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Robustness testing of a chiral NACE method for *R*-timolol determination in *S*-timolol maleate and uncertainty assessment from quantitative data

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Abstract

A robustness test of a capillary electrophoresis method for the chiral separation of timolol in nonaqueous acidified media was performed. A two-level Plackett–Burman design was applied in which one qualitative and six quantitative factors were examined. Resolution, migration times and relative migration times to pyridoxine (selected as internal standard) were examined as qualitative responses to evaluate electrophoretic performance. A quantitative response, the content of *R*-timolol in *S*-timolol maleate sample, was also considered. Even though some significant factor effects were observed on the qualitative responses, it was still possible to quantify the *R*-timolol in the *S*-timolol maleate samples properly. The quantitative response was not significantly affected by the selected factors, demonstrating the robustness of the procedure. However, the use of different HDMS-β-CD batches seemed to affect both types of responses necessitating to introduce a warning in the procedure.

Since the experiments of the Plackett–Burman design can be assimilated to laboratories in an interlaboratory study, uncertainty can be evaluated using the robustness test data. The robustness test was set-up in such a way that the required variances could be estimated.

Keywords: Robustness testing; Nonaqueous capillary electrophoresis; *R*-timolol content; Uncertainty assessment

1. Introduction

According to the analytical method lifecycle [1], robustness is a part of method validation. However, in current practice, it is tested during method development. Compared to the validation process that only considers quantitative results, robustness deals with both quantitative and qualitative responses. During this study, following the definition of robustness [1], one is testing whether the qualitative and/or quantitative results obtained applying the optimized (and usually not yet validated) method are not affected by small deliberate and judicious changes in analytical parameters. The robustness test also provides an indication of the reliability of the method during normal usage [1–6]. During the robustness testing, one can also prospect any factor that was not necessarily studied during the method optimization but may influence the results. One thus can anticipate the problems that may occur during later use of the method, i.e. in a collaborative study or during routine analysis [2,5,6]. If measures are sensitive to variations of analytical conditions, it is required to maintain the conditions constant or to introduce a warning in the method description.

The capillary electrophoresis (CE) technique, with its high efficiency, simplicity, selectivity and versatility [7], has been found a suitable orthogonal technique to liquid chromatography because of its different separation mechanism and is widely applied in the field of enantiomeric separations. The use of various cyclodextrins (CDs) as chiral selectors allowed separating of almost any chiral drug compound.

The first aim of this study is to test the robustness of a chiral nonaqueous CE (NACE) method. Both qualitative and quantitative responses were focused on. Very often, only qualitative responses, such as electrophoretic performance criteria,

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are considered during robustness testing in order to determine whether the analytical procedure remains stable [8]. However, quantitative responses should also be considered according to the analytical method objective, i.e. the determination of a substance in a sample. The NACE method was developed for the chiral separation of timolol in the presence of pyridoxine, used as an internal standard [9]. Suitable enantioseparation was obtained by combining heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β-cyclodextrin (HDMS-β-CD) as chiral selector with potassium camphorSO_3^- in methanol acidified with formic acid. *S*-timolol maleate, a β-adrenergic blocker, is used in the treatment of hypertension, arrhythmia and angina pectoris. It is also used for the prevention of myocardial infarctions and for the topical treatment of increasing intraocular pressure [10–12].

A major drawback of CE is its relatively poor precision [13]. However, the quality of quantitative results is obligatory with regard to important decisions that might be taken considering governmental regulations or limits for international trade. Therefore, the analysts are under increasing pressure to demonstrate the quality of their results. Very few studies in CE have been conducted taking into account the quality of the results in terms of precision or uncertainty [14–16]. The knowledge of the uncertainty of the measurement [17,18] is very important to demonstrate the quality and the fitness for purpose of the results [17,19]. Several strategies for evaluating and expressing uncertainty can be found in the literature [17,19–23] including the evaluation of uncertainty from robustness testing [21,23].

Following the importance of the above requirements, a second aim of this study was to assess the uncertainty of the *R*-timolol content obtained from the analysis of *S*-timolol maleate samples under the different CE conditions of the experimental design executed during the robustness test. Thus, a set-up illustrated in Fig. 1A was drawn and applied to execute the robustness study. This set-up was modelled on that commonly applied in an interlaboratory study where "Laboratories" are replaced by the "Experiments". Therefore, the "Experiments" which represent the experimental conditions elaborated by means of a Plackett–Burman design in the robustness test, can be assimilated to the laboratories in an interlaboratory study. Their number ($r = 8$) is in good agreement with the ISO 5725-2 guide [24] which recommends a minimum of eight laboratories for an interlaboratory study. Each experiment is independently executed twice ($c = 2$ series or batches) which can roughly be assimilated to assessment on different days or in this case to the use of different batches of CD (Fig. 1B). For each series, the test sample of *S*-timolol maleate is analysed twice, i.e. two sample solutions are prepared and analysed independently ($g = 2$ replicates) under repeatability conditions. This can be considered as replicate measurements.

By adopting the ISO 5725-2 guide [24], the results issued from this set-up were used to determine, for each of the four *S*-timolol maleate samples, the repeatability variance, s_r^2, the between-series variance, s_{Series}^2, the between-experiment variance, $s_{\text{Experiments}}^2$, and the reproducibility variance, s_R^2 [24].

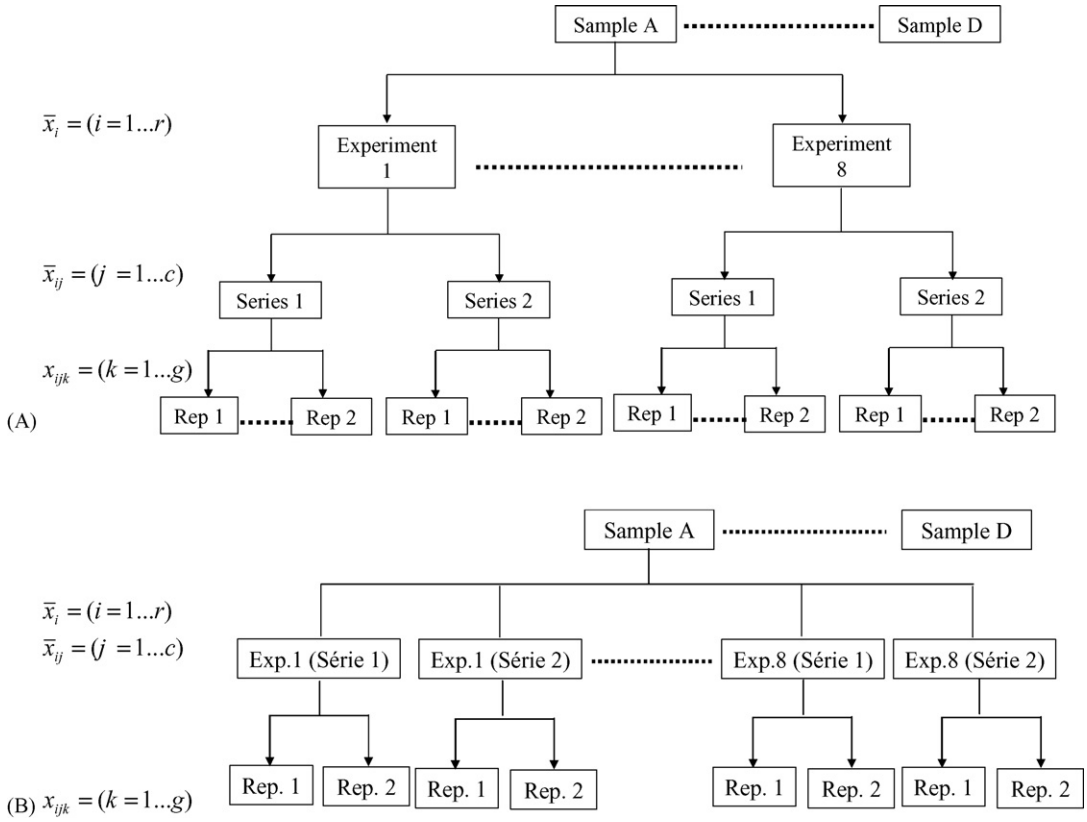

Fig. 1. Set-up of the robustness study adapted from an interlaboratory set-up (A), and applied in the present case (B): $r = 8$ experiments, $c = 2$ series (or two different batches of HDMS-β-CD) and $g = 2$ replicates.

2. Experimental

2.1. Chemicals and reagents

One *S*-timolol maleate sample (batch No. 107200204), donated by Prosintex Industrie Chimiche Italiane (Milan, Italy), and three (batches No. 11484, 11483 and 11351), obtained from the European Pharmacopoeia Secretariat (Strasbourg, France) were analysed. *R*-timolol *SCR* (batch No. 11381) was kindly afforded by Merck (Rahway, NJ, USA) and pyridoxine by SMB Technology (Marche-en-Famenne, Belgium).

Heptakis(2,3-di-*O*-methyl-6-*O*-sulfo)-β-cyclodextrin (HDMS-β-CD), obtained from Antek Instruments (Houston, TX, USA), was a new batch and coded as batch 2 while another, kindly provided by Professor Gyula Vigh (Texas A&M University, Texas, TX, USA), was an old one and coded as batch 1.

Potassium formate, ammonium formate and (1*R*)-(−)-10-camphorsulfonic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA) while formic acid (98–100%) of analytical grade and methanol of "liquid chromatography" grade were obtained from Merck (Darmstadt, Germany).

2.2. Apparatus

Two HP^{3D}CE systems, A and B (Agilent, Waldbronn, Germany), both equipped with an autosampler, an on-column diode-array detector and a temperature control system (15–60 ± 0.1 °C) were used to carry out the different experiments. They were equipped with CE ChemStation (Agilent) Softwares version Rev. A.05.03 (CE system A) and version Rev. A.08.03 (CE system B), respectively, for instrument control, data acquisition and data handling.

The experimental design was created and the statistical calculations were performed using the JMP software Version 5.1 for Windows (SAS Institute, Cary, NC, USA).

Uncoated fused silica capillaries were purchased from ThermoSeparation Products (San Jose, CA, USA).

Before their use, the solutions of background electrolyte (BGE) and those containing cyclodextrin (BGE-CD), as well as the samples to be analyzed were filtered through Polypure polypropylene membrane filters (0.2 μm) from Alltech (Laarne, Belgium).

2.3. Electrophoretic conditions

The nominal CE conditions [25] consisted in the use of uncoated fused silica capillaries having 50 μm internal diameter and 48.5 cm length (40 cm to the detector). The concentrations of HDMS-β-CD and potassium camphorsulfonate (camphorSO_3^-) were 30 mM. Both were used in combination in 0.75 M methanolic formic acid solution. Other CE conditions are indicated in Table 1. The resolution (Rs) was calculated according to the standard expression based on the peak width at half height [26]. A new capillary had to be conditioned at 15 °C with methanol for 15 min.

Table 1
Experimental domain

Factors	Limits	Level (−1)	Level (+1)	Nominal
Type of CE equipment		A	B	B
Capillary temperature (°C)	0, +2	15	17	15
Detection wavelength (nm)	±5	290	300	295
Voltage (kV)	±2	23	27	25
Injection time (corresponding volume in nL) (s)	±1	7 (20.5 nL)	9 (26.3 nL)	8 (23.4 nL)
HDMS-β-CD (mM)	±2.5	27.5	32.5	30
Camphorsulfonate (mM)	±2.5	27.5	32.5	30

2.4. Preparation of solutions

2.4.1. Background electrolyte solutions

The BGE consisted in a methanolic formic acid solution with potassium camphorSO_3^-. The potassium salt of (1*R*)-(−)-10-camphorsulfonic acid is obtained by dissolving simultaneously the required quantities of (1*R*)-(−)-10-camphorsulfonic acid and potassium formate in methanolic formic acid. The aid of an ultrasonic bath was necessary (about 10 min).

The BGE-CD solution was prepared by dissolving the corresponding amount of the required concentration of HDMS-β-CD in the BGE solution. The ultrasonic bath was used for dissolution (about 5 min).

2.4.2. Internal standard solution

The internal standard solution was prepared by dissolving about 12.5 mg of pyridoxine hydrochloride in methanol and dilute to 25.0 mL with the same solvent. Then, the solution was diluted 50-fold with the same solvent.

2.4.3. Reference solutions used for the system suitability tests (SST)

Two stock solutions containing 1 mg/mL of *S*-timolol and 1 mg/mL of *R*-timolol, respectively, were prepared in methanol. A mixture solution was prepared by mixing and diluting those stock solutions and that of the internal standard to obtain a working solution containing 5 μg/mL of pyridoxine, 10 μg/mL of *S*-timolol and 2 μg/mL of *R*-timolol. This solution was used for the SST to evaluate the performance of the electrophoretic separation under each experimental condition. The SST consisted in the determination of the resolution values between consecutive peaks.

2.4.4. Solutions used for assay

2.4.4.1. Reference solution. A stock solution of *R*-timolol was prepared in a 10.0 mL volumetric flask by dissolving, in methanol, an accurately weighted amount of approximately 20 mg of *R*-timolol maleate. This stock solution was then diluted one hundred fold to obtain a reference solution representing 1.0% (20 μg/mL) of impurity level and containing the internal standard at 5 μg/mL. This reference solution was used for the determination of the *R*-timolol maleate content in the *S*-timolol maleate samples.

2.4.4.2. Test sample solutions. Test sample solutions were prepared in 10.0 mL volumetric flasks by dissolving an accurately weighted amount of approximately 20 mg of *S*-timolol maleate sample in methanol. For complete dissolution of the substances, the solutions were sonicated in an ultrasonic bath for minimum 15 min. The test sample solutions contained the internal standard at 5 μg/mL. Two independent test solutions (two replicates) were prepared per sample and per series. Each test solution was analysed under repeatability conditions.

3. Results and discussion

3.1. Robustness study

For the performance of the robustness study, the procedure described in Ref. [2] was followed.

3.1.1. Identification and selection of the factors

Since the robustness study simulates interlaboratory changes, the factors selected have to reflect potential changes that may occur between different laboratories. Different CE equipment, injection times, concentrations of both potassium camphorSO_3^- and HDMS-β-CD, detector wavelengths, capillary temperatures and voltages were considered.

In this study, only one qualitative factor [5], the CE instrument (from the same manufacturer), was examined. In fact, not only the instrument, but also the data management software were concerned. The remaining six factors were quantitative factors [5].

3.1.2. Definition of the factor levels

The factor levels (−1, +1) tested in the robustness are indicated in Table 1. Their selection was done in such a way to reflect the potential variations that may occur. Quantitative factors are generally designed to situate the extreme levels symmetrically around the nominal, as was the case for detection wavelength, voltage, injection time, concentrations of HDMS-β-CD and of camphorSO_3^-. However, for the capillary temperature, since the CE equipment is not dedicated to cool the capillary below the nominal value (15 °C), the selected values (−1, +1) were not symmetrical around the nominal.

3.1.3. Selection of the experimental design

Several designs can be applied to execute a robustness test of analytical methods. The Plackett–Burman designs are frequently used. A two-level Plackett–Burman design [2,27] was elaborated for the seven factors. Eight experimental conditions were generated as indicated in Table 2.

3.1.4. Definition of the experimental set-up

The execution of the study, illustrated in Fig. 2, indicates that firstly, the reference mixture solution was analysed followed by the reference solution (a) and the sample test solutions. For each *S*-timolol maleate sample, two replicate mea-

Table 2
Experimental design

Experiments	Factors						
	Equipment CE	Temperature (°C)	Wavelength (nm)	Voltage (kV)	Injection time (s)	HDMS-β-CD (mM)	Camphorsulfonate (mM)
1	+1	+1	+1	−1	+1	−1	−1
2	−1	+1	+1	+1	−1	+1	−1
3	−1	−1	+1	+1	+1	−1	+1
4	+1	−1	−1	+1	+1	+1	−1
5	−1	+1	−1	−1	+1	+1	+1
6	+1	−1	+1	−1	−1	+1	+1
7	+1	+1	−1	+1	−1	−1	+1
8	−1	−1	−1	−1	−1	−1	−1

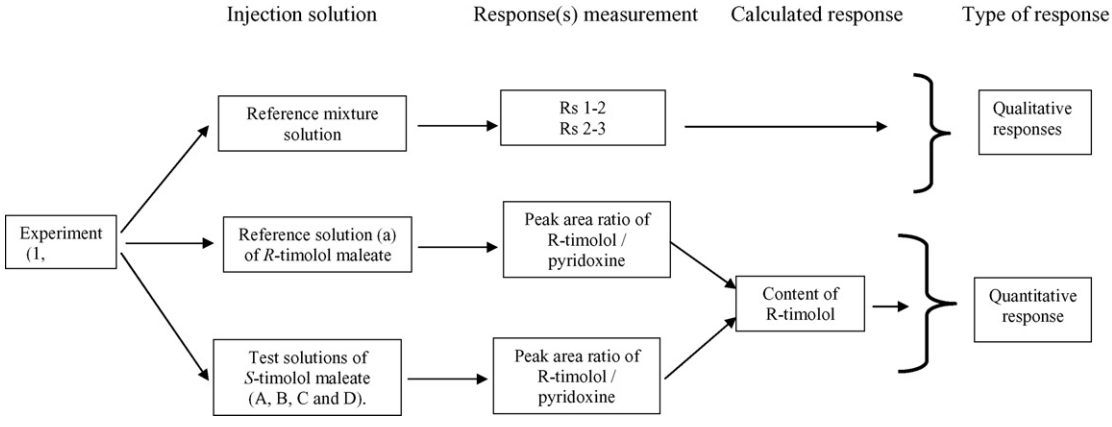

Fig. 2. Schematic representation of the execution of the robustness study.

surements were analysed applying the eight experimental conditions of Table 2. The overall analysis was repeated twice constituting two series. In fact, in the two series, different batches of HDMS-β-CD were used. The design experiments were executed in a random order and in parallel on the two CE instruments.

The temperature required by the design conditions was also used during the prior rinsing and conditioning processes. Before running a given design experiment, the capillary was rinsed with methanol for 5 min, then, conditioned with the corresponding BGE and BGE-CD solutions for 5 min each. Between runs, the capillary was rinsed with methanol for 2 min and conditioned with the corresponding BGE-CD solution for 4 min. Both BGE-CD and BGE solutions were renewed after about 70 min of analysis. At the end of each working day, the capillary was rinsed at 25 °C with methanol for 30 min, with 20 mM ammonium formate methanolic solution for 20 min and again with methanol for 30 min. Capillary wash cycles were performed at a pressure of approximately 1 bar.

3.1.5. Identification and selection of the responses

In this study, the most important response is the quantitative. It concerned the content of *R*-timolol impurity in the four samples of *S*-timolol maleate. The qualitative responses were also considered and were related to the electrophoretic separation between *S*-timolol and pyridoxine (Rs1–2) as well as between the timolol enantiomers (Rs2–3).

3.1.6. Results of the experiments

3.1.6.1. Adequacy of the CE equipment and qualitative responses. At nominal NACE conditions, the three substances are well separated with migration times of 9.8, 11.2 and 12.2 min for pyridoxine, *S*-timolol and *R*-timolol, respectively (Fig. 3). From additional experiments at nominal conditions ($n = 3$), mean values of the resolutions were 9.0 for Rs1–2 and 5.5 for Rs2–3. Those values were considered as starting points to examine the suitability of the CE system under the different experimental conditions of Table 2. It can be noticed that due to the differences in peak heights, it was possible to identify the three peaks and their migration order.

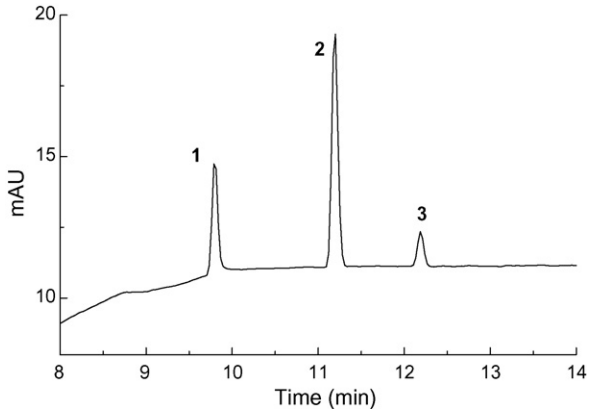

Fig. 3. Typical electropherogram of a reference mixture solution obtained by carrying out the CE separation using a BGE containing 30 mM HDMS-β-CD and applying a voltage of 25 kV (other conditions see in Section 2). Peaks and concentrations: 1, pyridoxine (5 μg/mL); 2, *S*-timolol (10 μg/mL); 3, *R*-timolol (5 μg/mL).

The results for the qualitative responses, presented in Table 3, show that in series 1, the enantioresolution (Rs2–3) value was above the nominal in only one experiment (No. 6), while in experiments 4 and 8 they were close. On the other hand, all Rs1–2 values were above the nominal. In series 2, half of the results (experiments 1, 2, 4 and 6) for Rs2–3 were above the nominal while in experiments 5 and 7 they were near. For Rs1–2, only the values obtained with two experiments (2 and 4) were below the nominal. It can be reminded that in series 1, an old HDMS-β-CD batch was used, and in series 2 a new. In terms of resolution, the separation was found to be affected by the HDMS-β-CD batches. Nevertheless, in spite of some lower Rs2–3 values observed, it still was possible to quantify the *R*-timolol peak in both series.

The migration times for most peaks, except in experiment 2, were somewhat higher in series 1. A possible explanation could be related to the difference in purity between both HDMS-β-CD batches. No certificate of analysis was available to check the purity of the old HDMS-β-CD batch, which moreover, has been stored a long time (about 2 years) at ambient temperature. The migration order observed in both series was the same.

Table 3
Electrophoretic performance: migration times (MT) and resolutions (Rs) calculated for pyridoxine/*S*-timolol and for *S*-timolol/*R*-timolol

Exp. no.	Series 1 (HDMS-β-CD batch 1)					Series 2 (HDMS-β-CD batch 2)				
	Migration time (min)			Rs1–2	Rs2–3	Migration time (min)			Rs1–2	Rs2–3
	1	2	3			1	2	3		
1	11.5	13.8	14.7	13.8	4.9	10.3	11.9	12.9	9.9	5.8
2	8.4	9.3	10.1	16.1	3.5	8.9	9.9	10.8	8.2	6.1
3	9.1	10.9	11.6	12.8	4.2	8.3	9.7	10.2	10.0	4.2
4	9.8	11.4	12.2	10.2	5.1	9.1	10.1	11.1	7.3	5.6
5	11.9	14.4	15.3	14.0	4.7	10.4	12.0	12.9	9.1	5.2
6	11.6	13.7	14.7	12.4	5.8	10.7	12.5	13.5	10.7	5.5
7	8.9	10.7	11.2	12.7	3.8	8.3	9.6	10.3	10.1	4.9
8	11.0	13.0	13.9	12.4	5.2	10.5	12.4	13.1	11.9	4.6

Legend: 1, Pyridoxine; 2, *S*-timolol; 3, *R*-timolol.

Table 4
Repeatability of the migration times (min) evaluated per experiment and per series

	Experiments							
	1	2	3	4	5	6	7	8
Series 1								
I.S.								
Mean	11.1	9.71	8.98	9.73	11.51	11.60	8.92	10.98
R.S.D. (%)	0.87	0.16	1.28	0.28	0.66	0.06	0.17	0.59
R-timolol								
Mean	14.11	12.11	11.42	12.06	14.79	14.73	11.33	13.93
R.S.D. (%)	0.79	0.13	1.53	0.15	0.58	0.14	0.23	0.49
RMT[a]								
Mean	1.27	1.25	1.27	1.24	1.28	1.27	1.27	1.27
R.S.D. (%)	0.12	0.09	0.33	0.13	0.12	0.17	0.22	0.16
Series 2								
I.S.								
Mean	10.38	9.14	8.75	9.30	11.11	11.35	8.56	10.74
R.S.D. (%)	1.09	1.13	2.05	1.14	2.18	1.30	2.66	0.50
R-timolol								
Mean	12.98	11.23	11.06	11.38	14.03	14.46	10.81	13.43
R.S.D. (%)	1.17	1.44	2.72	1.37	2.55	1.49	2.85	0.69
RMT[a]								
Mean	1.25	1.23	1.26	1.22	1.26	1.27	1.26	1.25
R.S.D. (%)	0.13	0.31	0.53	0.24	0.39	0.20	0.53	0.21

[a] RMT, relative migration times.

The repeatability of the migration times was also examined as system suitability test criterion. This was only done for the peaks of pyridoxine and *R*-timolol in the test sample solutions, and was estimated from the duplicates of the eight design experiments. Results, presented per series in Table 4, indicate for the majority of the migration times in series 1 a very constant value (most R.S.D. values below 1.0%), while in series 2 it is somewhat less good (most R.S.D. values above 1.0%). In series 1, only one experiment (No. 3) had a higher variability. In series 2, different variability patterns were seen. Constant migration times were noticed only with experiment 8 (R.S.D. below 1.0%). The migration times observed in experiments 1, 2, 4 and 6 were more varying but still below an R.S.D. value of 1.6%. For experiments 3, 5 and 7, high variabilities were noticed (R.S.D. above 2.0%).

The precision in the eight NACE experimental conditions was also examined in terms of the relative migration times between *R*-timolol and pyridoxine ($t_{M,R\text{-timolol}}/t_{M,\text{pyridoxine}}$). The relative migration times can be considered, in the internal standardisation process, as a suitable indicator to examine the constant velocity of a peak of interest (*R*-timolol) against that of an internal standard (pyridoxine) through the detector under repeatability conditions. It is known that differences in velocities affect the peak area and therefore, the quantification of analyte [28]. As can be seen in Table 4, the % R.S.D. of the relative migration times obtained are below 1%, indicating a good constant velocity within each experiment and for each series.

3.1.6.2. Quantitative responses. From the product specifications obtained from the manufacturer and from previous studies [29], only one impurity (*R*-timolol) was signalled to be present in the *S*-timolol maleate samples. Obviously, the electropherograms obtained (Fig. 4) presented only one impurity peak. Its content was determined by comparing the normalized ratio (NRT) to that from the normalized ratio of *R*-timolol in reference solution (NRR) using the following equation:

$$\text{Content of } R\text{-timolol (\%)} = \frac{\text{NRT}}{\text{NRR}} \times 1\% \quad (1)$$

The ratio of *R*-timolol in the test or reference samples was normalized as follows:

$$\text{Normalized ratio} = \frac{\text{Ratio of test or reference sample} \times 20}{\text{weighed mass (mg)}} \quad (2)$$

The ratio of test or reference samples was obtained using the following calculation:

$$\text{Ratio} = \frac{\text{Corrected area of } R\text{-timolol in test or reference sample}}{\text{Corrected area of pyridoxine in test or reference sample}} \quad (3)$$

the corrected area being obtained by dividing the peak area over the peak migration time.

The individual contents estimated for *R*-timolol in the two series (Table 5) are fluctuating between 0.54–0.72, 0.26–0.39, 0.18–0.32 and 0.04–0.15% in samples A, B, C and D, respectively, and were distributed around the mean values obtained under the nominal conditions (0.67, 0.37, 0.33 and 0.12% for samples A, B, C and D, respectively). These values were all below 1.0%, the maximum allowable content of this impurity as specified in the European Pharmacopoeia monograph of *S*-timolol maleate [26]. However, the variability sometimes was found high, particularly for sample D, the sample with the lowest *R*-timolol content. Indeed, the ratio value between the highest

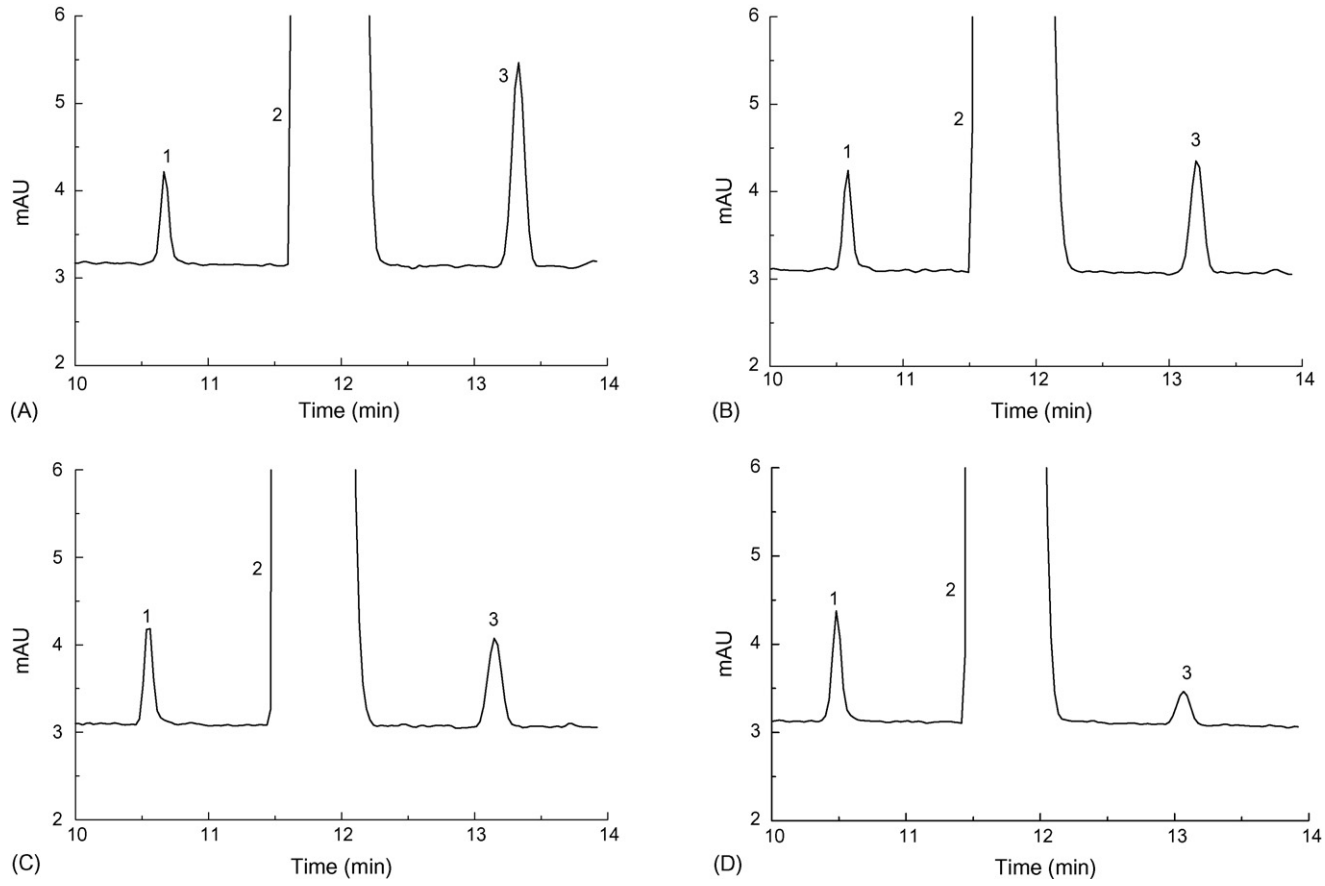

Fig. 4. Typical electropherograms of test sample solution A (A), test sample solution B (B), test sample solution C (C) and test sample solution D (D) obtained by carrying out the separation using the nominal NACE conditions (see in Section 2). Peaks and concentrations: 1, pyridoxine (5 μg/mL); 2, *S*-timolol (about 2.0 mg/mL); 3, *R*-timolol (A = 12.4 μg/mL, B = 6.8 μg/mL, C = 5.2 μg/mL and D = 2.2 μg/mL).

and the lowest *R*-timolol content is about 3.8 while in the other samples it is lower (1.3, 1.5 and 1.7, in samples A, B and C, respectively). Thus, a closer examination of those results was done considering the precision in terms of relative standard deviations per experiment and per series.

The precision of *R*-timolol content for each sample was evaluated by considering the pooled R.S.D. per experiments and the R.S.D. between series. One can see from Table 5 that the pooled R.S.D.'s are inferior to the acceptance limits of 10% [30] for all the experiments for samples A and B but not for samples C and D. Furthermore, for the sample D only one experiment (No. 2) had a value inferior to 10% [30]. This illustrates that the quantification of *R*-timolol in samples C and D is variable within each experiment whatever the series represented here by the difference of HDMS-β-CD batch. However, when looking at the R.S.D. between series, one can see that the values exceeded 10% even for the samples with small pooled R.S.D. Therefore, the use of different HDMS-β-CD batches was found to affect the precision of *R*-timolol content. It can be noticed that very high values of the R.S.D. value (i.e. 31%) between series were observed in sample D which is largely above the acceptance limits [30]. For that sample, the effect of the cyclodextrin batch could be explained by the very low concentration of *R*-timolol. Thus, in term of precision of *R*-timolol content, the robustness of the NACE method was found to be affected once more by the difference of HDMS-β-CD batches.

3.1.7. Statistical evaluation of the factor effects

The effect of each factor *X* was calculated using the equation:

$$E_x = \frac{\sum Y(+1)}{n_i} - \frac{\sum Y(-1)}{n_j} \quad (4)$$

where $\sum Y(+1)$ and $\sum Y(-1)$ are the sums of the responses when the factor *X* is at level +1 and −1, respectively, n_i and n_j the number of design runs with the factor at levels +1 and −1, respectively. The significance of each factor effect was evaluated statistically by comparison with a critical effect (E_{critical}) derived from a *t*-test [5,6,31]:

$$E_{\text{critical}} = t_{\text{critical}}(\text{S.E.})_{\text{est}} \quad (5)$$

where $(\text{S.E.})_{\text{est}}$ is the estimated standard error on an effect and t_{critical} the relevant tabulated *t*-value. An effect is considered significant if its absolute value is larger than the critical effect (or the corresponding *p*-value < α). The standard error (S.E.) was estimated from replicated experiments at nominal conditions [5,6,31].

Table 5
Contents (in %) of *R*-timolol in test samples A, B, C and D

	Sample A				Sample B				Sample C				Sample D			
	Series	Results	Pooled R.S.D. (%)	Between series R.S.D. (%)	Series	Results	Pooled R.S.D. (%)	Between series R.S.D. (%)	Series	Results	Pooled R.S.D. (%)	Between series R.S.D. (%)	Series	Results	Pooled R.S.D. (%)	Between series R.S.D. (%)
1	1	0.62	3.3	4.1	1	0.36	4.0	18.1	1	0.27	17.2	15.5	1	0.11	31.8	30.9
	1	0.66			1	0.34			1	0.29			1	0.11		
	2	0.59			2	0.27			2	0.27			2	0.10		
	2	0.62			2	0.27			2	0.18			2	0.04		
2	1	0.63	1.9	1.2	1	0.35	5.0	3.7	1	0.29	1.2	7.7	1	0.11	5.5	12.2
	1	0.64			1	0.37			1	0.29			1	0.11		
	2	0.65			2	0.33			2	0.26			2	0.10		
	2	0.63			2	0.36			2	0.26			2	0.09		
3	1	0.72	3.7	8.9	1	0.39	1.5	13.0	1	0.32	4.2	9.6	1	0.11	13.0	9.6
	1	0.71			1	0.39			1	0.29			1	0.11		
	2	0.60			2	0.32			2	0.27			2	0.11		
	2	0.65			2	0.33			2	0.27			2	0.09		
4	1	0.63	4.9	7.3	1	0.38	6.3	19.8	1	0.26	10.1	18.9	1	0.10	26.8	21.8
	1	0.65			1	0.38			1	0.30			1	0.10		
	2	0.61			2	0.30			2	0.23			2	0.10		
	2	0.55			2	0.26			2	0.20			2	0.05		
5	1	0.72	3.1	2.9	1	0.37	3.9	4.5	1	0.28	5.9	2.6	1	0.12	13.1	10.9
	1	0.70			1	0.39			1	0.31			1	0.11		
	2	0.70			2	0.35			2	0.29			2	0.12		
	2	0.66			2	0.37			2	0.28			2	0.15		
6	1	0.66	1.5	14.5	1	0.34	3.5	12.1	1	0.28	3.5	19.4	1	0.12	13.0	18.1
	1	0.68			1	0.36			1	0.30			1	0.11		
	2	0.55			2	0.29			2	0.22			2	0.10		
	2	0.54			2	0.30			2	0.22			2	0.08		
7	1	0.64	2.6	4.0	1	0.37	5.9	11.1	1	0.29	2.0	20.4	1	0.11	10.4	10.3
	1	0.67			1	0.38			1	0.30			1	0.11		
	2	0.63			2	0.30			2	0.22			2	0.11		
	2	0.61			2	0.34			2	0.22			2	0.09		
8	1	0.65	4.4	3.3	1	0.38	2.7	7.4	1	0.29	2.4	5.7	1	0.11	19.0	4.0
	1	0.69			1	0.37			1	0.30			1	0.11		
	2	0.66			2	0.35			2	0.28			2	0.14		
	2	0.62			2	0.33			2	0.27			2	0.10		
General mean (%) (R.S.D.)	0.64 (7.1%)				0.34 (10.72%)				0.27 (12.51%)				0.10 (20.01%)			

Table 6
Statistical significance (p-value*) of the factor and the series effects on the quantitative responses

Factors	*R*-timolol content			
	Sample A	Sample B	Sample C	Sample D
A—Type of CE equipment	**0.0002**	**0.0002**	**0.0005**	**0.0240**
B—Capillary temperature	0.2416	0.6660	1.0000	0.6335
C—Detection wavelength	0.1627	0.0392	0.7494	0.2216
D—Voltage	0.5537	0.3460	0.6323	0.2216
E—Injection time	0.1627	0.6660	0.8730	0.5055
F—HDMS-β-CD	0.4088	0.9311	0.6323	0.9239
G—CamphorSO_3^-	0.0526	0.1101	0.3421	0.1140
Series 1–2	**<0.0001**	**<0.0001**	**<0.0001**	0.0793

* The significant values at the 5% level are printed in boldface type.

3.1.8. Conclusions from the statistical analysis

3.1.8.1. Quantitative responses. Table 6 indicates that only the equipment has a significant effect on the content of *R*-timolol which was observed for all samples. Since in the two instruments, different software is used, it can be assumed that the parameters related to the software could be the sources of the significance of the quantitative results [32]. However, the chemical (concentrations of BGE and BGE-CD) and physical parameters (capillary temperature, detection wavelength, voltage and injection time) do not have significant effect on the quantitative results. Thus, it can be concluded that for those parameters, the NACE assay for the content of *R*-timolol is robust.

Since the precision of *R*-timolol content was found to be affected by the difference of HDMS-β-CD batches, it was interesting to examine statistically the effects of series. Table 6 shows that only the content of *R*-timolol in sample D was not significantly affected, confirming that the NACE method is affected by the HDMS-β-CD batches.

3.1.8.2. Qualitative responses. Graphical evaluation was done for the qualitative responses. From the effect plots (Fig. 5), it can be noticed that the most important effects on the enantiomeric resolution were observed mainly with voltage (negative effect), concentration of HDMS-β-CD (positive effect) and equipment, and to a lesser extent with temperature and the camphorSO_3^- concentration. The wavelength and the injection time seem not to affect that resolution.

For the resolution between pyridoxine and *S*-timolol, the effects observed are smaller. The concentration of camphorSO_3 seems not to affect that resolution.

In particular, an opposite effect of HDMS-β-CD concentration on both resolutions was observed.

3.2. Uncertainty assessment

One can consider each of the eight experiments elaborated from the Plackett–Burman design as eight laboratories that have worked under conditions slightly different from the nominal. Each different batch (series) executed for a given design experiments (Fig. 1A) can be considered as simulating another laboratory, resulting in 8 different values. Then, consequently, by adapting the ISO 5725-2 guide [24], the uncertainty of the *R*-timolol content can be determined from the robustness study. The following steps described in the ISO guide were applied: (i) detection of outliers, (ii) test of the variance homogeneity, (iii) calculation of the variance estimates and (iv) estimation of the different uncertainty components.

3.2.1. Detection of outliers

In an interlaboratory study, when suspected deviating values are observed, it is recommended to perform several tests for scrutiny consistency and outliers. In this study, since some remarkable deviation values were observed for the *R*-timolol content, the tests described in the ISO 5725-2 guideline were applied to evaluate whether those values are outliers. Firstly, laboratories (here design experiments) can have deviating values compared to the other laboratories. These outlying laboratories

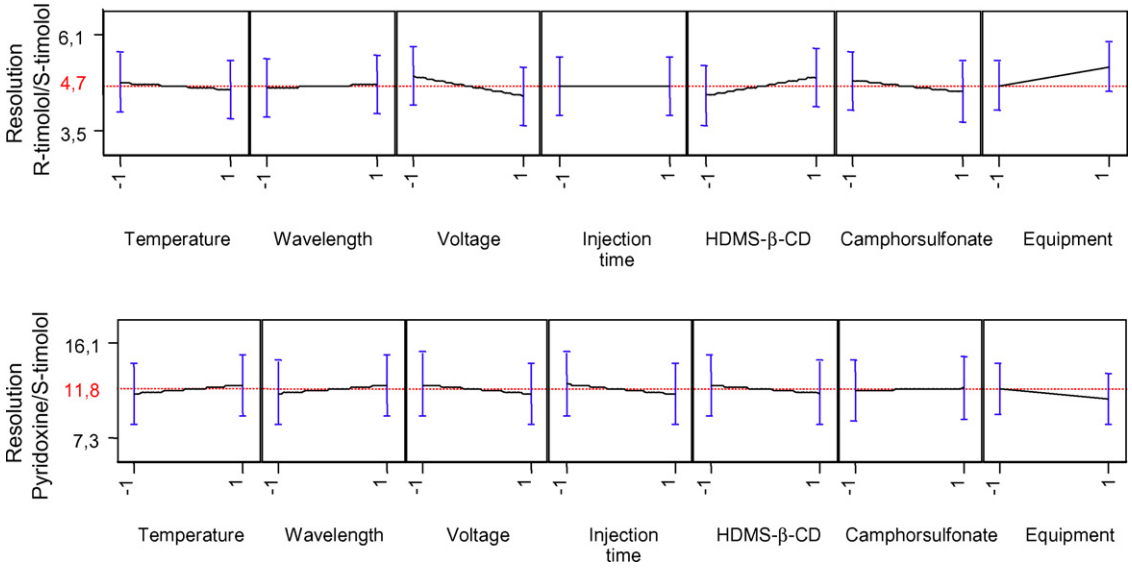

Fig. 5. Effect plots for the different factors on the resolution between *R*-timolol/*S*-timolol and pyridoxine/*S*-timolol.

Table 7
Results of the Grubbs' tests

Grubbs' tests	Calculated values				Critical values, $r = 8$	
	Sample A	Sample B	Sample C	Sample D	5%	1%
G_1	0.55	0.86	0.56	0.94	2.13	2.27
G_r	1.07	0.70	0.59	0.90	2.13	2.27
$G_{1,2}$	1.03	1.03	1.02	1.03	0.11	0.06
$G_{r,r-1}$	1.01	1.01	1.01	1.01	0.11	0.06
G	18.5	10.2	11.8	15.6	64.5	74.9
Outliers	–	–	–	–	–	

Legend: (–) no outliers nor stragglers.

(design experiments) are identified by Grubbs' tests. The different formulas applied in those tests are detailed elsewhere [24,33–36]. The test for one suspected outlier, i.e. smallest values G_1 or largest G_r, or for the polar pair ($G_{1,r}$) is significant if the calculated G-value is larger than the corresponding critical value. In the case of two suspected extreme observations ($G_{1,2}$ and $G_{r,r-1}$), the test is significant when the calculated G-value is smaller than the corresponding critical value.

As can be seen in Table 7, the calculated G-values were found not to be significant. It can be concluded that there are no outlying values in the set of data values reported. Therefore, all data remained for further statistical analysis.

3.2.2. Test of the variance homogeneity

A second type of outlying values are those for which the within-laboratory variance (here between-replicates or the within-experiments) is larger than that in the other laboratories (here experiments). Such outlying values are identified by means of a Cochran test [24]. For a given set of p standard deviations, s_i, all computed from the same number (n) of replicate results, the Cochran test statistic, C, is calculated as:

$$C = \frac{s_{\max}^2}{\sum_{i=1}^{p} s_i^2} \quad (6)$$

where $s_{\max}$ is the highest standard deviation of the set. When the calculated value of C is larger than the 1% critical value, the standard deviation (variance) is considered to be an outlier, while it is considered to be a straggler when the C value is smaller than the 1% value but larger than the 5% one.

For the four *S*-timolol maleate samples (A, B, C and D), the calculated C values being 0.41, 0.21, 0.25 and 0.34, respectively, were found to be below the critical 5% (0.52) and 1% (0.62) values, respectively. It can be concluded that the within-experiment variabilities observed for the four samples are similar and consequently all results could be used for the calculation of the variance estimates.

3.2.3. Calculation of the variance estimates

The variance estimates were calculated from an Anova table (Table 8). First the different mean squares are calculated, i.e. between-experiments mean square ($MS_{Experiments}$), between-series mean square (MS_{Series}) and between-replicates mean square ($MS_{Replicates}$) for the content of *R*-timolol in the four analyzed samples. Then, the estimates of the different variances could be calculated from these mean squares using the formulas indicated in Table 8 and taking into account the set-up of the study illustrated in Fig. 1A.

The different estimated variances presented in Table 9 show that different patterns were noticed. As logically expected because of the deliberate variations applied in the eight experimental CE conditions, the between-experiments variance ($s^2_{Experiments}$) was expected to be the highest, however, it was the lowest in all *S*-timolol maleate samples (values below 0.1%). This means that the overall variability of *R*-timolol content is not due to the experiment but to other sources of variability (Table 9). The deviations observed in the content of *R*-timolol from one experimental condition to another one are lower than those observed in those sources of variability. However, when considering the difference in series that are representing, namely the difference in HDMS-β-CD batches, it can be remarked that the variance of series was of first importance in samples B and C and of second importance in samples A and D, indicating that the difference in the HDMS-β-CD batch-to-batch is affecting the overall variability of *R*-timolol content. On the other hand, concerning the replicates, the estimated variances ($s^2_{Replicates}$)

Table 8
Analysis of variance components

Sources of variability	Mean squares	Estimated variances
Experiments	$MS_{Experiments} = \frac{cg\sum(\bar{x}_i - \bar{x})^2}{r-1}$	$s^2_{Experiment} = \frac{MS_{Experiment} - MS_{series}}{cg}$
Series	$MS_{Series} = \frac{g\sum\sum(\bar{x}_{ij} - \bar{x}_i)^2}{r(c-1)}$	$s^2_{Series} = \frac{MS_{Series} - MS_{Replicates}}{g}$
Replicates	$MS_{Replicates} = \frac{\sum\sum\sum(x_{ijk} - \bar{x}_{ij})^2}{rc(g-1)}$	$s^2_{Replicates} = MS_{Replicates}$

g, number of replicates per series; c, number of series per experiment; r, number of experiments.

Table 9
Estimation of the variance components

Sources of variability	Sample A	Sample B	Sample C	Sample D
Variance component				
Experiments ($s^2_{Experiment}$)	$<0.01 \times 10^{-4}$	0.019×10^{-4}	$<0.01 \times 10^{-4}$	0.075×10^{-4}
Series (s^2_{Series})	11.55×10^{-4}	13.68×10^{-4}	9.72×10^{-4}	0.485×10^{-4}
Replicates ($s^2_{Replicates}$)	15.16×10^{-4}	6.94×10^{-4}	6.52×10^{-4}	3.53×10^{-4}
Reproducibility variance (s^2_R)	26.72×10^{-4}	20.64×10^{-4}	16.23×10^{-4}	4.088×10^{-4}
Ratio reproducibility/repeatability	1.8	3.0	2.5	1.2

were not negligible at all. They were even of first importance in samples A and D. In this last situation, the noise due to analysis seems to influence highly the quantification of *R*-timolol at low concentration near to the limit of detection that is 0.1%.

3.2.4. Estimation of the different uncertainty components

The variances for repeatability (s^2_r) and reproducibility (s^2_R) were calculated using the following equations:

$$s^2_r = s^2_{Replicates} \quad (7)$$

$$s^2_R = s^2_{Replicates} + s^2_{Series} + s^2_{Experiments} \quad (8)$$

The s^2_r values obtained were 16.16×10^{-4}, 6.94×10^{-4}, 6.52×10^{-4} and 3.53×10^{-4} for the *R*-timolol content in samples A, B, C and D, respectively. From the literature, the ratio between the reproducibility and repeatability variances is expected to be between 2 and 4 [37] meaning that the between-laboratories variance (here represented by the between-experiments variance) is similar in magnitude or larger than the repeatability variance. Here, the calculated ratio was within the expectancy for the samples B (3.0) and C (2.5), and near for the sample A (1.8) while it was below the expectancy for the sample D (1.2). In this sample, the variability observed for replicates is somewhat similar to the overall variability indicating that at lower concentration near to the limit of detection (0.1%), the precision of NACE method is more affected by other sources of variability than those evaluated in this study.

The reproducibility represented by s^2_R, allowed calculating the standard uncertainty u_x using the equation:

$$u_{\bar{x}} = s_R \quad (9)$$

The standard uncertainty is $u_x = s_R = 0.0517$, 0.0449, 0.0403 and 0.0215%, and the expanded uncertainty using a coverage factor of $k = 2$ [20] becomes $U_x = 2u_x = 0.103$, 0.091, 0.081 and 0.040% for *R*-timolol content in samples A, B, C and D, respectively. Considering the nominal content of *R*-timolol in those samples that are 0.66, 0.35, 0.29 and 0.10% [9], respectively, the uncertainty seems to be concentration dependent.

According to the ISO guideline [24], one has to establish a functional relationship between precision values and the mean content by concentration. As suggested by ISO, the following relationships were investigated namely the straight line through the origin, the straight line with a positive intercept and the exponential relationship. Only the last one (logarithmic transformation) was found to correlate adequately the relationship between the precision value (s_R) and the content of *R*-timolol. Indeed, the corresponding equation ($y = 1.75x + 4.47$) presents an acceptable correlation coefficient ($R = 0.963$), with y the neperian logarithm of the standard deviation (s_R) and x the mean content of *R*-timolol. The linear correlation was also acceptable ($R = 0.891$).

Finally, for a single result x obtained with the tested NACE method, the results for *R*-timolol content in *S*-timolol maleate samples containing this impurity with a concentration similar to that of sample A (i.e. about 0.66%) is expected to be $x \pm 0.103\%$. For sample B (i.e. about 0.35%), sample C (i.e. about 0.29%) and sample D (i.e. about 0.10%), the expected single result will be $x \pm 0.09\%$, $x \pm 0.08\%$ and $x \pm 0.04\%$, respectively. This means that when analysing *S*-timolol maleate samples for which the content of *R*-timolol are similar to those of samples A, B, C and D, 95% of the measurement should be encompassed within the interval 0.56–0.76, 0.26–0.44, 0.21–0.37, and 0.06–0.14%, for those samples, respectively. Considering the results of Table 4 it can be noticed that the measurements fulfilled this expectation since measurements outside of these ranges occurred only in 3 out of 32 times for the *R*-timolol concentration of samples A and D, and only in 2 out of 32 times in sample B, while for sample C all the measurements were included in the range.

3.2.5. Comparison of the different uncertainty components from different studies

To confirm the appropriateness of the obtained uncertainty estimates, they were compared with those obtained from an interlaboratory study while analyzing the same or similar samples under the same analytical procedure. The expanded uncertainties obtained in the interlaboratory study were 0.160, 0.081, 0.084 and 0.082% for *R*-timolol content in samples A, B, C and D, respectively [15]. These estimates were compared by mean of an *F*-test. The *F* values calculated for samples A ($F = 1.56$), B ($F = 0.91$), C ($F = 1.05$) and D ($F = 3.82$) were found to be below the critical value ($F = 3.44$ at $\alpha = 0.05$). Therefore, it can be concluded that no significant difference was observed between the four estimates obtained in the two studies at the four *R*-timolol concentration levels involved. The robustness study could be proposed in this case as an alternative of the interlaboratory study to estimate the measurement uncertainty.

4. Conclusion

In this paper the robustness of the NACE method developed for the enantiomeric purity determination of *R*-timolol in

S-timolol maleate samples was evaluated. The qualitative and quantitative selected factors were found to have an influence on the resolutions but still, the *R*-timolol impurity was quantified without problem. Except for the type of instrument, the *R*-timolol contents determined were not significantly affected by the selected factors demonstrating the robustness of the analytical method. It was noticed that the different HDMS-β-CD batches are influencing both qualitative and quantitative results. Thus, a warning related to that parameter has to be introduced in the procedure as well as related to the use of different equipment.

Since the eight experiments elaborated from the Plackett–Burman design could be assimilated to laboratories from an interlaboratory exercise, different uncertainty components were estimated using the data obtained from the robustness test. The observed uncertainty values were found to be appropriate. Their comparison with the interlaboratory uncertainty estimates were found not significantly different. Thus this robustness test based approach for the estimation of the uncertainty is advantageous as no interlaboratory study is required anymore.

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