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Authors

McLoughlin, Nicole M

Keane, Romy E

Tidy, Rebecca J

et al.

Publication Date

2025

DOI

10.1007/978-1-0716-4298-6_6

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Authors:

Nicole M. Mc Loughlin
California Department of Fish and Wildlife, Wildlife Forensic Laboratory, Sacramento, CA, 95834 USA
ORCiD: 0009-0005-0893-5657
E: nmslattengren@ucdavis.edu
Romy E. Keane
ChemCentre, Forensic Science Laboratory, Bentley, Western Australia, 6102 Australia
ORCiD: 0009-0005-6270-8682
E: r.keane@ecu.edu.au
Rebecca J. Tidy
ChemCentre, Forensic Science Laboratory, Bentley, Western Australia, 6102 Australia
ORCiD: 0009-0007-7439-7775
E: r.tidy@ecu.edu.au
Zachary C. Goecker
University of California – Davis, Department of Environmental Toxicology, Davis, CA 95616 USA
ORCiD: 0000-0002-8788-5264
E: zachary.goecker@nist.gov
Robert H. Rice
University of California – Davis, Department of Environmental Toxicology, Davis, CA 95616 USA
ORCiD: 0000-0003-2508-4405
E: rhrice@ucdavis.edu
Joel P. A. Gummer
ChemCentre, Forensic Science Laboratory, Bentley, Western Australia, 6102 Australia
ORCiD: 0000-0002-3609-3398
E: JGummer@chemcentre.wa.gov.au
Ashley M. Spicer
California Department of Fish and Wildlife, Wildlife Forensic Laboratory, Sacramento, CA, 95834 USA
ORCiD: 0000-0002-2344-7062
E: Ashley.Spicer@wildlife.ca.gov
Glendon J. Parker*
University of California – Davis, Department of Environmental Toxicology, Davis, CA 95616 USA
ORCiD: 0000-0003-2937-5853
E: gjparker@ucdavis.edu
P: 801-243-4323
*Corresponding author

Running Head: *Hair Shaft Proteome*

i) Chapter Title:

Proteomic Analysis of Single Hairs

ii) Summary / Abstract:

Hair is a ubiquitous and robust mammalian tissue with biological, clinical, forensic, social and economic significance. The hair shaft proteome reflects both structural proteins, dominated by cuticular intermediate filament keratins and associated proteins, and proteins involved in the final cellular processes of terminally differentiating corneocytes prior to cornification. These distinct biological processes involve cell maintenance, biosynthesis, senescence and xenobiotic response. Because growth occurs rapidly and predictably, there is also temporal organization. In addition to physiological information, the hair shaft proteome also contains genetic and phylogenetic information in the amino acid sequence. Chemically the shaft is highly robust, the result of a highly structured protein matrix with abundant isopeptide and disulfide intermolecular bonds. Sample processing is therefore a challenge that requires robust chemistries but also minimizes the introduction of additional chemical modifications. This protocol depends on the combination of sodium dodecanoate and high levels of reductant to denature the matrix. The result is a proteome that is both readily accessible and can provide biological information to geneticists, developmental biologists, toxicologists, human and wildlife forensic scientists, scientists in the cosmetics industry, and wildlife ecologists.

iii) Key Words:

hair, hair shaft protein, cuticular corneocytes, cornification, shotgun mass spectrometry, tissue proteomics, forensic proteomics, species identification.

1. Introduction

Hair is a ubiquitous and robust mammalian tissue with biological, clinical, forensic and economic significance. The hair shaft contains the remains of multiple different cell types***(1, 2)***. Because of this, the hair shaft is relevant for investigators as a biological model of differentiation, and of a range of biological conditions***(3)***, including senescence***(4, 5)***, genetic mutations***(6)***, and environmental exposure***(7)***. The end result is a proteome that is both readily accessible and can provide a wealth of biological information to geneticists, developmental biologists, wildlife biologists, toxicologists, forensic scientists, as well as scientists in the cosmetics industry***(3)***.

Proteomic processing of hair shafts is a challenge due to the highly cross-linked nature of the keratin matrix***(8)***. Type-I and type-II keratin proteins are the basis of a highly organized hierarchical structure of intermediate filaments, maintained by extensive hydrogen bonding and reinforced by intra- and inter-molecular covalent bonds***(9-11)***. These cross-links fall into two categories: isopeptide bonds, the result of transglutaminase activity, and disulfide bonds, the result of disulfide bond formation between adjacent cysteines. The latter is reinforced by an extensive network of cementing keratin-associated proteins ***(12)***. This highly structured matrix accounts for the physical robustness and tensile properties of the hair shaft***(13)***.

Unlike most tissues, the hair shaft is not readily solubilized by standard proteolytic methods, particularly the outer cuticle***(8)***. Normally, full solubilization and peptide extraction is a precondition for maximum sensitivity in proteomic mass spectrometry. However, the conditions required to achieve this can introduce peptide modifications that reduce the concentration of native tryptic peptides and reduce the information gained from downstream bioinformatic analyses***(14-16)***. This method addresses these concerns in two ways: it incorporates gentler

chemistries, such as lower temperatures and shorter incubation times, and it specifically targets disulfide bonds by increases in the level of reductant in the extraction and digestion buffers, bringing the final dithiothreitol (DTT) concentration to 100 mM. This protocol uses a strong detergent, sodium dodecanoate, that is insoluble at or below pH 5, but unlike the chemically similar and popular sodium dodecyl sulfate, is uncharged and can be extracted from the sample using repeated phase extraction with ethyl acetate. Data yields over 2,000 peptides typically occur in samples that see a substantial swelling, often to greater than 1 mm in diameter, and softening of the hair shaft during the extraction phase. These shafts soften to the extent that a magnetic micro stir bar can disrupt the shaft and therefore create a suspension of keratinized matrix***(8)***.

Other hair shaft protocols make different choices to optimize peptide yields and balance the demands of solubilization and downstream compatibility. While in our hands the above method gave the best results and was time efficient***(8)***, other approaches have been developed that use different strategies to maximize peptide yields and achieve equivalent results***(17)***. These include physical disruption through milling of the hair shaft to maximize the surface area to volume ratio***(2)***, the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to resolve and fractionate hair shaft proteins for great depth of coverage***(18)***, use of urea/thiourea instead of detergent in the 'Shindai method'***(17, 20)***, or the use of additional proteolytic enzymes with different specificities. These protocols incorporate other priorities, such as time, cost, or depth of coverage***(2, 17-19)***. Strategies that use harsher chemistry may result in complete solubilization with shorter incubation times, but they also tend to have poorer information outcomes***(19)***.

2. Materials

1. Distilled water or water purified by reverse osmosis to be used to generate *Solid-Phase Extraction (SPE) - purified water*. (*see* **Note 1**).

2. *1M Ammonium Bicarbonate (ABC) in SPE-purified water* (*see* **Note 2**).

3. *Wash Solution containing* 2% Sodium Dodecanoate (SD) - 50 mM ABC working solution (*see* **Note 3**).

4. *1 M Dithiothreitol (DTT) in SPE-purified water* (*see* **Note 4**).

5. *0.5M Iodoacetamide (IAA) in SPE-purified water* (*see* **Note 5**).

6. *Ammonium Hydroxide (NH_4OH, 3% w/w) in SPE-purified water (see* **Note 6***).*

7. *Ethyl Acetate*. Mass-spectrometry grade.

8. *Trifluoroacetic acid (TFA)*. Mass-spectrometry grade, in 1 mL ampules.

3. Methods

3.1 Solution Preparation, Decontamination and Quality Control.

In solution preparation, chemical precision may conflict with the need to maintain a maximum level of cleanliness to minimize environmental contamination. The protocols below ensure a maximum level of cleanliness. Human skin proteins are in the air, on all laboratory surfaces, including the interior surfaces of disposables and instrumentation, as well as within reagents. Practices that reduce this contamination risk include the use of standard laboratory personal

protective equipment (PPE), a protected workspace such as a polymerase chain reaction (PCR) workstation, SPE of aqueous reagents (section 3.2.1), and the use of mass spectrometry (MS) - grade reagents for the preparation of stocks and solutions (*see* **Note 7**). Quality control of environmental contamination can occur through inclusion of reagent blanks in all sample sequences. The inclusion of pre-wash and post-wash chromatography conditioning runs can also be used to monitor and measure sample-to-sample carry-over on the instrument.

3.2 Proteomic Digestion of Single Hairs

3.2.1 Preparation of SPE-Purified Water

1. Disassemble a 10 mL syringe barrel from the plunger and attach a LuerLok SPE (ie. C18) column onto the syringe barrel.

2. Condition the column by passing about 5 to 10 mL of methanol through it. Pour the methanol into the syringe barrel with the attached SPE filter, reattach the syringe plunger. Discard the eluted methanol.

3. Ensure that air is not inserted into the SPE C18 matrix. Precondition a column by running water through the preconditioned column. The water should be the cleanest available, either distilled, or purified by reverse osmosis.

4. Discard the first 2 mL before collecting SPE-water into unused pristine, sealable glassware or pristine polypropylene vials.

5. Store the eluted water at room temperature (*see* **Note 1**). Use this SPE-purified water to generate all aqueous solutions for the protocol.

3.2.2 Hair Shaft Washing

1. Cut a 2 cm (20 mm) section of a single hair shaft. Reserve any remaining hair for additional analyses.

2. In a 1.5 mL tube, rinse hair with 500 µL of the Wash Solution (2% SD – 50 mM ABC). Vortex well. Discard solution and repeat with fresh Wash Solution two (2) more times, for a total of three (3) washes.

3. Remove and allow hairs to dry. Cut into 2 mm pieces using a razor blade or scissors and forceps (*see* **Notes 8 to 9**). Place all sections into a fresh 1.5 mL low binding polypropylene microcentrifuge vial.

3.2.3 Protein Reduction

1. Add 100 µL of Wash solution (2% SD – 50 mM ABC) to each sample tube.

2. Add 10 µL 1 M DTT to each sample vial.

3. Add a single (1) micro stir bar to each sample vial.

4. Centrifuge vials briefly to collect the contents at the bottom of the vial and ensure the sample hair is fully submerged in the solution.

5. Stir samples on stir plate on low-medium speed for 6 hours. Ensure hair stays fully submerged in the solution. Manually submerge the sections as required with a clean pipette tip.

3.2.4 Protein Alkylation and Detergent Extraction

1. Add 40 µL of 0.5 M IAA to each sample tube.

2. Continue stirring for another 45 minutes. Ensure the samples are protected from light.

3. Add 2 µL TFA to each sample tube (to adjust pH to ~3). Aliquot 2 to 5 µL onto a pH strip to confirm pH.

4. Add 175 µL ethyl acetate to each sample. Vortex well.

5. Centrifuge the samples at maximum speed for 3 minutes to decrease the volume of the interphase.

6. Remove the ethyl acetate (top phase) by pipetting and then discarding. If any of the lower phase is drawn into the pipette tip, gently dispense any lower phase material back into the tube. This will be visible in the tip since there is an obvious interphase line between the two layers.

7. Repeat the extraction two more times (3 extractions total). Take care to pipette out as much as possible of the upper ethyl acetate phase on the last extraction.

8. Add 6.3 µL concentrated SPE-filtered ammonium hydroxide (2.8-3.0% w/w) and 6.3 µL 1 M ABC to each sample tube. Gently mix (to adjust pH to ~8.5). Aliquot 2 to 5 µL onto a pH strip to confirm pH.

9. Store the samples overnight or as long as needed at ≤ -20˚C.

3.2.5 Sample Digestion

1. Remove samples from the freezer to thaw.

2. Resolubilize trypsin (20 µg) with 20 µL SPE water from aliquoted microcentrifuge tubes.

3. Add the entire 20 µL of trypsin to sample (one vial per sample). This is equivalent to a 1:5 ratio of protease to protein. Lower ratios of protease to protein (1:10 or 1:20) may work as well (*see* **Note 10**).

4. Stir the samples on a magnetic stir plate on a low-medium speed for 6 hours (*see* **Note 11**). Ensure hair remains fully covered by the solution. Push down as needed (*see* **Note 12**).

5. After the digestion is completed, remove the stir bars using an external magnet.

6. Centrifuge the samples at maximum speed (at least 13,000 *g*) for 15 minutes in a benchtop centrifuge at 4 ℃.

7. Pipette each supernatant into new tubes with centrifugal filters.

8. Centrifuge the samples at maximum speed (at least 13,000 *g*) for 5 minutes at 4 ℃.

9. Aliquot 60 µL of each sample into a fresh pre-labeled low-binding polypropylene microcentrifuge vial for storage at -20℃ until nanoLC-MS analysis. (*see* **Notes 13 to 15**).

3.3 Sample Analysis by nanoLC-MS

Proteomic analysis by shotgun mass spectrometry should be conducted according to the standard methods and protocols of liquid chromatography and mass spectrometry manufacturers***(21)***. A raw data file is produced and then analyzed for peptide spectra matching using any one of a range of established bioinformatic peptide spectra matching algorithms.

3.4 Bioinformatic Analysis.

In setting the search parameters, a human reference proteome should be used, such as the curated UniProt human reference protein database (proteome = UP000005640, www.uniprot.org), or the

equivalent protein FASTA file downloaded from the National Center of Bioinformatic Information based on human genome assembly GRCh38.p14 (www.ncbi.nlm.nih.gov/datasets/taxonomy/9606/). Since many hair proteins overlap with the CRAPome***(22)***, this bioinformatic filter should not be used. In conducting a search, the peptide and fragment parameters should reflect those of the instrument used. For example, on a Thermo Scientific Orbitrap Exploris 480 Mass Spectrometer, a 10 ppm and 0.04 Da threshold is appropriate for primary and secondary (*i.e.* fragmentation) masses respectively. In addition, many peptides from keratin-associated proteins may have over 10 cysteines per peptide. If the search algorithm does not have a more permissive search phase for post-translational modification (PTM) number and type per peptide, such as with PEAKs™***(23)*** or X!TANDEM***(24)***, highly modified peptides will be lost from the analysis and a maximum number of variable PTM assignments and missed cleavage number per peptide should be used, depending on the capacity of the software to tolerate a larger search space. Otherwise, default criteria may be used. As a minimum, the first stage should include variable deamidation of asparagine and glutamine (+1 NQ) and oxidized methionine (+16 M). Typically, most practitioners assume that all cysteines are modified with carbamidomethylation (+57 C). This may not be the case in hair because of the high number of cysteines. If it is important to identify the maximum number of peptides then variable carbamidomethylation should be assumed. As with any peptide spectral matching (PSM) run, standard internal quality control thresholds based on peptide sequence assignments using decoy databases should be used. Typically, this is a 1% false discovery rate for peptides, but a lower threshold of 0.1% can be used if higher stringency is necessary.

4. Notes

1. To minimize contamination, only pour from dry chemical stocks into a pristine 50 mL vial. Ideally attempt to weigh the reagent by pouring from the vial, only using a spatula if necessary. Use pristine weigh paper on the analytical balance. Care should be given to regular cleaning of the workspace with water and 70% ethanol, as well as ensuring protection of reagents and disposables from environmental dust. Unused vials, pipette tips and reagents should be covered at all times.

2. Add 949 mg of ABC to a 15 mL polypropylene vial and fill to the 12 mL mark with SPE-purified water. Vortex to dissolve and pass the entire solution through a preconditioned SPE column with filter. Discard the first 2 mL of the wash before collecting the remaining 10 mL by dispensing the stock into 10 x 1 mL aliquots in 1.5 mL low-binding microcentrifuge vials. Store at ≤ -20˚C.

3. Weigh 166.0 mg of ABC, fill to the 42 mL mark with SPE-purified water and vortex to dissolve. Pass the entire solution through a preconditioned SPE cartridge column with filter. Discard the first 2 mL of the wash before collecting the remaining 40 mL in a new 50 mL polypropylene vial. Add 800 mg of SD to the tube. Seal the cap with parafilm before mixing by vortexing and placing in an incubator at 70 ℃ for a few minutes to solubilize and gently mix. The addition of SD will slightly lower the concentration of ABC, below 50 mM. Warming the 2% SD – 50 mM ABC detergent solution in an incubator for a few minutes may be needed to allow the sodium dodecanoate to fully dissolve and go into solution. Parafilm on the cap of the falcon tube will help prevent leakage.

4. Weigh 1,851.0 mg DTT and transfer into a 15 mL falcon tube. Fill to the 12 mL mark with SPE-purified water. Vortex to dissolve. Pass the entire solution through a preconditioned SPE column with filter. Discard the first 2 mL of the wash before collecting the remaining 10 mL by aliquoting them into roughly 10 x 1 mL aliquots in 1.5 mL pristine low-binding microcentrifuge vials. Store at ≤ -20°C.

5. Weigh 1,110 mg IAA and transfer to a 15 mL falcon tube. Fill to the 12 mL mark with SPE-purified water. Vortex to dissolve. Pass the entire solution through a preconditioned SPE column with filter. Discard the first 2 mL of effluent before collecting the remaining 10 mL by dispensing roughly 1 mL into 10 x 1.5 mL low-binding microcentrifuge vials. Store at ≤ -20˚C. Iodoacetamide is light sensitive. It is best to work quickly and efficiently when handling this reagent and always keep stock solutions under cover and out of the exposure of light as much as possible when using. If necessary, cover incubators and equipment in foil.

6. Add 0.3 g of solid ammonium hydroxide to a 15 mL polypropylene vial and add SPE- purified water to a total of 12 mL. Mix or vortex to dissolve. Pass the entire solution through a preconditioned SPE column with filter. Discard the first 2 mL of effluent before collecting the remaining 10 mL by dispensing roughly 1 mL into 10 x 1.5 mL low-binding polypropylene microcentrifuge vials. Store at 4˚C.

7. Once solvent has been added to the syringe, take care not to push air through the column. If this does occur, the air can be removed by washing with water and repeating the MeOH preconditioning step. Only do this step if it is difficult to force the solution through the SPE column. Because this step employs a C18 solid phase resin cartridge, impurities cannot be removed from aqueous stocks or solutions containing organics or detergents/surfactants.

8. Cutting hairs into small pieces can be challenging. It is best to work on a dark surface when handling light colored hairs, and a light surface when working with dark colored hairs.

9. Depending on humidity, hair pieces may attract static electricity and ‘jump’ around while being cut.

10. The trypsin amount used in this protocol is high, 20 µg of trypsin is roughly a protease to protein ratio of 1 to 5. We found that the typical ratio of 1 to 50 as recommended in fully soluble protein mixtures was not as efficient for a partially insoluble digest.

11. The rounded/oval shaped micro stir bars are more efficient at mixing and disrupting the softened hair shaft than the square edged ones.

12. Samples may stir best when placed in a tube rack that is two inches above the stir plate and set to low-medium speed.

13. If working with less soluble keratin-based samples (such as skin and thicker hair shafts), then allow the samples to stir longer than 6 hours to maximize the digestion process.

14. We find that a sub-fraction of human hairs shafts (less than 5%) are intractable, failing to denature in the presence of even harsher chemistries. A systematic analysis of these samples is difficult due to the small sample size. Anecdotally we observe a higher likelihood of this phenomenon in female African Americans who often include heat treatments in their personal grooming. These may increase the prevalence of cross-linking through Maillard chemistries.

15. Use of microcentrifuge filters (0.22 or 0.45 µm) is necessary to remove particulates that may interfere with downstream chromatography. These filters should be employed when remixing frozen aliquots of trypsin digests since precipitation may occur during the freeze / thaw cycle.

Acknowledgements.

This work is or has been supported by the National Institute of Justice (2015-DN-BX-K065 and 15PNIJ-22-GG-03566-SLFO), California Department for Fish and Wildlife (CDFW, P2195005, P2295005, P2395001), and the West Australian Government (ChemCentre). The authors thank members of the Parker, Rice, CDFW and ChemCentre laboratories for assistance and advice in protocol development and manuscript preparation.

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