Proteomic method to extract, concentrate, digest and enrich peptides from fossils with coloured (humic) substances for mass spectrometry analyses

Elena R. Schroeter, Kevin Blackburn, Michael B. Goshe and Mary H. Schweitzer

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Final acceptance: 23 July 2019

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Note: This manuscript was transferred from another Royal Society journal without peer review.

Review History
RSOS-181433.R0 (Original submission)

Review form: Reviewer 1

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Is it clear how to access all supporting data?
Yes
Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No

Recommendation?
Major revision is needed (please make suggestions in comments)

Comments to the Author(s)
Schroeter et al. build in the current manuscript on their earlier works in an effort to mitigate a potentially major issue in palaeoproteomics - the presence of large amounts of humics in fossil bone interfering during protein extraction and MS analysis. This is a relevant question to resolve, as is indicated by the recent literature on this topic as well.

The major issue with the manuscript is that an internal control is lacking. That is, claims of relevance made in the paper of enhanced protein extraction through either Method 1 and Method 2 refer to extractions and data presented in a previous paper (Cleland et al., 2016, PRSB; Cleland et al., 2012, Plos One). However, recent research in protein preservation has shown remarkable variation of within-bone protein preservation, necessitating that extraction comparisons are performed on homogenized bone powders. Further issues with comparing the enhanced results derive from an absence in protein or peptide quantification, differences in MS set-up, and column chemistry. Therefore, the main conclusion of the paper could be seen as suggestive.

Minor comments:
- Lines 54-65. This is one massive sentence. Please break up in smaller sentences.
- Lines 85-98. The four elements of the used extraction methods are presented as novel. However, each of these has already appeared in the palaeoproteomic literature at least once. To avoid the suggestion that all four elements are introduced here for the first time, references to relevant literature are justified.
- Line 96: The re-occurring claim that this bone specimen has a particularly high humic content requires quantification.
- Line 126 vs line 147: You mention "a small piece", but later refer to approx. 250 mg., which is a large piece of bone, especially of this age. These two statements are in conflict, especially as "small" frequently refers to samples under 20mg in the isotope, collagen, and bone proteome literature.
- Line 257: I think you mean smaller than 3 kDa, not larger than 3 kDa.

Review form: Reviewer 2

Is the manuscript scientifically sound in its present form?
No

Are the interpretations and conclusions justified by the results?
No

Is the language acceptable?
Yes
Is it clear how to access all supporting data?
Not Applicable

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
I do not feel qualified to assess the statistics

Recommendation?
Major revision is needed (please make suggestions in comments)

Comments to the Author(s)
It is suggested that the methods section of the article should be revised to improve its clarity.

Decision letter (RSOS-181433.R0)

21-Nov-2018

Dear Dr Schroeter,

The editors assigned to your paper ("A proteomic method to extract, concentrate, digest, and enrich peptides from fossils with high humic content for mass spectrometry analyses") have now received comments from reviewers. We would like you to revise your paper in accordance with the referee and Associate Editor suggestions which can be found below (not including confidential reports to the Editor). Please note this decision does not guarantee eventual acceptance.

Please submit a copy of your revised paper before 14-Dec-2018. Please note that the revision deadline will expire at 00.00am on this date. If we do not hear from you within this time then it will be assumed that the paper has been withdrawn. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office in advance. We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Editors, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available, we may invite new reviewers.

To revise your manuscript, log into http://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision. Revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you must respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". Please use this to document how you have responded to the comments, and the adjustments you have made. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response.
In addition to addressing all of the reviewers’ and editor's comments please also ensure that your revised manuscript contains the following sections as appropriate before the reference list:

• Ethics statement (if applicable)
If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

• Data accessibility
It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article's supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data have been deposited in an external repository this section should list the database, accession number and link to the DOI for all data from the article that have been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

If you wish to submit your supporting data or code to Dryad (http://datadryad.org/), or modify your current submission to dryad, please use the following link: http://datadryad.org/submit?journalID=RSOS&manu=RSOS-181433

• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.

• Authors’ contributions
All submissions, other than those with a single author, must include an Authors’ Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

All contributors who do not meet all of these criteria should be included in the acknowledgements.

We suggest the following format:
AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.
Once again, thank you for submitting your manuscript to Royal Society Open Science and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Kind regards,
Andrew Dunn
Royal Society Open Science Editorial Office
Royal Society Open Science
openscience@royalsociety.org

on behalf of Prof Kevin Padian (Subject Editor)
openscience@royalsociety.org

Comments to Author:

Reviewers' Comments to Author:
Reviewer: 1

Comments to the Author(s)
Schroeter et al. build in the current manuscript on their earlier works in an effort to mitigate a potentially major issue in palaeoproteomics - the presence of large amounts of humics in fossil bone interfering during protein extraction and MS analysis. This is a relevant question to resolve, as is indicated by the recent literature on this topic as well.

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-Line 126 vs line 147: You mention "a small piece", but later refer to approx. 250 mg., which is a large piece of bone, especially of this age. These two statements are in conflict, especially as "small" frequently refers to samples under 20mg in the isotope, collagen, and bone proteome literature.
-Line 257: I think you mean smaller than 3 kDa, not larger than 3 kDa.

Reviewer: 2

Comments to the Author(s)
It is suggested that the methods section of the article should be revised to improve its clarity.
Author's Response to Decision Letter for (RSOS-181433.R0)

See Appendix A.

RSOS-181433.R1 (Revision)

Review form: Reviewer 2

Is the manuscript scientifically sound in its present form?  
No

Are the interpretations and conclusions justified by the results?  
No

Is the language acceptable?  
Yes

Is it clear how to access all supporting data?  
Yes

Do you have any ethical concerns with this paper?  
Yes

Have you any concerns about statistical analyses in this paper?  
No

Recommendation?  
Major revision is needed (please make suggestions in comments)

Comments to the Author(s)  
The additional supplemental table does not address the reviewer's previously expressed concerns.

Review form: Reviewer 3

Is the manuscript scientifically sound in its present form?  
Yes

Are the interpretations and conclusions justified by the results?  
Yes

Is the language acceptable?  
Yes
Decision letter (RSOS-181433.R1)

12-Mar-2019

Dear Dr Schroeter:

Manuscript ID RSOS-181433.R1 entitled "A proteomic method to extract, concentrate, digest, and enrich peptides from fossils with abundant humics for mass spectrometry analyses" which you submitted to Royal Society Open Science, has been reviewed. The comments of the reviewer(s) are included at the bottom of this letter.

Please submit a copy of your revised paper before 04-Apr-2019. Please note that the revision deadline will expire at 00.00am on this date. If we do not hear from you within this time then it will be assumed that the paper has been withdrawn. In exceptional circumstances, extensions may be possible if agreed with the Editorial Office in advance. We do not allow multiple rounds of revision so we urge you to make every effort to fully address all of the comments at this stage. If deemed necessary by the Editors, your manuscript will be sent back to one or more of the original reviewers for assessment. If the original reviewers are not available we may invite new reviewers.

To revise your manuscript, log into http://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with
Decisions." Under "Actions," click on "Create a Revision." Your manuscript number has been appended to denote a revision. Revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you must respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". Please use this to document how you have responded to the comments, and the adjustments you have made. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response.

In addition to addressing all of the reviewers' and editor's comments please also ensure that your revised manuscript contains the following sections before the reference list:

- **Ethics statement**
  If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

- **Data accessibility**
  It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article's supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data have been deposited in an external repository this section should list the database, accession number and link to the DOI for all data from the article that have been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

- **Competing interests**
  Please declare any financial or non-financial competing interests, or state that you have no competing interests.

- **Authors’ contributions**
  All submissions, other than those with a single author, must include an Authors’ Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

All contributors who do not meet all of these criteria should be included in the acknowledgements.

We suggest the following format:
AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.
• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.

Once again, thank you for submitting your manuscript to Royal Society Open Science and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Kind regards,
Andrew Dunn
Royal Society Open Science Editorial Office
Royal Society Open Science
openscience@royalsociety.org

on behalf of Prof Kevin Padian (Subject Editor)
openscience@royalsociety.org

Editorial office comments:
Unfortunately, in an earlier round of review, the comments supplied by a reviewer that were intended for the author as well as the Editor were not communicated to the authors, as the comments had been included in the comments to the Editor. Following our standard processes, these comments were not forwarded to the authors (comments supplied in confidence to the Editor are not generally communicated to the authors); however, from the comments of the reviewer, it would seem these comments were intended for the author as well. With this in mind, we now supply the substantive elements of these comments immediately below, with the current round of comments provided below this. We apologise for this state of affairs, and hope you’ll address the comments in this round of review.

==Original comments==
there are inconsistencies between different statements in the materials and methods section. For example, on page 6 it is stated that the bone sample was divided into 10 aliquots of which 5 were treated with NaOH and then all 10 samples were treated with the 400/200/4 solution. But on the following page (p7) it is stated that there were three sample sets, one of which had NaOH but no 400/200/4 treatment, which is inconsistent with the description given on the preceding page. Figure 1 is of little help in understanding the experimental protocol. Overall, this article if revised could make a useful contribution to the palaeoproteomics literature but as submitted I think that it fails to meet the journal's requirement that published work should be "scientifically sound, in which the methodology is rigorous and the conclusions fully supported by the data".

Reviewer comments to Author:
Reviewer: 3

Comments to the Author(s)
I have reviewed the revisions made by the authors in order to address the first round of reviews. The authors have responded in an adequate way to all queries.

I do not wish to add any more comments as it would not be fair on the authors, but I would strongly suggest to change the title as follows "A proteomic method to extract, concentrate, digest
and enrich peptides from sub-fossil bones with colored (humic) substances for mass spectrometry analyses". This is because: a) the paper only deals with bone, which has different characteristics than other substrates for paleoproteomics (for example skipping demineralization is not appropriate in all cases); b) "fossil" is a difficult word and often implies no organics and old age - using "sub-fossil" is useful to indicate that you do have organics; c) "abundant" still implies quantitation, which is not available in the paper; d) technically the authors do not provide chemical evidence that the colored substances are in fact "humics" - better to focus on the color aspect.

Reviewer: 2

Comments to the Author(s)
The additional supplemental table does not address the reviewer's previously expressed concerns.

Author's Response to Decision Letter for (RSOS-181433.R1)

See Appendix B.

RSOS-181433.R2 (Revision)

Review form: Reviewer 4

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No

Recommendation?
Accept with minor revision (please list in comments)

Comments to the Author(s)
In the manuscript entitled "A proteomic method to extract, concentrate, digest, and enrich peptides from fossils with colored (humic) substances for mass spectrometry analyses", Schroeter
and co-authors describe an protocol to extract ancient protein residues from bone samples highly infiltrated with coloured (humic) substances. As the authors point out, the challenge the method aims at addressing is significant. The study clearly describes how the method presented clearly leads to higher recoveries in terms of proteins peptides and PSMs. In the current version the manuscript is clearly readable, the experimental protocol is described with enough detail to guarantee its reproducibility and the literature review is pertinent and exhaustive. The evidence presented is solid and reliable and it fully supports the conclusions reported. I have no major comments, just a very few minor suggestions to marginally improve clarity. Specifically:

Line 121: "Two extraction methods were tested; one that..." could be replaced with: "Two extraction methods were tested: one that..."

It could be useful to specify the pH of some of the buffers and solutions used for sample preparation, e.g. ABC.

Line 194: "... an adapted, centrifugal..." could be replaced with: "... an adapted centrifugal...".

Line 317: The title "Efficiency and Modifications" is in my opinion a bit vague and does not effectively describe the content of the following paragraph. I suggest to replace it with something a bit more specific, such as: "Efficiency and Possible Variations of the Experimental Workflow".

Globally, I think the authors did a very good job.

Decision letter (RSOS-181433.R2)

10-Jul-2019

Dear Dr Schroeter:

On behalf of the Editors, I am pleased to inform you that your Manuscript RSOS-181433.R2 entitled "A proteomic method to extract, concentrate, digest, and enrich peptides from fossils with colored (humic) substances for mass spectrometry analyses" has been accepted for publication in Royal Society Open Science subject to minor revision in accordance with the referee suggestions. Please find the referees' comments at the end of this email.

The reviewers and Subject Editor have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the comments and revise your manuscript.

• Ethics statement
If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

• Data accessibility
It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article's supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials.
such as statistical tools, protocols, software etc can be accessed. If the data has been deposited in an external repository this section should list the database, accession number and link to the DOI for all data from the article that has been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

If you wish to submit your supporting data or code to Dryad (http://datadryad.org/), or modify your current submission to dryad, please use the following link: http://datadryad.org/submit?journalID=RSOS&manu=RSOS-181433.R2

• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.

• Authors’ contributions
All submissions, other than those with a single author, must include an Authors’ Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

All contributors who do not meet all of these criteria should be included in the acknowledgements.

We suggest the following format:
AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.

Please note that we cannot publish your manuscript without these end statements included. We have included a screenshot example of the end statements for reference. If you feel that a given heading is not relevant to your paper, please nevertheless include the heading and explicitly state that it is not relevant to your work.

Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript before 19-Jul-2019. Please note that the revision deadline will expire at 00.00am on this date. If you do not think you will be able to meet this date please let me know immediately.

To revise your manuscript, log into https://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under “Manuscripts with Decisions”. Under "Actions," click on "Create a Revision." You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.
When submitting your revised manuscript, you will be able to respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referees.

When uploading your revised files please make sure that you have:

1) A text file of the manuscript (tex, txt, rtf, docx or doc), references, tables (including captions) and figure captions. Do not upload a PDF as your "Main Document".
2) A separate electronic file of each figure (EPS or print-quality PDF preferred (either format should be produced directly from original creation package), or original software format)
3) Included a 100 word media summary of your paper when requested at submission. Please ensure you have entered correct contact details (email, institution and telephone) in your user account
4) Included the raw data to support the claims made in your paper. You can either include your data as electronic supplementary material or upload to a repository and include the relevant doi within your manuscript
5) All supplementary materials accompanying an accepted article will be treated as in their final form. Note that the Royal Society will neither edit nor typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details where possible (authors, article title, journal name).

Supplementary files will be published alongside the paper on the journal website and posted on the online figshare repository (https://figshare.com). The heading and legend provided for each supplementary file during the submission process will be used to create the figshare page, so please ensure these are accurate and informative so that your files can be found in searches. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

Once again, thank you for submitting your manuscript to Royal Society Open Science and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Kind regards,
Alice Power
Editorial Coordinator
Royal Society Open Science
openscience@royalsociety.org

on behalf of Kevin Padian (Subject Editor)
openscience@royalsociety.org

Reviewer comments to Author:
Reviewer: 4

Comments to the Author(s)
In the manuscript entitled "A proteomic method to extract, concentrate, digest, and enrich peptides from fossils with colored (humic) substances for mass spectrometry analyses", Schroeter and co-authors describe an protocol to extract ancient protein residues from bone samples highly infiltrated with coloured (humic) substances. As the authors point out, the challenge the method
Aims at addressing is significant. The study clearly describes how the method presented clearly leads to higher recoveries in terms of proteins, peptides, and PSMs. In the current version, the manuscript is clearly readable, the experimental protocol is described with enough detail to guarantee its reproducibility, and the literature review is pertinent and exhaustive. The evidence presented is solid and reliable, and it fully supports the conclusions reported. I have no major comments, just a very few minor suggestions to marginally improve clarity. Specifically:

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Globally, I think the authors did a very good job.

Author’s Response to Decision Letter for (RSOS-181433.R2)

See Appendix C.

Decision letter (RSOS-181433.R3)

23-Jul-2019

Dear Dr Schroeter,

I am pleased to inform you that your manuscript entitled "A proteomic method to extract, concentrate, digest, and enrich peptides from fossils with colored (humic) substances for mass spectrometry analyses" is now accepted for publication in Royal Society Open Science.

You can expect to receive a proof of your article in the near future. Please contact the editorial office (openscience_proofs@royalsociety.org and openscience@royalsociety.org) to let us know if you are likely to be away from e-mail contact. Due to rapid publication and an extremely tight schedule, if comments are not received, your paper may experience a delay in publication.

Royal Society Open Science operates under a continuous publication model (http://bit.ly/cpFAQ). Your article will be published straight into the next open issue and this will be the final version of the paper. As such, it can be cited immediately by other researchers. As the issue version of your paper will be the only version to be published I would advise you to check your proofs thoroughly as changes cannot be made once the paper is published.
On behalf of the Editors of Royal Society Open Science, we look forward to your continued contributions to the Journal.

Kind regards,

Lianne Parkhouse
Editorial Coordinator
Royal Society Open Science
openscience@royalsociety.org

on behalf of Kevin Padian (Subject Editor)
openscience@royalsociety.org

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Read Royal Society Publishing's blog: https://blogs.royalsociety.org/publishing/
We thank both reviewers for their comments and suggestions. Below, we have detailed the edits we’ve made to the manuscript in response to their suggestions. Additionally, we have uploaded a “tracked changes” version of the current manuscript that shows all of these changes in the document (as well a few minor typos we have corrected).

Reviewer: 1

Comments to the Author(s)
Schroeter et al. build in the current manuscript on their earlier works in an effort to mitigate a potentially major issue in palaeoproteomics - the presence of large amounts of humics in fossil bone interfering during protein extraction and MS analysis. This is a relevant question to resolve, as is indicated by the recent literature on this topic as well.

The major issue with the manuscript is that an internal control is lacking. That is, claims of relevance made in the paper of enhanced protein extraction through either Method 1 and Method 2 refer to extractions and data presented in a previous paper (Cleland et al., 2016, PRSB; Cleland et al., 2012, Plos One). However, recent research in protein preservation has shown remarkable variation of within-bone protein preservation, necessitating that extraction comparisons are performed on homogenized bone powders. Further issues with comparing the enhanced results derive from an absence in protein or peptide quantification, differences in MS set-up, and column chemistry. Therefore, the main conclusion of the paper could be seen as suggestive.

We thank reviewer 1 for their comments. We agree that a direct comparison is needed to quantify the relative contributions the many differences between the old study and the new study each had to the increase in protein identification, and to account for intra-bone variation. However, the main contribution of this paper is that this combined method allows protein extracts dark with humics to be concentrated and clarified without losing low-abundance proteins (e.g., NCPs). Thus, to address the reviewer’s concerns we have softened the language to shift focus from relative “improvement” over an older method, to the fact that this combined workflow allows sensitive analyses of low-abundance proteins in fossils containing abundant humics (as supported by its retrieval of 12 non-collagenous proteins). Additionally, we have added a statement acknowledging the limits of the comparison that can be made between the two methods.

Modifications to the text include:

Abstract—We have changed “This method allows better and more sensitive analyses of low-abundance proteins in fossils containing humics” to “This workflow allows analyses of low-abundance proteins in fossils containing humics.”

Results—We have added the following sentence to the paragraph comparing the 2015 study with the current study:

“A direct comparison of the HCl-ABC method employed in Cleland 2015¹ and this combined method is needed to quantify the relative contributions to the observed increase in efficiency from the various differences in their workflows (e.g., extraction buffers, MS instrument, column packing) and to fully account for possible intra-bone variation in
protein preservation in this specimen.\(^2\,^3\) Regardless, this combined workflow obtained 15 additional proteins previously unreported for this specimen, supporting its overall improvement in recovery.”

Conclusion—We have changed “Because this method allows better and more sensitive MS analyses of low-abundance proteins in fossils with a high humic content” to “Because this workflow allows MS analyses of low-abundance proteins in fossils with abundant humic content”

Minor comments:
- Lines 54-65. This is one massive sentence. Please break up in smaller sentences.

We have separated this list into individual sentences. That section now reads as follows:

“For example, these substances interfere with colormetric assays used for protein quantitation (e.g., Bradford and bicinchoninic acid (BCA) assays),\(^8\) preventing accurate measurement of protein for subsequent analyses. They also bind silver-stain, generating false-positives in SDS-PAGE gels, or can pre-stain gel lanes and obscure chemical stains applied to separated proteins.\(^9\,^10\) They can contaminate the isotopic content of ancient bone and interfere with stable isotope analyses.\(^2\,^11\) In mass spectrometry experiments, humic substances can cause ion-suppression during electrospray ionization (ESI),\(^3\,^12\) leading to either depressed signal intensity or no signal (regardless of the presence of protein in the sample). Further, they can clog analytical columns or spray tips during liquid chromatography,\(^3\) causing unstable spray and/or the loss of samples, columns, or spray tips. Even when these substances do not clog analytical columns, they can generate a build-up of particulate matter on the ion optics, which can suppress or prevent signal during analyses of all subsequent samples, necessitating expensive and time-consuming instrument cleaning.”

- Lines 85-98. The four elements of the used extraction methods are presented as novel. However, each of these has already appeared in the palaeoproteomic literature at least once. To avoid the suggestion that all four elements are introduced here for the first time, references to relevant literature are justified.

To clarify that this is a method combined from elements previously utilized in paleoproteomics and adapted for humic removal, we have added the following sentence to the introduction:

“Although the individual components of this method have been variously applied in previous paleoproteomic studies (e.g., non-demineralizing buffer,\(^4\,^5\) FASP\(^6\,^7\)) their combination and adaptation for humic removal has not yet been explored.”

Additionally, because during the course of this review a new paper was published discussing the removal of humics from fossil bones for proteomics, we have added the following sentence to the paragraph of the introduction that talks about previous methods to remove humics from protein extracts:
Cleland et al.\(^5\) employed a method utilizing both a non-demineralizing buffer and magnetic beads to separate humics from proteins in unconcentrated protein extracts.

-Line 96: The re-occurring claim that this bone specimen has a particularly high humic content requires quantification.

A quantitative comparison of the humic content of this moa fossil versus typical fossil specimens is beyond the scope of this paper. Thus, we have modified sentences in the abstract, introduction, and conclusion to avoid suggesting the humic content of this fossil is exceptionally high rather than simply abundant.

Modifications to the text include:

- **Title**—We have changed “fossils with high humic content” to “fossils with abundant humics”
- **Abstract**—We have changed the phrase “a moa fossil with very high humic content” to “a moa fossil dark with humic content”
- **Abstract**—We have changed the phrase “in fossils with high humic content” to “fossils with humic content”
- **Introduction**—We have changed the phrase “a moa fossil with high humic content” to “a moa fossil with humic content”
- **Conclusion**—We have changed the phrase “protein extracts from a moa fossil with a very high humic content” to “protein extracts from a moa fossil inferred from their dark color to have a high humic content”
- **Conclusion**—We have changed “fossils with high humic content” to “fossils with abundant humic content.”

-Line 126 vs line 147: You mention "a small piece", but later refer to approx. 250 mg., which is a large piece of bone, especially of this age. These two statements are in conflict, especially as "small" frequently refers to samples under 20mg in the isotope, collagen, and bone proteome literature. We have removed “small” from line 126, and now simply refer to “a piece of dark cortical bone.”

-Line 257: I think you mean smaller than 3 kDa, not larger than 3 kDa. We did indeed mean “less than.” We have corrected this typo.

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Reviewer: 2

Comments to the Author(s)

It is suggested that the methods section of the article should be revised to improve its clarity. We thank reviewer 2 for this recommendation. Because the reviewer did not provide specific instructions or recommendations, to improve clarity, we constructed a supplemental table (Supplemental Table 1) that breaks down each phase of the extraction and sample preparation procedures into a series of steps. The location of each action (e.g., in tube, in filter), the reagent used and the volume of it added, the incubation time and temperature, the centrifugation time and speed, and the number of times an action is performed/repeated, are listed in a concise format, to complement the more detailed description provided in the text.

To direct readers to this table, we have added the sentence, “Supplementary Table 1 provides a concise breakdown of the steps comprising the extraction, FASP, and stage tipping procedures,” to the end of the first paragraph in the Protein Extraction Section of the Methods.

Additionally, the previous Supplementary Table 1 has now been changed to Supplementary Table 2.
A proteomic method to extract, concentrate, digest, and enrich peptides from fossils with abundant humic for mass spectrometry analyses

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ABSTRACT

Humic substances are breakdown products of decaying organic matter that co-extract with proteins from fossils. These substances are difficult to separate from proteins in solution, and interfere with analyses of fossil proteomes. We introduce a method combining multiple recent advances in extraction protocols to both concentrate proteins from fossil specimens with high humic content, and remove humics, producing clean samples easily analyzed by mass spectrometry (MS). This method includes: 1) a non-demineralizing extraction buffer that eliminates protein loss during the demineralization step in routine methods; 2) filter-aided sample preparation (FASP) of peptides, which concentrates and digests extracts in one filter, allowing the separation of large humics after digestion; 3) centrifugal stage-tipping, which further clarifies and concentrates samples in a uniform process performed simultaneously on multiple samples. We apply this method to a moa fossil (~800–1000 yr) dark with humic content, generating colorless samples and enabling the detection of more proteins with greater sequence coverage than previous MS analyses on this same specimen. This workflow allows analyses of low-abundance proteins in fossils containing humics, and thus may widen the range of extinct organisms and regions of their proteomes we can explore with MS.

Keywords: paleoproteomics, fossils, humic substances, protein, mass spectrometry, moa

BACKGROUND
Humic substances are breakdown products of decaying organic matter\(^1\) that, to the frustration of many protein researchers, co-extract with proteins from fossils (e.g.,\(^2\)) and soil (e.g.,\(^3\)). These complex, hydrophilic, dark-colored substances have a large molecular weight range (< 10 kDa to > 100 kDa), and are notoriously difficult to separate from proteins in solution.\(^1,4,7\) This causes a host of problems for a number of downstream chemical analyses when conducting paleoproteomics of fossil tissues. For example, these substances interfere with colorimetric assays used for protein quantitation (e.g., Bradford and bicinchoninic acid (BCA) assays),\(^8\) preventing accurate measurement of protein for subsequent analyses. They also bind silver-stain, generating false-positives in SDS-PAGE gels, or can pre-stain gel lanes and obscure chemical stains applied to separated proteins.\(^9,10\) They can contaminate the isotopic content of ancient bone and interfere with stable isotope analyses.\(^2,11\) In mass spectrometry experiments, humic substances can cause ion-suppression during electrospray ionization (ESI),\(^3,12\) leading to either depressed signal intensity or no signal (regardless of the presence of protein in the sample). Further, they can clog analytical columns or spray tips during liquid chromatography,\(^3\) causing unstable spray and/or the loss of samples, columns, or spray tips. Even when these substances do not clog analytical columns, they can generate a build-up of particulate matter on the ion optics, which can suppress or prevent signal during analyses of all subsequent samples, necessitating expensive and time-consuming instrument cleaning.

Although the analytical problems generated by humic substances can be quite severe, fossilized bone containing high amounts of humic substances may potentially be valuable sources of paleoproteomic information. Humic acids can play a role in diagnostically cross-linking collagen...
molecules in a manner similar to leather tanning or fixation with formaldehyde. This suggests that fossils with humic content may be more likely to preserve original, minimally altered collagen molecules incorporated into diagenetic complexes with exogenous humics. However, any such preserved proteins are not readily accessible by MS, requiring them to be both extracted and separated from interfering humic substances.

A number of approaches have been employed to remove humic substances from bone and soil samples. For example, Szpak et al. tested the efficiency of both sodium hydroxide (NaOH) and ultrafiltration to remove humics from archaeological bone for isotopic analyses, and found that post-demineralization with HCl, sequential incubations with NaOH could be used to remove the alkali-soluble fraction of humic substances from samples. Qian et al. developed a method that, subsequent to enzymatic digestion of soil protein extracts, used ultrafilters to remove humics that had been precipitated from an acidified (~pH 3) peptide digest. Cleland et al. employed a method utilizing both a non-demineralizing buffer and magnetic beads to separate humics from proteins in unconcentrated protein extracts.

Here, we introduce a method that builds on these findings and several recent advances in protein extraction protocols to optimize the recovery of ancient proteins for paleoproteomics from fossils that contain a high amount of humic substances. This method: 1) utilizes a non-demineralization extraction buffer, because the demineralization phase of traditional bone protein extractions has been shown to be a source of loss, particularly for non-collagenous proteins; 2) allows for the concentration of a relatively large amount of extraction supernatant into a small sample volume, which is crucial for fossil specimens with a low abundance of preserved protein, but challenging
for those that also contain humic substances, which co-elute with protein; 3) uses a modified filter-aided sample preparation (FASP) protocol\textsuperscript{16} to filter humic substances from proteins/peptides both before and after digestion; 4) removes remaining humic substances with centrifugal stage-tip protocol\textsuperscript{17} that can prepare multiple samples simultaneously and consistently. \textit{Although the individual components of this method have been variously applied in previous paleoproteomic studies (e.g., non-demineralizing buffer,\textsuperscript{13,18} FASP\textsuperscript{19-20}) their combination and adaptation for humic removal has not yet been explored.} We apply this combined method to a moa fossil\textsuperscript{21} with humic content, from which preserved proteins were previously characterized by liquid chromatography-tandem mass spectrometry (LC-MS/MS),\textsuperscript{22} to demonstrate its utility for preparing fossils samples for paleoproteomic analyses.

\textbf{MATERIALS & METHODS}

\textbf{Specimen}

We analyzed fossilized (800–1000 yr) moa cortical bone (MOR OFT255, courtesy J. Horner and Museum of the Rockies) recovered from New Zealand cave deposits.\textsuperscript{21} This specimen was used because: 1) despite its relatively young age, the dark coloration of both the whole cortical fragments (described as very dark brown to nearly black)\textsuperscript{23} and the extraction supernatants produced in previous studies\textsuperscript{22-23} suggests a high content of humic substances; 2) Previous studies of this specimen using different extraction methods\textsuperscript{23} and MS analyses\textsuperscript{22} successfully produced evidence of protein preservation, including collagen I alpha 1 and alpha 2 sequences with biological and diagenetic post-translational modifications (PTMs). Thus, MOR OFT255
represents an ideal test case, in which both bone proteins and humic substances are known to be present in the tissue, allowing us monitor the effect of humic removal on peptide recovery.

Protein Extraction

Two extraction methods were tested; one that used a single incubation in a non-demineralization reagent for protein solubilization, and one that incorporated a brief pre-treatment of the bone powder with NaOH, followed by an extraction identical to the first in all other respects. NaOH has been used to remove “pigments” from bone prior to collagen extraction, humic acids from archaeological bone, or to improve the efficiency of some bone protein extraction methods. Therefore, we tested this extraction method both with and without an NaOH pre-treatment step to assess its usefulness in both removing humics and extracting peptides. Figure 1 illustrates the basic workflow of the developed extraction procedure (NaOH pretreatment step not shown).

Supplementary Table 1 provides a concise breakdown of the steps comprising the extraction, FASP, and stage tipping procedures.

A piece of dark cortical bone was ground to a fine powder in a sterilized mortar and pestle. Bone powder (50-55 mg/tube) was aliquoted into 10, 1.5 ml Protein LoBind centrifugation tubes (Eppendorf). An equal number of empty tubes, containing no bone powder but treated identically to bone samples in every way, were also prepared (i.e., “blank” control samples). Five tubes of each set received 1 ml of 0.1 M NaOH, were vortexed to mix, then were incubated at 4°C for 4 h with rocking. The remaining five aliquots and blanks were stored dry at 4°C for the same length
of time. Samples were then centrifuged at 15,000 rcf for 15 min, and NaOH fractions were collected and frozen at -80˚C to await concentration.

All 10 aliquots of bone powder then received 600 µL of a solution containing 400 mM ammonium phosphate dibasic (APD), 200 mM ammonium bicarbonate (ABC), and 4 M guanidine HCl (GuHCl), hereafter referred to as “400/200/4” for brevity. Samples were vortexed to mix, incubated at 75˚C in a heat block overnight, then subsequently centrifuged at 15,000 rcf for 15 min and the 400/200/4 supernatant was collected. Fractions of 400/200/4 that followed NaOH pretreatment were kept separate from those that had been incubated with untreated bone to enable comparison. Supernatants were frozen at -80˚C to await concentration.

**Extract Concentration and Digestion**

Supernatants were pooled into three distinct samples, each representative of the extract from ~250 mg of bone powder (or five blank tubes): 1) 0.1 M NaOH (~5 ml of supernatant), 2) 400/200/4 that had been applied after the NaOH pretreatment (~3 ml, hereafter “NaOH-400/200/4”), and 3) 400/200/4 from untreated bone (~3 ml). The NaOH and NaOH-400/200/4 samples, together, comprise “Method 1,” and the 400/200/4 sample from untreated bone represents the sole incubation of “Method 2.” All samples were concentrated and buffer exchanged at RT using 3 kDa MWCO centrifugal filters (Amicon-Ultra; Millipore). For each sample, ~400 µL of supernatant was added to a filter, then centrifuged for 20 min at 14,000 rcf. The flow-through was then discarded and the process repeated in increments of 300 µL (to accommodate sample held back in filter without overfilling to the lip of the seal) until all
supernatant (3–5 ml) had been concentrated into a single filter unit. Only one filter was used per sample to reduce the adsorptive loss of protein on the filter membranes. After the last centrifugation, filters containing concentrated sample received 300 µL of 50 mM ABC and were then stored overnight at 4˚C, in a humidity chamber wrapped in parafilm to prevent filters from drying. Samples were centrifuged at 14,000 rcf for 30 min at RT, then buffer exchanged into 300 µL ABC twice more, mixing the ABC in the solvent reservoir of the filtration device prior to centrifugation by pumping the solution in and out of the pipette tip several times to ensure the whole sample had been mixed.

Concentrated samples were prepared for LC-MS/MS analysis using a modified version of the standard filter aided sample preparation (FASP) protocol. Each sample received 50 µL of 10 mM DTT, were incubated in a heating oven at 60˚C, then centrifuged at 14,000 rcf for 15 min, followed by two exchanges of 200 µL of 8 M urea (in 100 mM Tris, pH 8.5) and centrifugation for 15 min at 14,000 rcf. Samples were alkylated with 100 µL of 50 mM ioadacetamide (in 8 M urea) for 20 min in the dark at RT, then incubated with 3 exchanges of 200 µL of 8 M urea followed by centrifugation for 20 min at 14,000 rcf. Samples were then exchanged three times into 300 µL of 50 mM ABC followed by centrifugation for 30 min at 14,000 rcf.

Filters were transferred into new collection tubes, and each sample received 500 ng of trypsin solubilized in 50 µL of 50 mM ABC (well-mixed with the sample in the filter by pumping the pipette as described above). Samples were allowed to digest overnight in a humidity chamber at 37˚C in a heating oven. After digestion, filters were centrifuged at 14,000 rcf for 30 min. Then, 40 µL of 50 mM ABC were added to the filter, mixed with the pipette to suspend any sediment.
at the bottom, and samples were centrifuged again at 14,000 rcf. This step was then repeated
twice, and the final filtered sample of ~150 µL was transferred to a new 1.5 ml Protein LoBind
centrifugation tube and received 3 µL of formic acid (FA) to quench any further digestion and
precipitate any remaining acid-insoluble humics out of solution while allowing peptides to
remain suspended. Samples were then frozen at -80°C to await stage-tipping.

Sample Purification and Concentration

Samples were stage-tipped using self-made tips following a protocol by Rappspilber et al. and
an adapted, centrifugal protocol proposed by Yu et al. Briefly, stage tips were assembled by
placing two discs of C18 membrane (Empore, 3M), perforated from a larger sheet with a blunt-
point, 16 gauge needle (I.D. 1.19 mm, Hamilton), in the tip of a 200 µL pipette tip (Figure S1A).
Stage tips were then placed in a collection tube made by perforating the top of a 1.5 ml Protein
LoBind tube with a heated glass pasture pipette (Fisher) (Figure S1B). By placing stage tips in
collection tubes (Figure S1C), multiple samples were stage tipped simultaneously and uniformly
using a centrifuge. Three stage-tip assemblies received 20 µL of methanol, then centrifuged for
30 s at 1,000 rcf. Tips were wetted with 20 µL of 80% acetonitrile (ACN)/0.2% FA followed by
centrifugation for 30 s at 1,000 rcf, then equilibrated with 20 µL of 0.2% FA and centrifuged for
1 min at 1,000 rcf. Tips were placed in new collection tubes, and each concentrated sample
(~150 µL each) was then passed through a stage tip by centrifugation at 1,000 rcf for 3 min. The
filtered sample from the collection tube was removed and passed through the same stage tip to
allow an additional opportunity for peptides to bind to the C18 membrane. After this second
pass, the flow-through (2x filtered sample) was collected and stored at 80°C. Stage tips were then
washed with 40 µL of 0.2% FA, centrifuged for 2 min at 1,000 rcf, then the tips were moved to
new collection tubes and peptides were eluted with 20 µL of 80% ACN/0.2% FA. The peptide
elution step was repeated, resulting in a final sample volume of 40 µL. The final sample was
dried under vacuum centrifugation, then stored at -80°C until LC-MS/MS analysis could be
performed.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) Analysis

Samples were analyzed by LC-MS/MS analysis using an Easy-nLC 1000 UHPLC (Thermo
Scientific) coupled to an Orbitrap Elite mass spectrometer (Thermo Scientific). Samples were
reconstituted in 25 µL of 0.1% FA, then 5 µL injections were loaded onto a new nanoViper nano
trap column (Thermo Scientific; I.D. 100 µm, 2 cm, PepMap C18, 5 µm particles) and in-line
separated with a new PicoFrit capillary column (New Objective; I.D. 75 µm x 25 cm) with an
integrated PicoTip emitter, packed in-house with Reprosil C18-AQ (3 µm) stationary phase
using a flow rate of 300 nl/min. For separation the following linear gradients were applied,
where mobile phase A is 0.1% FA and mobile phase B is 100% ACN/0.1% FA: t = 0 min, 0% B;
t = 1 min, 7% B; t = 120 min, 40% B; t = 121 min, 95% B, t = 136 min, 95% B. The MS
acquisition was performed in positive ion mode with the following parameters: the 10 most
intense precursor ions were fragmented using HCD (35% normalized collision energy); isolation
window was 3 m/z; precursor and product ion resolution was set to 60,000 and 15,000,
respectively; precursor scan range was m/z 400–2000; both precursor and product ions were
analyzed in the orbitrap. Dynamic exclusion was enabled, with a repeat count of 2, a repeat
duration of 30 s, and an exclusion duration of 120 s. Three injections of each sample were performed, and identification results across these injections were combined for each sample.

Data Analyses

Raw data files from the LC-MS/MS acquisitions were processed using Proteome Discoverer 1.4.1.14 (Thermo Scientific) and searched against the NCBI Aves protein database (retrieved May 19, 2015) using Mascot (Matrix Science, version 2.5) and X!Tandem (The GPM, version CYCLONE 2010.12.01.1) using the following parameters: precursor mass tolerance = 10 ppm, fragment ion tolerance = 0.02 Da; missed cleavages = 2; non-specific cleavage = one end; fixed PTMs = carbamidomethylation (C); variable PTMs = oxidation (P), oxidation (K), deamidation (NQ). X!Tandem searched the additional variable PTMs: Glu > pyro-Glu @N; Gln > pyro-Glu @N; ammonia-loss @N. Protein/peptide identifications were validated in Scaffold (Proteome Software, version Scaffold_4.8.6). Peptide identifications established at > 95% probability were accepted; protein identifications established at > 99% probability and containing at least 2 unique peptides were accepted.

RESULTS & DISCUSSION

Sample Clarity

Although the initial moa bone extract, particularly the 400/200/4 supernatants, were dark in color even before concentration, the final MS-ready samples were nearly colorless (Figure 2) and
virtually indistinguishable from blank controls that were processed using (initially) empty tubes as samples (Figure S2). Initial humic-containing supernatants (Figure 2, Row 1) became increasing dark and opaque during concentration, and the flow-through during filtering was markedly lighter than the portion of the supernatant held back in the filter (Figure 3).

Additionally, although 3 kDa MWCO, 500 µL Amicon Ultra filter units can concentrate 500 µL samples into a ~30 µL volume (and did so in the negative controls), the in-filter volume of concentrated 400/200/4 bone samples was approximately ~150 µL (Figure 3). These observations indicate that the humic substances that co-extracted with the protein from the bone largely remained in the filter instead of passing through, which is consistent with the reported size of these substances (e.g., mostly >10 kDa) and the findings of Szpak et al. that pre-digestion ultrafiltration is not useful for separation of humics from protein extract. These humic substances remained in the filter even after digestion using the FASP protocol, allowing tryptically digested peptides (now ≤ 3 kDa) to be separated from these substances when passed through the filter during centrifugation. This separation was indicated by the drastic reduction in color and opaqueness between the concentrated samples before (Figure 3) and after (e.g., Figure 2E-F) digestion.

Post-FASP (and pre-stage tip) concentrated samples, while lighter in color than the initial extracts, still possessed some coloration, suggesting that some humic and/or other substances that might cause interference in MS were still present. Subsequent stage tipping of the samples removed these last vestiges of color, and the final eluted samples were nearly colorless (Figure 2, Row 3).
Notably, although NaOH pretreatment has been reported as an effective method of removing humic substances from fossil bone, the single NaOH pretreatment incubation employed here was not effective at removing a substantial amount of humics from the moa bone prior to protein solubilization, as evidenced by the pale color of this fraction (Figure 2A), and the lack of difference in color between pretreated and non-pretreated 400/200/4 fractions (Figure 2B-C). This difference may be because Szpack et al. applied the NaOH treatment to demineralized bone, whereas we applied it directly to ground bone powder.

Mass Spectrometry

Across all injections of moa extractions analyzed by LC-MS/MS, 20 bone proteins were identified, including eight collagen alpha chains (six different collagen types) and 12 non-collagenous proteins (NCPs) (Table 1). The total number of unique peptides, total peptide spectral matches (PSMs), and sequence coverage for each fraction and method by protein are listed in Supplementary Table 2. Only keratins (and no bone proteins) were identified in any of the blank (reagent) control extract injections.

Nearly all the proteins identified were recovered from both methods, which shared 18 of the 20 proteins (Table 1). The exceptions were periostin, which was only found in the “NaOH-400/200/4” fraction of Method 1, and prolargin, which was found in the “400/200/4 only” extract, or Method 2. However, because these proteins were both identified by a relatively low number of PSMs from their respective fractions (3–4 PSMs; Table 1), this is likely a stochastic variation caused by data dependent acquisition and not a true difference between the methods.
In fact, when the number of unique (non-overlapping) peptides and percent sequence coverage identified from each protein is considered, the results from Methods 1 and 2 are nearly identical, with only slight variations that do not consistently favor either method (Figure 4). This suggests that both methods recover roughly the same subset of the moa bone proteome.

When the two fractions of Method 1 are considered separately, the “NaOH-400/200/4” fraction contains all the proteomic diversity obtained by this method; analysis of the “NaOH” pretreatment extract did not identify any unique proteins compared to the subsequent fraction (Table 1). Although the “NaOH” fraction possessed a greater number of collagen I PSMs than the NaOH-400/200/4 fraction (Table 1), the number of unique peptides and percent sequence coverage for all proteins were either substantially greater in the NaOH-400/200/4 fraction, or roughly equal between the two (STable 1). This suggests that unlike demineralization treatments, the NaOH pretreatment is not extracting a different subset of proteomic information than the following solubilization step. Further, the nearly identical results obtained from the pretreated (NaOH-400/200/4) and non-treated (400/200/4) fractions (Table1 and STable 1) indicate that NaOH pretreatment under these conditions does not improve the capacity of the 400/200/4 to solubilize proteins from the bone matrix.

Efficiency and Modifications

Previous LC-MS/MS analyses of this fossil produced sequences of collagen I, collagen II, and collagen V. The current method resulted in 15 additional proteins, including 12 NCPs, which ranged from 1.3% to 18.4% sequence coverage for a given protein (STable 1, Figure 4B). This
represents a substantial improvement over the previous study, which used a more common HCl
demineralization followed by ammonium bicarbonate solubilization, and utilized an entire gram
of fossil tissue as opposed to 250 mg. A direct comparison of the HCl-ABC method employed
in Cleland 2015 and this combined method is needed to quantify the relative contributions to
the observed increase in efficiency from the various differences in their workflows (e.g.,
extraction buffers, MS instrument, column packing) and to fully account for possible intra-bone
variation in protein preservation in this specimen. Regardless, this combined workflow
obtained 15 additional proteins previously unreported for this specimen, supporting its overall
improvement in recovery.

The design of this protocol as described here could potentially be modified to suit the needs of
individual studies. For example, we used conical 3 kDa MWCO ultrafilters (Milipore), to ensure
that small bone proteins or protein fragments were not potentially being lost. However,
researchers that utilize 10 kDa MWCO filters for their studies (e.g., ) might consider using
flat-bottomed filters, as there is some evidence that flat-bottomed ultrafilters may produce better
results by eliminating the dead space in the bottom of the filter. Additionally, larger centrifugal
filters (e.g., 2 ml or 4 ml) may be used to reduce the concentration time, though the depth of the
larger filters may be more difficult for performing FASP.

CONCLUSIONS

The method presented here combined a non-demineralization extraction buffer, filter-aided
concentration and digestion of extracts, and a centrifugal stage tip protocol to successfully
concentrate protein extracts from a moa fossil inferred from their dark color to have a high humic content, then remove those humics to allow LC-MS/MS analysis without mechanical or ionic interference from these substances. These analyses produced peptides from 20 bone proteins, 15 of which are newly identified in this specimen versus previous analyses using a more standard approach. Because this workflow allows MS analyses of low-abundance proteins in fossils with abundant humic content, it has the potential to widen the range of extinct organisms amenable to MS analyses, and increase regions of the proteome we can explore.

Although NaOH has been shown to aid in the removal of humic substances from a fossil bone pellet that has been demineralized with HCl prior to NaOH treatment, we found that a version of this method that incorporates a NaOH pre-treatment step to the protocol (Method 1) does not remove more humics, recover more proteins, or recover a substantially greater portion of the sequence of any protein than the method utilizing only 400/200/4 (Method 2). This indicates that NaOH pretreatment is not effective for humic removal in methods that utilize non-demineralizing solubilization reagents to avoid loss of NCPs in the demineralization fraction. We therefore recommend Method 2, as it produces nearly identical results with less cost in terms of time, supplies, and instrument fees.

DATA ACCESSIBILITY

All RAW mass spectrometry data and Scaffold results files are available at Dryad (http://dx.doi.org/10.5061/dryad.3tv1523).

All data files can be reviewed at: https://datadryad.org/review?doi=doi:10.5061/dryad.3tv1523.
COMPETING INTERESTS

The authors declare they have no competing interests.

AUTHOR’S CONTRIBUTIONS

MHS obtained the specimen, ERS performed molecular lab work and prepared samples, ERS, KB, and MBG performed MS analyses, ERS analyzed and interpreted data, and drafted the manuscript. All authors contributed to experimental design and gave final approval for publication.

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Figure 1. Diagram illustrating the extraction workflow of Method 2 (400/200/4 only). NaOH pretreatment step of Method 1 is not illustrated; however, NaOH supernatants were also concentrated, digested, and stage tipped as shown.

Figure 2. Progression of extracts through preparation for MS. (Row 1) Supernatant (NaOH or 400/200/4) immediately upon collection. Each tube represents one of five that were subsequently concentrated together. (Row 2) Supernatants after concentration and FASP using 3 kDa MWCO
Despite combining five tubes with high humic content, passing samples through the filter after digestion removed a large portion of the high-weight humics from the smaller peptides. (Row 3) Supernatants after stage tipping. Upon elution from the stage tips, samples were nearly colorless, suggesting that most inferring humic substances have been removed.

Figure 3. Concentrated 400/200/4 extract, before FASP digestion. Extracts dark with humic substances became increasingly dark and opaque during concentration, indicating that humic substances were too large to pass through the filter and co-concentrated with proteins. After proteolytic digestion, these humics remained in the filter while digested peptides passed through.

Figure 4. Comparison of the number of unique peptides (A) and sequence coverage (B) identified for each protein between Method 1 (NaOH + NaOH-400/200/4 fractions) and Method 2 (400/200/4 only). Both methods recover nearly identical amounts of unique peptides and sequence coverage for most proteins, suggesting that the inclusion of a NaOH pretreatment step in Method 1 does not provide additional proteome information over the sole 400/200/4 incubation of Method 2. These data represent the total, non-overlapping peptides and coverage relative to the mature form of the protein (i.e., without pro-peptide regions), combined from all three injections of each sample. See STable 1 for more details.
Appendix B

We thank both reviewers for their comments and suggestions. Below, we have detailed the edits we’ve made to the manuscript.

Reviewer: 2

There are inconsistencies between different statements in the materials and methods section. For example, on page 6 it is stated that the bone sample was divided into 10 aliquots of which 5 were treated with NaOH and then all 10 samples were treated with the 400/200/4 solution. But on the following page (p7) it is stated that there were three sample sets, one of which had NaOH but no 400/200/4 treatment, which is inconsistent with the description given on the preceding page. Figure 1 is of little help in understanding the experimental protocol. Overall, this article if revised could make a useful contribution to the palaeoproteomics literature but as submitted I think that it fails to meet the journal's requirement that published work should be "scientifically sound, in which the methodology is rigorous and the conclusions fully supported by the data."

We apologize for the lack of clarity on how our sample sets were obtained. First, we will explain here where we think the confusion arose from:

This paper tests two different methods for extraction.

- **Method 1:** incubates 5 aliquots of bone in NaOH, then collects and pools that NaOH. Then, the 400/200/4 solution is added to the same 5 aliquots of NaOH-treated bone, which is then collected and pooled into a separate sample. This generates 2 types of samples; a NaOH sample that was incubated with fresh, untreated bone (because it was the first step), and a 400/200/4 sample that is derived from the same, now NaOH-treated bone.

- **Method 2:** incubates 5 aliquots of fresh, untreated bone in the 400/200/4 solution and collects it. This generates 1 type of sample, a 400/200/4 sample that is derived from fresh, untreated bone.

Overall, Method 1 generates two sample types (NaOH treatment and NaOH-400/200/4 treatment) and Method 2 only one sample type (400/200/4 treatment). This is how we generated 3 sample types from 2 methods, and this is how we have a sample that has NaOH with no 400/200/4—it is generated as part of a two step method in which the first step (NaOH) and the second (400/200/4) are analyzed separately. Importantly, the NaOH-400/200/4 sample generated by this method does not combine the NaOH fraction and the 400/200/4 fraction of Method 1, it is only labeled this way to distinguish the 400/200/4 sample from bone that has had a NaOH pre-treatment from the 400/200/4 sample from bone that did not have the NaOH pre-treatment (Method 2).

We agree this has the potential to be confusing, and is not elucidated by the workflow figure we provided previously. Thus, to illustrate the origin of these three samples more plainly, we have added the following diagram and figure caption to the supplemental material:
Figure S1. Diagram of proteomic samples generated by this study. (A) Method 1: 5 aliquots of 50 mg ground moa bone powder were incubated in 0.1 M NaOH. The NaOH supernatant was subsequently collected and concentrated into a single sample, “NaOH.” These NaOH-treated bone pellets were then incubated in 400/200/4 solution, which was collected and concentrated into a second sample, “NaOH-400/200/4.” This label denotes that it was generated from bone that received 400/200/4 after pretreatment with NaOH. (B) Method 2: 5 aliquots of 50 mg ground moa bone powder were incubated directly in 400/200/4. The 400/200/4 supernatant was subsequently collected and concentrated into a solitary sample, “400/200/4.”

To direct readers to this diagram, we have added the following sentence to the first paragraph of the “Protein Extraction” section (lines 130-131):

“Supplementary Figure 1 illustrates a diagram of supernatant collection steps and sample generation for each method.”
We hope that this figure, combined with the previously provided workflow diagram and supplementary table detailing the steps of the procedure, is sufficiently clear for all readers.

Reviewer 3

I have reviewed the revisions made by the authors in order to address the first round of reviews. The authors have responded in an adequate way to all queries.

I do not wish to add any more comments as it would not be fair on the authors, but I would strongly suggest to change the title as follows "A proteomic method to extract, concentrate, digest and enrich peptides from sub-fossil bones with colored (humic) substances for mass spectrometry analyses". This is because: a) the paper only deals with bone, which has different characteristics than other substrates for paleoproteomics (for example skipping demineralization is not appropriate in all cases); b) "fossil" is a difficult word and often implies no organics and old age - using "sub-fossil" is useful to indicate that you do have organics; c) "abundant" still implies quantitation, which is not available in the paper; d) technically the authors do not provide chemical evidence that the colored substances are in fact "humics" - better to focus on the color aspect.

We thank the reviewer for this suggestion, and have adopted most of these comments into the new title:

“A proteomic method to extract, concentrate, digest, and enrich peptides from fossils with colored (humic) substances for mass spectrometry analyses”

We agree with the reviewer that “fossil” is a difficult word. There is not really a consensus in the paleo community whether “fossil” implies the complete lack of organics, or how much time can be encompassed by the term “sub-fossil.” Thus, we are concerned that if we use the term “sub-fossil” it will give readers the mistaken impression that this method is only applicable to very recently extinct species (i.e., the moa itself, as this sample is only 1000 years old). Because proteomic methods are increasingly applied to specimens tens to hundreds of thousands of years old without controversy, which few would categorize as “sub-fossil,” we think it is more appropriate to leave the term “fossil” in the title. We have made the other changes in both the main text and the supplement.
We thank the reviewer for their comments and suggestions. Below, we have detailed the edits we’ve made to the manuscript in accordance with their recommendations.

Reviewer comments to Author:
Reviewer: 4

Comments to the Author(s)
In the manuscript entitled "A proteomic method to extract, concentrate, digest, and enrich peptides from fossils with colored (humic) substances for mass spectrometry analyses", Schroeter and co-authors describe an protocol to extract ancient protein residues from bone samples highly infiltrated with coloured (humic) substances. As the authors point out, the challenge the method aims at addressing is significant. The study clearly describes how the method presented clearly leads to higher recoveries in terms of proteins peptides and PSMs. In the current version the manuscript is clearly readable, the experimental protocol is described with enough detail to guarantee its reproducibility and the literature review is pertinent and exhaustive. The evidence presented is solid and reliable and it fully supports the conclusions reported. I have no major comments, just a very few minor suggestions to marginally improve clarity.

Line 121: "Two extraction methods were tested; one that..." could be replaced with: "Two extraction methods were tested: one that..."

The semicolon in line 121 has been replaced with a colon.

It could be useful to specify the pH of some of the buffers and solutions used for sample preparation, e.g. ABC.

We agree, and have added the following pH information to the methods section:

- The pH for the 0.1 M NaOH solution has been added to line 127. The sentence now reads: Five tubes of each set received 1 ml of 0.1 M NaOH (pH ~12.8), were vortexed to mix, then were incubated at 4˚C for 4 h with rocking.

- The pH for the 400/200/4 solution has been added to line 144. The sentence now reads: “All 10 aliquots of bone powder then received 600 µL of a solution14 containing 400 mM ammonium phosphate diabasic (APD), 200 mM ammonium bicarbonate (ABC), and 4 M guanidine HCl (GuHCl) (pH 8.2), hereafter referred to as “400/200/4” for brevity.”

- The pH for the 50 mM ABC solution has been added to line 164. The sentence now reads: After the last centrifugation, filters containing concentrated sample received 300 µL of 50 mM ABC (pH 7.8) and were then stored overnight at 4˚C, in a humidity chamber wrapped in parafilm to prevent filters from drying.

Line 194: "... an adapted, centrifugal..." could be replaced with: "... an adapted centrifugal...".

Appendix C
The comma between ‘adapted’ and ‘centrifugal’ in line 194 has been removed.

Line 317: The title "Efficiency and Modifications" is in my opinion a bit vague and does not effectively describe the content of the following paragraph. I suggest to replace it with something a bit more specific, such as: "Efficiency and Possible Variations of the Experimental Workflow".

We agree, and have changed this heading (line 317) to the following:

“Efficiency and Potential Variations of the Experimental Workflow”

Globally, I think the authors did a very good job.

We thank the reviewer for their time and insights!

Editoral Comments:

• Ethics statement
If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

Please note that we cannot publish your manuscript without these end statements included. We have included a screenshot example of the end statements for reference. If you feel that a given heading is not relevant to your paper, please nevertheless include the heading and explicitly state that it is not relevant to your work.

We have added the following section heading and text to the end of the manuscript:

ETHICS
No humans, extant animals, or specimens requiring ethical approval were used in this study.

Thank you for resubmitting your paper. Before we proceed, we require for you to include a reference to your Dryad dataset in the reference list of your manuscript. Please include this reference, along with the DOI and both the 'for review' and 'for publication' URLs where they differ.

We have added a citation to the Dryad dataset to line 235 of the manuscript (“Raw data files$^26$ from the LC-MS/MS acquisitions…). The data set now appears as citation #26 in the reference list.