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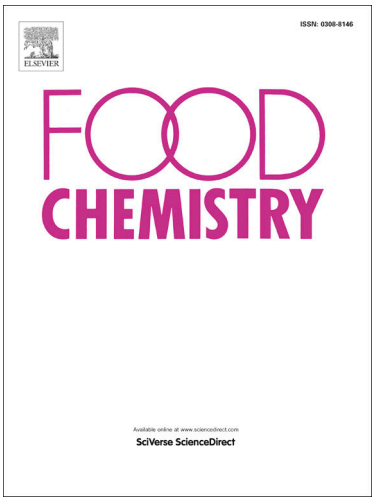

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Tracking the provenance of octopus using isotopic and multi-elemental analysis

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ABSTRACT

Octopus are an increasingly important seafood commodity, yet traceability techniques to validate the origins of octopus products are sorely lacking. For the first time, we investigate whether chemical profiling can identify geographical origins of octopus on international and domestic scales. Our samples consisted of wild-caught octopus from south-east Asia and southern Australia, regions with high seafood trade. We used a novel combination of stable carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotope analyses (Isotope-Ratio Mass Spectrometry) of internal calcified structures called statoliths, with elemental analyses (X-Ray Fluorescence using Itrax) of muscle tissue. We found that multivariate profiles had distinctive regional signatures, even across species, with high classification success (~95%) back to region of origin. This study validates isotopic and multi-elemental profiling as an effective provenance tool for octopus, which could be used to support transparency and accountability of seafood supply chains and thus encourage sustainable use of ocean resources.

Keywords: Geographical origin; wild resource; chemical profiling; traceability; seafood; octopus

1. INTRODUCTION

In a world of increasingly conscious consumers, demand is rising for seafood that is sustainable and ethical. However, the seafood industry has become an increasingly global marketplace, with lengthy, opaque supply chains, and with products undergoing multiple forms of processing and packaging before reaching a final destination (Iles, 2007). The volume and complexity of the seafood trade increases avenues for overexploitation driven by fraudulent and illegal activities (Fox, Mitchell, Dean, Elliott, & Campbell, 2018). Fraud is one of the highest risks to the seafood industry, with evidence of substitution of inferior products, product from unsustainable or illegal fisheries, presence of pathogens or banned substances such as some antibiotics, and links to human rights abuses. Seafood fraud can undermine the value of seafood products, diminish consumer confidence, and encourage unsustainable and unhealthy use of ocean resources. Tracing the provenance of seafood safeguards the industry from illegal and unsustainable activities by increasing the transparency and accountability of supply chains.

Chemical profiles within biological tissues can provide insights into geographical origins and are carried unaltered within the product itself across the supply chain, making them difficult to tamper with. These naturally-occurring chemical signatures, such as isotope ratios and elemental concentrations, can reflect regional conditions by being directly incorporated from the seawater, as mediated by environmental conditions such as temperature and salinity (Katerinopoulou, Kontogeorgos, Salmas, Patakas, & Ladavos, 2020), or from local diet signatures (Martino, Doubleday, & Gillanders, 2019). To date, chemical profiling is mostly undertaken on soft-tissues, including in fish (Camin, Perini, Bontempo, Galeotti, Tibaldi, & Piasentier, 2018; Gopi, Mazumder, Sammut, Saintilan, Crawford, & Gadd, 2019b), crustaceans (Luo, Jiang, Chen, Zheng, Liu, & Yang, 2019), bivalves (Zhang, Cheng, Han, Zhao, Chen, & Liu, 2019), and echinoderms (Kang et al., 2020). Chemical profiling of hard, biomineralised tissues can also useful for tracking the origins of marine animals. Biomineralised tissues are acellular and metabolically-inert, meaning chemical profiles of the environment are permanently trapped within the carbonate matrix (Arkhipkin, 2005; Campana, 1999). For example, stable oxygen isotopes ($\delta^{18}O$) in internal calcium carbonate ($CaCO_3$) structures, such as the ear stones (otoliths) of fish, have known and predictable relationships with environmental temperature and salinity, potentially revealing geographical information (Chung, Chen, Shiao, Lin, & Wang, 2020). Chemical profiling of biomineralised tissues has been extensively and successfully used to answer ecological questions (Martino, Doubleday, Fowler, & Gillanders, 2020), but to date, have had little application in the field of food provenance.

Octopus are an important fishery species in over 40 countries and are renowned for short lifespans, rapid growth, and ability to adapt to environmental changes (Sauer et al., 2019). Despite rising exploitation, many cephalopod species are increasing in global abundance, potentially due to high adaptability coupled with the release from predation and competition pressure due to the depletion of some traditional fishery stocks (Costello et al., 2016; Zoë A. Doubleday et al., 2016). Under climate change, the highly adaptable and resilient octopus are predicted to become an increasingly important fishery product (Zoë A Doubleday & Connell, 2018). Only a limited number of studies have investigated traceability of octopus using molecular techniques for species authentication (Espiñeira & Vieites, 2012; Maldini, Nonnis Marzano, Fortes, Papa, & Gandolfi, 2006) and chemical profiling for provenance has only recently investigated for other cephalopods, such as for cuttlefish (Varrà, Husáková, Patočka, Ghidini, & Zanardi, 2021). Octopus are an ideal test species for provenance studies as they are primarily site-attached with limited dispersion, compared to many migratory species, so chemical profiles in tissues reflect the environmental signatures of harvest locations (Semmens et al., 2007). Chemical proxies within octopus have only been explored for fishery applications, whereby elemental concentrations of stylets (a cartilaginous vestigial shell) were used to identify stock structure on a localised scale (Z. A. Doubleday, Pecl, Semmens, & Danyushevsky, 2008a, 2008b). Chemical profiling using fish otoliths has revolutionised our understanding of fish populations in ecology and fishery science (Campana, 1999; Campana & Thorrold, 2001), yet chemistry in the octopus ear stone equivalent (statoliths) has never before been analysed. Not only does chemical profiling of octopus have the potential to identify geographical origins, but accurate provenancing would improve common identification issues associated with octopus. Unlike many fish and shellfish species, octopus are hard to visually identify and are principally sold under the broad label '*Octopus spp'*. Provenance labelling would provide more information for the consumer to make sustainable and ethical choices.

Our overarching aims were to investigate whether chemical profiling can determine geographical provenance of octopus on international and domestic scales and test the efficacy of new traceability approach by combining isotopic analysis of statoliths with elemental analyses in soft-tissues. Commercially harvested octopus were sourced from southern Australia, representing the principle octopus harvesting grounds and fisheries in Australia, and south-east Asia, where there is a high degree of both import and export trade with Australia. The chosen spatial scale is relevant to the octopus trade industry in these regions, so this study is directly applicable to local seafood fraud and traceability questions. Our specific objectives were: 1) test regional- or species-specific influences on chemical profiles by comparing between two species caught from the same location and the same species from

multiple locations; 2) compare the two analytical methods to measure tissue chemistry; and 3) determine whether variation in chemical profiles can be used to accurately predict provenance of octopus on international and domestic scales.

2. MATERIALS AND METHODS

2.1 Sample collection and preparation

Octopus samples from South Australia were collected from research fishing operations in August 2019, as part of a previous study (Martino, Steer, & Doubleday, 2021). As such, their origins were verified and known. *Octopus berrima* were collected from Port Lincoln (*n* = 11) in South Australia (Figure 1) and *Octopus pallidus* were collected from Port Lincoln in South Australia (*n* = 10). All other octopus samples were collect from seafood markets in July - August 2019, with *Octopus pallidus* sourced from Victoria (*n* = 8) and Tasmania (*n* = 9); while *Amphioctopus aegina* were sourced from Vietnam (*n* = 11) and Indonesia (*n* = 9). While market samples do not have exact known origins, species distributions and known information on fisheries in listed countries are consistent with product labels (Reid, 2016; Sauer et al., 2019), and are suitable for our current purpose of differentiating between samples on an international-scale.

All octopus samples were frozen and transported to Adelaide at the Future Industries Institute, University of South Australia. Octopus were thawed, measured, weighed, and sexed. Muscle tissue samples were then taken from the third right or left arms, rinsed in Milli-q water, stored in individual vials and frozen. Ear stones (statoliths) were dissected, cleaned of adhering tissue, rinsed in Milli-q water, air-dried overnight in a laminar flow cupboard and stored until processing.

2.2 Elemental and isotopic analysis

Muscle tissues were analysed using X-ray Fluorescence (XRF) spectrometry on the Itrax high-resolution core scanner at Australia's Nuclear Science and Technology Organisation (ANSTO), in Sydney, New South Wales. Itrax determined the relative abundance of 26 different elements within sample material including Aluminium (Al), Silicon (Si), Phosphorus (P), Sulphur (S), Chlorine (Cl), Potassium (K), Calcium (Ca), Titanium (Ti), Vanadium (V), Chromium (Cr), Manganese (Mn), Iron (Fe), Nickel (Ni), Copper (Cu), Zinc (Zn), Arsenic (As), Bromine (Br), Rubidium (Rb), Strontium (Sr), Yttrium (Y), Zirconium (Zr), Antimony (Sb), Barium (Ba), Cerium (Ce), Samarium (Sm) and Lead (Pb) (Gadd, Gopi, Sammut, Saintilan, Crawford, & Mazumder, 2018). To prepare the thawed tissues for Itrax, the octopus arms were sliced in half longitudinally using a sharp blade. Samples were then placed in a line on Perspex sample holder with muscle tissue facing upwards, covered in a thin plastic layer, and flattened.

Opaque masking tape was used to detect beginning and end of run. Seven to ten samples were analysed per run. The Molybdenum target tube was used to continuously scan the samples at 1mm intervals with 30 s exposure time. A55 mA current and 30 kV voltage was used. A third of the samples were reran to assess precision and accuracy. The output spectra were fit to the model spectra using Q-Spec 8.6.0, which also accounted for elemental inferences and sum peaks.

Stable carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotope ratios in statoliths were analysed at the Biogeochemistry and Stable Isotope Facility at the University of Adelaide. Statoliths were crushed and approximately 100ug of powder was precisely weighed and transferred to sterilised glass vials. Samples were analysed using a NuCarb carbonate preparation system (Nu Instruments) in-line with a Perspective isotope ratio mass spectrometer (IRMS) equipped with a dual inlet. Each sample vial was individually evacuated to high vacuum in the autosampler (heated to 70°C) and 130 µL of 105% phosphoric acid was then automatically dispensed to digest the sample. The resultant CO_2 was then analysed for $\delta^{18}O$ and $\delta^{13}C$ composition using the dual inlet and IRMS. Isotope values were reported relative to Vienna Pee Dee Belemnite (VPDB) and expressed in standard delta (δ) parts per thousand (‰):

$$\delta = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) x\ 1000\ (‰) \qquad \text{(Equation 1)}$$

where R is the ratio of ^{13}C:^{12}C or ^{18}O:^{16}O. Data quality was monitored by bracketing samples with laboratory standards ANU-P3 ($\delta^{18}O$ = -0.32 ‰, $\delta^{13}C$ = + 2.24 ‰), UAC-1 ($\delta^{18}O$ = -18.4 ‰, $\delta^{13}C$ = -15.0 ‰) and IAEA CO-8 ($\delta^{18}O$ = -22.7 ‰, $\delta^{13}C$ = -5.76 ‰), and external reproducibility was ±0.023‰ and ±0.026‰ (1SD) for $\delta^{13}C$ and $\delta^{18}O$ respectively.

2.3 Statistical analysis

Single factor permutational analysis of variance PERMANOVA (Primer v.7) was used to assess whether individual isotopes or elements were significantly different between regions (Anderson, 2001). PERMANOVA is non-parametric and non-sensitive to heteroscedasticity. Non-transformed data were normalised and converted to Euclidean distance matrices with analyses performed using unrestricted permutations of the data with 9999 repeats. Region was the single fixed factor. Where significant differences were detected, *post hoc* pairwise comparisons were performed to identify between which regions the group means were significantly different.

A stepwise discriminant function analysis (DFA) was performed to determine which isotopes and elements were most important in discriminating between regions. Analysis was performed using the SPSS Statistics software package Version 26 (www.ibm.com/software/au/analytics/spss/products/statistics/). Variables were entered into the model using a stepwise forward method, using Wilk's Lambda and F-statistic probabilities to determine sequence of variable addition and evaluate model improvement, respectively. Identified isotopes and elements were then combined into a multivariate dataset. Using similar steps to the univariate PERMANOVA analyses, the multivariate dataset was analysed via a permutational MANOVA to assess whether combined chemistry was significantly different between regions. Canonical analysis of principle coordinates (CAP), using a leave-one-out data fitting approach, was used to assess spatial discrimination among sampling sites and generate classification statistics. *O. berrima* and *O. pallidus* from South Australia were initially included as separate groups to investigate species-specific variation. CAP analysis was then rerun to assess species influence on classification success back to region.

3. RESULTS

Oxygen isotopes ($\delta^{18}O$) in statoliths significantly decreased from lower latitudes near the equator to higher latitudes in southern Australia (Figure 2; Table S2). Carbon isotopes ($\delta^{13}C$) in statoliths showed a similar pattern, except for the values from the Vietnamese samples (Figure 2). For the elemental abundances in the muscle tissues, Australian octopus were comparatively higher in S, K, As, Br and Sb than south-east Asian octopus, and significant differences between regions were observed in abundances of 14 of the 26 elements analysed (P, S, Cl, K, Ca, Cr, Fe, Zn, Br, As, Br, Sb, Ba, Ce) (Figure 2; Table S2). No significant differences between regions were observed for the remaining elements (Al, Si, Ti, V, Mn, Ni, Cu, Rb, Sr, Y, Zr, Sm, Pb).

Stepwise discriminant function analysis suggested that $\delta^{18}O$ and $\delta^{13}C$ isotope ratios from statoliths, in addition to As, Zn, Cl, Ca, K, Sb, S, and Br from muscle tissues, was the best combination of chemical markers to discriminate between regions. Based on this reduced multivariate dataset, significant differences were detected between regions (Table S2). The CAP analysis further indicated that regional differences in multi-chemical signatures were largely driven by variation in $\delta^{18}O$ and $\delta^{13}C$ followed by Br, K and As (Figure 3). The two octopus species, *Octopus berrima* and *Octopus pallidus,* from South Australia displayed similar multi-chemical profiles. In contrast, *O. pallidus* exhibited different profiles between South Australia, Victoria and Tasmania, and *Amphioctopus aegina* displayed different profiles between Indonesia and Vietnam. Classification of octopus back to their region of origin was high at a total of 94.7%, with 54 out of 57 samples correctly classified (Table 1). The three samples incorrectly classified were either between the two South Australia species or between octopus from South Australia and Victoria. When the two species from South Australia were merged and classification statistics reran, classification success remained at 94.7% of samples correctly classified (Table S3).

4. DISCUSSION

Chemical profiling displayed exciting potential for tracking provenance of octopus. For the first time, a novel combination of $\delta^{13}C$ and $\delta^{18}O$ in statoliths combined with elemental signatures from soft-tissues were shown to accurately identify origins of octopus on international and domestic scales. Additionally, chemical profiles were primarily influenced by regional variation across a latitudinal gradient, rather than species-specific variation, supporting environmental conditions being the dominant drivers of chemical signatures in chosen tissues.

Chemical profiles, comprised of isotopic signatures in statoliths combined with elemental signatures in muscle tissues, were shown to be effectively identify geographical origin, with distinctive separation by region and high classification success (~95%). To our knowledge, this is the first study to analyse chemistry in octopus statoliths and demonstrate their potential as retrospective environmental tracers. The octopus in this study were harvested from regions of contrasting environmental conditions, having been collected from three colder temperate regions in southern Australia and two tropical south-east Asian regions closer to the equator. These divergent environmental conditions produced distinctive chemical profiles within the biological tissues of the octopus. While the three southern Australian regions are comparatively similar in latitude and environmental conditions, differences in chemistry were evident even on this domestic scale. A clear decreasing trend from higher to lower latitudes was observed in both $\delta^{13}C$ and $\delta^{18}O$ in statoliths. These $\delta^{18}O$ gradients align with well-established theory that $\delta^{18}O$ in seawater decreases towards the poles (Schmidt, R., & J., 1999). The trends in statoliths arise as $\delta^{18}O$ in seawater is mediated by salinity and then incorporated into internal calcium carbonate structures of marine animals in a predictable linear relationship with temperature (Chung et al., 2020). A latitudinal gradient of $\delta^{18}O$ in calcified structures has also been observed in other taxa such as brachiopods (Brand et al., 2013) and fish (Stephenson, Edmons, Moran, & Caputi, 2001). The trends in $\delta^{13}C$ observed likely reflects the regional specific dissolved inorganic carbon (DIC) of the ocean waters (Landman, Cochran, Cerrato, Mak, Roper, & Lu, 2004), although there may a minor physiological or diet influence (Chung, Trueman, Godiksen, & Grønkjær, 2019; Martino, Doubleday, Chung, & Gillanders, 2020). The relatively consistent relationships of $\delta^{13}C$ and $\delta^{18}O$ in the ocean combined predictable fractionation trends into carbonate tissues suggests the potential to create isotopic maps (“isoscapes”) of predicted values to be used for geolocation of marine animals and seafood. While ocean isoscapes are being routinely developed and applied in the northern hemisphere (Trueman & St John Glew, 2019), they are more limited in the southern hemisphere (Pearson, van de Merwe, Gagan, Limpus, &

Connolly, 2019), suggesting untapped potential here to inform ecology and seafood provenance.

While many isotopic traceability studies focus on $\delta^{13}C$ and nitrogen isotopes ($\delta^{15}N$) in soft-tissues (Kim, Kumar, & Shin, 2015; Zhang et al., 2019), here we show the value of using and applying $\delta^{13}C$ and $\delta^{18}O$ in biomineralised tissues. Whereas $\delta^{13}C$ and $\delta^{15}N$ in soft-tissues are incorporated from diet and provide region-specific diet signatures, $\delta^{18}O$ and $\delta^{13}C$ are primarily incorporated from the seawater and contribute parallel environmental information that can be used as a complementary provenance technique. Additionally, while some variability in the elemental trends were seen here, our results show that combining multiple elemental markers, particularly coupled with isotopic signatures, is highly effective at separating out geographical origins of seafood.

Regional-specific chemical signatures (related to localised environment or diet) were shown to be more dominant than species-specific signatures (related to uptake processes and physiology). In this study, we compared chemical signatures between two species caught from the same location and the same species from multiple locations. The two species, *Octopus berrima* and *Octopus pallidus* from South Australia showed distinctively similar chemical profiles. In contrast, *O. pallidus* from South Australia, Victoria, and Tasmania displayed distinctively different chemical profiles, and so too did *Amphioctopus aegina* from Indonesia and Vietnam. Species-specific variation may influence uptake processes of isotopic or elemental incorporation but overall, our trends indicate that these species-specific physiological effects had a relatively minor influence on chemical signatures compared to regional-specific environmental or diet effects. The absence of species-specific variation is relevant for octopus as it is often sold as '*Octopus spp.*' Or 'baby octopus' in markets, rather than under species names. For most commercially-harvest octopus species, there are difficulties with visually identifying octopus to species, and taxonomic and phylogenetic relationships are poorly described (Reid, 2016; Van Nieuwenhove, Ratsimbazafy, & Kochzius, 2019). Our results suggest that even when species is unclear or unknown for market octopus, chemical markers still act as effective tracer of geographic origins.

Here we show how octopus can be successfully classified back to their region of origin, on both domestic and international scales, using a novel combination of chemical profiling methods. The methods developed herein could be useful for a range of industry and trade needs. For example, larger, spatially complex fisheries may want to analyse a wide range of harvest locations across a coastline, while other seafood businesses may simply be interested in identifying if a product is imported or exported. The specific application will influence the

required sample size and spatial spread of sampling locations. Octopus fisheries in Australia, for example, are primarily artisanal, but with a high degree of international and domestic trade within the Australasian region. Consequently, the regional scale that this study was conducted was therefore relevant in this example. Burdensome sample sizes may not be necessary for traceability studies depending on the statistical approach, as shown by this study and others with only a handful of samples (<10) needed to characterise isotope or elemental signatures of local populations (Mazumder, Williams, Reid, Saintilan, & Szymczak, 2008) or distinguish the origin and production method of marine invertebrates (Gopi, Mazumder, Sammut, Saintilan, Crawford, & Gadd, 2019a). Although, repeat sampling may be needed in some instances to develop a reference database and account for temporal variability in environmental signatures. Overall, the potential of the presented provenance technique is wide-ranging and could handle both larger-scale (international) or localised traceability. This study validates a tool that could be wielded by aggregators or retailers to validate provenance of their product and satisfy certification requirements. Alternatively, it could be used by regulators to identify substituted and falsely labelled products to discourage fraudulent activity in the supply chains.

4.1 Conclusions

This study showed that chemical profiling is an effective provenance tool on both domestic and international scales for an important and highly-traded seafood product. As both interest in octopus fisheries and abundance of wild octopus increase, they are likely to grow as an important fishery resource. However, octopus can be particularly vulnerable to exploitation and localised depletion due to limited dispersal and non-overlapping generations. Chemical profiling could be developed as a widely-used traceability technique, even across multiple species, for assuring that only sustainable and well-managed populations of octopus are harvested and sold.

Seafood is the most traded food commodity in the world and at high risk to fraud. Effective provenance technology empowers the seafood industry to identify fraudulent activities and increase the transparency and accountability of supply chains. Accurate provenance supports the branding and reputation of high-value, sustainable seafood products, increases consumer confidence, and ultimately encourages the sustainable and healthy use of ocean resources.

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DATA AVAILABILITY - The data are available from the corresponding author, JM, upon reasonable request.

AUTHOR CONTRIBUTIONS - Conceptualization: J.M., and Z.D.; Methodology: J.M., D.M., P.G., and Z.D.; Formal Analysis: J.M., and P.G.; Investigation: J.M.; Resources: J.M., D.M., P.G., and Z.D.; Writing – Original Draft: J.M.; Writing – Review & Editing: : J.M., D.M., P.G., and Z.D.; Visualization: J.M.; Supervision: Z.D.; Project Administration: J.M.; Funding Acquisition: J.M., and Z.D.

DECLARATION OF INTERESTS - The authors declare no competing interests.

TABLES

Table 1 - Leave-one-out classification success for the regional comparison of combined tissue chemistry of octopus. Data represents the percentage of individuals from region (rows) classified to each predicted region (columns). Bold values are correctly classified.

	Predict. Region	Tasmania	Victoria	South Australia	South Australia	Vietnam	Indonesia
Orig. Region	**Species**	*O. pallidus*	*O. pallidus*	*O. berrima*	*O. pallidus*	*A. aegina*	*A. aegina*
Tasmania	*O. pallidus*	**100**	0	0	0	0	0
Victoria	*O. pallidus*	0	**87.5**	12.5	0	0	0
South Australia	*O. berrima*	0	12.5	**81**	0	0	0
South Australia	*O. pallidus*	0	0	9	**100**	0	0
Vietnam	*A. aegina*	0	0	0	0	**100**	0
Indonesia	*A. aegina*	0	0	0	0	0	**100**

FIGURES

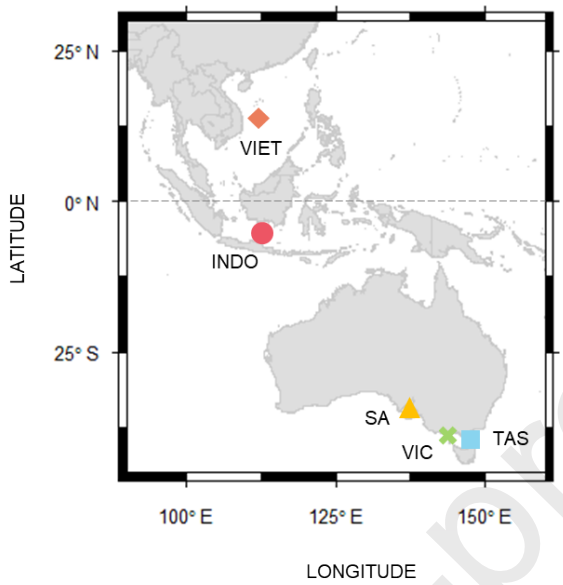

Figure 1 – Region of origin of octopus samples from Indo-Pacific Asia and Australasia: Vietnam (VIET), Indonesia (INDO), South Australia (SA), Victoria (VIC) and Tasmania (TAS) used in geographical provenance. SA samples are known-origin and were collected by the lead author from a previous study (Martino, Steer, & Doubleday, 2021). All other samples are indicated for illustrated purposes and were collected from fish markets, with indicated icons representing predicted fishing location from information known about species characteristics and local octopus fisheries (see methods for more details).

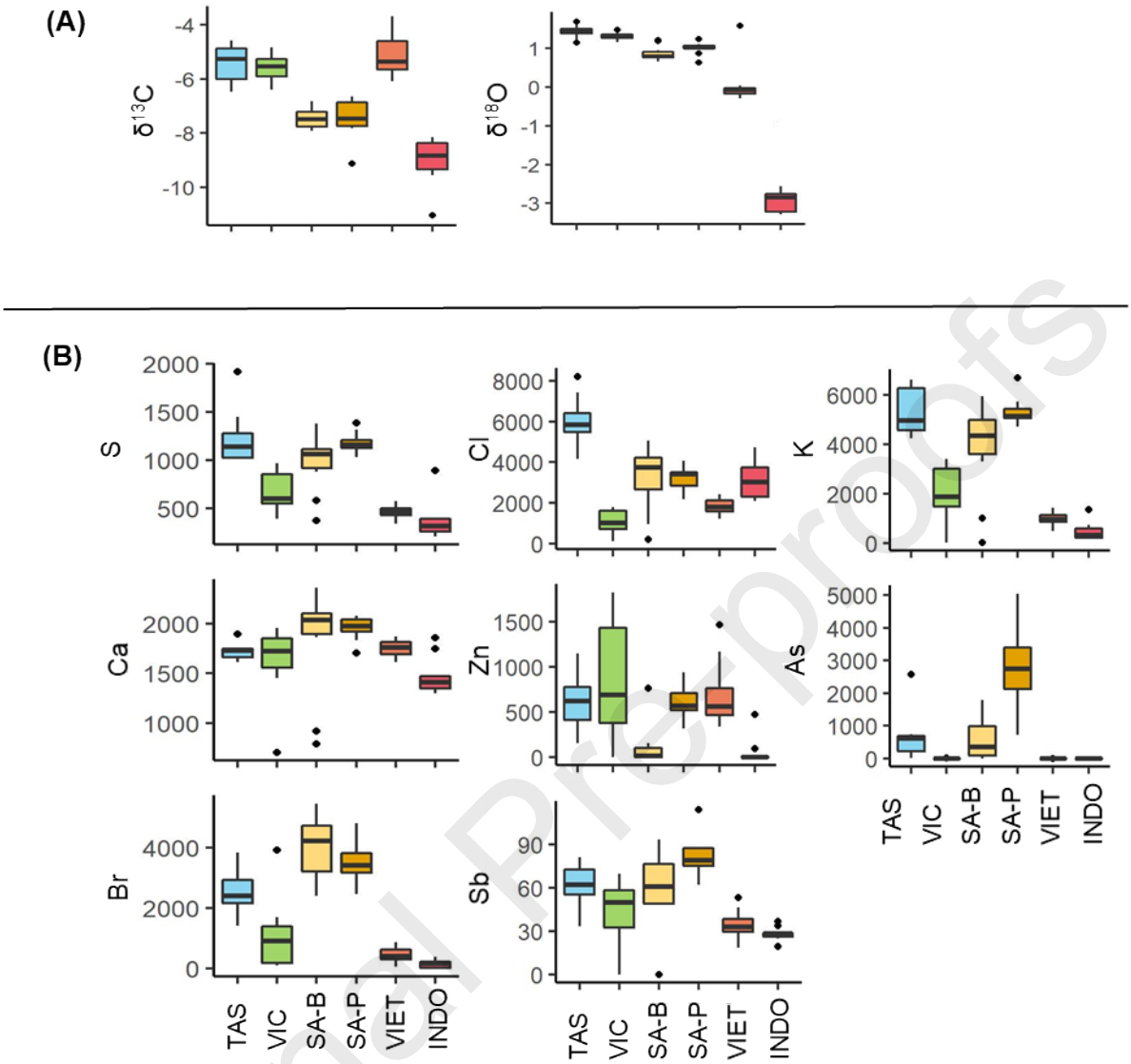

Figure 2 – Boxplots of (A) carbon ($\delta^{13}C$) and oxygen isotopes ($\delta^{18}O$) in octopus statoliths and (B) elemental abundances of Sulphur (S), Chlorine (Cl), Potassium (K), Calcium (Ca), Chromium (Cr), Zinc (Zn), Arsenic (As), Bromine (Br), and Antimony (Sb) in muscle tissue. The regions are arranged from high to low latitude and include Tasmania (TAS), Victoria (VIC), South Australia (SA), Vietnam (VIET), and Indonesia (INDO). South Australian samples includes two species – *Octopus berrima* (SA-B) and *Octopus Pallidus* (SA-P) – to elucidate species-specific effects.

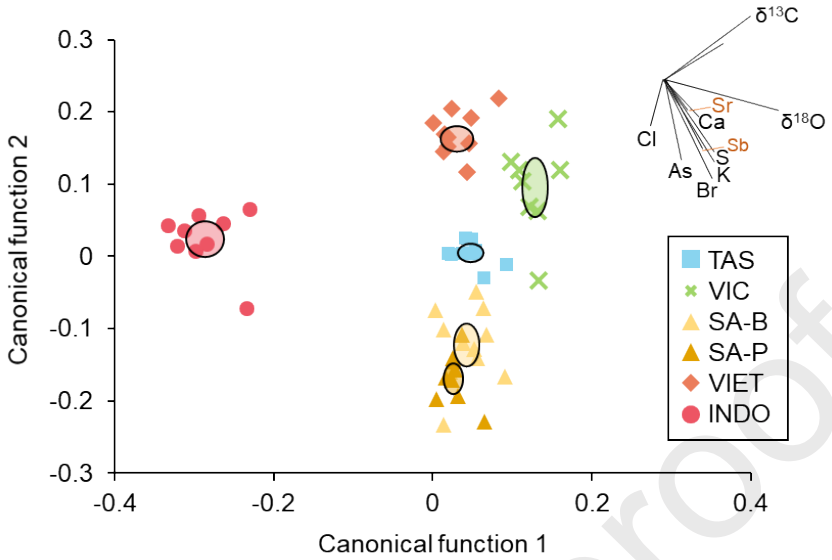

Figure 3 - Canonical analysis of principle coordinates (CAP) plot showing variation in chemical signatures in octopus from Tasmania (TAS), Victoria (VIC), South Australia (SA), Vietnam (VIET) and Indonesia (INDO). South Australian samples includes two species – *Octopus berrima* (SA-B) and *Octopus Pallidus* (SA-P) - to elucidate species-specific effects. Ellipses represent 95% confidence intervals around group means while vector diagram shows the direction and weight of individual chemical markers.

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Tracking the provenance of octopus using isotopic and multi-elemental analysis

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Highlights:

- Provenance and traceability tools can support long-term seafood security

- Traceability techniques have never been tested for octopus.
- A novel combination of isotopes in statoliths and elements in muscle were analysed
- Chemical profiling accurately identified origins of octopus, even across species
- This technique can support accountability and sustainability of seafood

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