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Review

Coupled-column reversed-phase liquid chromatography in environmental analysis

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Abstract

An overview is given of the applicability of coupled-column reversed-phase liquid chromatography (LC–LC) to the trace analysis of organic micropollutants in environmental samples, based on typical examples of recent and current work. Although LC–LC, as applied to the analysis of polar pesticides in environmental samples, is usually based on the use of two separation columns with almost similar stationary phase selectivity, it will be shown that a considerable gain in the overall selectivity of methods is attainable. Moreover LC–LC provides sensitivity and potential for automation by utilisation of large volume injections. Due to the large number of parameters involved in the development of analytical procedures a systematic approach towards methodology is discussed. Two basic types of applications are treated: (i) single-residue methodology focused at the rapid determination of one analyte and (ii) multi-residue methodology for the simultaneous determination of a number of analytes.

Contents

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1. Introduction

The attractiveness of coupled chromatographic techniques can be found in enhancement of sensitivity, selectivity and potential for automation. As regards selectivity, multidimensional chromatography, involving orthogonal separation principles on two or more coupled columns seems attractive. It must be stressed, however, that orthogonality in coupled-column systems also has its drawbacks in terms of selectivity. For example, the differences in the physico-chemical bases of the separation processes involved often leads to poorly compatible mobile phase systems, thus rendering complex interfaces. Furthermore, the use of two different separation principles may lead to an inversion of the elution order on the two subsequent columns. Hence, the separation obtained on the first column can, at least partly, be diminished on the second column. For this reason multidimensional coupled-column systems require small transfer volumes and a limited number of target analytes. The application range of coupled-column techniques will be determined by the separation power of the first column: low resolution favours multi-residue methods (MRMs) while high resolution leads to methods for single analytes or a group of compounds with fairly similar properties. This aspect has been discussed in some detail previously [1].

In comparison to true multidimensional techniques, the coupling of two reversed-phase liquid chromatography systems would offer less selectivity. This may be a major reason for the fact that, today, in contrast to the automated on-line sample treatment applying precolumn switching (PC–LC) [2–4], LC–LC is still a relatively unexplored field in environmental trace analysis. PC–LC does not offer too much selectivity since the precolumn is merely used for sample enrichment, providing an increase in sensitivity by loading large sample volumes. In PC–LC, selectivity can be enhanced by using (i) selective sorbent materials for trapping such as cation exchangers [5–7], metal-loaded phases [8,9] and immobilised antibodies [10,11], (ii) selective detection such as electrochemical detection [12], mass spectrometry [13,14] or (iii) fluorescence detection in combination with post-column derivatisation [15,16].

Another way towards more selectivity is to replace the precolumns by a full-size analytical column. This approach has proved to be successful in several applications in the biomedical field [16–24]. In this paper we will discuss the potential of LC–LC in the field of environmental analysis. The main features of LC–LC are considered in view of the application range of the technique, and moreover, some of the basic approaches to method development using this technique are treated.

2. Coupled-column liquid chromatography (LC–LC)

2.1. General aspects

In comparison to GC-based techniques, reversed-phase liquid chromatography (RPLC) is a more suitable technique for the determination of polar analytes in water, since derivatisation is usually superfluous and in the analysis of aqueous samples the mobile phase system is fully compatible resulting in a high potential for automation. The wide application range, long-term stability, ease of use, low cost and improved selectivity (diode array) makes that UV detection is widely used in residue analysis. However, UV detection does not deliver high sensitivity. Hence, in trace analysis it usually requires on-line or off-line concentration procedures. In environmental analysis by RPLC–UV of e.g. ground water, surface water and soil one is usually confronted with a large excess of polar interferences, e.g. anions and humic and fulvic acids, making the determination of more polar analytes eluting in first part of the chromatogram difficult or impossible. In such cases, selectivity can be improved by using selective sorbents for preconcentration, on-line [5–9] or off-line [25,26] or by the changing the mode of detection, e.g. to mass spectrometry [13,14,27].

As regards to cost effectiveness and speed of analyses, coupled-column RPLC–UV can serve a viable way towards fast and reliable trace analysis of polar organic pollutants in environmental samples.

It is assumed that the principle of LC–LC using heart-cutting is well known; a scheme of LC–LC using the heart-cutting technique is given in Fig. 1. In comparison to automated on-line sample enrichment on precolumns, one of the most favourable aspect of automated sample processing using LC–LC is the utilisation of the separation power of the first column (C-1). Besides the opportunity to enlarge the sample injection volume for the improvement of the sensitivity as used in precolumn technology, it offers the possibility to remove a large excess of early-eluting polar interferences encountered in environmental analysis (removal of S1, see Fig. 1). This pre-separation on C-1 contributes largely to the enhancement of the selectivity. For polar compounds even some multidimensionality caused by a size-exclusion effect can be observed, since a difference in migration speeds of the compounds on the two columns, caused by the ionic strength of the sample, plays a role [28].

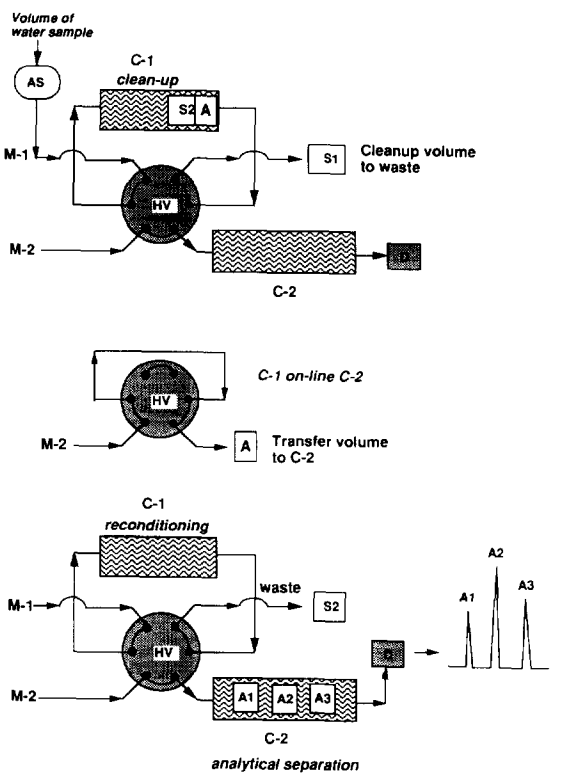

Fig. 1. Schematic presentation of separation procedure involved in coupled-column RPLC. AS = Autosampler; C-1, C-2 = first and second separation column, respectively; M-1, M-2 = mobile phases; S1, S2 = interferences; A = target analytes; HV = high-pressure valve; D = detector.

It should be emphasised that in our approach the forward-flush mode in column switching is advocated. Although sometimes the application of backflush is described [29], this mode has, in the case of polar to moderately polar analytes, two drawbacks: for the most polar compounds it will lead to additional band broadening, while for more retained analytes reversing the flow will diminish the separation obtained earlier in the process.

Another crucial feature of LC–LC is the transfer volume i.e. the time that C-1 is coupled on-line to C-2. In complex samples it is unavoidable that part of the interferences will be transferred together with the analytes. Hence, the attainable selectivity will be determined by (i) the effectiveness of clean-up on C-1 (clean-up volume) and (ii) the volume of the analyte fraction (transfer volume, A) which limits the transfer of S2. In other words, optimal selectivity will be obtained at a minimal transfer volume, while part of the required separation should have taken place before transfer.

The key to success in the enhancement of the selectivity in the analysis of polar analytes in environmental samples is the effective removal of early-eluting interferences. Therefore, the development of multi-residue methods involving larger transfer volumes, which would lead to less selectivity, can still be quite attractive.

Based on previous and current work, the next two sections will be devoted to the applicability of LC–LC for both the assay of a single analyte (single-residue methods, SRMs) and the simultaneous determination of a number of compounds

having a wider range of retention (MRMs). In the latter, computer-aided optimisation procedures will be demonstrated.

2.2. Single-residue methodology

2.2.1. General approach

In the analysis of water samples the application of RPLC-based stationary phases can provide efficient on-line trace enrichment, as has been shown in precolumn switching procedures [2–4]. However, PC–LC using hydrophobic precolumns has its limitations for the enrichment of highly polar analytes, since retention is small, causing the analyte to elute even during large-volume introduction of the aqueous sample. Moreover, these type of precolumns do not provide selectivity because only limited separation capacity is available to separate the analyte(s) from the excess of early-eluting UV-detectable interferences. As indicated in Fig. 1, using the enhanced separation power of a full-size LC column, rather than a short precolumn, coupled-column RPLC–UV can be a viable technique for the direct analysis of very polar analytes in environmental samples. Sensitivity and selectivity depend largely on the RPLC–UV properties of the analyte. In UV detection both selectivity (wavelength, nm) and sensitivity (ϵ, l mol^{-1} cm^{-1}) play a role; the analyte's C_{18} retention is important, since it influences both the maximum tolerable sample injection volume (sensitivity) and the potential for separation between analytes and interferences (selectivity).

In practical method development this leads to two rules of thumb which should be borne in mind in the process of method development:

(i) The clean-up volume, which is the volume of mobile phase M-1 used on column C-1, should at least be twice the dead volume of that column; in practice this means that the capacity factor, k, should be greater than 1.

(ii) The capacity factor of the analyte in the mobile phases M-1 and M-2 should not exceed 5, in order to achieve short times of analysis and good sensitivity.

2.2.2. Applications: water samples

2.2.2.1. Neutral compounds

Early applications of RPLC column-switching procedures were devoted to the determination of three very polar analytes, chloroallyl alcohol (CAAL) [30], ethylenethiourea (ETU) [31], and methylisothiocyanate (MITC) [28]. Direct large-volume injection of these compounds provides detection limits of approx. 1 μg/l. As an example a chromatogram of ETU obtained without sample pretreatment is shown in Fig. 2 indicating clearly the advantages of coupled-column RPLC:

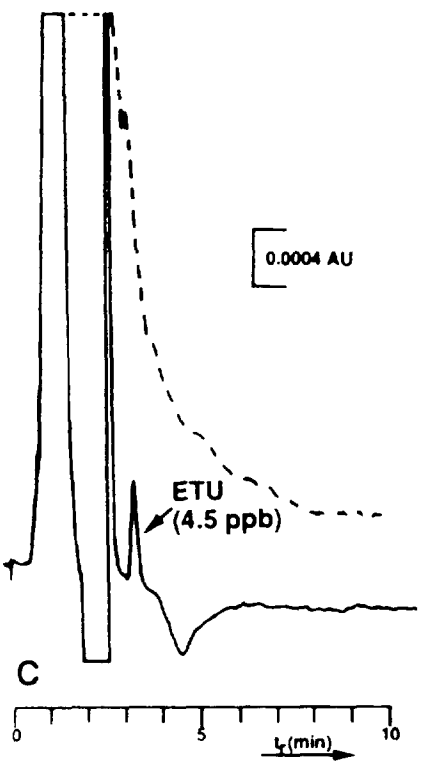

Fig. 2. Direct coupled-column RPLC–UV (232 nm) analysis (200-μl injection) of a spiked ground water sample (ETU, 4.5 μg/l). LC conditions: C-1, 5-μm Hypersil ODS (60 × 4.6 mm I.D.); C-2, 5-μm Hypersil ODS (150 × 4.6 mm I.D.); M-1 and M-2, acetonitrile–0.2% ammonia in water (1:99, v/v) with flows of 1 ml/min; clean-up volume, 1.5 ml; transfer volume, 0.50 ml. Dashed line corresponds to chromatogram obtained using the two columns connected in series without column switching.

improved selectivity and high sample throughput (run times of less than 10 min).

The RPLC–UV characteristics of these highly polar analytes are given in Table 1. As can be seen from these data, all three analytes possess one less-than-optimal parameter for its determination by RPLC–UV. The C_{18} retention of ETU is very poor, the UV detection wavelength of CAAL is non-selective, and MITC has a low UV sensitivity. Nevertheless, direct analysis of ground, surface and drinking water samples for these analytes yields an limit of detection (LOD) of 1 μg/l, indicating that one can handle one unfavourable property and still obtain trace-level sensitivity. By the use of a relatively simple manual concentration steps the LODs can be lowered to the EC drinking-water-limit of 0.1 μg/l [28,30,31]. Unfortunately no uniform sample preparation procedures could be found to enrich these compounds prior to LC–LC analysis. All three analytes represent different problems arising during conventional enrichment procedures. CAAL and MITC are volatile making evaporation steps troublesome. In the case of CAAL the advantages of RPLC were used by applying water as an holder. For MITC a phase switch over a silica solid-phase extraction (SPE) cartridge was used. In the analysis of ETU the main problem was caused by sorption of the compound to glassware during concentration steps. In this case the addition of thiourea as a competitive agent proved to alleviate this problem. A schematic representation of the sample treatment procedures used for ETU, CAAL and MITC are given in Fig. 3.

It can be expected that direct analysis of analytes with more favourable retention and UV properties as the very polar ones mentioned above immediately leads to 0.1 μg/l limits of detection. This assumption was investigated for the analysis of several compounds employing coupled-column RPLC with direct sample injection. Applying the straightforward approach and considerations given in [28,32] methods were developed for methabenzthiozuron and isoproturon within a few days (see Table 1). Both pesticides could be assayed within 10 min with direct sample injection (volumes, see Table 1) at a level of 0.1 μg/l [32]. The high molar extinction coefficients combined with the relatively high C_{18} retention, provides sufficient sensitivity, while the application of small transfer volumes yields the required selectivity. Chromatograms obtained with a rapid RPLC–UV analyser for the direct determination of methabenzthiozuron in surface water are given in Fig. 4.

Numerous samples of ground, surface, rain and drinking water were investigated using the coupled-column methods described above. Especially the ETU method [31] has been used frequently in survey programs monitoring the occurrence of ETU in ground water samples taken from agriculture locations in the Netherlands where dithiocarbamates, from which ETU

Table 1
Relevant information of analytes involving SRMs with direct large-volume injection

Compound	k^a	λ (nm)	ϵ ($l\,mol^{-1}\,cm^{-1}$)	Sample injection volume (ml)
Chloroallyl alcohol (CAAL)	7.0	205	10 000	0.20
Ethyleenthiourea (ETU)	1.6	233	18 000	0.20
Methylisothiocyaant (MITC)	20	237	3 000	0.77
Metabenzthiouzuron	>100	267	14 000	4.0
Isoproturon	>100	240	22 000	4.0
Bentazone	>100	220	25 000	2.0
Clenbuterol	>100	245	7 000	1.5

[a] C_{18} capacitity factor using a 100% aqueous mobile phase.

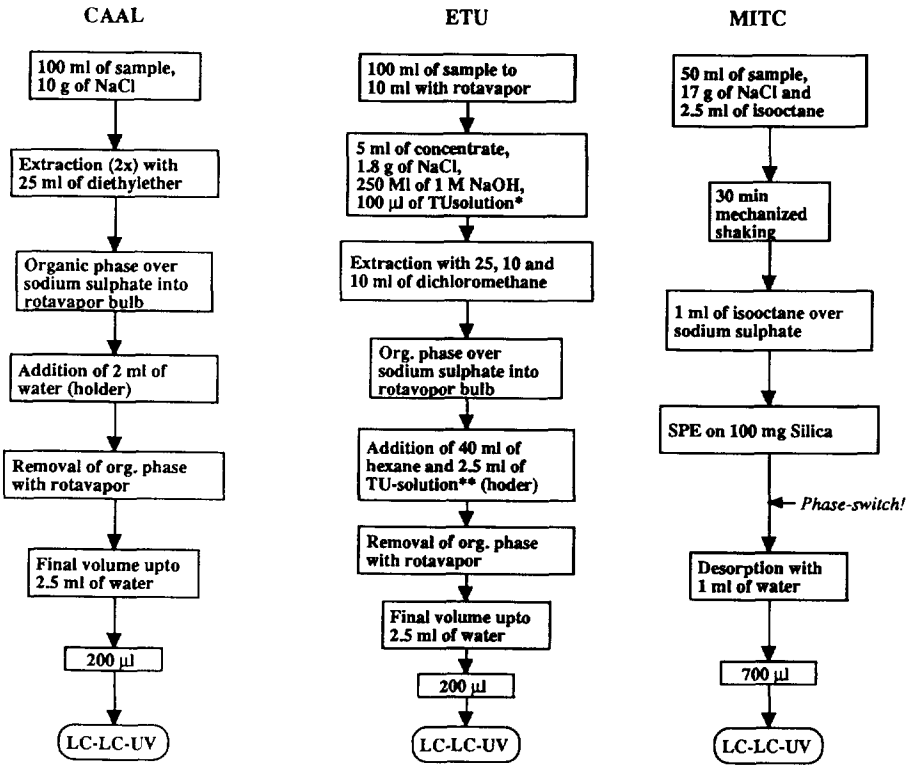

Fig. 3. Schemes of enrichment procedures for the determination of chloroallyl alcohol (CAAL), ethylenethiourea (ETU) and methylisothiocyanate (MITC) in water samples to a level of 0.1 µg/l. *Aqueous thiourea solution of 4 mg/ml. **Aqueous thiourea solution of 1 µg/ml.

is the main metabolite, are intensively used for soil sterilisation. During this period (1992–1994) the method has shown to be very robust during the analysis of more than 1000 samples. The results of positive samples were confirmed with an efficient GC–MS method employing derivatisation [33]. Both methods were in good agreement: a comparison between LC–UV (x) and GC–MS (y) made for 15 positive samples (ETU concentration range: 0.17–1.2 µg/l) provided a correlation of $y = 1.11x - 0.15$ with a correlation coefficient (r) of 0.989.

2.2.2.2. Acidic compounds

The RPLC analysis of acidic compounds may lead to the interference as indicated in Fig. 5; the co-elution of humic acids which show-up in the chromatogram as a broad (tailing) hump, eluting in the same region as the analytes, leads to severe losses in sensitivity. Therefore modifier-based gradients should be avoided since they cause large interfering peaks due to the renewed release of humic and/or fulvic acids from the column each time after a mobile phase change. Gradients based on pH changes are less disadvantageous in this respect. Using this type of gradients, the pH of mobile phase M-1 should be as low as possible in order to get a maximum peak compression of the analyte during sample load and create a maximum applicable pH range for subsequent steps. A pH of 2.3 is about the best one can achieve with alkyl-modified silicas.

An impression of the problems encountered in the development of the coupled-column procedure for bentazone (pK_a 3.2) is shown in Fig. 6. It clearly shows that small changes in the mobile phase composition can have a dramatic impact on the chromatogram. The more or less trial-

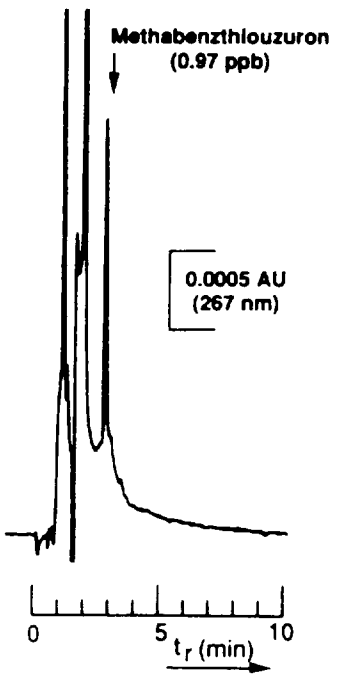

Fig. 4. Direct coupled-column RPLC–UV (267 nm) analysis (4.0 ml injection) of a spiked ground water sample (methabenzthiozuron, 0.97 μg/l). LC conditions: C-1, 3-μm Microspher C_{18} (50 × 4.6 mm I.D.); C-2, 3-μm Microspher C_{18} (100 × 4.6 mm I.D.); M-1 and M-2, acetonitrile–water (45:55, v/v) with flow-rates of 1 ml/min; clean-up volume, 5.9 ml; transfer volume, 0.45 ml.

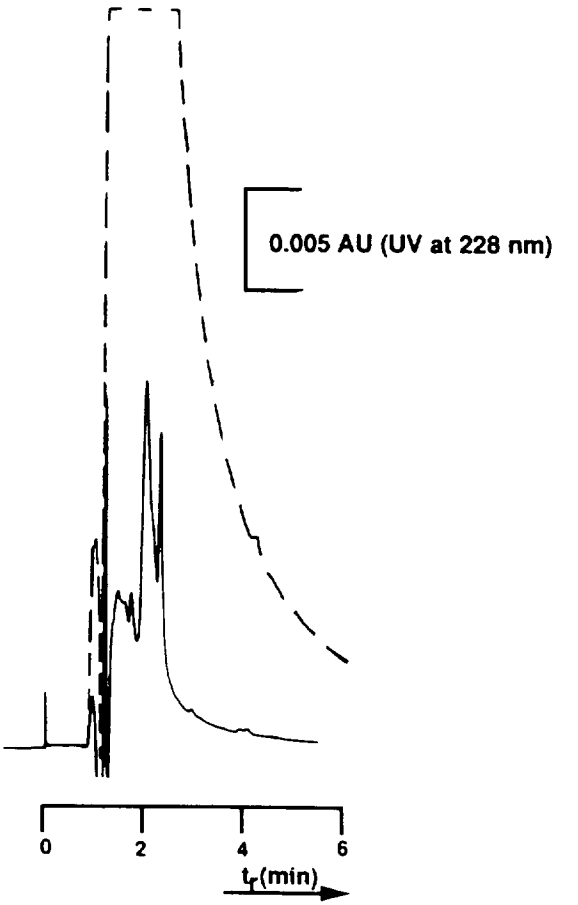

Fig. 5. Two RPLC–UV (228 nm) chromatograms of extracts of a ground water sample obtained after loading of 25 ml of sample adjusted at pH 7 (solid line) and pH 2 (dashed line) on 100-mg SPE C_{18} cartridges. After drying off the 1 ml of acetone used for desorption, the residue is dissolved in 1 ml of 0.05% aqueous trifluoroacetic acid (TFA). Injection of 100 μl of extract on a 3-μm C_{18} column (100 × 4.6 mm I.D.) using a mobile phase of methanol–0.05% TFA in water (60:40; v/v) and a flow-rate of 1 ml/min.

and-error optimisation shows that pH-based gradients (Fig. 6D) are superior over modifier-based gradients in terms of selectivity.

In other application fields pH tuning also proved to be a powerful tool the in the improvement of selectivity for the direct analysis of the β-agonist clenbuterol in samples of urine [34]. In this case the use of a pH gradient, pH 3.6 (in M-1) and pH of 7.5 (in M-2) provides remarkable selectivity. The obtained coupled-column RPLC–UV method using large volume injection (1.5 ml of urine) allowed the detection (UV at 254 nm) of clenbuterol in samples of urine at the ppb level in less than 10 min [34].

The examples discussed above show that coupled-column RPLC–UV can offer fast SRMs for the direct analysis of a wide variety of compounds in environmental water samples. The technique of course has its limitations, for analytes with insufficient UV absorption/selectivity (ε values of less than ca. 1000 and $\lambda < 200$ nm) and/or the absence of C_{18} retention (k value less than ca. 1), the successful development of a column-switching RPLC procedure for trace-level analysis becomes highly unlikely. Nevertheless, even in such cases, advantages of LC–LC, viz. large-volume injection and selectivity, can still be used effectively as has recently been shown for the determination of glufosinate in

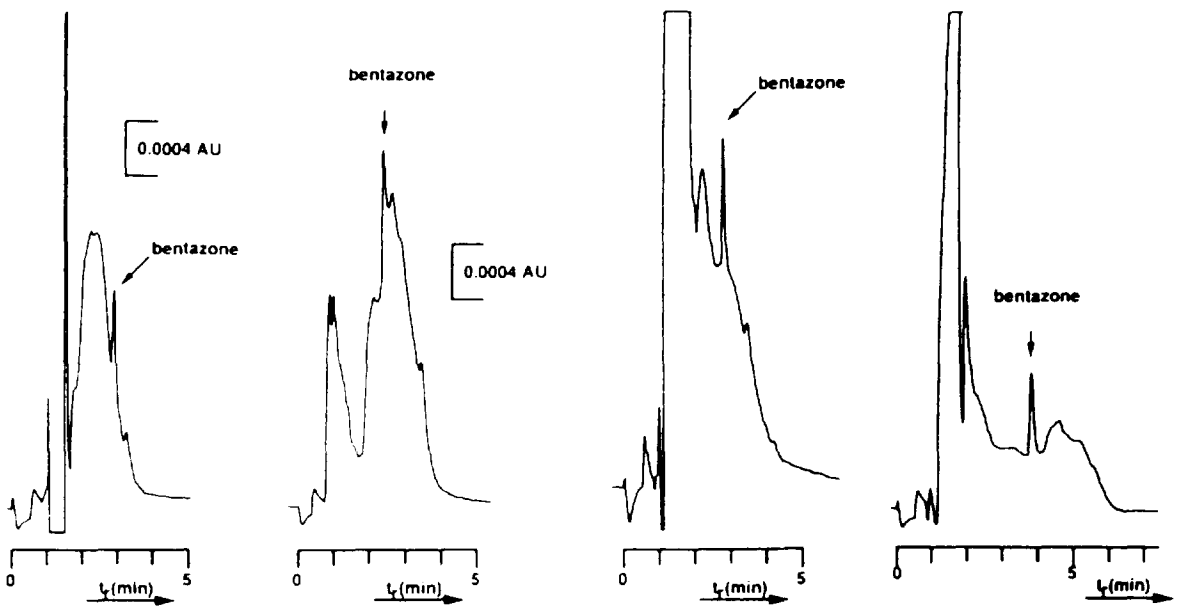

Fig. 6. Selectivity performance of different mobile phase compositions on coupled-column RPLC–UV (220 nm) analysis of a surface water sample containing 0.40 μg/l of bentazone and using direct sample (2.0 ml) injection. LC conditions: cleanup volumes: A, C and D, 4.65 ml and B, 3.75 ml; transfer volumes: A, C and D, 0.50 ml and B, 0.40 ml; columns, see Fig. 4; MeOH = methanol used as modifier in 0.03 *M* phosphate buffer; flow-rates 1 ml/min.

environmental water samples [35]. Glufosinate is an amphoteric analyte (water solubility, 200 g/l at 20°C) with poor UV properties. The application of 9-fluorenylmethoxycarbonyl (FMOC) derivatisation prior to coupled-column LC with fluorescence detection using a C_{18} column (C-1) coupled to an ion-exchange amino column proved to be useful. In this case the C_{18} column provides enrichment of the FMOC-glufosinate during large volume injection (up to 2.0 ml) while the analyte elutes as an almost unretained compound after injection. Under these conditions only a small transfer volume (300 μl) is required. In comparison to the previous coupled-column procedures this approach is somewhat different: beside the application of a second column with a different separation mechanism and fluorescence detection enhancement of selectivity is now obtained by transferring a small unretained fraction. With this procedure glufosinate could be assayed at a level of 0.25 μg/l in various type of water samples with a sample throughput of more than 50 per day. The performance of the procedure is illustrated in Fig. 7, which shows the LC analysis of surface water spiked with glufosinate at the 1.0 μg/l. A study describing the simultaneous determination of glufosinate, glyfosate and its major metabolite aminomethylphosphonic acid (AMPA) in environmental samples is currently underway.

2.2.3. Applications: soil samples

Recently our laboratory attributed more attention to the analysis of pesticides in soil. In this section some preliminary results will be discussed to show the potential of LC–LC in this particular application field. In comparison to water, soil will contain more interferences. Hence, clean-up will be even more important in the analysis of soil extracts, therefore coupled-column RPLC is

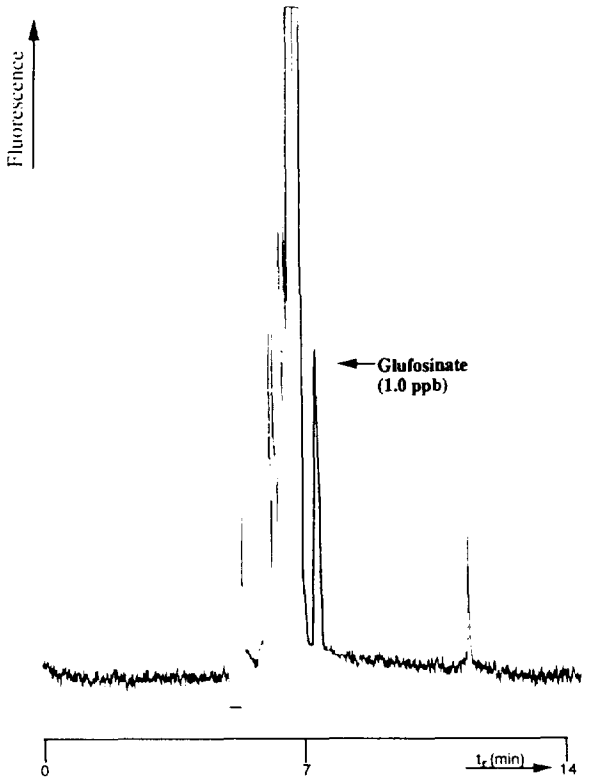

Fig. 7. Column-switching LC–fluorescence detection (λ_{ex} = 263 nm, λ_{em} = 317 nm) with large-volume injection (2.0 ml) of a spiked surface water sample (glufosinate, 1 μg/l) obtained after precolumn derivatisation procedure with FMOC [35]. LC conditions: C-1, 5-μm Nucleosil C_{18} (30 × 4.6 mm I.D.); C-2, 5-μm Adsorbospher NH_2 (250 × 4.6 mm I.D.); M-1, acetonitrile–0.05 *M* phosphate in water, pH 5.5 (35:65, v/v); M-2, acetonitrile–0.1 *M* phosphate in water, pH 5.5 (35:65, v/v); flow-rates 1 ml/min; cleanup volume, 2.25 ml; transfer volume, 0.30 ml.

an attractive technique to perform efficient on-line cleanup. In a monitoring program aimed at the occurrence of the more persistent pesticides, two SRMs were developed for the analysis of the pesticides pencycuron and fenpropimorph in soil. Fenpropimorph is a non-polar compound (large C_{18} retention) with good UV sensitivity (ϵ, 20 000 l mol^{-1} cm^{-1}) but poor UV selectivity (UV maximum at 205 nm). Sample preparation consisted of an overnight standing extraction of 50 g soil with 150 ml of acetonitrile. The extract is poured over a büchner filter and rinsed with about 50 ml of acetonitrile. After volume adjustment to 200 ml with acetonitrile, 100 ml of extract are taken and concentrated to a volume of about 10 ml by means of rotating film evaporation at 40°C. The concentrate is then transferred to a 25-ml volumetric flask and made up by volume with acetonitrile. Prior to LC analysis 2 ml of extract and 2 ml of water are mixed and passed through a 0.20-μm filter. From this solution 100 μl are injected into the LC system. The gain in selectivity provided by coupled-column RPLC is demonstrated in Fig. 8 which shows the analysis of soil spiked with 52 μg/kg of fenpropimorph.

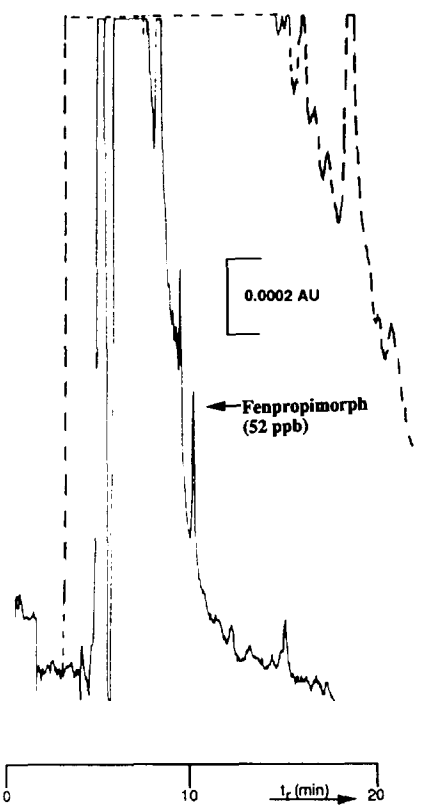

Fig. 8. Coupled-column RPLC–UV (215 nm) analysis of 100 μl of extract of a spiked soil sample (fenpropimorph, 0.052 mg/kg). LC conditions: C-1, 5-μm Hypersil SAS (60 × 4.6 mm I.D.); C-2, 5-μm Hypersil ODS (150 × 4.6 mm I.D.); M-1, acetonitrile–0.5% ammonia in water (50:50, v/v); M-2, acetonitrile–0.5% ammonia in water (90:10, v/v); flow-rates 1 ml/min; clean-up volume, 5.9 ml; transfer volume, 0.45 ml. Dashed line corresponds to chromatogram obtained using the two columns connected in series without column switching.

For the determination of very polar analytes, such as ETU, in soil LC–LC has its limitations. Hence, additional clean-up will be necessary for polar analytes. One can advantageously make use the analytes low C_{18} retention by applying a rapid SPE procedure as was firstly applied to apple juice [36]. In this case the application of SPE is reversed: the C_{18} cartridge traps the less polar components, while the very polar ones start to elute immediately. These analytes are than collected during sampling, thus providing some kind of a polarity cut-off filter. It must be mentioned that after the SPE step, LC–LC is still required to obtain sufficient selectivity. Recently we also applied this SPE approach to the determination of ETU soil samples. ETU was extracted from the soil with water by shaking 5 g of sample with 10 ml of water for 1 h. Fig. 9 shows a typical chromatogram of the ETU analysis in soil extract obtained after SPE indicating that LODs of approx. 0.01 mg/kg are feasible.

2.3. Multi-residue methodology

2.3.1. Introduction

In the SRM approach discussed above the use of small transfer volumes is essential to obtain selectivity and sensitivity. In multi-residue analysis, dealing with more analytes with a wide polarity range, larger transfer volumes will be required. Hence, the attainable selectivity will be lower. However, as discussed earlier, the major source of interferences in environmental samples are usually the more polar compounds. Hence, a major advantage of coupled-column RPLC, the removal of a large excess of early-eluting interferences will also be feasible for MRMs. The effect of widening the transfer window is shown in Fig. 10: enlarging the transfer volume opens the possibility of MRMs but gives away the possibility of optimising selectivity with this parameter.

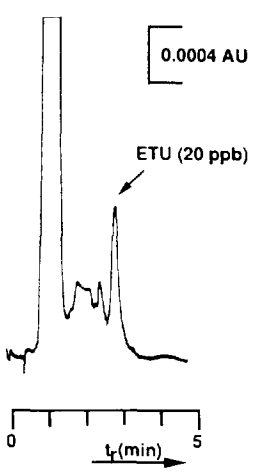

Fig. 9. Coupled-column RPLC–UV (233 nm) analysis of 200 μl of extract of a spiked soil sample (ETU, 0.020 mg/kg). LC conditions: C-1 and C-2, 5-μm Hypersil ODS (150 × 4.6 mm I.D.); M-1 and M-2, acetonitrile–0.2% ammonia in water (1:90, v/v) both with a flow-rate of 1 ml/min; clean-up volume, 2.5 ml; transfer volume, 0.44 ml.

MRMs usually involves the determination of analytes with different detection and retention properties, making the direct injection of aqueous samples in coupled-column RPLC–UV less attractive. Moreover, operational MRMs as applied in monitoring programmes are often applied in combination with GC-based multi-residue methods to be applied on the same sample. In this case it is often more convenient to use a unified extraction/concentration procedure with an organic solvent or (semi) automated SPE, so that the same extract can be used for all MRMs to be applied on a sample.

Several MRMs using coupled-column RPLC have been described. For example, the application of a 15 × 3.2 mm I.D., 7 μm C_{18} column as first separation column was used for the determination of herbicides in well water and maize [37], N-methylcarbamates insecticides in total diet extracts [38] and dinitrophenol pesticides in soil samples [39]. For the determination of a very heterogeneous group of nine pesticides (neutral, basic and acidic analytes) in cereals an MRM was described using a larger column as C-1 (50 × 3 mm I.D., 5 μm C_{18}) and one-step gradient elution profile for the adequate separation of all analytes [40].

Recently coupled-column RPLC–UV was also used for the determination of thirteen low-molecular-mass carbonyl compounds in air [41]. In this application, air sampling is performed on reagent-impregnated C_{18} SPE cartridges. During sampling the corresponding 2,4-dinitrophenylhydrazone derivatives of carbonyl com-

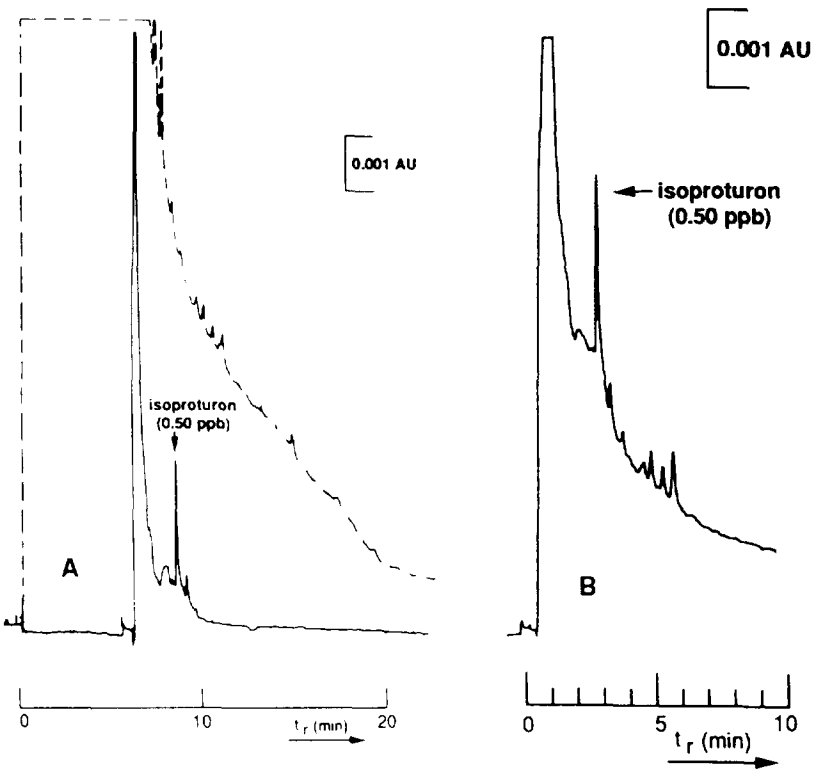

Fig. 10. Selectivity performance of applied cleanup and transfer volume on the coupled-column RPLC–UV (240 nm) analysis of a spiked surface water sample (isoproturon, 0.50 μg/l) using 4.0 ml of direct sample injection. LC conditions: columns as in Fig. 4; M-1 and M-2, acetonitrile–water (47.5:52.5, v/v) both with a flow-rate of 1 ml/min. (A) Cleanup volume of 5.85 ml and transfer volume of 0.45 ml; dashed line corresponds to chromatogram obtained using the two columns connected in series without column switching. (B) Cleanup volume of 5.85 ml and transfer volume of 10 ml.

pounds are formed. The cartridges are processed on-line with an automated SPE system coupled to a reversed-phase LC–LC system. A 3-μm C_{18} column (100 × 4.6 mm I.D.) as C-1 provided separation between the excess of unreacted reagent and the early-eluting analytes. In this set-up sensitivity could be improved by the injection of 2 ml of the sample obtained after desorption from the C_{18} cartridge.

The limitations observed in SRMs for acidic analytes are also encountered in MRMs for this class of compounds. In a study [42] directed to the simultaneous analysis of eight chlorophenoxy acids, dicamba and bentazone in ground and surface water samples it appeared that coupled-column RPLC could not eliminate the broad interference peak arising from co-extracted humic substances (see Fig. 5) obtained from SPE (100 mg C_{18}) extracts of ground and surface water. An additional clean-up over silica SPE cartridges removed an important part of the humps in the chromatogram, but problems were even then still observed in the first part of the chromatogram. The use of an internal surface reversed-phase (ISRP) column as C-1 was able to remove the remainder of the interfering early eluting material. Fig. 11 shows a chromatogram of the analysis of acidic compounds using this material in combination with a one-step gradient.

2.3.2. Systematic approach to method development

Development of MRMs with coupled-column RPLC–UV starts with the selection of initial mobile and stationary phases followed by some

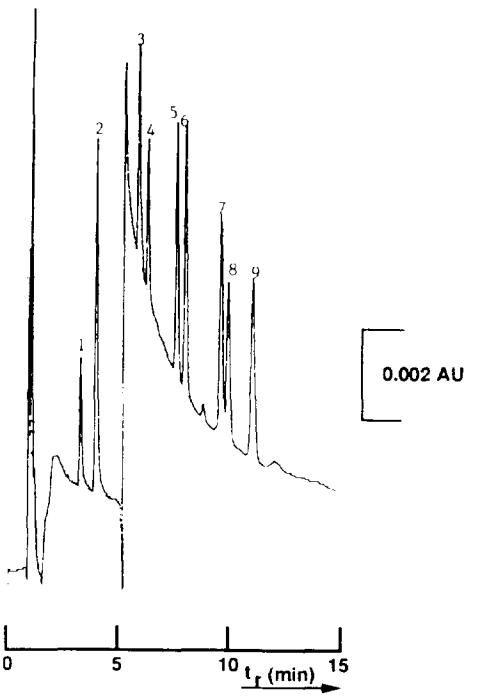

Fig. 11. Column-switching RPLC–UV (228 nm) and step-gradient elution on C-2 of a surface water sample spiked at a level of 0.9 μg/l with acidic herbicides. Injection of 400 μl of extract obtained after SPE sample pretreatment of 25 ml of sample on C_{18} and silica cartridges [42]. LC conditions: C-1, 5-μm ISRP GFF-II (10 × 3 mm I.D.); C-2, 3-μm Microspher C_{18} (100 × 4.6 mm I.D.); M-1, methanol–0.05% TFA in water (5:95, v/v); M-2A, methanol–0.03 *M* phosphate in water, pH 2.5 (50:50, v/v); M-2B, methanol–0.03 *M* phosphate in water, pH 2.5 (60:40, v/v); flow-rates 1 ml/min; clean-up volume, 1.0 ml; transfer volume, 0.50 ml. After transfer, the precolumn was cleaned with 3.0 ml of methanol and a one-step gradient on C-2 was applied consisting of 4.0 ml of M-2A and 11.0 ml of M-2B. Peaks: 1 = dicamba; 2 = bentazone; 3 = (2,4-dichlorophenoxy)acetic acid (2,4-D); 4 = (4-chloro-2-methylphenoxy)acetic acid (MCPA); 5 = 2-(2,4-dichlorophenoxy)propanoic acid (2,4-DP) = dichlorprop; 6 = 2-(4-chloro-2-methylphenoxy)propanoic acid (MCPP) = mecoprop; 7 = 4-(2,4-dichlorophenoxy)butanoic acid (2,4-DB); 8 = 4-(4-chloro-2-methylphenoxy)butanoic acid (MCPB); 9 = 2-(2,4,5-trichlorophenoxy)propionic acid (2,4,5-TP) = fenoprop.

scouting experiments providing detection characteristics of the compounds to be analysed. It must be stressed that from an efficiency point of view, one should not optimise the method beyond a certain degree: the LC system must be kept as simple as possible. For example, if proper LC conditions regarding the time of chromatographic run and the separation between analytes can be easily derived by the chromatographer on the basis of the preliminary experiments, a search for even more optimal separation conditions becomes less attractive. Obviously, if sample extracts are clean enough, column switching can be omitted. In practice, however, most environmental or food samples are rather complex, therefore method development usually requires quite some experimental trial-and-error.

In order to avoid excessive experimental work we developed method development procedures based on computer simulation and empirically established boundary conditions [43–45]. The mobile phase system to be optimised in column switching is a (multi) step-gradient elution over the first column. Step gradient elution can also serve as a good alternative for gradient elution since it shortens the run-time for analytes with large differences in retention [40]. As a first instrument towards the development of dynamic MRMs for pesticides with LC—LC a simulation programme was devised [43]. It was based on a diffusion model, the main objective was an accurate prediction of retention times and peak volumes. As input the program requires experimentally obtained retention data of the target compounds and, if possible, interfering matrix peaks described by the capacity factor k, as a function of the mobile phase composition φ, as well as the total plate number of the column(s). The ln k–φ data were converted to second-order polynomial relations. The program enables the analyst to search for suitable cleanup and transfer conditions in coupled-column RPLC. In this stage, no formal optimisation criteria were formulated and suitable conditions were found step by step with selected elution profiles making the final choice of conditions dependent on the expert judgment of the analyst. Consequently, boundary conditions were formulated for development of MRMs using coupled-column RPLC [44].

Another step forward was the development of a computer spreadsheet for the automated optimisation of step gradient elution conditions utilising optimisation criteria [45]. The procedure is based on the use of derived analytical equa-

tions for the prediction of chromatographic data (retention and peak volume) of analytes eluting under isocratic, one-step and/or two-step gradient elution profiles. Using the same input data as the ones used for the simulation procedure, the spreadsheet calculates for a given set of analytes the maximal resolution of the least efficiently separated pair of peaks in a three-dimensional space defined by the eluotropic strength of the first and second mobile phase and the time at which the step gradient takes place. In comparison to simulation, the developed calculation procedure enables a more rapid search to suitable conditions for on-line cleanup and separation of analytes using coupled-column RPLC and is therefore highly productive and flexible.

In order to explain the steps applied in our method development approach, one application [44] will be discussed in more detail. The RPLC–UV information of the involved compounds given in Table 2 reveals the large differences in polarity.

For these type of analytes the first parameter to be considered is the pH of the mobile phase. Initial experiments revealed that at pH 3.3 fair peak-shapes and reasonable retention could be obtained on a 100 × 4.6 mm I.D. column packed with 3-μm Microspher C_{18}. We prefer these column dimensions, because after optimisation of the separation one can easily apply two 50 × 4.6 mm I.D. columns with the same material to perform column switching.

Table 2
RPLC–UV characteristics of nine polar pesticides

Pesticide	C_{18} retention (k)	λ_{max} (nm)	ϵ_{max} ($l\,mol^{-1}\,cm^{-1}$)
Metamitron	2.4	308	10 000
Bentazone	3.8	220	25 000
Monuron	6.0	244	16 600
Metribuzin	6.6	294	9 200
DNOC	9.2	265	9 500
Diuron	18	244	17 000
Linuron	31	244	17 400
Dinoseb	99	265	8 100
Dinoterb	116	265	8 100

Data experimentally determined on a 3-μm Microspher C_{18} column with a mobile phase of methanol–0.03 M phosphate buffer, pH 2.9 (40:60; v/v) and UV photodiode array detection. DNOC = 4,6-dinitro-o-cresol.

Our approach of method development is visualised in Fig. 12. On the basis of our earlier experience the selection of useful coupled-column separation conditions should include the four criteria given below.

(i) The first-eluting analyte must have a retention which is at least twice as large as that of the unretained compound (similar to SRM approach).

(ii) The total time of the chromatographic run should be kept relatively short (for sensitivity and sample throughput).

(iii) The resolution (R_s) between two adjacent peaks must be at least 1.2 to prevent problems due to UV wavelength switches and/or changes in the mobile phase compositions.

(iv) The number of steps during gradient elution should be minimised to reduce baseline distortions and the complexity of the LC system.

An interpretation of the ln k–φ curves (Fig. 12) can already provide the outlines for the method to be developed. Criteria i and ii largely determine the applicable mobile phase composition(s). In terms of retention, criteria i and ii can simply be looked upon as $1 < k < 10$, therefore more or less determining the eluotropic strengths of the subsequent step gradient. The duration of a single mobile phase composition is governed by criterion iii. On these considerations suitable conditions can be read directly from the ln k–φ plots as indicated in Fig. 12, involving in this case a two-step gradient elution. If necessary optimisation of the gradient elution profile can be supported by computer simulation [43,44].

However, during analysis at low levels it appeared that because of baseline distortions a two-step gradient elution profile was inconvenient, moreover, it also remains unclear whether criterion iv is met. Meeting criteria i and ii, the selection of a suitable one-step gradient elution based on a graphical interpretation becomes less obvious and requires large numbers of chromatogram simulations. Hence, from both a practical and chromatographic point of view optimisation should preferably start with the search of a suitable one-step gradient elution profile, meet-

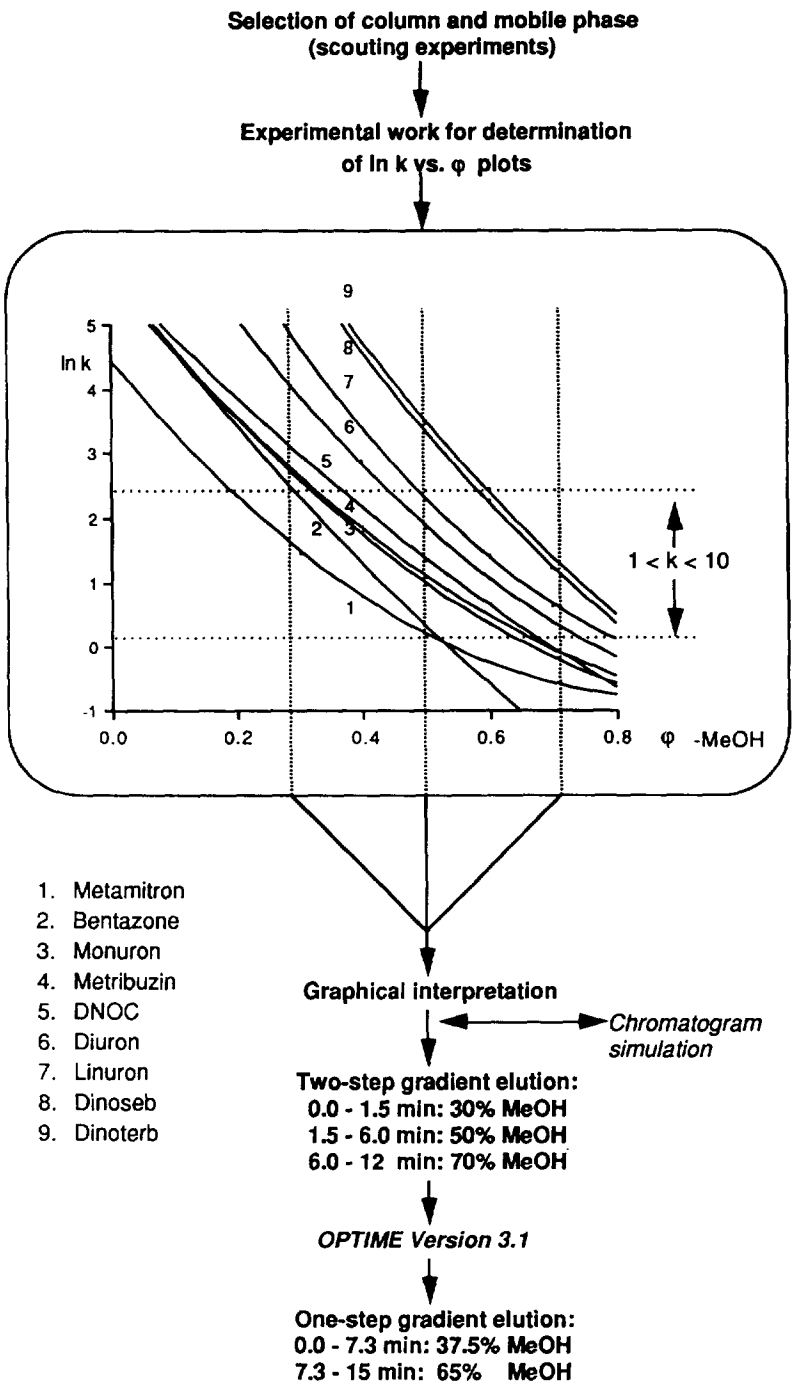

Fig. 12. Scheme of systematic method development. For further explanation, see text.

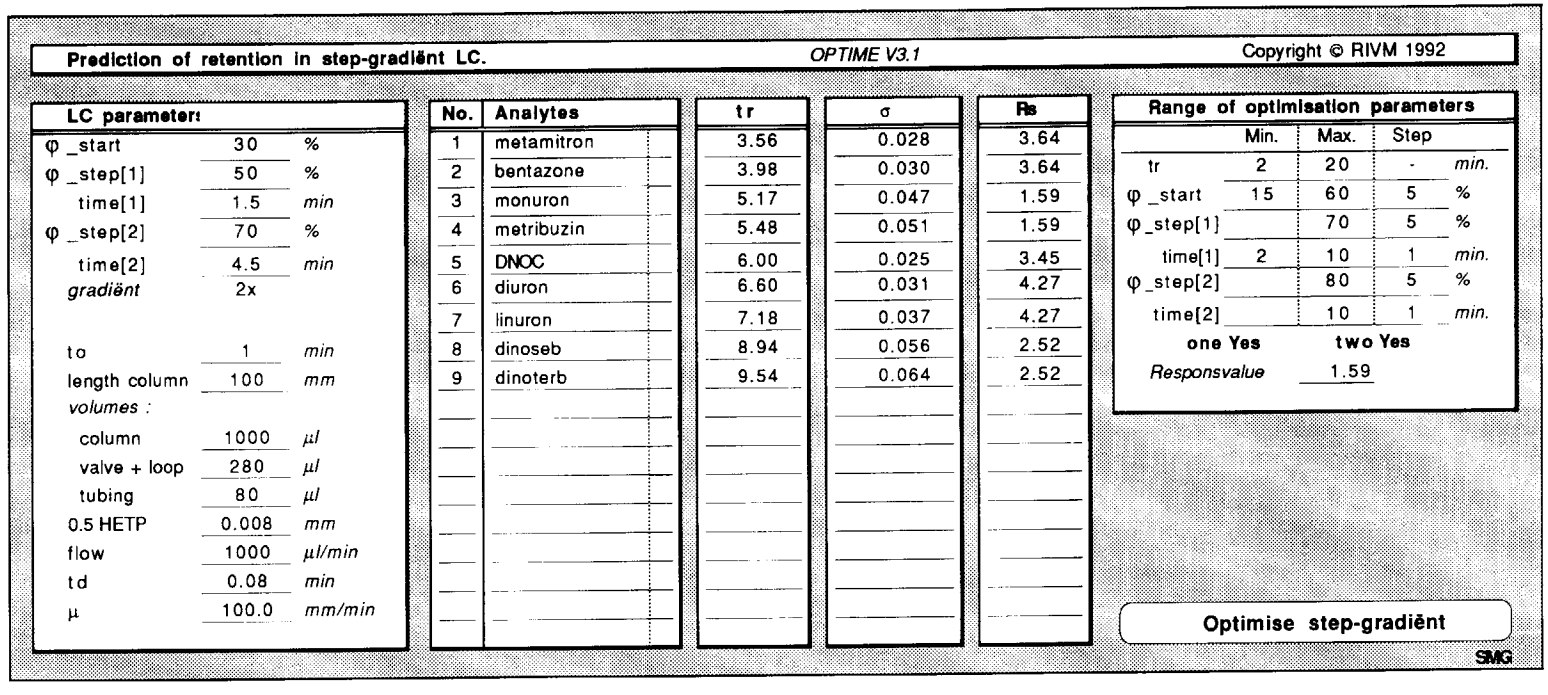

Fig. 13. Display of the in- and output screen of the calculation and optimisation procedure OPTIME version 3.1. For further explanation, see text.

ing criterion iv. As regards this requirement the optimisation procedure described in Ref. [45] is very convenient. An example of the worksheet of the procedure, OPTIME version 3.1, including in- and output ranges, is shown in Fig. 13 The first five columns correspond to the calculation procedure and show the flexibility and speed of this approach: changing one of the LC parameters immediately updates the chromatographic information of the analytes.

The optimisation parameters displayed in the last part of Fig. 13 are described in Table 3. The procedure activated by the Optimise step-gradient compartment displays, after calculation, the found optimum (maximal resolution of the least efficiently separated pair of peaks) as the Response value. On a separated sheet an optimisation report is made, including the best ten maximum responses with corresponding LC conditions and updated retention times.

As regards the production of chromatographic data and optimal separations conditions the results obtained by OPTIME version 3.1 were in good agreement with the simulation procedure and experimental verification [45]. An example of chromatographic performance is given in Table 4, which emphasises the usefulness of this tool in method development.

Very recently [34], OPTIME version 3.1 has been applied to the optimisation of coupled-column RPLC conditions for the direct analysis of eight β-agonists with a wide range in RPLC retention in samples of urine. OPTIME version 3.1 provided easily suitable conditions for the automated processing of urine samples involving (i) on-line cleanup, (ii) a two-step gradient elution profile and (iii) a time of analysis of less than 20 min.

Table 4
Comparison of experimental and calculated (OPTIME version 3.1) chromatographic data for the optimised one-step gradient elution conditions given in Fig. 12

Pesticide	Experimental		Calculated	
	t_r (min)	σ (min)	t_r (min)	σ (min)
Metamitron	3.20	0.060	3.24	0.041
Bentazone	4.70	0.055	4.69	0.059
Monuron	6.80	0.080	6.74	0.085
Metribuzin	7.30	0.085	7.30	0.092
DNOC	8.70	0.030	8.97	0.030
Diuron	9.60	0.045	9.95	0.038
Linuron	10.70	0.050	10.87	0.047
Dinoseb	13.90	0.075	14.04	0.081
Dinoterb	15.00	0.075	15.04	0.097

t_r = Retention time; σ = peak volume at 0.6 of the peak height.

3. Conclusions

It is demonstrated that coupled-column RPLC with UV detection is a versatile tool for the

Table 3
Description of optimisation parameters

Parameter	Description
Input by user	
tr – min	Minimal time of the first-eluting analyte (clean-up)
tr – max	Maximal time of the last-eluting analyte
a, b, c	Coefficients of second order ln k vs. φ plots
Range by user, optimum by OPTIME version 3.1	
φ – start	Minimum and maximum % MeOH of mobile phase 1
φ – step[1]	Minimum and maximum % MeOH of mobile phase 2
time[1]	Minimum and maximum time of the solvent switch 1
φ – step[2]	Minimum and maximum % MeOH mobile phase 3
time[2]	Maximum time of solvent switch 2

determination of polar and moderately polar organic micropollutants in environmental samples. In aqueous samples sub-μg/l detection limits are easily attainable. Coupled-column RPLC covers a wide range of pollutants to be determined in both multi- and single-residue methods. A systematic approach to method development renders the technique to be an important means in the operation of flexible monitoring programmes.

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