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Peak Separation by Adventitious or Added Water in Normal-Phase Chiral HPLC

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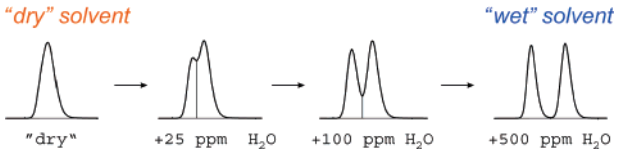

Trace amounts of water in eluents for normal-phase chiral HPLC can affect peak retention time, tailing, and resolution. Adventitious water can cause irreproducible analyses. Deliberate addition of water to the eluent can improve peak resolution and save analysis time and solvent needs.

The quantification of enantiomer mixture composition is essential to determine the success of any enantioselective synthesis.[1] Liquid chromatography on chiral stationary phases determines the relative quantity of both enantiomers as opposed to an enantiomeric excess; consequently, raw materials or mixtures can be analyzed. In a synthetic organic environment with moderately polar, nonvolatile test substrates, normal-phase HPLC[2,3] is the preferred method of analysis. The commercial availability of chiral stationary phases, particularly those based on polysaccharide-coated silica, has added to the widespread regular use of the technique.[4] The reproducibility of analytical separations on a given phase ("column") depends principally on the eluent composition, flow rate, and temperature.[2] Other, less obvious factors[5,6] may remain unrecognized by the analyst, and their unintentional variation can lead to puzzling results, as embraced by the phrase *"user to user, and column to column variations"*. We recently encountered surprising cases of apparently irreproducible separations which turned out to be connected to adventitious water in the mobile phase.[7] Since the trace water content of eluents is highly variable, difficult to control, and not regularly analyzed, many HPLC users may be affected by its effects without realizing it. While the influence of trace water in *nondonor eluents* for HPLC on SiO_2 or Al_2O_3 is well recognized,[2d,8] its relevance for alcohol-containing eluents and polysaccharide coated chiral stationary phases is not evident. We now present case studies illustrating the effects of adventitious or added water on peak resolution in normal-phase chiral HPLC, which are more general than previously recognized,[5,7] and conclude that water, which is actually a "forbidden" solvent in normal-phase chiral HPLC, is a useful additive for finding reliable separation conditions.

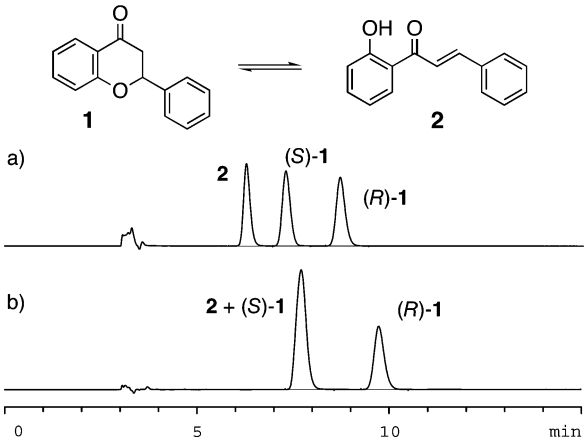

FIGURE 1. HPLC chromatogram illustrating an "irreproducible" peak separation of *rac*-**1** and **2**, due to adventitious water in the *n*-heptane/^{i}PrOH (90:10) eluent:[12] (a) measurement with "aged" eluent; (b) measurement with freshly prepared eluent.

Specific effects of adventitious or added water on the retention times of individual components in a mixture are illustrated by the analysis of mixtures of flavanone (**1**) and 2′-hydroxychalcone (**2**) (Figure 1). Since **1** can interconvert with **2** under a range of conditions, the chiral HPLC method must separate **2** from both enantiomers of **1**, or ee determinations might be inaccurate. The stationary phase Chiralcel-OD is recommended for analysis of nonhydroxylated flavanones including **1**.[4,9,10] First runs gave satisfactory resolutions both for the flavanone enantiomers ($\alpha = 1.33$, $R = 3.36$)[11] and the pair **2**/*S*-**1** ($\alpha = 1.31$, $R = 2.85$) (Figure 1a).

(1) Eliel, E. L.; Wilen, S. H.; Mander, L. N. *Stereochemistry of Organic Compounds*; John Wiley & Sons: New York, 1994; pp 214−295.

(2) (a) *HPLC Made to Measure*; Kromidas, S., Ed.; Wiley-VCH: Weinheim, 2006. (b) *Chiral Separation Techniques*, 3rd ed.; Subramanian, G., Ed.; Wiley-VCH: Weinheim, 2006. (c) *Chiral Separations by HPLC*; Krstulovic, A. M., Ed.; Ellis Horwood Limited: Chichester, U.K., 1989. (d) Unger, K. K.; Weber, E. *A Guide to Practical HPLC*; GIT Verlag: Darmstadt, Germany 1999.

(3) *H*igh *P*erformance *L*iquid *C*hromatography.

(4) (a) Okamoto, Y.; Yashima, E. *Angew. Chem., Int. Ed.* **1998**, *37*, 1020. (b) Francotte, E. R. *J. Chromatogr. A* **2001**, *906*, 379. (c) Yashima, E.; Yamamoto, C.; Okamoto, Y. *Synlett* **1998**, 344.

(5) Persson, B.-A.; Andersson, S. *J. Chromatogr. A* **2001**, *906*, 195.

(6) Meyer, V. R. *Pitfalls and Errors of HPLC in Pictures*, 2nd ed.; Wiley-VCH: Weinheim, Germany 2006.

(7) Effects of water on chiral HPLC separations: (a) Balmér, K.; Persson, A.; Lagerström, P.-O.; Persson, B.-A.; Schill, G. *J. Chromatogr.* **1991**, *553*, 391. (b) Balmér, K.; Lagerström, P.-O.; Persson, B.-A.; Schill, G. *J. Chromatogr.* **1992**, *592*, 331. (c) Svensson, S.; Vessman, J.; Karlsson, A. *J. Chromatogr. A* **1999**, *839*, 23. (d) Wang, F.; O'Brien, T.; Dowling, T.; Bicker, G.; Wyvratt, J. *J. Chromatogr. A* **2002**, *958*, 69. (e) Bielejewska, A.; Duszczyk, K.; Zukowski, J. *Acta Chromatogr.* **2005**, *15*, 183.

(8) (a) Snyder, L. R.; Kirkland, J. J. *Introduction to Modern Liquid Chromatography*; John Wiley: New York, 1979; p 374. (b) Snyder, L. R. *J. Chromatogr. Sci.* **1969**, *7*, 595.

(9) (a) *Application Guide for Chiral Column Selection*, 2nd ed.; Daicel Chemical Industries: Tokyo, 1989. (b) http://www.chiral-application-guide.com/, accessed July 2007.

(10) Okamoto, Y.; Kawashima, M.; Hadata, K. *J. Chromatogr.* **1986**, *363*, 173.

(11) α is the separation factor ($\alpha = k_2/k_1$ for $k_n = \{t_n - t_0\}/t_0$) and R the resolution ($R = 2\{t_2 - t_1\}/\{w_1 + w_2\}$) for t_n = retention time and w_n = baseline peak with of component n. A resolution of $R \geq 1$ is usually required for satisfactory peak−area ratio determinations.

(12) Conditions: Chiralcel OD (4.6 × 250 mm, *n*-heptane/^{i}PrOH 90:10 (v/v), 1 mL min^{-1}, 20 °C, $\lambda = 254$ nm).

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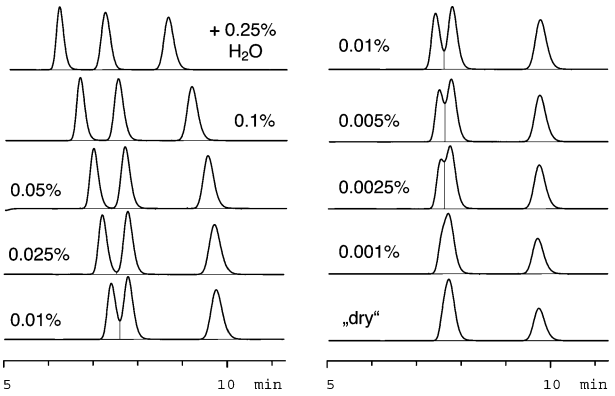

FIGURE 2. Chromatogram illustrating the effect of variable amounts of added water (v/v) on peak separation of *rac*-**1** and **2**.[12]

TABLE 1. Effect of Trace Water on Peak Separation of *rac*-1 and 2[12 a]

entry	added H_2O,[b] ppm	α (*S*-**1**/**2**)	*R*	α (*R*/*S*-**1**)[c]	*R*[c]
1	2500	1.31	2.85	1.33	3.36
2	1000	1.23	2.27	1.36	3.82
3	500	1.17	1.81	1.39	4.24
4	250	1.13	1.45	1.40	4.25
5	100	1.08	0.92	1.41	4.27
6	50	1.06	0.52	1.42	3.93
7	25	1.04	-	1.42	3.52
8	10	-	-	1.42	3.78
9	− („dry")[d]	-	-	1.43	4.02

[a] For conditions, see ref 12. [b] ppm for (v/v). [c] Reference 10 gives α = 1.41, *R* = 3.08 for hexane/^{i}PrOH = 90:10 (v/v), 0.5 mLmin^{-1}, 25 °C. [d] 23 ppm.

After a time break of 3 months, another batch of samples was analyzed, but, perplexingly, the peaks of *S*-**1** and **2** now overlapped (Figure 1b)! Attempts to reproduce the initial separation of **1** and **2**[13] on the same stationary phase included testing of several commercial "columns" available in-house prior to and after adequate washing procedures, using HPLC-quality solvents from freshly opened bottles; however, the earlier separation could no longer be realized. As it turned out, the decisive factor had been the replacement of a nearly empty solvent bottle of isopropyl alcohol shortly after the initial measurements by a freshly opened one; this had the effect of reducing the water content of the eluent. A series of measurements with "dry" eluent, spiked with defined quantities of water, was undertaken (Figure 2 and Table 1).

Moist solvent (0.025−0.25% H_2O, or 250−2500 ppm (v/v)) led to satisfactory peak resolution. Trace quantities of water (0.0025% = 25 ppm) induced a peak splitting, and a mere 10 ppm of added water gave a noticeable deformation of the combi peak (Figure 2). The reproducibility of peak forms and retention times after wet/dry changes was excellent if stabilization of the UV-absorption baseline (λ = 230, 254, and 280 nm) was achieved before subsequent measurements (typically 0.5−1 h of equilibration).

Water is readily absorbed by organic solvents from humid air, so it was necessary to determine the trace water level of *dry* HPLC-grade solvents by coulometric Karl Fischer titration (Table 2).[14] Freshly opened solvent bottles of several suppliers contained less than the maximal specified value of water (entries 1, 4−6). Freshly mixed *n*-heptane/isopropyl alcohol (90:10) eluent only contained 23 ppm of water, which explains why addition of a similar small quantity of water could already have a noticeable effect.

TABLE 2. Trace Water Content of Solvents Used for HPLC

entry	solvent	grade	specified H_2O (ppm)	measured H_2O (ppm)
1	^{i}PrOH	HPLC	<500	38[a]
2	^{i}PrOH, "wet"	HPLC	<500	18700[b]
3	EtOH abs	p.a.[c]		235
4	*n*-heptane	p.a.	<200	21
5	*n*-heptane	HPLC	<40	21
6	*n*-hexane	p.a.	<200	19

[a] Freshly opened bottle. [b] Almost empty 2.5 L bottle attached to a HPLC unit. [c] p.a. = for analysis.

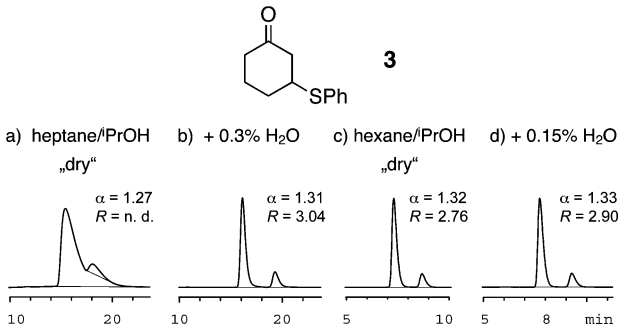

FIGURE 3. Illustrating the effects of added water on peak broadening in the analysis of **3** (70% ee) on Chiralpak-AD.[19]

In most laboratories, solvent bottles are attached to the HPLC unit by means of a cap containing a small hole for pressure-exchange, without protection toward absorption of moisture from the air. An almost empty 2.5 L bottle of isopropyl alcohol that we analyzed had accumulated a remarkable 1.8% (v/v) water (entry 2)! In practice, the trace water level of HPLC eluents is variable and increases with time. Going back to the example of Figure 1, a water content of 0.25% in the isopropyl alcohol (0.025% in the mixed eluent) is sufficient to induce satisfactory peak separation; for reliable analyses, we prefer to use a premixed "dry" eluent to which 0.25% of water is added. Trace water plays a major role in peak separation of *S*-**1** and **2**, but the α-value for separation of *S*-**1** and *R*-**1** was almost unaffected (Table 1, entries 3−9). Nevertheless, water can also affect the resolution of enantiomers. 3-Phenylthiocyclohexanone (**3**), the product of a cinchonine catalyzed addition of thiophenol to 2-cyclohexenone,[15] was analyzed on Chiralpak AD[16] with *n*-heptane/isopropyl alcohol (90:10) as the eluent; overlapping peaks precluded an accurate determination of enantiomeric excess (Figure 3a). The peak broadening was independent of flow rate (0.5 or 1 mLmin^{-1}), but after addition of 0.3% of water (v/v) to the eluent, the peaks were baseline separated (Figure 3b). The success is mainly due to a reduction of tailing. The surprising effect of changing from *n*-heptane[17] to *n*-hexane as hydrocarbon component may be noted: Added water has almost no effect on the latter separation (Figure 3b,c).[18]

(13) Test-mixture: *rac*-**1** (10.8 mg) and **2** (6.9 mg) in *t*-BuOMe (50 mL); injection volume = 20 μL.

(14) Analyses were carried out on a Mettler KF-coulometer DL-37.

(15) Hiemstra, H.; Wynberg, H. *J. Am. Chem. Soc.* **1981**, *103*, 417.

(16) Saito, M.; Nakajima, M.; Hashimoto, S. *Chem. Commun.* **2000**, 1851. These authors used *n*-hexane as the hydrocarbon component.

(17) We tend to use *n*-heptane in place of neurotoxic *n*-hexane as eluent.

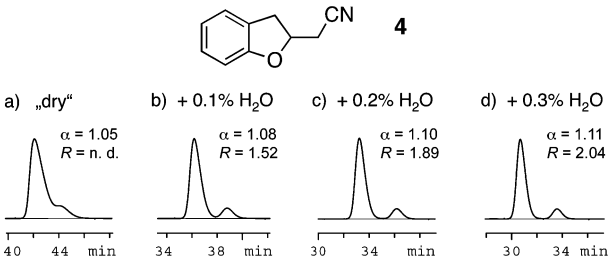

FIGURE 4. Illustrating the effects of added water in the analysis of **4** (76% ee) on Chiralcel-OJ.[21]

A similar behavior was noted in the separation of benzodihydrofuranyl-acetonitrile **4** on Chiralcel-OJ (Figure 4) where the incremental addition of water to the eluent *n*-heptane/isopropyl alcohol (90:10) gradually led to a better resolution by both decreasing broadening and increasing the separation factor (Figure 4).[20] However, the peak retention times decreased strongly upon addition of water, whereas they had slightly increased in the separation of **3**.

The aspect of reducing total analysis time by added water is highlighted in the HPLC analysis of naringin (**5**), a flavonoid which occurs in grapefruit peel and epimerizes during the ripening of the fruit.[22,23] Caccamese and co-workers have recently shown that this highly polar glycoside is conveniently separated into diastereomers (with respect to C-2) on a common cellulose-carbamate stationary phase (Chiralcel-OD), in an *n*-hexane/EtOH (60:40) eluent where ethanol was doped with 0.1% of CF_3CO_2H.[23] We find that satisfactory analyses and shortened retention times are also achieved by addition of water to the mobile phase (Figure 5).

Finally, we have also[5,7] found an example where added water not only led to complete peak separation but also changed the order of elution of enantiomers (Figure 6). In the case of a dry *n*-heptane/isopropyl alcohol (90:10) eluent, the enantiomers of phospholane **6** are incompletely separated. With increasing water levels, the peaks start to overlap, give a single peak, and then separate again (Figure 6).

The above examples illustrate that adventitious or added water in eluents for normal-phase chiral HPLC separations on polysaccharide coated silica has important practical consequences. These effects are apparently little recognized even by regular users. In the course of studies on the resolution of amino alcohols on polysaccharide stationary phases, Persson analyzed the effects of water in the 200−1400 ppm range and found that increasing water levels decreased peak resolution by selectively affecting the retention of one enantiomer.[5,7] Our examples highlight a different aspect: trace- or added water can increase peak resolution of commonly functionalized substrates by

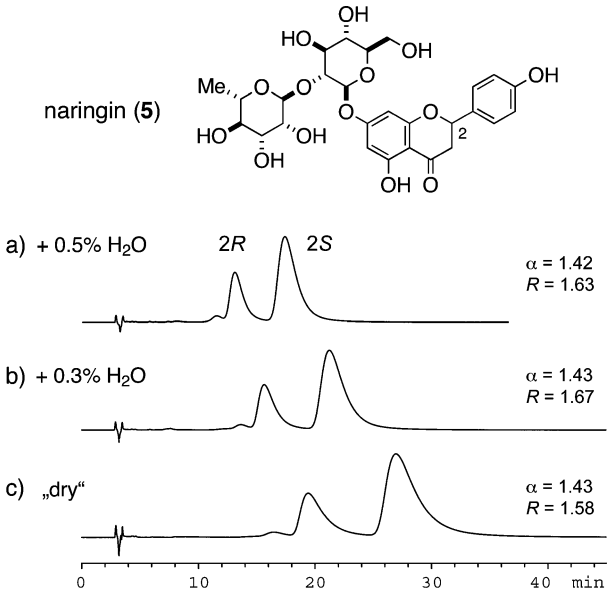

FIGURE 5. Effect of water additive on peak retention in the analysis of naringin[24] on Chiralcel-OD.[25]

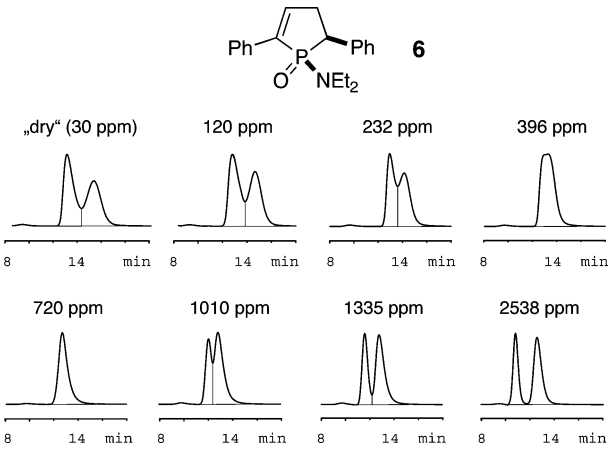

FIGURE 6. Effect of eluent water content on peak separation of **6** (15% ee): inversion of enantiomer elution order.[27]

reducing tailing or by differentially affecting retention times of components in mixtures. In the examples of Figures 1−5, the separation factor α of enantiomers is little affected, which excludes a structural modification of the chiral phase by water.[26]

The observed effects are probably a consequence of competitive binding of analyte and water to the SiO_2-support. The effects discussed relate to chiral stationary phases (CSP) coated on silica gel, and it remains to be seen whether immobilized CSPs[28] will be affected.

In conclusion, we have shown that added trace water (down to levels of 25 ppm) in eluents for normal phase HPLC can

(18) The water effects presented here are not specific to *n*-heptane mixtures. Figure 1 was also reproduced with *n*-hexane/^{i}PrOH as eluent with trace water additions; for another example with hexane, see ref 20.

(19) Conditions: Chiralpak-AD (4.6 × 250 mm, alkane/^{i}PrOH = 90/10 (v/v), 0.5 mLmin^{-1} for a/b; 1.0 mL min^{-1} for c/d; 20 °C, λ = 254 nm).

(20) Resolution enhancement in the analysis of **4** was also observed with an eluent based on hexane instead of heptane (conditions as in Figure 4): "dry", α = 1.07, *R* = 1.27; with 0.15% of water, α = 1.11, *R* = 2.18.

(21) Conditions: Chiralcel-OJ (4.6 × 250 mm, *n*-heptane/^{i}PrOH = 90/10 (v/v), 0.7 mL min^{-1}, 20 °C, λ = 280 nm).

(22) Gaffield, W.; Lundin, R., E.; Gentili, B.; Horowitz, R., M. *Bioorg. Chem.* **1975**, *4*, 259.

(23) (a) Caccamese, S.; Manna, L.; Scivoli, G. *Chirality* **2003**, *15*, 661. (b) Caccamese, S.; Bianca, S.; Santo, D. *J. Agric. Food Chem.* **2007**, *55*, 3816.

(24) For this naringin sample: (2*S*/2*R*) = 71:29. The diastereomer ratio in commercial samples is variable; see ref 23a.

(25) Conditions: Chiralcel-OD (4.6 × 250 mm, *n*-heptane/EtOH = 60:40 (v/v), 1 mLmin^{-1}, 20 °C, λ = 280 nm).

(26) Structural modification of chiral stationary phases by alcohol additives: Wang, T.; Wenslow, R. M. *J. Chromatogr. A* **2003**, *1015*, 99.

(27) Conditions: *Chiralcel-OJ* (4.6 × 250 mm, *n*-heptane/^{i}PrOH = 90/10 (v/v), 0.7 mLmin^{-1}, 20 °C, λ = 254 nm).

(28) Zhang, T.; Nguyen, D.; Franco, P.; Murakami, T.; Ohnishi, A.; Kurosawa, H. *Anal. Chim. Acta* **2006**, *557*, 221.

affect peak resolution and retention to a remarkable degree. Failure to account for this phenomenon may be the cause behind perplexing cases of irreproducible analytics. The deliberate addition of water (0.05−0.3% (v/v)) to eluents is a simple option in HPLC method development, especially in cases where peaks are insufficiently separated because of tailing. Since we have become aware of this effect, the probability of finding resolution conditions on our available CSPs has considerably increased. The shorter analysis times lead to higher productivity and savings of solvents—all at the expense of a few drops of water.

Experimental Section

HPLC analyses were performed at 20 °C on analytical columns (4.6 × 250 mm) using premixed solvents and isocratic pumping for best reproducibility. A multidiode array detector was used for detection. Solvent mixtures (v/v) were prepared by volumetric mixing: water was added either by weighing out on a balance (0.5−0.05% concentration range) or as a stock solution (0.1% H_2O (v/v) in eluent) for lower concentrations. For best reproducibility, the latter eluents are mixed directly before use, using dry solvents. Eluents containing 0.1−0.3% of water can be stored for several weeks in closed vessels and are attached to the HPLC system only during measurements.

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