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Short communication

Short communications: Exploring temporal fluorescent changes in the composition of human semen stains

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ABSTRACT

Semen traces are considered important pieces of evidence in forensic investigations, especially those involving sexsual offenses. Recently, our research group developed a fluorescence-based technique to accurately determine the age of semen traces. However, the specific compounds resonsible for the fluoresescent behaviour of ageing semens remain unknown. As such, in this exploratory study, the aim is to identify the components associated with the fluorescent behavior of ageing semen traces. In this investigation semen stains and various biofluorophores commonly found in body fluids were left to aged for 0, 2, 4, 7, 14 and 21 days. Subsequently, thin-layer chromatography (TLC) and ultra-performance liquid chromatography (UPLC) mass spectrometry were performed to identify the biofluorophores present in semen. Several contributors to the autofluorescence could be identified in semen stain, these include tryptophan, kynurenine, kynurenic acid, and norharman. The study sheds light on the.

1. Introduction

Sexual assault crimes are a serious issue in society, and recent reports have highlighted their significant societal impact [1–3]. One critical aspect of sexual assault investigations is the time of deposition of biological traces. The time of deposition can be crucial in supporting or refuting a suspect's alibi. Although many techniques have shown potential to estimate the age of biological traces, none have resulted in a practical procedure yet [4–8]. Recently, our research group has made significant progress by developing a fluorescence-based technique for accurately estimating the age of semen traces using fluorescence spectroscopy [9,10]. To further optimize and develop the technique, knowledge about the compounds that are responsible for these fluorescent properties is needed. Therefore, the aim of this study is to explore the fluorescent changes in semen over time and to identify the components that may contribute to these changes. Understanding the kinetics and patterns of these changes is vital for the development of reliable forensic age determination analysis. Building on earlier studies that identified tryptophan as the primary contributor to autofluorescence in fresh fingermarks and its derivatives in aged fingermarks, our research was extended to semen stains [11,12]. Here, we explore the potential role of tryptophan and its derivatives in the fluorescent characteristics of semen stains. As such, these biofluorophores were included as reference standards for comparative analysis. Thin layer chromatography and ultra-performance liquid chromatography (UPLC) mass spectrometry were used to identify the fluorophores in semen stains.

2. Material and methods

Table 1 lists all the materials and instruments, and their supplier that have been used in this study.

2.1. Sample preparation

From the donors that participated in this study, informed consents

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Table 1
Materials and instruments used and their supplier.

Material	Supplier
Chloroform, Acetonitrile, L-Tryptophan, L-Kynurenine, 3-Indoleacetic acid, Xanthurenic acid, Norharman, Riboflavin, Flavin Adenine Dinucleotide, Kynurenic acid, Squalene, Albumin from human serum, Cholesterol, Arachidonic acid, Fructose, TLC Silica gel 60 aluminium sheets	Sigma Aldrich, Zwijndrecht, the Netherlands
Methanol	Merck KGaA, Darmstadt, Germany
Trans-B-Carotene	Janssen Chimica, Essex, UK
Crime-lite ® 2 torches: UV (365 nm, 10 % band width 350–380 nm) Plastic goggle: Clear (D-21000) Camera filter: Clear (GG420, 1 % nom 406 nm)	Foster and Freeman, Worcestershire, UK

were obtained. Research protocols were performed in accordance with the Declaration of Helsinki and relevant guidelines and regulations of the Netherlands Code of Conduct for Research Integrity and the research code of the Amsterdam University Medical Centers (UMC). Semen was collected from three volunteers and each sample was pipetted in duplicate. Semen samples were collected from the fertility clinic of the Amsterdam UMC and directly stored at −80 °C.

2.2. Preparation of TLC plates

The silica-coated TLC plates were first pre-washed with methanol to remove any impurities and then activated by heating at 120 °C for 30 min. Three distinct groups of TLC plates were prepared to investigate how fluorescence patterns changed over time and to identify which compounds contributed to the observed fluorescence in semen samples. For the first group, two μl of semen from each donor (n = 3) were pipetted on a TLC plate, with each sample being prepared in duplicate. Additionally, a mixture of squalene/HSA (20 %/5mg/ml) and squalene/tryptophan (20 %/5 mg/ml) were included. The other two groups of TLC plate consisted of well-known bio-fluorophores which could contribute to the fluorescence pattern of semen. As such, for the second group, two μl of eight reference compounds, including tryptophan, kynurenic acid, 3-indoleacetic, xanthurenic acid, norharman, riboflavin, FAD and tryptophan/cholesterol (80/20 %), were pipetted onto another TLC plate, with each sample being prepared in duplicate. The third TLC plate, also prepared in duplicate, contained the remaining eight reference compounds: fructose, kynurenine, cholesterol, arachidonic acid, HSA, beta-carotene, HSA/cholesterol (80/20 %) and squalene. All plates, were aged in a dark environment at room temperature for intervals of 0, 2, 4, 7, 14, and 21 days. The mixtures were included to simulate protein and lipid interactions and to explore the fluorescent patterns generated by these reference compounds. After ageing, the TLC plates were developed using a chloroform/methanol (30 ml/120 ml) mobile phase for 75 min at each specified time intervals. An overview of the reference compounds, used concentrations and excitation and emission wavelengths found in literature are listed in Table 2.

2.3. Visualization of the compounds

After the TLC plates were developed, the plates were left to dry. To detect and visualize the fluorescent spots, the plates were illuminated with an ultraviolet (UV) Crime-lite® 2 torch (365 nm and 10 % bandwidth 350–380) which was placed at an angle of 45 °C at 40 cm above the plates. Images of the plates were obtained with a Canon EOS 40D and a Canon Macro Lens EF 100 mm f/2.8 USM, which was placed above the plates at a height of 120 cm. The settings used on the camera were aperture f/5.0 and *iso*-200. In front of the camera a clear (GG420) filter was used, respectively, to detect the emitted fluorescence. The exposure time for UV was set to 10 and 15 s. The fluorescent spots were evaluated by determining the retention factor (Rf) of each spot.

Table 2
Overview of Referenced Compounds, Concentrations, and Excitation/Emission Wavelengths from literature.

Reference compounds	concentration (mg/ml)	Excitation/emission wavelengths (nm)
Tryptophan	5	295–305/350 [13]
Kynurenic acid	5	344/404 [14]
3-Indoleacetic acid	10	340/480 [12]
Xanthurenic acid	1	375/440 [12]
Norharman	1	300, 370/440 [12]
Riboflavin	0.001	270, 370, 450/525 [15]
Flavin Adenine Dinucleotide (FAD)	0.01	450/530 [16]
Kynurenine	10	365/480 [17]
Arachidonic acid	50	310–340, 366/420, 450, 470 [18]
Human serum albumin (HSA)	5	280/340 [19]
Beta-carotene	10	457/534 [20]
Squalene*	100 %	
Cholesterol*	5	
Fructose*	50	

* are not fluorescent, but are highly abundant in semen and were therefore included as a control.

2.4. The retention factor (Rf)

The Rf value was determined by dividing the distance traveled by the compound by the distance traveled by the mobile phase. ImageJ was used to measure the distance of these two points, providing a pixel count for each distance. To compare the Rf values, the following procedure was conducted: For each duplicate set of semen samples, the Rf values of semen stains at each time point were individually compared to the Rf values of each reference in the set. If the Rf values of the semen stain at a specific time point matched within the 0.05 threshold for at least one reference in the set, it was considered a potential candidate. This predetermined cutoff value of 0.05 was established by considering the minimum and maximum differences in Rf values between different references and within the duplicate references, respectively.

2.5. UPLC Mass spectrometry

Three μL of semen were pipetted onto a TLC plate for each time interval. A methanol/chloroform mobile phase was then used to separate the spots. For mass spectrometry analysis, the eluents were perforated at locations B, C/D, and D/E (see Fig. 1). For the analysis of tryptophan metabolites, ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) was applied. The spots were dissolved in 100 μL of 100 % methanol, vortexed, and centrifuged for 5 min at 1000*g*. The positive control consisted of semen pipetted directly onto a washed TLC plate. These samples were sent to our Core Facility Metabolomics lab. In the lab, the spots were dried under a stream of nitrogen and reconstituted in 100 μL of water, of which 50 μL was used for analysis. To this solution, 10 μL of internal standard (0.6 pmol tryptophan-d5) was added, followed by deproteinization with 500 μL of acetonitrile. The samples were then centrifuged, dried with nitrogen, and reconstituted in 100 μL acetonitrile. Finally, 10 μL of this solution was injected into the UPLC-MS/MS system, which utilized an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm) at 50 °C on an Acquity XEVO TQ-XS system (Waters, Milford, MA). The eluent gradient was as follows: 0.1 % heptafluorobutyric acid in purified water (A) and acetonitrile/water (4:1, B) with a flow rate of 0.4 mL/min over a 6-minute run. The gradient profile was: initial 100 % A, 1.00 min 100 % A, 3.00 min 60 % A/40 % B, 4.00 min 100 % B, 4.10 min 100 % A. The desolvation temperature was set to 550 °C with a capillary voltage of 3.00 kV. MRM (Multiple Reaction Monitoring) transitions were

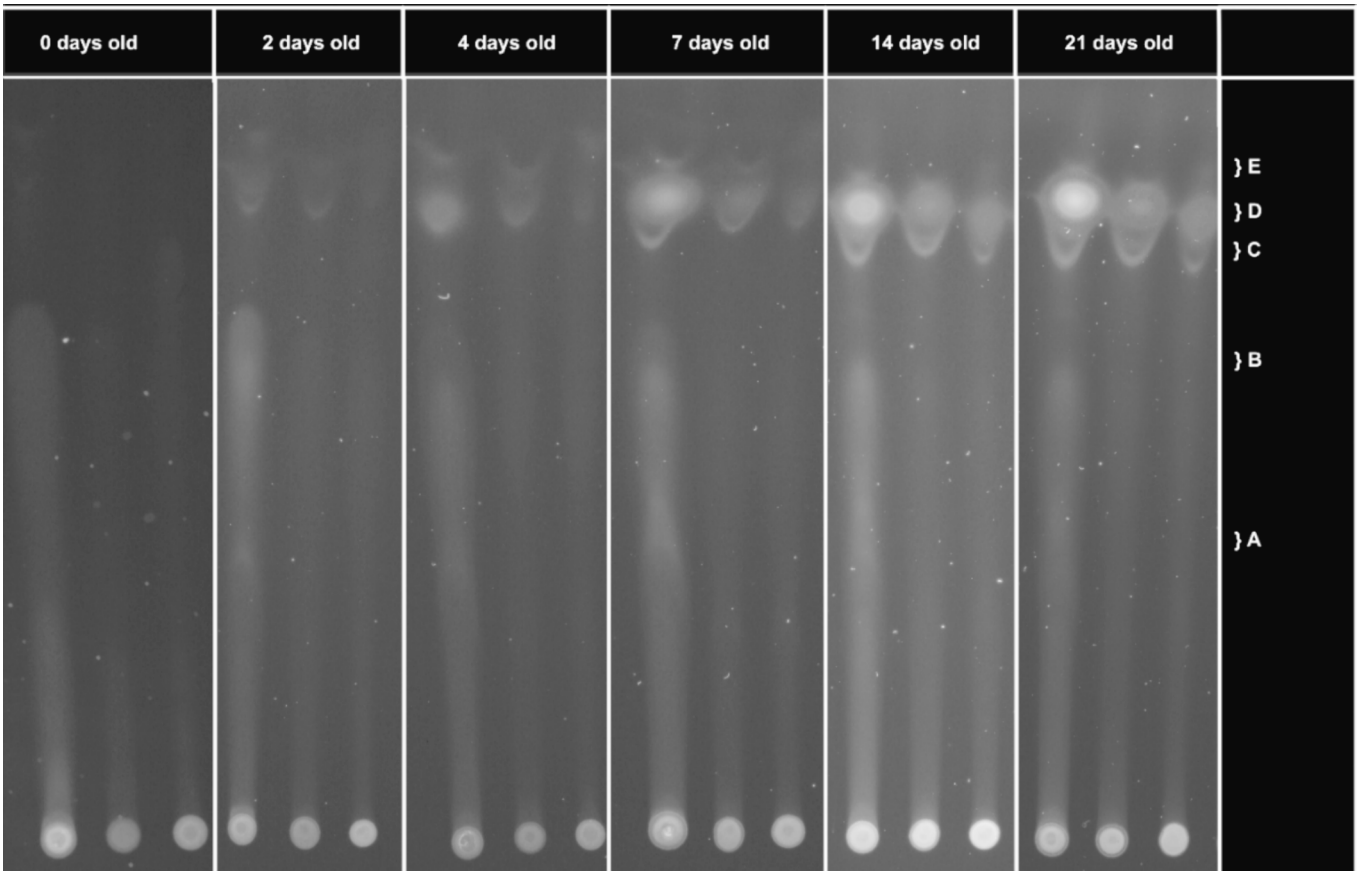

Fig. 1. Semen stains of three different donors were aged for 0, 2, 4, 7, 14, and 21 days on TLC plates in a dark environment. The plates were developed using chloroform/methanol (1:4) as mobile phase. Over time, both the clarity of the fluorescence pattern and the number of fluorescents spots that could be observed, increased. Spots were visualized using a UV Crime-lite®2 torch and a clear filter.

monitored for anthranilic acid (138 > 120), hydroxy-anthranilic acid (154 > 136), quinolinic acid (168 > 78), kynurenic acid (190 > 144), tryptophan (205 > 146), xanthurenic acid (206 > 132), kynurenine (209 > 192), and hydroxy-kynurenine (225 > 208), using tryptophan-d5 as the internal standard (210 > 193). A standard curve of the metabolites was used to calculate the abundance of the analytes using Quanlynx software (Waters, Milford, MA).

3. Results and discussion

3.1. Semen stains developed on TLC plate

A consistent pattern of fluorescent spots was observed after running the TLC plates with duplicate semen samples from three different donors that were aged for 0, 2, 4, 7, 14 and 21 days, see Fig. 1. The aged semen stains were visualized using the Crime-lite 2 torch, revealing distinct bright fluorescent spots labeled as A to E. Spot A and B exhibited faint visibility at each time point. In the fresh semen samples, characteristic spots C, D, and E were not visible, but they appeared at later time points. Specifically, spots C and E became clearly visible after 2 days, with faint visibility observed in one donor even at t0. Spot D could only be observed after 7 days. The variation in spot appearance may be attributed to inter-donor variability in the initial composition of semen samples or differences in sample processing times. Furthermore, differences in fluorescence intensity and the number of fluorescent spots were observed over time for each sample. Fresh semen stains exhibited weak fluorescent spots, whereas aged semen stains appeared brighter. After imaging the developed fluorescent spots under UV light, the retention factors of all semen spots were determined.

3.2. Matching fluorescence spots with the reference compounds based on Rf values

As expected, cholesterol and fructose did not show any fluorescence as they do not possess intrinsic fluorescent properties. The other eleven reference compounds (photographs of these TLC plates available upon request from the author), showed fluorescence spots that have similar Rf values as the fluorescence spots of semen stains. Fig. 2 provides an overview of the references that showed matching Rf values with semen samples over time. Tryptophan is considered one of the major contributors of the intrinsic fluorescence of body fluids and fingermarks [9–12,21]. The structure of tryptophan contains an indole ring, which can absorb UV light at a wavelength around 280 nm. The intensity and spectrum of tryptophan fluorescence can be influenced by its microenvironment [22]. Tryptophan showed matching Rf values with three out of six semen samples on day zero and six out of six semen samples on the other time intervals. Fresh tryptophan showed one weak visible fluorescent spot, characterized by a prolonged tail, which is in agreement with observations previously reported by van Dam et al. under conditions where tryptophan was exposed to office light for an hour [12]. Tryptophan derivatives including kynurenic acid, 3-indoleacetic acid and kynurenine were also expected to contribute to the fluorescence of semen stains. Kynurenic acid is derived from tryptophan metabolism and showed matching Rf values with four out of six semen samples on day zero and six out of six at all other days. The derivate 3-indoleacetic acid has two out of six matches of the Rf values on day zero, which increased to six after two days. Xanthurenic acid has been reported to contribute to the fluorescence of fresh fingermarks [12]. However, its role in the fluorescence of semen samples appears to be limited, especially on day zero and fourteen, where no matching Rf values were

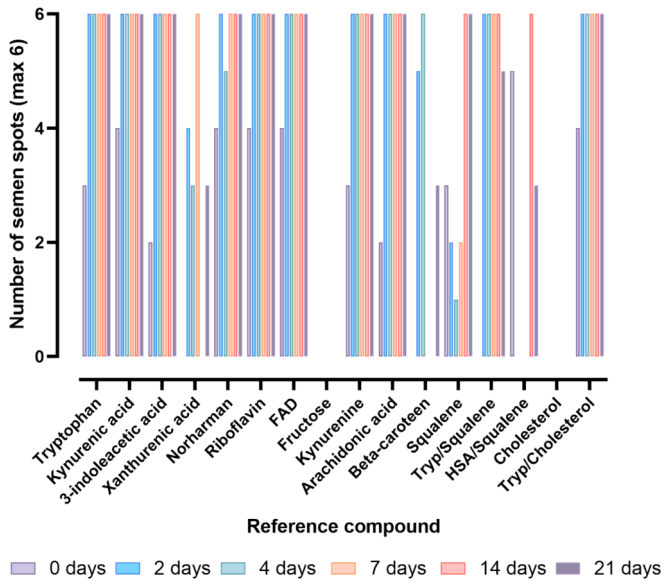

Fig. 2. Overview of the number of matching semen spots (including the duplicates) with the reference compounds based on the retention factor over the course of 21 days. The retention factors of the semen spots were compared with the retention factors of different reference compounds. When the differences in retention factor was smaller than 5 percent, the spots were identified as a match. The mixtures were included to simulate protein and lipid interactions and to investigate the fluorescent patterns produced by the reference compounds.

observed. Over the course of four and twenty-one days, a total of three matching Rf values were observed. The highest number of matches occurred on day seven, where six semen spots exhibited fluorescence consistent with xanthurenic acid. Norharman, also known as beta-carboline, is another fluorescent compound that can be formed from tryptophan through various biosynthetic processes. For fresh norharman, four out of six semen samples showed a matching Rf value. After two days, this increased to six matches, which remained at the same number over time, except on day four where the number of matches was reduced to five. Riboflavin, also known as vitamin B2, is present in semen and could be measured at each time interval in all six semen stains, except on day zero where only four semen stains showed matching Rf values. The derivative of riboflavin, FAD showed the exact same number of matches for each time interval. Kynurenine, a degradation product of tryptophan oxidation, showed matching Rf values with three out of six semen stains at day zero and then increased to six matches for all other time intervals. Arachidonic acid is an unsaturated lipid, that possesses a fluorophore structure. For fresh semen stains, two out of six semen stains showed similar Rf values as arachidonic acid. On all other time intervals, six of the semen stains showed matching Rf values. Another vitamin, which was compared to semen, is beta-carotene. After two days of ageing, five out of six semen samples showed matching retention factors with those of beta-carotene, while on the fourth day six semen samples had matching retention factors. At the other time intervals (zero, seven, fourteen, and twenty-one days) no fluorescence spots with similar retention factors as beta carotene were observed for the semen samples. Finally, squalene, an abundant polyunsaturated lipid, showed a decrease in the number of matches in the initial four days (from 3 to 1 matches). However, the remaining period showed an increase, resulting in a total of six matches after fourteen days. For fingermarks, studies have reported that squalene undergoes degradation or alteration as the fingermarks age [23–26]. Compounds such as squalene, wax esters and fatty acids are known to be susceptible to oxidation processes [6,27–29]. The observed increase in fluorescence after four days could be attributed to an elevation in carbonyl-containing oxidation products, as squalene can undergo rapid oxidation, resulting in products containing a carbonyl group [30]. Furthermore, the formation of different emissive structures over time, possible due to increased rigidity of molecules, could also account for the observed increase in fluorescence [30].

3.3. Mass spectrometry

Molecular and proteomic analyses have been used to study the composition of semen [31,32]. However, there have been no reported studies identifying the source of fluorescence in aged semen stains. In this study tryptophan and its metabolites were investigated using UPLC-MS. As such, after TLC, the spotted semen sample and its eluents B, C/D and D/E were analyzed with UPLC-MS. Eluent A was excluded from further analysis as it showed very weak fluorescence, see Fig. 1. As a positive control, a semen sample in its original state was included to ensure that sufficient sample was available for analysis. Tryptophan in fresh and old samples could be measured in all of the eluents. Therefore, it can be concluded that tryptophan is a contributor to the fluorescence spectra of semen samples. Furthermore, the presence of norharman could be confirmed in the eluents of semen stains. Over time, the levels of norharman tended to increase, however since this observation was based on three samples, more data is needed to draw any definite conclusions. Kynurenine could be measured in the eluents B, C/D and D/E, and showed the highest abundance in eluent B. Low signals of anthranilic acid, kynurenic acid and quinolinic acid could be detected in some of the eluents. The mass spectrometry analysis did not detect OH-kynurenine and xanthurenic acid in the samples. This suggests that while xanthurenic acid might show fluorescence on TLC plates, it could be present in concentrations below the detection limits of our MS/MS method, or the fluorescent spots observed might be due to other compounds with similar Rf values. Similarly, OH-kynurenine could not be detected and is therefore excluded as a main contributor to the fluorescence behavior of semen stains. The kynurenine pathway involves multiple oxidation reactions during the metabolic conversion of tryptophan, resulting in various metabolites, including OH-kynurenine and, further along the pathway, xanthurenic acid and quinolinic acid. The presence of quinolinic acid but not OH-kynurenine or xanthurenic acid in the mass spectrometry results could be attributed to factors such as methodological limitations, the stability of the metabolites, the sensitivity of the analytical method, or variability in the sample. Furthermore, we observed that each spot contains traces of different molecules. This could be explained by incomplete separation, which causes co-elution of multiple compounds that then fragment into overlapping residues in the mass spectra. While not optimal, it is a first step towards identifying the source of fluorescence in semen. For future research we would recommend to increase the concentration of the samples to apply mass spectrometry directly on to the plate, by coupling MALDI-TOF MS to TLC. Fuchs et al. performed such a combination method for the analysis of phospholipids, which helps prevent protein loss during the elution process while maintaining comparable resolution and sensitivity to spectra obtained using conventional methods [33]. Moreover, considering the difficulties involved in analyzing seminal fluid, an alternative approach could involve utilizing a model system that represents protein-lipid reaction in semen [25]. This approach allows for the examination of the fluorescence patterns exhibited by individual semen residue components and the potential investigation of chemical modifications within these specific components. With regard to the TLC, we recommend enhancing the resolution between compounds with similar polarities by adjusting the solvent system. In the current research, a mobile phase consisting of chloroform/methanol (1:4) was used, representing a relatively polar solvent system. The methanol will disrupt the bonds between protein and lipids and make the lipids more accessible for extraction by chloroform [34]. We would recommend to add an aqueous phase to the solvent system to solubilize the proteins and amino acids [34]. Furthermore, the consideration of using a longer TLC plate and extending running times has the potential to enhance

resolution. These adjustments allow each compound more time to interact with the mobile phase, particularly beneficial when dealing with compounds of similar polarities or those that exhibit slower migration, as observed in our research. In addition to these improvements, future experiments should include fluorescence spectroscopy to quantify the fluorescence signal. This approach would not only enhance the accuracy of spot detection and assignment but also provide a quantitative measure of fluorescence intensity, offering a more robust analysis than visual inspection alone. By combining improved TLC resolution with precise fluorescence quantification, the overall accuracy and reliability of detecting and identifying fluorescent compounds in semen stains will be further enhanced.

4. Conclusion

The method successfully identified tryptophan and its derivatives as fluorescing compounds in semen. However, optimization of our method is necessary to unravel the ageing process of dried semen samples.

CRediT authorship contribution statement

Nihad Achetib: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Susanne Danser:** Methodology. **Kirsa Min:** Investigation. **Zehra Köksal:** Writing – review & editing, Conceptualization. **Maurice C.G. Aalders:** Writing – review & editing, Supervision, Project administration. **Annemieke van Dam:** Writing – review & editing, Supervision, Project administration, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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