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Analysis of amino acid enantiomers from aged fingerprints†

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Fingerprints found at a crime scene can be key in criminal investigations. A method to accurately determine the age of the fingerprint, potentially crucial to linking the fingerprint to the crime, is not available at the moment. In this paper, we show that the use of the enantiomeric ratio of D/L-serine in fingerprints could pose as interesting target for age estimation techniques. We developed a UPLC-MS/MS method to determine the enantiomer ratios of histidine, serine, threonine, alanine, proline, methionine and valine from fingerprint residue. We found a significant change only in the relative ratio of D-serine with increasing fingerprint age after analysis of fingerprints up to 6 months old.

Introduction

Fingerprints can be crucial evidence in criminal investigations. The unique ridge detail can be used to individualize a perpetrator, if the fingermark is of sufficient quality and the reference fingerprint is present in the database. It is generally accepted that a fingermark found on an object is established by contact of the donor's finger and the object. However, the time at which this contact has taken place, which can be crucial to link the perpetrator to the crime, can at present not be derived from the fingermark. Therefore, fingerprint age estimation has been a topic of interest in the past decades.[1]

The main focus to estimate the time of deposition has been on using chemical changes in the composition of fingermark residue. After deposition, the molecules that make up a fingerprint are subject to degradation, such as hydrolysis and oxidation reactions.[1] Several investigations aimed at these changes to predict the age of a fingerprint. Studying fingerprint ageing using gas chromatography mass spectrometry (GC-MS), Archer *et al.* described the degradation of fatty acids and squalene in fingerprints after deposition on a surface.[2] Weyermann *et al.*, also based on GC-MS analyses, suggested a ratio between squalene and cholesterol as potential predictor for fingerprint age.[3] In subsequent research, Koenig *et al.* proposed to add wax ester compounds to the equation to reduce variability in initial composition.[4] Pleik *et al.* focused on the identification of degradation products of common fatty acids in fingerprints as potential tool for age determination.[5] Van Dam *et al.* used fluorescence spectroscopy to determine the relative amount of fluorescent oxidation products to estimate the age of fingerprints from male donors up to three weeks old, within several days' accuracy.[6] Alternatively, Oonk *et al.*, using a proteomics approach, suggested several potential protein markers to estimate fingerprint age.[7] More recently, Hinners *et al.*, suggested the ozonolysis of triacylglycerols as a means of determining the age of a fingerprint, and showed its potential as age marker in fingerprints up to one week old.[8] However, parameters often complicating accurate fingerprint age estimation are the influences of environmental factors such as temperature, humidity and light exposure.

Another potential drawback in many age estimation methods is that the starting concentrations at deposition are generally unknown and may vary largely, which could greatly affect the accuracy of the estimation. Targeting relative concentrations between fingerprint components could potentially overcome these issues, as was suggested by Van Dam *et al.* and Weyermann *et al.*[3,6] A method widely used in the fields of geochemistry and archaeology as dating tool for samples such as fossil bones and sediments, is amino acid racemization.[9–11] These methods are based on the fact that the biologically predominant and optically active L-enantiomer usually racemizes over time when it is isolated from the biological processes that maintain the optical activity, eventually leading to a racemic and optically inactive mixture.[12] Commonly used age determination methods are using the ratio of D/L-enantiomers of aspartic acid.[13]

In the aforementioned fields, separation of the amino acid enantiomers has been achieved using various analytical techniques. GC, capillary electrophoreses (CE) and (ultra) high performance liquid chromatography ((U)HPLC) are the most used methods to separate amino acid enantiomers.[11,14–17] When

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not using chiral stationary phase columns in liquid chromatography, derivatization of the amino acids prior to analysis is often essential, which is based on the formation of diastereomers by reaction with a chiral derivatizing agent.[15] Commonly used agents are 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA or Marfey's reagent), 1-(9-fluorenyl)ethyl chloroformate (FLEC), *N*-(4-nitrophenoxycarbonyl)-L-phenylalanine 2-methoxyethyl ester (*S*-NIFE), 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) and *o*-phthaldialdehyde (OPA) with chiral thiols.[15,18]

As amino acids are a commonly found component in fingerprint residue,[19] presumably in the naturally predominant L-enantiomer, we investigated if amino acid racemization could be a viable option for fingerprint age estimation. We developed a method to separate and relatively quantify amino acid enantiomers from fingerprints using FLEC and ultra-high-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). We chose FLEC as derivatization reagent as the reaction is relatively fast, and formed products are stable.[20] FLEC reacts with primary and secondary amines to form diastereomers, adding 236 Da to the amino acid molecular mass. As a proof of principle, we analyzed fingerprints from 6 different donors up to 6 months old.

Materials and methods

Solvents and chemicals

L-Alanine (≥98%), D-alanine (≥98%), L-serine (≥99%), D-serine (≥98%), L-threonine (≥98%), D-threonine (≥98%), L-valine (≥98%), D-valine (≥98%), L-histidine monohydrochloride monohydrate (≥98%), D-histidine monohydrochloride monohydrate (≥98%), L-methionine (≥98%), D-methionine (≥98%), L-proline (≥98%), D-proline (≥99%), L-phenylalanine (≥98%), D-phenylalanine (≥98%), *cis*-4-hydroxy-D-proline (≥98%), (−)-(9-fluorenyl)ethyl chloroformate solution and sodium tetraborate decahydrate were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). UPLC-grade acetonitrile (ACN), methanol (MeOH) and formic acid were purchased at Biosolve (Valkenswaard, the Netherlands). Ultrapure water was obtained by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C.

Fingerprints and standards

For a baseline measurement of fresh fingerprints (composition at $t = 0$), fingerprints (left and right thumb) from 40 donors (20 male, 20 female, age ranging from 20–69) were collected on 76 × 26 mm glass microscope slides (Thermo Scientific, Breda, the Netherlands). Similarly, for aging experiments, fingerprints (left and right thumb) from 6 donors (3 male, 3 female, age ranging from 20–45) were collected on glass microscope slides and subsequently stored in the dark in a temperature-controlled room (at 21 °C). All fingerprint donors gave informed consent. Fingerprints were aged for 0, 7, 14, 21, 30, 60, 90, 120, 150 and 180 days. Fingerprint residue was collected from the surface using polyester swabs (CleanTips Polyester Alpha, Texwipe, NC, USA). Prior to swabbing, the swab was wetted with 50 μL methanol. After swabbing the swab was placed in a 0.5 mL Eppendorf tube and 200 μL methanol was added. The tube was vortexed for 1 minute and subsequently placed in an ultrasonic bath for 10 minutes. After sonication, the sample solution was transferred to a 2 mL Eppendorf tube while the swab was transferred to a spin basket and subsequently placed on the Eppendorf tube. The tube was centrifuged for 10 minutes at 13 000 rpm. After centrifugation, the sample was transferred to a 2 mL LC injection vial and 20 μL of internal standard (50 mg L^{-1} hydroxy-D-proline in methanol) was added. Subsequently, the sample was evaporated under nitrogen flow at room temperature. After evaporation, 50 μL of 0.16 M borate buffer and 50 μL of 18 mM FLEC solution in acetone were added. After 20 minutes of incubation at room temperature, 100 μL 70 : 30 acetonitrile : ultrapure water (containing 1.5% (v/v) formic acid) was added. Prior to LC-MS/MS analysis, the samples were filtrated using a 0.45 μm syringe filter. The calibration standards were prepared out of a 10 mg L^{-1} stock solution containing a 50 : 50 mixture of each of the D- and L-amino acid enantiomers in methanol. Calibration series were prepared in duplicate ranging from 0.2 to 1.0 mg L^{-1} and were prepared by transferring the required volume of stock solution directly to an injection vial. The addition of internal standard and the derivatization using FLEC were executed as described above. After derivatization, 550 μL acetonitrile, 350 μL ultrapure water and 15 μL formic acid were added and the solution was vortexed for 1 minute. All samples were filtrated using a 0.45 μm syringe filter. All samples were analyzed in triplicate. The percentage of D-amino acid is calculated by dividing the peak area of the D-enantiomer by the sum of the D- and L-enantiomer:

$$\%\ \text{D-enantiomer} = \frac{\text{peak area}_{\text{D-enantiomer}}}{(\text{peak area}_{\text{D-enantiomer}} + \text{peak area}_{\text{L-enantiomer}})} \times 100\%$$

To test the accuracy of the determination of the ratio of D- and L-amino acid enantiomers, a calibration set with varying ratios of D- and L-amino acids was prepared from stock solutions consisting of 7 samples with L/D ratios of 100 : 0, 95 : 5, 90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50 approximately, adjusted for enantiomeric purity of amino acids.

UPLC-MS/MS

Separation was performed using an Acquity UPLC with an ethylene bridged hybrid (BEH) C18 1.7 μm, 2.1 × 150 mm, column (Waters, Milford, MA, USA). Solvents used were acetonitrile containing 0.4% (v/v, %) formic acid (A) and H_2O : MeOH (95 : 5) containing 0.4% (v/v, %) formic acid (B). A flowrate of 400 μL min^{-1} was used and a gradient starting at 75% A was programmed. A linear decrease of solvent A to 71% after 10 minutes, followed by a decrease to 67% after 20 minutes was programmed. Solvent A was then decreased to 61% after 25 minutes and held constant for 20 minutes. Finally, the column is flushed for 4 minutes by decreasing solvent A to 20%, followed by re-equilibrating the column for 5 minutes to 75% A (total run time of 55 minutes). An injection volume of 1 μL was

used. The column eluent was directly analyzed using MS/MS using a triple quadrupole (QqQ) mass spectrometer (Thermo Scientific Quantiva, Breda, the Netherlands), operated in positive mode. The ESI conditions were as follows: a spray voltage of 3.5 kV was used, the sheath gas, aux gas, and sweep gas were set to 45, 13 and 1 (Arb), respectively. The ion transfer tube was set to 342 °C and the vaporizer temperature was set to 358 °C. The cycle time was set to 1 second, the Q1 and Q3 resolution were set to 0.7 FWHM. The collision gas pressure was 1.5 mTorr and the source fragmentation was set to 0 V. The optimized MRM parameters can be found in Table S1.†

Results

Using FLEC derivatization and the developed UPLC-QqQ method, baseline separation of the D/L-enantiomers of histidine, serine, threonine, alanine, proline and valine was achieved (Fig. 1). Complete separation of the enantiomers of methionine and phenylalanine was not achieved. The baseline-separated amino acids were included in this study as well as methionine, even though no complete baseline separation was obtained. The limit of detection (LOD) and limit of quantification (LOQ) of the 7 amino acids used in this study are presented in Table S2.†

To verify the ability of the method to accurately quantify different ratios of L- and D-amino acids, standards with varying ratios of these enantiomers were analyzed, ranging from 100% L-amino acid to a 50 : 50 mixture (racemic equilibrium, Fig. 2). For all 7 amino acids included in the method, reasonable linearity was achieved ($R^2 > 0.98$). Especially important for the application of this method is the performance in the low range, which seems to be slightly poorer for alanine. Moreover, the percentage of D-enantiomer seems to be slightly overestimated in case of threonine, proline and valine. Subsequently, we determined the abundance of the D-enantiomer of these 7 amino acids in freshly deposited fingerprints from 40 donors (Fig. 3), as the D/L-ratio of the amino acids in freshly deposited fingerprints is an important factor in this study. To be suitable for age estimation, the percentage of D-amino acids in different fingerprints at the time of deposition would ideally be close to zero with low variability between donors.

The average percentage of D-amino acid directly after deposition is generally low for threonine, serine and histidine, combined with a relatively low variability. Proline, valine and methionine have a slightly higher content of D-amino acid in fresh fingerprints. Lastly, alanine appeared to have the highest percentage of D-amino acid in fresh fingerprints combined with a high variability among donors. To study the effect of fingerprint ageing on the ratio of L- and D-amino acids, the enantiomer ratios of the included amino acids were determined from fingerprints aged for up to 6 months. In the 6 month period, significant changes in D/L-ratio were only observed for serine. In case of serine (Fig. 4), a steady increase is observed for all donors with increasing fingerprint age during the first 30 days. After 30 days, D-serine has increased to over 1%, and further increasing to over 5% for 3 of the 6 donors in the 6 month period. For the other 3 donors, the % D-serine seems to eventually level-off, and even decrease after 120 days. As fingerprint age increases, variability in % D-serine increases as well, as can be deduced from the increasing standard deviation. The large deviation at 180 days however, is mainly caused by one donor (D4). No significant increase in D-enantiomers with time was found in case of the other 6 amino acids, resulting from problems with detectability and variability for these amino acids in aged fingerprints (data not shown).

Discussion

The aim of this study was to investigate the feasibility of using amino acid racemization to determine fingerprint age. In the developed method, using FLEC and UPLC-MS/MS, separation of 8 amino acids was achieved within 46 minutes. In comparison, Einarsson and Josefsson, the first to describe the enantiomeric separation of amino acids using FLEC, achieved baseline

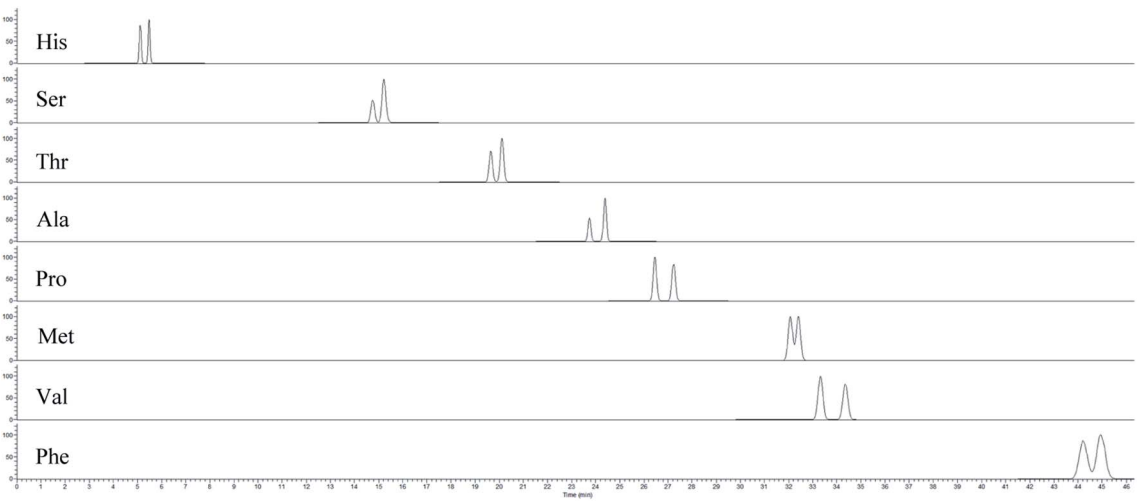

Fig. 1 UPLC-MS/MS chromatograms of the 8 amino acid enantiomers, showing their separation in the 46 minute gradient.

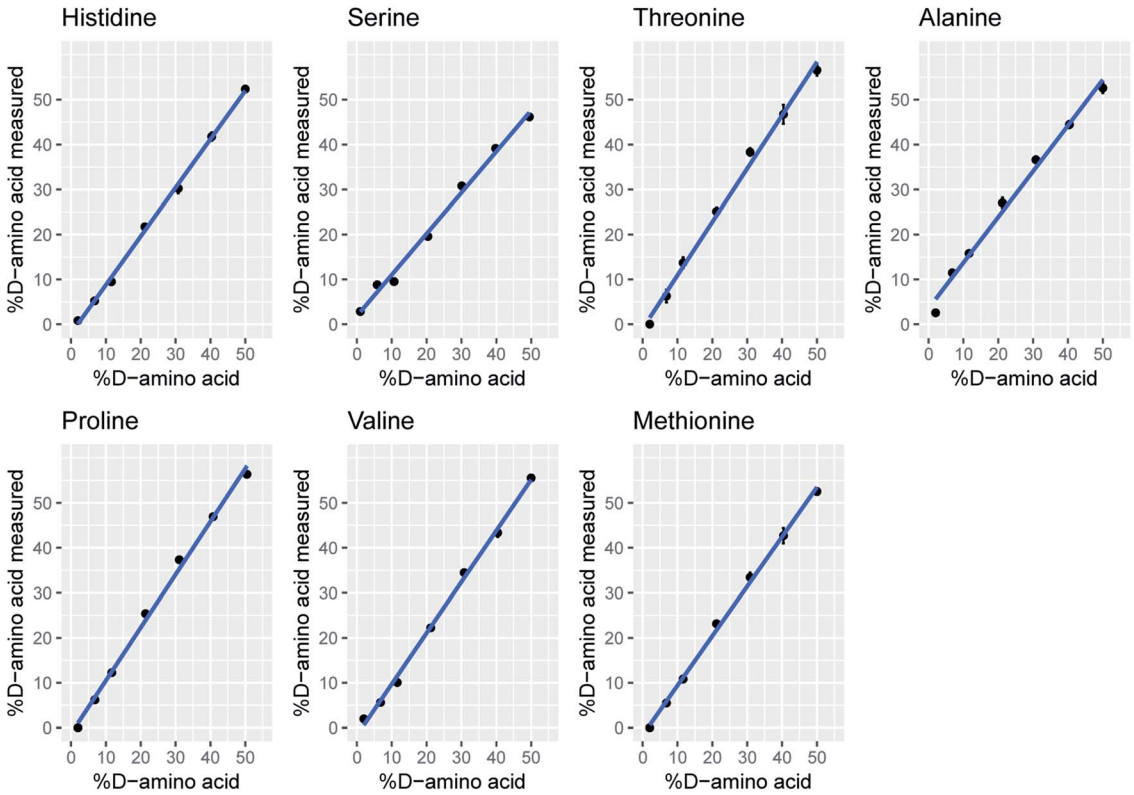

Fig. 2 Calibration results of analysis of varying ratios (L : D = 100 : 0, 95 : 5, 90 : 10, 80 : 20, 70 : 30, 60 : 40, 50 : 50, adjusted for purity) of D- and L-amino acids of histidine ($R^2 = 0.996$), serine ($R^2 = 0.996$), threonine ($R^2 = 0.987$), alanine ($R^2 = 0.992$), proline ($R^2 = 0.988$), valine ($R^2 = 0.991$) and methionine ($R^2 = 0.994$).

separation of 17 pairs of D/L-amino acids in 70 minutes.[21] In the study presented here we were able to determine the D/L-ratio of 7 amino acids from fingerprints. When analysing the enantiomeric ratios of fresh fingerprints based on a set of 40 donors, threonine, serine and histidine showed a low variability combined with a low concentration of the D-enantiomer. After analysis of fingerprints from 6 donors up to 6 months old, only an increasing trend of the D-enantiomer was seen in case of serine for all fingerprints up to 30 days. Thereafter, for 3 donors a further increase to over 5% relative D-serine was observed, while for the other 3 donors, the initial increase is levelling off, even followed by a decrease after 150 days. Also evident is the increase in variability after the first 30 days. A decrease, however, could be detrimental for the use of D-serine as age

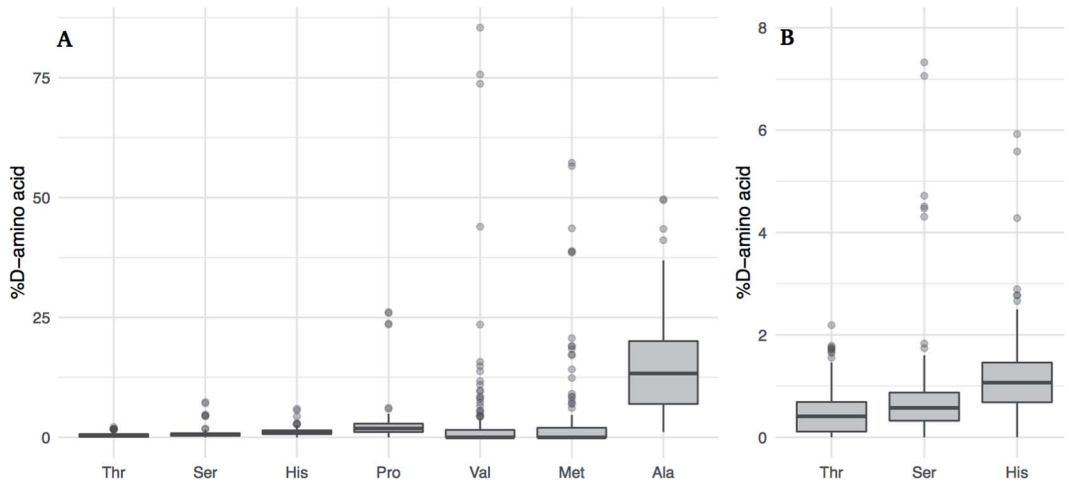

Fig. 3 (A) The percentage D-amino acid of threonine, serine, histidine, proline, valine, methionine and alanine in freshly deposited fingerprints of 40 donors. (B) Enlargement of the percentage D-amino acid of threonine, serine and histidine.

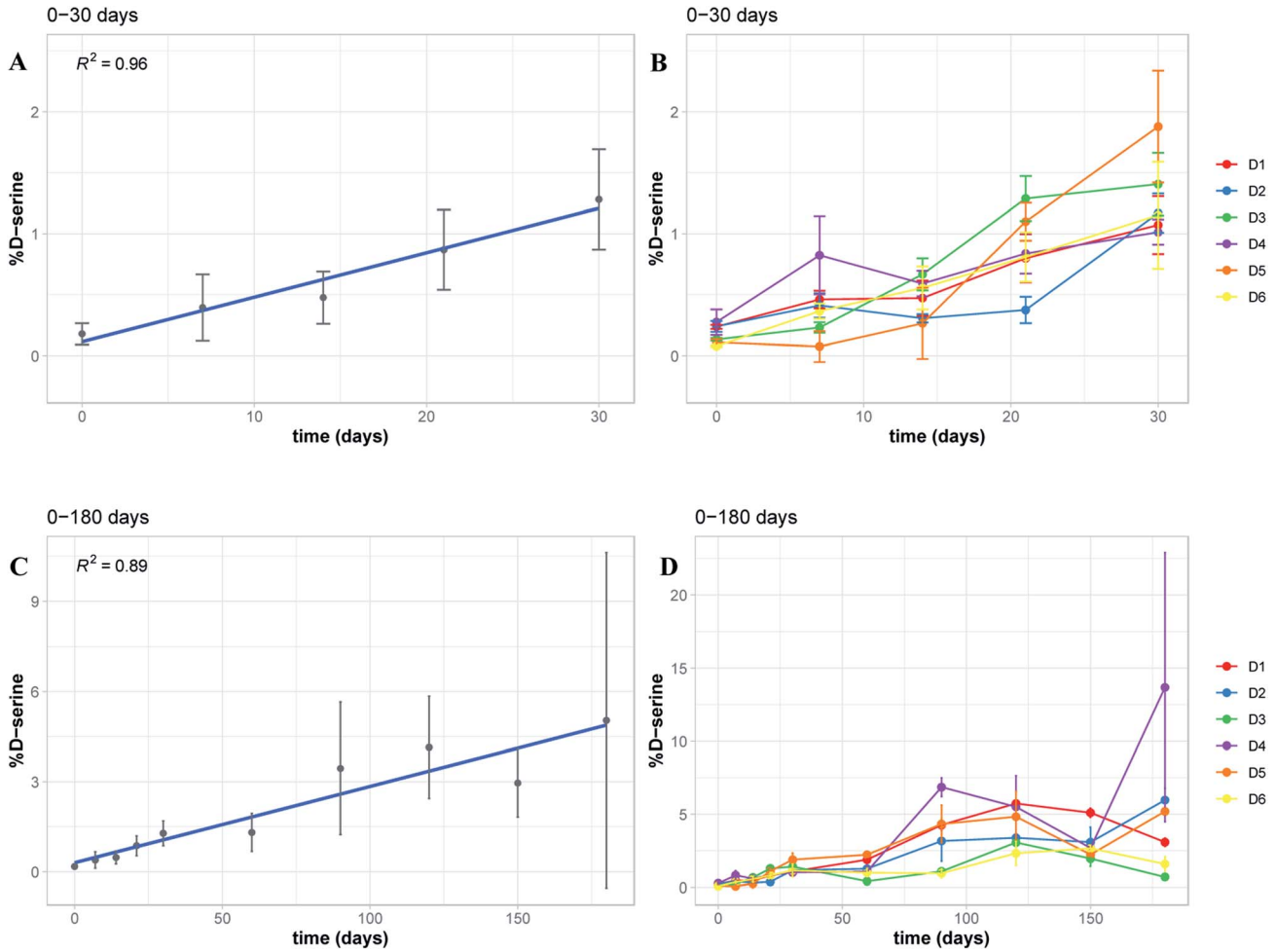

Fig. 4 The percentage D-serine in aged fingerprints of 6 donors, aged up to 1 month (A and B) and up to 6 months old (C and D), displayed as overall for the 6 donors (A and C), and per individual donor (B and D).

marker, as this would logically complicate the distinction between fingerprints with different ages. Further research is needed to develop a method which is more sensitive to the detection of the amino acids in order to accurately quantify the ratio. As each amino acid likely racemizes with a different rate, possibly different amino acids would be suitable for age estimation depending on the precise fingerprint age. In the fields of geochemistry and paleontology however, estimation is commonly done based on the enantiomers of a single amino acid, such as isoleucine or aspartic acid in quaternary science.[22]

Compared to previously suggested fingerprint age determination methods, the developed method offers similar advantages as described by Van Dam *et al.* and Weyermann *et al.*, by looking at ratios of potential age markers.[3,6] Looking at the enantiomers of an amino acid however, offers the additional advantage of correcting for the unknown starting amount and possible degradation that has taken place. When it comes to the timescale, the age estimation methods described by Hinners *et al.* and Van Dam *et al.* analyzed fingerprints up to 1 week and 3 weeks old, respectively.[6,8] The ratio of serine enantiomers could potentially extend this timescale of fingerprint age estimation methods, possibly up to several months. It is important to note that, eventually, the concentration of amino acid enantiomers will drop below the LOQ and thus analysis of the D/L-ratio will no longer be possible.

We found a significantly higher amount of the D-enantiomer for alanine compared to the other amino acids in freshly deposited fingerprints. This was not observed when amino acid stock solutions, containing different D/L-ratios, were analysed. Interference with other fingerprint constituents could potentially influence accurate determination of the D/L-ratio. Additionally, the variability in D-alanine in fresh fingerprints was found to be large, and as such, D-alanine was not a reliable marker for age estimation of the fingerprint deposition. This possibly is a result of environmental contamination, *via* consumption of food or the use of cosmetics, although it is unlikely this would only affect alanine.

Amino acid racemization in fingerprint residue is an unexplored area. It is well-known that the acidity plays an important role in the amino acid racemization rate in general.[23] The pH of fingerprint residue however, is unknown, and likely is variable both within and between donors. Additionally, fresh

fingerprints consist of 20–70% water, as was recently reported by Keisar *et al.*,[24] but will eventually dry up, since evaporation will start right after deposition. The precise mechanism of amino acid racemization in fingerprints therefore remains unknown and requires further research.

Overall, D-serine shows a promising trend for all fingerprints up to 30 days old. In older fingerprints, variability increases as for some donors a further increase is seen, whereas for others a decrease is observed. More research is needed, using larger data sets based on more donors, to elucidate the precise trend of D-serine with fingerprint age, while simultaneously investigating the behaviour of the other amino acid enantiomers. Additionally, it must be noted that the deposition pressure and time were not controlled in this study. The fact that a trend for D-serine was still observed, shows the potential of fingerprint dating based on amino acid racemization in practice. Some key parameters, such as temperature, humidity, light exposure and substrate were controlled. The influence of these factors thus remains unknown, and whereas the use of the serine enantiomer ratio in fingerprint dating could potentially overcome the issue of having unknown starting amounts, these factors likely influence the racemization rate as well. Next to confirming the potentially useful trend of D-serine, future studies should thus investigate the influence of parameters such as temperature, humidity and light exposure as well, to gain more insight in the applicability of the D/L-amino acid ratio for fingerprint dating.

Conclusion

In this paper, we present the development of an UPLC-MS/MS method to determine the D/L-ratios of 7 amino acids from fingerprints, after derivatization using (−)-(9-fluorenyl)ethyl chloroformate. In order to investigate the potential of D/L-amino acid ratios for use in fingerprint age estimation, freshly deposited fingerprints from 40 donors as well as fingerprints aged up to 6 months old from 6 donors were analyzed. In case of threonine, serine and histidine, a low concentration of the D-enantiomer in freshly deposited fingerprints was found. Analysis of aged fingerprints only showed a potentially useful trend for D-serine, which increased with fingerprint age for all donors up to 30 days. Thereafter, a further increase was seen in case of 3 donors, while an eventual levelling off followed by a decrease was detected for the other 3 donors. Further studies are needed, using larger dataset (*i.e.* more donors), to confirm the potentially useful trend seen for D-serine and investigate the behavior of the other amino acid enantiomers. Additionally, analysis should also focus on investigating the influence of temperature, humidity and substrate, and extend the timescale of the study. The use of D-serine poses as an interesting target for fingerprint age determination methods, as it overcomes the issue of having an unknown amount at the time of deposition.

Conflicts of interest

There are no conflicts to declare.

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