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Simultaneous authentication of species identity and geographical origin of shrimps: Untargeted metabolomics to recurrent biomarker ions

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A B S T R A C T

Mandatory disclosure of the species identity, production method, and geographical origin are embedded in the regulations and traceability systems, governing international seafood trade. A high-resolution mass spectrometry-based metabolomics approach could simultaneously authenticate the species identity and geographical origin of commercially important shrimps. The highly innovative approach spared the need for multiple testing methods which are in routine use currently. A robust chemometric model, developed using the metabolite fingerprint dataset, could accurately predict the species identity of the shrimp samples. Subsequently, species-specific biomarkers were discovered and a tandem mass spectrometry method for authentication of the species was developed. Two other chemometric models from the metabolomics experiment accurately predicted the geographical origin of king prawns and tiger prawns. The study has shown for the first time that food-metabolomics along with chemometrics can simultaneously check for multiple seafood fraud issues in the global seafood supply-chain.

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1. Introduction

Fisheries and aquaculture products are a major source of livelihood and sustenance for billions of people globally. The Food and Agriculture Organisation of the United Nations (FAO) has estimated the annual export trade of seafood alone to US$150 billion [1]. Unfortunately, seafood is also one of the most prominent food categories associated with food fraud, undermining the credibility of the whole seafood supply chain [2,3]. Seafood fraud not only threatens health and safety of consumers, also puts our oceans and other water resources at risk [4]. Shrimps and prawns are considered as a valuable seafood product that accounts for about 15% of the total value of internationally traded fishery and aquaculture products amounting to about US$43 billion [5]. Scandals, such as fraudulent labeling of low value species as premium species of shrimps and labeling aquaculture white leg shrimp (Litopenaeus vannamei) as premium wild-caught shrimp have been reported by international environmental nonprofit groups [6]. Disturbing reports of slave labour in Thailand shrimp industry have surfaced in the media, prompting the food industry giant Nestle to initiate stringent measures to prevent human rights abuses in the seafood supply chain in Thailand [7].

International laws entitle consumers to know the commercial identity of the species, production method (wild caught or farmed) and geographical origin for all categories of fishery and aquaculture products. Together, these three pieces of information constitute the “Traceability” of a seafood product [8,9]. DNA profiling based techniques are considered gold standard for species authentication of seafood. However, success of the approach heavily relies on the availability of comprehensive reference sequence libraries. Authentication of geographical origin and production method using DNA based techniques is highly challenging and reports of such application are very few [10–12]. Similarly proteomics strategies are mostly suitable for authentication of species identity and

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detecting adulteration of ingredients [13–15]. Among the techniques used to determine the geographical origin and production method of seafood, multi-element and stable isotope ratio analysis approaches are the most successfully employed [5,16]. However, these different analytical techniques often require complex sample preparation, different types of analytical platforms and often long assay run times. This is a significant disadvantage while trying to manage traceability in a fast-moving, complex supply chain of perishable seafood.

The complete set of metabolites synthesized in a biological system constitutes its ‘metabolome’ and is directly linked to an organism’s genetic make-up, food intake and changes in the environment, it lives in [17]. The targeted metabolomics approach emphasizes on detection and quantification of a few classes of compounds, mostly using a unit resolution mass spectrometer. Unit resolution mass spectrometer with triple quadrupole mass analyzer has the advantage of using selective reaction monitoring (SRM) or multiple reaction monitoring (MRM) mode, detecting the target compounds at trace level in complex matrices. Untargeted metabolomics which intends to study the global ‘metabolite fingerprint’ of a sample have several advantages over targeted approach such as combining targeted and untargeted screening, novel biomarker discovery and retrospective data analysis. Over the last five years, high-resolution mass spectrometer (HRMS) has established itself as the preferred analytical choice in untargeted metabolomics research, driven by increased affordability, unsurpassed sensitivity and high resolution [18–20]. HRMS based untargeted metabolomics coupled with the power of chemometrics data analysis can potentially investigate multiple authenticity issues within a single experiment [21,22].

HRMS based metabolite fingerprinting and chemometrics have been used for authentication of seafood/fish only on two previous occasions. Metabolite fingerprinting on a two dimensional gas chromatography and time of flight mass spectrometry was used to distinguish two bivalve species [23]. A Rapid evaporation ionisation mass spectrometry (REIMS) based metabolomics profile approach was reported recently for accurate identification of species identity of five different white fish fillet [24]. To our knowledge, HRMS based untargeted metabolomics approach has not been so far applied to test multiple food fraud issues in a single metabolomics experiment.

In this study, we aim to explore the feasibility of authenticating species identity, geographical origin, and production method of commercially important shrimps and prawns in a single untargeted metabolomics experiment.

2. Materials and methods

2.1. Samples collection

Authentic samples were collected either directly from aquaculture farms linked to the ICAR-Central Institute of Fisheries Technology in India or through a number of supermarket supply chains in the United Kingdom. Five commercially important species of shrimps; tiger prawn (Peneaus monodon), king prawn (Litopenaeus vannamei), Indian white shrimp (Fenneropenaeus indicus), Indian pink shrimp (Metapenaeus monoceros) and Argentinian red shrimp (Pleoticus muelleri) were considered for developing chemometric models for species authentication. The tiger prawn samples included wild caught prawns from India and Madagascar; and farmed prawns from Vietnam and Sri Lanka. Farmed king prawns were obtained from India, Thailand, Vietnam, and Honduras. The samples were transported to the laboratory within 24 h in insulated polystyrene boxes with dry ice and stored frozen at –80 °C following removal of the head and outer shell. The samples were freeze-dried immediately afterwards and stored in labelled polypropylene containers at –80 °C.

2.2. Samples preparation

Ten individual shrimp samples were pooled together to obtain one representative sample for a class of shrimp, labeled based on species identity and country of origin. Likewise, for a particular class of shrimp three representative samples were obtained. As pre-treatment, the freeze-dried samples were homogenized in a planetary ball mill (Retsch GmbH PM 200, Haan, Germany). Then, 0.05 g (±1%) of pulverized samples was weighed out on a Discovery DV215CD Analytical Balance (Ohaus Europe GmbH, Nanikon, Switzerland) into 1.5 mL micro centrifuge tubes. Next, the samples were extracted with 1 mL of aqueous methanol containing one part ultra-pure water (18.2 MΩ/cm, Merck Millipore, Billerica, USA) and four parts LC–MS grade Chromasolv methanol (Sigma-Aldrich, St Louis, MO, USA) by mixing at 2500 rpm with DVX-2500 Multitube Vortexer (VWR International, Lutterworth, UK) for 10 min, followed by sonication for 30 min at maximum frequency in a camSonix C1274 water bath sonicator (Camlab, Cambridge, UK) at room temperature. After centrifugation at 10,000 g for 10 min at 4 °C in a MIKRO 200R centrifuge (Hettich UK, Salford, UK), 0.8 mL of the supernatant was transferred into a fresh microcentrifuge tube and dried overnight in a miVac QUP-23050-A00 (Genevac, Ipswich, UK) centrifugal sample concentrator. The dry extracts were then reconstituted in 0.8 mL of ultra-pure water and filtered through a 0.22 µm Costar® cellulose acetate Centrifuge Tube Filter by centrifugation at 10,000xg, 4 °C for 10 min. Filtered extracts were transferred into LC vials (Waters, Manchester, UK) for LC–MS analysis.

2.3. Untargeted LC-HRMS analysis

Analyses were carried out on a Waters Acquity UPLC I-Class system (Milford, MA, USA) coupled to a Waters Xevo G2-S QToF mass spectrometer (Manchester, UK) with an electrospray ionisation source operating in a positive or negative mode with lock-spray interface for real time accurate mass correction. Instrument settings were as follows: source temperature was set at 120 °C, cone gas flow at 50 L h⁻¹, desolvation temperature at 450 °C, and desolvation gas flow at 850 L h⁻¹. The capillary voltage was set at 1.0 kV in positive mode and 0.5 kV in negative mode, respectively. Source offset was 80 (arbitrary unit). Mass spectra data were acquired in continuous mode using MS² function (low energy: 4 eV; high energy: ramp from 20 to 30 eV) over the range m/z 100–1200 with a scan time of 0.1 s A lock-mass solution of Leucine Enkaphalin (1 ng mL⁻¹) in methanol/water containing 0.1% formic acid (1:1, v/v) was continuously infused into the MS via the lock-spray at a flow rate of 10 μL min⁻¹. The chromatographic separation was conducted on a Waters Cortecs T3 column (100 mm x 2.1 mm, 1.6 μm). The column oven temperature was set at 45 °C, injection volume at 3.5 μL and flow rate at 0.4 mL min⁻¹. The mobile phase consisted of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid. The gradient was set as follows: 2.0 min of 99% (A) followed by a linear increase from 1% to 99% (B) over 16 min, isocratic cleaning step at 99% (B) for 0.5 min, then returned to initial conditions 99% (A) over 0.1 min and column equilibration step at 99% (A) for 1.4 min. Each sample was injected three times in order to assure reproducibility. At the beginning of the experiment, 10 pooled conditioning samples (QCs) were included. For quality control, QCs were also injected at intervals of every 10 samples throughout the entire experiment to determine the chromatographic reproducibility of retention times and peak intensities [25].
2.4. Chemometric data analysis

Raw data generated were imported to Progenesis QI 2.0 software (Waters, Newcastle, UK). After data conversion to the appropriate format using a filter set at 1.5, data were aligned to the best QC sample selected and peak picking from 0.6 to 18 min was carried out with sensitivity set at absolute ion intensity of 1000 (arbitrary unit) and chromatographic peak width to 0.08 min. A data matrix of detected metabolite features and corresponding normalised abundance was generated and then exported to SIMCA 14 (Umetrics, Malmo, Sweden) for multivariate analysis. To assess the general quality of the acquired spectral data (univariate/Pareto scaled) principal component analysis (PCA) and model assessment were performed. Next, data were mean centred, either Pareto or univariate scaled and grouped into respective classes prior to orthogonal partial least square discriminant analysis (OPLS-DA). The variable importance in projection (VIP) plots of the metabolite features associated with OPLS-DA, and subsequent cross-checking of the features in Progenesis QI for peak quality and intensity ensured selection of reliable metabolite features. R2 (cumulative), Q2 (cumulative) values and RMSECV were used to determine the validity of the models, with R2 (cum) employed as an indicator of the variation described by all components in the model and Q2 & RMSECV as measures of how accurately the model can predict class membership.

2.5. Biomarker discovery and method transfer to LC–MS/MS

The discovery of characteristic biomarkers for each shrimp species was achieved by generating individual OPLS-DA models during binary species comparison. Associated S-plots and variable importance in projection (VIP) plots enabled identification of a set of most promising ions in both ionisation modes responsible for class separation among all species. Selected ions were thoroughly investigated in both the raw data and Progenesis QI 2.0 for the peak quality and intensity as well as selectivity between assessed species. Accurate mass of the biomarker ions was searched against the metabolite databases ChemSpider, LipidBlaster, Metlin, Human Metabolome Database and FooDB to reveal putative identities.

Biomarkers, which were selective to the species identity were further investigated by targeted analysis. The retention time of biomarkers was confirmed on a Xevo TQ-S LC–MS/MS in selected ion monitoring mode applying the same chromatographic conditions (described in Section 2.3) as in the untargeted analysis. Next, the fragmentation spectrum obtained in daughter ion scan was compared to respective spectrum acquired on Q-ToF to confirm markers chemical identity. After optimising collision energies by repeated on-column injections, the most prominent fragment(s) of each biomarker’s precursor were selected for its respective MRM window and chromatographic conditions adjusted to decrease analysis time. None of these selected biomarkers were successfully identified; nevertheless, the minimal requirements of reporting for unknown metabolites (retention time, prominent ion and fragment ion) specified by the Chemical Analysis Working Group within Metabolomics Standards Initiative (MSI) have been fulfilled [26]. To assure correct identification up to three fragment ions were included in the final MS method, however, some of the selected markers only yielded one fragment ion, thus decreasing their reliability due to lack of possibility of ion ratios monitoring [27].

2.6. Targeted LC–MS/MS analysis

The analysis was performed on an Acquity UHPLC1–1 Class system (Waters, Milford, MA, USA) coupled to Xevo TQ-S triple quadrupole mass analyser (Waters, Manchester, UK) operating in positive electrospray ionisation mode. The following settings were applied: capillary voltage was set at 1.0 kV, the desolvation and source temperatures were set at 450 and 130 °C, respectively, while nitrogen cone and desolvation flow rates were set to 145 and 1000 L/h. Argon was employed as a collision gas, with a flow of 0.15 mL/min, yielding a collision cell pressure of 2.4 × 10⁻⁴ mbar. Inter-scan and -channel delays were both set to 3 ms while dwell times ranged from 20 to 163 ms.

Analytes’ separation was performed on a Waters Cortecs T3 column (100 mm × 2.1 mm, 1.6 μm), maintained at 45 °C with a 5 μL injection of a sample extract. The pump was operated at a flow rate of 0.4 mL/min with mobile phases consisting of A, 0.1% formic acid in water and B, 0.1% formic acid in methanol. The final gradient was isocratic 0–1.0 min 80% A, linear 1.0–2.0 min 75% A, linear 2.0–4.0 min 20% A, linear 4.0–6.5 min 10% A, linear 6.5–7.0 min 1% A for column flush, going back to initial conditions during 0.1 min and finishing with isocratic column equilibration 7.1–9.0 min. After each injection, the needle was washed with 0.1% formic acid in H₂O/MeCN/MeOH (2:1:1) and purged mobile phase A.

LC–MS/MS method assessment was performed by analysing test samples, representing each sample class, on three different days. An initial run consisted of 33 samples - three samples per class while last two runs consisted of 55 samples – five samples per class. Samples were randomized in each run with ‘solvent-QC-solvent’ sequence injected every 8 samples to monitor for possible carry-over, ion ratios alteration or sensitivity loss throughout the run. The initial test samples were part of the authentic samples collected but were not used in developing the chemometric models. Test samples, purchased from local supermarkets were tested using the targeted assay and authenticated against the label claim.

The relative abundance of the species specific markers of king prawn and tiger prawn, varying with geographical origin, was evaluated. Raw data were processed by Target Lynx v.4. (Waters, Milford, MA, USA) while statistical analysis (one-way ANOVA, based on absolute response) and associated graphs were prepared in GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, USA). A linear discriminant analysis (LDA) model for prediction of geographical origin, using the Unscrambler X software (Camo), was developed for the specific markers of king prawns. The model was evaluated to predict the geographical origin of 60 test samples of king prawns which were part of the authentic samples gathered for the project.

3. Results and discussion

3.1. High resolution mass spectrometry data processing and quality valuation

Liquid chromatography hyphenated to a QToF mass spectrometer, with data acquired in continuum mode, is a powerful tool for unbiased record of accurate mass data of every single detectable metabolite in complex biological samples. However, the major bottleneck in such “global metabolomics” experiments is the unmanageable amount of data generated which necessitates the employment of robust tools for data visualization, pre-processing and metabolite identification to ensure quality and reproducibility of the data [28,29]. The untargeted metabolomics experiments, in positive and negative ionisation mode, generated more than 1200 gigabytes of raw data each. The data was imported into the Progenesis QI software and was checked for quality by reviewing the retention time alignment of each sample against a randomly selected QC sample. Alignment scores of the samples ranged from 82.9 to 98.2% and 88.5 to 98.0% in positive and negative ionisation mode respectively; indicating excellent reproducibility of the data for the entire duration of the mass spectrometry exper-
iment. Various “minimum intensity” values for the “absolute ion intensity” filter were tried to optimise peak peaking sensitivity of the Progenesis QI software. This optimisation was important to ensure that the detected molecular features are authentic and at the same time relevant features are not missed. For each setting, the data matrix for all eleven different types of shrimps was imported in SIMCA 14 software for PCA analysis and subsequent evaluation. A minimum intensity value of 1000, both in positive and negative ionisation mode was found to be optimum. Nevertheless, a total of 24,411 molecular features were detected in positive ionisation mode whereas in negative ionisation mode a total of 4921 molecular features were detected. All QCs were found to be tightly clustered within the centre of respective PCA scores plots. PCA scores plot in ESI+ and ESI− mode showed clear indication of separation between different classes of shrimp, using second & fourth and second & fifth principal components respectively (Fig. 1A, B). The first six principal components explained 80 and 81% of variation in positive and negative ionisation mode respectively.

Though, farmed king prawn samples from different geographical origin grouped close by, there were clear indication of discrimination based on geographical origin. Similarly, there was clear indication of separation between farmed and wild caught tiger prawns. The recorded high values of R2X (cum) and Q2 (cum) for the PCA models (Table 1; No. 1 and 2) in the ESI+ and ESI− mode, indicate well explained cumulative variation of the data by the principal components and excellent prediction capability of the models [30]. Representative total ion chromatograms (TICs) of QC samples in ESI+ and ESI− mode, presented in the Fig. 2., show the extent of complex and rich information obtained from the untargeted metabolomics experiment.

### 3.2. Chemometric models for shrimp species authentication

For building the models for shrimp species authentication, samples of the species originating from different geographical origin and harvesting method was grouped as one species class. This resulted in total five species classes of shrimps. The datamatrix was exported to SIMCA 14 and exploratory PCA and OPLS-DA models were built using either univariate or Pareto scaling. The VIP values of the molecular features in an OPLS-DA plot was used to select the most relevant features. Metabolite ions with a VIP > 1 generally represent those features carrying the most relevant information for class discrimination [31]. Hence, the molecular features with VIP score of more than 1.5 was tagged and re-imported to Progenesis QI software to crosscheck the peak quality and intensity of the features. A number of rounds of such filtering of the detected molecular features ensured selection of most relevant metabolite features that contribute in classification of different classes of shrimp.

Finally, PCA (No. 3 & 4) models were generated considering 4914 and 900 molecular features in positive and negative ionisation mode respectively (Table 1). The PCA scores plots (Fig. 3A, B), both in positive and negative ionisation mode, showed clear separation based on the species identity of the shrimp samples. The species class of “Black tiger prawn” and “King prawn” represent samples of different origin or production method. Despite this variability of origin/production method, the samples of king prawn and black tiger prawn formed distinctive clusters in the PCA plots. Whilst first four components accounted for around 80% cumulative variation in the data, separation was mostly achieved along PC1 and PC2. The better values of R2X and Q2 for the PCA models (No. 3 & 4) as compared to the PCA models No 1 and 2 (Table 1), indicated reliable selection of most relevant features.

![Fig. 1.](image-url)Eleven different types of shrimps and pool QC samples clustering on PCA scores plot in ESI+ (A) and ESI− (B) mode.
Fig. 2. Representative total ion chromatograms (TICs) of pool QC samples in ESI+ (A) and ESI− (B) mode showing extent of metabolite signature obtained from the shrimp samples.

Fig. 3. Five different shrimp species and pool QC samples clustering on PCA scores plot in ESI+ (A) and ESI− (B) mode; OPLS-DA scores plot of species discrimination of shrimp in ESI+ (C) and ESI− (D) mode.
Table 1  
Values of different statistical parameters for developed chemometric models from detected metabolite features in positive and negative ionisation mode, where “A” is number of multivariate component, “N” is number of samples, “R2X” is the fraction of the variance of the X variables explained by the model, “R2Y” is the fraction of the variance of the Y variables explained by the model, Q2 is the fraction of the variance of the X and Y variables that denotes the prediction ability of the model.

| No. | Type      | A    | N   | R2X (cum) | R2Y (cum) | Q2 (cum) | Description                               |
|-----|-----------|------|-----|-----------|-----------|----------|-------------------------------------------|
| 1.  | PCA-X     | 12   | 125 | 0.962     | 0.975     | 0.944    | Positive mode; Univariate scaling; All classes of shrimp |
| 2.  | PCA-X     | 12   | 125 | 0.985     | 0.975     | 0.953    | Negative mode; Univariate scaling; All classes of shrimp |
| 3.  | PCA-X     | 12   | 125 | 0.988     | 0.982     | 0.991    | Positive mode; Univariate scaling; Species of shrimp |
| 4.  | PCA-X     | 16   | 121 | 0.995     | 0.995     | 0.991    | Negative mode; Univariate scaling; Species of shrimp |
| 5.  | OPLS-DA   | 4+1+0| 99  | 0.911     | 0.996     | 0.995    | Positive mode; Univariate scaling; Discrimination of Species |
| 6.  | OPLS-DA   | 5+2+0| 99  | 0.927     | 0.989     | 0.981    | Negative mode; Univariate scaling; Discrimination of Species |
| 7.  | OPLS-DA   | 3+1+0| 36  | 0.925     | 0.998     | 0.994    | Positive mode; Univariate scaling; Discrimination of Tiger Prawn origin |
| 8.  | OPLS-DA   | 3+1+0| 36  | 0.975     | 0.994     | 0.993    | Negative mode; Paretto scaling; Discrimination of Tiger Prawn origin |
| 9.  | OPLS-DA   | 3+1+0| 36  | 0.966     | 0.999     | 0.999    | Positive mode; Univariate scaling; Discrimination of King Prawn origin |
| 10. | OPLS-DA   | 3+1+0| 36  | 0.989     | 0.997     | 0.996    | Negative mode; Paretto scaling; Discrimination of King Prawn origin |

For predictive analysis, an OPLS-DA model (No. 5) was then auto-tifed with four predictive X-Y components and one orthogonal component, which resulted in R2X = 91.1%, R2Y = 99.6%, Q2 = 99.5% and RMSECV of 4% for the ESI+ data (Table 1). Another OPLS-DA model (No 6) with five predictive X-Y components and two orthogonal components was generated with resulting R2X = 92.7%, R2Y = 98.9%, Q2 = 98.8% and RMSECV of 5.2% for the ESI+ data (Table 1). A high value for R2Y and Q2 (closer to 1) indicate high explained variation and predictive ability of an OPLS-DA model respectively. Whereas, a lower value of RMSECV indicate better predictive ability of an OPLS-DA model [25]. The different classes of shrimp species appeared as better separated and tightly grouped clusters in the OPLS-DA scores plots in ESI+ as compared to ESI- mode (Fig. 3C, D), in accordance to the better statistical parameters obtained in ESI+ mode.

3.3. Chemometric models for shrimp origin authentication

OPLS-DA models for the dataset in ESI+ than ESI- mode were developed for prediction of geographical origin of the “Tiger prawns” and the “King prawns”. A similar strategy, followed for species authentication of shrimp samples, was adopted for selection of the most relevant molecular features that contribute to discrimination of geographical origin of tiger prawns and king prawns. The OPLS-DA models for tiger prawns in ESI+ than ESI- mode were finally built on datasets containing 1602 and 2081 molecular features respectively. The OPLS-DA models in ESI+ mode was auto-tifed with three predictive X-Y components and one orthogonal component resulting in R2X (cum) = 92.5%, R2X (cum) = 99.8%, Q2 (cum) = 99.8% and RMSECV = 2.5%. The OPLS-DA model in ESI- mode, fitted with similar numbers of predictive and orthogonal components resulted in equally good values of R2X (cum) = 97.5%, R2X (cum) = 99.4%, Q2 (cum) = 99.3% and RMSECV = 4% (Table 1, No 7 & 8). Interestingly, the samples of wild caught tiger prawns from India and Madagascar clustered closer together (shown within ellipse) in the OPLS-DA scores plots in ESI+ and ESI- mode (Fig. 4A, B); strongly indicating discrimination based on harvesting method. Samples of wild caught and farmed tiger prawns from the same country of origin were not available to further explore such discrimination in detail. Similarly, OPLS-DA scores plots for king prawns displayed well separated and tightly grouped clusters of king prawns originated from India, Thailand, Vietnam and Honduras (Fig. 4C, D). Again, the values of R2Y (cum) and Q2 (cum) was close to 1 establishing strong predictive capabilities of the models in ESI+ and ESI- mode (Table 1, No 9 & 10).

3.4. Validation of the chemometric models

The recognition ability for all the classification models both in positive and negative ionisation mode was 100% as presented in the corresponding misclassification tables (Supplementary material Figure S1). A misclassification table which provides a quantitative measure of the performance of a class or discriminant analysis model is a summary of the number of correctly classified observations, with known class belonging. All the OPLS-DA classification models were then validated using the response permutation option in SIMCA. The Y-data in the training set is permuted by randomly shuffling their position while the numeric value remains same. The permutation procedure can be repeated a number of times between 20 and 100. The R2Y and Q2Y values of the derived models from the permuted Y-data are then compared with the R2Y and Q2Y values of the real model to check the validity of the classification model. The permutation plots for the OPLS-DA models presented in supplementary material Figure S2-S4 summarizes the result of response permutation testing. It can be observed that even after 100 permutations, the R2Y and Q2Y values of the developed OPLS-DA models are substantially higher than the corresponding permuted values indicating validity of the models. The R2Y intercept below 0.3 and Q2Y intercept below 0.05 for the developed OPLS-DA models are another strong indication of the validity of the models. To further confirm the prediction ability of the developed models, 20 percent of the samples from the original training set was taken out and used as test sample set in the corresponding refitted model. It can be observed in the classification list (Supplementary Table S1-S6) that all the test samples of known class identity was recognised accurately. The untargeted metabolomics experiment was repeated three times with fresh sample sets and each time rugged chemometric models were derived establishing reproducibility of the approach.

3.5. Biomarker discovery and putative identification

A total of 36 biomarkers were putatively identified in ESI+ mode while a total of 33 ions were putatively identified in ESI- mode based on various accurate mass database search (Supplementary Table S7-S8). Putative identification of metabolite corresponds to Identification Level 3 of metabolomics standard initiative [26]. A metabolite may produces multiple accurate mass signals corresponding to iso- topes (12C, 13C) adducts (M+H+), (M+Na+), multimers ([M+2H]+, [M+2Na]+), and neutral loss fragments ([M+H2O]+) in a HRMS. Signal annotation was performed in Progenesis QI software clustering iso- topes, based on the isotopic pattern of a given molecule. The adducts of a molecule sharing the same retention time was clustered and translated into one value (molecular ion). However, where only one adduct is available for a feature the m/z value of the pseudo molecular ion was reported. These accurate masses can be searched against database(s) within a defined window to retrieve potential candidates. An in-built search engine in Progenesis QI was used to search the accurate masses against various databases. A mass error of less than 2 ppm and isotope similarity of more than 80% was considered as criteria for reporting identity. A meta-library developed within Progen-
esis QI using the identified biomarkers was successfully used to identify the metabolites in shrimp samples during subsequent metabolomics experiments. A set of 34 biomarkers exclusive to the species identity of a shrimp samples were discovered following the workflow presented in supplementary material Figure S5. Presence of these exclusive biomarkers were further confirmed by analysing the individual raw data files in Masslynx 4.1 software and was further investigated in LC–MS/MS for developing a targeted method of shrimp authentication. Unfortunately identity of these exclusive biomarkers could not be established with reasonable confidence by accurate mass database search.

3.6. LC–MS/MS targeted method for shrimp authentication

During the development and assessment of LC–MS/MS targeted method 34 markers were initially evaluated for selectivity in the first run. Overall, 17 biomarkers were deemed species specific (Table 2). Due to lack of full chemical identification, all biomarkers were assigned arbitrary IDs stemming from the species names and a consecutive number in which they were analysed. For each of the analysed shrimps species at least one specific molecule was selected i.e. king prawn (KP1), tiger prawn (TP1, TP 4–7), Indian pink shrimp (IPS1 and 4), Indian white shrimp (IWS4, 5, 7, 11) and Argentinian red shrimp (ARS 5 and 6). Nevertheless, other, less specific markers were also included in the method due to relatively low cross-talk or to aid geographical origin elucidation i.e. KP2 and 3 for king prawn and TP3 for tiger prawn (Table 2).

The selective markers provided a species specific response in three assessment runs, whereby sample’s species was assigned only in the presence of all assigned markers transitions with compliant ion ratios. The LC–MS/MS method proved to be robust with no carry-over or sensitivity loss (QCs response RSD <10% for all markers, for 100 injection runs). Additionally, ion ratios were in the range of ±20% of the mean QCs value for the three analytical runs. Representative XIC of selected species specific markers have been presented in supplementary material Figure S6. To trial the assay with market place samples, three additional blind runs consisting of total 76 samples of king prawns, 10 samples of tiger prawns and 3 samples of each Argentinian red shrimp, Indian white shrimp, and Indian pink shrimp were performed. Species of the samples were correctly predicted for all the species assessed yielding 0% false positives and negatives rate.

Three markers for king (KP1, KP2, and KP3) and tiger prawns (TP3, TP4 and TP6) showed significant (p < 0.001) differences in response between geographical origin within the assessed species groups (Fig. 5a–f). The difference in relative response of the said markers based on geographical origin is a strong indication that it might be also possible to employ those markers for geographical origin authentication of shrimp on a unit resolution triple quadrupole mass spectrometer platform.

Over the years the sensitivity of high resolution mass spectrometers has improved tremendously, enabling detection of even low concentration compounds. However, a sizable number of recurrent molecular features in any metabolomics experiment remain unknown. Chromatographic isolation of these unknown components for identification is often not practical due to low abundance. Guessing the identity and then synthesizing the compound for confirmation might also fail and is an expensive affair [32]. Defining these recurrent unidentified metabolites with accurate mass and fragment ions/spectra is a practical solution to this problem. In this study we have demonstrated that a class specific recurrent unidentified biomarker can be used successfully to develop food authentication assays. The success of this approach was also demonstrated for detection of adulteration in oregano [33].

4. Conclusions

The level of fraud in fisheries globally is a huge issue. There are many measures in place and initiatives being developed to try and lessen the impact this has on the integrity of seafood that is a staple in the diet of billions of citizens around the world. In terms of the laboratory testing methods that support traceability systems, there are quite a number of these and while they are fit for purpose in terms of uncovering particular aspects of fraudulent practise they can only provide evidence that one particular form of malpractice may have occurred. Here we report a highly innovative approach using high resolution mass spectrometry and chemometrics that can distinguish the species identity and geographical origin of shrimp in a single metabolomics experiment. Recurrent species specific exclusive markers were identified from the unta-
## Table 2
Details of the LC–MS/MS method for the 18 markers employed in species and geographical origin elucidations. Where: “cone voltage for all the compounds was set to 20V,” markers deemed exclusive to the associated species.

| Species                  | Marker | Retention Time [min] | Accurate mass [m/z] | Molecular ion [m/z] | Transitions [m/z] | Dwell time [sec] | Collision Energy * [V] | Ion ratios |
|--------------------------|--------|----------------------|---------------------|--------------------|-----------------|------------------|----------------------|------------|
| King Prawn               | KP1*   | 1.73                 | 820.4384            | 820.5              | 398.2           | 0.063            | 40                    | 0.551      |
|                          | KP2    | 4.34                 | 292.1575            | 292.2              | 416.0           | 0.065            | 40                    | 0.0022     |
|                          | KP3    | 0.91                 | 310.1377            | 310.0              | 513.3           | 0.108            | 20                    | N/A        |
|                          | TP1*   | 3.18                 | 967.4512            | 967.5              | 615.0           | 0.016            | 40                    | 0.261      |
|                          | TP3    | 5.48                 | 518.3234            | 518.3              | 633.0           | 0.016            | 40                    | 0.212      |
| Tiger Prawn              | TP4*   | 2.96                 | 543.2995            | 543.3              | 131.0           | 0.108            | 20                    | 0.959      |
|                          | TP5*   | 2.13                 | 549.2211            | 549.2              | 235.3           | 0.237            | 20                    | N/A        |
|                          | TP6*   | 3.16                 | 726.1178            | 726.1              | 427.2           | 0.231            | 20                    | N/A        |
|                          | TP7*   | 3.53                 | 855.1091            | 855.1              | 247.2           | 0.044            | 20                    | N/A        |
| Indian Pink Shrimp       | IPS1*  | 2.22                 | 804.4489            | 804.4              | 638.2           | 0.016            | 40                    | 0.947      |
|                          | IPS4*  | 4.19                 | 864.965             | 864.9              | 499.0           | 0.044            | 40                    | 0.650      |
|                          | IWS4*  | 3.53                 | 569.3147            | 569.3              | 416.2           | 0.027            | 20                    | N/A        |
| Indian White Shrimp      | IWS5*  | 3.85                 | 599.0004            | 599.0              | 213.0           | 0.014            | 20                    | 0.605      |
|                          | IWS7*  | 3.09                 | 702.3815            | 702.4              | 240.2           | 0.027            | 20                    | 0.329      |
|                          | IWS11* | 3.11                 | 1016.9582           | 1017               | 268.9           | 0.016            | 20                    | 0.344      |
| Argentinian Red Shrimp   | ARS5*  | 3.40                 | 449.592             | 449.6              | 515.9           | 0.016            | 20                    | 0.258      |
|                          | ARS6*  | 3.67                 | 485.3513            | 485.3              | 666.1           | 0.016            | 20                    | 0.920      |
targeted metabolomics study and a LC–MS/MS assay was developed for rapid authentication of species identity of the shrimps. The assay was tested on unknown shrimp samples from the market and all the samples tested were accurately classified based on the species identity. Even though the identity of the recurrent exclusive markers could not be established, the markers were reproducible and the MSI requirement for reporting an unknown metabolite was satisfied. Mislabelling of species identity and geographical origin are the most prevalent forms of fraud in the seafood sector and cut across many issues about the economics around fraud. The need to ensure food being consumed around the world is not linked to the use of modern day slavery and child labour is of critical importance. While the presented method will not directly show if shrimps have been produced using such practises but can provide invaluable evidence that claims being made are false and may lead back to supply chains that use such abhorrent practises. The models developed have been shown to be very robust but can be considered as a first proof of principle. A wide range of species of shrimps originated from many regions are now required to build a unique database that can be used as a tool by industry and regulatory agencies to police the world shrimp trade.

Conflicts of interest

There are no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2019.04.001.

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