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Kinetic optimisation of the reversed phase liquid chromatographic separation of proanthocyanidins on sub-2 μm and superficially porous phases

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ABSTRACT

Phenolic compounds, and proanthocyanidins in particular, are important natural molecules which are of significant importance due to their sensory and biological activities. The analysis of proanthocyanidins in natural products is very challenging due to their complex nature. In this study, the kinetic performance of a range of recently developed C18 columns, including sub-2 μm fully porous and 2.6 μm superficially porous particle-packed columns, was evaluated for improved proanthocyanidin analysis. The kinetic plot method was employed to compare the ultimate performance limits of each column in terms of efficiency and speed for different maximum pressures and temperatures using representative proanthocyanidins comprising a range of molecular weights and functionalities as test analytes. By combining plate height data with relevant parameters such as column permeability and mobile phase viscosity, plots of practically attainable efficiencies as a function of analysis time for specific experimental configurations were obtained and performance limits for all investigated supports could accurately be compared. Both fully- and superficially porous particles provided significant speed and/or efficiency gains compared to conventional 5 μm particle packed columns. Analyte properties, particle size and packing quality as well as analysis temperature were all found to have a significant influence on the performance of the presently investigated chromatographic supports. For smaller compounds, higher optimal linear velocities and better performance in the low-efficiency range were observed, while the lower diffusion coefficients of larger procyanidins resulted in lower optimal linear velocities and better performance in the high-efficiency regime. Faster analyses become possible at higher temperatures due to decreased eluent viscosity and faster mass transfer, which was especially beneficial for larger compounds and resulted in dramatic improvement in efficiency. A possible explanation of the abnormal behaviour of oligomeric procyanidins is presented. Our findings indicate that new column formats, when used under optimal conditions, significantly improve the speed and/or efficiency of reversed phase liquid chromatographic analyses of proanthocyanidins.

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1. Introduction

Phenolic compounds are among the most studied, yet complex group of natural compounds. These molecules are of particular interest due to their wide range of health benefits, including antioxidant, anti-hypertensive, anti-depressant and anti-inflammatory activities [1–3], as well as the influential roles they are known to play in the determination of food quality [4,5]. Despite recent advances in phenolic analysis [6], the high complexity and diversity of chemical properties of these molecules continues to hamper their detailed analysis. In fact, there is a continuous demand for faster and more efficient methods for the routine and in-depth analysis of phenolic compounds.

Proanthocyanidins are a subclass of phenolic compounds, which, due to their complexity, constitute a severe analytical challenge. Natural proanthocyanidins vary widely in terms of molecular weight (MW) and isomeric structures, with the result that current chromatographic methods for their analysis are unable to provide complete resolution. In order to design improved methods for proanthocyanidin analysis, a better understanding of the chromatographic behaviour of these compounds is required.

With recent developments in HPLC column technology and stationary phase chemistries, newly designed and improved HPLC columns continue to be introduced on the market. Of these, the most important developments which have found application for phenolic analysis include the use of smaller particle-packed columns and/or elevated temperatures to improve the HPLC

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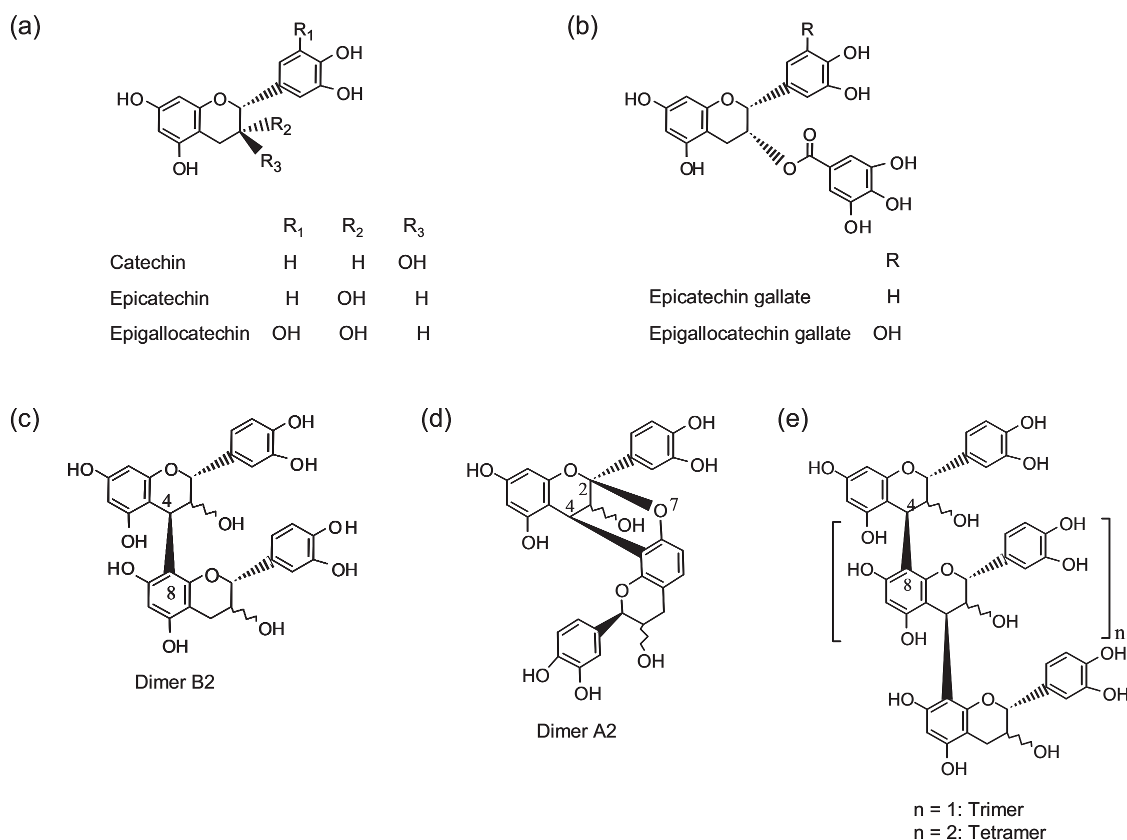

Fig. 1. Chemical structures for different phenolic compounds under investigation.

separation of phenolic compounds [7–21]. However, there remains uncertainty in recent literature about the quantitative benefits of these approaches compared to conventional HPLC methods for phenolic determination.

Given the versatility of new column and instrumental developments, analysts are presented with the challenge of having to select a column and instrument that are best suited for an intended application, as they are faced with a wide range of selectivities and column formats to choose from [22]. As a result, method development becomes a lengthy process and column selection a difficult task.

The availability of methods such as the kinetic plot method (KPM) that allows one to gain a rapid in-depth understanding of the performance limits of different chromatographic supports [23–26] strongly facilitates these assessments. The most attractive feature of the KPM, in relation to classical methods, lies in its ability to combine plate height data with other relevant parameters such as column permeability, mobile phase viscosity and maximum operating pressure to provide plots from which attainable efficiencies and corresponding analysis times for specific system configurations can easily be deduced [23,24]. Inclusion of this information becomes especially relevant when a comparison of the performance of differently shaped and sized chromatographic supports is sought, as there is no need to specify a common reference length [23,24]. This knowledge is invaluable when designing methods for targeted groups of compounds as it helps the analyst to make an informed decision regarding the choice of column and/or analysis conditions. Also of importance is the fact that the chemical composition of real world samples can differ significantly, resulting in widely varying chromatographic behaviours as a function of the physico-chemical properties of the target analytes [7].

In view of the precedent discussion, the current study was aimed at investigating the potential benefits of a new generation of C18 columns packed with both fully porous and superficially porous particles for proanthocyanidin analysis by means of a detailed kinetic evaluation. For this purpose, selected proanthocyanidin compounds covering a range of functionalities and molecular weights were used as test analytes (Fig. 1). A critical comparison of the kinetic performance of the different columns as well as a discussion on the practical implications for proanthocyanidin analysis is presented.

2. Experimental

2.1. Reagents and materials

Standards of (−)-epicatechin (EC) and (±)-catechin (C), (−)-epigallocatechin (EGC) and (−)-epicatechin gallate (ECG) were purchased from Sigma Aldrich (Steinheim, Germany). Procyanidin A2 (dimer A2), procyanidin B2 (dimer B2) and (−)-epigallocatechin gallate (EGCG) standards were purchased from Extrasynthèse (Genay, France). Trimeric and tetrameric procyanidin standards were preparatively isolated and purified in our laboratory by hydrophilic interaction chromatography (HILIC) and reversed-phase liquid chromatography (RP-LC) and characterised using mass spectrometry (MS). HPLC grade acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). Deionised water was obtained using a Milli-Q water purification system (Millipore, Milford, MA, USA). All solutions were filtered through 0.45 μm HVLP membrane filters (Millipore) and degassed in an ultrasonic bath (Branson Model 3510, Danbury, USA) before use.

Table 1
Retention factors, molecular masses and diffusion coefficients for the phenolic standards used in the study.

Compound	Retention factor (k)	Molecular mass (g/mol)	Diffusion coefficients[a] (m^2/s)
Epigallocatechin (EGC)	3.5 ± 0.2	306	5.15×10^{-10}
Catechin (C)	4.7 ± 0.4	290	5.24×10^{-10}
Epicatechin gallate (ECG)	7.5 ± 0.4	442	4.08×10^{-10}
Procyanidin dimer B2 (dimer B2)	9.2 ± 0.7	578	3.48×10^{-10}
Procyanidin dimer A2 (dimer A2)	9.3 ± 0.6	576	3.49×10^{-10}
Epicatechin (EC)	11.4 ± 0.7	290	5.24×10^{-10}
Epigallocatechin gallate (EGCG)	13.1 ± 1.9	458	4.05×10^{-10}
Procyanidin trimer (trimer)	6.4 ± 0.4	866	2.73×10^{-10}
Procyanidin tetramer (tetramer)	8.3 ± 0.6	1155	2.30×10^{-10}

[a] Diffusion coefficients as calculated at 25 °C according to [27].

2.2. Instrumentation

Plate height data for 5 cm columns were experimentally determined on an Acquity UPLC system consisting of a binary pump, degasser, autosampler, column oven, photodiode array (PDA) detector (500 nL flow cell, 10 mm path length), controlled by Waters Empower software (Waters, Milford, MA, USA). Sample volumes of 5 μL were injected using the full loop injection mode. The extra-column- and dwell volumes for this system were ~20 and 145 μL, respectively. An average external variance ($\sigma^2{}_{ext}$) of 0.11 s^2 was measured at 0.8 mL/min.

Plate height measurements for a 25 cm column were performed on an Alliance 2690 HPLC system equipped with a binary pump, degasser, autosampler, column oven, 996 PDA detector (8 μL flow cell, 10 mm path length), controlled by Millenium software (Waters). The pump and column outlet tubings were replaced with minimal lengths of 0.127 mm i.d. PEEK tubing to minimise the extra-column volume. Stainless steel tubing (83 cm, 0.01 in. i.d.) was connected between the injector and the column to preheat the mobile phase. The extra-column- and dwell volumes for this system were ~96 and 812 μL, respectively. An average external variance ($\sigma^2{}_{ext}$) of 2.22 s^2 was measured at 0.8 mL/min.

2.3. Chromatographic conditions

2.3.1. Plate height and system contribution measurements

Plate heights were measured for nine phenolic compounds on four different C18 columns using an isocratic mobile phase consisting of acetonitrile (ACN) and 0.1% formic acid in water (%v/v). Conditions were selected such that the retention factors for all compounds were between 3.5 and 15 (Table 1). The mobile phase compositions were adjusted in order to keep the retention factors constant on all columns. The column characteristics and the mobile phase compositions used for each column are presented in Table 2.

Stock solutions of 1000 mg/L of each standard were prepared in acetonitrile and individual samples with final concentrations of 25 mg/L of each standard and 1 mg/L of uracil (the unretained marker) were prepared such that the final solvent composition was similar to the mobile phase. Flow rates were systematically increased until the maximum column or instrument pressure was reached to ensure a good fitting of the data in the B- and C-term regions of the plate height curves. The maximum allowable pressures (ΔP_{max}) and permeabilities (K_{v0}) for individual columns are listed in Table 2. Measurements were performed in triplicate at each flow rate and experiments were performed at 25 °C and 50 °C. The average relative standard deviations for linear velocity- and plate height values measured for all compounds on all columns were 0.08 and 1.38%, respectively. For the determination of the system contribution, the column was replaced with a zero-dead volume union and uracil was injected under the same conditions.

2.3.2. Gradient analyses

Peak capacities for the gradient separation of a cocoa extract were calculated at different flow rates by injecting 2 μL of a cocoa extract (prepared using a modified method of Kelm et al. [28]) on the Kinetex column. The relevant gradient profiles are summarised in the Supplementary Information (Table S1). The mobile phases were 0.1% formic acid in water (% v/v) (A) and acetonitrile (B) and the column temperature 50 °C.

2.4. Data analysis and kinetic plots construction

For every component in the sample, the number of theoretical plates was calculated using the peak width at half height with Chemstation software (Agilent, Waldbronn, Germany) and an average of three measurements was used for each point. The linear velocity was calculated using the elution time (t_0) of uracil. All reported plate count (N), linear velocity (u_0, mm/s), column pressure (P, bar) and column permeability (K_{v0}, m^2) values were corrected for the system contribution in accordance with the following equations:

$$N_{corrected} = \frac{(t_R - t_{ext})^2}{(\sigma_{tot}^2 - \sigma_{ext}^2)} \quad (1)$$

Table 2
Columns and mobile phase compositions used at different temperatures.

Column	ΔP_{max} (bar)	K_{v0} (m^2)[d]	Mobile phase composition (% ACN)					
			25 °C			50 °C		
			Set 1[a]	Set 2[b]	Set 3[c]	Set 1[a]	Set 2[b]	Set 3[c]
Phenomenex Kinetex C18 (50 mm × 4.6 mm, 2.6 μm)	600	0.72×10^{-14}	8.3	14.7	12.3	5.7	11.9	9.4
Waters UPLC C18 (50 mm × 4.6 mm, 1.7 μm)	1000	0.34×10^{-14}	7.1	13.4	10.6	4.2	10.0	7.4
Waters Xbridge C18 (250 mm × 4.6 mm, 5 μm)	400	1.92×10^{-14}	7.5	13.7	10.8	4.0	9.9	7.3
Agilent Zorbax C18 (50 mm × 4.6 mm, 1.8 μm)	600	0.42×10^{-14}	9.1	15.7	13.0	6.5	12.8	10.0

[a] Set 1 contained EGC, C, procyanidin dimer B2, EC and EGCG.
[b] Set 2 contained ECG and procyanidin dimer A2.
[c] Set 3 contained procyanidin trimer and tetramers.
[d] Values given were calculated for set 1 at 25 °C on each of the columns.

where t_R denotes the solute retention time, t_{ext} is the time spent by the solute in the connecting tubing, σ^2_{tot} and σ^2_{ext} represent the total peak variance and external variance, respectively.

$$u_{0corrected} = \frac{L}{t_0 - t_{ext}} \quad (2)$$

where L is the column length.

$$K_{v0} = \frac{d_p^2}{\phi_0} = \frac{u_0 \eta L}{\Delta P_{col}} \quad (3)$$

in which d_p represents the particle diameter (m), ϕ_0 is the column flow resistance (dimensionless) and ΔP_{col} is the column pressure ($Pa = N/m^2$), obtained by subtracting the pressure drop in the connecting tubing (ΔP_{ext}) from the total pressure drop (ΔP_{tot}).

The reduced (dimensionless) plate heights (h) and linear velocities (ν) were calculated using:

$$h = \frac{H}{d_p} \quad (4)$$

$$\nu = \frac{u d_p}{D} \quad (5)$$

where H and u are the experimental plate height and linear velocity, respectively, while D is the diffusion coefficient (D, cm^2/s), calculated using the Wilke–Chang equation [27]:

$$D_{A,B} = 7.4 * 10^{-8} \frac{(\psi_B MW_B)^{0.5} T}{\eta_B \bar{V}_A^{0.6}} \quad (6)$$

where subscripts A and B symbolise the solute and the solvent, respectively, ψ is the association factor of the solvent (dimensionless), MW is the molecular weight of the solvent (g/mol), T is the temperature (K), η_B is the viscosity of the solvent (cP) and $\bar{V}$ is the molar volume of the solute (cm^3/g mol). The mobile phase viscosity for acetonitrile–water mixtures was calculated according to [29] based on the relationship:

$$\eta_{\chi,T} = 10^{(-2.063+(602/T)+0.071\chi+(62/T)\chi+0.504\chi^2-(346/T)\chi^2)} \quad (7)$$

where χ is the volumetric fraction of the organic solvent.

For the construction of kinetic plots, the following two equations, based on experimental parameters, were employed:

$$N = \frac{\Delta P_{\max}}{\eta} \left(\frac{K_{v0}}{u_0 H} \right)_{experimental} \quad (8)$$

$$t_0 = \frac{\Delta P_{\max}}{\eta} \left(\frac{K_{v0}}{u_0^2} \right)_{experimental} \quad (9)$$

Data were fitted using the Giddings model:

$$H = \frac{A u^n}{1 + D u^m} + \frac{B}{u} + C u \quad (10)$$

where n and m are equal to 1.

3. Results and discussion

The goal of this study was to evaluate a set of new generation support materials for the improved separation of proanthocyanidins. Since it is known that the potential benefits of these phases depend to a large extent on the properties of the analytes under investigation [7], plate height curves and kinetic plots were constructed for a range of standard proanthocyanidins with different properties.

3.1. Van Deemter curves

The kinetic performance of four columns, an Acquity UPLC C18 and an Agilent Zorbax C18-SB, both packed with fully porous sub-2 μm particles, a Kinetex C18 packed with superficially porous 2.6 μm particles and an Xbridge C18 packed with fully porous 5 μm particles, were compared. Each column was operated close to its maximum allowable pressure in order to maximally exploit its ultimate performance limits. To assess the chromatographic performance of the different columns, Van Deemter (VD) curves for different phenolic compounds were constructed and are shown in Fig. 2. Upon inspection of the VD curves for the test analytes, no significant variations were observed in the chromatographic behaviour of low molecular weight compounds C, EC, EGC, ECG and EGCG (data not shown). Notable differences were, however, obvious for high molecular weight compounds (dimeric, trimeric and tetrameric procyanidin isomers) relative to the low molecular weight compounds. For visual clarity, only data for one representative small molecule and those of higher oligomers are therefore presented. The reader is referred to Tables 3a and 3b for summaries of optimal plate heights (H_{min}, h_{min}) and linear velocities (u_{opt}, ν_{opt}) for the individual compounds on each of the columns.

From the H_{min} data, it is evident that comparable performances are obtained for the 2.6 μm superficially porous Kinetex column and the sub-2 μm fully porous particle-packed columns, corresponding to minimum plate heights between 3.74 and 5.20 μm for smaller molecules, and values of 6.62–7.70 μm for the tetrameric procyanidin. On the other hand, the 5 μm column shows minimum plate heights ranging between 10.86 and 14.79 μm. Although these values are well within the theoretically expected H_{min} limits ($H_{min} = 2d_p$) for the respective fully porous particles sizes, the H_{min} values achieved on the Kinetex column (with the exception of the value obtained for the tetrameric procyanidin) are much lower than theoretically expected, indicating a better column packing quality and/or uniform particle size distribution for the superficially porous particles [30–32].

To compare particles with different sizes, columns should preferentially be evaluated in terms of their reduced plate heights ($h = H/d_p$) and reduced linear velocities ($\nu = ud_p/D$) [25,33]. The reduced plate height curves presented in Fig. 2 and the data summarised in Tables 3a and 3b confirm that the 2.6 μm superficially porous column performs significantly better than all other columns, with h_{min} values ranging between 1.4 and 2.0. The sub-2 μm and 5 μm fully porous particles display a similar performance with h_{min} values in the range of 2.0 and 3.0 for small molecules and higher values for the procyanidin tetramer. Previous studies have reported similar low h_{min}-values for the superficially porous particles and have ascribed this to the low eddy dispersion (A-term), resistance to mass transfer (C-term) and recently also to the reduced B-term band broadening associated with the morphology of this particle type [32,34–36].

It is further noted that smaller compounds exhibit higher optimal linear velocities and very flat C-term (resistance to mass transfer) slopes, implying that linear velocities higher than the optimum values can be used without significant losses in efficiency. This trend reverses as the size of the molecule increases. These differences may be partially attributable to inaccuracies in the estimation of diffusion coefficients of especially the larger molecules according to the Wilke–Chang equation. Another possible cause for this unexpected behaviour of the high MW procyanidins may lie in the rotational isomerism of these molecules (refer to Section 3.4). This trend is observed on both small and large particle columns, although the effect is much more detrimental on the 5 μm column due to the dependence of the C-term on the particle size.

Compared to the 5 μm XBridge column, the small-particle packed columns provide minimum plate heights for the small phenolic compounds which are 2.6–2.9 times lower, and optimal linear velocities which are 2.0–2.1 times higher (Table 3a). This implies that by using shorter columns packed with sub-2 μm or superficially porous particles operated at optimal linear velocities, speed gains in the order of 4.7–5.0 times can be obtained compared to

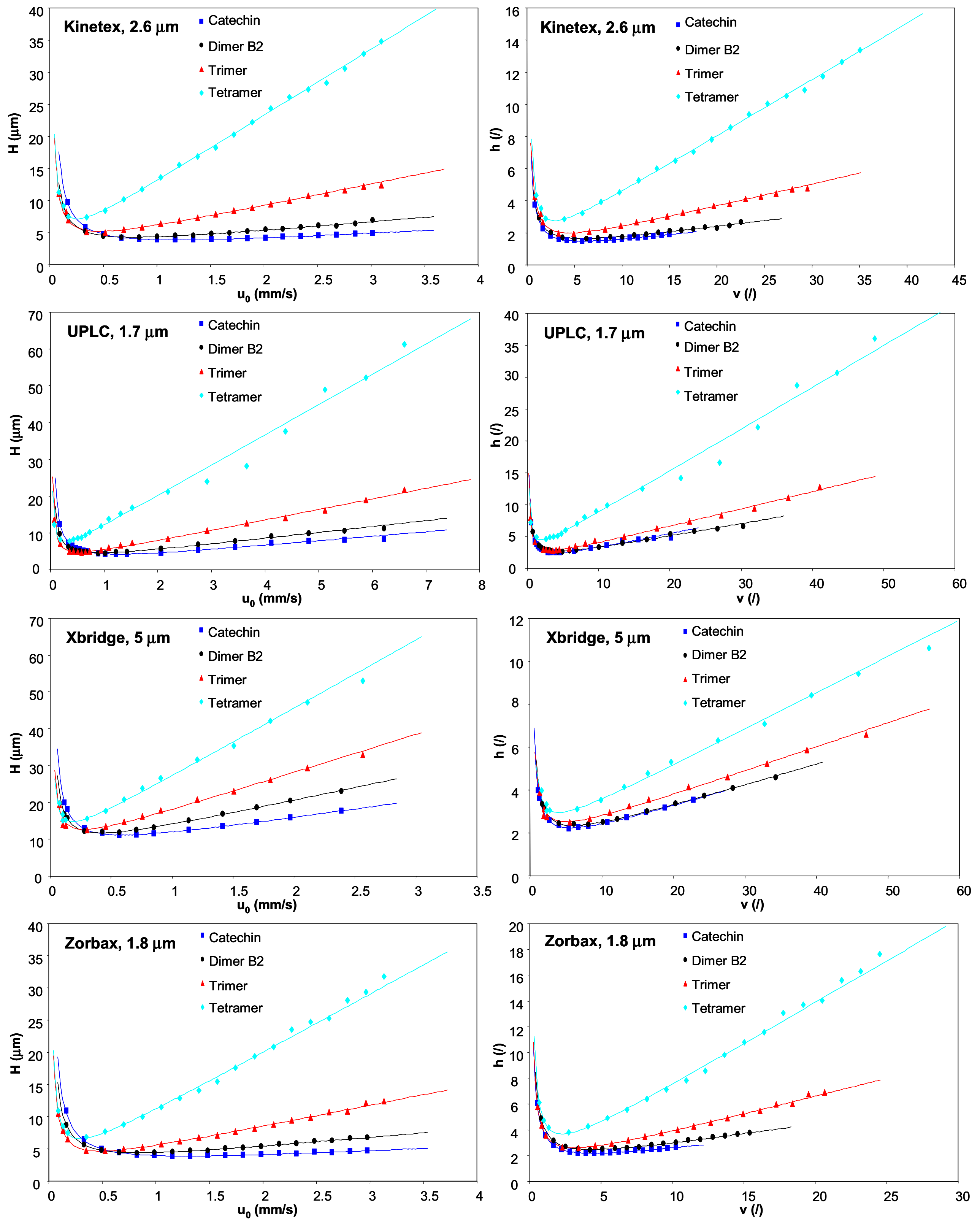

Fig. 2. Van Deemter and reduced plate height curves for catechin (blue), procyanidin dimer B2 (black), trimeric procyanidin (red) and tetrameric procyanidin (cyan) on the four columns under investigation at 25 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

Table 3a
Optimum experimental data obtained for the different phenolic compounds on four different columns at 25 °C.

Compound	Kinetex C18 (d_p = 2.6 μm)				UPLC C18 (d_p = 1.7 μm)				Xbridge C18 (d_p = 5 μm)				Zorbax C18 (d_p = 1.8 μm)			
	H_{min} [a]	u_{opt} [b]	h_{min}	ν_{opt}	H_{min} [a]	u_{opt} [b]	h_{min}	ν_{opt}	H_{min} [a]	u_{opt} [b]	h_{min}	ν_{opt}	H_{min} [a]	u_{opt} [b]	h_{min}	ν_{opt}
EGC	3.74	1.07	1.45	5.07	4.14	1.12	2.27	3.37	10.86	0.51	2.18	5.51	3.75	1.06	2.09	4.05
C	3.86	1.18	1.49	5.83	4.24	1.27	2.48	3.98	11.16	0.59	2.21	5.42	3.90	1.30	2.17	4.55
Dimer B2	4.29	0.79	1.63	6.26	4.62	0.90	2.57	4.32	11.72	0.43	2.38	8.15	4.42	0.85	2.43	4.27
EC	4.16	1.53	1.57	8.35	4.94	1.78	2.91	6.69	11.95	0.85	2.39	6.77	4.18	1.76	2.26	6.26
EGCG	4.53	1.36	1.71	7.54	5.06	1.41	2.94	5.98	12.98	0.68	2.57	8.75	4.39	1.51	2.44	6.61
ECG	4.29	1.25	1.63	8.15	4.68	1.31	2.73	4.68	12.25	0.65	2.44	9.57	4.37	1.37	2.41	6.47
Dimer A2	4.16	1.06	1.60	8.17	4.51	1.15	2.65	4.76	12.08	0.56	2.40	6.71	4.37	1.22	2.41	6.62
Trimer	5.20	0.48	1.94	4.93	4.88	0.51	2.76	3.62	12.67	0.28	2.49	5.58	4.67	0.49	2.60	3.46
Tetramer	7.16	0.26	2.85	3.90	7.70	0.27	4.60	2.67	14.79	0.19	3.02	2.30	6.62	0.30	3.82	2.74

[a] H_{min} is given in μm.
[b] u_{opt} is given in mm/s.

Table 3b
Optimum experimental data obtained for the different phenolic compounds on four different columns at 50 °C.

Compound	Kinetex C18 (d_p = 2.6 μm)				UPLC C18 (d_p = 1.7 μm)				Xbridge C18 (d_p = 5 μm)				Zorbax C18 (d_p = 1.8 μm)			
	H_{min} [a]	u_{opt} [b]	h_{min}	ν_{opt}	H_{min} [a]	u_{opt} [b]	h_{min}	ν_{opt}	H_{min} [a]	u_{opt} [b]	h_{min}	ν_{opt}	H_{min} [a]	u_{opt} [b]	h_{min}	ν_{opt}
EGC	4.06	1.15	1.54	4.28	4.55	1.85	2.51	2.95	10.65	0.84	2.13	5.24	4.46	1.60	2.41	3.82
C	4.25	1.18	1.64	3.69	4.59	2.20	2.66	2.91	10.91	1.00	2.21	5.16	4.62	1.82	2.52	3.76
Dimer B2	4.24	0.91	1.64	4.75	4.63	1.70	2.65	4.37	11.24	0.75	2.25	6.45	4.66	1.18	2.52	4.14
EC	5.16	1.39	1.97	4.21	4.96	3.54	2.94	8.26	11.59	1.36	2.32	8.55	5.20	2.02	2.83	4.09
EGCG	5.13	1.15	1.93	4.77	4.99	2.76	2.96	7.14	12.20	1.14	2.42	8.87	4.95	1.60	2.67	4.42
ECG	4.54	1.31	1.73	5.20	5.02	2.40	2.96	5.99	11.65	1.08	2.36	9.80	4.79	1.66	2.62	4.93
Dimer A2	4.43	1.20	1.68	6.09	4.77	2.17	2.84	7.02	11.58	0.96	2.34	8.60	4.62	1.51	2.53	4.62
Trimer	4.41	0.69	1.72	5.25	5.11	1.12	2.97	5.18	12.06	0.58	2.37	5.30	5.28	0.74	2.87	3.68
Tetramer	5.16	0.48	2.09	4.99	6.39	0.81	3.74	5.12	12.68	0.46	2.51	6.29	6.04	0.52	3.42	2.62

[a] H_{min} is given in μm.
[b] u_{opt} is given in mm/s.

conventional 5 μm phases (provided that the columns are operated within their respective pressure limitations). This gain is reduced for the larger procyanidins, corresponding to a value of 3.3–3.8 for the tetrameric compound.

3.2. Kinetic plots of phenolic compounds on different columns

Kinetic plots represent one of the best tools currently available to compare the performance of different chromatographic supports [25]. Plotting the plate number (N) as a function of analysis time (t_R), the type of support that offers the fastest separation for a given efficiency or the highest number of plates in a given analysis time can easily be deduced. For an intrinsic evaluation of columns with different packing properties, separation impedance plots representing the separation impedance (E_0) vs the plate count (N), or the reduced form E_0 vs N/N_{opt}, are more suited as they reflect the pure packing quality of the columns, independent of the applied pressure and/or the mobile phase properties [23]. Kinetic plots of t_R vs N and E_0 vs N/N_{opt} for two representative analytes on the four columns are shown in Fig. 3A and B, respectively.

From Fig. 3A, it can be seen that for a fixed analysis time, the 2.6 μm Kinetex column performs better than all other columns over the entire range of practically relevant efficiencies (~25 000–250 000). The 1.7 μm UPLC column provides very fast separations for efficiencies ranging between 10 000 and 25 000. Fig. 3A also shows that optimum performance for small molecules is obtained in the C-term dominated region using short columns packed with small particles. Large molecules are optimally separated at low flow rates (B-term dominated region) and highly efficient separations can be achieved on very long columns, however at the cost of long analysis times. In addition, Fig. 3A shows that efficiencies greater than 160 000 are not attainable on columns packed with sub-2 μm particles under the given pressure constraints when analysing small phenolic molecules. However, when

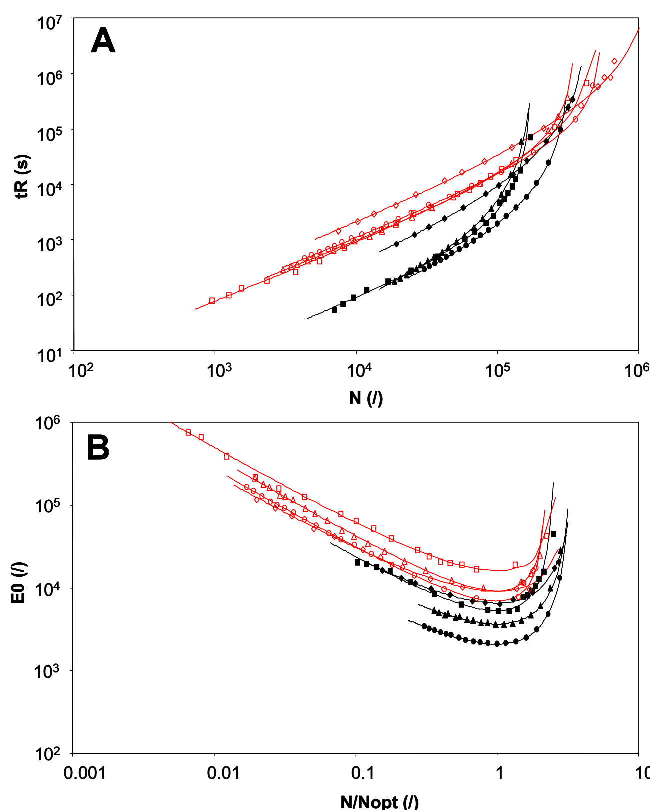

Fig. 3. Kinetic plots of (A) t_R vs N and (B) E_0 vs N/N_{opt} for catechin (black, closed symbols) and the tetrameric procyanidin (red, open symbols) on the 2.6 μm Kinetex (circles), 1.7 μm UPLC (squares), 5 μm Xbridge (diamonds) and 1.8 μm Zorbax (triangles) columns at 25 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

these columns are operated at the optimal flow rate for the larger procyanidins, they outperform the larger particles in the efficiency range up to 250 000 plates. Because of the low backpressures associated with the superficially porous particles, long (2.9 m) Kinetex columns can also be used for small molecule analysis to reach efficiencies up to ∼280 000 in an analysis time of roughly 26 h, while this efficiency range can be extended to ∼485 000 (on a 3.6 m column) when dealing with large molecules. It is only for efficiencies in excess of 300 000 for small molecules and 500 000 for large molecules that the 5 μm Xbridge column outperforms all other columns. These efficiencies are, however, outside the practical range of most HPLC applications as they require column lengths longer than 5 m. It is clear from Fig. 3A that for separations requiring conventional efficiencies ($N \sim 25\,000$), all three small particle columns will provide much faster analyses compared to the 5 μm phase when operated at optimal conditions and maximum pressure. For small molecules, this translates to a 4.3–5.2 times reduction in analysis time, whereas for the tetrameric procyanidin the gain is reduced to 2.0–2.3. Alternatively, the benefits of these phases (within the range of optimal efficiencies) can be exploited to increase the efficiency for a given analysis time.

Fig. 3B shows reduced separation impedance plots for catechin and the tetrameric procyanidin on the four columns. From these plots, it is clear that the Kinetex column provides the lowest E_0 values, with minimum values of 2084 and 6911 for catechin and the tetramer, respectively, while these values range between 3640 and 6505 for catechin and 9041 and 16 137 for the tetramer on the other columns. From the observed trend, it can be generalised that the separation impedance number for a given column increases as the size of the molecule increases. The E_0 value of around 2000 obtained for the Kinetex column is generally considered to be very good, reflecting the higher permeability and good packing quality of this column [30]. This good performance has been accredited to the uniform particle size distribution achieved during the manufacturing process of these particles, which leads to a very homogeneous packing [37].

3.3. Effect of temperature on plate height behaviour

The benefit of using elevated temperatures in liquid chromatographic separations is well documented in literature. An increase in temperature is associated with a reduction in mobile phase viscosity and a faster analyte diffusion, which improves the mass transfer of analytes between the mobile and stationary phases and therefore results in better efficiency at higher flow rates [38–43]. To study the effect of temperature in phenolic analysis, two temperatures, 25 and 50 °C, were selected for evaluation. A maximum temperature of 50 °C was selected to compare all columns at equivalent temperatures (the maximum temperature of the Kinetex column is 60 °C according to the manufacturer) and to reduce the risk of thermal degradation, since proanthocyanidins are known to be susceptible to thermal degradation and epimerisation reactions [44,45]. Fig. 4 represents Van Deemter plots for phenolic standards on four different columns as obtained at the two temperatures. From these plots, it can immediately be seen that the C-term slope flattens as temperature increases. This results in a broadened range of optimal linear velocities towards high flow rates, implying that faster analyses can be performed at higher temperatures without a significant loss in efficiency. For small molecules, an increase in analysis temperature was only found to be useful for increasing analysis speed, since the maximum attainable efficiencies were roughly unchanged. This behaviour is in agreement with previous reports where it was attributed to decreased resistance to mass transfer due to increased diffusion coefficients at high temperatures as well as improved secondary interaction kinetics [39,40,46–48]. Interesting to note is the differences in reduced plate height curves as a function of temperature for the Kinetex and Zorbax columns in Fig. 4. Since the reduced velocity takes changes in D into account, curves at both temperatures are expected to overlap, as is indeed observed for the small molecules on the UPLC and XBridge columns. The abnormal increase in reduced plate height has previously been observed for superficially porous (Halo) phases, where this behaviour was tentatively ascribed to "unexpected variation of the eddy dispersion with the linear velocity at high temperatures, which might be related to the roughness of the external surface of the superficially-porous particles" [49]. For the Zorbax column, this phenomenon may be a result of the larger internal diameter of this column (4.6 mm i.d.), resulting in less effective preheating of the mobile phase at high flow rates.

Temperature elevation proved to be much more beneficial for larger molecules, leading to a dramatic improvement in efficiency and a significant gain in analysis speed on all particle sizes. This is especially clear from the reduced plate height curves for the larger molecules on all columns. The minimum plate heights for the trimeric and tetrameric procyanidins were reduced, while the optimal linear velocities shifted to higher values at 50 °C compared to 25 °C. A comparison of the kinetic data obtained for the trimeric and tetrameric procyanidin isomers at 25 and 50 °C shown in Tables 3a and 3b indicate efficiency gains ranging between 5 and 18% for the trimer and 17 and 39% for the tetramer, with the highest gain on the superficially porous particles. In addition, analysis speed increased by factors between 1.4 and 2.2 for the trimer and 1.8 and 3 for the tetramer, with the highest gain on the UPLC (1.7 μm) column. Possible reasons for the unexpected decrease in minimum plate height for oligomeric procyanidins as a function of temperature, which cannot be explained based on increased diffusion and lower viscosity, will be discussed in the next section.

In summary, both sub-2 μm fully porous and 2.6 μm superficially porous particles delivered superior performance compared to the 5 μm particles, a fact accredited to the good mass transfer properties of small particles. For 5 μm particles, an increase in temperature resulted in improved chromatographic performance in the high linear velocity region for both small and large molecules. The fact that optimum column performance was only achieved at a much higher temperature for larger compounds indicates that high temperature is necessary for faster, efficient separation of high MW procyanidins, provided that analyte and column stability are ensured.

3.4. Effect of analyte properties and retention factor on kinetic performance

It is well known that the kinetic performance of a chromatographic support is highly influenced by the properties of the analyte under investigation [7]. To better understand the effect of analyte molecular weight on kinetic performance, plots of t_0/N^2 vs N were constructed for four compounds with molecular weights ranging between 290 and 1155 g/mol. From the curves shown in Fig. 5, it is evident that the optimum plate count (N_{opt}) is gradually shifted to higher N values as the weight of the molecule increases. This means that large molecules are optimally separated at low flow rates on long columns (B-term dominated region), while small molecules are optimally separated on short columns at high flow rates (C-term dominated region). These variations are expected for analytes with smaller diffusion coefficients. However, the increase in the minima of the curves observed especially for the tetrameric procyanidin is unexpected.

The abnormal plate height behaviour of the high MW procyanidins (Figs. 2–5), and especially the decrease in minimum plate height observed for these molecules as a function of temperature (Fig. 4), indicates that secondary equilibria may play a role in their chromatographic behaviour [50]. Similar kinetic behaviour was

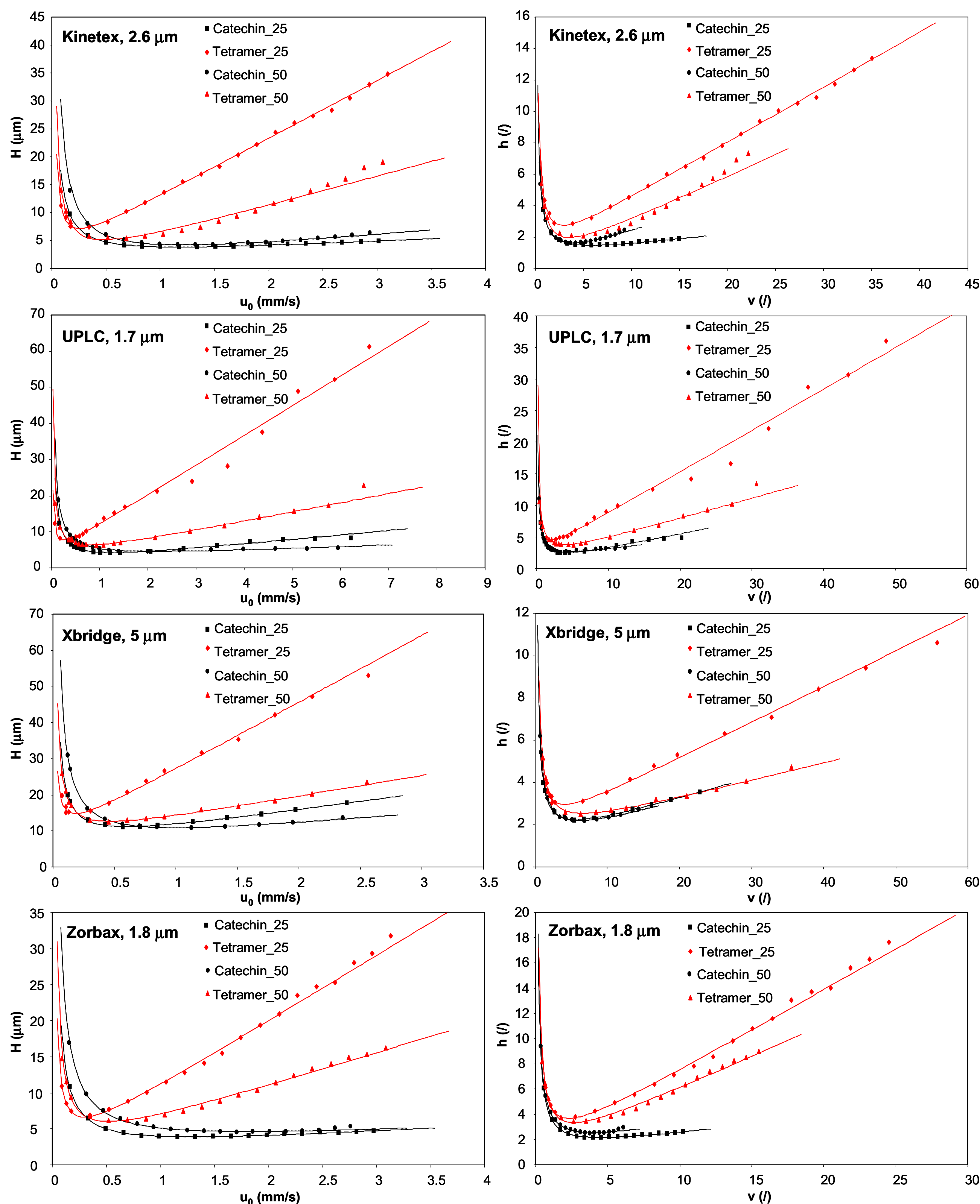

Fig. 4. Experimental Van Deemter plots and reduced VD plots for a small (catechin, black, ■/●) and a big (tetrameric procyanidin, red, ♦/▲) molecule on four columns at 25 and 50 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

previously observed for anthocyanins [12,13], which are known to exist in several chemical forms in solution. We have shown how the pH- and temperature dependant equilibria between these forms are responsible for higher minimum plate heights for anthocyanins [12]. In essence, the interconversion between chemical species on the same time scale as the chromatographic separation is responsible for additional band broadening. In the case of anthocyanins, an increase in temperature results in faster

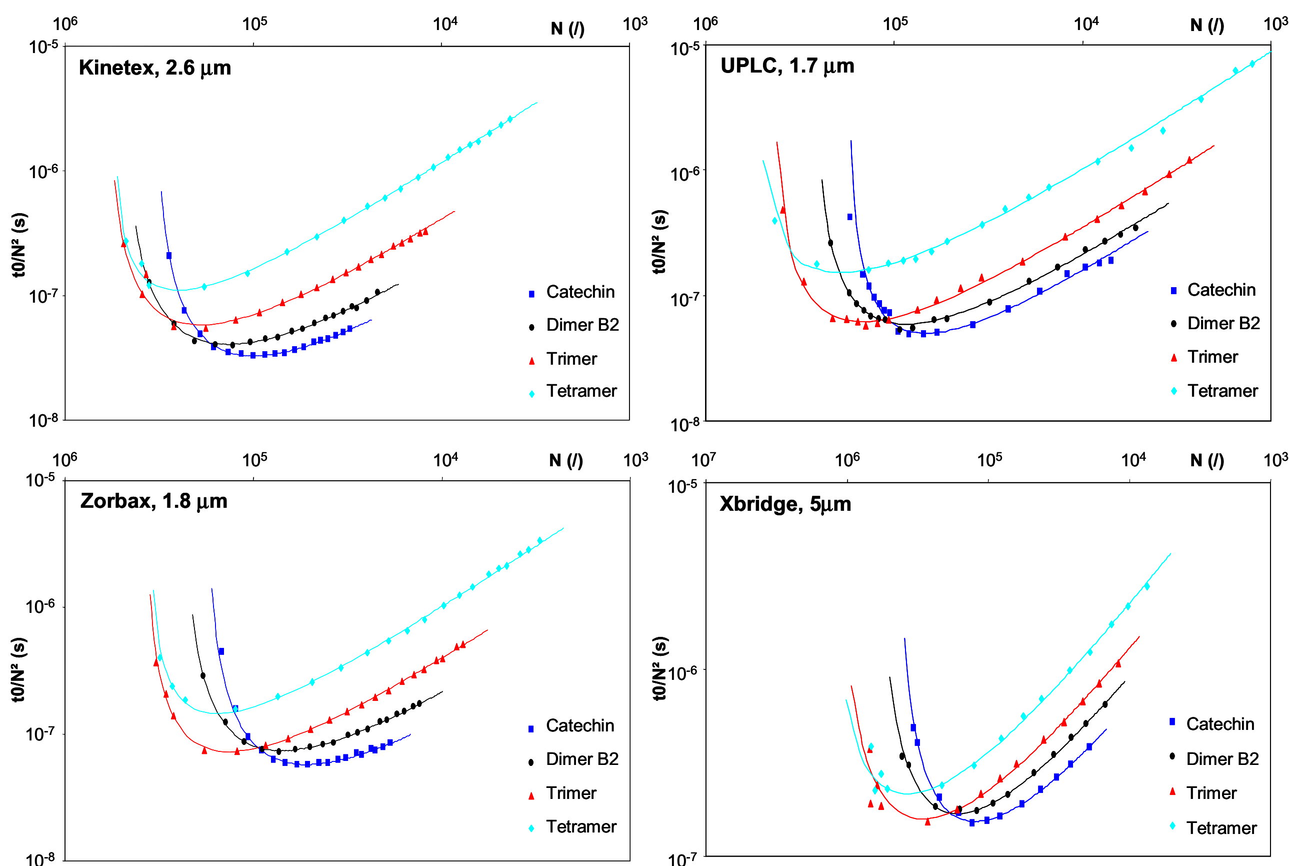

Fig. 5. t_0/N^2 vs N plots for catechin (blue, squares, MW = 290 g/mol), procyanidin B2 (black, circles, MW = 579 g/mol), a procyanidin trimer (red, triangles, MW = 866 g/mol) and a procyanidin tetramer (cyan, diamonds, MW = 1155 g/mol) on the Kinetex, UPLC, Zorbax and Xbridge columns obtained at 25 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

kinetics of the interconversion reaction, and therefore a reduction in the minimum plate height at higher temperatures. A similar phenomenon may be responsible for the same behaviour observed here for the procyanidins.

Proanthocyanidins are subject to epimerisation reactions involving the C-2 carbon (Fig. 1) [51,52]. No kinetic data on epimerisation reactions involving oligomeric procyanidins are available, although for monomeric proanthocyanidins this reaction is very slow (compared to the time scale of chromatographic separation) [52]. It can be confirmed using the approach reported in [12,50] that this reaction is much too slow to affect chromatographic peak shapes. This is supported by the fact that individual isomers are easily separated in RP-LC, which would not be the case if the reaction occurred in the timeframe of the chromatographic separation.

Another more likely explanation for this behaviour may lie in the temperature-dependant rotation of (epi)catechin units around the inter-flavonoid bond of oligomeric B-type procyanidins [53]. This phenomenon may be observed in the detection of rotational isomers of peracylated dimeric procyanidins at ambient temperatures by nuclear magnetic resonance (NMR) [54–56]. For native procyanidins, first-order NMR spectra are observed due to the relatively fast (on the NMR time-scale) interconversion between these rotational isomers, although they may be detected in the fluorescence time domain. Due to a lack of kinetic data on this rotation reaction for higher MW procyanidins, we could not quantitatively confirm that this phenomenon is responsible for band broadening at low temperature chromatographic separations. However, since the individual isomers of procyanidins (catechin and epicatechin) are easily separated in RP-LC, and for higher MW procyanidins retention is primarily affected by the properties of the bottom terminal unit [18,57], it is hypothesised that the individual rotational isomers will display different retention under RP-LC conditions. In this case, rotational isomerism on the same time scale of the chromatographic separation will lead to band broadening. This hypothesis may be tested by comparing the chromatographic behaviour of B-type procyanidins (where rotation occurs freely around the inter-flavonoid bond) with that of the corresponding A-type procyanidin (Fig. 1), where the additional ether linkage is expected to hinder free rotation of the subunits.

Fig. 6A compares the plate height curves measured for A-type and B-type procyanidin dimers on the Zorbax column at 25 °C. A slightly higher minimum plate height and C-term slope are observed in this figure for procyanidin B2. Note that these compounds have virtually identical retention factors and diffusion coefficients (Table 1), and therefore the effect of these parameters on the chromatographic behaviour of these molecules is negligible. Also note that the discrepancy between the plate height behaviour of monomeric- and tetrameric procyanidins is significantly lower for the 5 μm XBridge column. This can be ascribed to the much longer length (and therefore t_R values) of this column, which reduces the effect of secondary equilibria compared to the shorter 50 mm small-particle packed columns [12,13].

Analogously to anthocyanins, it is expected that an increase in temperature will lead to faster interconversion (in this case between rotational isomers) and therefore less band broadening due to secondary equilibria. Indeed, as observed in Fig. 6B, H_{min} and u_{opt} values for the B-type procyanidin dimer are closer to those of the corresponding A-type molecule at 50 °C.

If, as seems likely, this phenomenon is indeed responsible for the abnormal chromatographic behaviour of oligomeric procyanidins,

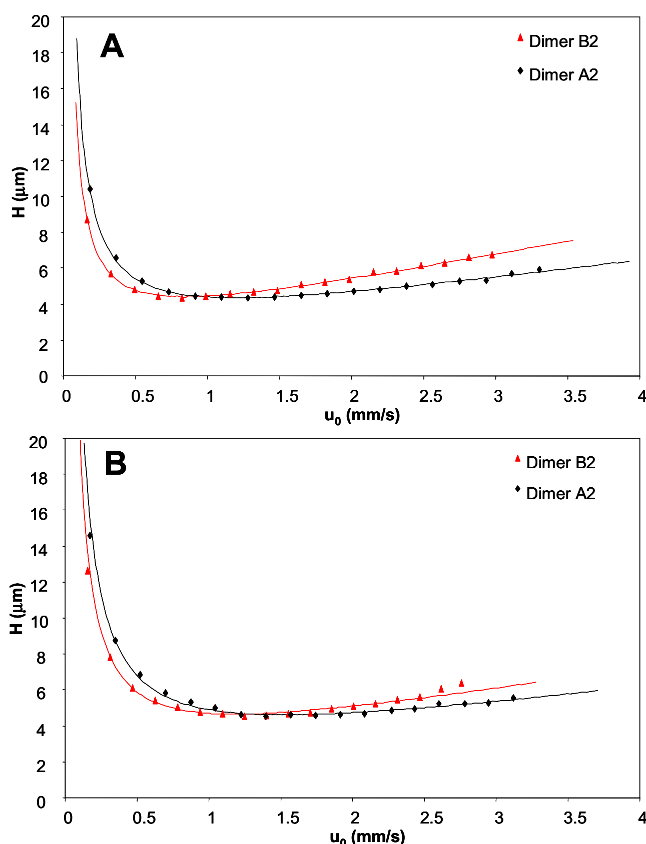

Fig. 6. Experimental Van Deemter plots for dimer B2 (▲) and dimer A2 (♦) obtained on the 1.8 μm Zorbax column at 25 °C (A) and 50 °C (B).

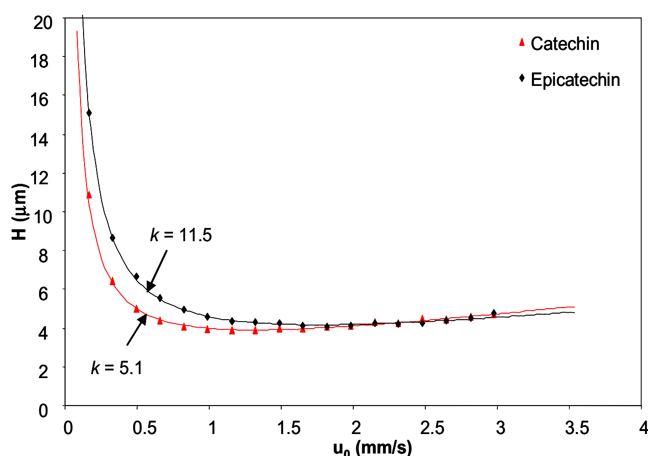

Fig. 7. Experimental Van Deemter plots for catechin (▲) and epicatechin (♦) obtained on the 50 mm × 4.6 mm, 1.8 μm Zorbax column at 25 °C.

it is expected that the effect will be exacerbated with an increase in the degree of polymerisation (DP) (and therefore the number of interflavonoid bonds). Indeed, this is supported by the increasing H_{min} values observed in the sequence catechin ∼ dimer A2 < dimer B2 < trimer < tetramer.

These data confirm that analyte properties significantly influence the performance of a chromatographic support, demonstrating the risk associated with making assumptions on the optimal chromatographic conditions based on a single analyte, even when working with compounds that have similar structures. Rather, when designing chromatographic methods for specific samples, it is essential to base decisions on kinetic data for as many as possible representative analytes of the mixture.

3.5. *Effect of retention and the system contribution on plate height*

To demonstrate the effect of the retention factor on chromatographic performance, two compounds (catechin (k = 5.1) and epicatechin (k = 11.5) with comparable diffusion coefficients and molecular weights (Table 1) were selected. In the absence of molecular weight and/or diffusion coefficient differences, it is expected that these compounds will exhibit very similar chromatographic behaviour. Van Deemter plots for these compounds on a Zorbax column at 25 °C and 50 °C are presented in Fig. 7. Note that this 4.6 mm i.d. column was selected for this comparison, since the effect of extra-column band broadening is significantly reduced (see further). Comparing the curves for catechin and epicatechin, it is evident that the latter shows a slightly flatter C-term slope at high flow rates, and especially much higher plate heights in the low linear velocities (B-term) region. These discrepancies can be attributed to the differences in retention factors: an increase in retention is expected to lead to an increased B-term, as more time is available for longitudinal diffusion to occur [7], as seen for epicatechin with a k value of 11.5. Higher retention will also lead to a slight reduction in the C-term for the range of k values used here due to the dependence of this term on k. It should be noted that these trends were observed on all columns and that highly retained analytes displayed slightly higher B-terms compared to weakly retained ones. It is also worth mentioning that the differences in the C-term region were greatly reduced at 50 °C, for reasons discussed in Section 3.3.

In a chromatographic system, analyte zones broaden as a result of both the physical processes occurring inside the column, extra-column causes such as injection effects, detector volume, dwell volume between injector and detector as well as time-related effects such as acquisition rate and detector time constant [58–60]. Since the efficiency (N) of a peak in isocratic separations is directly related to its retention volume (V_n) or time (t_R) (as given by [59,61]):

$$N = \left[\frac{V_n}{\sigma}\right]^2 = a\left[\frac{V_n}{w}\right]^2 \quad \text{or} \quad N = \left[\frac{t_R}{\sigma}\right]^2 = a\left[\frac{t_R}{w}\right]^2 \tag{11}$$

where w denotes peak width (a is a constant determined based on the height where the peak width was measured), it is expected that peaks exhibiting smaller elution volumes (earlier eluting peaks with small k values) will be more affected by extra-column contributions. Furthermore, given that the peak elution volume decreases with decreasing column length and internal diameter, the detrimental effects of the extra-column contributions will also be much more pronounced on shorter, small i.d. columns than on conventional i.d. columns.

To quantify the effect of the column internal diameter on column performance, two 5 cm columns, a 1.7 μm, 2.1 mm i.d. UPLC and a 1.8 μm, 4.6 mm i.d. Zorbax column, were compared. Since the experiments were performed for compounds with similar retention factors on the same instrument and the column lengths and particles sizes of the two columns are similar (as per manufacturers specifications), it is expected that differences in performance will mainly be caused by differences in column diameter. Fig. 8 presents VD curves for weakly (EGC, k = 3.5), averagely (ECG, k = 7.5) and strongly retained analytes (EGCG, k = 13.1) on the two columns before and after accounting for extra-column contribution to band broadening according to Eq. (1). Comparing the plots of EGC on the two columns, no significant differences in performance are observed for the Zorbax column between data obtained with and without correction for the system contribution. Noteworthy differences can, however, be seen for the 2.1 mm i.d. UPLC column, where an efficiency loss of more than 40% is obtained for EGC, while the extra-column contribution

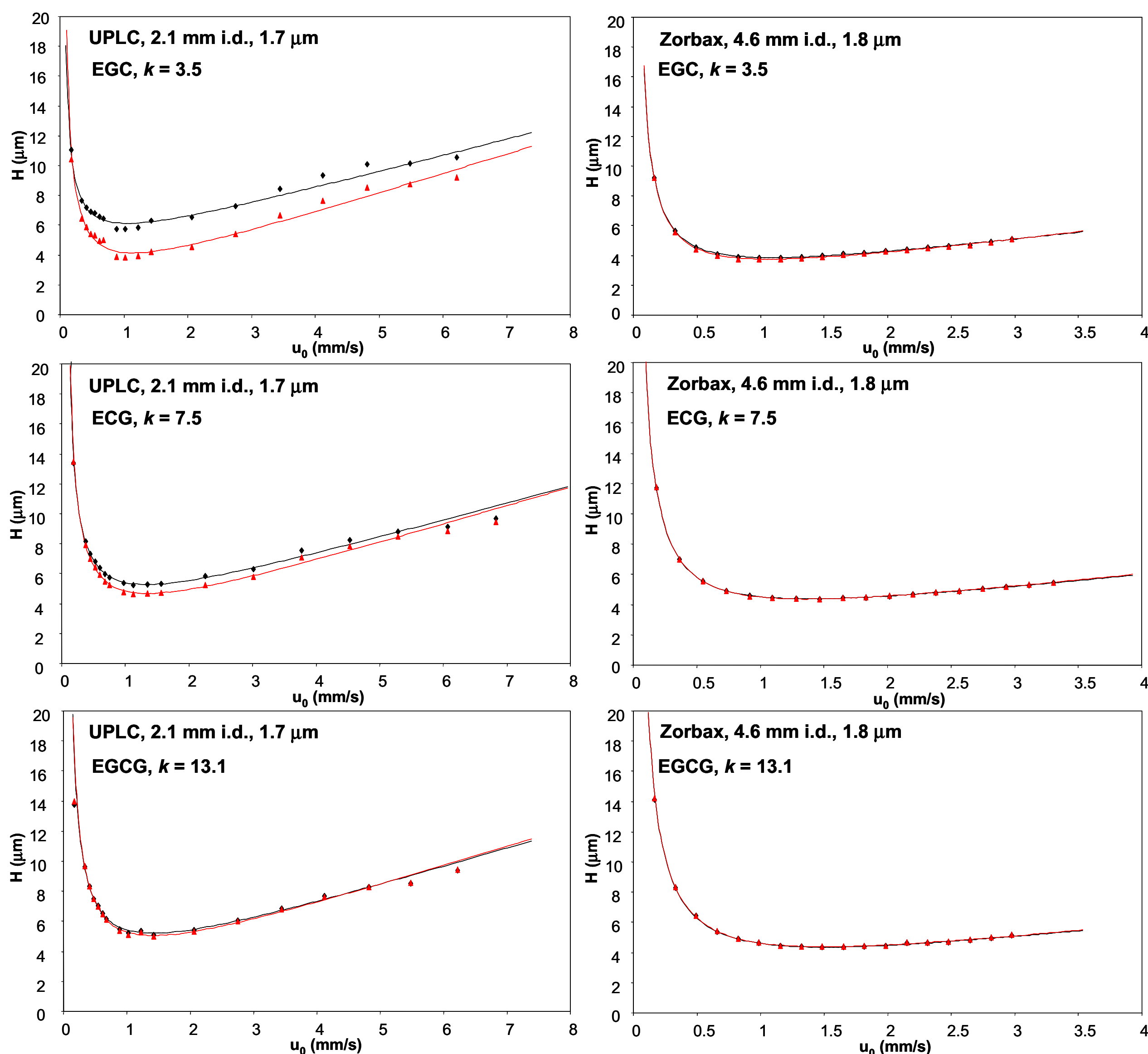

Fig. 8. Experimental Van Deemter curves obtained for the weakly retained (EGC, k = 3.5), averagely retained (ECG, k = 7.5) and well retained (EGCG, k = 13.1) analytes on the 1.7 µm UPLC and 1.8 µm Zorbax columns before (black, diamonds) and after (red, triangles) accounting for system contribution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

steadily decreases as retention increases. It can also be seen that the extra-column effects on column performance are virtually negligible for the most retained analyte on both columns.

While the efficiency of larger bore columns is not greatly affected by the extra-column effects, particular attention needs to be paid when working with narrow bore columns as significant loss in efficiency can be obtained for small k values ($k \leq 7$). Note that these experiments were performed on an Acquity UPLC system, which has a very low extra-column volume (∼20 µL in the set-up used here). This approximates the lowest extra-column volumes of commercially available instrumentation [62,63], which further highlights the importance of using suitable instrumentation when employing short small particle-packed columns. The importance of this aspect is however somewhat reduced in gradient operation due to focussing of analytes at the head of the column.

While extra-column band broadening is of less importance for conventional bore columns (such as the 4.6 mm Zorbax column used here), this benefit is offset by the detrimental effects of frictional heating occurring on wider bore columns operated at high flow rates [64].

3.6. Practical implications of kinetic data on phenolic analysis

The data obtained for proanthocyanidins in this study show that the optimal linear velocity decreases as the particle size and size of the molecule increase. The dependence of the optimal linear velocity on the analyte molecular weight has severe practical implications for isocratic separations, as working outside the optimal velocity range will result in a significant loss of efficiency. As a consequence, for samples containing compounds spanning a wide range of molecular weights such as often encountered in

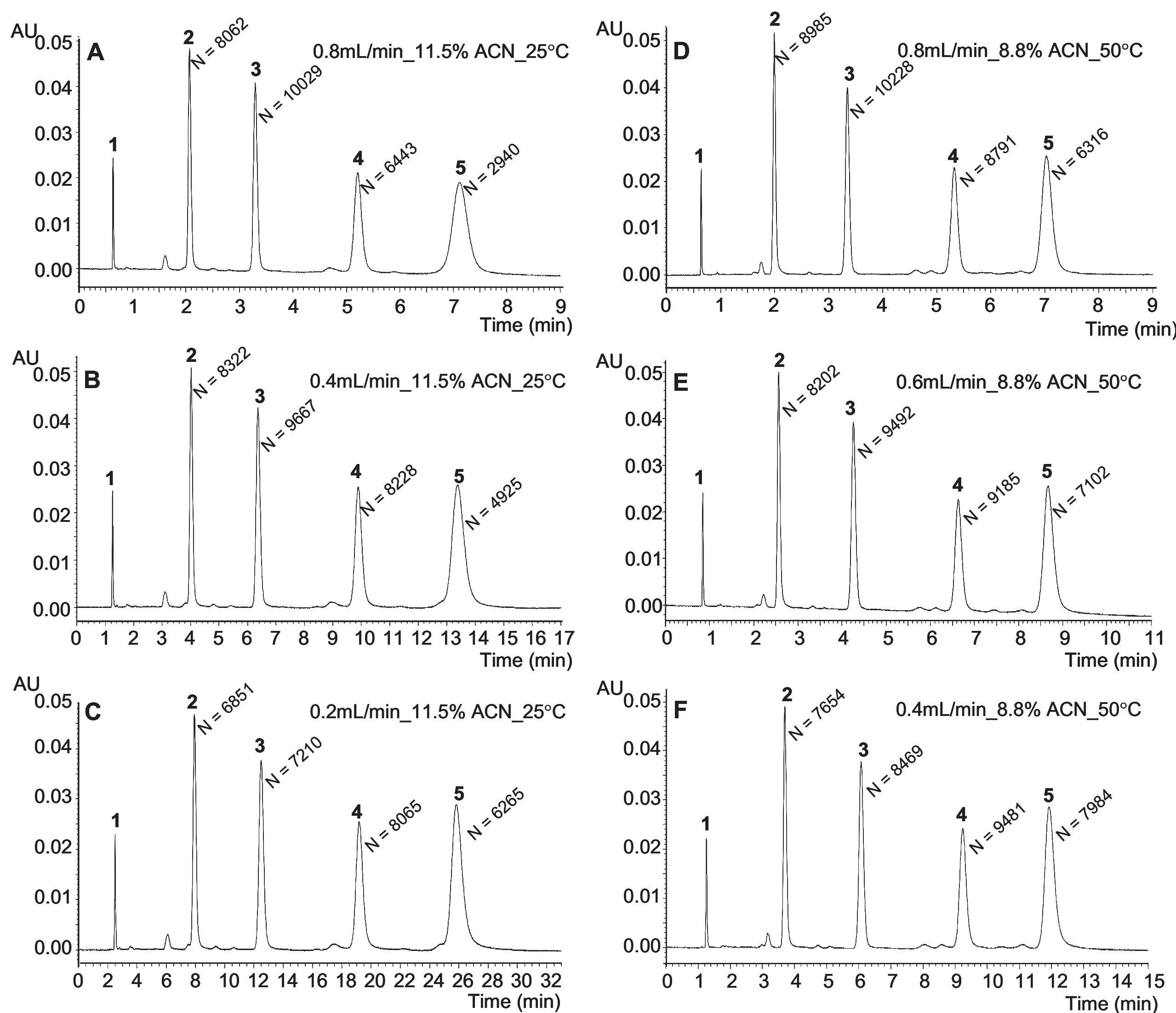

Fig. 9. Chromatograms for isocratic separations of a mixture of large and small procyanidins obtained on a Kinetex column (50 mm × 4.6 mm, 2.6 μm) at 25 and 50 °C using flow rates specified in the figure. Labels—1: uracil; 2: catechin; 3: epicatechin; 4: trimeric procyanidin and 5: tetrameric procyanidin.

phenolic extracts, it will be impossible to find a linear velocity where all compounds are optimally separated. This implies that a compromise between speed and efficiency will have to be made when dealing with samples of this nature.

To quantify this effect, a mixture of low- and high MW procyanidins were analysed at flow rates close to optimal for each set of compounds as well as at an intermediate flow rate (deduced from the kinetic data obtained at the two analysis temperatures used in this study). From the results presented in Fig. 9, it can be seen that an efficiency loss of 28% is obtained for epicatechin when working at (or close to) the optimal conditions for the large procyanidins (F = 0.2 mL/min), while efficiency losses of 20 and 53% are observed for the trimeric and tetrameric procyanidins, respectively, when working at the optimal flow rate for the smaller molecules (F = 0.8 mL/min) at 25 °C. However, when working at an intermediate flow rate (F = 0.4 mL/min), only about 4% and 21% efficiency-loss occurred for epicatechin and the tetrameric procyanidin, respectively.

A similar trend, albeit smaller, is obtained at 50 °C, where efficiency losses of 7 and 20% are obtained for the trimeric and tetrameric procyanidins, respectively, at 0.8 mL/min (close to optimal for small molecules) and 17% for epicatechin when working at 0.4 mL/min (close to optimal for large molecules). However, when an intermediate linear velocity (F = 0.6 mL/min) is used, efficiency losses of about 7, 3 and 11% were obtained for epicatechin and the trimeric and tetrameric procyanidins, respectively. From these results, it seems preferable to tune the chromatographic parameters to the optimal values for higher MW compounds when a mixture of low- and high MW phenolics are analysed, since the loss in efficiency is consistently less for the small molecules.

Most HPLC methods of phenolic analysis are performed on 5 μm columns using flow rates between 0.8 and 1.0 mL/min. While these flow rates are close enough to the optimal linear velocity for smaller molecules, they are clearly above the optimal linear velocity for larger molecules, which, in light of the unique chromatographic behaviour of these compounds, could explain the poor resolution commonly observed for high MW phenolics and procyanidins in particular. For small particle-packed columns, the trend is to further increase the flow rate due to the favourable plate height behaviour for small molecules. However, our results indicate that this will lead to a further reduction in efficiency, and therefore the benefits of these phases for proanthocyanidin analysis are best exploited at relatively low linear velocities, which would still deliver improved performance compared to conventional HPLC methods.

Fig. 10. Chromatograms for the gradient RP separation of a cocoa extract on a Kinetex column (50 mm × 4.6 mm, 2.6 μm) at 50 °C using flow rates specified in the figure. For experimental details, please refer to Section 2.3.2. Peak numbers correspond to the degree of polymerisation of individual procyanidin isomers as identified by LC–MS [17].

Because the optimal linear velocity for high molecular weight compounds is shifted to higher values at high temperatures, elevated operating temperature can be beneficial when analysing samples containing analytes spanning a wide range of molecular weights. A slightly higher linear velocity can then be employed for a faster, efficient separation of high molecular weight compounds while the loss in efficiency for low molecular weight compounds is kept to a minimum.

It should be noted that most routine HPLC methods employ gradient analysis. It has been shown that optimisation of isocratic efficiency leads to equivalent gains in gradient peak capacity (provided the gradient steepness is kept constant), albeit that these values are related via the square root of N [13,65]. For mixtures containing proanthocyanidins comprising a wide range of MWs, the gain in performance will vary due to the divergent effect of chromatographic parameters on the different compounds. In order to evaluate the effect of flow rate on the gradient separation of proanthocyanidins, a cocoa extract containing a wide range of procyanidins of different degrees of polymerisation was analysed under gradient conditions on the Kinetex column at 50 °C. The chromatograms, together with the gradient peak capacities (n_p) calculated according to Neue [66], are presented in Fig. 10A–C. Peak capacity values were calculated using peak widths for a range of molecules of differing DP (indicated in Fig. 10). For this sample, therefore, the average improvement in chromatographic performance is measured. Note that for these analyses, the gradient slope was kept constant for each of the studied flow rates. A gain in peak capacity of 6 and 21% is obtained by decreasing the flow rate from 0.8 to 0.6 and 0.4 mL/min, respectively. Slight changes in selectivity observed in these chromatograms may potentially have been caused by increased longitudinal temperature gradients due to frictional heating at the higher flow rates [64]. Note especially the improvement in resolution obtained under gradient conditions for the higher MW (DP 4–8) compounds in Fig. 10C. These results confirm that the conclusions about the effect of the flow rate drawn from the isocratic kinetic evaluation are also valid for gradient analysis on the same columns. Clearly, for complex mixtures of proanthocyanidins, chromatographic conditions should preferably be selected based on the optimal conditions for the high MW compounds.

4. Conclusions

In light of the increasing interest in the determination of phenolic compounds in natural products, methods aimed at improving the speed and/or resolution of standard RP-LC methods are required. Especially recent developments in sub-2 μm and superficially porous phases show promise for this purpose, although detailed information on the benefits of these phases for phenolic analyses is still lacking. In this study a comprehensive kinetic evaluation of two sub-2 μm and a superficially porous C18 phase was performed using a standard 5 μm phase as reference. The kinetic plot method was used to compare the chromatographic supports and determine optimal analysis conditions for proanthocyanins of differing MWs and chemical properties. Superficially porous 2.6 μm particles provided efficiencies comparable to sub-2 μm fully porous particles, although at lower pressures. It was further shown that analysis temperature, analyte physico-chemical properties, particle size and packing properties all have a strong influence on the performance of a chromatographic support.

Oligomeric procyanidins in particular show much more conservative plate height behaviour (lower H_{min} and u_{opt} values) than expected based on their lower diffusion coefficients alone. We have demonstrated that this behaviour may be explained by the on-column interconversion between rotational isomers. This has important implications for the analysis of higher MW procyanidins, and might indeed be partially responsible for the current lack of suitable 1-dimensional LC methods for the analysis of these compounds. Our results suggest that higher temperatures, longer column lengths and lower flow rates are required for improved procyanidin analysis.

The fact that both the maximum efficiency and the optimum linear velocity decrease as the size of the molecule increases, suggests that a compromise between chromatographic efficiency and analysis speed will have to be made when analysing phenolic extracts containing analytes with widely varying physico-chemical properties if optimum performance is to be achieved. The use of high

temperature is, however, much more beneficial for high molecular weight compounds. Extra-column band broadening was shown to have a large influence on the performance of narrow bore (2.1 mm i.d.) columns for compounds displaying a retention factor below 7, whereas this effect was negligible on conventional bore (4.6 mm i.d.) columns. For the latter columns, however, the detrimental effects of frictional heating have to be considered for analyses performed at very high flow rates.

In summary, our results indicate that the combination of high temperature and new column formats, when used under optimal kinetic conditions for the target analytes, provide significant promise to improve speed and/or efficiency of RP-LC analyses of phenolic compounds compared to conventional HPLC methods.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.02.067.

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