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Dependence of the solute retention on the column pressure in reversed-phase HPLC

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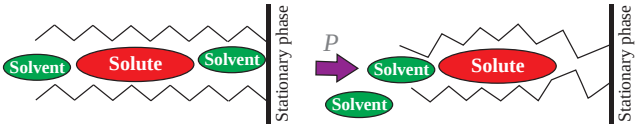
In the course of reversed-phase HPLC analysis, increase in pressure at the column inlet can affect the retention of sorbates only when they penetrate into the grafted phase. Due to conformational changes in the alkyl groups of the loose grafted layer, the solvation of sorbates by these alkyl groups would increase, leading to a growth of the retention factors. In the case of the surface sorption of solutes, the effect is not observed.



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In column chromatography, the only measurable parameter is the retention time of a sorbate i, $t_R(i)$. However, such a characteristic is not convenient for broad use since it depends on the size of the column dimensions and on the velocity of the mobile phase at the given mobile phase composition and temperature. To exclude this dependence, the concept of the retention factor,[1] $k(i)$, is introduced, for the calculation of which an additional measurement of the so-called 'dead' column time, t_0, is required:

$$k(i) = \frac{t_R(i) - t_0}{t_0}. \quad (1)$$

For calculation of thermodynamic parameters of solute transfer from mobile phase into stationary phase, use is made of the dependence of natural logarithms of the retention factor on the reversed absolute temperature:[2]

$$\ln k(i) = -\frac{\Delta H^0(i)}{R}\frac{1}{T} + \frac{\Delta S^0(i)}{R} + \ln\phi, \quad (2)$$

where $k(i)$ is the solute i retention factor, ΔH^0 and ΔS^0 are the corresponding standard changes of enthalpy and entropy, R is the gas constant, T is the absolute temperature of the column, and ϕ is the phase ratio of the column. When the measurement temperature is raised, the pressure at the column inlet drops due to the normal decrease in the viscosity of the mobile phase. Luckily, the thermodynamic parameters are beyond doubt when the retention factors do not depend on the pressure at the column inlet. However, the dependence of the retention factor on the pressure at the column inlet not only for macro- and oligomers,[3–5] but also for small-sized molecules[6–9] has been recently indicated. A thorough analysis of some possible reasons for the dependence of retention on pressure was carried out;[10] however, the retention mechanism was left unattended.

The purpose of this work is to evaluate the effect of pressure on the solute retention in reversed-phase chromatography as a consequence of loose packing of the grafted layer, and to discuss the dependence of the effect on the mechanism of sorbate retention. The dependence of retention on the pressure at the chromatographic column inlet was studied for the separation of six 3-glucosides of the main natural anthocyanidins, such as pelargonidin (Pg3G), cyanidin (Cy3G), peonidin (Pn3G), delphinidin (Dp3G), petunidin (Pt3G), malvidin (Mv3G), and chlorogenic acid (5-caffeoylquine, 5CQA). Isocratic conditions were applied with the eluent containing 10 vol% formic acid and 10 vol% acetonitrile in water on a 150 × 4.6 mm Symmetry C18 column, 3.5 μm. To determine the 'dead' time, oxalic acid was used since the retained volume of this compound practically did not depend on the flow rate of the mobile phase (the discrepancy did not exceed 1%). The retention time measurements were repeated until the last two consecutive chromatograms coincided completely. According to our experience, with the autosampler of the Agilent 1200 Infinity chromatograph when the device entered the mode (the thermostat temperature stabilization is critical), the error in the calculations of the natural logarithm of

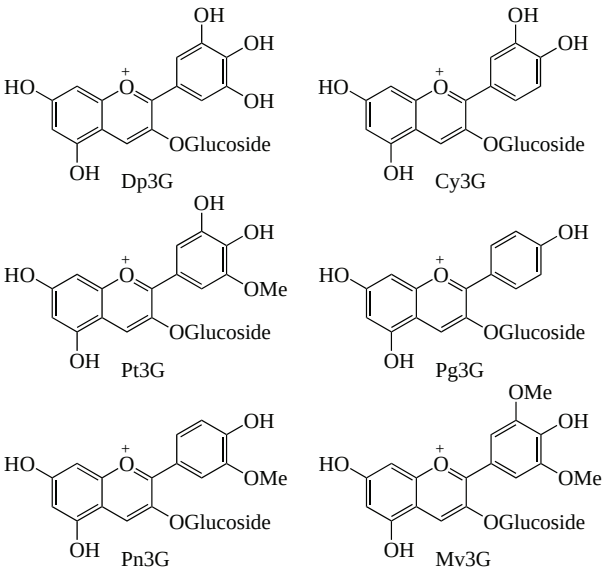


Table 1 Natural logarithms of sorbate retention factors at different column inlet pressures *P* and temperatures *T* (± 0.002, *n* = 3, *p* = 0.95).

Compound	P/u^a at T = 30 °C			Δ_{max} (%)	P/u^a at T = 50 °C			Δ_{max} (%)
	171/0.8	86/0.4	43/0.2		152/0.8	76/0.4	38/0.2	
Dp3G	–0.243	–0.294	–0.333	8.9	–0.975	–0.976	–0.991	1.6
Cy3G	0.407	0.362	0.326	8.0	–0.258	–0.260	–0.271	1.4
Pt3G	0.804	0.755	0.716	8.8	0.129	0.125	0.115	1.4
Pg3G	0.981	0.940	0.907	7.4	0.333	0.335	0.328	0.5
Pn3G	1.384	1.338	1.302	8.2	0.743	0.741	0.734	1.0
Mv3G	1.718	1.667	1.627	9.2	1.057	1.053	1.045	1.3
5CQA	–0.049	–0.063	–0.084	3.5	–0.349	–0.353	–0.359	1.0

[a] Parameter *P* denotes pressure (bar); *u* is mobile phase rate (ml min^{-1}).

the retention factor did not exceed 0.002 logarithmic units (see Table 1). The maximum change in the natural logarithm of the retention factor (Δ_{max}) with increasing pressure was calculated using formula (3):

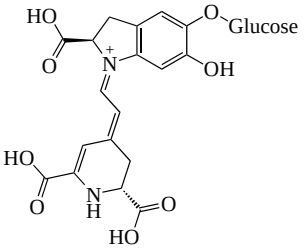
$$\Delta_{max} = \frac{k(P_{max}) - k(P_{min})}{k(P_{min})} \cdot 100\%. \quad (3)$$

For all the substances studied in this work, the change in the natural logarithm of the retention factor was many times greater than the experimental error in determining this parameter at 30 °C, but at 50 °C the increase in retention significantly decreased. In this case, the minimum change in retention at both temperatures was found for 5CQA. This important fact may indicate the cause for this phenomenon. Thus, anthocyanins are retained by the float mechanism, in which the flavylium part is completely immersed in the bonded phase, while the strongly hydrophilic carbohydrate part remains on the surface of the C18 phase.[11] However, the analysis of the chromatographic behavior of chlorogenic acids led to the conclusion that in the case of 5CQA, the penetration of the caffeic acid substituent into the bonded phase is limited, so the retention is mainly due to hydrophobic repulsion onto the surface of the bonded phase, although the influence of residual silanol groups is noticeable.[12]

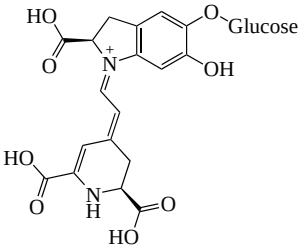
Thus, the effect of pressure on retention depends on the retention mechanism and turns out to be the highest for sorbates penetrating into the grafted layer. To confirm this idea, the dependence of betacyanin retention on pressure was investigated. The analysis of betacyanin retention[13] showed that the mechanism of hydrophobic repulsion of sorbates onto the surface of the grafted phase was most likely. If this hypothesis is true, the pressure should not affect the retention of betacyanins. However, betacyanins have a higher hydrophilicity compared to that of anthocyanins, so their retention in reversed-phase chromatography should be suitable for the analysis only when the acetonitrile content in the mobile phase decreases below 8–5 vol%, and with such a water-rich eluent, a phase collapse is observed for conventional octadecylsilane sorbents.[14] Therefore, to record chromatograms of betacyanins, it is necessary to use only stationary phases resistant to the collapse, *e.g.*, Reprosil-Pur AQ-C18.[13]

Indeed, our experiments have shown that the retention factors of betacyanins on the stationary phase 100 × 4.6 mm Reprosil-Pur AQ-C18, 3 μm in the mobile phase containing 8 vol% of acetonitrile and 1 vol% of phosphoric acid in water did not depend on the pressure at the inlet into the column even at 30 °C (see Table 2).

To explain the results obtained, it is necessary to take into account that the surface density of alkyl groups at C18 phases is two times lower than the packing density of alkyl groups in the solid phase of alkanes.[15] Therefore, there is a lot of 'empty' space in the grafted layer, which is partially filled with molecules



Betanin



Isobetanin

Table 2 Natural logarithms of betacyanin retention factors at different pressures at the inlet into the Reprosil-Pur C18-AQ column at T = 30 °C (± 0.002, *n* = 3, *p* = 0.95).

Compound	P/u^a			Δ_{max} (%)
	98/0.8	49/0.4	24/0.2	
Betanin	1.238	1.236	1.231	0.7
Isobetanin	1.688	1.687	1.682	0.6

[a] *P* is pressure (bar); *u* is mobile phase rate (ml min^{-1}).

of organic solvent of the mobile phase. In this case, the pressure can promote conformational changes inside the grafted layer, increasing the contact area of alkyl groups with the sorbate penetrating into the grafted layer. This can increase the solvation energy in the stationary phase, which leads to an increase in the retention factor.

To summarize, one of the possible reasons for the dependence of sorbate retention on pressure in reversed-phase HPLC may be a change in the solvation of sorbates penetrating from the bonded phase due to conformational changes in loosely packed grafted alkyl groups with increasing pressure. Therefore, it is necessary to develop methods that level the effect of pressure on the thermodynamic characteristics of the transfer of substances from mobile phase into stationary phase. In the case of routine surface sorption, this effect may be absent.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2023.04.044.

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