Supplementary Information on "ANALYST" publication entitled

ECL sensor combined with a paper electrode for the determination of phenylalanine

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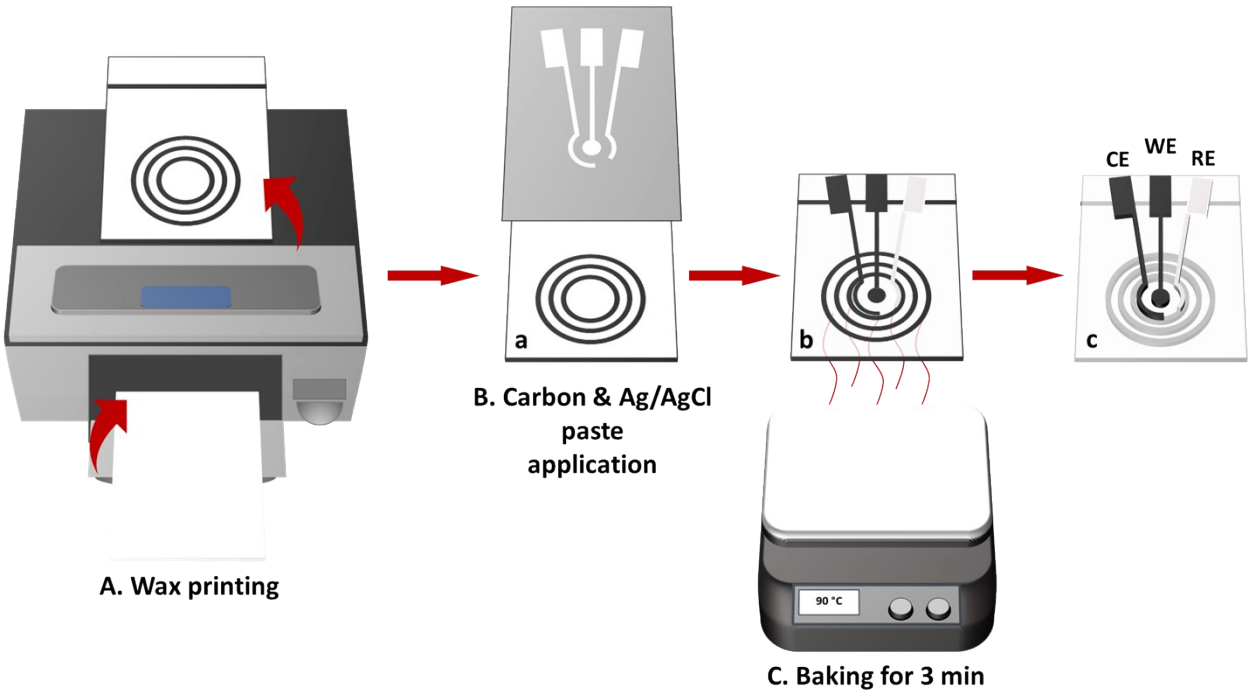

Schema S1. The fabrication procedure of the paper electrode; **a)** the wax printed NC membrane, **b)** the paper electrode assemble with CE, WE, and RE, **c)** the baked paper electrode

AuNP modification of the paper electrodes

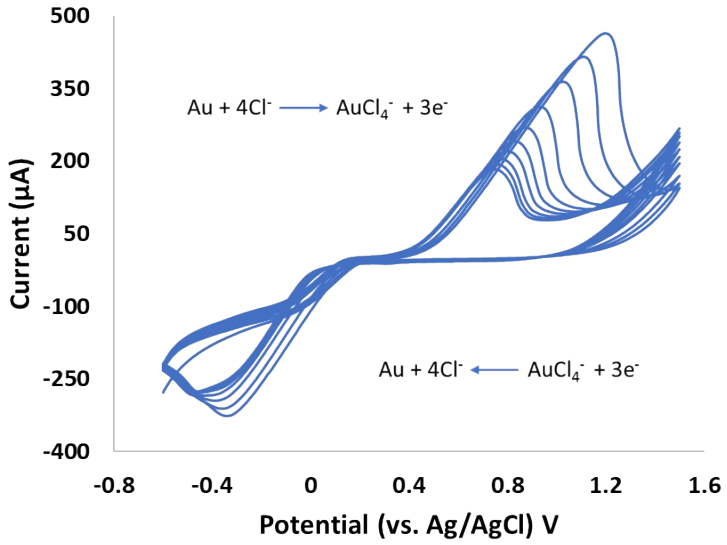

Fig. S1. Cyclic voltammograms of 10.0 mM $HAuCl_4$ in 100.0 mM KCl and 100.0 mM H_2SO_4, on the WE for 10 cycles at a scan rate of 100 mV/s in the range of -0.6 to +1.5 V.

The electrodeposition of gold nanoparticles (AuNPs) onto the working electrode surface of PEs was carried out from 20.0 µl of 0.01 M $HAuCl_4$ solution including 0.1 M KCl and 0.1 M H_2SO_4, using CV in the range of -0.6 to +1.5 V for several numbers of cycles at various scanning rates. Typical voltammograms were shown in Fig. S1.

Characterization of the paper electrodes using EIS

Table S1. The R_{ct} values recorded by unmodified PE and AuNPs@PE

	R_s (ohm)	**R_{pore} (ohm)**	**R_{ct} (ohm)**	**W (ohm.$s^{-1/2}$)**
PE	63.4	220.4	4704	$3.502x10^{-4}$
AuNPs@PE	243.7	$5.643x10^{7}$	191.4	$2.298x10^{-3}$

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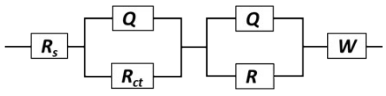

$R_s(QR_{ct})(QR)W$

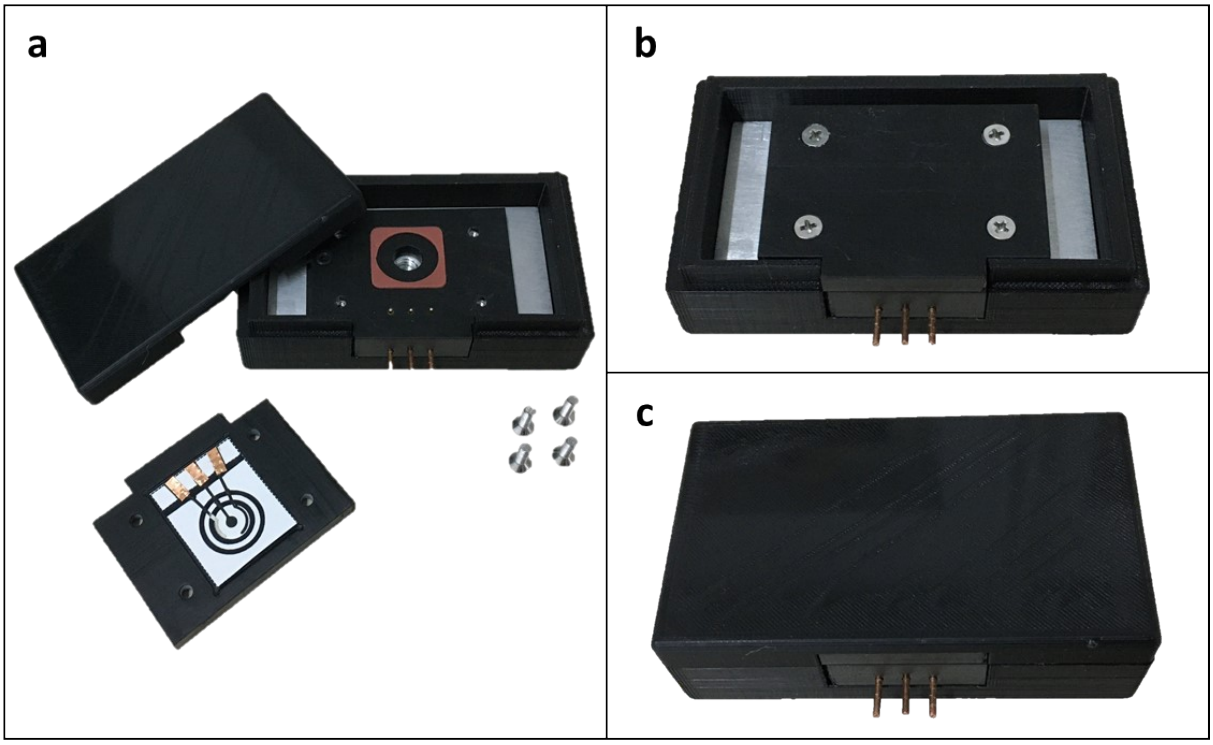

Fig. S2. The photograph of the paper-based ECL sensor; **a)** all components of the paper-based ECL sensor, **b)** the top and bottom components assembled by screwing together **c)** the photograph showing the closed version of the system

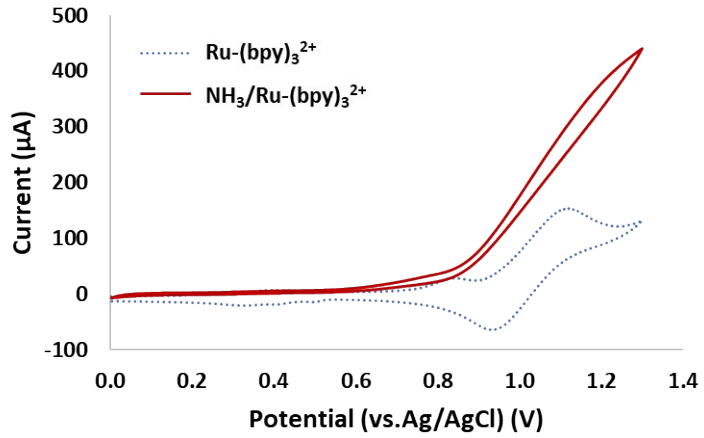

Fig. S3. Cyclic voltammetry responses of 10.0 mM Ru-$(bpy)_3^{2+}$ (dash line) and NH_3/Ru-$(bpy)_3^{2+}$ in the presence of 0.605 mM L-Phe, at a scan rate of 100 mV s^{-1}, from 0 V to 1.3 V.

The optimization of the sensing system

It is known that the emission wavelength of the $Ru(bpy)_3^{+2}$ is about 620 nm. However, the emission wavelength may vary depending on the coreactant of $Ru(bpy)_3^{+2}$ which is used in the experiment. Therefore, the optimization of the emission wavelength was carried out before starting the experiments. For this purpose, 10.0 mM $Ru(bpy)_3^{+2}$ and $1.0x10^{-3}$ M ammonia solution were used to obtain ECL signals. The ECL signals were recorded by setting the emission wavelength of the PMT detector to be 610, 620, and 625 nm. Here, the ECL measurements were carried out using CV for 4 cycles at a potential range from 0 to +1.3 V. As shown in Fig. S4, the ECL signal intensities obtained at 620 and 625 nm were very close to each other and approximately twice higher than the ECL signals obtained at 610 nm. According to the results in Fig. S4, it was decided to record the ECL signals of Ru-$(bpy)_3^{2+}$/NH_3 at 625 nm in the developed system.

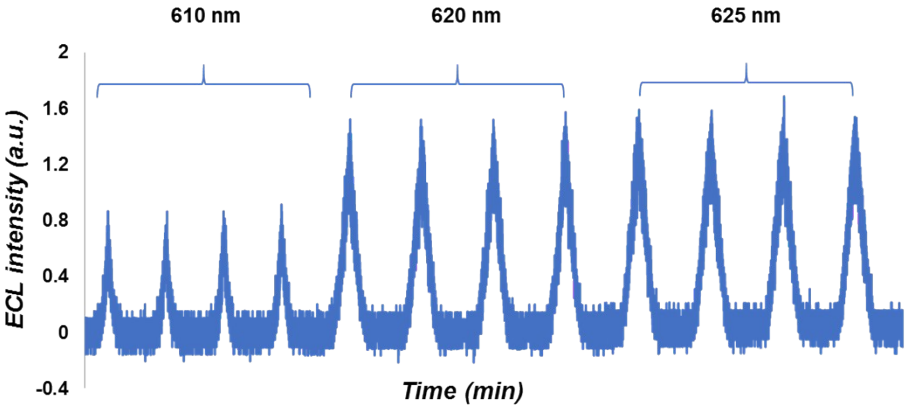

Fig. S4. The ECL signal-time curve of 10.0 mM Ru-$(bpy)_3^{2+}$ in the presence of $1.0x10^{-3}$ M NH_3 at three different emission wavelengths of 610, 620, and 625 nm, ECL signals were generated using CV from 0 V to 1.3 V for 4 cycles at a scan rate of 100 mV s^{-1}

In order to investigate the effect of the emission slit width of the PMT detector on the ECL signals, the slit widths were changed in the range of 2.5-20 and the ECL measurements were carried out using CV for 4 cycles at a potential range from 0 to +1.3 V at a scan rate of 100 mV s^{-1}. According to the results, shown in Fig. S5, the slit width of 20 which provided the highest ECL signal, was adjusted to acquire the ECL data.

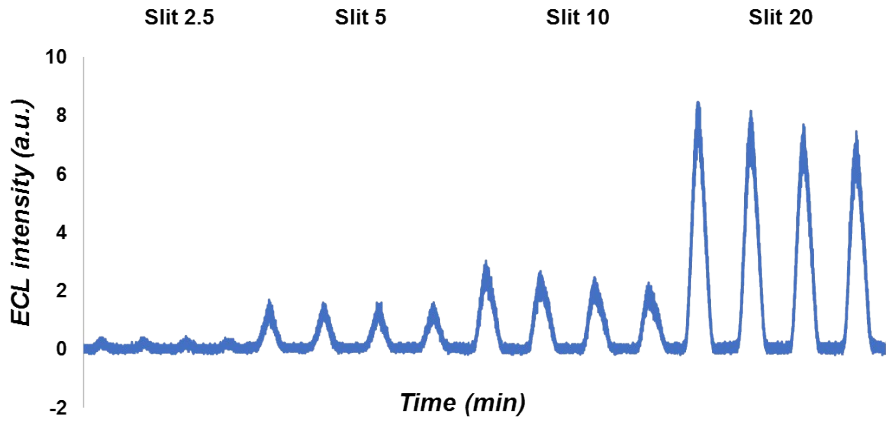

Fig. S5. The ECL signal-time curve of 10.0 mM Ru-$(bpy)_3^{2+}$ in the presence of $1.0x10^{-3}$ M NH_3 using four different slit widths in the range of 2.5 to 20, ECL signals were generated using CV from 0 V to 1.3 V for 4 cycles at a scan rate of 100 mV s^{-1}

Although the maximum slit width was used to acquire the ECL signals, the intensity of the signals of Ru-$(bpy)_3^{2+}$/NH_3 was not high enough. In order to increase the sensitivity of the developed system, the ECL measurements were carried out by increasing the PMT detector voltage. Therefore, the detector voltage was changed from 600V to 800V and 1000V, and the signals were obtained by setting the emission wavelength and the slit width to 625 nm and 20, respectively. While the intensity of the ECL signal acquired at 600V was observed at about 7 units, the ECL signal increased approximately 20 fold at 800V and 150 fold at 1000V, as shown in Fig. S6. As a result, the optimum ECL signal was obtained by adjusting the slit width to 20, the emission wavelength to 625 nm, and the detector voltage to 1000V. Accordingly, all experiments were carried out under the specified conditions.

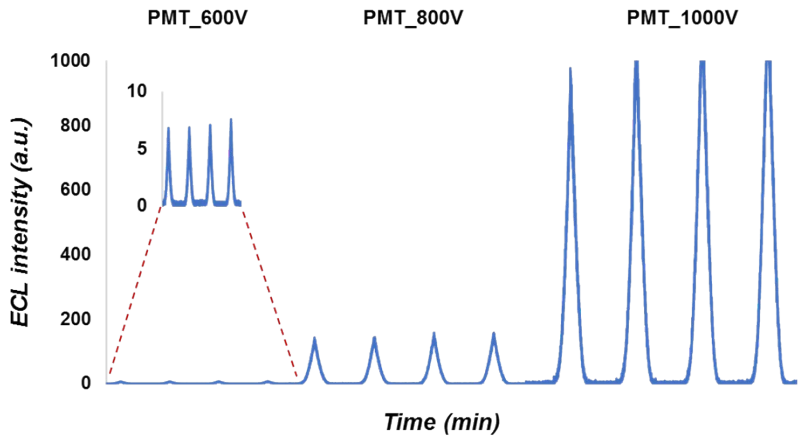

Fig. S6. The ECL signal-time curve of 10.0 mM Ru-$(bpy)_3^{2+}$ in the presence of $1.0x10^{-3}$ M NH_3 at the PMT detector voltages of 600V, 800V, and 1000V, ECL signals were generated using CV from 0 V to 1.3 V for 4 cycles at a scan rate of 100 mV s^{-1}

The optimization of the diameter of the WE

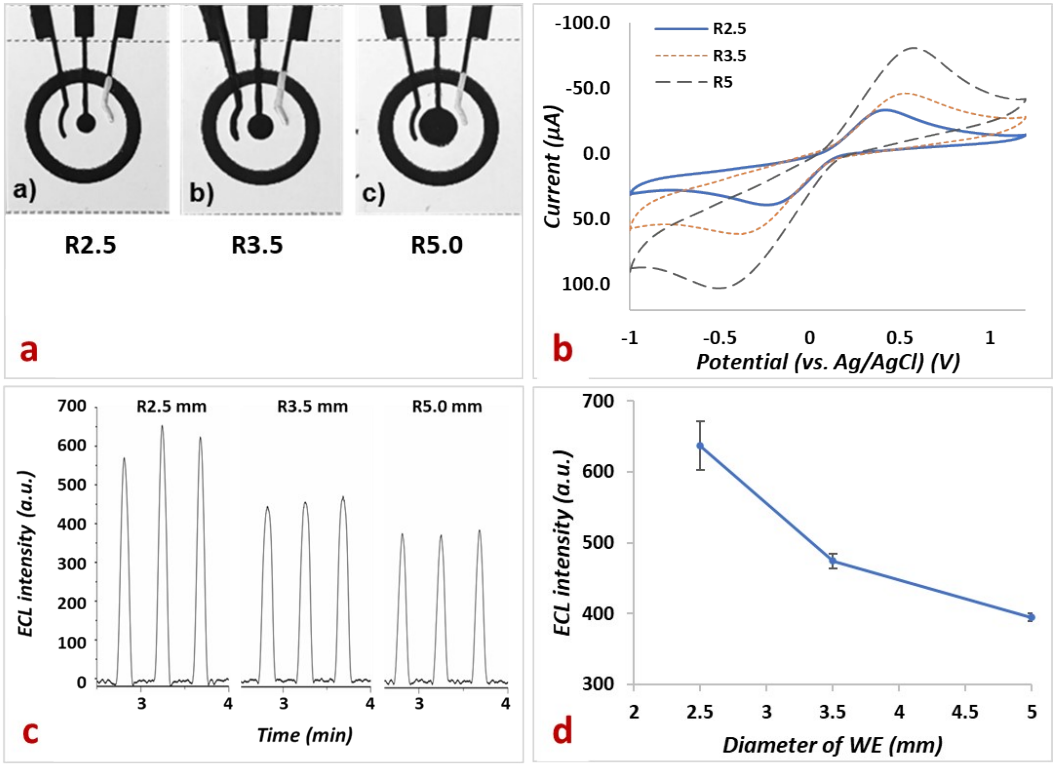

Fig. S7. a) The photographs of the PEs with three different WE diameters of 2.5, 3.5, and 5.0 mm, and **b)** the cyclic voltammetry responses of them at a scan rate of 100 mV s^{-1}, from -1.0 V to 1.2 V. **c)** The ECL signals of 10.0 mM Ru-$(bpy)_3^{2+}$ in the presence of 0.605 mM L-Phe by CV, at a scan rate of 100 mV s-1, from -0.5 V to 1.3 V, **d)** the comparison of ECL signal intensities using the PEs with three different WE diameters of 2.5, 3.5, and 5.0 mm

The recovery studies

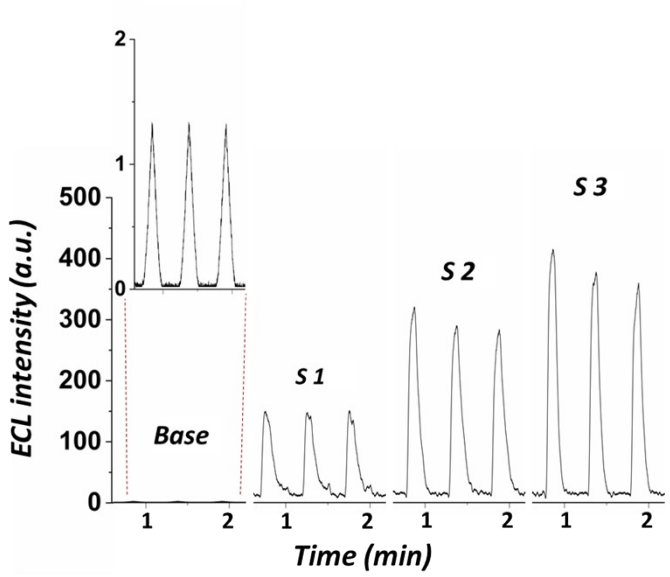

Fig. S8. The ECL signals of Ru-$(bpy)_3^{2+}$ in the presence of NH_3 generated as a consequence of the enzymatic reaction of L-Phe at three different concentrations of 0.061 mM (S1), 0.121 mM (S2), 0.303 mM (S3) in blood samples and blood sample without L-Phe (base) by CV in the potential between -0.5 V and 1.3 V, for 3 cycles, at a scan rate of 100 mV/s

HPLC-UV analysis for the determination of PAL enzyme activity

While the stock solution of L-Phe (10.0 mg/mL) was prepared by dissolving 100.0 mg of L-Phe powder in 10.0 mL of 25.0 M Tris-HCl buffer (pH 8.5), the stock solution of TCA (1.0 mg/mL) was prepared by dissolving 10.0 mg of TCA powder in 5.0 mL of ethanol solution and then diluted with 5.0 mL of Tris-HCl buffer. Before use, the stock solutions were stored in the refrigerator at 4°C. A mixture of L-Phe and TCA diluted 10-fold was injected into the HPLC system. The HPLC-UV chromatogram of the mixture was shown in Fig. S9, and the elution order was L-Phe (2.98 min.) and TCA (5.41 min.)

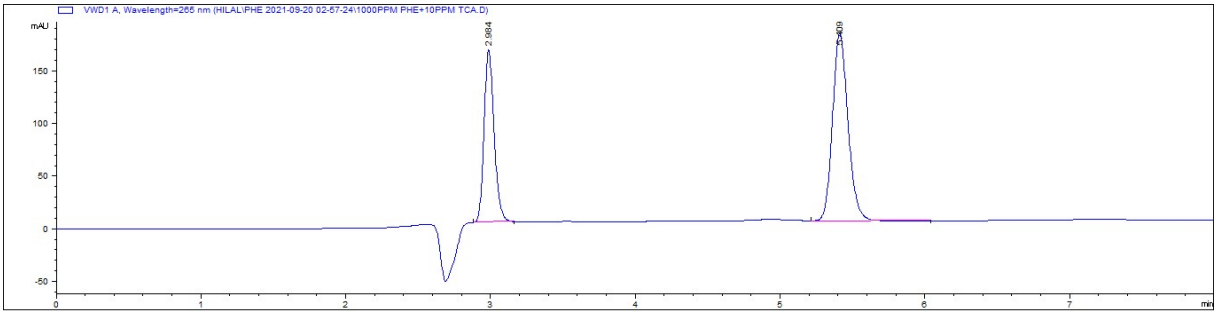

Fig. S9. The HPLC-UV chromatogram of L-Phe (1.0 mg/mL) and TCA (0.1 mg/mL)

In order to evaluate the enzyme activity of PAL, the L-Phe was interacted with a certain amount of PAL enzyme and the formation of TCA was monitored by a UV detector at 265 nm using HPLC analysis. For the analysis, the stock solution of L-Phe was diluted with Tris-HCl buffer to prepare the standard solutions at seven different concentrations from 2.5 µg/mL to 200.0 µg/mL. The enzyme activity of the commercial product (PAL from *Rhodo**torul**a glutinis*) was defined as the amount of enzyme required to convert 1.0 µmol of L-Phe to ammonia and TCA per minute. According to this product information, the amounts of required enzyme were calculated for 50.0 µL of L-Phe standards at variable concentrations (2.5-200.0 µg/mL).

Table S2. The amounts of PAL enzyme required to convert 50.0 µL of L-Phe to TCA

The concentration of L-Phe (µg/mL)	**Amount of PAL enzyme**	
	Calculated (µL)	**Added (µL)**
2.5	0.09	**1.0**
5.0	0.18	**1.0**
10.0	0.36	**2.0**
20.0	0.72	**2.0**
50.0	1.80	**2.0**
100.0	3.60	**4.0**
200.0	7.20	**8.0**

The L-Phe/PAL solutions were prepared by adding the amounts of enzyme indicated in Table S1 to the 50.0 mL of L-Phe solutions and incubated at 40°C for 30 minutes. Here, the enzyme solutions were added a little more than the calculated amounts to ensure that L-Phe was completely converted to TCA, as shown in Table S1. At the end of the incubation, 5.0 µL of 0.5 M HCl solution was added to the L-Phe/PAL solutions for terminating the reaction. These solutions were immediately injected into the HPLC-UV system, and the chromatograms were obtained under the aforementioned conditions.

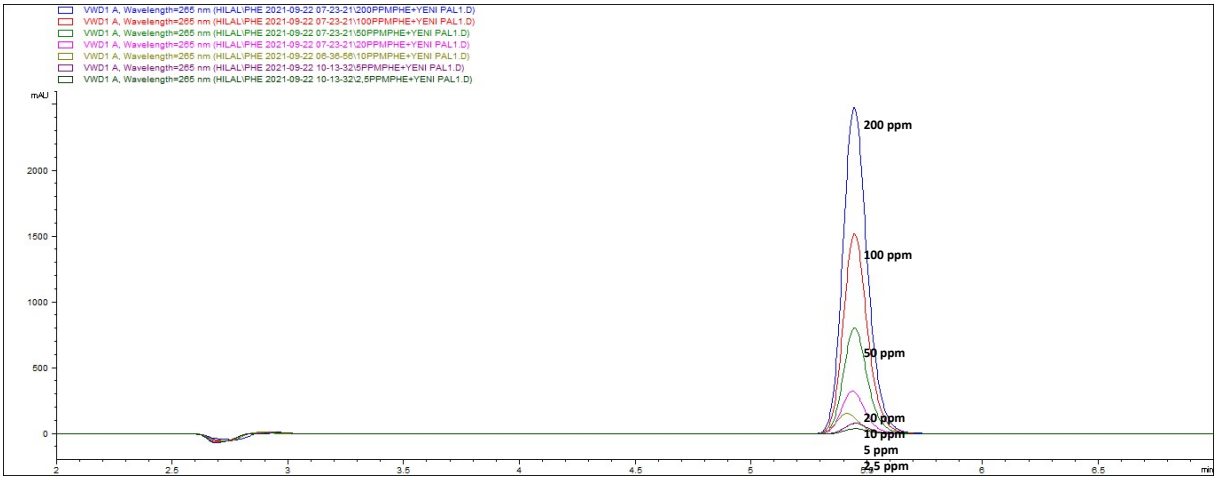

Fig. S10. The HPLC-UV chromatograms of TCA as an enzymatic reaction product of L-Phe (2.5-200.0 µg/mL) with PAL

The calibration curve was obtained in the range of 1.0-200.0 µg/mL for TCA standards to calculate the amounts of TCA formed as an enzymatic reaction product of L-Phe. For this purpose, the TCA standards at varying concentrations were prepared by diluting the stock solution of TCA (1.0 mg/mL) with Tris-HCl buffer. Then, the standard solutions were injected into the HPLC-UV system and the chromatograms were presented in Fig. S11a. The analytical figures of merit for the HPLC-UV based detection and assay variability of TCA were presented in Table S2 and Table S3, respectively.

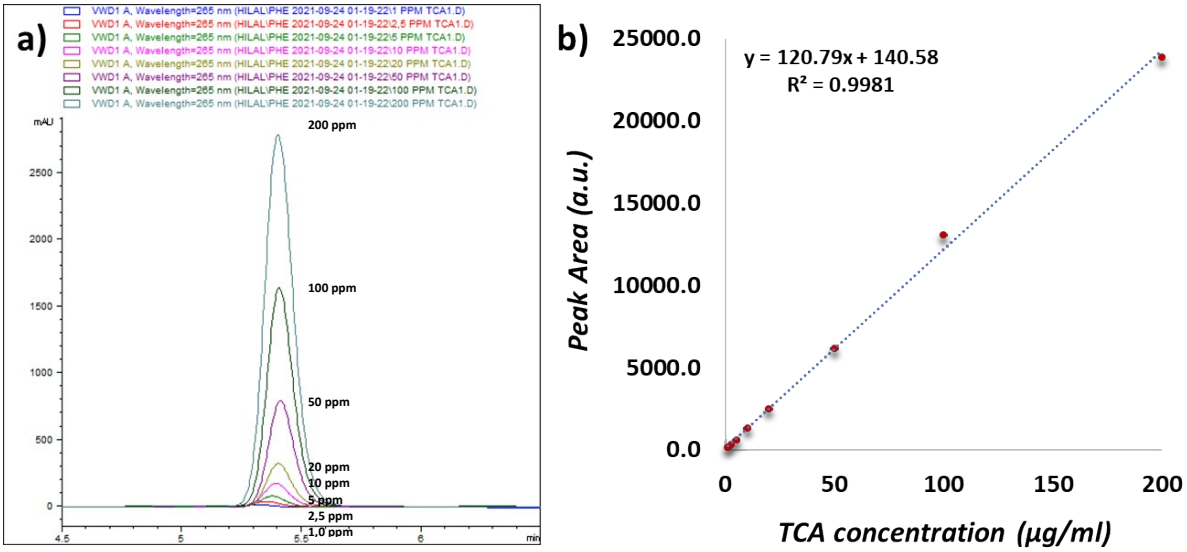

Fig. S11. a) The HPLC-UV chromatograms, **b)** the calibration plot of TCA in pH 8.5 Tris-HCl buffer at different concentrations in the range of 1.0-200.0 µg/mL

Table S3 Quantitative parameters for the HPLC-UV detection of TCA

	Regression equation	Correlation coefficient (r^2)	Linear range (µg/mL)	LOD (µg/mL)
TCA	y = 120.79x + 140.58	0.9981	1.0-200	0.14

Table S4 Assay variability of TCA in buffer solution (n=3), using the HPLC-UV method

TCA (µg/mL)	1.0	2.5	5.0	10.0	20.0	50.0	100.0	200.0
$\bar{x}$*	135.9	314.2	627.3	1335.0	2503.2	6176.6	13078.3	23881.6
SD	5.71	0.58	2.13	7.13	6.35	15.88	4.63	13.75
RSD, %	4.20	0.18	0.34	0.53	0.25	0.26	0.04	0.06

$\bar{x}$: Mean peak area*

The amounts of TCA formed as an enzymatic reaction product of L-Phe were calculated by using the regression equation and the results were compared with the theoretically calculated values. The recoveries of TCA were found in the range of 103.0-109.8%, except for 2.0 µg/ml of TCA standard. When this concentration was omitted from the range, the average recovery was found as 106.4%, with an RSD of 3.03%. These results indicated that the PAL from *Rhodo**torul**a glutinis* worked at approximately a hundred percent efficiency. Moreover, the chromatogram as shown in Fig. S10 illustrated that the conversation ratio was 100% after the incubation since the peak of L-Phe was not observed. Therefore, it was decided that the PAL from *Rhodo**torul**a glutinis* could be used in the developed ECL system for the determination of L-Phe.

Table S5 The recoveries of TCA formed as an enzymatic reaction product of L-Phe, using the HPLC-UV method

TCA concentration (µg/mL)			
Theoretical	**Found**	**Recovery %**	**Parameters**
2.0	1.3 ± 0.05	64	
4.0	4.1 ± 0.52	102	$\bar{x}$=106.4[a]
7.9	8.1 ± 0.28	102	SD = 3.23
15.9	17.4 ± 1.73	110	RSD. % = 3.03
39.6	43.1 ± 6.79	109	CI =109.8 – 103.0[b]
76.6	83.6 ± 5.25	109	(*P* = 0.05)
143.5	152.8 ± 1.00	106	

[a]$\bar{x}$ = Mean

[b]CI = Confidence interval

L-Phe detection using the HPLC-UV method in a blood sample

We compared the results obtained using the developed method with an HPLC method. For this purpose, stock solution of the L-Phe was spiked into the reference blood sample as to be the concentrations of 10.0, 20.0 and 50.0 µg/mL. The final volume of the solutions was adjusted to 0.5 mL with Tris-HCl buffer. In order to remove blood cells and proteins, the prepared samples were transformed into centrifugal devices (Amicon® Ultra 0.5 mL Centrifugal Filters, 10K-MWCO) and centrifuged for 10 min at 14000 g. The filtered samples were injected directly into the HPLC-UV system. The obtained chromatogram was shown in Fig. S12.

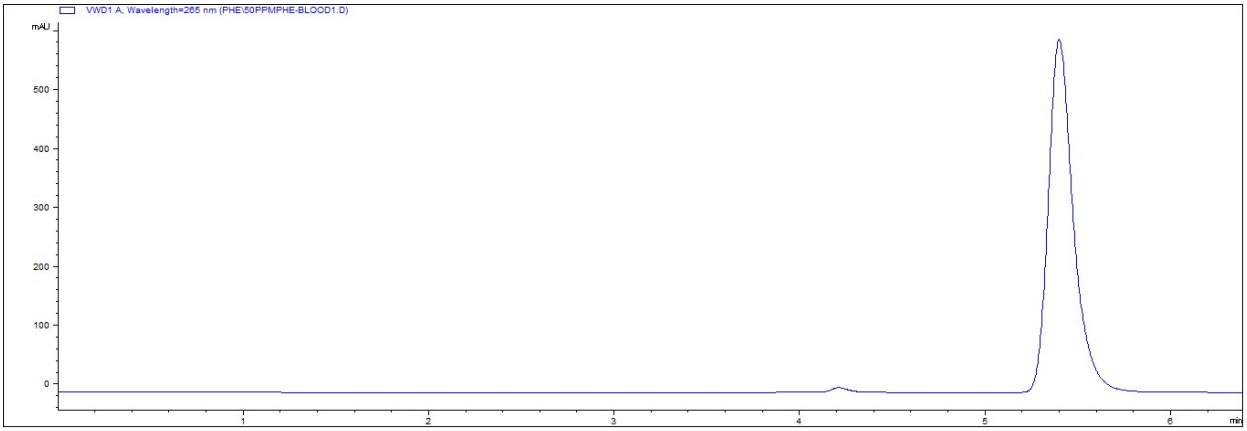

Fig. S12. The HPLC-UV chromatogram of TCA as an enzymatic reaction product of 50.0 µg/mL of L-Phe with PAL in a blood sample