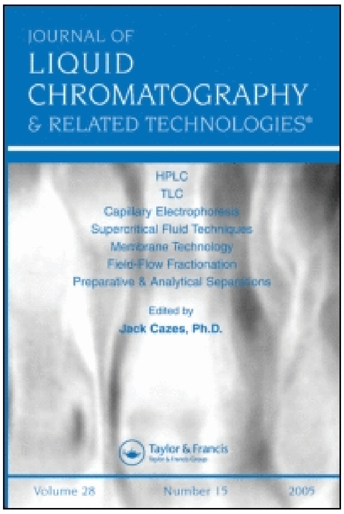

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THE HPLC SEPARATION OF TWO MIXTURES OF NEUTRAL PESTICIDES: EFFECTS OF TEMPERATURE, GRADIENT STEEPNESS, AND FLOW RATE

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ABSTRACT

The application of a practical approach for the optimization of the separation of a mixture of triazine and substituted urea herbicides and a mixture of organophosphorous insecticides is presented. The separation is achieved with reversed-phase HPLC and acetonitrile/water mobile phase mixtures. The separation method is based on the simultaneous optimization of temperature (T) and gradient steepness (b) as a means of varying selectivity and achieving an acceptable resolution.

The effect of flow rate is also examined. Triazine and substituted urea herbicides exhibit greater changes in resolution than organophosphorous insecticides with variation in T and b. This is attributed to the more diverse and polar molecular nature of the herbicide mixture. Flow rate is used as an additional parameter that could facilitate resolution. The overall separation is achieved within a few experiments.

INTRODUCTION

The reversed-phase HPLC separation of sample mixtures could be achieved by optimization of sample retention (retention factor, k), column efficiency (plate number, N), and selectivity (separation factor, α).[1] In the past, variables that were believed to have the largest effect on selectivity (e.g. solvent type, column type, pH) were chosen for the optimization of a separation.[1,2]

Solvent strength is defined as the %B of the strong solvent in isocratic elution and it is analogous to gradient steepness parameter b in gradient elution.[3,4] The retention factor k increases as solvent strength decreases,[1] and the resolution R_s of the components of a sample initially increases and then levels off.[1,5] However, this is not true for all sample mixtures, because solvent strength also influences selectivity α[1] and the resolution is determined by the combined effect of these two factors.

Column heating at elevated temperatures is applicable and widely employed in some areas of liquid chromatography, such as size exclusion and ion exchange.[6] Column heating is used to improve retention data reproducibility and column efficiency, reduce the column pressure drop and the separation time.[1,6] The possibility of employing temperature variations to optimize selectivity in reversed-phase liquid chromatography has achieved less attention.[1,4,6] A change in selectivity with temperature is expected whenever two or more distinct processes contribute to sample retention.[1,7]

Favorable temperature selectivity effects are more likely to occur for the separation of ionizable samples[7] and compounds with multiple polar substituents,[8] but some neutral samples have shown significant changes in selectivity with temperature.[1,8] For neutral samples, it appears that gradient steepness induced selectivity is generally stronger than temperature selectivity.[1,6,8]

While changes in either T or b have a smaller effect on selectivity than changes in other parameters like solvent or column type,[1,9] this is less true when T and b are simultaneously optimized for a given sample.[5] This is due to that, temperature and gradient steepness effects are uncorrelated or weakly correlated, which means that these parameters affect selectivity differently and will be complementary during method development.[4,7,8] Additionally, changes in T or b are usually more convenient and free of practical problems than changes in solvent or column type.[2,9]

The flow rate (F) of the mobile phase is correlated to column plate number N that affects resolution R_s. According to Snyder et al.[1] the variation of flow rate has a modest effect on band spacing, but could be a useful parameter for fine adjustment of resolution.

While previous research has concentrated on the individual effects of temperature and gradient steepness, only recently has the effect of their combined use been explored.[2,4,5,7,,8,10,11,12]

This study describes the simultaneous variation of temperature and gradient steepness as a means for separating a mixture of 14 triazine and substituted urea herbicides and a mixture of 15 organophosphorous insecticides. The effect of flow rate on further improving the separations is also examined. This new approach is aimed at increasing the efficiency of the method development process.

EXPERIMENTAL

Reagents

Acetonitrile for UV and methanol for HPLC were supplied by Lab – Scan, Ireland. HPLC grade water, was prepared in the laboratory with a MilliQ/MilliRO system (Millipore, USA). Analytical standards of all the pesticides and degradation products, were supplied by Dr Ehrenstorfer, Germany and ChemService, USA.

Stock solutions of individual pesticides were prepared in methanol, at 1 mg/mL, except simazine (0.1 mg/mL). The concentration of the herbicide working solutions was 0.8 μg/mL, while the insecticide solutions were prepared at a concentration of 1–2 μg/mL.

Apparatus

The HPLC system consisted of a 9012 pump, associated with a Polychrom 9065 diode-array detector (Varian, USA) and a Rheodyne 7161, 100 μL, loop injector (Rheodyne, USA). The wavelength of the detector was set at 234 nm for the detection of triazine/substituted urea herbicides and at 215 nm for the detection of organophosphorous insecticides.

The column was a Zorbax SB – C18 4.6 mm x 15 cm (5 μm) connected with a Zorbax SB – C18 precolumn (Hewlett Packard, USA). The mobile phase consisted of acetonitrile and water.

Jones Chromatography (England) supplied a 7980 column block heater with a temperature accuracy of ± 0.5°C. The mobile phase was preheated by a preheating wire that was wrapped around the tube entering the column.

Procedure

Gradient steepness b was varied by changing gradient time t_G. Experiments (a) and (b) (Figure 1) were conducted in order to examine the effect of gradient

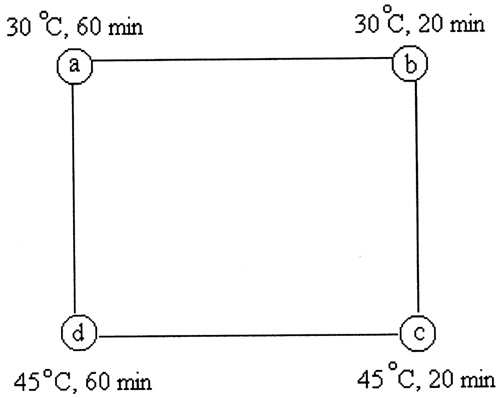

Figure 1. Four experiments required for optimizing temperature and solvent strength.

steepness on the separation. The next two experiments (Figure 1, experiments (c) and (d)), were carried out using high temperature in order to examine its effect on band spacing.

The flow rate during the first four experiments was 2 mL/min. Optimum conditions of temperature and gradient time were selected and two additional experiments ((e) and (f)), were conducted at flow rates of 1 mL/min and 2 mL/min in order to evaluate the effect of flow rate on the separation. Finally, taking into consideration the results of the preceding experiments, optimum conditions that would result in an acceptable separation were selected. All the experiments were repeated twice.

RESULTS

In order to check the efficiency of the column during the two sets of experiments, the average peak width at half height for the four conditions tested was calculated. There was no significant difference in $w_{1/2}$ for the two classes of compounds in similar chromatographic conditions. This result indicates that the efficiency of the column was stable.

Problems in resolution are mainly encountered for the late eluting compounds. From the chromatograms (Figures 2 and 3), it appears that the herbicides are evenly distributed throughout the chromatogram, while some of the organophosphorous insecticides are bunching in a rather narrow time window. The last nine organophosporous compounds elute at a time interval of 6.4-min during experiment (a) (Figure 2(a)), while the last eight herbicides elute at an interval of 8.2 minutes (Figure 3(a)).

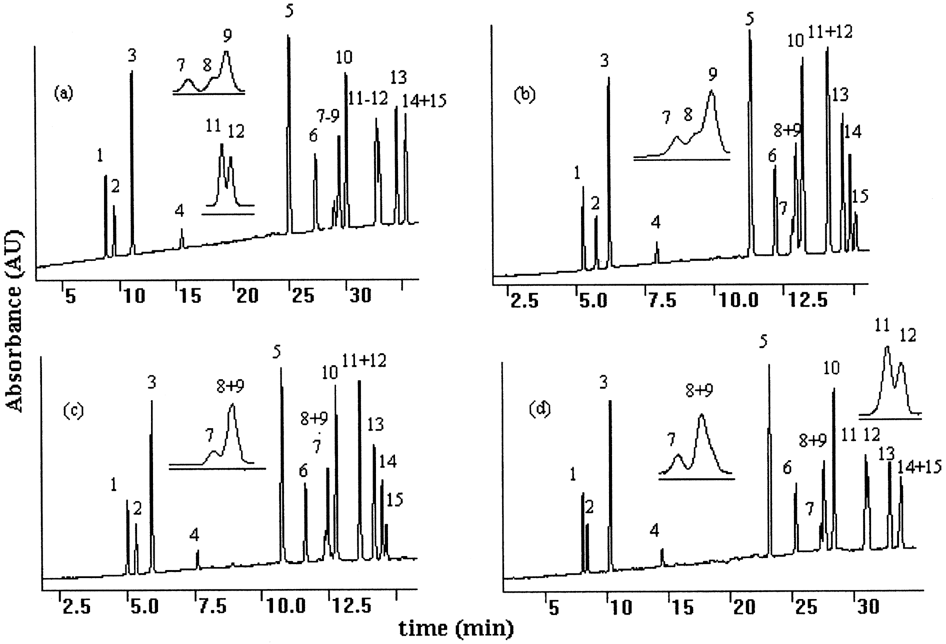

Figure 2. Four-method development experiments for separation of the 15-insecticide sample. (a): 30°C, 60 min, (b): 30°C, 20 min, (c): 45°C, 20 min, (d): 45°C, 60 min. Peaks: 1 – Z-mevinphos, 2 – dimethoate, 3 – E-mevinphos, 4 – demeton-s-methyl, 5 – azinphos methyl, 6 – parathion methyl, 7 – malathion, 8 – triazophos, 9 – fenitrothion, 10 – azinphos ethyl, 11 – fenthion, 12 – parathion ethyl, 13 – coumaphos, 14 – phoxim, 15 – disulfoton. Other conditions are presented in the text.

Concerning the separation of the organophosphorous insecticides, there are four band pairs with a resolution <1.5 in the conditions tested (Table 1, Figure 2). The decrease of the temperature in most cases enhances the separation of the unresolved band pairs. The increase of gradient time (decrease of gradient steepness) in most cases facilitates, or does not affect, the separation of all band pairs, except phoxim and disulfoton that are resolved only at shorter gradient times. Similarly, the increase in flow rate improves separation of all band pairs, except phoxim and disulfoton (Table 1).

Concerning the separation of triazine/phenylurea herbicides, there are five band pairs with a resolution <1.5 in certain conditions (Table 2, Figure 3). The variation of temperature and gradient time significantly influences the separation of this group of compounds. All the herbicides are resolved with $R_s>1.5$ at the conditions of experiment (d), except terbutylazine and linuron ($R_s=1.2$). It is interesting to notice that chloridazon and desethyl atrazine are resolved only at higher temperatures.

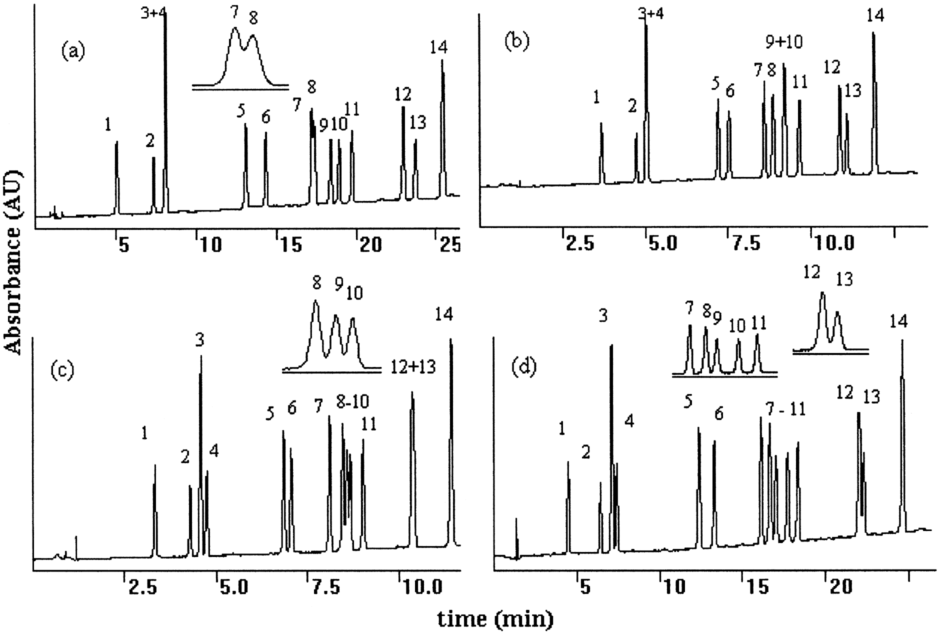

Figure 3. Four-method development experiments for separation of the 14-herbicide sample. (a): 30°C, 60 min, (b): 30°C, 20 min, (c): 45°C, 20 min, (d): 45°C, 60 min. Peaks: 1 – deisopropyl atrazine, 2 – metamitron, 3 – chloridazon, 4 – desethyl atrazine, 5 – simazine, 6 – cyanazine, 7 – chlortoluron, 8 – atrazine, 9 – monolinuron, 10 – diuron, 11 – metobromuron, 12 – terbuthylazine, 13 – linuron, 14 – prometryne. Other conditions are presented in the text.

In this set of experiments, the effects of gradient time and flow rate also follow similar qualitative trends. In cases where the resolution is favored by higher flow rates, it is also enhanced by longer gradient times (Table 2).

Taking into account the effect of the three parameters tested, conditions that would result in an acceptable separation were selected. For the organophosphorous pesticides mixture, the final experiment was carried out with a flow rate of 1.5 mL/min, a gradient of 5 to 100% ACN in 65 min, at ambient temperature (≈22°C). The run time of the final separation was 40 min. The achieved resolution was 1.0 for malathion and triazophos, 1.6 for triazophos and fenitrothion, 0.8 for fenthion and parathion ethyl, and 0.8 for phoxim and disulfoton.

For the triazine/phenylurea mixture, a final experiment was carried out with a flow rate of 1.5 mL/min, a gradient of 5 to 100% ACN in 45 min, at a temperature of 40°C. The run time of the final separation was 23 min. All peaks have a resolution factor ≥ 1.5, except chloridazon and desethyl atrazine (R_s = 1.3).

Table 1. Effect of Temperature *T*, Gradient Time t_G, and Flow Rate on the Resolution R_s of the Mixture of Organophosphorous Insecticides[a]

	30 °C	45 °C	1 mL/min (Exp. e)	2 mL/min (Exp. f)
Malathion/triazophos				
60 min	1.4	1.1	0.7	1.2
20 min	0.7	1.0		
Triazophos/fenitrothion				
60 min	0.7	0.0	1.3	1.7
20 min	0.6	0.0		
Fenthion/parathion ethyl				
60 min	0.9	0.7	0.0	1.0
20 min	0.0	0.0		
Phoxim/disulfoton				
60 min	0.0	0.0	1.6	0.0
20 min	1.9	1.5		

[a] The conditions in experiments e and f for the optimization of flow rate were 5–100% ACN in 60 min at ambient temperature (≈22°C).

Table 2. Effect of Temperature *T*, Gradient Time t_G, and Flow Rate on the Resolution R_s of the Mixture of Triazine/phenylurea Herbicides[a]

	30 °C	45 °C	1 mL/min (Exp. e)	2 mL/min (Exp. f)
Chloridazon/desethyl atrazine				
60 min	0.0	1.8	1.5	0.9
20 min	0.0	2.0		
Chlortoluron/atrazine				
60 min	0.7	2.6	3.5	2.0
20 min	2.6	3.8		
Atrazine/monolinuron				
60 min	4.9	1.7	2.0	2.6
20 min	3.0	1.1		
Monolinuron/diuron				
60 min	2.6	3.4	0.6	1.9
20 min	0.0	1.0		
Terbuthylazine/linuron				
60 min	3.3	1.2	1.1	1.8
20 min	1.9	0.0		

[a] The conditions in experiments e and f for the optimization of flow rate were 5–100% ACN in 40 min at 38°C.

DISCUSSION

The findings of this study generally concurred with previous research concerning the effectiveness of the simultaneous variation of temperature and gradient steepness (gradient time) in achieving an acceptable separation of mixtures of non-ionic compounds.[5,8,10,11,12]

Due to the complex nature of the molecules and the chromatographic system, it is difficult to have insight into the mechanisms that mainly contribute to the separation. However, some general remarks could be made.

The method is more efficient in separating the triazine/phenylurea herbicides than the organophosphorous insecticides. It appears that, the organophosphorous compounds exhibit more similar chromatographic behavior, indicating similarity in their structures, sizes, and physicochemical properties. The mixture of herbicides is more diverse because it contains compounds from different chemical classes. Eight of the herbicides belong to the triazine chemical group, five of the compounds belong to the substituted urea group, and chloridazon is a pyridazinone.[13]

Triazine and substituted urea herbicides have some different physicochemical properties: for example, all the triazines are weak bases; with pK_b values ranging from 10 to 12,[14] while the substituted ureas are not.[14] On the other hand, the 15 organophosphourous insecticides are esters of the phosphoric, phosphorothioic, and phosphorodithioic acid[13] and, therefore, their chemical structure is more similar.

According to Zhu et al.,[7] among the situations favoring temperature, selectivity is when two molecules possess different functional groups and the temperature dependence for the retention of these groups is not the same. Dolan et al.[5] and Zhu et al.,[8] report that the variation of temperature and gradient steepness is more effective in achieving an acceptable separation, when the sample mixture contains multiple polar functional groups.

In Table 3, the type and number of polar substituents for the two mixtures of compounds is presented. The triazine/phenylurea herbicides contain more polar moieties than the organophosphorous insecticides. This probably results in greater changes in band spacing during the four method development experiments, and a better final separation. All the critical band pairs, except monolinuron – diuron, belong to different chemical categories. The triazines typically possess two >NH groups, while the phenylurea herbicides contain the -CO-NH- group.

It is concluded that separation for these band pairs is mainly due to polar selectivity effects. On the other hand, monolinuron and diuron are very similar molecules. They differ in that diuron contains one atom of chlorine more than monolinuron and a methyl group instead of a methoxy group. According to Tanaka,[15] chlorine and methoxy groups contribute to hydrophobic group selectivity, which in this case is probably the main mechanism of the separation.

Table 3. The Type and Number of Polar Substituents of the Insecticide and Herbicide Mixtures

Triazine/Phenylurea Herbicides	No. Polar Groups	Organophosphorous Insecticides	No. Polar Groups
>NH	12	>C=O	8
—CONH—	5	$—NO_2$	3
>C=O	2	>NH	1
$—NH_2$	4	—C≡N	1
—C≡N	1		

Concerning the insecticide mixture, it is interesting to notice that only one of the molecules of every critical band pair contains polar substitution. Malathion contains two >C=O groups, fenitrothion and parathion ethyl contain a $-NO_2$ group, and phoxim contains a -CN group. Triazophos, fenthion, and disulfoton do not possess any polar substitution. The lack of polar substitution is a possible cause of the moderate response of this mixture to temperature and gradient steepness changes.

The effect of the varying flow rate is considerable for the two sample mixtures. In these experiments, the variation in F affected both column plate number and gradient steepness. There is the same qualitative effect of the flow rate and gradient time on the resolution of the mixtures. When the separation is facilitated by long gradient times (small b) it is also facilitated by higher flow rates.

This observation is justified by the equation $b = \frac{S\Delta\phi V_m}{t_G F}$, where $\Delta\phi$ is the change in volume fraction of the organic solvent during gradient, V_m the column dead volume, t_G the gradient time, F the flow rate, and $S=-d\log k/d\phi$.[3] It follows, that either changing the gradient time or the flow rate varies the gradient steepness parameter. It should be marked, that if it is desirable to change flow rate without affecting gradient steepness and the band spacing that resulted in optimizing T and b, the halving of flow rate should be accompanied by a doubling in gradient time, so as to maintain $t_G F/L$ constant.[16]

The modest trend for improved separation of the organophosphorous compounds at lower temperatures, suggests that the use of sub-ambient temperatures would facilitate the separation. Sub-ambient temperatures have been less commonly used to improve the resolution of difficult to separate compounds by increasing band spacing at the expense of poor column efficiency and long separation times.[6] Moreover, the resolution of band pairs such as chloridazon/desethyl-atrazine and triazophos/fenitrothion changes in response to temperature and not to gradient steepness variation. Consequently, these experiments stress the importance of temperature control in reversed-phase HPLC for the separation of sample mixtures.

When temperature and gradient steepness are changed in this systematic way, predictions could be made for the separation that would result by any combination of the two factors between the tested values. In a case like the organophosphorous-insecticide mixture, the components of the sample could be effectively separated if two separations with optimized (and different) conditions were developed.[5]

For example, a run at long gradient time with high flow rate and low temperature (experiment (f), Table 1) would sufficiently separate all the bands ($R_s \geq 1$), except phoxim and disulfoton, which could be separated in a short run, at low flow rate and low temperature (experiment (e), Table 1). Moreover, with samples like this, further changes in conditions could be used (e.g. change in solvent or column type).[16]

The simultaneous variation of temperature and gradient steepness results in better separation of the mixture with more polar nature, indicating that generally polar group selectivity is the main mechanism that promotes separation. The method is very useful for separating sample mixtures in an easy, fast, and systematic way. It could be used as an initial step for separating more demanding samples.

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REFERENCES

1. Snyder, L.R.; Kirkland, J.J.; Glajch, J.L. *Practical HPLC Method Development*, John Wiley and Sons: New York, 1997.
2. Zhu, P.L.; Snyder, L.R.; Dolan, J.W.; Djodjevic, N.M.; Hill, D.W.; Sander, L.C.; Waeghe, T.J. J. Chromatog. A **1996**, *756***,** 21–39.
3. Snyder, L.R.; Dolan, J.W.; Gant, J.R. J. Chromatogr. **1979**, *165*, 3–30.
4. Zhu, P.L.; Dolan, J.W.; Snyder, L.R. J. Chromatogr. A **1996**, *756*, 41–50.
5. Dolan, J.W.; Snyder, L.R.; Djodjevic, N.M.; Hill, D.W.; Saunders, D.L.; Van Heukelem, L.; Waeghe, T.J. J. Chromatog. A **1998**, *803*, 1–31.
6. Bolliet, D.; Poole, C.F. Analyst **1998**, *123*, 295–299.
7. Zhu, P.L.; Dolan, J.W.; Snyder, L.R.; Hill, D.W.; Van Heukelem, L.; Waeghe, T.J. J. Chromatogr. A **1996**, *756*, 51–62.
8. Zhu, P.L.; Dolan, J.W.; Snyder, L.R.; Djodjevic, N.M.; Hill, D.W.; Lin, J.-T.; Sander, L.C.; Van Heukelem, L. J. Chromatogr. A **1996**, *756*, 63–72.

9. Snyder, L.R. J. Chromatogr. B **1997**, *689*, 105–115.
10. Hancock, W.S.; Chloupek, R.C.; Kirkland, J.J.; Snyder, L.R. J. Chromatogr. A **1994**, *686*, 31–43.
11. Chloupek, R.C.; Hancock, W.S.; Marchylo, B.A.; Kirkland, J.J.; Boyes, B.E.; Snyder, L.R. J. Chromatogr. A **1994**, *686*, 45–59.
12. Guillaume, Y.; Guinchard, C. J. Liq. Chromatogr. **1995**, *18*(17), 3409–3422.
13. Matolcsy, G.; Nadasy, M.; Andriska, V. In *Pesticide Chemistry;*. Elsevier: Amsterdam, 1988; 652–727.
14. Hornsby, A.G.; Wauchope, R.D.; Herner, A.E. In *Pesticide Properties in the Environment;*Springer-Verlag: New York, 1995.
15. Tanaka N.; Goodel H.; Karger, B.L. J. Chromatogr. **1978**, *158*, 233–248.
16. Dolan, J.W.; Snyder, L.R.; Saunders, D.L.; Van Heukelem, L. J. Chromatogr. A **1998**, *803*, 33–50.

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