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A Systematic Approach for the Development of Liquid Chromatographic Methods

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Abstract:
Developing chromatographic methods can be laborious, time-consuming, and expensive. The definition of a strategy to find rapid separation conditions has therefore become of prime importance since the analytical development has to follow the rhythm of the generation of new compounds. To meet this demand, chemists clearly need the utilization of automation and standardized strategies of work. This paper reports a standardized and fully automated approach dedicated to the development of liquid chromatographic (LC) methods, which are widely used to provide information about chemical reactions, such as impurity profiles and structure or potency determinations. The present approach consists of automatically screening samples with predefined chromatographic conditions. Chromatography is performed under the gradient mode, and the whole dataset is generated in less than 1 day. Even if the screening does not directly provide the final conditions for the separation of the compounds of interest, it generally gives sufficient information to allow a rapid optimization of the chromatographic separation (typically less than 24 h). Different examples of development of LC methods using this strategy are presented.

Introduction

In the pharmaceutical industry, analytical chemistry is of prime importance in the development of a new drug since the different decisions, at all the considered steps of the development, are taken on the basis of results generated by one or several analytical methods. This is particularly true in the field of process chemistry. Indeed, at each step of the chemical development, synthesized molecules are subjected to very sophisticated analyses that are of prime importance since they provide the process chemist with information about the chemical reaction. Impurity profile and potency determinations are generally necessary to better understand the chemical processes. Moreover, analytical information is always essential to study the scaling-up of chemical processes. Finally, the characterization of impurities in drugs is also a very important control that needs to be performed in order to guarantee a high level of quality.

Presently, liquid chromatography (LC) is probably the most frequently used analytical technique for in-process control. However, developing LC methods can be very time-consuming since the mixtures of compounds can be very difficult to separate, especially when these compounds present similar molecular structures as is the case in process chemistry. It is therefore important to have available some robust and rapid LC methods. Different technical possibilities to reduce time, such as the utilization of short length columns, monolithic columns associated with high flow rates, or even very high-pressure liquid chromatography, are available. However, these approaches will reduce the analysis time but have less impact on the time needed to develop the method. A possible way to reduce the time dedicated to method development can be the utilization of a standardized approach in which several columns are screened for their separation efficiency and selectivity[1,2] consisting of automatically injecting compounds or mixtures of compounds to separate. Testing these samples on standardized stationary phases, using preselected mobile phases and gradients, will therefore provide enough information to optimize the separation if it is necessary.

The objective of this paper is to describe and demonstrate that a standardized and automated approach can be used to rapidly develop LC methods (typically less than 1 day) dedicated to the analysis of samples generated in process chemistry and subsequently isolate impurities and reference materials from early development batches.

Materials and Methods

The study was conducted using a liquid chromatograph (Alliance 2695, Waters, Milford, MA) equipped with an autosampler and a photodiode array detector (2996 PDA detector, Waters). The detection was carried out in the present work at 220 nm, and the temperature of the column was kept at 40 °C. The flow rate was set to 1.2 mL/min. A column and solvent switching valves (Waters) were added to the liquid chromatograph in order to have an automated selection of column and mobile phase. The system, coupled to a solvent selector, allows choosing automatically which column and which solvent can be connected to the LC system by simply rotating the column and solvent selection valves.

For the isolation of compounds, a FractionLynx UV with a 2767 Sample Manager, a 2525 binary gradient module, an XP Workstation, and a PDA detector M2996, all from Waters, were used.

The different analytical columns used were a Symmetry shield RP8, an Atlantis dC18, both from Waters and a Zorbax Extend C18 from Agilent Technologies (Palo Alto, CA). The dimensions of all columns were 100 mm × 4.6 mm i.d (3.5

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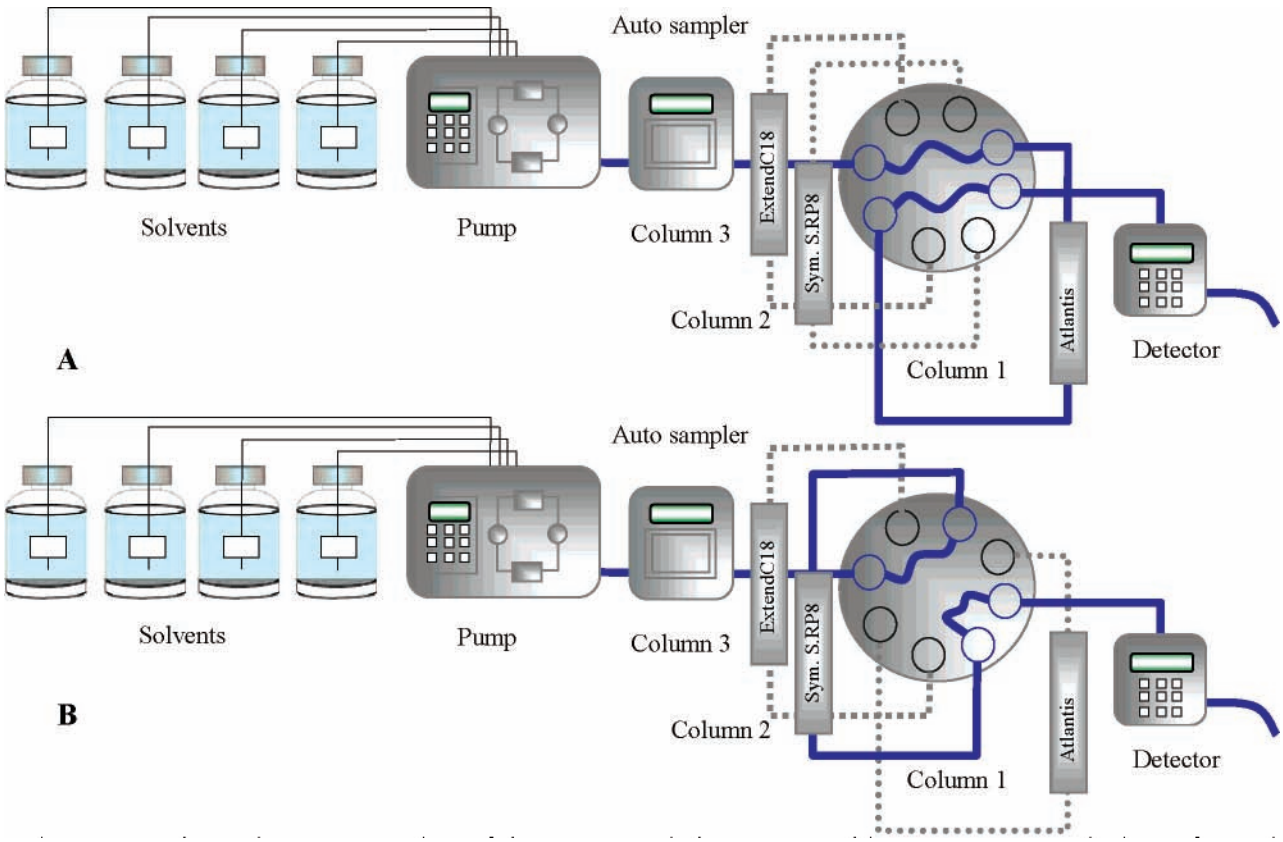

Figure 1. **Schematic representation of the automated chromatographic system. (A) Analysis performed on column 1. (B) Analysis performed on column 2.**

μm). For the preparative chromatography the same columns were used with the preparative equipment.

For preliminary experiments, seven other stationary phases were used: XTerra MS-C18, XTerra RP8, and Sunfire C18, all from Waters were 100 mm $\times$ 4.6 mm i.d. (3.5 μm). Interchim HSC, ODB, and NEC columns were also 100 mm $\times$ 4.6 mm i.d. (3.0 μm) from Interchim (Montluçon, France). The Zorbax CN from Agilent Technologies was the only stationary phase with a 150 mm length and 4.6 mm i.d (5.0 μm).

Methanol Ultra HPLC grade and acetonitrile Ultra HPLC grade, both from J.T. Baker (Deventer, Holland), were used as organic modifiers. Trifluoroacetic acid (TFA, J.T. Baker) and ammonium acetate (Merck, Darmstadt, Germany) were used to prepare pH 2.0 and pH 6.8 mobile phases, respectively. The LC experiments were carried out using a linear gradient ranging from 5% to 95% of organic modifier in 10 min. Including the re-equilibration time needed before a second injection, the total run analysis was 16 min.

Results and Discussion

The objective of the standardized approach is to test the compounds to separate or a mixture of these compounds in different chromatographic conditions in order to obtain different separations. To be efficient, the approach should generate highly different chromatograms, allowing selection of the best chromatographic conditions. Moreover, this testing step should be as rapid as possible, in order to reduce the time of development and costs and to increase productivity. It should also be automated in order to be run without human oversight. The most frequently used parameters to modify for developing a new chromatographic method are the nature of the stationary phase, the composition of the mobile phase (pH and organic modifier), and the gradient mode.

The selection of the stationary phases to include in the standardized approach was performed on the basis of a wide set of experiments, including 10 stationary phases with different characteristics. Many of different compounds (acidic, basic, and neutral) were tested on these columns, and the selection was made by considering the stability and the efficiency of the columns, their ability to give reproducible results, and their ability to generate high differences in retention times (RT) when parameters are changed. The 10 columns tested on the preliminary study were selected regarding their stationary phase characteristics. Different stationary phases such as C18, C8, or CN were used in order to observe their influence on the retention times of a wide range of compounds with different structures and physicochemical characteristics, i.e., different pK_a, log P. For confidential reasons and since these compounds correspond to intermediates and API in a very early stage of development, any structures, even partially, cannot be shown. Numerous chromatograms corresponding to the different chromatographic conditions tested and to the different columns were collected. After having eliminated the columns that have given some chromatographic problems such as repeatability during the screening or stability of the stationary phase, the data were used to compare the ability of each column to give differences in retention times. A statistical

Table 1. **Number of Peaks Observed in the Different Conditions Tested[a]**

	pH 2. 0 MeCN	pH 2.0 MeOH	pH 6.8 MeCN	pH 6.8 MeOH
I	4	6	5	6
II	6	4	5	4
III	5	6	7	6

[a] Linear gradient: 5 to 95% of organic modifier in 10 min, flow rate 1.2 mL/min, UV detection at 220 nm, seven compounds in the sample mixture. Column I: Atlantis dC18 100 mm × 4.6 mm i.d. (3.5 μm). Column II: Symmetry Shield RP8 100 mm × 4.6 mm i.d. (3.5 μm). Column III: Zorbax Extend C18 100 mm × 4.6 mm i.d. (3.5 μm).

Table 2. **Number of Peaks Observed in the Different Conditions Tested[a]**

	pH 2. 0 MeCN	pH 2.0 MeOH	pH 6.8 MeCN	pH 6.8 MeOH
I	3	2	2	2
II	2	3	2	2
III	3	2	2	2

[a] Linear gradient: 5 to 95% of organic modifier in 10 min, flow rate 1.2 mL/min, UV at 220 nm, three compounds in the sample. Column I: Atlantis dC18 100 mm × 4.6 mm i.d. (3.5 μm). Column II: Symmetry Shield RP8 100 mm × 4.6 mm i.d. (3.5 μm). Column III : Zorbax Extend C18 100 mm × 4.6 mm i.d. (3.5 μm).

study was conducted to regroup the columns presenting similar behaviors. The XTerra MS-C18 and the Extend C18 were found to provide similar results, Atlantis dC18 and Sunfire C18 seemed to have similar influences on retention times, and the Symmetry Shield RP8 was found sufficiently different from the two pairs cited before. Some repeatability issues were observed when using the Zorbax CN, while some stability issues were observed with the 3 HSC, ODB, and NEC columns. The XTerra RP8 was also discarded because of the low efficiency observed in chromatograms generated with this stationary phase:[3] Finally, Atlantis dC18, Symmetry Shield RP8, and Zorbax Extend C18 were selected to be used in the standardized screening. The Atlantis dC18 column is known to be compatible with 100% aqueous mobile phases and is particularly well suited for polar compounds. Symmetry Shield RP8 has a C8 embedded polar group, offering a different selectivity than that of other C8 stationary phases, and finally the Zorbax Extend C18 was the third column selected.

A 10 mM ammonium acetate buffer (pH 6.8) and a 0.1% v/v solution of trifluoroacetic acid (pH 2.0) were selected in order to cover a sufficient range of pH to take advantage of a possible ionization of the compounds to analyze. The maximum limit of pH was set to 6.8 since Atlantis dC18 and Symmetry Shield RP8 stationary phases may be damaged at higher pH values. Since methanol and acetonitrile are the most frequently used organic modifiers and since they are known to offer differences in selectivity, they were both included in the screening. Finally, only one linear gradient with the organic modifier concentration ranging from 5 to 95% in 10 min was selected for the standardized approach. Indeed, it was observed from previous experiments[3] that, even if it can be improved when using a longer gradient (i.e., 30 min instead of 10 min), the separation is generally obtained when a shorter gradient (10 min) is used. The information gathered is usually enough to select initial conditions that possibly need to be optimized.

The standardized approach dedicated to the development of LC methods consists in systematically testing the mixture of compounds on three stationary phases, with two mobile phase pH's, two organic modifiers, and one gradient mode. This represents 12 experiments. These 12 experiments are

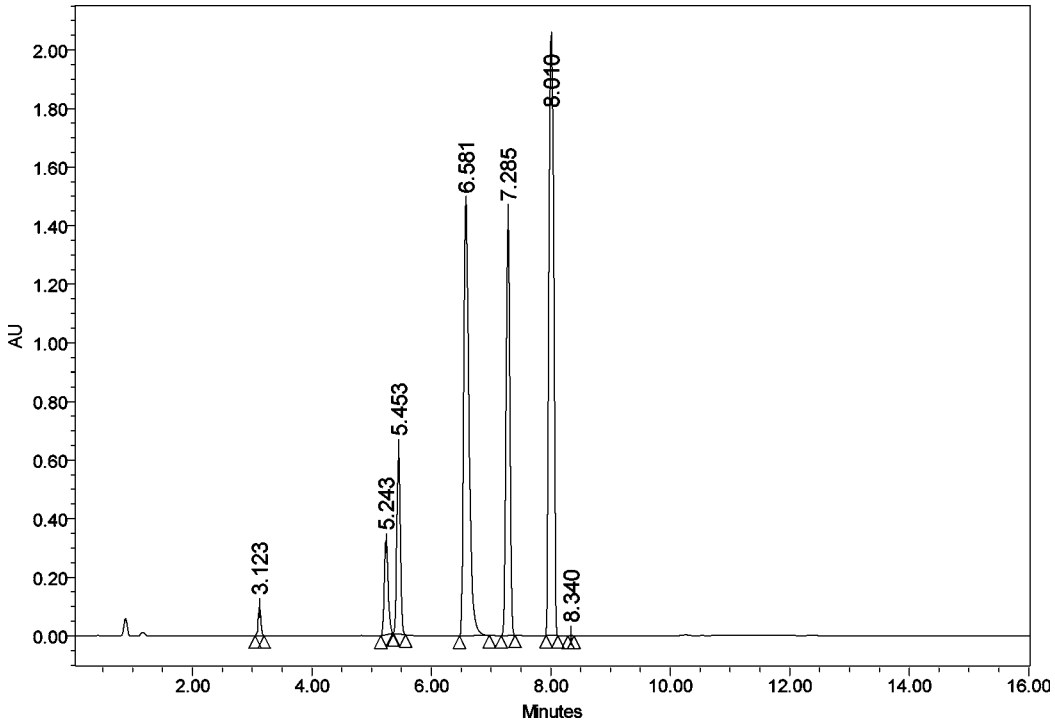

Figure 2. **Chromatogram illustrating the separation of seven compounds on a Zorbax Extend C18 column (150 mm × 4.6 mm i.d., 3.5 μm), pH 6.8, gradient 5 to 95% of acetonitrile in 10 min, UV at 220 nm, flow rate 1.2 mL/min.**

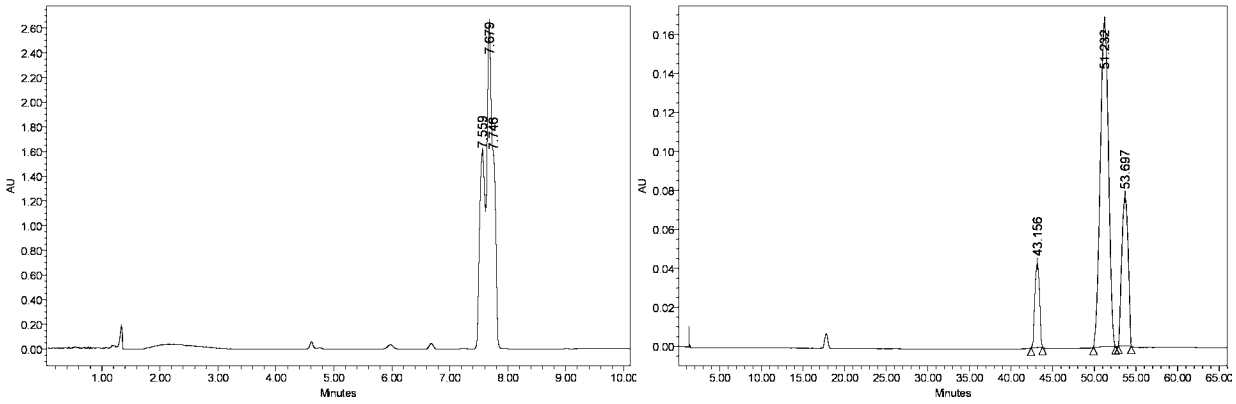

Figure 3. **Chromatograms illustrating the separation of three position isomers on a Symmetry Shield RP8 column (150 mm × 4.6 mm i.d., 3.5 μm). (A) Screening result: pH 2.0, 10 min linear gradient 5 to 95% for methanol, UV at 220 nm, flow rate 1.2 mL/min. (B) After optimization: pH 2.0, 60 min linear gradient 5 to 15% for methanol, UV at 220 nm, flow rate 1.2 mL/min.**

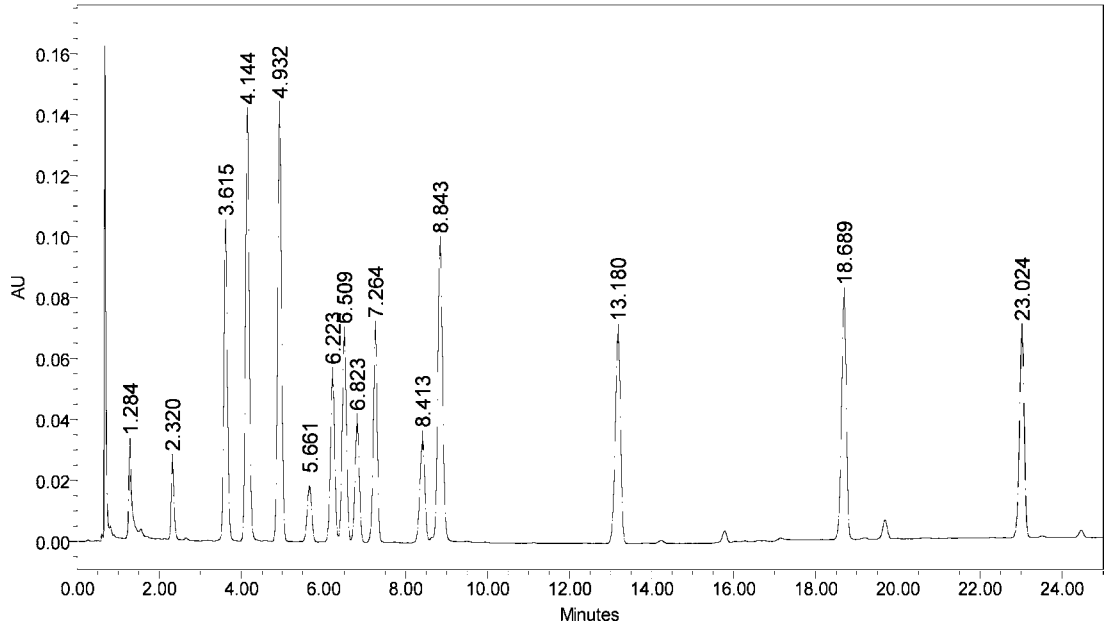

Figure 4. **Chromatogram illustrating the separation of 15 compounds on a Symmetry Shield RP8 column (150 mm × 4.6 mm i.d., 3.5 μm), pH 6.8, linear gradient 5 to 95% in acetonitrile in 30 min, UV at 235 nm, flow rate 1.2 mL/min.**

performed in an automatic way by using one autosampler for the injection of samples and two automatic switching valves for selecting mobile and stationary phases. 12 different chromatograms are therefore obtained, and the best one, i.e., the one that gave the best separation, can be selected. If it is necessary, the chromatographic conditions can be optimized in order to improve the LC separation. 10 min are needed to perform the analysis, and 6 min, to re-equilibrate the column and prepare for a second injection. A chromatogram is therefore generated every 16 min and, a complete screening for one sample is performed in a little bit more than 3 h.

Figure 1A illustrates the functioning of the selection of the column. The quaternary LC pump selects the suitable solvents to run the desired gradient, and the sample is injected on column 1 (Atlantis). The different combinations of buffers (0.1% TFA solution or 10 mM ammonium acetate pH 6.8) and solvent (acetonitrile and methanol) are successively tested on the first column. When all combinations are tested, the column selection valve is automatically switched to analyze samples on column 2 (Figure 1B). In the same way, column 3 is switched when all experiments are completed on column 2. It must be noted that washing mobile phases and periods of equilibration are necessary when chromatographic conditions are changed.

This standardized approach was used to develop new LC methods for generating analytical information about the actual chemical synthesis of new active pharmaceutical ingredients (APIs), including reagents, reaction products, and byproducts.

The first example consists of the separation of seven compounds, all involved in a two-step synthesis reaction. Table 1 illustrates the results of the standardized approach in terms of the number of peaks observed in the chromatograms generated in the different conditions.

As can be shown in Table 1, the standardized approach gives a very rapid estimation of what could be the best chromatographic conditions for the separation of the different compounds. Several tested conditions offered a six-peak

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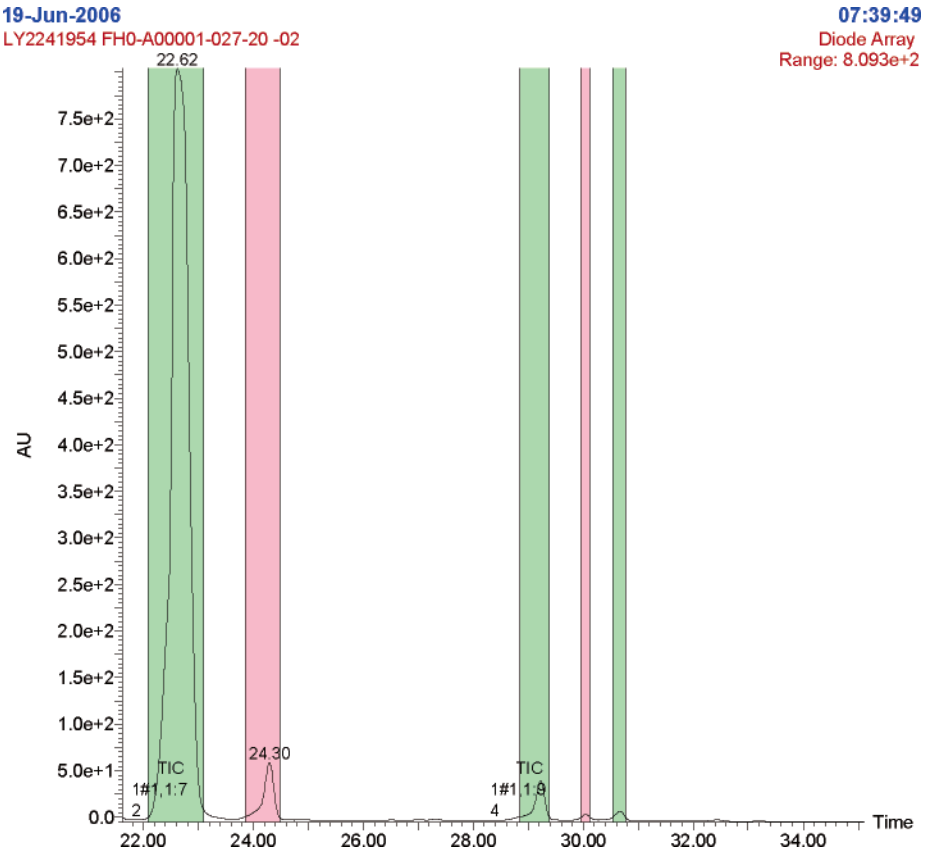

Figure 5. **A typical chromatogram from a preparative run in the Lynx fraction collector with the use of a PDA detector as used in the LC screening system.**

separation, meaning that two compounds coeluted. However, when using a Zorbax Extend C18 and an acetonitrile and pH 6.8 mobile phase, it is possible to obtain the separation of the seven peaks. Figure 2 illustrates the chromatogram of the separation obtained using the developed approach. The resolution between the different peaks is complete, and these conditions can be considered as satisfactory and directly usable.

The systematic approach was also used for developing an LC method to separate some positional isomers. The separation of positional isomers is frequently considered a difficult problem to solve in chromatography, and in the present study, the ortho, meta, and para isomers were used to evaluate the screening. As shown in Table 2, the separation obtained using pH 6.8 mobile phases never presents three peaks, regardless of the stationary phase or the organic modifier used. However, using the acidic (pH 2.0) mobile phase, the three peaks were observed on the three stationary phases tested but the separations observed were not completed and needed a subsequent optimization.

The chromatograms illustrated in Figure 3A illustrate the separation obtained on the Symmetry Shield RP8 column under the screening conditions. Even though the three compounds are visible, the conditions are not usable since the three peaks show a significant overlap. However, as previously mentioned, the main objective of the standardized approach consists of discovering chromatographic conditions that separate, even partially, the compounds of interest. With this information, i.e., the mobile phase composition and the column, it is therefore relatively easy to optimize the separation by modifying the gradient slope or the temperature. Starting from the results generated during the screening approach, the optimization of the separation was performed by changing the gradient parameters. The methanol concentration gradient was first set to 5 to 95% in 60 min, giving rise to a better but not complete separation. A second gradient with methanol concentration ranging from 5 to 50% in 60 min was then tested. Finally, a complete separation of the three isomers was obtained by reducing the final methanol concentration from 50 to 15%, the gradient time being kept at 60 min. Resolutions of 5.5 between the first and second peaks and 1.5 between the second and third peaks (Figure 3B) were observed. After the standardized approach has been run, only three additional steps were needed to obtain a complete and optimized separation.

The same standardized approach was used to develop an LC method dedicated to the support of a nine-step chemical synthesis of an API. The number of compounds involved in this synthesis was 15. The first tests were performed using the screening approach, and considering the results obtained, we selected the Symmetry Shield RP8 as the most suitable stationary phase when used with an ammonium acetate pH 6.8 mobile phase combined with acetonitrile. The gradient initially tested was a 5 to 95% linear gradient in 10 min. However, as the separation was not complete, a further optimization was conducted by modifying the gradient. The same linear gradient, from 5 to 95% in acetonitrile, was tested, first in 20 min and then within 30 min. By modifying only this parameter and using a quick two-step optimization, it was possible to obtain the separation illustrated in Figure

4. 15 compounds are completely separated, providing analytical information over the whole synthesis using a single LC method.

Moreover, it must be noted that this application may also be used for analyzing complex mixtures containing an unknown amount of compounds. Indeed, running a chemical synthesis can generate some unwanted reaction products such as byproducts or untypical impurities. Analyzing those types of samples with the standardized approach can be a very instructive way to know more about the efficiency of the chemical reaction studied.

The method was also found to be very useful for the analysis of the first screening reaction mixtures of a new project. This screening gives a good overview of the impurity profile of the different reaction screening mixtures. Additionally, the use of the best LC screening conditions for the isolation of the products from the best reaction conditions gives an early and very useful insight into the structure of the different products obtained from the reaction.

To efficiently do this the best analytical method is transferred to the Fraction Lynx Collector (Waters), which is then operated with the same column material in the corresponding preparative equipment.

The crude product mixture was inserted as a sample, and the best LC method is programmed as a new method in the Inlet Method. The instrument is programmed to collect the different peaks in the chromatogram in different fractions. It is easily programmable to avoid the isolation of unwanted peaks or solvent in between peaks. Figure 5 illustrates a chromatogram obtained during the isolation of some materials coming from a reaction mixture. The isolated peaks are indicated with colored segments alternating green and red. The first green segment corresponds to the isolation of the reference material. In this particular case we were able to isolate 150 mg of material. The second peak is unreacted starting material, and the following three are impurities.

This screening approach is very simple to set up and does not necessitate complex equipment. The minimum configuration needed only consists of a complete LC system (pump, autosampler, and UV or ideally photodiode array detector), two automatic switching valves for the selection of columns and mobile phases, and the suitable software for acquiring chromatograms. If an LC system is available, the major investment consists of acquiring one automatic column switching valve (ca. $7000) and one automatic solvent selector (ca. $3500). The fraction collector (ca. $90 000) may be very useful in collecting material such as impurities or byproducts that could be difficult to isolate, which certainly provides a lot of information about the studied chemistry.

The setup of the screening consists essentially of defining the gradient slopes and the switching times for the selection of stationary phases and mobile phases, which is very simple to achieve by means of any LC software. On the other hand, when mobile phases and samples are prepared, the system can run without oversight 24 h per day. Human intervention is only requested for starting the screening and for interpreting the data.

This means that for a low financial investment—all chemistry laboratories are generally equipped with an LC system—a lot of time can be saved by running this systematic screening to develop suitable LC methods and the risk of missing hidden impurities can be reduced. Finally, it must be noted that if this approach was developed for providing analytical support to organic chemists, it should be useful for any process research laboratory involved in analyzing a large number of samples.

Conclusion

An automated approach was developed in order to standardize and reduce the time needed for the development of LC methods. The main objective of this screening approach consists of generating information about the separation of compounds in a given mixture. From that point, the subsequent optimization of the separation can be performed, if necessary, in order to achieve a complete separation that can be used to generate analytical data and/or to isolate the compounds. The screening is based on the utilization of three stationary phases, two mobile phase pH's, and two organic modifiers. Chromatography is performed under a linear gradient mode in 10 min. The screening may not directly provide the final conditions for the separation of the compounds of interest but generally provides sufficient information to allow optimization of the chromatographic separation. In the present study, a complex mixture of 15 compounds was resolved in only two additional steps after the completion of the screening. This development was conducted in less than 24 h. Moreover, this application may also be used in order to generate information about the quality of a chemical reaction since it is important to know if impurities or byproducts are generated. Finally, it must be mentioned that this automated methodology not only saves time for the analytical chemist but also gives the route selection organic chemist an easy tool to develop LC methods on his own. It also generates valuable information from the first screening experiments concerning structures of impurities and reference material for quantitative analysis.

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