Supplementary material to New Journal of Chemistry

# Dual design spaces for micro-extraction together with core–shell chromatographic determination of dorzolamide and timolol in rabbit plasma: An example of quality by design method development.

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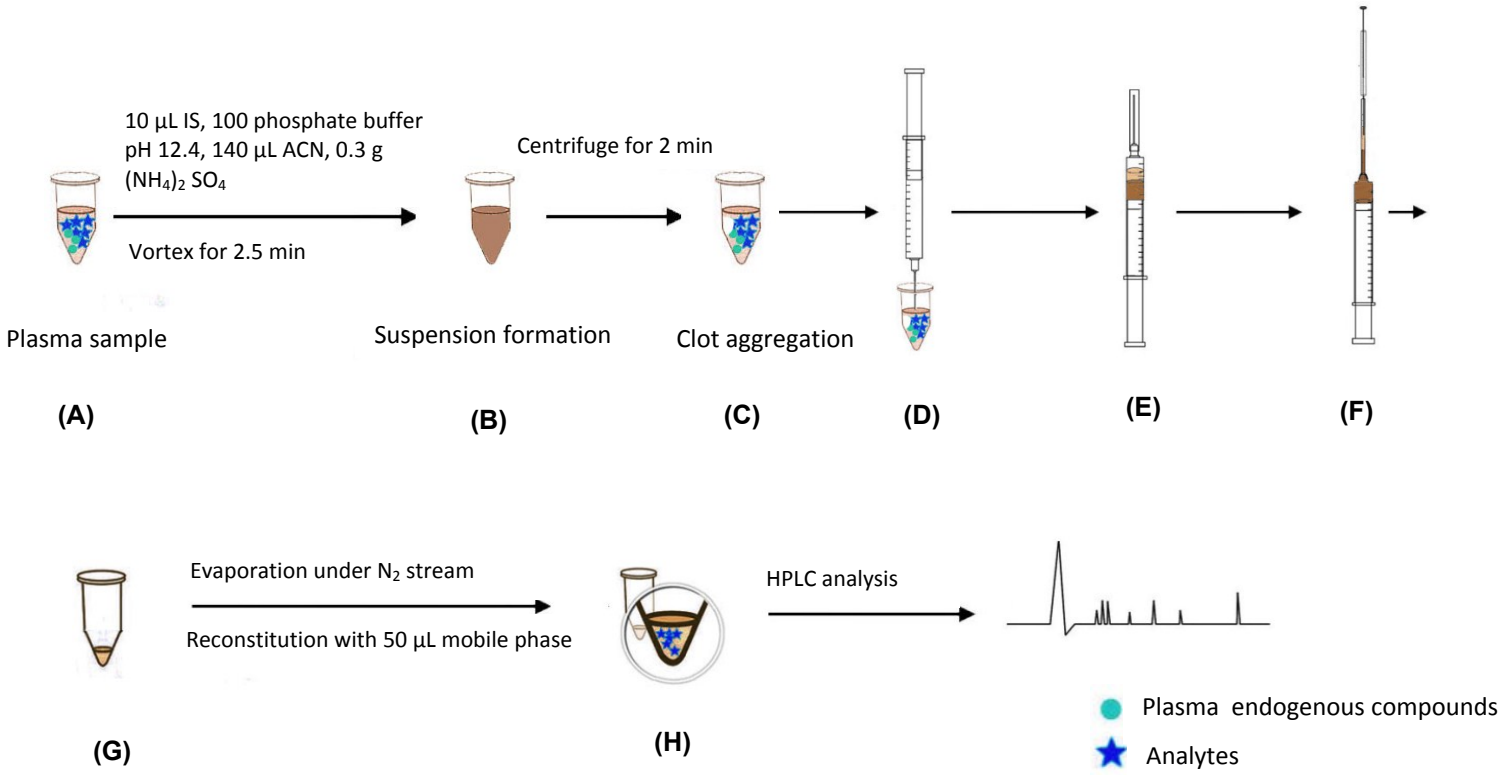


**Fig. S1.** Schematic representation of the VA-SALLME procedure.

**(A):** Plasma sample.

**(B):** A suspension was formed.

**(C):** The precipitates were aggregated.

**(D):** All the solution was withdrawn into a 1-mL syringe.

**(E):** The syringe left to stand statically upside down, two separate phases could be easily observed.

**(F):** The plunger was slowly pushed to move the upper layer phase to the narrow capillary tube and was sucked using a 100 µL micro-syringe.

**(G):** The organic phase was sucked into an Eppendorf vial.

**(H):** The dried residue was reconstituted with 50 µL of the mobile phase.

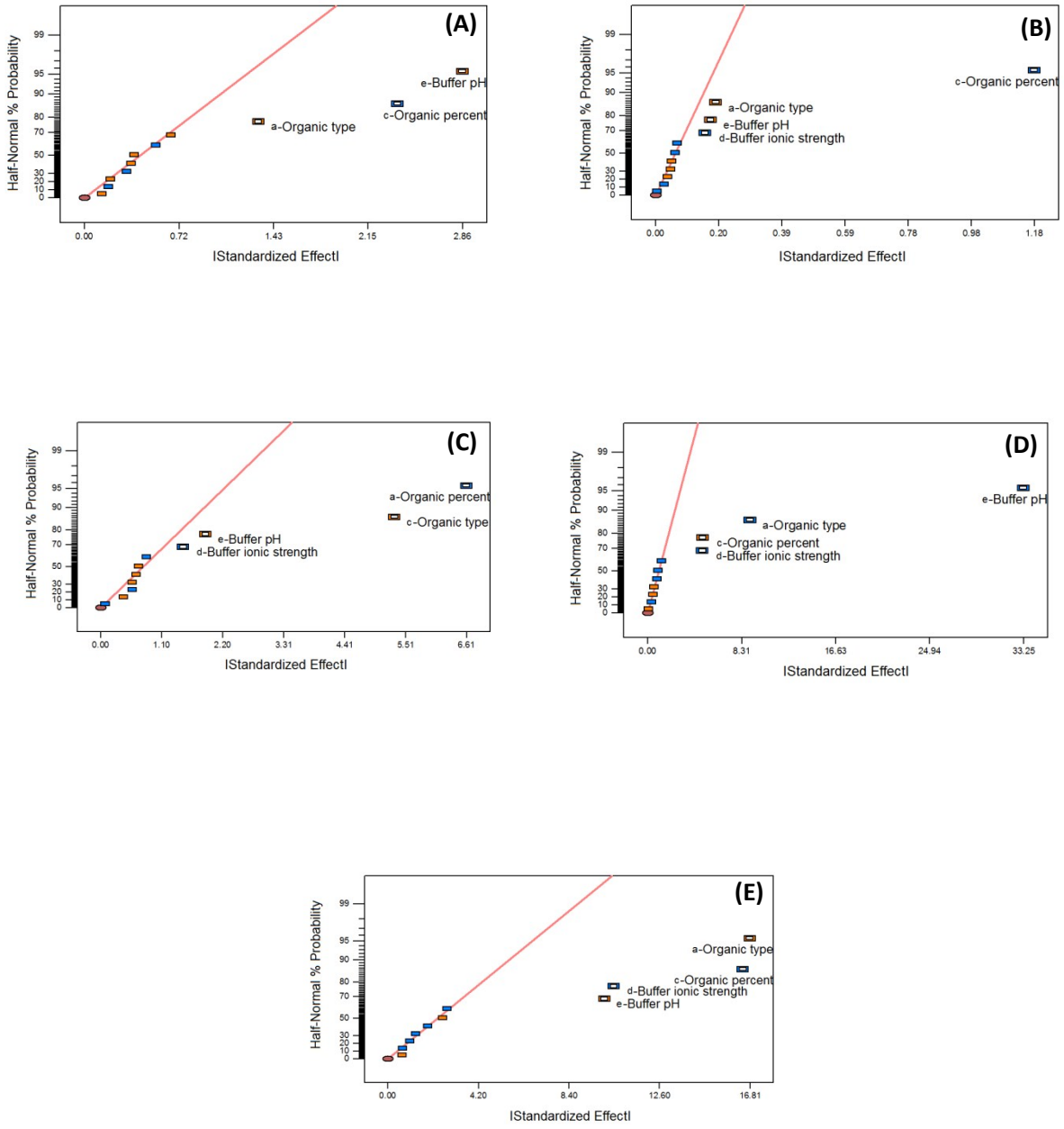


**Fig. S2.** Half normal probability plots of the standardized effects in a Plackett–Burman screening design of the proposed HPLC method for (A): DOR retention time, $T_1$; (B): IS retention time, $T_2$; (C): TIM retention time, $T_3$; (D): resolution between DOR and IS, $R_1$ and (E): resolution between IS and TIM, $R_2$.

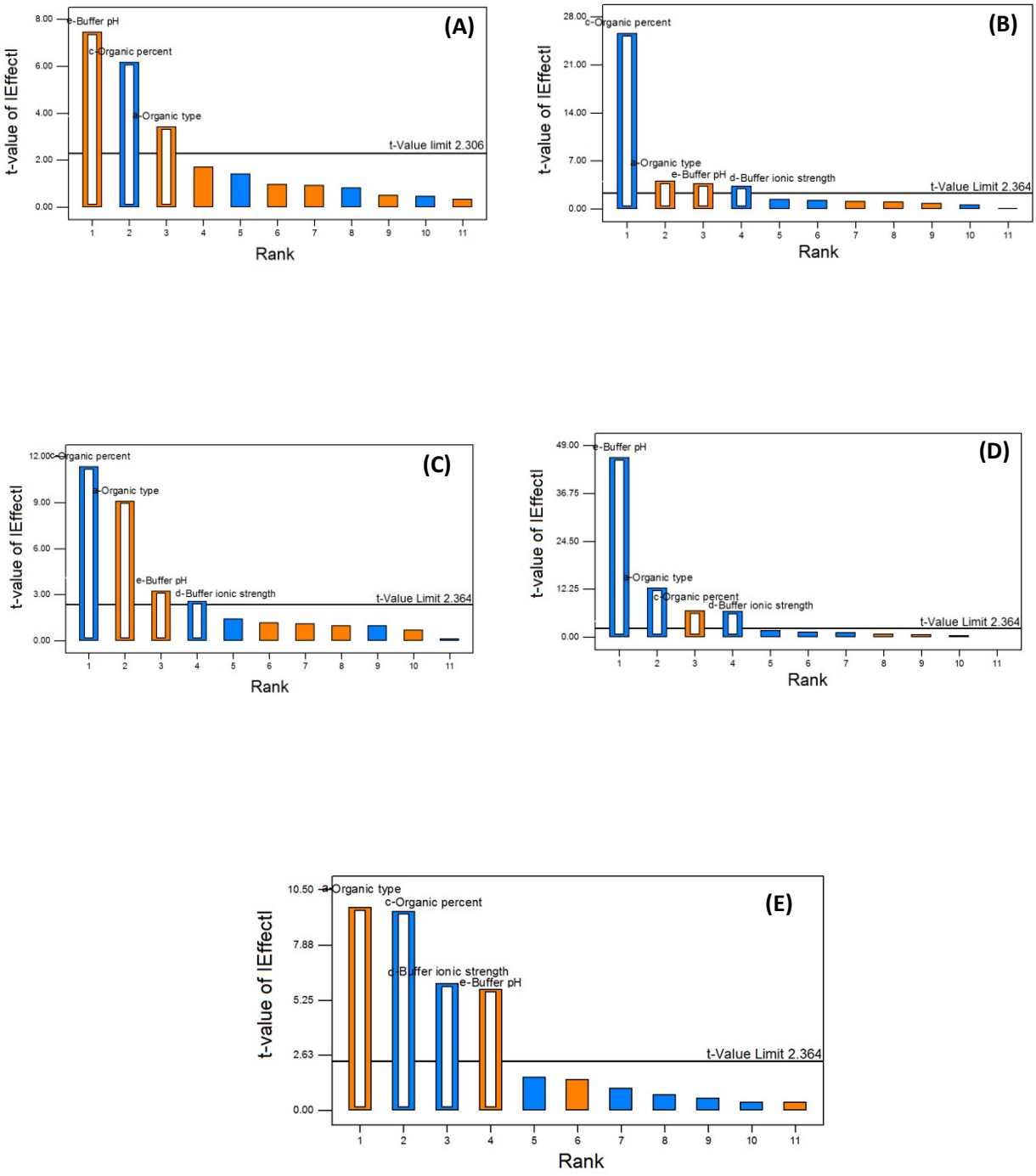


**Fig. S3**. Pareto charts of the main effects in a Plackett–Burman screening design of the proposed HPLC method for (A): DOR retention time, $T_1$; (B): IS retention time, $T_2$; (C): TIM retention time, $T_3$; (D): resolution between DOR and IS, $R_1$ and (E): resolution between IS and TIM, $R_2$.

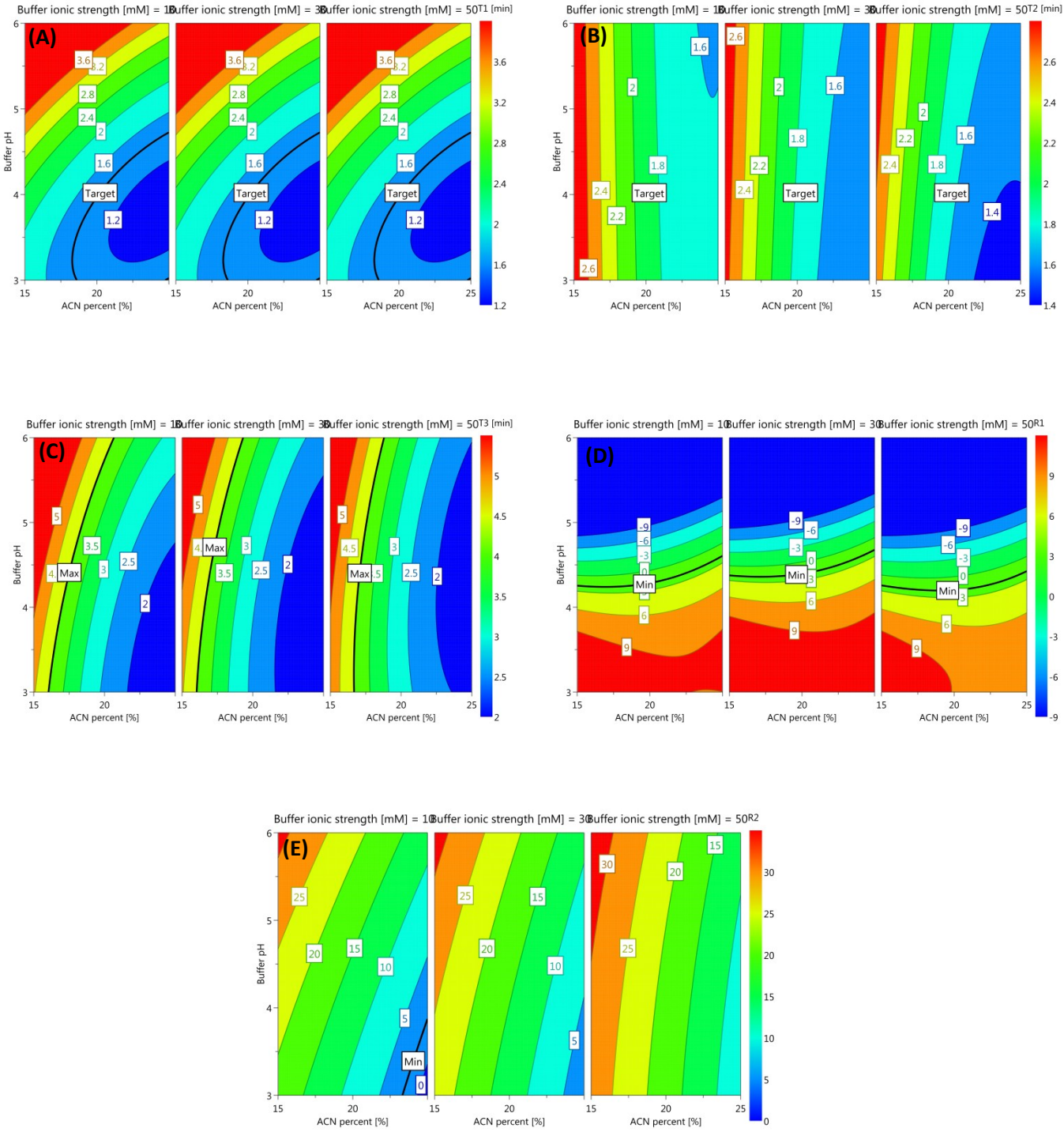


**Fig. S4.** Response contour plots in a Box-Behnken design for the CQAs of the proposed HPLC method; (A): DOR retention time, $T_1$; (B): IS retention time, $T_2$; (C): TIM retention time, $T_3$; (D): resolution between DOR and IS, $R_1$ and (E): resolution between IS and TIM, $R_2$; obtained by plotting ACN percent versus buffer pH, while buffer ionic strength kept constant at 10, 30 or 50 mmol $L^{-1}$.

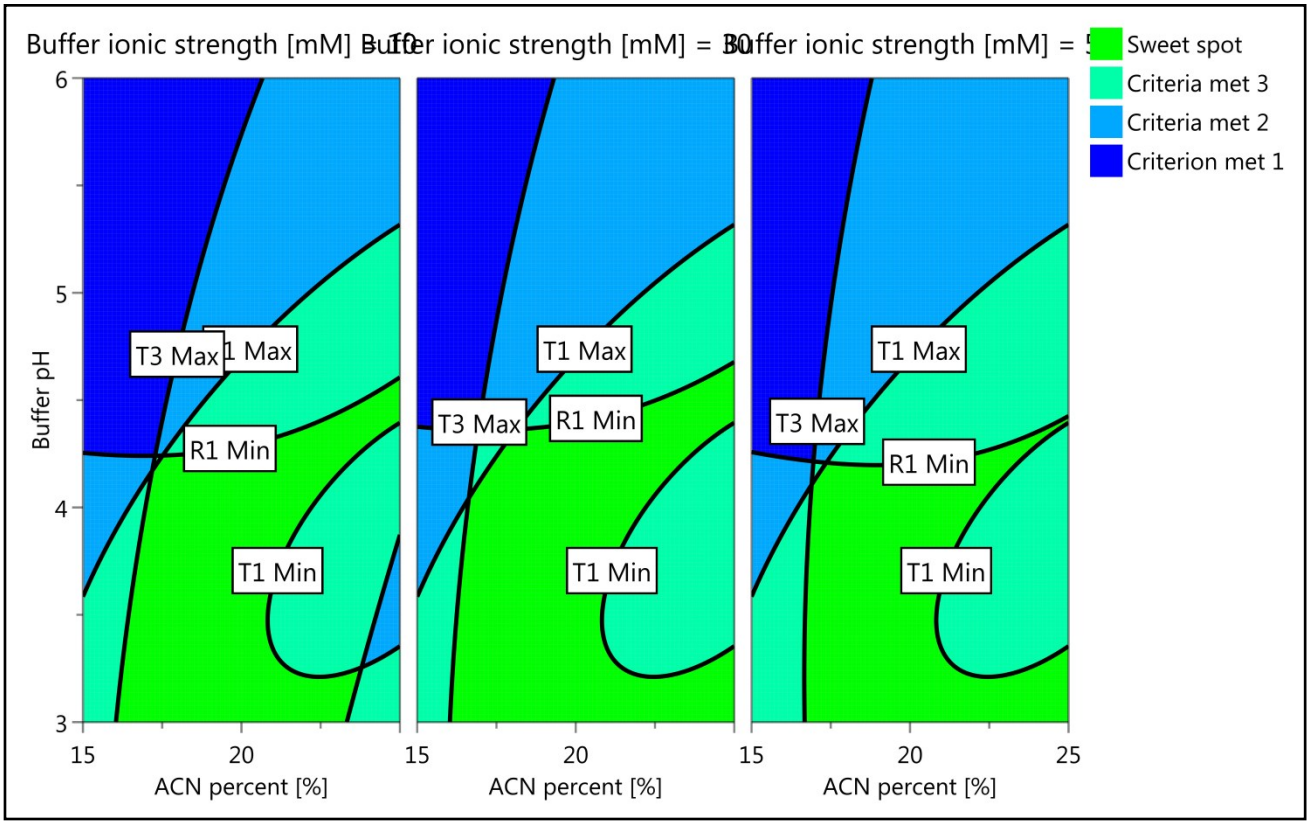


**Fig. S5.** Sweet spot plots for the CQAs of the proposed HPLC method obtained by plotting ACN percent versus buffer pH, while buffer ionic strength kept constant at 10, 30 or 50 mmol $L^{-1}$.

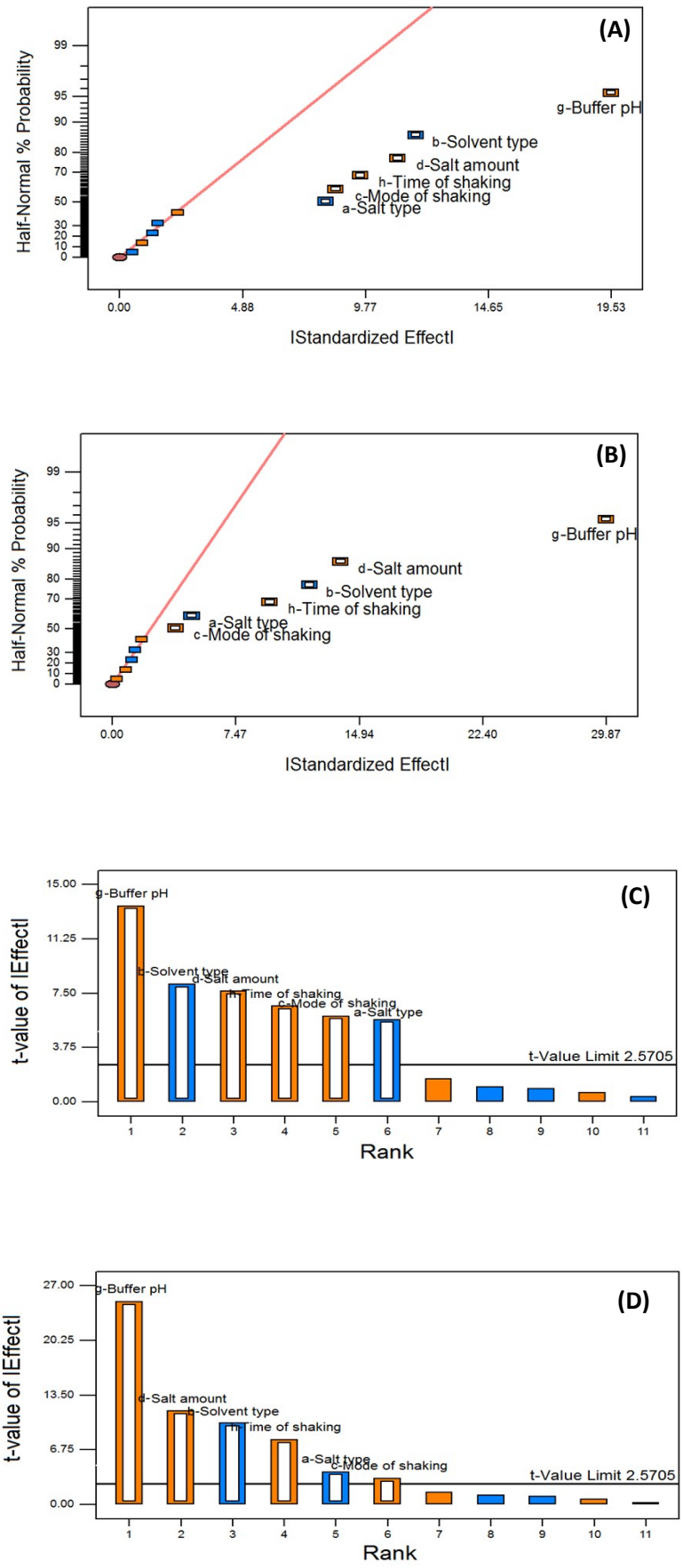


**Fig. S6**. Half normal probability plots of the standardized effects in a Plackett–Burman design of the proposed VA-SALLME method for (A): DOR and (B) TIM and their corresponding Pareto charts (C) and (D), respectively.

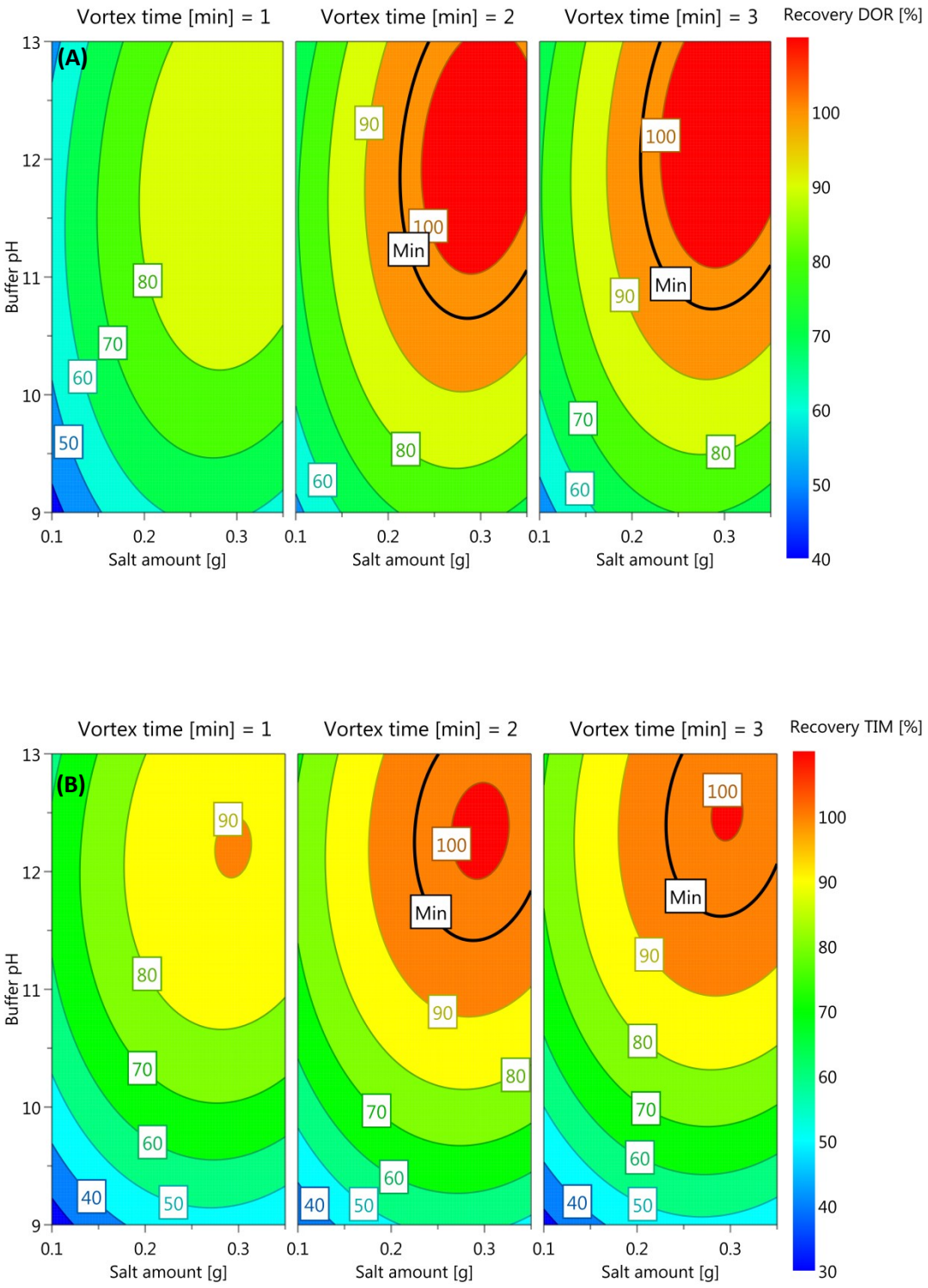


**Fig. S7.** Response contour plots in a Box-Behnken design for the CQAs of the proposed VA-SALLME method; the extraction recoveries of (A) DOR and (B) TIM from rabbit plasma, obtained by

plotting $(NH_4)_2SO_4$ amount versus buffer pH, while vortex time kept constant at 1 min, 2 min or 3 min.

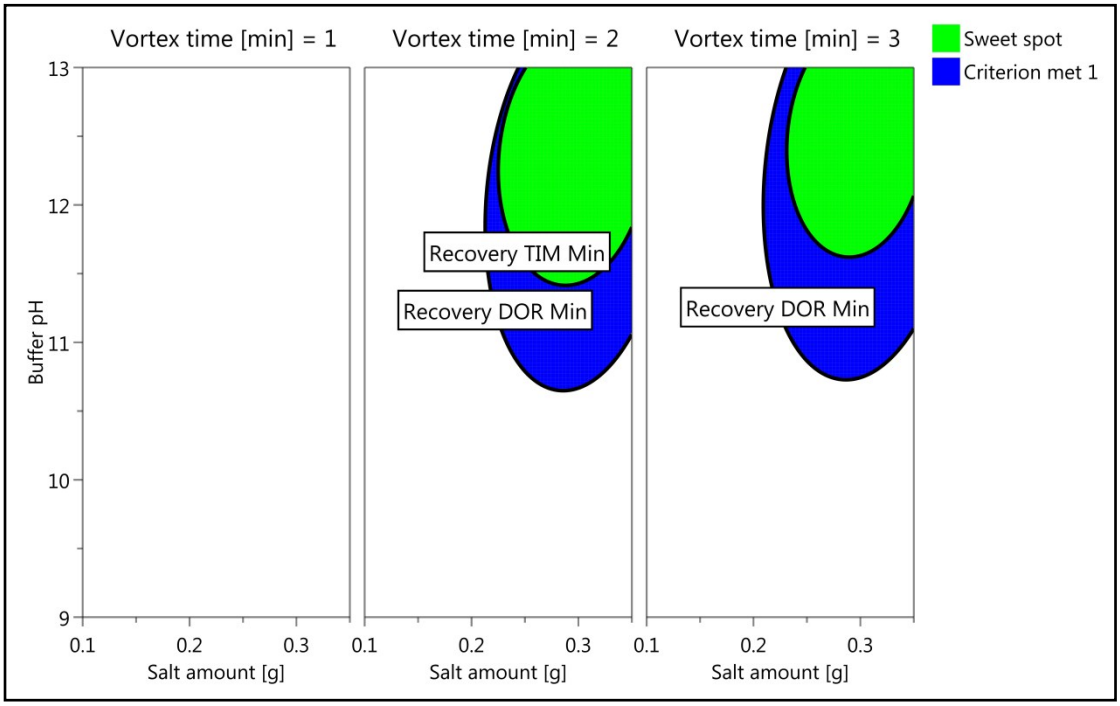


**Fig. S8.** Sweet spot plots for the CQAs of the proposed VA-SALLME method obtained by plotting $(NH_4)_2SO_4$ amount versus buffer pH, while vortex time kept constant at 1 min, 2 min or 3 min.

**Table S1**

Plackett–Burman design screening matrix of the studied factors and critical quality attributes for the proposed HPLC method.

| Run | Coded variables[a] | | | | | | | | | | | Critical quality attributes[b] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $x_1$ | $x_2$ | $x_3$ | $x_4$ | $x_5$ | $x_6$ | $x_7$ | $x_8$ | $x_9$ | $x_{10}$ | $x_{11}$ | $T_1$ | $T_2$ | $T_3$ | $R_1$ | $R_2$ |
| 1 | MeOH | Citrate | 30 | 50 | 6 | 0.0 | 0.9 | -1 | +1 | -1 | +1 | 3.39 | 1.67 | 5.93 | -31.27 | 29.48 |
| 2 | ACN | Citrate | 15 | 50 | 3 | 0.1 | 1.1 | -1 | +1 | 1 | +1 | 2.05 | 2.40 | 5.11 | 5.11 | 18.07 |
| 3 | MeOH | Phosphate | 15 | 10 | 3 | 0.1 | 0.9 | +1 | +1 | -1 | +1 | 2.80 | 2.91 | 13.11 | 1.56 | 42.59 |
| 4 | ACN | Citrate | 30 | 10 | 6 | 0.1 | 0.9 | +1 | +1 | +1 | -1 | 2.61 | 1.68 | 2.78 | -18.23 | 22.92 |
| 5 | MeOH | Citrate | 15 | 10 | 6 | 0.0 | 1.1 | +1 | -1 | +1 | +1 | 7.11 | 2.87 | 15.1 | -34.33 | 52.26 |
| 6 | ACN | Phosphate | 30 | 50 | 3 | 0.0 | 0.9 | +1 | -1 | +1 | +1 | 0.98 | 1.24 | 1.02 | 8.39 | -4.11 |
| 7 | MeOH | Phosphate | 30 | 10 | 3 | 0.0 | 1.1 | -1 | +1 | +1 | -1 | 1.34 | 1.55 | 5.22 | 6.18 | 30.46 |
| 8 | MeOH | Citrate | 30 | 50 | 3 | 0.1 | 1.1 | +1 | -1 | -1 | -1 | 1.44 | 1.50 | 3.77 | 1.48 | 18.31 |
| 9 | MeOH | Phosphate | 15 | 50 | 6 | 0.1 | 0.9 | -1 | -1 | +1 | -1 | 7.01 | 2.79 | 13.55 | -36.54 | 42.61 |
| 10 | ACN | Phosphate | 15 | 50 | 6 | 0.0 | 1.1 | +1 | +1 | -1 | -1 | 5.33 | 2.67 | 6.88 | -27.42 | 29.44 |
| 11 | ACN | Citrate | 15 | 10 | 3 | 0.0 | 0.9 | -1 | -1 | -1 | -1 | 1.95 | 2.62 | 6.82 | 11.26 | 29.79 |
| 12 | ACN | Phosphate | 30 | 10 | 6 | 0.1 | 1.1 | -1 | -1 | -1 | +1 | 2.27 | 1.56 | 2.17 | -17.75 | 18.77 |

[a] Coded variables: $x_1$, organic type; $x_2$, buffer type; $x_3$, organic percent (%, v/v); $x_4$, buffer ionic strength (mmol $L^{-1}$); $x_5$, buffer pH; $x_6$, TEA percent (%, v/v); $x_7$, flow rate (mL $min^{-1}$); $x_8$, $x_9$, $x_{10}$ and $x_{11}$; dummies 1, 2, 3 and 4; respectively.

[b] $T_1$, $T_2$, $T_3$ corresponds to the retention times of DOR, IS and TIM, respectively (min), $R_1$, $R_2$ corresponds to the resolution between DOR and IS and that between the IS and TIM, respectively.

**Table S2**

Box–Behnken design optimization matrix of the studied three critical process parameters and the critical quality attributes for the proposed HPLC method.

| Run | Critical process parameters[a] | | | Critical quality attributes[b] | | | | |
|---|---|---|---|---|---|---|---|---|
| | $X_1$ | $X_2$ | $X_3$ | Retention time (min) | | | Resolution | |
| | | | | $T_1$ | $T_2$ | $T_3$ | $R_1$ | $R_2$ |
| 1 | 15 | 3.0 | 30 | 1.80 | 2.65 | 4.62 | 15.89 | 22.77 |
| 2 | 25 | 3.0 | 30 | 1.27 | 1.52 | 1.59 | 9.80 | 1.73 |
| 3 | 15 | 6.0 | 30 | 6.05 | 2.75 | 6.45 | -38.82 | 31.90 |
| 4 | 25 | 6.0 | 30 | 2.98 | 1.60 | 2.20 | -24.42 | 11.65 |
| 5 | 15 | 4.5 | 10 | 3.01 | 2.87 | 5.41 | -2.22 | 24.19 |
| 6 | 25 | 4.5 | 10 | 1.40 | 1.57 | 1.82 | 3.43 | 4.72 |
| 7 | 15 | 4.5 | 50 | 2.77 | 2.64 | 5.19 | -2.28 | 29.82 |
| 8 | 25 | 4.5 | 50 | 1.35 | 1.40 | 1.76 | 1.23 | 8.47 |
| 9 | 20 | 3.0 | 10 | 1.47 | 1.93 | 2.43 | 10.00 | 8.70 |
| 10 | 20 | 6.0 | 10 | 4.30 | 1.90 | 4.36 | -34.78 | 21.48 |
| 11 | 20 | 3.0 | 50 | 1.33 | 1.61 | 2.63 | 8.75 | 17.59 |
| 12 | 20 | 6.0 | 50 | 4.25 | 1.90 | 3.44 | -35.34 | 22.32 |
| 13 | 20 | 4.5 | 30 | 1.67 | 1.70 | 2.66 | 0.67 | 16.55 |
| 14 | 20 | 4.5 | 30 | 1.75 | 1.77 | 2.74 | 0.46 | 16.72 |
| 15 | 20 | 4.5 | 30 | 1.76 | 1.78 | 2.77 | 0.41 | 15.97 |

[a] $X_1$: acetonitrile percent (%, v/v); $X_2$: buffer pH; $X_3$: buffer ionic strength (mmol $L^{-1}$).

[b] $T_1$, $T_2$, $T_3$ correspond to the retention times of DOR, IS and TIM, respectively, $R_1$, $R_2$ correspond to the resolution between DOR and IS and that between the IS and TIM, respectively.

**Table S3**

Plackett–Burman design screening matrix of the studied factors and critical quality attributes for the proposed VA-SALLME method of DOR and TIM from rabbit plasma.

| Run | Coded variables[a] | | | | | | | | | | CQA[b] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | a | b | c | d | e | f | g | h | j | k | l | $Y_1$ | $Y_2$ |
| 1 | $ZnSO_4$ | ACN | Vortex | 0.45 | 250 | 50 | 9 | 1 | 3 | -1 | +1 | 30.8 | 20.2 |
| 2 | $ZnSO_4$ | ACN | Vortex | 0.45 | 100 | 150 | 13 | 4 | 1 | -1 | -1 | 65.5 | 63.1 |
| 3 | $ZnSO_4$ | IPA | Ultrasound | 0.08 | 100 | 150 | 9 | 4 | 3 | -1 | +1 | 12.6 | 3.1 |
| 4 | $(NH_4)_2SO_4$ | ACN | Vortex | 0.08 | 250 | 150 | 9 | 4 | 3 | +1 | -1 | 42.2 | 24.1 |
| 5 | $(NH_4)_2SO_4$ | IPA | Ultrasound | 0.45 | 250 | 50 | 13 | 4 | 3 | -1 | -1 | 49.2 | 50.0 |
| 6 | $(NH_4)_2SO_4$ | IPA | Vortex | 0.08 | 250 | 150 | 13 | 1 | 1 | -1 | +1 | 38.5 | 30.1 |
| 7 | $(NH_4)_2SO_4$ | ACN | Ultrasound | 0.45 | 100 | 150 | 13 | 1 | 3 | +1 | +1 | 54.4 | 54.2 |
| 8 | $(NH_4)_2SO_4$ | ACN | Ultrasound | 0.08 | 100 | 50 | 9 | 1 | 1 | -1 | -1 | 22.7 | 9.1 |
| 9 | $ZnSO_4$ | IPA | Ultrasound | 0.45 | 250 | 150 | 9 | 1 | 1 | +1 | -1 | 15.7 | 7.0 |
| 10 | $ZnSO_4$ | ACN | Ultrasound | 0.08 | 250 | 50 | 13 | 4 | 1 | +1 | +1 | 41.6 | 41.4 |
| 11 | $(NH_4)_2SO_4$ | IPA | Vortex | 0.45 | 100 | 50 | 9 | 4 | 1 | +1 | +1 | 39.5 | 23.2 |
| 12 | $ZnSO_4$ | IPA | Vortex | 0.08 | 100 | 50 | 13 | 1 | 3 | +1 | -1 | 31.2 | 27.1 |

[a] Coded variables: a, salt type; b, solvent type; c, mode of shaking; d, salt amount (g); e, solvent volume (µL); f, buffer volume(µL); g, buffer pH; h, shaking time (min); j, centrifugation time (min), k and l; dummies 1, 2, 3; respectively.

[b] Critical quality attributes (CQAs); $Y_1$, $Y_2$ correspond to the extraction recoveries of DOR and TIM, respectively; each result is average of triplicate extractions.

**Table S4**

Box–Behnken design optimization matrix of the studied three critical process parameters and the observed and predicted critical quality attributes for the proposed VA-SALLME method of DOR and TIM from rabbit plasma.

| Run | Critical process parameters[a] | | | Critical quality attributes[b] | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | % Recovery of DOR ($Y_1$) | | | % Recovery of TIM ($Y_2$) | | |
| | A | B | C | Observed | Predicted | %Er[c] | Observed | Predicted | %Er[c] |
| 1 | 0.100 | 9 | 2 | 48.2 | 47.4 | 0.80 | 29.9 | 31.0 | -1.07 |
| 2 | 0.350 | 9 | 2 | 67.5 | 67.3 | 0.20 | 46.7 | 47.6 | -0.90 |
| 3 | 0.100 | 13 | 2 | 61.8 | 62 | -0.20 | 69.2 | 68.3 | 0.90 |
| 4 | 0.350 | 13 | 2 | 98.4 | 99.2 | -0.80 | 98.1 | 97.0 | 1.08 |
| 5 | 0.100 | 11 | 1 | 53.5 | 54.8 | -1.33 | 59.2 | 58.1 | 1.09 |
| 6 | 0.350 | 11 | 1 | 83.9 | 83.4 | 0.52 | 78.6 | 80.8 | -2.19 |
| 7 | 0.100 | 11 | 3 | 68.3 | 67.6 | 0.72 | 63.9 | 64.8 | -0.91 |
| 8 | 0.350 | 11 | 3 | 96.2 | 96.1 | 0.07 | 89.5 | 87.5 | 2.01 |
| 9 | 0.225 | 9 | 1 | 60.1 | 60.2 | -0.10 | 46.2 | 44.7 | 1.54 |
| 10 | 0.225 | 13 | 1 | 80.1 | 79.2 | 0.90 | 82.8 | 83.2 | -0.44 |
| 11 | 0.225 | 9 | 3 | 67.8 | 68.7 | -0.90 | 47 | 46.6 | 0.44 |
| 12 | 0.225 | 13 | 3 | 96.3 | 96.2 | 0.10 | 93.2 | 94.7 | -1.54 |
| 13 | 0.225 | 11 | 2 | 95.3 | 96.2 | -0.87 | 89.1 | 90.2 | -1.17 |
| 14 | 0.225 | 11 | 2 | 96.1 | 96.2 | -0.07 | 90.5 | 90.2 | 0.26 |
| 15 | 0.225 | 11 | 2 | 97.1 | 96.2 | 0.93 | 91.2 | 90.2 | 0.91 |

[a] A, $(NH_4)_2SO_4$ amount (g); B, buffer pH; C, vortex time (min).

[b] Extraction recoveries, average of triplicate extractions.

[c] %Er: Observed-predicted.

**Table S5**

System repeatability with intra- and inter-day precision for DOR and TIM in rabbit plasma analyzed by the developed VA-SALLME-HPLC method.

| **Matrix** | **Concentration (ng mL$^{-1}$)** | **Intra-day assay (n = 6)** | | **Inter-day assay (n = 6)** | |
|---|---|---|---|---|---|
| | | % Recovery ± SD[a] | Precision (RSD)[b] | % Recovery ± SD[a] | Precision (RSD)[b] |
| DOR | 2 (LQC) | 99.7 ± 1.803 | 1.809 | 101.1 ± 1.475 | 1.459 |
| | 25 (MQC) | 100.2 ± 1.787 | 1.783 | 99.3 ± 1.595 | 1.606 |
| | 50 (HQC) | 98.7 ± 1.239 | 1.255 | 99.5 ± 1.967 | 1.976 |
| TIM | 2 (LQC) | 99.8 ± 1.506 | 1.509 | 99.9 ± 1.576 | 1.578 |
| | 25 (MQC) | 99.6 ± 1.416 | 1.422 | 100.2 ± 1.640 | 1.637 |
| | 50 (HQC) | 98.5 ± 1.208 | 1.226 | 98.8 ± 1.227 | 1.242 |

[a] Standard deviation, n=6.

[b] Relative standard deviation.

**Table S6**

Linearity data of DOR and TIM calibration curves in aqueous solution and rabbit plasma obtained by the developed method.

| Compound | Linearity range[a] (ng $mL^{-1}$) | Correlation coefficient (r) | Intercept ± SD[b] | Slope ± SD[b] | LOD (ng $mL^{-1}$) | LOQ (ng $mL^{-1}$) |
|---|---|---|---|---|---|---|
| ***Aqueous solution*** | | | | | | |
| DOR | 2 - 200 | 0.9999 | 0.006 ± 0.003 | 0.015 ± 3.36 x $10^{-5}$ | 0.60 | 1.83 |
| TIM | 3 - 200 | 0.9999 | 0.005 ± 0.003 | 0.009 ± 2.99 x $10^{-5}$ | 0.97 | 2.94 |
| ***Plasma*** | | | | | | |
| DOR | 0.9 - 50 | 0.9999 | 0.013 ± 0.005 | 0.061 ± 2.00 x $10^{-4}$ | 0.29 | 0.87 |
| TIM | 1.5 - 50 | 0.9999 | 0.004 ± 0.005 | 0.037 ± 1.97 x $10^{-4}$ | 0.46 | 1.40 |

[a] Peak area ratio of the analyte/IS versus corresponding concentration (ng $mL^{-1}$).
[b] Standard deviation, n=7.

**Table S7**

Stability study data of DOR, TIM and IS in aqueous solutions and rabbit plasma analyzed by the developed VA-SALLME- Core-shell chromatogrphic method.

| **Condition** | **Percentage of initial concentration (%) ± SD[a]** | | | | |
|---|---|---|---|---|---|
| | **DOR** | | **TIM** | | **IS** |
| ***Aqueous solutions' stability*** | **LQC (5 ng mL$^{-1}$)** | **HQC (200 ng mL$^{-1}$)** | **LQC (5 ng mL$^{-1}$)** | **HQC (200 ng mL$^{-1}$)** | **200 ng mL$^{-1}$** |
| Refrigeration for 12 h (at 4 °C) | 100.1 ± 0.709 | 99.5 ± 0.706 | 99.6 ± 0.767 | 99.6 ± 0.831 | 99.5 ± 0.765 |
| Refrigeration for 24 h (at 4 °C) | 100.3 ± 0.800 | 99.8 ± 0.632 | 100.0 ± 0.692 | 99.5 ± 0.681 | 99.5 ± 0.730 |
| ***Plasma stability*** | **LQC (2 ng mL$^{-1}$)** | **HQC (50 ng mL$^{-1}$)** | **LQC (2 ng mL$^{-1}$)** | **HQC (50 ng mL$^{-1}$)** | **200 ng mL$^{-1}$** |
| Three freeze–thaw cycles (−20 °C) | 99.2 ± 1.340 | 99.6 ± 1.760 | 99.3 ± 1.201 | 99.2 ± 1.415 | 99.1 ± 1.332 |
| Room temperature (12 h) | 98.5 ± 1.302 | 98.7 ± 1.452 | 98.8 ± 1.440 | 98.9 ± 1.657 | 98.9 ± 1.538 |
| Room temperature (24 h) | 98.3 ± 1.370 | 98.6 ± 1.545 | 98.6 ± 1.336 | 98.7 ± 1.365 | 98.5 ± 1.375 |
| Refrigeration for 24 h (4 °C) | 99.6 ± 1.551 | 100.0 ± 1.593 | 100.6 ± 1.771 | 99.7 ± 1.484 | 99.2 ± 1.370 |
| Freezer at −20 °C for 1 month | 99.7 ± 1.562 | 99.8 ± 1.539 | 99.6 ± 1.711 | 99.6 ± 1.762 | 99.1 ± 1.517 |

[a] Standard deviation, average of three determinations.