Title

Improved radiocarbon analyses of modern human hair to determine the year-of-death by cross-flow nanofiltered amino acids: common contaminants, implications for isotopic analysis, and recommendations

Journal

Rapid Communications in Mass Spectrometry, 29(19)

ISSN

0951-4198 1097-0231

Authors

Santos, Guaciara M

De La Torre, Hector A. Martinez

Boudin, Mathieu

et al.

Publication Date

2015-08-25

DOI

10.1002/rcm.7273

Peer reviewed

Research Article

Rapid Communications in Mass Spectrometry

Received: 25 April 2015 Revised: 3 July 2015 Accepted: 10 July 2015 Published online in Wiley Online Library

Rapid Commun. Mass Spectrom. **2015**, *29*, 1765–1773
(wileyonlinelibrary.com) DOI: 10.1002/rcm.7273

Improved radiocarbon analyses of modern human hair to determine the year-of-death by cross-flow nanofiltered amino acids: common contaminants, implications for isotopic analysis, and recommendations

Guaciara M. Santos[1]*, Hector A. Martinez De La Torre[1], Mathieu Boudin[2,3], Marco Bonafini[2] and Steven Saverwyns[2]

[1]Department of Earth System Science, University of California, Irvine, Irvine, CA 92697, USA
[2]Royal Institute for Cultural Heritage, Jubelpark 1, B-1000 Brussels, Belgium
[3]Ghent University, Faculty of Bioscience Engineering, Coupure Links 653, B-9000 Ghent, Belgium

RATIONALE: In forensic investigation, radiocarbon (^{14}C) measurements of human tissues (i.e., nails and hair) can help determine the year-of-death. However, the frequent use of cosmetics can bias hair ^{14}C results as well as stable isotope values. Evidence shows that hair exogenous impurities percolate beyond the cuticle layer, and therefore conventional pretreatments are ineffective in removing them.
METHODS: We conducted isotopic analysis (^{14}C, $\delta^{13}C$, $\delta^{15}N$ and C/N) of conventionally treated and cross-flow nanofiltered amino acid (CFNAA)-treated samples (scalp- and body-hair) from a single female subject using fingernails as a reference. The subject studied frequently applies a permanent dark-brown dye kit to her scalp-hair and uses other care products for daily cleansing. We also performed pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) analyses of CFNAA-treated scalp-hair to identify contaminant remnants that could possibly interfere with isotopic analyses.
RESULTS: The conventionally treated scalp- and body-hair showed ^{14}C offsets of ~21‰ and ~9‰, respectively. These offsets confirm the contamination by petrochemicals in modern human hair. A single CFNAA extraction reduced those offsets by ~34%. No significant improvement was observed when sequential extractions were performed, as it appears that the procedure introduced some foreign contaminants. A chromatogram of the CFNAA scalp-hair pyrolysis products showed the presence of petroleum and plant/animal compound residues, which can bias isotopic analyses.
CONCLUSIONS: We have demonstrated that CFNAA extractions can partially remove cosmetic contaminants embedded in human hair. We conclude that fingernails are still the best source of keratin protein for year-of-death determinations and isotopic analysis, with body-hair and/or scalp-hair coupled with CFNAA extraction a close second.

Scalp-hair samples have been widely used in the fields of human forensic science and anthropology. Applications can vary from toxicology screens to evaluate long-term exposure to illegal substances[1–4] to isotopic measurements to detect dietary preferences and nutritional status,[5,6] as well as spatial distributions and migratory behavior.[7,8]

In cases involving unidentified human remains whose year-of-death is within the past 60 years, direct ^{14}C measurements of scalp-hair can in principle precisely determine the year-of-death (YOD).[9] The method relies on the excess of atmospheric ^{14}C produced by the above-ground nuclear weapon tests performed during the mid-20th century. This anthropogenic disturbance in atmospheric ^{14}C concentrations has been well documented. Atmospheric bomb ^{14}C peaked in the Northern Hemisphere in 1964 and has been falling since 1965, after international treaties banned nuclear weapons tests and the excess ^{14}C worked its way into the carbon cycle.[10] Since all living organisms incorporate carbon through CO_2 fixation (new plant growth tissue) or nutritional pathways (food chain), the excess ^{14}C is locked within tissues during their formation, preserving a time-specific signature from the bomb-pulse. Although the use of ^{14}C bomb-pulse methodology by the Accelerator Mass Spectrometry (AMS) technique is costly, it has been successfully used in the past to reveal modern forgeries, identify poaching, validate the annual pattern of tropical tree species and determine the birth- and/or death-age of subjects in forensic cases.[11–15] The fast turnaround time of hair development (~2.5 days from the 'birth' of the hair cell until full keratinization[16]) makes this tissue an ideal material for YOD determinations on modern humans, as well as for isotopic longitudinal studies. For YOD determinations the

* *Correspondence to:* G. M. Santos, Department of Earth System Science, 1222 Croul Hall, University of California, Irvine, Irvine, CA 92697–3100, USA.
E-mail: gdossant@uci.edu

keratinized tissue is isolated and processed before undergoing ^{14}C measurements. The resulting ^{14}C signatures can then be compared with those of pre-established ^{14}C age calibration curves from where the subject has spent their last few months.[9,10] Once a match between the ^{14}C from the tissue and the atmospheric curve is found, a calendar age for the year of tissue formation can be determined.

However, sociocultural factors and the pursuit of an ideal appearance drive modern humans to constantly change aspects of their scalp-hair; subjecting it to any number of cosmetic products and treatments for regular cleaning and grooming. Cosmetic ingredients can include hundreds of petrochemical derivatives that interact with hair to give various effects.[17–20] Solvents such as benzyl and n-butyl alcohol, and phenol derivatives (inexpensive carbolic compounds extracted from coal tar or petroleum), are regularly used in the preparation of dyeing agents to better facilitate the production of darker shades.[21–24] Petrochemicals are also the cheapest way to produce artificial coloring and fragrances in care products. Botanical and animal by-products are also frequently used in the hair care industry. Amino acids, proteins, fatty acids, oils and waxes are just some of the substances added to shampoos and conditioners to provide texture, assist in moisture retention, strengthen hair strands, and help chemical reagents from dye-kits fuse beyond the hair cuticle layer.[17–32] Shampoos formulated for children also contain plant extracts (e.g., betaines to help in cleansing[33]) and petroleum derivatives, such as white petroleum jelly, and mineral oil for softening and soothing the scalp. Since hair care formulations are also designed to penetrate and treat the scalp tissue, even the developing hair follicles are exposed to exogenous impurities.[27,34]

For decades, the alteration of human hair strands due to the use of cosmetic products and treatments has been well documented. In an early work, Robbins and Kelly demonstrated that amino acids in human hair fibers (i.e., cystine, cysteic acid, tyrosine, and methionine) can be altered when subjected to bleaching and permanent waving *in situ*,[35] an effect that was confirmed by others.[36] Regarding dyes and dye precursors (components to assist in the oxidation, diffusion and locking of the dye into the hair matrix) special attention has been devoted to permanent hair coloring and its final effects.[37,38] For instance, Chen *et al.* showed the fate of hair dye precursors in the cuticle, cortex and medulla of human hair using time-of-flight secondary ion mass spectrometry (TOF-SIMS).[39] Using a similar approach, Ruetsch *et al.* studied coconut and mineral oils, and subsequently a set of vegetable oils with different types of unsaturation in the fatty acid components and cationic conditioning compounds.[40–42] The penetrating effects of polymers into the hair cortex region to assist in dye-color retention have also been shown by optical microscopy of cross-sections of hair strands.[21] In quantitative studies, Jones and Chahal[29] showed that over 50% of botanical and protein extracts in cosmetic products can be retained in the hair structure, while Zimmerley *et al.*[43] showed that ~7% of externally applied d-glycine percolates into hair fibers.

The ability of extraneous compounds to remain trapped in hair and the detrimental effects of this ability on forensic analysis have also been studied. Alcohol-containing hair care products (such as hair sprays, permanent wave solutions, dyeing and bleaching agents, and herbal lotions) are known to alter the concentration of fatty acid ethyl esters – a marker for chronic excessive alcohol consumption in humans.[44,45] Broecker *et al.* used liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry (LC/QTOFMS) to study scalp-hair from 90 dead subjects.[46] Their findings revealed the presence of several toxic substances, including panthenol (a pro-vitamin B_5 extracted from natural sources that is regularly used in hair care products and creams).

Chemical compounds containing carbon and nitrogen once transferred to and embedded in the scalp-hair shaft of adults and children can bias isotopic results, especially ^{14}C signatures and quality control indicators (e.g., %C, %N, and C/N). De La Torre *et al.* demonstrated that scalp-hair strands were ^{14}C-depleted (~5–13‰) compared with the subjects' fingernail reference, and that the C/N values appeared anomalous.[47] Moreover, the ^{14}C offsets increased as the chemical treatments applied to hair strands evolved from conventional procedures (i.e., methanol/chloroform[48] and acid/alkaline/acid[9]) to those capable of stripping the hair surface.[47] The fact that chemical compounds found in hair care products incorporate themselves deeply into the hair structure makes it difficult to distinguish and remove these products. In addition, since some of the contaminants can be derived from present-day plant or animal sources, contamination by fossil fuel (and therefore old derived carbon) can be a hurdle in determining the YOD by ^{14}C pulse-labelling methodology (e.g., from 1953 onward).[9]

We applied cross-flow nanofiltered amino acid (CFNAA) extractions[49] to keratinized tissues from a single human subject, including scalp-hair samples contaminated by a permanent coloring from a dark-brown dye-kit. In order to investigate whether the significant discrepancies between contaminated and non-contaminated keratinized tissues could be resolved, we conducted isotopic analysis (^{14}C, $\delta^{13}C$, $\delta^{15}N$ and C/N) of solvent-treated and CFNAA-extracted fingernails, scalp- and androgenic-hair (termed 'body-hair'). We also performed pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) analyses of CFNAA-treated scalp-hair to identify contaminant remnants that might interfere with isotopic analyses.

EXPERIMENTAL

Keratinized tissues analyzed

Fingernails, scalp- and body-hair were sampled from a single human subject (subject B in De La Torre *et al.*).[47] The subject is a middle-aged female, who has consistently used the same dark-brown permanent coloring dye kits for over 3 years (same coloring formula and brand). Fingernails were collected to serve as references for both types of hair analyzed, as they were not directly exposed to any cosmetic products (such as nail polish). All samples were collected together at the end of the summer of 2012. Subject B's scalp-hair was previously confirmed to be contaminated by petroleum-based derivatives, which altered its ^{14}C-isotopic signatures and its quality control indicators (%C, %N, and C/N).

Once collected, the samples were wrapped in clean aluminum foil and stored in small plastic bags to be shipped to the Royal Institute of Cultural Heritage (Brussels, Belgium) to undergo CFNAA extractions and Py-GC/MS analyses.[49] The subject gave informed consent to participate in the study. The participant's samples were collected during regularly scheduled grooming. Personal identifiers were completely removed for anonymity.

Rapid Communications in Mass Spectrometry

Chemical laboratory procedures

Keratin protein conventional pretreatment

In order to remove superficial contaminates (residual lipids, oils, fats, waxes and grease) prior to analysis, the samples were sonicated in an ultrasonic bath for 15 min, and immersed in successive solvent washes (hexane, ethanol and acetone; Merck, Brussels, Belgium). Once the solvent delipidation treatment was complete, the samples were subjected to a conventional treatment for organic samples, i.e., sequential baths of 1% HCl, 1% NaOH, 1% HCl (Merck), followed by rinsing with pure water until all contaminant chemicals were completely removed. In order to avoid disintegration of the keratinized material under alkaline conditions, this chemical treatment was performed at room temperature.[50] The samples were then dried at 40°C. This pretreatment procedure is designed to remove molecules adsorbed or transferred onto the surface of the keratinized material by endogenous-exogenous pathways. Previous findings based on C/N ratios and the isotopic ratio values of horse hair treated with shampoo (rich in protein) and human hair dyed with henna (a plant-derived dye) indicated that the effect of exogenous chemicals on the stable isotope ratios of hair was mostly insignificant.[48]

Cross-flow nanofiltered amino acid extraction analyses

Sub-samples of keratinized materials (15–50 mg) treated by the conventional procedure described above were then subjected to CFNAA extraction.[49] First, the amino acids of the protein material were released by subjecting it to hot acid hydrolysis with 2 mL 6 M HCl in a sealed tube in a nitrogen atmosphere at 110°C for 24 h. The hydrolysate was then filtered over a 0.7-µm glass fiber filter (APFF03700; Merck Millipore, Brussels, Belgium). The concentrate was re-dissolved into a 100 mL solution with Milli-Q water (Merck Millipore), and then run through a cross-flow nanofiltration unit loaded with a filter membrane having a 360 Da MWCO (molecular weight cut-off)[51] in order to collect the amino acids. Finally, the amino acids were freeze-dried and reserved for further analyses.

Analytical procedures

Radiocarbon determinations, and stable isotope and atomic C/N analyses

Before the analyses could be performed, the freeze-dried amino acids were reconstituted in 1 mL Milli-Q water. For the ^{14}C analysis the reconstituted amino acids were transferred into pre-baked 6-mm OD quartz tubes and dried once more before evacuation and sealing. Carbon dioxide (CO_2) was produced from the extracts by combustion at 900°C in evacuated, sealed quartz tubes with pre-baked cupric oxide (CuO) and silver wire.[52] The CuO provides oxygen during sample combustion, while the silver prevents sulfur and halogens from later interfering with graphitization. The CO_2 produced was then cryogenically cleaned using a vacuum line and collected within glass vessels for graphitization and analysis.

Graphitization of the cryogenically clean CO_2 and the ^{14}C measurements of the graphite targets were carried out at the Keck Carbon Cycle Accelerator Mass Spectrometry (KCCAMS) Facility, at the University of California Irvine (Irvine, CA, USA). The evolved CO_2 was reduced to filamentous graphite using hydrogen and pre-baked iron powder as catalysts, following established protocols.[53] High-precision ^{14}C-AMS measurements were performed using a modified compact AMS system (model NEC 0.5MV 1.5SDH-1; National Electrostatics Corporation, Middleton, WI, USA). This instrument routinely achieves 0.2–0.3% on precision and accuracy and can also provide the $^{13}C/^{12}C$ values of the respective graphite samples measured.[54] Thereafter, the ^{14}C results were corrected using the online $\delta^{13}C$ values, following the instrumental analysis described in Santos *et al*.[55] For background corrections, coal samples were processed and analyzed alongside the unknown samples. The radiocarbon results shown in the figures, tables and text are expressed as a Fraction of Modern Carbon (FmC or $Fm^{14}C$).

Stable isotope and atomic C/N analyses

Approximately 50 µL of permeate (cross-flow nanofiltered amino acids) were used for stable isotope and C/N analysis. The stable isotope and C/N values were determined from amino acid extracts preloaded in tin capsules (5 × 8 mm capsules; OEA Laboratories Ltd, Callington, UK). Analyses were performed in duplicate on a Thermo Flash EA/HT elemental analyser coupled to a Thermo Delta V Advantage isotope ratio mass spectrometer via a Conflo IV interface (all supplied by Thermo Fisher Scientific, Bremen, Germany). During measurements, the samples were interspersed with in-house standards, such as caffeine and leucine, International Atomic Energy Agency (IAEA, Vienna, Austria) reference materials, IAEA-N1 and IAEA-C6, and internally calibrated acetanilide. The analytical precision of the measurements was better than 0.25‰ for both $\delta^{13}C$ and $\delta^{15}N$ values. The stable isotope results are measured as the ratio of the heavier isotope to the lighter isotope ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) and reported as δ values in parts per 1000 or 'per mil' (‰) relative to internationally defined standards for carbon (Vienna Pee Dee Belemnite, vPDB) and air for C and N, respectively.[56]

Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS)

In order to characterize some of the complex compounds in the treated scalp-hair after the CFNAA extractions, a Py-GC/MS analysis was performed using a Trace gas chromatograph coupled to a ThermoFinnigan Polaris Q quadrupole ion trap mass spectrometer (Thermo Scientific, Erembodegem, Belgium). A single-shot pyrolysis was conducted in a helium atmosphere (1 mL/min) at 550°C (EGA/PY-3030D pyrolysis unit; Frontier Laboratories Ltd, Fukushima, Japan) in the presence of 2.5% tetramethylammonium hydroxide in methanol as a derivatization compound. A Supelco SLB-5ms column (length 20 m, ID 0.18 mm, film thickness 0.18 µm; Sigma-Aldrich, Diegem, Belgium) was used for chromatographic separation. The oven temperature was held at 35°C for 1 min, increased to 240°C at 10°C/min, then from 240°C to 315°C at 6°C/min and finally held isothermally for 5 min at 315°C. The interface temperature was 290°C. Electron ionization (EI) mass spectra were acquired in positive ion mode at 70 eV in the *m/z* 35–650 mass range, with a cycle time of 0.5 s.

RESULTS AND DISCUSSION

Radiocarbon results

Petrochemical byproduct contamination into the structure of scalp- and body-hair is clearly demonstrated by the ^{14}C-AMS analyses of individual aliquots of keratinized materials following conventional pretreatment (Fig. 1(A)). The ^{14}C values of fingernails from the same subject were used as a reference point for all the comparisons.

As expected, the ^{14}C results show that the fossil C contamination was more severe in scalp- than in body-hair. The highest ^{14}C results from the subject's keratinized tissues yielded FmC = 1.0421 ± 0.0010 (the mean value obtained from the fingernail tips subjected to the conventional chemical pretreatment; Supplementary Table S1, Supporting Information). This FmC value is equivalent to a $\Delta^{14}CO_2$ value of ~32‰, associated with the end of the 2011 calendar year to the beginning of 2012,[57] in close agreement with the time of the fingernail tip formation (4–6 months before sampling). The hair ^{14}C offsets relative to the ^{14}C reference value of the fingernails were 8.7‰ for body-hair and 20.9‰ for scalp-hair.

A significant improvement in the CFNAA analysis ^{14}C values from the scalp-hair is observed, as the nano-filtration process removed substantial amounts of the petrochemical byproducts (Fig. 1(B)). However, direct comparisons between the ^{14}C results from the fingernails treated by CFNAA and those from the conventional pretreatment revealed the presence of some contamination introduced during the CFNAA sample processing, thus biasing the ^{14}C results towards depleted values. It is still unclear whether this contamination was introduced during the CFNAA procedure itself, or from the dehydration step, as the amino acids were loaded into the quartz tubes prior to combustion. This procedural contamination was, however, observed on all the keratinized tissues undergoing CFNAA processing. Nevertheless, the single CFNAA procedure shows encouraging results compared with the conventional and specialized chemical treatments tested previously.[47,48] The nanofiltration process applied here is an intermediate process of filtration between reverse osmosis (RO) and ultrafiltration (UF) that can reject particles in the approximate size range of 1 nanometer.[58] However, it cannot discriminate contaminants from molecules of a similar size such as those of the amino acids that comprise keratinized tissue.

We also attempted to obtain more accurate ^{14}C results from the keratinized materials by performing multiple filtrations. This was done by re-dissolving the amino acids after the first CFNAA collection and by forcing the solution through the cross-flow nanofiltration unit two or three times (Fig. 2). Multiple filtrations did not show any significant improvements in terms of removing C contaminants from scalp-hair, or from any of the other keratinized tissues, and did not move the ^{14}C values of hair any closer to the fingernail reference point (using the conventional protocol).

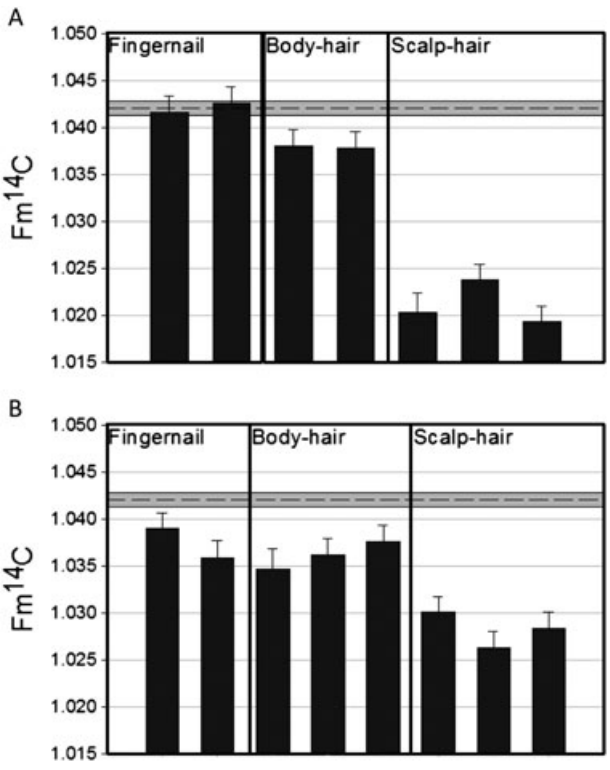

Figure 1. $Fm^{14}C$ signatures of subject's aliquots of keratinized materials following: (A) conventional chemical pretreatments and (B) CFNAA analyses. The horizontal baseline is the average of $Fm^{14}C$ (±SD) of the fingernails, and serves as the specific reference point for the hair analyses shown in this work.

Isotopic analyses and atomic C/N determinations

Isotopic analyses and C/N determinations were carried out on fingernails and hair samples (scalp and body), and the results are listed in Table 1. Previously, the $\delta^{13}C$ values from the scalp-hair of Subject B were lower than those of the fingernail by 0.8‰ on average, while the atomic C/N values ranged from 4.0 to 4.6 (scalp to hair-tip) with a total %C of 45.8–50.4.[47] An apparent loss on the %N was also observed, probably due to hair protein alterations at the molecular level, an issue already reported by others.[35,36] Based on the atomic C/N value of 3.4 for the fingernail of Subject B, we estimated that the contamination by exogenous carbon into scalp-hair was of the order of 18 to 35%. Here, however, the overall stable isotopic values of all the keratinized materials (fingernails, scalp- or body-hair) were very similar to each other, whether processed by the conventional or CFNAA

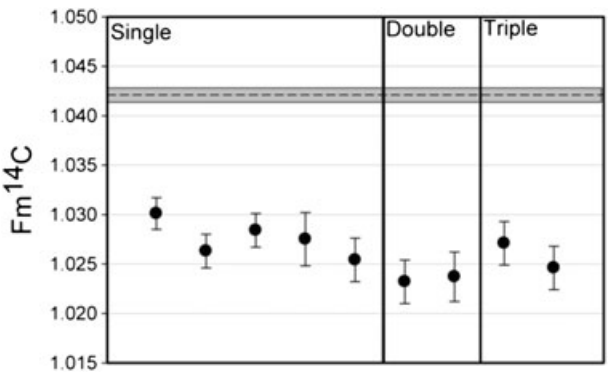

Figure 2. $Fm^{14}C$ signatures of scalp-hair subjected to single, double or triple filtration in the CFNAA analyses. The horizontal baseline is the average of $Fm^{14}C$ (±SD) of the fingernails pretreated by the conventional procedure to serve as reference.

Rapid Communications in Mass Spectrometry

Table 1. Isotopic analyses (as $\delta^{13}C$, $\delta^{15}N$ and C/N values) for keratinized materials (fingernails, scalp- and body-hair samples) from a single female who makes use of permanent coloring from a dark-brown dye kit

Sample ID	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	C/N (Atomic)
Conventional pre-treatment			
Fingernail	−18.9	9.7	3.6
Body-hair	−18.8	9.0	3.6
Scalp-hair	−18.8	9.1	3.7
CFNAA pre-treatment			
Fingernail	−18.9	9.6	3.4
Body-hair	−18.4	8.8	3.4
Scalp-hair	−18.5	9.0	3.4

chemical pre-treatments. The atomic C/N ratios, however, were slightly higher for the keratinized materials treated using the conventional cleaning than for those treated using the CFNAA (e.g., average C/N = 3.6 versus 3.4, respectively).

Previous time-series experiments using ^{14}C-AMS to address the deposition and accumulation effects of shampoo contaminants in scalp-hair on an infant indicated that, once the contaminant was deposited into the hair, it remained for a period of several months.[47] Since previous C/N ratios from Subject B were also anomalous (as mentioned above), we expected to see the same phenomenon, although all the keratinaceous samples for this study were taken 1 year apart. Therefore, we suspect that the lack of an apparent pattern in Subject B's scalp-hair (an adult who makes use of a single dark dye-kit) can be attributed to: (a) constant change of shampoos and conditioners. Since a single cosmetic hair care product can have hundreds of ingredients with a broad range of $\delta^{13}C$ values (e.g., −50 to −10‰, from petrochemical byproducts to animal and plant extracts; Meier-Augenstein[59]), the cumulative effects of contaminants in hair would be hard to reproduce or properly quantify; and (b) an adult should experience a loss of approximately 30% of their previously grown hair in 1 year, due to continuous growth and shedding.[60] Therefore, we speculate that a combination of changes in hair-care products and constant shedding could promote shifts in the effects of C and N contaminants into the scalp-hair, making it impossible to trace contaminants using stable isotope values alone, including the C/N ratios.

Micro-analytical identification of the components in treated human hair by Py–GC/MS

Several human hair microchemical analyses have been performed with the purpose of evaluating occupational and environmental exposure to toxic elements or drug abuse. Py–GC/MS was used in this study to identify the pyrolysis marker compounds that are embedded in the CFAA extract of the subject's scalp-hair. It should be stressed that this test was not intended to be exhaustive. Rather, it examined whether some of the exogenous contaminants within hair might have molecular weights similar to those of primary amino acid sequences of human hair keratin, and therefore could not possibly be isolated by the CFAA procedure.

A chromatogram of the pyrolysis products from the scalp hair is shown in Fig. 3. The large peak at R_t (retention time) 19.29 min is margaric acid (heptadecanoic acid) methyl ester, which is the internal standard. Our analysis allowed us to identify several chemical compounds by comparing their mass spectra with those from reference libraries (Table 2). Many of the chemical compounds listed are regularly added to conditioner/shampoo or hair bleaching dye-kit products. These compounds have molecular weights smaller than the 360 Da MWCO of the filter, and therefore it is safe to conclude they were also present in the permeate that was later measured by isotope analysis. The identified compounds can be divided into two groups: (1) those derived from petrochemical byproducts that consequently contain a ^{14}C age close to infinite/fossil (or

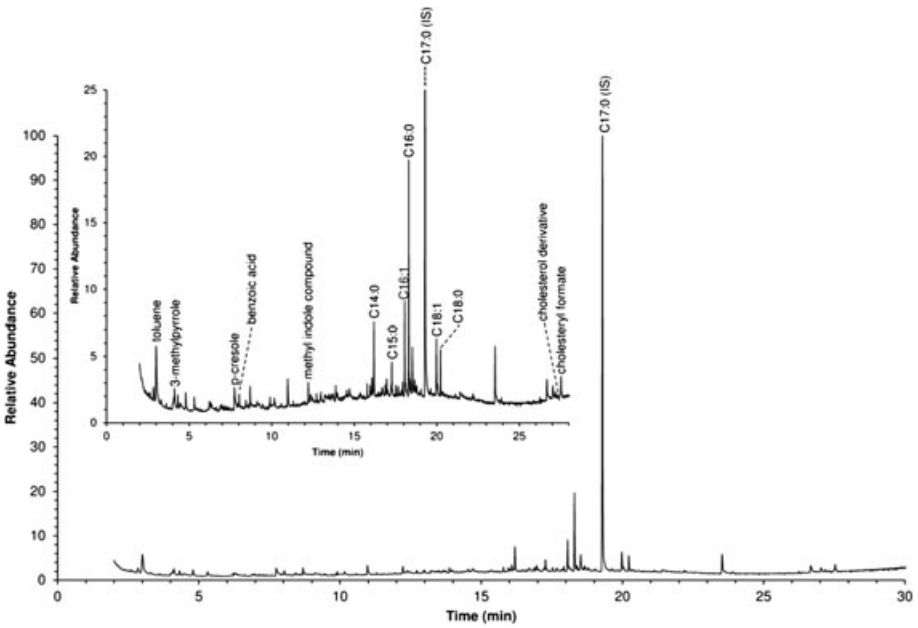

Figure 3. Pyrolysis–GC/MS chromatogram of the CFNAA-treated scalp-hair. For peak identification, see Table 2.

Table 2. Pyrolysis products of the CFNAA-treated scalp-hair

Retention time (min)	Chemical compound
	PETROLEUM SOURCE
3	toluene
4.1	3-methylpyrrole
7.74	*p*-cresol
8.03	benzoic acid, methyl ester
12.22	methylindole*
	PLANT/ANIMAL SOURCE
	Lipids
16.19	tetradecanoic methyl ester (C14:0)
17.28	pentadecanoic acid methyl ester (C15:0)
18.06	hexadecenoic acid methyl ester (C16:1)
18.3	hexadecanoic acid methyl ester (C16:0)
19.98	octadecenoic acid methyl ester (C18:1)
20.23	octadecanoic acid methyl ester (C18:0)
27.19	cholesterol derivative
27.53	cholesteryl formate

Peak numbers as in Fig. 3.
*GC/MS analysis could not clarify the location of the methyl group, which could be any of the following 3

FmC = 0) and thus biasing hair FmC signatures towards 'depleted older' values; and (2) those from a plant or animal source possibly from a present-day extract, and therefore undetectable by ^{14}C-AMS, as their FmC values are close to those expected from the protein keratin of the subject tested.

The peaks listed in Table 2 comprise ~40% of all peaks obtained. The additional unlabeled peaks in Fig. 3 could not easily be identified, but it is highly probable that they are also compounds derived from the hair-care solutions.

Implications for forensic year-of-death determination and isotopic studies

Several studies on toxicology and isotopic composition of body tissues and its association with diet have used human scalp-hair samples, due to the non-invasive sampling methodology compared with other human materials. Recently, modern human scalp-hair has also been proposed as an alternative and less invasive material for determining the YOD.[9]

However, as we have demonstrated most of the ambiguities in ^{14}C results between the subject's nail-hair parings were attributable to contaminants embedded within certain types of keratinized samples. Scalp- and body-hair contains more fossil C contaminants (e.g., petrochemical byproducts) than fingernails due to the constant use of cosmetic products designed to penetrate the hair structure. The contamination is more prevalent in scalp- than in body-hair. The highest offset between nail and hair was ~21‰, suggesting that at least 2% of fossil fuel contamination was present. Since the atmospheric bomb ^{14}C level has been falling at a rate of 5–6‰ per year, this offset can cause an error equivalent to ~4 years in the YOD determination, which is unacceptable in view of the accuracy expected for applications of the bomb-pulse methodology from 1955 onward.[11–15] These findings also suggest that, regardless of the decontamination procedure employed (conventional pretreatments *vs* CFNAA analyses), contaminants embedded in hair cannot be fully removed. This conclusion is supported by the Py–GC/MS analyses (Fig. 3 and Table 2). Nevertheless, a single CFNAA treatment was able to eliminate ~34% of the contaminants from scalp-hair strands (Fig. 1(B)).

While it is accepted that any additional carbon of a different age in a sample undergoing ^{14}C measurements will cause the measured date to be inaccurate, contamination that cannot be removed but has an equivalent ^{14}C value to that of the sample being studied will pass unnoticed. For instance, the animal and plant extracts added to shampoos, conditioners and hair-dye kits should have close to modern ^{14}C values, and therefore they cannot be effectively detected or quantified from present-day hair samples.

Due to the nature of the present-day extracts added to cosmetic products, one can also expect that the stable isotopic results of hair strands alone (δ^{13}C, δ^{15}N and C/N values) cannot help to distinguish signals built into the hair through metabolic pathways via diet (which also makes use of plants and animal products) from those of contamination. Indeed, the stable isotope results and C/N determinations from all the keratinized tissues obtained here were not dramatically shifted (e.g., they did not fall outside of the C/N range of 2.9–3.6 of DeNiro[61]), except for the scalp-hair undergoing conventional treatment (Table 1). It is argued herein that the unexplained variability in the stable isotope values of keratinized tissues (nail-hair parings) reported by O'Connell *et al.*[62] could be partially due to intermittent C and N contamination from plant and animal extracts (in addition to petrochemicals) embedded in hair-care products, although this was not clearly inferred from their previous tests on animal and human hair subjected to shampoo and henna treatments.[48] It also appears that for those early tests the researchers used a circular reasoning, as they used the quality control atomic C/N range of 3.0–3.8[48,62] for comparisons (rather than the C/N range of 2.9–3.6 of DeNiro, which is narrower[61]) leading to the conclusion that shampoo and henna could not interfere with the stable isotope signals. Interestingly, the quality control C/N range of O'Connell and Hedges was empirically produced using human subject scalp-hair treated with a conventional solvent pretreatment (i.e., methanol/chloroform and pure water).[48] This conventional pretreatment was not designed to penetrate into the hair shaft to remove all exogenous impurities from the hair matrix. Even the CFNAA treatment is incapable of isolating the amino acids solely from the scalp-hair. Consequently, it is not currently possible to apply any systematic correction to trace back the isotopic signatures derived solely from the compounds that formed the scalp-hair keratinized tissue.

It should be stressed that most of the methods used to evaluate the sorption of cosmetics into hair strands of all textures (from infants to adults, straight to curly, oiled to dry, or normal to damaged) have been limited to their overall effects.[33,34,63,64] Nevertheless, some attempts to quantify individual compounds from cosmetics transferred into hair have been reported, with values varying from 7 to >50%.[29,43] We find that even if these contributions to scalp-hair cannot be properly detected by measurements of δ^{13}C, δ^{15}N or C/N values, they are still significant. Biased stable isotopic results due to the presence of exogenous impurities in scalp-hair may be analytically limited, if the amino acids of origin (from diet) and from hair cosmetics (petrochemicals, plants and animal extracts) are characterized

Rapid Communications in Mass Spectrometry

by similar isotopic signatures, but significant if they are not, which can be hard to assess on living or deceased human groups. Consequently, we conclude that scalp-hair is also not suitable for use in isotopic studies. Nevertheless, since the growth of human hair throughout the body is cyclic, body-hair, which can be less contaminated by care products, can in principle serve as a replacement material, if fingernails from the subjects are not available to undergo conventional keratin chemical cleaning for isotopic analyses.

CONCLUSIONS AND FURTHER RECOMMENDATIONS

For decades human hair has been used in isotopic analyses, based on the assumption that contaminants are limited to the surface of the hair strands. Based on this assumption conventional chemical pretreatments were designed, and a range of 3.0–3.8 for the atomic quality control C/N ratio (specifically for hair samples) was considered adequate.

Recently, scalp-hair has also been tested in YOD determinations for forensic science applications with limited success. Although there is a considerable amount of published information on hair-care product formulations and their interactions with scalp-hair, a gap in information between interdisciplinary research fields (cosmetic industry *vs* isotopic analyses) is still apparent.

In this study, we provide a brief overview on the chemicals found in the hair-care treatments, their penetration and alteration effects in hair and scalp, and some of their detrimental impacts on forensic and isotopic analysis. We also validate previous findings that human hair is contaminated with 'older' C due to the use of cosmetic products that contain petrochemical byproducts. No chemical pretreatments applied to hair have yet been found to completely remove contaminants, which would enable ^{14}C values derived from hair keratin to match those of fingernails. Nevertheless, we demonstrate that CFNAA extractions can help partially remove those contaminants. The technique is straightforward, and can be easily implemented as a routine sample pretreatment for hair analyses.

These results could have an impact on studies that continue to make use of living and/or deceased human scalp-hair samples. We have demonstrated that the C/N ratio of bulk or chemically clean hair samples alone is not always a reliable indicator of contamination built in the hair strands, as some of those contaminants have the same isotopic signature as those from a regular diet. This conclusion is supported by the Py–GC/MS chromatogram of the CFNAA-treated scalp-hair, as both petroleum and plant/animal remnants were still found to be present after treatment.

Since identifying a scenario in which hair analysis may be appropriate is difficult, especially in forensic science applications, we can at least confidently recommend the use of the following combinations: body-hair pretreated by conventional or CFNAA treatments, or scalp-hair coupled with CFNAA extractions. Generally speaking, fingernails are still the best source of keratin protein for overall isotopic analysis and YOD assessments from 1955 onward. Standardization on one chemical pretreatment method for fingernails in laboratory studies will be important, and one possible standard is the conventional chemical treatment described here. In addition, we recommend applying the technique to the nail matrix when sampling the deceased rather than to the nail tip, so that the directly proportional signal of ^{14}C of the subject's 'last meal' and keratinized tissue can be evaluated against the atmospheric ^{14}C bomb peak for a precise calendar age estimate.

REFERENCES

[1] G. A. A. Cooper, R. Kronstrand, P. Kintz. Society of hair testing guidelines for drug testing in hair. *Forensic Sci. Int.* **2011**, *218*, 20.

[2] H. Sachs. History of hair analysis. *Forensic Sci. Int.* **1997**, *84*, 7.

[3] C. M. Selavka, F. Rieders. The determination of cocaine in hair: a review. *Forensic Sci. Int.* **1995**, *70*, 155.

[4] C. Staub. Analytical procedures for determination of opiates in hair: a review. *Forensic Sci. Int.* **1995**, *70*, 111.

[5] J. H. Williams, T. C. O'Connell. Differential relations between cognition and ^{15}N isotopic content of hair in elderly people with dementia and controls. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences* **2002**, *57*, M797.

[6] K. J. Petzke, B. T. Fuller, C. C. Metges. Advances in natural stable isotope ratio analysis of human hair to determine nutritional and metabolic status. *Curr. Opin. Clin. Nutr. Metab. Care* **2010**, *13*, 532.

[7] L. O. Valenzuela, L. A. Chesson, S. P. O'Grady, T. E. Cerling, J. R. Ehleringer. Spatial distributions of carbon, nitrogen and sulfur isotope ratios in human hair across the central United States. *Rapid Commun. Mass Spectrom.* **2011**, *25*, 861.

[8] R. Bol, J. Marsh, T. H. Heaton. Multiple stable isotope (^{18}O, ^{13}C, ^{15}N and ^{34}S) analysis of human hair to identify the recent migrants in a rural community in SW England. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2951.

[9] G. Hodgins. Measuring atomic bomb-derived ^{14}C levels in human remains to determine Year of Birth and/or Year of Death. *The National Criminal Justice Reference Service - NCJRS Report 227839.* **2009**, p 97 (available electronically).

[10] Q. Hua, M. Barbetti, A. Z. Rakowski. Atmospheric radiocarbon for the period 1950–2010. *Radiocarbon* **2013**, *55*, 2059.

[11] L. Caforio, M. E. Fedi, P. A. Mandò, F. Minarelli, E. Peccenini, V. Pellicori, F. C. Petrucci, P. Schwartzbaum, F. Taccetti. Discovering forgeries of modern art by the ^{14}C Bomb Peak. *Eur. Phys. J. Plus* **2014**, *129*, 1.

[12] K. T. Uno, J. Quade, D. C. Fisher, G. Wittemyer, I. Douglas-Hamilton, S. Andanje, P. Omondi, M. Litoroh, T. E. Cerling. Bomb-curve radiocarbon measurement of recent biologic tissues and applications to wildlife forensics and stable isotope (paleo) ecology. *Proc. Natl. Acad. Sci.* **2013**, *110*, 11736.

[13] L. Andreu-Hayles, G. M. Santos, D. A. Herrera-Ramírez, J. Martin-Fernández, D. Ruiz-Carrascal, T. E. Boza-Espinoza, A. F. Fuentes, P. M. Jørgensen. Matching dendrochronological dates with the Southern Hemisphere ^{14}C Bomb Curve to confirm annual tree rings in *Pseudolmedia Rigida* from Bolivia. *Radiocarbon* **2015**, *57*, 1.

[14] E. M. Wild, K. A. Arlamovsky, R. Golser, W. Kutschera, A. Priller, S. Puchegger, W. Rom, P. Steier, W. Vycudilik. ^{14}C dating with the bomb peak: an application to forensic medicine. *Nucl. Instrum. Methods Phys. Res. B* **2000**, *172*, 944.

[15] N. Lynnerup, H. Kjeldsen, R. Zweihoff, S. Heegaard, C. Jacobsen, J. Heinemeier. Ascertaining year of birth/age at death in forensic cases: a review of conventional methods and methods allowing for absolute chronology. *Forensic Sci. Int.* **2010**, *201*, 74.

[16] C. F. Bengtsson, M. E. Olsen, L. Ø. Brandt, M. F. Bertelsen, E. Willerslev, D. J. Tobin, A. S. Wilson, M. T. Gilbert. DNA

from keratinous tissue, part I: hair and nail. *Annals of Anatomy* **2012**, *194*, 17.
[17] C. S. Whewell. The chemistry of hair. *J. Soc. Cosmet. Chem.* **1961**, *12*, 207.
[18] A. Hunting. *Encyclopedia of Shampoo Ingredients*. Micelle Press, Cranford, **1983**, pp. 480.
[19] A. Hunting. *Encyclopedia of Conditioning Rinse Ingredients*. Micelle Press, Cranford, **1987**, pp. 506.
[20] J. F. Corbett. Chemistry of hair colorant processes – science as an aid to formulation and development. *J. Soc. Cosmet. Chem.* **1984**, *35*, 297.
[21] A. Kuzuhara, T. Hori. New method of dyeing keratin fibers using poly (ethylene imine) and its coloring mechanism. *J. Appl. Polymer Sci.* **2003**, *90*, 3806.
[22] FDA/USA. Food and Drug Administration/USA, Hair Dyes: Fact Sheet. Available: http://www.fda.gov/Cosmetics/ProductsIngredients/Products/ucm143066.htm (last updated 03/19/2014).
[23] R. H. DeSelms. *UV-Active Phenol Ester Compounds*. Enigen Science Publishing, Washington, **2008**.
[24] H. E. Cox. Hair dyes II. The functions and reactions of phenols. *Analyst* **1940**, *65*, 393.
[25] M. Gamez-Garcia. Effects of some oils, emulsions and other systems on the mechanical properties of hair at small deformations. *J. Soc. Cosmet. Chem.* **1993**, *44*, 69.
[26] E. Oshimura, M. Ino. Effects of arginine on hair damage via oxidative coloring process. *J. Cosmet. Sci.* **2004**, *55*, S155.
[27] E. Oshimura, H. Abe, R. Oota. Hair and amino acids: the interactions and the effects. *J. Cosmet. Sci.* **2007**, *58*, 347.
[28] A. L. Villa, M. R. Aragão, E. P. Dos Santos, A. M. Mazotto, R. B. Zingali, E. P. de Souza, A. B. Vermelho. Feather keratin hydrolysates obtained from microbial keratinases: effect on hair fiber. *BMC Biotechnol.* **2013**, *13*, 15.
[29] R. Jones, S. Chahal. The use of radiolabelling techniques to measure substantivity to, and penetration into, hair of protein hydrolysates. *Int. J. Cosmet. Sci.* **1997**, *19*, 215.
[30] A. Mainkar, C. Jolly. Formulation of natural shampoos. *Int. J. Cosmet. Sci.* **2001**, *23*, 59.
[31] H. Feigenbaum. *The Use of Cationizing Reagents in the Preparation of Conditioning Polymers for Hair and Skin Care*. SKW QUAB Chemicals, Inc., Saddle Brook, **2009**.
[32] T. Evans, R. R. Wickett. Practical Modern Hair Science. Allured Pub Corp., **2012**, p. 562.
[33] Z. D. Draelos. Essentials of hair care often neglected: Hair cleansing. *Int. J. Trichol.* **2010**, *2*, 24.
[34] B. Bhushan. *Biophysics of Human Hair: Structural, Nanomechanical, and Nanotribological Studies*. Springer, New York, **2010**, IX, p. 192. ISBN: 978-3-642-15900-8.
[35] C. R. Robbins, C. H. Kelly. Amino acid analysis of cosmetically altered hair. *J. Soc. Cosmet. Chem.* **1969**, *20*, 555.
[36] N. Baba, Y. Nakayama, F. Nozaki, T. Tamura. Denaturation and release of amino acids in hairs treated with cold-waving lotions, hair-dyes, hair-bleaching solutions and depilatories. *Journal of Hygienic Chemistry* (*in Japanese*) **1973**, *19*, 4.
[37] A. Guerra-Tapia, E. Gonzalez-Guerra. Hair Cosmetics: Dyes. *Actas Dermo-Sifiliográficas (English Edition)* **2014**, *105*, 833.
[38] Y. Song, M. Srinivasarao, A. Tonelli, C. M. Balik, R. McGregor. Laser scanning confocal microscopy study of dye diffusion in fibers. *Macromolecules* **2000**, *33*, 4478.
[39] B. J. Chen, P. L. Lee, W. Y. Chen, F. D. Mai, Y. C. Ling. Hair dye distribution in human hair by ToF-SIMS. *Appl. Surface Sci.* **2006**, *252*, 6786.
[40] S. B. Ruetsch, Y. K. Kamath, A. S. Rele, R. Mohile. Secondary ion mass spectrophotometric Investigation of penetration of coconut and mineral oils into human hair. *J. Cosmet. Sci.* **2001**, *5*, 169.
[41] S. B. Hornby, Y. Appa, S. Ruetsch, Y. Kamath. Mapping penetration of cosmetic compounds into hair fibers using time-of-flight secondary ion mass spectrometry (TOF-SIMS). *Int. J. Cosmet. Sci.* **2005**, *27*, 299.
[42] S. B. Ruetsch, Y. K. Kamath. Penetration of cationic conditioning compounds into hair fibers: A TOF-SIMS approach. *J. Cosmet. Sci.* **2005**, *56*, 323.
[43] M. Zimmerley, C. Y. Lin, D. C. Oertel, J. M. Marsh, J. L. Ward, E. O. Potma. Quantitative detection of chemical compounds in human hair with coherent anti-Stokes Raman scattering microscopy. *J. Biomed. Optics* **2009**, *14*, 044019.
[44] S. Hartwig, V. Auwärter, F. Pragst. Effect of hair care and hair cosmetics on the concentrations of fatty acid ethyl esters in hair as markers of chronically elevated alcohol consumption. *Forensic Sci. Int.* **2003**, *131*, 90.
[45] P. Kintz, D. Nicholson. Testing for ethanol markers in hair: Discrepancies after simultaneous quantification of ethyl glucuronide and fatty acid ethyl esters. *Forensic Sci. Int.* **2014**, *243*, 44.
[46] S. Broecker, S. Herre, F. Pragst. General unknown screening in hair by liquid chromatography–hybrid quadrupole time-of-flight mass spectrometry (LC–QTOF-MS). *Forensic Sci. Int.* **2012**, *218*, 68.
[47] H. A. M. De La Torre, K. M. Ormsby, B. T. Fuller, G. M. Santos. ^{14}C measurements elucidate isotopic differences between nails and hair in modern humans. *Radiocarbon* **2014**, *56*, 53.
[48] T. C. O'Connell, R. E. M. Hedges. Investigations into the effect of diet on modern human hair isotopic values. *Am. J. Phys. Anthropol.* **1999**, *108*, 409.
[49] M. Boudin, P. Boeckx, P. Vandenabeele, M. Van Strydonck. Improved radiocarbon dating for contaminated archaeological bone collagen, silk, wool and hair samples via cross-flow nanofiltrated amino acids. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 2039.
[50] G. M. Santos, K. Ormsby. Behavioral variability in ABA chemical pretreatment close to the ^{14}C age limit. *Radiocarbon* **2013**, *55*, 534.
[51] A. Buekenhoudt, F. Bisignano, G. De Luca, P. Vandezande, M. Wouters, K. Verhulst. Unravelling the solvent flux behaviour of ceramic nanofiltration and ultrafiltration membranes. *J. Membrane Sci.* **2013**, *439*, 36.
[52] G. M. Santos, J. R. Southon, K. C. Druffel-Rodriguez, S. Griffin, M. Mazon. Magnesium perchlorate as an alternative water trap in AMS graphite sample preparation: a report on sample preparation at KCCAMS at the University of California, Irvine. *Radiocarbon* **2004**, *46*, 165.
[53] G. M. Santos, M. Mazon, J. R. Southon, S. Rifai, R. Moore. Evaluation of iron and cobalt powders as catalysts for 14 C-AMS target preparation. *Nucl. Instrum. Methods Phys. Res. Sect. B* **2007**, *259*, 308.
[54] R. K. Beverly, W. Beaumont, D. Tauz, K. M. Ormsby, K. F. von Reden, G. M. Santos, J. R. Southon. The Keck Carbon Cycle AMS Laboratory, University of California Irvine: Status report. *Radiocarbon* **2010**, *52*, 301.
[55] G. M. Santos, R. B. Moore, J. R. Southon, S. Griffin, E. Hinger, D. Zhang. AMS ^{14}C sample preparation at the KCCAMS/UCI Facility: status report and performance of small samples. *Radiocarbon* **2007**, *49*, 255.
[56] Z. Sharp. *Principles of Stable Isotope Geochemistry*. Prentice Hall, USA, **2007**.
[57] I. Levin, B. Kromer, S. Hammer. Atmospheric $\Delta^{14}CO_2$ trend in Western European background air from 2000 to 2012. *Tellus B* **2013**, *65*.
[58] P. Eriksson. Nanofiltration extends the range of membrane filtration. *Environ. Prog.* **1988**, *7*, 58.
[59] W. Meier-Augenstein. Applied gas chromatography coupled to isotope ratio mass spectrometry. *J. Chromatogr. A* **1999**, *842*, 351.
[60] W. Bergfeld, F. Mulinari-Brenner. Shedding: How to manage a common cause of hair loss. *Cleveland Clinic J. Med.* **2001**, *68*, 256.

[61] M. J. DeNiro. Postmortem preservation and alteration of in vivo bone collagen isotope ratios in relation to palaeodietary reconstruction. *Nature* **1985**, *317*, 806.
[62] T. C. O'Connell, R. E. M. Hedges, M. A. Healey, A. H. R. W. Simpson. Isotopic comparison of hair, nail and bone: Modern analyses. *J. Archaeol. Sci.* **2001**, *28*, 1247.
[63] M. V. R. Velasco, T. C. de Sá Dias, A. Z. de Freitas, N. D. Vieira Júnior, C. A. S. de Oliveira Pinto, T. M. Kaneko, A. R. Rolim Baby. Hair fiber characteristics and methods to evaluate hair physical and mechanical properties. *Brazilian J. Pharmaceut. Sci.* **2009**, *45*, 1.
[64] Y. Zhou, R. Rigoletto, D. Koelmel, G. Zhang, T. W. Gillece, L. Foltis, D. J. Moore, X. Qu, C. Sun. The effect of various cosmetic pretreatments on protecting hair from thermal damage by hot flat ironing. *J. Cosmet. Sci.* **2011**, *62*, 265.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website.

