Journal of Chromatography A, 1473 (2016) 48–55

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# On the feasibility to conduct gradient liquid chromatography separations in narrow-bore columns at pressures up to 2000 bar

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ARTICLE INFO

*Article history:*
Received 10 August 2016
Received in revised form 3 October 2016
Accepted 6 October 2016
Available online 6 October 2016

*Keywords:*
Ultra-high pressure
Narrow-bore columns
Viscous heating
2.1 mm ID
Gradient elution
Preloaded gradient

ABSTRACT

The limits in operating pressures are extended for narrow-bore columns in gradient elution up to 2000 bar. As the required pumps for these pressures are incompatible with common chromatographic solvents and are not suitable to apply a mobile phase composition gradient, a mobile phase delivery and injection system is described and experimentally validated which allows to use any possible chromatographic solvent in isocratic and gradient elution. The mobile phase delivery and injection system also allows to perform multiple separations without the need to depressurize the column. This system consists out of 5 dual on/off valves and two large volume loops in which the gradient and equilibration volume of initial mobile phase are loaded by a commercial liquid chromatography pump. The loops are then flushed toward the column at extreme pressures. The mobile phase delivery and injection system is first evaluated in isocratic elution and shows a comparable performance to a state-of-the-art commercial flow-through-needle injector but with twice the pressure rating. Distortion of the loaded gradient by dispersion in the gradient storage loop is studied. The effect of the most important parameters (such as flow rate, pressure and gradient steepness) is experimentally investigated. Different gradient steepnesses and volumes can be applied at different flow rates and operating pressures with a good repeatability. Due to the isobaric operation of the pumps, the gradient is monitored in real-time by a mass flow meter installed at the detector outlet. The chromatograms are then converted from time to volume-base. A separation of a 19-compound sample is performed on a 300 × 2.1 mm column at 1000 bar and on a 600 × 2.1 mm column at 2000 bar. The peak capacity was found to increase from 141 to 199 and thus scales with $\sqrt{L}$ as is predicted by theory. This allows to conclude that the inlet pressure for narrow-bore columns in gradient elution can be increased up to 2000 bar without fundamental pressure-induced limitations.

## 1. Introduction

To achieve faster and better separations, reducing the particle diameter provides a time-proven method. However, columns packed with small particle sizes (sub-2 µm) can only be operated around the optimal velocity if the maximal operating pressure for liquid chromatography (LC) devices is sufficiently high. So-called ultra-high-pressure instrumentation is now widely available from different instrument manufacturers, but an upper pressure limit in operating pressure appears to have been reached around 1200–1300 bar due the mechanical challenges in the construction of reliable instrumentation and problems with column bed stability and lifetime. Additionally, concerns arose regarding the effect of viscous heating which leads to radial temperature and thus velocity profiles, that in turn result in additional band broadening [1–13]. Only recently one vendor introduced an LC device with an increased operating pressure of 1500 bar [14].

Possibilities beyond this pressure limitation were first illustrated by Jorgenson and co-workers [15–19] in their seminal work on capillary LC instrumentation capable of working at pressures in the range of 4000–6000 bar, a tenfold increase versus state-of-the art commercial instruments at that time. In the work by Jorgenson and co-workers, capillary columns were used to cope with the viscous heating effect at ultra-high pressures. This has extensively been examined up to 7000 bar for isocratic as well as gradient elution [15–18].

In previous work, a prototype LC set-up was realized, allowing to operate at pressures up to 2600 bar (260 MPa) for large separation volumes (>5 mL) using Haskel pumps [20]. The performance of an in-house-built injector was compared at 800 bar to commercially-available injectors, yielding equal performance but twice the maximum pressure rating. The performance of (coupled) custom columns (2.1 mm ID packed with fully-porous and superficially-porous particles) were assessed at extreme-pressure

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http://dx.doi.org/10.1016/j.chroma.2016.10.008
0021-9673/

conditions. Increasing the inlet pressure from 800 to 2400 bar and scaling the column length proportionally (from 150 mm to 450 mm), resulted in the theoretically expected linear increase in plate count from 20,000 to 59,000. Viscous-heating effects were diminished by insulating coupled columns and applying an intermediate-cooling strategy in a forced-air oven [21]. As a proof-of-concept, the system allowed to investigate fundamental effects during isocratic separations.

However, to further illustrate the feasibility of inlet pressures beyond 2000 bar for normal/narrow-bore columns, several improvements must be made:

- The Haskel pump seals are only compatible with methanol and thus other chromatographic solvents (such as acetonitrile) are not possible.
- Enabling the possibility of gradient elution: the Haskel pumps are pressure-controlled and have a high internal volume (2 mL versus 100 μL for state-of-the-art instrumentation). Thus, it is not possible to control the volumetric fraction as a function of time by e.g. changing the feed to the Haskel pump (e.g. via a commercial HPLC system).
- As the Haskel pumps work in a so-called constant pressure mode, a system must be developed which allows to measure both the flow rate as well as the composition of the mobile phase [22,23]. This allows to transform the measured time-based chromatograms and gradients to volume-based ones.

The most straightforward way to produce a gradient with the system described in [20], would be by connecting the pumps to two different solvent reservoirs filled with a different solvent and to mix the streams in a high pressure mixer at pressures up to 2000 bar. Unfortunately, in order to control the delivered gradient with these pneumatic amplifier Haskel pumps, mass flow controllers which are able to operate at these pressures would be required. Another solution is using the so-called exponential dilution method for generating a gradient [16]. In this method, a high pressure solvent vessel is filled with a weak solvent and is flushed at extreme pressures with a stronger solvent. This system works as a continuous stirred tank reactor and thus delivers an exponential gradient. Whereas this system allows to generate gradients up to 5000 bar and beyond, it has also some drawbacks. The most important drawbacks are a lack of flexibility to change gradient programs, impossibility to reequilibrate the column and it required careful inspection of the components in order to produce the desired gradient [16]. The reservoir and connection tubing has to be rinsed and refilled with the initial solvent after each gradient. This means that performing multiple measurements without depressurizing the system is not possible. Additionally, both methods above do not allow to use chromatographic solvents other than methanol and water due to pump seal incompatibility unless modifications are made to the seals. The method proposed further on is similar to a system designed by Link [24] and described in literature [25,26], where a gradient is preloaded by a commercial system in a storage loop at low pressures and then is flushed out at extreme pressure by the Haskel pumps. The loaded gradient can be any kind of mixture of solvents since there is no direct contact with the Haskel pump seals.

Recent studies have investigated the possibilities of a constant pressure approach in various chromatographic systems [22,23,27]. When a gradient of e.g. acetonitrile in water is run from 5 to 90%, the viscosity decreases from 1.044 to 0.457 cP [28]. In constant flow rate mode, the flow rate is set such that the system works at its maximal operating pressure in the beginning of the gradient. As a consequence, it will only use 50% of its available operating pressure near the end of the gradient. Since the used pumps work isobaric, it is only possible to work in a constant system pressure drop mode. Luckily, in liquid chromatography, a system reaches its maximal kinetic performance at its maximal pressure drop and thus the constant flow rate mode (cF-mode) inherently does not use the full capabilities during the whole gradient [29]. However, in an isobaric system (cP-mode), the maximal pressure drop is maintained and time gains up to 15-25% were found [22].

A comparison between these two modes is only possible if the same volumetric-based gradient program is used. In this case, the elution volumes of the compounds will be the same for both operating modes if there is no significant influence of pressure or viscous heating on retention [30]. In the B-term and around the optimum of the Van Deemter-curve, cP-mode yields the highest kinetic performance but the two modes converge toward each other in the C-term area [22,23].

The most convenient way to describe separation performance in gradient elution is by using the peak capacity, $n_{p,V}(/)$, which, in volumetric units is given by [22]

$$n_{p,V} = 1 + \sum_{i=1}^{n} \frac{V_{R,i} - V_{R,i-1}}{4 \cdot \sigma_{V,i}} \tag{1}$$

where $n$, $V_{R,i}$ ($m^3$) and $\sigma_{V,i}$ ($m^3$) are the number of components, the retention volume of component $i$ and the volume-based standard deviation, respectively. In order to obtain the volume-based standard deviation, the chromatograms were transformed from time to volume-base. Afterwards, the chromatograms were integrated using an in-house developed algorithm [31].

## 2. Experimental system

### 2.1. Columns and chemicals

Hypersil columns (fully porous C18, 150 × 2.1 mm, 1.9 μm, packed at 3000 bar) were provided by Thermo Scientific. Titan C18 UHPLC columns (fully porous C18, 150 × 2.1 mm, 1.9 μm) were provided and custom packed by Supelco at pressures up to 19,000 psi. As the system was operated at pressures beyond the intended pressure range for the columns, column lifetime was limited and performance was lower than for their commercial counterparts operated at or below their specified pressure rating.

Methanol (MeOH) and Acetonitrile (ACN) (Supra-Gradient grade) were purchased from Biosolve (Valkenswaard, Netherlands). HPLC grade water was prepared in the laboratory using a Milli-Q gradient water purification system (Millipore, Bedford, MA, USA). A 19-compound small molecule mixture, that eluted evenly over the applied gradients, was used to evaluate the mobile phase delivery and injection system containing: Uracil, valero-, heptano-, aceto-, octano-, benzo- and propiophenone, indene, pyrene, iodobenzene, bromobenzene, naphtalene, methyl-, ethyl-, butyl- and propylparaben, aspirine, ketoprofen and ibuprofen. The sample was dissolved in 40/60 ACN/$H_2O$.

### 2.2. Set-up

The set-up for delivering large solvent volumes at extreme pressures is fully described in [20]. In brief, two separately controlled Haskel pumps are installed and fine-tuned to obtain a stable pressure signal. Since the pumps are pneumatic amplifiers, the operating mode is isobaric (constant pressure) as opposed to the commercial constant flow rate mode in UHPLC. Methanol (MeOH) is used as flushing solvent. An Agilent 1100 system (G1312) was used for loading the loops and detection was done by a 1100 DAD detector (flow cell with a volume of 1.7 μL). The performance of the injector described in this work was compared with an Agilent 1290 autosampler (G4226A).