**SUPPLEMENTARY INFORMATION for**

**A facile lyophilisation-based sample preparation approach for the determination of selected wastewater-borne antiretroviral drugs and metabolites by SFC-MS/MS.**

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**Sample collection**

A detailed sample collection protocol was described in detail in ut for the present study –evaluation of SFC-MS/MS as an alternative method to UHPLC-MS/MS – a raw wastewater sample was collected during a severe drought episode (April 2018) in the Western Cape region of South Africa. For this purpose, several grab samples were collected from a wholly domestic wastewater-receiving treatment plant. Several 500 mL samples were collected at ~15 minutes intervals over 2 hours and pooled into a 5L pre-cleaned volumetric flask for homogenisation. The sampling expedition was timed to coincide with high daily inflows (~1430–1530 hrs.) into the plant to maximise chances of obtaining a representative sample. The homogenised sample was then aliquoted into several 500 mL precleaned amber bottles without addition of preservatives, placed in an insulated box containing ice packs and transported to the lab where it was processed within 24 h.

**Mobile phase chemistries**



**Figure S2:** Stationary phase chemistries for the five columns evaluated in the present study: (A) 2-ethylpyridine (2-EP), 1-aminoanthracene (1-AA), 2-picolylamine (2-PIC), high strength silica octadecyl (HSS C18), and ethylene bridged hybrid (BEH) silica. BEH silica is employed as base material for 2-EP, 1-AA and 2-PIC phases.

**Multiple reaction monitoring data**

**Table S1**: Optimised SFC-ESI-MS/MS MRM conditions used for the analysis of the target ARVs and their metabolites.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Compound | MW (gmol-1) | tR (±SD) | Q ion transition(tdwell, sec) | CV/CE(V/eV) | Q ion transition(tdwell, sec) | CV/CE(V/eV) | q/Q (±SD) |
| efavirenz | 315.7 | 2.76 (±0.01) | 316.1 > 168.0(0.018) | 20/30 | 316.1 > 244.1(0.018) | 20/25 | 0.41 (±0.1) |
| nevirapine | 266.3 | 3.97 (±0.004) | 267.3 > 226.1(0.003) | 20/25 | 267.3 > 107.1(0.003) | 20/30 | 0.67 (±0.1) |
| nevirapine-D3 | 269.0 | 3.97 (±0.002) | 270.0 > 110.0(0.003) | 20/25 | a– | a– | a– |
| zidovudine | 267.2 | 3.98 (±0.02) | 268.0 > 110.0(0.003) | 20/20 | 268.0 > 42.0(0.003) | 20/30 | 0.29 (±0.04) |
| 12-hydroxy nevirapine | 282.3 | 5.04 (±0.01) | 283.2 > 223.0(0.003) | 20/25 | 283.2 > 196.1(0.003) | 20/30 | 0.77 (±0.1) |
| ritonavir | 720.9 | 5.85 (±0.004) | 721.5 > 296.1(0.003) | 15/15 | 721.5 > 426.5(0.003) | 15/15 | 0.26 (±0.03) |
| emtricitabine | 247.3 | 5.87 (±0.01) | 248.0 > 130.6(0.046) | 15/15 | 248.0 > 113.5(0.046) | 15/30 | 0.13 (±0.02) |
| lamivudine | 229.3 | 6.55 (±0.01) | 230.0 >112.2(0.003) | 30/15 | 230.0 > 95.1(0.003) | 30/25 | 0.04 (±0.1) |
| desthiazolylmethyloxycarbonyl ritonavir | 579.8 | 6.64 (±0.01) | 580.1 > 268.1(0.003) | 20/25 | 580.1 > 410.1(0.003) | 20/25 | 0.11 (±0.02) |
| 8,14 dihydroxy efavirenz | 347.7 | b– | 346.0 > 261.8(0.003) | 20/15 | 346.0 > 241.8(0.003) | 20/15 | b– |
| zidovudine glucuronide | 443.4 | c– | 442.0 > 125.0(0.003) | 20/20 | 442.0 > 113.0(0.003) | 20/20 | c– |

a The qualifier ion for Nevirapine-D3 was not measured.

b & c The SFC-MS/MS was not suitable for the analysis of Zidovudine glucuronide and 8,14-dihydroxy Efavirenz.

c Values in parenthesis denote the standard deviations measured for the ion ratios (q/Q) (n = 9).

**Extracted ions chromatograms.**



**Figure S3:** Typical MRM chromatograms obtained for the target analyte standards at 52.1 ng/mL illustrating the optimised MS/MS acquisition conditions. Experimental conditions as specified in Section 2.2 and Table S1.

**Asymmetry factors (As).**

**Table S2:** Asymmetry factors at 10% peak height for the test analytes on the columns evaluated for this study

|  |  |
| --- | --- |
| Columns | Test compounds |
| EFV | NVP | NVP-D3 | AZT | NVPM | RTV | FTC | 3TC | RTVM |
| BEH 2-EP | 1.00 | 1.10 | 1.00 | 1.05 | 1.00 | 1.05 | 1.67 | 1.67 | 1.80 |
| 1-AA | 1.00 | 1.13 | 1.05 | a– | 1.05 | 1.10 | 1.20 | 1.50 | 1.55 |
| 2-PIC | 1.05 | 1.00 | 1.00 | 0.80 | 1.05 | 1.00 | 1.05 | 1.15 | 1.60 |
| HSS C18 SB | 1.05 | 1.10 | 1.05 | 1.05 | 1.10 | 1.05 | 1.10 | 1.20 | 1.20 |
| BEH | 1.00 | 1.05 | 1.00 | 1.00 | 1.05 | 1.05 | 1.15 | 1.20 | 1.20 |

a AZT peak below signal-to-noise ratio

**Method validation procedures**

Limits of detection (LODs) and limits of quantification (LOQs) were calculated according to Eq.’s S1 and S2 (Evard et al., 2016; Kruve et al., 2015b, 2015a; SANTE, 2015; Van Loco et al., 2002).

LOD = $3.3×\frac{s\_{y}}{b}$ Equation S1

LOQ = $10×\frac{s\_{y}}{b}$ Equation S2

Method detection limits and Method quantification limits were calculated according to Eq.’s S3 and S4 (Evard et al., 2016; Kruve et al., 2015b, 2015a; SANTE, 2015; Van Loco et al., 2002).

MDL = $\frac{LOD×100}{Rec×PF}$ Equation S3

MQL=$ \frac{LOQ×100}{Rec×PF}$ Equation S4

where Rec is the analyte recovery and EF the pre-concentration factor. Recoveries (%) and matrix effects (%) for fortified samples (lyophilisation/SPE) were calculated according to Eq.’s S5 and S6

Recovery (%) = $\frac{blank subtracted pre-extraction spike concentration}{blank-subtracted post-extracted spike concentration }$×100 Equation S5

Matrix effects (%) = $\frac{blank subtracted post-extraction spike concentration}{neat standard concentration}$×100 Equation S6

**Table S3**: A worked out example for the calculation of Cochran’s test for homogeneity of variance using calibration data for ritonavir (RTV) (Raposo and Barcelo, 2021).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Conc.(mg/L) | Replicates | SD | SD2 | SD2max. | ∑SD2*i* | Cochran’s *C* |
| 1 | 2 | 3 |  |  |  |  |  |
| 0.00061 | 0.010716 | 0.0109121 | 0.0108558 | 0.000100969 | 1.01948E-08 | 0.002796417 | 0.056076976 | **0.049867478** |
| 0.00244 | 0.0438772 | 0.0433985 | 0.0430681 | 0.000406809 | 1.65493E-07 |  |  |  |
| 0.00488 | 0.0863698 | 0.0863937 | 0.0867902 | 0.000236121 | 5.57533E-08 |  |  |  |
| 0.03906 | 0.6888036 | 0.698809 | 0.7077046 | 0.009455929 | 8.94146E-05 |  |  |  |
| 0.07813 | 1.4049522 | 1.4066224 | 1.4288149 | 0.013321193 | 0.000177454 |  |  |  |
| 0.15625 | 2.8121929 | 2.8163371 | 2.79471 | 0.011478659 | 0.00013176 |  |  |  |
| 0.31250 | 5.793796 | 5.7007722 | 5.7037045 | 0.052881163 | **0.002796417** |  |  |  |
| 0.62500 | 11.9612747 | 12.3096379 | 11.8754036 | 0.229960211 | 0.052881699 |  |  |  |
| ***C*Crit.** (3,5) **0.5157** |  |  |  |  |  |  |  |

**Example of ANOVALOF Calculation** (Raposo and Barcelo, 2021)**.**

Calculation for AOVALOF for linearity using the calibration data for ritonavir (RTV).

$$F\_{Calc.}= \frac{MS\_{LOF}}{MS\_{ε}}$$

$F\_{Tab. }= F\_{∝, n-2,n\left(p-1\right)}=4.0662$ at $∝$ = 0.05, p = 3 and n = 8.

$MS\_{LOF}= \frac{SS\_{LOF}}{n-2}=8.67681×10^{-8}$ and $SS\_{ε}=\frac{SS\_{ε}}{n(p-1)}=0.00701$

Therefore, $F\_{Calc.}= 1.24× 10^{-5}$is less than $F\_{Tab.}=4.0662$ then linearity is confirmed.

**Method recovery data**

**Table S4:** ILIS- and non-ILIS-corrected recoveries measured for the target analytes in wastewater effluent and influent samples at the 0.03 ng/mL.

|  |  |  |
| --- | --- | --- |
| Matrix | Sample preparation method | Analytesa |
| EFV | NVP | AZT | NVPM | RTV | FTC | 3TC | RTVM |
| Effluent | ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 95.8 (±5) | 74.0 (±7) | n.d.b | 76.4 (±5) | 63.9 (±5) | 73.1 (±6) | 98.9 (±3) | n.q.c |
|  | 1. SPE
 | 92.7 (±8) | 104 (±5) | n.d. | 99.6 (±2) | 78.4 (±4) | 22.8 (±8) | 41.2 (±7) | 56.3 (±6) |
|  |  |  |  |  |  |  |  |  |  |
|  | Non-ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 50.2 (±3) | 90.2 (±3) | n.d. | 80.8 (±2) | 73.4 (±6) | 88.0 (±3) | n.d. | n.q. |
|  | 1. SPE
 | 49.5 (±5) | 89.8 (±2) | n.d. | 89.5 (±5) | 72.4 (±7) | 22.2 (±4) | 20.3 (±1) | 33.1 (±6) |
|  |  |  |  |  |  |  |  |  |  |
| Influent | ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 104 (±6) | 94.8 (±10) | n.d. | 87.8 (±4) | 84.1 (±2) | 103 (±6) | 102 (±5) | n.q. |
|  | 1. SPE
 | 72.0 (±1) | 103 (±9) | n.d. | 111 (±3) | 87.8 (±6) | 66.3 (±3) | 51.7 (±6) | 90.1 (±4) |
|  |  |  |  |  |  |  |  |  |  |
|  | Non-ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 110 (±3) | 85.3 (±7) | n.d. | 86.5 (±6) | 70.5 (±5) | 67.5 (±7) | 100 (±6) | n.d. |
|  | 1. SPE
 | 71.9 (±2) | 89.5 (±10) | n.d. | 83.7 (±2) | 76.1 (±4) | 12.7 (±8) | 10.2 (±7) | 73.9 (±2) |

a mean value (n = 4), with standard deviation in parenthesis.

b n.d. – not detected.

c n.q. – not quantified.

**Table S5:** ILIS- and non-ILIS-corrected recoveries measured for the target analytes in wastewater effluent and influent samples at the 0.3 ng/mL.

|  |  |  |
| --- | --- | --- |
| Matrix | Sample preparation method | Analytesa |
|  | EFV | NVP | AZT | NVPM | RTV | FTC | 3TC | RTVM |
| Effluent | ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 90.7 (±4) | 72.7 (±5) | n.d.b | 75.5 (±3) | 81.0 (±9) | 64.5 (±7) | 83.0 (±7) | n.q.c |
|  | 1. SPE
 | 86.2 (±3) | 98.1 (±1) | n.d. | 101 (±4) | 80.7 (±3) | 31.4 (±2) | 25.0 (±1) | 58.7 (±2) |
|  |  |  |  |  |  |  |  |  |  |
|  | Non-ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 118 (±9) | 76.2 (±1) | n.d. | 86.4 (±1) | 86.3 (±14) | 58.7 (±2) | 66.1 (±3) | n.q. |
|  | 1. SPE
 | 91.0 (±6) | 100 (±1) | n.d. | 103 (±5) | 80.7 (±1) | 30.3 (±3) | 18.1 (±1) | 57.8 (±8) |
|  |  |  |  |  |  |  |  |  |  |
| Influent | ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 103 (±6) | 94.5 (±2) | n.d. | 97.7 (±14) | 89.9 (±6) | 100.5 (±2) | 99.3 (±4) | n.q. |
|  | 1. SPE
 | 63.2 (±1) | 99.2 (±1) | n.d. | 100 (±6) | 95.6 (±5) | 54.3 (±3) | 19.5 (±6) | 78.1 (±2) |
|  |  |  |  |  |  |  |  |  |  |
|  | Non-ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 64.3 (±9) | 88.5 (±10) | n.d. | 921.3 (±11) | 86.1 (±9) | 71.7 (±11) | 92.5 (±4) | n.q. |
|  | 1. SPE
 | 94.9 (±8) | 103 (±3) | n.d. | 105 (±2) | 95.9 (±3) | 29.6 (±5) | 20.0 (±1) | 80.0 (±2) |

a mean value (n = 4), with standard deviation in parenthesis.

b n.d. – not detected.

c n.q. – not quantified.

**Table S6:** ILIS- and non-ILIS-corrected recoveries measured for the target analytes in wastewater effluent and influent samples at the 3 ng/mL.

|  |  |  |
| --- | --- | --- |
| Matrix | Sample preparation method | Analytesa |
| EFV | NVP | AZT | NVPM | RTV | FTC | 3TC | RTVM |
| Effluent | ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 83.4 (±3) | n.q.b | 82.0 (±2) | n.q. | 84.4 (±12) | 75.6 (±4) | 74.1 (±5) | n.q.c |
|  | 1. SPE
 | 96.0 (±6) | n.q. | 114 (±5) | n.q. | 88.5 (±5) | 41.7 (±4) | 31.3 (±1) | 56.3 (±7) |
|  |  |  |  |  |  |  |  |  |  |
|  | Non-ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 90.8 (±8) | n.q. | 85.8 (±6) | n.q. | 78.0 (±5) | 80.4 (±4) | 82.4 (±3) | n.q. |
|  | 1. SPE
 | 98.3 (±4) | n.q. | 89.6 (±7) | n.q. | 88.1 (±1) | 40.4 (±6) | 30.8 (±5) | 56.2 (±1) |
|  |  |  |  |  |  |  |  |  |  |
| Influent | ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 100 (±3) | n.q. | 99.0 (±6) | n.q. | 88.0 (±6) | 67.8 (±9) | 99.6 (±6) | n.q. |
|  | 1. SPE
 | 100 (±4) | n.q. | 106 (±4) | n.q. | 91.7 (±1) | 23.1 (±9) | 19.6 (±1) | 79.0 (±6) |
|  |  |  |  |  |  |  |  |  |  |
|  | Non-ILIS-corrected |  |  |  |  |  |  |  |  |
|  | 1. Lyophilisation
 | 94.7 (±9) | n.q. | 96.8 (±8) | n.q. | 90.1 (±5) | 81.8 (±7) | 96.9 (±6) | n.q. |
|  | 1. SPE
 | 98.7 (±1) | n.q. | 110 (±9) | n.q. | 99.3 (±4) | 33.9 (±4) | 21.6 (±8) | 80.5 (±3) |

a mean value (n = 4), with standard deviation in parenthesis.

b not quantified due to detector saturation.

c not quantified.

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