: Classes of oil analysed by 60MHz NMR.

Pure Cold Pressed Rapeseed oil (CPRSO)	Pure Sunflower oil (SFO)	Pure Refined Rapeseed oil (RRSO)	Cold Pressed Rapeseed Oil and Refined Rapeseed oil (CPRSO + RRSO)	Cold Pressed Rapeseed oil and Sunflower Oil (CPRSO+ SFO)

There are currently no analytical methods to detect CPRSO adulteration. There has been extensive work done with edible oil authentication with Fourier Transformed-Infrared Spectroscopy (FT-IR) (Rohman and Che Man 2012), Raman (Consuelo Lopez-Diez et al. 2003) and High field NMR (Vigli et al. 2003). It could therefore be assumed that similar techniques could be applied to the CPRSO sector. The innovation of the benchtop NMR has provided a new tool to explore edible oil adulteration. The benchtop NMR has some advantages over the high field alternative. The rare-earth magnets used in the benchtop NMR mean it is much smaller and can be transported easily, as long as no other magnetic equipment is in close proximity. The body of work already done with high field NMR means that information obtained from the spectrum of high field NMR can be transposed to benchtop NMR, e.g. some peak identification.

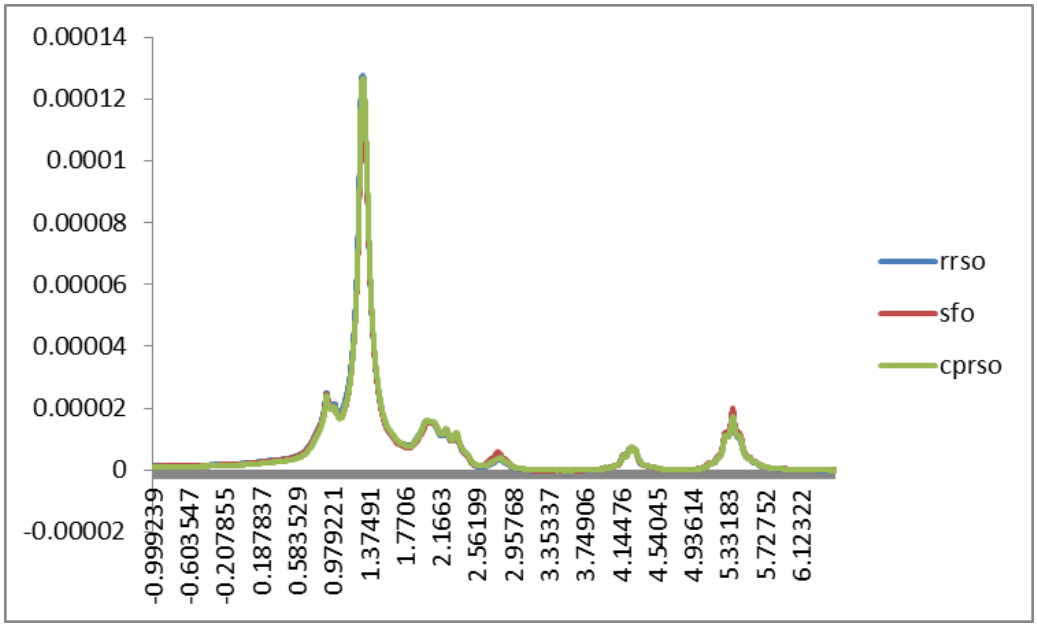
The aim of this study was to establish if a 60Hz NMR coupled with chemometic analysis could be used to detect when CPRSO has been adulterated with either RRSO or SFO, and if so to what extent. For a full description of the materials and methods see Appendix A. Further data on mixtures and oils used can be found in the Appendix B

Results and discussion

Spectral exploration

All vegetable oil consists of triglycerides (~98%) which give a very characteristic NMR profile dominated by the magnetic resonance of hydrogens near a double bond, i.e. acyl chain [=(CH2)n] at 1.23 ppm. Here, the signal acquired by the low-field 60MHz NMR instrument consisted of sharp peaks and minimal noise. When the NMR spectra of RRSO, SFO and CPRSO samples are superimposed (Figure 2.2), it is possible to distinguish some small differences. NMR sample signals are almost identical with the green colour (CPRO) dominating because it is the upper colour superimposing. The different unsaturated fatty acid composition of SFO (red colour) is highlighted in the magnetic resonance of 5.4 (olefinic protons) and 2.7 (bis allylic carbon protons) ppm and therefore differs from the rest of the oil samples. A blue colour (representing RRSO) is not present because it is identical in composition and in the magnetic resonance of its protons to the CPRO sample (green). This indicates that only multivariate analysis can assist in the interpretation of the spectra and provide a method for the discrimination of these samples that are almost identical in composition.

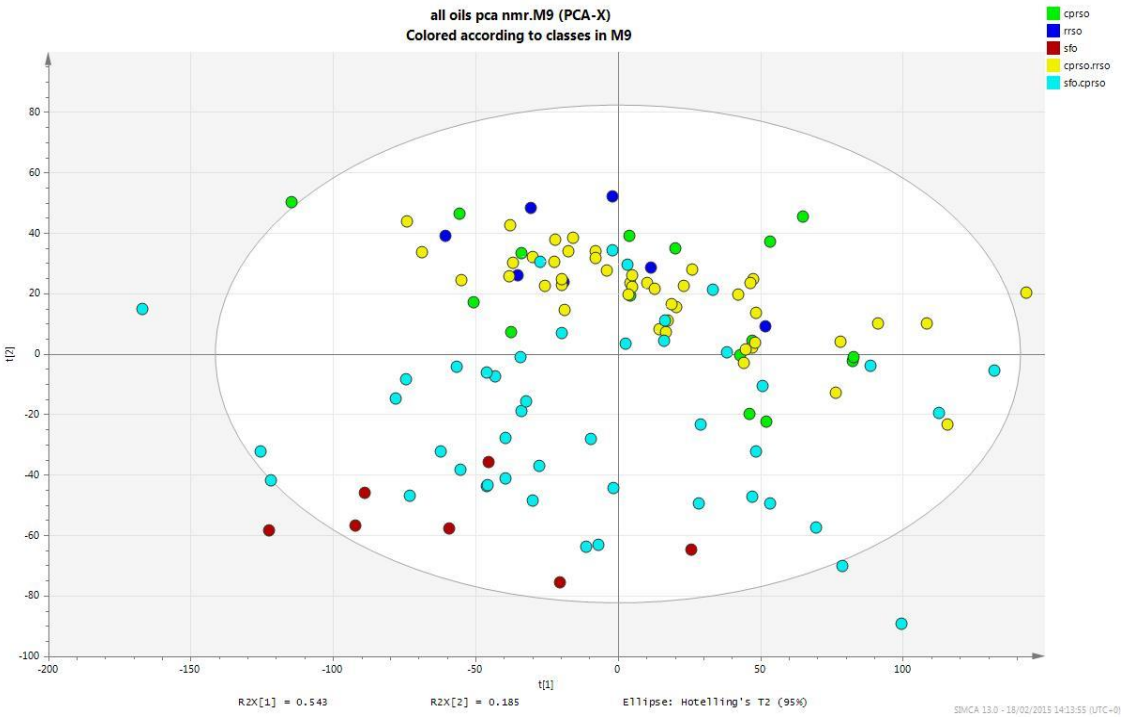
Figure 2.2: Superimposed spectra of RRSO, SFO and CPRSO



Data exploration: Principal Component Analysis

A Principal Component Analysis[1] (PCA) of all the oils was performed to illustrate the differences between each oil and each class of oil. Between the three pure oils of CPRSO, RRSO and SFO, both types of rapeseed oil were found very close to one another. This was to be expected as they would have very similar fatty acid compositions. Refined sunflower was found much further away on the PCA graph which confirms the assumption that of the three oils, SFO differs the most from the others.

Figure 2.3: Principal Component Analysis (PCA) of all pure oils and oil mixes. CPRSO-CPRSO (green); RRSO (navy blue); SFO – SFO (red); CPRSO-RRSO – CPRSO (yellow) and SFO-CPRSO – SFO CPRSO mixes (turquoise).



[1] Principal component analysis (PCA) is a statistical procedure that uses an orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components (also called principal modes of variation). PCA is mostly used as a tool in exploratory data analysis and for making predictive models.

Also as expected, the SFO and CPRSO mixes were situated close to the pure SFO samples and were also quite separate from the other oils in the PCA (Figure 2.3). The pure cold-pressed rapeseed oils, the RRSO and the CPRSO/RRSO mixes were all found within the same area with no noticeable spacing between the three groups. It would appear that the differences in minor compound composition between the oils are not a large enough factor to successfully differentiate between them. This would suggest that further classification analysis may be difficult with these three classes of oils.

Classification analysis

Following spectral exploration and the confirmation of the different oil classes present, the dataset was randomly divided into a calibration (70%) and validation set (30%). The calibration set was used to train the chemometric model using classification analysis. The accuracy of the method was determined using the validation set. Both Soft Independent Modelling by Class Analogy (SIMCA) and Partial Least Squares Discriminant Analysis (PLS-DA) classification techniques were carried out on 3 datasets (original, expanded and combined).

Table 2.1: Example of SIMCA table output: Sunflower oil (SFO) and cold pressed rapeseed oil (CPRSO) mixes

	SFO	SFO and CPRSO	CPRSO
100% CPRSO	1.47E-05	0.121006	0.033719
100% CPRSO	1.30E-05	0.0176664	0.015885
100% CPRSO	0.00058928	0.188833	0.01958
93% CPRSO	6.00E-05	0.0583584	0.050435
86% CPRSO	7.25E-05	0.281929	0.127003
79% CPRSO	0.00019945	0.165188	0.02259
72% CPRSO	0.00080699	0.0452297	0.005968
65% CPRSO	0.00042723	0.0162916	7.59E-05
58% CPRSO	0.00452133	0.221029	0.000175
51% CPRSO	0.0129635	0.18206	5.77E-05
44% CPRSO	0.0482765	0.333792	4.01E-06
37% CPRSO	0.0302516	0.131454	2.18E-07
30% CPRSO	0.0284955	0.0710267	7.97E-08
23% CPRSO	0.0611765	0.240332	5.99E-09
16% CPRSO	0.105662	0.284673	3.68E-09
9% CPRSO	0.0552296	0.181632	1.33E-10
2% CPRSO	0.100121	0.0011332	1.69E-11
100% SFO	0.00082182	6.45E-08	4.90E-13
100% SFO	0.00034471	6.14E-07	4.67E-13

Table 2.1 shows a typical SIMCA output, where green highlights that the model has a high confidence that the prediction is accurate. An amber coloured box denotes that the model has predicted that there is a possibility the sample is classified correctly, whilst a colourless box shows no significant confidence in prediction. Table 2.1 shows the areas where errors occurred in all three columns at some point. It may be possible that these errors could be reduced with larger class sizes to further enhance the difference between classes (Brereton 2006).

With regards to the performance of the different classifiers, overall, SIMCA consistently produced results with higher sensitivity than PLS-DA (Table 2.2). For the original CPRSO and SFO scored relatively high (37%) compared to CPRSO and RRSO (71%). The expansion of the dataset greatly helped to increase the sensitivity for CPRSO and RRSO classification. When all 5 classes were combined in a SIMCA analysis, two thirds of the dataset were classed correctly. The output for PLS-DA was less accurate than SIMCA for all datasets. There was no difference in performance between RRSO and SFO prediction. They both scored consistently low scores of 33% for the original and expanded sets. The score was slightly higher when 5 classes were combined (44%) though still lower than 50%.

Table 2.2: Summary of classification performance

		Correct Classification rate (%)		
		Original set	Expanded set	Combined dataset
SIMCA	CPRSO vs RRSO	37%	**77%**	**66%**
	CPRSO vs SFO	71%	64%	
PLS-DA	CPRSO vs RRSO	33%	33%	40%
	CPRSO vs SFO	33%	33%	

Conclusions

Initial data exploration showed that the pulsar NMR instrument was able to sufficiently differentiate SFO from the other classes of oils. The signal from the instrument was discriminative enough to also identify a proportion of mixed vegetable oils (i.e. adulterated samples). When 3 classes were analysed (CPRSO vs RRSO vs mixtures of the two and CPRSO vs SFO vs mixtures of the two) the sensitivity was higher than 5 classes (all oil types present in the same problem). This was to be expected as more classes result in more possibilities for oil classification. In other words the simplest the analytical problem i.e. the adulteration investigation, the better the system is at predicting the nature of unknown samples. Limitations in the current design with regards to the number of oils used in the calibration set is a contributing factor to the classification error found in results. In most cases in multivariate analysis, a larger dataset should significantly improve correct classification rate.

Although a 60 MHz NMR has been used before to analyse edible oil mixtures to the same standard as an FT-IR machine (Parker et al 2014), this study found it was slightly less successful than FT-IR (66-76%). This should be taken in the context that the success rate of FT-IR for the "SIMCA combine dataset" was 78%, illustrating that no technique was able to get close to 100% sensitivity. As mentioned earlier, the sensitivity of the NMR would be likely to increase with a larger training dataset and prediction set. There seems to be potential for 60MHz NMR to become a common appliance in the food authentication sector. Its ease of use and quick analysis time make it comparable to other spectroscopic techniques. Vegetable oils are an excellent matrix to explore with this type of NMR.

To be fair, the authenticity problems related to vegetable problems are often too complex and too challenging even for established chemical techniques such as chromatography. Other simpler problems such as prediction of a particular fatty acid or even the fatty acid profile, oxidation status of the oil or other quality parameters can be easily predicted with the correct calibration models and the use of low frequency NMR.

3 Evaluation of spectroscopic techniques to detect the authenticity of cold pressed rapeseed oil

Introduction

The three spectroscopic techniques used in this study all produce spectra dependent on the oil composition. The 400 MHz NMR produces peaks (Figure 3.1) specific to different components of the triacylglyceride compound e.g. peaks between 4 - 4.5 correspond to the glycerol fraction of the compound. As these triacyglyceride peaks correspond to about 97% of the oils composition, the peaks corresponding to minor compounds are often lost behind these much larger peaks (Hidalgo and Zamora 2003). The FT-IR spectrum (Figure 3.2) is produced when the subject (oil) interacts with an IR beam to produce an emission spectrum based on the composition of the subject tested. For example the peaks between 2700-3100 are caused by C-H stretching. Raman spectroscopy (Figure 3.3) is different to IR spectroscopy as it uses a light beam which interacts with the subject (oil) to shift the energy in the beam up or down. Raman and FT-IR could be seen as complementary some molecules are not FT-IR active (will not produce a peak) but Raman active (will produce a peak) and *vice versa*. For a full description of the materials and methods see Appendix C. Further data on mixtures and oils used can be found in the Appendix D.

The aim of this study was to establish if any of the three types of spectroscopic analysis could be used to detect when CPRSO which has been adulterated with either RRSO or SFO, and if so to what extent?

Figure 3.1: Example of CPRSO 400MHz NMR spectra

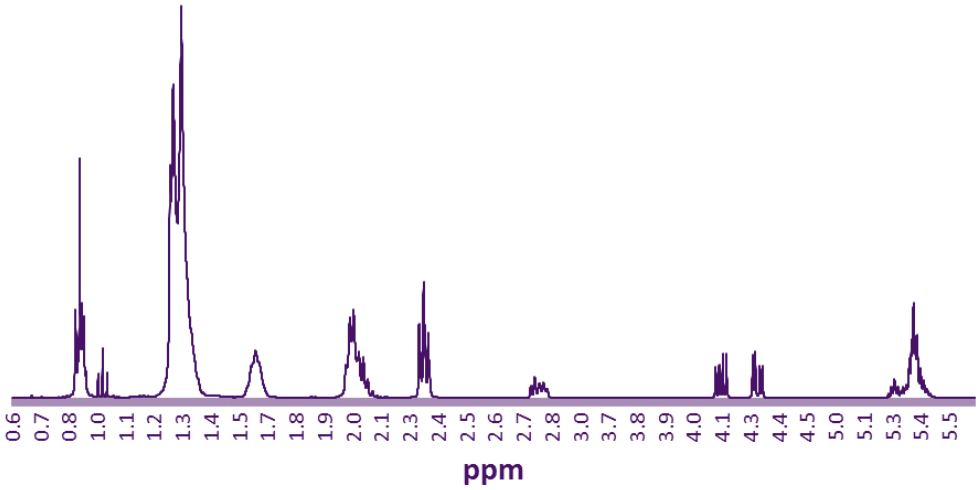


Figure 3.2: Example of CPRSO FT-IR spectra

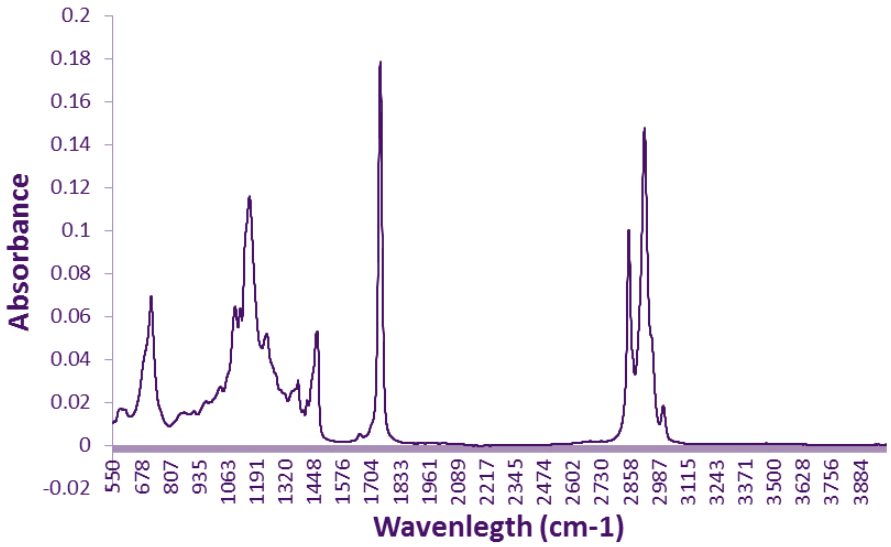
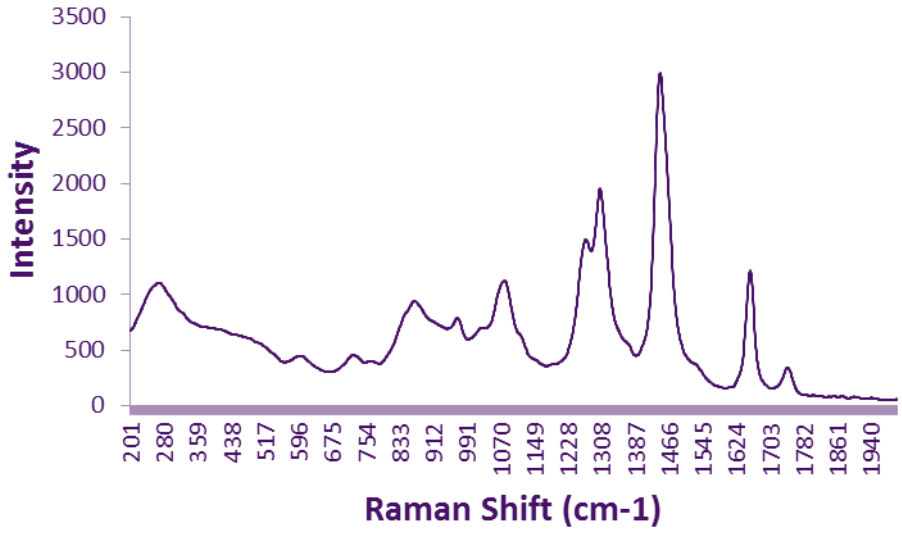


Figure 3.3: Example of CPRSO Raman spectra



Results and discussion

Data exploration using Principal Component Analysis

PCA was performed to indicate the level of separation between the different oil classes. A good spread between oil groups and clustering within groups would indicate that classification is likely to be more successful.

The FT-IR PCA (Figure 3.4) shows good separation of SFO and sunflower/CPRSO mixes from the oil groups. The diagram shows that the pure rapeseed oils and the refined/CPRSO mixes, were less separated, therefore highlighting the similarities that refined and CPRSO. Raman spectroscopy PCA (Figure 3.5) again shows good separation regarding SFO and its mixtures but the rapeseed oils. The PCA also shows some points outside of the confidence zone, these outliers were removed before the classification step of the analysis took place. The 400 MHz NMR PCA (Figure 3.6) showed good separation with SFO but not with rapeseed oil. This highlights the problem of refined and CPRSO differentiation, as even a high field NMR seems to struggle to produce spectra which contain enough differences between the two groups.

Figure 3.4: PCA score plot using FT-IR spectra. CPRSO (light blue), SFO (yellow), RRSO (dark blue), RRSO and CPRSO mix (green), SFO and CPRSO mix (red).

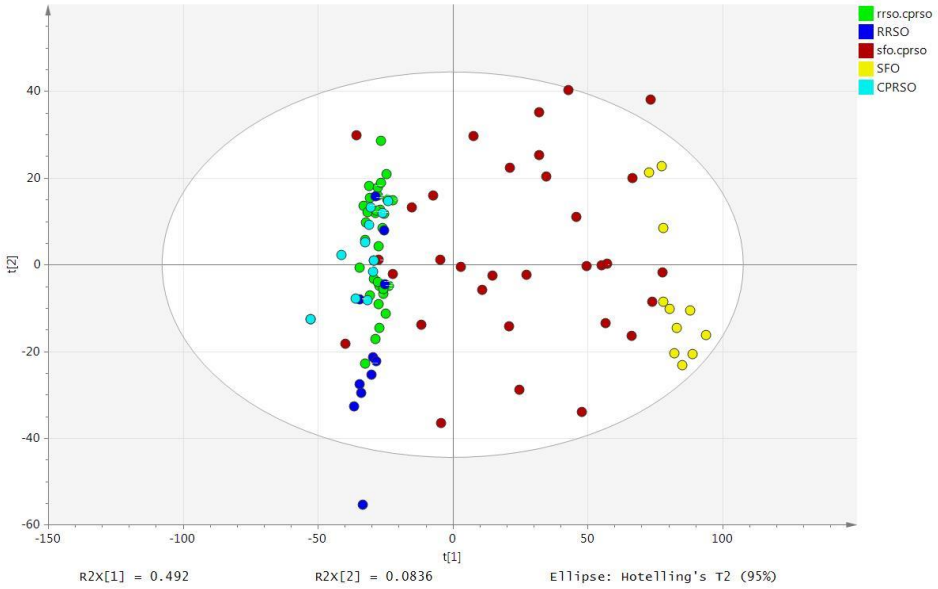


Figure 3.5: PCA score plot using Raman spectra. CPRSO (dark blue), SFO (light blue), RRSO (red), RRSO and CPRSO mix (green), SFO and CPRSO mix (yellow).

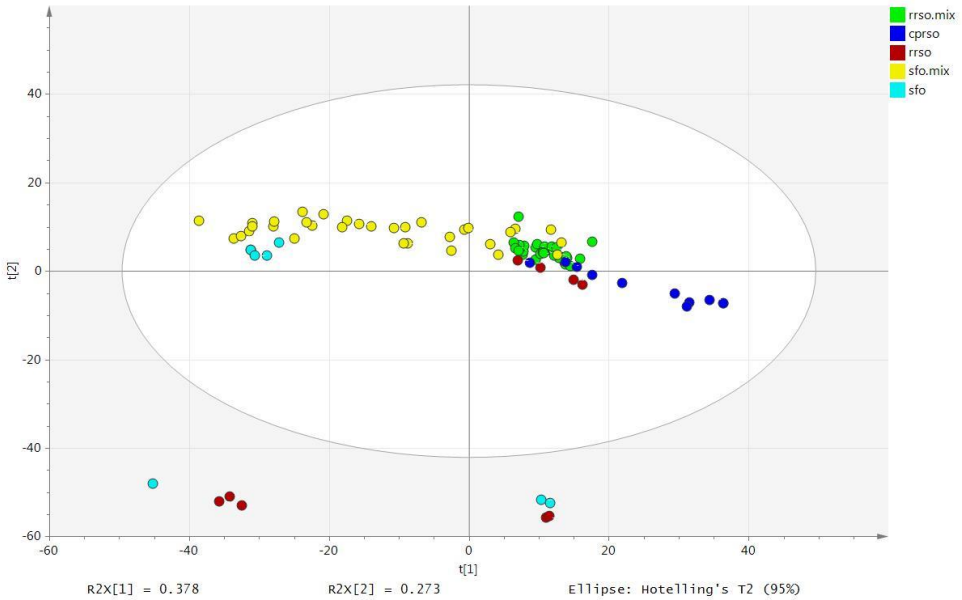
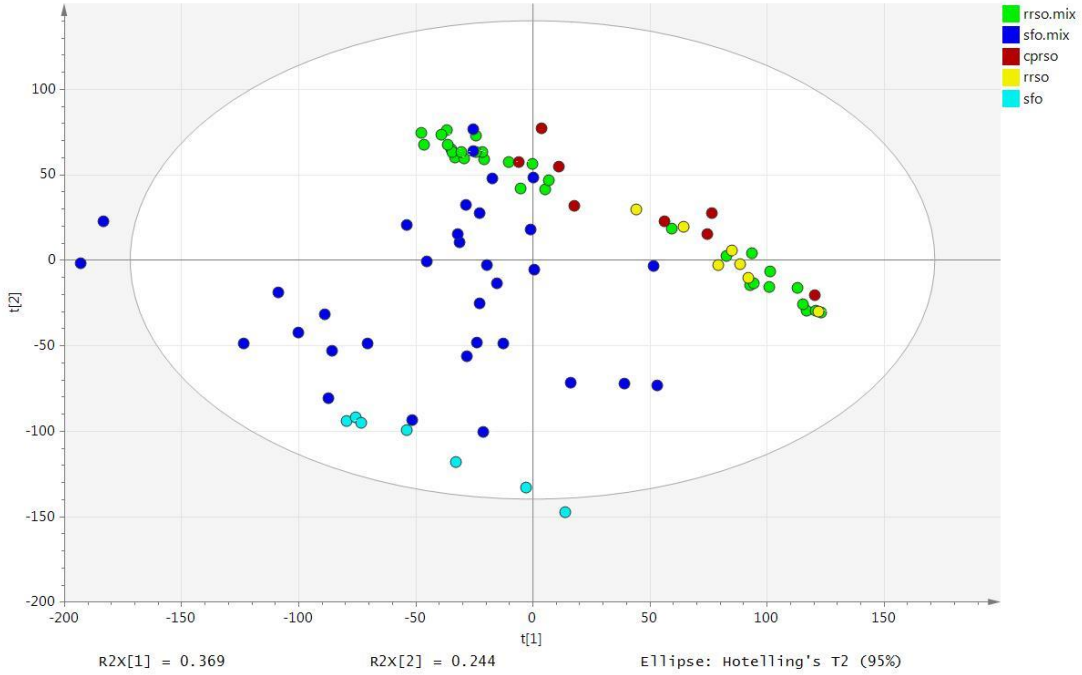


Figure 3.6: PCA score plot using 400 MHz NMR spectra. CPRSO (red), SFO (light blue), RRSO (yellow), RRSO and CPRSO mix (green), SFO and CPRSO mix (dark blue).



Example of classification output

Table 3.1: Example of classification output: 5 Class dataset

Primary	Class I.D.	File	RRSO.CPRSO	RRSO	SFO.CPRSO	SFO	CPRSO
86	RRSO.CPRSO	37 av	0.540432	0.106533	0.520077	-0.23345	0.06641
87	RRSO.CPRSO	38 av	0.234738	-0.00798	0.626698	-0.19714	0.343687
88	RRSO.CPRSO	39 av.	0.281821	0.280868	0.631334	-0.25198	0.057952
89	RRSO.CPRSO	40 av.	0.173994	0.029835	0.965762	-0.32829	0.158703
90	RRSO.CPRSO	41 av.	0.437888	0.271854	0.665187	-0.30342	-0.07151
91	RRSO.CPRSO	42 av.	0.396241	0.080986	0.792512	-0.28751	0.01777
92	RRSO.CPRSO	43 av.	0.515494	0.283625	0.658013	-0.34168	-0.11545
93	RRSO.CPRSO	44 av.	0.3794	0.12716	0.688811	-0.25691	0.061541
94	RRSO.CPRSO	45 av.	0.56566	0.166022	0.622553	-0.27681	-0.07743
95	RRSO.CPRSO	46 av.	0.242172	0.251769	0.529154	-0.24913	0.226034
96	RRSO.CPRSO	47 av.	0.443816	0.279926	0.824553	-0.34807	-0.20023
97	RRSO.CPRSO	48 av.	0.379234	0.293768	0.622395	-0.25264	-0.04276
98	RRSO.CPRSO	49 av.	0.17137	0.385513	0.621463	-0.26332	0.084973
99	RRSO.CPRSO	50 av.	0.306457	0.48753	0.339783	-0.1475	0.013731
100	RRSO	p52	0.368379	0.533661	0.530554	-0.21568	-0.21692
101	RRSO	p53	0.0533025	0.568866	0.851236	-0.36427	-0.10914
102	RRSO	p60	0.451671	0.525885	-0.34503	0.213899	0.15357
103	RRSO	p61	0.205219	0.874764	0.145315	0.01305	-0.23835
104	RRSO	p62	0.307115	0.593497	-0.22894	0.111716	0.216609
105	SFO	p50	-0.06301	0.001009	0.47162	0.663455	-0.07307
106	SFO	p51	0.0031804	-0.02974	0.617401	0.609096	-0.19994
107	SFO	p69	-0.221334	0.08671	0.015541	0.847206	0.271877
108	SFO	p70	-0.341929	0.519668	0.231055	0.667837	-0.07663
109	SFO	p71	-0.280524	0.099762	0.164269	0.795488	0.221006
110	SFO.CPRSO	88av.	0.634035	0.187291	0.042363	0.053893	0.082418
111	SFO.CPRSO	89av.	0.629492	-0.02691	0.569581	-0.18555	0.013385
112	SFO.CPRSO	90av.	0.328834	0.151753	0.434802	-0.05908	0.143691
113	SFO.CPRSO	91av.	0.385067	0.168706	0.511977	-0.01956	-0.04619
114	SFO.CPRSO	92av.	0.236314	0.14153	0.352838	0.13728	0.132039
115	SFO.CPRSO	93av.	0.0266089	0.165728	0.758576	-0.00738	0.056472
116	SFO.CPRSO	94av.	0.26377	-0.05542	0.846025	-0.04274	-0.01163
117	SFO.CPRSO	95av.	0.245441	-0.13871	0.83515	0.150225	-0.09211
118	SFO.CPRSO	96av.	-0.09402	0.149921	0.382325	0.393595	0.168177
119	SFO.CPRSO	97av.	0.128096	-0.13725	0.53307	0.341864	0.134221
120	SFO.CPRSO	98av.	-0.139902	0.154243	0.586025	0.393803	0.005831
121	SFO.CPRSO	99av.	-0.098635	0.035624	0.497786	0.46169	0.103534
122	SFO.CPRSO	100av.	0.14484	-0.22841	0.90388	0.309697	-0.13001
123	SFO.CPRSO	101av.	-0.050254	-0.11433	0.950114	0.326075	-0.1116
124	CPRSO	p33	0.144353	0.371479	-0.03088	0.00508	0.509969
125	CPRSO	p34	0.330162	0.302214	-0.26276	0.134757	0.495622
126	CPRSO	p24	0.039033	0.158238	-0.11709	0.139695	0.780122
127	CPRSO	p43	-0.425474	-0.36184	0.497956	-0.00096	1.29032

Table 3.1 shows an example of a classification model generated from the Umetrics software. The class I.D. column indicates the true nature of the oil, while the colour in each column indicated the strength of prediction in the model of a correct classification. Green indicates the model calculated a high possibility that the oil has been correctly classified, amber signifies that there is medium strength in the prediction, while no colour indicates no confidence.

3.2.3 Classification analysis

Table 3.2: Summary of classification performance using FT-IR

		Correct Classification rate (%)	
		3 Class dataset	5 Class dataset
SIMCA	CPRSO vs RRSO	39%	24%
	CPRSO vs SFO	87%	
PLS-DA	CPRSO vs RRSO	78%	57%
	CPRSO vs SFO	87%	

Table 3.3: Summary of classification performance using Raman

		Correct Classification rate (%)	
		3 Class dataset	5 Class dataset
SIMCA	CPRSO vs RRSO	30%	31%
	CPRSO vs SFO	48%	
PLS-DA	CPRSO vs RRSO	65%	57%
	CPRSO vs SFO	39%	

Table 3.4: Summary of classification performance using 400 MHz NMR

		Correct Classification rate (%)	
		3 Class dataset	5 Class dataset
SIMCA	CPRSO vs RRSO	53%	66%
	CPRSO vs SFO	89%	
PLS-DA	CPRSO vs RRSO	79%	71%
	CPRSO vs SFO	94%	

The rate of correct classification varied widely with regards to the spectroscopic instruments, chemometric techniques and type of data set analysed. The FT-IR spectroscopy classification varied hugely regarding chemometric technique. When classifying CPRSO and RRSO (3 column analysis)

SIMCA gave a low rate of 39% while PLS-DA was much more successful with 78% correctly classified. This rate of 78% correct classification seems impressive as the PCA diagram (Figure 3.1) illustrated how close the two types of oil are in their composition. The chemometric technique did not have any effect with regards to SFO and rapeseed oil classification, as both SIMCA and PLS-DA achieved 87% correct classification rate. When the classes were combined into a 5 class analysis the correct classification rate dropped to 24% for SIMCA and 57% for PLS-DA (Table 3.2). This would indicate that FT-IR can be a useful tool in CPRSO identification but that it has limitations regarding the number of classes in the model.

The classification results when using Raman spectroscopy were similar to FT-IR when 5 classes were analysed but the technique was not as good as FT-IR when 3 classes were used. Unexpectedly the best classification rate for a Raman dataset came from a CPRSO and RRSO dataset. When using PLS-DA the model was able to produce a classification rate of 65% for this dataset (Table 3.3). Much like with the FT-IR results, when 5 classes were analysed in the Raman model, the correct classification rate dropped to lower levels (31% SIMCA and 57% PLS-DA).

The 400 MHZ NMR spectra produced the highest classification rates out of all three techniques. The highest classification rate was 94% for CPRSO and SFO 3 class analysis (Table 3.4). This would be expected as NMR spectra give detailed information regarding the fatty acid composition of oils. As SFO differs in its fatty acid composition from rapeseed oil, especially in the polyunsaturated C18 groups, it is understandable why correct classification rate is high in this instance. With regards to the CPRSO and RRSO 3 class analysis, a PLS-DA model was able to correctly classify 79% correctly. This is 1% higher than FT-IR was able to do with the same oils. When all 5 classes were analysed the NMR was able to produce much higher levels of correct classification (SIMCA 66% AND PLS-DA 71%) than FT-IR or Raman. The low field NMR classification results can be found in Chapter 3. The results are not in par with the high filed NMR and are in line with the vibrational spectroscopy instruments.

Conclusions

The initial PCA diagrams showed the clear differences between rapeseed oil and SFO. They also showed the similarities between CPRSO, RRSO and mixtures of the two. Constructing a model of classification of these three rapeseed classes is clearly challenging. The SFO mixtures were expected to produce higher classification rates due to their differing composition as highlighted in Figures 3.1-3.3. With regards to vibrational spectroscopy techniques, FT-IR was found to be superior to Raman spectroscopy, achieving good prediction rates when 3 classes were analysed. Neither the analysis with Raman nor FT-IR spectroscopy were able to achieve classification rates above 57% when using a 5 class dataset. It would therefore appear that benchtop techniques are currently limited to dealing with two pure oils and one binary mixture. The 400MHz (high filed) NMR provided the best classification rate for all the types of datasets. It was particularly better at 5- class PLS-DA analysis, 14% higher than either FT-IR or Raman. With regards to the 3 class analysis, the high filed NMR was better than FT-IR but only by 1% for CPRSO+RRSO and 7% for CPRSO+SFO. The analysis with FT-IR competed well against the analysis with NMR, considering the gap in capital cost and resources for maintenance between both instruments. The FT-IR would provide a quick, low cost and easy method to screen for oil datasets with 3 oil classes. An interesting development is the recent introduction of the low-field NMR (Chapter 2). It has the potential to offer 'the best of both worlds', the low instrument footprint and low maintenance cost of an FT-IR) and resolution and molecular structure information of a NMR instrument. In practice and especially in oil analysis that is studied here, the low field NMR delivers similar, and in most cases slightly lower, classification rates compared to FTIR. In fact, the low resolution of 60 MHz instrument, deriving from a weaker magnet, is simply not specific enough compared to the rich molecular vibration information captured by the FT-IR and thus, coupled with the cost, sample acquisition procedure, speed of analysis and versatility of the instrumentation, FTIR remains the obvious choice when conducting untargeted vegetable oil classification analysis.

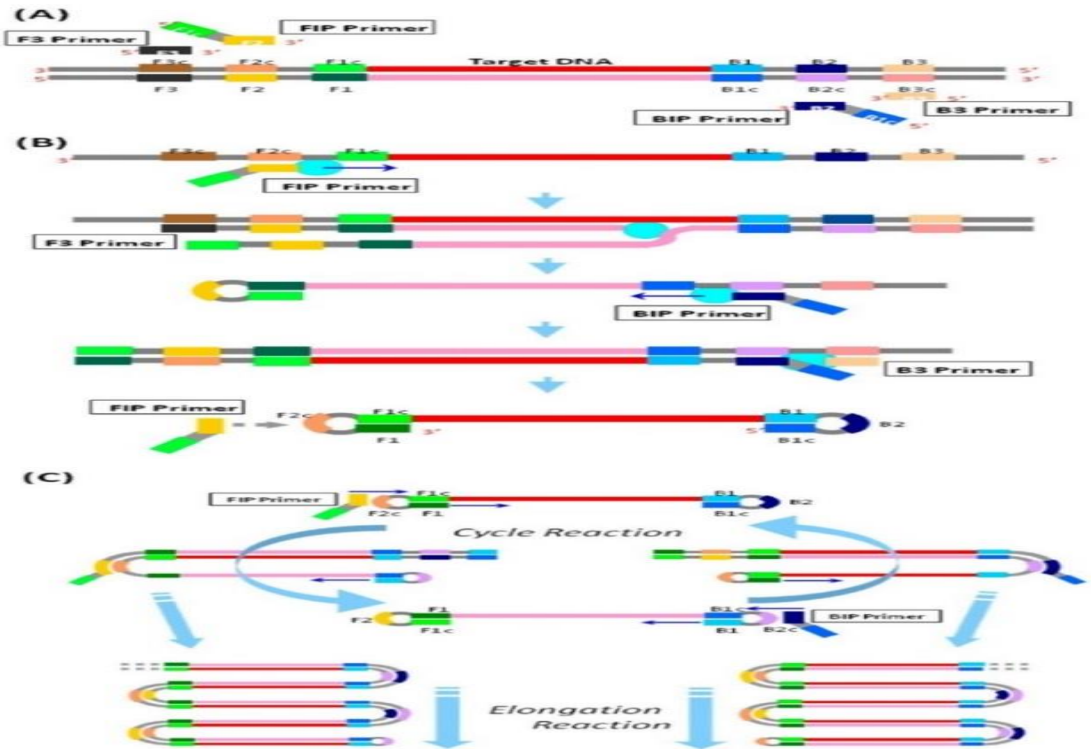
4 Loop-mediated Isothermal Amplification Assay

Goats cheese authenticity by LAMP assay: validation and survey

Introduction

The LAMP assay is a nucleic acid amplification method that rapidly amplifies target DNA with high specificity and efficiency (Ahmed et al., 2010). A major advantage of the technique is the ability to amplify specific sequences of DNA under isothermal conditions, decreasing the analysis time. A schematic representation on how LAMP works is shown in Figure 4.1. For a full description of the materials and methods see Appendix E and the LAMP assay validation method see Appendix H. The present study was designed to validate a commercially LAMP method to assess the authenticity of goats' cheese within the Great Britain and the IoI.

Figure 4.1: Schematic representation of the mechanism of LAMP.



The steps in the procedure are characterized by the use of carefully designed primers, specifically used to recognize distinct regions on the target gene (A). The reaction process proceeds at a constant temperature (eliminating the use of a thermal cycler commonly used in PCR) using strand displacement reaction. Thus, amplification and detection of DNA can be completed in a single step (What-When-How, 2014 and Optigene, 2014).

Results and discussion

4LAMP assay validation (cheese)

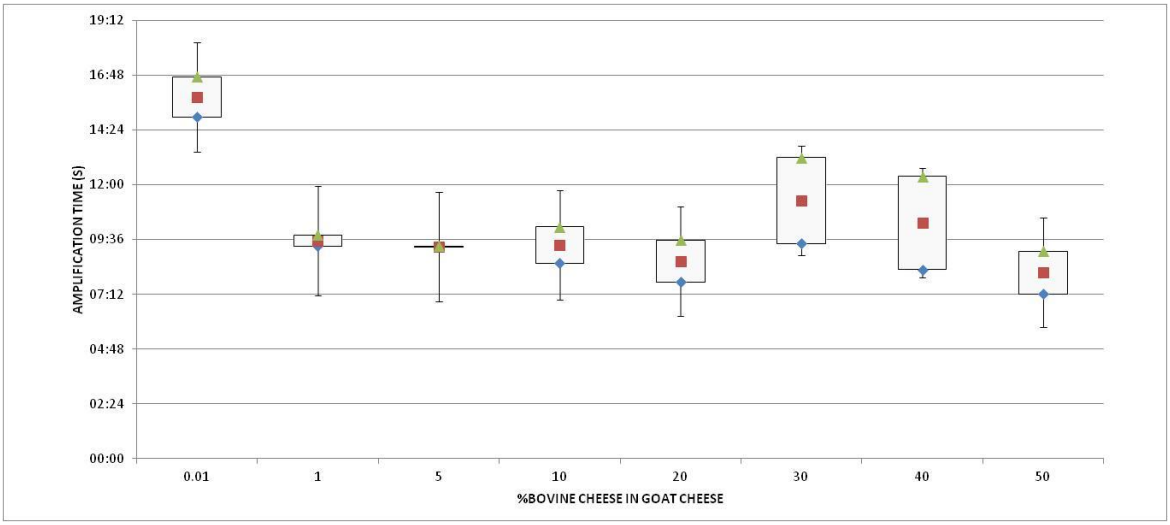
The LAMP assay was validated in terms of specificity, selectivity and Limit of Detection (LoD). Using cow, goat and sheep specific primers, ten cow, goat and sheep cheese samples were correctly identified as positive. No amplification of other non-declared species was found, demonstrating no detectable cross reactivity. Hard and soft goat cheese was analysed by melting curve analysis, of which there was no differences were noted (standard deviation 0.17; 84.61ºC ±0.12 ºC). The signature melting temperature was given as 84.3ºC (±0.5ºC) for the specific goat amplicon (Table 4.1).

Table 4.1: LAMP assay annealing temperatures

Reaction Mix	'Signature' annealing temperature (±0.5°C)
Cow	84.7°C
Sheep	83.6°C
Goat	84.3°C
Positive Control	89.2°C

In order to determine the LoD and selectivity of the LAMP assay, goat cheese was analysed with varying additions of cow and sheep cheese (with 0.01%, 1%, 5%, 10%, 20%, 30%, 40% and 50%). The limit of detect was 0.01%. The detection time ranged from 08.11 (±1.2) min for concentrations containing 50% cows' cheese and 16.43 min for concentrations containing 0.01% cows' cheese (Figure 4.2). A longer amplification time was found for goat samples with 30% and 40% cow cheese added. This may be due to different efficiencies of the primers, meaning each assay is unique it terms of its speed and sensitivity. The goats' cheese adulterated with sheep cheese demonstrated a faster reaction time of between 7 and 9 (±1.0) min over the same concentration range. The faster reaction time for the sheep assay was expected in line with manufacturer's guides.

Figure 4.2: Box plots of detection time of LAMP assay with a detection limit of 0.01% adulteration with cows' cheese



LAMP analysis

The results from the LAMP analysis demonstrate no issue in terms of goat cheese adulteration with cow milk (Table 4.3). Trace amounts of cow DNA were found in 17 goats' cheese samples (18%), of which the majority (69%) where purchased in delicatessen's or farmers markets. It is probable that these samples became contaminated with cow's cheese post production as opposed to intentionally adulterated.

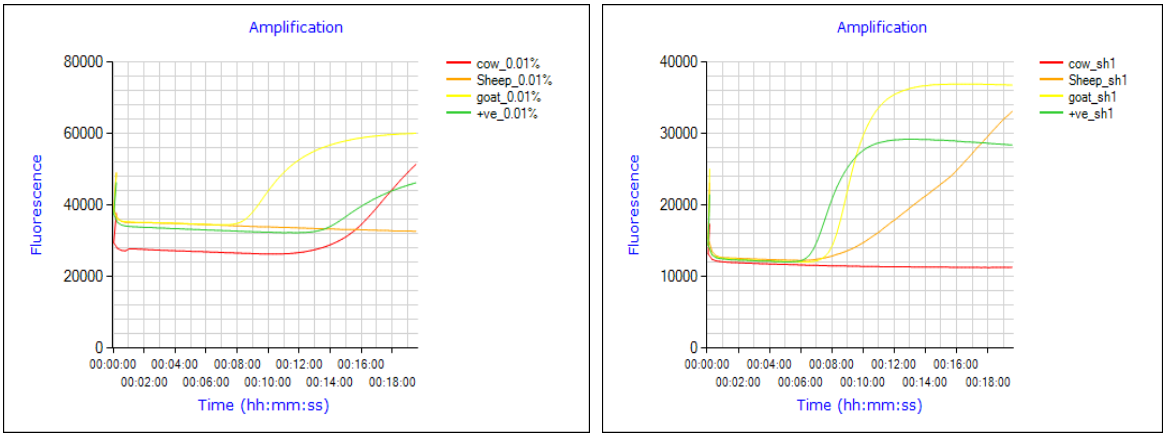
Table 4.3: Goats' cheese samples containing cow or sheep DNA (non-labelled) determined from the LAMP analysis and estimated quantities (%)

Positive Sample	Cow DNA	Sheep DNA	Estimated Quantity
PP2	-	+	80% sheep
PP3	-	+	trace sheep
PP4	+	-	trace cow
PP7	+	+	trace sheep and cow
PP8	+	-	trace cow
PP9	+	-	trace cow
PP10	+	+	trace cow, 5-10% sheep
PP11	-	+	trace sheep
SH1	-	+	trace sheep
SH7	+	-	trace cow
SH9	-	+	trace sheep
BH3	-	+	trace sheep
BH5	-	+	trace sheep
BH9	+	+	trace cow and sheep
BH10	-	+	trace sheep
GW2	+	-	trace cow
GW4	-	+	trace sheep
GW8	+	-	trace cow
GW9	+	+	trace cow, ~80% sheep
BY1	-	+	trace sheep
BY8	+	-	trace cow
BY9	+	-	trace cow
BY10	+	+	trace cow, definite sheep >goat
RH3	-	+	trace sheep
RH4	-	+	trace sheep
RH5	+	+	trace cow and sheep
RH6	-	+	85% sheep
RH7	-	+	Definite sheep more than goat
RH9	-	+	50% sheep
OX2	+	-	trace cow
OX3	+	+	trace cow, 5-10% sheep
OX10	-	+	trace sheep
NI1	-	+	trace sheep
NI3	-	+	5-10% sheep
NI5	-	+	trace sheep
FET	+	-	trace cow
SHF	-	+	trace sheep

+ indicates the presence of that species: - indicates the absence of that species

The most interesting finding of the study was the number of goats' cheese samples found to contain sheep DNA. Of the 94 goats' cheese samples analysed, 28 (30%) were found to contain sheep DNA in varying quantities. Of the 28 samples positive for sheep DNA, 67% of these were determined to be trace amounts. From the amplification curve in Figure 4.3a, the gradient of the orange line indicating sheep DNA is much lower in comparison to both the yellow and green line (goat and positive respectively). This suggests that there is only trace amounts of sheep DNA in the samples. Again it can be concluded that these samples were not intentionally adulterated but have become contaminated with sheep cheese or milk.

Figure 4.3: Comparison of the amplification curve obtained for (a) 0.01% adulteration of goat cheese and (b) goat cheese sample analysed determined to have only trace quantities of sheep DNA.

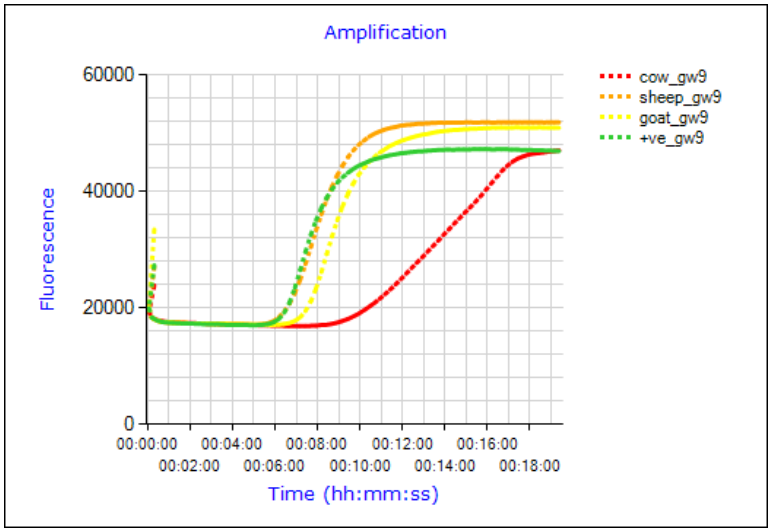


Furthermore, it was determined that 9 out of the 28 goats' cheese samples (33%) positive for sheep DNA contained over 5%. Six of the goats' cheese samples contained significant quantities, between 50 and 85% sheep DNA. Figure 4.4 shows a positive result. The height of the amplification curve, consistent with the goat (yellow) and positive (green) line, demonstrates a significant level of sheep DNA. If this is compared to the red line, showing the level of cow DNA, the gradient is much lower indicating only trace amounts are in the sample.

Interestingly, 50% of the samples containing significantly high quantities (50-85%) were imported from other countries within the EU, with three samples produced in the United Kingdom. From this data it is evident that the risk of goat cheese adulteration is significant both within the UK and Europe, with major significance for the importation of speciality cheese, especially from

France. The three positive goat cheese samples produced in the UK contained between 50% and 85% sheep DNA and were all purchased in delicatessens or farmers markets. These findings could potentially have implications for small scale processors as cheese purchased in the above outlets is indicative of low scale, farm production, possibly from own herd. However, the fraudulent action could also be down to adulterated milk from third party milk processors. It is also interesting to note that sample PP2 (labelled as 'French Goat Cheese'), containing 80% sheep cheese was made from UK milk, produced in France but packed in the UK. This type of activity could be used in the future to highlight potential risk areas, but also highlights the complexity and length of the supply chain.

Figure 4.4: Goats' cheese sample analysed using the LAMP method demonstrating a positive result for cow DNA (trace) and sheep DNA (80%).



As already discussed, the major motivation behind food adulteration is additional profit. The issue addressed in the current study is the adulteration of goats' cheese with sheep milk. From an economical point of view the substitution of goat milk for sheep milk is not viable as they have similarly high primary production costs. However, unlike goat milk, it is apparent that sheep milk is not widely available on a commercial scale within the UK. This would indicate that the majority, if not all, of the sheep milk produced in the UK goes towards the production of secondary sheep milk products including cheese, or is exported. Figures from the EU indicate that there is 69% more sheep milk produced in comparison to goat milk, however this is not the case for global figures (FAO, 2012 data (2014)). It is apparent that sheep milk is available commercially in some EU countries. This could highlight potential problems for goat milk or cheese imported from other EU countries. UK figures

are not available, but if similar statistics are assumed the question remains of where the surplus of sheep milk is going. The increase in demand for goats' cheese (Mowlem, 2005), merged with supply issues caused by fluctuations in the lactation period (Khanzadi et al., 2013) could potentially lead to fraudulent adulteration and explain the findings of this study. This supply and demand issue has also been highlighted several times by the Grocer Magazine (Grocer Magazine, 2014). Sheep milk has a higher solids content compared to goat or cow milk (Raynal-Ljutovac et al., 2008), resulting in a 10-15% greater cheese yield (Haenlein, 2002). This could further explain the motivation behind the adulteration of goats' cheese with sheep milk, as a greater cheese yield would ultimately be economically beneficial in comparison to adulteration with cow milk. From this information it can also be suggested that the cheese may have a more pleasing texture and taste, indicating that any adulteration may go unnoticed, whilst adulterating with cow milk may highlight potential quality issues.

Conclusions

The results from the current study highlight some potential areas for concern in terms goats' cheese adulteration with milk from other species. Unexpectedly, some goats' cheese was found to contain significant levels of sheep DNA, which is an area requiring further surveying. Only trace levels of cow DNA were found in the goats' cheese samples, which is probably an issue of contamination as opposed intentional adulteration. The LAMP method was easy to use, was highly sensitive and had the additional benefit of being multiplex. With regards to goats' cheese, increasing the sample set and incorporating more European samples could allow for greater intelligence gathering. It is evident from the current study that more analysis must be carried out in order to gain a wider understanding of the extent of fraud in this area and the potential impact on the economics and safety of the food supply chain.

Meat and fish authenticity by LAMP assay: validation and survey

Results and discussion: Fish

Screening of each species type with all Optigene provided kits. Samples from each species were screened against all species specific kits in order to identify if cross reactivity between species existed (Table 4.4).

Table 4.4: Screening of each species with each species specific kits

	Cod Samples	Coley Samples	Haddock Samples	Whiting Samples	Pollock Samples
Cod master mix	✓	×	×	×	×
Haddock master mix	×	×	✓	×	×
Coley master mix	×	✓	×	×	×
Pollock master mix	×	×	×	×	✓
Whiting master mix	×	×	×	✓	×

Check on synthetic controls

Synthetic controls (Table 4.5) were purchased from Optigene for the fish validation. When the controls were received they were screened against each master mix. This highlighted the fact that the cod control would work on both the cod master mix and haddock master mix and vice versa. This was also true of the pollock and whiting control. Only the coley control was species specific. This was relayed back to Optigene who were aware of this.

Table 4.5: Synthetic control check

	Cod Samples	Coley Samples	Haddock Samples	Whiting Samples	Pollock Samples
Cod master mix	✓	✓			
Haddock master mix	✓	✓			
Coley master mix			✓		
Pollock master mix				✓	✓
Whiting master mix				✓	✓

Screening of cod samples pre-contaminated with known concentration of other species

Cod material was used as the base material which was then spiked with other species at different percentages (Table 4.6). This was completed at 5 different percentages 20%, 10%, 5%, 1% & 0.1%. This work was conducted to determine if mixing multiple species affected detection of target. It also permitted us to identify that all mixing/processes method rarely permit a homogenous sample to be created (see Figures 4.12 – 4.15). No cross reactivity was detected during this process.

Table 4.6: Screening of spiked cod samples

	Coley Samples	Haddock Samples	Whiting Samples	Pollock Samples
20%	✓	✓	✓	✓
10%	✓	✓	✓	✓
5%	✓	✓	✓	✓
1%	✓	✓	×	×
0.1%	✓	×	×	×

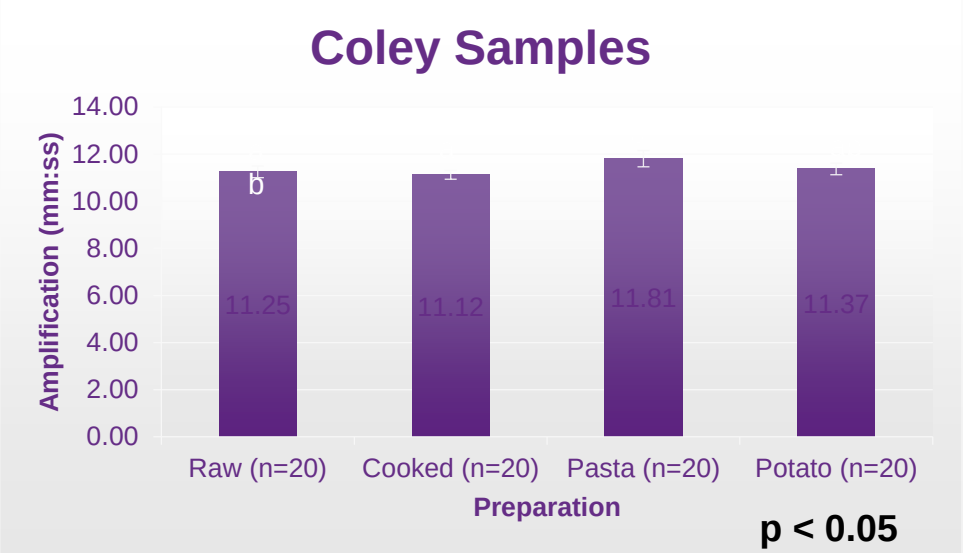
Fish processing/treatments

Twenty samples from each species were adulterated four ways. Samples were screened raw, cooked, cooked with pasta and cooked with potato. This was completed in order to identify if any species was affected by common food industry treatment/processes. Cod, haddock, whiting showed little statistical difference in result recovery between all four treatments, whereas coley and pollock did (Table 4.7 and Figure 4.5). It should be noted that coley showed a statically difference in recovery between cooked and cooked with pasta.

Table 4.7: Statistical analysis of coley adulteration

	Coley					
	N	Minimum	Maximum	Mean		Std. deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Raw (n=20)	20	10.03	13.39	11.25	.24736	1.10622
Cooked (n=20)	20	9.55	12.45	11.12	.18655	.83429
Pasta (n=20)	20	9.48	15.50	11.81	.34343	1.53587
Potato (n=20)	20	9.57	13.56	11.37	.24452	1.09352

Figure 4.5: Coley samples



As can be seen from Table 4.8 and Figure 4.6, pollock showed statistically significant higher values when prepared with potato which is also highlighted in the delayed amplification time (Ct value) in Figure 4.7.

Table 4.8: Statistical analysis of pollock adulteration

	Pollock					
	N	Minimum	Maximum	Mean		Std. deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Raw (n=20)	20	8.18	11.03	9.57	.19905	.89019
Cooked (n=20)	20	8.22	11.32	9.49	.19041	.85155
Pasta (n=20)	20	8.22	13.02	9.81	.26719	1.19492
Potato (n=20)	20	8.21	14.18	10.93	.33388	1.49314

Figure 4.6: Pollock samples

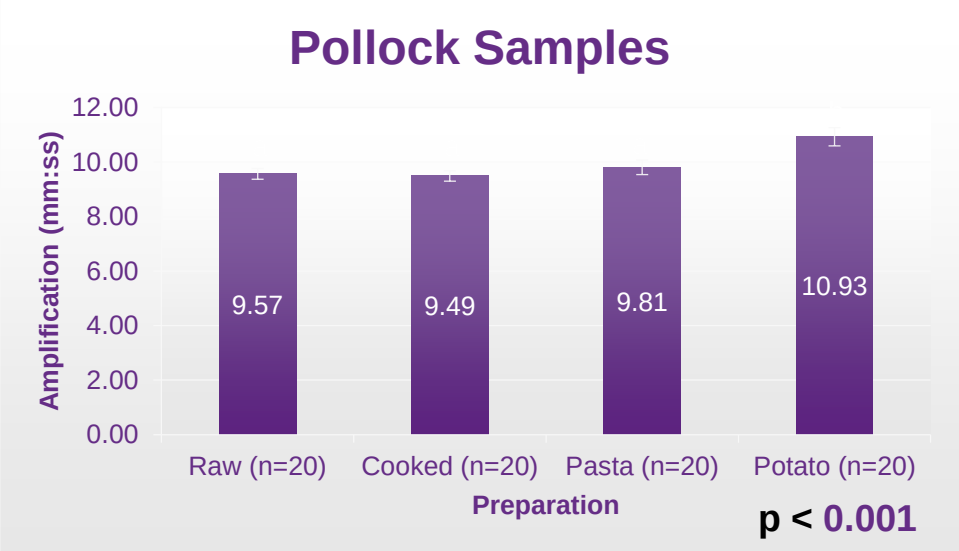
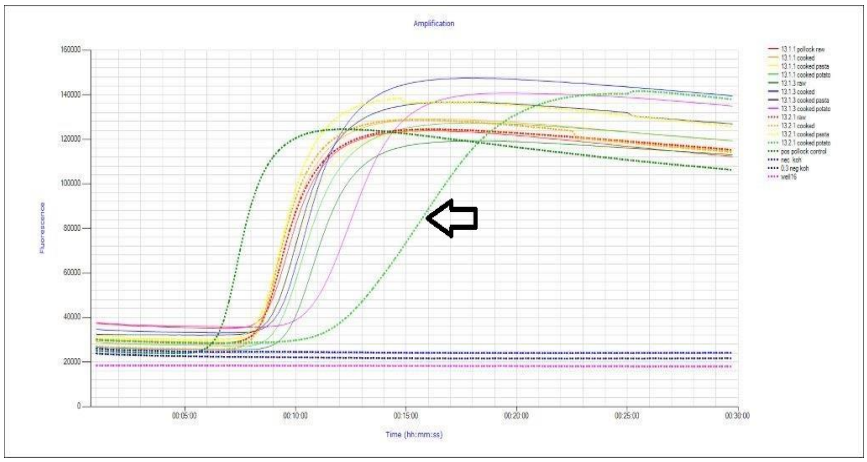


Figure 4.7: Amplification curve for adulterated pollock

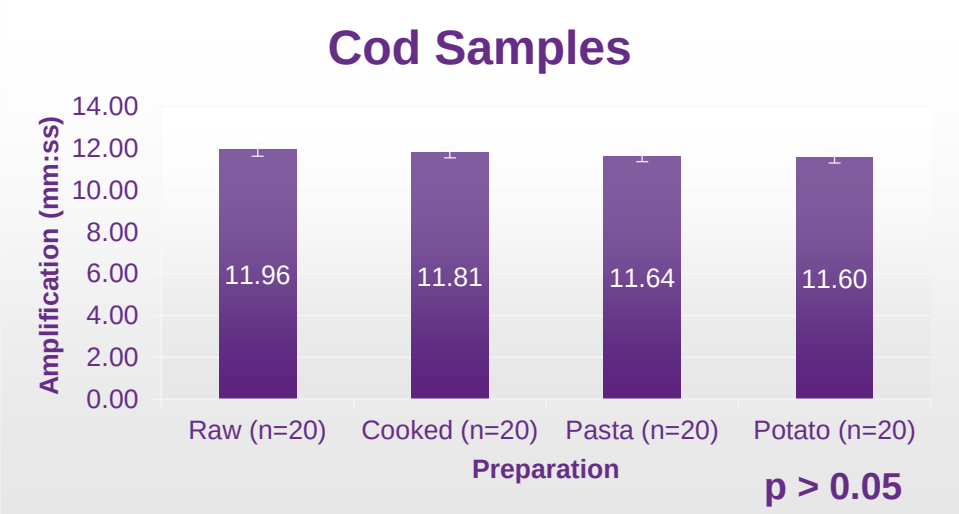


For cod there was little statistically difference between the adulterated processes (Table 4.9 and Figure 4.8). See Appendix I for an example of the amplification curve and annealing derivative.

Table 4.9: Statistical analysis of cod adulteration

	Cod					
	N	Minimum	Maximum	Mean		Std. deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Raw (n=20)	20	9.20	15.09	11.96	.32243	1.44195
Cooked (n=20)	20	9.20	13.47	11.81	.24689	1.10414
Pasta (n=20)	20	10.04	14.22	11.64	.26196	1.17153
Potato (n=20)	20	9.23	14.09	11.60	.28795	1.28776
Valid N (listwise)	20					

Figure 4.8: Cod samples

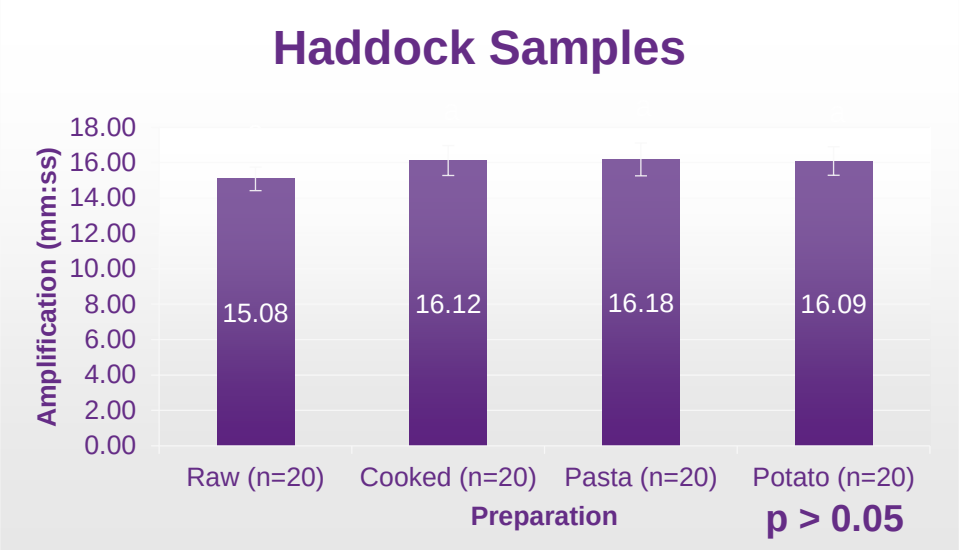


Haddock species showed no statistical significant difference between raw, cooked, cooked with pasta and cooked with potato (Table 4.10 and Figure 4.9).

Table 4.10: Statistical analysis of haddock adulteration

	Haddock			
			95% confidence interval	
	Mean	Std. Error	Lower bound	Upper bound
Raw (n=20)	15.08	.662	13.693	16.464
Cooked (n=20)	16.12	.846	14.348	17.889
Pasta (n=20)	16.18	.926	14.241	18.119
Potato (n=20)	16.09	.807	14.402	17.781

Figure 4.9: Haddock samples

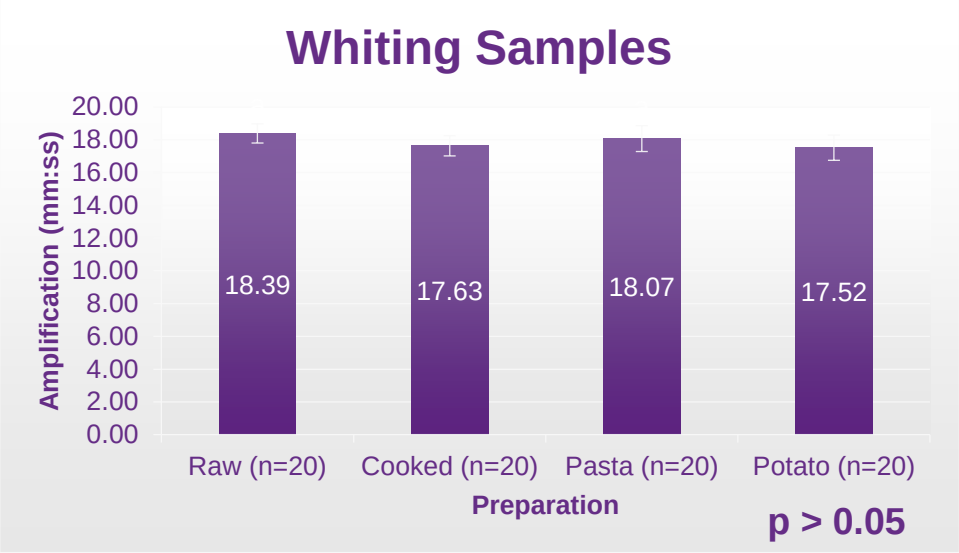


Whiting also displayed little variation statistically following adulteration (Table 4.11).

Table 4.11: Statistical analysis of whiting adulteration

	Whiting					
	N	Minimum	Maximum	Mean		Std. deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Raw (n=20)	20	15.42	24.11	18.39	.58549	2.61841
Cooked (n=20)	20	13.45	24.08	17.63	.61563	2.75320
Pasta (n=20)	20	13.36	26.09	18.07	.78865	3.52694
Potato (n=20)	20	13.52	27.38	17.52	.77013	3.44414

Figure 4.10: Whiting samples



This work demonstrated that the adulteration process had little effect on the specificity of each assay. All samples were detected in a raw, cooked, cooked with pasta and cooked with potato with little variation to CT values.

Screening of the fish speciation library

Upon validation of the LAMP assays for each species, the technique was employed to screen the stored library. A total of 378 samples were screened during this process. Samples were received from QUB in labelled bags. All samples were correctly identified (Table 4.12).

Table 4.12: Screen of known samples

	Cod samples	Coley samples	Haddock samples	Whiting samples	Pollock samples
No. Samples screened	175	20	121	20	42
No. correctly identified	100%	100%	100%	100%	100%
No. incorrectly identified	0%	0%	0%	0%	0%

Determination of limits of detection for fish species assays

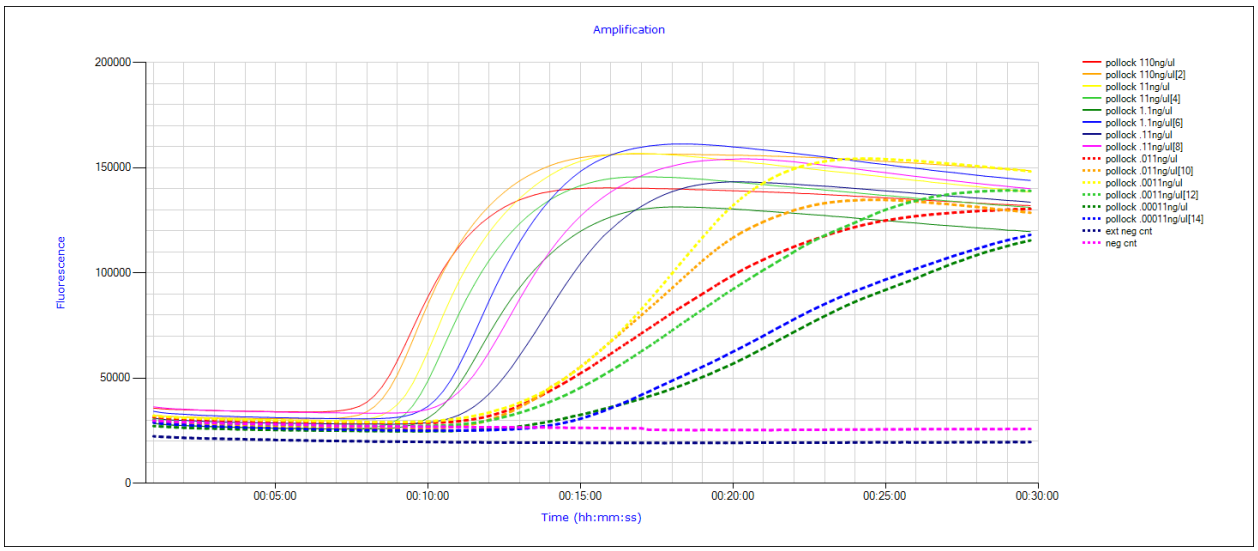
Determination of assay sensitivity using a 10 fold dilution factor

To ascertain the sensitivity capabilities of each species' specific assay, a comprehensive screen of serial dilution was completed. A fresh piece of pre identified fish species was cut and extracted. Recovered molecular material was checked for purity and yield by spectrophotometry prior to use. Each species sample was screened in triplicate, its CT values recorded and plotted (see Appendix J). The limits of detection for each species assay is identified below in Table 4.13. The limits of detection varies between master mixes for each species, with some being significantly more sensitive (pollock assay: Figure 4.11) to that of others (Haddock assay).

Table 4.13: Sensitivity capabilities: Limit of Detection for each species

Dilution	Cod assay	Coley assay	Haddock assay	Whiting assay	Pollock assay
Neat	232 ng/µl	210 ng/µl	511 ng/µl	112 ng/µl	112 ng/µl
10-1	23 ng/µl	21 ng/µl	51 ng/µl	11.2 ng/µl	11.25 ng/µl
10-2	2.3 ng/µl	2.1 ng/µl	5.1 ng/µl	1.12 ng/µl	1.125 ng/µl
10-3	0.232 ng/µl	0.21 ng/µl	0.511 ng/µl	0.112 ng/µl	0.125 ng/µl
10-4	0.0232 ng/µl	0.021 ng/µl			0.0125 ng/µl
10-5	0.00232 ng/µl				0.00125 ng/µl
10-6					0.000125 ng/µl

Figure 4.11: Amplification curve for pollock dilutions



Determination of assay sensitivity for Spiked/Contaminated samples

In an attempt to artificially replicate a mixed species food sample a pre identified fish species was mixed with cod (base material). The screening of these spiked fish samples to ascertain sensitivity levels identified a difficulty incurred when trying to prepare a homogenous sample type with fish. Processed fish products are rarely minced and most often mixed, it was deemed important to replicate a similar process with the spiked samples. However, by doing so a sample when tested can represent significantly differing results dependant on the piece chosen for screening. Although samples were thoroughly mixed, a true representative sample of each percentage is impossible due to the non-homogeneous nature of the mixture (Tables 4.14a-d; Figures 4.12a-d).

Table 4.14a: Pollock spike

	Mean	Std. Dev.	CV
Cod with Pollock 20%	10.28667	0.035119	0.341402
Cod with Pollock 10%	12.62667	1.682033	13.32127
Cod with Pollock 5%	12.85	1.891851	14.72258

Figure 4.12a: Pollock spike

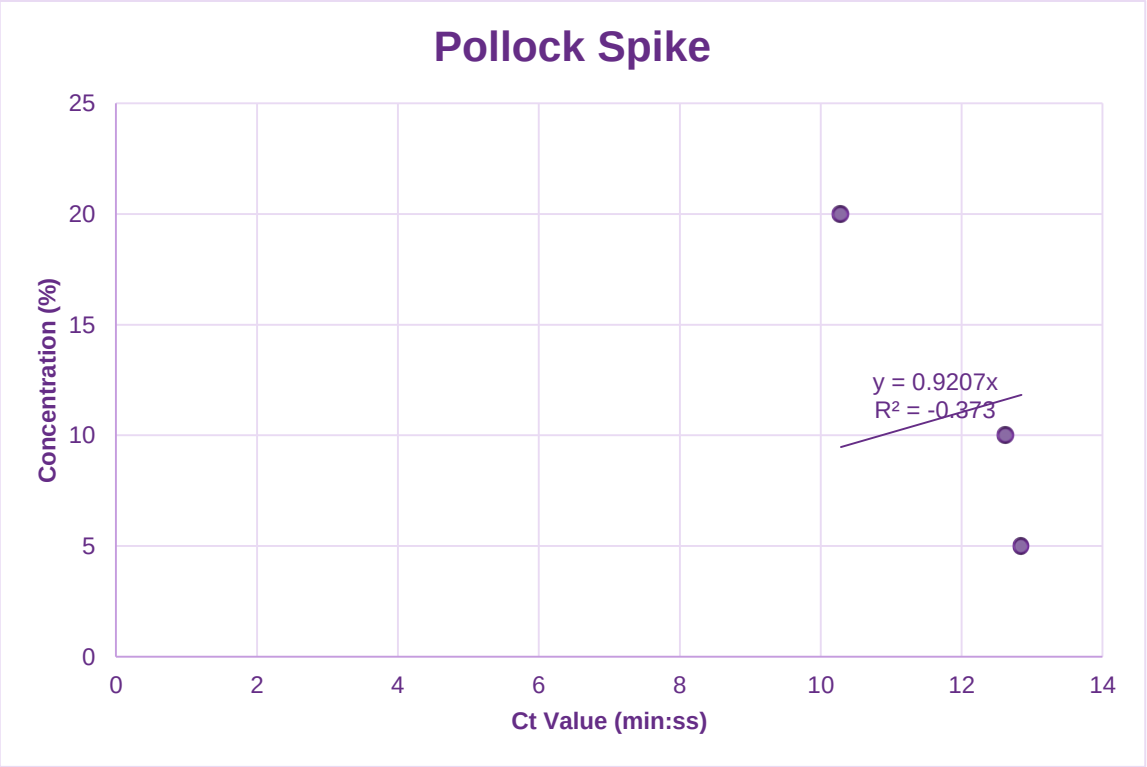


Table 4.14b: Whiting spike

	Mean	Std. Dev.	CV
Cod with Whiting 20%	22.79667	2.496524	10.95127
Cod with Whiting 10%	25.98	1.664962	6.408631
Cod with Whiting 5%	16.67333	0.413078	2.477476

Figure 4.12b: Whiting spike

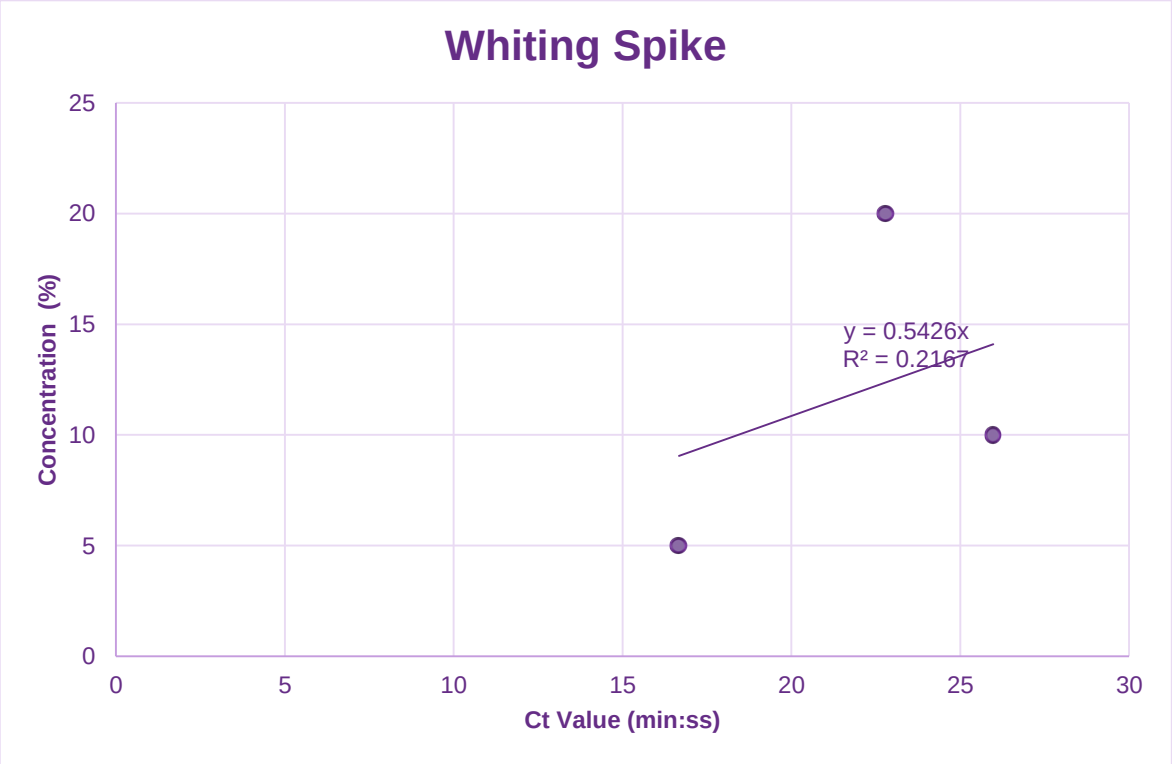


Table 4.14c: Haddock spike

	Mean	Std. Dev.	CV
Cod with Haddock 20%	19.29	4.582052	23.75351
Cod with Haddock 10%	20.03		
Cod with Haddock 5%	17.02333	0.593408	3.485852
Cod with Haddock 1%	25.65	2.05061	7.99458

Figure 4.12c: Haddock spike

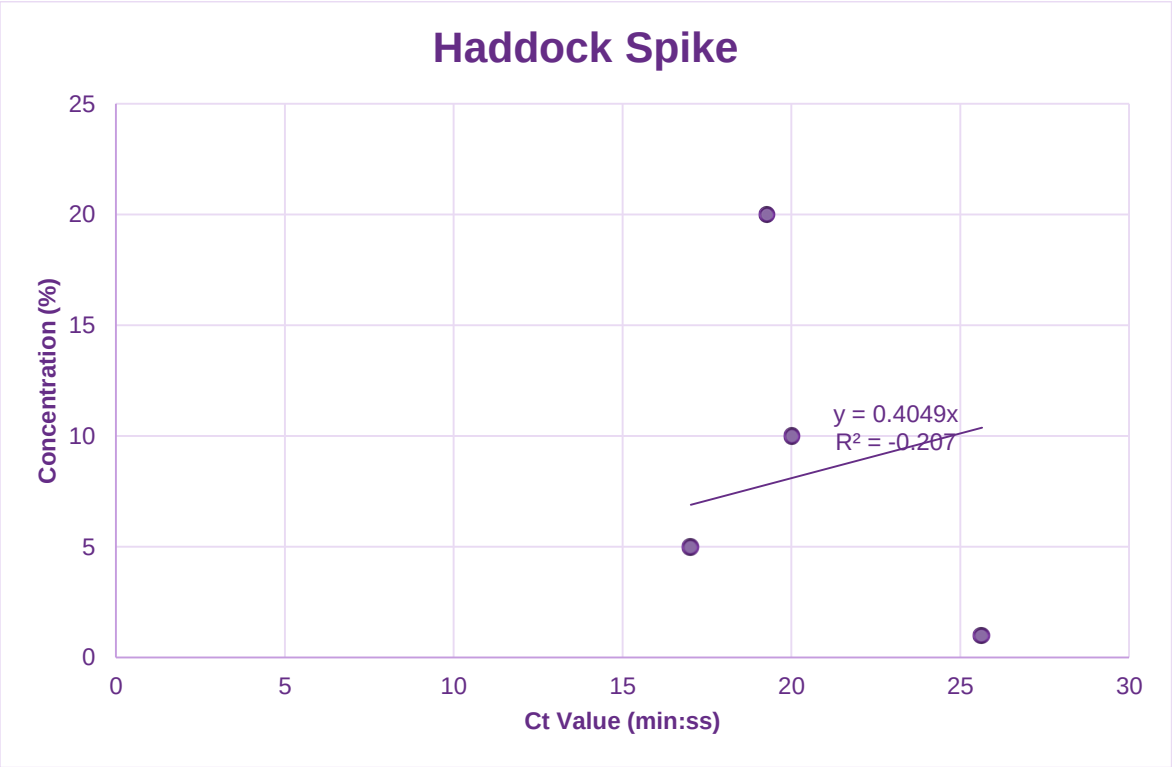
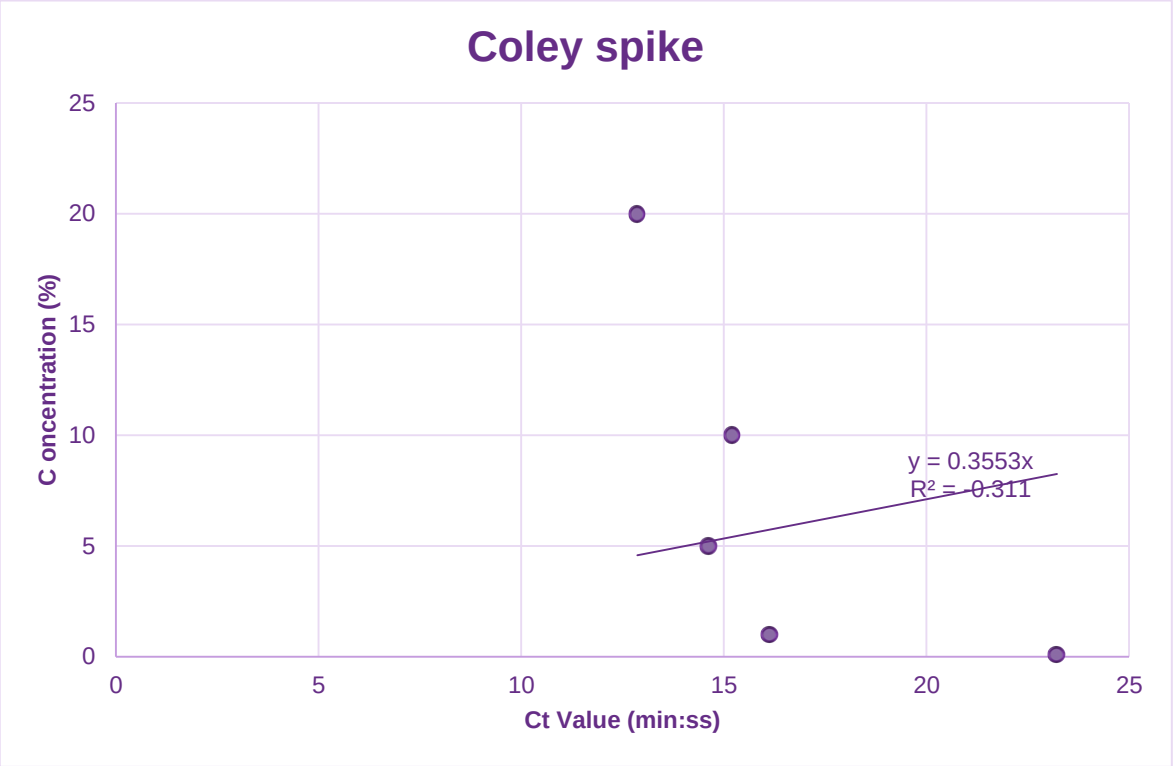


Table 4.14d: Coley spike

	Mean	Std. Dev.	CV
Cod & Coley 20%	12.86667	0.292632	2.27434
Cod & Coley 10%	15.20667	0.196554	1.292549
Cod & Coley 5%	14.62333	0.527004	3.603858
Cod & Coley 1%	16.13333	1.254286	7.7745
Cod & Coley 0.1%	23.22		

Figure 4.12d: Coley spike



Results and discussion: Meat

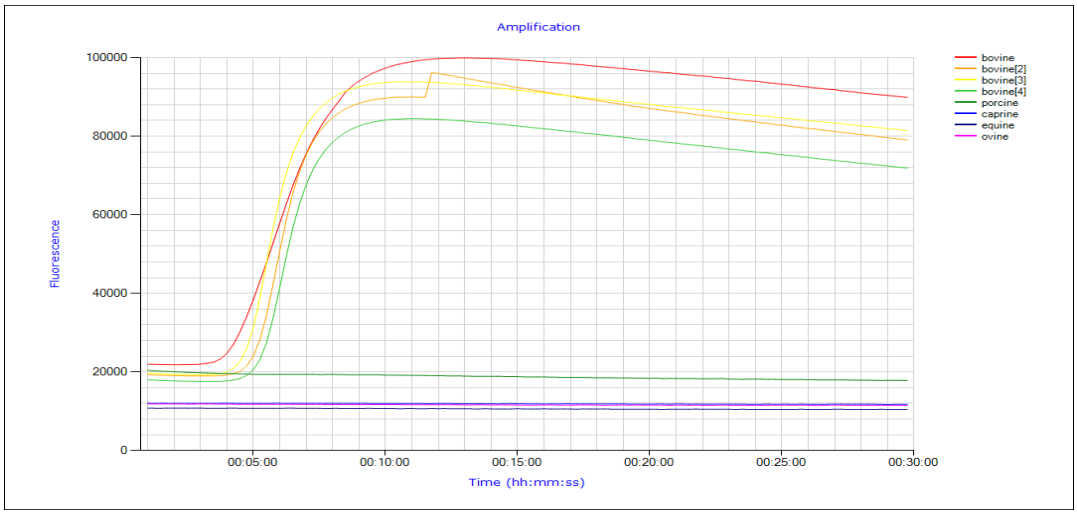
Screening of each species type with all Optigene provided species specific kits

Samples from each species were screened against all meat master mixes in order to identify if cross reactivity between species existed (Table 4.15).

Table 4.15: Screening of each species with each reaction kit

	Bovine Samples	Ovine Samples	Porcine Samples	Caprine Samples	Equine Samples
Bovine Master mix	✓	×	×	×	×
Porcine Master mix	×	×	✓	×	×
Ovine Master mix	×	✓	×	×	×
Equine Master mix	×	×	×	×	✓
Caprine Master mix	×	×	×	✓	×

Figure 4.13: Amplification of bovine species only with bovine species master mix



Screening of bovine samples pre-contaminated with known concentration of other species

Bovine material was used as the base material which was then spiked with 4 other species at different percentages. This was completed at 5 different percentages 20%, 10%, 5%, 1% & 0.1%. This work was conducted to determine if mixing multiple species affected detection of target. It also permitted us to identify that all mixing methods permit a more homogenous sample to be created than with fish samples. No cross reactivity was detected during this process.

Meat processing/treatments

Twenty samples from each species was adulterated four ways. Samples were screened raw, cooked, cooked with pasta and cooked with potato. This was completed in order to identify if specificity could be affected by common food industry treatment/processes. Bovine, ovine, caprine, equine and porcine showed little statistical difference in results recovered between all 4 treatments (Tables 4.16a-e; Figures 4.14a-e)

Table 4.16a: Statistical analysis of ovine adulteration

	N	Minimum	Maximum	Mean		Std. deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Raw (n=20)	20	0.00	7.28	4.61	.34394	1.53813
Cooked (n=20)	20	3.40	9.03	4.99	.29394	1.31455
Pasta (n=20)	20	3.43	8.45	4.62	.29580	1.32284
Potato (n=20)	20	3.38	13.33	4.82	.51255	2.29218
Valid N (listwise)	20					

Figure 4.14a: Ovine samples

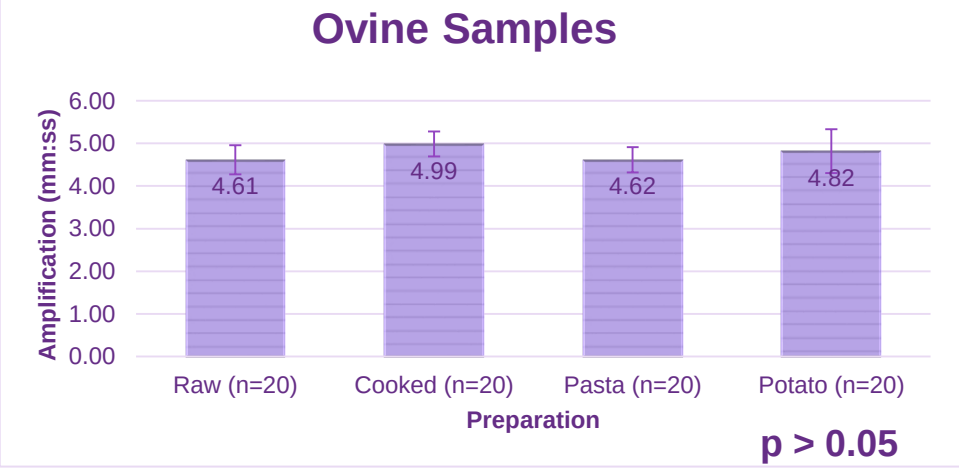


Table 4.16b: Statistical analysis of caprine adulteration

	N	Minimum	Maximum	Mean		Std. deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Raw (n=20)	20	6.22	25.09	12.93	1.18063	5.27994
Cooked (n=20)	20	6.34	19.28	10.14	.72214	3.22950
Pasta (n=20)	20	6.20	26.19	11.00	1.32151	5.90999
Potato (n=20)	20	6.45	19.24	11.06	.76331	3.41365
Valid N (listwise)	20					

Figure 4.14b: Caprine samples

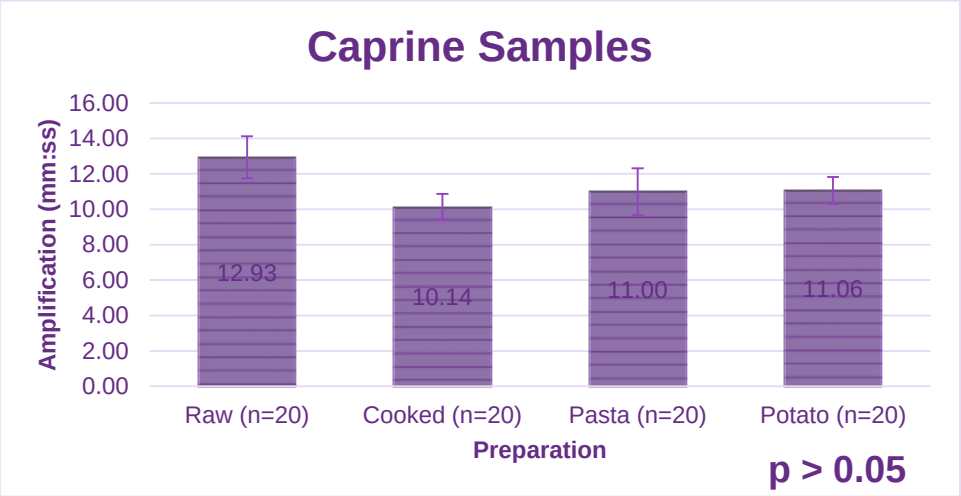


Table 4.16c: Statistical analysis of porcine adulteration

	N	Minimum	Maximum	Mean		Std. deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Raw (n=20)	20	6.50	24.33	12.60	1.07304	4.79878
Cooked (n=20)	19	8.06	25.25	12.18	1.14719	5.00049
Pasta (n=20)	20	7.29	19.39	11.30	.85767	3.83562
Potato (n=20)	20	7.55	27.15	10.74	1.02292	4.57464
Valid N (listwise)	19					

Figure 4.14c: Porcine samples

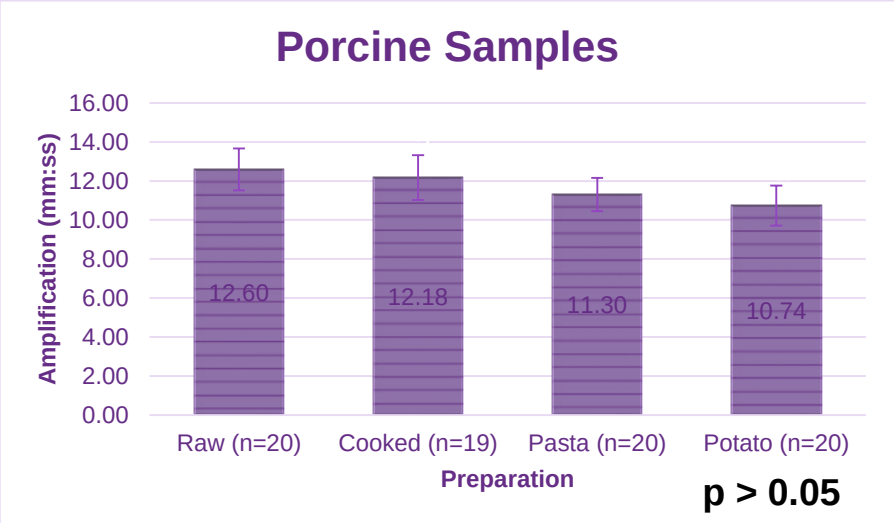


Table 4.16d: Statistical analysis of equine adulteration

	N	Minimum	Maximum	Mean		Std. deviation
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic
Raw (n=20)	20	6.17	19.57	10.70	.85134	3.80729
Cooked (n=20)	20	6.15	15.38	9.50	.55323	2.47412
Pasta (n=20)	20	6.36	19.34	10.25	.87954	3.93342
Potato (n=20)	20	6.42	12.35	9.53	.42660	1.90782
Valid N (listwise)	20					

Figure 4.14d: Equine samples

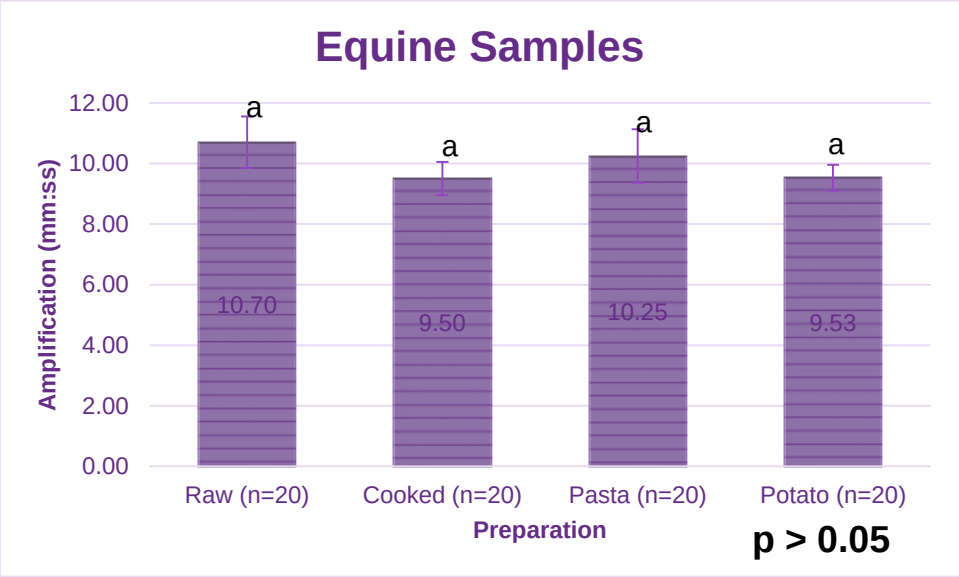
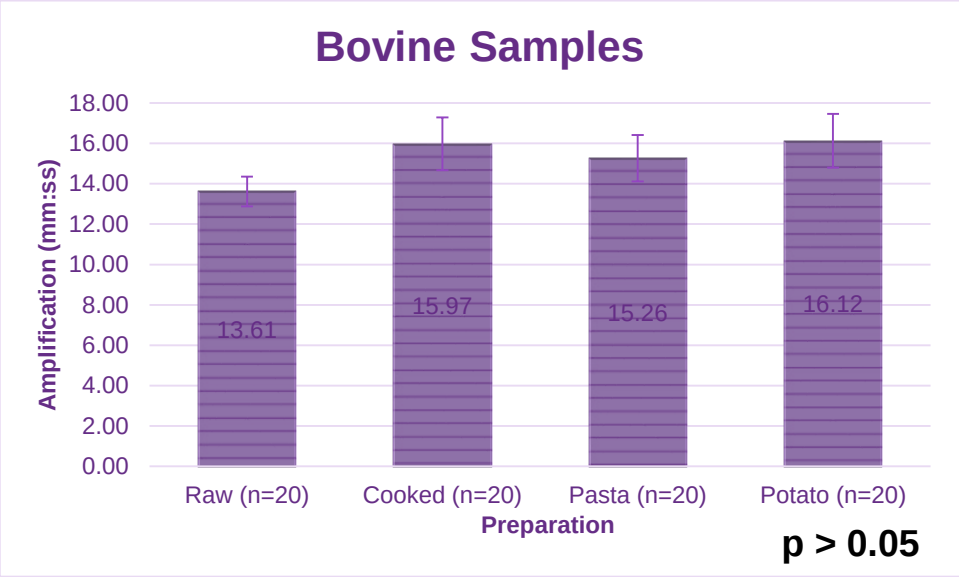


Table 4.16e: Statistical analysis of bovine adulteration

			95% Confidence Interval	
	Mean	Std. Error	Lower Bound	Upper Bound
Raw (n=20)	13.61	.740	12.063	15.158
Cooked (n=20)	15.97	1.309	13.235	18.714
Pasta (n=20)	15.26	1.147	12.863	17.664
Potato (n=20)	16.12	1.334	13.333	18.916

Figure 4.14e: Bovine samples



Screening of the Meat Speciation Library

After validation of the LAMP assays for each species, the technique was employed to screen the stored library of samples with a total number of 104 samples were screened during this process (Table 4.17).

Table 4.17: Screen of known samples

	Bovine Samples	Ovine Samples	Porcine Samples	Equine Samples	Caprine Samples
No. samples screened	35	20	20	24	4
No. correctly identified	100%	100%	100%	100%	100%
No. incorrectly identified	0%	0%	0%	0%	0%

Determination of limits of detection for meat species assays

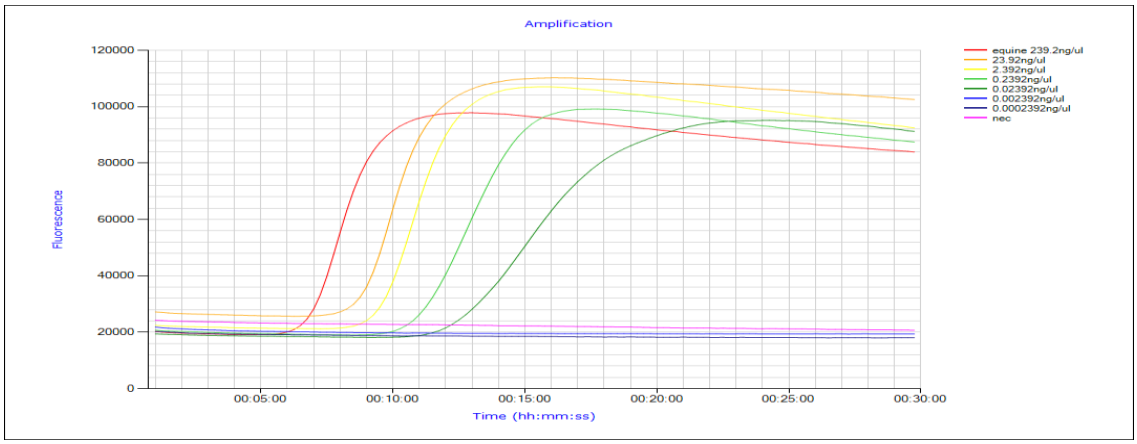
Determination of assay sensitivity using a 10 fold dilution factor

To ascertain the sensitivity capabilities of each species specific assay a comprehensive screen of serial dilution was completed in triplicate. A fresh piece of pre identified meat species was cut and extracted. Recovered molecular material was check for purity and yield by spectrophotometry prior to use. The limits of detection for each species is identified below in Table 4.18. The detection limits vary only slightly between master mixes for each species, the exception being porcine samples that are more sensitive.

Table 4.18: Sensitivity capabilities

	Bovine Assay	Ovine Assay	Porcine Assay	Equine Assay	Caprine Assay
Neat	225 ng/µl	210 ng/µl	230 ng/µl	230 ng/µl	425 ng/µl
10-1	25 ng/µl	21 ng/µl	23 ng/µl	23 ng/µl	42.5ng/µl
10-2	2.5 ng/µl	2.1 ng/µl	2.3 ng/µl	2.3 ng/µl	4.25 ng/µl
10-3	0.25 ng/µl	0.21 ng/µl	0.23 ng/µl	0.23 ng/µl	0.45 ng/µl
10-4	0.025 ng/µl	0.021 ng/µl	0.023 ng/µl	0.023 ng/µl	0.045 ng/µl
10-5	0.0025 ng/µl	0.0021 ng/µl	0.0023 ng/µl	0.0023 ng/µl	0.0045 ng/µl
10-6	ng/µl	ng/µl	0.00023 ng/µl	ng/µl	ng/µl

Figure 4.15: Amplification curve of equine specific assay



Determination of each assays sensitivity with spiked/contaminated samples

In an attempt to artificially replicate a mixed species food sample a pre identified meat species was mixed with bovine (base material). The screening of these spiked meat samples to ascertain sensitivity levels identified a lesser difficulty than that incurred with fish when trying to prepare a homogenous sample type. However, each sample when tested can offer significantly differing results dependant of the piece chosen for screening, even when assessed in triplicate. This again is most likely due to the un-homogenous nature of the mixed sample (Tables 4.19a-d; Figures 4.16a-d).

Figure 4.16a: Pork spike graph

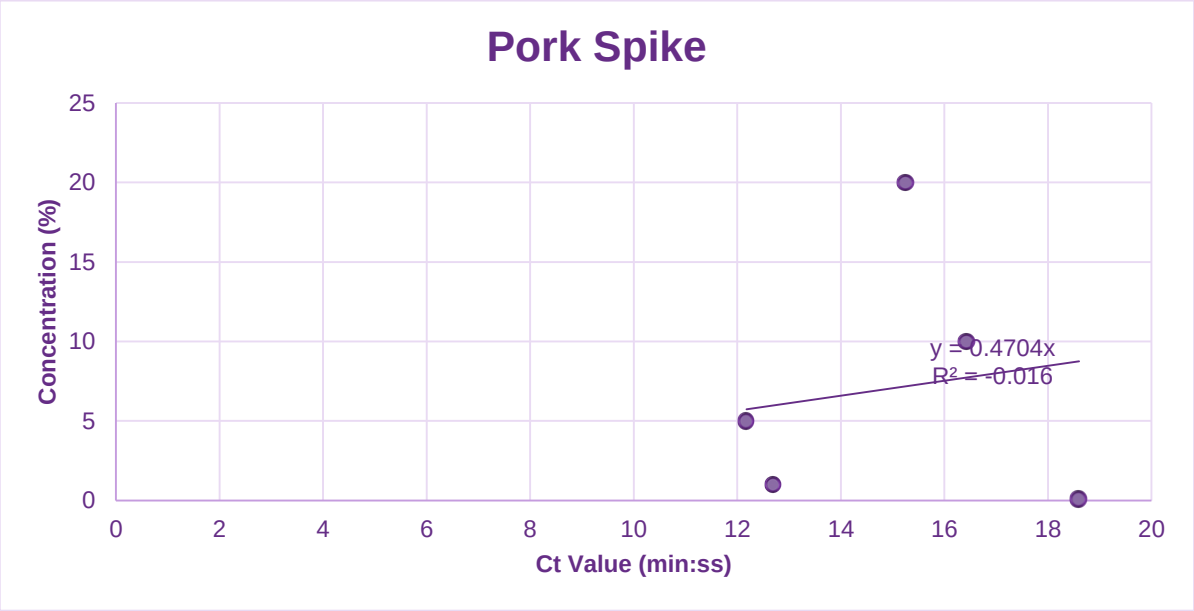


Table 4.19a: Pork spiking

	Mean	Std. Dev.	CV
Pork 20%	15.26	0.325269	2.131515
Pork 10%	16.435	2.835498	17.2528
Pork 5%	12.18	0.226274	1.857752
Pork 1%	12.7	0.537401	4.231505
Pork 0.1%	18.6	0.424264	2.28099

Figure 4.16b: Goat spike graph

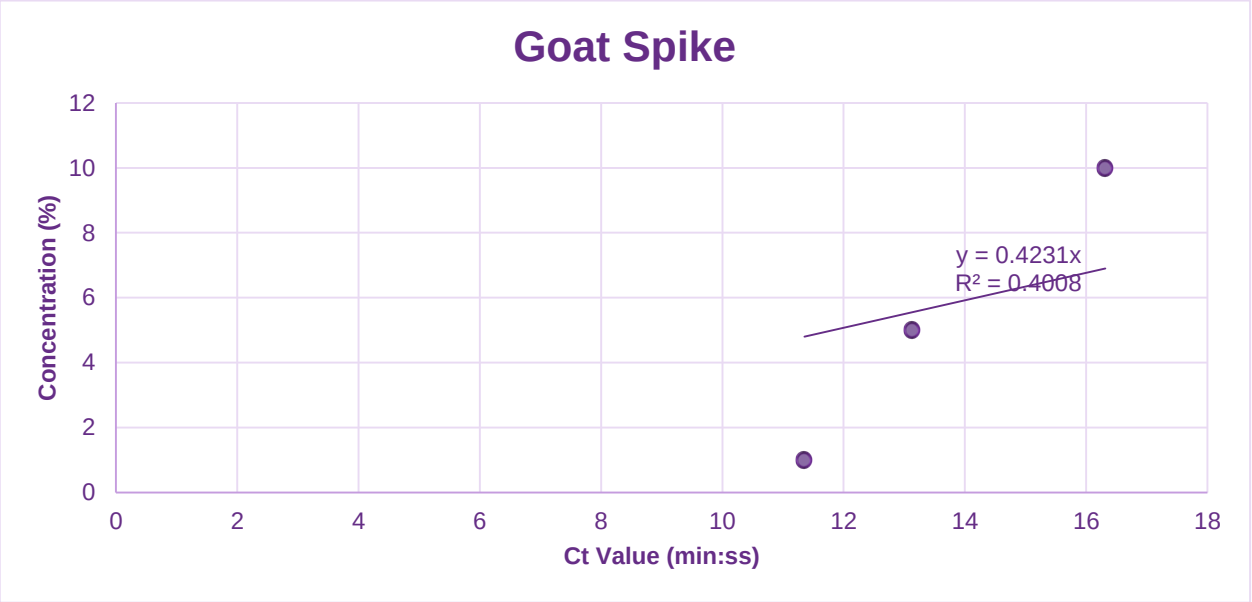


Table 4.19b: Goat spiking

	Mean	Std. Dev.	CV
Goat 20%	8.496667	0.669353	7.87783
Goat 10%	16.31667	9.380002	57.48724
Goat 5%	13.13667	7.912221	60.23005
Goat 1%	11.35333	1.811776	15.9581
Goat 0.1%	13.19333	1.800454	13.64669

Figure 4.16c: Horse spike graph

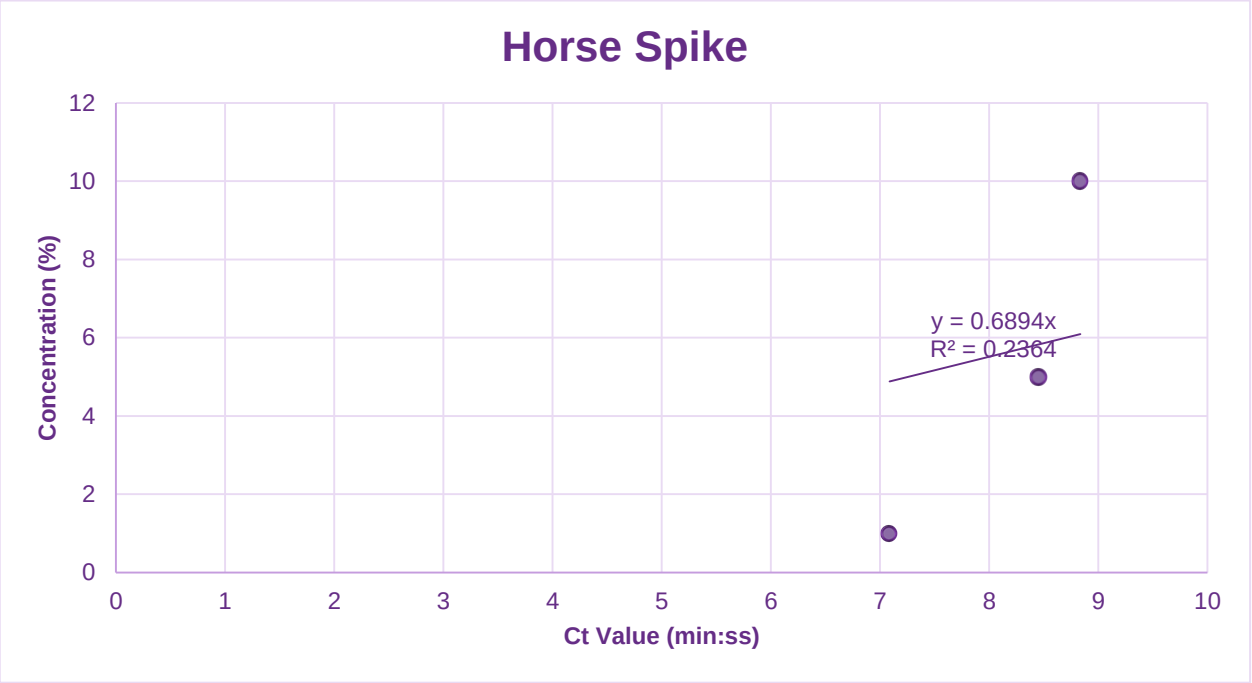


Table 4.19c: Horse spiking

	Mean	Std. Dev.	CV
Horse 20%	8.92	0.432666	4.850517
Horse 10%	8.836667	1.13143	12.80381
Horse 5%	8.453333	1.851522	21.90286
Horse 1%	7.086667	0.533791	7.532335
Horse 0.1%	8.916667	0.702662	7.880317

Figure 4.16d: Sheep spike graph

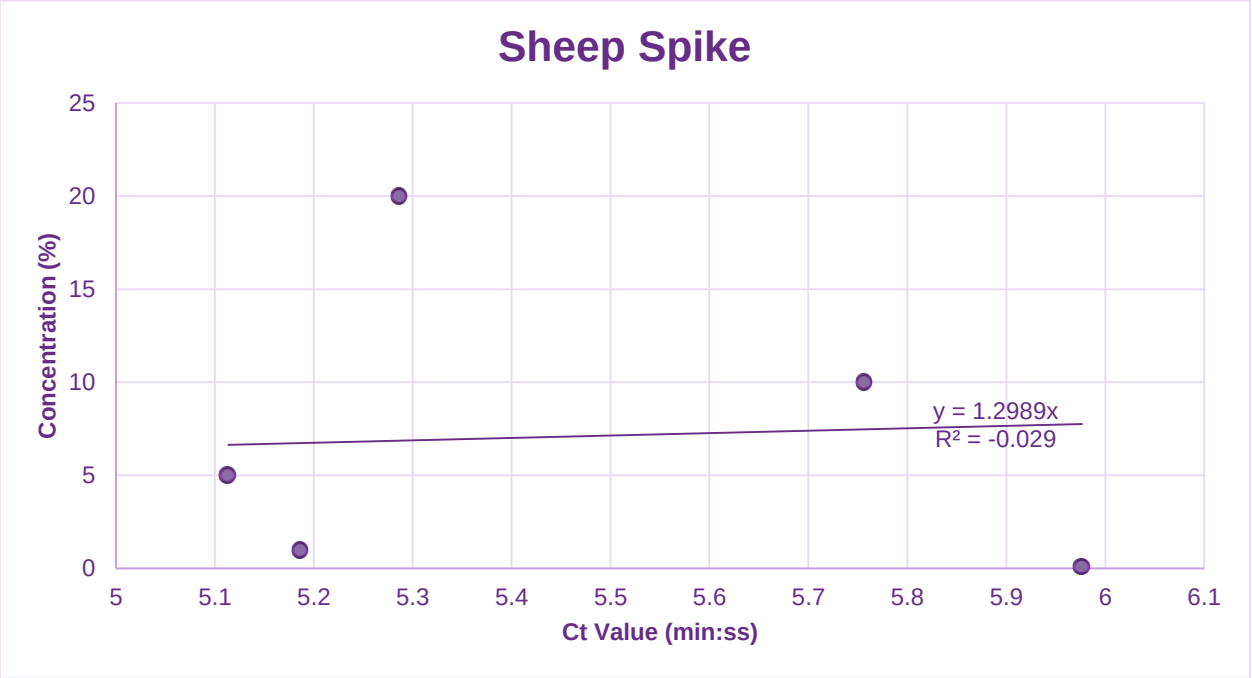


Table 4.19d: Sheep spiking

	Mean	Std. Dev.	CV
Sheep 20%	5.286667	0.900685	17.03692
Sheep 10%	5.756667	2.377316	41.29675
Sheep 5%	5.113333	0.090738	1.774532
Sheep 1%	5.186667	0.516946	9.966828
Sheep 0.1%	5.976667	0.556447	9.310325

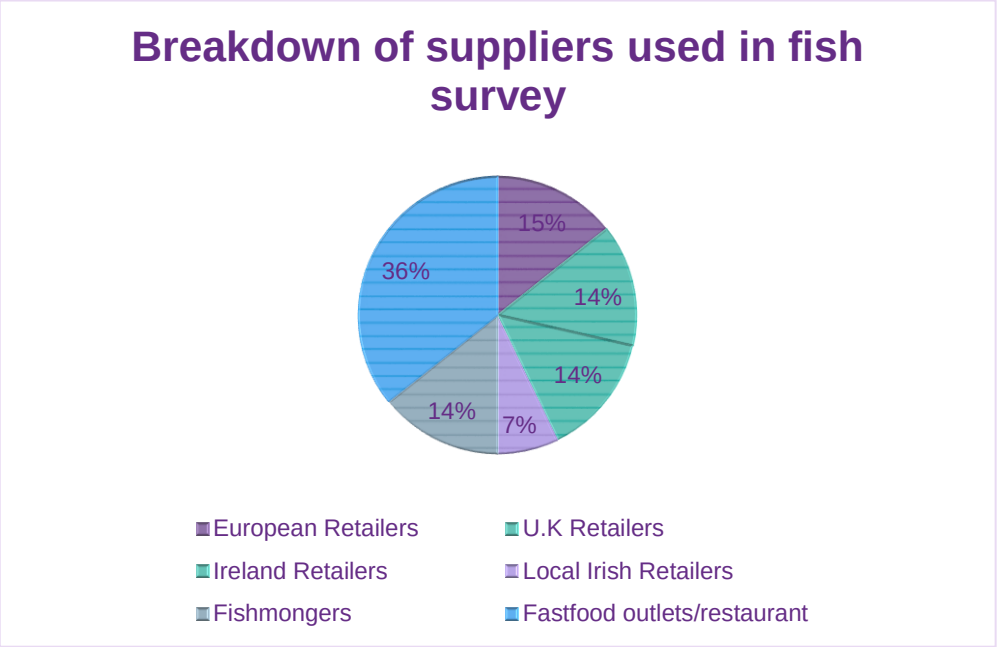
Survey of retail fish and meat products on the Island of Ireland

A wide variety of fish and meat samples were collected and screened by the previously validated species specific LAMP assays to determine any indication of food fraud occurring on the IoI.

Fish Surveillance

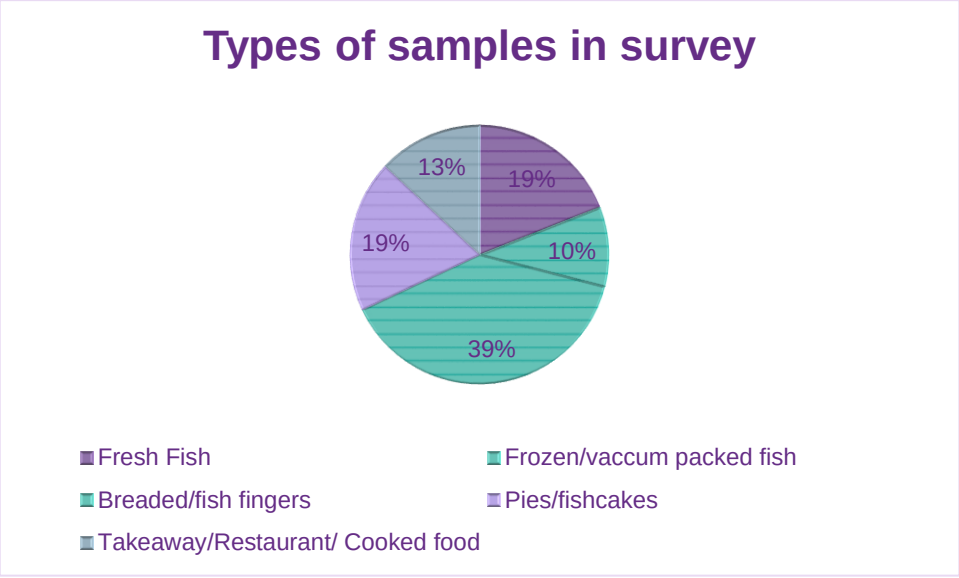
One hundred fish products readily available in retail outlets and restaurants were screened in this survey. Each duplicated sample was screened using the five pre-validated fish species assay in one thousand tests, producing five hundred test results. Fish products were obtained from a variety of retail sources across the IoI, the UK and the European Union (EU). These are listed in Figure 4.17.

Figure 4.17: Breakdown of suppliers used in fish survey



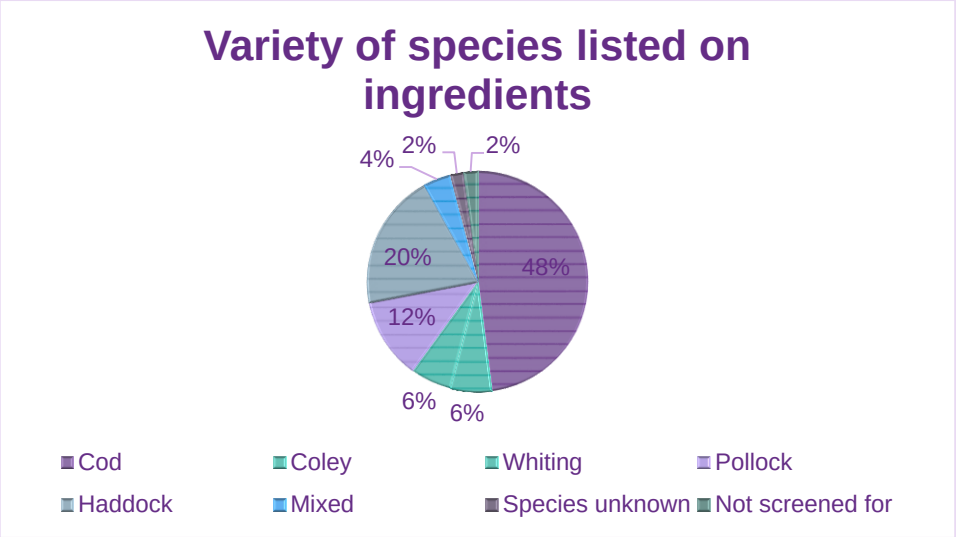
A variety of fish products detailed below (see Figure 4.18) were purchased from an assortment of retail sources on the IoI.

Figure 4.18: Types of samples in the survey



In Figure 4.19 is a breakdown of the fish species listed on the ingredients for all samples. Some samples contained more than 1 type of fish. 2 samples came under the unknown species these were samples from takeaways where the samples were described as fillet o' fish and fish burger. Two samples also came under species not screened for; these were scampi and salmon fishcakes. These were included so as to check for possible food fraud using some of the species checked in the assay.

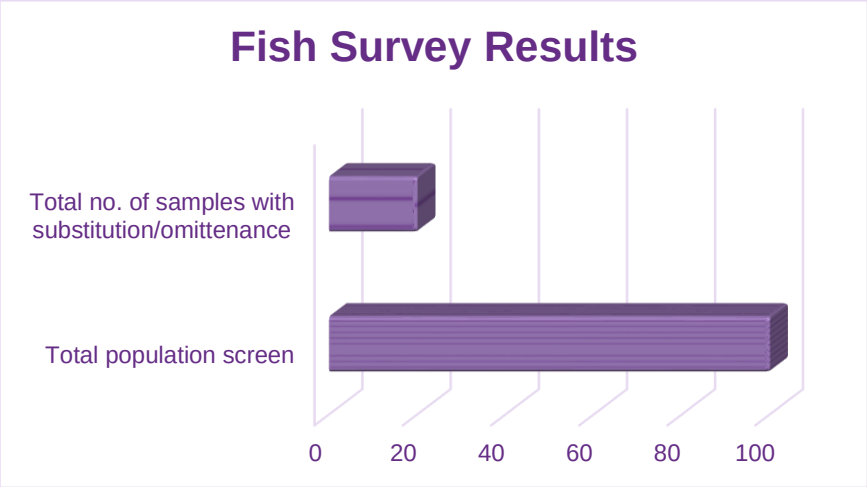
Figure 4.19: Variety of species listed on ingredients



Fish survey results

As can be seen below in Figure 4.20 in the fish survey, twenty one samples had either substitution or omittance fraud – that is, 19 of these were positive for undeclared species and 2 were negative for the declared species.

Figure 4.20: Fish survey results

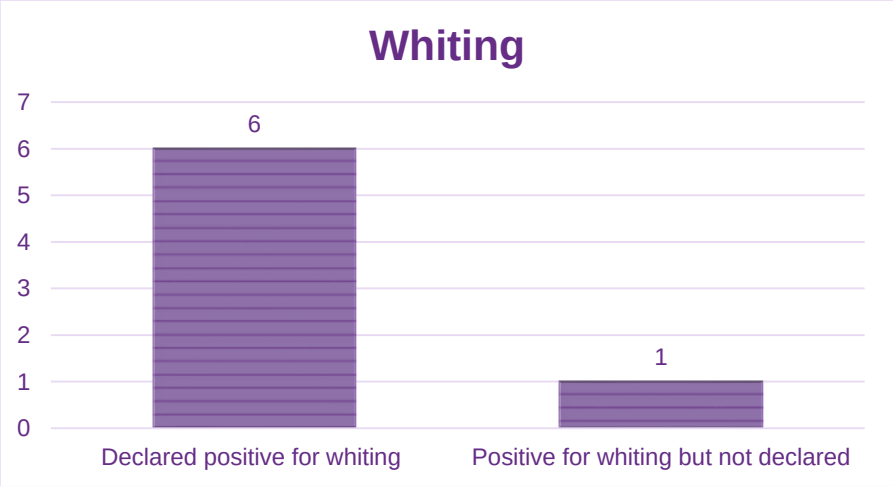


Each species has been broken down into two to three categories – (1) declared positive, (2) positive but not declared, and (3) negative for the target but declared as positive on the ingredients.

4.3.2.1 Whiting results

In Figure 4.21, one sample was positive for whiting but not declared.

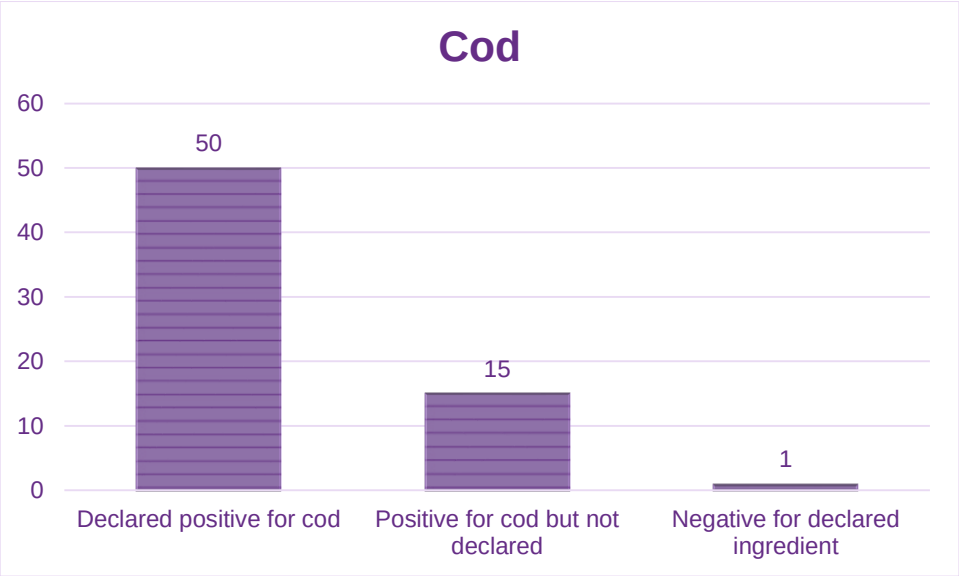
Figure 4.21: Whiting results



Cod results

Fifteen samples were positive for cod but not declared. One sample was negative for the declared ingredient (See Figure 4.22). This sample was also negative for the four other markers.

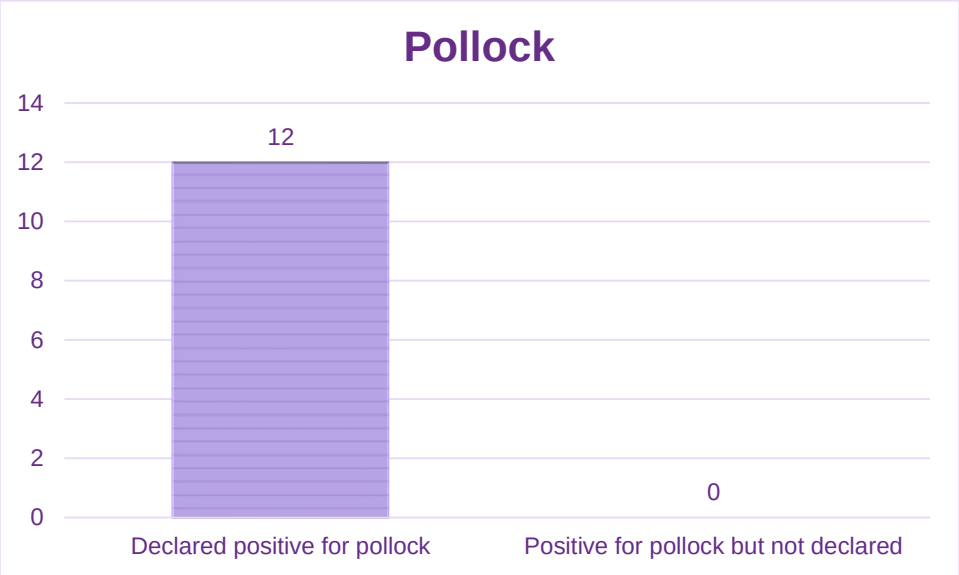
Figure 4.22: Cod results



Pollock results

No pollock samples were deemed part of substitution or omittance fraud. All samples declared as positive for pollock gave positive results for the pollock target (Figure 4.23).

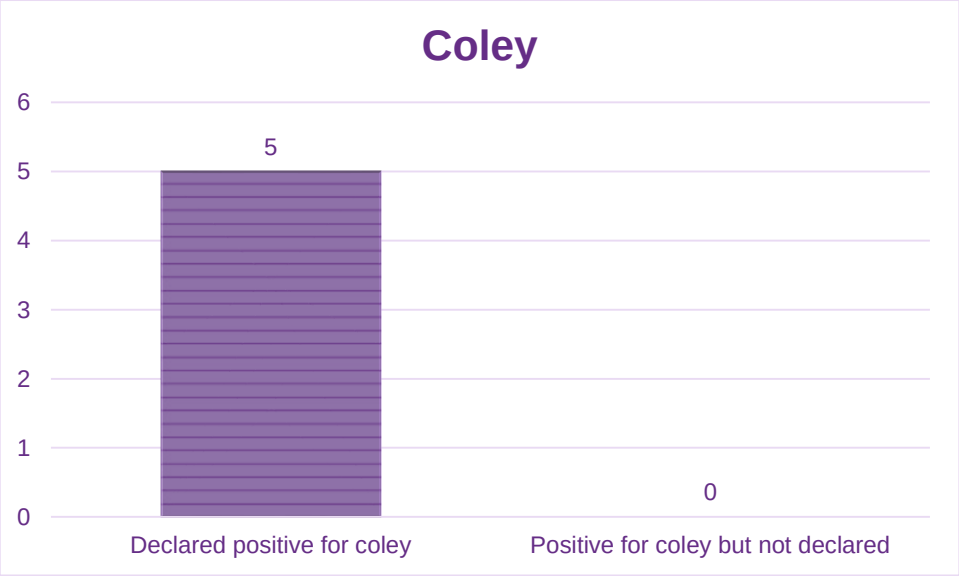
Figure 4.23: Cod results



Coley results

Five samples tested positive for the declared ingredient of coley. There was no coley found that was not declared (Figure 4.24).

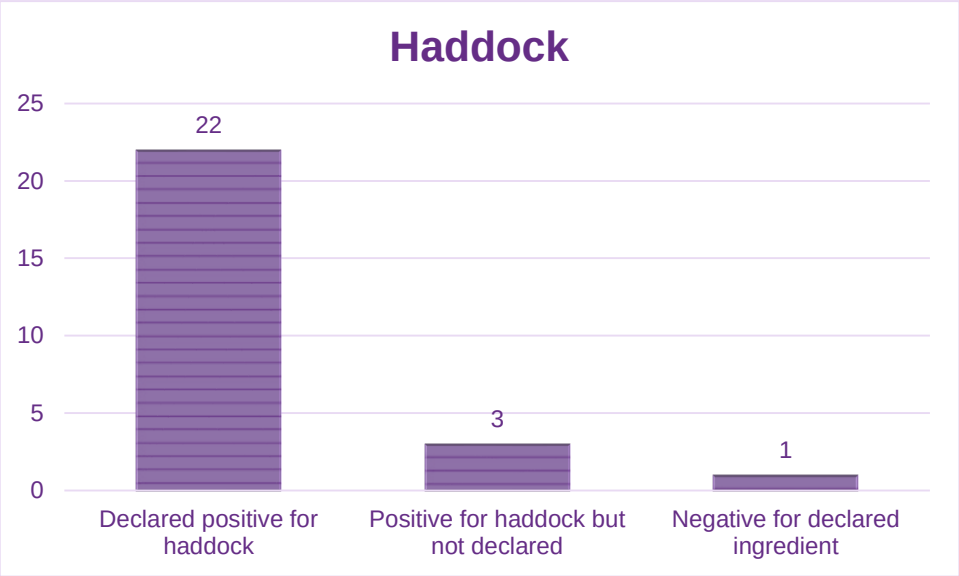
Figure 4.24: Coley results



Haddock Results

Haddock was identified in three samples but not declared (Figure 4.25). One sample was negative for haddock, which was declared within ingredients.

Figure 4.25: Haddock results



Discussion of the fish results

A number of whole/intact/unprocessed fish samples were positive for species other than that with which it was declared and identified. Since the samples were intact fillets of fish and not processed, these undeclared positives are most likely attributed to cross contamination during the handling/storage of multiple species at a retail outlet. To overcome the issue of cross contamination of handling it is recommended that all future sampling by scientific analysts omit the exterior of the fish from the test sample (taking interior flesh only) in an aim to reduce surface contamination. See Table 4.20 for all samples and result details.

Seven processed samples screened positive for the presence of undeclared species. It is most probable that the addition of extra fish species occurs during the food processing phase, whether intentional or accidental. As each of the Optigene fish assays are not quantitative, we cannot ascertain the severity of the adulteration, only that it exists (Table 4.20). Two samples were listed as positive but screened negative for their species. These samples demonstrate substitution fraud within the fish retail sector, with particular emphasis on processed (cake/finger/pies) samples types.

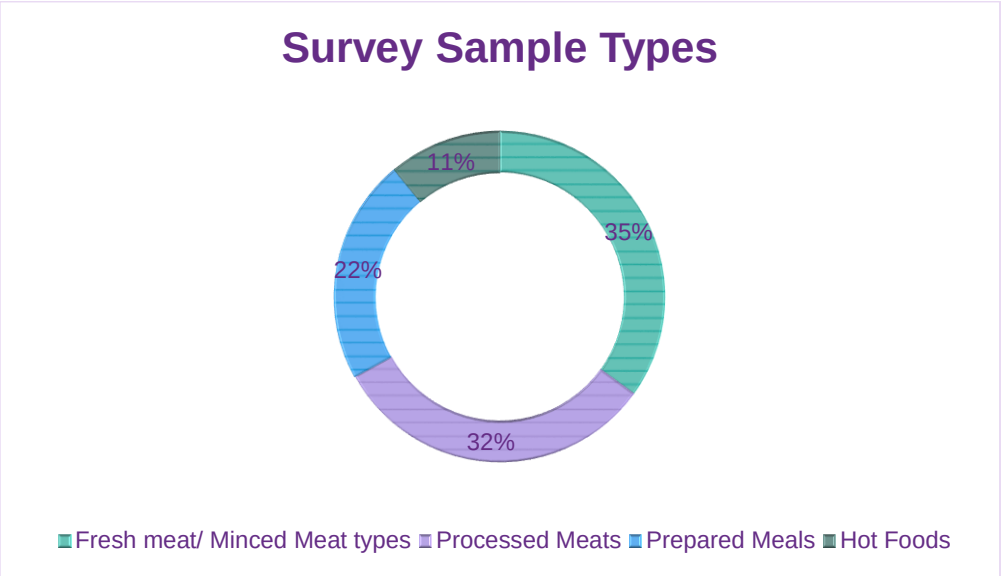
Meat surveillance

One hundred meat type products readily available for procurement in retail outlets and restaurants were collected. Each replicated sample was screened with earlier validated meat species specific LAMP assays in one thousand tests producing five hundred surveillance test results. Meat based products were obtained from a selection of retail outlets on the IoI, UK and the EU (Figure 4.26).

Figure 4.26: Retail outlets

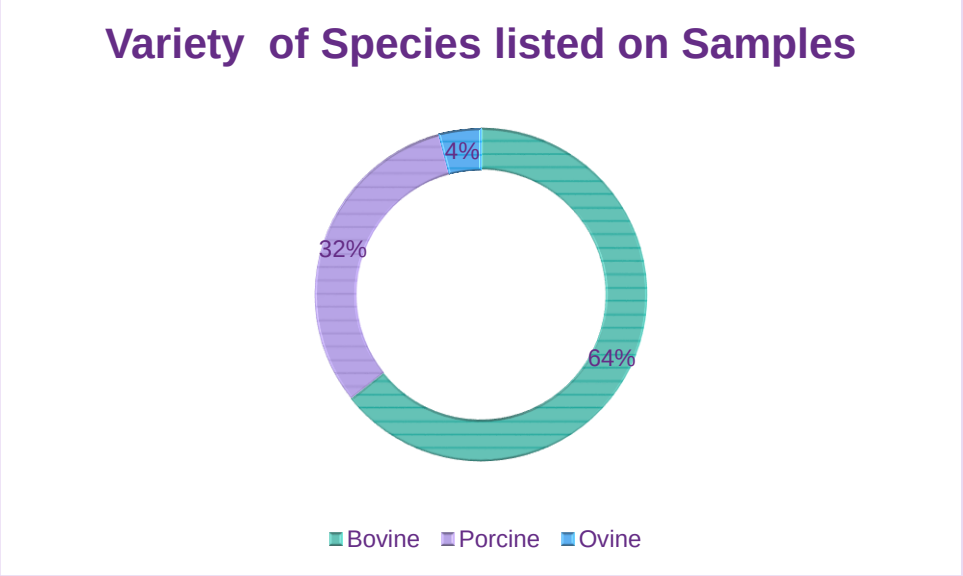
Five categories of samples were identified for inclusion in the meat survey population (Figure 4.27).

Figure 4.27: Survey sample types



Only three red meat species – bovine, porcine and ovine – were listed on the contents of ingredients contained within the 100 samples surveyed (Figure 4.28).

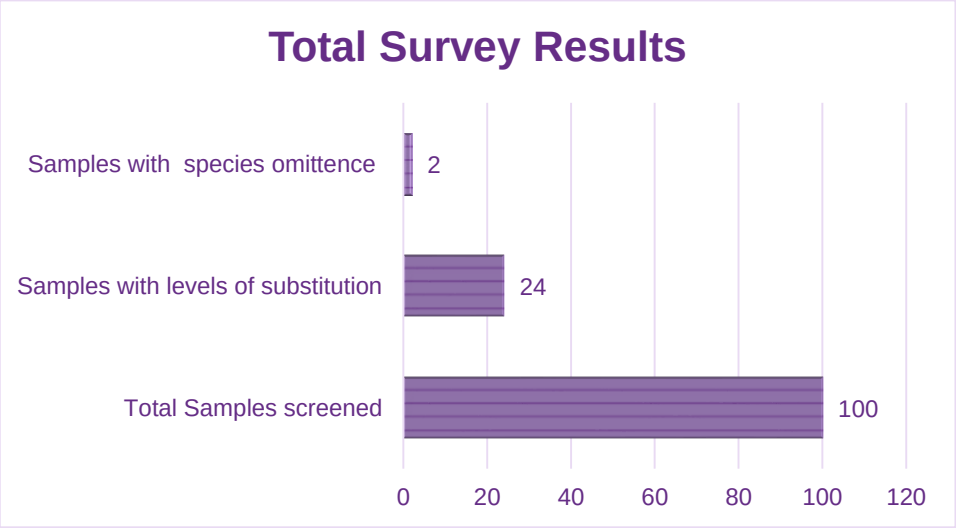
Figure 4.28: Variety of species listed on samples



Meat survey results

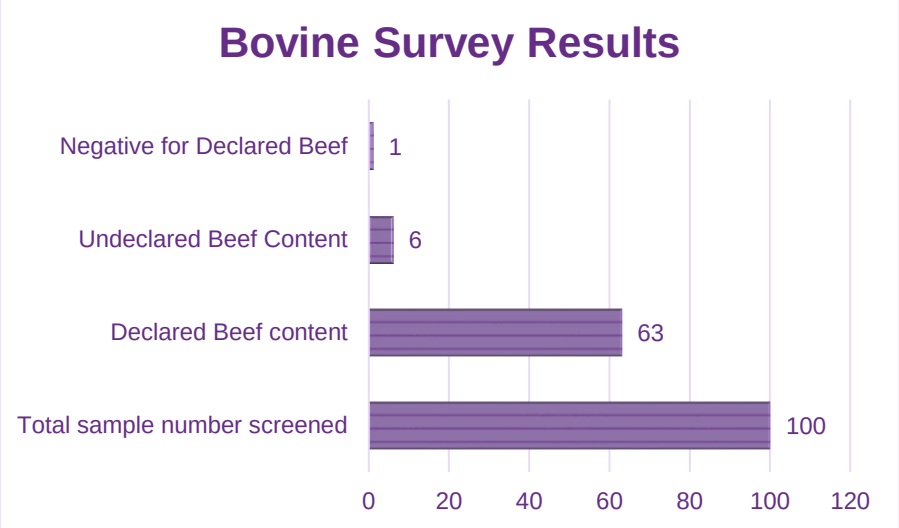
Of the 100 samples surveyed, it was observed 24 contained varying levels of species addition and a further 2 samples identified with species omittance (Figure 4.29).

Figure 4.29: Total survey results



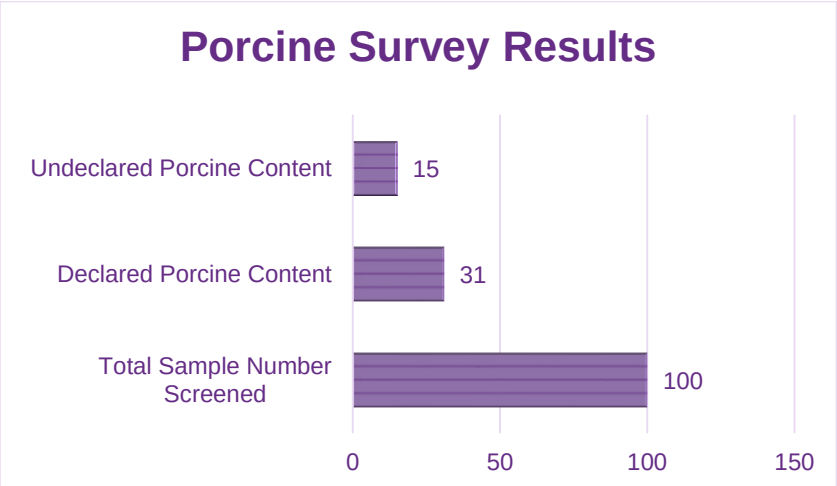
Post LAMP analysis of bovine screening recovered 6 samples previously undeclared for its presence and 1 sample negative for bovine presence where stated (Figure 4.30).

Figure 4.30: Bovine survey results



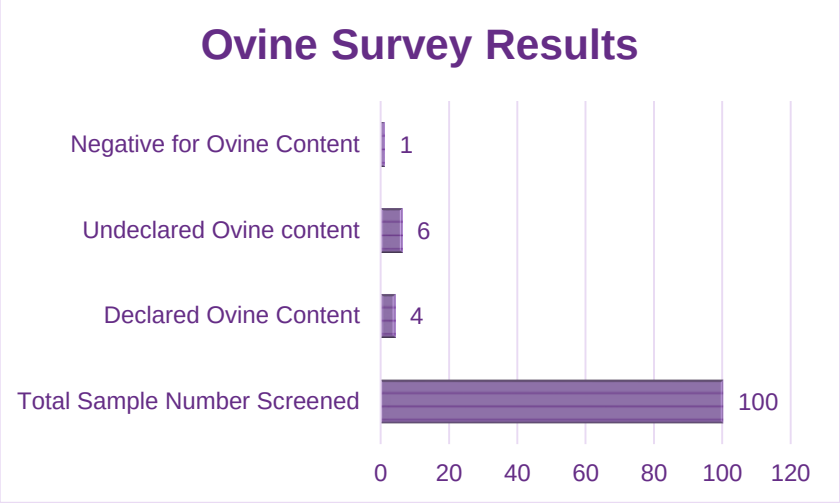
On examination of porcine screening, 15 samples were identified as positive where not stated in contents and deemed to contain a level of addition food fraud (Figure 4.31).

Figure 4.31: Porcine survey results



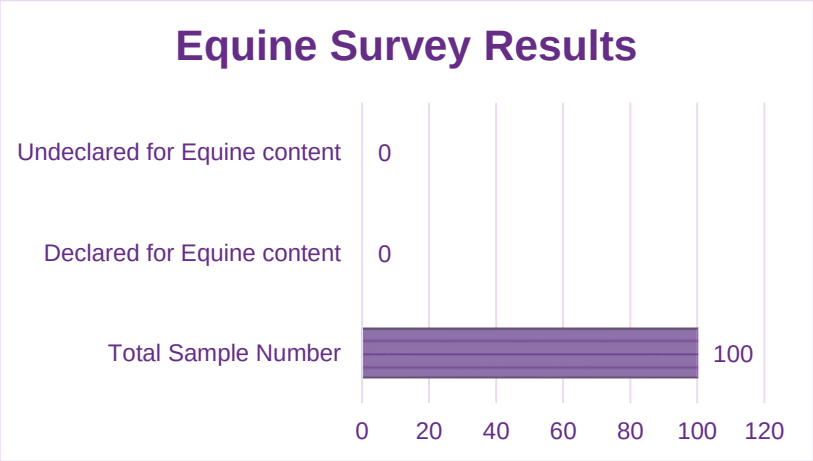
A single sample stated as containing ovine species was screened negative for its presence, and previously positive for undeclared bovine target, this demonstrates the substitution on a single species with a diverse species mixture. Six further samples were identified as positive for addition fraud with ovine content as undeclared (Figure 4.32).

Figure 4.32: Ovine survey results



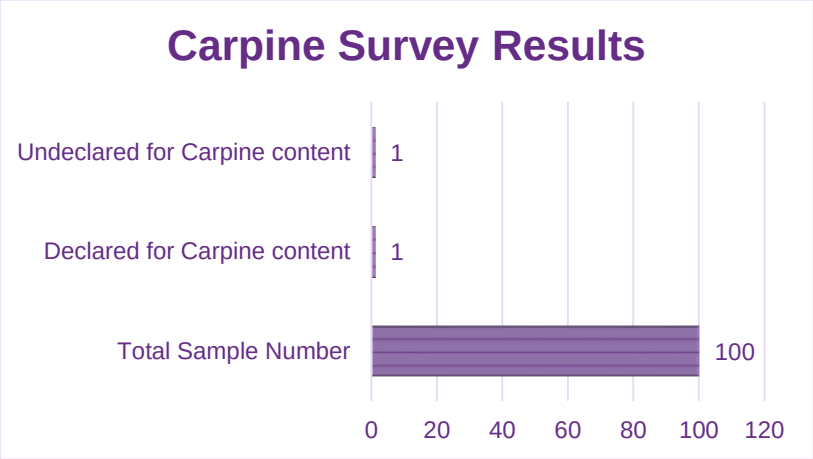
All one hundred samples screened negative for the presence of equine target. Two samples repeatedly gave annealing and amplification values but because these values were above our established LoD's they were reported as negative (Figure 4.33).

Figure 4.33: Equine survey results



A solitary sample was identified as positive for caprine species which was not declared, this sample was also recognised as containing additional undeclared species on previous screening for bovine and ovine too (Figure 4.34).

Figure 4.34: Caprine survey results



Discussion of the meat survey recoveries

Two samples (62 and 83) were listed positive but screened negative for their respective species. These samples exhibit substitution fraud within the meat processing sector, with particular emphasis on manipulated meat samples (minced products/pies) as both fell within this category. See Table 4.21 for all samples and result details. Twenty four samples (No.'s 6, 8, 9, 11, 12, 13, 14, 15, 16, 17, 23, 52, 53, 58, 61, 62, 63, 70, 72, 74, 80, 87, 90 and 91) comprising of minced products, ready-made meals and hot foods were identified as positive for the occurrence of undeclared species. The addition of extra meat types most likely arises during the food processing phase, whether intentional or accidental. Due to the non-quantitative nature of each LAMP method, we cannot ascertain the severity of the adulteration with other meat, only that it exists (Table 4.21).

Conclusions regarding the use of Optigene species specific LAMP assays

The use of Optigene LAMP assays for multiple speciation identification in red meat and fish samples is unattainable. Even a solitary sample cannot be screened for multiple targets in a single run. This is owing to the single target nature of each assay and the limited capacity of the Optigene Genie II LAMP machine. Post validation and sample survey has revealed the most suitable use for Optigene's species specific assays and LAMP machine is for use with single species identification in small sample volumes.

Table 4.20: Fish sample detail and results

Sample No	Shop	Sample type	Cod	Whiting	Pollock	Coley	Haddock
1	The Fishman's Market	Fresh Whiting					
2	The Fishman's Market	Fresh Haddock	16.17				
3	The Fishman's Market	Fresh Cod					
4	The Fishman's Market	Fresh Coley					
5	Aldi	Cod Fish Fingers					
6	Aldi	The Fishmonger Frozen Haddock Fillets					
7	Aldi	Skellig Bag Cod Fillets					
8	Aldi	Specially Seleted Smoked Haddock Fishcakes with cheese & leek					
9	Aldi	Skellig Bag Smoked Coley					
10	Aldi	The Fishmonger Frozen Atlantic Cod Fillets					
11	Aldi	Specially Seleted Smoked Cod Fishcakes with cheddar					
12	Eurospar	Donegal Catch 12 fish fingers 100% fish fillet					
13	Eurospar	Birds Eye 10 Fish Fingers Cod					
14	Eurospar	XSELL Breaded Haddock fillet portions					
15	Eurospar	XSELL Breaded Cod fillet portions					16.29
16	Tesco	Smoked Coley					
17	Tesco	Haddock Fillet					
18	Tesco	Tesco Battered Haddock Fillets	21.26				
19	Tesco	Tesco Battered Cod Fillets					
20	Tesco	Tesco Everday Value Fish fingers frozen					
21	Tesco	Tesco Fish Fingers					
22	Tesco	Tesco 2 Cod Fillet Fish Cakes					
23	Tesco	Tesco 2 lighly dust cod fillets					
24	Tesco	Whiting Fillet					
25	Lidl	Deluxe 3 fish roast cod salmon& haddock					
26	Lidl	Ocean Trader 4 battered cod fillets					
27	Lidl	Ocean Trader 4 battered haddock fillets					
28	Lidl	Ocean Trader 4 chuncky cod fillets (improved receipe)					
29	Lidl	Ocean Sea 15 Fish fingers from alaska pollock					
30	Lidl	Ocean Sea 10 cod fish fingers					
31	Lidl	Ocean Trader 4 breaded cod fishcakes					
32	Lidl	Ocean Trader 4 breaded haddock fishcakes					
33	Lidl	Inismara Breaded haddock fillets					
34	Lidl	Inismara Breaded cod fillets					
35	Lidl	Inismara haddock Fillets	23.06				
36	Lidl	Inismara cod Fillets					
37	East coast seafood	Cod					
38	East coast seafood	Whiting	11.38				
39	East coast seafood	Haddock	16.2				
40	East coast seafood	Coley	20.1				
41	Supervalu	Haddock	11.1				
42	Supervalu	Whiting	23.02				17.45
43	Supervalu	Cod					12.2
44	Supervalu	Smoked Coley					
45	Supervalu	Simon's Naturally Good Fish Pie, smoked coley, white fish & salmon					
46	Supervalu	Birds Eye Fish Fillets in Crispy Batter	17.4				
47	Supervalu	Donegal Catch Atlantic Cod fillets (Creations Brand)					
48	Supervalu	Youngs Breaded Fish Cakes with Alaska Pollock	17.52				
49	Supervalu	Supervalu Breaded Cod Fillets					
50	Supervalu	Donegal Catch Breaded Atlantic Cod Fillets					

51	Dunnes	Donegal Catch 4 atlantic haddock breadcrumb					
52	Dunnes	Dunnes Stores Breaded haddock fillets					
53	Dunnes	Blue Vitality fish nuggets gluten & dairy free					
54	Dunnes	Dunnes white fish goujons					
55	Dunnes	Youngs chopshop 2 extra large fish fillets					
56	Dunnes	Birds Eye Simply breaded 2 fish fillets					
57	Dunnes	Birds Eye 10 fish fingers					
58	Dunnes	Youngs 10 fish fingers free from gluten, dairy & wheat					
59	Dunnes	Donegal catch 10 cod fish fingers					
60	Dunnes	Birds Eye 4 fish fillets					
61	Dunnes	Donegal catch 4 atlantic whiting					
62	Dunnes	Dunnes breaded cod fillets					
63	Dunnes	Dunnes seafood pie					Negative
64	Dunnes	Dunnes breaded cod fillets crispy crumb					
65	Dunnes	Dunnes battered cod fillets					
66	Dunnes	Dunnes my family favourites 4 breaded cod fillets					
67	Dunnes	Whiting	17.15				
68	Dunnes	Cod		16			
69	Dunnes	Smoked Haddock					
70	Dunnes	Haddock fillet	23.31				
71	Dunnes	Smoked Coley					
72	Marks and Spencers	Potato Topped ocean pie					
73	Marks and Spencers	Potato topped fisherman pie					
74	Marks and Spencers	Count on us fish pie haddock prawns salmon					
75	Marks and Spencers	Melt in middle 2 cod fishcakes					
76	Marks and Spencers	Melt in middle 2 haddock fishcakes	16.02				
77	Marks and Spencers	Made withour wheat gluten free scottish lochmuir salmon fillet fishcakes	22.58				
78	Marks and Spencers	Fish pie & juicy peas					
79	Marks and Spencers	2 smoked haddock fillet fishcakes	21.15				
80	Marks and Spencers	Made withour wheat gluten free 2cod fillets					
81	Marks and Spencers	6 jumbo breaded cod fish fingers					
82	Marks and Spencers	Beer battered cod & scraps					
83	Marks and Spencers	Made without wheat gluten free cod fillet fishcakes					
84	Marks and Spencers	9 chunky breaded cod fish fingers					
85	Marks and Spencers	2 cod fillet fishcakes (in crumb)					
86	Marks and Spencers	Breaded 2 cod fillets (in crumb)					
87	Marks and Spencers	Lightly dusted 2 haddock fillets					
88	McDonalds	Filet o fish	Negative	Negative	Negative	Negative	Negative
89	Genoa Sallins	Scampi	Negative	Negative	Negative	Negative	Negative
90	Genoa Sallins	Fresh Cod					
91	Marcaris Clane	Cod portion	Negative				
92	Genoa Sallins	Piece of cod					
93	Marcaris Clane	Fish Burger	Negative	Negative	Negative	Negative	Negative
94	Carragh	Cod					
95	Carragh	Cod					
96	Carragh	Cod					
97	Newbridge	Cod					
98	Newbridge	Cod					
99	Newbridge	Cod					
100	Newbridge	Cod					

Table 4.21: Meat sample details and results

			LAMP RESULTS				
Sample No	Sample Name	supplier	BEEF	HORSE	PIG	GOAT	SHEEP
1	Mince meat burger	tesco	Y	N	N	N	N
2	Aungus beef burger	tesco	Y	N	N	N	N
3	Value saver Lasagne	tesco	Y	N	N	N	N
4	Finest Lasagne	tesco	Y	N	N	N	N
5	Lamb Pie	tesco	Y	N	N	N	Y
6	Minced meat	local butcher Limerick1	Y	N	13.52	N	N
7	Beef meatballs	local butcher Limerick1	Y	N	N	N	N
8	Beef Burger	local butcher Limerick1	Y	N	13.22	N	N
9	Meatballs	local butcher Limerick2	Y	N	14.53	N	N
10	Beef burger	local butcher Limerick2	Y	N	N	N	N
11	Kebab stick	local butcher Limerick 1	Y	N	11.56	N	N
12	Meat balls	local butcher Kildare 1	Y	N	12.1	N	N
13	Beef burger	local butcher Kildare 1	Y	N	13.45	N	N
14	Meat balls	local butcher Kildare 2	Y	N	11.39	N	10.4
15	Beef burger	local butcher Kildare 3	Y	N	12.05	N	N
16	Meat balls	local butcher Kildare 3	Y	N	11.37	N	N
17	Beef burger	local butcher Kildare 3	Y	N	13.03	N	N
18	Big Eat Oak Hurst burger	Aldi	Y	N	N	N	N
19	Hunters bockhurst pork sausage	Aldi	N	N	Y	N	N
20	Beef grill steaks	Aldi	Y	N	N	N	N
21	Oakhurst chargrill quarter pounder	Aldi	Y	N	N	N	N
22	Oakhurst original irish beef burger	Aldi	Y	N	N	N	N
23	Brannans sweet chilli sausages	Aldi	10.1	N	Y	N	N
24	Bertie bear ham	Aldi	N	N	Y	N	N
25	Specially selected goat cheese tartlet	Aldi	Y	N	N	Y	N
26	Specially selected quarter pounders	Aldi	Y	N	N	N	N
27	100% pork meatballs	Aldi	N	N	Y	N	N
28	100% beef meatballs	Aldi	Y	N	N	N	N
29	Beef lasagne	Aldi	Y	N	N	N	N
30	Vegetarian Quarter pounder	Aldi	N	N	N	N	N
31	kellys peppered steak slices	Aldi	Y	N	N	N	N
32	Quickster sausage and egg muffin	Aldi	Y	N	Y	N	N
33	Quickster chargrilled quarter pounder	Aldi	Y	N	N	N	N
34	Quickerster flamegrill pork rib	Aldi	N	N	Y	N	N
35	simple bistro spaghetti bolognese	Aldi	Y	N	N	N	N
36	Smeatons pork sausage	Aldi	Y	N	Y	N	N
37	Sausage rolls	Aldi	N	N	Y	N	N
38	Vegetarian minced meat	Aldi	N	N	N	N	N
39	Mogerley chichen and mushroom pie	tesco	Y	N	N	N	N
40	Bolognese	tesco	Y	N	N	N	N
41	Everday value beef burgers	tesco	Y	N	N	N	N
42	Flame grilled beef burger	tesco	Y	N	N	N	N
43	Pulled Pork Pasty	tesco	N	N	Y	N	N
44	Beef hotpot	tesco	Y	N	N	N	N
45	Vegetarian Sausage	tesco	N	N	N	N	N
46	Beef lasagne	tesco	Y	N	N	N	N
47	Sausage rolls	tesco	N	N	Y	N	N
48	Steak and kidney pie	Dunnes Stores	Y	N	N	N	N
49	Spice Burgers	Dunnes Stores	Y	N	N	N	N
50	Beef Curry Pie	Dunnes Stores	Y	N	N	N	N

51	Minced steak and Onion Pie	Dunnes Stores	Y	N	N	N	N
52	Mixed Veg Soup	Dunnes Stores	N	N	15.3	N	N
53	Peppered Steak Slice	Dunnes Stores	Y	N	N	N	8.44
54	Chargrilled quarter pounder	Dunnes Stores	Y	N	N	N	N
55	Meat balls in onion gravy	Dunnes Stores	Y	N	N	N	N
56	Stewed steak	Dunnes Stores	Y	N	N	N	N
57	Steak and gravy pie	Dunnes Stores	Y	N	N	N	N
58	Steak and kidney pie	Dunnes Stores	Y	N	9.02	N	N
59	Minced Beef and pastry slices	Dunnes Stores	Y	N	N	N	N
60	Quarter pounders	Dunnes Stores	Y	N	N	N	N
61	Scooby doo meatballs	Dunnes Stores	Y	N	15.34	N	N
62	100% lamb Mince	Dunnes Stores	9.37	N	N	N	N
63	100% pork meatballs	Dunnes Stores	11.15	N	Y	N	N
64	Jumbo Sausage Rolls	Dunnes Stores	N	N	Y	N	N
65	Pork hot dog	Dunnes Stores	N	N	Y	N	N
66	Pork hot dog	Dunnes Stores	Y	N	Y	N	N
67	Hot deli counter sausage rolls	Centra	N	N	Y	N	N
68	Hot deli counter sausage	Centra	N	N	Y	N	N
69	Quorn Burger	Centra	N	N	N	N	N
70	Beef Burgers	Centra	Y	N	14.11	N	N
71	Lamb kebabs	Centra	N	N	N	N	Y
72	Chicken nuggets h	Mc Donalds	N	N	N	N	8.4
73	Beef Burger	Mc Donalds	Y	N	N	N	N
74	Meatballs	Supervalue	Y	N	12.35	N	N
75	Beef mince	Supervalue	Y	N	N	N	N
76	Deli pudding	Supervalue	N	N	Y	N	N
77	Aungus beef burger	Supervalue	Y	N	N	N	N
78	BBQ rib	Supervalue	N	N	Y	N	N
79	Deli sausage roll	Supervalue	N	N	Y	N	N
80	Pepperoni sausage	Supervalue	11.53	N	Y	N	N
81	Superquinn sausage	Supervalue	N	N	Y	N	Y
82	Chrizo	Supervalue	N	N	Y	N	N
83	Steak and onion pie	Supervalue	N	N	N	N	N
84	Beef burgers	Centra	Y	N	N	N	N
85	Kabanos	Centra	Y	N	Y	N	N
86	Hot dogs	Centra	N	N	Y	N	N
87	Black pudding	Centra	9.23	N	Y	N	N
88	Whiting pudding	Centra	N	N	Y	N	N
89	Steak and kidney pie	Fastfood outlet	Y	N	N	N	N
90	Hot pocket	Fastfood outlet	Y	N	N	N	9.23
91	Deli Sausage rolls	tesco	12.37	N	Y	16.02	N
92	Spice Bean Burgers	tesco	N	N	N	N	N
93	Mushroom burger	tesco	N	N	N	N	N
94	New York pulled pork	tesco	N	N	Y	N	N
95	Spanish Chrizo	tesco	N	N	Y	N	N
96	Microwave chicken burger	tesco	N	N	N	N	N
97	Steak Pie	tesco	Y	N	N	N	N
98	Deli Vegatable Samosa	tesco	N	N	N	N	N
99	Deli Vegatable spring roll	tesco	N	N	N	N	N
100	Deli Beef pastry	tesco	Y	N	N	N	N

5 Rapid Evaporative Ionisation Mass Spectrometry (REIMS)

Introduction

The recently developed ambient ionization mass spectrometric (MS) methods lifted a number of intrinsic constraints of traditional mass spectrometric analysis schemes, allowing *in-situ*, real-time analysis of a wide variety of samples. Although this feature of ambient MS methods has been demonstrated successfully, the early enthusiasm was curbed by problems regarding the interpretation of the resulting data. Since ambient sampling of unmodified objects does not allow reproducible analysis conditions (for instance in the case of the proposed luggage screening application at airports), the sensitivity of the method changes not only from sample type to sample type, but even in the course of the analysis of a single object (Ewing et al, 2001).

Ambient profiling has been gradually introduced in the last few years, mostly for the analysis of biological material including tissues and unicellular organisms (Huang et al, 2010). While Desorption Electrospray Ionization (DESI) (Takats et al, 2004) has offered an excellent solution for the metabolic/lipidomic profiling of cells and biological tissues, DESI analysis requires frozen section samples for optimal performance. This requirement is easily fulfilled in case of tissue imaging analysis, however it is not suitable for rapid analysis. As a response to this problem, a number of alternative solutions were developed, following fundamentally two distinct strategies. In the case of Probe Electrospray Ionization (PESI) (Hiraoka et al, 2007) and related techniques, a solid probe is immersed into the tissue specimen and the liquid phase residues are directly ionised from the sharp tip of the probe by direct electrospray ionization. The alternative approach is based on the mechanical or thermal ablation of tissue material that leads to the formation of an aerosol containing charged particles. Introduction of this aerosol into commercially available atmospheric ionization mass spectrometers results in the ionization of certain constituents producing characteristic spectral profile. Since both mechanical and thermal ablation methods are widely used in surgery for resection and coagulation of minor bleeds, these applications enabled the development of the so-called 'intelligent' surgical devices, where the surgical aerosol ('surgical smoke') is analysed using a mass spectrometer. The 'intelligent' surgical devices are able to identify tissues in real-time using unmodified surgical tools as ion sources. Due to its abundant use, electrosurgery-based 'intelligent' surgical devices were developed to practical level and are being tested in human tumour surgery environments. The underlying ionization method – i.e. the thermal ablation of largely aqueous

samples by electric current – was termed Rapid Evaporative Ionization Mass Spectrometry (REIMS) (Schafer et al, 2009) while the surgical application became widely known as 'iKnife', referring to the combined identification and cutting functions of the device. REIMS-based tissue analysis generally takes a few seconds and can provide histological tissue identification with 90-98 % correct classification performance which makes it a valid alternative to intraoperative frozen section histology. The feedback is given within seconds with potentially as good an accuracy compared to the duration of frozen histology of 20-40 minutes for a single specimen (Balog et al, 2013). Since almost any arbitrary biological sample can be analysed by means of REIMS, the applicability of the technique goes well beyond oncological surgery or just the analysis of human tissues. It has recently been demonstrated that the REIMS spectrum of bacterial colonies (obtained by using standard bipolar electrosurgical tools as an ion source) shows excellent taxonomical specificity, allowing also the differentiation of strains at sub-species (e.g. serotype) level (Strittmatter et al, 2014). Further advantage of the REIMS-based bacterial identification (e.g. compared to the widely used MALDI-MS based approach) is that the metabolic/lipidomic markers detected by the REIMS method remain selectively detectable in a mammalian tissue environment, i.e. using the REIMS approach bacteria can theoretically be detected in human or animal samples without culturing.

In course of the development of the iKnife method, most systematic characterisation studies were performed on food-grade animal tissues including mostly porcine, bovine and ovine organs, due to the ethical constraints associated with the use of human tissues. As a by-product of these studies, it was concluded that not only different mammalian species can be identified using the REIMS method, but the individual breeds also give distinctively different spectra and even subtle differences as the diet of the animals can have a measurable impact on the data. Based on these observations, REIMS technology is expected to find its application niche in the field of food security with special emphasis on food authenticity and food microbiology applications.

Mass spectrometric techniques have been widely used for food security/authenticity applications, mostly in the form of LC-MS, GC-MS and isotope ratio MS (IRMS) assays (Schipilliti et al, 2010; Levinson& Gilbride, 2011). GC-MS and LC-MS are largely used for the detection of xenobiotics (environmental pollutants, drug residues, illegal food additives, pesticides, etc.) in various foods or food ingredients (Handford, Elliott & Campbell, 2015; Botitsi et al, 2011; Lebedev, 2013; Nzoughet et al, 2013). More recently, biomarker-driven food authenticity testing has been gaining more attention, especially due to the high cost and time demand of the more traditional IRMS assays. Certain food types were found to contain chemically well-defined marker compounds (e.g. leptosin in Manuka honey) (Kato et al, 2012), which can easily be detected using regular HPLC-MS/MS approaches. However the gold standard still remains IRMS where sub-ppm shifts in the 13C/12C, 14N/15N and 16O/18O ratios of certain organic constituents are associated with the biological and geographical

origin of food components (Gentile et al, 2015). The most serious disadvantage of these methods is that the associated analytical schemes all require field sampling, transport of samples to the analytical laboratory, storage, extensive sample preparation, chromatographic separation and eventually mass spectrometric analysis of the individual molecular constituents of interest. As a result, the reporting times vary in the range of a few days to a few weeks, while the associated costs can reach several hundreds of dollars per individual sample. REIMS technology may solve some of these issues, as no sample preparation is needed, and the analysis can be done within a couple of seconds with a simple monopolar hand piece and the mass spectrometer with no extra cost, leaving the only issue of transporting the samples to a lab containing a suitable mass spectrometer. Since food counterfeiting (including mislabelling) is an emerging problem worldwide, the field of food authenticity testing requires new, cost-efficient, preferably on-site and real-time analytical approaches. This challenge was partially responded by the widespread introduction of infrared profiling techniques, which can also be performed by handheld infrared probes (He et al, 2007; Prieto et al, 2014). While infrared spectral profiles show excellent sensitivity to a broad range of variability including origin, presence of macroscopic constituents or method of preparation, the link between a detected anomaly and its chemical origin cannot be easily linked. For example, if a new spectral feature is detected, the spectral information does not give sufficient information for the identification of its molecular origin. In principle, REIMS profiling can solve this and the previously mentioned problems by providing in-situ, real-time molecularly resolved information. However these were not demonstrated by the current study. The main purpose of the current study is to explore the capabilities of REIMS profiling with regard to the species- and breed-level differentiation of raw meat products. Due to the scandal associated with horse meat found in various meat-containing food products in the UK in 2013, one of the specific aims was to demonstrate the capabilities of REIMS with regard to the identification of horse meat.

Meat speciation using REIMS

Results and discussion

The REIMS analysis of various animal tissues was found to yield spectra dominated by fatty acids and complex glycerophospholipids as it is shown in Figure 5.1 a, b and c. Although the spectra for the two different types of beef features are almost identical, a few characteristic differences can also be observed. In contrast, there is a clear difference between horse and beef spectra. The delay between sampling and appearance of the signal is less than a single second, while 2-3 second analysis time provides sufficient signal-to-noise ratio for the subsequent data analysis and identification of the sample. Carryover effects were studied by investigating the attenuation of the signal after stopping the evaporation of the sample. The effect of alternating the sample (eg. beef-horse-beef) on the was

also investigated. The signal was observed for less than 2s following the end of tissue evaporation and no carryover effects were observed when the samples were alternated without cleaning the setup in between the individual analyses, although visible contamination was observed on the inner wall of the ion transfer tube. The seemingly contradictory results (visible contamination – no signal carryover) were explained by the nature of the ionization phenomenon. As it was described earlier, the charged aerosol particles are formed upon the thermal evaporation of tissues and they are subsequently dissociated to produce the observed molecular ions. Since the ionic species used for tissue identification (i.e. glycerophospholipids) are non-volatile and they cannot undergo desorption ionization from the inner wall of the transfer tube, the observed contamination does not interfere with the recorded signal. The only surface where phospholipid desorption can occur is the jet disruptor (Appendix G, Figure G.1). However the jet disruptor surface is kept at 800°C and exposed to oxygen, hence the long-term survival of organic species is unlikely there.

With regard to the age and diet of the animals, previously we have shown that although age and diet does have an effect on the phospholipid profile, it doesn't affect the classification accuracy. The deviation due to age and diet differences is significantly lower than the distance between different classes (Balog et al, 2010).

Comparison of equine and bovine meat

The first series of experiments were aimed at establishing the difference between the spectral data obtained from the REIMS analysis of beef and horse meat (*Bos taurus* and *Equus caballus*, respectively). In order to assess the real-life applicability of the method, 50 gram burgers were fried in a pan with or without the use of oil. Different seasonings were also tested. The cooked samples were analysed alongside with the original raw specimens. Frying was done in a Teflon coated frying pan with or without vegetable (Venus sunflower) oil and two different seasoning was studied, a simple with salt and pepper and a complex using seasoning mix (containing: salt, dried vegetables (tomatoes, garlic with sulfite, onion), sugar, seasoning (paprika, black pepper, celery seed, nutmeg, cumin, coriander, chili), flavor intensifiers (sodium-glutamate, sodium-guanylate, sodium-inosinate), yeast extract, vegetable oil and silicone-dioxide). The resulting spectral data was pre-processed and subjected to PCA. The results are shown in Figure 5.2a. The figure clearly demonstrates that data points referring to different species of origin separate clearly along the first principal component, while data points show partial separation along the second principal component as a result of processing (cooking). The key message of the PCA analysis is that processing of the meat does not interfere with the identification of the species of origin. A Principal Component-Linear discriminant Analysis (PCA-LDA) model was built from a training set consisting of pure horse and Angus beef

spectra acquired from both raw and fried mince patties. The cross-validation of this PCA-LDA model resulted in a 99.5% correct classification of horse and beef, nevertheless the raw and cooked samples could be separated with an 80.1%-97.8% accuracy. The preparation of sample has clearly no effect on the differentiation of the species. Most well-established tests available for the identification of horse meat in food items employ either immunochemical or molecular genetic methods and both of these methods suffer from the hydrolysis of protein or DNA targets associated with heat treatment (Bertolini et al, 2015; Wang et al, 2015; Martin et al, 1988), however a number of novel ELISA-based and LC-based methods provide excellent results for heat-treated samples (Hsieh & Ofori, 2014; Balin, Vogensen & Karlsson, 2009; von Bergen, Brockmeyer & Humpf, 2014). In this aspect, the REIMS-based identification methods are also largely insensitive to heat treatment.

Figure 5.1: Mass spectra acquired from equine, and two different breed bovine meat with REIMS. The spectra mainly feature fatty acids in the m/z range 100-500 and glycerophospholipids in the m/z range 600-900.

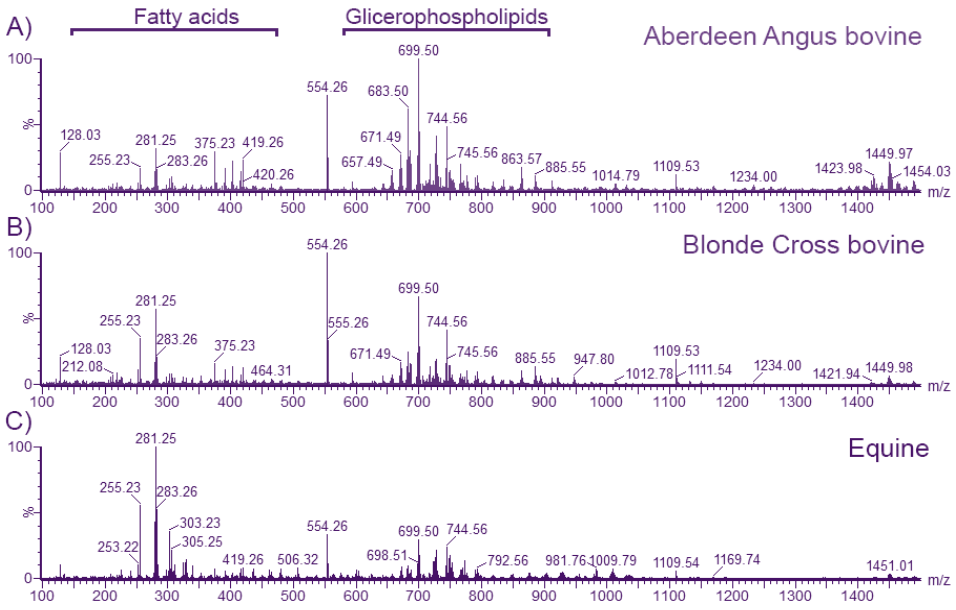
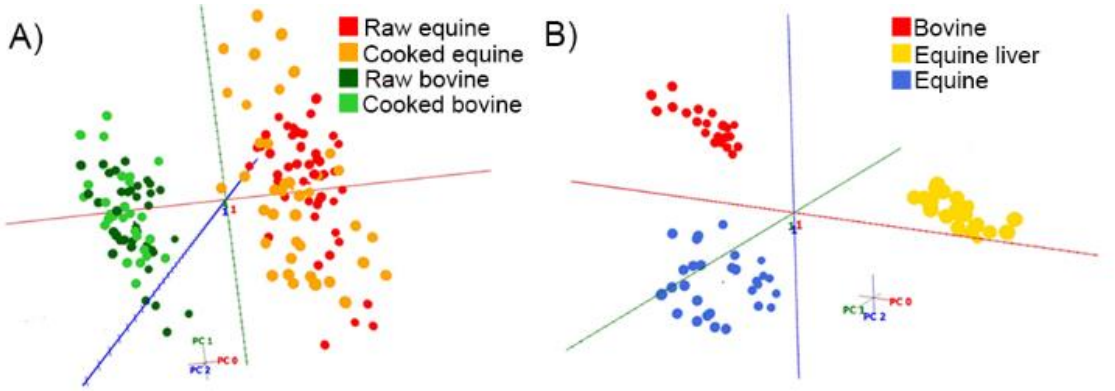


Figure 5.2: Identification of different products and effect of cooking on mass spectra. A) Three-dimensional PCA plot of raw and cooked Angus beef and horse meat. The preparation of sample has clearly no effect on the differentiation of the species. B) Three-dimensional PCA plot of two different horse organ products and beef. A clear separation can be observed on the PCA plot.

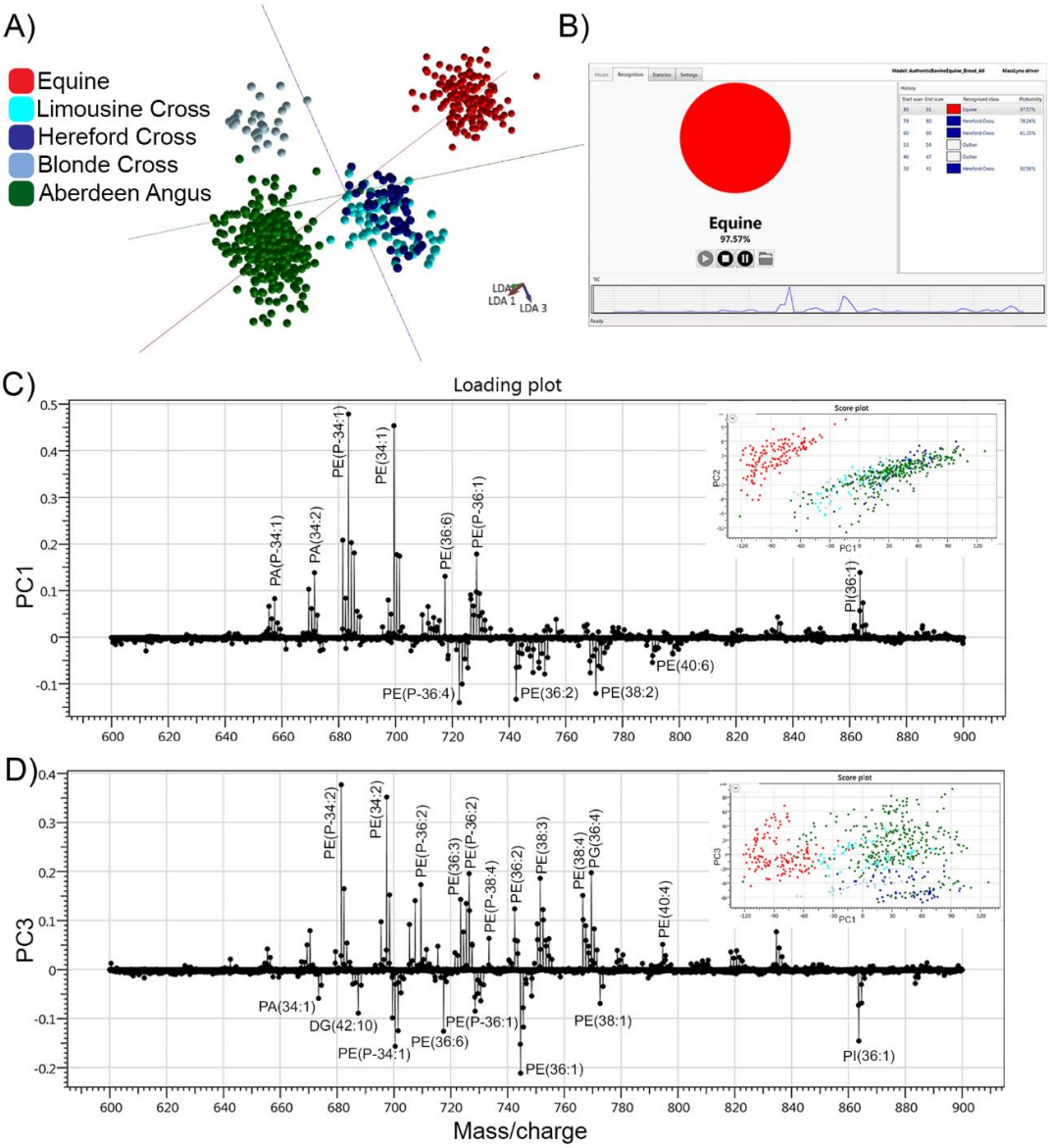


Following the proof of concept study, 15 authentic bovine and 5 equine samples were obtained. All samples were divided into 4 pieces and a total of 30 sampling points were taken in 4 separate experiments. A multivariate model was built from all sampling points (e.g. a total of 600 spectra including 300 Aberdeen Angus beef, 30 Blonde cross beef, 60-60 Hereford cross – Limousine cross and 150 Equine) and the classification performance was evaluated using leave-one animal out cross validation resulting in 100% separation between equine and bovine samples. A second, leave 20% out cross validation was calculated for different breed types, as there was only one Blonde cross animal sample, thus leave-one animal out was not applicable. There was a 97.48% correct classification rate as shown on the confusion matrix in Table 5.1. The pseudo 3-D LDA plot is shown on Figure 5.3a. A small portion of samples were ground and 4 10g patties were created containing 2.5-5-10% of equine meat in Hereford cross bovine meat and 25% equine in 25% Limousine cross meat. The patties were tested subsequently by sampling each of them 10 times and classified immediately using the multivariate model built from all 600 sampling points. The breed of the bovine meat was correctly classified in all 4 cases, while the horse meat was detected in 3 cases out of 4 (Figure 5.3b). In case of 5% equine – 95% bovine, the horse meat was not detected.

Table 5.1: Confusion matrix of leave-one animal out cross validation of equine – bovine multivariate model.

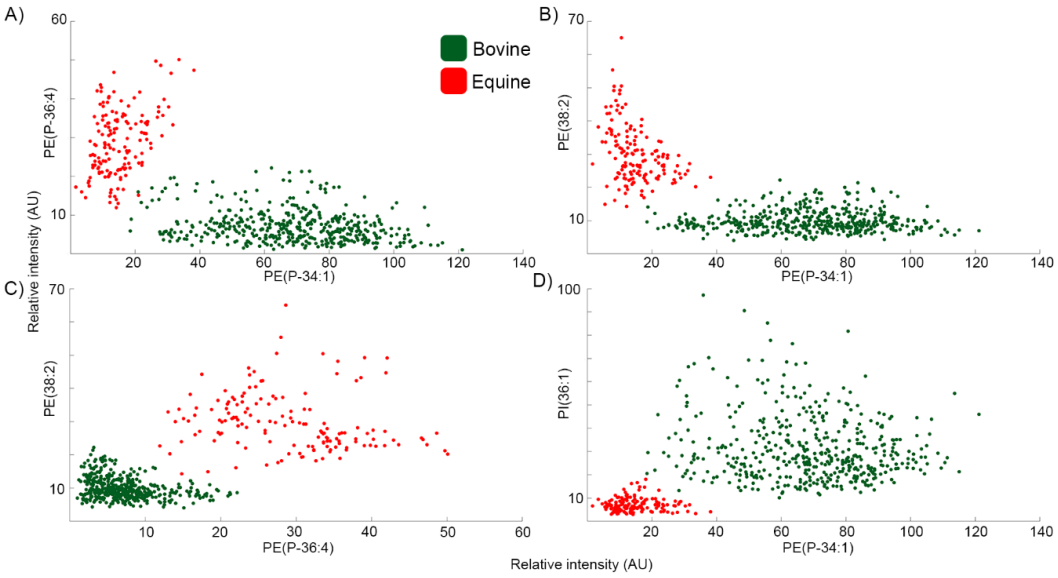
	Aberdeen Angus	Blonde Cross	Equine	Hereford Cross	Limousine Cross	Outlier	Total
Aberdeen Angus	294	3	0	0	0	3	300
Blonde Cross	0	29	0	0	0	1	30
Equine	0	0	150	0	0	0	150
Hereford Cross	0	0	0	54	6	0	60
Limousine Cross	0	0	0	6	54	0	60

Figure 5.3: A) Pseudo 3-D plot of data obtained by the analysis of 4 different bovine breed and equine samples. B) Real-time analysis of meat with home-build classification software. C) First principal component loading plot with an insert showing the first two principal components. D) Third principal component loading plot with an insert containing the first and third principal component. PC3 was chosen because the separation between different cattle breed was more specific to PC3, while the separation between equine and bovine was more specific to PC1 and PC2.



PCA not only allows us to look at the similarities and differences in large multivariate datasets, but can also shed light on the background of the separation of data groups in the form of loading functions explaining the composition of individual principal components. In case of mass spectrometric data, the loading functions show what the contribution of individual mass spectrometric peaks is to the given principal component, as it's shown in Figure 5.3c and d. The loading function responsible for the separation of the REIMS lipidomic profiles of horse and cattle skeletal muscle shows clear biochemical information. The base peaks in both directions (i.e. positive and negative) correspond to phosphatidyl-ethanolamine species including plasmalogens with 34-36-38 carbon atom cumulative acyl chain length, however horse tends to produce stearoyl-linoleyl-phosphatidyl-ethanolamines (PE 36:2, PE 38:2), while cattle produces PE 34:1, PE 36:1 in larger amounts, as it was revealed by the loading plot and MS/MS fragmentation of the corresponding ions (Figure 5.3c). Similar saturation/desaturation patterns were observed for other PE molecular ions and their [M-NH4]- counterparts and phosphatidil-inositol ions (PI) produced by the REIMS process. The 6 most abundant peaks of PC1 and PC2 was selected for further "marker ratio" analysis. All of them were coupled and the 4 combinations with the highest discriminating power were selected e.g. PE(P-34:1)/PE(P-36:4) and PE(P-34:1)/PE(38:2) and PE(P-36:4)/PE(38:2) and PE(P-34:1)/PI(36:1) and tested (Figure 5.4). The cross-validation based uniquely on these peaks between equine and bovine resulted in 100% separation. Figure 5.4a,b both show the combination of one bovine and one equine specific peak, thus the ratio of the peaks could be used for the separation of the two species. Figure 5.4c shows a combination of two peaks more abundant in equine, while Figure 5.4d shows the combination of two peaks more abundant in bovine. Both combinations have the power to differentiate between the species, however the ratio would not be suitable for separation.

Figure 5.4: Comparison of level of unique phosphatidil-ethanolamine and phosphatidil-inositol species in bovine and equine. The relative intensity of different phospholipid species in each sample spectrum is shown on the figure.



Comparison of different tissue types within the same species. Two different tissue types of the same species and a given tissue type of two species were analysed in an experiment and the results were subjected to PCA analysis in order to demonstrate the relative magnitudes of the difference between species and tissue types. The results are shown in Figure 5.2b, demonstrating that there are comparable differences in both species and organ level, however these do not interfere with each other. There was a 100% correct classification of the three different products based on the cross-validation underlying the clear separation observed on the PCA plot. Results imply that the REIMS technique, given that sufficient amount of reference data is available, is able to identify not only the species of origin, but also the type of tissues. This is not surprising in the light of earlier human studies, where REIMS was used for the identification of various healthy and cancerous human histological tissue types.

Comparison of different type of cattle, horse, venison and their mixtures. In food counterfeiting cases where ground meat is involved, the actual product is rarely pure with regard to its species of origin. In most of the cases the mixtures contain variable amount of the inappropriate component, hence food testing assays are expected to detect the 'contaminant' down to 1% concentration. While the multivariate model is not ideal for the trace detection of a tissue with a certain taxonomical origin in

the matrix of the other, we tested the capabilities of the method in this direction by constructing a PCA/LDA supervised model featuring horse (*Equus caballus*), venison (roe deer; *Capreolus capreolus*) and two types of cattle (Black angus, Wagyu; *Bos taurus*) skeletal muscle and the 1:1 binary mixture of all pairs. The model featuring complete separation among the individual groups already in the demonstrated three dimensions is shown in Figure 5.5. Leave-20% out cross-validation of all pure and 50-50% mixed minced samples resulted in 97.79% correct classification, the confusion matrix is shown in Table 5.2. Although the localisation of binary mixtures in between their components is reassuring regarding the linearity of the model, the relative dispersion of the homogeneous data groups already indicates the lack of feasibility of proper quantification using the model. The model is likely to allow the detection of 10-20% contribution of tissue produced by another species in the sample, however these numbers are not satisfactory from a legal/regulatory aspect yet.

Table 5.2: Confusion matrix of leave-one animal out cross validation of grain fed beef (GR), horse (HO), vension (VE) and WAGYU beef (WA) multivariate model and the 1:1 mixtures. The overall accuracy was 97.79%.

	50HO 50GR	50VE 50GR	50VE 50HO	50VE 50WA	50WA 50GR	50WA 50HO	GR	HO	VE	WA	Outl.	Total
50HO50GR	79	0	0	0	0	0	1	3	0	0	1	84
50VE50GR	0	21	0	0	0	0	0	0	0	0	0	21
50VE50HO	0	0	17	0	0	0	0	1	3	0	0	21
50VE50WA	0	0	0	22	0	0	0	0	0	0	0	22
50WA50GR	0	0	0	0	82	0	0	0	0	0	1	83
50WA50HO	0	0	0	0	0	81	0	0	0	0	1	82
GR	0	0	0	0	1	0	93	0	0	0	1	95
HO	0	0	0	0	0	2	0	113	0	0	0	115
VE	0	0	0	0	0	0	0	0	50	0	1	51
WA	0	0	0	0	4	0	0	0	0	107	0	111

Figure 5.5: Pseudo 3-dimensional plot of products from 4 different species or breed and the 1:1 mixture. The axes represent the projected coordinates of linear discriminant 1, LD1 (red), LD2 (green) and LD3 (blue).

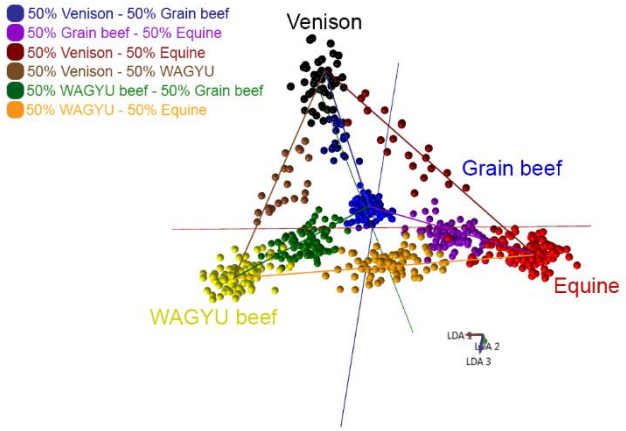


Table 5.3: Classification of patties containing one or more different meat type. The detection limit was based on the false negative results.

% of different meat types in the patty	Horse (HO) meat detected	Wagyu (WA) meat detected	Venison (VE) meat detected	Grain beef (GR) meat detected	False positive/ negative
1.25HO98.75GR	-	-	-	+	FN
1.25HO98.75VE	-	-	+	-	FN
1.25WA98.75GR	-	-	-	+	FN
1.25WA98.75VE	-	-	+	+	
2.5HO97.5GR	-	-	-	+	FN
2.5HO97.5VE	-	-	+	-	FN
2.5WA97.5GR	-	-	-	+	FN
2.5WA97.5VE	+	+	+	-	FP
5HO95GR	+	+	-	+	
5HO95VE	+	-	+	+	FP
5WA95GR	-	+	-	+	
5WA95VE	-	+	-	+	
10H090GR	+	-	-	+	
10HO90VE	+	-	+	+	FP
10WA90GR	-	+	-	+	
10WA90VE	-	+	+	-	
GR	-	-	-	+	
33GR33HO33WA	+	+	-	+	
HO	+	-	-	-	
33HO33VE33GR	+	-	+	+	
VE	-	-	+	+	FP
33VE33HO33WA	+	+	+	+	
25VE25HO25WA25GR	+	+	+	+	
WA	-	+	-	+	
33WA33VE33GR	-	+	+	+	

Detection limit

In order to test the feasibility of the detection of various amounts of different meat present, the various types of tissues were finely ground and thoroughly mixed together at different ratios (1.25-2.5-5-10%-equal mix of 3, equal mix of 4 different meat). Mince patties were prepared using 25 g (~6 oz) of sample and were sampled 11 times (in an approximate timeframe of 3 s/sampling point). Spectra

were classified using a model containing pure horse/beef/venison/Wagyu and different mixtures (e.g. 25-50-75% of each). The results of this study are shown in Table 5.3. In case of 1.25% and 2.5% patties, in only 1 out of 4 cases was the meat detected, however in all other cases all meat types mixed in the patty was detected. The results show that detection limit down to 5% concentration level is feasible using the REIMS technology. In real life examples, most of the food fraud cases will contain more than 5% of illegal mixture within the product, if not the whole species is changed to a less expensive alternative. In order to assess the feasibility of the detection of even lower concentration levels (preferably down to 0.1%) of tissue of another species present in meat products, acquisition of considerably more data is necessary. Furthermore, trace detection will require a different bioinformatics approach, most likely by the discovery of species-specific biomarkers which can be used as presence/absence marker for a given species.

Conclusion

Application of the REIMS technique for the rapid lipidomic profiling of food-grade meat products was successfully performed for the first time. The described results clearly demonstrate that the lipidomic profiles can be recorded in a few seconds timeframe and the profiles show good animal species-level specificity. Furthermore, the results obtained for Angus and Wagyu beef implies that the method show some sub-species (e.g. breed) selectivity and can potentially also be used to detect even finer differences, e.g. geographic origin of the animals.

Fish analysis using REIMS: Speciation and catch method

Introduction

In the present study REIMS was applied to five commercially popular white fish species (cod, coley, haddock, pollock and whiting) and investigated as to whether REIMS based lipidomics could be applied to fish speciation measuring the phospholipid profiles of each species. To date, there are very few studies which have investigated fish speciation using a lipidomic approach and/or an accelerator mass spectrometry (AMS) technique. The REIMS technology has the potential to fulfil both criteria, whilst also addressing another more complex issue of fish fraud which to date has never been detected using any form of analytical systems. The 'seven sins of fish' are widely regarded as the seven different ways in which fish fraud can manifest itself (Elliott, 2014). Substitution of one species of fish for another is by far the most commonly reported. Another, but much less well known form of fraud is 'catch method' which is a mandatory requirement on the labels of all unprocessed and prepacked products sold commercially within the EU (EC Pocket Guide, 2014). To date, the scientific investigation of the identification of different catch methods within the same species has never been

undertaken. However, using the innovative REIMS technology the possibility of differentiating between line and trawl caught haddock by measuring and comparing the phospholipid profiles of these two types of catch was undertaken to determine if discrimination could be achieved.

Results

REIMS fish speciation

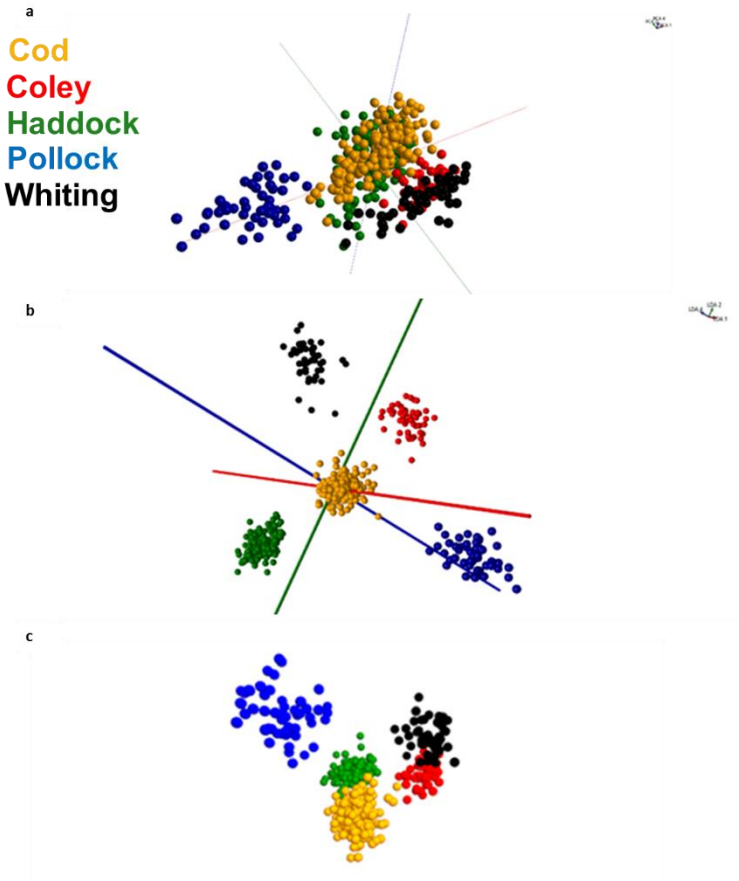
Raw spectroscopic data obtained from authenticated samples of cod (n=194), coley (n=51), haddock (n=133), pollock (n=50) and whiting (n=50) were dominated by intact fatty acids (m/z 200-500) and phospholipids (m/z 600-950). The REIMS data were pre-processed and subjected to multivariate analysis where PCA, LDA and Orthogonal OPLS-DA were applied. 80 PCA components and 4 LDA components were used to generate the chemometric models which were based upon the phospholipid profiles (m/z 600-950) of each fish sample. Clustering was identified within the 3-D PCA score plot using components 1,2 and 4 (Figure 5.6a). However, clear separation between the five species of fish was obtained within the 3-D LDA score plot using components 1,2 and 4 (Figure 5.6b) and the OPLS-DA score plot where 4 latent and 4 orthogonal components were used (Figure 5.6c). The effects of various freeze/thawing cycles were also investigated within this study in which samples (n=32) were frozen/thawed up to four times. All models establish that the varying cycles do not interfere with the quality of the raw data and thus species identification. A leave-20%-out cross validation of the PCA-LDA models, where one average spectrum per sample was used resulted in a 99.37% correct classification (Table 5.4).

Table 5.4: Results from the leave-20%-out cross validation of the PCA-LDA speciation models generated using the prototype software package in which an overall correct classification rate of 99.37% was achieved. Of the 478 samples analysed, only 3 were not assigned the correct species classification.

	Cod	Coley	Haddock	Pollock	Whiting	Outlier	Total	Correct classification rate (%)
Cod	193	0	0	0	0	1	194	99.48
Coley	0	51	0	0	0	0	51	100.00
Haddock	0	0	132	0	0	1	133	99.25
Pollock	0	0	0	50	0	0	50	100.00
Whiting	0	1	0	0	49	0	50	98.00
Total							478	99.37

Additionally, a correct classification rate of 99.37% was obtained for the OPLS-DA model (supplementary information) which was due to two cod samples being identified as coley and whiting, and one coley sample being identified as whiting.

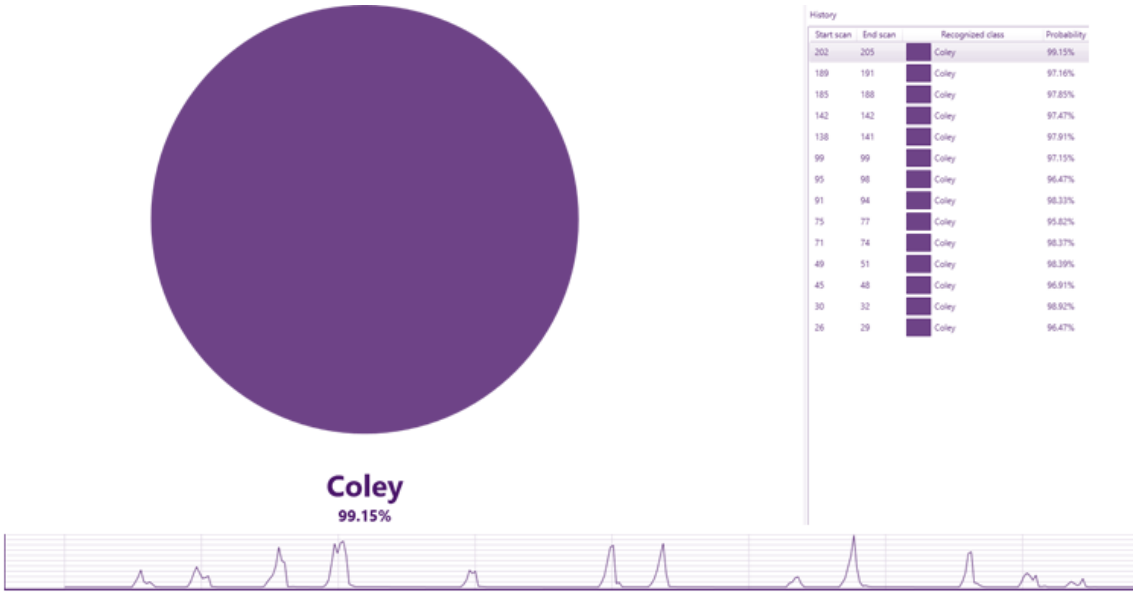
Figure 5.6: (a) PCA, (b) LDA and (c) OPLS-DA models generated using the prototype software package and SIMCA 14. All models were generated using the phospholipid profiles (m/z 600-950) of the fish samples with clear separation of the five fish species of fish; cod (orange), coley (red), haddock (green), pollock (blue) and whiting (black) visible within the 3-D LDA and OPLS-DA models.



External validation of speciation models. Validation of the PCA-LDA speciation models was carried out using an external set of authenticated cod (n=22), coley (n=20), haddock (n=20), pollock (n=20) and whiting (n=17) samples which had not been previously used to generate the chemometric models. The PCA-LDA models were exported to a prototype recognition software package allowing REIMS spectral

data to be both acquired and run live through the software providing a near instantaneous identification (Figure 5.7). Of the 99 samples analysed, 98 (98.99%) were correctly identified with one cod sample being assigned as an outlier. However, to ensure validity the samples were also cross validated in a similar manner to that of the leave-20%-out cross validation (Table 3). An overall correct classification rate of 98.99% was achieved.

Figure 5.7: Validation of the speciation models using the prototype recognition software package and a further set of authenticated fish samples. In this scenario, the sample under investigation is correctly identified as coley near instantaneously excluding the delay between sampling and appearance of a signal which was ~2s. Twelve cuts were taken from this sample which is identified in the chromatogram and a standard deviation of 5 was used for class assignment. Of the 99 samples analysed, 98 were correctly identified with one cod sample being assigned as an outlier.



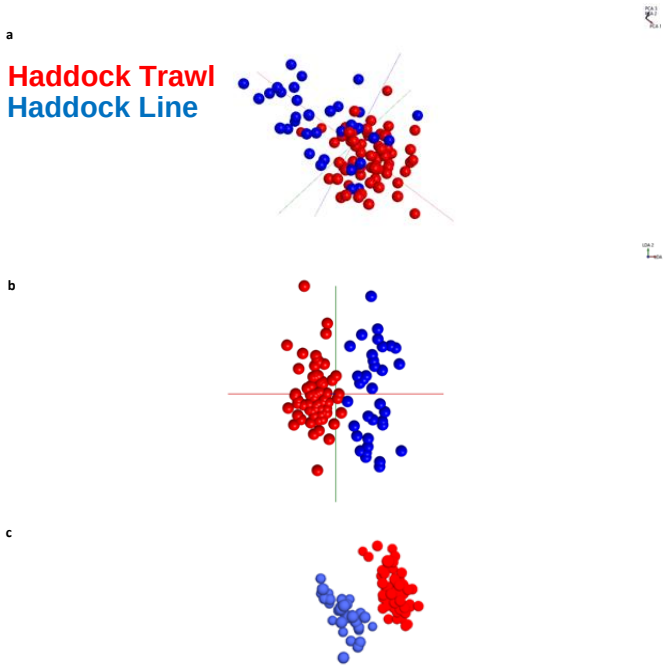
PCR testing of suspect 'haddock' samples. Whilst generating the speciation models, six suspect samples labelled as haddock were identified as cod using both the real-time recognition software and the prototype software cross validation. Additionally, both the LDA and OPLS-DA models grouped the six 'haddock' samples within the cod clusters. As a result, the samples were analysed using PCR. Mitochondrial cytochrome c oxidase subunit I gene (COI) was used as genetic marker for the six

samples which all showed 99% similarity with Gadus morhua species (a cod fish) on both Genbank and BOLD. No significant similarities were found with Melanogrammus aeglefinus (haddock).

Analysis of seabass and seabream samples. Raw spectroscopic data obtained from authenticated samples of seabass (n=6) and seabream (n=8) were simultaneously run live through the prototype recognition software providing a near-instantaneous classification. Of the 14 samples analysed, 13 (92.86%) were correctly identified as outliers with one sample being identified as both an outlier and coley sample. However, 66% of the cuts within the sample were identified as outliers with the other 34% being identified as coley. When carrying out a cross validation similar to that of the external validation, an overall correct classification rate of 100% for all 14 samples was obtained as the cross validation uses one average spectrum of all the cuts per sample.

REIMS catch method – haddock line v trawl. Raw spectroscopic data obtained from both line caught (n=35) and trawl caught (n=65) haddock samples were exposed to multivariate analysis allowing PCA, LDA and OPLS-DA models to be generated. Twenty PCA components and 3 LDA components were used to generate the catch method models. Some separation was apparent within the 3-D PCA score plot using components 1,2 and 3 (Figure 5.8a). However, clear separation was attained in the two-dimensional (2-D) LDA score plot using components 1 and 2 (Figure 5.8b), and the OPLS-DA score plot (Figure 5.8c) in which 1 latent and 4 orthogonal components were used. A leave-20%-out cross validation of the PCA-LDA models resulted in a 96.00% correct classification with three trawl caught samples and one line caught sample being misidentified. However, a correct classification rate of 100% was obtained for the OPLS-DA model.

Figure 5.8: (a) PCA, (b) LDA and (c) orthogonal partial least squares-discriminant analysis (OPLS-DA) models generated using the prototype software package and SIMCA 14. Clear separation of the two catch methods; haddock trawl (red) and haddock line (blue) is visible within the 2-D LDA and 3-D OPLS-DA models.



Discussion

Predominately REIMS is a technique which has been utilised for medical and bacterial applications. However, recent studies indicate that this innovative technology may also find an application towards the detection of food fraud. This proof of concept study aimed at differentiating between five commercially popular white fish species further demonstrates this. The raw REIMS spectral data of the five species of fish are visually very similar, yet it is evident within the chemometric models that fish speciation is achievable based upon their phospholipid profiles (m/z 600-950). Consequently, this suggests that separation of the fish classes is attained through the varying intensities and ratios of the ions and not the presence of unique markers. The absence of species specific markers is understandable, especially as the five species of fish under investigation are all very similar and classified under the same genus; gadidae. More importantly, a binning of 0.5 Da was used to generate the chemometric models. The REIMS technology produces a peak at each Dalton which is extremely beneficial when carrying out an untargeted metabolomics study as large amounts of data can be acquired. However, it can also be an inconvenience when using an external lockmass such as Leucine Enkephalin (m/z 554.2615) and can occasionally result in a peak either competing with or in some cases engulfing the lockmass ion. This was identified at various stages of our study and meant that a binning lower than 0.5 Da could not be applied to generate the chemometric models. The use of an internal lockmass using a peak within the raw data can circumvent the issue, however, it requires that peak to be present in all species and samples. Such a broad binning would not often be associated with the majority of metabolic or lipidomic profiling experiments. In saying this, our fish speciation

study clearly identifies that the separation of five similar white fish species is achievable. As a result, this may imply that the use of high resolution mass spectrometry (HRMS) is not necessarily a requirement for a qualitative screening study aimed at identifying the species of a fish fillet, but may be necessary when attempting to identify unique species specific markers.

Lipidomic profiling is an extremely popular approach with regards to food analysis. When it comes to fish and meat analysis it is less prominent and is often side-lined for a genomic or proteomic approach as proteins and DNA remain relatively unchanged. This makes them appropriate molecules for species detection and identification within fresh, frozen, cooked and processed food products. Oxidation and hydrolysis of phospholipids can occur during storage and freeze/thawing cycles which may result in quality deterioration of a fish sample thus making it difficult to analyse samples. Therefore, as well as establishing that speciation is achievable using a lipidomics approach, it was also fundamental to determine that the number of freeze/thawing cycles did not have an impact on the quality of the data and thus, the models. The PCA, LDA and OPLS-DA models clearly exhibit that; (1) storage at -80°C does not affect the lipid profiles of the fish samples; (2) the varying freeze/thawing cycles do not compromise the integrity of the phospholipid intensities and ratios and (3) if there are any changes within the phospholipids then they are overcome by differences amongst the varying species, i.e. speciation. Perhaps this, and in turn the overall stability of the models is reflected in the leave-20%-out cross validation in which an overall correct classification rate of 99.37% was achieved (Table 4.1). This was in relative accordance with the misclassification table of the OPLS-DA model using SIMCA 14, in which a 99.37% correct classification was achieved. R2 and Q2 values of 0.829 and 0.809 were obtained respectively indicating that the OPLS-DA model had both a good quality of fit and predictivity towards new data. A large Q2 value also suggests that the multivariate data points are fairly clustered with there being very few outliers within the dataset as exemplified in all of the chemometric models within Figure 5.3.

Whilst the internal leave-20%-out cross validation using the prototype software and the misclassification table using SIMCA 14 indicate that the relevant models are robust and stable, they do not necessarily reveal how the models would perform when using an external set of samples which have not been previously used to generate the chemometric models. Although the OPLS-DA model has a Q2 value of 0.809, this could still be a result of over fitting. However, there is not a large discrepancy between the two values which is often the case with over fitted models. The relevant permutation tests (supplementary information) also justify this stance.

Therefore, validation using further authenticated samples of cod, coley, haddock, pollock and whiting was essential. In order to fulfil this, a two-tier approach was carried out. The first involved exporting the PCA-LDA model(s) generated by the prototype software to a prototype recognition software. This allowed REIMS spectral data to be simultaneously acquired and run live through the software enabling

a near-instantaneous identification of each sample (Figure 5.4). Of the 99 samples analysed, 98 (98.99%) were correctly identified with one cod sample being classified as an outlier. The second approach was carried out to ensure the validity of the results from the recognition software. The raw data were subjected to a cross validation similar to that of the leave-20%-out cross validation, in which a model was created using the training set and then the 99 validation samples were assigned a fish species classification. The results were in coherence to that of the recognition software and a correct classification rate of 98.99% was obtained (Table 5.5).

The two-tier validation with further samples not only established the stability and reliability of the chemometric models, but it also verified that they were not over fitted. Perhaps more impressively and one of the core reasons for carrying out this research was the fact that the correct identification of a fish species was obtained near instantaneously, excluding the delay between sampling and appearance of a signal which was ~2s. Considering that no sample preparation was required, which is a major pitfall for most conventional techniques such as ELISA, LC-MS, NMR and PCR, coupled with the speed at which an accurate species identification was obtained, it is significant that within this study we have demonstrated that REIMS, and AMS in general, has a prominent role to play in tackling fish fraud.

Table 5.5: Validation of the speciation models using the prototype software package cross validation ensuring the results from the prototype recognition software were accurate. All samples were assigned the correct fish species except one cod sample which was identified as an outlier resulting in an overall correct classification rate of 98.99%.

	Cod	Coley	Haddock	Pollock	Whiting	Outlier	Total	Correct classification rate (%)
Cod	21	0	0	0	0	1	22	95.45
Coley	0	20	0	0	0	0	20	100.00
Haddock	0	0	20	0	0	0	20	100.00
Pollock	0	0	0	20	0	0	20	100.00
Whiting	0	0	0	0	17	0	17	100.00
Total							99	98.99

In order for ambient ionisation techniques to play a more significant role towards the investigation of the mislabelling of fish, they have to be thoroughly scrutinised against so called conventional techniques. This is essential, particularly with regards to quantitative studies. During our investigation and generation of the speciation models it was found that six samples labelled as haddock where clustered within the cod samples. Therefore, we analysed them using PCR to establish whether they were indeed haddock or whether they had accidentally been mislabelled. Mitochondrial

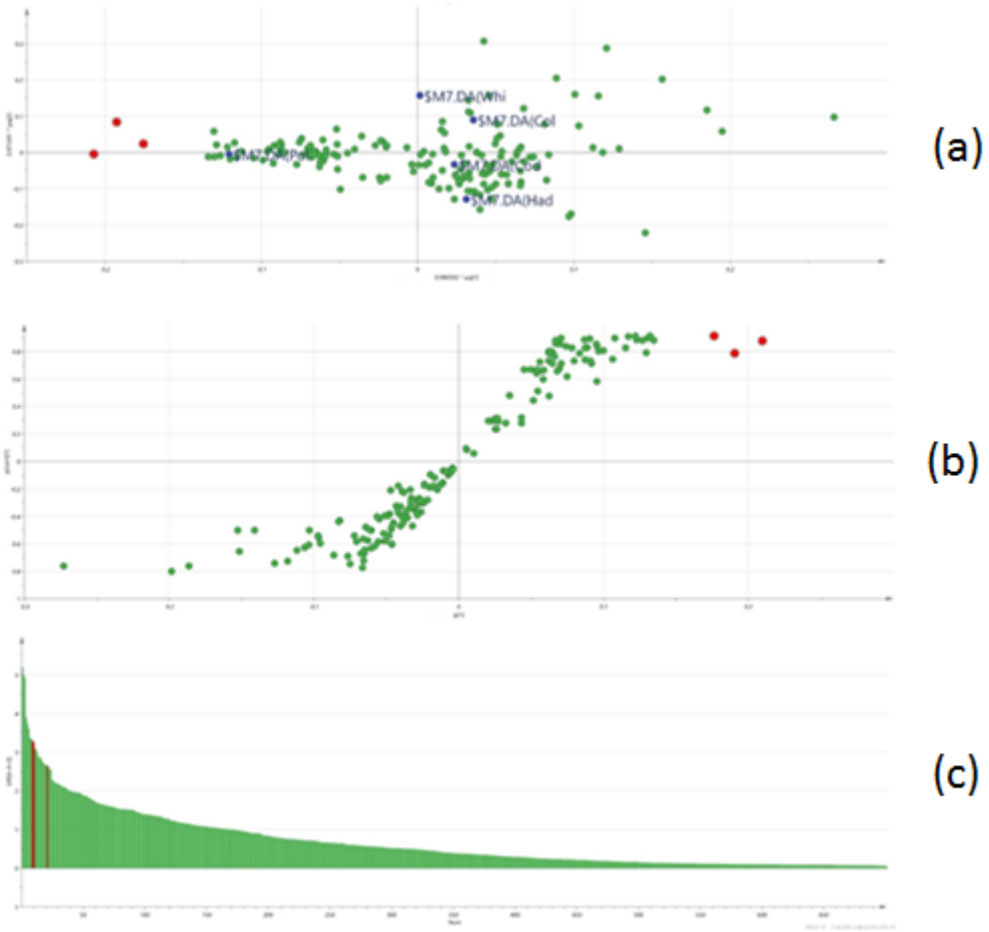
cytochrome c oxidase subunit I gene (COI) was used as genetic marker for the six samples, in which all showed 99% similarity with Gadus morhua species (a cod fish) on both Genbank and BOLD. No significant similarities were found with Melanogrammus aeglefinus (haddock). Albeit six samples, this demonstrates why AMS techniques are beginning to play a more prominent role in food analysis. Both PCR and REIMS analysis provided identical speciation results, but the time taken to achieve them were very different. Whereas the REIMS technology in conjunction with the real-time recognition software provided a result within seconds, PCR analysis provided results of the six samples within 24 hours, including time taken for sample preparation. REIMS has the capability to analyse many samples within the timeframe taken for a PCR result. These comparisons are hugely significant for the development of AMS, but they undoubtedly show how companies with fast moving supply chains will be operating quality checks in the near future, with fast and accurate results attainable within seconds. It should be stated that the mislabelling of the six cod samples within our model building process do not fall under the same category as the majority of the studies identified, in which economic gains were the motivation. In this scenario, genuine misidentification or human error are to blame which can sometimes occur due to the large size of the fish genus.

Correct identification of a fish species is imperative. However, it is equally important that the models and recognition software do not assign a species classification to a sample whose species was not used in the model building process. As a result, 8 seabream and 6 seabass samples were analysed using the prototype recognition software. 92.86% of the samples were correctly identified as outliers with one seabream sample being classified as both an outlier and coley sample. However, further analysis of the sample indicated that 66% of the cuts were identified as an outlier with the other 34% being identified as coley. Similar to the external validation of the PCA-LDA speciation models, the samples were subjected to a cross validation where they were left out of the model building process and then assigned a fish species classification. Due to the fact that the one suspect sample was predominately assigned an outlier classification and that the software takes an average spectrum of all the cuts for each sample, this resulted in the sample being assigned as an outlier which led to a 100% correct classification of the 14 samples. This further demonstrates along with the external validation of the speciation models and the PCR testing of the six suspect ‘haddock’ samples that fish speciation is achievable using both REIMS and a lipidomics approach, with fast and accurate results attainable.

Separation of the five white fish species is evident within the chemometric models. However, as eluded to previously this is most likely not a result of unique species specific markers. Instead there are several ions which are found at significantly higher levels in certain species compared to that of others. Whilst PCA score plots illustrate the similarities and differences within the large multivariate data set (Figure 5.3a), PCA loading plots interpret which mass spectrometric peaks contribute the

most to each individual principal component (PC). Figure 5.9 demonstrates the method which was taken to identify ions which were found to occur more prominently in pollock samples. This included; (a) a loading plot demonstrating which ions led to the distribution of the PCA score plot (b) a S-plot identifying which ions are found more prominently in pollock compared to the other species of fish and (c) a variable importance for the projection (VIP) plot which identifies which ions have the greatest influence on the large multivariate dataset. Those labelled in red signify the ions which contribute most to pollock being separated from the other species. In order to generate S-plots individual OPLS-DA models of each species of fish against the other four species were generated. The VIP plot of all 701 ions (Figure 6.7c) in the large dataset clearly demonstrates that the ions labelled in red are of great importance with all of them having a VIP >2 and therefore contribute significantly to the outcome of the PCA score plot. It is important to state though that individual OPLS-DA models for each species of fish can be generated excluding ions with a VIP <1 as they bear little importance to the large dataset. This would result in the models and S-plots being generated from 169 of the 701 ions in the large dataset. To identify ions which were significant to the other four species of fish, the same workflow was carried out for all. In total xx number of ions were identified and a putative identification was assigned.

Figure 5.9: Method to identify ions which are found predominately within pollock compared to that of the other species of fish. (a) Loading plot identifying the average position of each species of fish and the relevant ions that contribute most to their positioning; (b) a S-plot of pollock v the other species of fish identifying the ions that are found predominately in pollock. The S-plot was generated excluding ions with a VIP <1 resulting in it being made up of 169 ions; (c) a VIP graph of all 701 ions analysed in the multivariate dataset. The three ions identified within the loading and S-plots (red) have great significance towards the dataset and explain the positing of the PCA score plots.



Substitution of one species of fish for another is by far the most commonly reported with regards to fish fraud. However, there are six other formats in which fish fraud can manifest itself; IUU fishing; fishery substitution; processed raw material authenticity (species adulteration); chain of custody abuse; undeclared product content and catch method. To date, the scientific investigation of

different catch methods within the same species of fish has never been reported. In our study, 100 haddock samples made up of both line (n=35) and trawl (n=65) caught were investigated. Both the LDA and OPLS-DA models identified clear separation between the two catch methods. A leave-20%-out cross validation using the prototype software resulted in a 96% correct classification of the PCA-LDA models, whilst a 100% correct classification was obtained for the OPLS-DA model (Table 5.6). R2 and Q2 values of 0.906 and 0.788 were obtained respectively suggesting that the OPLS-DA model was both robust and had good predictability towards a new set of data.

Table 5.6: Results from the misidentification table of the catch method OPLS-DA model generated using SIMCA 14 in which a 100% correct classification was obtained.

	Haddock Line	Haddock Trawl	Total	Correct classification rate (%)
Haddock Line	35	0	35	100.00
Haddock Trawl	0	65	65	100.00
Total			100	100.00

Separation of the two haddock catch methods was achieved but it is questionable as to whether this was due to genuine differences in which the fish samples were caught. REIMS is a lipidomic based technique dominated by intact phospholipids and fatty acids. The different methods in which a fish can be caught is most likely to affect secondary metabolites (stress markers) within a fish sample and not the lipidome. Therefore, the results from this study could be a consequence of three different possibilities; (1) The REIMS technology, although it is dominated by lipids may also pick up secondary metabolites capable of differentiating between line and trawl caught samples; (2) as well as secondary metabolites being effected, the lipidome of fish samples is also effected through the differing catch methods, although this is extremely unlikely; (3) perhaps in this study we have identified differing diets between the two methods? Line caught fish are likely to be caught at shallower depths compared to that of trawl caught samples. As a result, this may result in them having varied diets which would affect the lipid profile of the fish samples thus explaining the separation within the chemometric models. Whichever scenario it may be, separation between the two catch methods has been achieved and therefore, this is the first scientific study to demonstrate that differentiating between line and trawl caught samples within the same species is possible. However, in order to

categorically confirm this a through validation using further authenticated samples is required and studies using techniques such as LC-MS, PCR and ELISA are necessary to provide comparative results.

Conclusion

This proof of concept lipidomic study using the REIMS technology has identified that multiple fish fraud concepts can be investigated. It is evident that REIMS has the capability to accurately differentiate between five similar white fish species rapidly using their phospholipid profiles. This is demonstrated within the various chemometric models. Vigorous and thorough testing of the models has established that they are robust, capable of correctly assigning a fish speciation classification, stable enough to the point where they can identify samples as outliers if the species is not present within the models and most importantly can identify mislabelled samples near instantaneously as shown with the six suspect 'haddock' samples. Therefore, it is reasonable to suggest that the REIMS technology could be applied to detect fish fraud within the global seafood supply chain, with fast and accurate results being obtainable. Additionally, our study has further exemplified why AMS techniques are beginning to play a more prominent role in investigating food fraud cases. The large gulf in sample preparation and assay running times between the REIMS technology and PCR perhaps indicates where the industry is heading with regards to companies carrying out their own quality checks with fast, accurate and reliable results being obtainable. As well as this, we have for the first time reported the scientific investigation of differing catch methods within the same species of fish. Early results within this study indicate that it is feasible to differentiate between line and trawl caught haddock samples. However, it is unclear yet whether these differences are due to the catch method of a fish or dietary differences.

Meat analysis using REIMS: Geographical origin

Introduction

One of the current analytical challenges for food authenticity application is the determination of geographical origin. Isotope ratio MS (IRMS) assay is the gold standard methodology to achieve this but it is also recognised as delicate, time-consuming and expensive technique to perform. REIMS technology could help to solve these issues as no (or minimum) sample preparation is required and analysis can be performed in matter of seconds with minimum cost. In this proof of concept study, rapid lipids profiling was investigated for its potential to determine if geographical origin can be achieved with REIMS.

Results

Raw spectrometric data obtained from NI beef (n=21), RoI beef (n=3) and Scottish beef (n=18) samples were exposed to multivariate analysis allowing PCA and LDA models to be generated. 20 PCA components and 3 LDA components were used to create the geographical origin method models. Some separation could be observed on the PCA plot (Figure 5.10) and so a LDA model was then generated (Figure 5.11). A 20% leave out validation on the model created was then carried out and showed some potential in separating the various samples via their geographical origin (Table 5.7). However, results showed only 80% accuracy for this model and more investigation will be required in order to confirm and validate the present results. Such separation could be attributed to various factors such as environment, feeding or processing for instance. As it stands for this particular application, REIMS technique could be used in conjunction with other fast and simple technique such as spectroscopy techniques in order to refine the current result.

Figure 5.10: PCA models generated using the prototype software package.

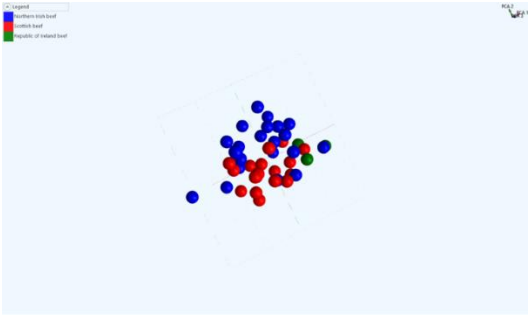


Figure 5.11: LDA models generated using the prototype software package. Separation from geographical origin; Scottish beef (red), Republic of Ireland (RoI) beef (green) and NI beef (blue) is visible within the 2-D LDA.

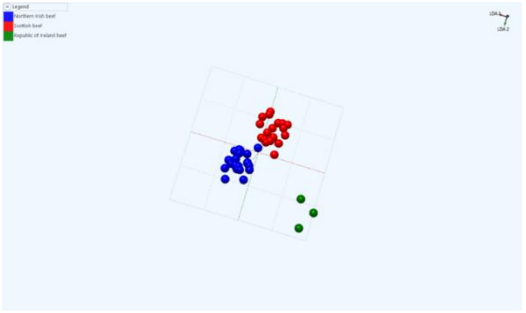


Table 5.7: Validation of the geographical origin models using the prototype software package (20% cross validation carried out).

	NI beef	RoI Beef	Scottish beef	Outlier	Total	Correct classification rate (%)
NI beef	18	1	1	1	21	85.71
RoI beef	0	0	1	2	3	0.00
Scottish beef	0	0	16	2	18	88.88
Total	21	3	18	5	42	80.95

Conclusion

Considering the limited number of samples available for this proof of concept study, these preliminary results are promising but will require more future work in order to validate the results and assess further the full potential of REIMS to determine geographical origin.

Meat analysis using REIMS: Drug treated meat detection

Introduction

Another potential application of REIMS technique can be the detection of effect-based drug response by studying the lipid profile. Such metabolomics/lipidomic approach have been already investigated using hyphenated technique such as LC or GC-High Resolution Mass Spectrometry and proved to be promising and successful. Applying REIMS technology for this type of analysis will be extremely beneficial as it requires limited or no sample preparation and is able to supply a results in seconds. Objectives of this study was to investigate if full lipid profile could provide information about drug treatment and no attempt to search for the drug or its residues and/or metabolites were carried out.

Results

Samples used for this short study were treated with nitroimidazole at low dose as detailed in the paper by Arias et al (2016). Unfortunately, these preliminary results showed poor separation on PCA plot (Figure 5.12) and after careful examination of the raw data no significant difference between the various samples used in this short study could be found. Despite this, a LDA model was generated (Figure 5.13) in order to perform 20% cross validation. Results of this validation are extremely poor (around 20%) and are in agreement with observation from PCA plot (Table 5.8). However this negative

result should not rule this technology for this type of analysis as it might be dependent on the drugs used or on the tissue itself to be analysed.

Figure 5.12: PCA models generated using the prototype software package between control day 5 (red), treated day 5 (green) and treated day 0 (blue).

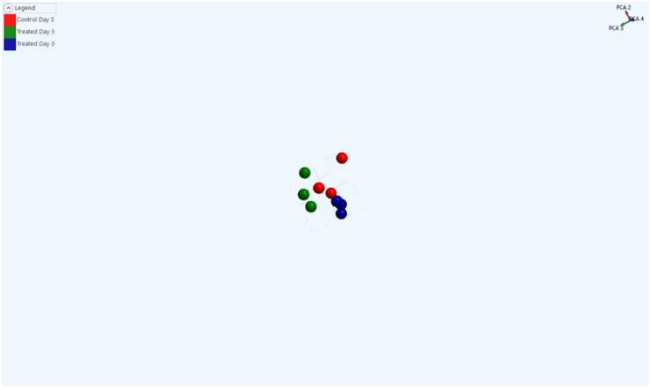


Figure 5.13: LDA models generated using the prototype software package with control day 5 (red), treated day 5 (green) and treated day 0 (blue).

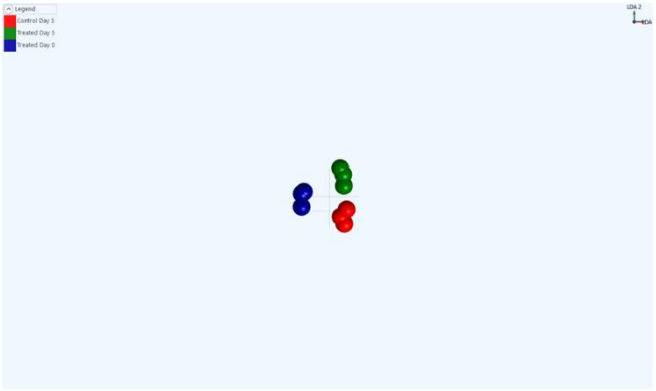


Table 5.8: Validation of the drug treated animal models using the prototype software package (20% cross validation carried out)

	Control day 5	Treated day 0	Treated day 5	Outlier	Total	Correct classification rate (%)
Control day 5	1	1	0	1	3	33.33
Treated day 0	0	1	2	0	3	33.33
Treated day 5	3	0	0	0	3	0
Total	4	2	2	1	9	22.22

Conclusion

REIMS technology was unable in this case to differentiate between the 2 type of tissue analysed. However, this type of work should be investigated further using for instance other type of controlled substances such as corticosteroid in order to fully rule out the potential of REIMS for this type of application.

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7 Appendices

Appendix A: Authenticity of Cold Pressed Rapeseed Oil using 60Hz Pulsar NMR: Materials and methods

A.1 Instrumentation

The instrument used was an Oxford Instruments 60 MHz benchtop NMR. Calibration was performed with 14% TMS (tetramethylsilane) before samples were analysed. A sealed tube of TMS was inserted into the instrument, where it underwent "tune and match" function and "optimize XYZ". Then a NMR tube containing a diluted oil sample underwent "tune and match" and "optimize XYZ".

A.2 Sample preparation

All samples were diluted with analytical standard chloroform as this was shown to produce a more defined spectrum than pure oil alone. 300µl of oil was pipetted directly into a Aldrich Color Spec NMR tube, followed by 700µl of chloroform. The tube was inverted several times until the oil was completely dissolved in the chloroform.

A.3 Spectra acquisition

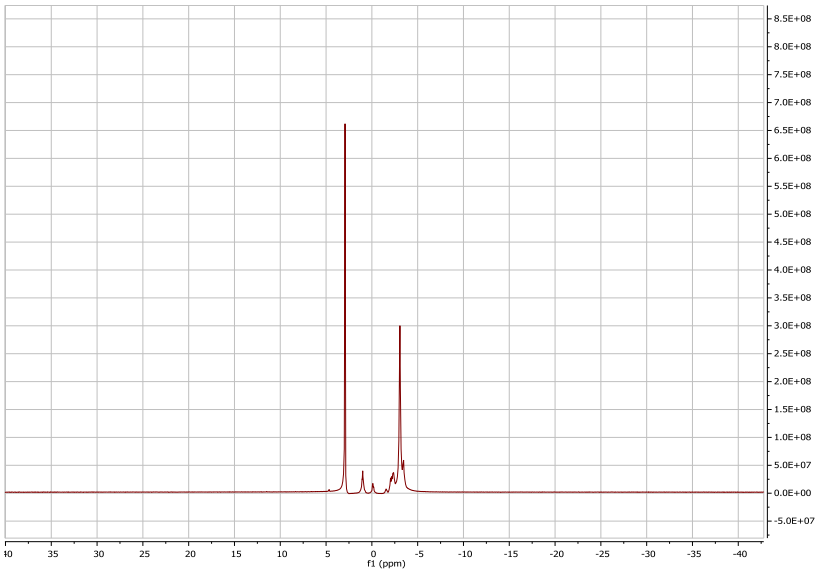
The NMR tubes were kept at a constant temperature in a heating block at 25°C. Each sample was then measured with the machine settings set to:

- No Scans: 16
- Filter: 5,000
- Recycle delay: 10 sec

A.4 Spectral data pre-processing

Prior to any processing the raw signal looks like the example of Figure A.1. Guidance for sample data analysis was provided by Dr Kate Kemsley and her team at the Institute of Food Research, Norwich. In brief, in MNOVA software package, each spectra was aligned at 4.26 ppm on the triglyceride peak (Figure A.2).

Figure A.1: Raw MNOVA spectra from CPRSO



Then the spectra was cut using the "manual zoom" function to eliminate the linear sections found either side of the spectra and the large chloroform peak (Figure A.3). Each spectrum was exported as a csv file where further pre-processing could take place.

Figure A.2: CPRSO aligned on triglyceride peak

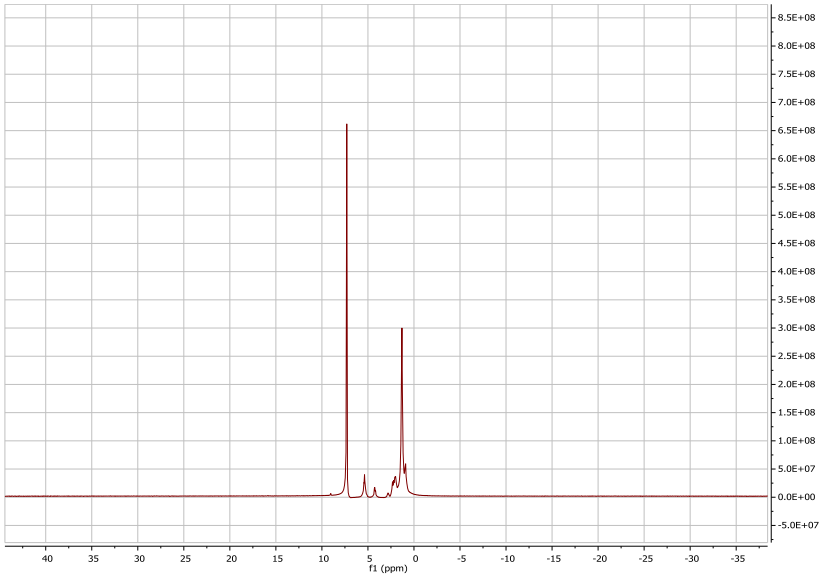
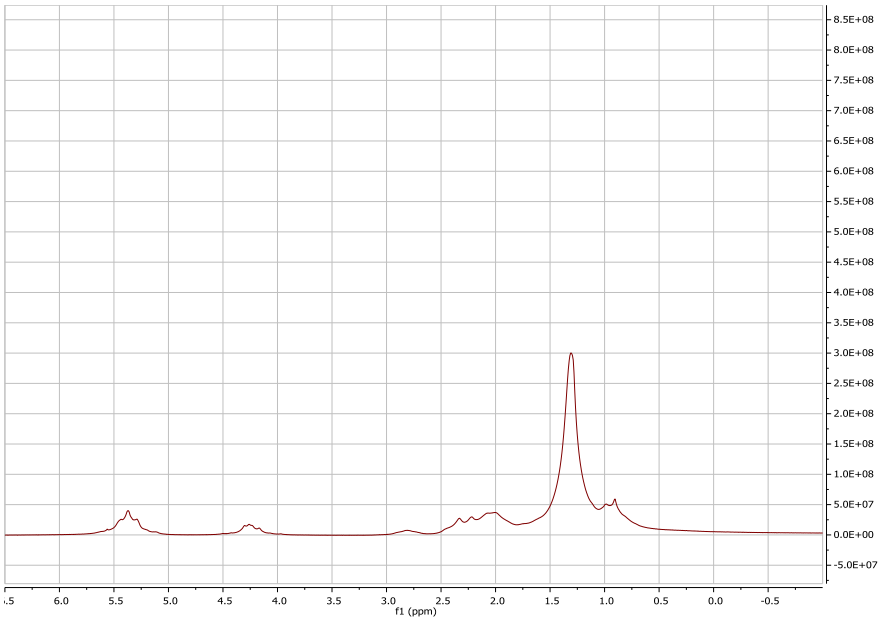
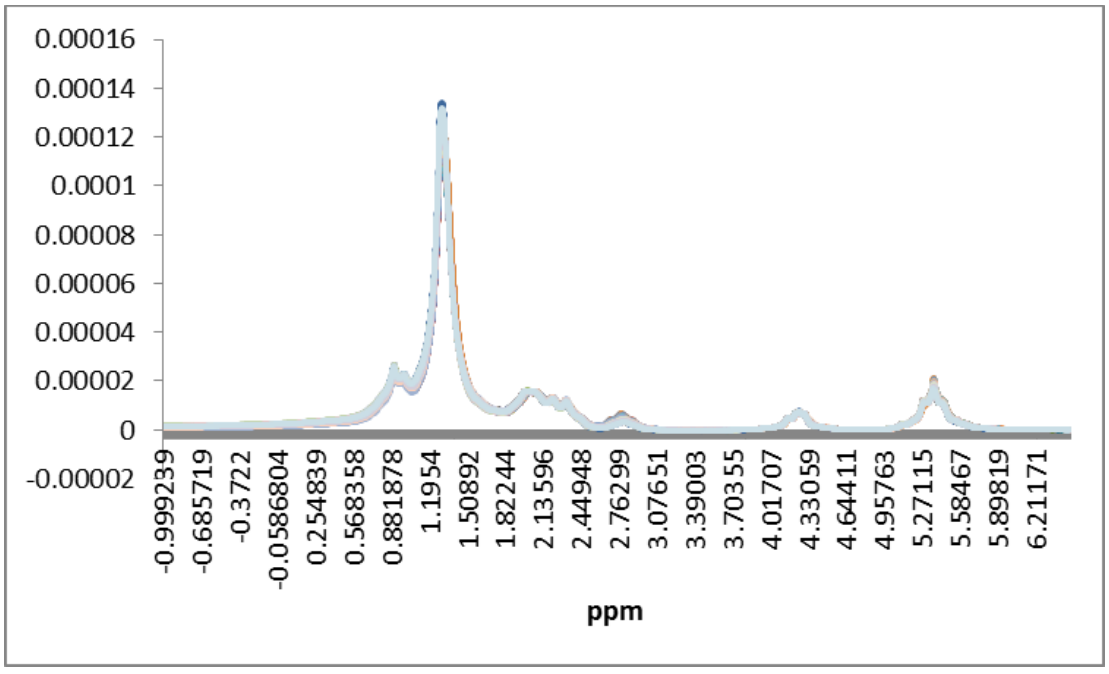


Figure A.3: CPRSO spectra cut to remove chloroform peak and linear stretches at either side



The spectra were normalised in excel by calculating the area of each triglyceride peak. Each point on the spectrum was then divided by this value (Figure A.4).

Figure A.4: All pure oil and oil mixes normalised in Microsoft Excel



A.5 Multi-Variate classification analysis and datasets

Following pre-processing, all spectra were imported into Umetrics Soft Independent Modelling by Class Analogy (SIMCA) Software, a data analysing software package. The spectra were subject to 3 filters (standard normal variate, derivatives and savitzky-golay). In this way, various chemometric techniques could be carried out with the oils including primary component analysis (PCA), (SIMCA) and Partial Least Squares Discriminant Analysis (PLS-DA). These are standard classification techniques which are used to reduce noise and are utilised extensively in science and in the food industry.

A range of different datasets were tested for their performance using both SIMCA and PLS-DA analysis in order to establish if one form of data analysis produced superior results.

Original Set: The first data set analysed was:

- 46 samples of CPRSO and refined rapeseed mix
- 10 samples of CPRSO
- 7 samples of RRSO
- 46 samples of CPRSO
- 10 samples of CPRSO
- 7 samples of SFO

Expanded Set: In an attempt to increase the differences between the classes, the number of pure CPRSO samples in the original set was increased (class - CPRSO).

- 46 samples of CPRSO and RRSO
- 13 samples of CPRSO
- 7 samples of RRSO
- 46 samples of CPRSO and SFO
- 13 samples of CPRSO
- 7 samples of SFO

Combined set: This is a combination of both expanded sets, which results in five classes;

1. CPRSO – 13 samples
2. RRSO – 7 samples
3. SFO – 7 samples
4. CPRSO and SFO mix – 92 samples
5. CPRSO and RRSO mix – 92 samples

Having 5 classes as opposed to 3 classes would in theory make prediction more challenging.

Appendix B: Data on mixtures and oils used in the evaluation of the authenticity of cold pressed rapeseed oil using 60Hz Pulsar NMR

B.1 Cold pressed rapeseed oil (CPRSO) and refined rapeseed oil (RRSO) mixes calibration set:

CPRSO.RRSO Calibration set							
% CPRSO	Oil name	% RRSO	Oil name	% CPRSO	Oil name	% RRSO	Oil name
4%	CPRSO 7	96%	RRSO 3	52%	CPRSO 4	48%	RRSO 5
7%	CPRSO 3	93%	RRSO 5	55%	CPRSO 7	45%	RRSO 2
10%	CPRSO 2	90%	RRSO 5	58%	CPRSO 7	42%	RRSO 4
13%	CPRSO 5	87%	RRSO 5	61%	CPRSO 3	39%	RRSO 5
16%	CPRSO 6	84%	RRSO 5	64%	CPRSO 7	36%	RRSO 4
19%	CPRSO 4	81%	RRSO 2	67%	CPRSO 6	33%	RRSO 1
22%	CPRSO 1	78%	RRSO 4	70%	CPRSO 5	30%	RRSO 3
25%	CPRSO 3	75%	RRSO 1	73%	CPRSO 5	27%	RRSO 1
28%	CPRSO 1	72%	RRSO 4	76%	CPRSO 2	24%	RRSO 2
31%	CPRSO 4	69%	RRSO 2	79%	CPRSO 5	21%	RRSO 3
34%	CPRSO 6	66%	RRSO 3	82%	CPRSO 1	18%	RRSO 3
37%	CPRSO 6	63%	RRSO 1	85%	CPRSO 1	15%	RRSO 3
40%	CPRSO 3	60%	RRSO 4	88%	CPRSO 1	12%	RRSO 1
43%	CPRSO 5	57%	RRSO 4	91%	CPRSO 2	9%	RRSO 4
46%	CPRSO 2	54%	RRSO 5	94%	CPRSO 7	6%	RRSO 2
49%	CPRSO 4	51%	RRSO 1	97%	CPRSO 4	3%	RRSO 2

B.2 Cold pressed rapeseed oil (CPRSO) and refined rapeseed oil (RRSO) mixes validation set:

CPRSO.RRSO Validation set			
% CPRSO	Oil name	% RRSO	Oil name
93%	CPRSO 9	7%	RRSO 6
86%	CPRSO 8	14%	RRSO 7
79%	CPRSO 10	21%	RRSO 7
72%	CPRSO 8	28%	RRSO 7
65%	CPRSO 9	35%	RRSO 6
58%	CPRSO 8	42%	RRSO 6
51%	CPRSO 9	49%	RRSO 6
44%	CPRSO 10	56%	RRSO 7
37%	CPRSO 8	63%	RRSO 6
30%	CPRSO 9	70%	RRSO 7
23%	CPRSO 10	77%	RRSO 6
16%	CPRSO 9	84%	RRSO 6
9%	CPRSO 10	91%	RRSO 7
2%	CPRSO 8	98%	RRSO 6

B.3 As well as oil mixes, pure oils were also measured as part of the calibration and validation set:

Pure oils of calibration		Pure oils of validation	
Oil	%	Oil	%
RRSO 1	100%	RRSO 6	100%
RRSO 2	100%	RRSO 7	100%
RRSO 3	100%	CPRSO 11	100%
RRSO 4	100%	CPRSO 12	100%
RRSO 5	100%	CPRSO 13	100%
CPRSO 1	100%		
CPRSO 2	100%		
CPRSO 3	100%		
CPRSO 4	100%		
CPRSO 5	100%		
CPRSO 6	100%		
CPRSO 7	100%		
CPRSO 8	100%		
CPRSO 9	100%		
CPRSO 10	100%		

B.4 Cold pressed rapeseed oil (CPRSO) and sunflower oil (SFO) mixes calibration set:

CPRSO.SFO Calibration set							
% CPRSO	Oil name	% SFO	Oil name	% CPRSO	Oil name	% SFO	Oil name
4%	CPRSO 1	96%	RRSO 5	52%	CPRSO 7	48%	RRSO 4
7%	CPRSO 2	93%	RRSO 3	55%	CPRSO 1	45%	RRSO 5
10%	CPRSO 6	90%	RRSO 4	58%	CPRSO 4	42%	RRSO 5
13%	CPRSO 6	87%	RRSO 1	61%	CPRSO 5	39%	RRSO 4
16%	CPRSO 4	84%	RRSO 3	64%	CPRSO 1	36%	RRSO 5
19%	CPRSO 3	81%	RRSO 2	67%	CPRSO 7	33%	RRSO 1
22%	CPRSO 3	78%	RRSO 2	70%	CPRSO 6	30%	RRSO 3
25%	CPRSO 4	75%	RRSO 1	73%	CPRSO 3	27%	RRSO 4
28%	CPRSO 5	72%	RRSO 2	76%	CPRSO 7	24%	RRSO 5
31%	CPRSO 3	69%	RRSO 1	79%	CPRSO 5	21%	RRSO 2
34%	CPRSO 7	66%	RRSO 1	82%	CPRSO 3	18%	RRSO 3
37%	CPRSO 5	63%	RRSO 5	85%	CPRSO 2	15%	RRSO 4
40%	CPRSO 2	60%	RRSO 2	88%	CPRSO 6	12%	RRSO 2
43%	CPRSO 1	57%	RRSO 3	91%	CPRSO 6	9%	RRSO 1
46%	CPRSO 2	54%	RRSO 4	94%	CPRSO 4	6%	RRSO 2
49%	CPRSO 4	51%	RRSO 3	97%	CPRSO 7	3%	RRSO 1

B.5 Cold pressed rapeseed oil (CPRSO) and sunflower oil (SFO) mixes validation set:

CPRSO.SFO Calibration set			
% CPRSO	Oil name	% SFO	Oil name
93%	CPRSO 8	7%	RRSO 6
86%	CPRSO 10	14%	RRSO 6
79%	CPRSO 9	21%	RRSO 7
72%	CPRSO 10	28%	RRSO 6
65%	CPRSO 8	35%	RRSO 7
58%	CPRSO 8	42%	RRSO 6
51%	CPRSO 9	49%	RRSO 6
44%	CPRSO 10	56%	RRSO 7
37%	CPRSO 9	63%	RRSO 7
30%	CPRSO 8	70%	RRSO 6
23%	CPRSO 10	77%	RRSO 7
16%	CPRSO 10	84%	RRSO 6
9%	CPRSO 8	91%	RRSO 7
2%	CPRSO 9	98%	RRSO 6

B.5 As well as oil mixes, pure oils were also measured as part of the calibration and validation set:

Pure oils of calibration		Pure oils of validation	
Oil	%	Oil	%
SFO 1	100%	SFO 6	100%
SFO 2	100%	SFO 7	100%
SFO 3	100%	CPRSO 11	100%
SFO 4	100%	CPRSO 12	100%
SFO 5	100%	CPRSO 13	100%
CPRSO 1	100%		
CPRSO 2	100%		
CPRSO 3	100%		
CPRSO 4	100%		
CPRSO 5	100%		
CPRSO 6	100%		
CPRSO 7	100%		
CPRSO 8	100%		
CPRSO 9	100%		
CPRSO 10	100%		

Appendix C: Evaluation of Spectroscopic Techniques to Detect the Authenticity of Cold Pressed Rapeseed Oil: Materials & methods

C.1 Instruments

The high field NMR was a Bruker Ultrashield 400 Plus 1H NMR (Burker, Rheinstetten, Germany). The number of scans was set at 16 and the chemical shifts were referenced by setting the TMS peak to 0 ppm. The FT-IR used was a Thermo Scientific Nicolet IS5 with Id5 ATR attachment. The spectral range recorded was from 550-4000 cm-1 against air as a background. The ATR was thoroughly cleaned with methanol after each use to ensure a clean sampling surface. Samples were collected in replicas of three and averaged. The Raman spectra were recorded on a DeltaNu Advantage 1064 spectrometer. The machine was calibrated with a polystyrene tube were a calibration value below 0.1 was achieved before acquisition. Integration time was set to 10 secs. And the number of scans set to 2. Samples were taken in triplicate and averaged.

C.2 Sample preparation

For the high field, all oil samples were diluted in deuterated chloroform in the ratio 60 µl of oil: 500 µl deuterated chloroform in a standard Aldrich ColorSpec NMR tube. The FT-IR and Raman samples were pure oil and required no dilution. Samples were kept at 25^0C in a heating block before acquisition. Further data on oils used can be found in the Appendix D.

C.3 Multi-variate classification analysis and datasets

FT-IR and Raman: All spectra were imported into Umetrics SIMCA Software, a data analysing software package. The spectra were subject to 3 filters (standard normal variate, derivatives and savitzky-golay). In this way, various chemometric techniques could be carried out with the oils including PCA, SIMCA and PLS-DA. These are standard classification techniques which are used to reduce noise and are utilised extensively in science and in the food industry. The spectra were also cut to reduce waste spectra being analysed. For FT-IR the spectra was analysed between 654.232-1875.43 and 2520.02-3120.74. The raman spectra was analysed form 800.314-1800.22. The spectra were then subject to Pareto scaling.

NMR: The NMR spectra received no spectral pre-processing, only pareto scaling. This is in line with other scientific papers which do not use spectral filters for NMR data (Mannina et al. 2001).

A range of different datasets were tested for their performance using both SIMCA and PLS-DA analysis in order to establish if one form of data analysis produced superior results.

3 Class dataset

- 46 samples of CPRSO and RRSO
- 13 samples of CPRSO
- 7 samples of RRSO
- 46 samples of CPRSO and SFO
- 13 samples of CPRSO
- 7 samples of SFO

5 Class dataset

- CPRSO – 13 samples
- RRSO – 7 samples
- SFO – 7 samples
- CPRSO and SFO mix – 92 samples
- CPRSO and RRSO mix – 92 samples

Having 5 classes as opposed to 3 classes would in theory make prediction more challenging.

Appendix D: Data on mixtures and oils used in the evaluation of spectroscopic techniques to detect the authenticity of cold pressed rapeseed oil

D.1 Cold pressed rapeseed oil (CPRSO) and refined rapeseed oil (RRSO) mixes calibration set:

Calibration			
CPRSO 1	Yellowfeilds	RRSO 3	Spar
CPRSO 2	Broighter	RRSO 4	Co-Op
CPRSO 3	Ola	RRSO 5	Heritage
CPRSO 4	Just	VALIDATION	
CPRSO 5	Hillfarm	RRSO 6	Asda
CPRSO 6	Cotwold Gold	RRSO 7	Princes
CPRSO 7	Lakeshore	CPRSO 8	Donegal
RRSO 1	Sainsburys	CPRSO 9	Boarderfeilds
RRSO 2	Tesco	CPRSO 10	Farringtons

D.2 Cold pressed rapeseed oil (CPRSO) and refined rapeseed oil (RRSO) mixes calibration set:

CPRSO .RRSO Calibration set							
% CPRSO	**Oil name**	**% RRSOFO**	**Oil name**	**% CPRSO**	**Oil name**	**% RRSOFO**	**Oil name**
4%	CPRSO 7	96%	RRSO 3	52%	CPRSO 4	48%	RRSO 5
7%	CPRSO 3	93%	RRSO 5	55%	CPRSO 7	45%	RRSO 2
10%	CPRSO 2	90%	RRSO 5	58%	CPRSO 7	42%	RRSO 4
13%	CPRSO 5	87%	RRSO 5	61%	CPRSO 3	39%	RRSO 5
16%	CPRSO 6	84%	RRSO 5	64%	CPRSO 7	36%	RRSO 4
19%	CPRSO 4	81%	RRSO 2	67%	CPRSO 6	33%	RRSO 1
22%	CPRSO 1	78%	RRSO 4	70%	CPRSO 5	30%	RRSO 3
25%	CPRSO 3	75%	RRSO 1	73%	CPRSO 5	27%	RRSO 1
28%	CPRSO 1	72%	RRSO 4	76%	CPRSO 2	24%	RRSO 2
31%	CPRSO 4	69%	RRSO 2	79%	CPRSO 5	21%	RRSO 3
34%	CPRSO 6	66%	RRSO 3	82%	CPRSO 1	18%	RRSO 3
37%	CPRSO 6	63%	RRSO 1	85%	CPRSO 1	15%	RRSO 3
40%	CPRSO 3	60%	RRSO 4	88%	CPRSO 1	12%	RRSO 1
43%	CPRSO 5	57%	RRSO 4	91%	CPRSO 2	9%	RRSO 4
46%	CPRSO 2	54%	RRSO 5	94%	CPRSO 7	6%	RRSO 2
49%	CPRSO 4	51%	RRSO 1	97%	CPRSO 4	3%	RRSO 2

D.3 Cold pressed rapeseed oil (CPRSO) and refined rapeseed oil (RRSO) mixes validation set:

CPRSO.RRSO Validation set			
% CPRSO	Oil name	% RRSO	Oil name
93%	CPRSO 9	7%	RRSO 6
86%	CPRSO 8	14%	RRSO 7
79%	CPRSO 10	21%	RRSO 7
72%	CPRSO 8	28%	RRSO 7
65%	CPRSO 9	35%	RRSO 6
58%	CPRSO 8	42%	RRSO 6
51%	CPRSO 9	49%	RRSO 6
44%	CPRSO 10	56%	RRSO 7
37%	CPRSO 8	63%	RRSO 6
30%	CPRSO 9	70%	RRSO 7
23%	CPRSO 10	77%	RRSO 6
16%	CPRSO 9	84%	RRSO 6
9%	CPRSO 10	91%	RRSO 7
2%	CPRSO 8	98%	RRSO 6

D.4 As well as oil mixes, pure oils were also measured as part of the calibration and validation set:

Pure oils of calibration		Pure oils of validation	
Oil	%	Oil	%
RRSO 1	100%	RRSO 6	100%
RRSO 2	100%	RRSO 7	100%
RRSO 3	100%	CPRSO 11	100%
RRSO 4	100%	CPRSO 12	100%
RRSO 5	100%	CPRSO 13	100%
CPRSO 1	100%		
CPRSO 2	100%		
CPRSO 3	100%		
CPRSO 4	100%		
CPRSO 5	100%		
CPRSO 6	100%		
CPRSO 7	100%		
CPRSO 8	100%		
CPRSO 9	100%		
CPRSO 10	100%		

D.5 Cold pressed rapeseed oil (CPRSO) and sunflower oil (SFO) mixes calibration set:

Calibration		Validation	
CPRSO 1	Co-Op	SFO 6	Asda
CPRSO 2	Tesco	SFO 7	Spar
CPRSO 3	Farmington's	CPRSO 8	Cotswold Gold
CPRSO 4	Boarderfeilds	CPRSO 9	Hillfarm
CPRSO 5	Donegal	CPRSO 10	Ola
CPRSO 6	Yellowfeilds		
CPRSO 7	Broighter		
SFO 1	Tesco		
SFO 2	Centra		
SFO 3	Flora		
SFO 4	Borges		
SFO 5	Sainsburys		

D.6 Cold pressed rapeseed oil (CPRSO) and sunflower oil (SFO) mixes calibration set:

CPRSO .SFO Calibration set							
% CPRSO	**Oil name**	**% SFO**	**Oil name**	**% CPRSO**	**Oil name**	**% SFO**	**Oil name**
4%	CPRSO 1	96%	SFO 5	52%	CPRSO 7	48%	SFO 4
7%	CPRSO 2	93%	SFO 3	55%	CPRSO 1	45%	SFO 5
10%	CPRSO 6	90%	SFO 4	58%	CPRSO 4	42%	SFO 5
13%	CPRSO 6	87%	SFO 1	61%	CPRSO 5	39%	SFO 4
16%	CPRSO 4	84%	SFO 3	64%	CPRSO 1	36%	SFO 5
19%	CPRSO 3	81%	SFO 2	67%	CPRSO 7	33%	SFO 1
22%	CPRSO 3	78%	SFO 2	70%	CPRSO 6	30%	SFO 3
25%	CPRSO 4	75%	SFO 1	73%	CPRSO 3	27%	SFO 4
28%	CPRSO 5	72%	SFO 2	76%	CPRSO 7	24%	SFO 5
31%	CPRSO 3	69%	SFO 1	79%	CPRSO 5	21%	SFO 2
34%	CPRSO 7	66%	SFO 1	82%	CPRSO 3	18%	SFO 3
37%	CPRSO 5	63%	SFO 5	85%	CPRSO 2	15%	SFO 4
40%	CPRSO 2	60%	SFO 2	88%	CPRSO 6	12%	SFO 2
43%	CPRSO 1	57%	SFO 3	91%	CPRSO 6	9%	SFO 1
46%	CPRSO 2	54%	SFO 4	94%	CPRSO 4	6%	SFO 2
49%	CPRSO 4	51%	SFO 3	97%	CPRSO 7	3%	SFO 1

D.7 Cold pressed rapeseed oil (CPRSO) and sunflower oil (SFO) mixes validation set:

CPRSO .SFO Calibration set			
% CPRSO	Oil name	%SFO	Oil name
93%	CPRSO 8	7%	SFO 6
86%	CPRSO 10	14%	SFO 6
79%	CPRSO 9	21%	SFO 7
72%	CPRSO 10	28%	SFO 6
65%	CPRSO 8	35%	SFO 7
58%	CPRSO 8	42%	SFO 6
51%	CPRSO 9	49%	SFO 6
44%	CPRSO 10	56%	SFO 7
37%	CPRSO 9	63%	SFO 7
30%	CPRSO 8	70%	SFO 6
23%	CPRSO 10	77%	SFO 7
16%	CPRSO 10	84%	SFO 6
9%	CPRSO 8	91%	SFO 7
2%	CPRSO 9	98%	SFO 6

D.8 As well as oil mixes, pure oils were also measured as part of the calibration and validation set:

Pure oils of calibration		Pure oils of validation	
Oil	%	Oil	%
SFO 1	100%	SFO 6	100%
SFO 2	100%	SFO 7	100%
SFO 3	100%	CPRSO 11	100%
SFO 4	100%	CPRSO 12	100%
SFO 5	100%	CPRSO 13	100%
CPRSO 1	100%		
CPRSO 2	100%		
CPRSO 3	100%		
CPRSO 4	100%		
CPRSO 5	100%		
CPRSO 6	100%		
CPRSO 7	100%		
CPRSO 8	100%		
CPRSO 9	100%		
CPRSO 10	100%		

Appendix E: Loop-mediated Isothermal Amplification Assay

E.1 Materials and methods for cheese sample analysis

E.1.1 Sampling

Seventy-six goat cheese samples were purchased from eight separate locations throughout the UK & Ireland. The samples were purchased in retail outlets, delicatessens, cheese shops and markets. Sampling took place over a period of seven days and all samples were stored at 4°C prior to analysis. Samples were analysed and an A and B sample was frozen stored, in separate freezers. The A and B sample is important in protecting the producer against false-positive results, as well as allowing confirmation of a positive sample.

E.1.2 Materials

A LAMP assay was carried out using the Genie® II, a portable, real-time fluorometer and four species specific reaction mixes (sheep, cow, goat and a positive control) obtained from Optigene Ltd., United Kingdom. A dry block heater, Genie® test strips and bijous were also supplied by Optigene Ltd., UK. 0.3M Potassium Hydroxide (KOH) lysis solution was prepared for the extraction of DNA in the cheese samples (Riedel de Haen, Germany).

E.1.3 LAMP method for cheese

An aliquot (0.25cm3) of cheese sample was added to 1ml 0.3M KOH in a bijou tube. The sample solution was heated to 95^{0}C for 10 m using a dry block heater. The solution was allowed to cool. Samples which showed an 'orange' colour were diluted 1:10 in 0.3M KOH. LAMP reaction was carried out with a total volume of 20 µl of each reaction mix (sheep, cow, goat and a positive control) and 5 µl of the prepared sample. The LAMP assay was run at 65^{0}C for 20 minutes, with a melting curve analysis step (annealing curve 98^{0}C - 78^{0}C ramping at 0.05^{0}C/s) using a Genie® II fluorometer.

E.1.4 Data analysis

Annealing temperatures obtained from the LAMP assay were compared to 'signature' annealing temperatures outlined in the assay handbook. The method was qualitative, however analysis of the amplification curves (time and peaks) were used to determine trace amounts of adulteration. Further estimations to determine the % range of adulteration was possible through the development of standard curves using pure DNA, however this was outside of the objectives of the study.

E.1.5 LAMP validation (cheese)

Cross reactivity between species DNA was determined by analysing cow, sheep and goats' cheese (hard and soft) samples in triplicate. To determine the detection limit of the assay, goats' cheese was prepared with 0.01%, 1%, 5%, 10%, 20%, 30%, 40% and 50% of cows' cheese. The samples were homogenised and analysed in two runs with the LAMP conditions mentioned above.

E.2 Materials and methods for meat and fish sample analysis

The LAMP assay method and validation for meat and fish was carried out in a similar manner to that indicated in Chapter 5.1 but with the following additional information.

E.2.1 Positive and negative controls

Positive controls were run along with meat and fish samples. For meat samples, a known pre identified positive was extracted. For fish samples a synthetic positive control for each species was added to the plate. A negative extraction control (NEC) was extracted with each run. This consisted of 1ml of 0.3M KOH being extracted alongside samples.

E.2.2 False positive ratio

During the entire validation process, each of the 10 Optigene species specific assays (fish and meat) displayed no cross reactivity between species, thus minimising the incidence of false positive results. It should also be noted that post validation, when assays were implemented in the screening of 200 collected fish and meat samples this was also observed (See Chapter 4.4).

Three food products gave rise to recovery of equine target above determined LoD. The samples were re-extracted and rescreened using the Optigene equine assay on the Genie II LAMP machine and upon post assay analysis 2 of the 3 samples reproduced positive signal for equine target (once again above the determined LoD).

Due to the unexpected recovery of any equine target in food samples being sold to the public, it was deemed significant to have these 2 results confirmed by an independent lab using alternative screening methods. Subsequently these samples gave negative recovery for equine presence when tested by species specific real time PCR (see Appendix F for reports issued). This highlights the minimal risk of false positive occurrence with limits of detection. It should also be noted that dependant on what portion of each sample tested, differing results can be recovered due to the un homogeneous nature of

processed samples (as demonstrated during validate with results obtained from screening spiked samples).

E.2.3 False negative ratio

In an effort to minimise false negative results a species positive sample (EPC: external positive control) was extracted and screened within each sampling run as a quality assurance check on both extraction and screening processes. To further reduce this risk it would be optimal if Optigene create an internal positive control (IPC) which could be used to spike each sample prior to extraction, identifying any samples where extraction may have failed or sample inhibitors presence inhibited the LAMP reaction, thus eliminating the reporting of false negative results.

In an additional attempt to minimise false negative incidences, it was deemed essential to extract in triplicate from each of the samples being screened. This is essential due to the un-homogenous nature of most processed food types, fish dishes particularly. As described within this validation report, processed fish foods (pies, cakes, fingers etc.) present a roughly mixed preparation to keep a flake like texture; this rough mixture inhibits the creation of a homogenous sample. By testing multiple sites from each single sample, the occurrence of false negative results is thus reduced.

E.2.4 Test method

The test method is described in P5.132 Rev (00) and P5.133 Rev (00). See Appendix H.

E.2.5 Assay robustness

Pros:

- KOH boil extraction protocol yields good quality DNA for use in assay
- Extraction procedure is quick, cheap and requires minimal technical input
- Assay turnaround time is short (50 minutes from extraction to result)
- Species specific master mixes are pre prepared requiring minimal expertise
- Procedure is labour minimal and technically simple
- Screening for single species is effortless.
- Minimal post analysis required

Cons:

- Small sample size necessary presents difficulty when cutting and repeatability

- Molecular yields from extraction protocol can be unstable a after a short period of time, therefore sample numbers must be limited and extracted in small batches.
- Food products with high fat levels (which many processed meat and fish products contain) must be extracted and screened promptly (cooled fats had an inhibitory effect on the LAMP assay).
- Sensitivity capabilities between species vary.
- Multiple species screening in a single run is not possible due to the limited capacity of the Optigene LAMP Genie II machine and the single nature of all species specific master mixes. To achieve multiple species screen of a single sample or more, a larger capacity LAMP machine would need to be manufactured by Optigene and the production of a multiple species master mixes with appropriate controls considered.

E.2.6 Repeatability

Two technically competent analysts, conducted all procedures in pre-designated work areas appropriate to the work stage. All equipment with particular emphasis on those that require precision/accuracy such pipettes, balances, heat blocks were calibrated prior to use. All of these elements combine to produce repeatability within each of the 10 assays.

Appendix F: Confirmatory reports by an independent laboratory using alternative screening methods for the Optigene equine assay

WEATHERBYS

IEC Animal Species Detection Report

Lab Number	Sample ID	Date Received
B624285	Sample 91	12/10/2016

Species Identification was carried out using a real time PCR assay specific for equine and porcine.

RESULT: No Equine DNA detected.

RESULT: Porcine DNA detected – Major parts.

Detection Categories - Major parts (50-100 %), medium parts (10-50 %), minor parts (1-10 %). LOD=0.01% LOQ >1.0%

pp Paul Flynn

Rebecca M. Weld PhD

8th December 2016

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WEATHERBYS

IEC Animal Species Detection Report

Lab Number	Sample ID	Date Received
B624288	Sample 94	12/10/2016

Species Identification was carried out using a real time PCR assay specific for equine and porcine.

RESULT: No Equine DNA detected.

RESULT: Porcine DNA detected – Major parts.

Detection Categories - Major parts (50-100 %), medium parts (10-50 %), minor parts (1-10 %). LOD=0.01% LOQ >1.0%

p.p. Paul Flynn

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Appendix G: Rapid Evaporative Ionisation Mass Spectrometry (REIMS)

G.1 Materials and methods for meat speciation using REIMS

The proof of concept study was based on Venison, Wagyu beef, Grain-fed beef and horse minced meat samples obtained from Kezie Foods Ltd. (Burnhouses Farm Duns Berwickshire TD11 3TT) in 2x0.5 kg packs. Horse meat and horse liver samples were obtained from the University of Veterinary Sciences (Budapest, Hungary). The reproducibility and authenticity study was performed on 5 equine, 5 bovine samples supplied by Irish abattoir (ABP_Waterford), including 2 Hereford Cross, 2 Limousin Cross and 1 Blonde Cross breed, and 10 Scottish bovine (Aberdeen Angus) samples supplied by Scottish abattoir, all samples were from different animals. All measurements were carried out using a modified Xevo G2-S Quadrupole Time-of-flight mass spectrometer (Waters Corp., Wilmslow, UK). The experimental setup was identical to the earlier reported one, originally developed for the intraoperative identification of biological tissues (Balog et al, 2010). Briefly, REIMS analysis of tissue specimens was performed by electrosurgical evaporation and on-line mass spectrometric analysis of the aerosol produced. The monopolar electrosurgical handpiece was connected to an Erbotom ICC 300 (Erbe Elektromedizin GmbH, Tübingen, Germany) electrosurgical generator providing power-controlled sinusoidal 330 kHz alternating current. The generator was used in 'cut' mode at a 40 W power setting. All specimens were deposited on the 'neutral' or return electrode of the electrosurgical setup and incisions were made using a custom-built monopolar hand piece that was equipped with a smoke evacuation line. The 4 m long 3.97 mm outer diameter 2.38 mm inner diameter Tygon PVC smoke evacuation line was connected to an air driven Venturi pump, which was mounted on the atmospheric interface of the mass spectrometer. The Venturi pump was driven by 2 bar nominal inlet pressure of zero grade pressurized air or Nitrogen. The exhaust of the Venturi pump device was sampled orthogonally by the inlet capillary of the mass spectrometer. In order to avoid the contamination of the instrument and enhance the sensitivity of the analytical setup, the atmospheric interface was equipped with a heated jet disruptor surface as it is shown in Figure G.1. The jet disruptor was a Kanthal S (Kanthal, Hallstahammar, Sweden) coil and was kept at 800 °C. The depicted setup efficiently stops larger aerosol particles entering the StepwaveTM ion guide and all material deposited on the Kanthal surface undergoes subsequent carbonization and combustion into carbon dioxide and water. An additional 0.2ml/min 2ng/ul leucine-enkephalin solution in isopropyl-alcohol was introduced directly into the atmospheric interface to provide internal reference peak for lock-mass calibration of data. Negative ion mass spectra were acquired in the mass range of 150-1500 m/z at a mass spectrometric resolution of 15,000 FWHM at m/z 600. The chemical identity of detected ionic species was determined by accurate mass measurement, MS/MS fragmentation of molecular ions and comparison of this data to data obtained by the analysis of authentic standards under identical conditions. Phospholipid standards were obtained from Cayman Chemical (Ann Arbor, MI, USA) and Avanti Polar Lipids (Alabaster, Alabama, US). All acquired data files

were pre-processed using a custom-built software package (Waters Research Centre, Hungary) containing standard Masslynx pre-processing algorithms (Waters, UK). First the recorded scans were combined into average spectra containing 3-5 scans, resulting in 5-10 replicate spectra of each sample. Each meat sample was divided into 4 pieces in order to obtain replicates for reproducibility, while burger samples were recorded on 6 separate days, 4 batches per day. The spectra were background subtracted, and lockmass corrected using lockmass m/z 699.497 in case of burgers or m/z 554.2516 in case of meat pieces. After lockmass correction, spectra were TIC normalised and rebinned to 0.1 Da bin.

The resulting data vectors were subjected to multivariate statistics in order to obtain a classifier for the identification of the species of origin. PCA was used to eliminate chemical noise and reduce the dimensionality of the dataset. Following PCA, the first 25 principal components were subjected to linear discriminant analysis (LDA). The LDA classifiers were tested with leave 20%-out cross validation in case of minced meat patties and leave one animal out cross-validation in case of authentic meat samples, i.e. each data file containing spectra acquired from one animal was left out of the training set, a model was built on all other datasets and the data files left out were classified using the training model. Each animal was left out and classified exactly once. Each spectrum was classified to the closest class in the LDA space, however if the spectrum was farther from the closest class average than 5 x standard deviation of the class, the spectrum was marked 'outlier' and excluded from analysis. The correct classification rate was calculated based on number of spectra classified correctly/ all spectra of the full dataset. An LDA classifier was built containing 0-25-50-75-100% of pure and mixed minced samples (venison, Wagyu beef, grain fed beef and equine) acquired on 6 different days, then used for on-line meat detection on samples containing 0-1.25-2.5-5-10-25 and 33% of Wagyu beef and horse meat mixed into venison and grain fed beef meat. The acquired spectrum was tranSFO rmed by the PCA-LDA projection matrix to the LDA space of the training set, where Mahalanobis squared distance was calculated to each class average. The test specimen was classified to the tissue type for which the Mahalanobis distance is minimal, or marked 'outlier' as previously described and excluded from data analysis.

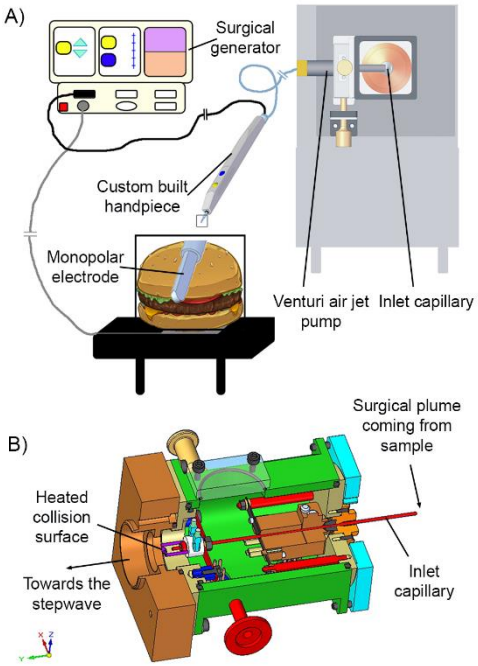


Figure G.1: REIMS experimental setup used for sampling. A) The setup using a custom built monopolar hand piece for sampling the different meat types. The hand piece was equipped with a smoke evacuator connected to the REIMS source of the mass spectrometer on the distal end. B) The REIMS source without the venturi chamber. The heated collision surface or jet disruptor element stops larger particles to enter the mass spectrometer and facilitates ion formation with the impact of large droplets on its surface.

G.2 Materials and methods for fish speciation using REIMS

G.2.1 Sampling

This proof of concept study was based upon five commercially popular white fish species. All authenticated tissue samples of cod, coley, haddock, pollock and whiting were sourced from trusted suppliers and stored at -80°C. Samples of seabass and seabream were sourced and also stored at -80°C. Prior to REIMS analysis the samples were thawed at room temperature for two hours.

G.2.2 REIMS setup

The experimental setup for this study was similar to that reported previously in Chapter 5.2. A Medimass REIMS source (Medimass, Budapest, Hungary) was mounted orthogonally to the interface of a Xevo G2-XS QTof mass spectrometer (Waters Corporation., Wilmslow, UK) which was operated in

negative ion and sensitivity mode. Mass spectra data were acquired over the range m/z 200-1200 with a scan time of 0.5s. The REIMS source was connected to a monopolar electrosurgical knife (Model PS01-63H, Hangzhou medstar technology Co, Ltd, Jiaxing City, China) through a 3m long, 1cm. diameter ultra-flexible tubing (evacuation/vent line). Electrosurgical dissection in all experiments were carried out using an Erbe VIO 50 C generator (Erbe Medical UK Ltd, Leeds, UK). The generator was operated in 'autocut' mode with a power setting of 30W. All samples were cut on the return electrode and a venturi gas jet pump driven by nitrogen (1 bar) evacuated the aerosol produced at the surgical site towards a heated coil which was set at 2.8A, 2.3V. A lockmass solution of Leucine Enkephalin (m/z 554.2615) (2ng / µL) in isopropanol (IPA) was infused using a Waters Acquity UPLC I-class system (Waters Corporation., Milford, MA, USA) at a continuous flow rate of 0.1 ml/min for real-time accurate mass correction. Prior to analysis the mass spectrometer was calibrated using 5mM sodium formate solution (90% IPA) at a flow rate of 0.2 ml/min for two minutes. Dependent on the size, each tissue sample was cut 8-12 times for reproducibility with each cut lasting approximately 3-5s. The delay between sampling and appearance of a signal was ≈2s, with no carry-over effects visible between each burn and/or sample.

G.2.3 REIMS data pre-processing and analysis

PCA, an unsupervised technique, LDA and Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA), supervised techniques, were used to build the qualitative speciation and catch method models within this study. Raw data generated by the mass spectrometer were pre-processed using a prototype software package (Waters Research Centre, Budapest, Hungary) containing standard Masslynx pre-processing algorithms (Waters). The recorded scans for each sample were combined to give an average spectrum and thus one spectrum for each sample was used to build the chemometric models. The resulting data were lock massed (m/z 554.2615) and normalised before being exposed to multivariate analysis. The chemometric models were built using the phospholipid region (m/z 600-950), a spectral intensity threshold of 2e6 and a binning of 0.5 Da. PCA was used to eliminate chemical noise before exploiting the data to LDA analysis using the first 25 PCA components. The prototype software enabled a leave-20%-out cross validation of the PCA-LDA score plots in which one average spectrum per sample was analysed. A model was built using 80% of the samples and data files left out were classified using the training model. This was repeated five times enabling each sample to be left out once from the model building process. Using a standard deviation of 5, each sample was classified to the closest class. If a sample was outside the standard deviation range of 5 for all classes, then they were marked as outliers.

The PCA-LDA models created using the prototype software package were exported to a prototype recognition software package (Waters Research Centre, Budapest, Hungary) allowing for real-time

identification of samples. Raw data files were acquired and ran live though the software providing a near-instantaneous identification, excluding the delay between sampling and appearance of a signal which was ~2s. A standard deviation of 5 was used for class assignment. The spectral intensity limit was set at 1e8 thus ensuring that only the cuts were assigned a species classification and not any background noise. The processed matrix generated within the prototype software package was exported to SIMCA 14 (Umetrics, Sweden) allowing the data to be exposed to further chemometric functions such as OPLS-DA. All data was mean-centered, pareto scaled and grouped accordingly into the five species of fish. R2 (cumulative), Q2 (cumulative) and a misclassification table were used to determine the validity of the models. R2 (cum) indicates the variation described by all components in the model and Q2 is a measure of how accurately the model can predict class membership. Permutation tests were carried out to ensure the models were not over fitted. Individual OPLS-DA models of each species of fish against the other species were generated to obtain S-plots for each species and identify which ions influenced the data the most.

G.2.4 PCR setup

Mitochondrial cytochrome c oxidase subunit I gene (COI) was used as genetic marker for the examination of the six suspect 'haddock' samples. DNA extraction was performed using a commercial kit (NucleoSpin Tissue – Macherey Nagel) according to the manufacturer guidelines. A fragment of approximately 655bp of COI was amplified using the primer pair COIfish_F1 (5'-TCAACYAATCAYAAAGATATYGGCAC-3') and COIfish_R1 (5'-ACTTCYGGGTGRCCRAARAATCA-3') in a PCR reaction. The sequences were determined by direct DNA sequencing on both strands of the PCR products by BigDye Terminator v3.1 cycle sequencing kit using the amplification primer pair and analysed on ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Sequences were compared with those deposited in GenBank and in BOLD databases too. Results were considered valid above 98% of similarity.

G.3 Materials and methods for Geographical origin analysis using REIMS

G.3.1 Sampling

This proof of concept study was based upon three set of beef samples from different geographical origin (NI, RoI and Scotland). All authenticated tissue samples of meat were sourced from trusted suppliers and stored at -80°C. Prior to REIMS analysis the samples were thawed at room temperature for two hours.

G.3.2 REIMS setup

The experimental setup for this work was identical to that reported previously Chapter 5.3.2.

G.4 Materials and methods for analysis of drug-treated meat using REIMS

G.4.1 Sampling

This proof of concept study was designed using pork samples sourced from a previous experiment where animals were treated with nitro imidazole drugs derivative. Samples were stored at -80°C prior to REIMS analysis the samples and were thawed at room temperature for two hours.

G.4.2 REIMS setup

The experimental setup for this work was identical to that reported previously Chapter 5.5.2.

Appendix H: LAMP assay validation method

P5.132

SIGNATURE PAGE

The signatures below apply to pages 1 to 3 inclusive:

Written by:..... Date:.....

Approved by:..... Date:.....

Professor Thomas C. Buckley, MSc., FIBMS, FAMLS,

Head of Microbiology

QA Review by:..... Date:.....

QA Manager / Deputy

Authorised by:..... **Effective Date:**.....

Professor Thomas C. Buckley, MSc., FIBMS, FAMLS,

Head of Microbiology

Distribution Codes: 1, 3, 11, 12.

1. Scope:

This method is intended for the extraction of DNA from cheese / meat / fish samples using simple KOH boil extraction method.

2. Responsibility

It is the responsibility of the laboratory analyst to understand and carry out this procedure.

3. Equipment:

3.1 Measuring callipers

3.2 Sterile scalpels

3.3 Sterile forceps

3.4 Disposable gloves

3.5 Dry heating block

3.6 2 ml sterile micro-centrifuge tubes

3.7 1000 µl pipette and tips

3.8 Magnetic stirrer

4. Chemicals / Reagents:

4.1 KOH

4.2 Microsol 10%

4.3 Sterile purified water.

5. Procedure:

5.1 Clean all work surfaces and equipment with suitable cleaning reagent (10% Microsol) prior to all work commencement.

5.2 Prepare 0.3M KOH daily prior to use. Weigh 0.168 g of KOH, add to 10 ml of sterile purified water and place on magnetic stirrer until completely dissolved prior to use.

5.3 Label all 2 ml tubes with sample identification on the lid with permanent marker.

5.4 Measure each sample to an approximate size of (0.25 cm^3) using a measuring callipers and cut using a separate sterile sample for each. Clean measuring callipers between each sample.

5.5 Place sample in a labelled 2 ml tube and add 1 ml 0.3M KOH.

5.6 The sample solution is heated to 95°C for 10 mins using a dry heating block.

5.7 Samples are forwarded to the LAMP set-up area where they are stored on ice for the short period prior to screening.

6. References

7. Sop / Worksheet Cross Referral:

8. 8. Health And Safety:

8.1 L coats and gloves must be worn at all times during the process and special attention given during the handling of samples and chemicals.

8.2 Reference MSDS for details on chemicals and reagents used.

P5.133

SIGNATURE PAGE

The signatures below apply to pages 1 to 3 inclusive:

Written by:..... Date:.....

Approved by:..... Date:.....

Professor Thomas C. Buckley, MSc., FIBMS, FAMLS,

Head of Microbiology

QA Review by:..... Date:.....

QA Manager / Deputy

Authorised by:..... **Effective Date**:.....

Professor Thomas C. Buckley, MSc., FIBMS, FAMLS,

Head of Microbiology

Distribution Codes: 1, 3, 11, 12.

1. Scope:

This method is intended for detection of predetermined species specific targets in meat and fish samples using LAMP technology.

2. Responsibility:

It is the responsibility of the laboratory analyst to understand and carry out this procedure.

3. Equipment:

3.1 PCR station
3.2 Pipettes
3.3 Pipette tips with filter
3.4 Centrifuge
3.5 Vortex
3.6 Fridge & Freezer
3.7 2 ml micro-centrifuge tubes
3.8 0.5ml micro-centrifuge tubes
3.9 Genie 0.5ml strip tubes
3.10 Gloves
3.11 Optigene Genie II machine

4. Chemicals / Reagents:

4.1 Microsol 10%.
4.2 0.6m KOH.
4.3 Master mix for equine, bovine, caprine, ovine & porcine.
4.4 Master mix for haddock, cod, coley, pollock & haddock.
4.5 Positive for controls for fish species.

5. Procedure:

5.1 Clean all work surfaces and equipment with suitable cleaning reagent (10% Microsol) prior to all work commencement.
5.2 Lab coat and gloves are changed between each designation work station.

5.3 Each species specific master mix is stored at -20°C. The master mix is allowed to thaw on ice in a DNA free room. 20ul is required per reaction. A total of 16 reactions can take place at one time in the lamp machine. One extra reaction is added giving a total of 17 reactions and a volume of 340ul. This is then pipetted into a 0.5ml tube.

5.4 Following this the master mix is taken to the designated DNA work area where the pre extracted samples have already been transferred to.

5.5 Extracted samples are pulse centrifuged. 20ul of the appropriate master mix is added to each well. 5ul of DNA is then added. For the positive control 2.5ul of 0.6m KOH is added to the 20ul of master mix and 2.5ul of the specific species control giving the same volume as the other samples.

5.6 Upon completion the area is thoroughly cleaned.

5.7 The strip tubes are then transferred to a separate work area where they are loaded into the LAMP machine and run on a pre-installed programme (See figure 1).

5.8 Following completion of the run controls are checked in order to make sure the run is valid. The amplification Ct value along with the annealing temperature are checked and recorded for all.

Genie	Temperature	Time
Amplification	65°C	30 minutes
Annealing	98-80°C	5 minutes

6. References:

Genie handbook, species specific master mix kit pamphlet, control insert.

7. Sop / Worksheet Cross Referral:

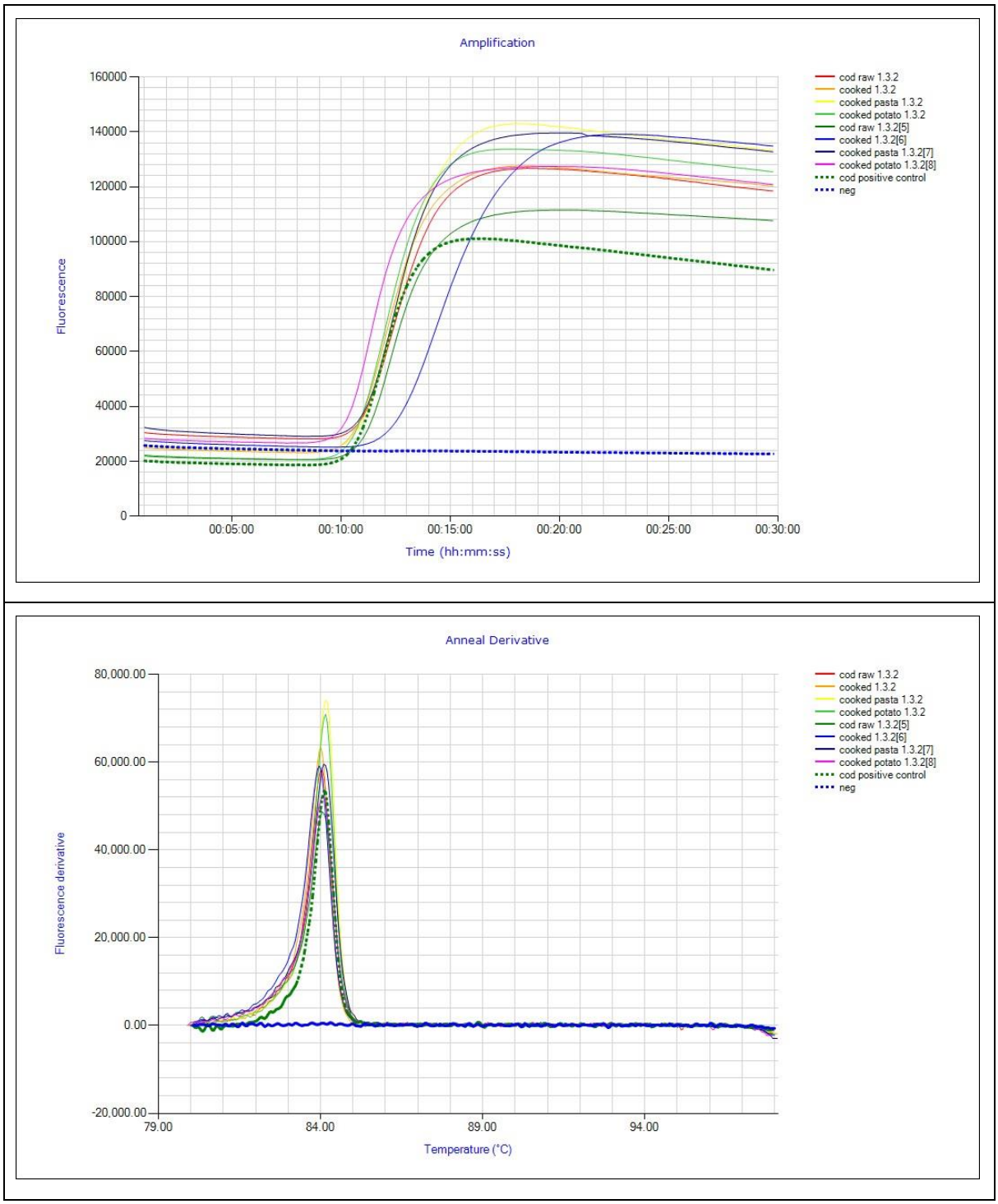
P5.132

8. Health And Safety:

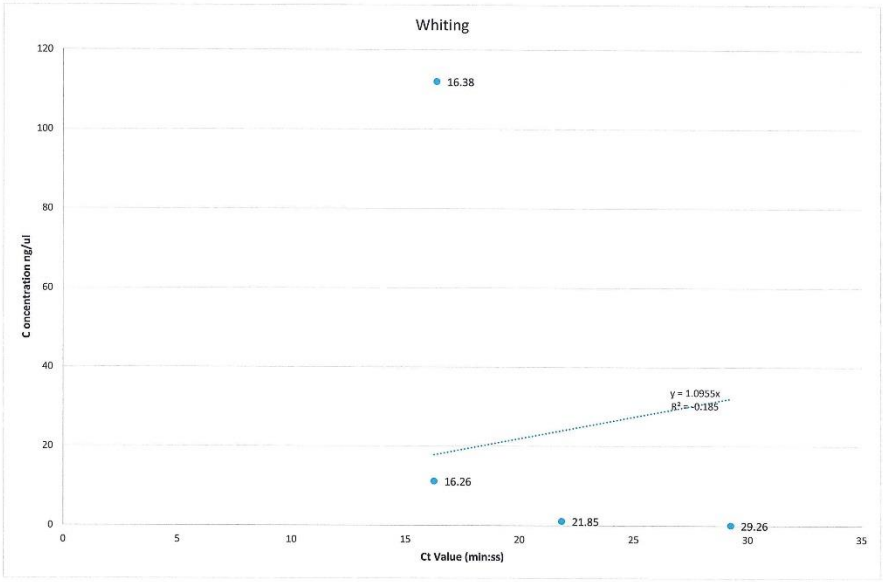
8.1 Gloves and a white coat must be worn at all times when handling samples and chemicals.

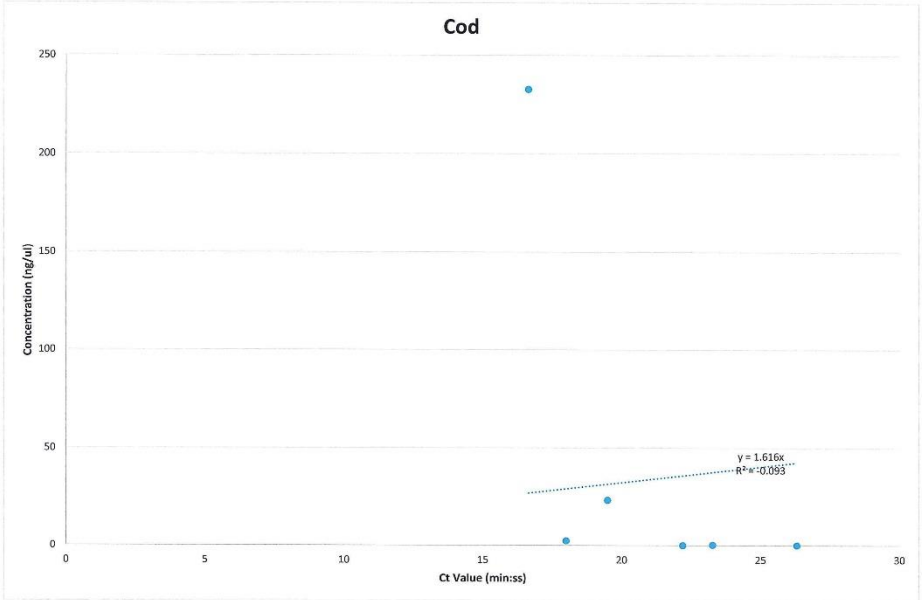
8.2 Reference MSDS for details on chemicals and reagents used.

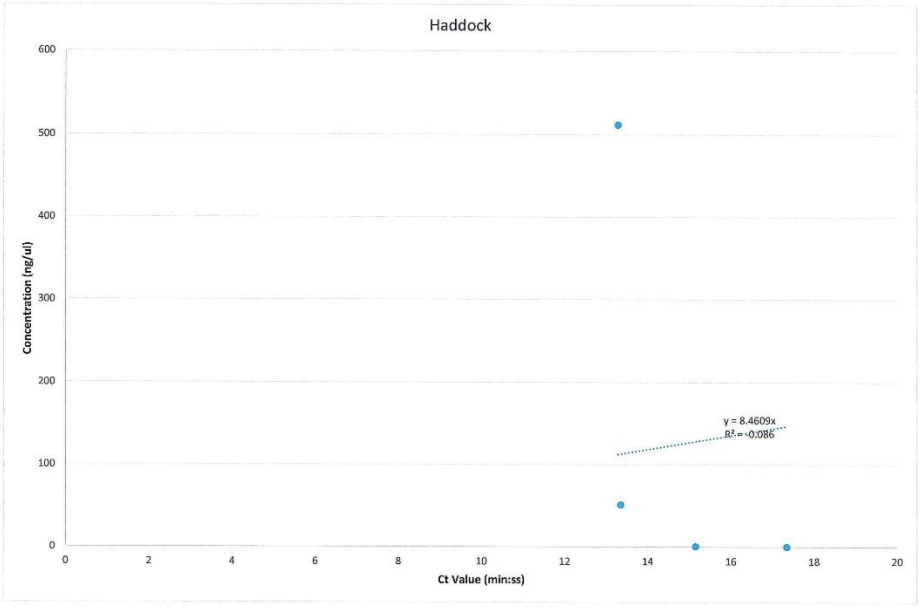
Appendix I: Example of amplification curve and annealing derivative

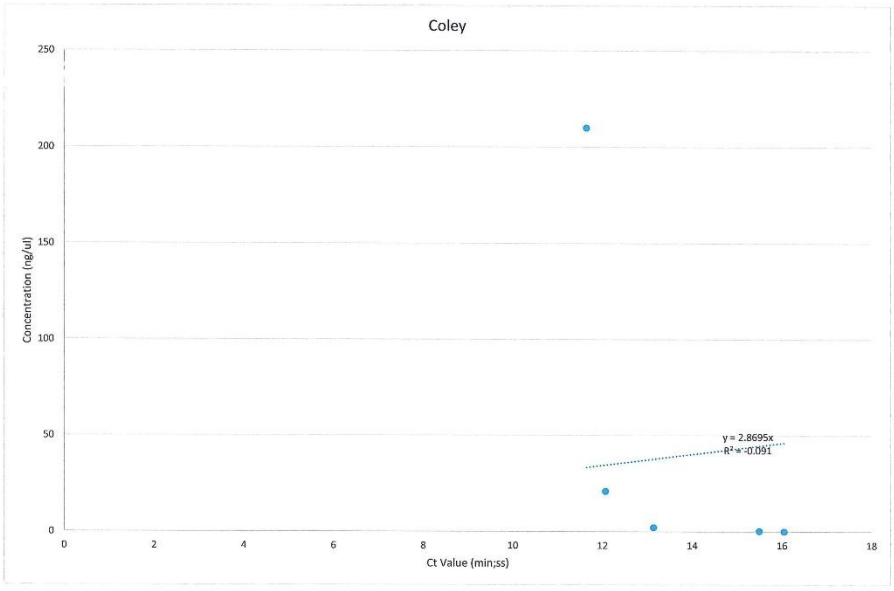


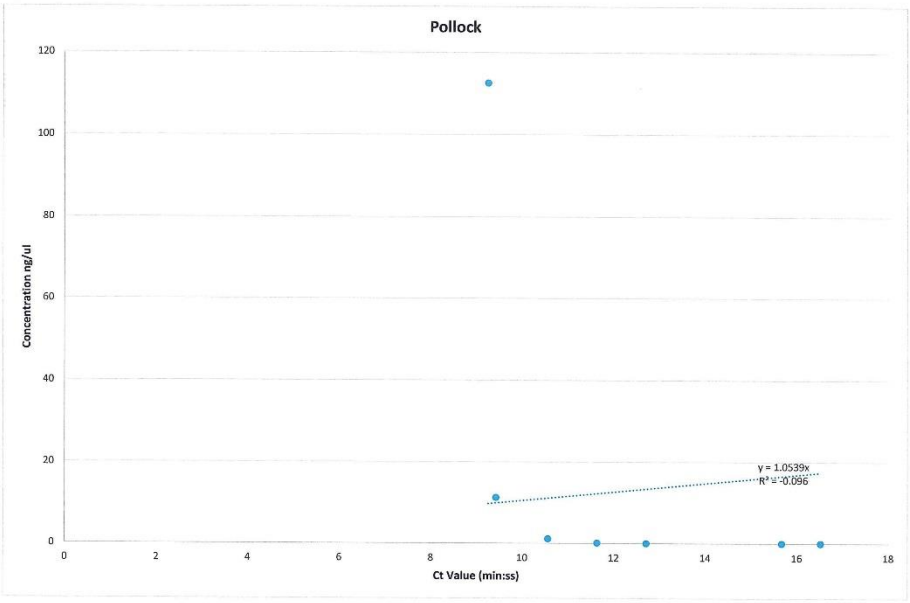
Appendix J: Determination of limits of detection for Fish species Assays: CT values

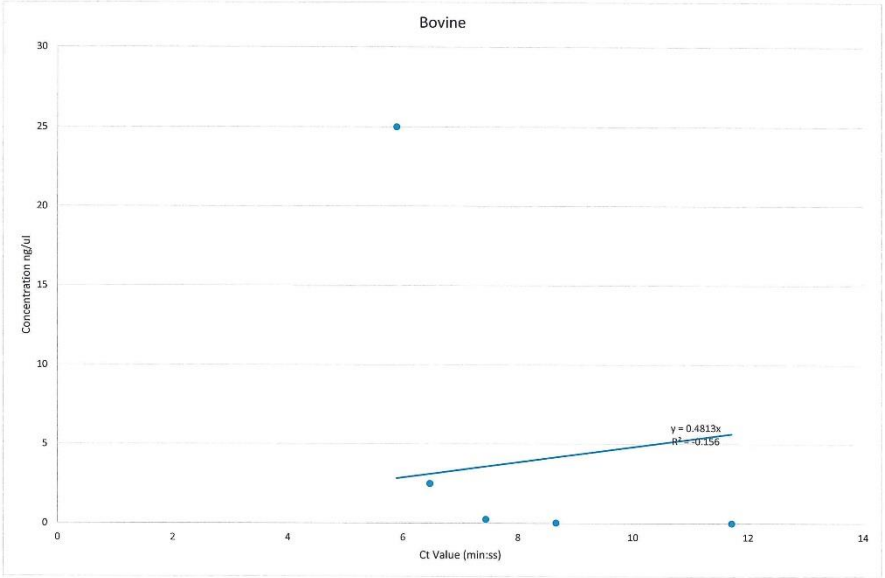


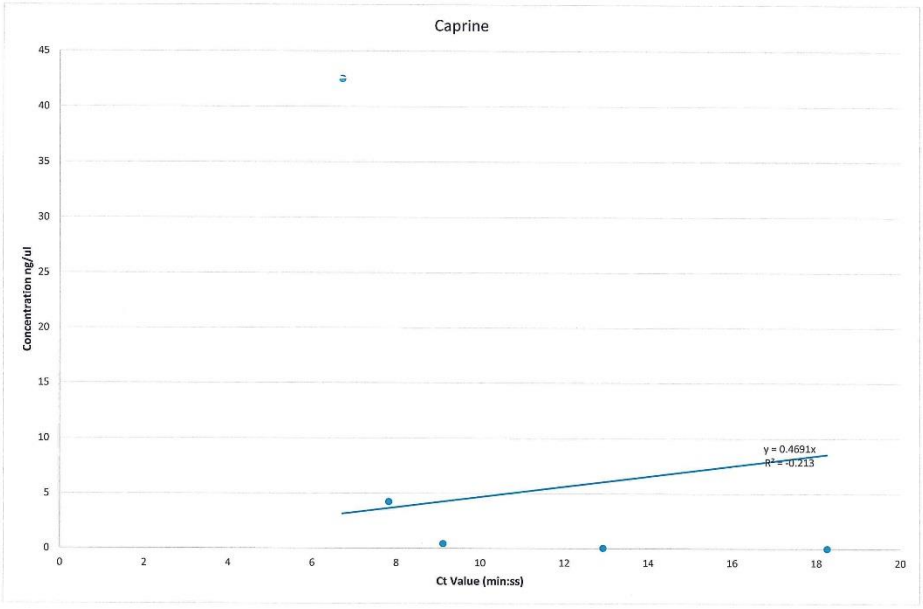


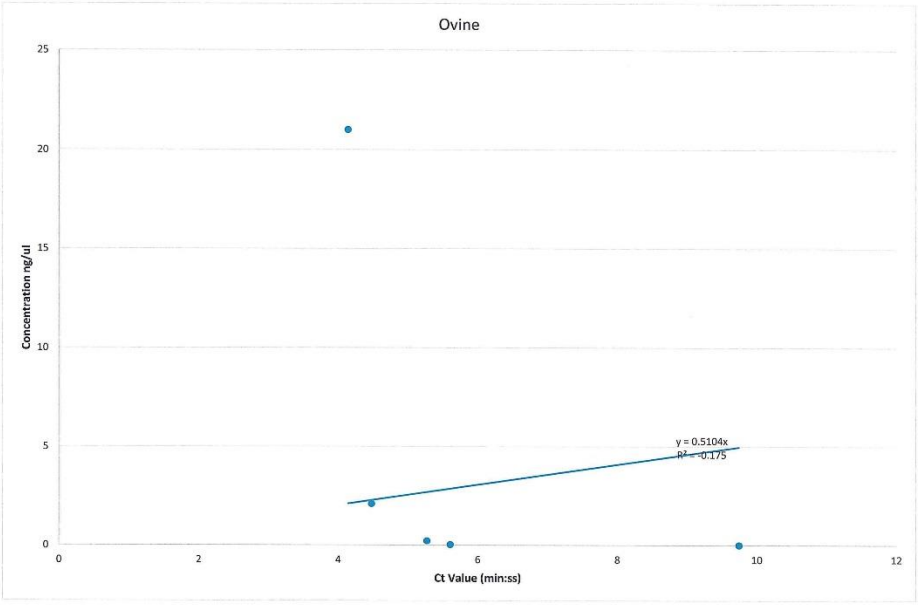

Haddock
600
500
400
300
200
100
0
Concentration (ng/ul)
y = 8.4609x
R² = 0.086
0
2
4
6
8
10
12
14
16
18
20
Ct Value (min:ss)

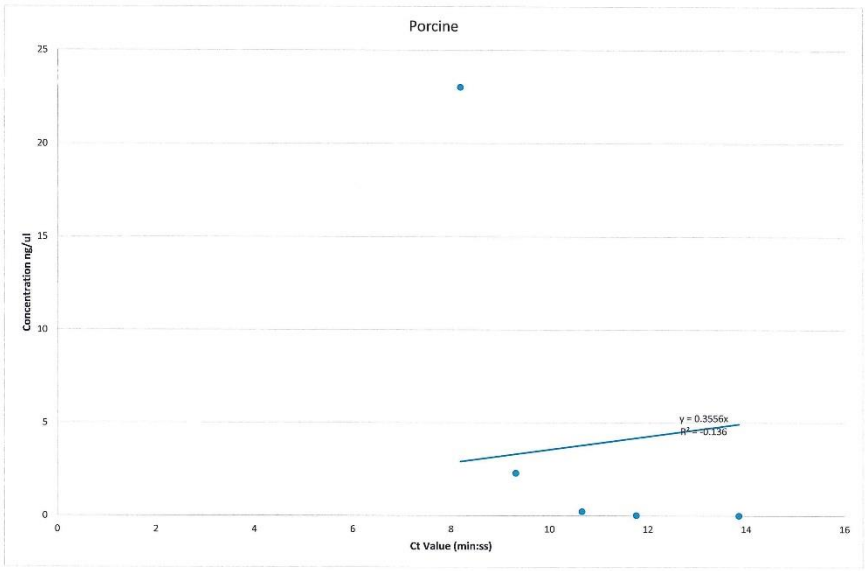

Coley
250
200
150
100
50
0
Concentration (ng/ul)
y = 2.8695x
R² = 0.091
0
2
4
6
8
10
12
14
16
18
Ct Value (min;ss)

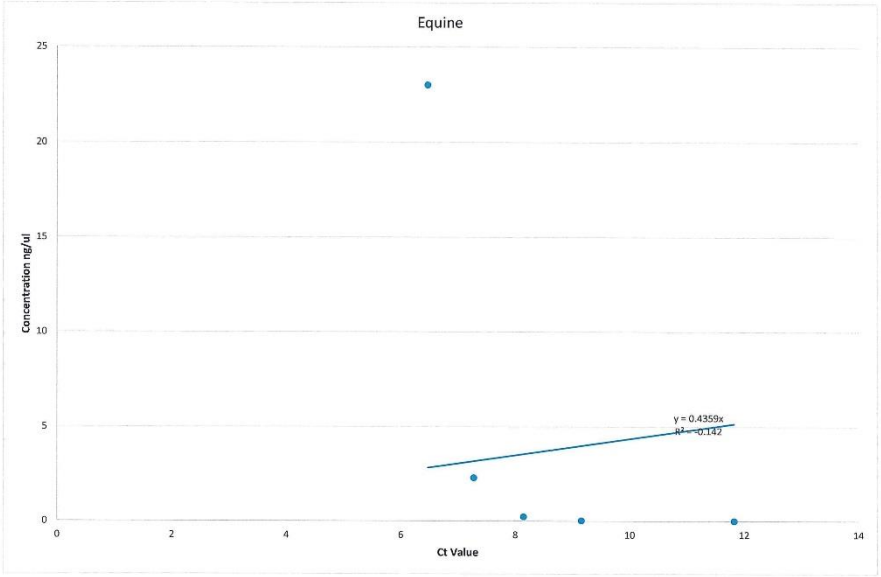

Pollock
Concentration ng/ul
Ct Value (min:ss)
y = 1.0539x
R² = -0.096


Bovine
Concentration ng/ul
Ct Value (min:ss)
y = 0.4813x
R² = -0.156


Caprine
Concentration ng/ul
Ct Value (min:ss)
y = 0.4691x
R² = 0.213


Ovine
Concentration ng/ul
Ct Value (min:ss)
y = 0.5104x
R² = 0.175


Porcine
Concentration ng/ul
Ct Value (min:ss)
y = 0.3556x
R² = -0.136
0
5
10
15
20
25
0
2
4
6
8
10
12
14
16


Equine
Concentration ng/ul
Ct Value
y = 0.4359x
R² = -0.142
0
5
10
15
20
25
0
2
4
6
8
10
12
14

(0)21 ***safe*food:**
7 Eastgate Avenue, Eastgate, Little Island, Co. Cork
7 Ascaill an Gheata Thoir, An tOiléan Beag, Co. Chorcaí
7 Aistyett Avenue, Aistyett, Wee Isle, Co. Cork
Tel: +353 (0)21 230 4100 ***Fax:*** +353 230 4111
Email: info@safefood.eu ***Web:*** www.safefood.eu