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Accurate measurements of the true column efficiency and of the instrument band broadening contributions in the presence of a chromatographic column

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ABSTRACT

A rapid and simple validated experimental protocol is proposed for the accurate determination of the true intrinsic column efficiency and for that of the variance of the extra-column volume of the instrument used, the latter being obtained without requiring the removal of the chromatographic column from the HPLC system. This protocol was applied to 2.1 mm × 100 mm columns packed with sub-3 (2.7 μm Halo Peptide ES-C_{18}) and sub-2 μm (1.6 μm prototype) core–shell particles. It was validated by observing the linear behavior of the plot of the apparent column plate height *versus* the reciprocal of $(1+k')^2$ for at least three homologous compounds, with a linear regression coefficient R^2 larger than 0.999. Irrespective of the contribution of the several, different instruments used to the total band broadening, the same column HETP value was obtained within 5%. This new protocol outperform the classical one in which the chromatographic column is replaced with a zero dead volume (ZDV) union connector to measure the extra-column volume variance, which is subtracted from the variance measured with the column to measure the intrinsic HETP. This protocol fails because it significantly underestimates the system volume variance.

1. Introduction

Less than ten years ago, before the advent of ultra-fine particles and the development of very high pressure liquid chromatography (vHPLC), the contribution of extra-column band broadening to column efficiency was barely discussed in practical applications of HPLC, although it had already been tackled from a theoretical viewpoint in GC and had been considered in LC [1–5]. Until the early 2000s, the column dimensions (4.6 mm i.d. and 5–25 cm long) and the particles used (5 μm) were such that the instrument contributions to the widths of recorded peaks were negligible (<5%) for small retention factors ($k' \simeq 1$), as they had been for twenty years. Actually, the influence of extra-column band broadening on chromatographic performance was known since the early 1960s, when GC was the most used chromatographic method [1] and many users built their own instruments and packed their own columns. That was because the diffusion coefficients of tracer molecules in gases are about four orders of magnitude larger than those observed in liquids. Each subunit of a chromatograph (injector, valves, tubes, detector, *etc.*) contributes to broaden the sample band and affects the chromatographic resolution. The minimization of these contributions was sought early by designing low-dispersive instruments.

The impact of the extra-column band broadening on the performance of LC columns became a major concern after the emergence of the vHPLC column technology [6]. The particle size shrunk down to 1.7 μm while these fine particles were packed in 2.1 mm i.d. and 5–15 cm long columns. In order to conserve most of the resolution power that these new columns could provide (about 300 000 plates/m), new HPLC systems were designed that provided low band broadening contributions. New instruments providing small variance contributions due to their small extra-column volumes were developed and provided significantly improved performance during the last decade. The volume variance contributions of instruments decreased from about 50–100 μL^2 for classical 400 bar LC instruments to only 1–15 μL^2 for the most recent 1000 bar vHPLC instruments [7–12].

A decade after their introduction, the performance of vHPLC columns is still improving with the generation of new sub-2 μm core–shell particles [13,14]. The potential efficiency of these columns approaches now 450 000 plates per meter. Unfortunately, instrument performance is lagging behind that of modern column technology. At best, analysts using the most recent vHPLC systems, which generate volume variances of 8 (the optimized 1290 Infinity system), 3.5 (the classic Acquity instrument), and 0.7 μL^2

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(the I-class Acquity instrument), respectively [15], can observe efficiencies of 120 000, 200 000, and 360 000 plates/m provided that they use weakly retained compounds ($k' \simeq 1$). This considerable progress causes new problems by making obsolete the classical methods used to determine the intrinsic efficiency of highly efficient vHPLC columns. Analysts must now measure accurately the column efficiency and the extra-column volume variances of their instruments. In the recent past, a fair estimate of the instrument variance contribution was provided by replacing the column with a zero dead volume (ZDV) union connector. It was recently shown that this method may underestimate by about 20% the volume variance of some instruments [15], meaning that the intrinsic column efficiencies given by this invasive method are somewhat smaller than the true ones, hence incorrect. Previous investigations suggested that this was due to the high pressure in the pre-column volume, which slows down the radial equilibration across the capillary diameter [15]. Therefore, the peak variance measured at low pressures in the presence of a ZDV connector is smaller than the true value at high pressure in the presence of the column.

The purpose of this work was to develop an alternative method for the accurate determinations of the extra-column band variances of instruments in the presence of the chromatographic column and of the intrinsic efficiencies of modern narrow-bore columns packed with sub-2 μm core–shell particles. The new technique will be non-invasive since the column remains connected to the instrument during the analysis and no ZDV union connector should be used to estimate the volume variance of the instrument used. The method must be validated. Its application to the determination of the true intrinsic efficiencies of new prototype columns packed with 1.6 μm core–shell CORTECS (TM) particles will be investigated to demonstrate its usefulness.

2. Theory

2.1. Band variance under isocratic and quasi-isocratic conditions

The total volume variance includes the contributions of the chromatograph (injector, valve, connecting tubes, detector, electronics) and of the column to the recorded band width. Under strictly isocratic conditions, it is given by [1,7,9]:

$$\sigma^2_{v,Exp.} = \sigma^2_{v,ex} + \frac{V_0^2}{N_{intrinsic}}(1+k')^2 \quad (1)$$

where $\sigma^2_{v,Exp.}$ is the total volume variance measured by the analyst, $\sigma^2_{v,ex}$ is the extra-column volume variance, V_0 is the column void volume, $N_{intrinsic}$ is the intrinsic column efficiency, and k' is the retention factor. Note that Eq. (1) assumes that the sample is dissolved in the same eluent as the one used as the mobile phase.

If the sample is dissolved in either a weaker or a stronger solvent than the mobile phase, the initial retention factor, k'_I, of the compound at the column inlet is then $k'_I \neq k'$ and the corrected total volume variance is written [10]:

$$\sigma^2_{v,Exp} = \sigma^2_{v,ex,pre-column}\left[\frac{k'}{k'_I}\right]^2 + \frac{V_0^2}{N_{intrinsic}}(1+k')^2 + \sigma^2_{v,ex,post-column} \quad (2)$$

where $\sigma^2_{v,ex,pre-column}$ and $\sigma^2_{v,ex,post-column}$ are the pre- and post-column extra-column volume variances, respectively. Such analyses are made under so-called "quasi-isocratic" conditions because the retention factor of the compound at the column inlet is not rigorously constant. For instance, Eq. (2) applies well for performance optimizing the injection sequence (or POISe [16]) in order to minimize the contribution of the injection process to the overall extra-column band broadening. This approach is based on the physical phenomenon of band compression [17–22], which eliminates most of the pre-column band broadening by dissolving the sample molecules in a much weaker solvent ($k'_I \ll k'$) than the mobile phase. This method is not used in this work to simplify the protocol.

2.2. Relationship between the apparent and the intrinsic HETP

Under strict isocratic conditions, the elution volume V_R is given by:

$$V_R = V_{ex} + V_0(1+k') \quad (3)$$

where V_{ex} is the extra-column volume.

By definition, the apparent height equivalent to a theoretical plate (HETP) H (*e.g.*, the overall performance of the column and chromatograph as measured by the analyst) is written:

$$H = L\frac{\sigma^2_{v,Exp.}}{V_R^2} \quad (4)$$

Assuming that $V_{ex} \ll V_0$, substitution of Eq. (1) into Eq. (4) provides the relationship between H, $H_{intrinsic} = L/N_{intrinsic}$, the column dimensions (V_0 and L), the system variance ($\sigma^2_{v,ex}$), and the retention factor k':

$$H(k') = H_{intrinsic} + L\frac{\sigma^2_{v,ex}}{V_0^2}\frac{1}{(1+k')^2} \quad (5)$$

This result shows that, if under certain conditions of flow rate, temperature, and mobile phase composition, the intrinsic HETP depends weakly on k' for a series of closely related compounds (same class of compounds such as an homologous series of analytes), the plot of the apparent HETP *versus* the reciprocal of $(1+k')^2$ is expected to be linear. Its y-axis intercept will provide $H_{intrinsic}$ while its slope will give an estimate of the true extra-column volume variance $\sigma^2_{v,ex}$. The main advantage of this approach for the determination of $H_{intrinsic}$ and $\sigma^2_{v,ex}$ is that the column does not need to be replaced with a zero dead volume (ZDV) connector. The main disadvantage is that the conditions mentioned above are not systematically met under certain RPLC conditions.

3. Experimental

3.1. Chemicals

The mobile phase used was a binary mixture of acetonitrile and water (50/50, v/v). Tetrahydrofuran (THF) was used to measure the column hold-up volumes. All these solvents were HPLC grade from Fisher Scientific (Fair Lawn, NJ, USA). Acetonitrile was filtered before use on a surfactant-free cellulose acetate filter membrane, 0.2 μm pore size (Suwannee, GA, USA). The standard RPLC checkout sample (1 mL ampoule) was purchased from Agilent technologies. It contains 100.3 μg/mL (±0.5%) of acetophenone, propiophenone, butyrophenone, valerophenone, hexanophenone, heptanophenone, octanophenone, benzophenone, and acetanilide. The solvent of this sample is a mixture of acetonitrile and water (35/65, v/v). Toluene, naphthalene, and acetophenone (>99% purity) were all purchased from Sigma–Aldrich (Suwannee, GA, USA).

3.2. Instruments

Four different very high pressure liquid chromatographs (vHPLC) were used in this work, two 1290 Infinity system (Agilent Technologies, Waldbroon, Germany), a classic Acquity system, and an I-class Acquity system (Waters, Milford, USA) in order to generate four significantly different values of the extra-column volume variances, in the range of 40, 10, 3, and 1 μL^2 at a flow rate of 0.4 mL/min for small molecules.