Simplified Fabrication of Laminated Paper-Based Analytical Device (LPAD) with Color-Palette Mobile App for Analysis of Salicylic Acid in Pharmaceutical Products

Nakarin NOIRAHANG,*1,2 Kanchana URAISIN,*1,2 Panwadee WATTANASIN,*3,4† and Phoonthawee SAE TEAR*1,2†

*1 Flow-Innovation Research for Science and Technology Laboratories (Firstlabs),

*2 Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

*3 Center of Excellence for Trace Analysis and Biosensor, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.
*4 Division of Physical Science, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.

† To whom correspondence should be addressed.

E-mail: panwadee@gmail.com (P.W.); phonthawee.sae@mahidol.edu (P.S.)
Abstract

In this work, we present for the first time, a simplified fabrication of a laminated paper-based analytical device (LPAD) with a free-mobile app, Palette Cam, for image analysis. A filter paper is cut in rectangular shape (9 cm × 3 cm) and placed between a top laminating sheet with punched holes and a bottom laminating sheet. The holes allow accessibility of liquid on the paper. Thermal lamination is then employed to complete fabrication of LPAD. Our simplified design reduces a tedious alignment of small pieces of paper to the holes. We demonstrate the LPAD with analysis of salicylic acid in pharmaceutical products. Each 4 μL of ferric reagent and sample is dispensed on the LPAD. Smartphone was used to capture the images. The RGB (red green blue) color intensity from the Palette Cam is converted into a logarithm color ratio. Our LPAD is simplified, cost-effective and able to be portable device.

Keywords: Laminated paper-based analytical device, LPAD, Palette Cam, Salicylic acid, Mobile app
Introduction

Laminated paper-based analytical device (LPAD) is categorized as one in a group of microfluidic paper-based analytical device (µPAD). LPAD was first proposed by Cassano and co-worker in 2013.\(^1\) Unlike general fabrication of the µPAD, creation of patterned hydrophobic boundaries, by photolithography,\(^2\) wax printing\(^3\) and screen printing\(^4-6\) is not required. LPAD is barrier-free µPAD with simple fabrication by craft-cutting of paper in desired shape and dimension, followed by thermal lamination in the way similar to making an identification (ID) card. Process of lamination addresses a common problem that paper has relatively low mechanical strength, particularly when paper is wet.

Due to the benefits of LPAD in terms of mechanical strength and overall durability, several detections on LPAD have been proposed. Colorimetric assays were reported for detections of BSA and glucose\(^1\) and detection of histidine\(^7\). Image analysis for colorimetric assay was carried out by using either scanner or compact camera with ImageJ or Adobe Photoshop software. Chemiluminescence assays was also proposed with origami procedure for cotinine analysis\(^8\) with a microplate reader as a detection tool. Gold
nanoparticles (AuNPs)\textsuperscript{9} and copper nanocluster (CuNC)\textsuperscript{10} were also applied to LPAD with using a photomultiplier tube (PMT)\textsuperscript{9} and a compact camera\textsuperscript{10} as detection tools, respectively. Electrochemical sensing with low-voltage bipolar electrode incorporated on LPAD was presented for demonstrating detections of hydrogen peroxide and glucose.\textsuperscript{11}

Capillary electrophoresis (CE) was also incorporated on LPAD for proofing a concept by separation of mixture of food colorants.\textsuperscript{12}

Regarding the first fabrication of LPAD, a cutting plotter equipped with a craft cutter is originally employed for cutting a filter/chromatography paper to obtain the paper strips, depending on the design and analytical procedure.\textsuperscript{1,7-9} The paper strips are then laminated with a cover sheet and a bottom sheet by thermal laminator. The laminated cover sheet has a cutout pattern same as the paper strip with slightly smaller dimension, resulting in accessibility for reagent and sample dispensing on the paper strip. The laminated plain bottom sheet is used as mechanical support as received.

Later on, the lamination process has been adopted to fabricate the \(\mu\)PADs, even later works have not been used the terms of LPAD. Paper platform was not strip, but designed to cut the paper as circle discs\textsuperscript{13} and square discs\textsuperscript{14} by using a hole puncher and an office
cutter/scissors, respectively. For circular disc design, multilayer alignment, including the top laminating sheet, circular paper discs and hydrophobic membrane discs, was proposed for analysis of ammonia based on gas-diffusion\textsuperscript{13}. The top sheet of the laminating pouch was perforated with circular holes of 3-fold smaller diameter than the circular disc, to ensure that punched holes are aligned on the circular paper disc. However, precise alignment for multilayer assembly is crucial. Square disc was later designed to reduce the time-consuming alignment by placing square disc with arranging under the circular hole-punched laminating sheet.\textsuperscript{14} With same concept, punched holes are slightly smaller than square disc. This design was applied to analysis of antioxidant activity.

In our point of view, cutting small pieces of paper strip/disc is simple for fabrication of LPAD.\textsuperscript{1,7-14} However precise alignment of paper strip/disc to the hole-punched laminating sheet is still required and time-consuming. In this work, we therefore simplify for the first time to fabricate the LPAD by insertion of a piece of rectangular filter paper (9 × 3 cm\textsuperscript{2}) between the cover laminating sheet with perforated holes and the bottom laminating sheet. This way, precise alignment of paper strip/disc, can be avoided resulting in a simpler fabrication of LPAD. Our simplified LPAD was demonstrated to determine
the salicylic acid, SA (as beta hydroxy acid, BHA) in pharmaceutical products. Ferric reagent was employed to chemically react with salicylic acid, forming a violet complex of \([\text{Fe}^{3+}-(\text{SA})_2]^+\). Smartphone was used to obtain an image of the violet complex on the detection zone of the LPAD. We also simplified the step of image analysis by using a free color-palette mobile application, named Palette Cam, instead of using an ImageJ processing software. Palette Cam app was manipulated for simplified analyzing in the RGB (red, green, blue) color mode. The color intensity obtained from the Palette Cam is converted into a color ratio as \(\log I_0/I\) (\(I_0\): the intensity of blank; \(I\): intensity of standard or sample). Our proposed fabrication and detection on the LPAD were evaluated and validated by a comparative spectrophotometric method.

**Experimental**

**Reagents and chemicals**

All chemicals and reagents used were analytical reagent grade. Deionized water (DI) from Milli-Q® Advantage A10 Water Purification System (resistivity 18.2 MΩ cm)
system were used for preparation of all solutions.

A stock salicylic acid (SA) of 10,000 mg L$^{-1}$ was freshly prepared by dissolving 0.1 mg of salicylic acid (Sigma-Aldrich, USA) in 5.0 mL of 99.9 % (v/v) ethanol (RCI Labscan, Thailand), followed by 5.0 mL of DI water. A working standard SA was also freshly prepared from the 10,000 mg L$^{-1}$ stock SA solution by aliquoting appropriate volume to give a series of SA solution (200-1000 mg L$^{-1}$).

A color-forming reagent is ferric reagent. Four solutions of ferric reagent are followings: (1) 6% (w/v) FeCl$_3$ in DI water, (2) 6% (w/v) FeCl$_3$ in 1% (v/v) HCl, (3) 6% (w/v) Fe(NO$_3$)$_3$ in DI water, and (4) 6% (w/v) Fe(NO$_3$)$_3$ in 1% (v/v) HNO$_3$. Each solution was carried out by dissolving 0.3 g of either ferric (III) chloride hexahydrate (Merck, country) or ferric (III) nitrate nonahydrate (Merck, country) in either 5.0 mL of DI water or in 5.0 mL of 1% HCl (RCI Labscan, Thailand)/1% HNO$_3$ (Merck, country), respectively. The ferric reagent was stored at 4°C and used within one week.

Samples

Pharmaceuticals for acne (S1 and S2), pain (S3 and S4) and wart (S5 and S6)
treatments were purchased from the drug stores in Bangkok, Thailand. Each sample was prepared by weighting 0.50 g and dissolving in 2.0 mL of dimethyl sulfoxide (DMSO). Then the solution was twice filtered with 0.45-µm nylon membrane. The filtered sample was finally 20-fold diluted by pipetting 100.0 µL sample and making up volume to 2.00 mL with DI water. Recovery study was carried out by spiking an 80 µL of the 10,000 mg L⁻¹ stock SA solution into pretreated sample prior making up volume to 2.00 mL with DI water. Percentages of recoveries were determined by the following equation;

\[
\%\text{Recovery} = \left( \frac{C_{\text{spiked sample}} - C_{\text{sample}}}{C_{\text{standard}}} \right) \times 100
\]

where \(C_{\text{spiked sample}}\): concentration of SA found in sample spiked with standard; \(C_{\text{sample}}\): concentration of SA found in sample and \(C_{\text{standard}}\): concentration of standard.

Fabrication of laminated paper-based analytical device (LPAD)

Components and dimensions of LPAD is illustrated in Fig. 1a. LPAD consists of a 3-layer assembly with a piece of a Whatman filter paper No. 4 (9.0 cm × 3.0 cm in the middle layer) and two top-and-bottom layer of polyester lamination sheets (125 µm thick, 9.5 cm × 3.5 cm). Holes for liquid loading and detection zone were one-side punched on
the top lamination sheet. The laminated plain bottom sheet is used as mechanical support as received.

A procedure of fabrication of the LPAD device is extremely simple as making the ID card, as shown in Fig. 1b. The top lamination sheet was punched as perforated design with circular holes (Step 1 in Fig. 1b) using a Silhouette-CAMEO® Digital Cutting Tool (CAMEO 3, China). A rectangular filter paper was then inserted between two lamination sheets (Step 2 in Fig. 1b). A thermal laminator with roller was employed (Step 3 in Fig. 1b) to complete fabrication of the LPAD (Step 4 in Fig. 1b).

Operating procedure of LPAD for analysis of salicylic acid as BHA

The analysis of salicylic acid using the LPAD can be performed as Steps 1-4 shown in Fig. 2. A 4.0 μL of 6% (w/v) Fe(NO$_3$)$_3$ in DI water was dispensed on the detection zone (Step 1). Drying with blow drier (Step 2) and ambient temperature (Step 3) was carried for 30 s each. A series of standard salicylic acid (0 - 1,000 mg L$^{-1}$) was then dispensed with volume of 4.0 μL to each detection zone (Step 4). The color in the detection zones were changed from pale-yellow to purple. Image of the LPAD was captured in the light.
control box (Step 5) by utilizing iPhone 8 plus (Dual 12MP Wide and Telephoto cameras, Wide: f/1.8 aperture and Telephoto: f/2.8 aperture). The intensity of color was analyzed in the RGB color mode by using the Palette Cam, free mobile applications on iOS software. Total analysis time is 2 min.

The color intensity was obtained from reading position at the center point of the detection zone of the LPAD. The reading color intensity was then converted into a logarithm ratio (log I₀/I), where I₀: the intensity of blank using DI water; I: intensity of standard or sample. With using Palette Cam app, the center of the image was selected to be pointed for acquisition of the RGB intensity. Triplicate measurement of each standard SA and sample was carried out. A calibration graph was plotted between color ratio (log I₀/I) of green color (G) and concentration of standard SA in unit of mg L⁻¹).

Our proposed fabrication and detection on the LPAD were evaluated and validated by a visible spectrophotometric method. Briefly, a solution of ferric chloride was used as color-forming reagent to produce the violet complex. A bench-top spectrophotometer set at 528 nm was employed with a conventional 1-cm plastic cuvette. A same set of standard solutions and samples were aliquoted for analysis of SA by the LPAD method and
batchwise colorimetric method.

Results and Discussion

Relationship between RGB-color intensity and concentration of salicylic acid

As mentioned in the operating procedure, the smartphone was used to capture the images of the LPAD in the light control box and the Palette Cam app was then applied to convert the color intensities of RGB into a color ratio (log $I_0/I$). After dispensing the standard SA to the detection zone, the color was immediately changed from pale-yellow to purple, indicating the $[\text{Fe}^{3+}-(\text{SA})_2]^+$ complex was formed. The purple color appearing on the detection zones intense accordingly to the increasing of SA concentration from blank to 1,000 mg L$^{-1}$ (see Fig. 3a). With captured images, color appearance is slightly different from the actual color seen by naked eyes due to the filter compartment equipped in smartphone camera.

Fig. 3b shows the linear calibration plots of RGB from standard SA (0-1,000 mg L$^{-1}$).
Signals in $y$-axis is a color ratio of $I_0$ and $I_R$, $I_G$ or $I_B$. As seen in the calibration plots, intensities of RGB colors gave the good linear plots and satisfied coefficient of determination ($r^2$). Sensitivities from red and blue intensities were comparable. However, it was found that the color ratio for green intensity gave highest slope and $r^2$. Therefore, we chosen green intensity for the plotting as color ratio in $y$-axis for the calibration graph.

In addition, we also demonstrated the precision of the reading the color intensity at several reading position on the detection zone of the LPAD. Results show in Table S1 in the supplement information, indicating that reading signals were comparable even shifting from the center point of the detection zone.

**Investigations of type and concentration of ferric reagent**

The determination of SA is based on the measurement of purple color of the [Fe$^{3+}$-(SA)$_2$]$^+$ complex. Therefore, type and concentration of ferric reagent are important parameters as they will affect the complexation. This investigation was carried out using a series of standard SA from 200 to 1,000 mg L$^{-1}$.

Two types of ferric salt FeCl$_3$ and Fe(NO$_3$)$_3$ were chosen and prepared in DI water.
and acid. Both ferric salts gave clear solutions after entirely dissolved in either DI water and 1 %(v/v) acid. Table 1 shows the results of calibration plots from 4 solutions of ferric reagent (mentioned in the Experimental section). All ferric reagents gave good linear plots with comparable sensitivities (slopes of the calibrations), except ferric reagent from FeCl$_3$ in acid. With FeCl$_3$ reagents in both water and acid, we observed that color of the complex on the detection zone of LPAD fades from purple until it disappears within 1 min. On the other hand, Fe(NO$_3$)$_3$ reagents performed a longer lasting of color appearance (2 - 5min).

Regarding the analytical procedure in the Step 5 in Fig. 2, appearance purple color on the LPAD should last long enough to allow taking photo of the LPAD. In this work, ferric reagent of ferric nitrate in DI water was selected for further experiments.

Then, we optimized the concentration of the Fe(NO$_3$)$_3$ in DI water. The investigated range was 2 - 8 %(w/v). It was found that all concentrations of Fe(NO$_3$)$_3$ gave comparable sensitivities, but the 6 %(w/v) gave the $r^2 > 0.99$. Thus we selected the optimal concentration of Fe(NO$_3$)$_3$ at 6 %(w/v).

**Drying process after loading of ferric reagent on the detection zone of the LPAD**
In this investigation of drying process, we initially tested a simplest procedure without drying the detection zone after liquid dispensing (see procedure A in Table 2). The standard SA was immediately dispensed after loading the ferric reagent. Purple color was observed with $0.156 \pm 0.013$ a.u. The color distribution on the detection zone was inhomogeneous, leading to the poor precision (8.3 % RSD). This may be caused by the wetting of the paper where liquid drop of standard SA impels the preloaded drop of ferric reagent out from the center of the detection zone.

To reduce the error from the inhomogeneous distribution, drying step was then applied after dispensing the standard SA and the ferric reagent. Air dry (procedure B in Table 2) and a combination of blow and air dries (procedure C in Table 2) gave the homogeneity of the color distribution and improved the precision (4.1-4.6 % RSD). In this work, we chosen the drying step with use of blow and air dries (procedure C) after loading the ferric reagent on the detection zone since it gave the higher analytical signal.

**Reaction time**

In this work, reaction time is defined as a timing after dispensing the standard SA.
on the detection zone (a timing between operational steps 4 and 5 in Fig. 2). The term of reaction time is related to a moment of image capturing (step 5 in Fig. 2). Fig. 4 shows the results of varying reaction time from 0-5 min. For 0 min reaction time, we immediately observed the violet color on the LPAD and then took a photo after dispensing the standard SA. It was found that the color ratio was higher than other reaction times (1-5 min). Regarding the reaction time of 1 to 5 min, the color ratio was comparable and relatively lower than this from immediate capturing. Thus we selected to immediately take a photo of the LPAD (step 5 in Fig. 2) after loading the standard SA on the detection zone (step 4 in Fig. 2).

Analytical features and application to samples

Under the optimal condition, green intensity of blank ($I_0$) and standard/sample ($I_G$) was used to convert to color ratio. The calibration curve is linear in the range of 200 to 1,000 mg L$^{-1}$ ($\log I_0/I_G = (3.0 \times 10^{-4} \pm 7.02 \times 10^{-6})[\text{SA, mg L}^{-1}] + (0.099 \pm 0.001), r^2 = 0.998$), with fit to the propose for the measurement of SA in pharmaceutical products. Detection limit (3SD of regression line/slope) and quantitation limit (10SD of regression
line/slope) were found to be 50.5 and 168 mg L\(^{-1}\), respectively. Precisions as RSD were acceptable with ten replicate measurements of three standard SA solutions (1.3 % for 200 mg L\(^{-1}\), 1.9 % for 600 mg L\(^{-1}\) and 1.3 % for 1,000 mg L\(^{-1}\)). Total analysis time is 2 min, giving possible analysis of 30 samples per hour. LOD and LOQ could be improved by multi-aliquot of standard or sample using the same dispense volume (our case: 4.0 µL) with blow dry process. However current LOD is already fit to the purpose in the analysis of SA in pharmaceutical products.

Effect of possible interfering substances in pharmaceutical products was investigated. In this investigation, the operating analytical procedure in Fig. 2 was employed for comparison of the signals of a standard solution 200 mg L\(^{-1}\) SA with and without the presence of each interfering substance. The results in Table 3 show that our LPAD method can tolerate for citric acid and glycerol at 300 mg L\(^{-1}\) and for benzoic acid, lactic acid and EDTA at 600 mg L\(^{-1}\). However, these interfering substances are usually present at very low concentration in pharmaceutical products. Moreover, these interfering substances can be diminished by diluting the sample (20 folds) prior analysis. Therefore, our LPAD method can be applicable to detect SA in pharmaceutical products.
Six samples (S1-S6) were analyzed by using our LPAD method as compared with batchwise visible spectrophotometric method. The quantitative results as bar plots are shown in Fig. 5. According to the paired t-test, the results in Fig. 5 show significant agreement between our LPAD and comparative method for all samples ($t_{\text{stat}} = 2.10$, $t_{\text{crit}} = 2.57$ at $P = 0.05$). Pearson's correlations also confirmed that our LPAD method gave results that did not differ significantly from values using the batchwise spectrophotometry ($r^2 = 0.998$). Recoveries of 77 – 97 % for 6 samples were satisfactorily obtained. Practically, the recoveries were acceptable in the range of 80 -120 %. In this work, our recoveries were agreed with the criterion. In addition, recoveries in this work were from entire operating analytical procedure using our LPAD method.

Comparison with other paper-based devices

Table 4 lists the analytical features for the rapid determination of salicylic acid, obtained from our LPAD device as compared with two other types of the paper-based device.

Based on the voltammetric electroanalytical method, Device A was designed using
a circle piece of filter paper placed on the detection area for serving as liquid reservoir.

An advantage of use of the circle piece of filter paper is that could dramatically decrease the required volume of the buffer solution. Device A was applied to plant sample including leaves,\textsuperscript{15,16} and seeds.\textsuperscript{17,18}

Regarding the colorimetric method, Device B designed with wax printing of hydrophobic barrier\textsuperscript{19} was applied to food sample for analysis of chemical food additives (borax, nitrite, nitrate and SA). In contrast, our LPAD method with hydrophobic barrier-free was fabricated and applied to pharmaceutical products. LOD was comparable to the PAD from Device B. Our LPAD saves the liquid volume per analysis (8 $\mu$L) and analysis time (2 min per sample).

Conclusions

We present a simplified fabrication of laminated paper-based analytical device (LPAD) by placing a plain filter paper between a cover and a bottom laminating sheet. Punched holes are created on the cover laminating sheet to obtain accessibility of liquid dispensing.
on the paper. Detection zones is defined as paper area within the boundary of the punched holes. We see that confined liquid volumes of sample and reagent are required even fabrication based on use of small paper strips/discs. Thus, our fabrication of LPAD is simpler than the previous design since precise alignment and/or assembly is not required. We demonstrated the LPAD to determine the salicylic acid (SA) in pharmaceuticals for wart, pain and acne treatments. Small volumes of sample/standard SA and ferric reagent were dispensed. Violet complex was immediately formed on the detection and smartphone was used to capture the images. A free-mobile app Palette Cam was also used, for the first time in LPAD, to analyze in the RGB color mode. The color intensity obtained from the Palette Cam is converted into color ratio ($\log \frac{I_0}{I}$). Our LPAD is simplified, cost-effective and able to be portable device.

Acknowledgements

Financial support was from the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation (NN).
The Young Scientists scholarship from Faculty of Science, Mahidol University given to NN was supported his M.Sc. study. This research project was also supported by the CIF and CNI grant, Faculty of Science, Mahidol University. The National Research Council of Thailand (IRN/502/2563) chaired by Assoc. Prof. Dr. Duangjai Nacapricha was also acknowledged.
References

1. C. L. Cassano, and Z. H. Fan, Microfluid. Nanofluidics, 2013, 15, 173.
2. A. W. Martinez, S. T. Phillips, M. J. Butte, and G. M. Whitesides, Angew. Chem. Int. Ed., 2007, 46, 1318.
3. M. Younas, A. Maryam, M. Khan, A. A. Nawaz, S. H. I. Jaffery, M. N. Anwar, and L. Ali, Microfluid. Nanofluidics, 2019, 23, 38.
4. K. Sirivibulkovit, T. Pimklang, P. Pakawatpanurat, A. Sabarudin, D. Nacapricha, and P. Saetear, Key Eng. Mater., 2019, 824, 197.
5. J. Sitanurak, N. Fukana, T. Wongpakdee, Y. Thepchuay, N. Ratanawimarnwong, T. Amornsakchai, and D. Nacapricha, Talanta, 2019, 205, 120113.
6. Y. Sameenoi, P. N. Nongkai, S. Nouanthavong, C. S. Henry, and D. Nacapricha, Analyst, 2014, 139, 6580.
7. A. Kugimiya, A. Fujikawa, X. Jiang, Z. H. Fan, T. Nishida, J. Kohda, Y. Nakano, and Y. Takano, Appl. Biochem. Biotechnol., 2020, 192, 812.
8. W. Liu, C. L. Cassano, X. Xu, and Z. H. Fan, Anal. Chem., 2013, 85, 10270.
9. W. Liu, J. Luo, Y. Guo, J. Kou, B. Li, and Z. Zhang, Talanta, 2014, 120, 336.
10. C.-Y. Chen, C.-L. Chen, C.-M. Wang, and W.-S. Liao, Nanomaterials, 2018, 8, 97.
11. C.-M. Wang, C.-H. Hsieh, C.-Y. Chen, and W.-S. Liao, Anal. Chim. Acta, 2018, 1015, 1.
12. C. Xu, M. Zhong, L. Cai, Q. Zheng, and X. Zhang, Electrophoresis, 2016, 37, 476.
13. Y. Thepchuay, R. B. Mesquita, D. Nacapricha, and A. O. Rangel, Anal. Bioanal. Chem., 2020, 412, 3167.
14. K. Sirivibulkovit, S. Nouanthavong, and Y. Sameenoi, Anal. Sci., 2018, 34, 795.
Xu, N. Bao, and H.-Y. Gu, *Biosens. Bioelectron.*, 2014, 60, 154.

16. K.-C. He, H.-R. Wang, H. Yang, L.-J. Sun, W. Liu, and N. Bao, *Anal. Chim. Acta*, 2020, 1120, 59.

17. L.-J. Sun, Y. Xie, Y.-F. Yan, H. Yang, H.-Y. Gu, and N. Bao, *Sens. Actuators B Chem.*, 2017, 247, 336.

18. L.-J. Sun, J.-J. Zhou, J.-L. Pan, Y.-Y. Liang, Z.-J. Fang, Y. Xie, H. Yang, H.-Y. Gu, and N. Bao, *Sens. Actuators B Chem.*, 2018, 276, 545.

19. R. Nalin, and D. Wijitar, *J. Anal. Chem.*, 2020, 75, 487.
Table 1. Calibration plots of standard SA ranging 200-1,000 mg L$^{-1}$ SA with various type of ferric salts and medium.

| Type of ferric salt | Medium |            |            |
|--------------------|--------|------------|------------|
|                    | DI water | Acid$^b$ | Acid$^b$ |
| FeCl$_3$           | y = 0.0019x + 0.1319, r$^2$ = 0.9821 | y = 0.0028x + 0.056, r$^2$ = 0.9890 |
| Fe(NO$_3$)$_3$     | y = 0.0021x + 0.1021, r$^2$ = 0.9999 | y = 0.0019x + 0.025, r$^2$ = 0.9995 |

$^a$ 6% ferric salt (w/v)

$^b$ 1% (v/v) HCl for FeCl$_3$ and 1% (v/v) HNO$_3$ for Fe(NO$_3$)$_3$
Table 2. Effect of drying procedure on homogeneity of color distribution on the LPAD.

| Drying procedure for reagent and sample | Image of LPAD for 500 mg L\(^{-1}\) SA |
|----------------------------------------|--------------------------------------|
| A. No drying                           | | log \(I_0/I_G\) = 0.156 ± 0.013 |
| B. Air dry 60 s                        | | log \(I_0/I_G\) = 0.147 ± 0.006 |
| C. Blow dry 30 s + Air dry 30 s (operated same as in Fig. 2) | | log \(I_0/I_G\) = 0.153 ± 0.007 |
Table 3. Tolerance limit of the LPAD method for potential interfering substance in pharmaceutical products.

| Interfering substance | Concentration range tested (mg L$^{-1}$) | Tolerance limit$^a$ (mg L$^{-1}$) |
|-----------------------|------------------------------------------|----------------------------------|
| Benzoic acid          | 500 - 800                                | 600                              |
| Citric acid           | 100 - 500                                | 300                              |
| Glycerol              | 100 - 500                                | 300                              |
| Lactic acid           | 500 - 800                                | 600                              |
| EDTA                  | 500 - 800                                | 600                              |

$^a$ Tolerance limit is defined as the maximum concentration of the interfering substance that gives < 5% change in the signal of a standard solution at 200 mg L$^{-1}$ SA.
Table 4. Comparison of analytical features of paper-based analytical device for determination of salicylic acid.

| Device and Detection Method | Sample | Analytical System | Signal Plot on y-Axis | Linear Range (mg L⁻¹ SA) | LOD (mg L⁻¹ SA) | Liquid Volume Per Analysis (Sample Vₛ, Reagent Vᵣ, Buffer V₆) | Analysis Time (min) |
|----------------------------|--------|-------------------|------------------------|--------------------------|-----------------|-------------------------------------------------------------|--------------------|
| A. Paper-based electroanalytical device |        |                   |                        |                          |                 |                                                             |                    |
| A1. DPVᵃ ¹⁵ | Living tomato leaves | WEᵇ: carbon tape electrode, REᵣ: Ag/AgCl wire, CE₆: Pt wire | Current | 0.08 – 13.81 | < 0.007ᵇ | Vᵣ: 7 μL PBS⁰ | N.R. |
| A2. DPVᵇ ¹⁷ | Pea seeds | WEᵇ: carbon tape electrode, REᵣ: Ag/AgCl wire, CE₆: Pt wire | Current | N.R. | N.R. | Vᵣ: 10 μL PBS⁰ | N.R. |
| A3. DPVᵇ ¹⁸ | Pea seedlings | WEᵇ: graphene oxide modified carbon tape electrode, REᵣ: Ag/AgCl wire, CE₆: Pt wire | Current | 0.14 – 13.81 | < 0.014ᵇ | Vᵣ: 10 μL PBS⁰ | < 5 |
| A4. SWVᵇ ¹⁶ | Arabidopsis thaliana leaves | WEᵇ: pencil traces modified carbon tape electrodes, REᵣ: Ag/AgCl wire, CE₆: Pt wire | Current | 0.014 – 13.81 | N.R. | N.R. | N.R. |
| Device and Detection Method | Sample | Analytical System | Signal Plot on y-Axis | Linear Range (mg L\(^{-1}\) SA) | LOD (mg L\(^{-1}\) SA) | Liquid Volume Per Analysis (Sample V\(_S\), Reagent V\(_R\), Buffer V\(_B\)) | Analysis Time (min) |
|-----------------------------|--------|-------------------|-----------------------|----------------------------------|----------------------|-------------------------------------------------|---------------------|
| A. Paper-based electroanalytical device | Meat ball, sausage, pickled foods | Image analysis: scanner, 600-dpi and ImageJ software | \(\Delta\) Intensity\(^h\) | 100 – 9000 | 35\(^i\) | \(V_S: 25 \mu L\) | 10 |
| B. PAD\(^c\) by wax printing of hydrophobic barrier with colorimetry | Skin medication products | Image analysis: iPhone8 plus, 300-dpi and Pallete Cam Apps | \(\log{(I_B/I_S)}\)\(^i\) | 200 – 1000 | 50.5\(^a\) | \(V_S: 4 \mu L\) | 2 |

N.R.: not reported.  
\(^a\) Differential pulse voltammetry.  
\(^b\) Square wave voltammetry.  
\(^c\) Paper-based analytical device.  
\(^d\) Laminated paper-based analytical device.  
\(^e\) Working electrode.  
\(^f\) Counter electrode.  
\(^g\) Reference electrode.  
\(^h\) Difference in the intensities of blank and intensities of standard/sample.  
\(^i\) Log based 10 of intensities of blank divided by standard/sample.  
\(^j\) LOD of salicylic acid based on 5 times of signal/noise ratio.  
\(^k\) LOD of salicylic acid based on 6 times of signal/noise ratio.  
\(^l\) LODs of the salicylic acid by ImageJ Program.  
\(^m\) LODs of salicylic acid by naked eye.  
\(^n\) LOD of salicylic acid 3SD regression/slope.  
\(^o\) Phosphate-buffered saline.
Figure Captions

Fig. 1 Laminated paper-based analytical device (LPAD) with (a) three-layer exploded view and (b) steps for fabrication.

Fig. 2 Schematic representation of the operating procedure of LPAD for quantifying salicylic acid as BHA in pharmaceutical and acta treatment products.

Fig. 3 Calibration plots using the LPAD for the determination of salicylic acid and the corresponding images of the purple [Fe³⁺-(SA)₂]⁺ complex.

Fig. 4 Effect of reaction time on color intensity appearing on the LPAD.

Fig. 5 Bar plots showing the salicylic acid as BHA contents in products for acne treatment (S1-S4) and wart treatment (S5 and S6), as determined by the LPAD and the comparative visible spectrometry. Triplicate measurement was carried out. *Note: The salicylic acid results for S1-S4 are five-fold of the actual concentrations.
Fig. 1

a.
Top lamination sheet with detection hole
Filter paper sheet
Bottom lamination sheet

Punched hole

9.0 cm
9.5 cm

b.
Step 1
Punching holes on the top lamination sheet

Step 2
Insertion of filter paper between two lamination sheets

Step 3
Assembly by heated laminator

Step 4
Laminated paper-based analytical device (LPAD)
Fig. 2

1. **Step 1**: 4.0 μL of ferric reagent (30 s)
2. **Step 2**: Blow dry 30 s
3. **Step 3**: Air dry 30 s
4. **Step 4**: Taking photo
5. **Step 5**: 4.0 μL of a series SA standard or sample (30 s)
a. mg L$^{-1}$ SA Blank 200 400 600 800 1,000

b. 

| Color  | Equation                  | $r^2$  |
|--------|---------------------------|--------|
| Green  | $y = 1.67x 	imes 10^{-4} + 0.107$ | 0.994  |
| Red    | $y = 0.80x 	imes 10^{-4} + 0.116$ | 0.993  |
| Blue   | $y = 0.66x 	imes 10^{-4} + 0.112$ | 0.969  |

Fig. 3
Fig. 4
Fig. 5
Graphical Index

Simplified Fabrication of LPAD