Fingermarks, more than just a ridge pattern
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**ABSTRACT**

The autofluorescence of fingermarks is used for their detection. The components responsible for this autofluorescence are largely unknown. Thin layer chromatography and fluorescence spectroscopy were used to identify autofluorescent components and evaluate their forensic value. Based on our results, tryptophan is hypothesized to be a major contributor to the autofluorescence when part of peptides or proteins, *id est*, not in its free form. Part of the autofluorescence could be assigned to a kynurenine derivative. Pheophorbide A, a metabolite of chlorophyll, is inferred as a red fluorescent fingermark component. Chlorophyll is a plant pigment which implies that dietary information can potentially be retrieved from fingermarks.
1. INTRODUCTION
Fingermarks play an important role in forensic research. When found at a crime scene, ridge groove pattern and DNA analysis can contribute to the identification of a person. The property of fingermarks to fluoresce when excited by UV and visible light is useful for detection [1-2]. Up to this moment it is not well known what the nature of this intrinsic fluorescence is [3]. Fingermarks are made up of desquamated cells, sebum, sweat and external components such as soap, skin care products and dirt [4-5]. Personal hygiene, behavior/occupation, diet, time of the day, type of contact are just a few factors that influence the composition of fingermarks. This, on the one hand, is the cause of the poor reproducibility of fingermarks but, on the other hand may offer opportunities to profile the fingermark donor.

We intend to identify fluorophores in fingermarks in order to use this knowledge to optimize fluorescence imaging and to retrieve more information about the fingermark donor. Thin layer chromatography (TLC) and fluorescence spectroscopy were used to identify the fluorophores in fingermarks. Fingermarks were deposited directly upon the TLC plate for analysis [3, 6]. As reference compounds, fluorophores were selected known to be found in sweat, sebum and skin [3, 7-12]. The list was subsequently narrowed down by only considering compounds that display fluorescence upon ultra violet A (UVA) (365 nm, 10% band width 350–380 nm) and blue light (445 nm, 10% band width 420–470 nm) excitation.

2. METHODS
2.1. Materials
Bovine serum albumin (BSA), chloroform, protoporphyrin IX, pyridoxal hydrochloride, thiamine hydrochloride, L-tryptophan, tyrosine, riboflavin, bilirubin, L-kynurenine, xanthurenic acid and flavin adenine dinucleotide disodium (FAD) salt hydrate were purchased from Sigma Aldrich, USA. Acetic acid, ethanol, methanol, chloroform, D,L-phenylalanine and TLC Silicagel 60 aluminum sheets were obtained from Merck, Germany. Pheophytin A was purchased from DHI group, Denmark. Pheophorbide A was obtained from TAMA biochemical CO, LTD, Japan. Trans-b-carotene was acquired from Janssen Chimica, Belgium. Ninhydrin (Nin-print) was purchased from BVDA International B.V., the Netherlands.

2.2. Apparatus
Crime-lite® 2 torches of different colors were obtained from Foster and Freeman, UK: UV (365 nm, 10% band width 350–380 nm), violet (410 nm, 10% band width 400–430 nm) blue (445 nm, 10% band width 420–470), blue-green (480 nm, 10% band width 460–510), green (530, 10% band width 500–560). Plastic goggles used were provided with the Crime-lite® 2 torches: clear (D-21000) yellow (I-505500), orange (I-505600), and red (I-505700). Camera filters used were provided with the Crime-lite® 2 torches: light yellow (GG455), yellow (GG495), red (G590), orange (OG550), clear (GG420) and
a polarizer. Digital images were taken with a Nikon D40 digital camera (Japan). Emission and excitation spectra were obtained with a LS 55 Luminescence spectrometer (Perkin Elmer, USA). In order to measure the emission and excitation spectra of the eluted fingerprint spots and reference compounds on TLC plates, a fiber optic accessory to the spectrometer was used (Perkin Elmer, USA).

2.3. Visualization of fingerprint

The UV, violet, blue, blue-green and green Crime-lite® 2 torches were used to visualize fingerprints on an undeveloped TLC plate. Digital images were taken using the long pass filters supplied with Crime-lite® 2 torches. The digital camera was placed at a distance of approximately 25 cm of the samples and the following settings were employed: aperture f/5.6, iso-800, focal length 50 mm. The amount of fluorescence originating from the background and fingerprints depended on the Crime-lite® 2 torches used which influenced the exposure times used: UV: 25 s, violet: 25 s, blue: 8 s, blue-green: 30 s, green: 30 s.

2.4. Thin layer chromatography

The TLC method was used as introduced by Bramble et al. and modified by Jones et al. [3, 6]. Fingermarks were placed on a methanol, pre-washed and activated (30 min, 120 8C) silica coated TLC plate. The mobile phases [2] studied were: MP1: chloroform/methanol (1:4), MP2: chloroform/methanol (4:1) and MP3: ethanol/glacial acetic acid (9:1). Stock solutions of reference compounds were prepared in 1% methanol except PPIX (0.1 M NaOH) and pheophytin (90% acetone as supplied by manufacturer). Milli-Q water was used for stock solutions. All stock solutions were stored at -20⁰C and kept for no longer than 7 days. Riboflavin photodegradation products were generated by exposing riboflavin (0.01 mg ml⁻¹, 10 ml) in an open 94 mm Petri dish to light from the blue Crime-lite® 2 torches for 10 min at a distance of 25 cm. Concentrations of solutions depended on the MP used and are listed in table 1 and 2. Samples of 2 µl were applied to the TLC plate. For analysis dried plates were studied with UV and blue Crime-lite® 2 torches in combination with the clear and yellow goggles. Next, spectra of the observed spots were obtained using the fiber optic accessory of the fluorimeter. The plates with stacked fingerprints were sprayed with ninhydrin. 20 µl aliquots of 50 mg ml⁻¹ BSA were pipetted onto a TLC plate to serve as fluorescence reference for fingerprint residues.

2.5. Fingerprint deposition

Volunteers were asked to wash their hands with water and soap, rinse well with water, dry their hands with a paper towel, rinse with 70% ethanol and dry by waving their hands through the air. Afterwards volunteers rubbed their fingertips from the bridge of the nose to the cheeks and across the forehead. Volunteers wearing facial products were asked to rub behind their ears. Next they pressed a finger on the TLC plate. Volunteers donated a maximum of two fingerprint marks which had to be placed at different days. To obtain a stronger fluorescence signal volunteers deposited ten fingerprints on top of
each other (one for each finger). The total number of volunteers that deposited marks was 19 for MP1 and MP3 and 18 for MP2. Prints were run within an hour of deposition.

3. RESULTS AND DISCUSSION
The main challenge in studying the composition of fingermarks is that they are diverse, as their composition depends on factors such as personal hygiene and diet, time of day and the type of contact. During test experiments it was noted that fingermarks left in the morning displayed a stronger fluorescence emission than those left in the afternoon. For this reason fingermarks for TLC analysis were deposited before noon. The origin of this phenomenon is not yet clear. It could be due to the reduction of fluorophores resulting from oxidative stress of daily life or perhaps it has to do with the circadian rhythm. Fingermarks were placed on TLC plates and fluorescence images were obtained prior to running (data not shown). We found that images obtained with the UV and blue Crime-lite® 2 showed the best visibility and highest contrast. The UV Crime-lite® 2 torches were used in combination with the polarizer filter instead of the supplied clear filter because with this particular experimental set-up it resulted in the best visualization of the fingermarks. Developed TLC plates were thus inspected with both the UV and blue Crime-lite® 2 using clear and yellow goggles respectively. The fluorescent spots were categorized into four different categories: red, yellow-x, blue and purple fluorescence. Yellow-x is a category for spots showing fluorescence in the orange-yellow-green range. Spots displaying very weak fluorescence were also added into this category. The same method was applied to the reference compounds. It must be noted that none of our reference compounds displayed purple fluorescence.

The MPs were selected from literature. The chloroform/methanol 4:1 MP was obtained from a TLC study on the content of visibly excited fluorescing compounds in fingermarks; relative amounts were modified for better extraction [3]. The ethanol/acetic acid MP was selected to separate amino acids [13]. On developed TLC plates, fingermark residues were observed at the location where the fingermarks had been placed. These residues mostly displayed a blue or yellow autofluorescence occasionally combined with red/orange autofluorescence (data not shown). The residues reacted positive with ninhydrin. Based on these observations we hypothesized that the fluorescence originated from protein bound tryptophan. Fluorescence spectra of the fingerprint residues were consistent with those of BSA (Fig. 1) which indicates that the fingerprint residue fluorescence is caused by peptides and/or proteins. Tryptophan derivates are known to show stronger fluorescence than tryptophan [14-16], thus plates were run with the tryptophan derivates kynurenine and xanthurenic acid as reference compounds using MP1. While conducting the experiments we noted that fingerprint autofluorescence initially increases in time upon deposition on the TLC plate. The same observation was made for kynurenine and tryptophan when used as reference compounds. The increase of fluorescence was not studied quantitatively or followed closely. It was observed that tryptophan fluorescence increased steeply within a few days after deposition. In addi-
tion the fingermarks fluoresced stronger when they were a few weeks old than when they were freshly deposited. No decrease in fluorescence intensity was observed within 6 months.

From 25 single and 6 stacked fingermarks eluted a total of 81 spots with MP1. These spots are categorized based on their fluorescence color and a compilation is shown in Fig. 2. No reference compounds could be excluded from the yellow-x category. Some of the eluted spots from the stacked fingermarks reacted strongly with ninhydrin. From 26 single fingermarks plus 6 stacked fingermarks developed with MP2, 24 fluorescent spots were eluted. The development of 26 single fingermarks plus 6 stacked fingermarks with MP3 yielded 50 fluorescent spots. Both inter and intra donor variation was observed, both in fingerprint fluorescence intensity and in the type and number of spots eluting from the deposited fingerprints. The inter person variation was more pronounced than the intra person variation. The number of spots eluting from single fingermarks varied between zero and six spots. None of the reference compounds categorized as yellow-x could be excluded based on their Rf-value. Below we will describe

<table>
<thead>
<tr>
<th>Compound</th>
<th>chloroform/methanol (1:4)</th>
<th>chloroform/methanol (4:1)</th>
<th>ethanol/glacial acid (9:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml Rf-value (sd)</td>
<td>mg/ml Rf-value (sd)</td>
<td>mg/ml Rf-value (sd)</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>1 0.98 (0.01)</td>
<td>1 1.00 (0.00)</td>
<td>10 0.97 (0.01)</td>
</tr>
<tr>
<td>β carotene</td>
<td>10 0.99 (0.01)</td>
<td>1 0.99 (0.01)</td>
<td>1 0.97 (0.01)</td>
</tr>
<tr>
<td>Flavinadenine di-nucleotide (FAD)</td>
<td>0.01 0.89 (0.03)</td>
<td>0.01 0.48 (0.05) t</td>
<td>1 0.08 (0.02) t 0.45 (0.02) t 0.61 (0.03) t 0.86 (0.00)</td>
</tr>
<tr>
<td>Pheophorbide a</td>
<td>0.1 0.97 (0.01)</td>
<td>0.1 0.98 (0.01)</td>
<td>0.1 0.88 (0.05)</td>
</tr>
<tr>
<td>Pheophytin</td>
<td>3×10⁻³ 0.72 (0.02)</td>
<td>3×10⁻³ 1.00 (0.00)</td>
<td>3×10⁻³ 0.98 (0.01)</td>
</tr>
<tr>
<td>Protoporphyrin IX</td>
<td>0.01 0.99 (0.01)</td>
<td>0.01 0.65 (0.15)</td>
<td>0.01 0.96 (0.02)</td>
</tr>
<tr>
<td>Pyridoxal HCL (vitamin B6)</td>
<td>0.1 0.76 (0.03)</td>
<td>0.1 0.70 (0.05) t</td>
<td>0.1 0.67 (0.04) t</td>
</tr>
<tr>
<td>Riboflavin (vitamin B2)</td>
<td>0.001 0.74 (0.02)</td>
<td>0.001 0.46 (0.07) t</td>
<td>0.001 0.56 (0.03) t</td>
</tr>
<tr>
<td>Riboflavin photo-product</td>
<td>0.01 0.65 t 0.73 (0.00) 0.85 (0.02)</td>
<td>0.01 0.44 (0.06) t 0.66 (0.04) t 0.95 (0.03) 0.98</td>
<td>0.01 0.64 (0.07) t 0.80 (0.12) 0.88 (0.03)</td>
</tr>
<tr>
<td>Thiamin (vitamin B1)</td>
<td>10 0.55 (0.02)</td>
<td>10 0.05 (0.02) t 0.61 (0.07) 0.79 (0.01)</td>
<td>10 0.09 (0.01)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>10 0.67 (0.01) t</td>
<td>10 0.09 (0.04) t</td>
<td>10 0.56 (0.03) t</td>
</tr>
</tbody>
</table>
Table 2. Rf-values of reference compounds (n=3), t: spot is tailed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chloroform/methanol (1:4)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/ml</td>
<td>Rf-value (sd)</td>
<td></td>
</tr>
<tr>
<td>Kynurenine</td>
<td>1</td>
<td>0.56 (0.01) t</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.93 (0.02)</td>
<td></td>
</tr>
<tr>
<td>Xanthurenic acid</td>
<td>1</td>
<td>0.93 (0.02)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.98 (0.01)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Fluorescence excitation and emission spectra of BSA (black line) and two fingermark residues left on TLC plate after development with MP1 (grey and dotted line). Emission wavelength: 340 nm, excitation wavelength: 265 nm.

Figure 2. Compilation of fluorescent spots eluted with MP1 from single and stacked fingermarks. The latter were treated with ninhydrin. A, B and C indicate the position of spots that displayed Rf-values and fluorescence spectra consistent with those of reference compounds.
the reference compounds studied, their eluting behavior and matches with eluted fingermark spots. Rf-values and standard deviations can be found in table 1 and 2. Reference compounds were considered to match with eluted fingermarks when there was an overlap with the eluted spots.

**Bilirubin** is the yellow break down product of heme and displays an orange-yellow fluorescence. Its Rf-values in all three MPs lay around 0.97–1.00 which is in the solvent front. The color of bilirubin changed from yellow to green when run on the TLC plate with MP2, none of the eluted fingermark spots did, indicating that bilirubin is not present in fingermarks.

**β-Carotene** is an orange pigment found in fruit and vegetables; it shows an orange-yellow fluorescence. Carotenes are lipophilic and are present in the stratum corneum [17]. β-Carotene displayed comparable Rf-values with all three investigated MPs. The Rf-values were high, 0.97–1.00, which is in the solvent front. β-Carotene could not be excluded as a fingermark component.

**Pheophorbide A and pheophytin** are red fluorescent metabolites of chlorophyll. Demetallation of chlorophyll leads to the formation of pheophytin. Pheophorbide A is a dephytylated pheophytin [9, 12, 18] which is known to reach the skin, sometimes leading to phototoxicity [12, 18]. In MP1 their Rf-value differed, 0.97 and 0.72 respectively. Red fluorescent fingermark spot A with Rf-value 0.98 (Fig. 2) was inferred to be pheophorbide A, based on Rf-value and emission spectrum (Fig. 3). The spot was weak and no excitation spectrum could be obtained due to photobleaching. In MP2 the Rf-values were comparable, 0.98 and 1.00 respectively. One of the red fluorescent fingermark spots with Rf-value 0.98 yielded fluorescence spectra corresponding well with the emission and excitation spectra of both pheophorbide A and pheophytin (Fig. 4). In MP3 pheophorbide A eluted into an elongated spot (Rf-value 0.88) and pheophytin did not (Rf-value 0.98). A red fluorescent eluted fingermark spot (Rf-value 0.76) displayed an emission spectrum comparable to that of pheophytin and pheophorbide A and was elongated. This spot was thus inferred to be pheophorbide A.
Protoporphyrin IX (PPIX) is an orange-red fluorescent porphyrin produced by acne caused by skin bacteria [10]. In MP2 it had an Rf-value of 0.65 which corresponds with the Rf-value of one red fluorescent fingermark spot observed in MP2, indicating the presence of PPIX. With the other two MPs it eluted into the solvent front.

Riboflavin (vitamin B2) has been proposed as the major fluorophore in fingermarks [19-20], but Jones et al. excluded it by TLC [3] and it was also excluded as the main fluorophore that causes the fluorescence in sweat [7]. However the fluorescence characteristics of riboflavin and other B vitamins inspired us to include riboflavin, thiamin, pyridoxal HCl and FAD into our investigation. Riboflavin and its photoproduct displayed yellow fluorescence under the conditions studied. The riboflavin photo-product eluted into several yellow fluorescent spots. As stated above none of the yellow fluorescent spots could be excluded based on their Rf-value and fluorescence color, but no spectral confirmation could be obtained. If present in fingermarks, riboflavin is inferred not to be a major contributor to the autofluorescence of fingermarks. Pyridoxal HCL (vitamin B6) displayed a non-specific yellowish green fluorescence. Pyridoxal HCL could not be excluded as a fingermark component.

Thiamin (vitamin B1) was the only blue fluorescent reference compound in our series. In MP1 its Rf-value (0.55) was consistent with that of some eluted fingermark spots. In MP2 it eluted into three spots, with only one spot (Rf-value 0.79) corresponding to an eluted fingermark spot. No match was observed with MP3. No spectra could be obtained from any of the spots corresponding with thiamin. Thiamin could not be excluded as a fingermark component.

Figure 4. Excitation and emission spectra of a red fluorescent spot (grey line), pheophorbide A (black line) and pheophytin (dotted line) eluted with MP2. Excitation wavelength: 390 nm, emission wavelength: 680 nm.
Flavin adenine dinucleotide (FAD) is a riboflavin containing biomolecule. It displays a yellow fluorescence and can be found in the skin [21]. In MP3 FAD eluted into four different spots all consistent with eluted fingermark spots. FAD could not be excluded as a fingermark component.

Tryptophan is a fluorescent amino acid and is potentially one of the most important fluorophores in fingermarks due to its abundance in the human body. Tryptophan has been suggested to be a major contributor to the intrinsic fluorescence of fingermarks, although this was not published in a full article [8]. Tryptophan displayed a very weak fluorescence and reacted positively with ninhydrin. In MP1 and 2 an Rf-value consistent with eluted fingermark spots was observed and categorized as yellow-x and in MP3 with a blue spot. However, none of the Rf-values of the ninhydrin positive spots eluted from the stacked fingermarks developed with the MP1 corresponded to the Rf-value of tryptophan. Free tryptophan is thus not expected to be a major contributor to fingermark autofluorescence.

Tyrosine and phenylalanine are fluorescent amino acids. However under the experimental conditions used they showed no visible fluorescence at 10 mg ml⁻¹. For this reason we decided they were not major contributors to the autofluorescence of fingermarks and removed them from the study.

Kynurenine is a blue fluorescent tryptophan metabolite [14-16]. With MP1 kynurenine eluted into a strong fluorescent main spot (Rf-value 0.56) and a weaker fluorescent spot (Rf-value 0.93). The top spot displayed fluorescence spectra that was consistent with spectra from the eluted fingermark spots C (Rf-value 0.93) and B (Rf-value 0.95) (Fig. 5).

Figure 5. Fluorescence excitation and emission spectra of a kynurenine derivative (Rf-value 0.93, black line) and two blue fluorescent fingermark spots (spot B = grey and spot C = dotted line) eluted with MP1. Emission wavelength: 440 nm, excitation wavelength: 365 nm.
The color of the eluted spots was inconsistent; they could be blue, yellow or green. The spectra obtained from the spots changed quickly when taken repetitively. All these observations imply that kynurenine is photo-unstable.

**Xanthurenic acid** has been described as a blue fluorescent tryptophan metabolite [14-16]. Xanthurenic acid eluted into two spots (Rf-values 0.93 and 0.98) with MP1. It must be noted that xanthurenic acid spots fluoresced yellow-white upon UV excitation instead of the reported blue fluorescence [14, 16]. The shape of the fluorescence spectra obtained from the spot with Rf-value 0.98 is similar to those of eluted spots B and C but the match is not conclusive (data not shown). Xanthurenic acid cannot be excluded as a fingermark component.

The combination of TLC and fluorescence spectroscopy has yielded new information on the nature of fluorescing compounds in fingermarks. With three MPs, a total of 77 single and 18 stacked fingermarks were developed yielding 155 eluted fluorescent spots. Sixteen (10%) of the eluted spots fluoresced brightly enough in order to obtain fluorescence spectra. These spots represent compounds that either fluoresce very brightly or are present in abundance. There was a large variation in the fluorescent properties of the studied fingermarks. Some donors consequently donated highly fluorescent fingermarks while others left no or invisible fingermarks. Other donors donated fingermarks of varying fluorescence intensity.

All of the TLC-reference compounds studied were fluorescent when excited with UVA and blue light, the concentrations used depended on the MP but did not exceed 10 mg ml\(^{-1}\). Riboflavin, tryptophan, phenylalanine and tyrosine were expected to be major contributors to the autofluorescence of fingermarks based on their abundance in the body. Riboflavin is highly fluorescent and only 0.001 mg ml\(^{-1}\) was necessary to obtain reference spectra measured directly from the TLC plate, yet none of the spectra obtained from the eluted fingermarks spots matched. In addition no spectroscopic match could be obtained for riboflavin photoproducts or the riboflavin containing FAD. Tryptophan could barely be observed on the plate at a concentration of 10 mg ml\(^{-1}\), phenylalanine and tyrosine displayed no visible fluorescence at this concentration. For these reasons we excluded riboflavin, free tryptophan, phenylalanine and tyrosine as major contributors to the autofluorescence of fingermarks. Yet the fingermark residues on developed plates displayed fluorescence spectra similar to that of BSA (Fig. 1) and reacted positively with ninhydrin. It is thus inferred that bound tryptophan is an important contributor to the fluorescence in the residue.

Kynurenine is a tryptophan metabolite and eluted into two spots in MP1, the main spot (Rf-value 0.56) fluoresced brightly but could not be matched spectroscopically to any of the eluted fingermark spots. The fluorescence spectra of the weaker spot (Rf-value 0.93) were consistent with the spectra of two eluted fingermark spots. It is inferred
that a kynurenine derivative is present in fingermarks. The spectrum of xanthurenic acid was similar but did not match as well with the spectra of eluted fingermark spots. Thiamin and carotene could not be excluded based on the obtained Rf-values, yet no spectroscopic confirmation could be obtained, indicating that they are no major contributors to the autofluorescence of fingermarks. The color of bilirubin changed from yellow to green when developed with MP2, none of the eluted fingermark spots did, indicating that bilirubin is not present in our sets of fingermarks. The red autofluorescence that can be observed in some fingermarks was inferred to be most likely pheophorbide A and/or pheophytin. In our studied fingermark set, one eluted red fluorescent spot was suspected to be the result of (proto)porphyrin.

4. CONCLUSION

The combination of TLC and fluorescence spectroscopy provided a strong indication that the chlorophyll decomposition product, pheophorbide A, is present in some fingermarks. This indicates that it may be possible to retrieve information about a person's diet based on the autofluorescence of their fingermark. Further research is needed to obtain information on the time span between dietary intake and the appearance in fingermarks. Bilirubin appeared not to be present in our set of fingermarks. The evidence for the presence of riboflavin, FAD, pyridoxal HCl and thiamin was inconclusive. This leads us to conclude that if they are present in fingermarks, they are not major contributors to intrinsic fingermark fluorescence. Tryptophan is hypothesized to be a major contributor to the autofluorescence only when part of peptides or proteins, id est, not in its free form. The fluorescence spectra and Rf-value of one of the eluted kynurenine spots were consistent with those of two eluted fingermark spots. We thus infer that a kynurenine derivative is responsible for some of the fingermark autofluorescence. The method used proved to be successful in identifying fluorescing compounds in fingermarks. Of ten percent of the eluted spots usable fluorescence spectra were obtained but the majority of these spectra remained unmatched. A different set of reference compounds needs to be established to identify more fluorescing compounds in fingermarks. Based on our results we suggest that tryptophan derivatives should form the next set of reference compounds to be studied. High quality TLC plates should increase the number of fluorescence spectra obtainable from eluted fingermarks.

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Dr. J.-W. Handgraaf is acknowledged for providing a matplotlib script to generate Fig. 2.

REFERENCES


