Measurement of the elution strength and peak shape enhancement at increasing modifier concentration and temperature in RPLC

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Abstract Two approaches are proposed to measure the effect of different experimental factors (such as the modifier concentration and temperature) on the elution strength and peak shape in reversed-phase liquid chromatography (RPLC), which quantify the percentage change in the retention factor and peak width (referred to the weakest conditions) per unit change in the experimental factor. The approaches were applied to the separation of a set of flavonoids with aqueous micellar mobile phases of the surfactant Brij-35 (polyoxyethylene(23)dodecanol), in comparison with acetonitrile-water mixtures, using a Eclipse XDB-C18 column. The particular interaction of each flavonoid with the oxyethylene chains of Brij-35 molecules (adsorbed on the stationary phase or forming micelles) changed the elution window, distribution of chromatographic peaks, and partitioning kinetics, depending on the hydroxyl substitution in the aromatic rings of flavonoids. At 25ºC, peak shape with Brij-35 mobile phases was significantly poorer with regard to acetonitrile-water mixtures. At increasing temperature, the efficiency with Brij-35 increased, approaching at 80ºC the values obtained at equilibrium conditions, already reached with acetonitrile at 25ºC.

Keywords: Reversed-phase liquid chromatography · Elution strength · Peak shape enhancement · Flavonoids · Brij-35 · Temperature

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Introduction

In reversed-phase liquid chromatography (RPLC), the separation performance (i.e. elution strength, selectivity, peak shape and robustness) depends on the analytes, the column nature, the mobile phase composition and other factors as the temperature and pH (with ionisable compounds). There is, however, still a need to develop parameters that allow a fair comparison of the effects among factors. In this work, we propose two general approaches to measure the effect of different experimental factors (as the modifier concentration and temperature) on the elution strength and peak shape. The approaches were applied to the separation of a set of flavonoids using two RPLC modes: micellar liquid chromatography (MLC) with aqueous solutions of the surfactant Brij-35 (polyoxyethylene(23)dodecanol), and the classical RPLC with acetonitrile-water mixtures. In both cases, an Eclipse XDB-C18 column in the range 25−80ºC was used.

In MLC, surfactant micelles are formed in the mobile phase and the alkyl-bonded stationary phase is coated with a layer of surfactant monomers [1]. The variety of new interactions between the solutes, micelles and stationary phase gives rise to a particular chromatographic behaviour with respect to acetonitrile-water mixtures. Micellar mobile phases confer also a large solvent power allowing the direct injection of biological fluids (such as urine, plasma or milk), which simplifies and accelerates the analytical procedures [2]. However, pure micellar mobile phases (without addition of organic solvent) are usually weak and yield poor chromatographic efficiencies, due to the increase in the stationary phase film thickness by adsorption of the surfactant, which slows down the stationary phase diffusion.

The most common surfactant in the MLC literature is the anionic sodium dodecyl sulphate (SDS), used in more than 70% of the reports [1,3]. Brij-35 (polyoxyethylene(23)dodecanol, $CH_3(CH_2)_{10}CH_2(OCH_2CH_2)_{23}OH$), which is the most suitable non-ionic surfactant for MLC owing to its commercial availability, high purity, low cost, low toxicity, high cloud

temperature and low background absorbance [4], has been studied in a significantly lesser extent [1,3]. This surfactant increases the polarity of the stationary phase, and changes the selectivity in comparison to the classical mode with acetonitrile-water or methanol-water mixtures. Brij-35 is biodegradable and does not require an organic solvent in the mobile phase to elute the flavonoids in practical analysis times, giving rise to an example of a “green” chromatographic procedure.

HPLC analysis is usually carried out at room temperature. However, by working at higher temperature, the chromatographic performance may be improved (i.e. shorter analysis times and enhanced peak shapes are achieved), since some physical parameters playing an important role in HPLC, such as viscosity, mobile phase polarity, or diffusivity depend strongly on temperature [5]. Accordingly, in order to improve the poor efficiency observed with micellar mobile phases, the effect of an increasing temperature on the peak shape was studied by several authors [6–9]. The effect of temperature on the partitioning equilibria of solutes (and consequently on the retention) was also checked [10–12]. The authors concluded that at higher temperature the peak shape is enhanced, due to the reduction of the surfactant layer on the stationary phase, and the increase in the entrance and exit rates of solutes to and from the micelles. In most of these reports, hybrid mobile phases of the anionic surfactant SDS and an alcohol were used, and the change in the chromatographic behaviour for a few selected mobile phases was examined. Particularly interesting is the report of Lavine and Hendayana [7], who observed that the poor efficiencies for several aromatic compounds obtained with SDS at room temperature approached those with methanol above 55^{o}C.

Description of the retention and peak shape

Modelling the retention and peak shape in liquid chromatography upon changes in the experimental factors is of great interest to find optimal conditions for a given separation

problem. It also allows a reliable and global comparison of the behaviour of different compounds, columns, or experimental conditions. The models indicated below were used in this work to describe the retention and peak shape behaviours with acetonitrile-water mixtures (hydro-organic RPLC) and Brij-35 micellar aqueous mobile phases (MLC).

Modelling the retention behaviour

Effect of the concentration of organic solvent on retention in hydro-organic RPLC

Among the models proposed to describe the retention times with changes in the concentration of organic modifier in the mobile phase (φ) [13], we have used the most popular:

$$\ln k = \ln k_w - b\,\varphi + c\,\varphi^2 \quad (1)$$

where k is the retention factor for a mobile phase containing a given amount of modifier (acetonitrile in this work), and k_w is the extrapolated value of the retention factor with water (i.e. in the absence of organic solvent); b and c are fitting parameters. This model can be simplified in narrow concentration ranges to a linear equation:

$$\ln k = \ln k_w - S\,\varphi \quad (2)$$

where S is the so-called Snyder elution strength.

Effect of the surfactant concentration on retention in micellar RPLC

The retention in micellar RPLC can be described by [14]:

$$\frac{1}{k} = \frac{1}{K_{AS}} + \frac{K_{AM}}{K_{AS}}\mu = a + b\,\mu \quad (3)$$

where K_{AS} and K_{AM} are the solute-stationary phase and solute-micelle association constants, respectively, and μ the molar concentration of monomers of surfactant forming micelles. This

105 equation has been demonstrated to give accurate descriptions for micellar mobile phases
106 without organic solvent, or containing a fixed amount of organic solvent.
107
108 *Effect of temperature on retention*
109 The influence of temperature on retention is described from thermodynamic considerations by
110 the Vant'Hoff equation [15,16]:

111 $$\ln k = \frac{\Delta H^{\circ}}{RT} - \frac{\Delta S^{\circ}}{R} + \ln \phi \qquad (4)$$

112 where ϕ is the phase ratio, ΔH° and ΔS° are the standard enthalpy and entropy changes in
113 the system, respectively, R is the gas constant and T the absolute temperature. For sufficiently
114 narrow temperature ranges, where ΔH° and ΔS° are constant, Eq. (4) can be transformed in a
115 simple two-parameter equation:

116 $$\ln k = a - \frac{b}{T} \qquad (5)$$

117 where a and b are fitting parameters.
118
119 Dependence of the peak width on retention time
120 The description of the changes in the peak half-widths with the experimental conditions
121 allows the prediction of the peak shape (i.e. width and asymmetry) for each analyte, and the
122 evaluation of the effect of different factors on column performance. Once the location and
123 shape of the chromatographic peaks for the compounds in a sample are known at any
124 experimental condition in a selected range, the resolution between peaks can be calculated in
125 a reliable way, and finally optimised to get the best separation [17].
126 A linear dependence has been demonstrated to predict with good accuracy the left (A) and
127 right (B) half-widths at a given retention time, in practical ranges [18–20]:

$$A = m_A t_R + A_0 \quad (6)$$

$$B = m_B t_R + B_0 \quad (7)$$

where t_R is the retention time, and A_0 and B_0 the intercepts of the straight-lines. The sum $m_A + m_B$ indicates the broadening rate at increasing retention time; B/A measures the peak asymmetry.

Experimental

Mobile phases were prepared with acetonitrile (Scharlab, Barcelona, Spain) in the hydro-organic mode, and with Brij-35 (99% purity, Merck, Darmstadt, Germany) in the micellar mode. The pH was buffered at 3.5 with 0.01 M citric acid monohydrate and sodium hydroxide (Panreac, Barcelona). Eight flavonoids (flavones and flavonols), purchased from Sigma-Aldrich (St. Louis, MO, USA), were used as probe compounds: baicalein, chrysin, fisetin, flavone, 3-hydroxyflavone, 5-hydroxyflavone, 7-hydroxyflavone, and quercetin.

Uracil (Acros Organics, Geel, Belgium) was used as dead time marker. Eluted with and without column, its mean retention time was 1.28 min and 0.076 min in the hydro-organic mode, and 1.32 min and 0.073 min in the micellar mode, respectively.

In the hydro-organic mode, the probe compounds were eluted with four mobile phases at different acetonitrile levels (35, 40 and 45 and 50 v/v percentage), each one at four temperature levels (25, 35, 45 and 55ºC). In the micellar mode, four mobile phases were used at four different Brij-35 levels: 20, 30, 40 and 50 mM, each one at four temperature levels (25, 35, 45 and 55ºC). Additional measurements were made at 65, 75 and 80ºC for 35% acetonitrile and 20 mM Brij-35. Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout.

The HPLC system (Agilent, Waldbronn, Germany) was equipped with an isocratic pump (Series 1200), a temperature controller (Series 1200, maximal temperature: 80ºC), an

autosampler (Series 1100), and a UV-visible detector (Series 1100) set at 254, 274, 315 and 350 nm. The same Eclipse XDB-C18 column (125 mm×4.6 mm i.d., 5 μm particle size) (Agilent) was used, together with a C18 guard column (30 mm×4.6 mm i.d., 5 μm particle size, Scharlab), in both hydro-organic and micellar modes. The flow-rate was 1 mL/min.

Results and discussion

Experimental conditions and chromatographic data

The effect of the modifier concentration and temperature on the retention and shape of chromatographic peaks was studied for a set of eight flavonoids, which are polyhydroxy compounds able to interact selectively with Brij-35. Flavonoids are natural compounds found in plants, which are receiving considerable attention in recent years due to their bioactivity, and antioxidant, antiinflammatory and antimicrobial properties. The extraction, purification and characterisation of these compounds from plant extracts have been extensively studied [21]. In addition, an important work has been made to develop sensitive and selective analytical methods for their characterization and determination [22]. Among these, chromatography has played an important role [23,24].

The probe compounds were eluted with acetonitrile-water mixtures and micellar mobile phases of Brij-35. The range of concentrations of the modifiers was selected so that the most polar solute was sufficiently retained to be measured, and the retention time of the most retained solute was not excessive at 25°C. In the micellar mode, care was also taken to adopt a concentration of surfactant above the critical micellar concentration (CMC), and to saturate the stationary phase with surfactant monomers. The CMC of Brij-35 is small (9.0×10^{-5} M [1]), so that it easily meets the first condition. However, the adsorption of Brij-35 on the stationary phase has been found to continue largely after its CMC, exceeding ca. 50 times [25]. Finally, in both chromatographic modes, the concentration of the weakest mobile phase

was adapted to get similar retention times for the least retained solute (ca. 75 min). Accordingly, the selected concentration ranges were 35–50% (v/v) for acetonitrile, and 20–50 mM for Brij-35.

The changes in the retention factors with the concentration of modifier at 25 and 55ºC are depicted in Figs. 1a and d, for the hydro-organic and micellar modes, respectively. Similar curves, shifted to shorter retention times, were obtained at higher temperature. Next, the chromatographic behaviour of the set of flavonoids is discussed, based on the experimental data obtained at varying concentration of both modifiers and temperature.

Interaction of flavonoids with Brij-35

In the micellar mode, the plot of $1/k$ versus the concentration of surfactant (Fig. 1e) gives information about the strength of the interactions of solutes with the modified stationary phase and micelles. The plots were linear for all compounds eluted with Brij-35, with correlation coefficients $r > 0.99$ (even $r > 0.999$), which means that the stationary phase is saturated with surfactant. The extrapolation of the linear segment ($1/k$ vs. μ) yielded intercepts ($1/K_{AS}$) close to zero, indicating a strong interaction of flavonoids with the non-ionic surfactant adsorbed on the stationary phase.

The chemical structures of the flavonoids studied in this work are shown in Table 1. Although the elution order in the hydro-organic and micellar modes was similar, the distribution of peaks (i.e. selectivity) and elution strength was different in both chromatographic modes. The interaction of flavonoids with the stationary phase, and consequently the retention behaviour, can be explained if the number and position of hydroxyl groups in the aromatic rings of flavonoids are considered [26]. Hydroxyl substitution at X1 and X2 positions allows the formation of intramolecular hydrogen bonding, preventing their availability for further interactions, and reducing the polarity of the ketone group.

This increases the retention of the flavonoids bearing these groups, which should be more retained than flavone (without OH substitution). It is the case of 3-hydroxyflavone (with OH at X1 position) and 5-hydroxyflavone (with OH at X2). The late elution of 5-hydroxyflavone (73 min with 35% acetonitrile at 25°C), relative to 3-hydroxyflavone (40.5 min) suggests that the OH substitution at X2 is more effective than at X1 to produce an intramolecular hydrogen bond. Also, chrysin with OH at X2, despite containing an additional hydroxyl at X4, is more strongly retained than 7-hydroxyflavone (with a unique OH substitution at X4). The least retained compounds have OH substitutions at two or three of the X4, X5 and X6 positions (fisetin, quercetin, 7-hydroxyflavone and baicalein). Three of these compounds contain also OH groups at X1 or X2.

On the other hand, Brij-35 is adsorbed on the C18 stationary phase modifying its properties. According to NMR studies carried out by Lavine et al., using a C18 column and micellar mobile phases of SDS, the dodecyl chain of this surfactant is inserted in the bonded organic layer with the sulphate group oriented outside [27]. This was confirmed by the increased retention of cationic compounds with SDS-modified columns [28]. The situation should be similar for Brij-35, which should expose its oxyethylene chain with terminal OH group to the mobile phase. This increases the polarity of the stationary phase. The surfactant forms also micelles in the mobile phase containing an apolar dodecyl core (similar to SDS) and a relatively polar surface with oxyethylene chains. Consequently, hydrophobic and hydrophilic sites of interaction are provided in both stationary phase and mobile phase. The strong interaction of the available OH groups at X3, X4, X5 and X6 positions of the least retained flavonoids with the oxyethylene chain of Brij-35 explains the significant increase in retention in the micellar mode (e.g. the retention time of fisetin is 2.6 min with 35% acetonitrile, against 22.4 min with 20 mM Brij-35), and the high elution strength of Brij-35 mobile phases as detailed below.

The elution window is significantly reduced in the micellar mode: 22.4–75.6 min (k = 16.2–57.2) with 20 mM Brij-35, against 2.6–71.9 min (k = 1.0–54.3) with 35% acetonitrile, both at 25°C. In these conditions, the micellar/hydro-organic retention times ratio was: 8.54, 7.65, 4.13, 3.96, 2.46, 2.06, 1.35 and 1.05, for fisetin, quercetin, 7-hydroxyflavone, baicalein, chrysin, flavone, 3-hydroxyflavone, and 5-hydroxyflavone, respectively. Note that the retention time for the most retained compound (5-hydroxyflavone, which has no available OH groups) was similar in both chromatographic modes for the weakest mobile phases (35% acetonitrile and 20 mM Brij-35). In contrast, for the most polar flavonoids (fisetin, quercetin, 7-hydroxyflavone and baicalein) it was below 8 min and above 20 min with 35% acetonitrile and 20 mM Brij-35, respectively. The accordion effect has been described consistently in the MLC literature: the retention times for the most apolar compounds are relatively closer to those for the polar compounds compared to the classical RPLC mode [29]. This makes the chromatographic peaks be more evenly distributed in the chromatograms, and gradient elution less necessary. It should be indicated, however, that the observed effect of increased retention times for the early eluting compounds in MLC is particularly strong for Brij-35 and flavonoids.

Elution strength

In RPLC, the addition of organic modifiers to the aqueous mobile phase decreases its polarity, and therefore, the retention. Certain additives, such as Brij-35, can establish new interactions between solutes and stationary phase and mobile phase that modify the elution behaviour. Figs. 1b and e correspond to the descriptions in Eqs. (1) and (3), respectively, obtained at 25 and 55ºC: the elution strength decreases with increasing modifier concentration. Also, the partitioning equilibrium shifts to the mobile phase with increasing temperature, decreasing the retention according to Eq. (4). Figs. 1c and f depict the linear variation between ln k and 1/T

for mobile phases containing acetonitrile and Brij-35, respectively, at the limits of the concentration ranges.

Several approaches can be suggested to quantify and compare the elution strength with regard to different factors (e.g. modifier concentration and temperature). The simplest approach would measure the change in the absolute values of the retention factor in a selected range of the experimental factor, *f*:

$$r_k = -\frac{k_u - k_l}{f_u - f_l} \tag{8}$$

where the subscripts u and l indicate the upper and lower values of a selected experimental range. This approach has the drawback of depending highly on the solute retention. To avoid this problem, the elution strength can be calculated as the percentage variation of the retention factor per percentage variation of the experimental factor (e.g. with respect to the lowest limit of the experimental range, f_l, which would yield the largest retention, k_l):

$$r_k = -\frac{k_u - k_l}{k_l}\frac{f_l}{f_u - f_l} = -\frac{k_u - k_l}{f_u - f_i}\frac{f_l}{k_l} \tag{9}$$

However, Eq. (9) has the drawback that the percentage change in the experimental factor depends on the selected value (in Eq. (9), f_l). A third approach, which we propose here, is the calculation of the percentage change in the retention factor (referred to the largest retention) per unit of experimental factor. We will call this parameter “elution capability”:

$$C(\%) = -\frac{k_u - k_l}{k_l}\frac{100}{f_u - f_l} = -\frac{100}{k_l}\frac{k_u - k_l}{f_u - f_l} \tag{10}$$

In fact, Eq. (10) is only independent from the retention when the ln *k* vs. modifier concentration relationship is linear (Eq. (2)). Note that when the interval of modifier concentration approaches zero, the parameter *C*(%) is given by the following expression:

$$S = -\frac{100}{k}\frac{dk}{df} = -100\frac{d\ln k}{df} \tag{11}$$

Consequently, for infinitesimal changes in the concentration of acetonitrile, *C*(%) matches the parameter *S* (×100) in Eq. (2), which defines the elution strength of an organic solvent in RPLC.

With the definition of *C*(%) in Eq. (10), and in order to obtain a practical measurement that facilitates comparisons among different factors, a similar magnitude of the factors in the studied experimental range is convenient. This is the case with the v/v percentage of acetonitrile, the millimolar concentration of Brij-35, and the Celsius scale, whose working ranges in this work were: 35–50% (v/v), 20–50 mM and 25–55° C, respectively.

Table 2 shows the elution capability for the experimental ranges selected in this work. As observed, when the concentrations of acetonitrile and Brij-35 are expressed in the selected units (v/v percentage of acetonitrile and mM Brij-35), the strength of the former is twice that of Brij-35. Thus, if the acetonitrile percentage is increased in one unit (e.g. from 35 to 36%), the retention factor will be reduced by an average value of 4.9% along the experimental range, whereas an increase in one unit in the millimolar concentration of Brij-35 (e.g. from 20 to 21 mM) will yield a 2.1% decrease. On the other hand, an increase in one Celsius degree will yield a 1.3% decrease in the retention factor for the hydro-organic mode and 0.8% for the micellar mode.

The comparison of the elution strength of the modifiers using the same concentration units is also convenient. Table 2 also shows the results when the concentration of acetonitrile and Brij-35 are expressed in g/L and millimolar concentration. Brij-35 reveals as the strongest eluent, since an increase in 1 g/L decreases the retention factor by 1.7%, while an increase in 1 g/L of acetonitrile decreases it by only 0.6%. Considering the relative masses of Brij-35 (1198 g/mol) and acetonitrile (41 g/mol), the elution capability expressed as millimolar concentration is much higher for Brij-35: 2.1% versus 0.025% for acetonitrile.

299 It can be observed that the elution strength of acetonitrile is slightly larger for the most
300 retained compounds with regard to those less retained, whereas in the micellar mode it does
301 not seem to depend on the compound nature (Table 2), so that the selectivity should be
302 unaffected by changes in the concentration of surfactant, while it decreases with acetonitrile.
303 From Eq. (10), when the experimental factor increases in one unit, the retention factor of a
304 compound *i* will change as follows:

305 $$k_i = k_{i,0}\left(1-\frac{C_i}{100}\right) \quad (12)$$

306 where $k_{i,0}$ refer to the initial conditions and C_i to the elution capability for compound *i*.
307 Therefore, the selectivity for a pair of compounds when the experimental factor increases in
308 one unit will be:

309 $$\alpha = \frac{k_{1,0}(100-C_1)}{k_{2,0}(100-C_2)} = \alpha_0 \frac{(100-C_1)}{(100-C_2)} \quad (13)$$

310 where α_0 is the selectivity at the initial conditions. For $C_1 > C_2$, the selectivity will decrease,
311 since ~~the peak of~~ the most retained compound will decrease its retention time in a larger
312 extent, which will shift the peaks closer. The opposite will happen for $C_2 > C_1$.

313 Thus, for example, the *C* values for the pair baicalein/7-hydroxyflavone eluted in the
314 hydro-organic mode were rather similar (Table 2). Therefore, the selectivity practically did
315 not change, going from 1.008 at 35% acetonitrile and 25ºC to 1.004 at 50% acetonitrile and
316 25ºC, and ~~to~~ 1.009 at 35% acetonitrile and 55ºC. In the micellar mode, the surfactant did not
317 either influence the selectivity, going from 0.966 to 0.968 in the range 20 to 50 mM Brij-35.
318 In contrast, an increased temperature had a greater effect on 7-hydroxyflavone, with the
319 selectivity going from 0.966 to 1.025 in the range 25–55ºC for 20 mM Brij-35, with a reversal
320 in the elution order. On the other hand, for the pair chrysin/flavone, the selectivity increased
321 in the hydro-organic mode, going from 1.20 to 1.37 in the 35–50% acetonitrile range at 25ºC,
322 and from 1.20 to 1.42 in the 25–55ºC range for 35% acetonitrile. In the micellar mode, the

pair flavone/3-hydroxyflavone increased its selectivity with temperature, going from 1.08 to 1.15 in the 25–55ºC range at 20 mM Brij-35, and decreased it slightly at increasing surfactant concentration, going from 1.15 to 1.12 in the range 20–50 mM at 25ºC.

Peak shape enhancement

Effect of the modifier concentration and temperature on the peak shape

The nature of the modifier added to the mobile phase affects the shape of chromatographic peaks (i.e. their width and symmetry). The peak shape in the micellar mode with Brij-35 at 25ºC was appreciably poorer with regard to the hydro-organic mode with acetonitrile (Figs. 2a and c, respectively). In order to discuss the observed differences, instead of comparing the characteristics of individual peaks in each chromatographic mode, we will use a global approach based on the fitting to linear plots of the left (*A*) and right (*B*) half-widths of chromatographic peaks versus the retention time. These plots give a visual representation of the changes in the width and symmetry of chromatographic peaks at increasing retention times.

It should be first indicated that the peaks were highly symmetrical in the hydro-organic mode, except for those flavonoids eluting close to the dead time. In micellar RPLC, the left and right halfwidths were also similar for all flavonoids, except for flavone, whose plots diverged (i.e. the asymmetry increased with the retention time). The interaction of the ketonic group (which is not estabilised in this flavonoid) with the surfactant can be the reason for this behaviour.

The slopes of the linear half-width plots are similar for compounds showing similar partition kinetics with the stationary phase [20]. This is the case of the hydro-organic mode: the behaviour of all flavonoids is described by the same linear trend. In contrast, in the micellar mode, the slopes of the linear trends for different compounds were different,

indicating particular interactions between Brij-35 and each flavonoid. Figs. 2a and b show the width variation with the retention time for the set of flavonoids, when the concentration of acetonitrile was changed and the temperature was kept constant at 25 and 55°C, respectively. Figs. 3a and b depict the data at several temperatures (25, 35, 45 and 55°C) and fixed acetonitrile concentration (35 and 55%, respectively). Some data for fisetin were not included in the plots due to problems in their measurement, since the peaks appeared close to the dead time. As observed, in the hydro-organic mode, the changes in both modifier concentration and temperature yielded a similar linear pattern. This suggests a partitioning kinetics between mobile phase and stationary phase similar for all flavonoids. Also, the partitioning must be close to equilibrium, since the kinetics is scarcely affected by temperature.

The behaviour in micellar RPLC was far different (see Figs. 2c and d, and 3c and d). First, the peak widths and the broadening rate with the retention time were significantly larger at 25ºC with respect to the hydro-organic mode. Also, the plots showed an increasing pattern for the peak width with the retention time, but the linearity was lost. This should be explained by the variety of interactions that flavonoids experience with the stationary phase modified by adsorption of Brij-35, resulting in different partitioning kinetics (each compound showed a different linear trend). However, the differences among compounds decreased significantly at 55ºC (compare Figs. 2c and d): the cloud of points on the plot was less dispersed, approaching the behaviour in the hydro-organic mode. As we discussed in a previous work [30], a slow kinetics in the distribution equilibria gives rise to an increase in the peak width without affecting the peak asymmetry. At increasing temperature, the process is closer to equilibrium, which decreases the peak width.

The linear trends between peak width and retention time for each compound depicted in Figs. 3c and d (where the data at two Brij-35 concentrations and varying temperature are plotted) should also be remarked, together with the fact that the lines are shifted in the order:

fisetin < quercetin < 7-hydroxyflavone ≈ baicalein < chrysin < flavone ≈ 3-hydroxyflavone < 5-hydroxyflavone, which is also the elution order for these compounds in the assayed mobile phases. This means that the least retained compounds showed relatively wider peaks. Also, the partitioning kinetics is different for each compound: the slope of the lines decreased from fisetin to 5-hydroxyflavone. This agrees with the results in Figs. 2c and d, where differences in behaviour are observed at 25ºC when the concentration of surfactant is changed: the rate of decrease in the peak width with increasing modifier concentration is higher for the most polar compounds. Finally, note that the plots in Figs. 3a and b corresponding to 25 and 50% acetonitrile are similar, and agree with the those in Figs. 2a and b, obtained each at different temperature.

Fig. 4 summarises the effect of the modifier concentration and temperature on the peak width. The change in peak width for flavone and 5-hydroxyflavone, at increasing modifier concentration or temperature (in the 25–80ºC range), is shown taking as reference the weakest eluent in the experimental design at 25°C (corresponding to the top point for each modifier): 35% acetonitrile in the hydro-organic mode, and 20 mM in the micellar mode. For the hydro-organic mode, the effect of both factors (acetonitrile concentration and temperature) on the peak width follows a similar trend: the measured widths are practically located on the same straight-line, indicating that they only depend on the retention time. In contrast, in the micellar mode, the changes in the concentration of Brij-35 and temperature show different trends.

At increasing temperature, the peak widths with Brij-35 are significantly reduced (curvilinear trend in Fig. 4), getting closer to the hydro-organic behaviour (linear trend at the bottom of the figure), which is consistent with the data presented by Lavine and Hendayana for SDS [7]. However, at 80ºC the peaks for Brij-35 were still somewhat wider than those for acetonitrile, which was the top temperature in the column temperature controller. The results

398 should be interpreted by considering that the partitioning rate in the micellar mode with Brij-
399 35 increases with temperature, approaching equilibrium conditions practically reached in the
400 hydro-organic mode at 25ºC.
401
402 *Measurement of the peak shape enhancement*
403 The quantification of the effect of different experimental factors on the peak width due to a
404 change in the column kinetics is problematic, since the peak width depends on the retention
405 time, which in turn changes with the experimental conditions (it depends on the elution
406 strength). In order to isolate the kinetics changes produced by the change in the experimental
407 factor, the measurements should be corrected to eliminate the effect of the elution strength of
408 the factor. For this purpose, a parameter similar to that in Eq. (10) is here proposed. For the
409 left half-width:
410 $$E_A(\%) = -\frac{100}{A_l}\frac{A_u - A_l^c}{f_u - f_l} \quad (14)$$
411 where u and l refer again to the upper and lower levels of the factor *f*. The superscript c
412 indicates that the value is corrected to eliminate the effect of the retention time. The parameter
413 E_A in Eq. (14) (and another similar for the right half-width, E_B), which will be called “peak
414 shape enhancement”, measures the change in width attributable to the effect of the
415 experimental factor on the partitioning kinetics, once the change due to the elution strength
416 has been corrected. This can be achieved by comparing the widths for the upper and lower
417 levels of the factor at the same retention time. We suggest the calculation of the widths at the
418 retention time for the upper limit conditions, $t_{R,u}$, assuming the behaviour at the lower limit
419 conditions, according to:
420 $$A_l^c = \frac{A_l - A_0}{t_{R,l} - t_0}(t_{R,u} - t_0) + A_0 \quad (15)$$

where A_0 and t_0 are the left half-width and retention time of a peak obtained without column. Eq. (15) assumes a linear behaviour between the peak at the lower limit conditions and the peak obtained without column.

The values of peak shape enhancement for the eight flavonoids, corresponding to the peak half-widths, are given in Table 3. From the mean values, it can be concluded that the effect of temperature on kinetics is significantly smaller in the hydro-organic mode than in the micellar mode: in the hydro-organic mode, the reduction in peak width by increasing the temperature in one degree amounts 0.24% and 0.16% for the left and right half-widths, respectively, while the reductions in the micellar mode would be 0.78% and 0.83%, respectively. Note that this represents an increase in the asymmetry in the hydro-organic mode (from 0.98 to 1.03 for flavone in the 25–55ºC range), but a decrease in the micellar mode (from 1.32 to 1.15 for flavone). On the other hand, an increase in one unit v/v percentage of acetonitrile in the hydro-organic mode will yield 0.41 and 0.42% reductions in *A* and *B* (0.0021 and 0.0022% per one mM acetonitrile), respectively, while a one mM increase in the concentration of surfactant in the micellar mode will produce a similar increase in *A* and *B* of around 0.23% (in this case, peak shape deteriorates).

Therefore, the recommended strategy in micellar RPLC with Brij-35 should be, at least for the analysis of flavonoids, minimising the concentration of surfactant and increasing the temperature to reduce the retention times and enhance the efficiency. An increasing temperature decreases also the asymmetry. Meanwhile, in hydro-organic RPLC, the retention should be preferably reduced by increasing the modifier concentration, since this has a greater beneficial effect on the efficiency. Note that in this case an increased temperature, in general, deteriorates the peak asymmetry. It should be also noted that the effect of temperature on the peak width for Brij-35 is stronger at lower temperature, and a moderate temperature increase is enough to obtain a significant improvement in the efficiency.

Chromatograms for a mixture of the eight flavonoids obtained at low and high temperature, in the hydro-organic and micellar mode, are shown in Fig. 5. The concentration of both modifiers (acetonitrile and Brij-35) was adapted to make the retention time for the most retained compound similar at both temperatures. In the hydro-organic mode, no significant difference was observed by increasing the modifier concentration and temperature, regarding the efficiency and selectivity (i.e. distribution of the peaks in the chromatogram). In contrast, in the micellar mode, an increased temperature yielded a significant improvement in the efficiency, together with a small change in the selectivity, resulting in enhanced peak resolution. Thus, the peaks of quercetin and 7-hydroxyflavone, on the one hand, and chrysin, flavone and 3-hydroxyflavone, on the other, which were highly overlapped at 25ºC, were resolved close to the baseline at 80ºC (also at 55ºC). In addition, the peaks of baicalein and 7-hydroxyflavone, which were fully overlapped in the hydro-organic mode, are partially resolved at 80ºC.

Conclusions

The addition of modifiers in RPLC decreases the polarity of the mobile phase, giving rise to a reduction in the retention times. When specific interactions between solutes and stationary phase are added to the hydrophobic interactions (as is the case with Brij-35 in the analysis of flavonoids), particular behaviours are obtained: the retention times of flavonoids are changed, but at different degree depending on the OH substitution in the aromatic rings. This yields significant changes in the elution window and distribution of chromatographic peaks. The partitioning kinetics (which is also particular for each flavonoid) is, however, negatively affected by the interaction of flavonoids with the stationary phase modified with Brij-35. This work shows that an increased temperature makes the behaviour in the micellar mode closer to

the hydro-organic mode regarding the peak shape. Meanwhile, its particular selectivity is maintained.

In order to evaluate the effect of the modifier concentration and temperature on the retention and peak shape in both hydro-organic and micellar modes, two parameters were proposed, which have been called “elution capability” and “peak shape enhancement”. These parameters quantify in a practical and reliable way the elution strength for compounds of different polarity, and the change in the widths of chromatographic peaks due to a change in the partitioning kinetics, respectively. These parameters can be used in any other situations found in liquid chromatography.

The results indicated that the addition of acetonitrile to the mobile phase scarcely affected the partitioning kinetics of flavonoids, which seems to be a fast equilibrium, since the peak shape was neither affected by an increased temperature in the range 25–80^{o}C. Meanwhile, the addition of Brij-35 deteriorated the kinetics, whereas an increased temperature accelerated the partitioning equilibria, yielding narrower peaks. Although at room temperature the peaks obtained with Brij-35 are significantly wider with regard to those obtained with acetonitrile, the two behaviours become closer at increasing temperature.

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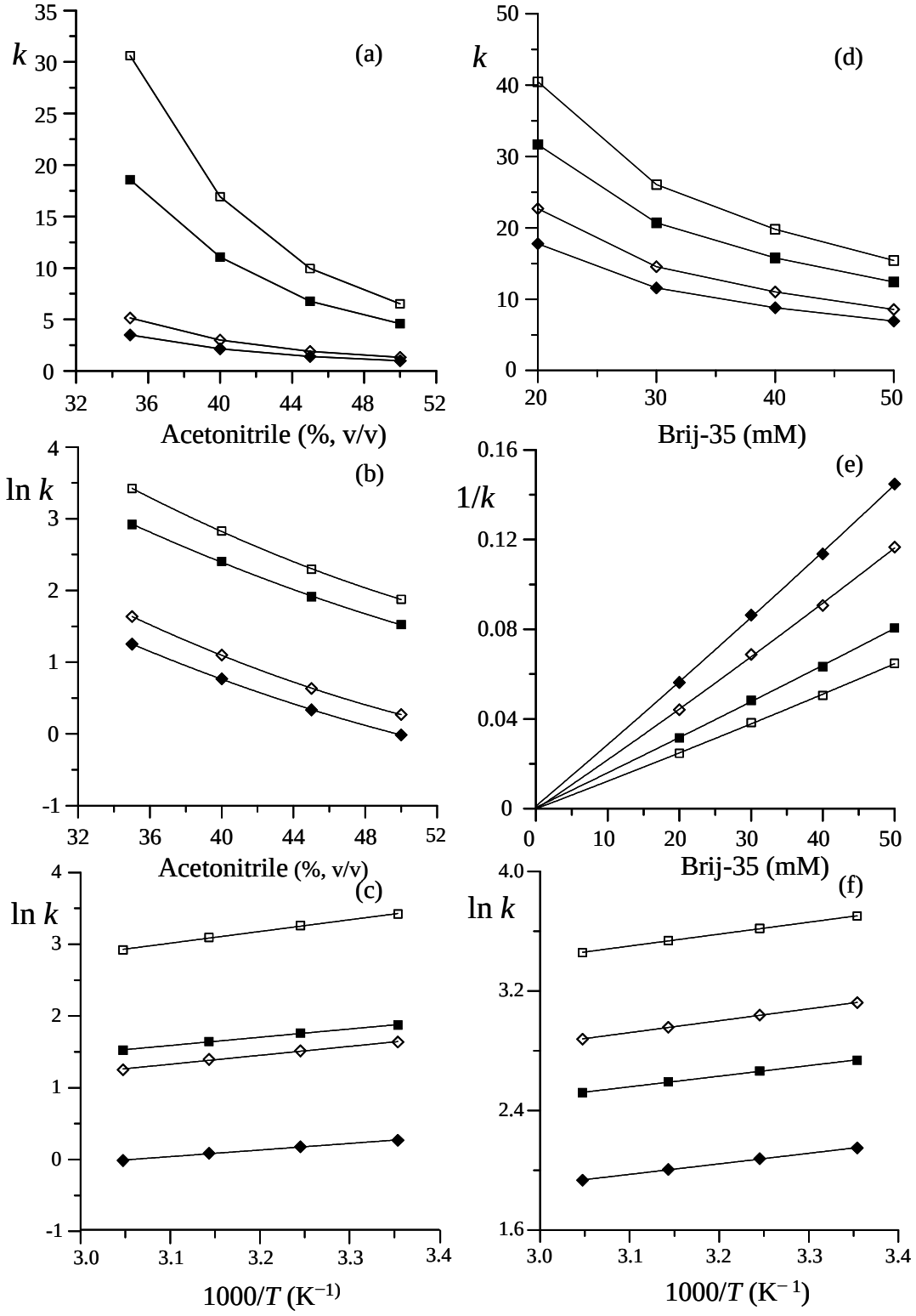


Fig. 1. Retention behaviour for two selected compounds: 3-hidroxyflavone (□ and ■), and baicalein (◊ and ♦), in the hydro-organic (a,b,c) and micellar (d,e,f) modes. In (a,b,d,e), the empty symbols correspond to 25ºC, and the full symbols to 55ºC. In (c,f), the empty symbols correspond to 35% acetonitrile and 20 mM Brij-35, and the full symbols to 50% acetonitrile and 50 mM Brij-35.

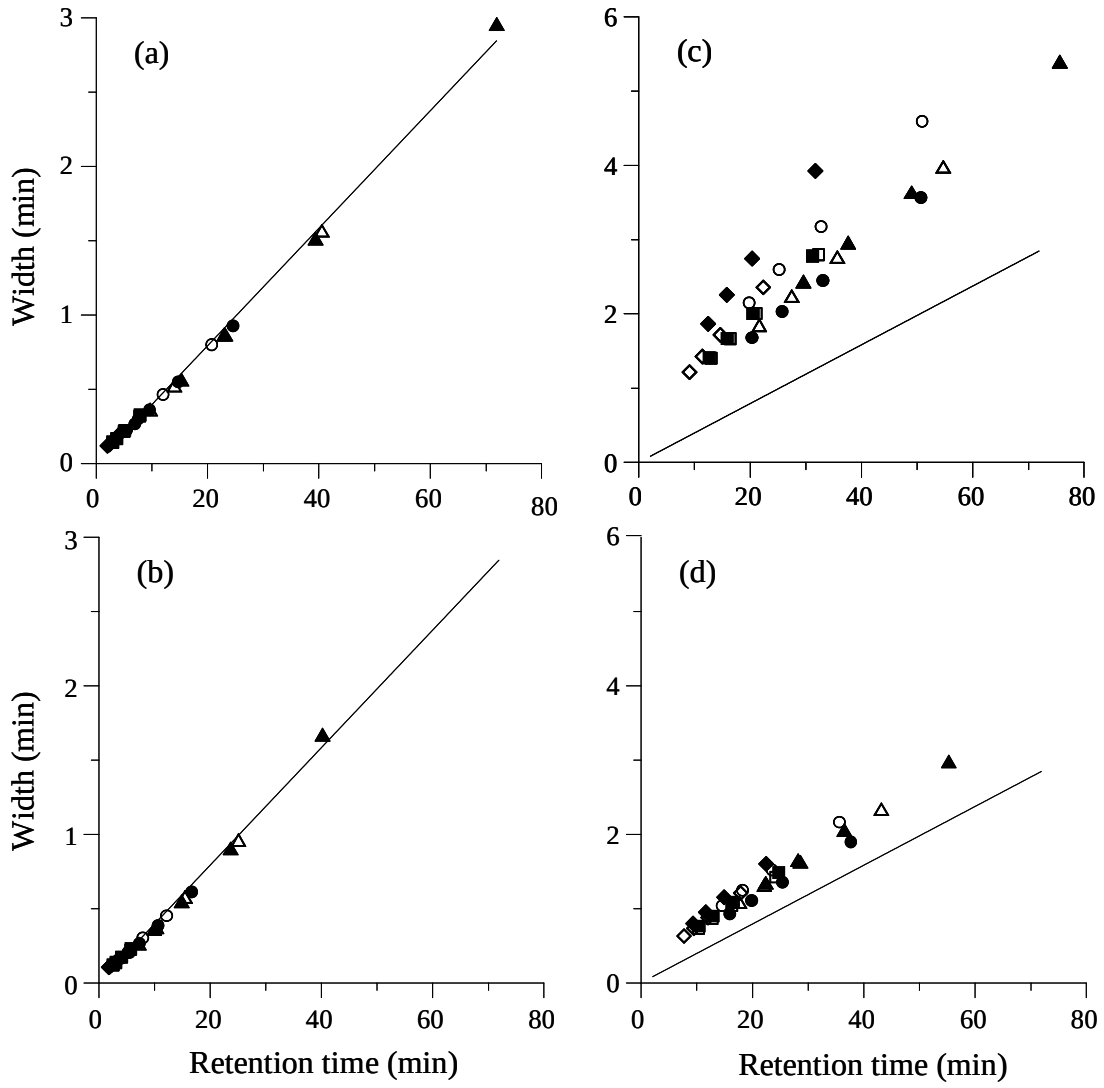


Fig. 2. Widths (*A* + *B*) for the set of flavonoids at increasing modifier concentration in the assayed experimental ranges at 25ºC (a,c) and 55ºC (b,d), for the hydro-organic (a,b) and micellar (c,d) modes. For comparison purposes, the fitted dependence of the widths on the retention time for the hydro-organic mode at 25ºC was depicted in all figures (a–d). Symbols: baicalein (■), chrysin (○), fisetin (◊), flavone (●), 3-hydroxyflavone (△), 5-hydroxyflavone (▲), 7-hydroxyflavone (□), and quercetin (♦).

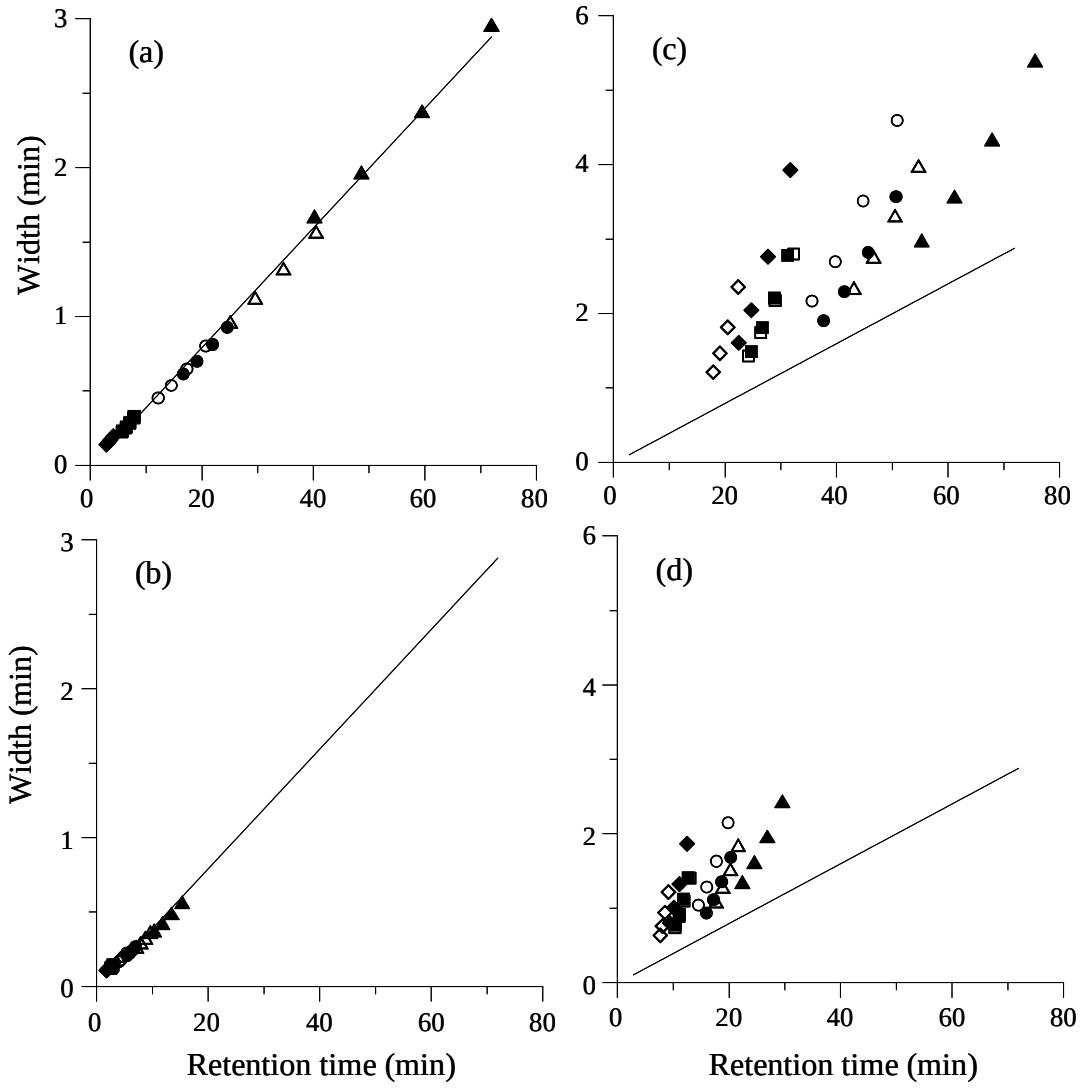


Fig. 3. Widths (*A* + *B*) for the set of flavonoids at increasing temperature in the assayed experimental ranges for 35% (a) and 50% (b) acetonitrile, and 20 mM (c) and 50 mM (d) Brij-35. See Fig. 2 for other details.

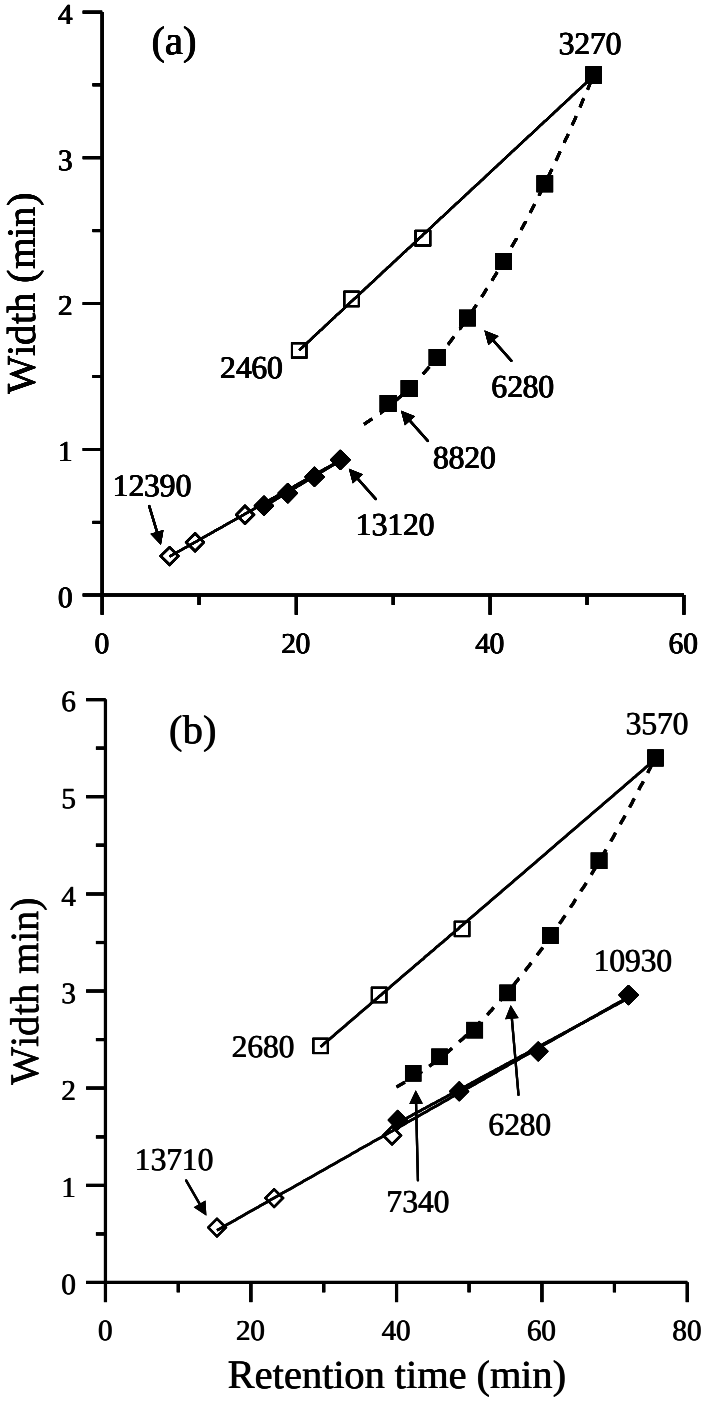


Fig. 4. Peak widths for flavone (a), and 5-hydroxyflavone (b), at several modifier concentrations (empty symbols) and temperatures (full symbols). The value at the longest retention time was obtained with 35% acetonitrile in the hydro-organic mode, and 20 mM Brij-35 in the micellar mode, both at 25ºC. Peak widths decreased at: (◊) increasing acetonitrile concentration at 25ºC, (♦) increasing temperature at 35% acetonitrile, (□) increasing Brij-35 concentration at 25ºC, and (■) increasing temperature at 20 mM Brij-35. Some efficiency values (*N*), calculated according to Foley and Dorsey [31], are given at selected times.

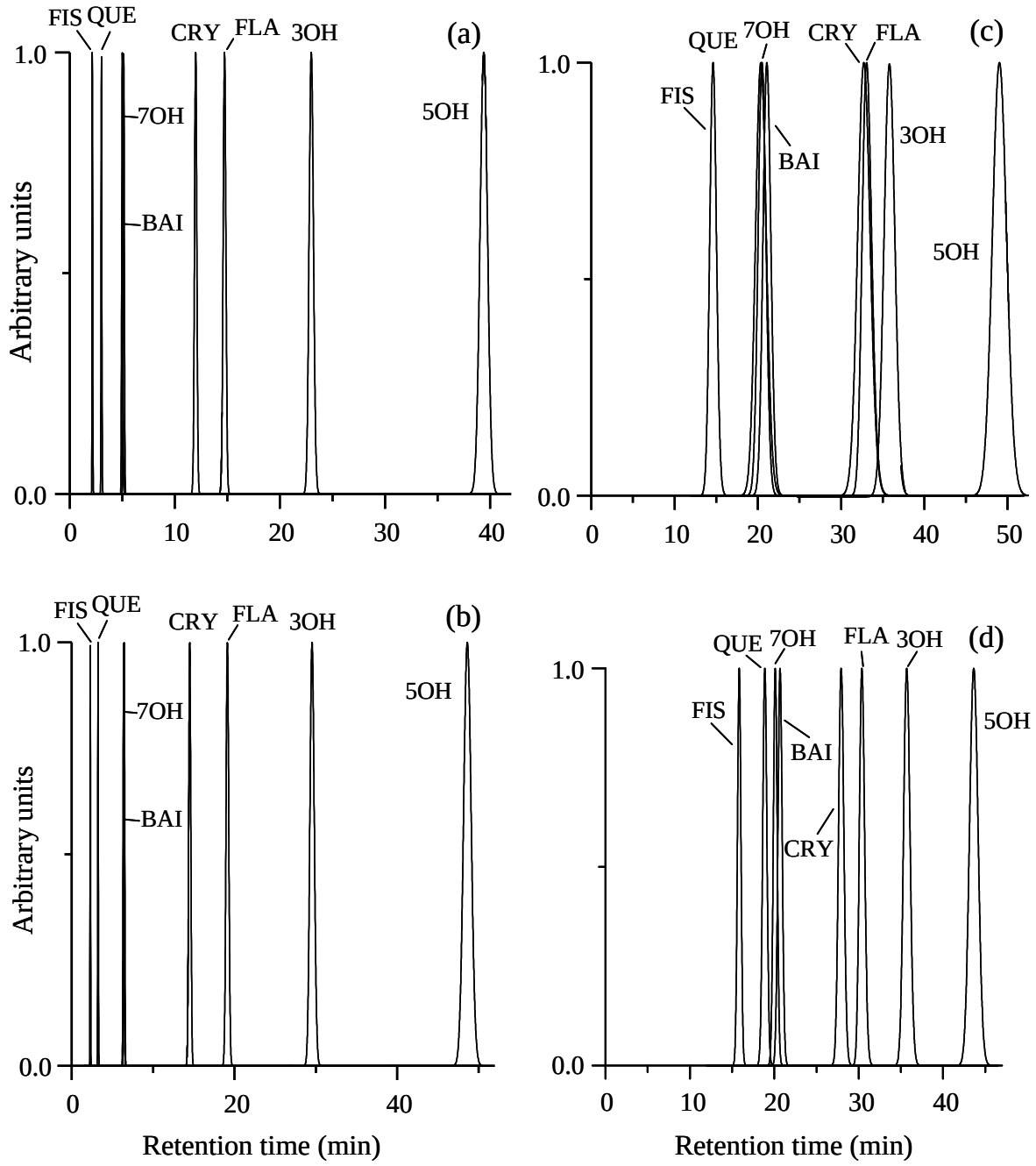
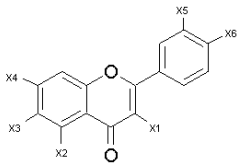


Fig. 5. Normalised chromatograms with regard to the peak height, showing the peaks in the hydro-organic (a,b) and micellar (c,d) modes. Experimental conditions: (a) 40% acetonitrile at 25ºC, (b) 35% acetonitrile at 45ºC, (c) 30 mM Brij-35 at 25ºC, and (d) 20 mM Brij-35 at 80ºC. Compounds: baicalein (BAI), chrysin (CRY), fisetin (FIS), flavone (FLA), 3-hydroxyflavone (3OH), 5-hydroxyflavone (5OH), 7-hydroxyflavone (7OH), and quercetin (QUE).

Table 1. Structure and polarity of the flavonoids studied in this work.



Compound	X1	X2	X3	X4	X5	X6
Fisetin	OH	H	H	OH	OH	OH
Quercetin	OH	OH	H	OH	OH	OH
7-Hydroxyflavone	H	H	H	OH	H	H
Baicalein	H	OH	OH	OH	H	H
Chrysin	H	OH	H	OH	H	H
Flavone	H	H	H	H	H	H
3-Hydroxyflavone	OH	H	H	H	H	H
5-Hydroxyflavone	H	OH	H	H	H	H

Table 2. Elution capability (%) calculated according to Eq. (10) for each experimental factor in the working range.[a]

Compound	Hydro-organic mode				Micellar mode		
	$C(\varphi)$ v/v %	$C(\varphi)$ g/L	$C(\varphi)$ mM	$C(T)$ °C	$C(\mu)$ mM	$C(\mu)$ g/L	$C(T)$ °C
Fisetin	4.08	0.52	0.021	1.07	2.08	1.73	0.70
Quercetin	4.60	0.58	0.024	1.39	2.09	1.74	1.01
7-Hydroxyflavone	4.84	0.61	0.025	1.04	2.06	1.72	0.86
Baicalein	4.85	0.62	0.025	1.04	2.05	1.71	0.72
Chrysin	5.17	0.66	0.027	1.46	2.09	1.75	0.99
Flavone	4.95	0.63	0.026	1.11	2.05	1.71	0.84
3-Hydroxyflavone	5.17	0.66	0.027	1.30	2.06	1.72	0.68
5-Hydroxyflavone	5.27	0.67	0.028	1.48	2.07	1.73	0.88
Mean value	4.9±0.3	0.62±0.04	0.025±0.002	1.26±0.19	2.08±0.02	1.74±0.17	0.82±0.13

[a] Working ranges: φ = 35–50% acetonitrile, μ = 20–50 mM Brij-35 and T = 25–55°C.

Table 3. Peak shape enhancement (%) according to Eq. (14) for each experimental factor in the working range.[a]

Compound	Hydro-organic mode				Micellar mode			
	E_A (φ)[b]	E_A (T)	E_B (φ) [b]	E_B (T)	E_A (μ)[c]	E_A (T)	E_B (μ)[c]	E_B (T)
Fisetin	–	–	–	–	-0.28	0.96	-0.31	0.99
Quercetin	0.64	0.37	0.49	0.31	-0.22	1.02	-0.25	1.02
7-Hydroxyflavone	0.59	0.35	0.53	0.27	-0.27	0.78	-0.28	0.87
Baicalein	0.55	0.35	0.52	0.26	-0.26	0.88	-0.30	0.87
Chrysin	0.32	0.24	0.37	0.16	-0.21	0.76	-0.24	0.79
Flavone	0.32	0.19	0.39	0.11	-0.25	0.58	-0.14	0.83
3-Hydroxyflavone	0.23	0.12	0.34	0.04	-0.19	0.69	-0.18	0.67
5-Hydroxyflavone	0.25	0.04	0.32	-0.01	-0.17	0.60	-0.17	0.62
Mean value	0.41±0.17	0.24±0.13	0.42±0.09	0.16±0.12	–0.23±0.04	0.78±0.16	–0.23±0.06	0.83±0.14

[a] Working ranges: φ = 35–50% acetonitrile, μ = 20–50 mM Brij-35 and T = 25–55ºC.

[b] Expressed per one unit percentage of acetonitrile. [c] Expressed per one mM Brij-35.