Highly Sensitive and Selective Water-Soluble Fluorescent Probe for the Detection of Formaldehyde in Leather Products†

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Abstract

Excess formaldehyde (**FA**) provides a significant threat that causes irreversible damage to both the environment and human health, and long-term **FA** emission from commercial leather products is a significant problem. Therefore, to help control the **FA** content, we propose a fluorescence-based method to detect **FA** selectively using a reaction-based probe **FAP-1**. Water-soluble **FAP-1** was simply synthesized through two steps and the optical properties of **FAP-1** towards species relevant to leather making were evaluated. Then a number of genuine leather samples were analyzed to reveal the **FA** content. Compared with the international standards, the fluorescence method was competitive to the 2,4-dinitrophenylhydrazine (DNPH) reaction-based HPLC method, and superior to the acetylacetone (ACAC) reaction-based UV-Vis method. As a result of its reliability,

low-cost, simple synthesis, ease of operation with reduced measurement time compared with standard methods, our method exhibits great potential applications for the analysis of **FA** found in other industrial products.

Keywords: Formaldehyde, fluorescent probe, derivatization methods, leather industry

1. Introduction

Formaldehyde (**FA**) is a toxic chemical involved in aldehyde-tanned leather and the release from its crosslinking collagen fibers or surface finishing agent is inevitable, during production, storage, and use of the finished products in households [1,2]. In recent years, great strides have been made by our group to reduce excess **FA** emissions from leather, since it can cause severe irritation or health risks by irreversibly destroying proteins and DNA in organisms [3–7]. Owing to the carcinogenic properties as well as possible leukemogenicity, **FA** has been officially classified as a carcinogen by the International Agency for Research on Cancer (IARC) and United States Environmental Protection Agency (EPA)[8]. For these reasons, the Chinese Leather Industry Committee Organization has adopted similar environmental requirements to those from the EU, USA and Japan in order to restrict and control the amount of **FA** used in the leather industry. For example, limits of 20 mg/kg for baby products, 75 mg/kg and 300 mg/kg for products with and without direct skin contact, respectively have been regulated[9]. Hence, it is important to develop new approaches to precisely detect free **FA** in leather and help control this toxic chemical, in order to meet ever more stringent regulatory requirements.

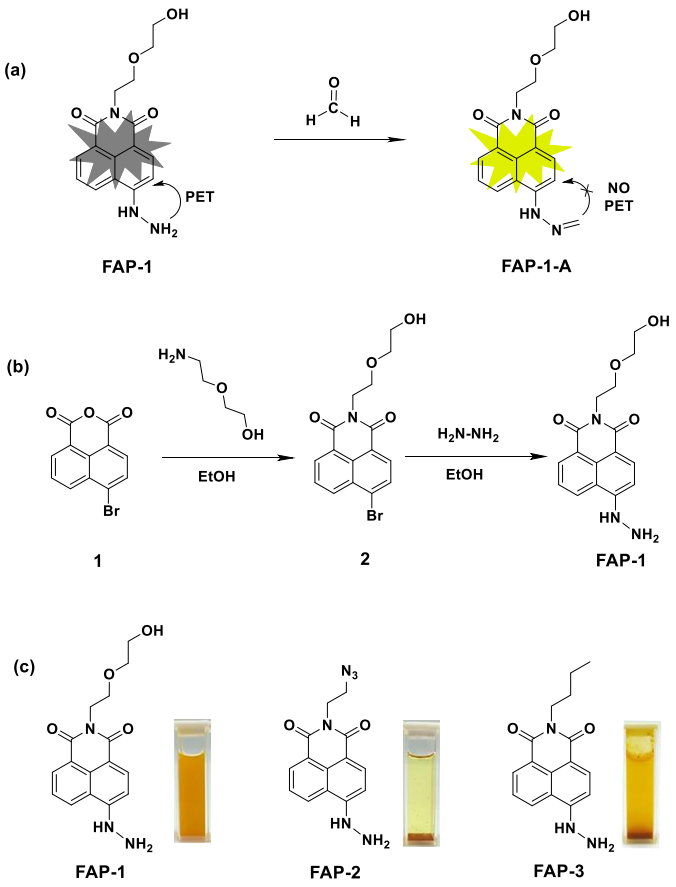
Over recent years, methods developed for **FA** detection include in situ measurements and derivatization methods, etc[10]. Up to now, chemical derivatization methods are still the

most widely used for **FA** detection in leather and related materials[11–13], as well as for food, cosmetic, drug and water analysis [14–18]. For example, the extracted **FA** from leather products reacts with 2,4-dinitrophenylhydrazine (DNPH) or acetylacetone (ACAC) to form derivatives, which are then detected by high performance liquid chromatography (HPLC) or a UV-Vis spectrophotometer. However, the time-consuming procedures and the lack of detection performance remain. Specifically, the HPLC method using DNPH has the requirement of pre-acid treatment, solvent-dependence and uses expensive equipment. As for the colorimetric method using ACAC, pre-heating of samples is required (Hantzsch reaction) [19], and results in low selectivity for **FA** [13,15]. Therefore, a simple, low cost, sensitive and selective analytical method to accurately measure the amount of **FA** in leather or other extended merchandise is required.

Reactivity-based fluorescent probes are valuable tools to actively recognize and detect important species in biology and the environment, owing to their high sensitivity, selectivity and ease of operation[20–27]. As the simplest reactive carbonyl species, HCHO has been detected and imaged in living cells using reactivity-based fluorescent probes [28,29]. Due to the significant role of **FA** in biological systems any additional probes have been developed[30–35]. Furthermore, probes have been developed to detect **FA** in real samples such as food and in the air. For example, Ding et al.[36] distinguished the fish, shrimp, octopus and chicken in normal and contaminated states using a **FA** probe and Gu et al.[37] observed the color and fluorescence changes under sunlight and 365 nm UV light, caused by **FA** vapors and probe (loaded ono a TLC plate) [38–42]. These preliminary studies indicate the broad and potential applications in monitoring **FA** in other extended

commercial or industrial materials. Herein, we aimed to develop a fluorescence method to detect **FA** in leather from sheep.

Previous probes for **FA** have been generally classified based on aza-cope rearrangement, formation of formimine or methylenehydrazine, respectively[43–45]. The mechanism of hydrazine (-NH-NH_2) on naphthalimide scaffold of probe is one of the priority candidates. With the basic advantages mentioned previously, we developed a new probe **FAP-1** to detect **FA** in leather extracted from sheep, based on photo-induced electron transfer (PET). As shown in Scheme 1, this probe was synthesized simply in ethanol in tow steps and the structure contains a hydrophilic moiety for improving water solubility. The optical properties associated with detection sensitivity, pH stability and selectivity were then investigated. After exploring the reliable detection of **FA** for leather applications, we compared our new approach with traditional methods of DNPH and ACAC derivatization.



Scheme 1 (a) Mechanism for the fluorescence-based probe **FAP-1** towards **FA**; (b) Synthetic procedures for **FAP-1**; (c) Solubility of **FAPs** (20 mM) in water, presented as **FAP-1**, **FAP-2**, and **FAP-3**.

2. Experimental section

2.1. Chemicals and instruments

All chemical reagents and materials were purchased from commercial sources and used according to standard procedures. Aldehyde-tanned leather product (hairy skin) of sheep provided by Prosper Skins & Leather Enterprise Co. Ltd. (Henan, China). PBS (pH 7.4, 10 mM) solution was made manually using ultra-pure water. NMR solvents ($CDCl_3$, DMSO-d_6) were purchased from Cambridge Isotope Laboratories, *etc*. The synthesis reactions were monitored by TCL. NMR spectra were acquired on a JNM-ECS 400M spectrometer, using TMS as the internal standard. UV-Vis absorption spectra were observed using a Cary 5000

UV spectrophotometer from Agilent Technology. Fluorescence emission spectra changes were obtained using an Edinburgh Instruments FS5 fluorescence spectrometer. For all the spectra, the excitation/emission wavelength were 440/553 nm, and the slit widths were set as 1.5/1.2 nm. HPLC purification of DNPH derivatives were performed on a Breeze HPLC system equipped with Waters 1525 Binary HPLC pump, 2489 UV-Vis detector and Symmetry C18 column (4.6 × 150 mm) with 5.0 μm packing material. The mobile phase was water-acetonitrile (60:40, v/v) and the detection wavelength was 350 nm. The injection volume was 20 μL and the flow rate was set at 1.0 mL/min. UV absorbance of ACAC derivatives were performed on a LabTech UV BlueStar A and the absorbance measured at 412 nm.

Synthesis of probe FAP-1. The probe was synthesized according to the method shown in Scheme 1(b). 2-(2-Aminoethoxy)-ethanol (0.454 mL, 4.56 mmol) was added to 4-bromo-1,8-naphthalic anhydride (1 g, 3.61 mmol) in ethanol (20 mL) and heated under reflux for 3 h. After cooling to room temperature, the resulting mixture was poured into water, filtered and washed with water and cold-ethanol to generate yellow solid **1a** (1.06 g, 80.64 %)[46]. Then a mixture of compound **1a** (182 mg, 0.5 mmol) and 1.6 mL of hydrazine hydrate (80 %) in ethanol was heated for 4 h at 70 ℃. After cooling to room temperature the ethanol was removed by rotary evaporation, and the crude product was purified by flash chromatography on silica gel (DCM: MeOH = 20: 1) to afford **FAP-1** as an orange solid (129.5 mg, 82 %). ^{1}H NMR (400 MHz, DMSO-d_6) δ: 9.25 – 9.07 (m, 1H), 8.61 (d, J = 8.0 Hz, 1H), 8.42 (t, J_1 = J_2 = 6.5 Hz, 1H), 8.28 (dd, J = 8.6, 2.0 Hz, 1H), 7.63 (dd, J = 9.2, 5.9 Hz, 1H), 7.24 (d, J = 8.6 Hz, 1H), 5.73 (s, 1H), 4.69 (d, J = 5.8 Hz, 2H),

4.59 (d, *J* = 5.4 Hz, 1H), 4.20 (t, *J* = 6.6 Hz, 1H), 3.61 (t, *J* = 6.6 Hz, 2H), 3.45 (d, *J* = 2.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 164.28, 163.34, 153.68, 134.71, 131.06, 129.78, 128.76, 124.53, 122.07, 118.87, 107.68, 104.46, 72.55, 67.59, 60.67, 38.80. HR-MS calculated for $C_{16}H_{17}N_3O_4$ $[M+Na]^+$ m/z 338.11113, found 338.11117. Note: the synthetic procedures and spectra for **FAP-2** and **FAP-3** are described in the supplementary information.

2.2. Spectroscopic analysis

Probe **FAP-1** (6.31 mg, 0.02 mmol) was dissolved in water or ethanol (2mL) and 200 μL pipetted into water (19.8 mL) in order to prepare a stock solution (100 μM). The optical test solution using **FAP-1** (5 μM) or different concentrations of **FAP-1** for the real leather samples using different concentrationswer e prepared by diluting the stock solution. **FA** solutions of different concentrations (1~400 μM) were prepared from 10 mM stock solution. For the selectivity experiment, the probe **FAP-1** (5 μM) was incubated with relevant leather making chemicals and protein species from sheep skin (aldehydes, salt, metal ions, anions, surfactant, amino acids, polypeptides of collagen, lanolin, fatliquor, retanning filler) of 50 μM for 90 mins before recording the spectra. All the solutions detected by fluorescence were prepared in PBS (pH 7.4, 10 mM).

*2.3. **FA** detection in leather products*

FA extracted from leather products. For extracting **FA** from leather or fur, a (2 ± 0.1) g sample and 50 mL sodium lauryl sulfonate solution (0.1 wt%, 40 ℃) were added to 100 mL glass bottles and capped. The bottles were then shaken smoothly in a (40 ± 1) ℃ water bath for (60 ± 2) mins, the warm extract was then immediately filtered using a polyamide

membrane (0.22 μm) and cooled to room temperature (18 ℃ to 26 ℃)[47–49]. (Extra information can be found in Fig. S12 of the supporting information.)

FA standard solution. To a 200-mL volumetric flask was added 1 mL of 37 wt% **FA** solution and diluted to the mark with water. This stock solution was labeled as 1 mg/mL. To a 50-mL volumetric flask was pipetted 50 μL of **FA** stock solution into pre-filled 10 mL glass bottles and pipetted to the mark with water. This standard solution was labeled as 2.016 μg/mL. To seven 10-mL glass bottles, different volumes of standard solution were pipetted to generate calibration curves for the three derivatization methods in the supporting information.

Fluorescence method using FAP-1. A mixture of leather extract (5 mL) and **FAP-1** from the stock solution was added to a glass bottle for incubation. The blank solutions were prepared using the same procedures, including both extract (5 mL) and **FAP-1** in water (5 mL), for subtracting the fluorescence background from the extract and probe in aqueous solution.

HPLC method using DNPH. A mixture containing leather extract (5 mL), acetonitrile (4 mL) and 0.5 mL DNPH solution (0.2 wt% in phosphoric acid solution) were added into 10 mL glass bottles and pipetted to the mark with water, shaken evenly and left for (60~180) mins and filtered using a polyamide membrane (0.22 μm).

Colorimetric method using ACAC. To a 200 mL volumetric flask was added ammonium acetate (30 g), which was dissolved in water, followed by the addition of acetic acid (0.6 mL) and ACAC (0.4 mL), and then diluted to the mark and stored in dark. To acquire the blank solution, to a 200 mL volumetric flask was added ammonium acetate (30

g), which was dissolved in water, followed by the addition of acetic acid (0.6 mL), which was then diluted to the mark with water and placed in dark. Note: the ACAC solution was stored for 12 h before use and used within one week.

A mixture of the leather extract (5 mL) and ACAC solution (5 mL) was added to a 25 mL flask, and the capped mixture was shaken gently in a (40 ± 1) ℃ water bath for (30 ± 1) mins and cooled to room temperature at least 30 mins in the dark. To avoid the background interferences, a mixture of blank solution (5 mL) and extract (5 mL) was treated in the same way to provide a baseline correction for the absorbance measurements.

3. Results and Discussion

Construction of fluorescent probe FAP-1. Herein, a PET mechanism was used to design three facile sensors, FAPs, which all contain a hydrazine (-NH-NH_2) group for **FA** detection, as shown in Scheme 1(a). These probes, contain a hydrazine unit on the 1,8-naphthalimide core, and PET results in fluorescence quenching. By following the reaction between **FA** and hydrazine, the PET was suppressed and the fluorescence was enhanced, thus a turn-on signal could be detected[50]. Moreover, several groups were evaluated for water solubility Scheme 1(c), the result indicated that 2-(2-aminoethoxy)-ethanol endows good solubility to the probe, over systems with an azide group or simple alkyl chain. The solubility enhancement can be attributed to the oxygen atoms of the ether and hydroxyl, both of which form hydrogen bonds with H_2O in aqueous solution. As such probe **FAP-1** can be used to detect **FA** in aqueous solution.

Optical responses of FAP-1 towards FA. We firstly investigated the optical properties of **FAP-1** by observing the UV-Vis absorption and fluorescence intensity. As shown in Fig.

1(a), a solution of **FAP-1** (5 μM) in PBS buffer (pH 7.4) displayed a maximum absorption (A_{max} = 0.030, ε = 6.0 × 10^3 M^{-1} cm^{-1}) at 440 nm. Upon addition of 100 μM **FA** for 3 mins, an enhanced absorption was observed (A_{max} = 0.042, ε = 8.4 × 10^3 M^{-1} cm^{-1}). For the fluorescence of **FAP-1**, prior to treatment with **FA**, probe **FAP-1** (5 μM) exhibited weak fluorescence at 553 nm, due to photo-induced electron transfer from hydrazine to the naphthalimide fluorophore. After addition of **FA**, the intensity of fluorescence centered at 553 nm gradually enhanced and reached a maximum 100-fold increase within 90 mins (Fig.1b), indicating that hydrazine has reacted with **FA** to form a hydrazone product **FAP-1-A**, thus the fluorescence was recovered from the naphthalimide fluorophore. Meanwhile, the fluorescence changes from dark to bright yellow can be easily observed under 365 nm ultraviolet irradiation, inset of Fig. 1(b).

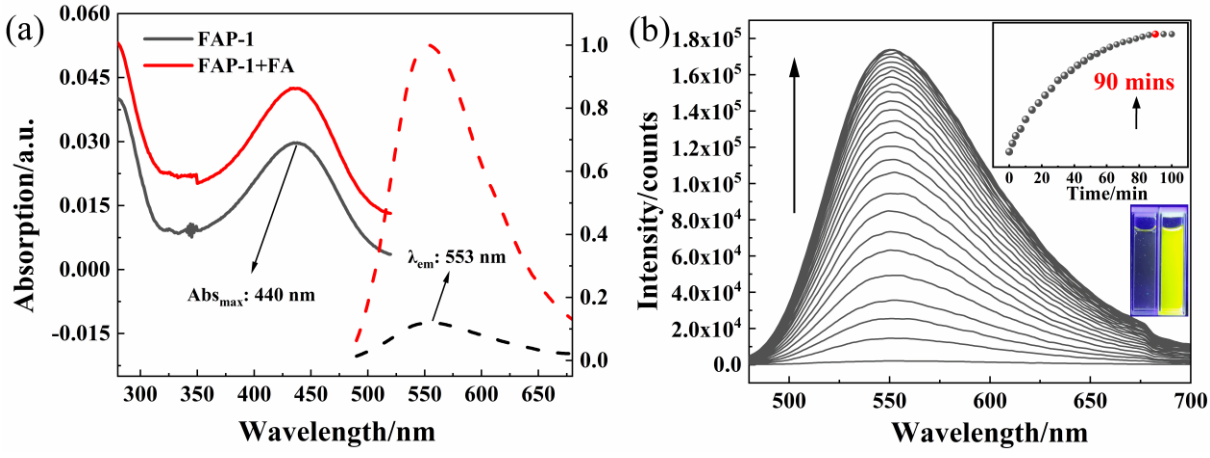


Fig. 1 (a) UV-Vis absorption (solid line) and normalized fluorescence spectra (dashed line) for **FAP-1** (5 μM) in PBS (pH 7.4) before (black) and after (red) treatment with 100 μM **FA** after 3 mins; (b) Reaction profiles of 5 μM **FAP-1** after the addition of 100 μM **FA** in 100 mins, λ_{ex} = 440 nm. Inset: the continuous time record of fluorescence intensities at 553 nm and photograph of **FAP-1** in the absence and presence of **FA** under 365 nm ultraviolet irradiation.

To assess the changes using fluorescence, dose-dependent titrations by increasing levels of **FA** (0~400 μM) were recorded and shown in Fig. 2(a). The fluorescence intensity at 553 nm for 5 μM of probe **FAP-1** reached a maximum at about 200 μM **FA**, and it was linearly (R^2 = 0.9921) related to **FA** concentrations over a range from 0~80 μM (inset of Figure 2b), equating to a sensitivity of 0~60 mg/kg for **FA** from leather samples. The limit of detection (LOD) was calculated as 0.76 μM according to the equation (LOD = 3δ/k, shown in the ESI), which is sensitive enough to detect **FA** from leather leather extracts.

The effects of pH on the UV-Vis absorbance and fluorescence response of **FAP-1** in the absence or presence of **FA** were also investigated. As shown in Fig. S10, in the absence of **FA**, the fluorescence intensity of the free probe displayed only a very minor variation in different buffer solutions with pH ranging from 4.0 to 9.0, indicating that the changes of pH have minimal effect on the free probe. When treated with **FA**, the obvious fluorescent signal change was noted in the pH range of 4.0~8.0, so did the measurement of UV-Vis absorbance, suggesting that **FA** can be detected by the probe in weak acid and neutral water.

Next, to determine the selectivity, **FAP-1** was treated with relevant species, including aldehydes, salt, metal ions, anions, surfactants, amino acids, polypeptides of collagen, lanolin, fatliquor, retanning agent and HCHO in PBS (pH 7.4), respectively. All these species can be involved in the leather making process or biomolecules, which can be directly extracted from the skin tissue matrix. As shown in Fig. 2(c) and Fig. S11, only **FA** could elicit an enhancement in emission intensity, while the other analytes hardly affected **FAP-1**, except for a slight response from retanning agent that possibly contains HCHO.

Moreover, the fluorescence intensities were recorded after **FA** addition in competitive analytes and shown in Fig. 2(d). These results indicate that the fluorescent probe **FAP-1** displayed high selectivity for **FA** over other potential interferences in a complex mixture.

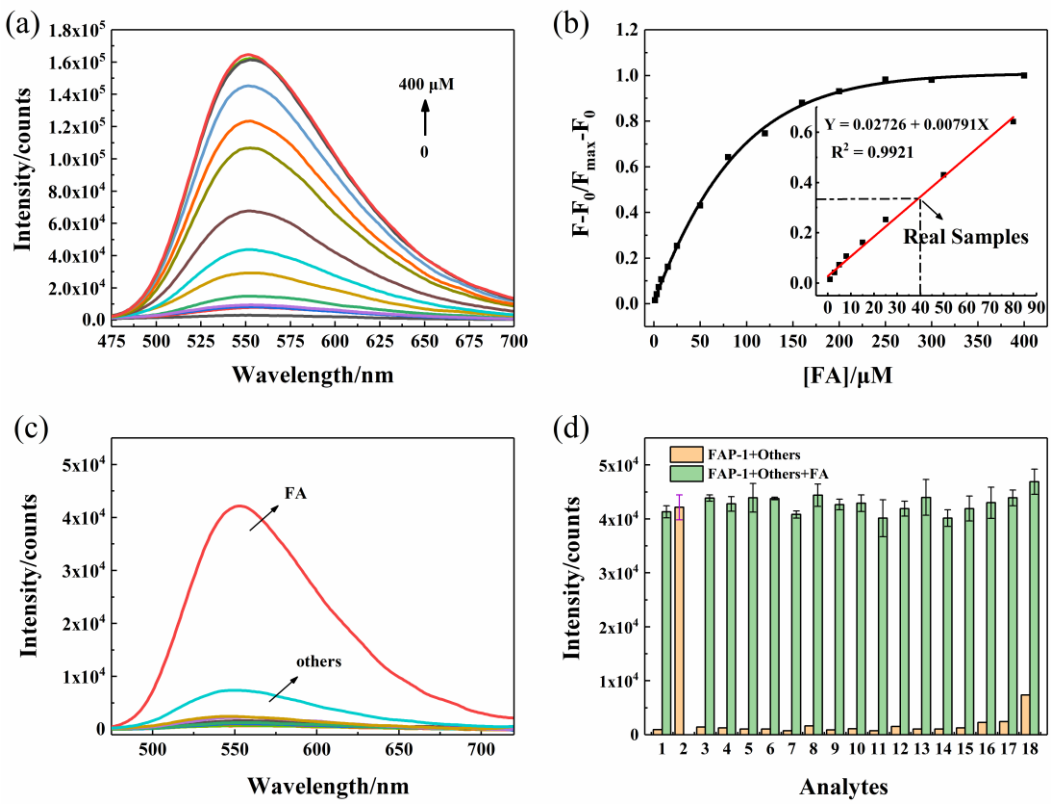


Fig. 2 (a) The fluorescence response of the probe **FAP-1** (5 μM) to **FA** at different concentrations (0~400 μM) in PBS (pH 7.4). λ_{ex} = 440 nm; (b) The plot of fluorescence intensity ($F-F_0/F_{max}-F_0$) at 553 nm as a function of FA concentration (0~400 μM) and inset (0~80 μM); (c) Fluorescence response of FAP-1 (5 μM) incubated with relevant analytes (50 μM); (d) Fluorescence intensities at 553 nm (orange), with the addition of 50 μM FA into the solution (Green). 1: blank, 2: FA, 3: acetaldehyde, 4: glutaraldehyde, 5: NaCl, 6: $Cr(NO_3)_3$, 7: $MgSO_4$, 8: $Ca(ClO)_2$, 9: NH_4Cl, 10: sodium lauryl sulfonate, 11: L-cysteine, 12: glycine, 13: alanine, 14: L-glutamic acid, 15: polypeptides, 16: lanolin, 17: fatliquor, 18: retanning agent, λ_{ex} = 440 nm.

Detections of FA in leather products. Encouraged by the response to **FA** of probe **FAP-1** described above, the fluorescence method was then evaluated for the determination

of **FA** from a real sample, which was provided from a company in leather manufacturing, inset of Fig. 3(b). The extracted solution was obtained using protocols from the International Standard Organization (ISO) drafted with International Union of Leather Technologists and Chemists Societies (IULTCS) or Chinese Standards (GB/T) to obtain the **FA** from leather[47–49]. Then, the response of probe **FAP-1** to **FA** extracted in water was investigated. As shown in Fig. 3(a, b), a turn-on signal of fluorescence appeared as soon as probe **FAP-1** (5 μM) was incubated with the extracted **FA** solution (5 mL), and the intensity enhancement reached a plateau within 60 mins. This observation demonstrated that the solution contained a certain amount of **FA**, which could be determined by the probe **FAP-1**. In addition, by changing the amount of the **FAP-1**, differences of fluorescence intensity enhancement were observed. We prepared a range of **FAP-1** concentrations (0.2~20 μM) to incubate with the **FA** extractions. As shown in Fig. 3(c), before and after the incubation, the fluorescence intensities increased rapidly as **FAP-1** increases from 0.2~8.0 μM. However, over 8.0 μM, the values tended to a plateau with a constant difference (inset), which indicates that an appropriate concentration of **FAP-1** is required for accurate **FA** detection.

Based on the desirable pre-test and optimization of probe **FAP-1**, we then measured the extracted samples from three different pieces (P_1, P_2 and P_3) of a certain leather and a blank solution, with three replicates on each of the samples, as shown in Fig. S12. The average fluorescence intensity for three extracts were substituted into the linear equation inset of Fig. 2(b), to calculate the corresponding concentrations of **FA**. Using this method, the resulting average **FA** content was 29.6 mg/kg in leather after unit conversion from liquid to solid, according to the original sample weight (2 g).

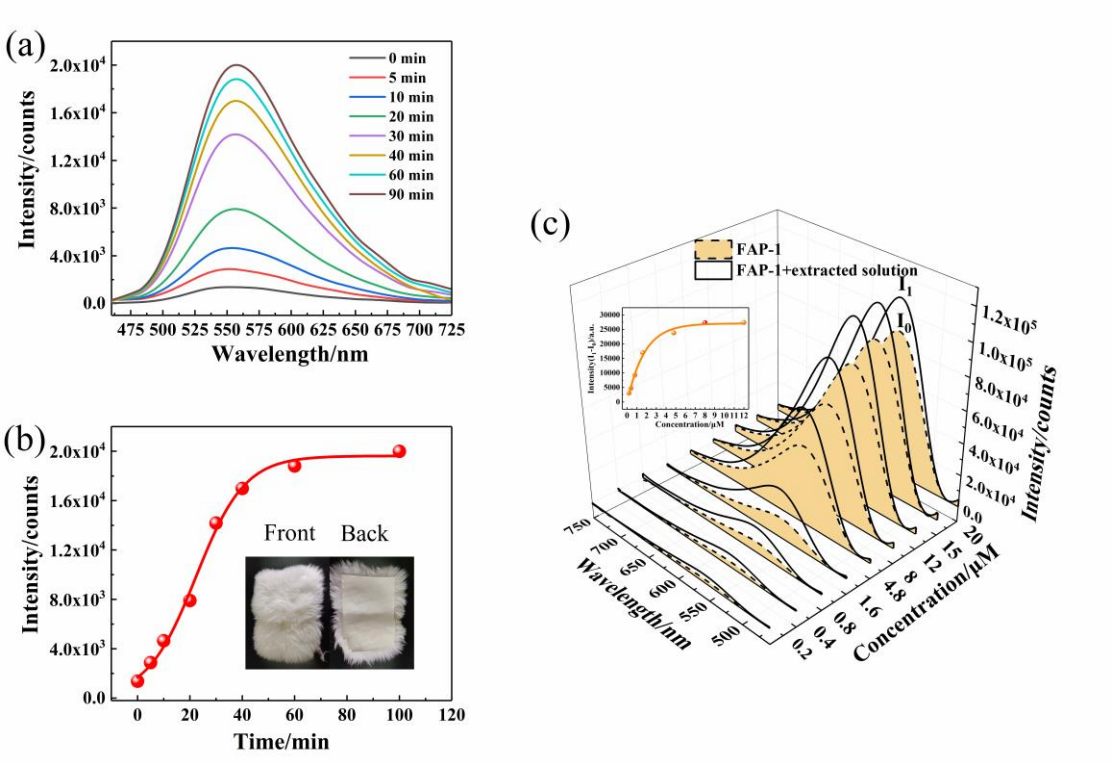


Fig. 3 (a) Reaction profiles of 5 μM **FAP-1** to the **FA** extract. λ_{ex} = 440 nm; (b) The corresponding plot of fluorescence intensity at 553 nm as a function of time; (c) Fluorescence changes before and after **FAP-1** (0.2~20 μM) incubation with **FA** extract (5 mL) after 60 mins. Inset: fluorescence intensity differences at 553 nm versus probe concentrations.

Analytical comparisons using standard methods. For leather products, the standard methods for **FA** content determination are provided by ISO 17226-1-2018, ISO 17226-2-2018 and GB/T 19941 1-2-2019 [47–49]. Which are methods using high-performance liquid chromatography (HPLC) and colorimetric analysis, with 2,4-dinitrophenylhydrazine (DNPH) and acetylacetone (ACAC) as chemical derivatizing agents, respectively. Herein, we used these two methods on 3 pieces (P_1, P_2 and P_3) of leather, which were also used for fluorescence detection as above.

Firstly, the DNPH-derivative of 3 samples were analyzed using HPLC and compared with the retention time of standard **FA** for qualification, as shown in Fig. 4(a). The standard

FA-DNPH-derivative peak was confirmed as peak 2, which had a retention time at 3.9 mins. The area of peaks 2 were plotted to a good linear correlation (R^2 = 0.9912), inset of Fig. 4(a). By using this calibration curve, the average **FA** content from the extracts was 0.67 μg/mL or 33.7 mg/kg of leather after unit conversion, which was nearly equal to the result of 29.6 mg/kg by fluorescence detection. However, the detection value was 2.79 μg/mL or 134.9 mg/kg (over 4-fold higher than HPLC) using the ACAC method using a UV-Vis spectrometer and the same samples, as shown in Fig. 4(b). The result indicates that the DNPH derivatization approach from the standard methods is highly sensitive and selective for the **FA** content detection in leather, while the ACAC derivatization method still exhibits problems in selectivity, due to the interferences with aldehyde analogues in aldehyde-tanned leather [13,15].

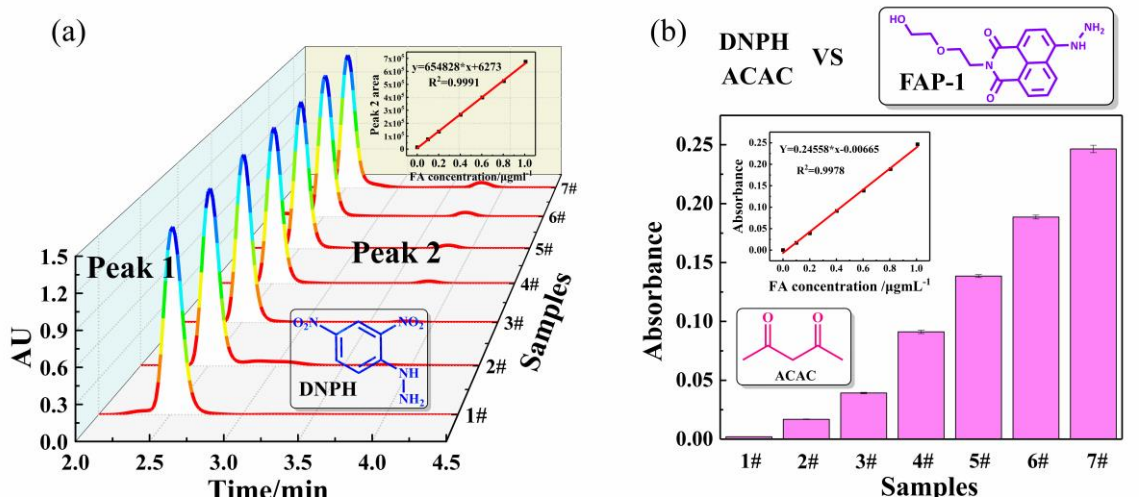


Fig. 4 (a) HPLC: chromatograms of standard solutions of **FA** (7 samples, 0-1.0 μg/mL) reacted with DNPH (Peak 1) to form derivative (Peak 2), plotted in the linear regression (R^2 = 0.9991) of inset; (b) UV-Vis: absorbance changes of standard solutions of **FA** (7 samples, 0-1.0 μg/mL) after reacting with ACAC, plotted in the linear regression (R^2 = 0.9978) of inset.

Therefore, the ACAC method may not be suitable as a reliable method for evaluating a products quality. For example, the **FA** in leather has a safe limit (75 mg/kg, in many countries), the detection results using **FAP-1** and DNPH were both in the safe zone, while that ACAC generated a value almost 2-fold above the safe limit (Fig. 5). Thus, the reactivity-based fluorescent probe **FAP-1** was capable of detecting **FA** with high selectivity in leather, and could provide an alternative method to the ACAC method. The raw data obtained using these methods are collated in Table S1. The calculations for LOD and recovery for **FAP-1**, DNPH and ACAC are shown in the table of Fig. 5.

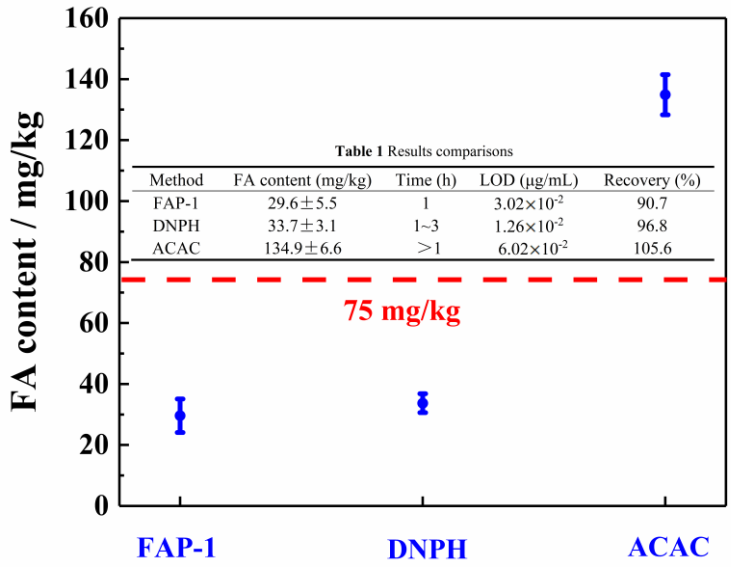


Table 1 Results comparisons

Method	FA content (mg/kg)	Time (h)	LOD (μg/mL)	Recovery (%)
FAP-1	29.6±5.5	1	3.02×10^{-2}	90.7
DNPH	33.7±3.1	1~3	1.26×10^{-2}	96.8
ACAC	134.9±6.6	>1	6.02×10^{-2}	105.6

Fig. 5 Methods of **FAP-1**, DNPH and ACAC for **FA** determination in leather with comparisons.

In addition, probe **FAP-1** can be directly used in water or ethanol under mild conditions, avoiding the addition of extra chemicals or requiring complex procedures as described in the sample preparation for the standard methods, such as concentrated phosphoric acid (DNPH) or or 12 hours storage in the dark and heating before use (ACAC). Importantly, the time required for derivatizing using **FAP-1** is one hour, which is significantly shorter than using DNPH and ACAC, as shown in the table of Fig. 5. The low-cost and simple synthesis

of probe **FAP-1** make it a promising tool for further fluorescence based detection applications.

4. CONCLUSIONS

In summary, we have successfully developed a fluorescent probe **FAP-1**, which displays favorable properties, such as water solubility and is sensitive, and selective for **FA** amongst many other species found in leather making, thus providing a desirable method for trace **FA** detection in leather production. More importantly, the detection precision attributed to the fluorescence method is similar to the HPLC method and preferable to the standard colorimetric UV-Vis method. Additionally, the method using probe **FAP-1** is facile, low-cost and time-saving and can provide reliable data for **FA** detection within the leather industry. Therefore, this research indicates that fluorescence-based probes are powerful molecular tools for versatile detection methods of products derived from an industrial context. As such we envision that small-molecular fluorescence probes can provide a completely new approach within the leather chemical industry and have great potential for applications of quality control beyond formaldehyde.

Notes

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material is available in the online version of this article at http://dx.doi.org/[DOI] and is accessible for authorized users.

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