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The reproducibility of liquid chromatography separation technology and its potential impact on large scale plant metabolomics experiments

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ABSTRACT

Unraveling the constituents of biological samples using HPLC is a central core technology in metabolomics experiments. Consistency in retention time across many samples is a critical criterion for judging the quality of a data set, which must be met before further analysis are possible. Here, the performance of two ultra high-performance liquid chromatography (UHPLC) systems has been compared using an established separation protocol optimized for phenylpropanoids, a class of secondary compounds found in plants displaying intermediate polarity. The two systems differed markedly with respect to their reproducibility and pressure stability. The standard deviation of the retention time of representative peaks differs up to 30-folds between the systems. Adjustments made to the gradient profiles succeeded in equalizing their level of performance. However, the modifications made to the separation protocol reduced the quality of the separation, particularly of the more rapidly eluting components, and lengthened the run time.

1. Introduction

Advances in chromatography and computational technology in the recent years enabled the routine measurement and analysis of metabolite profiles of large sample sets by liquid chromatography coupled to mass spectrometry (LC–MS) [1–6].

Metabolomics approaches can be divided into two [6] main strategies. Targeted experiments aim to quantify known metabolites, whereas untargeted approaches are used to detect as many metabolites as possible in a single sample. The latter type, in which, typically, the identification of molecular markers is attempted by contrasting between the metabolome of a wild type and a mutant, or between that produced in response to a particular treatment against that from an non-treated control, requires the use of a number of different technology platforms for a comprehensive view on various groups of metabolites (e.g. LC–MS for primary and secondary metabolites and gas chromatography–MS for primary and volatile metabolites) [3].

A number of factors, like experimental design, LC–MS analysis methods and data analysis strategies can heavily influence the validity of the obtained results [7]. Metabolomics experiments require often the analysis of many replicate samples to achieve sufficient statistical power. However, it is understood that the retention time of a given metabolite can vary slightly as a result of column degradation, instability of the mobile phase pH or variation in the column temperature, all of which are difficult to control when large numbers of samples need to be analyzed [8]. Various data analysis software packages (e.g. XCMS, MetAlign and Profile Analysis) have been designed to correct for non-linear retention time shifts to a certain extent through the application of alignment algorithms [9–11]. The consequence of large shifts in retention time, which cannot be dealt with by alignment algorithms, can be data loss or an unacceptable level of type 1 error. Furthermore [12] revealed source contamination as a major factor impacting on day-to-day data variability, showing significant loss of sensitivity after three days of continuous use.

Our approach to analyze the effect of stress on the plant metabolome had focused on the behavior of the phenylpropanoids, based on UHPLC (ultra high performance liquid chromatography) separation technology [13]; in attempt to improve annotation power, we shifted to a linked U(HPLC)/MS platform. The intention was to enable the robust metabolic profiling of up to several thousand complex plant samples, and this required the elaboration of a highly stable separation platform. Here, we report a comparison between two commercially available HPLC systems with respect to their separation reproducibility for the given protocol.

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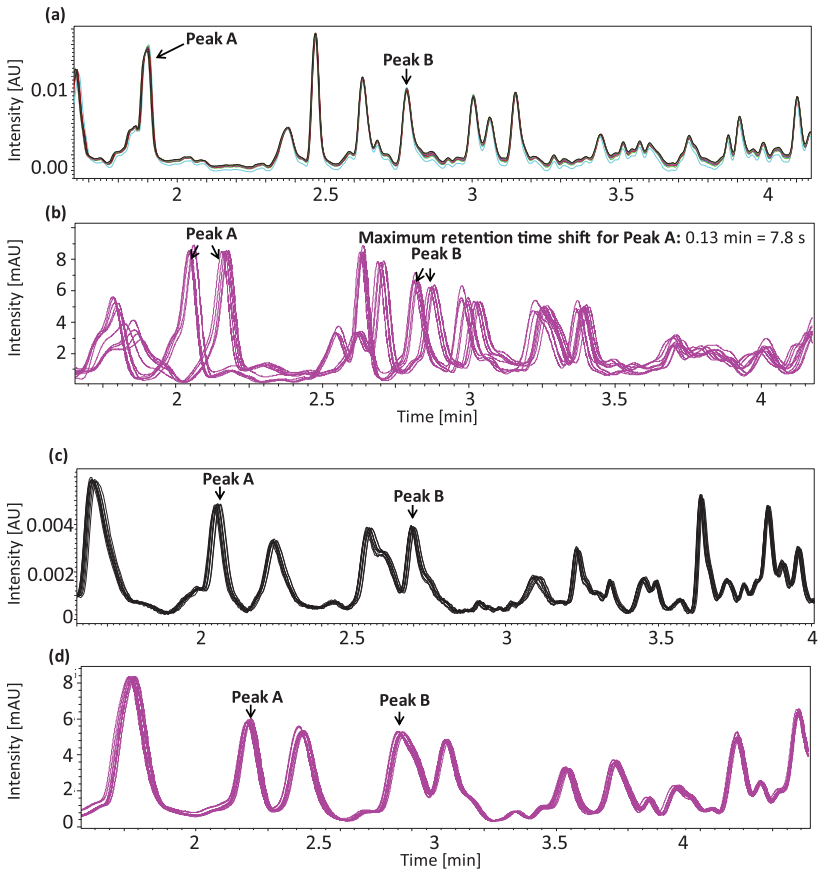

Fig. 1. Chromatograms produced following ten consecutive injections of a sugar beet leaf extract into two LC-systems using (a,b) gradient 1 and (c,d) gradient 2. (a) Consistent retention times (system 1), (b) retention time shifts of up to 6.6 s for peak A (system 2), (c,d) consistent chromatograms produced by both systems 1 and 2.

2. Material and methods

2.1. Methanol-based extraction of sugar beet leaves

Sugar beet leaves were snap-frozen and lyophilized, and a 15 mg sample was extracted in 900 μL methanol (75% v/v), formic acid (0.1% v/v). Zirconia beads (58%) (1.0–1.2 mm diameter, Muehlmeier, Baernau, Germany) were added to the sample to faciliate homogenization in a commercial bead mill (45 s; speed: 4 m/s, FastPrep, MPBio, Solon, USA). The samples were then centrifuged (27,500 × *g*, 10 min, 4 °C) and 80 μL of the resulting supernatant was supplemented with 20 μL 0.1% formic acid (solvent A) and the sample re-centrifuged (27,500 × *g*, 5 min, 4 °C). The final supernatant was injected into the LC system.

2.2. HPLC instruments, column and gradient

The two LC platforms compared were Acquity UPLC® (Waters, Milford, USA) ("system 1") and Ultimate 3000 RSLC (Dionex, Thermo Fisher Scientific Inc., Waltham, USA) ("system 2"). System 1 comprised the pump module 186015001, the sample manager 186015006, the column manager 186015007, the photo diode array detector 186015026 and the fluorescence detector 186015029. System 2 was fitted with a solvent rack SRD-3200 including a degasser, a HPG-3200RS pump, a DAD-3000RS UV-detector, a TCC-3000RS column thermostat and a WPS-3000TRS autosampler module. System 2 was coupled to an ultra high resolution time-of-flight MS device (maxis Impact, Bruker, Billerica, USA). Both systems were operated with an Acquity UPLC® BEH Phenyl column (130 Å, 1.7 μm 2.1 × 100 mm), fitted with a Acquity UPLC BEH Phenyl VanGuard Pre-Column (130 Å, 1.7 μm, 2.1 × 5 mm). Each experiment was performed twice on each system, using the same pair of replicate columns to monitor the extent of performance variation due to the column. The column temperature was maintained at 35 °C. The initial solvent gradient applied ("gradient 1") was following [13]: During the first step from 0 to 10 min solvent B (acetonitril with 0.1% (v/v) formic acid) was linearly increased from 0 to 40%, in the second step B was increased and maintained at a high level to wash the column (10–11.5 min: 40–97% B, 11.5–13 min: 97% B, 13–13.5 min: 97–0% B). In the third step the column was re-equilibrated to starting conditions 13.5–15 min: 0% B (flow rate of 500 μL/min).

The gradient was later adjusted to isocratic starting conditions whereby 5% B were maintained from 0 to 1 min. Then the mobile Phase was linearly increased from 5 to 40% until 10 min, followed by the same washing step from 10 to 11.5 min with

Table 1
Standard deviation of the retention time [ms] for representative peaks.

Peak	System 1		System 2	
	Gradient 1	Gradient 2	Gradient 1	Gradient 2
A	120	420	3780	420
B	60	300	1800	300

Variability of the retention time of two compounds eluting during the time window in which shifts occurred. The table shows the values obtained with system 1 and system 2 applying the two different gradients. The standard deviation when using gradient 1 differed considerably between systems 1 and 2. Using gradient 2, the two systems' performances were comparable.