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Deferred standards, an on-line qualification, validation and system stability probe for chromatographic assay ☆

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Abstract

Specific programming of automated HPLC systems allows total on-line qualification, validation and stability monitoring using the concept of deferred standards. Setting up such a process for routine analyses in an automated HPLC system requires specific autosampler programming as well as specific monitoring software. With an autosampler, a double injection procedure is programmed, the first introducing the sample, and the second, a few minutes deferred, the deferred control standard. Two additional compounds are therefore added to the sample before and during the chromatographic process: the internal standard for sample quantification and the deferred standard for system control. Specific methodologies are described of how to obtain classical quantitative analysis information as well as system qualification validation stability information. Experiments were performed to develop specified methodologies to monitor the quality of quantitative analysis during the life of the column by using the deferred standard concept to probe the effects of column ageing on separation characteristics.

Keywords: Deferred standards; Internal standards; Validation; Stability studies; Automation; Polynuclear aromatic hydrocarbons

1. Introduction

The economic importance of chromatographic separations is evidenced by the use of validation procedures. Chromatographic assays have become technologically and methodologically mature, spreading from health and environmental sciences to quality control in chemistry. However, separation and chromatographic assay validation are time-consuming processes which are ready for simplified procedures that provide enhanced information.

1.1. Qualification validation stability

The scientific, technological and regulatory development of chromatographic methods to be used for assays is dependent on both the instrumentation (mostly commercial) and the associated separation

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methodology [1,2]. Separation development is well described in the scientific chromatographic literature. The economic impact of tracking separation quality through each step of the process has led to the development of specific scientific approaches [3–5]: most are grouped under the generic term of ''Quality'', and include the QVS concept (qualification, validation, stability). The QVS process is divided into three steps.

The first involves chromatographic system tools: pump, solvent, autosampler, column temperature control, detector and integration systems (machines and software). Guidelines have been established by harmonisation conferences (such as the International Conference on Harmonisation: ICH) or by governmental institutions, such as the US Food and Drug Administration (FDA). However, fields are still open for research strategies to enhance precision or improve the existing recommended and regulatory rules.

The second is related to separation and assay validation. The major limitation of validation guidelines is linked to the ''snapshot-like'' nature of information obtained at a given time or step of the separation or of the assay series. It is obvious that, during large series of assays, revalidation steps or validation criteria control must be performed according to established rules [3–5]. If a validation control step does not indicate the presence of a problem, the process goes on carrying the risk of sub-detection problems, the impact of which may be detected a posteriori, leading to expensive and time-consuming re-assays. From a legal point of view, these sub-detection problems may lead to polemical situations (drug enforcement, environmental and food pollution). At the laboratory level, complications arise when these problems begin to appear; diagnosis of the origin of a posteriori detected problems can be complex. Therefore, there is a need to find and quantify these biases at early stages, in order to limit or avoid costly re-assays. Moreover, if early or a priori probes can be defined, vast areas open up to enhance the ''ruggedness'' of not only the assay series, but also of the entire validation control process. Such developments of ''early or immediate'' validation probes constitute the third step of the QVS concept.

The major fundamental and costly limitation of chromatographic assays is the dependence of the validation criteria on the characteristics of the separation system (involving instrumentation and mobile phase/stationary phase couple). Determination of the status of the column is of key importance, because a missed column replacement decision (based on column retirement criteria) can lead to degraded assay productivity. Ageing or poisoning of the column can alter the elution characteristics of the sample, i.e. change its retention and band spreading. Numerous parameters are available to monitor the quality of the separation, including peak capacity, efficiency and resolution. However, there is a need for a global methodology, in particular when the problems originate outside of the column, such as detection, injection or flow-rate instabilities (sensitivity, linear range, injected volume, flow-rate variations).

The major objective of this report is to demonstrate that a global method is methodologically possible and will facilitate, at the expense of limited instrumental modifications, the setting up of life tracking of the overall chromatographic system. The general principle is to introduce into the chromatogram a specific compound, called the deferred standard (DS), the purpose of which is to monitor the characteristics of the separation system in terms of its compliance with validation criteria. This independent ''watcher'' of the status of the separation system is not involved in the assays, but is present in all the chromatograms. Its elution peak profile and quantitative characteristics can be described precisely in each assay.

1.2. The deferred standard concept and quantitative analysis

The principle is the following: a double injection is performed, the first involving the mixture to be analysed and the internal standard, and the second involving the pure deferred standard. A short delay is set up between the injections to elute the deferred standard in an empty baseline zone of the sample [5–10].

Two different strategies can be used. The first considers this double injection process as a method to control and track the characteristics of a chromatographic system. If the characteristics of the deferred

standard are reproducible (injected mass and elution profile) the system is considered suitable for the chromatographic assay. The second is to use the DS procedure as a ''quality probe'' in the chromatographic assay series. If, during the assay series, the elution characteristics of the DS are reproducible (even with different batches of mobile phase), validation of the assay with limited or classical systematic and repetitive elution of standard mixtures is authorised.

In order to set up the DS method, the autosampler program must be modified to inject, with a delay of a few minutes, the double sequence of the sample and the deferred standard. In order to demonstrate the effectiveness of the DS concept in the monitoring of a long-term assay, we have chosen to use laboratory packed columns. They were assumed to have a lower stability than commercial columns, and to age faster, an advantage for this study.

2. Experimental

2.1. Chemicals

HPLC-grade methanol was purchased from Prolabo (Paris, France) as was carbon tetrachloride. Freshly double-distilled water was used for the mobile phase. All polycyclic aromatic hydrocarbons (PAHs: toluene, naphthalene, biphenyl, fluorene and anthracene) and thiourea (void volume probe) were purchased from Sigma (St. Louis, MO, USA).

2.2. Chromatographic system

The HPLC system consisted of an SP 8800 pump, an SP8875 autosampler fitted with a 20 μL sample loop and a Spectra 200 UV detector set at 254 nm (Spectra-Physics, San Jose, CA, USA). The column temperature was controlled at 40±1 °C by means of a Waters (Milford, MA, USA) temperature control system. For all experiments, the mobile phase flow-rate was controlled at 0.50±0.01 mL/min. In a preliminary step, all devices of the HPLC system were independently qualified (using accreditation and certification procedures) [3].

2.3. Columns and mobile phases

Empty columns (150×3 mm I.D.) were purchased from Upchurch Scientific (Oak Harbor, WA, USA), and packed in our laboratory with Hypersil ODS 5 μm (Thermo Hypersil, Runcorn, UK). The column packing procedure was identical to that recommended by Hypersil [11,12]. A silica paste was prepared using a binary mixture of methanol–carbon tetrachloride (90:10, v/v). This paste was sonicated prior to injection into the plastic column tube. A packing station model Chromatem (Touzart et Matignon, Les Ulis, France) was used with pure methanol as packing solvent. The maximum pressure never exceeded 350 bar. Column equilibration was finished when a constant flow-rate was measured at the end of the column for 10 min; this needed approximately 300 mL of methanol.

The performances of these columns were evaluated in a preliminary step in terms of theoretical plate numbers (13 000±500) and selectivity (1.65±0.15) using a mixture of five PAHs. During the experiments, column void volumes were determined daily by triplicate injection of a 5.65 g/L thiourea solution. Mobile phases were made of binary mixtures of methanol–water (65:35, v/v), then filtered with a Millipore filter model HVLP 0.45 μm (Millipore, Molsheim, France) and sonicated before use.

2.4. Sample preparation

All PAH mixture solutions [13–16] were prepared in the mobile phase; the final concentrations are given in Table 1. In order to eliminate possible bias due to concentration or injection order, a randomisation process [17] was applied to the solute mixture compositions and injection sequences during the entire experimental program using software listed in Appendix A (Programs 1 and 2). Sixty mixtures, varying in composition (number of PAHs) and concentration, were prepared according to ''Program 1'' with only one constraint: each compound at 1:50 dilution must be preceded or followed by a 1:1 dilution compound in every mixture. The injection sequences were randomly defined by means of ''Program 2''. For quantitative analyses, biphenyl

Table 1
Concentrations of PAHs used for routine analyses

Sample	Concentration (g/L)				
	Toluene	Naphthalene	Biphenyl	Fluorene	Anthracene
1	1.7500	0.1860	0.0610	0.1000	0.0100
2	0.8750	0.0930	0.0305	0.0500	0.0050
3	0.1750	0.0186	0.0061	0.0100	0.0010
4	0.0350	0.0037	0.0012	0.0020	0.0002

was added to each PAH mixture as an internal standard at a final concentration of 0.061 g/L.

2.5. Peak profile analysis

Each chromatogram was recorded by a data acquisition system described elsewhere [18] and developed in our laboratory operating at a frequency of 2 Hz. These digital chromatograms allow complex peak analysis by means of statistical moments theory [19] and a posteriori quantitative recalculations. In addition, retention times and peak areas were determined from each chromatogram.

2.6. Deferred standard injection procedure

Toluene was chosen as DS and injected 2.8 min after each sample injection. Such a delay ensured that the DS peak did not interfere, and that there was sufficient resolution between the DS and the sample components. Two rinse cycles were programmed between these injections (sample and standard) to avoid sample loop contamination.

3. Results and discussion

3.1. Method validation

3.1.1. Separation validation

To avoid a possible bias in data analyses, repeatability and reproducibility of the chromatographic method was tested using Table 1 mixtures at concentration number 1. Expanded uncertainties as RSD (%) of both capacity factors and peak areas calculated for five injections of each PAH daily during a week are listed in Table 2 [20]. These values never exceeded 1.50%, indicating a good stability for our system (at the chosen confidence level of 95%).

3.1.2. Quantitative analysis validation

Only one example, anthracene, will be described, but analogous determinations were performed for all the other PAHs. Four concentrations with dilution factors varying from 1:75 to 1:2 in triplicate injections were used in the calibration study. The calibration curve in terms of peak area and injected amount led to the straight line $y = 5 \cdot 10^6 x - 1797.5$ and a corresponding correlation coefficient of

Table 2
Expanded uncertainties as RSD (%) of retention factors and peak areas of five injections of PAH solutions on 7 days. These were calculated using a coverage factor of 2.8 for repeatability and 2 for reproducibility, which gives a level of confidence of approximately 95%

		Toluene (1.7500 g/L)	Naphthalene (0.1860 g/L)	Biphenyl (0.0610 g/L)	Fluorene (0.1000 g/L)	Anthracene (0.0100 g/L)
Repeatability, RSD (%)	k'	1.20	1.06	1.04	0.98	0.90
	Area	1.06	0.82	1.26	1.18	1.03
Reproducibility, RSD (%)	k'	1.07	0.82	1.32	1.11	0.92
	Area	1.22	1.18	1.37	1.03	1.09

Table 3
Classical F-test applied to the quantitative study of anthracene. Four different dilution factors were used, and each solution was injected in triplicate (see also Table 4)

Dilution factor	Q. injected (μg)	Replicates		
		Area 1	Area 2	Area 3
1:75	0.005	21 730	19 954	24 150
1:30	0.013	57 333	57 298	58 261
1:15	0.027	118 307	121 397	120 047
1:2	0.200	909 127	908 503	911 037

0.9999, y expressed in arbitrary units, x in μg. The validity of the linear model was assessed using the classical F-test comparison, the major characteristics of which are summarised in Tables 3 and 4. A Cochran test was also performed to verify the variance homogeneity of the linear model. The limit of detection (LOD) and limit of quantification (LOQ) were $2\cdot10^{-4}$ and $8\cdot10^{-4}$ μg at 3× and 10× the noise level, respectively.

3.1.3. Solution stability

Solute injection is critical for the stability of the HPLC system; extra-column variability must be eliminated. After the autosampler was qualified at the delivered volume level (qualification procedure), injected samples were evaluated in terms of degradation (retention times) and response coefficients (peak areas). During 14 days, six PAH mixtures were injected according to two different sample preparation processes:

1. Stock solutions prepared on day 0 of the experimental program: type 1 solutions.
2. Stock solutions prepared each day: type 2 solutions.

The six PAH mixtures contained three or all PAH (five) in two different concentration ranges: concentrations 1 and 2 described in the validation procedure (Table 1). This process was employed to avoid a possible bias due to the type of PAH or a concentration effect.

Averages and variances obtained for both solution types were compared statistically using Student and Fisher tests. No differences between these two populations were observed. The peak area and k' graphs of toluene monitored over 14 days are shown in Fig. 1A and B. Once the stability of all solutions was established in the 14-day test, all stock solutions were renewed every week.

Table 4
F-Test applied to the quantitative study of anthracene (see also Table 3). Variance analysis testing the regression model curvature at a 1% error risk, taking into account linearity and non-linearity

	Degrees of freedom	Variance	$F_{calc.}$	F_{limit} ($\alpha = 0.01$)
Linearity	1	$1.6\cdot10^{12}$	$7.3\cdot10^{5}$	11.26
Non-linearity	2	$4.0\cdot10^{6}$	1.81	8.65
Residuals	8	$2.2\cdot10^{6}$		
Total	11			

3.2. Systematic analysis

Once the methodological precautions were formalised, a quantitative analysis was simulated over 3 months, which corresponds to approximately 2500 injections of each PAH, which is more than 3000 analyses per column. This study consisted of the systematic monitoring of column dead volume, theoretical plate number (N), peak width (W) and retention factor (k') for every PAH as separation parameters. The injected solutions and operating procedures were identical to those described in the preceding sections.

3.2.1. Column void volume

Column void volume was monitored to avoid bias in retention factor and column efficiency measurements. The average daily measured void volume values are shown in Fig. 2, where the x-axis described as ''Cumulative injected quantity'' represents the cumulative amount of thiourea introduced into the column during all the measurements. The data demonstrate good stability, indicating the absence of stationary phase compression; no geometrical factors perturbed the chromatographic parameters during the experiments.

3.2.2. Peak parameters

Fig. 3A and B present data obtained for fluorene during the systematic assays. Fig. 3A shows column plate number values calculated with seven different

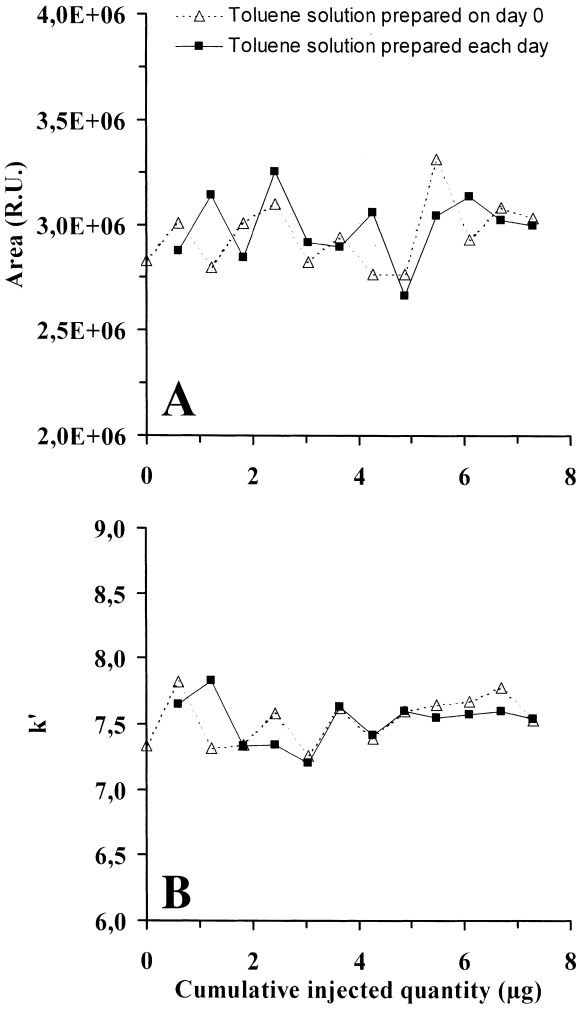

Fig. 1. Stability of toluene stock solutions. Comparison of a toluene stock solution at a given concentration. (A) Peak area, (B) retention factor. *x*-Axis correspond to the cumulative amount of toluene eluted from the column for each type of solution (8 μg corresponds to 14 days).

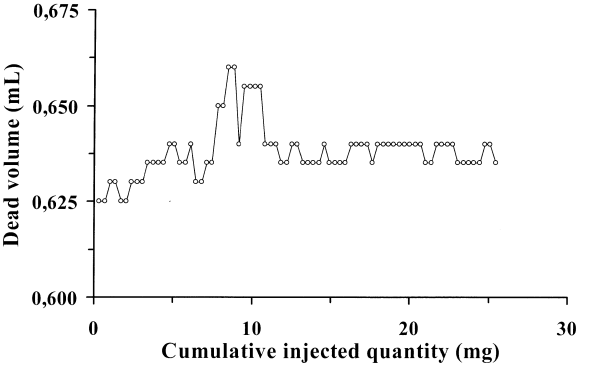

Fig. 2. Column void volume monitoring. *y*-Axis values are calculated from triplicate injections of a 5.65 g/L thiourea solution. The *x*-axis corresponds to the cumulative amount of thiourea eluted from the column during the experimental program.

methods described in the literature [2] as a function of the cumulative injected amount of sample. The first three methods (Ni, Nh and N3) calculate *N* values from the upper part of the peaks (at 60.7, 50.0 and 32.4% of the peak height) and show a particular behaviour that divides the lifetime of the column into two different stages, up to and after a cumulative injected fluorene mass of 60 μg. On the contrary, the four other *N* calculation methods (N4, N5, Ntan and Nsyst), which utilise the lower part of the peak (13.4, 4.4, 0.0 and 10.0% of the peak height), describe a regular and monotonic decrease of column efficiency. This result was confirmed by systematic measurements performed at 10% of the total peak height for all PAHs during the experiment. The band width increased with the injection number in a logarithmic fashion.

The stability of the C_{18} coating is monitored by k' measurements. The pattern obtained for fluorene was similar for all other PAHs.

The major conclusion of this methodological study concerning column stability is that the column rapidly reaches a maturation stage after the first few injections. In the case of toluene, this stage was reached at a cumulative compound quantity of 10 μg. Once this maturation stage was reached, a remarkable stability was observed over 2500 analyses (3 months), where no significant loss of retention factor or separation efficiency was observed.

In terms of band width, the classical *H* and *N* terms must be used cautiously: measured variance is of more interest and not biased by retention. A column may be assumed to be significantly advanced in age when *N* values decrease due to increased variance. Decreasing retention factors can only be considered at best a second order probe of column ageing.

3.3. Quantitative analysis and system survey

Two types of standards were added to samples

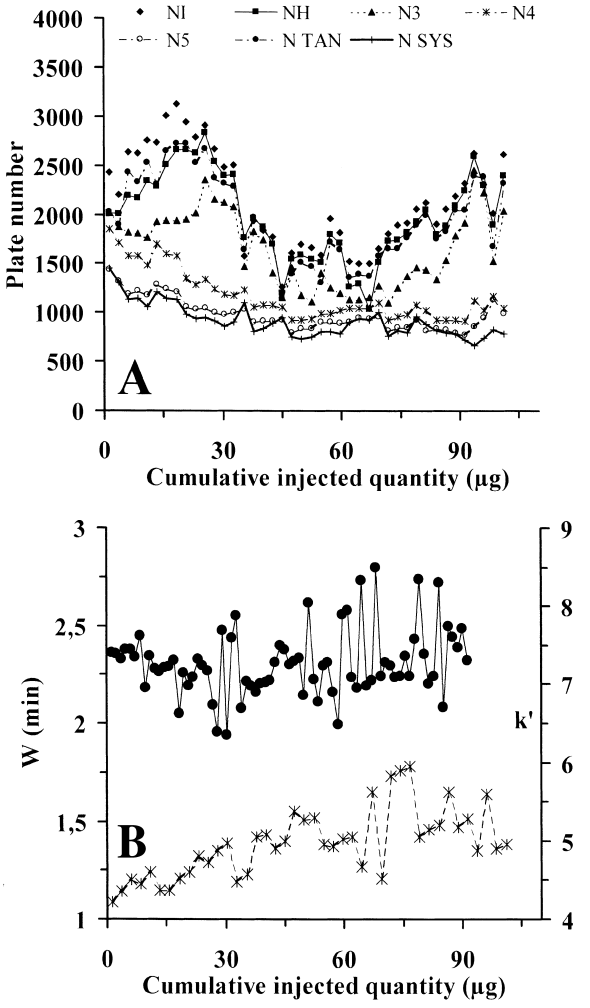

Fig. 3. Monitoring separation efficiency of the column for fluorene. *x*-Axis corresponds to the cumulative amount of fluorene eluted from the column. *y*-Axis is plate number calculated from fluorene peak. (A) Plate number measurements. (B) Peak width measurements at 10% of the peak height (· · ·) and retention factors (———) calculated from the void volume and peak apex volume.

prior to chromatographic assay, the internal and the deferred standard. The DS is injected pure in every analytical sequence, with a time delay calculated to properly position it compared to the analysed compounds. This pure solute, soluble in the mobile phase, must be chosen to have a good detector response and a compatible injection volume over the entire range of the calibration curves.

To set up the quantitative analysis of PAHs, an internal standard (biphenyl, 0.061 g/L) was added to randomised solution mixtures and the relative responses were determined.

The deferred standard was used to evaluate the health of the chromatographic system on-line. An example of the double injection procedure is shown in Fig. 4 for three different mixtures. Two elution zones can be observed in these chromatograms, the first including the sample with the internal standard and the second includes only the DS. The elution characteristics of the DS are ubiquitous and clearly identify its specificity. In isocratic elution, its band width is smaller than that of the adjacent sample peaks. In the different chromatograms, the retention of anthracene varies due to slight variations in the mobile phase composition. Such variations are only noticeable for well-retained peaks. Variations in the peak area of DS in Fig. 4 reflect injection volume variations.

Once this instrumental and methodological development has been completed, a large number of chromatographic analyses can be performed classically. Analysis time is not increased by adding the DS to the assay. The only additional cost is the cost of the DS compound and the complexity of the autosampler injection procedures. It is possible to monitor the quality of the separation process by monitoring the characteristics of the DS peak, as shown in Fig. 5 for a large number of assays. The separation efficiency profiles measured at the upper part of the peak of the DS were different from those observed in Fig. 3. However, the Nsys pattern (the

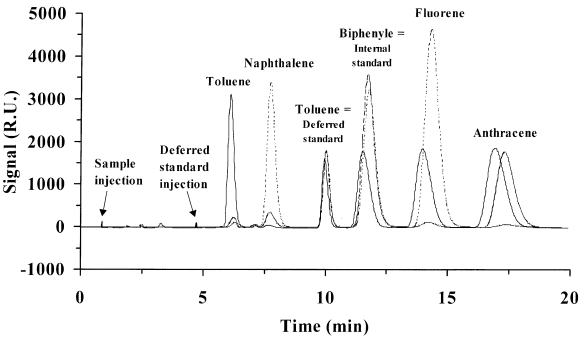

Fig. 4. Deferred standard in a classical internal standard-based chromatographic assay. Double injections of three different PAH mixtures at different concentrations, mobile phase batches and column ages.

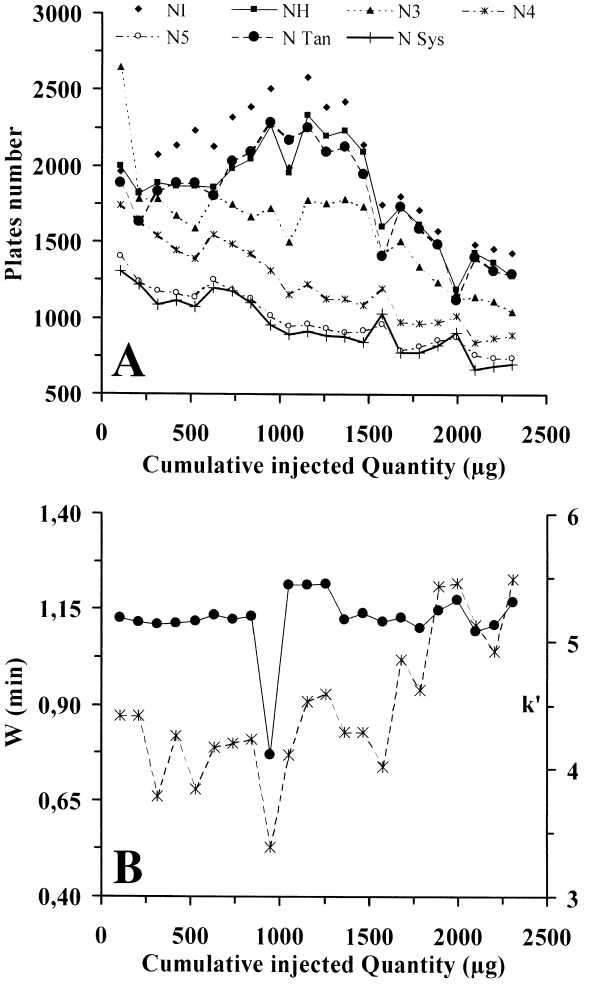

Fig. 5. Elution characteristics of the deferred standard during the experimental series. *x*-Axis corresponds to the cumulative amount of toluene eluted during the assay series. (A) Plate number calculation using different algorithms. (B) Band width and retention factor measurements for the deferred standard. Left *y*-axis (*), peak width; right axis (- - -) retention factor.

only one which takes into account peak asymmetry) is analogous in Figs. 3 and 5. In Fig. 5, k' changes can be linked to slight solvent composition changes, as indicated by the widely distributed values which led to a continuous drift during the experimental program.

Monitoring of the characteristics of the DS peak during the life time of the column reveals tendencies not evidenced by simple ''snapshot'' validation procedures. Moreover, it provides an ageing model for the System/Analysis couple, which may be used for a posteriori revalidation procedures. This point is of major economic value, as validation criteria of the system can be monitored cumulatively. For example, as long as the analysis matches the validation criteria, no problems arise. When the validation criteria are no longer met, it is possible, by means of the DS concept, to restore the history of the entire analysis cycle and diagnose exactly what sample needs to be re-assayed. It is also possible to redefine a posteriori the validation criteria during the entire analytical cycle. Thus, a time-dependent dimension of the validation criteria can be defined. This concept is in total agreement with the general problem of carrying out assays with a system in which some parts are thermodynamically unstable, the most evident being the column. The signal produced by the deferred standard can also be used to monitor the response of the spectrophotometric detector (lamp ageing) and the injection volume. A positive drift in the area of the deferred standard indicates an increase in the injected volume, while a negative shift can be generated by either a decreasing injection volume or an energy loss of the detector lamp.

4. Conclusion

A deferred standard in the chromatographic assay can be used to monitor the flow-rate and mobile phase composition, both important validation criteria for the assay, by noting the retention time, separation efficiency and peak resolution characteristics of the DS. Column efficiency validation criteria can also be observed continuously, and injection or detection biases can be identified. It is also possible to imagine the combined use of the deferred standard and the internal standard for automatic recalibration of peak detection and quantification, an unmatchable advantage because it does not reduce assays productivity and incurs only incremental development costs to properly position the DS.

Qualification and validation procedures as well as quality assessment strategies represent a necessary maturing step toward high-quality analytical results. The deferred standard concept permits continuous monitoring of the separation characteristics and can

be considered as a ''good health'' indicator for the validation criteria.

It is rather astonishing that the deferred standard concept has failed to capture the attention of the separation science community. The economic and legal impact of producing assays of the highest quality may, in the future, focus more attention on it again.

Acknowledgements

Professor Georges Guiochon (Knoxville University, TN, USA) is acknowledged for suggesting the principle of this report (Deferred Standard Concept for Separation Quality Control Tracking).

Appendix A

Program 1

```
5 PRINT ''CONCENTRATION PREPARATION PROGRAM''
7 CLS
10 PRINT ''RANDOM SEQUENCE''
12 PRINT ''SOL 1 SOL 2 SOL 3 SOL 4 NUMBER''
15 i=0
20 FOR w=1 TO 4
30 FOR x=1 TO 4
40 FOR y=1 TO 4
50 FOR z=1 TO 4
51 IF w*x=4 THEN GOTO 52 ELSE GOTO 56
52 IF w=2 THEN GOTO 56 ELSE GOTO 59
56 IF y*z=4 THEN GOTO 57 ELSE GOTO 65
57 IF y=2 THEN GOTO 65 ELSE GOTO 59
59 i=i+1
60 PRINT ''w x y z i''
65 NEXT z
70 NEXT y
80 NEXT x
90 NEXT w
```

Program 2

```
CLS
DIM B(1 TO 60)
DIM A(1 TO 60)
LPRINT ''MIXTURE SEQUENCE PROGRAM''
RANDOMIZE
I=0
DO
   present=0
   value=INT(RND*60)+1
   j=0
   DO
      j=j+1
      IF B(j)=value THEN present=1
   LOOP UNTIL j=i+1 OR present=1
   IF present=0 THEN
      i=i+1
      B(i)=value
      IF A(value)>0 THEN PRINT ''Redundance value''
      A(value)=i
   END IF
LOOP UNTIL i=60
LPRINT '' ''
LPRINT ''idx gives the sequence order, B gives the solution number''
LPRINT ''idx associated with A is only a first trial verification''
LPRINT ''idx B idx B idx A idx A idx A''
FOR i=1 TO 20
   LPRINT USING ''# #, # #, # #, # #, # #, # #, # #, # #, # #, # #'';
   i,;B(i);i+20,B(i+20);i+40;B(i+40);i;A(i);i+20;A(i+20);i+40;A(i+40)
NEXT i
```

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