Journal of Natural Fibers

ISSN: (Print) (Online) Journal homepage: www.tandfonline.com/journals/wjnf20

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To cite this article: Theresa Schmidt, Jacqueline Koch, Nils Puchalla & Annemarie E. Kramell (2024) Characterization of Recent and Historical Silks: Effects of Silk Processing on Chemical Composition and Amino Acid Racemization, Journal of Natural Fibers, 21:1, 2361312, DOI: 10.1080/15440478.2024.2361312

To link to this article: https://doi.org/10.1080/15440478.2024.2361312

JOURNAL OF NATURAL FIBERS
2024, VOL. 21, NO. 1, 2361312
https://doi.org/10.1080/15440478.2024.2361312

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Characterization of Recent and Historical Silks: Effects of Silk Processing on Chemical Composition and Amino Acid Racemization

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ABSTRACT

In this work, the influence of dyeing, mordanting and degumming techniques on the amino acid (AA) composition and the AA racemization (AAR) of (non-)mulberry silks is studied by a chiral GC-MS method. The tested dyeing and mordanting procedures as well as a pre-treatment of the silk filaments with EDTA-DMF, which enables a parallel dyestuff analysis by HPLC-DAD, do not cause any significant change in the AA composition. However, an increased proportion of e.g. (D)-Asx can be observed for some of the mordanted BM silks. Increased D/L Asx ratios can also be observed in indigo dyed silks and after an alkaline treatment at 110°C ($pH > 9$). Furthermore, the GC-MS method was used for the characterization of historical silk samples from finding sites in the Tarim Basin (China). The analysis of these silk fabrics reveals the presence of degraded *Bombyx* silk with significantly increased D/L Val, D/L Glx and D/L Asx ratios. Including microscopic and spectroscopic investigations, the results of this multi-analytical approach offer insights into the silk processing techniques and raw materials used.

摘要

采用手性气相色谱-质谱联用技术研究了染色、媒染和脱胶工艺对（非）桑蚕丝氨基酸（AA）组成和AA外消旋（AAR）的影响. 所测试的染色和媒染程序以及用EDTA-DMF对丝线进行预处理，这使得能够通过HPLC-DAD进行平行染料分析，不会导致AA组成的任何显著变化. 然而，对于一些媒染的BM丝，可以观察到例如（D）-Asx的比例增加. 在靛蓝染色的丝绸中以及在110°C（$pH > 9$）的碱性处理后，也可以观察到D/L Asx比率的增加. 此外，采用气相色谱-质谱联用技术对塔里木盆地发现地的历史丝绸样品进行了表征. 对这些丝绸织物的分析揭示了降解的家蚕丝绸的存在，其D/L-Val、D/L-Glx和D/L-Asx比率显著增加. 包括显微镜和光谱研究在内，这种多分析方法的结果为丝绸加工技术和所用原材料提供了见解.

KEYWORDS

Silk; *Bombyx mori*; silk processing; amino acid racemization; chiral GC-MS; historical textiles

关键词

丝; 家蚕; 丝绸加工; 氨基酸外消旋化; 手性GC-MS; 历史纺织品

Introduction

Silk is an exclusive material with unique properties that has been used for thousands of years in the production of textiles. Various silk producers have been used throughout history, but the most important is the domesticated silkworm *Bombyx mori* L. (BM), which feeds exclusively on mulberry leaves of the genus *Morus* and is descended from the Chinese wild silkworm *Bombyx mandarina* Moore (Arunkumar, Metta, and Nagaraju 2006; Xia et al. 2009). Studies on the genetic diversity of *Bombyx* silkworm strains indicate that silkworms were initially domesticated in China and then subjected to independent spreads along the Silk Road that gave rise to the development of most local strains (Xiang et al. 2018). The transfer of the sericulture from China westwards to the kingdoms of the Tarim Basin must have taken place between the second-century BCE and the third-century CE,

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/15440478.2024.2361312

which correlates with China's Han Dynasty (202 BCE–220 CE) and the Three Kingdoms period (220–80) (Wu 2024). In the following centuries, sericulture spread further westwards to Byzantium and Europe, probably reaching Byzantium in the mid-sixth century (Wu 2024).

Cocoons are a multi-layered composite material consisting of two proteins, namely the water-insoluble fibroin and the water-soluble, glue-like sericin, which coats the fibroin filaments and binds them together to form an intact cocoon. The production of silk fabrics from cocoons is very complex and involves a large number of steps. To prevent the butterfly from hatching and thus destroying the cocoon, cocoons are first treated with steam or in a vacuum at 65–75°C (Veit 2022). After removing the outer, loose cocoon layer, softening the sericin in hot water and searching for the outer end of the filament with a brush, cocoons can be unwound, whereby an approximately 700–2000 m long thread can be unreeled from a single cocoon (Veit 2022). To obtain soft, smooth and glossy silk fabrics, the reeled raw silk is degummed. Enzymes, organic acids, alkaline degumming agents and/or surfactants such as sodium carbonate (Na_2CO_3) or Marseille soap are often used to remove the sericin coating (Biswal et al. 2022). In order to compensate for the weight loss during degumming, which would lower the selling price, metal salts, vegetable or synthetic substances can be incorporated into the fibroin filament in a process known as weighting (Garside, Wyeth, and Zhang 2010). Damaged or hatched cocoons and non-reeling parts of the cocoons can be used to produce spun silk yarns, which are known as Bourette, Schappe or floret silk (for a further distinction see (Veit 2022)). In the manufacture of textiles, silk is also frequently bleached, printed or dyed, whereby the dyeing can take place in the piece, i.e. as a fabric, or in strand form. In the latter case, dyed yarns are processed on various looms, e.g. treadle looms, to create colorful and luxurious fabrics.

Expensive silk fabrics, which have been a symbol of wealth and luxury for many centuries, especially in Europe, are common in museum collections. Interdisciplinary studies of these textile testimonials help to understand and reconstruct ancient technologies, trade routes and cultural exchanges. In addition to the examination of, for example, weaving patterns, fiber and dyestuff analyses are a key issue of these interdisciplinary studies (Gao et al. 2024; Ge et al. 2023; Karadag and Oraltay 2022; Kramell et al. 2014). For instance, microscopic and Fourier-transform infrared (FTIR) or Raman spectroscopic investigations can reveal the degradation state of the fibroin filaments or the presence of a sericin coating (Deveoglu et al. 2019; Koperska et al. 2014; Vilaplana et al. 2015). These methods can also be used to obtain initial indications of the presence of BM or non-mulberry silk, e.g. from *Antheraea mylitta* Drury (AM) (Boulet-Audet, Vollrath, and Holland 2015; de Palaminy, Daher, and Moulherat 2022; Gao et al. 2024). Since the amino acid (AA) sequence and composition of silk fibroin from non-mulberry silkworms differs from BM, AA analyses as well as proteomic approaches can provide further information with regard to the silk producer (Ge et al. 2023; Kmet et al. 2023; Li et al. 2015; Reddy, Zhao, and Yang 2013; Zheng et al. 2021). Nano-flow liquid chromatography tandem mass spectrometry (nanoLC-MS/MS) in combination with a calcium nitrate solubilization and a suitable enzyme digestion enables, for example, the determination of AA sequences and thus the identification of species-specific fibroin proteins. Corresponding analyses of silk fabrics from the archaeological site of Palmyra revealed the use of wild silks derived from AM (Lee et al. 2022). Lee et al. thus provide the first biochemical evidence for long-standing archaeological speculation about the production of Indian wild silk in antiquity and international trade between ancient Palmyra and the Indian subcontinent (Lee et al. 2022). Information on the structure of silk can also be obtained by ^{13}C CP-MAS NMR spectroscopy (Deveoglu et al. 2019). In addition, Deveoglu et al. use ^{27}Al MAS NMR spectra to characterize silk fabrics mordanted with alum and dyed with different flavonoids (Deveoglu et al. 2019).

Furthermore, AA racemization (AAR) rates can be used to characterize historical silks. Moini et al. introduced capillary electrophoresis MS (CE-MS) for age estimation of silk textiles based on the racemization rate of asparagine (Asn) and aspartic acid (Asp) (Moini and Rollman 2017; Moini, Klauenberg, and Ballard 2011; Shimada et al. 2023). This technique is suitable for dating BM silks from museum collections, ranging in age from the present to ~2500 years ago. At approximately 100 µg (or less), the amount of sample used is significantly less than the amount required for accelerator mass spectrometry (AMS) dating (^{14}C dating) (Moini, Klauenberg, and Ballard 2011). In addition, the costs of authenticating silk using AMS dating are relatively high and the presence of dyestuffs or other difficult-to-remove contaminants such as humic substances can falsify the ^{14}C age (Boudin et al. 2013). In contrast, AAR is affected by environmental factors such as pH value, temperature, humidity, UV radiation, impurities and the presence of metal ions (Bada 1985; Bright and Kaufman 2011; Goodfriend 1992; Kaufman 2006; Smith, Williams, and Wonnacott 1978). The influence of factors such as UV radiation, pH value and humidity on the D/L ratio of AAs and thus on the age determination can be minimized if archaeological remains are protected, e.g. by an inorganic template. When examining silks, it should be noted that silk processing, e.g. degumming or dyeing, can accelerate AAR. Initial studies by Moini et al. and Shimada et al. have already shown that treatment of BM silk with alkaline solutions at elevated temperatures can have a significant effect on the racemization rate of Asp, Asn, phenylalanine (Phe) and tyrosine (Tyr) (Moini and Rollman 2017; Shimada et al. 2023). These studies also showed that CE-MS measurements enable the identification of artificially aged silk, obtained by a chemical treatment with a base at elevated temperatures, and thus silk forgery (Moini and Rollman 2017). Furthermore, Moini et al. investigated the effect of UV exposure on (L) to (D) conversion of silk proteins (Moini, Klauenberg, and Ballard 2011). However, systematic studies on the influence of degumming, mordanting and dyeing techniques on AAR rates are still pending. The influence of mordants is of particular interest as it is known that the presence of metal ions such as Cu^{2+}, Cd^{2+} and Fe^{2+}, which are typically used for mordanting (Cardon 2007; Schweppe 1992), can a) catalyze degradation reactions of AAs or proteins and b) affect AAR rates by stabilizing the carbanion intermediate (Johnson and Pratt 2010; Smith, Williams, and Wonnacott 1978). Fe^{2+} in particular leads to powerfully oxidizing hydroxide radicals in a so-called Fenton's reaction. Furthermore, when examining proteinaceous materials by GC or LC, it should be noted that metal ions and polyvalent cations such as Ca^{2+} can reduce the efficiency of AA derivatization by forming strong cation ion-AA complexes (Bonaduce, Cito, and Colombini 2009; Colombini and Modugno 2004). These complexes can selectively subtract some AAs from the analysis and thus prevent the identification of the proteins (Bonaduce, Cito, and Colombini 2009). The effect of polyvalent cations on the identification of proteinaceous binders in paintings has already been investigated, including the development of analytical procedures with purification steps to remove inorganic components (Bonaduce, Cito, and Colombini 2009; Lluveras et al. 2010). However, the authors are not aware of any corresponding studies on silk. In addition to the use of metal salts such as copper(II) sulfate or stannic chloride for mordanting or weighting, the conditions of use, storage or conservation may also result in a higher content of metal ions in historical silk samples. A high content of metal ions is to be expected, especially in archaeological samples with soil contact.

In this study, we investigate the influence of different dyeing and mordanting techniques on the AA composition of mulberry and non-mulberry silks. The investigations were carried out on BM, AM and *Attacus atlas* L. (AtA) silk samples, which differ in terms of their morphology, chemical composition and physical structure. In addition, the AAR rates of mordanted and dyed samples were determined, whereby the effect of the degumming method was also considered. In this context, a gas chromatography-MS (GC-MS) method for the identification and quantification of 11 enantiomeric AA pairs in silk fibroin was developed and validated. In the course of method development, clean-up steps using organic-aqueous systems and the chelating agent EDTA were tested, which enable the extraction of natural dyes and thus a parallel dyestuff analysis by high-performance liquid chromatography-diode array detection (HPLC-DAD). Furthermore, the influence of the derivatization and hydrolysis procedure on the racemization of AAs was studied. The developed and validated GC-MS method was used to characterize historical silk samples from finding sites in the Tarim Basin, covering a period from the first millennium BC to third century AD. Furthermore, these samples were analyzed by optical microscopy (OM), scanning electron microscopy (SEM), SEM in combination with energy-dispersive X-ray spectroscopy (SEM-EDX), FTIR spectroscopy in attenuated total reflection mode (ATR-FTIR spectroscopy), HPLC-DAD and liquid chromatography-electrospray ionization-MS/MS (LC-ESI-MS/MS). On the one hand, the aim was to evaluate the influence of silk processing on the AA composition and the AAR of recent silks. On the other hand, a multi-analytical technique-based approach was established, which enables a comprehensive characterization of historical silk samples and thus provides information on ancient silk processing techniques and processed raw materials, especially on colorants and silk used. In addition, degradation processes are characterized on a molecular level.

Material and methods

Chemicals and materials

L-Alanine (L-Ala; 99%), L-isoleucine (L-Ile; 99%), L-proline (L-Pro; 99%), L-cysteine (L-Cys; >98%), L-lysine (L-Lys; 98%) and L-norleucine (L-Nle; 99%) were obtained from Alfa Aesar. D-Alanine (D-Ala, 99%), D-aspartic acid (D-Asp, 99%), D-cysteine (D-Cys, 98%), D-isoleucine (D-Ile, >98%), D-methionine (D-Met, 99%), D-norleucine (D-Nle, 99%), D-phenylalanine (D-Phe, 99%), D-threonine (D-Thr, 99%), D-tyrosine (D-Tyr, 99%), D-tryptophane (D-Trp, 99%) and chloroform (HPLC grade) were purchased from Thermo Fisher GmbH; glycine (Gly; ≥99%) and D-histidine (D-His; >99%) from TCI (Tokyo Chemical Industry); L-leucine (L-Leu; ≥99%), L-valine (L-Val; ≥99%), D- and L-glutamic acid (Glu; ≥99%), L-aspartic acid (L-Asp; ≥99%), L-threonine (L-Thr; ≥99%), L-phenylalanine (L-Phe; ≥99%), L-methionine (L-Met; ≥99%), D-serine (D-Ser; >99%), D-leucine (D-Leu; >99%), calcium oxalate (≥98%) and iron(II) sulfate heptahydrate ($Fe(II)SO_4 \cdot 7\,H_2O$, ≥99%, p.a.) from Carl Roth GmbH + Co. KG; L-serine (L-Ser; >99%), L-histidine (L-His; ≥99%), D-lysine (D-Lys, >98%), sodium carbonate (Na_2CO_3; anhydrous), pyridine (≥99.0%), ethylene diamine (≥99%), phenol (≥96%), ethylenediaminetetraacetic acid (EDTA, 99.4–100.6%), 3,3′-dithiodipropionic acid (DTDPA, 99%) and indigo (95%) from Sigma-Aldrich; L-tryptophane (L-Trp; 99%) and D-proline (p-Pro, >99%) from Acros Organics; D-valine (D-Val, >99%) from MP Biomedicals LLC.; isooctane from VWR Chemicals [ACS (American Chemical Society), Reag. Ph. Eur. (*Pharmacopoea Europaea*)] and Supelco (for liquid chromatography; ACS reagent, ≥99.0%); *N,N*-dimethylformamide (DMF, 99.5%), isopropanol (for HPLC), concentrated hydrochloric acid (HCl; 37%) and sodium hydroxide (NaOH, 98%, reinst, pellets) from Fisher Scientific; methanol (MeOH, HPLC gradient grade) and acetonitrile (ACN, HPLC super gradient grade) from VWR Chemicals; ethylene diamine (≥99.5%; GC), potassium hydrogen tartrate and sodium dithionite from Merck KGaA; potassium aluminum sulfate dodecahydrate ($KAl(SO_4)_2 \cdot 12\,H_2O$, 99.0–100.5%) from Riedel-de Haën; copper(II) sulfate pentahydrate ($Cu(II)SO_4 \cdot 5\,H_2O$, >99%, p.a.) from Fluka. 2,2,3,3,4,4,4-Heptafluorobutyl chloroformate (HFBCF) was brought from Apollo Scientific Ltd (97%, Cheshire, United Kingdom) and Carbolution Chemicals GmbH (95%, St. Ingbert, Germany). Dried and ground madder roots (*Rubia tinctorum* L.) were bought from Kremer Pigmente GmbH & Co KG (Aichstetten, Germany).

Cocoons, recent silk and wool samples

BM cocoons were obtained from the World of Butterflies & Moths (Lincolnshire, England), the Francke Foundations (Halle/Saale, Germany) and a Chinese web store. Degummed BM silk and cocoons of AM (Indian tasar silkmoth) were purchased from Seidentraum (organic peace silk, Sternenfels, Germany), a sheep wool fleece (100% merino wool) from Galerie Smend (Köln, Germany). Cocoons of AtA were obtained from the butterfly house of Jonsdorf (Germany); *Antheraea pernyi* Guérin-Méneville (APe) silkworm cocoons from the World of Butterflies & Moths (Lincolnshire, England).

Historical textile samples

Silk and wool samples from archaeological sites of Niya, Yanghai and Wupu in the Xinjiang Uyghur Autonomous Region (Tarim Basin, China) were taken within the framework of an interdisciplinary

Chinese-German research project (for a further description of the samples and finding sites, see Beck et al. 2014; Kramell et al. 2014; Kramell et al. 2016; Wertmann et al. 2020).

Degumming of cocoons (Schmidt et al. 2023)

Before degumming, pupae, plant parts and coarse impurities were removed with tweezers. Cocoons were degummed at 95°C for 60–120 min (reaction time for BM cocoons: 30–90 min; reaction time for AM and APe cocoons: 90 min, reaction time for AtA cocoons: 120 min) in an aqueous solution containing Na_2CO_3 (1 g/L) and ethylenediamine (2.5% w/w). After degumming, the fibers were immediately rinsed thoroughly with distilled water until a neutral pH value was achieved and dried at 100°C until constant weight was reached. All experiments were repeated three times. The efficiency of the degumming process was monitored by microscopic and ATR-FTIR spectroscopic studies, as well as by determining the degumming ratio D_r (%), as reported by (Schmidt et al. 2023).

To investigate the influence of alkaline degumming procedures, BM cocoons were treated with aqueous solution containing Na_2CO_3 (1 g/L or 2.5 g/L) at 95°C for 30–120 min. Furthermore, BM cocoons were degummed with ethylenediamine (2.5% w/w) at 95°C for 120 min as described by (Schmidt et al. 2023).

Treatment of silk with Na_2CO_3 solutions (modified according to (Moini and Rollman 2017))

To simulate the artificial aging described by Moini and Rollman (Moini and Rollman 2017), degummed BM silk samples and cocoons (200 mg) were treated in sealed tubes with aqueous Na_2CO_3 solutions (3 mL, pH 9, 11 and 13) at 110°C for 60–120 min. The pH of the solutions was measured using a digital pH meter and adjusted to a pH of 9, 11 and 13 with HCl (6 M) or NaOH (1 M). After alkaline treatment, the filaments were then rinsed with MeOH and distilled water and dried at room temperature. Dried silk samples were hydrolyzed and then analyzed by GC-MS as described below.

Sample treatment with calcium oxalate

In order to determine the influence of a possible residue of calcium oxalate, which is found on some wild silk cocoons, BM, AtA and AM silk samples were treated with calcium oxalate. For this purpose, degummed silk (200 mg) was moistened with water and treated with an aqueous calcium oxalate suspension (100 mL; 15% based on the dry weight of the silk) for 60 min at 40°C. Subsequently, the silk fibers were rinsed two times with water and dried at room temperature.

Dyeing and mordanting procedure (modified according to (Cardon 2007) and (Schweppe 1992))

In the course of the mordanting procedure, degummed silk (200 mg) was first moistened with water to ensure uniform and complete wetting with the metal salt solution. During the mordanting and dyeing process, the silk is turned occasionally.

All experiments were repeated three times with degummed silk of BM, AtA and AM.

Mordanting procedure with potassium alum

The mordant bath was prepared by adding $KAl(SO_4)_2 \cdot 12\,H_2O$ (15% - based on the dry weight of the silk) in water (100 mL) at 40°C. Degummed and moistened silk was immersed into the bath, which was then heated to 60°C and left at this temperature for 60 min. After the mordant bath had cooled to room temperature, the silk was removed, rinsed several times with water, and dried at room temperature or used while still wet for the subsequent madder dyeing process.

Mordanting procedure with potassium alum and potassium hydrogen tartrate

Mordanting procedure described above was carried out in the presence of $KAl(SO_4)_2 \cdot 12\ H_2O$ (15% - based on the dry weight of the silk) and potassium hydrogen tartrate (4% - based on the dry weight of the silk).

Mordanting procedure with copper(II) sulfate or iron(II) sulfate

The mordant bath was prepared by adding $Cu(II)SO_4 \cdot 5\ H_2O$ (6% based on the dry weight of the silk) or $Fe(II)SO_4 \cdot 7\ H_2O$ (3% based on the dry weight of the silk) in water (100 mL) at 40°C. Afterward, degummed and moistened silk was immersed into the bath and was treated for 60 min at 60°C with this solution. After the mordant bath had cooled to room temperature, the silk was removed, rinsed several times with water, and dried at room temperature.

Mordant dyeing with madder (Rubia tinctorum *L.*)

Preparation of the dye bath: A filter containing dried and ground madder roots (10 g) was soaked in water (200 mL) overnight. The extract was then heated slowly within 60 min until reflux began, and the temperature was kept constant for a further 10 min. After removing the heat source, water (100 mL) was added and the plant material was removed. Degummed and moistened silk mordanted with potassium alum or potassium alum and potassium hydrogen tartrate was added to the dye bath. The dye bath was heated to 60°C and left at this temperature for 30 min. Afterward, the dye bath was cooled to room temperature, the silk was removed, rinsed several times with water until the wash water appeared clear and dried at room temperature.

Vat dyeing with indigo

For a suspension of synthetic indigo (2 g, powder) in water (8 mL), an aqueous NaOH solution (7%, m/v) was added and the suspension was heated to 60°C. Then sodium dithionite was added until the solution began to turn yellow. Degummed silk (200 mg) was moistened with water and immersed into the dye bath at 55°C for 2–3 min. The silk was then removed, squeezed vigorously and air-dried. After drying, the silk was thoroughly rinsed with water and dried at room temperature. If necessary, the dyeing process can be repeated so that deeper shades can be achieved.

Sample pre-treatment with an EDTA solution

Sample preparation with EDTA-ACN-MeOH (modified according to (Manhita et al. 2011; Zhang and Laursen 2005))

For some silk samples, an additional purification step was performed prior to the hydrolysis (see Conventional hydrolysis of silk samples) to eliminate interfering components such as metal ions or organic impurities. For this purpose, silk samples (20 mg) were treated with an EDTA solution [4 mL, H_2EDTA (aqueous, 0.1%)/ACN/MeOH, 2:10:88, v/v/v] for 30 or 60 min at 60°C. Subsequently, the sample material was rinsed with MeOH (2 × 4 or 10 mL) and dried at room temperature. In the course of method development, the procedure was tested on recent BM, AM and AtA silk samples (non-mordanted/undyed and mordanted/dyed samples).

Sample preparation with EDTA-DMF (modified according to (Manhita et al. 2011))

For some dyed silk samples, an additional extraction step was performed prior to the hydrolysis to allow parallel dyestuff analyses. For this purpose, red and blue dyed samples (recent and historical materials, 20 mg) were extracted with an EDTA solution (4 mL, 0.1%, aqueous-organic system consisting of water and DMF, 1:1 v/v) for 30 and 60 min at 95°C. The fibers were then rinsed with MeOH (2 × 4 or 10 mL) and dried at room temperature. Dried silk samples were hydrolyzed and then analyzed by GC-MS as described below. EDTA-DMF extracts were analyzed for dyestuffs by HPLC-DAD as described in previous studies (Kramell et al. 2014; Kramell et al. 2016). In the course of method development, the procedure was tested on BM, AM and AtA silk, dyed with indigo or madder.

AA analysis by GC-FID

AA analyses using GC-FID were performed as reported by (Schmidt et al. 2023).

AA analysis by GC-MS

Samples were analyzed with a Shimadzu gas chromatograph Nexis GC-2030, equipped with an AOC-20i injector (injection volume: 0.5 µL, splitless mode), an AOC-20s autosampler and a mass spectrometer (MS; GCMS-QP2020 NX, ion source temperature: 210°C, mass range: *m/z* 60–650, full scan mode, voltage conversion dynode: 70 eV). Chromatographic separation was performed on a CP-ChiraSil-L-Val column (25 m × 0.25 mm × 0.12 µm, Agilent). The chromatogram was recorded in the retention time range from 4 to 54 min. The injection port was held at 230°C, and the interface between the column and the detector source was set at 200°C. The column oven temperature was initially set to 92°C, held for 1 min and then ramped with 2.5°C min^{-1} to 200°C, held for 10 min; the carrier gas was hydrogen at a constant column flow of 1.20 mL/min (linear velocity: 66.0 cm/sec). All analyses were performed in triplicate. After use, the autosampler syringe should be rinsed with isopropanol followed by chloroform and isooctane to prevent corrosion of the plunger.

Preparation of standard solutions

Stock solutions (5, 10, 20 and 50 mM) of (L-)AAs and (D)-AAs were individually prepared in aqueous hydrochloric acid (0.1 M). (L)-Nle and (D)-Nle (20 mM) were used as internal standards.

Conventional hydrolysis of silk samples (modified according to (Vilaplana et al. 2015))

Hydrolysis of the silk samples (1–8 mg) was performed with HCl (1000 µL, 6 M) at 110°C for 24 h. As stabilization reagents and chelating agents, phenol in water (50 µL, 1%), DTDPA (50 µL, 1%) in an aqueous solution of sodium hydroxide (0.2 M) and EDTA (100 µL, 0.1%) in an aqueous solution of NaOH (pH 9) were added to all samples. The hydrolyzate was concentrated to dryness at 90°C using a nitrogen stream, and the residue was dissolved in HCl (200 µL, 0.1 M). Aliquotes (25–200 µL) were used for AA derivatization.

Hydrolysis with HCl assisted by microwave (modified according to (Colombini et al. 1998))

Various proteins (e.g. lupine flour protein, bovine serum albumin, ovalbumin, fibroin) and working solutions of (L)-Ala and (L)-Asp (14 µL, 50 mM) were treated with HCl (2000 µL, 6 or 7 M) at 150°C or 160°C for 10–180 min in a sealed tube using an Anton Paar Monowave 300 microwave system (max. power: 250 W, stirring speed: 600 rpm). The hydrolyzate was concentrated to dryness at 90°C using a nitrogen stream and the residue was dissolved in HCl (200 µL, 0.1 M). Aliquotes (50 µL) were used for AA derivatization.

AA derivatization with HFCBF (modified according to (Zahradníčková, Hušek, and Šimek 2009))

Aliquotes of AA stock solutions or hydrolyzates (25–200 µL) were diluted with distilled water (100 µL), a NaOH-Na_2CO_3 solution was added (50 µL, 1 M NaOH/0.5 M Na_2CO_3, 4:1 v/v), and this solution was mixed with a vortex for 20 sec. Then, the derivatization reagent solution (85 µL, HFCBF/isooctane, 1:3 v/v) was added and it was mixed again for 20 sec. Subsequently, the catalyst solution (50 µL, 50 mM Na_2CO_3/pyridine, 3:1 v/v) was added, the solution was thoroughly vortexed for 20 sec, and then remained at room temperature for about 5 min. Finally, isooctane (165 µL) was added and the solution was mixed for several minutes until the upper organic phase remained clear after mixing and showed no turbidity. An aliquot of the organic phase (0.5 µL) was injected into the gas chromatograph.

AA analysis by GC-MS – validation experiments

Specificity

To determine the specificity of the method, a mixture of all studied (L)- and (D)-AAs was measured by GC-MS after derivatization with HFBCF [for retention times see Table S1, Supplementary Material (SM)]. Furthermore, hydrolyzates of degummed BM and AM silks as well as blank samples (without AA or protein addition) were analyzed by GC-MS.

Linearity, limit of detection (LOD) and limit of quantification (LOQ)

For the calibration, solutions were prepared with different mixtures of (L)- and (D)-AAs [AAs: in the range of min 0.4 to max 50 mM with (L)- and (D)-Nle as internal standards (22 µL, 20 mM)]. Each calibration solution was measured three times, and analyses were executed with average peak area ratios of the respective (L)- or (D)-AA and (L)- or (D)-Nle. The linearity of each calibration function was tested with the Mandel's test. LOD and LOQ were determined by means of a calibration curve method according to DIN 32645 (Kromidas 2011). These validation data are shown in Table S1, SM.

Precision

To determine the repeatability, six replicate measurements were carried out for two different concentrations (Table S1, SM). For this purpose, working solutions containing respective (L)- or (D)-AAs as well as the internal standard [(L)- or (D)-Nle] were prepared, derivatized and analyzed with GC-MS. (L)-Ala and Gly were analyzed separately from the other AAs due to the high concentration differences. For the interpretation of the repeatability, the relative standard deviation (RSD) of the respective AA concentration was used. For determining the method precision, six BM as well as six AM cocoons were degummed with Na_2CO_3 (1 g/L) and ethylenediamine (2.5% w/w) at 95°C for 60 (BM cocoons) or 90 min (AM cocoons) as described above, degummed and dried BM and AM silk samples (max 6.5 mg) were treated with an EDTA-ACN-MeOH solution (see Sample preparation with EDTA-ACN-MeOH), hydrolyzed with HCl at 110°C for 24 h and independently derivatized with HFCBF in the presence of the internal standards (L)- and (D)-Nle. Aliquots (0.5 µL) of the organic phases were analyzed by GC-MS. For the interpretation of the method precision, the RSD of the AA concentrations was used (Table S1, SM). Furthermore, Dixon's Q test and Neumann trend test were applied for the identification of outliers or trends.

Recovery

The calculation of the recovery rate for each AA was performed for three concentration levels with a number of three replicates (Table S1, SM). For this purpose, degummed silken filaments of BM [max 6.5 mg, degumming method: Na_2CO_3 (1 g/L) and ethylenediamine (2.5% w/w), 95°C, 60 min] were spiked with defined (L)- or (D)-AA concentrations [(L)- and (D)-AAs were studied separately], hydrolyzed with HCl at 110°C for 24 h and derivatized with HFCBF as described above. Hydrolysis, derivatization and GC-MS analysis of the spiked silk samples were performed independently of each other on different days. The calculated AA concentrations were compared with the target concentrations, and the recovery rate was determined. The average AA concentrations of degummed silken filaments of BM, determined in the course of the method precision, were used for the calculation of the target concentrations.

Results and discussion

The subjects of the studies were degummed BM (family Bombycidae) filaments as well as non-mulberry silks of the species AM (family Saturniidae, tribe *Saturniini*) and AtA (family Saturniidae, tribe *Attacini*). In the course of the investigations, degummed silks were mordanted according to historical recipes with copper(II) sulfate, iron(II) sulfate, potassium alum as well as potassium alum and potassium hydrogen tartrate. Madder (*R. tinctorum* L.) roots and the blue vat dye indigo were used for dyeing. Both colorants were used in various regions of the world by different cultures to produce red, purple or blue colored textiles (Cardon 2007; Hofenk de Graaff 2004; Schweppe 1992). Madder, for example, was cultivated in various regions of Europe and Anatolia and was among the most demanded products in the Ottoman Empire (Ozdemir and Karadag 2023b). Since the second half of the 19th century, synthetic dyes were increasingly used in the textile industry. Today, a return to natural colorants can be observed. Interest in textile products dyed with natural dyes has increased in recent years, particularly among environmentally sensitive and conscious consumers (Karadag 2023). In this context, the economic and scientific interest is also growing in textiles dyed with natural indigo or madder and mordanted with natural, nontoxic metal salts such as potassium alum or plant-based mordants such as acorns (*Quercus* species) (Deveoglu et al. 2012; Ozdemir and Karadag 2023a, 2023b).

In addition to the recent samples mentioned above, textile samples from archaeological sites in the Tarim Basin (Xinjiang Uyghur Autonomous Region, Eastern Central Asia) – covering a period from the first millennium BC to third century AD (see Examination of historical samples) – were analyzed.

Effect of dyeing and mordanting procedures on the determined AA composition of recent silks

To investigate the effect of the mordanting and dyeing process on the AA composition, mordanted, dyed and untreated (i.e. degummed, undyed and non-mordanted) BM, AM and AtA silk samples were analyzed by GC-FID as described in a study of (Schmidt et al. 2023) and the determined AA composition was compared. BM silk was also treated with calcium oxalate to investigate the influence of Ca^{2+} ions, which are detectable on the surface of some wild silks in the form of calcium oxalate (Boulet-Audet, Vollrath, and Holland 2015). The dyeing and mordanting procedures as well as the presence of Ca^{2+} ions do not cause any significant change in the AA composition under the selected conditions (Figures S1–S3 and Tables S2–S4, SM). Consequently, no significant changes can be observed for structural indices such as the Gly/Ala or the long chain/short chain ratio (100LC/SC), which can be used to characterize silk fibroins (see (Freddi et al. 1994; Schmidt et al. 2023)). For instance, the 100LC/SC ratio of BM fibroin is 21–29, regardless of the mordanting and dyeing conditions (Table S2, SM). However, as already described by Ahmed and Darwish (Ahmed and Darwish 2012), some of the treated silks are characterized by a slight increase of the acidic AAs. For example, BM and AtA silks mordanted with alum and dyed with madder show a slightly higher Glx content (BM-Al/madder: 5.4 mol%; AtA-Al/madder: 2.3 mol%) compared to the untreated BM (3.5 mol%) and AtA samples (1.3 mol%, Figures S1B and S3B, for the assignment of the sample ID, see Tables S2–4, SM).

In addition to AA analysis, bluish and reddish silk samples were also examined for the colorants used. Since only a small sample amount is usually available, especially for precious historical samples, the following procedure is used: The silk samples are extracted, the extracts are examined for the presence of dyestuffs by HPLC-DAD and the remaining silk filaments are then analyzed for their AA composition. In this context, different extraction methods and their effects on the AA composition were tested (see next section).

Sample pre-treatment with an EDTA solution: clean-up step and dyestuff analyses

Dyed silk samples were treated with an aqueous EDTA-DMF or EDTA-ACN-MeOH solution prior to AA analysis by GC-FID, and the extracts were analyzed for the presence of organic dyestuffs by HPLC-DAD (see (Kramell et al. 2014; Kramell et al. 2016)). Alizarin and purpurin, dyes of the anthraquinone type, were detected, for example, in EDTA-DMF extracts of recent silk samples mordanted with potassium aluminum sulfate and dyed with madder. The EDTA-DMF extraction step is also suitable for the detection of vat dyes such as indigo. A slight discoloration was observed for dyed silk samples after treatment with the aqueous EDTA-ACN-MeOH solution. However, as already described by (Manhita et al. 2011), this milder method does not allow efficient extraction of anthraquinones and vat dyes such as indigo.

GC-FID studies on dyed and undyed BM, AM and AtA silks show that sample pre-treatment with EDTA-ACN-MeOH or EDTA-DMF does not cause any significant change in the AA composition and the resulting structural indices of indigo and madder dyed silks (Figures S4-S6 and Tables S2–S4, SM). However, an increased Glx content, comparable to that of dyed samples (see previous section), can be observed for undyed and non-mordanted AM and AtA silk samples after treatment with EDTA-ACN-MeOH or EDTA-DMF (AM: 2.2 mol%, AM EDTA-ACN-MeOH: 3.5 mol%, AM EDTA-DMF: 3.5 mol%, AM-Al/tartrate/madder EDTA-DMF: 3.6 mol%, AtA: 1.3 mol%, AtA EDTA-ACN-MeOH: 2.6 mol%, AtA EDTA-DMF: 2.9 mol%, AtA- Al/tartrate/madder EDTA-DMF: 2.8 mol%, for the assignment of the sample ID see Tables S2–S4, SM). Consequently, sample pre-treatment with EDTA-ACN-MeOH or EDTA-DMF of undyed and non-mordanted silks also leads to an enrichment of the acidic AA Glu in hydrolyzed samples (see previous section).

In summary, pre-treatment of dyed silk samples with EDTA-DMF enables the analysis of e.g. indigoid- and anthraquinone-type dyes without significantly changing the AA composition of the silk. Since sample material from historical textiles is usually only available to a very limited extent, a parallel dyestuff analysis is aimed for the characterization of these materials. In addition, interfering organic components and metal ions, especially from historical samples, can be removed by EDTA-ACN-MeOH or EDTA-DMF pre-treatment (see Introduction).

Chiral AA analyses on recent silks using GC-MS

In addition to the AA composition, D/L ratios of silk fibroin AAs were used to characterize silks.

For chiral analysis of AAs by GC-MS, degummed silk samples were hydrolyzed with 6 N HCl, then the free AAs were derivatized with HFBCF and enantiomeric AA pairs were separated on a Chirasil-Val capillary column. Eleven (DL)-AA pairs were identified and quantified in hydrolyzates of degummed silks, namely (DL)-Ala, (DL)-Val, (DL)-Leu, (DL)-Asx (Asp + Asn), (DL)-Met, (DL)-Ser, (DL)-Phe, (DL)-Glx (Glu + Gln), (DL)-His, (DL)-Lys and (DL)-Tyr.

Effect of hydrolysis, derivatization and treatment with an EDTA solution on the racemization rate

To investigate the influence of the derivatization procedure on AA racemization (AAR), stock solutions of (L)-AAs and (D)-AAs were derivatized with HFBCF at room temperature. The content of (D)- or (L)-AA derivatives after reaction of (L)- or (D)-AAs with HFBCF was below the detection limit (Table S1, SM). Thus, the derivatization procedure has no significant influence on the D/L ratio. Subsequently, the influence of silk fibroin digestion and sample pre-treatment was investigated. For this purpose, recent degummed BM and AM silk samples, treated with an aqueous EDTA-ACN-MeOH solution at 60°C for 30–60 min, were digested without microwave irradiation with 6 N HCl at 110°C for 24 h. HFBCF was also added to the hydrolyzates at room temperature. As shown in Figure 1 and Tables S5–S6 (SM), only the content of five (D)-enantiomers – namely (D)-Ala, (D)-Val, (D)-Ser, (D)-Glx and (D)-Asx – was above the LOQ. (L)-Ala and the achiral AA Gly are predominant in BM and AM fibroin and account for approximately between 70 and 80 mol% of the total AA content (for comparison see Tables S2–3). Under the above conditions, the conversion of (L)- to (D)-Ala is less than 1% (D/L Ala: 0.3–0.7%). D/L ratios between 5% and 12% were determined for the AAs Ser (BM: 12.0%, AM: 10.3%), Val (BM: 7.7%, AM: < LOQ), Glx (BM: 5.0%, AM: 8.5%) and Asx (BM: D-Asx < LOQ, AM: 7.4%), which occur with a significantly lower proportion in BM and AM fibroin. A comparison of BM silk before and after treatment with EDTA-ACN-MeOH shows no significant changes in the AAR rates (Figure 1). Thus, digestion and sample pre-treatment are suitable for a D/L analysis of silk.

In this context, different hydrolysis procedures using HCl with microwave irradiation were tested on stock solutions of (L)-AAs and various proteins. As expected, the hydrolysis time can be significantly reduced by microwave irradiation (for comparison see (Andreotti et al. 2006; Bonaduce, Cito, and Colombini 2009)). However, the use of microwave-assisted hydrolysis leads to greatly increased levels of (D)-AAs. For example, D/L AA ratios of almost 100%, i.e. racemic mixtures, were observed for the stock solutions of Ala and Asp after 40 min of hydrolysis in the microwave (Koch 2022; Puchalla 2022). The effect of digestion time on Asx racemization using 6 N HCl at 110°C without microwave irradiation was already investigated on BM silk by Moini et al. (Moini, Klauenberg, and Ballard 2011) The (L) to (D) conversion by heating was found to be about 1% after 2 h and 4% after 24 h, reaching a plateau after 24 h. Since we cannot observe complete hydrolysis for all silk samples after 2 h, all further experiments were carried out without microwave irradiation for 24 h with a 6 N HCl at 110°C.

Effect of dyeing and mordanting procedures on AAR

As described above, racemization rates and/or degradation reactions of AAs can be affected by the presence of metal ions. Since metal salts such as iron(II) sulfate, copper(II) sulfate and potassium alum are frequently used as mordants, the influence of dyeing and mordanting procedures on (L) to (D) conversion of silk fibroin AAs was investigated. For this purpose, degummed BM silk samples treated with various metal salts and colorants were prepared according to the procedure described above (see previous section and Tables S5–S6, SM). In this context, dyed and mordanted silks were examined before and after treatment with EDTA-ACN-MeOH or EDTA-DMF (see sample pre-treatment with an EDTA solution). Furthermore, calcium oxalate was treated in BM silks and thus the effect of divalent Ca^{2+} ions on AAR rates was analyzed. In most cases, the (D)-AA proportions as well as D/L ratios determined for dyed and/or mordanted silks are comparable to those of the undyed and unmordanted silk filaments (Figure 1 and Table S6, SM). However, a D/L Met ratio of 67% was determined for sample BM-Cu EDTA-ACN-MeOH. For all other dyed and/or mordanted BM

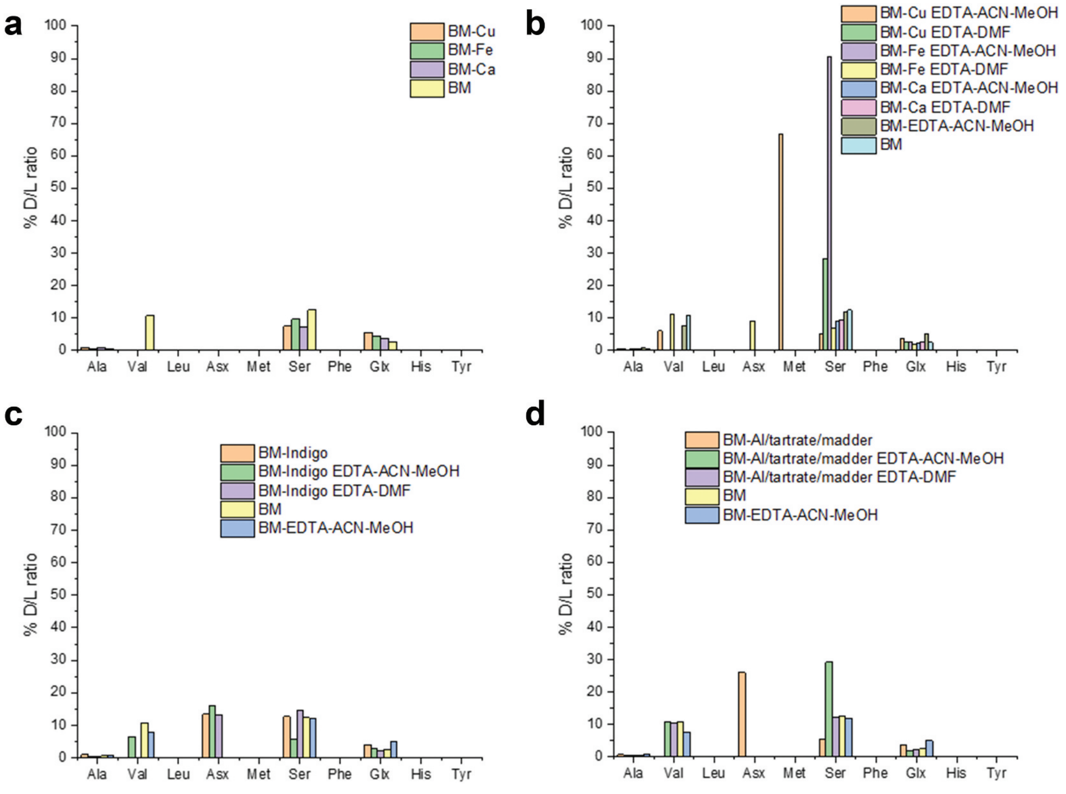

Figure 1. D/L ratios for (a–b) mordanted and calcium oxalate treated BM silks, (c) indigo dyed BM silks and (d) madder dyed BM silks before and after treatment with EDTA-ACN-MeOH or EDTA-DMF (degumming procedure: 0.1% Na_2CO_3 and 2.5% ethylenediamine, 95°C, 60 min, see Table S5 for assignment of the sample ID).

samples, the (D)- and/or (L)-Met content was below the LOQ. This is not surprising due to the low Met content in BM silk and the sensitivity of Met to oxidation. It is well known that Met can be oxidized to methionine sulfoxide and methionine sulfone when proteins are digested with 6 N HCl (Rutherfurd and Gilani 2009). The D/L ratio of Ser varies greatly between the samples tested, ranging from 5% to 90%. For instance, BM silk that has been mordanted with iron(II) sulfate and treated with EDTA-ACN-MeOH shows a D/L Ser ratio of 90.48%. In contrast, D/L Ser ratios of 6.80% and 28.35% were determined for BM silk mordanted with iron(II) sulfate or copper(II) sulfate and treated with EDTA-DMF. In addition, an increased (D)-Asx content was detected for the sample BM-Al/tartrate/madder (D/L Asx: 26.04%). However, the proportion of (D)-Asx in alum/tartrate-mordanted and madder-dyed samples treated with EDTA-DMF or EDTA-ACN-MeOH prior to hydrolysis is below the LOQ. In summary, an increased proportion of (D)-AAs can be observed for some mordanted samples, with thermodynamically unstable AAs such as Ser racemizing more readily than other AAs (see (Reischl and Lindner 2015) for studies on the mechanisms of Ser racemization) (Bada 1985). In addition, it should be noted that AAs such as Ser, Asn and Asp occur more frequently in amorphous fibroin regions, which are more susceptible to chiral rearrangements than crystalline fibroin regions (Shimada et al. 2023).

A (D)-Asx content of 0.1–0.2 ± 0.0005–0.02 mol% and thus D/L Asx ratios between 13% and 16% were found for BM samples dyed with indigo (Figure 1c). Since no metal salts were used for the preparation of these samples, the slightly increased (D)-Asx content is probably due to the alkaline conditions during the indigo dyeing process. In the present study, NaOH was added to the dye bath (pH 8–9) and silk dyeing was carried out for 2–3 min at 55°C, repeating the dyeing process several times to obtain a deep blue color. As described by Moini and Rollman, such alkaline conditions lead to an increased proportion of (D)-Asx (Moini and Rollman 2017). For instance, a previous study by Moini and Rollman shows that treatment of BM silk at pH 9.07 and a temperature of 110°C results in a 12% increase in D/L Asx after 120 min. However, the temperature of the dye bath and the previously used degumming process also appear to be important for changes in the D/L Asx ratio during the dyeing process. A study by Shimada et al. shows, for example, that indigo dyeing at 21°C and a pH of 10.5 does not change the D/L Asx ratio of a silk sample previously degummed at 85–90°C and a pH of 11–12 (Shimada et al. 2023). A D/L Asx ratio of approximately 17% was found for this alkaline degummed silk sample before and after indigo dyeing. Using the calibration curve of Moini et al., this value corresponds to an age of 500–600 years (Moini, Klauenberg, and Ballard 2011; Shimada et al. 2023).

Since the AAR rate is affected by the pH and temperature, various alkaline degumming methods are considered in the next step.

Effect of degumming procedures and artificial aging with Na_2CO_3 solutions on AAR

Studies by Shimada et al. show that the D/L Asx ratio of alkaline degummed silk is significantly higher than that of an untreated cocoon (Shimada et al. 2023). In comparison, Shimada et al. found that the D/L Asx ratio for enzymatically degummed silks did not change greatly. Consequently, the degumming conditions are crucial for the racemization of Asx.

To investigate the influence of alkaline degumming procedures in more detail, BM cocoons were treated with 0.1–0.25% Na_2CO_3 and/or 2.5% ethylenediamine at 95°C for 30–120 min (pH 8–10), using microscopic studies and the average degumming ratio D_r to evaluate the degumming efficiency. As already described in a previous study, under these conditions the degumming ratio of BM cocoons is about 30–35% (Table 1), indicating high degumming efficiency without destruction of the filaments (Schmidt et al. 2023). Harsher conditions, i.e. long treatment time and/or high concentration of the degumming agent, lead to partial degradation of fibroin fibers and a degumming ratio >35%. In order to induce a partial loss of silk fibroin, BM cocoons were treated with 10% ethylenediamine at 95°C for 120 min (sample ID: BM-4 120 min). This sample shows brittle fibers with a degumming ratio of 38%. However, as described by (Schmidt et al. 2023), the above-mentioned degumming conditions do not

Table 1. D/L ratios of the recent, undyed and non-mordanted BM silk degummed with alkaline-degumming agents and subsequently digested with 6 N HCl at 110°C for 24 h (derivatization with HFCBF).

			% D/L ratio									
Sample ID	Degumming procedure	Degumming ratio D_r[a]	Ala	Val	Leu	Asx	Met	Ser	Phe	Glx	His	Tyr
BM-1 30 min	Na_2CO_3 (1 g L^{-1}) and ethylenediamine (2.5%), 30 min, 95°C, pH 9–10	32	0.5	10.6	[c]	[c]	[c]	13.7	[c]	2.6	[b]	[c]
BM-1 60 min (BM)	Na_2CO_3 (1 g L^{-1}) and ethylenediamine (2.5%), 60 min, 95°C, pH 9–10	31	0.6	10.7	[c]	[c]	[c]	12.4	[b]	2.4	[b]	[c]
BM-1 90 min	Na_2CO_3 (1 g L^{-1}) and ethylenediamine (2.5%), 90 min, 95°C, pH 9–10	31	0.5	7.2	[c]	12.2	[c]	13.6	[c]	1.2	[b]	[c]
BM-2 30 min	Na_2CO_3 (1 g L^{-1}), 30 min, 95°C, pH 8–9	34	0.8	8.6	[c]	[c]	[c]	3.7	[c]	2.2	[b]	[c]
BM-2 60 min	Na_2CO_3 (1 g L^{-1}), 60 min, 95°C, pH 8–9	31	0.6	6.9	[c]	10.2	[c]	3.3	[c]	[b]	[b]	[c]
BM-2 90 min	Na_2CO_3 (1 g L^{-1}), 90 min, 95°C, pH 8–9	28	0.5	8.2	[c]	[c]	[b]	8.8	[c]	[b]	[b]	[c]
BM-2 120 min	Na_2CO_3 (1 g L^{-1}), 120 min, 95°C, pH 8–9	32	0.9	16.8	35.4	[b]	[b]	[b]	[c]	[b]	[b]	[b]
BM-3 120 min	Na_2CO_3 (2.5 g L^{-1}), 120 min, 95°C, pH 10	30	1.0	6.3	30.5	[b]	[b]	[b]	[c]	[b]	[b]	[b]
BM-4 120 min	Ethylenediamine (10%), 120 min, 95°C, pH 12–14	38	0.5	4.2	[c]	[b]	[b]	1.9	[c]	[b]	[b]	[c]

[a] (Schmidt et al. 2023); [b] (D)-enantiomer concentration is below the LOD; [c] (D)-enantiomer concentration is below the LOQ.

lead to a significant change in AA composition. The effects of the degumming procedure on the D/L ratios are considered below.

As shown in Table 1, D/L ratios are comparable to those of the undyed and unmordanted fibroin filaments (degumming method: 0.1% Na_2CO_3 and 2.5% ethylenediamine, 95°C, 60 min), with D/L Ala < 1%, D/L Val < 17%, D/L Ser < 14% and D/L Glx < 3% (Figure 2a). With the exception of the samples BM-1 90 min (0.1% Na_2CO_3 and 2.5% ethylenediamine, 95°C, 90 min, pH 9–10) and BM-2 60 min (0.1% Na_2CO_3, 95°C, 60 min, pH 8–9), the (D)-Asx concentration is below the LOQ or LOD. A D/L Asx ratio of 10–12% was determined for BM-1 90 min and BM-2 60 min, which is comparable to that of the indigo dyed samples (see previous section). For sample BM-4 120 min (degumming method: 10% ethylenediamine, 95°C, 120 min, pH 12–14), which shows brittle fibers due to the harsh degumming conditions, the (D)-Asx and (L)-Asx content was below the LOD. However, a treatment with 0.1% or 0.25% Na_2CO_3 for 120 min leads to an increased (D)-Leu content and thus to D/L Leu ratios of 31–35%.

To simulate the artificial aging as well as the alkaline processing described by Moini and Rollman (Moini and Rollman 2017), degummed BM silk samples and BM cocoons were treated with Na_2CO_3 solutions at pH 9, 11 and 13 at 110°C for 60–120 min. At pH 9 and 11, similar D/L ratios for Ala, Val, Ser and Glx were obtained as with the alkaline degumming procedures described above (Table 2 and Figure 2b). In this context, treatment times of 90–120 min also lead to increased D/L Leu ratios (26–28%). In agreement with the studies by Moini and Rollman (Moini and Rollman 2017), increased (D)-Asx formation is observed with increasing pH. As shown in Figure 2b–d, the D/L Asx ratio increases from 12% to 13% at pH 9 to 44–67% at pH 11, with the proportion of (D)-Asx at pH 11 increasing with increasing treatment time. In addition, a (D)-Tyr concentration above the LOQ was detected at pH 11 and thus a D/L Tyr ratio of 5.6% was determined. At pH 13, silk samples are almost completely dissolved and a further increase in the D/L Asx ratios to 85–100% can be observed. Furthermore,

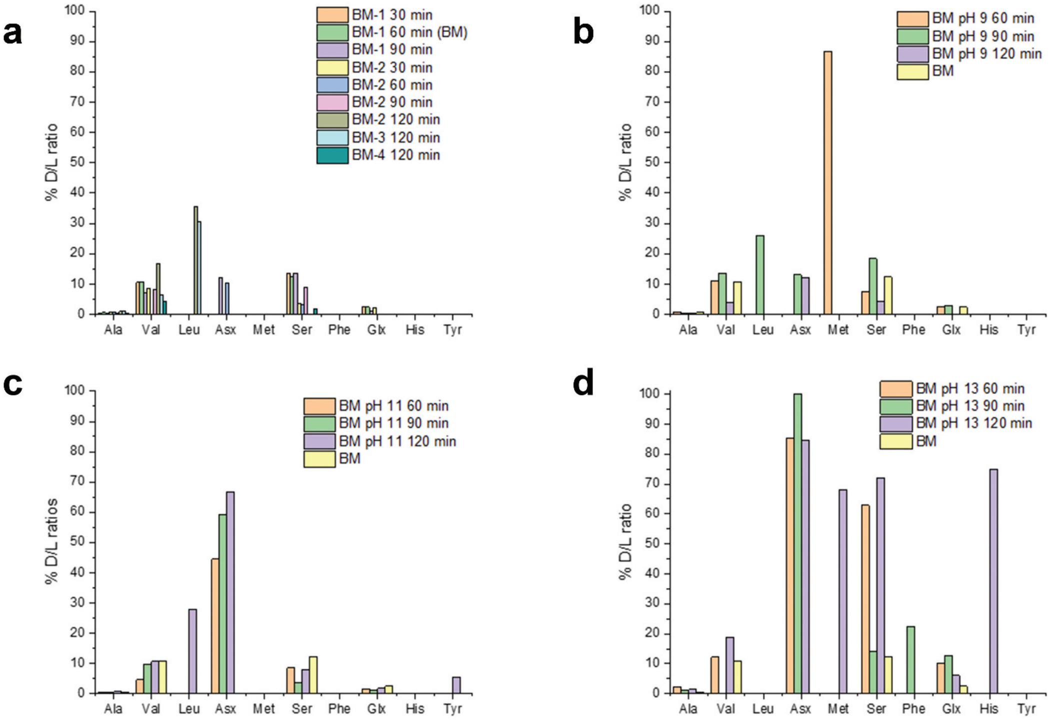

Figure 2. D/L ratios for (a) BM silks degummed with various degumming agents and (b–d) BM silks after treatment with Na_2CO_3 solutions at pH 9, 11 and 13 at 110°C (see Tables 1–2 for assignment of the sample ID).

Table 2. D/L ratios of the recent, undyed and non-mordanted BM silk degummed with alkaline degumming agents and subsequently digested with 6 N HCl at 110°C for 24 h (derivatization with HFCBF).

		% D/L ratio									
Sample ID	Alkaline treatment	Ala	Val	Leu	Asx	Met	Ser	Phe	Glx	His	Tyr
BM pH 9 60 min	Na_2CO_3, pH 9, 60 min, 110°C	0.9	11.1	[b]	[b]	86.7	7.4	[b]	2.5	[a]	[b]
BM pH 9 90 min	Na_2CO_3, pH 9, 90 min, 110°C	0.5	13.6	26.1	13.0	[b]	18.4	[a]	2.9	[a]	[a]
BM pH 9 120 min	Na_2CO_3, pH 9, 120 min, 110°C	0.3	4.1	[a]	12.1	[b]	4.4	[b]	0.8	[b]	[b]
BM pH 11 60 min	Na_2CO_3, pH 11, 60 min, 110°C	0.5	4.6	[b]	44.4	[b]	8.5	[b]	1.5	[a]	[b]
BM pH 11 90 min	Na_2CO_3, pH 11, 90 min, 110°C	0.4	9.7	[b]	59.3	[b]	3.7	[b]	1.2	[a]	[b]
BM pH 11 120 min	Na_2CO_3, pH 11, 120 min, 110°C	0.6	10.7	27.9	66.7	[b]	8.0	[b]	1.8	[a]	5.6
BM pH 13 60 min	Na_2CO_3, pH 13, 60 min, 110°C	2.3	12.1	[b]	85.2	[b]	62.9	[b]	10.2	[a]	[b]
BM pH 13 90 min	Na_2CO_3, pH 13, 90 min, 110°C	1.2	[b]	[b]	100	[b]	14.2	22.5	12.7	[a]	[b]
BM pH 13 120 min	Na_2CO_3, pH 13, 120 min, 110°C	1.4	18.9	[b]	84.7	68.2	72.2	[b]	6.1	[b]	[b]

[a] (D)-enantiomer concentration is below the LOD; [b] (D)-enantiomer concentration is below the LOQ.

increasing the pH to 13 also results in increased D/L Ala (1.2–2.3%), Ser (14–72%) and Glx (6–13%) ratios as well as a (D)-Phe and (D)-His content above the LOQ after a treatment period of 90–120 min (D/L Phe: 22.5%, D/L His: 75.0%). In summary, alkaline treatment at 110°C leads to increased D/L ratios, with the D/L Asx ratio being particularly affected. Consequently, not only the pH value but also the temperature is a decisive factor for the AAR pattern in alkaline treatments.

Chiral AA analyses by GC-MS - validation experiments

The results of the validation study for AA analyses using GC-MS are summarized in Table S1, SM. Retention times of derivatized AAs were determined using (L)- and (D)-AA standard solutions. Fifteen enantiomeric (DL)-AA pairs were separated on a Chirasil-Val capillary column under gradient temperature conditions. However, coelution was observed for (D-) and (L)-Pro derivatives, as already described by Zahradníčková et al., the content is therefore given as the sum of (D)- and (L)-Pro (Zahradníčková, Hušek, and Šimek 2009). Chromatograms of hydrolyzed silk samples of non-mulberry and mulberry silkworms as well as blank samples were free from interference. Calibration plots [concentration of (D)- or (L)-AA versus peak area ratio of (D)- or (L)-AA and (D)- or (L)-Nle as internal standards] are linear in the respective selected range (Ala and Gly: min 1.6 mM, max 11.8 mM; other AAs: min 0.3 mM, max 2.6 mM). For all AAs considered, an R^2 value >0.978 was obtained, and both LODs and LOQs were considered adequate for the purposes of the present study. The repeatability, expressed by relative standard deviations (RSD), ranged from 2.6% for (D)-Met and (D)-Leu to 28% for (D)-Lys. To determine the method precision, hydrolysis of degummed BM or AM silk, treated with an EDTA solution, was performed six times, and RSDs of AA concentrations were calculated. The RSD values range from 2.7% for (D)-Glx [(D)-Glu + (D)-Gln] to 23.7% for (L)-Ile. The (D/L)-Cys, (D/L)-Pro, (D)-Ile, (D)-Leu, (D)-Thr, (D)-Phe, (D)-His, (D)-Tyr, (D)-Lys and (D)-Met concentrations of the hydrolyzates are below the LOQ and LOD, respectively. Thus, no values for method precision are given for these AAs. Trp, a low abundance AA of BM silk (Moini, Klauenberg, and Ballard 2011), is decomposed during treatment with HCl (Rutherfurd and Gilani 2009) and cannot be determined using this method. In addition, it should be noted that Asn and Gln are deaminated during acid hydrolysis to Asp and Glu (Rutherfurd and Gilani 2009). Thus, after hydrolysis with 6 N HCl, values for (D/L)-Asp and (D/L)-Glu are reported as the sum of the acid and amide derivatives [(D/L)-Asx or (D/L)-Glx]. Recovery tests were carried out on spiking decummed BM silks with defined concentrations of (D)- and (L)-AAs, whereby (D)- and (L)-AAs were analyzed separately. The recovery rate of the different AAs after hydrolysis and derivatization ranged from 57% for (D)-Cys to 120% for Gly with an average recovery rate of 89% at the low, 84% at the medium and 82% at the high concentration level. (L)-Ile and (L)-Thr, AAs with two chiral carbon centers, which are only present in small amounts in silk fibroin, are converted into (D)-*allo*-Ile and (D)-*allo*-Thr by epimerization. The content of these diastereomers is below the LOD for all silk samples, and therefore these AAs are not included in the AAR studies described above. The studies on AAR rates focused on 11 (DL)-AA pairs that were identified and quantified in hydrolyzates of degummed BM and AM silks (see chiral AA analyses on recent silks using GC-MS). The developed and validated chiral GC-MS method was successfully used for the analysis of recent (see chiral AA analyses on recent silks using GC-MS) and historical silk samples (see next section).

Examination of historical samples – case study on silk fabrics from the Tarim Basin

Silk samples from archaeological sites of Niya and Yanghai in the Xinjiang Uyghur Autonomous Region (China) were analyzed using OM, SEM, SEM-EDX and ATR-FTIR spectroscopy as reported by (Kramell et al. 2014, 2016). In addition, dyestuff analyses were performed using HPLC-DAD and LC-ESI-MS/MS. As already described by Kramell et al., no dyestuffs were detected in these silk fragments (Table 3) (Kramell et al. 2014, 2016).

As shown in Figure 3, warps and wefts of the tabby weave fragments from grave M5 of the Niya site, which can be dated to the Han dynasty (206 BC –220 AD (Kramell et al. 2016)), are not twisted. These

Table 3. D/L ratios of historical silk and wool samples treated with EDTA-ACN-MeOH before hydrolysis with 6 N HCl at 110°C for 24 h (derivatization with HFCBF).

						% D/L ratio										
Archaeological graveyard sites	Object/object ID	Sample ID	Description of the sample[d]	Material	Detected dyestuffs[d]	Ala	Val	Leu	Asx	Met	Ser	Phe	Glx	His	Lys	Tyr
Niya (objects originate from grave M5), Han dynasty (206 BC –220 AD)[d]	Robe 95MNIM5–15	95MNIM5-15D	Fabric fragment (tabby weave) with beige/non-pigmented warps and wefts	Silk	-	0.6	51.8	[a]	76.5[b]	[c]	8.7	[a]	57.1	[a]	[b]	[b]
		95MNIM5-15G	Fabric fragment (tabby weave) with brownish warps and wefts,	Silk	No dyes detected	0.5	50.0	[b]	80	[c]	21.1	[a]	23.7	[c]	[b]	[b]
	Skirt 95MNIM5–18	95MNIM5-18A	Fabric fragment (tabby weave) with brownish warps and wefts	Silk	Traces of unknown UV active compounds (λ_{max} = 350 nm)	0.4	51.4	[b]	86.2	[c]	16.1	[b]	25.0	[a]	[b]	[b]
	Shirt 95MNIM5–43	95MNIM5-43A	Fabric fragment (tabby weave) with brownish warps and wefts	Silk	No dyes detected	0.3	59.3	[b]	66.7	[c]	17.8	[a]	19.4	[c]	[b]	[b]
Yanghai, Zone III, probable period of use: 7th-4th centuries BCE	Skirt 03SAYM376:13 (IIIM376:13)	03SAYM376:13-1	Fabric fragment with twisted beige/non-pigmented warps and wefts	Silk	-	0.5	50.0	[b]	59.5	[b]	25.9	[a]	55.2	[b]	[b]	[b]
Wupu, calibrated AMS radiocarbon date (95.4% probability) of the robe: 760–682 cal. BC[e]	Robe 86HWMNN–1 (no number)	86HWMNN-1D	Fabric fragment (twill) with twisted brownish warps and wefts	Wool	Traces of rubiadin and indigotin	[b]	10.8	[b]	47.7[b]	[c]	[b]	[b]	[c]	[c]	[b)]	[b]
Yanghai, calibrated AMS radiocarbon date (95.4% probability) of the textile fabric: 839–601 cal. BC[d,f]	Surface find without assignment to specific graves	20110615/OF11-B	Fabric fragment (twill) with blue warps and wefts (single yarn, Z-twist), 2:2 float	Wool	Indigo and indirubin	[b]	10.3	[b]	37.3[b]	[b]	100	[b]	54.2	[c]	[b]	[b]

[a](D)-enantiomer concentration is below the LOD; [b](D)-enantiomer concentration is below the LOQ; [c](L)- and (D)-enantiomer concentration is below the LOQ; [d](Kramell et al. 2014; Kramell et al. 2016); [e]A blue wool thread from the robe of tomb 86HWMNN–1 was radiocarbon dated by (Schröder et al. 2016). The wool fabric 20,110,615/OF11-B was radiocarbon dated by (A. Kramell et al. 2014). A recent merino wool sample was also analyzed by GC-MS (Tables S6 and S7, SM).

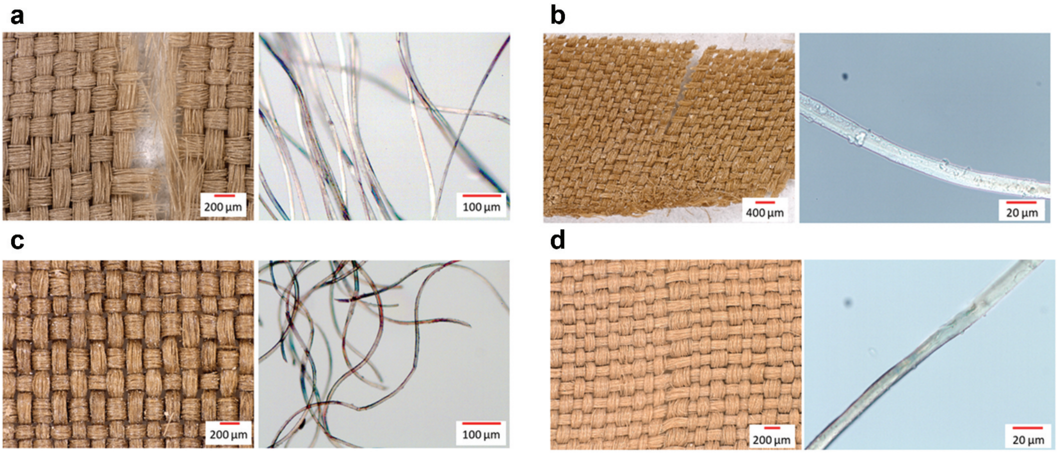

Figure 3. Tabby weave fragments from the Niya site and morphologies of the silk filaments; sample ID: (a) 95MNIM5-15D, (b) 95MNIM5-15G (c), 95MNIM5-18A (d) 95MNIM5-43A.

textile fragments consist of long silk filaments, revealing the use of reel silk and thus of intact cocoons for textile production. Long continuous silk filaments can be obtained by killing the pupae with boiling water or steam before hatching and unwinding the cocoon as a continuous thread. In contrast, the fabric fragment 03SAYM376:13-1 from a tomb of the Yanghai burial ground consists of twisted warps and wefts, whereby the diameter of the yarns varies greatly (Figure 4). Microscopic studies show that silk filaments from the Niya and the Yanghai site have a triangular cross-sectional shape typical of BM silk, with a filament cross-section of around 8–15 µm (Figure S7, SM). Compared to the silk fabrics from the Niya site, however, shorter silk filaments were used to produce textile fragment 03SAYM376:13-1. This indicates that this textile fragment was made of hatched and thus non-reelable cocoons. Another interpretation approach is the use of the so-called Bourette silk, produced from the waste of silk extraction or from damaged cocoons (see (Veit 2022)).

SEM-EDX studies on silk filaments and adhering particles reveal the presence of ubiquitous elements such as N, O, Si, Na, K, Al, Cl, Mg, Ca and Fe (Figure S8, SM), which can often be detected in samples with soil contact. ATR-FTIR spectra of silk samples from the finding sites of Niya and Yanghai show characteristic bands in the range 1600–1700, 1480–1560 and 1210–1270 cm^{-1} which are assigned to the amide I, amide II and amide III bands of silk fibroin (Figure S9, SM). Previous studies have shown that degummed BM silk has characteristic bands at 975 and 998 cm^{-1}, corresponding to

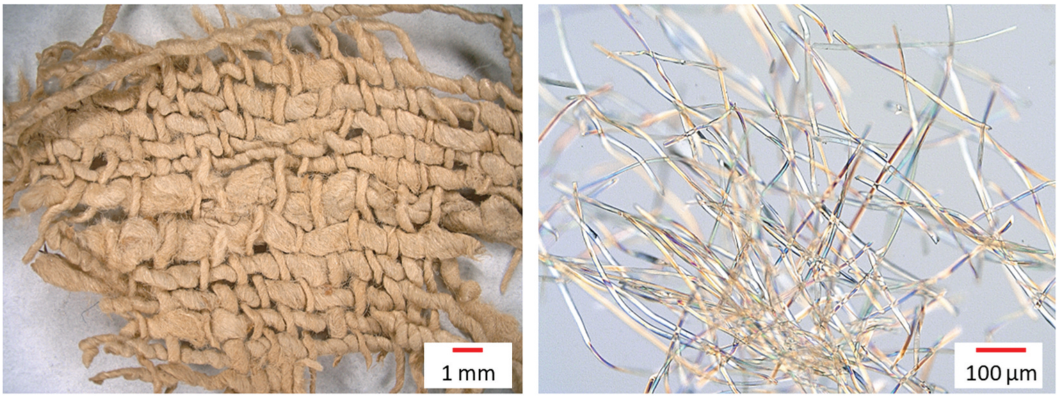

Figure 4. Textile fragment from the Yanghai site and morphologies of the silk filaments (sample ID: 03SAYM376:13-1).

the β-sheet conformation of polyalanineglycine $(AG)_n$ (Boulet-Audet, Vollrath, and Holland 2015; Koperska et al. 2014; Schmidt et al. 2023). In contrast, a prominent peak at 963 cm^{-1} can be observed for non-mulberry silks, e.g. from Saturniidae silk moths, which are assigned to polyalanine $(A)_n$ β-sheets (Boulet-Audet, Vollrath, and Holland 2015; Schmidt et al. 2023). These characteristic bands are masked in the historical samples by the presence of a broad and intense band in the range 940–1090 cm^{-1} displaying submaxima at 1000 and 1033 cm^{-1}, which are probably ascribed to vibrations of adhesions such as silicate minerals. In samples 03SAYM376:13-1 and 95MNIM5-43A, however, the peaks at 975 and 998 cm^{-1} characteristic of BM silk are detectable as submaxima. Another characteristic band for BM silk is a shoulder at 1260 cm^{-1}, corresponding to the β-sheet conformation in the amide III band (de Palaminy, Daher, and Moulherat 2022). This band can be detected in all historical samples, which indicates the presence of *Bombyx* silk. Distinct signature peaks for sericin (Aguayo et al. 2014) at around 1400 cm^{-1} and 1070 cm^{-1} cannot be observed in the historical samples, with the exception of sample 03SAYM376:13-1. ATR-FTIR spectra of sample 03SAYM376:13-1 show a characteristic band for sericin at 1403 cm^{-1} with an intensity comparable to recent degummed BM samples (Figure S9, SM). Differences in sericin content may be due to different storage conditions or different degumming methods. Due to the good water solubility of sericin, a low sericin content in historical silk samples with soil contact is not surprising. However, in the case of sample 03SAYM376:13-1, the increased sericin content may also indicate the presence of Bourette silk (see above), which usually has a high sericin content.

For further characterization, historical samples were analyzed for their AA composition and AAR rates using GC-MS (Tables 3–4). Ala and Gly are predominant in all historical silk samples and account for between 81 and 92 mol% of the total AA content. The Gly content of samples 95MNIM5-18A, 95MNIM5-43A and 03SAYM376:13-1 is between 47 and 54 mol%, which is higher than the Ala content of 38–43 mol%. Thus, the Gly/Ala ratio is >1, which is characteristic for BM fibroin. A Gly/Ala ratio of 1 was determined for samples 95MNIM5-15D and 95MNIM5-15 G, with a Gly content of 40–45 mol% and an Ala content of 41–47 mol%. Furthermore, all historical samples have a 100LC/SC ratio between 6 and 9. Thus, these samples have a lower 100LC/SC ratio than recent BM or non-mulberry silk, suggesting a higher structural regularity compared to recent silk (100LC/SC of BM fibroin: 17–26, 100LC/SC of Saturniidae fibroin: 14–53 (Schmidt et al. 2023)). This is not surprising, since poorly ordered and loose amorphous regions, containing a high proportion of bulky side groups, are degraded faster than ordered and compactly stacked crystalline regions. Since the crystalline regions of BM fibroin consist of Ala- and Gly-rich repetitive motifs, the degradation of amorphous regions also leads to an increased Ala and Gly proportion, which can be observed in particular for samples 95MNIM5-15 G, 95MNIM5-18A and 95MNIM5-43A (Gly+Ala: 91–92 mol%) (Li et al. 2013). At the same time, these historical samples have a very low amount of polar AAs as well as lower Tyr/Ala and Tyr/Gly ratios compared to recent fibroin (Tables 4 and S2, SM). The ratios of Tyr/Ala and Tyr/Gly are markers for oxidative changes in the silk fibroin structure, as Tyr is sensitive to degradation and oxidation reactions and can be transformed into Ala by loss of the phenol group (Vilaplana et al. 2015). Consequently, a decrease in the Tyr content is also observed for all historical silk samples examined (Table S7, SM). Compared to the recent BM samples, the historical silk samples show a lower Val and Glx content, with D/L Glx ratios of 19–57% and D/L Val ratios of 50–59% (Table S7, SM). Thus, the D/L Val ratio in particular is significantly increased compared to the recent samples, regardless of the silk processing. At 0.3–0.7 mol%, the Asx content of the historical samples is also slightly decreased than that of recent BM silk, which contains 1 mol% Asx. The Asx racemization ratio for these samples is between 60% and 86%, with D/L Asx ratios of 67–86% for the Niya samples from tomb 5. A D/L Asx ratio of 60% was determined for sample 03SAYM376:13-1 from the Yanghai site. Using the calibration curve of (Moini, Klauenberg, and Ballard 2011), which spans in time from the present to ~2500 years ago, these D/L Asx ratios result in AAR dates of over 2500 years (Moini, Klauenberg, and Ballard 2011). However, it should be noted that, Moini et al. used silk samples from museum collections, which were stored with extraordinary care, to create the calibration curve. Thus, many of the environmental factors that affect racemization rates remain fairly constant. The silk samples examined from the Niya and Yanghai sites were stored in tombs for a large part of the time and

Table 4. AA composition of historical silk and wool samples treated with EDTA-ACN-MeOH before hydrolysis with 6 N HCl at 110°C for 24 h (derivatization with HFCBF).

		Comparative AA composition									
Sample ID	Material	Ala+Gly [mol%]	Gly/Ala	100LC/SC[a]	P [mol%][b]	NP [mol%][c]	Ser+Pro+Thr+Tyr [mol%][d]	A [mol%][e]	B [mol%][f]	Tyr/Ala	Tyr/Gly
95MNIM5-15D	Silk	81	1.0	8	13	87	16	1	1	0.10	0.10
95MNIM5-15G	Silk	91	1.0	7	4	96	5	1	0	0.06	0.06
95MNIM5-18A	Silk	92	1.1	6	3	97	5	1	0	0.07	0.06
95MNIM5-43A	Silk	92	1.4	8	2	98	4	1	0	0.06	0.04
03SAYM376:13-1[g]	Silk	86	1.2	9	8	92	9	1	1	0.08	0.06
86HWMNN-1D	Wool		31	0.8	212	3	97	17	1	0.19	0.24
20110615/OF11-B	Wool		26	0.6	273	4	96	15	2	0.19	0.31

[a] Ratio between long chain (LC: other AAs) and short chain (SC: Ala, Gly, Ser, Thr) AAs; [b] Polar AAs: acidic, basic and hydroxyl AAs; [c] Non-polar AAs: other AAs; [d] AAs with hydroxyl groups: Ser, Thr, Tyr; [e] Basic AAs: Lys, His; [f] Acidic AAs: Asx, Glx; [g] Sample 03SAYM376:13-1 was also analyzed by GC-FID without treatment with EDTA-ACN-MeOH, yielding similar AA compositions (Ala+Gly: 85 mol%, Gly/Ala: 1.5, 100LC/SC: 11, P: 9 mol%, NP: 91 mol%, Ser+Pro+Thr+Tyr: 8 mol%, A: 2 mol%, B: 1 mol%, Tyr/Ala: 0.08, Tyr/Gly: 0.05, for GC-FID method see (Schmidt et al. 2023).

were therefore exposed to various environmental influences, which can affect the Asx racemization rate. It is difficult to estimate the influence of environmental factors such as pH, temperature or metal ion chelation on the D/L Asx ratio of archaeological silk samples. The authors advise against determining the age of these silk samples solely on the basis of the Asx racemization rate. The ages given in Table 3 are based on^{14}C dating and/or the archaeological context.

In summary, the textile fragments from archaeological sites of Niya and Yanghai were most likely made of silk produced by a moth of the genus *Bombyx*. At this point, it should be noted that silk of the Chinese wild silkworm *Bombyx mandarina* Moore has a similar AA composition to BM silk filaments, with a Gly content higher than that of Ala (Deng et al. 2021). This phenomenon, i.e. fibroin filaments with a Gly/Ala ratio of >1, is only presented in a few species worldwide, e.g. *Bombyx* species or *Pseudoips prasinana* L. (originally belonging to the genus of Bena, Nolidae family) (Boulet-Audet, Vollrath, and Holland 2015; Lucas, Shaw, and Smith 1960; Rindos et al. 2021). The latter is native to the Palearctic region, and its filaments of the main cocoon have a triangular cross-section, whereby *P. prasinana* filaments are a little thinner than BM silk (Rindos et al. 2021). The use of this species in the course of ancient textile production is not known to the authors.

For comparison, the AA composition of historical wool samples from the ninth to seventh century BC was determined. As expected, the silk and wool samples show significant differences in their AA composition and can therefore be easily distinguished (Tables 4, S7 and Figure S10, SM). In addition, the treatment with EDTA-DMF described above (see Sample pre-treatment with an EDTA solution) was successfully used to detect indigoid dyes in the blue sample 20,110,615/OF11-B (Table 3). As shown in Table 3, the D/L Asx ratios of the wool samples are 37% and 48%, respectively, and are thus increased in comparison to an undyed recent wool sample (D/L Asx: 5.2%, Table S6). Environmental influences could also have affected the D/L Asx ratio in these cases. Furthermore, AAR rates also depend on the AA composition, bond strengths and thus on the protein type (e.g. (Bright and Kaufman 2011)). Thus, investigations on AA racemization in wool samples are the subject of future studies.

Conclusion

Mulberry and non-mulberry silk samples, mordanted and dyed according to historical recipes, were analyzed for their AA composition using GC-FID and structural indices such as the 100LC/SC ratio were compared. With the exception of a slight increase in Glx, no significant differences were found in the mordanted and dyed samples compared to untreated silk. For dyeing and mordanting, madder roots and indigo as well as various metal salts were used, which – with the exception of copper(II) sulfate – are natural, eco-friendly and sustainable raw materials that have been used for centuries. Pre-treatment of the dyed samples with EDTA-ACN-MeOH or EDTA-DMF, which enables parallel detection of mordant and vat dyes and thus comprehensive characterization of the sample material, also does not cause any significant changes in the AA composition. To investigate the influence of silk processing on AA racemization, a chiral GC-MS method was developed and validated that allows the identification and quantification of 11 enantiomeric AA pairs in fibroin hydrolyzates. In the course of the method development, various hydrolysis procedures were tested. Since microwave-assisted hydrolysis leads to significantly reduced hydrolysis times but to greatly increased levels of (D)-AAs, the hydrolysis was carried out without microwave irradiation using 6 N HCl. Studies on mordanted BM silk samples, before and after treatment with EDTA-ACN-MeOH or EDTA-DMF, show that an increased proportion of (D)-Ser, (D)-Met and (D)-Asx can be observed for some of these samples. Furthermore, indigo dyeing carried out under basic conditions (pH 8–9) at 55°C led to a slightly increased (D)-Asx content and thus to D/L Asx ratios of 13–16%. In the next step, the effect of alkaline degumming procedures, using 0.1–0.25% Na_2CO_3 and/or 2.5% ethylenediamine at 95°C for 30–120 min as well as 10% ethylenediamine at 95°C for 120 min, was investigated. However, only two samples were found to contain (D)-Asx above the LOQ, with D/L Asx ratios of 10–12%. In addition, a treatment with 0.1% or 0.25% Na_2CO_3 for 120 min at 95°C led to an increase in the (D)-Leu content and thus to D/L Leu ratios of 31–35%. Harsh conditions, i.e. temperatures of 110°C and pH values >9, lead to a significant increase in the D/L Asx ratio to 44–67%, with the (D)-Asx content increasing with treatment time. At pH 13 and a temperature of 110°C, the silk filaments are almost completely dissolved and D/L Asx ratios of up to 100% can be observed, with increased (D)-Ala, (D)-Ser, (D)-Glx, (D)-Phe and (D)-His ratios also being observed.

The study of silk fabrics from the archaeological sites of Niya and Yanghai in the Tarim Basin (China) using the described GC-MS method as well as microscopic and spectroscopic techniques reveals the presence of degraded *Bombyx* silk with a low proportion of AAs with bulky side groups. A low sericin content was found in silk fabrics from the Niya site, which are dated to the Han dynasty (206 BC −220 AD) and consist of long silk filaments, indicating the use of intact cocoons for textile production. A silk fabric from the Yanghai site contains a higher sericin content than the samples from Niya and was made from spun warp and weft threads. D/L Asx ratios of 60–86% as well as significantly increased D/L Val and D/L Glx ratios were determined for the silk fabrics from both sites. The influence of environmental factors such as metal chelation or pH on the AA racemization of archaeological silk samples is difficult to estimate. The AA composition of a wool sample from the Yanghai site differs significantly from the silk samples. With the aid of EDTA-DMF extraction procedure, indigoid dyestuffs were detected in this sample. The presented multi-analytical technique-based approach enables the analysis of dyestuffs and silk filaments and thus a comprehensive characterization of historical silk textiles.

Highligths

- The influence of dyeing, mordanting and alkaline degumming techniques on the amino acid (AA) composition and the AA racemization of silks was studied.
- A chiral GC-MS method for the identification and quantification of 11 enantiomeric AA pairs in silk fibroin hydrolyzates was developed and validated.
- Sample pre-treatment with EDTA-DMF enables parallel dyestuff analysis using HPLC-DAD and thus a comprehensive characterization of dyed silks.
- Studies on historical silk fabrics from sites in the Tarim Basin show the presence of degraded *Bombyx* silk with significantly increased D/L Val, D/L Glx and D/L Asx ratios.

Acknowledgments

This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation, project number 425225219). Samples from the finding sites of Niya and Yanghai (Xinjiang Uyghur Autonomous Region, China) were taken within the framework of an interdisciplinary Chinese-German research project entitled “Silk Road Fashion – Clothes as a means of communication in the 1st millennium BC in Eastern Central Asia.” The authors are grateful to the Archaeological Institute in Urumqi and the Turpan Museum in Turfan, and especially to Prof. Dr M. Wagner, Dr P. Wertmann (Deutsches Archaeologisches Institut) and Prof. Dr. P. E. Tarasov (Freie Universität Berlin) for continuing support. The authors would like to thank Prof. R. Csuk (Martin-Luther-Universität Halle-Wittenberg) for continuous support, N. Quandt (working group of Prof. Ebbinghaus, Martin-Luther-Universitat Halle-Wittenberg) and U. Schwarzer (Landeskriminalamt in Magdeburg) for providing SEM- and SEM-EDX spectra. We also thank J. Werner and M. Schneider (Martin-Luther-Universität Halle-Wittenberg) for their valuable help in the laboratory and C. Jägern (Pflanzgarten School Garden, Francke Foundations, Halle) as well as the butterfly house of Jonsdorf (Germany) for providing hatched cocoons. We acknowledge the financial support of the Open Access Publication Fund of the Martin-Luther-University Halle-Wittenberg.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The work was supported by the Deutsche Forschungsgemeinschaft [425225219]; Open Access Publication Fund of the Martin-Luther-University Halle-Wittenberg .

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References

Aguayo, T., C. Araya, M. Icaza, and M. M. Campos-Vallette. 2014. “A Vibrational Approach for the Study of Historical Weighted and Dyed Silks.” *Journal of Molecular Structure* 1075:471–478. https://doi.org/10.1016/j.molstruc.2014.07.016.

Ahmed, H. E., and S. S. Darwish. 2012. “Effect of Museum Conditions on Historical Dyed Silk Fabric with Madder Dye.” *Journal of Polymers and the Environment* 20 (2): 596–606. https://doi.org/10.1007/s10924-012-0421-x.

Andreotti, A., I. Bonaduce, M. P. Colombini, G. Gautier, F. Modugno, and E. Ribechini. 2006. “Combined GC/MS Analytical Procedure for the Characterization of Glycerolipid, Waxy, Resinous, and Proteinaceous Materials in a Unique Paint Microsample.” *Analytical Chemistry* 78 (13): 4490–4500. https://doi.org/10.1021/ac0519615.

Arunkumar, K. P., M. Metta, and J. Nagaraju. 2006. “Molecular Phylogeny of Silkmoths Reveals the Origin of Domesticated Silkmoth, *Bombyx Mori* from Chinese *Bombyx Mandarina* and Paternal Inheritance of *Antheraea Proylei* Mitochondrial DNA.” *Molecular Phylogenetics and Evolution* 40 (2): 419–427. https://doi.org/10.1016/j.ympev.2006.02.023.

Bada, J. L. 1985. “Racemization of Amino Acids.” In *Chemistry and Biochemistry of the Amino Acids*, edited by G. C. Barrett, 399–414. Dordrecht: Springer Netherlands.

Beck, U., M. Wagner, X. Li, D. Durkin-Meisterernst, and P. E. Tarasov. 2014. “The Invention of Trousers and Its Likely Affiliation with Horseback Riding and Mobility: A Case Study of Late 2nd Millennium BC Finds from Turfan in Eastern Central Asia.” *Quaternary International* 348:224–235. https://doi.org/10.1016/j.quaint.2014.04.056.

Biswal, B., A. K. Dan, A. Sengupta, M. Das, B. K. Bindhani, D. Das, and P. K. Parhi. 2022. “Extraction of Silk Fibroin with Several Sericin Removal Processes and its Importance in Tissue Engineering: A Review.” *Journal of Polymers and the Environment* 30 (6): 2222–2253. https://doi.org/10.1007/s10924-022-02381-w.

Bonaduce, I., M. Cito, and M. P. Colombini. 2009. “The Development of a Gas Chromatographic–Mass Spectrometric Analytical Procedure for the Determination of Lipids, Proteins and Resins in the Same Paint Micro-Sample Avoiding Interferences from Inorganic Media.” *Journal of Chromatography A* 1216 (32): 5931–5939. https://doi.org/10.1016/j.chroma.2009.06.033.

Boudin, M., P. Boeckx, P. Vandenabeele, and M. Van Strydonck. 2013. “Improved Radiocarbon Dating for Contaminated Archaeological Bone Collagen, Silk, Wool and Hair Samples via Cross-Flow Nanofiltrated Amino Acids.” *Rapid Communications in Mass Spectrometry: RCM* 27 (18): 2039–2050. https://doi.org/10.1002/rcm.6652.

Boulet-Audet, M., F. Vollrath, and C. Holland. 2015. “Identification and Classification of Silks Using Infrared Spectroscopy.” *The Journal of Experimental Biology* 218 (19): 3138–3149. https://doi.org/10.1242/jeb.128306.

Bright, J., and D. S. Kaufman. 2011. “Amino Acids in Lacustrine Ostracodes, Part III: Effects of pH and Taxonomy on Racemization and Leaching.” *Quaternary Geochronology* 6 (6): 574–597. https://doi.org/10.1016/j.quageo.2011.08.002.

Cardon, D. 2007. *Natural Dyes: Sources, Tradition, Technology and Science*. London: Archetype Publications Ltd.

Colombini, M. P., R. Fuoco, A. Giacomelli, and B. Muscatello. 1998. “Characterization of Proteinaceous Binders in Wall Painting Samples by Microwave-Assisted Acid Hydrolysis and GC-MS Determination of Amino Acids.” *Studies in Conservation* 43 (1): 33–41. https://doi.org/10.1179/sic.1998.43.1.33.

Colombini, M. P., and F. Modugno. 2004. “Characterisation of Proteinaceous Binders in Artistic Paintings by Chromatographic Techniques.” *Journal of Separation Science* 27 (3): 147–160. https://doi.org/10.1002/jssc.200301625.

Deng, T., L. Cheng, Y. Peng, X. Tong, N. Guo, Z. Li, T. Zhao, and F. Dai. 2021. “Structure and Properties of *Bombyx Mandarina* Silk Fiber and Hybrid Silk Fiber.” *Journal of Natural Fibers* 18 (3): 330–342. https://doi.org/10.1080/15440478.2019.1623743.

de Palaminy, L., C. Daher, and C. Moulherat. 2022. “Development of a Non-Destructive Methodology Using ATR-FTIR and Chemometrics to Discriminate Wild Silk Species in Heritage Collections.” *Spectrochimica Acta Part A, Molecular and Biomolecular Spectroscopy* 270:120788. https://doi.org/10.1016/j.saa.2021.120788.

Deveoglu, O., R. Karadag, A. Spinella, and E. T. Guzel. 2019. “Examination of Dyeing Properties on Silk of Some Flavonoids by Spectroscopic Techniques.” *Journal of Natural Fibers* 18 (2): 238–249. https://doi.org/10.1080/15440478.2019.1616650.

Deveoglu, O., B. Y. Sahinbaskan, E. Torgan, and R. Karadag. 2012. "Investigation on Colour, Fastness Properties and HPLC-DAD Analysis of Silk Fibres Dyed with *Rubia Tinctorium* L. and *Quercus Ithaburensis* Decaisne." *Coloration Technology* 128 (5): 364–370. https://doi.org/10.1111/j.1478-4408.2012.00389.x.

Freddi, G., Y. Gotoh, T. Mori, I. Tsutsui, and M. Tsukada. 1994. "Chemical Structure and Physical Properties of *Antheraea Assama* Silk." *Journal of Applied Polymer Science* 52 (6): 775–781. https://doi.org/10.1002/app.1994.070520608.

Gao, S., M. Yao, Narenggaowa, D. Guo, Y. Li, K. L. Do, J. Liu, and F. Zhao. 2024. "Identification of Fibers and Dyes in Archaeological Textiles from Bazhou, Xinjiang (220-420 CE), and Their Silk Road Origins." *Journal of Archaeological Science: Reports* 164:105941. https://doi.org/10.1016/j.jas.2024.105941.

Garside, P., P. Wyeth, and X. Zhang. 2010. "Understanding the Ageing Behaviour of Nineteenth and Twentieth Century Tin-Weighted Silks." *Journal of the Institute of Conservation* 33 (2): 179–193. https://doi.org/10.1080/19455224.2010.501293.

Ge, R., L. Cong, Y. Fu, B. Wang, G. Shen, B. Xu, M. Hu, H. Yu, J. Zhou, and L. Yang. 2023. "Multi-Faceted Analysis Reveals the Characteristics of Silk Fabrics on a Liao Dynasty DieXie Belt." *Heritage Science* 11 (1): 217. https://doi.org/10.1186/s40494-023-01064-6.

Goodfriend, G. A. 1992. "Rapid Racemization of Aspartic Acid in Mollusc Shells and Potential for Dating Over Recent Centuries." *Nature* 357 (6377): 399–401. https://doi.org/10.1038/357399a0.

Hofenk de Graaff, J. H. 2004. *The Colourful Past: Origins, Chemistry and Identification of Natural Dyestuffs*. London: Abegg-Stiftung and Archetype Publications Ltd.

Johnson, A. P., and L. M. Pratt. 2010. "Metal-Catalyzed Degradation and Racemization of Amino Acids in Iron Sulfate Brines Under Simulated Martian Surface Conditions." *Icarus* 207 (1): 124–132. https://doi.org/10.1016/j.icarus.2009.11.028.

Karadag, R. 2023. "Establishing a New International Standard for Natural Dyed Textile Goods [Natural Organic Dye Standard (NODS)]." *Journal of Natural Fibers* 20 (1): 2162187. https://doi.org/10.1080/15440478.2022.2162187.

Karadag, R., and R. G. Oraltay. 2022. "Application of a New SEM–EDX Technique Associated with HPLC/DAD: Manufacturing Methods of Historical Textiles." *Archaeological and Anthropological Sciences* 15 (1): 8. https://doi.org/10.1007/s12520-022-01702-3.

Kaufman, D. S. 2006. "Temperature Sensitivity of Aspartic and Glutamic Acid Racemization in the Foraminifera *Pulleniatina*." *Quaternary Geochronology* 1 (3): 188–207. https://doi.org/10.1016/j.quageo.2006.06.008.

Kmet, P., L. Kucerova, H. Sehadova, B. Chia-Hsiang Wu, Y.-L. Wu, and M. Zurovec. 2023. "Identification of Silk Components in the Bombycoid Moth *Andraca Theae* (Endromidae) Reveals Three Fibroin Subunits Resembling Those of Bombycidae and Sphingidae." *Journal of Insect Physiology* 147:104523. https://doi.org/10.1016/j.jinsphys.2023.104523.

Koch, J. 2022. *Martin-Luther-Universität Halle-Wittenberg.*

Koperska, M. A., D. Pawcenis, J. Bagniuk, M. M. Zaitz, M. Missori, T. Łojewski, and J. Łojewska. 2014. "Degradation Markers of Fibroin in Silk Through Infrared Spectroscopy." *Polymer Degradation and Stability* 105:185–196. https://doi.org/10.1016/j.polymdegradstab.2014.04.008.

Kramell, A., X. Li, R. Csuk, M. Wagner, T. Goslar, P. E. Tarasov, N. Kreusel, R. Kluge, and C.-H. Wunderlich. 2014. "Dyes of Late Bronze Age Textile Clothes and Accessories from the Yanghai Archaeological Site, Turfan, China: Determination of the Fibers, Color Analysis and Dating." *Quaternary International* 348:214–223. https://doi.org/10.1016/j.quaint.2014.05.012.

Kramell, A. E., P. Wertmann, D. Hosner, R. Kluge, F. Oehler, C.-H. Wunderlich, P. E. Tarasov, M. Wagner, and R. Csuk. 2016. "A Multi-Analytical Techniques Based Approach to Study the Colorful Clothes and Accessories from Mummies of Eastern Central Asia." *Journal of Archaeological Science: Reports* 10:464–473. https://doi.org/10.1016/j.jasrep.2016.11.021.

Kromidas, S. 2011. *Handbuch Validierung in der Analytik*. Weinheim: John Wiley & Sons.

Lee, B., E. Pires, A. M. Pollard, and J. S. O. McCullagh. 2022. "Species Identification of Silks by Protein Mass Spectrometry Reveals Evidence of Wild Silk Use in Antiquity." *Scientific Reports* 12 (1): 4579. https://doi.org/10.1038/s41598-022-08167-3.

Li, L., Y. Gong, H. Yin, D. Gong, and F. Zhou. 2015. "Different Types of Peptide Detected by Mass Spectrometry Among Fresh Silk and Archaeological Silk Remains for Distinguishing Modern Contamination." *PLoS One* 10 (7): e0132827–e0132827. https://doi.org/10.1371/journal.pone.0132827.

Li, M.-Y., Y. Zhao, T. Tong, X.-H. Hou, B.-S. Fang, S.-Q. Wu, X.-Y. Shen, and H. Tong. 2013. "Study of the Degradation Mechanism of Chinese Historic Silk (Bombyx Mori) for the Purpose of Conservation." *Polymer Degradation and Stability* 98 (3): 727–735. https://doi.org/10.1016/j.polymdegradstab.2012.12.021.

Lluveras, A., I. Bonaduce, A. Andreotti, and M. P. Colombini. 2010. "GC/MS Analytical Procedure for the Characterization of Glycerolipids, Natural Waxes, Terpenoid Resins, Proteinaceous and Polysaccharide Materials in the Same Paint Microsample Avoiding Interferences from Inorganic Media." *Analytical Chemistry* 82 (1): 376–386. https://doi.org/10.1021/ac902141m.

Lucas, F., J. T. B. Shaw, and S. G. Smith. 1960. “Comparative Studies of Fibroins: I. the Amino Acid Composition of Various Fibroins and Its Significance in Relation to Their Crystal Structure and Taxonomy.” *Journal of Molecular Biology* 2 (6): 339–349. https://doi.org/10.1016/S0022-2836(60)80045-9.

Manhita, A., T. Ferreira, A. Candeias, and C. Barrocas Dias. 2011. “Extracting Natural Dyes from Wool—An Evaluation of Extraction Methods.” *Analytical & Bioanalytical Chemistry* 400 (5): 1501–1514. https://doi.org/10.1007/s00216-011-4858-x.

Moini, M., K. Klauenberg, and M. Ballard. 2011. “Dating Silk by Capillary Electrophoresis Mass Spectrometry.” *Analytical Chemistry* 83 (19): 7577–7581. https://doi.org/10.1021/ac201746u.

Moini, M., and C. M. Rollman. 2017. “Buyid Silk and the Tale of Bibi Shahrbanu: Identification of Biomarkers of Artificial Aging (Forgery) of Silk.” *Analytical Chemistry* 89 (19): 10158–10161. https://doi.org/10.1021/acs.analchem.7b02854.

Ozdemir, M. B., and R. Karadag. 2023a. “Anatolian Acorn Oak’s Economic Potential in the Application to the Textile and Leather Industries.” *Textile & Leather Review* 6:320–332. https://doi.org/10.31881/TLR.2023.044.

Ozdemir, M. B., and R. Karadag. 2023b. “Madder (*Rubia Tinctorum* L.) as an Economic Factor Under Sustainability Goals in the Textile Dyeing.” *Journal of Natural Fibers* 20 (1): 2128968. https://doi.org/10.1080/15440478.2022.2128968.

Puchalla, N. 2022. Bachelor Thesis, Halle (Saale): Martin-Luther-Universität Halle-Wittenberg.

Reddy, N., Y. Zhao, and Y. Yang. 2013. “Structure and Properties of Cocoons and Silk Fibers Produced by *Attacus Atlas*.” *Journal of Polymers and the Environment* 21 (1): 16–23. https://doi.org/10.1007/s10924-012-0549-8.

Reischl, R. J., and W. Lindner. 2015. “The Stereoselective Separation of Serine Containing Peptides by Zwitterionic Ion Exchanger Type Chiral Stationary Phases and the Study of Serine Racemization Mechanisms by Isotope Exchange and Tandem Mass Spectrometry.” *Journal of Pharmaceutical & Biomedical Analysis* 116:123–130. https://doi.org/10.1016/j.jpba.2015.02.014.

Rindos, M., L. Kucerova, L. Rouhova, H. Sehadova, M. Sery, M. Hradilova, P. Konik, and M. Zurovec. 2021. “Comparison of Silks from *Pseudoips Prasinana* and *Bombyx Mori* Shows Molecular Convergence in Fibroin Heavy Chains but Large Differences in Other Silk Components.” *International Journal of Molecular Sciences* 22 (15): 8246. https://doi.org/10.3390/ijms22158246.

Rutherfurd, S. M., and G. S. Gilani. 2009. “Amino Acid Analysis.” *Current Protocols in Protein Science*, 58 (1): 11–9. https://doi.org/10.1002/0471140864.ps1109s58.

Schmidt, T., N. Puchalla, M. Schendzielorz, and A. E. Kramell. 2023. “Degumming and Characterization of *Bombyx Mori* and Non-Mulberry Silks from Saturniidae Silkworms.” *Scientific Reports* 13 (1): 19504. https://doi.org/10.1038/s41598-023-46474-5.

Schröder, O., M. Wagner, S. Wutke, Y. Zhang, Y. Ma, D. Xu, T. Goslar, R. Neef, P. E. Tarasov, and A. Ludwig. 2016. “Ancient DNA Identification of Domestic Animals Used for Leather Objects in Central Asia During the Bronze Age.” *The Holocene* 26 (10): 1722–1729. https://doi.org/10.1177/0959683616641741.

Schweppe, H. 1992. *Handbuch der Naturfarbstoffe: Vorkommen, Verwendung, Nachweis*. Landsberg/Lech: ecomed.

Shimada, C. M., B. McCarthy, C. M. Rollman, A. Hare, J. Ueda, and M. Moini. 2023. “More Reliable Dating of Japanese Silk Mounting Textiles for Conservation Decisions: Effects of Silk Processing on Silk Surface Morphology and Aspartic Acid Racemization.” *Studies in Conservation*: 1–16. https://doi.org/10.1080/00393630.2023.2289720.

Smith, G. G., K. Williams, and D. Wonnacott. 1978. “Factors Affecting the Rate of Racemization of Amino Acids and Their Significance to Geochronology.” *The Journal of Organic Chemistry* 43 (1): 1–5. https://doi.org/10.1021/jo00395a001.

Veit, D. 2022. “Silk.” In *Fibers: History, Production, Properties, Market*, edited by D. Veit, 351–380. Cham: Springer International Publishing.

Vilaplana, F., J. Nilsson, D. V. P. Sommer, and S. Karlsson. 2015. “Analytical Markers for Silk Degradation: Comparing Historic Silk and Silk Artificially Aged in Different Environments.” *Analytical & Bioanalytical Chemistry* 407 (5): 1433–1449. https://doi.org/10.1007/s00216-014-8361-z.

Wertmann, P., X. Chen, X. Li, D. Xu, P. E. Tarasov, and M. Wagner. 2020. “New Evidence for Ball Games in Eurasia from Ca. 3000-Year-Old Yanghai Tombs in the Turfan Depression of Northwest China.” *Journal of Archaeological Science: Reports* 34:102576. https://doi.org/10.1016/j.jasrep.2020.102576.

Wu, G. 2024. “Mapping Byzantine Sericulture in the Global Transfer of Technology.” *Journal of Global History* 19 (1): 1–17. https://doi.org/10.1017/S1740022823000050.

Xia, Q., Y. Guo, Z. Zhang, D. Li, Z. Xuan, Z. Li, F. Dai, et al. 2009. “Complete Resequencing of 40 Genomes Reveals Domestication Events and Genes in Silkworm (*Bombyx*).” *Science* 326 (5951): 433–436. https://doi.org/10.1126/science.1176620.

Xiang, H., X. Liu, M. Li, Y. N. Zhu, L. Wang, Y. Cui, L. Liu, et al. 2018. “The Evolutionary Road from Wild Moth to Domestic Silkworm.” *Nature Ecology & Evolution* 2 (8): 1268–1279. https://doi.org/10.1038/s41559-018-0593-4.

Zahradníčková, H., P. Hušek, and P. Šimek. 2009. “GC Separation of Amino Acid Enantiomers via Derivatization with Heptafluorobutyl Chloroformate and Chirasil-L-Val Column.” *Journal of Separation Science* 32 (22): 3919–3924. https://doi.org/10.1002/jssc.200900400.

Zhang, X., and R. A. Laursen. 2005. “Development of Mild Extraction Methods for the Analysis of Natural Dyes in Textiles of Historical Interest Using LC-Diode Array Detector-MS.” *Analytical Chemistry* 77 (7): 2022–2025. https://doi.org/10.1021/ac048380k.

Zheng, H., H. Yang, Y. Zhou, T. Li, Q. Ma, B. Wang, Q. Fang, and H. Chen. 2021. “Rapid Enrichment and Detection of Silk Residues from Tombs by Double-Antibody Sandwich ELISA Based on Immunomagnetic Beads.” *Analytical Chemistry* 93 (43): 14440–14447. https://doi.org/10.1021/acs.analchem.1c02556.