Rapid identification of closely related muscle foods by vibrational spectroscopy and machine learning

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Muscle foods are an integral part of the human diet and during the last few decades consumption of poultry products in particular has increased significantly. It is important for consumers, retailers and food regulatory bodies that these products are of a consistently high quality, authentic, and have not been subjected to adulteration by any lower-grade material either by accident or for economic gain. A variety of methods have been developed for the identification and authentication of muscle foods. However, none of these are rapid or non-invasive, all are time-consuming and difficulties have been encountered in discriminating between the commercially important avian species. Whilst previous attempts have been made to discriminate between muscle foods using infrared spectroscopy, these have had limited success, in particular regarding the closely related poultry species, chicken and turkey. Moreover, this study includes novel data since no attempts have been made to discriminate between both the species and the distinct muscle groups within these species, and this is the first application of Raman spectroscopy to the study of muscle foods. Samples of pre-packed meat and poultry were acquired and FT-IR and Raman measurements taken directly from the meat surface. Qualitative interpretation of FT-IR and Raman spectra at the species and muscle group levels were possible using discriminant function analysis. Genetic algorithms were used to elucidate meaningful interpretation of FT-IR results in (bio)chemical terms and we show that specific wavenumbers, and therefore chemical species, were discriminatory for each type (species and muscle) of poultry sample. We believe that this approach would aid food regulatory bodies in the rapid identification of meat and poultry products and shows particular potential for rapid assessment of food adulteration.

Introduction

Whilst muscle foods, which include meat and poultry, play an important part in the human diet and have done so for several thousand years,[1] significant numbers of consumers eschew this group of foods, either in part or as a whole, for religious, moral, cultural or dietary health considerations. The dietary health considerations for example could involve factors as simplistic as variations in fat content between different species or by perceived health risks surrounding large-scale food safety issues such as BSE and foot-and-mouth disease.[2,3] As well as these considerations, it is also important for consumers, retailers and food regulatory bodies that the products concerned are of a high quality, authentic, and have not been subjected to adulteration by any lower-grade material either by accident or for economic gain.[4–9] Therefore, the ability to determine the authenticity of any foodstuff is important[10–14] and in muscle foods, species identification has been recognised as a significant issue.[15–18]

At present, a variety of methods have been developed for the identification and authentication of meat and poultry products. These include methods based on the detection of DNA or RNA,[19,20] immunological,[21–23] electrophoretic[24–26] and chromatographic[27] techniques. The disadvantage with these current techniques however, is that they are time consuming, labour intensive and require a considerable amount of background training. Conversely, vibrational spectroscopic techniques (including mid-infrared, near-infrared and Raman) are rapid, reagentless, non-destructive and would be ideally suited to this type of analysis.[28–33] Several studies have applied infrared (both mid and near) spectroscopic techniques and visible spectroscopy to the problem of species identification and authenticity in meat and poultry.[17,18,34] These have resulted in varying degrees of success, and none of these methods have been successful in differentiating between the closely related species chicken and turkey. In addition, no study has investigated Raman spectroscopy as a method for the speciation of muscle foods. Indeed, no vibrational spectroscopic method has been shown to discriminate between distinct muscle groups within a species.

The consumption of poultry has risen dramatically in recent years and this is partly as a consequence of the popularity of convenience foods, which have significantly increased its commercial value.[35] The objective of the series of experiments undertaken for this study was to apply vibrational spectroscopic methods, namely HATR (horizontal attenuated total reflectance) FT-IR and Raman spectroscopy, to the problem of authenticity in comminuted muscle foods; the samples

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(in particular those from poultry) were minced so as to make them visibly indistinguishable from each other.

Materials and methods

Sample preparation

Samples of pre-packed meat and poultry were purchased from national retail outlets on the morning of each experiment. For the preliminary analyses these included chicken (skinless breast fillets), pork (boneless steaks), turkey (skinless breast fillets), lamb (neck fillets), beef (rump steaks) and for the subsequent experiments, chicken (skinless breast fillets and legs with skin) and turkey (skinless breast fillets and legs with skin). These were stored at 4 °C until required. For reasons of brevity samples for the preliminary FT-IR analysis were not homogenised but cut into solid samples measuring approximately 60 × 10 × 10 mm.

For subsequent experiments, in order to 'disguise' the poultry samples (that is to say make them visibly identical), individual samples were first weighed aseptically into 30 g sub-samples (after removal of skin if this was present) and comminuted for 10 s in a Moulinex type 505, 180 W coffee mill (Moulinex UK Ltd, Birmingham, UK). The bowl of the coffee mill was washed and dried with a paper towel between each sample. The sample was removed from the coffee mill and placed into the upturned lid of a 90 mm Petri dish and pressed manually to a thickness of ~5 mm using the inverted base of a Petri dish as the press. A sterile upturned Petri dish base was used to cover each prepared sample. A total of 4 Petri dish samples were prepared for each group (*e.g.* chicken breast). These were then analysed immediately following preparation.

HATR FT-IR spectroscopy

FT-IR analysis was undertaken using a ZnSe HATR accessory (Spectroscopy Central Ltd, Warrington, UK) on a Bruker IFS28 infrared spectrometer equipped with a DTGS (deuterated triglycine sulfate) detector (Bruker Ltd, Coventry, UK) as described elsewhere.[30] For preliminary analysis, 12 whole-meat replicates were individually analysed from five species; beef, lamb, pork, chicken and turkey. Each whole-meat replicate was placed in intimate contact with the HATR crystal, with no preference given to which surface of the sample was to be measured, and a FT-IR absorbance spectrum collected. For the analysis of poultry species in subsequent experiments, 15 replicates of each type (chicken breast, chicken leg, turkey breast and turkey leg) were individually excised by scalpel from the prepared Petri dish samples and the upper surface placed in intimate contact with the HATR crystal and a spectrum collected.

The crystal surface was cleaned with distilled water and a soft tissue following collection of each spectrum and washed thoroughly with acetone, rinsed with distilled water and dried with a soft tissue at the end of each sampling interval. The IBM-compatible PC used to control the IFS28 spectrometer was also programmed (using OPUS version 2.1 software running under OS/2 Warp provided by the manufacturers) to collect spectra over the wavenumber range 4000 cm^{-1} to 600 cm^{-1}. The reference spectra were acquired from the cleaned blank crystal prior to the presentation of each sample replicate. All spectra were collected in absorbance mode with a resolution of 16 cm^{-1}, and to improve the signal-to-noise ratio 256 scans were co-added and averaged. Collection time for each sample spectrum was 60 s and a total of 60 spectra were collected for the preliminary analysis and 120 for the comminuted poultry experiments. Each experiment was undertaken in duplicate. Any CO_2 peaks were removed prior to analysis. Typical FT-IR spectra from poultry are shown in Fig. 1; each spectrum is represented by 441 wavenumbers.

Raman spectroscopy

Raman spectra were collected using a near infrared diode laser with an excitation at 785 nm, using a Renishaw 2000 Raman probe system together with the Renishaw WiRE Grams software package and a CCD detector (Renishaw PLC, Gloucestershire, UK)[36] as described elsewhere.[31,37,38] The probe had an optimal focusing distance of 12 mm from the sampling point and the laser power was set at 78 mW to measure spectra of chicken and turkey samples. Pure ethanol was used as a standard (it has a characteristic Raman shift from the C–C–O vibration at 882 cm^{-1}) and reference spectra were collected at the start of each experiment. Fifteen spectra (measurements) were randomly taken from each of the four samples, namely chicken breast and leg and turkey breast and leg, which were repeated in triplicate over three days. Raman spectra were collected for 10 seconds and 1 accumulation over the wavenumber range 100 cm^{-1} to 3000 cm^{-1}. Typical Raman spectra from poultry are shown in Fig. 2.

Cluster analysis

For FT-IR, ASCII data were exported from the Opus software used to control the FT-IR instrument and imported into Matlab version 6.1 (The MathWorks, Inc., Natick, MA) which runs under Microsoft Windows NT on an IBM-compatible PC. To minimise problems arising from unavoidable baseline shifts the spectra were scaled so that the smallest absorbance was set to 0 and the highest to +1.[39] In the case of Raman measurements, spectra were converted to multifiles, cosmic rays removed, and to account for photon count differences the spectra were scaled such that the offset = 0 and the height of the first line (where the laser line is cut out by the holographic filter) at 250 cm^{-1} = 1.

Matlab was then used to perform principal components-discriminant function analysis (PC-DFA) on both Raman and FT-IR spectral data sets as described elsewhere.[39] Briefly, the initial stage of the cluster analyses involved the reduction of the multidimensional spectroscopic data by principal components analysis (PCA).[40] PCA is a well known technique for reducing the dimensionality of multivariate data whilst preserving most of the variance, and Matlab was employed to perform PCA according to the NIPALS algorithm.[41] Discriminant function analysis (DFA; also known as canonical variates analysis (CVA)) then discriminated between groups on the basis of the retained principal components (PCs) and some *a priori* class structure.[42] Two strategies were used:

(a) For the analysis of chicken, pork, turkey, lamb and beef using FT-IR spectroscopy the class structure was two groups

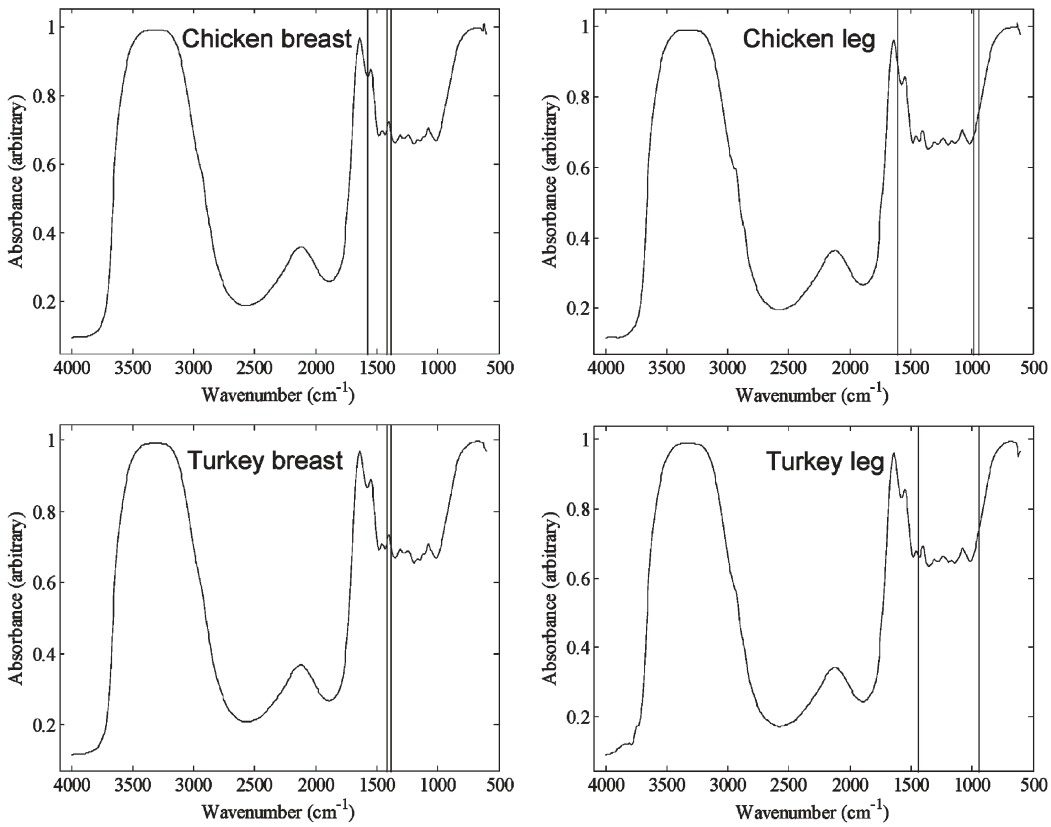

Fig. 1 Typical FT-IR absorbance spectra of chicken and turkey for leg and breast muscles. Also shown are the wavenumbers selected by GA as the most discriminatory bands. See Table 1 and text for details.

per meat type relating to the two duplicate experiments. Thus there were 10 classes in total.

(b) For the analysis of chicken breast, chicken leg, turkey breast and turkey leg using FT-IR and Raman spectroscopies the class structure was one per muscle and species type. That is to say there were 4 classes in total. In order to validate this process, data from a separate experiment were projected into PC-DFA space constructed from training data from other independent experiments.

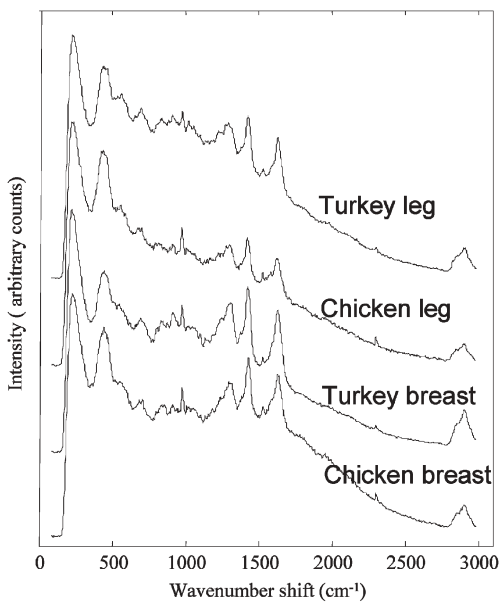

Fig. 2 Typical Raman spectra of chicken and turkey for leg and breast muscles.

Genetic algorithms

Having shown that there was discriminatory information within the FT-IR fingerprints, computational heuristic search methods, such as genetic algorithms (GA) were used to aid the discovery of important biochemical features in these spectra.

In this study a GA was applied to determine the subset of n wavenumbers, taken from the FT-IR data matrix, which when applied to a discriminant multiple linear regression (D-MLR) model[43] would optimally distinguish between two selected data

Table 1 Confusion matrices showing the predictions from PC-DFA projection analysis of the muscle and species type from Raman spectra

		Predictions			
	Identities	Chicken breast	Chicken leg	Turkey breast	Turkey leg
Training data	Chicken breast	30	0	0	0
	Chicken leg	0	29	0	1
	Turkey breast	0	0	30	0
	Turkey leg	0	2	0	28
Test data	Chicken breast	11	1	3	0
	Chicken leg	1	13	0	1
	Turkey breast	2	0	13	0
	Turkey leg	0	2	0	13

groups. Initially GA D-MLR analysis was performed in order to discriminate between the two general muscle types and the two species. The analysis was then extended such that four GA models were used to discriminate between each of the four muscle groups in turn (chicken breast, chicken leg, turkey breast and turkey leg) and the remaining 3 types. Training data were from the first FT-IR experiment, whilst test data were from the second independent experiment. All calculations were performed using in-house software written in C++ running under Microsoft Windows NT on an IBM-compatible PC and optimization was achieved by monitoring the residual mean square error of prediction (RMSEP) for each model. Full details of the GA-MLR algorithm are given in ref. 43, and a previous application using this technique to discriminate between control and salt-treated tomato fruit is described in Johnson *et al.*[44]

In this study the GA used proportional selection, and two-point crossover recombination with mutation, operating on a population of binary-encoded chromosomes, each chromosome representing v candidate wavelengths. The parameter v can be set to any integer value, between 2 and the total number of wavelengths used, prior to the execution of any single GA run. In order to select optimally the value of v, a set of GA experiments are performed where v is varied between 2 and v_{max} (v_{max} being the minimum number of wavenumbers allowed before overtraining in the regression model occurs).

The probability of mutating a given chromosome after recombination was set to 0.2, and the probability of changing a bit from a 0 to 1 (or *vice versa*), once a chromosome is selected for mutation, was set to 0.01. No two identical candidates were allowed in a given population and the top ten percent of each generation are automatically transferred unchanged to the next generation. A total of 100 independent GA runs were performed for each discrimination model in order to statistically validate the results of each genetic search.

Results and discussion

Preliminary analysis

Results from the preliminary FT-IR analysis on five different species, where samples were presented intact, illustrate that all 5 species could be clearly differentiated using PC-DFA (Fig. 3). Furthermore, the discrimination between mammalian and avian muscle types is evident as is the observation that chicken and turkey are closely related to each other, as are pork and lamb, whilst beef appears to be the least related to any of the other species. It is known that there are distinct differences between avian breast and mammalian muscle types, related, in the formers case, to adaptations to flight. Mammalian muscle for example has higher levels of myoglobin, numbers of mitochondria and a higher capillary density than those observed in avian breast muscle, whilst the avian muscle is adapted to obtain energy from large glycogen stores.[45] Whilst the ability to discriminate between these 5 species using FT-IR spectroscopy does not appear to be problematic in the present study, previous workers have failed to discriminate unequivocally between the closely related avian species.[17,18,34] The next stage was therefore to investigate this further with different muscle types using FT-IR and Raman spectroscopies.

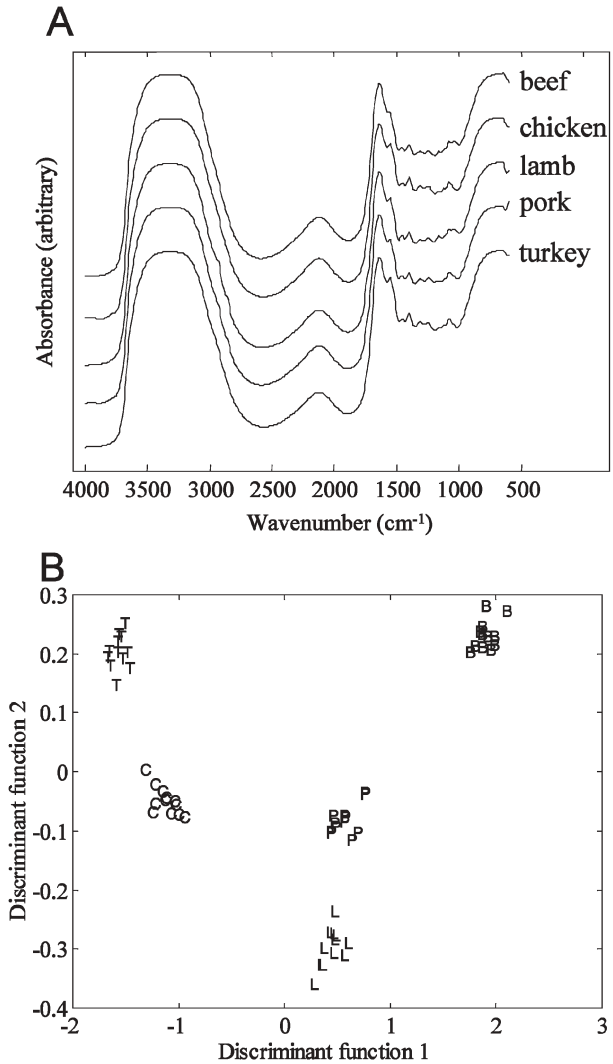

Fig. 3 (A) Typical FT-IR spectra from five meat species studied collected without comminution. (B) PC-DFA plot on FT-IR spectra showing the relationship between beef (B), lamb (L), pork (P), chicken (C) and turkey (T). Data were collected in duplicate with six samples from each species per experiment. For PC-DFA a total of 20 PCs were used with the *a priori* knowledge of 2 classes per species.

Analysis of poultry using Raman spectroscopy

In order to disguise the meat species, chicken and turkey meat from breast and leg muscles were comminuted as described above. All samples were analysed pure and no attempt in the present study was made to quantify mixtures of these muscle and species types; in part because of the conclusions made by ref. 17, 18 and 34 who found that whilst quantification of mixtures of different species (*viz*. pork from beef) was possible, the discrimination between poultry species was intractable.

All four meats were analysed first by Raman spectroscopy as described above. The Raman spectra are shown in Fig. 2, and visually are more complex than the FT-IR spectra (Fig. 1). It is clear that these Raman spectra contain a strong baseline shift that is likely to have arisen from fluorescence which has been produced when these samples have been analysed. Indeed, this is a common occurrence when analysing biological

material even with a near infrared laser.[36,47] Whilst the Raman spectra are qualitatively similar, subtle quantitative differences can be seen. However, the visual inspection of all these high dimensional spectra is not possible and hence cluster analysis was used.

As detailed above, each meat was analysed 15 times, 3 times over a period of 3 d. Data from two of those days were used in the construction of PC-DFA using the *a priori* information about the muscle type and poultry species (*i.e.*, there were four groups each containing 30 Raman spectra). After PC-DFA was performed, the test set from the remaining day was projected first into the PCA space and then the projected PCs projected into DFA space as detailed elsewhere.[46,47] The resulting PC-DFA projection plot is shown in Fig. 4, where it can be clearly seen that in the first discriminant function the major discrimination was between leg and breast muscle irrespective of species type. This is perhaps expected given the biochemical nature of these two muscle types. On closer inspection of Fig. 4 it is also possible to see discrimination between the poultry species in the second discriminant function; note that the scale in DF2 is much smaller than that for DF1 indicating that this is a much smaller spectral difference. For ease of interpretation, the PC-DFA has been converted into confusion matrices for both the training and test data (Table 1). It can be seen for the test data that only 2 of the 60 spectra are recovered to the wrong muscle type, while 8/60 are recovered to the wrong species type. All other spectra were correctly classified. These results again highlight that the discrimination between leg and breast muscle is more evident than discrimination at species level.

The GA-MLR method that is used below for the analysis of the FT-IR spectra could not be used on the Raman spectra because the ratio of the number of objects to variables was too low. As reported previously this constraint meant that the algorithm became unstable, and therefore the optimisation of MLR was not possible in this instance.[48]

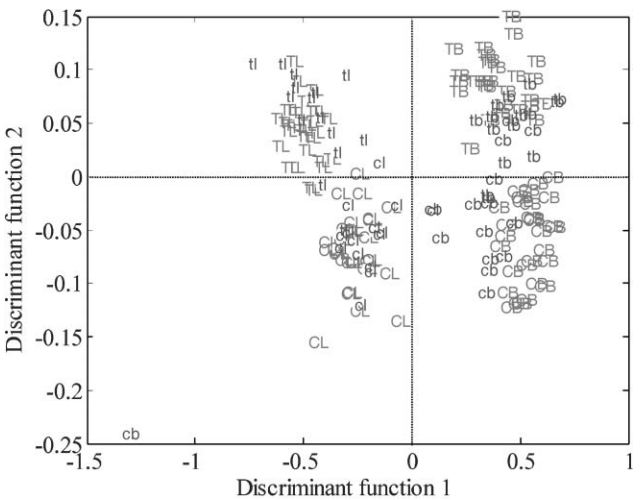

Fig. 4 PC-DFA plot constructed from the Raman spectra. 20 PCs accounting for 98.7% of the explained variance were used in DFA with *a priori* knowledge of the muscle and species type (*i.e.*, 4 classes in total). Upper case letters denote the data from two experiments that were used to construct the PC-DFA model, whilst lower case letters denote the test set from an independent experiment. CB + cb = chicken breast, CL + cl = chicken leg, TB + tb = turkey breast, TL + tl = turkey leg.

Analysis of poultry using FT-IR spectroscopy with GA-MLR

Initially cluster analysis was used as described above for the analysis of the FT-IR spectra from the four muscle types. Very similar results were seen as described for the Raman spectra (data not shown) indicating that FT-IR could also be used for the identification of the muscle type and species from which the meat had been obtained. The next stage was to ascertain if there were any spectral features (of those 441 wavenumbers collected) that could be used for discrimination, rather than using a full spectral chemometric approach. Therefore GA-MLR was used, as described above, to differentiate between (1) different poultry species, (2) different muscle types, and (3) each of the four meat samples.

It was notable for all GA-MLR models that very few wavenumbers, of the total 441 possible, were found to be highly discriminatory illustrating the power of GA for variable selection prior to MLR. Note that in all of these models one experiment was used for calibration (evolution) whilst the other was retained to test the models' ability to generalise. That is to say, be predictive for spectra not used in the training process.

A total of only 8 FT-IR wavenumbers were required by GA-MLR to discriminate between general muscle type, species and each of four separate muscle groups from the closely related muscle foods; the details of these FT-IR bands are shown in Fig. 1 and summarised in Table 2. In the case of discrimination of muscle type the three wavenumbers selected were 1413 cm^{-1}, 1444 cm^{-1} and 1729 cm^{-1} and these wavenumbers could be ascribed to C–N stretch from amides, N–H bend from amides and C=O stretch from saturated aliphatic aldehyde, respectively. Moreover, the wavenumber 1729 cm^{-1} was unique for the discrimination of muscle type. For discrimination at the species level only two wavenumbers were selected, 1575 cm^{-1} and 1606 cm^{-1}, which can be ascribed to a CNH combination vibration from amide II and NH_2 deformation from amines, respectively.

For the discrimination of chicken leg muscle from all other meat samples three wavenumbers were selected, and these were 942 cm^{-1}, 988 cm^{-1} and 1606 cm^{-1} which can be ascribed to O–H deformation from a carboxyl group, P–O–P stretch from phosphorous (likely from nucleic acids) and NH_2 deformation from amines respectively. Moreover, the wavenumber at

Table 2 Vibrations selected by GA-MLR as being the most discriminatory for various muscle types and species

	Wavenumber/cm^{-1} [a]							
	942	988	1382	1413	1444	1575	1606	1729
Muscle type				■	■			■
Species						■	■	
Chicken leg	■	■					■	
Chicken breast			■	■		■		
Turkey leg	■				■			
Turkey breast			■	■				

[a] Marked cells highlight the discriminatory wavenumbers.

988 cm^{-1} was only selected to discriminate for chicken leg muscle whilst 1606 cm^{-1} was also selected to discriminate at the species level.

In the case of chicken breast muscle three wavenumbers were selected, 1382 cm^{-1}, 1413 cm^{-1} and 1575 cm^{-1}, ascribable to C–N stretch from amines, C–N stretch from amides, and CNH combination vibration from amide II respectively. The wavenumber at 1413 cm^{-1} was previously selected to discriminate muscle type and 1575 cm^{-1} had also been selected to discriminate at the species level. From the analysis of turkey leg muscle only two wavenumbers were selected, 942 cm^{-1} and 1444 cm^{-1} ascribable to O–H deformation from a carboxyl group and N–H bend from amides. The wavenumber at 942 cm^{-1} was previously selected to discriminate chicken leg muscle and 1444 cm^{-1} was selected to discriminate at the species level. As with turkey leg muscle, only two wavenumbers were selected to discriminate turkey breast muscle from all others and these were 1382 cm^{-1} and 1413 cm^{-1}, attributable to C–N stretch from amines and C–N stretch from amides respectively. Finally, the two wavenumbers selected to discriminate turkey breast muscle were also both selected to discriminate chicken breast muscle, the only difference between the 2 muscle types being that an additional wavenumber was selected to discriminate chicken breast muscle at 1575 cm^{-1}, and significantly, this particular wavenumber was also selected for discrimination at the species level.

Several of the wavenumbers of interest could be readily ascribed to proteinaceous groups and indeed this is not surprising given that the substrate under analysis was muscle tissue. As previously described in the literature, muscle tissue contains mostly water ($\sim$75%) and protein (18–20%) with the remainder containing fats, carbohydrates and minerals.[45,49] Therefore, the major group of compounds in muscle tissue after water are proteinaceous and these can be further subdivided into myofibrillar, sarcoplasmic and connective tissue proteins which constitute approximately 60, 30 and 10% of the muscle proteins respectively.[45]

Two of the discrimination models contained a single wavenumber particular to that group, such as turkey leg and more importantly muscle type. The wavenumber assigned to muscle type at 1729 cm^{-1} and ascribable to saturated aliphatic aldehyde, for example, could be as a result of lipid oxidation.[50] Moreover, aldehyde has also previously been shown to be related to the metabolic type of skeletal muscle[51,52] and its levels can also vary according to feeding regime, such as the use of food supplements.[53–55] What was evident was the fact that all the models contained a combination of either 2 or 3 wavenumbers which were particular to a single muscle group of a particular species and which distinguished them from all of the others. An example of the wavenumbers selected by GA-MLR to discriminate chicken leg from the other muscle groups is shown in Fig. 5. In this figure the chicken leg samples are clearly recovered separately from the other three muscle types, and similar results were seen when each of the wavenumbers were plotted for the discrimination of each of the other three meat types (data not shown).

In conclusion, these data clearly demonstrate the utility of these analytical approaches, based on FT-IR and Raman spectroscopy, which in combination with appropriate machine learning-based strategies, provides a rapid, robust and accurate method for the authentication of closely related muscle foods. In particular, and as a result of FT-IR analyses

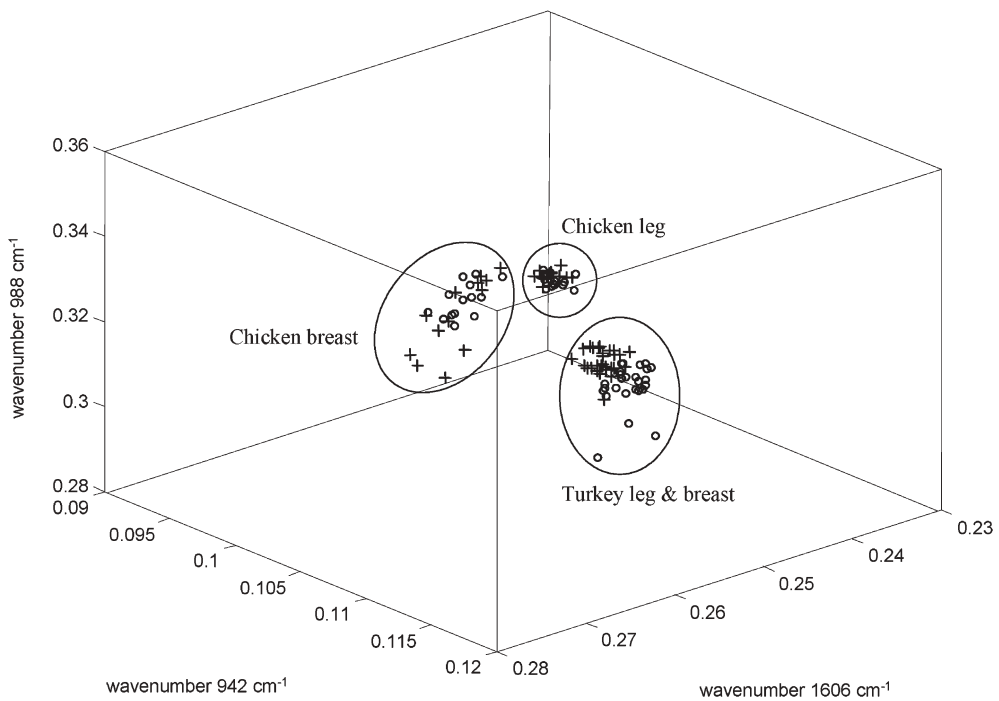

Fig. 5 Pseudo 3-dimensional plot of the intensity of 942 cm^{-1} plotted against 988 cm^{-1} and 1606 cm^{-1} from FT-IR data. Crosses denote data used in the construction of the GA-MLR model from experiment 1 to discriminate between muscle types from poultry. Circles are data from a separate second experiment. Other orientations show the same discrimination.

by GAs, it was possible to discriminate qualitatively between all four muscle groups and to find wavenumbers of particular relevance, between not only the muscle groups of a particular species, but those wavenumbers of interest which were responsible for the general discrimination at both the muscle type and species level. Finally, this is the first time that FT-IR and Raman spectroscopy have been used successfully for the discrimination of both closely related poultry species (chicken and turkey) and for the differentiation of leg from the more expensive breast muscle. We therefore believe that vibrational spectroscopies could aid in the identification of specific muscle groups and also assist in any future quantitative studies concerning the adulteration of one meat type by another.

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