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Emergency Diagnosis Made Easy: Matrix Removal and Analyte Enrichment from Raw Saliva using Paper-Arrow Mass Spectrometry

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Supplementary Methods

1. Chemicals and Reagents

Paracetamol (meets USP testing specifications, 98.0-102.0%), paracetamol-D4 (100 µg/mL in methanol), iron(III) chloride (reagent grade, 97%), potassium ferricyanide(III) (99%), and formic acid (reagent-grade, ≥ 95%), were purchased from Sigma–Aldrich (St. Louis, Mo, USA). Ammonium formate (99%), methanol (99.8%, HPLC grade) and hydrochloric acid (1M) were purchased from Fisher Scientific (Loughborough, UK). Ethyl acetate (≥ 99.5 %) was purchased from Merck KGaA (Darmstadt, Germany). Water was purified using a Milli-Q Advantage A10 water purification system (Millipore, MA, USA) before use in this study. Chromatography paper (25mm thick, Grade 1) was purchased from Whatman (Maidstone, UK).

2. Standard solution preparation

A standard stock solution of paracetamol (2 mg/mL) was prepared with pure methanol and stored at -20 °C. 100 µg/mL paracetamol diluted from 2 mg/mL stock solution using water or blank saliva was used as a working solution. Stock solutions of iron(III) chloride (0.2M), potassium ferricyanide(III) (0.02M) and ammonium formate (1M) were prepared with water and stored at 4°C.

3. Mass spectrometry analysis

3.1. Instruments and software for PS-MS/MS and PA-MS/MS

PS-MS/MS and PA-MS/MS were performed with a Thermo Scientific Orbitrap Exploris 240 mass spectrometer (Thermo Fisher, Waltham, MA, USA). For paper spray ionisation, the paper was held by a copper clip at its base to provide an electrical connection and placed 3 mm from the inlet of the mass spectrometer. The spray solvent, 9:1 methanol: water (v/v) with 0.5 % formic acid and 10 mM ammonium formate, was automatically pumped onto the centre of the paper at a rate of 500 µL/min during 0.01-0.09 min using the instrument's syringe pump. The ion source conditions were set as: spray voltage, +3.5 kV; ion transfer tube temperature, 320 ºC; without nebuliser gas supply. Nitrogen was used as the collision gas. Multiple reaction monitoring (MRM) transitions were: *m*/*z* 152.0706 → 110.060 (quantifier) and *m*/*z* 152.0706 → 65.071 (qualifier) for paracetamol, and *m*/*z* 156.0957 → 114.085 (quantifier) and *m*/*z* 156.0957 → 69.090 (qualifier) for paracetamol-D4. The normalised HCD collision energy was set at 70%. The spray voltage was applied to induce an electrospray event from 0.10 min and stopped at 0.45 min. The 0.35 min voltage application cycle was repeated three more times to generate a total of four peaks in one chromatogram. The total run time was 1.66 min. The data acquisition was under the control of Thermo Scientific Xcalibur software and data processing was completed using the Xcalibur Quan Browser.

3.2. Instruments and software for UPLC-MS/MS method

UPLC was carried out on a Waters ACQUITY UPLC system (Waters Corporation, Milford, MA). The injection volume was 3 µL. UPLC separation was performed on a Waters ACQUITY UPLC BEH C18 column (2.1 mm x 50 mm, 1.7 µm) with a BEH C18 guard column (2.1 mm x 5 mm, 1.7 µm). The mobile phase consisted of combinations of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in methanol, v/v) at a flow rate of 0.3 mL/min with an elution gradient as follows: 0 min, 5% B; 3 min, 30% B; 3.01-4.5 min, 95% B. A 2.5-min post-run time was set to fully equilibrate the column. Column and sample chamber temperatures were 40 ºC and 6 ºC, respectively. Mass spectrometry analysis was conducted by a Waters Xevo triple quadrupole mass spectrometer (Waters, Milford, USA) with electrospray ionisation in positive mode. Desolvation and cone gases used nitrogen set at 1000 L/h and 150 L/h, respectively. The desolvation and source temperatures were kept at 600 ºC and 150 ºC, respectively. The source capillary voltage was 3.7 kV. Argon was used as the collision gas. The MRM transitions were: *m*/*z* 151.94 → 109.95 as a quantifier and *m*/*z* 151.94 → 92.84 as a qualifier for paracetamol and *m*/*z* 155.96 → 114.05 as quantifier and *m*/*z* 155.96 → 96.69 as qualifier for paracetamol-D4. The optimised parameters for the four MRM transitions were: cone voltage, 46, 46, 38 and 38V, respectively; collision energy: 16, 22, 15 and 21 eV, respectively. The dwell time was 0.1 s per transition. Peaks were integrated using MassLynx V4.1 SCN 901 (Waters, Milford, USA) and the peak area ratio of the quantifiers of paracetamol and paracetamol-D4 were used for quantification.

4. Chromatography paper preparation

Chromatography paper was cut into different shapes (Figure S1) with a digital template using a 40 W laser cutter at 20% speed and 10% cutting power (HPC Laser Ltd, UK), and then these pieces of paper were cleaned under sonication with methanol for 5 min, followed by water for 5 min and then methanol for 5 min. They were dried in air overnight before use.

5. Human Resting Saliva Collection

The collection and preparation of human saliva was conducted with requisite ethical approval (approval number: 10058) by the ethical committee of the University of Liverpool. Informed consent from participants was obtained. Volunteers were restricted from intake of any food or drinks for at least 1 hr prior to sample collection. Human whole saliva was spat into a vial after being passively pooled at the bottom of the mouth for 2 min.[1] Saliva samples were collected and analysed on the same day without any sample storage for re-usage.

6. Staining method for visualisation of paracetamol movement on arrow-shaped paper

The migration of paracetamol on arrow-shaped paper after PC was compared with that of traditional triangular paper. It was visualised by a colourimetric method based on a redox reaction reported in the literature [2-4] with slight modifications. 20mM iron(III) chloride, 10mM potassium ferricyanide(III) ($K_3Fe(III)(CN)_6$) and 0.2M hydrochloric acid in water was sprayed onto the paper by a fine mist sprayer. The spray solution was freshly made from the stock solutions (Supplementary method 2) no longer than 2 hours in advance of the experiment. After drying under ambient

conditions, paracetamol present on paper assumed the colour of Prussian blue (potassium ferric ferrocyanide), whose chemical formula is $KFe(III)[Fe(II)(CN)_6]$. Photos in Figures 5b and S6 were taken within 2-5 mins after spraying with the dye solution; the colour appears slightly different due to time variation between photographs being taken.

Supplementary Figures

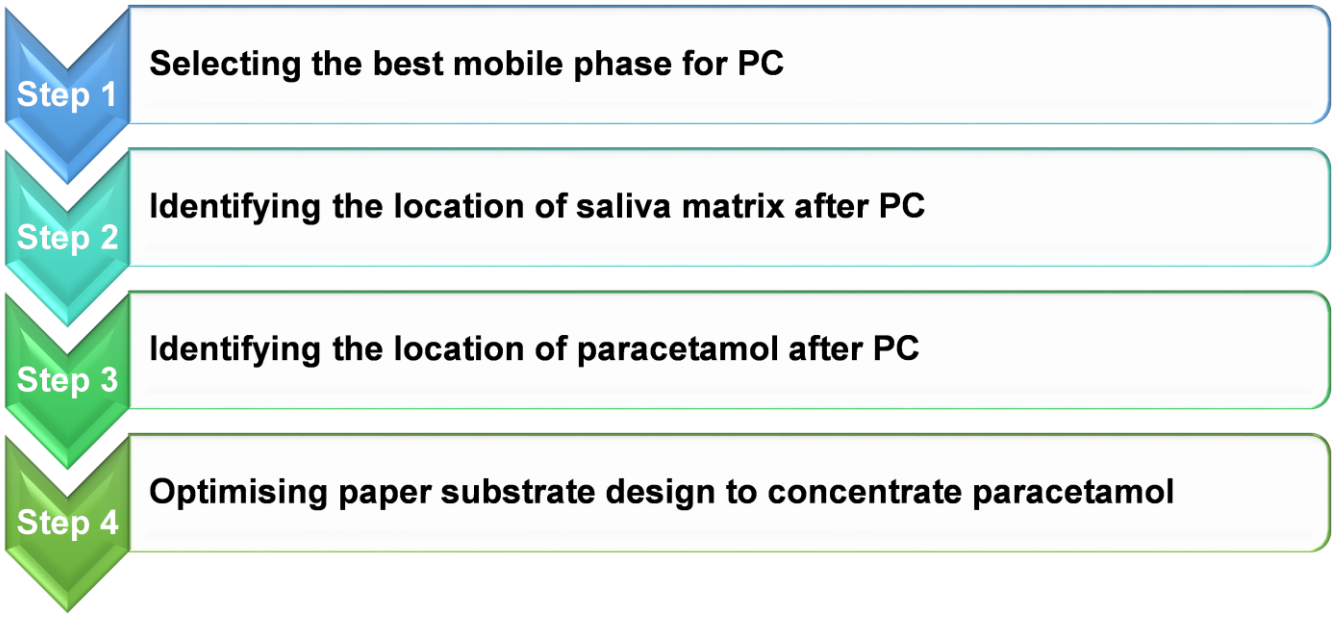

Figure S1. A flowchart showing the main method development steps for PA-MS. Whilst the flow chart relates to paracetamol in saliva, the general process could be applied generally to other analytes in a variety of matrices.

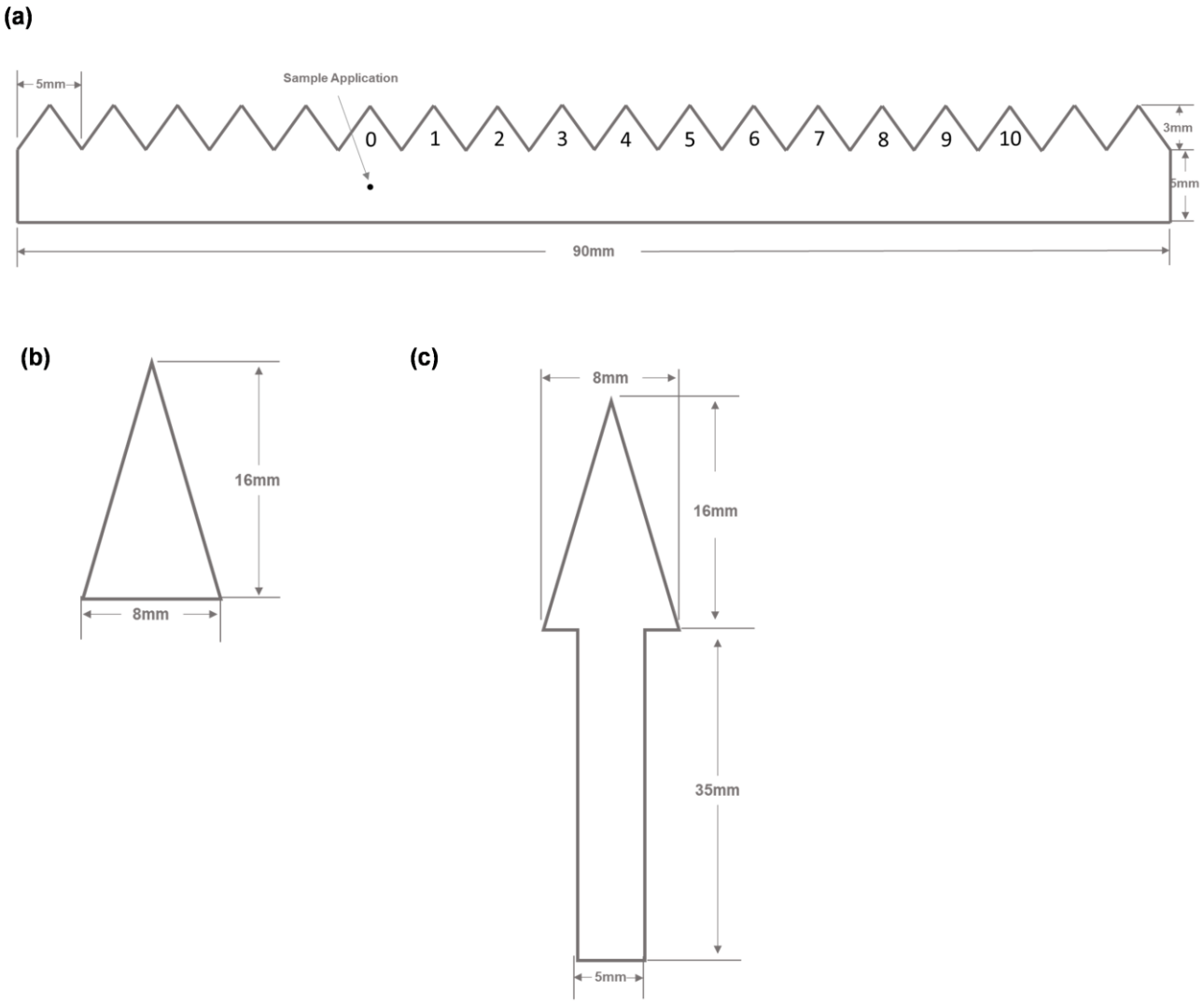

Figure S2. Dimensions of three shapes of chromatography paper used in this study. (**a**) Serrated paper strips used to determine the location of salivary components and paracetamol (for method development and paper arrow design purposes). (**b**) Triangular paper used for traditional PS-MS analysis. (**c**) Finalised arrow-shaped paper strips used for PA-MS.

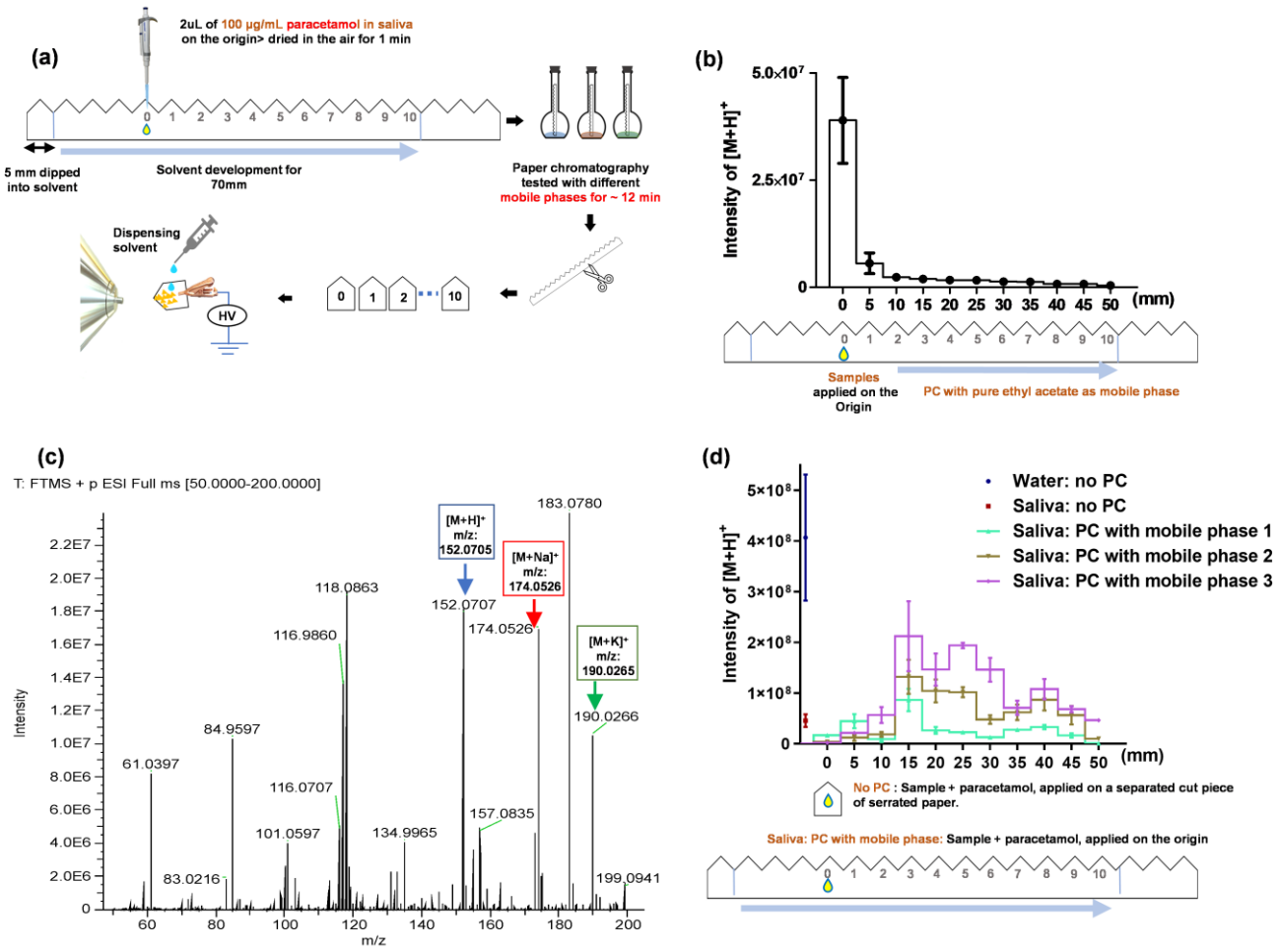

Figure S3. Workflow and result of mobile phase screening for PA-MS. (**a**) Workflow of mobile phase screening for PA-MS, where 2 µL of saliva spiked with 100 µg/mL paracetamol was applied at the origin and subjected to 12 min of PC using different mobile phases. When the front of the mobile phase reached the 10th region from the origin, the paper strip was taken out of the flask and dried in air for 1 min. Then individual regions, labelled 0-10, were cut apart manually and the signal intensities of the protonated paracetamol ion, $[M+H]^+$, on each piece were acquired by MS analysis. (**b**) Intensity of $[M+H]^+$ after PC with pure ethyl acetate. (**c**) Full scan mass spectrum at the origin after PC with pure ethyl acetate. (**d**) Intensities of $[M+H]^+$ in water or saliva with or without PC. 2 µL of 100 µg/mL paracetamol in water was applied on a separated piece of serrated paper, and then $[M+H]^+$ was detected without PC, which is indicated by the label of "Water-no PC". 2 µL of 100 µg/mL paracetamol in saliva was also applied on a separated piece of serrated paper, and then $[M+H]^+$ was detected without PC, which is shown with label of "Saliva-no PC". 2 µL of saliva spiked with 100 µg/mL paracetamol was applied at the origin. PC was conducted separately with three different mobile phases: mobile phase 1, 9:1(v/v) ethyl acetate: formic acid; mobile phase 2, 9:1(v/v) of ethyl acetate: formic acid with 10 mM ammonium formate; mobile phase 3, 9:1(v/v) of ethyl acetate: formic acid with 50 mM ammonium formate. The data are expressed as mean ± SD (n =3).

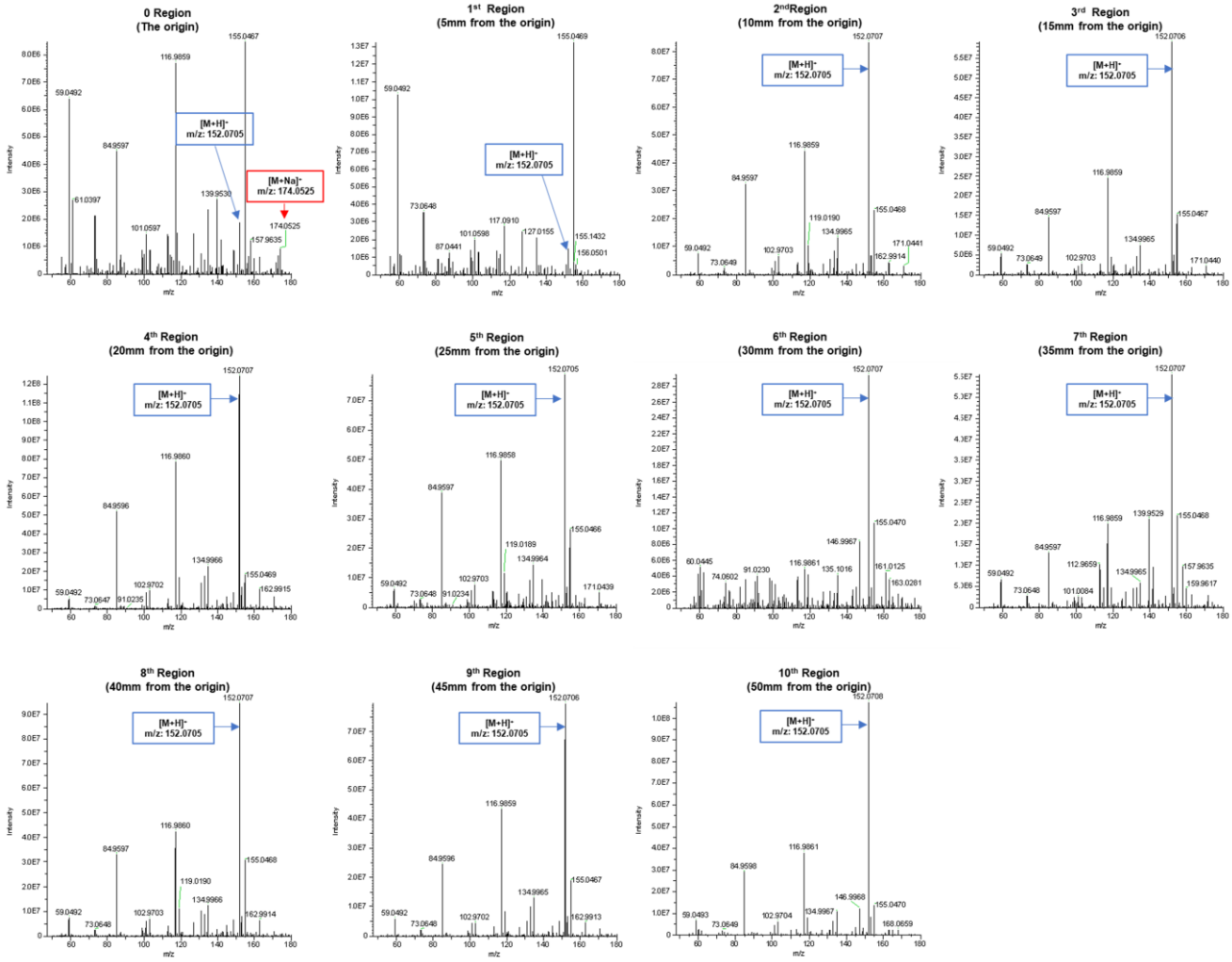

Figure S4. Average mass spectra of full scans from Regions 0-10. Blank saliva (2 µL) was applied at the origin and dried in air. After 12 min of PC with the optimised mobile phase [9:1 ethyl acetate: formic acid (v/v) with 50 mM ammonium formate], Regions 0-10 were cut apart. 2uL of 100 µg/mL paracetamol in water was added onto each piece of paper. After air-drying, MS analysis was conducted. Region 4 gave the highest signal intensity of the paracetamol ion.

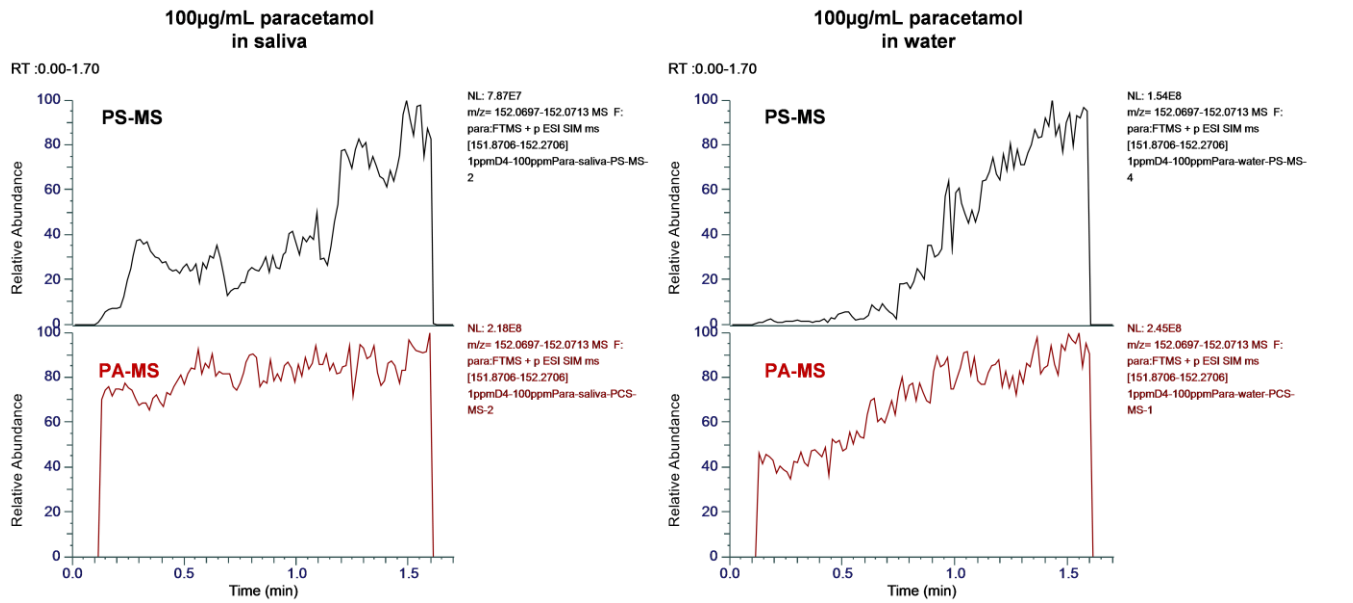

Figure S5. Selected ion monitoring (SIM) chromatograms of $[M+H]^+$ were obtained for 2µL of saliva and water spiked with 100 µg/mL paracetamol applied onto triangular paper for PS-MS and onto arrow-shaped paper for PA-MS. The chromatograms are shown with a mass tolerance of 5 ppm centred around the protonated molecular ion, *m*/*z* 152.0705.

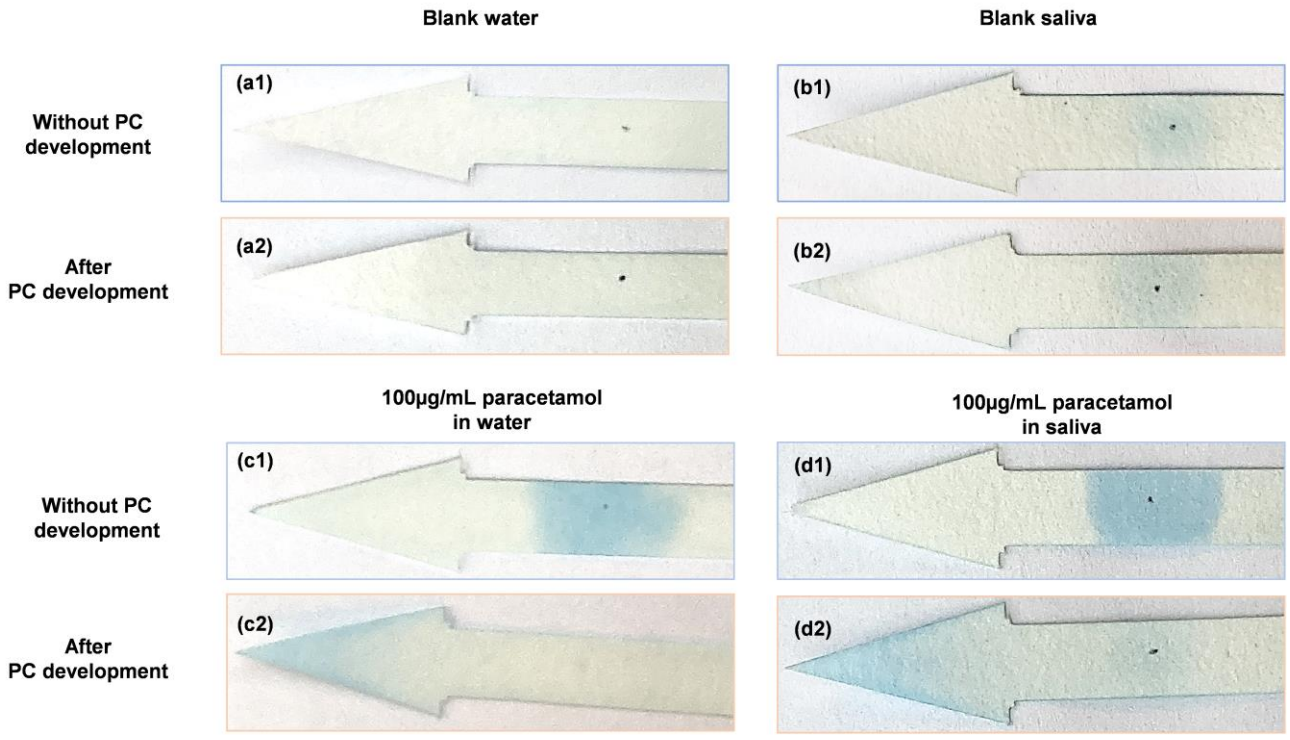

Figure S6. Staining results. 2µL of a sample was applied onto the site marked with a pencil: (**a**) blank water, (**b**) blank saliva, (**c**) blank water spiked with 100 µg/mL paracetamol, (**d**) saliva spiked with 100 µg/mL paracetamol. This was stained with Prussian Blue spray as described in Supplementary Method 6, (**1**) without or (**2**) after 5-min PC development.

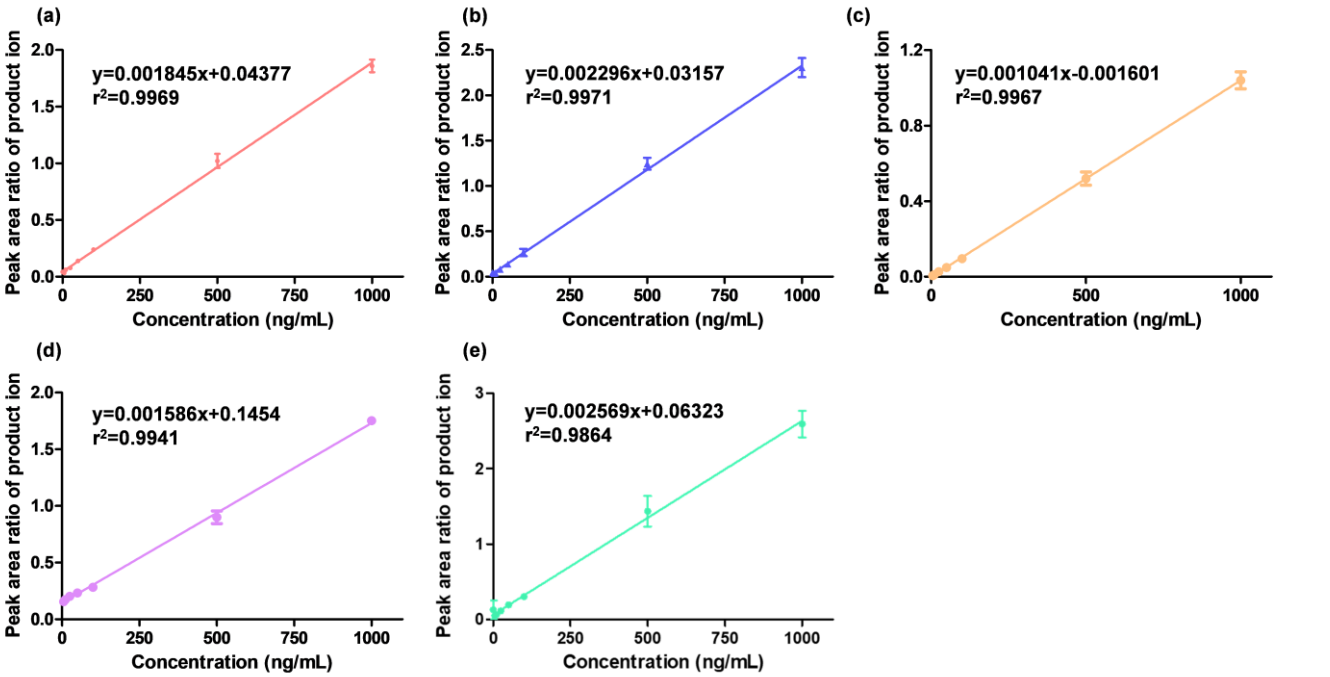

Figure S7. Comparison of calibration curves of three matrices spiked with paracetamol (5-1000 ng/mL) detected by different methods. The matrices and paracetamol detection methods were: (**a**) raw saliva detected by PA-MS/MS, (**b**) water detected by PA-MS/MS, (**c**) pre-treated saliva detected by UPLC-MS/MS, (**d**) pre-treated saliva detected by PS-MS/MS, and, (**e**) raw saliva detected by PS-MS/MS. The data points are expressed as mean ± SD (n =3).

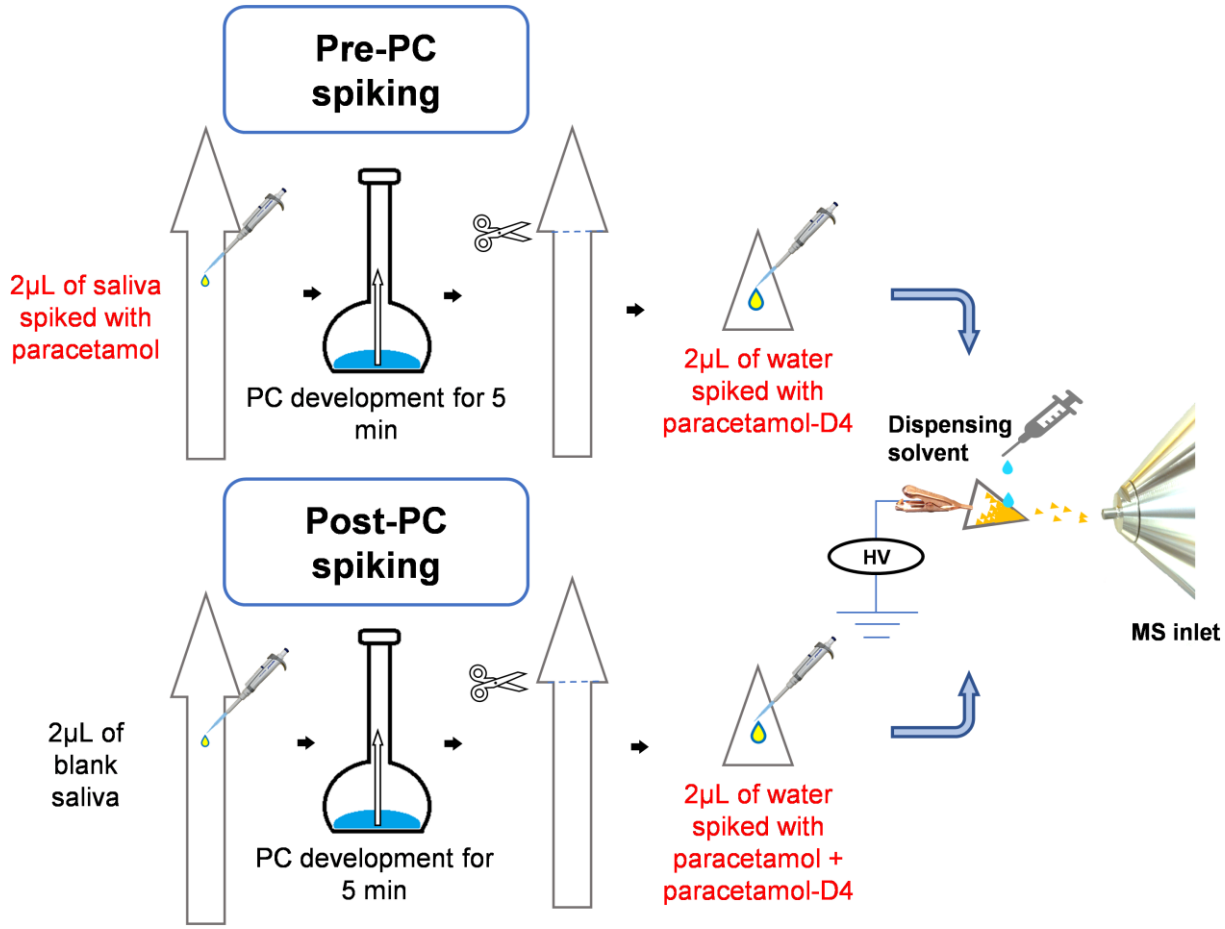

Figure S8. Procedure for extraction recovery experiment of spiked paracetamol in saliva by PA-MS/MS.

Supplementary Tables

Table S1. Summary of demographic information from seven participants.

Gender	Age (years)	Ethnicity	Resting saliva flow rate (g/min)
Female: 4 Male: 3	36 ± 11	Asian: 3 White: 3 Others: 1	0.28 ± 0.22

Table S2. Precision and accuracy of paracetamol in raw saliva detected by PA-MS/MS (5-1000 ng/mL).

Theoretical concentration (ng/mL)	Peak area ratio		CV	Calculated concentration (ng/mL)	Accuracy (bias)	Mean ratio of S/N[a]
	Mean	SD				
5	0.032	0.005	14.2%	-6.38	-228%	135.93
10	0.061	0.002	3.5%	9.07	-9.3%	82.022
25	0.078	0.003	4.0%	18.28	-26.9%	210.40
50	0.139	0.005	3.4%	51.62	3.2%	185.70
100	0.240	0.008	3.5%	106.36	6.4%	307.36
500	1.021	0.063	6.1%	529.84	6.0%	425.71
1000	1.860	0.055	3.0%	984.23	-1.6%	2.64*E16

[a] Ratio of S/N is the signal intensity of the product ion *m*/*z* 110.06 over the signal intensity of the background noise.

Table S3. Precision and accuracy of paracetamol in raw saliva, detected by PS-MS/MS (5-1000 ng/mL).

Theoretical concentration (ng/mL)	Peak area ratio Mean	SD	CV	Calculated concentration (ng/mL)	Accuracy (bias)
5	0.050	0.012	24.0%	-5.15	-203%
10	0.063	0.011	16.9%	-0.22	-102%
25	0.114	0.010	8.9%	19.83	-20.7%
50	0.195	0.037	19.1%	51.10	2.2%
100	0.304	0.016	5.4%	93.66	-6.3%
500	1.436	0.201	14.0%	534.17	6.8%
1000	2.591	0.177	6.8%	983.82	-1.6%

Table S4. Precision and accuracy of paracetamol in pure water, detected by PA-MS/MS (5-1000 ng/mL).

Theoretical concentration (ng/mL)	Peak area ratio Mean	SD	CV	Calculated concentration (ng/mL)	Accuracy (bias)
5	0.037	0.003	9.4%	2.36	-52.7%
10	0.041	0.006	14.0%	3.96	-60.4%
25	0.081	0.007	8.7%	21.64	-13.4%
50	0.138	0.009	6.1%	46.50	-7.0%
100	0.267	0.041	15.4%	102.54	2.5%
500	1.246	0.066	5.3%	528.72	5.7%
1000	2.306	0.106	4.6%	990.61	-0.9%

Table S5. Precision and accuracy of paracetamol in pre-treated saliva, detected by UPLC-MS/MS (5-1000 ng/mL).

Theoretical concentration (ng/mL) (n = 3)	Peak area ratio		CV	Calculated concentration (ng/mL)	Accuracy (bias)
	Mean	SD			
5	0.005	0.001	11.8%	6.68	33.5%
10	0.011	0.001	9.4%	11.72	17.2%
25	0.026	0.005	17.7%	26.62	6.5%
50	0.049	0.005	10.8%	48.47	-3.1%
100	0.096	0.004	4.6%	93.75	-6.2%
500	0.519	0.050	9.7%	500.57	0.1%
1000	1.040	0.063	6.1%	1000.10	<0.1%

Table S6. Precision and accuracy of paracetamol in pre-treated saliva, detected by PS-MS/MS (5-1000 ng/mL).

Theoretical concentration (ng/mL) (n = 3)	Peak area ratio		CV	Calculated concentration (ng/mL)	Accuracy (bias)
	Mean	SD			
5	0.156	0.044	28.3%	6.82	36.4%
10	0.172	0.0301	17.9%	16.77	67.7%
25	0.203	0.028	13.6%	36.30	45.2%
50	0.233	0.0189	8.1%	55.20	10.4%
100	0.282	0.021	7.5%	86.23	-13.8%
500	0.900	0.096	10.7%	476.06	-4.8%
1000	1.752	0.033	1.9%	1012.84	1.3%

Table S7. Precision and accuracy of salivary paracetamol detected by PA-MS/MS (0.2-200 μg/mL).

Theoretical concentration (μg/mL) (n =5)	Peak area ratio		CV	Calculated concentration (μg/mL)	Accuracy (bias)
	Mean	SD			
0	0.052	0.008	15.9%	0.011	-
0.2	0.265	0.003	1.1%	0.24	19.1%
1	1.118	0.007	0.7%	1.15	15.3%
5	5.037	0.10	1.9%	5.35	7.0%
10	9.972	0.20	2.0%	10.6	6.3%
25	24.10	0.27	1.1%	25.8	3.1%
50	44.49	1.87	4.2%	47.6	-4.8%
100	93.39	1.40	1.5%	100.0	-0.03%
200	187.2	5.89	3.1%	200.5	0.2%

Table S8. Comparison between PA-MS/MS and UPLC-MS/MS with regards to the sample volume, solvent volume and time required for analysis.

Experiment parameter	PA-MS/MS [a]	UPLC-MS/MS [b]
Sample volume per test	2 μL	50 μL
Sample preparation time	~5 min[a]	~55 min[b]
Solvent volume for sample preparation	25 μL	200 μL
MS running time	~1.6 min	~7.5 min
Solvent volume for MS analysis	40 μL	~2500 μL

[a] The sample was prepared by a 5-min process of paper chromatography before MS detection.
[b] 50 μL sample was deproteinated with 200 μL methanol (1:4, v/v), stood at -20 ºC for 30 min, and then centrifuged for 20 min at 14000 rpm at 4 ºC. The supernatant was diluted with water (1:4, v/v).

Supplementary References

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