Evaluation of chromatographic conditions in reversed phase liquid chromatography – mass spectrometry systems for fingerprinting of polar and amphiphilic plant metabolites

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**Fig. S1** The mean number of features extracted from each of the reversed phase systems by an in-house implementation of the second derivative Gaussian filter with median measure based thresholds (white bars), the XCMS procedure (grey bars) and their consensus (black bars). Error bars indicate one standard deviation (n=3). Upper plot originates from negative ion mode detection, lower from positive ion mode detection. Columns have been operated at three mobile phase pH-values: first 3.5 (left), then 10.0 (middle) and finally 5.5 (right), and in four combinations of gradient and temperature: 1-50%B at 40°C, 1-50%B at 60°C, 1-99%B at 40°C and 1-99%B at 60°C (from left to right). Missing bars indicate that the: i) the BEH Shield RP18 column was not operated at 60°C at pH 10.0 for positive ion mode, as these conditions represent the column tolerance limit, and ii) that we were unable to obtain stability in retention on the BEH Phenyl column for the last batch (pH 5.5 with positive ion mode detection).
Fig. S2 The mean consensus of feature detection between any two of three replicates (6 combinations) when using the in-house implementation (red bars) or the XCMS procedure (blue bars) with both negative ion mode detection (two upper plots) and positive ion mode detection (two lower plots). As some systems exhibited severe retention time shifts consensus was calculated with allowed shift of either ±2.5 s corresponding to 4 scans or ±6.25 s corresponding to 10 scans. The later increased consensus considerably for some systems, and indicated lower reproducibility. System characteristics (column, detection mode, mobile phase pH, gradient and column temperature) are described in Figure 3 or Fig. S1
**Fig. S3** The cumulative number of features as a function of width at zero-crossing visualised for a ‘good’ system *i.e.* many detected features with a hypothesised low background contamination (HSS T3, 1-50% B, pH 3.5) and a ‘poor’ system *i.e.* many detected features but some of these suspected to originate from background contamination (BEH Shield RP18, 1-99% B, pH 10.0). The systems are visualised with both negative ion mode detection (presumably less background detected) and positive ion mode (presumably a lot of background detected). Numbers are derived using the in-house implementation, and thus might differ slightly from those obtained using the XCMS procedure.
Supplementary Information Appendix A: Initial optimisation

Prior to the acquisition of the complete suite of experiments used for information assessment, a rough method optimization was performed with the Acquity BEH C18 column (2.1 mm i.d. × 100 mm, particle size 1.7 µm) with both acidic (pH 3.5) and alkaline mobile phase (pH 10.0) in 95:5 (mobile phase A) or 5:95 (mobile phase B) mixtures of distilled water: methanol (v/v). The acidic mobile phases buffered with ammonium formiate (0.16 mM) and formic acid (0.87 mM), and the alkaline mobile phases with ammonium formiate (0.16 mM) and ammonium hydroxide (0.87 mM).

The sample injection volume was selected to balance the accuracy of m/z measurement (at high ion currents the microchannel plate detector saturates, which result in slight underestimation of the m/z value) and detection of low abundance and/or poorly ionisable compounds. We decided to proceed with injection volumes of 4 µL (ESI-) and 2 µL (ESI+) respectively, which resulted in a chromatographic peak width at half height of app. 6 seconds for the tallest chromatographic peaks as recognised in the base peak ion chromatograms when the linear gradient evolves from 1-99% B in 20 min. Chromatographic peaks broadened by silanol interactions at high pH were not considered. Clearly, the tallest chromatographic peaks caused the microchannel plate detector to saturate, which resulted in m/z shifts of app. 0.03 m/z units.

Flow rates of 200, 250, 300 and 325 µL min⁻¹ were tested. In all cases, the higher flow rate improved peak shape and separation. However, for the water:methanol mixtures 325 µL min⁻¹ resulted in back pressures above 1000 bar, which often caused the system to leak. Thus, we settled for a flow rate of 300 µL min⁻¹. Column temperatures of 20°C, 40°C and 60°C were tested. In some cases, a column temperature of 20°C resulted in better separation than that of 40°C, but in order to keep the back pressure operational we decided against it. In all inspected m/z’s the column temperature of 60°C performed better with respect to separation and elution time. However, as the thermolability of the analytes was unknown, we choose to proceed with column temperature of both 40°C and 60°C in order to assess their contribution to the features detected and consensus of detection.

Desolvation temperatures of 300, 350, 400 and 420°C were tested. In general the lower temperature resulted in less efficient ionization, and at high contents of water in the mobile phase spray droplet formation at the back plate was observed. For the three higher desolvation temperatures day-to-day variation in ionization was more pronounced compared to inter-temperature variation. However, the higher temperature always resulted in maximum or near maximum ionization, and we settled for 420°C. Capillary voltages of 1.0, 1.5, 2.0, 2.5 and 3.0 kV were tested, and in the interval between 2.0 and 3.0 (ES⁺ only) all inspected m/z’s reached their maximum ionization efficiency. We settled for 2.5 kV as a compromise. Cone voltages of 20, 45, 50 and 65 V were tested, and no significant change in ionization efficiency was observed. Thus, we proceeded with the lower cone voltage in order to minimize system load. The source temperature was 120°C, the desolvation gas flow 700 L h⁻¹, and the source gas flow 50 L h⁻¹.
Supplementary Information Appendix B: Retention time × m/z plots

All runs have been plotted as m/z × retention time maps of detected features. No drift is present in the m/z-dimension since the time-of-flight axis was binned to nominal resolution. The 2D-maps are shifted slightly in the m/z dimension to be make apex position and peak width visible for all three replicates. For the retention time dimension drift is apparent. The three replicates have been plotted on top of each other: 1st in blue, 2nd in green and 3rd in red. Thus, low retention time drift systems appear very red, whereas in high drift systems also the green and blue colours are visible. Peak apex positions are indicated by horizontal lines (which do not represent the m/z region spanned), the peak widths are indicated by vertical lines. In general we observe that increasing the column temperature from 40°C to 60°C causes the peaks to elute around 1 minute earlier. Data obtained using the in-house implementation of the second derivative Gaussian filter with median measure based thresholds, and might differ slightly from those obtained using the XCMS procedure.
BEH C18 with low mobile phase pH, negative ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH Phenyl with low mobile phase pH, negative ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH Shield RP18 with low mobile phase pH, negative ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
HSS C18 SB with low mobile phase pH, negative ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
HSS T3 with low mobile phase pH, negative ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH C18 with high mobile phase pH, negative ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH Phenyl with high mobile phase pH, negative ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH Shield RP18 with high mobile phase pH, negative ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH C18 with intermediate mobile phase pH, negative ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH Phenyl with intermediate mobile phase pH, negative ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH Shields RP18 with intermediate mobile phase pH, 40°C, negative ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom).
BEH C18 with low mobile phase pH, positive ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH Phenyl with low mobile phase pH, positive ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH Shield RP18 with low mobile phase pH, positive ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
HSS C18 SB with low mobile phase pH, positive ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
HSS T3 with low mobile phase pH, positive ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
**BEH C18 with high mobile phase pH, positive ion mode detection** and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH Phenyl with high mobile phase pH, positive ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH Shield RP18 with high mobile phase pH, 40°C, positive ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom).
BEH C18 with intermediate mobile phase pH, positive ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom), and 40°C (left) and 60°C (right).
BEH Shield RP18 with intermediate mobile phase pH, 40°C, positive ion mode detection and other operating conditions: 1-50% B (top) and 1-99% B (bottom).