Automation and Validation of HPLC-Systems

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Key Words

Automation
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System suitability test ruggedness
Method transfer

Summary

The automation of chromatographic systems is of increasing interest to industry and research laboratories in routine applications. Besides potentially saving time or making better use of available instrumentation, automation also improves the quality of results by producing more precise and more reproducible HPLC data. The need for the validation of methods and qualification of instruments is increasingly recognised in order to ensure compliance with legal requirements (e.g. in the pharmaceutical industry) and to ensure the reliability of analytical results. Possibilities and requirements for automated HPLC systems are elaborated. Emphasis is placed on defining the goals of validation and on discussing different aspects of the validation of LC methods, system suitability tests, ruggedness of methods and the transfer of LC methods from laboratory to laboratory. Adequate strategies of HPLC method development provide very useful information on the validation and ruggedness of LC methods.

Introduction

During the last ten years, HPLC emerged as a generally accepted analytical tool for very broad areas of application. A prerequisite for such a breakthrough was the possibility of automating HPLC systems. Automated HPLC systems are used worldwide for routine applications at universities, in industry and in governmental laboratories. Wherever HPLC is used as a quality control tool, e.g. for release analysis of pharmaceutical preparations, the validation of HPLC methods is absolutely necessary. Validation is not only necessary for fulfilling governmental regulations, but is a vital task in serious analytical laboratories, where high quality work in research, development and quality assurance is performed. This paper will discuss applications of automated HPLC, its possibilities and limitations, as well as trends for future development. It will also discuss validation and show that validation is not just an administrative necessity performed for regulatory agencies, but is an extremely demanding scientific task for all chromatographers.

Automation

The goals of automation are:

- to save labour, time and money;
- to make better use of expensive instrumentation during nights and weekends;
- to achieve higher analysis output;
- to achieve higher quality results compared to manually operated HPLC systems.

Automation can be achieved by the mechanisation of sample handling and by data acquisition and processing via computer systems.

Mechanisation: Very early in the development of HPLC instrumentation, manual injectors were replaced by automatic sample injectors to allow for unattended operation and to achieve a higher potential for reproducibility of the injection process. Modern autosamplers can inject even small sample volumes without significant loss, with good precision and adequate reproducibility. More sophisticated samplers are able to inject variable amounts, dilute the sample prior to injection and even perform precolumn derivatisation [1]. In the past, autosamplers were successfully connected to continous flow systems (autoanalyser) [2] for online sample preparation. More complex sample handling can be done by laboratory robots [3]. The possibilities for utilizing laboratory robots for sample preparation in HPLC are collected in Table I. There are, however, some limita-

Table I. Unit operation of laboratory robots

Possibilities of Laboratory Robots for sample preparation in HPLC
Unit operations
inject into HPLC
dillute
add solvents/reagents
solvent extractions
filtration
weighing

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tions to be considered in the application of currently available robot systems:

- They are rather slow in operation;
- method development and the change from one method to another can be very time consuming;
- they use an enormous amount of laboratory space;
- there are still some limitations in the processing of powder samples;
- the installation cost of presently available robot systems is rather high, and the versatility and flexibility of the systems should be further improved.

Mechanisation is not always easy and probably has reached its limits. In the future, integrated approaches may prove to be more elegant. There are some simple solutions for dedicated tasks, such as the sample processer system for derivatisation of amino acids [1] and approaches for serum sample processing with special columns [4] or multidimensional HPLC systems [5].

Data Processing: Computers play an increasingly important part in the automation of HPLC systems. Table II shows the different steps and levels of HPLC data processing. Modern data systems can do more than just integrate peak areas. More and more, efficient tools are used in the evaluation and documentation of HPLC results. Graphic presentation of chromatogrammes is much more suitable for human data interpretation than huge tables of numeric data. A few good modern chromatography data systems help to routinely determine column characteristics (plate numbers, tailing factors, resolution of peaks and H/u-curves). Laboratory Information and Management Systems (LIMS) help in the documentation of chromatogrammes and in the establishment of huge data bases for optimal storage and retrieval. There is an increasing need for the integration of chromatographic data processing systems with widely-used spread sheet programmes like Lotus 123 [6] and RS/1 [7], with statistical packages like SAS [8], and with word processing systems for report generation. Hopefully it will soon be possible to really integrate text, tables, chromatogrammes, other graphical figures (like spectra) and chemical structures and reactions in a single computer system for the preparation of manuscripts or reports [9].

Trends and future developments: Expert systems will play an increasing role in HPLC laboratories. They help to optimally design experiments (Expert system for experimental design [10]); they automatically develop HPLC methods [11]; they simulate HPLC runs with modified experimental conditions (DryLab 1–5 [12]); and they even help locate origins of instrument failures (HPLC DOCTOR is an expert system for trouble shooting in HPLC [13]).

Table II. Possibilities of EDP in automation

Data Processing in HPLC
Raw data collection
Peak identification
Calibration/Standardisation
Report generation
Documentation
Evaluation
Graphic Presentation
Laboratory information management

The presently available hardware for automated HPLC systems provides nearly unlimited possibilities for automation if one is willing to pay the price. There is, however, much room for improvement and there is a real need for it. Compared to spectroscopic analytical instruments, the current HPLC systems still have a very unsatisfactory mean time of failure. Reliability should be improved. On-line diagnoses could be built into sophisticated HPLC systems so that the proper function could be documented. Under routine conditions the precision of autosamplers can be a problem. Very often the specifications of the instruments are only fulfilled with test mixtures under ideal conditions. Thereafter reproducibility becomes more difficult. The speed of laboratory data systems is far from optimal. HPLC is a very fast chromatographic method and will be even faster in the future [14] with the introduction of shorter columns and smaller column packing materials. However, what is the use of achieving a chromatographic separation within a couple of seconds if you have to wait minutes thereafter for data processing and report generation? A very important weakness of current instrumentation in big laboratories is the lack of communication standards for the integration of laboratory data systems in LIMS computer systems and corporate EDP. Experience has shown that the more intelligent the HPLC systems are, the more problems one will have to communicate with LIMS. Unfortunately there is little hope for fast improvement in the standardisation of data communication interfaces.

Validation

Many laboratory managers associate validation with increased workload in the laboratory, increased paperwork and missing capacity. However, validation is basically nothing new. Since the development of analytical methods, data have been elaborated to prove the reliability and precision of these methods. New to present validation processes is the consequent planning of validation and systematic documentation of all the experiments. What is validation?

> VALIDATION IS THE PROCEDURE USED TO PROVE THAT A TEST METHOD CONSISTENTLY YIELDS WHAT IT IS EXPECTED AND REQUIRED TO DO WITH ADEQUATE ACCURACY AND PRECISION

Validation consits of three important parts:

- planning
- experimentation
- documentation.

Planning experiments well can significantly reduce the number of experiments needed and can improve the quality of the results. Good documentation of the validation results helps in the transfer of methods from user to user and from laboratory to laboratory.

For HPLC a typical set of validation criteria for assay and purity determination is shown in Table III. The number of parameters that must be studied depends on the goal of the

Table III. Validation criteria for HPLC tests

Typical Validation criteria for HPLC
• LINEARITY of the analytical function
• Origin of the analytical function
• Detection limit/limit of quantification
• Precision
• Accuracy
• Selectivity
• Specificity
• Column to column variability

analytical method. For assay methods, the linearity of the analytical function is tested (Fig. 2). The origin should be within the confidence limits of the regression line, and the correlation coefficient should usually be greater than 0.99, except for trace analysis where lower correlation coefficients often must be tolerated. The precision of the HPLC determinations is measured by assaying the same sample several times, including all the sample preparation steps. The standard deviation of these independent determinations provides a good estimate of the precision of the HPLC assay. The accuracy of HPLC assays is much more complicated to prove. It has to be shown that no interfering compounds mimic overly high assay values of the compound of interest. Adequate specificity of the detection and selectivity of the separation have to be demonstrated. Like many other separation methods and spectroscopic quantitative assays, it is very time consuming and not at all trivial to show specificity and selectivity. Davis and Giddings [15], as well as Martin, Hermann and Guiochon [16], investigated the problems of peak distribution in chromatogrammes with a statistical approach. As shown in Table IV, the minimal peak capacity and the theoretical plates necessary for separation with a probability of only 90 per cent are excessive if more than 10 compounds are present in the sample. This demonstrates clearly that separation of multicomponent samples is not feasible with only plate numbers.

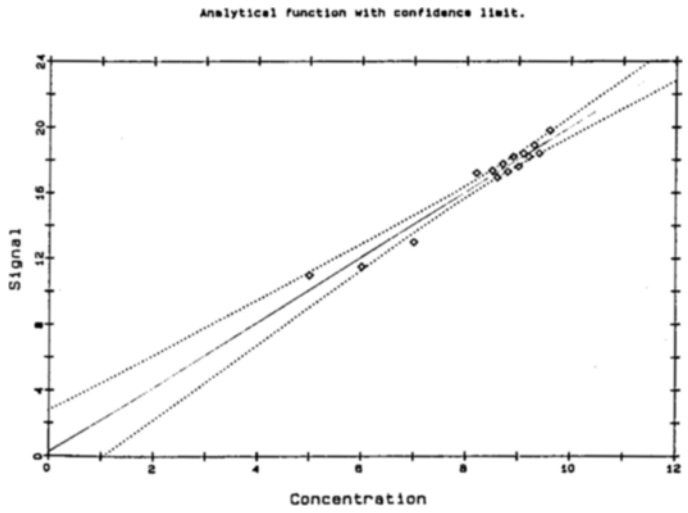

Fig. 1

Analytical Function of HPLC Assay: Linear Regression with confidence limits.

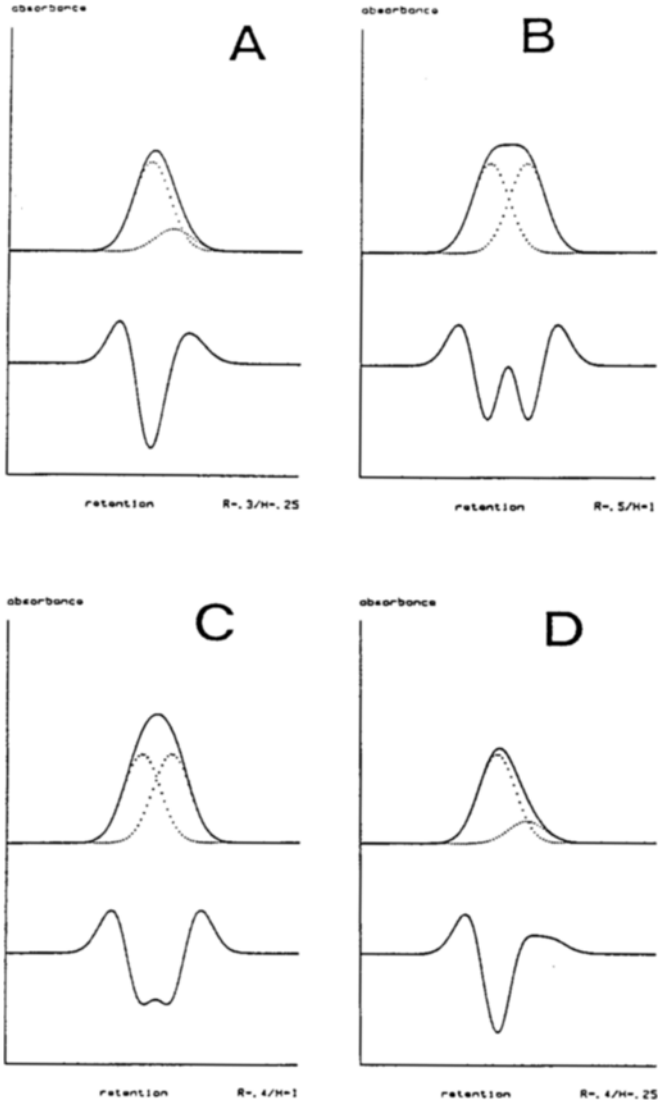

Fig. 2

Use of derivatives of chromatogrammes for the detection of overlapping peaks.

upper curve: simulated overlapping chromatographic peaks with Resolution R and peak high ratio H.

lower curve: second derivative.

Method optimisation usually is one of the ways to overcome this problem by adjusting the selectivity of the separation and perhaps the selectivity of the detection system as well [17]. However, the risk of interferences always remains [18]. This risk can be minimised by several methods (Table V). Using chromatographic means, the peak capacity can be enlarged either by using a more efficient column (with more theoretical plates) or by using multi-dimensional HPLC, where the overall peak capacity of independent chromatographic steps can be multiplied [19]. The selectivity of the system can be improved by systematic solvent optimisation. On the other hand, the selectivity of the detection and data processing can be improved. Broad peaks and tailing peaks indicate possible interferences [18]. This can be more easily detected by using derivatives of the chromato-

Table IV. Peak capacities and theoretical plates necessary to separate statistically distributed peaks with 90 per cent confidence with a single injection [15]

number of compounds	peak capacity	theoretical plates
3	21	1900
4	59	15000
8	402	6.8×10^5
16	2013	1.7×10^7
20	3254	4.4×10^7

Table V. Peak purity tests

Peak purity
A. Chromatographic Approaches
– higher peak capacity
• more plates
• multidimensional HPLC
– systematic method optimisation
B. Detection and Signal processing
– peak width
– tailing
– derivates of chromatograms
– dual wave length detection
– diodearray detection (LC-UV)
– LC-MS

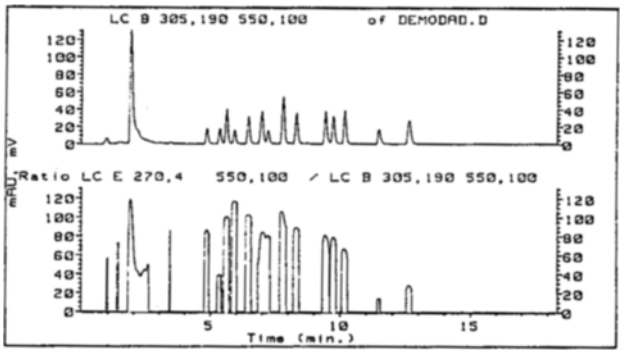

Fig. 3
Detection of peak overlapp by wavelength Ratio Chromatogrammes.

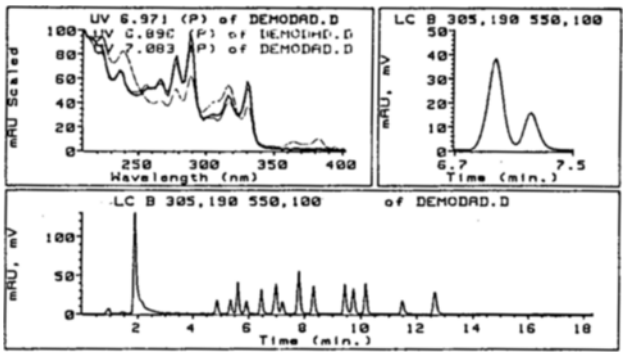

Fig. 4
LC-UV.

gramme [20, 21], as shown in Fig. 2. Dual wavelength detection [22] can give indications of overlapping peaks by examining the ratio of two or more wavelengths (see Fig. 3). Multi-dimensional detection, like LC-UV with diodearray detection (see Fig. 4) or LC-MS [23, 24] (see Fig. 5) gives additional information about the peak purity. Unfortunately, the general impression is that only a few of these methods are used in routine laboratories. Software tools to perform these methods efficiently on a routine basis are perhaps not available. The problem of interference is also related to the type of standardisation. Table VI shows the advantages and disadvantages of internal and external standardisation. Internal standards clearly have the advantage of not being sensitive to complex sample preparation, not even to sample losses during the sample preparation. On the other hand, the internal standard is an additional peak in the chromatogramme to be separated and there are additional possibilities for interferences. Generally, according to error addition rules, internal standardisation gives a higher standard deviation because of the two measurements. Table VII shows the advantages and disadvantages of peak height versus peak area evaluation. Practical experiences have shown that for gradient analysis, peak area measurements are to be preferred.

In the context of validation, method optimisation is of importance for two reasons. As mentioned, the problem of

Table VI. Advantages and disadvantages of internal and external standardisation

	internal Standard	external Standard
advantage	– not sensitive to loss during complex sample preparation	– simple – fast – more accurate for simple separations
disadvantage	– error addition – 2 possible interferences	– reliability of injection

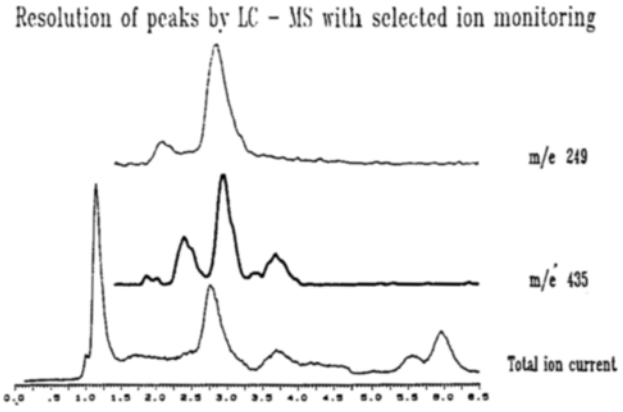

Fig. 5
Resolution of unresolved peaks by specific detection using LC-MS.

peak purity can be addressed by a good optimisation strategy. The second reason is that a good optimisation strategy produces excellent input for the validation of an HPLC method. Systematic evaluation of separation parameters ultimately provides the answer to what parameters are important, not important or critical. This information is useful for the transfer of HPLC methods from one laboratory to another. Good experimental design and reasonable documentation significantly improve the acceptance of an HPLC method in another laboratory and may even overcome the 'NIH-Syndrome' (Not invented here).

Ruggedness

The result of the optimisation also gives valuable information about the ruggedness of the method. Fig. 6 shows a contour-plot of an optimisation function with two parameters, where the maximum of the optimisation response is close to a steep slope. Under routine conditions it may be wise not to operate in the overall maximum, but in a flat region near the maximum. Such an operating condition is much more resistant to fluctuation in parameters and therefore more rugged. Many optimisation strategies are described in the literature, but from the point of view of validation only those methods that lead to a global survey of the response surface are valuable. For example, simplex optimisation does not produce a general survey and may be stuck in local maxima. In this respect the method described by Lankmeyer and Wegscheider [17] is very interesting because – at every step of the optimisation – it provides information on the expected global maxima, as well as on the region of the hyperplane, where more experiments are necessary in order to minimise the uncertainty of the global hyperplane.

Table VII. Peak height versus peak area for quantitation of HPLC

Peak Height or Peak Area
peak height
• not sensitive to flow variation
• sensitive to solvent composition
• sensitiv to temperature (k')
• sensitive to column degradation
• less sensitive to interferences than area
peak area
• sensitive to flow variation
• not sensitive to solvent composition, temperature, and retention
• not sensitive to column degradation
• preferred for gradient analysis

System Suitability Test (SST)

The goal of the system suitability test is to demonstrate adequate performance of the HPLC system prior to the use of the HPLC test. A prerequisite is a validated HPLC method. In HPLC usually the plate number, k'-factors and/or retention times, the detection limit, selectivities of critical pairs of peaks, peak tailing and the relative standard deviation of a number of standard injections are checked. Many pharmacopoea like the USP give detailed information about what is required for GC and HPLC system suitability tests. Fig. 7 demonstrates the importance of the SST and shows how bad column-to-column reproducibility may be (25). Nominally identical HPLC reversed phase columns RP18 (from the same manufacturer but from different batches) produce significantly different retention and selectivity. It may be necessary to discard such a column for this specific separation and look for another more adequate separation column. For this reason the system suitability test should contain limits and minimal requirements for the performance of the separation.

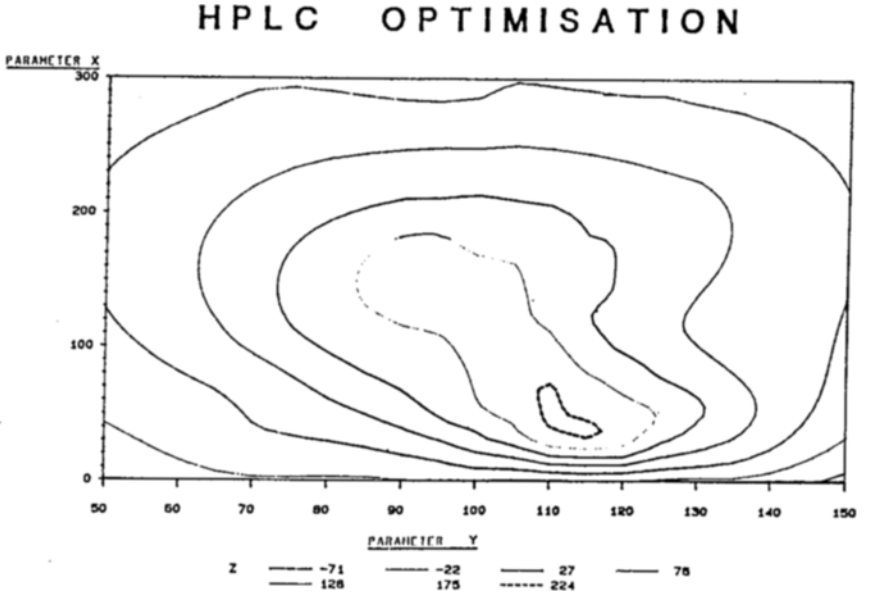

Fig. 6
Contour-plot of an optimisation function.

Qualifications of Instruments

It is obvious that HPLC instruments must work properly in order to insure reliable and valid results. This should be checked from time to time. This validation of instruments is called **QUALIFICATION**. Practical experience in our laboratory has shown that qualification in regular intervals is necessary. Many automatic injectors show excellent reproducibility of the injection volume when they are new. After a couple of months, however, this reproducibility can diminish. It is very important that a malfunction in the instrumentation is identified as early as possible. Qualification of instruments is also important if HPLC methods have to be transferred from laboratory to laboratory, especially for the transfer of gradient elution HPLC methods. Fig. 8 shows that the gradient delay-volume, as well as the gradient mixing-volume, of the HPLC instruments is important for the gradient characteristic. If either of these parameters is very different from the original HPLC instrument, significant differences in the retention times and even in the selectivity of separation may occur, especially if a gradient method has to be transferred from a high pressure gradient HPLC instrument to a low pressure side-gradient mixing instrument. Usually the low pressure mixing instruments have much higher delay- and mixing-volumes. In cases of very short columns with small inner diameters, the operation with a low pressure-side gradient instrument may be impossible.

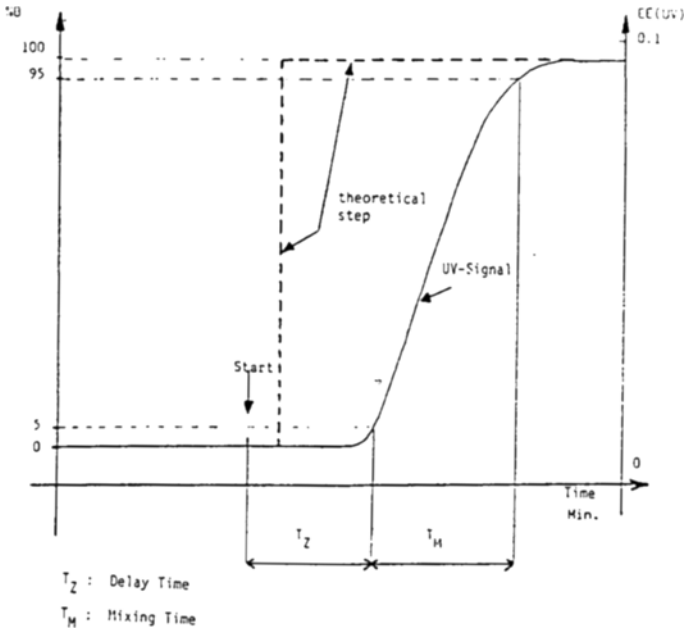

Fig. 8
Gradient characteristics: Delay volume and Mixing volume.

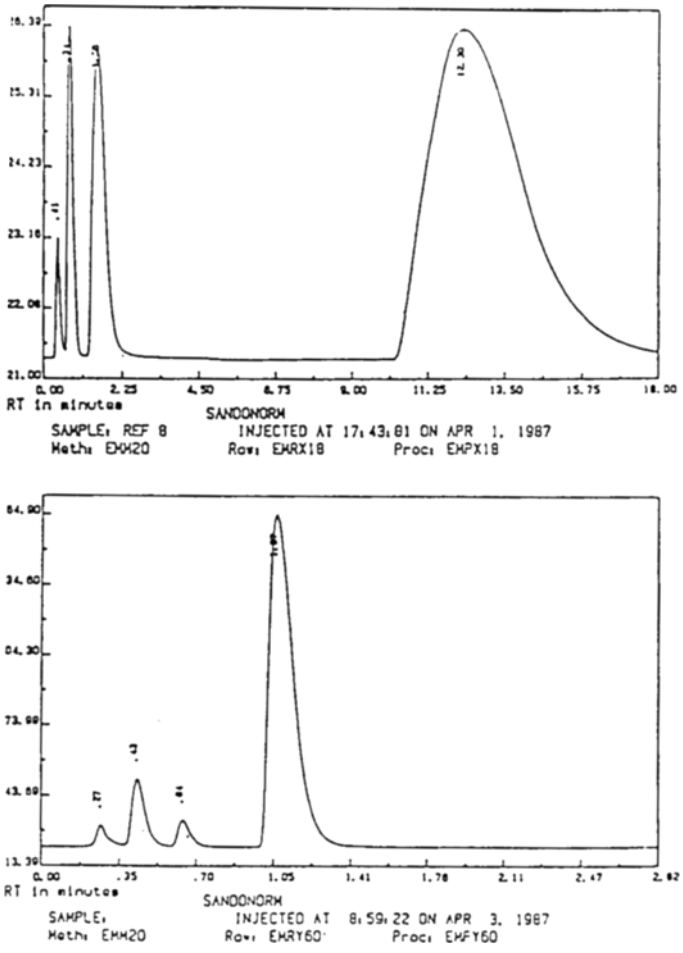

Fig. 7
Differences of retention and selectivities by column to column variability.

Conclusions

It has been shown that automation of HPLC systems was successful in routine applications. Mechanisation and laboratory robots make even complex sample preparations with unattended operation and high reproducibility possible. In the future, computer systems will also expand in areas of documentation and laboratory information management. Expert systems will soon help handle routine problems efficiently, without replacing the human expert. It was emphasised that the validation of HPLC methods, especially the proof of peak purity, is extremely demanding scientifically and may never come to an end. It was also shown that system suitability tests and qualification of instruments assure the reliability and validity of HPLC results.

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