Peer review history for:

Upton C, Prinsloo G, Steenkamp P, Okpeku M. Comparative metabolomic analyses of *Holothuria cinerascens* and *Pseudocnella sykion*. S Afr J Sci. 2026;122(1/2), Art. #21964. https://doi.org/10.17159/sajs.2026/21964

HOW TO CITE:
Comparative metabolomic analyses of *Holothuria cinerascens* and *Pseudocnella sykion* [peer review history]. S Afr J Sci. 2026;122(1/2), Art. #21964. https://doi.org/10.17159/sajs.2026/21964/peerreview

The original manuscript for review is appended below.

Reviewer 1: Round 1
Date completed: 31 July 2025
Conflicts of interest: None

Recommendation: Accept / Revisions required / Resubmit for review / Resubmit elsewhere / Decline / See comments
REVIEWER: Accept
Does the manuscript fall within the scope of SAJS? Yes/No
REVIEWER: Yes
Is the manuscript written in a style suitable for a non-specialist and is it of wider interest than to specialists alone? Yes/No
REVIEWER: Yes
Does the manuscript contain sufficient novel and significant information to justify publication? Yes/No
REVIEWER: Yes
Do the Title and Abstract clearly and accurately reflect the content of the manuscript? Yes/No
REVIEWER: Yes
Is the research problem significant and concisely stated? Yes/No
REVIEWER: Yes
Are the methods described comprehensively? Yes/No
REVIEWER: Yes
Is the statistical treatment appropriate? Yes/No/Not applicable/Not qualified to judge
REVIEWER: Yes
Are the interpretations and conclusions justified by the research results? Yes/Partly/No
REVIEWER: Yes
Please rate the manuscript on overall contribution to the field Excellent/Good/Average/Below average/Poor
REVIEWER: Good

Please rate the manuscript on language, grammar and tone Excellent/Good/Average/Below average/Poor	
REVIEWER: Excellent	
Is the manuscript succinct and free of repetition and redundancies? Yes/No	
REVIEWER: No	
The number of tables in the manuscript is Too few/Adequate/Too many/Not applicable	
REVIEWER: Adequate	
The number of figures in the manuscript is Too few/Adequate/Too many/Not applicable	
REVIEWER: Adequate	
Are the results and discussion confined to relevance to the objective(s)? Yes/No	
REVIEWER: Yes	
Is the supplementary material relevant and separated appropriately from the main document? Yes/No/Not applicable	
REVIEWER: Yes	
Please rate the manuscript on overall quality Excellent/Good/Average/Below average/Poor	
REVIEWER: Excellent	
Is appropriate and adequate reference made to other work in the field? Yes/No	
REVIEWER: Yes	
Is it stated that ethical approval was granted by an institutional ethics committee for studies involving human subjects and non-human vertebrates? Yes/No/Not applicable	
REVIEWER: Yes	
If accepted, would you recommend that the article receives priority publication? Yes/No	
REVIEWER: Yes	
Are you willing to review a revision of this manuscript? Yes/No	
REVIEWER: Yes	
With regard to our policy on 'Publishing peer review reports', do you give us permission to publish your anonymised peer review report alongside the authors' response, as a supplementary file to the published article? Publication is voluntary and only with permission from both yourself and the author. Yes/No	
REVIEWER: Yes	
Comments to the Author: This manuscript presents novel work, is well-written, and is relevant to the South African scientific community. Enough detail is provided in the methods and results for non-metabolomics experts to follow, and the interpretation was done with sufficient depth. There are a few areas which may be improved to increase the overall clarity and conciseness, especially for a general readership, these have been highlighted in the relevant sections below. Overall, the results and discussion might benefit from some consolidation to improve conciseness. While this is something that is likely not in the authors' power now, this type of study would be strengthened by having a commercially used species as a control group, in order to better understand the unique or novel aspects of these selected species. This may be mentioned as a direction for future studies. **General** This manuscript presents novel work, is well-written, and is relevant to the South African scientific	

community. Enough detail is provided in the methods and results for non- metabolomics experts to follow, and the interpretation was done with sufficient depth. There are a few areas which may be improved to increase the overall clarity and conciseness, especially for a general readership, these have been highlighted in the relevant sections below. Overall, the results and discussion might benefit from some consolidation to improve conciseness. While this is something that is likely not in the authors' power now, this type of study would be strengthened by having a commercially used species as a control group, in order to better understand the unique or novel aspects of these selected species. This may be mentioned as a direction for future studies.

By section: Introduction
While the problem this study aimed to address is clearly laid out, it would be useful for those not familiar with sea cucumbers or related organisms, to mention the species typically used for commercial purposes (L37-50), and explain the known or expected physiological/metabolic differences from the of the unique South African species that are the topic of this paper. E.g. An assessment of the known beneficial metabolites or attributes (if this exists in literature) of currently used species in relation to the expected differences in these species from SA would also assist the reader in determining the potential importance of these results.
Sample collection and preparation

L105-107: It is mentioned that the samples were frozen after collection, then cleaned and dissected, and frozen again before freeze-drying and storage at -80°C. Does this mean that the samples were thawed for cleaning and dissection, or do these processes happen while frozen? If thawed, handled, and refrozen, the metabolic profiles measured may no longer represent an accurate snapshot of the *in situ* metabolic conditions of the organism. As the topic of best practice for metabolic quenching is still debated, this is not meant to be a critique of the route chosen, but rather a request to explain the rationale behind it.

In relation to the sampling, and the observed metabolic heterogeneity mentioned in L176, it might be useful to explain whether there was any selection of particular areas of sea cucumber body tissues for analysis (e.g. body wall or specific organs), or if the entire organism was freeze-dried and homogenized, especially for those not familiar with the anatomy of these organisms. As the organisms have distinct organs, each of these will have different metabolic profiles.

NMR analysis
L124-127: There is inconsistency in capitalization in the full names of the PCA and OPLS-DA acronyms. Either all parts of the name must be capitalized, or none (the latter is more often
done in literature).

Results
L173: Why the 'X' in "PCA-X"?
L238-243 and 250-255: The details of the regions associated with each species may be better suited to inclusion in supplementary information, especially as this is also shown visually in figures 3 and 4 (especially given the general rather than metabolomics-expert readership).

Compound annotation
L281: What is meant by 'metabolic potential'? A greater range of metabolites? More metabolites overall? This is also used in the discussion, so an explanation of the term would be useful.

Discussion
As mentioned in the general comments, the overall messages of this manuscript may come across clearer if the writing were to be made more concise. There is some repetition of ideas, especially in the last paragraph of the discussion and the conclusion — some of this may be merged.

Author response to Reviewer 1: Round 1

This manuscript presents novel work, is well-written, and is relevant to the South African scientific community. Enough detail is provided in the methods and results for non-metabolomics experts to follow, and the interpretation was done with sufficient depth. There are a few areas which may be improved to increase the overall clarity and conciseness, especially for a general readership, these have been highlighted in the relevant sections below. Overall, the results and discussion might benefit from some consolidation to improve conciseness. While this is something that is likely not in the authors' power now, this type of study would be strengthened by having a commercially used species as a control group, in order to better understand the unique or novel aspects of these selected species. This may be mentioned as a direction for future studies.

While this is something that is likely not in the authors' power now, this type of study would be strengthened by having a commercially used species as a control group, in order to better understand the unique or novel aspects of these selected species. This may be mentioned as a direction for future studies.

AUTHOR: Thank you for your comment. We have revised the manuscript to include this in the conclusions and directions for future studies (Line 339-344).

Introduction

While the problem this study aimed to address is clearly laid out, it would be useful for those not familiar with sea cucumbers or related organisms, to mention the species typically used for commercial purposes (L37-50), and explain the known or expected physiological/metabolic differences from the of the unique South African species that are the topic of this paper. E.g. An assessment of the known beneficial metabolites or attributes (if this exists in literature) of currently used species in relation to the expected differences in these species from SA would also assist the reader in determining the potential importance of these results.

AUTHOR: Thank you for your comment. While we are limited by the word count and an editorial request to reduce length, we have incorporated references to commonly reported compounds in sea cucumbers and their associated beneficial properties (Lines 32–36). We also highlight some of the typically overexploited or commercialised species (Lines 7, 43–44). Given the untargeted nature of this study, our aim was not to predefine specific compounds but rather to establish a baseline metabolite profile of the investigated species for comparative purposes. Nonetheless, the text includes discussion of compounds and bioactivities commonly associated with sea cucumbers (Lines 32–36, 320-324, 328-329), as well as relevant physiological mechanisms of the species studied (Lines 60–66, 74–77, 187–193, 305-317). However, we would like to emphasise that due to the lack of scientific attention on the species investigated in this study, there is very limited information regarding their biology aside from basic morphological descriptions–particularly *P. sykion*, which is endemic to Southern Africa. This study therefore provides a valuable first step toward addressing this gap and sets the foundation for future targeted investigations.

Sample Collection and Preparation

L105-107: It is mentioned that the samples were frozen after collection, then cleaned and dissected, and frozen again before freeze-drying and storage at -80°C. Does this mean that the samples were thawed for cleaning and dissection, or do these processes happen while frozen? If thawed, handled, and refrozen, the metabolic profiles measured may no longer represent an accurate snapshot of the in situ metabolic conditions of the organism. As the topic of best practice for metabolic quenching is still debated, this is not meant to be a critique of the route chosen, but rather a request to explain the rationale behind it.

AUTHOR: Thank you for your comment. We fully recognise the importance of metabolic quenching in preserving metabolite integrity for metabolomics studies. In our workflow, samples were initially frozen to avoid the need to dissect live organisms. Dissection was then performed as rapidly as possible while the specimens remained partially frozen, thereby minimising metabolic activity and potential degradation. Following dissection, samples were snap-frozen in liquid nitrogen, then freeze-dried and processed with care to avoid repeated freeze–thaw cycles. Although ethical approval was not required for sea cucumbers at the time of this study, as they are lower-order marine invertebrates, this approach was selected to balance ethical considerations with the need to preserve the metabolic state of the specimens as much as possible.

Sample Collection and Preparation In relation to the sampling, and the observed metabolic heterogeneity mentioned in L176, it might be useful to explain whether there was any selection of particular areas of sea cucumber body tissues for analysis (e.g. body wall or specific organs), or if the entire organism was freeze-dried and homogenized, especially for those not familiar with the anatomy of these organisms. As the organisms have distinct organs, each of these will have different metabolic profiles.
AUTHOR: Thank you for your comment. We agree that distinct tissues may exhibit tissue-specific metabolic profiles. However, the aim of this study was to perform an overall comparative metabolomics analysis between *H. cinerascens* and *P. sykion*, rather than to investigate intra-organism variation. Accordingly, no within- or between-tissue comparisons were undertaken.
NMR Analysis L124-127: There is inconsistency in capitalization in the full names of the PCA and OPLS-DA acronyms. Either all parts of the name must be capitalized, or none (the latter is more often done in literature).
AUTHOR: Thank you for your comment. We have ensured that all appearances of PCA-X and OPLS-DA are consistently formatted in uppercase throughout the manuscript (Lines 126, 128, 131, 172, 179, 185, 245, Figure 1 heading, and Supplementary Figure 1 heading), in keeping with common practice in the metabolomics literature.
Results L173: Why the 'X' in "PCA-X"?
AUTHOR: Thank you for your comment. PCA-X refers to the model type used in the SIMCA software, where principal component analysis is performed on the data X-matrix. PCA-X is not a new type of PCA, but a specific application used to visualize sample clustering along principal components, with groups distinguished by colours or labels.
L238-243 and 250-255: The details of the regions associated with each species may be better suited to inclusion in supplementary information, especially as this is also shown visually in figures 3 and 4 (especially given the general rather than metabolomics-expert readership).
AUTHOR: Thank you for your comment. The results of the contribution loadings plots, detailing the regions associated with each species, have now been moved to the supplementary information as Supplementary Table 1 and has been referenced in the respective locations in the manuscript (Lines 221 and 225).
Compound Annotation L281: What is meant by 'metabolic potential'? A greater range of metabolites? More metabolites overall? This is also used in the discussion, so an explanation of the term would be useful.
AUTHOR: Thank you for your comment. In our manuscript, the term "metabolic potential" refers to the capacity of *H. cinerascens* and *P. sykion* to produce beneficial metabolites with ecological significance and potential applications in natural product discovery, and that may also inform the enhancement and expansion of sustainable aquaculture practices. We have revised the manuscript to more clearly represent this (Lines 258-260, 271-272, 327-329).
Discussion As mentioned in the general comments, the overall messages of this manuscript may come across clearer if the writing were to be made more concise. There is some repetition of ideas, especially in the last paragraph of the discussion and the conclusion — some of this may be merged.
AUTHOR: Thank you for your comment. We have revised the manuscript to remove repetitive statements and merge overlapping ideas, particularly in discussion and the conclusion.

Reviewer 2: Round 1
Date completed: 27 June 2025
Conflicts of interest: None

Recommendation: Accept / Revisions required / Resubmit for review / Resubmit elsewhere / Decline / See comments
REVIEWER: Revisions required

Does the manuscript fall within the scope of SAJS? Yes/No
REVIEWER: Yes
Is the manuscript written in a style suitable for a non-specialist and is it of wider interest than to specialists alone? Yes/No
REVIEWER: Yes
Does the manuscript contain sufficient novel and significant information to justify publication? Yes/No
REVIEWER: Yes
Do the Title and Abstract clearly and accurately reflect the content of the manuscript? Yes/No
REVIEWER: Yes
Is the research problem significant and concisely stated? Yes/No
REVIEWER: Yes
Are the methods described comprehensively? Yes/No
REVIEWER: Yes
Is the statistical treatment appropriate? Yes/No/Not applicable/Not qualified to judge
REVIEWER: Yes
Are the interpretations and conclusions justified by the research results? Yes/Partly/No
REVIEWER: Partly
Please rate the manuscript on overall contribution to the field Excellent/Good/Average/Below average/Poor
REVIEWER: Good
Please rate the manuscript on language, grammar and tone Excellent/Good/Average/Below average/Poor
REVIEWER: Good
Is the manuscript succinct and free of repetition and redundancies? Yes/No
REVIEWER: Yes
The number of tables in the manuscript is Too few/Adequate/Too many/Not applicable
REVIEWER: Adequate
The number of figures in the manuscript is Too few/Adequate/Too many/Not applicable
REVIEWER: Too many
Are the results and discussion confined to relevance to the objective(s)? Yes/No
REVIEWER: Yes
Is the supplementary material relevant and separated appropriately from the main document? Yes/No/Not applicable
REVIEWER: Yes
Please rate the manuscript on overall quality Excellent/Good/Average/Below average/Poor
REVIEWER: Good
Is appropriate and adequate reference made to other work in the field? Yes/No
REVIEWER: No

Is it stated that ethical approval was granted by an institutional ethics committee for studies involving human subjects and non-human vertebrates? Yes/No/Not applicable
REVIEWER: Not applicable
If accepted, would you recommend that the article receives priority publication? Yes/No
REVIEWER: No
Are you willing to review a revision of this manuscript? Yes/No
REVIEWER: No
With regard to our policy on 'Publishing peer review reports', do you give us permission to publish your anonymised peer review report alongside the authors' response, as a supplementary file to the published article? Publication is voluntary and only with permission from both yourself and the author. Yes/No
REVIEWER: Yes
Comments to the Author: 1- title: metabolomic analyses instead of metabolomics analysis 2- abstract lacks highlighting the novelty of the study and its main findings in terms of annotated metabolites 3- why did the authors chose these 2 species in particular? What is the common feature between both species that encourage performing a comparative study between them? Authors should clarify that in the abstract and the intro sections 4- add the names of the sea cucumbers to the key words 5- the cited references in text are wrongly numbered. The first cited reference in the intro section is numbered "34" instead of "1". This should be correctes throughout the text 6- the first mention of the analytical tools NMR and UPLC/MS in the intro section should be written in their full name 7- was the analysis of UPLC/MS analysis conducted by high resolution detector ?If yes, as inferred from text, then the abbreviation should be written UPLC/QTOF/HR-MS. 8- lack of tandem MS data hinders the annotation of metabolites and matching with references/database which makes Table 2 of identifications very questionable. 9- authors should use the term " tentatively identified" or "annotated" instead of "identified" for UPLC/MS derived metabolites 10- the error in ppm should be added to Table 2 for each predicted formulae 11- what are the number of replicates in pca and opls analyses? Mention in both "material and methods" and Fig. 1 legend 12- authors need to add references that support their conclusions on PCA and OPLS results concerning H. Cinerascens metabolic variability despite its closely located clusters to P. Sykion in lines 181-186 13- in figure 1, several codes are observed in yhe pca and opls plots such as S 1A, W 1a, S1c, w 3b...etc. what is the significance of these codes? Kindly explain in the figure legend. 14- the figure legends of fig. 3 and fig. 4 are abscent 15- figure 5 legend needa to be located below the figure and the authors need to add info that explains why there are 12 UPLC/MS chromatograms for analysing 2 specimens ? 16- the suppl. Figure legend is incomplete

Author response to Reviewer 2: Round 1
1- title: metabolomic analyses instead of metabolomics analysis
AUTHOR: Thank you for your comment, the title has been amended as recommended.
2- abstract lacks highlighting the novelty of the study and its main findings in terms of annotated metabolites
AUTHOR: Thank you for your comment. We have revised the abstract to clearly emphasize the novelty of

this study, and to provide greater detail on the main findings, particularly the annotated metabolites that distinguish the two species.
3- why did the authors chose these 2 species in particular? What is the common feature between both species that encourage performing a comparative study between them? Authors should clarify that in the abstract and the intro sections
AUTHOR: The two specimens were chosen as they are relatively understudied (in the case of *H. cinerascens*) and unknown in the case of *P. sykion.* P. sykion is an endemic species to the waters of southern Africa meaning it is a significant species to the country with little done on this species aside from basic morphology offering significant potential for research expansion on this holothurian species. The two species were chosen due to their abundance along the coastline of KwaZulu-Natal and their close geographical proximity (being found in the same location) which enabled the comparison to determine whether taxonomy or geographical proximity play a larger role in metabolite differentiation among these species which may inform future studies on holothurian metabolomics. This has been added into the abstract and introduction for clarity (Lines 11-12, 75-77, 83-86)
4- add the names of the sea cucumbers to the key words
AUTHOR: The names of the sea cucumbers have been added to the keywords list (Line 26).
5- the cited references in text are wrongly numbered. The first cited reference in the intro section is numbered "34" instead of "1". This should be corrected throughout the text
AUTHOR: Thank you for pointing this out. The citation numbers have been formatted to reflect order of appearance.
6- the first mention of the analytical tools NMR and UPLC/MS in the intro section should be written in their full name
AUTHOR: Response: Thank you for your comment. The first mentioning of the analytical tools ^{1}H-NMR and UPLC-QTOF-MS have been written out in full in the introduction section (Lines 81-83).
7- was the analysis of UPLC/MS analysis conducted by high resolution detector? If yes, as inferred from text, then the abbreviation should be written UPLC/QTOF/HR-MS.
AUTHOR: Thank you for your comment. The abbreviation has been updated throughout the manuscript to appear as UPLC-QTOF-HR-MS (Lines 10, 26, 83, 136, 137, 144, 159, 235, 325, 335, Supplementary Table 3 Heading, Figure 5 heading).
8- lack of tandem MS data hinders the annotation of metabolites and matching with references/database which makes Table 2 of identifications very questionable.
AUTHOR: Thank you for your comment. This study employed an untargeted metabolomics approach using full-scan UPLC-QTOF-HR-MS to broadly profile the baseline metabolite composition and compare the two species. All compounds were tentatively identified and annotated through comparison with multiple metabolite databases, including ChemSpider, PubChem, the Dictionary of Marine Natural Products, and the NIST 2014 Mass Spectral Library, as well as relevant literature (Lines 166-169). In cases with multiple potential matches, many corresponded to isomeric compounds, making definitive identification challenging. This limitation, including the impact of isomerism on compound confirmation, is discussed in the manuscript (Lines 238-244, 338-342). We acknowledge the importance of MS/MS data and have emphasised the need for future targeted fragmentation analyses and structural elucidation to confirm compound identities, particularly for highly isomeric metabolites or compounds lacking reference spectra (Lines 238-244, 257-263, 315-317,330-332, 338-344, 364-367). Nevertheless, the tentative annotations provided here serve as a baseline metabolite profile for these species, highlighting their potential value and underscoring the importance of expanding metabolomics research in this area.
9- authors should use the term " tentatively identified" or "annotated" instead of "identified" for UPLC/MS derived metabolites
AUTHOR: Thank you for your comment. We have revised the manuscript to include tentative annotation or annotation where present for the UPLC-QTOF-HR-MS results (Lines 166, 238-239, 247-248, Supplementary Table 3 heading)
10- the error in ppm should be added to Table 2 for each predicted formulae
AUTHOR: Thank you for your comment. The average mass error has already been included in the caption below Table 2 (Now Supplementary Table 3). However, we have included additional columns in Table 2 (Now Supplementary Table 3) to show the individual percentage error and ppm error values. In addition,

the average Mass Error (ppm) has been added to the caption below the figure.

11- what are the number of replicates in pca and opls analyses? Mention in both "material and methods" and Fig. 1 legend

AUTHOR: Thank you for your comment. The number of replicates has been added to both the Materials and Methods section (Line 110) and the Figure 1 legend (Line 371). The Materials and Methods section has also been revised for clarity.

12- authors need to add references that support their conclusions on PCA and OPLS results concerning H. Cinerascens metabolic variability despite its closely located clusters to P. Sykion in lines 181-186

AUTHOR: Thank you for your comment. The variability referred to reflects that *H. cinerascens* samples display greater overall variation compared to *P. sykion*, as indicated by the wider dispersal of *H. cinerascens* data points on the OPLS-DA plot (Figure 1b). This has been further clarified in the manuscript (Lines 173-175, 181-185). The PCA-X results show that while most *H. cinerascens* samples cluster closely with *P. sykion*, while some display wider dispersal, indicating higher intra-species variability and separation from *P. sykion* samples. The overall R^2 and Q^2 values from the PCA-X ($R^2X = 0.885$, $Q^2(cum) = 0.787$; Line 177-178) indicate good model performance and separation between the two species, which is reinforced by the OPLS-DA results ($R^2X(cum) = 0.856$, $R^2Y(cum) = 0.637$, $Q^2(cum) = 0.592$; Line 179), demonstrating clear metabolic differentiation between the two species. References supporting the interpretation of PCA-X and OPLS-DA analyses in SIMCA software have been added to support these conclusions (Lines 177-185).

13- in figure 1, several codes are observed in the pca and opls plots such as S 1A, W 1a, S1c, w 3b...etc. what is the significance of these codes? Kindly explain in the figure legend.

AUTHOR: Thank you for your comment. The alphanumeric codes in the PCA and OPLS plots represented internal sample identifiers used during cataloguing. To avoid ambiguity and improve clarity for readers, we have standardised the labels to reflect only the species and sample numbers in both the figures and their legends. This also ensures consistency across the manuscript and highlights the inter-species comparisons more effectively.

14- the figure legends of fig. 3 and fig. 4 are absent

AUTHOR: Thank you for your comment. The figure legends for Figures 3 and 4 are present in full and correctly positioned in our originally submitted version (as shown in the screenshot provided).

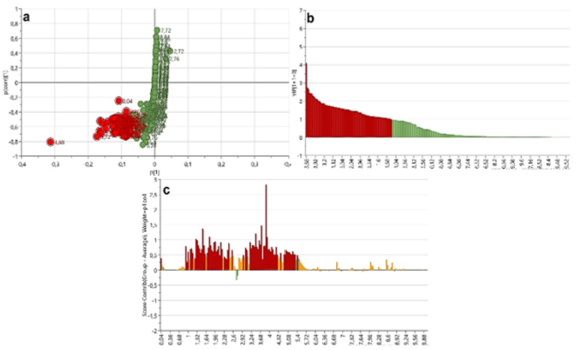

Figure 3: *S-plot (a), VIP score plot (b), and contribution loadings plot (c) showing the ¹H-NMR spectral regions positively associated with H. cinerascens that contribute to the clustering and separation of the tissue extracts between H. cinerascens and P. sykion. Bars above the x-axis represent chemical shift regions positively associated with H. cinerascens.*

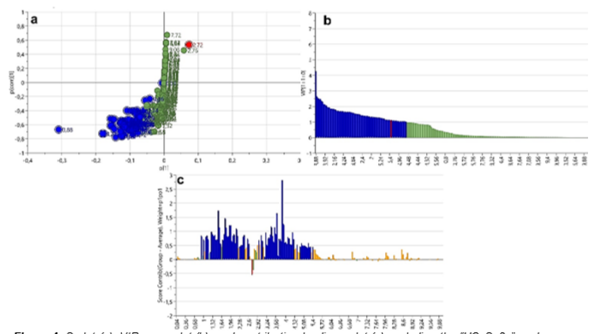

Figure 4: *S-plot (a), VIP score plot (b), and contribution loadings plot (c) excluding the "HC_S_3a" and "HC_W_3a" samples, showing the ¹H-NMR spectral regions positively associated with H. cinerascens that contribute to the clustering and separation of the tissue extracts between H. cinerascens and P. sykion. Bars above the x-axis represent chemical shift regions positively associated with H. cinerascens.*

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Following manuscript editing, we have also double-checked the submission files to ensure that the legends are included and clearly visible (as seen below).

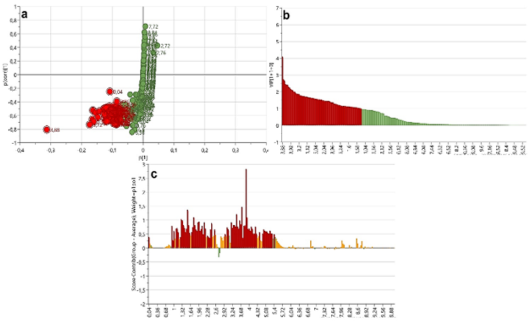

Figure 3: S-plot (a), VIP score plot (b), and contribution loadings plot (c) showing the ^{1}H-NMR spectral regions positively associated with *H. cinerascens* that contribute to the clustering and separation of the tissue extracts between *H. cinerascens* and *P. sykion*. Bars above the x-axis represent chemical shift regions positively associated with *H. cinerascens*.

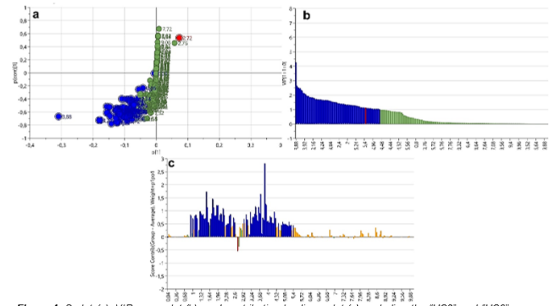

Figure 4: *S-plot (a), VIP score plot (b), and contribution loadings plot (c) excluding the "HC3" and "HC6" samples, showing the ^{1}H-NMR spectral regions positively associated with H. cinerascens that contribute to the clustering and separation of the tissue extracts between H. cinerascens and P. sykion. Bars above the x-axis represent chemical shift regions positively associated with H. cinerascens.*

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15- figure 5 legend needs to be located below the figure and the authors need to add info that explains why there are 12 UPLC/MS chromatograms for analysing 2 specimens ?

AUTHOR: Thank you for your comment. In our originally submitted document, the legend for Figure 5 does appear below the figure (as shown in the screenshot provided).

Initially, we included 12 chromatograms to illustrate variability within and between the samples analysed. However, we acknowledge that due to the high degree of similarity within species profiles, this may appear redundant. We have therefore revised the figure to display only the two most representative chromatograms from each species for clarity.

16- the suppl. Figure legend is incomplete

AUTHOR: Thank you for your comment. While the supplementary figure legend appears complete and untruncated on our side, we have revised it to provide more detailed and comprehensive phrasing for improved clarity.

Comparative Metabolomics Analysis of *Holothuria cinerascens* and *Pseudocnella sykion*

Abstract

Sea cucumbers are soft-bodied marine invertebrates revered for their potent health benefits derived from biologically active molecules. However, research has primarily focused on commercially valuable species, leading to overexploitation and neglect of other species, which may also hold potential for beneficial metabolites and nutritional composition. This study explored the metabolic composition between two sea cucumber species*, Holothuria cinerascens* and *Pseudocnella sykion*, from Southern Africa, using untargeted ^{1}H-NMR and UPLC-QTOF-MS. Our findings revealed distinctive metabolic profiles, with *H. cinerascens* exhibiting elevated levels of compounds associated with osmoregulation, energy and amino acid metabolism, and defence mechanisms compared to *P. sykion.* These disparities may stem from genetic, ecological, and reproductive variations between the species. While providing insights into sea cucumber metabolism, this study also underscores the challenges in holothurian metabolomics research that warrant further exploration. Expanding sea cucumber research to understand their metabolic pathways and composition offers prospects for discovering novel bioactive compounds, improving aquaculture practices, fostering economic opportunities, and advancing sustainable resource management and conservation efforts.

Keywords: *Holothurian, ^{1}H-NMR, UPLC-QTOF-MS, marine, metabolites, osmolytes.*

Tables, figures and supplementary material are inserted at the end of the document.

Introduction

Sea cucumbers, marine invertebrates of the phylum Echinodermata, are characterised by soft, leathery, cylindrical bodies and pentaradial symmetry[34]. These organisms inhabit diverse marine environments, with the highest abundance and diversity concentrated in the tropical and subtropical Asia-Pacific regions[1; 34; 51]. Harsh environmental conditions have driven the production of unique metabolites in marine species, with echinoderms emerging as novel sources of biologically beneficial biomolecules that exhibit a range of therapeutic anti-thrombotic, anti-coagulant, anti-microbial, anti-inflammatory, anti-oxidant, anti-hypertensive, and wound-healing properties[18; 25; 33; 44].

The health benefits of sea cucumbers are well recognised, with some species referred to as "marine ginseng" and used for centuries in Asian cultures as medicinal ingredients[14; 27; 35]. Their body walls are often dried into a highly valued, nutritionally dense dietary delicacy referred to as "Beche-de-Mer", "Trepang", or "Gamat"[34; 51]. The growing interest in natural pharmaceutical and nutritional sources has propelled marine alternatives into the forefront of scientific research as safer substitutes for synthetic or terrestrially derived components[25; 29]. However, the commercial demand for sea cucumber products is primarily focused on a few select species, leading to overexploitation and endangerment despite conservation efforts, catch limits, and legal action against poaching[4; 34; 51; 54]. While sustainable cultivation practices are being adopted, challenges persist, including high mortality rates in captivity and nutritional and metabolic variability influenced by environmental factors and species-specific differences[20; 29; 54]. Therefore, investigating the metabolic properties of alternative sea cucumber species is crucial to uncovering their biochemical and nutritional potential, and promoting sustainable resource management.

Metabolomics is an increasingly popular research field with broad applications spanning biomarker discovery, disease diagnosis, therapeutic monitoring, geographical determination, nutritional analysis, and environmental impact studies. Metabolomics aims to identify metabolites involved in an organism's physiological and metabolic responses, which represent the final products of gene expression and provide valuable insights into the complex metabolic responses to both internal and external factors[3; 37 3; 40].

In South Africa, *Holothuria cinerascens* is a prominent species within the Holothuriidae family under the Holothuriida order, commonly found inhabiting intertidal regions along the warmer Eastern coastlines and Indian Ocean[21; 34; 47]. This shallow-water species is primarily a deposit feeder but can also engage in suspension feeding[1; 21; 38]. It is characterised by a thick body wall, which ranges in colour from rusty red-brown to purple-black, and can grow to an average length of 16 cm[1; 29; 34]. *H. cinerascens* is one of the few known holothurian species to undergo asexual reproduction through transverse fission during winter or suboptimal environmental conditions[10]. In addition, *H. cinerascens* is highly sensitive to environmental stressors, expelling its internal organs–a process known as evisceration–as a defensive mechanism when threatened[18; 34].

Members of the Dendrochirotida, are predominantly located in temperate regions, often residing in soft sediment or attached to hard rock or coral surfaces[47]. *Pseudocnella sykion,* commonly known as "the black sea cucumber", is a Dendrochirotid species within the Cucumaridae family, endemic to the warm tropical waters along the Eastern coast of South

Africa, typically nestled within rock crevices along intertidal rocky shores[47; 48]. This suspension-feeding species is relatively small, reaching up to nine cm, with a plump, barrel-shaped body that ranges from dark olive-green to black[45; 46].

Despite the ecological and therapeutic significance of sea cucumbers, substantial gaps remain in our understanding of their metabolic profiles. *H. cinerascens* and *P. sykion* have received little scientific attention beyond basic morphology, with *P. sykion* being an endemic species with restricted geographical ranges, further limiting available information. This knowledge gap is largely due to the scarcity of research on non-commercially valued varieties, compounded by limited research resources and infrastructure in developing countries, like South Africa. This study aims to bridge these gaps by conducting an untargeted metabolomics comparison of *H. cinerascens* and *P. sykion* from KwaZulu-Natal, South Africa, using ^{1}H-NMR and UPLC-QTOF-MS techniques. By exploring the metabolic profiles of these species, this research highlights the untapped potential of non-commercial sea cucumber species and emphasises the importance of expanding research efforts to promote sustainable practices, conservation, and the discovery of novel metabolites with therapeutic applications. Moreover, investigating indigenous or endemic sea cucumber species could present significant economic opportunities for developing nations, fostering income generation and job creation through cultivation, processing, exportation, and research initiatives involving these species.

Methods and materials

Ethical statement

Currently, the Animal Research Ethics Committee (AREC) does not require ethics clearance for research involving sea cucumbers, as they are classified as lower-order marine invertebrates without a definitive spine or neural network. Nevertheless, it is essential to note that all procedures related to sample collection, handling, and processing complied with the regulations and guidelines provided by AREC and the Department of Forestry, Fisheries, and the Environment (DFFE).

Sample collection and preparation

Holothuria cinerascens and *Pseudocnella sykion* specimens were gathered from the intertidal rocky shores at Park Rynie, KZN (30°19′S, 30°44′ E) during the spring tide in August 2021 and January 2022. The samples were promptly frozen, followed by cleaning and dissection. The tissues were then frozen in liquid nitrogen, freeze-dried, ground into a powder, and stored at -80°C until analysis.

NMR analysis

For NMR analysis, 50 mg of the dried tissue were added into 2 ml Eppendorf tubes along with 600 µl each of deuterated methanol ($CH_3OH–d_4$) and 0.01% 3-(trimethylsilyl)-propionic acid (TSP), deuterium oxide–potassium dihydrogen phosphate ($D_2O–KH_2PO_4$) buffer. The samples underwent 15 minutes of sonication followed by 15 minutes of centrifugation at 12 000 rpm (10 625 xg). The supernatant was transferred into an NMR tube and analysed by performing 32 scans in a 600MHz NMR spectrometer.

The raw spectral data was pre-processed, including referencing, normalisation, and baseline correction, using MestReNova (v14.2.2). Binned regions spanning from 0.04 – 10.0 ppm at 0.04 ppm widths were generated to reduce the spectral intensities in preparation for subsequent multivariate analysis. These regions were exported as a CSV database file into SIMCA software v17.0.1. (Umetrics, Sweden), which was used to extract chemical information related to metabolic composition and differentiation. Prior to multivariate analysis, exclusion zones were defined for the water (4.6–5.0 ppm) and methanol (3.28–3.36 ppm) peaks to ensure that these areas did not affect the analysis. The data analysis consisted of two steps. First, an unsupervised Principal Component analysis (PCA) was performed to provide an unbiased data comparison, enabling the identification of trend clusters and potential data outliers. This was followed by a supervised Orthogonal Partial Least Square Discriminatory analysis (OPLS-DA). The OPLS-DA compared the chemical data based on predefined class identifiers to uncover further trends within the dataset. Spectral regions that had the most significant impact on metabolic differentiation were identified by creating an S-Plot, VIP scores plot, and contributions loading plot. The OPLS-DA results were validated through a permutation test involving 100 permutations. Subsequently, compound annotation was done in Chenomx (v9.02) by comparing the differentiated spectral regions identified in the contribution loadings plot with compound databases and relevant scientific literature.

UPLC-QTOF-MS

The UPLC-QTOF-MS analysis was conducted in triplicate, with metabolites extracted by combining approximately 45mg of freeze-dried tissue with 2 ml of cold 70% HPLC-grade methanol. The extraction process involved sonication followed by vortexing to ensure thorough homogenisation. Following extraction, insoluble tissue material was pelleted by centrifugation, and the resulting supernatant was filtered through a Pall acrodisc GHB 0.2-micron syringe filter (13 mm, 0.2 µm) equipped with a Teflon plunger head to remove particulates in preparation for UPLC analysis.

The UPLC-QTOF-MS analysis was performed using a Waters T3 C18 column (150 mm x 2.1 mm x 1.8 µm) integrated with the Waters Classic UPLC system, coupled to the Waters SYNAPT G1 Mass Spectrometer (Waters Corporation, Milford, MA, USA). Initial analyses revealed issues of signal saturation and co-elution, prompting subsequent experiments to utilise the Waters Premier UPLC paired with the Waters SYNAPT XS Mass Spectrometer (Waters Corporation, Milford, MA, USA). The UPLC system operated with a column temperature of 60°C. The mobile phase consisted of Solvent A (water with 10 mM formic acid) and Solvent B (Acetonitrile with 10 mM formic acid). The elution gradient began with 100% Solvent A for one minute, transitioning linearly to 1% Solvent A over 16 minutes, at a flow rate of 0.4 mL/min, with a total runtime of 20 minutes. QTOF-MS analysis was conducted in both positive and negative electrospray ionisation modes (ESI^+ and ESI^-). The capillary voltage was set to 0.6 kV, and the cone voltage was maintained at 30 V. The source and desolvation temperatures were set to 120°C and 450°C, respectively, with nitrogen (N_2) serving as the desolvation gas at a flow rate of 600 L/h. Accurate mass calibration was achieved using LockMass correction with leucine enkephalin (100 pg/uL) sampled every 20 seconds.

Data generated from UPLC-QTOF-MS analysis was processed using the integrated MassLynx 4.2 (SCN 1028) software, ensuring a mass accuracy below 1 mDa. Elemental composition analysis was performed using the software's embedded tools, with predefined ranges for carbon (C; 1–80), hydrogen (H; 1–200), oxygen (O; 0–35), and sulphur (S; 0–2). This approach was tailored to provide a comprehensive evaluation of elemental compositions relevant to saponins and other holothurian metabolites, while excluding atypical elements, such as chlorine (Cl), fluorine (F), phosphorous (P), and nitrogen (N). Monoisotopic mass data were accounting for both odd and even electron states, with a mass tolerance of 3 mDA. Metabolite annotation was achieved by comparing the mass spectral data to online databases, including ChemSpider, PubChem, The Dictionary of Marine Natural Products, and the NIST 2014 Mass Spectral Library, along with relevant literature.

Results

The results of the unsupervised PCA-X analysis of the ^{1}H-NMR data from body tissue extracts of *H. cinerascens* and *P. sykion* (Figure 1a) revealed closely grouped clusters for the body tissues from both species. However, the tissue extracts from *H. cinerascens* demonstrated the greatest metabolic variability, appearing more widely dispersed across the plot. Notably, four samples of *H. cinerascens* appeared outside the 95% confidence circle, with "HC_S_3a" and "HC_W_3a" appearing as outliers in both the Hotelling's and DmodX

Line Plot test. Overall, the model displayed a high goodness of fit and predictability, with R^2X = 0.885 and Q^2(cum) of 0.787.

The OPLS-DA results (Figure 1b) had an R^2X(cum) value of 0.856, R^2Y(cum) of 0.637, and Q^2(cum) of 0.592. The results show a complete separation between *H. cinerascens* and *P. sykion.* Furthermore, *H. cinerascens* displays a greater degree of metabolic variability, as evidenced by the higher level of sample dispersion towards the left of the y-axis. In contrast, *P. sykion* samples appear tightly clustered on the right side of the y-axis. The distinct horizontal separation between the two species underscores their metabolic differentiation. This varies from the overall PCA-X and could indicate that taxonomic grouping may play a more significant role in metabolic similarities than geographical location. Possible reasoning for this could be minor niche segregation between the *H. cinerascens* and *P. sykion* within the same location, whereby the *H. cinerascens* appear more exposed on rock surfaces and buried under sand, whilst the *P. sykion* are only found hidden in tight and narrow rock crevices. This could result in food source variation within the same location and the exposure of *H. cinerascens* to greater environmental stress factors that may influence the metabolic activity and body wall metabolites within this species, which is known to undergo evisceration when subjected to stressors. In addition, this distinction may also be linked to the ability of *H. cinerascens* to undergo asexual reproduction. This process involves metabolic changes that alter the mutable collagenous tissue within the body wall to facilitate fission, a process not known to occur in *P. Sykion.* A permutation plot was carried out with 100 permutations to validate the OPLS-DA model and had the intercepts of R^2 = (0.0; 0.145) and Q^2 = (0.0; -0.269) (Supplementary Figure 1).

The NMR profiles of *H. cinerascens* and *P. sykion* were stacked to visualise the chemical shift regions responsible for metabolic variation between these species (Figure 2a, b). Notable spectral differences were observed between the two species, with *H. cinerascens* demonstrating greater metabolic activity and potential than *P. sykion*. Additionally, distinctive spectral regions exclusively associated with each species were evident throughout the comparisons. In the region spanning 0.0–2.5 ppm, *H. cinerascens* exhibited higher intensity spectral peaks, while *P. sykion* displayed inconsistent, low peaks. However, *P. sykion* showed a distinct multiplet pattern around 2.7 ppm, absent in *H. cinerascens*. Both species displayed differentiated peaks between 3.0–4.0 ppm, with *H. cinerascens* showing less distinct peak patterns in higher concentrations than *P. sykion.* Notable variations were observed at 3.0 ppm, where the *H. cinerascens* displayed a multiplet, absent or in low concentration in *P. sykion,* while *P. sykion* exhibited a singlet in most samples. Distinct spectral peaks, likely correlating with glycerol, were observed between 3.5–3.8 ppm, with greater resolution in *P. sykion*. *H. cinerascens* samples contained a singlet at 4.4 ppm and

doublets between 4.5 and 4.6 ppm, absent in *P. sykion.* The abundant peaks in the sugar-aliphatic region may be related to holothurin compounds known to be abundant in the *Holothuria* genus to which *H. cinerascens* belongs. In the aromatic region (Figure 2b), peak variations between 5.1–5.5 ppm showed no distinct pattern between the two species. Several doublets were observed between 5.9 and 6.2 ppm, with a higher intensity doublet at 6.1 ppm in *H. cinerascens*. Both species exhibited a singlet at 6.8 ppm, more prominent in *H. cinerascens.* Additionally, between 7.9 and 8.05 ppm, *H. cinerascens* displayed a multiplet and doublet, absent within the *P. sykion*. A marked distinction was also noted within the 8.0–8.8 ppm region, with *P. sykion* possessing small peaks between 8.15–8.35 ppm, while a larger array of peaks was observed within *H. cinerascens* between 7.95–8.75 ppm.
The observed metabolic profiles within the tissue extracts of these two species suggest significant metabolic differentiation despite their collection from the same geographical location. This variation may be attributed to minor ecological distinctions resulting from the habitat preferences and feeding mechanisms of these species. Additionally, genetic factors associated with their distinct taxonomic orders could contribute to metabolic differences, as seen in the defensive mechanism of evisceration exhibited by *H. cinerascens,* which is absent in *P. sykion.*

The spectral regions influencing separation between *H. cinerascens* and *P. sykion* were determined by generating an S-plot and VIP scores plot, with influential scores identified from both ends of the S-plot and VIP score values ≥ 1.0 (Figure 3 a, b). A contribution loadings plot (Figure 3c) identified the spectral regions contributing to metabolic differentiation, with regions positively associated to *H. cinerascens* at 0.04, 0.92, 1.08, 1.12, 1.28, 1.32, 1.36, 1.4, 1.52, 1.56, 1.76, 1.8, 1.92, 1.96, 2.08, 2.16, 2.44, 3.2, 3.24, 3.4, 3.44, 3.48, 3.52, 3.56, 3.6, 3.64, 3.68, 3.72, 3.8, 3.84, 3.88, 3.92, 4.24, 4.56, and 5.04 ppm, with 0.96, 1, 1.16, 1.24, 1.44, 1.48, 1.6, 1.68, 1.72, 1.84, 1.88, 2, 2.04, 2.12, 2.2, 2.28, 2.32, 2.36, 2.4, 2.56, 2.96, 3.04, 3.08, 3.76, 3.96, 4, 4.04, 4.08, 4.12, 4.16, 4.2, 4.28, 4.4, 4.52, 5.08, 5.12, 5.16, 5.2, 5.24, 5.28, 5.32, and 5.4 ppm contributing to a lesser extent.

However, due to the “HC_S_3a” and “HC_W_3a” samples being identified as complete outliers, the contribution comparisons were repeated, excluding these samples for comparison. The resulting S-plot and VIP scores plot can be seen in Figure 4a, b, where influential scores were identified from both ends of the S-plot and VIP score values ≥ 1.0. A contribution loadings plot (Figure 4c) identified the spectral regions contributing to metabolic differentiation, with regions positively associated to *H. cinerascens* at 0.92, 1, 1.08, 1.12, 1.28, 1.32, 1.36, 1.4, 1.52, 1.56, 1.76, 1.8, 1.84, 1.92, 1.96, 2.08, 2.16, 2.2, 2.4, 2.44, 3.2, 3.24, 3.4, 3.44, 3.48, 3.52, 3.56, 3.64, 3.68, 3.72, 3.8, 3.84, 3.88, 3.92, and 4.24 ppm, with 0.04, 1.16, 1.24, 1.44, 1.48, 1.6, 1.68, 1.72, 1.88, 2, 2.04, 2.12, 2.28, 2.32, 2.36, 2.56, 2.6, 2.96, 3.04, 3.08, 3.16, 3.6, 3.76, 3.96, 4, 4.04, 4.08, 4.12, 4.16, 4.2, 4.28, 4.4, 4.52, 4.56, 5.04, 5.08, 5.12, 5.16, 5.2, 5.24, 5.28, 5.32, and 5.4 ppm contributing to a lesser extent. However, the results also identified the region at 2.72 ppm as negatively associated with *H. cinerascens* and positively associated with *P. sykion.* This correlates with the spectral pattern unique to *P. sykion* identified in the NMR stack (Figure 2).

Compound annotation

Compound annotation was carried out by comparing the spectral bin regions derived from S-Plots, VIP scores plots, and contribution loadings plots alongside compound the databases Chenomx NMR Suite software, the Human Metabolome Database (HMDB), and relevant literature. The annotated compounds resulting from this analysis are listed in Table 1 below.

The UPLC-QTOF-MS analysis (Figure 5) revealed distinct metabolic profiles between *H. cinerascens* and *P. sykion.* Table 2 provides a comprehensive summary of the UPLC profiles, detailing retention times, observed and monoisotopic masses, and calculated empirical formulas for each compound. Compound data were derived based on broad parameters set by the MS software, which necessitated referencing generalised matches from available literature and databases. Although numerous compounds were identified, precise confirmation was hindered by the significant degree of compound isomerism, the lack of holothurian-specific MS databases, and the limited availability of mass spectral data in current literature. The Dictionary of Marine Natural Products did not contain any information on *H. Cinerascens* and *P. sykion.* Consequently, many compounds had multiple potential matches, resulting in uncertainty about their precise identities. Compounds labelled as “unknown” did not correspond to any reference spectra, suggesting they may represent novel holothurian- or species-specific metabolites. The results depict a clear metabolic distinction between *H. cinerascens* and *P. sykion,* corroborating the OPLS-DA findings (Figure 1b), despite both species being collected from the same geographical region. *H. cinerascens* exhibited a higher metabolite potential compared to *P. sykion,* consistent with findings from the ^{1}H-NMR analyses (Figures 2-4, Table 1). These differences may be attributed to minor ecological variations, differences in food selectivity, and genetic factors linked to their taxonomic divergence. For instance, *H. cinerascens* possesses genetically encoded abilities to undergo evisceration and asexual reproduction, traits not observed in *P. sykion.*

Notably, *H. cinerascens* demonstrated a higher potential for triterpene glycosides, evidenced by the identification of compounds such as holothurin A (arenicolaside A), holothurin B, B3, B4; 17-hydroxyfuscocineroside B; 25-hydroxyfuscocineroside B; nobiliside B, nobiliside II (nobiliside C), not detected in *P. sykion.* Additionally, *H. cinerascens* showed potential for aliphatic organosulphates, like dibutyl sulphate, octyl sulphate, 6-methylheptyl sulphate. These findings suggest that *H. cinerascens* may possess more robust chemical defence mechanisms, with many of these compounds possessing significant biological potential. However, despite the greater metabolite diversity in *H. cinerascens,* some compounds were unique to *P. sykion,* such as rosmarinic acid – a phenolic compound commonly associated with plant species[13; 24], along with several unknown compounds which may represent novel sulphated triterpene glycosides. These findings underscore the unique metabolic profiles of the two species and highlight the importance of further investigations, including fragmentation analyses and metabolic pathway studies, to determine whether these compounds are endogenous metabolic products or derived from external sources, such as microbial interactions or dietary intake.

Both *H. cinerascens,* classified as a “low-value” species[34], and *P. sykion,* a lesser-known species endemic to Southern Africa, display considerable metabolic potential with prospective therapeutic and nutritional applications for commercialisation and natural drug discovery. However, fully characterising the structural properties and chemical identities of their metabolites requires extensive compound isolation, purification, and targeted analyses employing fragmentation data. Further research is also essential to deepen our understanding of the metabolic pathways underlying the biochemical diversity within these species. Moreover, evaluating the biological properties of their metabolites could inform the feasibility of integrating these species into aquaculture systems. Such an approach could provide a sustainable source of biological resources for downstream testing and industrial applications, promoting the utilisation of these underexplored species in environmentally sustainable ways.

Discussion

This study represents the first known metabolic comparison between *H. cinerascens* and *P. sykion*, providing novel insights into the metabolic profiles of these species. Our findings revealed distinct patterns in compound abundance, with *H. cinerascens* showcasing higher metabolic potential than *P. sykion* (Table 1). Notably, *H. cinerascens* displayed moderate to moderate-low levels of various amino acids, sugar-type compounds, and carboxylic acids, which were largely absent or in very low concentrations in *P. sykion.* Moreover, betaine, an osmoregulant, was present in high concentrations among both species but was significantly elevated in *H. cinerascens.* Previous studies have linked the upregulation of betaine, along with alterations in energy metabolism, to stress responses in sea cucumbers triggered by high temperatures, high salinity environments, hypoxia, and skin ulceration syndrome[20; 30; 31; 57]. Additionally, higher temperatures have been associated with disruptions in energy metabolism, resulting in heightened glucose levels[42]. Therefore, the higher levels of betaine and glucose in *H. cinerascens* may indicate a heightened need for water regulation and potential heat stress, attributable to greater sun and UV exposure compared to *P. sykion.*

Conversely, *P. sykion* demonstrated a higher potential for proline and sarcosine. Proline was detected at moderate-low concentrations in *P. sykion*, with minimal and inconsistent occurrences observed in *H. cinerascens.* Sarcosine, though present at very low concentrations in *P. sykion*, was absent in *H. cinerascens.* Furthermore, several compounds, including ascorbic acid, dimethyl sulfone, ethanolamine, glycerol, glycine, malonate, and mannose, were uniformly detected at moderate to moderate-low concentrations in both *H. cinerascens* and *P. sykion*.

It is evident that *H. cinerascens* and *P. sykion* exhibit distinct metabolic profiles despite being collected from the same location. This variation may stem from minor ecological distinctions arising from the habitat preferences of these species. *H. cinerascens*, being larger, is often found on rock surfaces more exposed to factors like wave action, sunlight and UV radiation, temperature fluctuations, and moisture loss during low tide[1; 34]. In contrast, *P. sykion* typically resides wedged deep within rock crevices, offering a more sheltered and stable environment[45].

Sea cucumbers are known as osmoconformers which utilise osmolytic compounds to maintain cellular osmotic pressure in response to their saline environments[1; 55]. Many of the identified compounds in this study are recognised as "compatible osmolytes"[30; 31; 55]. These compounds can be produced in high cellular concentrations without disrupting cell function, helping to stabilise protein structures, maintain osmotic pressure, and prevent water loss in organisms exposed to high salinity environments[30; 31; 55]. Additionally, these compounds possess various anti-oxidant and cytoprotective properties, making them valuable in natural drug research[31; 55].

Interestingly, certain compounds–including sugars, organic acids, and choline derivatives–can form stable liquids at room temperature without water when combined in specific compositions[8; 49]. These liquids, referred to as natural deep eutectic liquids or solvents (NaDES), have been shown to help maintain water balance in plants during periods of drought, cold, high temperatures, and increased salinity levels[49]. Therefore, it is plausible that many of the identified osmolytes in these sea cucumbers, constantly exposed to high salinity environments, function as NaDES to prevent desiccation in their saltwater habitats and when exposed to sunlight. This could also explain why these compounds are more abundant in *H. cinerascens,* which is more exposed to high heat and UV radiation during low tide. These findings underscore the adaptability of sea cucumbers to their harsh environments and highlight the potential for further research into the therapeutic and protective properties of their metabolites. The presence of NaDES and compatible osmolytes could offer novel applications for maintaining cellular stability under stress, both in marine organisms and potentially in other fields, such as agriculture and pharmacology.

Furthermore, dietary variation, influenced by feeding mechanisms and environmental niches, could introduce different nutrients and metabolic building blocks to each species, leading to compositional changes in the metabolome[19; 28; 39]. *P. sykion,* exclusively a suspension feeder, relies on water submersion to feed from the water column. In contrast, while *H. cinerascens* has a broader range of feeding mechanisms–being a surface deposit feeder that can also engage in suspension feeding when necessary–its exposure to food sources when attached to surfaces away from the sediment may be limited, especially during low tide[1; 21; 34; 38]. Therefore, it is likely that *H. cinerascens* consumes more detritus and sediment-based nutrients while *P. sykion* is likely more exposed to organic faecal matter, phytoplankton, or algae from the surrounding organisms in the rock crevices.

Moreover, the metabolic differentiation between these species may stem from their classification within genetically distinct taxonomic orders, Holothuriida and Dendrochirotida [47]. Genetic factors significantly drive metabolic variations by modulating gene expression in response to internal and external signals[9; 11; 12; 29; 33]. For example, *H. cinerascens* demonstrates evisceration of its internal organs, a defensive mechanism observed in other Holothuriida species that is triggered by heat stress, predation, or handling[10; 18]*.* This response may induce metabolic changes that differentiate *H. cinerascens* from *P. sykion* Notably, *H. cinerascens* demonstrated higher levels of xylose and glucose, with the NMR spectral data (Figure 2) suggesting the potential of additional sugar-type compounds. While these monosaccharides can contribute to various compounds, they are frequently found in triterpene glycosides, with xylose typically forming the first monosaccharide in the carbohydrate chain[23; 25; 36]. Numerous studies have highlighted the role of saponins in defence mechanisms, including evisceration, and it has been observed that species more exposed to harsh environmental conditions tend to possess higher concentrations of

saponins[18; 23; 25; 33; 35; 44]. Given the higher environmental exposure of *H. cinerascens* compared to *P. sykion,* it is reasonable to expect elevated metabolic activity and higher concentrations of these compounds in *H. cinerascens,* reinforced by the NMR spectral comparison (Figure 2).
These findings were corroborated by the UPLC-QTOF-MS analysis, which identified higher metabolic activity within *H. cinerascens* (Table 2)*.* The putative identification of various triterpene glycosides suggests significant therapeutic potential within both *H. cinerascens* and *P. sykion*. Although *H. cinerascens* displayed greater metabolic potential, the presence of rosmarinic acid, among other compounds, from *P. sykion* is noteworthy and further supports the bioactive potential of this species, as rosmarinic acid is known to have various beneficial properties, such as anti-cancer, anti-tumour, anti-inflammatory, anti-microbial, anti-viral, and anti-oxidant activities[13; 24]. While rosmarinic acid has been found in *H. forskali*[44], further studies are needed to determine whether this metabolite originates from *P. sykion* or the potential plant, faecal, or detritus-based material it consumes.

Additionally, genetically encoded reproductive strategies triggered by environmental conditions, can also influence metabolic variability among sea cucumber species[9-11]. While both *H. cinerascens* and *P. sykion* are capable of sexual reproduction, *H. cinerascens* is one of the few species capable of asexual reproduction through transverse fission. Fission, a process controlled by the nervous system, involves metabolic changes that rearrange internal organs and facilitate enzymatic interactions that lead to constriction of the body wall[10; 11]. Prior to fission, the organism ceases feeding, potentially inducing metabolic changes through nutrient and energy restriction[10]. Consequently, genetic factors governing reproductive strategies and the capacity for fission may contribute to metabolic variability during this process. As a result, fissiparous species like *H. cinerascens* are anticipated to display greater metabolic variability compared to species unable to undergo this process, such as *P. sykion.* However, further investigations are essential to elucidate the complete metabolic structures within these species and the physiological mechanisms involved in their production and utilisation.

This study represents the first untargeted metabolomics analysis on *H. cinerascens* and *P. sykion,* a largely unknown species from Southern Africa, providing new insights into their metabolic composition and contributing to our understanding of interspecies metabolic variability. Notably, *H. cinerascens* exhibited greater metabolic potential than *P. sykion,* with identified compounds implicated in osmoregulation, energy metabolism, amino acid metabolism, and defence mechanisms*.* The observed metabolite variability between these species suggests possible genetic, physiological, or ecological adaptations, indicating that geographical location may play a lesser a role in metabolic similarity compared to the metabolic differentiation influenced by taxonomic, dietary, or niche-specific factors. However, further investigations are imperative to fully understand the biochemical pathways within sea cucumbers and how environmental or physiological factors may influence these metabolic differences.

This study highlights the promise of untargeted ^{1}H-NMR and UPLC-QTOF-MS metabolomics in studying sea cucumber metabolism and facilitating baseline comparisons between species to identify common or species-specific metabolic patterns. Nonetheless, while untargeted ^{1}H-NMR and UPLC-QTOF-MS metabolomics can offer an unbiased view of the metabolome, it has limitations in the structural elucidation of metabolic compounds, particularly in situations with overlapping spectral peaks[3; 40; 52]. Additionally, metabolite annotation relied on comparisons with existing literature and databases, potentially limiting the identification of holothurian-specific compounds due to a lack of reference material or spectra. Therefore, future studies should employ compound isolation, characterisation, and targeted analyses using alternative metabolomics techniques and solvents to comprehensively assess the metabolic potential and structural composition of these species. Moreover, investigating metabolic stress responses and mitigation strategies in cultivation settings is crucial in understanding sea cucumber health, and could enhance the nutritional value and survivability of sea cucumbers in artificial environments, thereby incentivising sustainable cultivation practices. Furthermore, expanding sea cucumber research holds promise for discovering novel bioactive metabolites, improving cultivation practices, and fostering the integration of a broader range of species into commercial and pharmaceutical utilisation, while opening up economic pathways in third-world countries. However, ensuring sustainable resource management is essential to prevent the recurrence and creation of new endangerment cycles observed in high-value species.

Conclusion

Understanding sea cucumber metabolism and its biological potential can play a pivotal role in expanding species utilisation in aquaculture practices, thereby promoting sustainable utilisation of marine resources. Furthermore, exploring indigenous or endemic sea cucumber species could offer significant economic opportunities, particularly in developing nations, by fostering income generation and job creation through expanded cultivation practices, processing, exportation, and research into pharmaceutical and natural drug development.

This study highlights the metabolic variations between *Holothuria cinerascens* and *Pseudocnella sykion*, offering valuable insights into their physiological adaptations and biological processes. The findings underscore the influence of genetic, ecological, and reproductive factors on sea cucumber metabolism, underscoring the need to consider these aspects in future research and conservation efforts. By elucidating the baseline metabolic profiles of these understudied sea cucumber species, this research contributes to a broader understanding of marine ecosystems and the potential applications of sea cucumbers across various fields. Identifying key metabolites involved in osmoregulation, energy metabolism, and defence mechanisms provides a foundation for further exploration of their metabolic pathways, biological functions, and potential benefits for human health. While this study marks a significant advancement in marine metabolomics, it also highlights areas requiring further investigation. Addressing limitations, such as sample size, employing alternative metabolomics techniques for structural elucidation, and examining metabolic stress responses in cultivation settings are essential for deepening our understanding of sea cucumber metabolism and enhancing their commercial viability. Expanding research efforts in this domain holds promise for sustainable resource management and the development of effective conservation strategies. Integrating a wider range of species into commercial use, while prioritizing sustainable practices, can alleviate pressure on high-valued species and contribute to the preservation of marine biodiversity.

Declarations

This work appears in whole or in part in a thesis/dissertation.

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Tables and Figures

Table 1: Compound annotation table identifying key metabolites from the ^{1}H-NMR spectral comparison between *Holothuria cinerascens* and *Pseudocnella sykion*. HC represents *Holothuria cinerascens*; PS represents *Pseudocnella sykion.*

Metabolite	^{1}H-NMR Chemical shifts (ppm) – HC	^{1}H-NMR Chemical shifts (ppm) – PS	Chenomx (ppm)	Human Metabolome Database	Reference Chemical Shift (ppm)	Literature	Higher in:
Acetic acid	1.91	1.90	1.9	1.91	1.91	58	HC
Alanine	1.47 3.76	1.47 3.77	1.5 3.8	1.47 3.77	1.47 3.77	32; 43; 50	HC
Arginine	1.66 1.72 1.88 1.92 3.22 3.78	1.64 1.71 1.88 1.91 3.23 3.76	1.6 1.7 1.9 1.9 3.2 3.8 6.7 7.2	1.53 1.59 1.86 3.17 3.77	1.68 1.90 3.23 3.74	32; 50	HC
Ascorbic acid	3.70 3.74 4.05 4.52	3.71 3.75 4.02 4.51	3.7 3.8 4.0 4.5	3.44 3.73 4.72	4.03	7	EVEN
Betaine	3.25 3.84	3.25 3.84	3.3 3.9	3.25 3.89	3.25 3.89	50	HC
Butyric acid	0.87 1.51 2.14	0.87 1.51 2.14	0.9 1.5 2.2	0.88 1.54 2.14	0.86 2.18	2	HC
D-Glucose	3.23 3.38 3.40 3.45 3.48 3.52 3.70 3.72 3.75 3.82 3.83 3.88 4.64 5.21	3.23 3.38 3.40 3.45 3.48 3.52 3.70 3.72 3.75 3.82 3.83 3.88 4.64 5.21	3.2 3.4 3.4 3.5 3.5 3.5 3.7 3.7 3.8 3.8 3.8 3.9 4.6 5.2	3.39 3.52 3.72 3.81 4.03 5.21	3.23 3.40 3.46 3.52 3.73 3.82 3.88 4.63 5.22	50	HC
Dimethyl sulfone	3.16	3.16	3.1	3.14	3.14	58	EVEN
D-Xylose	3.21 3.31 3.42 3.48 3.60 3.61 3.66 3.69 3.88 4.52 5.19	3.21 3.31 3.42 3.48 3.60 3.61 3.66 3.69 3.88 4.52 5.19	3.2 3.3 3.4 3.5 3.6 3.6 3.7 3.7 3.7 3.9 4.6 5.2	3.52 3.68 3.68 3.87 4.00 4.63	3.68-3.72 4.07-4.10 4.08-4.11 4.29-4.33 4.43-4.46 4.78	41	HC
Ethanolamine	3.12 3.82	3.13 3.81	3.1 3.8	3.13 3.81	3.15 3.83	43; 53	Even
Galactose	3.47 3.64 3.69 3.71 3.73 3.73	3.48 3.64 3.69 3.71 3.73 3.73	3.5 3.6 3.7 3.7 3.7 3.7	3.52 3.74		43	HC

	3.76 3.79 3.84 3.93 3.98 4.07 4.57 5.27	3.76 3.79 3.92 3.98 4.07 4.57 5.17	3.8 3.8 3.8 3.9 4.0 4.1 4.6 5.3	3.82 3.95 4.12 5.21	3.81 4.07		
Glutamate	2.03 2.13 2.37 2.41 3.76	2.03 2.13 2.37 2.41 3.76	2.0 2.1 2.3 2.4 3.8	2.06 2.11 2.32 2.36 3.75	2.08 2.34 3.74	50	HC
Glutamine	2.11 2.14 2.38 2.40 3.76	2.11 2.14 2.41 2.46 3.76	2.1 2.1 2.4 2.5 3.8 6.9 7.6	2.12 2.42 2.46 3.76	2.12 2.44 3.70	2	HC
Glycerol	3.53 3.61 3.71	3.53 3.61 3.71	3.6 3.6 3.8	3.43 3.60 3.61 3.85	3.54 3.62	2	EVEN
Glycine	3.55	3.55	3.6	3.55	3.57	41; 43; 50	EVEN
Lysine	1.43 1.50 1.71 1.87 1.91 3.00 3.75	1.43 1.50 1.71 1.87 1.91 3.00 3.75	1.4 1.5 1.7 1.9 1.9 3.0 3.8	1.43 1.72 1.90 2.12 3.01 3.74	1.46 1.70 1.89 3.01 3.74	32	HC
Malonate	3.13/3.14	3.13	3.1	3.11	3.11	43	EVEN
Mannose	3.36 3.58 3.67 3.74 3.81 3.84 3.86 3.89 4.89 5.17	3.37 3.56 3.65 3.72 3.75 3.80 3.84 3.86 3.90 3.92 3.93 4.90 5.18	3.4 3.6 3.7 3.7 3.7 3.8 3.8 3.8 3.9 3.9 3.9 3.9 4.9 5.2	3.46 3.71 3.74 3.80 3.82 3.99 5.21	3.38 3.81 3.82 4.74 4.91 5.45	56	EVEN
Methylmalonate	1.24 3.18	1.24 3.18	1.2 3.2	1.23 3.16	1.21	7; 53	HC
Myo-inositol	3.27 3.52 3.60 4.04	3.26 3.52 3.61 4.06	3.3 3.5 3.6 4.1	3.27 3.54 3.54 3.61 3.61 4.05	3.52 3.60	7	HC
Proline	1.99 2.03 2.05 2.34 3.32 4.12	1.99 2.03 2.05 2.34 3.32 4.12	2.0 2.0 2.1 2.3 3.3 3.4 4.1	1.47 1.75 2.20 2.99 3.40 3.57	1.99 2.06 2.33 3.33 3.40 4.12	32	PS
Pyruvate	2.36	2.36	2.4	2.36	2.40	58	HC
Sarcosine	N/A	2.72 3.60	2.7 3.6	2.73 3.60 3.78	2.7 3.6	15	PS
Taurine	3.25	3.25	3.5	3.25	3.27	43	HC

	3.42	3.42	3.4	3.41	3.43		
Threonine	1.31 3.60 4.21	1.31 3.58 4.25	1.3 3.6 4.3	1.32 3.58 4.24	1.33 3.58 4.25	50	HC
Valerate	0.86 1.31 1.51 2.15	0.87 1.29 1.50 2.15	0.6 1.3 1.5 2.2	0.88 1.33 1.51 2.17	1.20 1.56	53	HC

Table 2: Comparative analysis of metabolites identified in ESI negative mode by UPLC-QTOF-MS in *Holothuria cinerascens* (HC) and *Pseudocnella sykion* (PS).

Rt	Observed m/z	Monoisotopic mass	Empirical formula (neutral)	Detected in Species HC	Detected in Species PS	Compound	References
6.36	359.0743	360.08452	$C_{18}H_{16}O_8$		X	Rosmarinic acid	44 ChemSpider ID: 4474888/445104
6.87	209.0834	210.09258	$C_8H_{18}O_4S$	X		Dibutyl sulfate or Octyl sulphate or 6-methylheptyl sulphate; DBE=0	26; 59 ChemSpider ID: 11738 Or 8547
6.90	195.0620	196.07693	$C_7H_{16}O_4S$	X		Heptyl hydrogen sulphate	ChemSpider ID: 158845
6.92	209.0836	210.09258	$C_8H_{18}O_4S$	X		Aliphatic organosulphate or Dibutyl sulfate or Octyl sulphate or 6-methylheptyl sulphate; DBE=0	26; 59 ChemSpider ID: 11738 Or 8547
7.08	209.0838	210.09258	$C_8H_{18}O_4S$	X			
7.16	209.0844	210.09258	$C_8H_{18}O_4S$	X			
7.55	209.0842	210.09258	$C_8H_{18}O_4S$	X			
7.59	209.0840	210.09258	$C_8H_{18}O_4S$	X			
7.75	209.0839	210.09258	$C_8H_{18}O_4S$	X			
7.84	209.0838	210.09258	$C_8H_{18}O_4S$	X			
8.25	237.1141	238.12388	$C_{10}H_{22}O_4S$	X		Diamyl sulphate or Decyl sulphate or 8-methylnoyl hydrogen sulfate; DBE=0	26 ChemSpider ID: 13674908 Or 8564
8.30	1199.5144	1200.52337	$C_{54}H_{88}O_{27}S$	X		Holothurin A1 or Holothurin A4 or Scabraside D; DBE=11	5; 35; 36 ChemSpider ID: 29368578
	1199.5177	1199.51915	$C_{66}H_{80}O_{19}Na$	X		Unknown; DBE=26	
8.39	223.0990	224.10823	$C_9H_{20}O_4S$	X	X	2,6-Dimethylheptyl hydrogen sulfate or 6-Methyloctyl sulfate or 7-methyloctyl hydrogen sulphate; DBE=0	26 ChemSpider ID: 23327239 or 141892 PubChem CID: 141892
8.48	1197.5016	1198.50772	$C_{54}H_{86}O_{27}S$	X		Holothurin A (Arenicolaside A) or 17-Hydroxyfuscocineroside B (Scabraside B) or 25-Hydroxyfuscocineroside B	5; 6; 35; 36
	1197.4994	1198.50882	$C_{70}H_{79}O_{14}Sna$	X		Unknown	
8.72	1181.5083	1182.51280	$C_{54}H_{86}O_{26}S$	X	X	Scabraside A or 24-dehydroechinoside A or 17-dehydroxyholothurin A (Fuscocineroside C) or Fuscocineroside B	5; 6; 16; 35; 36
	1181.5016	1182.50693	$C_{61}H_{82}O_{21}S$	X	X	Unknown; DBE=21	
8.92	237.1149	238.12388	$C_{10}H_{22}O_4S$	X	X	Diamyl sulphate or Decyl sulphate or 8-methylnoyl hydrogen sulfate; DBE=0	26 ChemSpider ID: 13674908 Or 8564

8.96	1199.5203	1200.52337	$C_{54}H_{88}O_{27}S$	X	X	Holothurin A1 or Holothurin A4 or Scabraside D; DBE=11	5; 35; 36
9.14	1197.4954	1198.50184	$C_{61}H_{82}O_{22}S$	X	X	Unknown; DBE=21	
	1197.4998	1197.50100	$C_{70}H_{78}O_{14}Sna$	X	X	Unknown; DBE=31	
9.30	367.2151	368.22326	$C_{17}H_{36}O_6S$	X	X	2-[2-(Tridecyloxy)ethoxy]ethyl hydrogen sulfate or C13-alkyl-2-ethoxysulfate (C13-AE2S); DBE=0	ChemSpider ID: 2289709 or PubChem CID: 3023544
9.36	1181.5095	1182.51280	$C_{54}H_{86}O_{26}S$	X	X	Scabraside A or 24-dehydroechinoside A or 17-dehydroxyholothurin A (Fuscocineroside C) or Fuscocineroside B; DBE=12	5; 16; 35; 36
	1181.5028	1181.50257	$C_{52}H_{86}O_{26}Sna$	X	X	Unknown; DBE=9	
9.39	1181.5133	1182.52806	$C_{58}H_{86}O_{23}S$	X		Unknown	
	1181.5016	1182.50693	$C_{61}H_{82}O_{21}S$	X		Unknown; DBE=21	
	1181.5017	1182.51040	$C_{52}H_{87}O_{26}Sna$	X		Unknown; DBE=9	
9.39	669.2791	670.28704	$C_{29}H_{50}O_{15}S$	X		32-([(4-Methylphenyl)sulfonyl]oxy)-3,6,9,12,15,18,21,24,27,30-decaoxadotriacontan-1-oic acid DBE=5	ChemSpider ID: 123961299
9.49	367.2159	368.22326	$C_{17}H_{36}O_6S$	X	X	2-[2-(Tridecyloxy)ethoxy]ethyl hydrogen sulfate or C13-alkyl-2-ethoxysulfate (C13-AE2S); DBE=0	ChemSpider ID: 2289709 or PubChem CID: 3023544
9.67	1147.5514	1148.55897	$C_{59}H_{88}O_{20}S$	X		Unknown, DBE=16	
	1147.5510	1148.55906	$C_{53}H_{89}O_{25}Na$	X		Unknown; DBE=9 **Note:** Co-elution detected	
9.75	1181.5153	1182.52806	$C_{58}H_{86}O_{23}S$	X		Unknown	
		1182.51040	$C_{52}H_{87}O_{26}Sna$	X		Unknown; DBE=9	
	1181.5050	1182.51280	$C_{54}H_{86}O_{26}S$	X		Scabraside A or 24-dehydroechinoside A or 17-dehydroxyholothurin A (Fuscocineroside C) or Fuscocineroside B; DBE=12	16; 35; 36
9.71	251.1201	252.13953	$C_{11}H_{24}O_4S$	X		Undecyl hydrogen sulfate or 4®,8-Dimethylnonyl Sulfate **Note:** Straight-chain or branched molecule; DBE=0	26 ChemSpider ID: 109828 or 34448657 PubChem CID: 24796507
9.75	251.1299	252.13953	$C_{11}H_{24}O_4S$	X			
9.84	251.1298	252.13953	$C_{11}H_{24}O_4S$	X	X		
9.94	1295.5319	1296.53862	$C_{66}H_{88}O_{24}S$		X	Unknown; DBE=23	
10.11	669.2792	670.28704	$C_{29}H_{50}O_{15}S$	X		32-([(4-Methylphenyl)sulfonyl]oxy)-3,6,9,12,15,18,21,24,27,30-decaoxadotriacontan-1-oic acid; DBE=5	ChemSpider ID: 123961299
10.12	251.1304	252.13953	$C_{11}H_{24}O_4S$		X	Undecyl hydrogen sulfate o®(R),8-Dimethylnonyl Sulfate; DBE=0	26 ChemSpider ID: ID: 109828 or 34448657 PubChem CID: 24796507
10.43	671.2966	672.30269	$C_{29}H_{52}O_{15}S$	X		Unknown; DBE=4	
10.60	1177.5172	1178.52376	$C_{48}H_{90}O_{30}S$	X		Unknown	
	1177.5054	1178.51202	$C_{62}H_{82}O_{20}S$	X	X	Unknown; DBE=22	
	265.1478	266.15518	$C_{12}H_{26}O_4S$	X	X	Lauryl sulfate/Dodecyl hydrogen sulphate; DBE=0	22 ChemSpider ID: 8448 PubMed CID: 8778

10.60	859.3828	860.38642	$C_{41}H_{64}O_{17}S$	X		Holothurin B (Axilogoside A) or Holothurin B4 or Nobiliside B or Nobiliside II (Ananaside C); DBE=10	5; 6; 16; 17; 35; 36 ChemSpider ID: 25052543 PubChem CID: 23674754
	859.3779	859.37619	$C_{39}H_{64}O_{17}SNa$	X		Unknown; DBE=7	
10.71	711.2894	712.29775	$C_{52}H_{40}O_3$	X		Unknown; DBE=33	
		712.29534	$C_{50}H_{41}O_3Na$	X		Unknown; DBE=30	
10.88	713.3018	714.31326	$C_{31}H_{54}O_{16}S$	X		35-([(4-Methylphenyl)sulfonyl]oxy)-3,6,9,12,15,18,21,24,27,30,33-undecaoxapentatriacontan-1-oic acid; DBE=5	17 ChemSpider ID: 123961295
10.96	843.3841	844.39151	$C_{41}H_{64}O_{16}S$	X		Holothurin B3 or 24-dehydroechinoside B; DBE=10	6; 35; 36 ChemSpider ID: 76818153
11.07	655.2979	656.30778	$C_{29}H_{52}O_{14}S$	X		Unknown; DBE=3	
11.23	1127.6241	1128.63363	$C_{67}H_{93}O_{11}SNa$		X	Unknown; DBE=21	
11.25	613.2875	614.29721	$C_{27}H_{50}O_{13}S$	X		Unknown; DBE=3	
11.65	655.2977	656.30778	$C_{29}H_{52}O_{14}S$	X		Unknown; DBE=3	
11.76	655.3008	656.30778	$C_{29}H_{52}O_{14}S$	X		Unknown; DBE=3	
11.78	619.2897	620.29613	$C_{34}H_{45}O_9Na$	X	X	Unknown; DBE=12	
12.05	619.2881	620.29613	$C_{34}H_{45}O_9Na$	X	X	Unknown; DBE=12	

"X" = compound presence; "DBE" = Double Bond Equivalent. Average percentage error = 0.0061%.

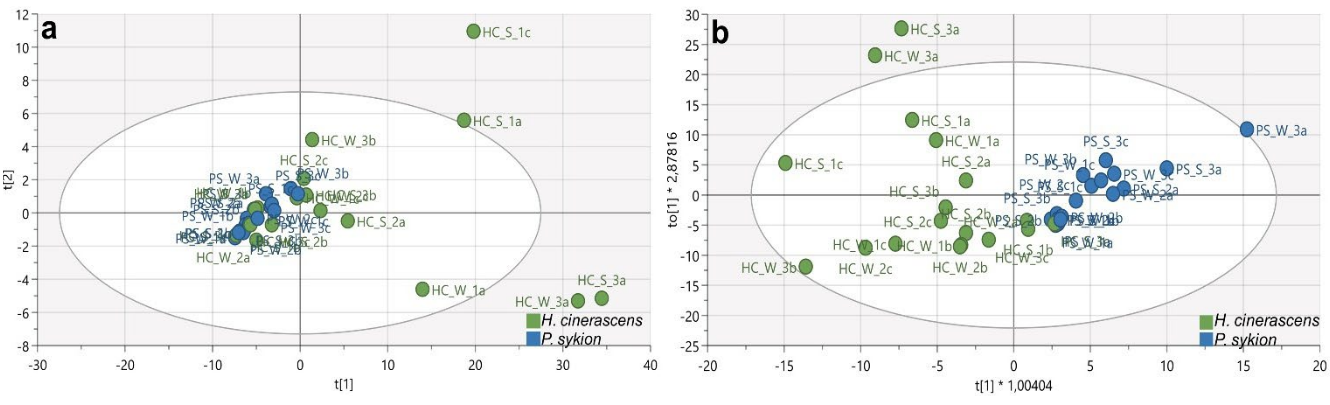


Figure 1: *PCA-X results (a) and OPLS-DA score plot (b) from the ^{1}H-NMR spectral data showing the metabolic separation between H. cinerascens and P. sykion.*

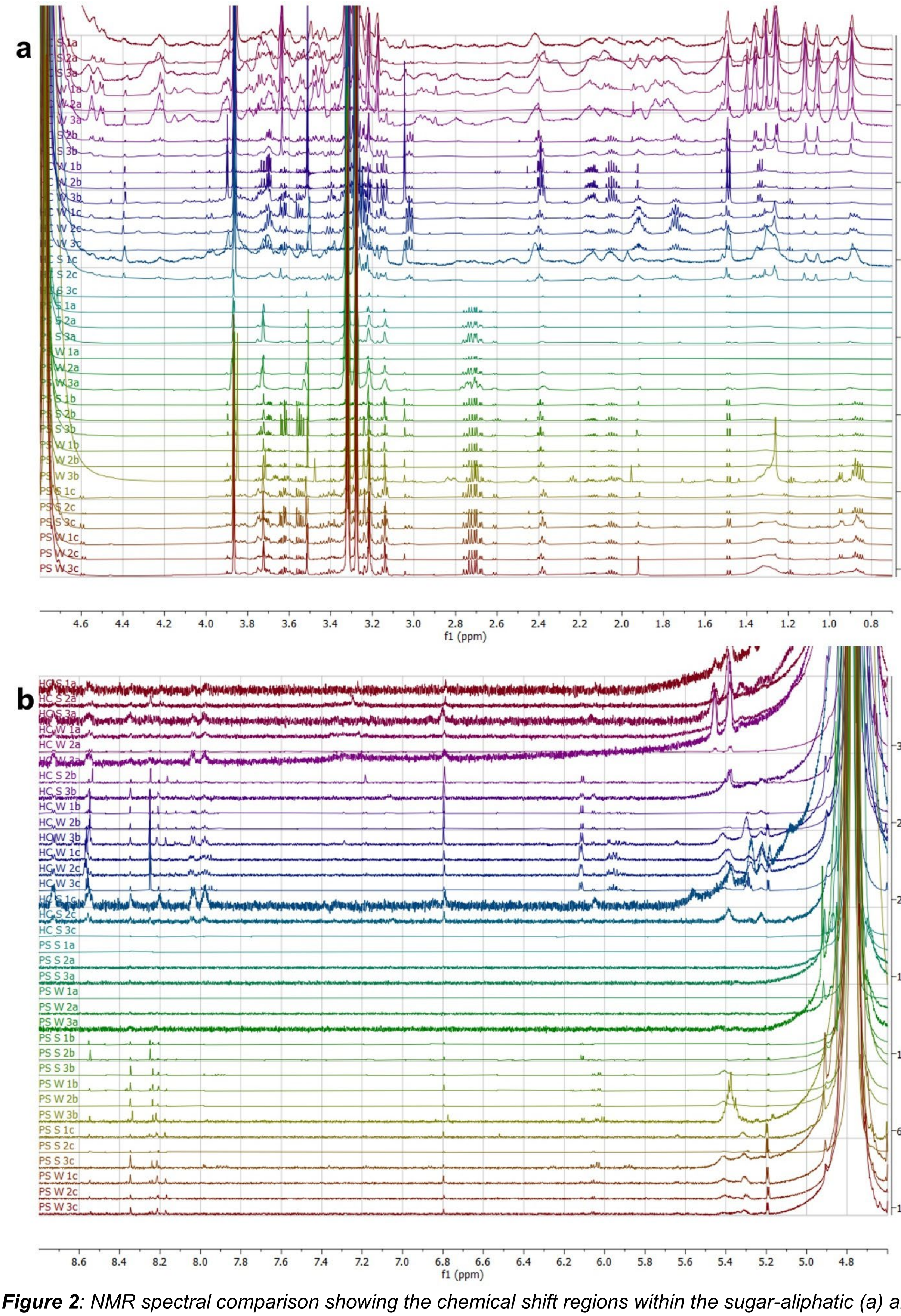


***Figure 2**: NMR spectral comparison showing the chemical shift regions within the sugar-aliphatic (a) and aromatic (b) zones that differentiate Holothuria cinerascens ("HC") and Pseudocnella sykion (“PS”).*

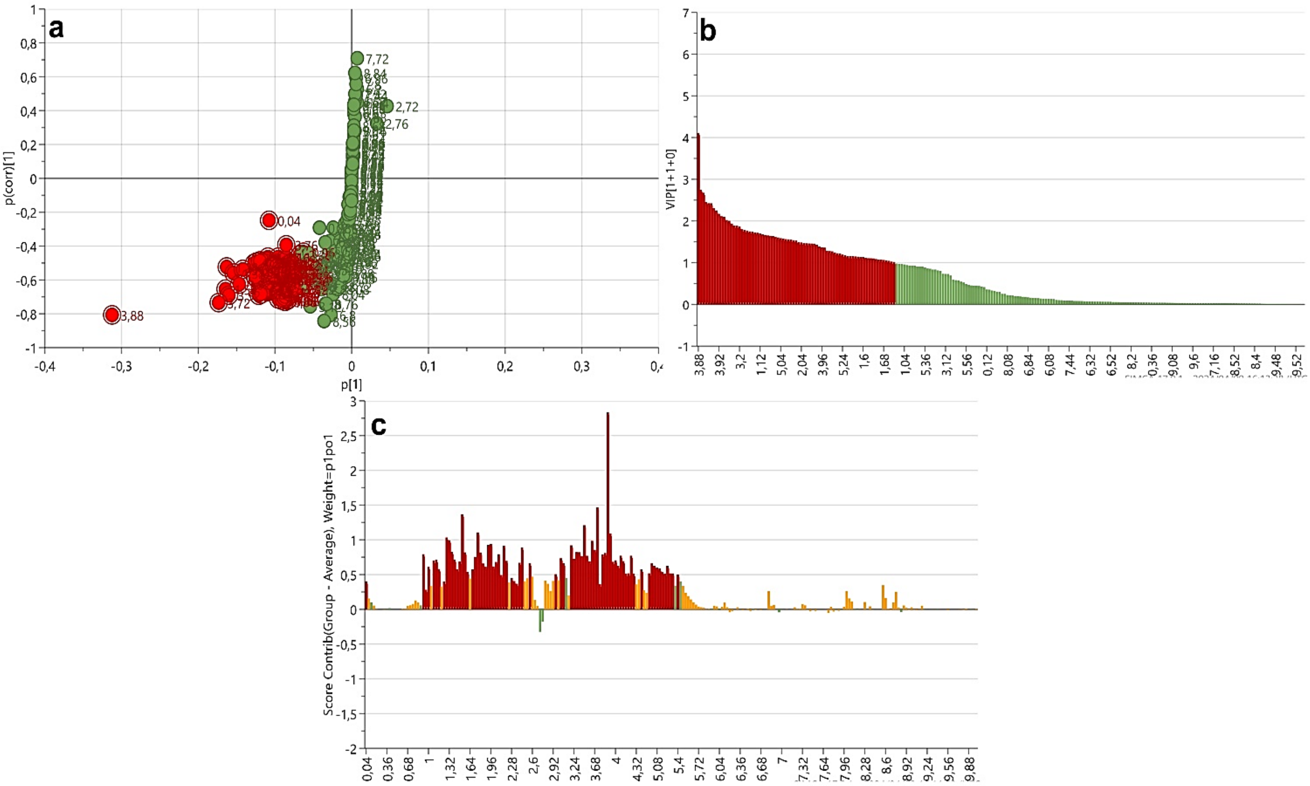


Figure 3: *S-plot (a), VIP score plot (b), and contribution loadings plot (c) showing the* [1]*H-NMR spectral regions positively associated with H. cinerascens that contribute to the clustering and separation of the tissue extracts between H. cinerascens and P. sykion. Bars above the x-axis represent chemical shift regions positively associated with H. cinerascens.*

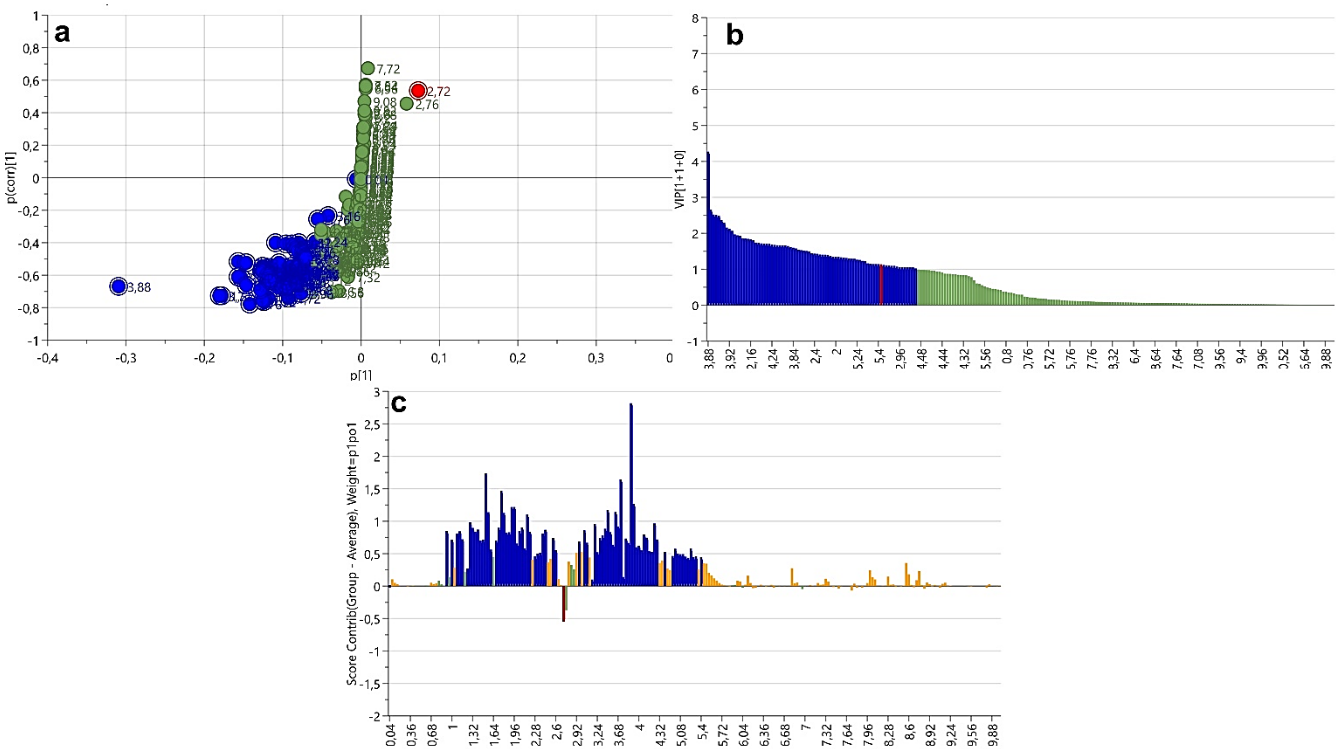


Figure 4: *S-plot (a), VIP score plot (b), and contribution loadings plot (c) excluding the "HC_S_3a" and "HC_W_3a" samples, showing the* [1]*H-NMR spectral regions positively associated with H. cinerascens that contribute to the clustering and separation of the tissue extracts between H. cinerascens and P. sykion. Bars above the x-axis represent chemical shift regions positively associated with H. cinerascens.*

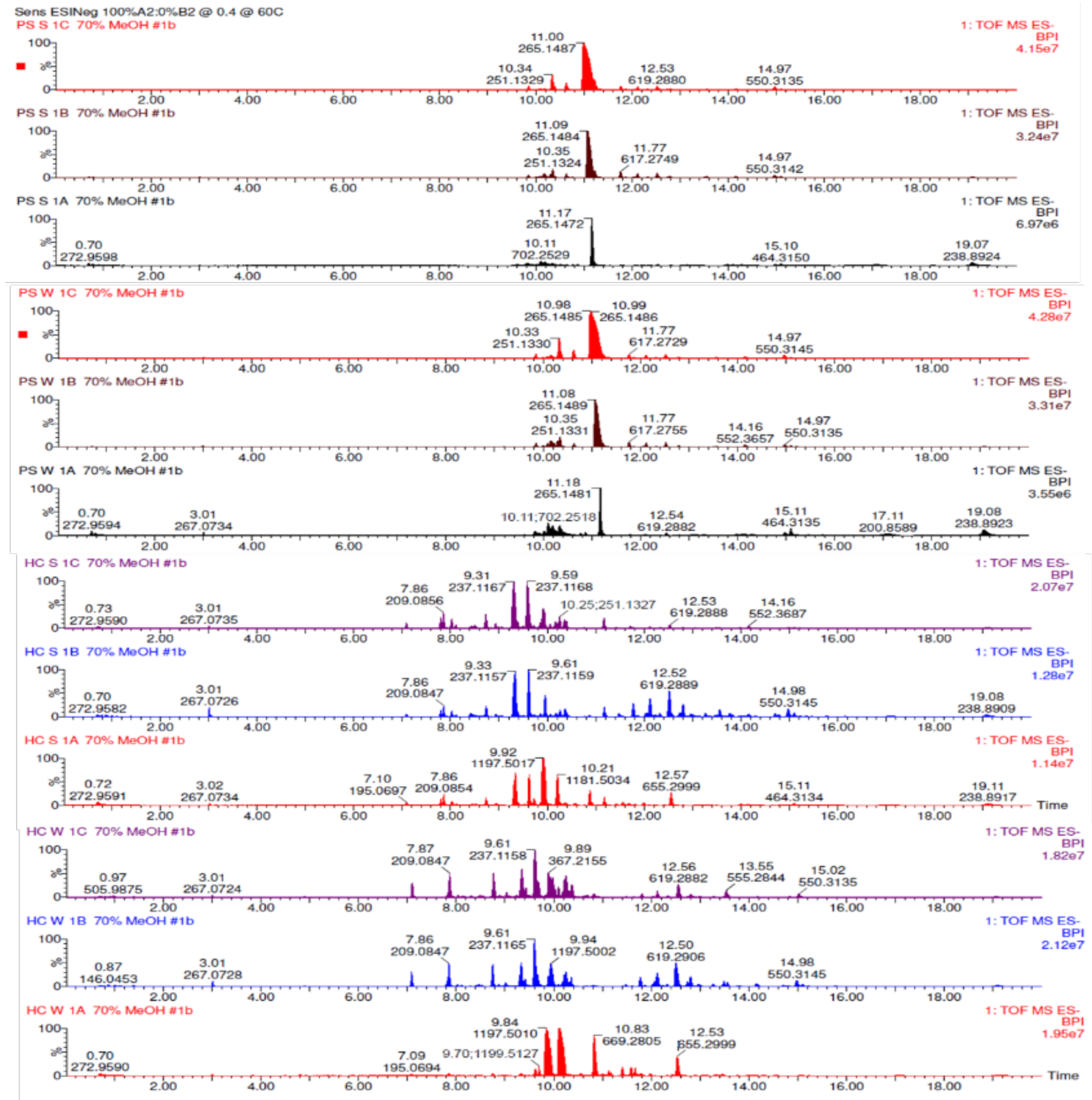
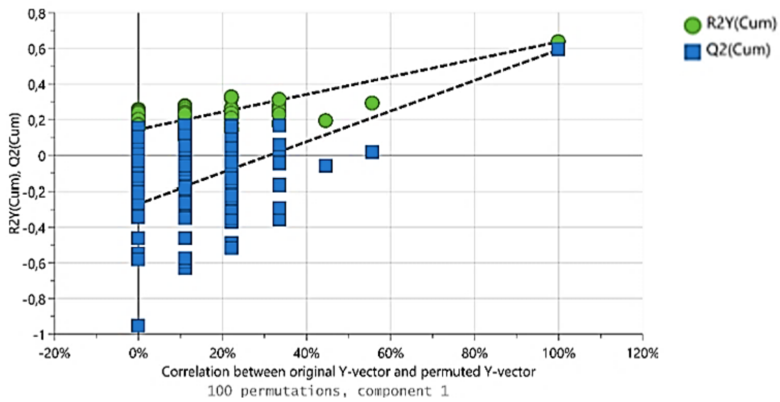


***Figure 5:** UPLC-QTOF-MS spectral comparison showing the spectral peaks that differentiate Holothuria cinerascens ("HC") and Pseudocnella sykion ("PS").*



***Supplementary Figure 1:** Permutation validation of the metabolic comparison between H. cinerascens and P. sykion. Permutation R^2 = (0.0; 0.145) and Q^2 = (0.0; -0.269)*

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