Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Development of an online-SPE--LC--MS-based assay using endogenous substrate for the investigation of soluble epoxide hydrolase (sEH) inhibitors

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Instrumental setup and LC-MS method

Online SPE-LC (Electronic Supplementary Material Fig. SI) was performed on an Agilent 1200 LC system (Agilent, Palo Alto, CA) comprised a G1379B degasser, two G1312B gradient HPLC pumps and a high-pressure, two-position six port valve implemented in a G1316B column oven set to 40 °C. 96-well plates were kept at 4 °C in a LEAP HTC-PAL auto sampler (Leap Technologies, Carrboro, NC) equipped with a 20 µL sample loop and 25 µL syringe. The samples (25 µL) were injected and 20 µL were introduced by full-loop injection into a flow of 1500 µl/min 0.1% acetic acid (HAc) in water delivered by pump 1. The analytes were extracted using a Cyclone RP-18 column (Thermo Fisher Scientific, Waltham, MA) with the dimensions 50 X 0.5 mm, a particle size of 50 µm and a pore size of 10 nM. After 30 sec the 6 port valve was switched, and the analytes were back flushed by the solvent stream of 500 µL/min delivered by pump 2 into a Phenomenex Kinetex reversed phase column (Phenomenex, Torrance, CA) with the dimensions 50 X 2.1 mm filled with core-shell particles of 1.7 µM and a pore size of 10 nm equipped with a 0.5 µm KrudKatcher inlet filter (Phenomenex). The analytes were separated by a binary gradient of 25 mM ammonium acetate containing 0.1 % acetic acid (HAc) as solvent A and 95/5 (v/v) ACN/water as solvent B. The gradient, flow rates and the six port switching times are displayed in the Electronic Supplementary Material Figure SI.
Mass spectrometric detection was carried out on an ABI 4000 TRAP tandem mass spectrometer equipped with a pneumatically assisted “turbo V” electrospray ionisation (ESI)-source (Applied Biosystems, Foster City, CA). The instrument was operated in negative ion mode with an ion-spray voltage of -4500V and an entrance potential of -10 V, using 35 psi curtain gas, 30 psi nebulizer gas and 50 psi drying gas at 500 °C. The analytes were detected with unit resolution in scheduled selected reaction monitoring mode (SRM). The following transitions were measured with optimized declustering potential (DP), collision energy (CE) and collision exit potential (CXP): 14(15)-EpETrE *m/z* 319/219, DP -65 V, CE -20 V, CXP -4 V ; 14,15-DiHETrE *m/z* 337/207, DP -65 V, CE -25 V, CXP -10 V; 10(11)-EpHep *m/z* 283/185 DP -90 V, CE -28 V, CXP -13 V and 10,11-DiHHep *m/z* 301/123 DP -110 V, CE -40 V, CXP -7 V. The collision-activated dissociation gas was set to “medium”. Analyst Software (version 1.5.1, Applied Biosystems) was used for controlling the online-SPE-LC-ESI-MS/MS system, data acquisition, integration and quantification. For calibration, the analyte to I.S. ratios were linearly fitted reciprocally weighted by concentration.

Table SI: Reproducibility and robustness of the LC-MS based assay. Shown are the mean IC50 values measured for sEHi (1) of three independent determinations on three different days, using two different batches of substrate and enzyme. Each IC50 value was calcuated based on three independent measurements on one plate

	Day 1	Day 2	Day 3
Plate 1	2.0 ± 0.4	1.9 ±0.2	3.2 ±0.2[a]
Plate 2	2.2 ± 0.5	2.0 ±0.2	3.5 ±0.8[b]
Plate 3	2.1 ± 0.2	1.9 ±0.4	2.6 ±0.4
Intraday mean	**2.1 ± 0.1**	**1.9 ± 0.1**	**3.1 ± 0.5**
Interday mean		**2.4 ± 0.6**	

[a] IC_{50} after 7 days storage at 4°C: 3.7 ± 0.3
[b] IC_{50} after 7 days storage at -20°C: 3.8 ±0.5

Fig. S1 Scheme of the online-SPE-LC-MS/MS system. The sample is transferred onto the SPE column by pump 1 (A). After this loading step, the six port valve is switched at 0.5 min so and the analytes are eluted from the SPE column towards the separation column by pump 2 (B). The valve is switched back after 0.9 min.The oxylipins elute, while the SPE column is cleaned and regenerated. In the diagrams the applied gradients (black line) and flow rates (grey line) of the LC-pumps are given, and switching points of the six port valve are indicated by dashed lines.

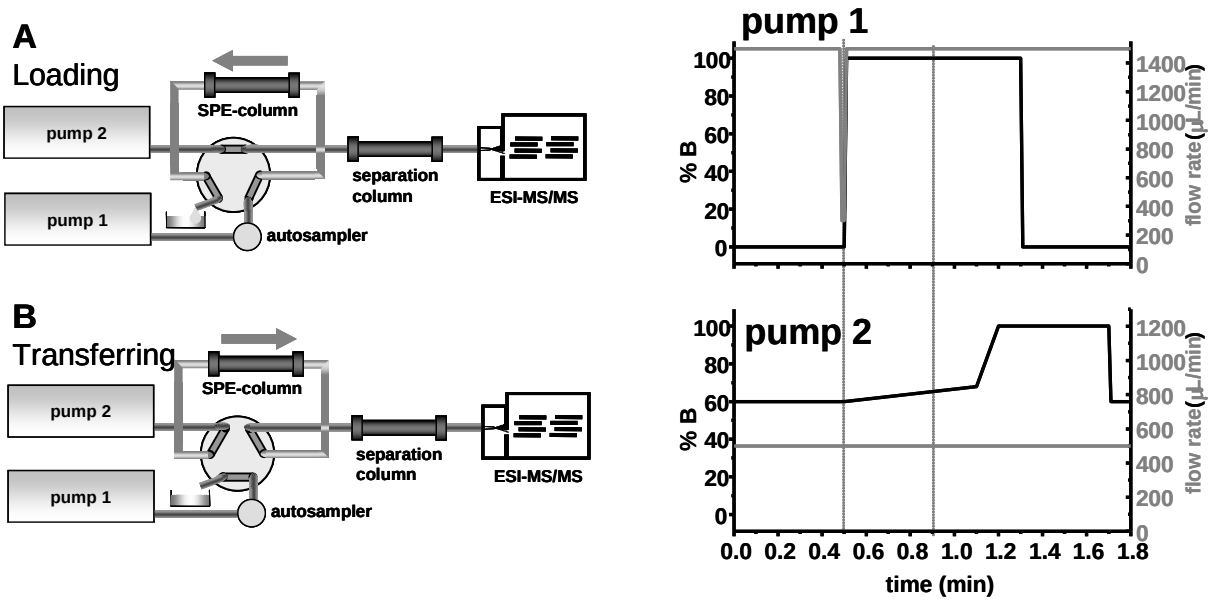

Fig. S2: Break through test of the SPE column. 20 µl of 500 nM standard solution (75% ACN) were injected in the loading flow 1500 µl/min of 100 % water containing 0.1 % HAc and the eluent of the SPE was monitored by ESI(-)-MS/MS. After 5 minutes the eluent was changed in 0.2 min to 100 % ACN in order to elute the loaded analytes. The SRM signal of the substrate 14(15)-EpETrE and the product 14,15-DiHETrE are shown.

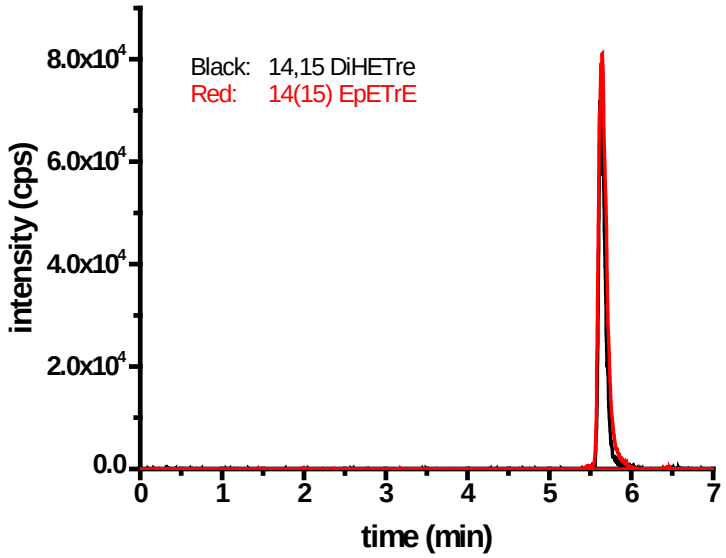

Fig. S3: Influence of the reaction buffer on the quantification. Shown is the determined concentration of spiked buffer (red) compared to the standard solution (black). Results are shown as mean and SD (error bars smaller than symbols) of the analysis of three samples per concentration in a 96 well plate **A** Product: 14,15-DiHETrE **B** Substrate 14(15)-EpETrE.