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SYSTEMATIC USE OF TETRAHYDROFURAN IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

AN EXAMPLE OF THE SELECTIVITY BENEFITS OF TERNARY MOBILE PHASES

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SUMMARY

Selectivity is described here as the ability of a solvent to exhibit specific solute interactions which another solvent of similar polarity does not exhibit. Selectivity differences are shown to provide an important and ready means of improving and controlling resolution. The selectivity effects of a water–methanol–tetrahydrofuran ternary system are discussed with respect to the separation of vanillin and syringaldehyde and that of eight cinnamic acid derivatives.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a powerful tool for solving difficult separations and subsequent quantitative analyses at trace levels and has therefore been extensively used, particularly in the biological and medical sciences. At present, reversed-phase HPLC using *n*-alkyl bonded phases is the most frequently selected separation mode. It is estimated that 60–80% of HPLC separations are accomplished using reversed-phase chromatography[4,5]. The advantage of reversed-phase HPLC compared with adsorption chromatography has been described by several workers[4,6,7] and indeed reversed-phase chromatography is used over a broad polarity range and in diverse applications.

The rôle of the mobile phase in reversed-phase chromatography and the expected solute interactions with the non-polar stationary phase have been described previously[3,8]. In particular, we have demonstrated[1,2,9] that mobile-phase control with three mixed solvents can be a potentially powerful tool for the optimization of selectivity. In those experiments several isocratic and gradient methods were used to study solvent selectivity of (1) binary mixtures of water–methanol, –acetonitrile and –tetrahydrofuran, and of (2) ternary mixtures of water, methanol and a third solvent chosen from acetonitrile, tetrahydrofuran, diethyl ether, methylene chloride, dimethylformamide, dimethyl sulphoxide and water 50% saturated with hexane. The results indicated that the retention of compounds containing certain functional groups can be systematically and precisely influenced by ternary solvent systems. Tetrahydro-

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furan, a solvent which is extensively used in reversed-phase HPLC[10–15], was found to be particularly useful as a ternary solvent in the separation of compounds which differ in their number of ether groups.

It should be mentioned at this point that compounds differing in the number of constituent ether groups can, as a rule, be easily separated by other chromatographic methods, *e.g.*, adsorption methods, including thin-layer chromatography (TLC). By contrast, separations of such compounds by reversed-phase HPLC are frequently poor, particularly for methoxy-containing compounds, on octyl- and octadecyl-bonded phases. However, reversed-phase HPLC has many methodological advantages which are of interest with respect to the solution of this separation problem.

The work of Tanaka *et al.*[3] illustrates vividly that the selectivity of some aromatic hydrocarbons seems to be greater in the tetrahydrofuran–methanol system than in acetonitrile–methanol and that a mixture of tetrahydrofuran, methanol and water would be an interesting ternary mobile phase for control of separation of substances with different functional groups. This work shows, among other things, that phenols are significantly retarded in tetrahydrofuran–water, relative to methanol–water, whereas in acetonitrile–water no special selectivity for phenols is observed relative to methanol–water.

The present work describes the reversed-phase separation of vanillin–syringaldehyde and eight cinnamic acid derivatives. The main difficulty was the complete resolution of all substances which differ only in the number of methoxy groups, because these compounds could not be separated by us on a reversed-phase system with a binary mobile phase of water–methanol or –acetonitrile. Based on experience gained from previous work[1,3], tetrahydrofuran in a ternary mobile phase with water–methanol was employed.

EXPERIMENTAL

Column, solvents and samples

Separations were performed on a 250 × 4.6 mm I.D. stainless-steel column packed with 10-μm LiChrosorb RP-8®, a totally porous granular material, with covalently bonded octyl groups. This column was obtained from Spectra-Physics, Santa Clara, CA, U.S.A.

The mobile phase consisted of different ternary mixtures of distilled water, methanol and tetrahydrofuran (THF) (E. Merck, Darmstadt, G.F.R.). No attempt was made to control the pH of the water in the analysis of vanillin–syringaldehyde. In the case of cinnamic acid metabolites, the pH was adjusted to 3 with acetic acid. All solvents were continuously degassed using helium. Samples were standards (commercial source), dissolved in methanol.

Control of flow, composition and temperature

A Spectra-Physics Model SP8000B liquid chromatograph was used. It employs a single pump, attached to a low pressure ternary proportioning valve. Up to three different solvents were mixed by the valve according to the operators instructions, in both isocratic and gradient elution. The temperature was controlled at 35°C in a forced-air oven.

Detection

The detector was a Spectra-Physics Model SP8310 multiple wavelength UV-photometer operated at 254 nm.

RESULTS AND DISCUSSION

Separation of vanillin and syringaldehyde

Vanillin and syringaldehyde differ only in the additional methoxy group in the *meta* position of syringaldehyde. As mentioned before, on a reversed-phase column with a binary mobile phase of water–methanol or water–acetonitrile, both substances are eluted with the same capacity factor, regardless of the relative composition of the mobile phase. Although simplified, the reasons for this behaviour lie in the retention increase due to the methyl group and the retention decrease due to the oxygen, the combined effect of which is no change in retention. With methanol–water (1:1) as mobile phase the capacity ratio (k') of vanillin and syringaldehyde was 1.3. As expected, water–methanol (3:2) as mobile phase increased the k' value to 1.5, but the separation was not improved. Fig. 1a shows the chromatogram with 70% water–30% methanol for which the k' value of both substances was 2.5.

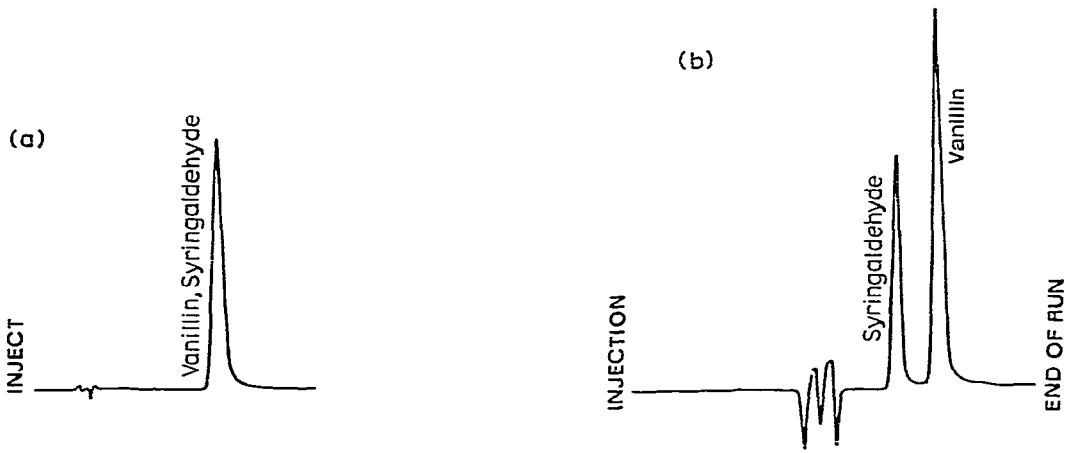


Fig. 1. Reversed-phase separation of vanillin and syringaldehyde. Conditions: SP8000B liquid chromatograph (Spectra-Physics); mobile phase, water–methanol (7:3) (a) or water–methanol–tetrahydrofuran (7:2:1) (b), flow-rate 1 ml/min; detector, SP8310 UV detector (Spectra-Physics), 254 nm; column, LiChrosorb RP-8, 10 μm.

The second step in improving the separation of vanillin and syringaldehyde was therefore the optimization of the selectivity of the mobile phase. Based on previous work, tetrahydrofuran was chosen because of its ability to accelerate compounds containing ether groups. Thus tetrahydrofuran causes syringaldehyde (two ether groups) to elute in front of vanillin (one ether group). Fig. 1b shows that a mixture of 10% tetrahydrofuran, 20% methanol and 70% water is the optimum mobile phase to achieve baseline separation. Another advantage of tetrahydrofuran is the shortening in retention time of both substances. The analysis is now completed in less than half the time of that required for the analysis with a binary mobile phase.

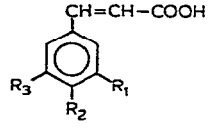
Separation of cinnamic acid metabolites

The metabolites of cinnamic acid (Table I) can be used as an indicator of the metabolism of drugs because the final amount of metabolites in urine gives an indication of body metabolism, and the activity of intestinal bacteria.

Preliminary work showed that about 75% water (pH 3) was required to achieve retention of the compounds on the RP-8 column; however, the binary water–methanol

TABLE I

CINNAMIC ACID METABOLITES



Cinnamic acid	R_1	R_2	R_3
m-Hydroxy	OH	H	H
p-Hydroxy	H	OH	H
3,4-Dihydroxy (caffeic acid)	OH	OH	H
m-Methoxy	OCH_3	H	H
p-Methoxy	H	OCH_3	H
3,4-Dimethoxy	OCH_3	OCH_3	H
3,4,5-Trimethoxy	OCH_3	OCH_3	OCH_3
3-Methoxy-4-hydroxy	OCH_3	OH	H

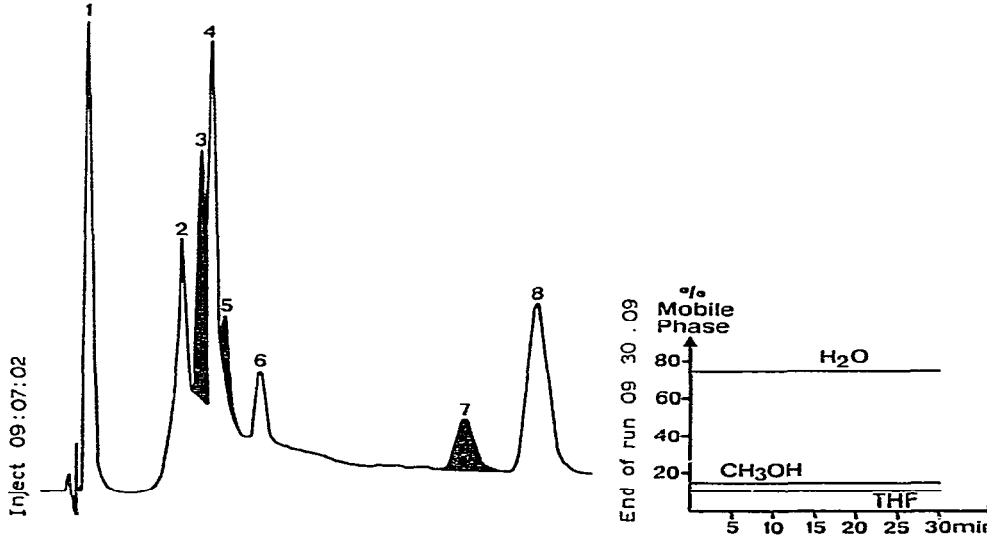


Fig. 2. Ternary isocratic separation of cinnamic acid metabolites. Mobile phase: water (pH 3)–methanol–THF. Other conditions as in Fig. 1. Cinnamic acids: 1 = 3,4-dihydroxy; 2 = 3-methoxy-4-hydroxy; 3 = 3,4-dimethoxy; 4 = *p*-hydroxy; 5 = 3,4,5-trimethoxy; 6 = *m*-hydroxy; 7 = *p*-methoxy; 8 = *m*-methoxy.

phase gave no separation of the mono-, di- and trimethoxy derivatives. From previous work involving methoxy-substituted compounds, as described above, this was to be expected. It followed that a selective separation of the metabolites could be achieved with a ternary mobile phase of water–methanol and THF. The separation was achieved in three steps.

Step 1. Fig. 2 shows the first analysis with THF in the ternary solvent. The water content was held constant at 75% to maintain retention and 10% THF was added as the modifier, the remainder being 15% methanol. The mono-, di- and trimethoxycinnamic acids were well separated but eluted together with hydroxy derivatives.

Step 2. Because of the complexity of the sample, a gradient was used. An initial composition consisting of 80% water, 10% methanol and 10% THF was held constant for 5 min to increase retention. The water content was then reduced to 70% and the methanol and the THF contents increased to 15%. Fig. 3 shows this ternary

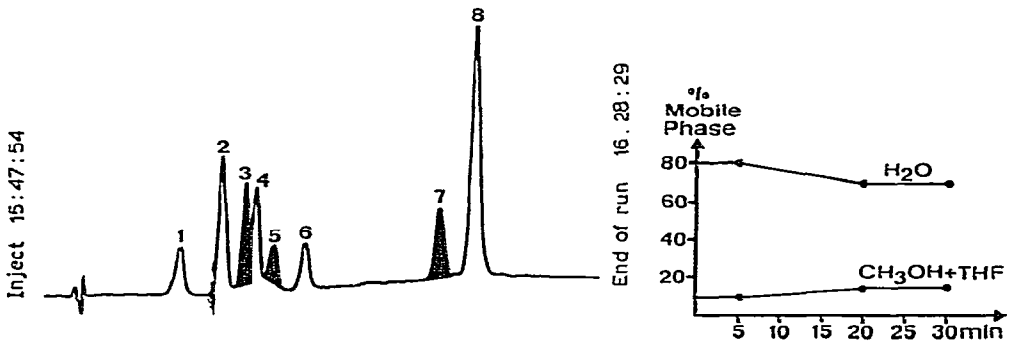


Fig. 3. Ternary gradient separation of cinnamic acid metabolites. For conditions see Fig. 2.

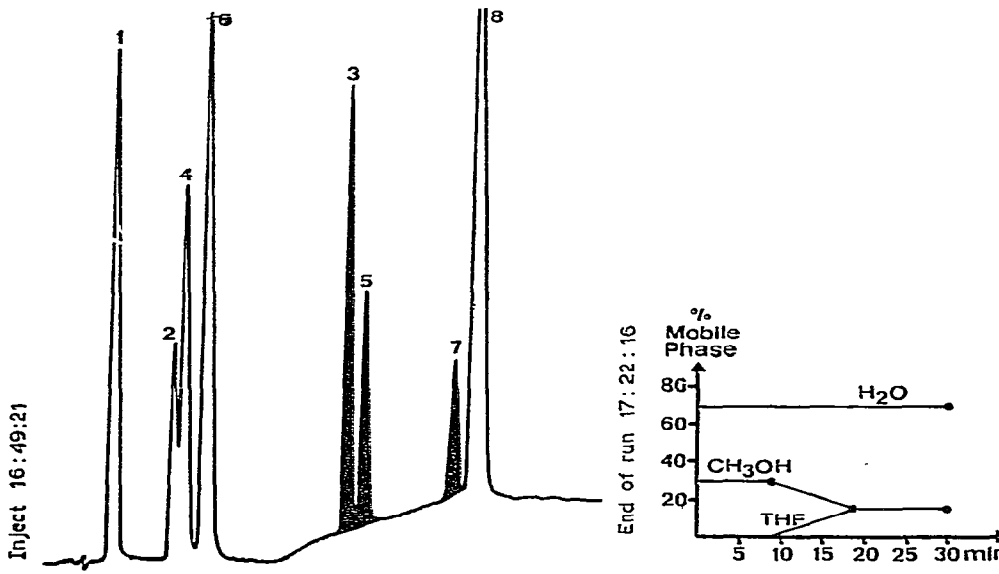


Fig. 4. Separation as in Fig. 3 but with a different gradient profile.

gradient separation where all three solvent concentrations were simultaneously changed. The three methoxycinnamic acids were preferentially accelerated, the monomethoxy more than either the tri- or dimethoxy. This may be explained by steric effects.

Step 3. The remaining difficulty was to improve the separation of *p*-hydroxycinnamic acid from the di- and trimethoxy derivatives. Here again, ternary solvent selectivity effects provided resolution.

Fig. 4 shows the HPLC separation of the metabolites, but in this case with a different gradient profile. Because of the absence of THF at the beginning of the analyses, the tri- and dimethoxy derivatives are selectively retained longer on the column while the first group of derivatives are eluted. THF is then rapidly added, causing the di- and trimethoxy derivatives to be eluted closely together but well separated from the mono-derivatives which of eluted last.

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