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ABSTRACT

Supercritical Fluid Chromatography (SFC) has known a strong regain of interest for the last 10 years, especially in the field of pharmaceutical analysis. Besides the development and validation of the SFC method in one individual laboratory, it is also important to demonstrate its applicability and transferability to various laboratories around the world. Therefore, an inter-laboratory study was conducted and published for the first time in SFC, to assess method reproducibility, and evaluate whether this chromatographic technique could become a reference method for quality control (QC) laboratories. This study involved 19 participating laboratories from 4 continents and 9 different countries. It included 5 academic groups, 3 demonstration laboratories at analytical instrument companies, 10 pharmaceutical companies and 1 food company. In the initial analysis of the study results, consistencies within- and between-laboratories were deeply examined. In the subsequent analysis, the method reproducibility was estimated taking into account variances in replicates, between-days and between-laboratories. The results obtained were compared with the literature values for liquid chromatography (LC) in the context of impurities determination. Repeatability and reproducibility variances were found to be similar or better than those described for LC methods, and highlighted the adequacy of the SFC method for QC analyses. The results demonstrated the excellent and robust quantitative performance of SFC. Consequently, this complementary technique is recognized on equal merit to other chromatographic techniques.

KEYWORDS

Supercritical Fluid Chromatography (SFC), inter-laboratory study, collaborative study, reproducibility, pharmaceutical impurities, salbutamol sulfate

1. INTRODUCTION

From its first commercialization in the 1980s, SFC has the reputation to be poorly reproducible and robust. However, the performance of modern SFC instruments has significantly improved since 2012 and it can now be considered as a well-established technology in pharmaceutical research/discovery environments [1,2]. Despite these instrumental advances, its application in more regulated laboratories still seems to be considered risky and this perception may continually hamper the implementation of routine SFC methods in QC environments.

Recently, several studies highlighted the excellent quantitative performance of SFC in the pharmaceutical domain [3-6]. The published methods were fully validated according to ICH Q2 guidelines and demonstrated the applicability of modern SFC in the context of pharmaceutical quality control. Nevertheless, the evaluation of method precision was limited to repeatability and intermediate precision, as the validation protocol included only one equipment/laboratory. To properly evaluate method reproducibility, the between-laboratories variability should also be studied by means of an inter-laboratory assay [7,8]. This evaluation is required when the analytical method has to be transferred and used in different laboratories, or is introduced as a reference method in a monograph. Inter-laboratory studies are well described in the literature for chromatographic techniques, especially liquid chromatography [9-11]. To the best of our knowledge, such data have never been published for SFC.

In a previous paper, a robust SFC method was developed for the determination of salbutamol sulfate related impurities according to the Quality by Design principles [6]. For this purpose, Design Space determination was employed to find out a robust working zone [12]. The optimization of a robust method was indeed a keystone to guarantee successful method transfer to several laboratories. Moreover, the developed method was fully validated according to the total error approach for the quantitative determination of impurities B, D, F and G, down to a concentration level of 0.3% of active pharmaceutical ingredient, in agreement with the

specifications of pharmacopoeai method. This predictive validation strategy follows the requirements of ICH Q2 (R1) guideline and ensures that every future analysis result will fall within the acceptance limits (i.e. +- 15 % for impurities determination) with at least a probability of 95 %. Method development and validation were performed considering the salbutamol impurities available as chemical reference standard (namely impurity B, D, F, I and G). To propose this method as a normative method, an inter-laboratory study is a mandatory step.

The objective of the present study was to estimate the precision (repeatability and reproducibility) of the results obtained for the determination of impurity D in salbutamol sulfate samples. The study protocol was proposed following the ISO 5725-2 international standard [8]. A detailed protocol was established to study the sources of variability at different levels, i.e. replicates, days and laboratories. In this study, all experiments were conducted on one single type of instrument to avoid potential problems related to the delivery of a compressible fluid, backpressure control, and injection mode, often observed in SFC. Several academic, demonstration and industrial laboratories equipped with SFC technology were selected to take part in this study.

To ensure proper instrument handling and method set-up, a preliminary method test was organized to get familiar with the method and to verify various criteria, i.e. method selectivity, sensitivity and system repeatability. The results of this test were collated and evaluated by the study coordinator before starting the quantitative study.

In the following study the content of impurity D was evaluated in three independent salbutamol sulfate samples. These samples at different concentration levels for impurity D aimed at covering the validated dosing range. The results of this study were analysed according to the ISO guidelines [8]. Finally, the quantitative data issued from this study were used to assess measurement uncertainty.

2. MATERIAL AND METHODS

2.1 Chemicals and reagents

Salbutamol hemisulfate (> 98.0 %) was purchased from TCI Europe (Zwijndrecht, Belgium) and used as salbutamol hemisulfate standard. Related impurities B, D, F, G and I were provided by EDQM (Strasbourg, France). Salbutamol hemisulfate was split in three batches and each batch was spiked with different amount of related impurity D to get three salbutamol samples. The minimal quality requirements for solvents and reagents were: methanol gradient grade, 2-propanol analytical grade, water ULC-MS/SFC grade, ammonium hydroxide 25 or 28 % w/w analytical grade, carbon dioxide 99.995 %.

2.2 Instrumentation

Each laboratory used a Waters Acquity UPC$^{2®}$ equipped with a PDA detector (Waters, Milford, MA, USA). If MS or another detectors were hyphenated to the chromatographic system, they were disconnected prior to the experiments. The injector was equipped with a 5 or 10 µL loop operating in the partial loop with needle overfill mode. 2-propanol (900 µL) and water/methanol (50/50, v/v) (500 µL) were used as weak and strong needle wash solvents, respectively. Chromatograms were recorded at 220 nm in compensated mode (310-410 nm) with an acquisition frequency of 20 Hz, a resolution of 1.2 nm and a filter time constant of 0.5 s. Masslynx^{TM} or Empower^{TM} software was used to control the system and acquire the data.

2.3 Chromatographic conditions

SFC conditions were reported in a previous publication [6]. The UPC^2 Torus Diethylamine (DEA) 100 × 3.0 mm (particle size of 1.7 µm) analytical column was used. One new column was provided to each laboratory and was used immediately to perform the present study. The experiments were executed at a flow rate of 1.5 mL/min and 0.1 % v/v ammonium hydroxide in methanol was used as modifier. The gradient mode was applied, with an initial modifier fraction of 2 %, followed by a linear increase to 35 % in 6.5 min. Post-run: the initial mobile

phase conditions were reached within 0.5 min followed by 3 min of re-equilibration (total run time 10 min). The backpressure regulator was set at 135 bar (1958 psi). The autosampler temperature and the injected volume were set at 6°C and 2 µL, respectively.

2.4 Sample preparation

All solutions were prepared in water/methanol 20/80 v/v. After preparation, all solutions were stored in the dark at 5°C (± 3°C).

2.4.1 Preliminary testing

Stock solution containing impurities was prepared by transferring accurately weighed amounts of 5 mg impurity B, 5 mg impurity D, 5 mg impurity F and 5 mg impurity G in a volumetric flask of 50.0 mL. Intermediate solution was prepared by weighing an accurate amount of 20 mg salbutamol sulfate and adding 600 µL stock solution in a volumetric flask of 10.0 mL. Then the content of one vial of impurity I was dissolved with 1.0 mL of intermediate solution. This latter solution containing salbutamol sulfate and all related impurities is used to perform the preliminary test (system suitability test (SST) solution).

2.4.2 Inter-laboratory study

Stock solution of impurity D was prepared by adding an accurately weighed amount of 5 mg of impurity D in a volumetric flask of 5.0 mL. Then, calibration standards at 4, 6 and 8 µg mL^{-1} were prepared by means of dilutions (40, 60 and 80 µL of stock solution respectively in a volumetric flask of 10.0 mL).

Each lab received three salbutamol samples labelled sample A, B and C. These latters contained different amount of impurity D to evaluate the validated dosing range during this study: 0.2 % of impurity D in salbutamol sulfate (sample B), 0.3 % of impurity D (sample C) and 0.4 % of impurity D (sample A). The study was performed in a blind way as the laboratories did not known samples concentration and level. Sample solution was prepared by adding an accurately weighed amount of 20 mg salbutamol sulfate unknown solid sample in a volumetric flask of

10.0 mL. Three independent solutions were prepared for each sample and this protocol was repeated on three days.

2.5 Preliminary testing

The first step of this collaborative study was to perform a familiarisation experiment, which allowed also checking the reliability of SFC instruments for further quantitative analysis. This preliminary testing was implemented to verify several performance criteria: selectivity, retention times stability, peak area variability and sensitivity. To verify method selectivity and sensitivity, SST solution was injected 6 times using the above described SFC method,. The RSD values should be < 1% for retention times (for all compounds) and < 2 % for peak areas (only for impurities). The signal-to-noise ratio (S/N) was calculated for impurity D as described by USP:

$$S/N = 2H/h \quad (1)$$

where H is the height of the peak measured from the peak apex to a baseline extrapolated over a distance ≥5 times the peak width at its half-height; and h is the amplitude of the noise values observed over a distance ≥ 5 times the peak width at half-height and, if possible, situated equally around the peak of interest. The S/N ratio should be higher than 25.

2.5 Set-up of the inter-laboratory study

The study involved 19 participating analytical laboratories (p =19): 5 academic (universities), 3 demonstration laboratories at analytical instrument company, 1 food and 10 pharmaceutical companies. These laboratories are located on 4 continents and in 9 countries. These 19 sites present different quality standard: GMP for the sending lab, ISO 9001 for the food company lab, GMP for several pharmaceutical companies and R&D instrument in GMP environment for the others. Academic and demonstration laboratories do not have any certification. Each laboratory performed the analyses in three different days (series) (c = 3). Per day, the samples were prepared and analysed independently in triplicate (g = 3) considering 3 concentrations

202 levels (q = 3) by means of samples A, B and C. The study layout per concentration (sample) is
203 summarized in fig. 1.
204 This study layout enables the inclusion of day/series variability as generally done in a method
205 validation protocol. It allows estimating the intermediate precision for each laboratory, which
206 is the sum of intra-day and inter-day variances. The study layout provides information on three
207 sources of variability (i.e. replicates, days, laboratories), which are the main components of
208 method reproducibility. Each laboratory reported raw data in a validated and locked Excel file.
209 The study coordinator performed all data and statistical analyses using Excel (Microsoft
210 Excel® for Mac 2011) followed by a report verification by the study supervisors.
211 2.6 Statistical analysis
212 2.6.1 Scrutiny of results for consistency and outliers
213 First, the results were critically examined for outliers and stragglers regarding between-
214 laboratory and within-laboratory consistency. This examination was done by graphical
215 consistency techniques and numerical outlier tests specified in the ISO guidelines [8,13]. Tables
216 with critical values for all mentioned tests can be found in the ISO guidelines [8]. Mandel's *k*
217 plotting and Cochran's test were used to verify whether the within-laboratory variances of some
218 laboratories were not considerably larger than in the other participating laboratories. Mandel's
219 *k* statistic was calculated as:

220 $$k_{ij} = \frac{s_{ij}\sqrt{p_j}}{\sqrt{\Sigma s_{ij}^2}} \quad (2)$$

221 where s_{ij} is the standard deviation within one cell (laboratory) at concentration level j and p_j is
222 the number of laboratory reporting test result for concentration level j.
223 Mandel's *k* values were plotted to graphically evaluate the within-laboratory variation. The
224 indicator values at 1% and 5 % significance levels were drawn on the Mandel's plots.
225 The Cochran's test was applied as numerical outlier test and calculated as followed:

$$C = \frac{s^2_{max}}{\sum_{i=1}^{p} s_i^2} \quad (3)$$

where s_{max} is the highest variance obtained for one sample and s^2_i is the variance within one laboratory for this sample. The variance is considered to be an outlier when C is larger than the 1 % critical value and a straggler when C is smaller than the 1% critical value but larger than the 5% one. Outliers were noted ** and stragglers * in the results tables.

Mandel's *h* plotting and Grubb's tests were used to verify whether laboratories with deviating results compared to those of the others (between-laboratory variance consistency) occur. Mandel's *h* statistic was calculated as:

$$h_{ij} = \frac{\overline{x_{ij}} - \overline{\overline{x_j}}}{\sqrt{\frac{1}{p_j - 1}\sum_{i=1}^{p_j}(\overline{x_{ij}} - \overline{\overline{x_j}})^2}} \quad (4)$$

where $\overline{x_{ij}}$ represents a cell (laboratory) mean and $\overline{\overline{x_j}}$ the general mean for concentration level j. Mandel's *h* values were also plotted to graphically evaluate the between-laboratory variation. The Grubb's tests were finally used as a numerical outlier tests. They are structured in four subsequent tests. First, the test to determine whether the largest observation ($\overline{x_p}$) is an outlier:

$$G_p = \frac{\overline{x_p} - \overline{\overline{x_j}}}{\sqrt{\frac{1}{p_j - 1}\sum_{i=1}^{p_j}(\overline{x_{ij}} - \overline{\overline{x_j}})^2}} \quad (5)$$

Simultaneously, the test is used to determine whether the smallest observation ($\overline{x_1}$) is an outlier:

$$G_1 = \frac{\overline{\overline{x_j}} - \overline{x_1}}{\sqrt{\frac{1}{p_j - 1}\sum_{i=1}^{p_j}(\overline{x_{ij}} - \overline{\overline{x_j}})^2}} \quad (6)$$

When the single Grubb's test is negative, then the equivalent double Grubb's test is performed. This Grubb's test is used to examine whether either the two largest ($G_{p-1,p}$) or two smallest ($G_{1,2}$) observations are outliers

$$G_{p-1,p} = \frac{\sum_{i=1}^{p-2}(\overline{x_{ij}} - \overline{\overline{x_{p-1,p}}})^2}{\sum_{i=1}^{p_j}(\overline{x_{ij}} - \overline{\overline{x_j}})^2} \quad (7)$$

$$G_{1,2} = \frac{\sum_{i=3}^{p}(\overline{x_{ij}} - \overline{\overline{x_{1,2}}})^2}{\sum_{i=1}^{p_j}(\overline{x_{ij}} - \overline{\overline{x_j}})^2} \quad (8)$$

where $\bar{\bar{x}}_{p-1,p}$ is the average of the two largest observations and $\bar{\bar{x}}_{1,2}$ of the two smallest observations in the data set. For the single Grubb's test, outliers and stragglers gives rise to values exceeding the 1 % and 5 % critical values respectively. For the double Grubb's test, outliers and stragglers gives rise to values smaller than the 1 % and 5 % critical values respectively.

2.6.2 Variances estimation

After testing and discarding the outliers, the mean squares between laboratories ($MS_{laboratories}$), between days (MS_{days}) and between replicates ($MS_{replicates}$) were calculated applying the variance analysis detailed in table 1.

From the means squares, the repeatability (s^2_r), between-laboratories ($s^2_{laboratories}$) and reproducibility variances (s^2_R) were estimated [9].

According to the ISO 5725-2 guidelines, the calculation of the repeatability (s^2_r) and reproducibility (s^2_R) estimates were performed using the following equations:

$$s_r^2 = s_{replicates}^2 \quad (9)$$

$$s_R^2 = s_{replicates}^2 + s_{laboratories}^2 \quad (10)$$

In the present study, the protocol layout involved three independent series for each laboratory by means of three different days. Consequently, reproducibility was estimated according to [9]:

$$s_R^2 = s_{replicates}^2 + s_{days}^2 + s_{laboratories}^2 \quad (11)$$

2.6.3 Uncertainty estimation

The reproducibility variance allowed the estimation of the standard uncertainty u_x using the following equation:

$$u_x = \sqrt{s_R^2} \quad (12)$$

Therefore, the expanded uncertainty U_x could be calculated as:

$$U_x = 2u_x \quad (13)$$

using a coverage factor k = 2 [14].

2.6.4 Trueness criterion

The z-score gives a bias estimate of the results. An absolute z-scores below 2 are acceptable. A zone of doubtful performance exists for absolute z-scores between 2 and 3. Those results do not necessarily have to be unacceptable, since there is some uncertainty on how close the assigned sample value is to the unknown true value. However, an absolute z-score of 3 or more can be interpreted as an unacceptable performance [15].

For the present study, z-score was calculated for each laboratory according to:

$$z = \frac{\bar{x_l} - \hat{x}}{\sigma} \quad (14)$$

where $\bar{x_l}$ is the mean value reported by an individual laboratory, $\hat{x}$ is the assigned sample value, σ is the standard deviation (without outlier lab).

3. RESULTS AND DISCUSSION

3.1 Preliminary testing – performance criteria

The first step of this collaborative study was checking the ability of each laboratory to perform quantitative analysis of the salbutamol sulfate samples. A preliminary testing was done to verify several performance criteria: selectivity, retention times stability, peak area variability and sensitivity. A typical chromatogram is presented in figure 2. In terms of sensitivity, the signal-to-noise ratio was measured for impurity D and was always higher than 25, as highlighted for each laboratory in supplementary data table 1. Nevertheless, a large variability in the S/N values was observed, varying from 34 to 918 with an average of 143. The UV lamp power (depending on its rated life) may partly explain this variability. A difference of noise measurement could also be suspected, especially considering that different software was used within the participant laboratories. As all laboratories fulfilled the sensitivity and variability criterion for impurity D (see below), this S/N difference was not further investigated.

Besides sensitivity, each individual laboratory also reported an adequate separation of API and related impurities (baseline separation of all peaks). The observed retention times variability with six replicates was always lower than 0.2%, as shown in supplementary data table 1. Finally, the RSD values on the peak area for six consecutive injections was below 2.0%, except for a few values. The higher variability observed for impurity F could be explained by the more difficult peak integration (more tailing of this peak). The integration of impurity B is harder due to the baseline slope at the end of the gradient leading to higher variability. Nevertheless, this criterion was satisfied by each lab for impurity D (RSD values between 0.27 and 1.89%), which is the target analyte in the present study. All laboratories successfully passed the preliminary step and performed the collaborative study.

3.2 Inter-laboratory study – quantitative results

Using a validated and locked Excel sheet, each laboratory reported the mass content (% m/m) of impurity D in salbutamol sulfate A, B and C samples. The results are summarized in supplementary data table 2 and supplementary figure 1.

3.2.1 Scrutiny of results for consistency and outliers

Within- and between-laboratories consistencies were examined by means of the graphical Mandel's methods and with numerical outlier tests.

3.2.1.1 Within-laboratory variance tests

Mandel's *k* plotting and Cochran's test were used to verify whether the within-laboratory variance (repeatability) of all laboratories can be considered equal. These tests were performed considering 9 measurements for each sample in each laboratory. Results have been reported in table 2 and figure 3.

On the Mandel's *k* plot (figure 3), indicator lines at 1 and 5 % significance levels were drawn. Laboratory 01 tends to show a higher repeatability variance. The same observation was made for laboratory 11 regarding sample A (borderline case). Numerical outlier testing was also

performed by means of a Cochran's test (see table 2). This test highlighted outlier values for laboratory 01 at all concentration levels (samples A, B, C). A second Cochran's test indicated an outlier for sample A at laboratory 11. The third Cochran's test did not suggest outlier or straggler. However, it is important to keep in mind that the repetition of statistical tests may lead to excessive rejection. Moreover, for laboratory 11, an outlier value was only observed for one sample and this value was much more acceptable than in laboratory 01. In this context, laboratory 01 can be considered as an outlying laboratory, while sample A analysed in laboratory 11 was not discarded at this stage.

3.2.1.2 Between laboratories variance tests

Mandel's *h* plotting and Grubbs' tests were used to verify whether laboratories with deviating results occur. Results have been reported in table 3 and figure 4. On the Mandel's *h* plot, indicator lines at 1 and 5 % significance levels were drawn.

As illustrated in figure 4, laboratories 01 and 11 tend to report higher concentrations than the other laboratories, but Mandel's *h* remains below the 5% significance level. The raw data of these two laboratories were thoroughly investigated. For laboratory 01, the three calibration curves obtained were not linear, probably due to a standard preparation issue when making the dilutions. For laboratory 11, a systematic lower AUC of calibration standards led to an overestimation of sample content. The preparation of impurity D stock solution seemed to be the source of this issue. It is important to notice that the root causes are not related to the analytical technique (SFC) but to sample and/or standard preparation.

This Mandel's *h* plot also highlighted the quite balanced distribution of reported values around the mean value. Grubb's tests were performed on the means of the values reported by each laboratory. As illustrated in table 3, no outlier or straggler value was reported.

These Grubb's tests could also be performed on the individual measurements (individual measurements reported) where the Cochran's test has shown the lab variance was suspicious.

These results were also reported in table 4. Grubb's test highlighted one outlying value for an individual measurement reported by laboratory 01. Consequently, this value was discarded and the Cochran's test was repeated on the remaining data set. Without this individual outlying value, the within-laboratory variance of laboratory 01 still remains significantly higher than in other laboratories, as shown in table 4.

As already mentioned, an in-depth evaluation of individual laboratory reports showed that laboratory 01 obtained non-linear calibration curves for two series, but the curve profiles for both series were different. According to the laboratory report, accurate balance and appropriate glassware and pipettes were used. However, regarding the calibration curves, an inadequate weighing of standards and/or inappropriate use of the automatic pipette were suspected The random errors were mainly explained by an operator training/ability to work with low mass weighing (5 mg) and accurate dilutions. This observation is also corroborated by the function of the operator, which was an not a qualified and fully trained analyst. Consequently, this laboratory data cannot be considered as reliable and the lab 01 was definitively discarded for the evaluation of method precision.

3.2.1.3 Results consistency

As required by the ISO guidelines, results were removed from the original data set when they were outliers with the numerical technique, or when they were stragglers with the numerical technique and they exceed the 1% critical level on the Mandel plot. Table 5 summarized the results and outliers values. As above explained, outlier values (lab 01) were discarded for method variances estimation. The outlier values obtained were mainly explained by samples and standards preparation. This step of the analytical protocol is similar whatever the analytical technique used for the quantitative analysis (i.e. LC or SFC or other technique).

3.2.2 Variances estimation

The final objective of this inter-laboratory study was to estimate the method variance and variance components. These results were summarized in table 6.

As shown in Table 6, the total method variability was mainly due to the "laboratory" factor (contribution around 70 % at all concentration levels). The contributions from the "day" and "replicate" factors were quite similar (10 to 15 % of the total variance), with a slightly larger impact of the day factor, except at the highest concentration level. It is often expected to have the reproducibility about 2 to 4 times higher than the repeatability, when considering the standard deviations [16]. In the present study, ratios close to 3 were observed for the whole dosing range (i.e. from 0.2 to 0.4 % of impurity D). Considering variances (s^2), ratios between reproducibility and repeatability were within the range 6 – 10 (a 4 – 9 range is often advised [13]). However, it is important to notice that both ratios within or above this range have reported in the literature for LC method, including ratios close to 80 for the determination of impurities [9].

The reproducibility variance was 2-3 times larger than the intermediate precision (repeatability variance + days variance), confirming the important contribution of the "laboratory" to the total variability. This laboratory contribution to the total variance could be explained by (i) the use of various SFC systems (not evaluated during method validation performed using only one equipment), (ii) the recent SFC implementation in many participating laboratories, (iii) the difficulty to handle low masses and low dilution volumes, (iv) the CO_2 supply that was not evaluated during method optimization and validation. The contribution of different equipment/systems and some technical aspects related to samples and standards preparation are analytical aspects that need to be considered independent of the separation techniques. Nevertheless the reproducibility values, that take into account all variability components, are close to or even lower than those reported for LC impurities determination. Our results obtained

with several modern SFC systems in several laboratories highlighted the reliability of this technique.

Finally, to present some more intuitive values, standard deviations and relative standard deviations were calculated for both repeatability and reproducibility. The relationship between standard deviation and impurity D concentration was presented in figure 5. As expected, the standard deviation was proportional to the concentration (linear relationship) while the relative standard deviation was rather constant within the validated dosing range (table 6).

RSD reproducibility values close to or below 10 % were obtained in this study. Considering all sources of variability, i.e. replicates, days and 18 laboratories (meaning 18 instruments and 18 operators), these good RSD values again clearly highlighted the reliability of this SFC method for the quantification of salbutamol sulfate impurity D.

3.2.3 Measurement uncertainty evaluation

The expanded uncertainty values are described in table 7. For a non-conform sample (0.4 % of impurity D), the result was expected to have an expanded uncertainty of 0.058 % m/m. Therefore, 95 % of the reported values are expected to be comprised between 0.342 and 0.458 %. As illustrated in supplementary table 2, the individual measurements fulfilled this expectation, since 9 out of 171 measurements in sample A (5 %) were outside the expanded-uncertainty range. Using the mean value of each laboratory, only one laboratory was outside the range for concentrations of 0.2 and 0.3 % (samples C and B) and two laboratories were outside the range for concentration of 0.4 %. The laboratory outside the range was the one previously discarded by the outlier statistical tests (lab 01). As observed for the variance estimation, the relative expanded uncertainty values were also lower or equivalent those described in the literature for LC methods using a similar study protocol [9].

3.2.4 Trueness criterion

In the present study, the “true” value of the impurity D content in the three salbutamol sulfate samples is unknown. Consequently, to estimate the trueness, Z-scores were calculated using the general mean (without outlier) as assigned value (see supplementary data table 3). Figure 6 demonstrates that the laboratories with the highest |z-scores| were those highlighted during the outliers evaluation. In conclusion, during the preliminary screening, none of the participating laboratories, except laboratories 01 and 11 showed a significant bias.

4. CONCLUSION

A collaborative study was carried out on the SFC method to determine the content of impurity D in salbutamol sulfate API. After the development and validation of a robust SFC method in one single laboratory (the development lab), the precision of this method in various laboratories around the world was demonstrated. It is important to mention that this step of reproducibility evaluation is mandatory to propose the method as an alternative to current normative methods. The method reproducibility was estimated by taking into account replicates, days and laboratories variances. The values obtained were compared with those published in the literature in the context of impurities determination [9,11]. For this SFC method, repeatability and reproducibility variances were similar or better than the ones described for LC methods. The reproducibility values highlighted the reliability of the method and its potential use in different labs for QC analysis. For the first time, the quantitative and robust performance of modern SFC was demonstrated by means of a collaborative study, showing its potential to replace to other chromatographic techniques for pharmaceutical quality control. Finally, as the study involved only Waters® instrumentation, an expanded study should be performed including different manufacturer’s equipment.

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FIGURES CAPTIONS

Figure 1. Set up of the collaborative study per sample: p = 19 laboratories, c = 3 series (days), g = 3 replicate measurements.

Figure 2. Representative SFC chromatogram of salbutamol sulfate and its related impurities. Experimental conditions: see text.

Figure 3. Mandel's *k* plotting – within-laboratory consistency. Samples A, B and C were represented in orange, purple and blue, respectively. Indicator lines at 1 % (red) and 5 % (black) significance levels.

Figure 4. Mandel's *h* plotting – between-laboratories consistency. Samples A, B and C were represented in orange, purple and blue, respectively. Indicator lines at 1 % (red) and 5 % (black) significance levels.

513 Figure 5. Standard deviations *vs.* concentration level relationship. Repeatability (blue triangles),

514 reproducibility (red crosses).

515 Figure 6. Z-scores of the participating laboratories. Samples A, B and C were represented in

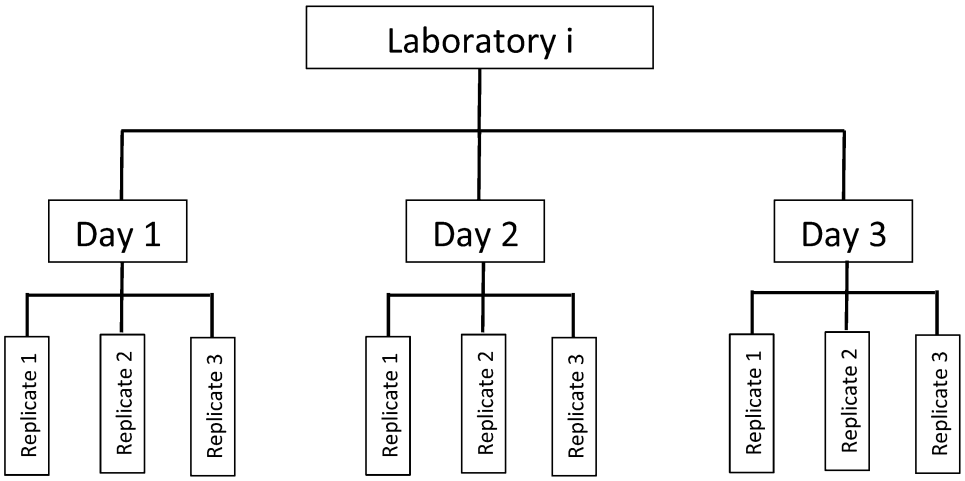
516 orange, purple and blue, respectively.

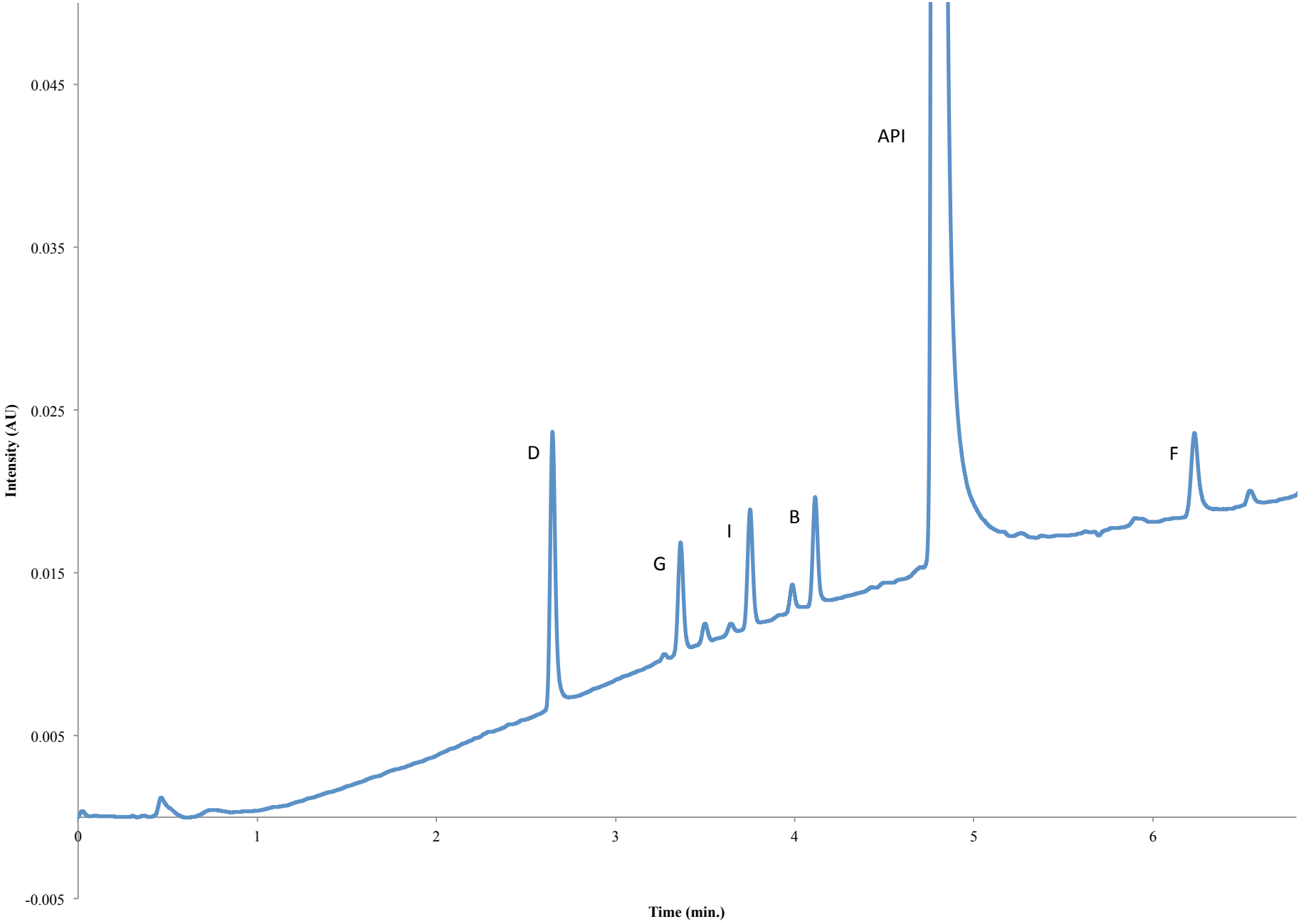
517

$X_i = (i = 1...p)$

$X_{ij} = (j = 1...c)$

$X_{ijk} = (k = 1...g)$

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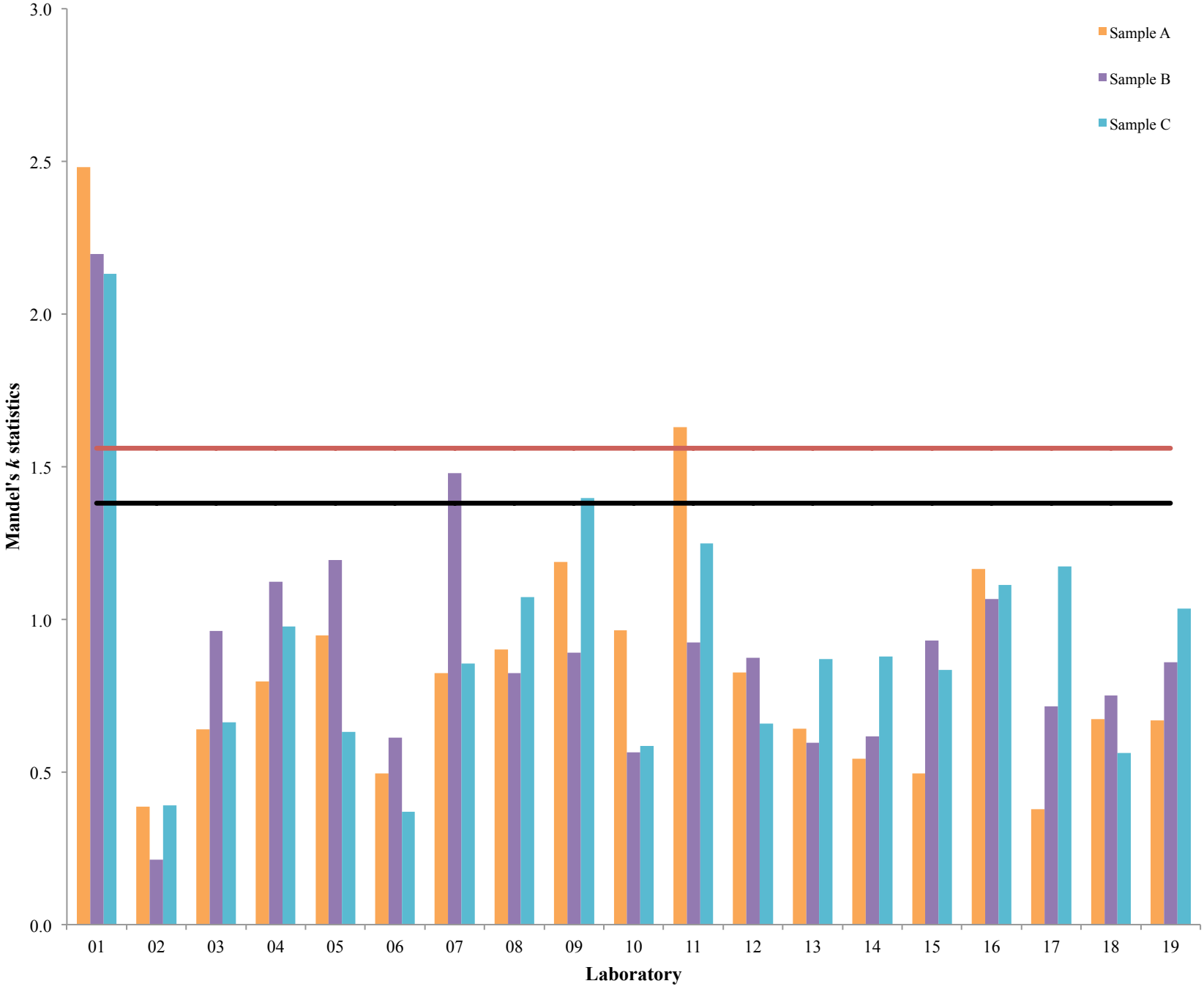
0.035
0.025
0.015
0.005
-0.005
Intensity (AU)
0
1
2
3
4
5
6
Time (min.)
D
G
I
B
API
F


Sample A
Sample B
Sample C
Mandel's k statistics
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2.5
2.0
1.5
1.0
0.5
0.0
01
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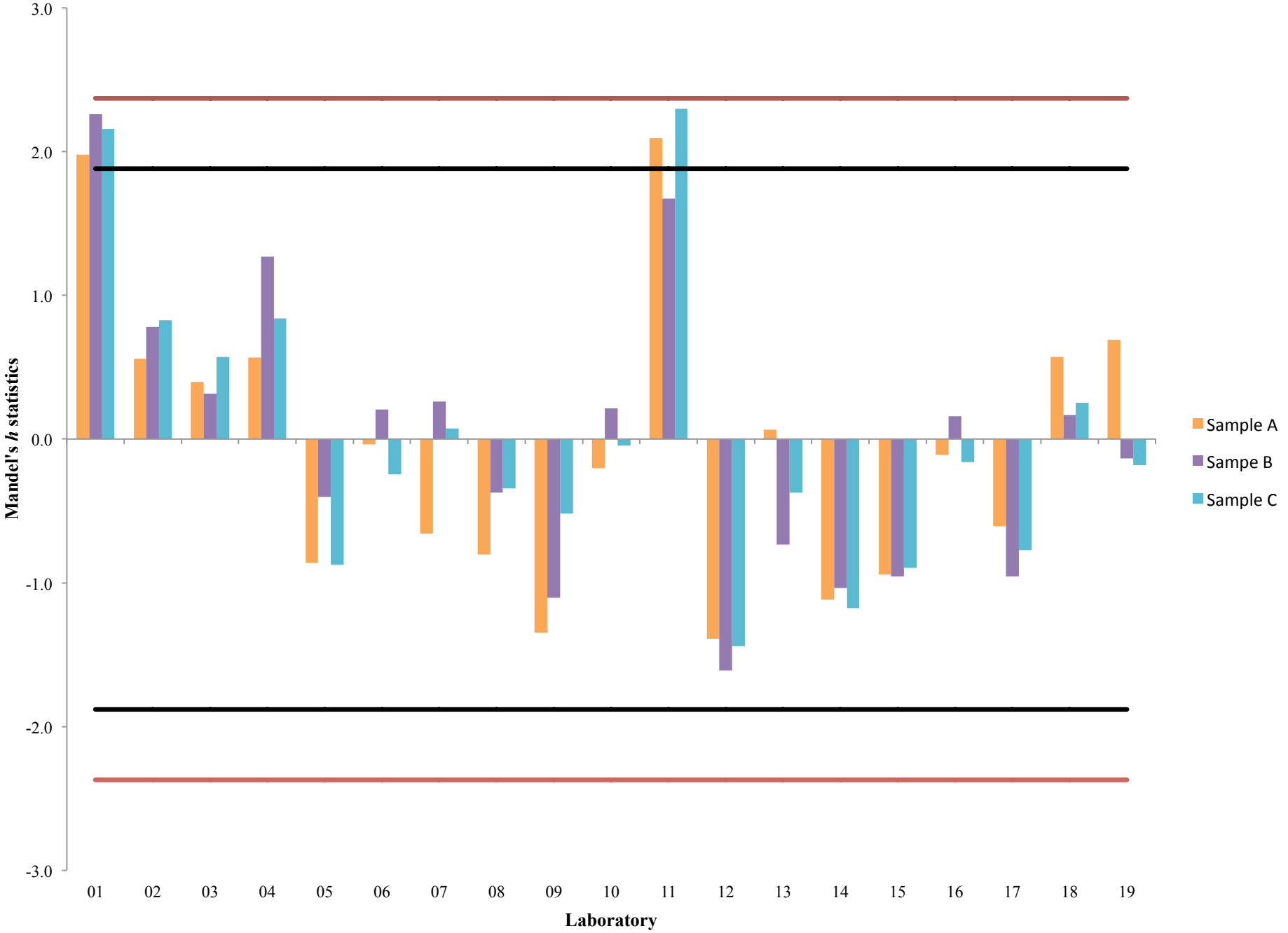
Laboratory


3.0
2.0
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-1.0
-2.0
-3.0
Mandel's h statistics
01
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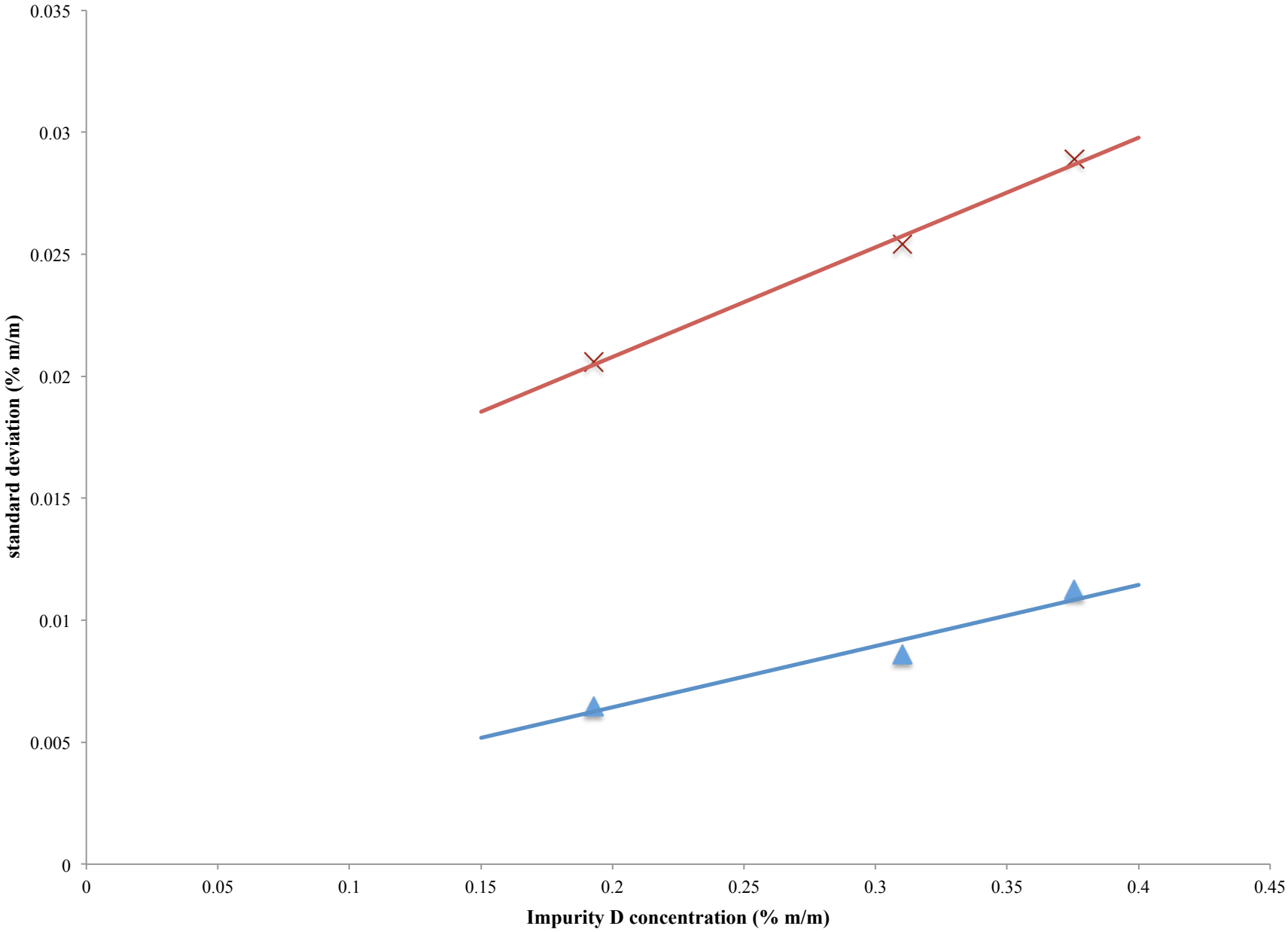
Laboratory
Sample A
Sampe B
Sample C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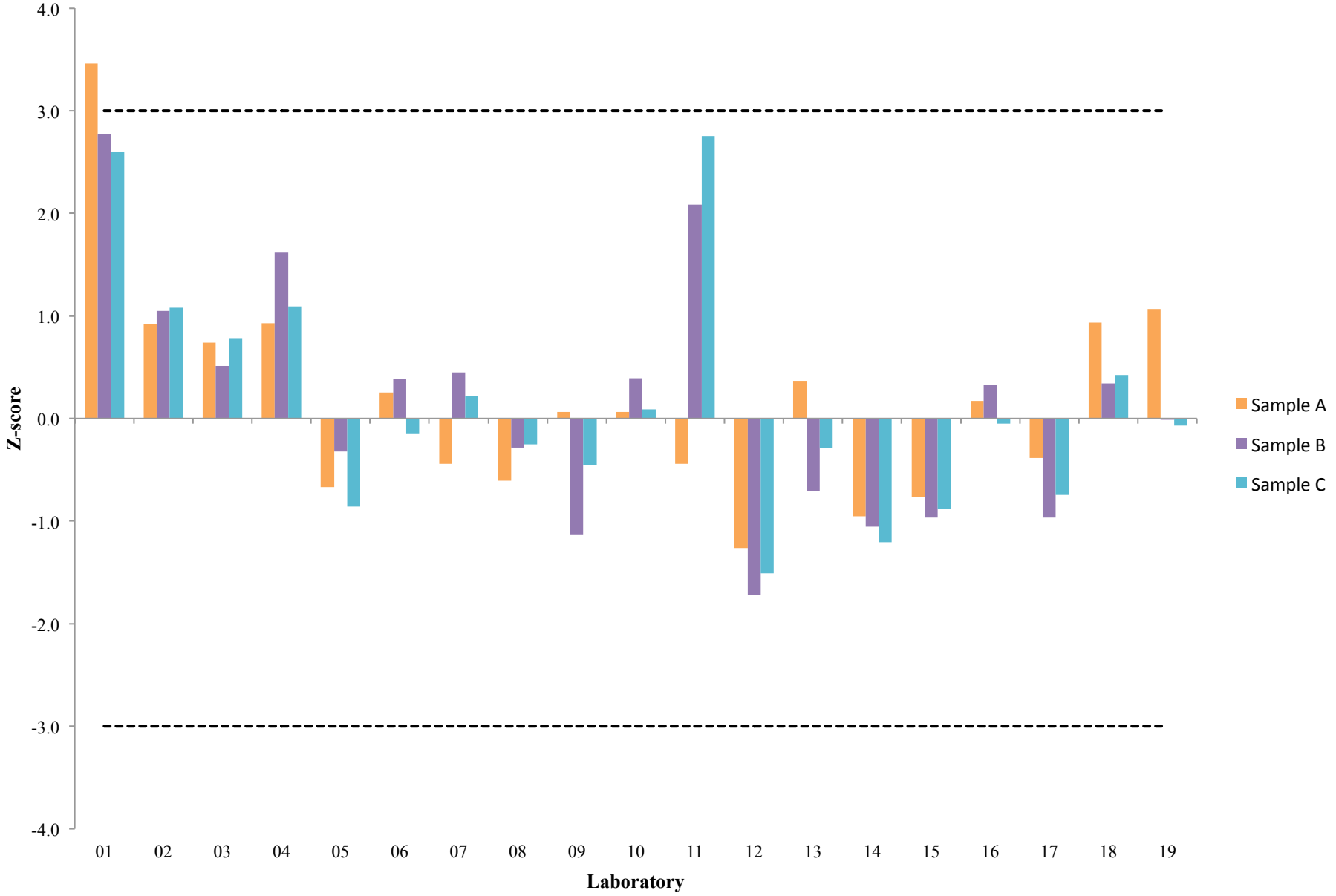
0.03
0.025
0.02
0.015
0.01
0.005
0
standard deviation (% m/m)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
Impurity D concentration (% m/m)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Z-score
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02
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14
15
16
17
18
19
Laboratory
Sample A
Sample B
Sample C

Table 1. Analysis of variance components (p= number of laboratories, c = number of days per laboratory, g = number of replicates per day)

Sources of variability	Mean squares	Estimated variance
Laboratories	$MS_{laboratories} = \frac{cg\Sigma(\bar{x}_i - \bar{x})^2}{p-1}$	$s^2_{laboratories} = \frac{MS_{laboratories} - MS_{days}}{cg}$
Days	$MS_{days} = \frac{g\Sigma\Sigma(\bar{x}_{ij} - \bar{x}_i)^2}{p(c-1)}$	$s^2_{days} = \frac{MS_{days} - MS_{replicates}}{g}$
Replicates	$MS_{replicates} = \frac{\Sigma\Sigma\Sigma(\bar{x}_{ijk} - \bar{x}_{ij})^2}{pc(g-1)}$	$s^2_{replicates} = s^2_r = MS_{replicates}$

Table 2. Within-laboratory results consistency (**outlier, *straggler)

	Mandel's *k* statistics		
Lab number	Sample A	Sample B	Sample C
01	2.481**	2.197**	2.130**
02	0.386	0.213	0.391
03	0.640	0.961	0.662
04	0.797	1.123	0.975
05	0.947	1.193	0.631
06	0.494	0.613	0.370
07	0.824	1.479*	0.854
08	0.900	0.823	1.074
09	1.188	0.891	1.396
10	0.963	0.565	0.585
11	1.628**	0.924	1.248
12	0.825	0.874	0.658
13	0.642	0.595	0.869
14	0.543	0.616	0.877
15	0.494	0.930	0.834
16	1.165	1.065	1.112
17	0.379	0.715	1.173
18	0.672	0.751	0.562
19	0.668	0.859	1.036
Indicator values for Mandel's *k* statistics (p=19, n=9)			
5 % level		1.38	
1 % level		1.56	
Cochran's test			
	Sample A	Sample B	Sample C
C	0.3240** (outlier lab 01)	0.2540** (outlier lab 01)	0.2389** (outlier lab 01)
Critical values (p=19, n=9)			
5 % level		0.1500	
1 % level		0.1738	
Second Cochran's test (after elimination of outliers)			
C	0.2064** (outlier lab 11)	0.1544	0.1347
Critical values (p=18, n=9)			
5 % level		0.1579	
1 % level		0.1829	
Third Cochran's test (after elimination of outliers)			
C	0.1386	nd	nd
Critical values (p=17, n=9)			
5 % level		0.1658	
1 % level		0.1920	

Table 3. Between-laboratories results consistency

	Mandel's *h* statistics		
Lab number	Sample A	Sample B	Sample C
01	1.978*	2.260*	2.159*
02	0.560	0.781	0.828
03	0.398	0.318	0.570
04	0.566	1.267	0.841
05	-0.861	-0.404	-0.872
06	-0.036	0.206	-0.246
07	-0.658	0.262	0.075
08	-0.801	-0.371	-0.342
09	-1.344	-1.102	-0.518
10	-0.203	0.213	-0.044
11	2.095*	1.672	2.299*
12	-1.388	-1.611	-1.441
13	0.064	-0.733	-0.374
14	-1.115	-1.035	-1.178
15	-0.942	-0.956	-0.896
16	-0.108	0.160	-0.161
17	-0.605	-0.957	-0.773
18	0.570	0.168	0.252
19	0.691	-0.136	-0.180
Indicator values for Mandel's *h* statistics (p=19)			
5 % level		|2.37|	
1 % level		|1.88|	
Grubb's test on lab mean (one outlying observation)			
	Sample A	Sample B	Sample C
G_p	2.159	2.624	2.299
G_1	1.331	1.871	1.441
Critical values (p = 19)			
1 % level		2.968	
5 % level		2.681	
Grubb's test on lab mean (two outlying observations)			
	Sample A	Sample B	Sample C
$G_{p-1,p}$	1.099	1.056	1.099
$G_{1,2}$	0.986	1.034	0.986
Critical values (p = 19)			
1 % level		0.3398	
5 % level		0.4214	
Grubb's test on individual measurements (one outlying observation)			
	Sample A	Sample B	Sample C
G_p	4.276**	3.223**	3.474**
G_1	1.863	1.988	1.877
Outlier	*Lab 01*	*Lab 01*	*Lab 01*
Critical values (p = 19)			
1 % level		2.968	
5 % level		2.681	
Grubb's test on individual measurements (two outlying observations)			
	Sample A	Sample B	Sample C
$G_{p-1,p}$	0.923	0.916	1.001
$G_{1,2}$	0.922	0.923	1.000
Critical values (p = 19)			
1 % level		0.3398	
5 % level		0.4214	

Table 4. Cochran's test results without outlying values (**outlier)

Cochran's test			
	Sample A	Sample B	Sample C
C	0.2148** (outlier lab 01)	0.2353** (outlier lab 01)	0.1918** (outlier lab 01)
Critical values (p=19, n=8)			
5 % level	0.1583		
1 % level	0.1844		

Table 5. Summary of labs results (**outlier)

	Average impurity D content in salbutamol sulfate (% m/m)		
Lab number	Sample A	Sample B	Sample C
01	0.4384**	0.2444**	0.3689**
02	0.3960	0.2124	0.3346
03	0.3912	0.2024	0.3280
04	0.3962	0.2229	0.3349
05	0.3536	0.1868	0.2908
06	0.3782	0.2000	0.3070
07	0.3596	0.2012	0.3152
08	0.3553	0.1875	0.3045
09	0.3680	0.1717	0.3000
10	0.3732	0.2001	0.3122
11	0.4419	0.2317	0.3725
12	0.3378	0.1607	0.2762
13	0.3812	0.1797	0.3037
14	0.3460	0.1731	0.2830
15	0.3511	0.1748	0.2902
16	0.3761	0.1990	0.3091
17	0.3612	0.1748	0.2934
18	0.3963	0.1992	0.3198
19	0.3999	0.1926	0.3087
Mean	0.3790	0.1955	0.3133

Table 6. Estimation of the variance components (p = 18)

Sources of variability	Impurity D at 0.2 % (sample B)	Impurity D at 0.3 % (sample C)	Impurity D at 0.4 % (sample A)
Variances			
Laboratories ($s^2_{laboratories}$)	3.23×10^{-4}	4.67×10^{-4}	5.83×10^{-4}
Days (s^2_{days})	5.96×10^{-5}	1.04×10^{-4}	1.27×10^{-4}
Replicates ($s^2_{replicates}$)	4.19×10^{-5}	7.37×10^{-5}	1.26×10^{-4}
Repeatability variance (s_r^2)	4.19×10^{-5}	7.37×10^{-5}	1.26×10^{-4}
Reproducibility variance (s_R^2)	4.24×10^{-4}	6.45×10^{-4}	8.36×10^{-4}
Ratio $(s_R^2)/(s_r^2)$	10.13	8.75	6.62
Repeatability sd (s_r)	6.47×10^{-3}	8.59×10^{-3}	1.13×10^{-2}
Reproducibility sd (s_R)	2.06×10^{-2}	2.54×10^{-2}	2.89×10^{-2}
Ratio $(s_R)/(s_r)$	3.18	2.96	2.57
Repeatability RSD (%)	3.36 %	2.77 %	2.99 %
Reproducibility RSD (%)	10.68 %	8.19 %	7.69 %

Table 7. Estimation of the measurement uncertainty

Uncertainty	Impurity D at 0.2 % (sample B)	Impurity D at 0.3 % (sample C)	Impurity D at 0.4 % (sample A)
Expanded uncertainty (% m/m)	4.12×10^{-2}	5.08×10^{-2}	5.78×10^{-2}
Relative expanded uncertainty (%)	21.36 %	16.37 %	15.39 %

Supplementary table 1. Results of preliminary performance testing (D, G, I, B, API, F refer to the different compounds).

Lab	Retention times (min) and RSD (%, n =6)						Peak area RSD (%, n=6)					S/N imp D
	D	G	I	B	API	F	D	G	I	B	F	
01	2.64 (0.10)	3.41 (0.09)	3.69 (0.06)	3.96 (0.05)	4.76 (0.04)	6.10 (0.05)	0.45	1.87	1.85	*4.36*	1.58	66
02	2.74 (0.04)	3.46 (0.02)	3.74 (0.02)	3.98 (0.02)	4.77 (0.02)	6.11 (0.01)	0.33	0.41	0.88	*3.30*	1.89	66
03	2.65 (0.05)	3.44 (0.03)	3.74 (0.01)	4.11 (0.01)	4.79 (0.01)	6.12 (0.01)	1.10	1.15	0.20	0.52	1.16	227
04	2.60 (<0.005)	3.36 (0.12)	3.66 (<0.005)	4.04 (0.13)	4.72 (<0.005)	6.06 (<0.005)	1.00	1.22	1.35	1.13	8.27	39
05	2.69 (0.13)	3.26 (0.11)	3.53 (0.08)	3.95 (0.07)	4.63 (0.07)	5.90 (0.06)	1.75	1.40	1.15	1.78	1.23	34
06	2.45 (0.10)	3.27 (0.07)	3.54 (0.04)	3.95 (0.03)	4.64 (0.03)	6.02 (0.01)	0.69	1.11	0.49	0.56	0.59	61
07	2.90 (<0.005)	3.63 (<0.005)	3.96 (0.10)	4.28 (<0.005)	4.95 (0.08)	6.38 (<0.005)	1.89	1.67	1.31	1.30	12.4	34
08	2.90 (<0.005)	3.40 (<0.005)	3.80 (<0.005)	4.20 (<0.005)	4.90 (<0.005)	6.30 (<0.005)	0.47	0.00	0.33	0.43	0.29	350
09	2.54 (0.03)	3.33 (0.03)	3.63 (0.02)	4.03 (0.02)	4.73 (0.03)	6.11 (0.01)	1.04	0.96	1.50	0.85	6.12	38
10	2.62 (0.05)	3.49 (0.02)	3.67 (0.04)	4.10 (0.04)	4.78 (0.02)	6.09 (0.06)	0.69	0.70	0.25	0.59	0.98	153
11	2.70 (0.03)	3.49 (0.02)	3.89 (0.02)	4.07 (0.01)	4.90 (0.03)	6.33 (0.01)	1.14	1.96	0.30	*3.59*	0.98	79
12	2.68 (0.04)	3.31 (0.08)	3.63 (0.10)	3.95 (0.07)	4.77 (0.05)	6.07 (0.05)	0.91	0.67	0.42	1.28	0.71	81
13	2.65 (0.04)	3.36 (0.04)	3.75 (0.02)	4.11 (0.03)	4.80 (0.02)	6.23 (0.01)	1.18	2.45	1.39	*2.03*	*2.55*	52
14	2.85 (0.03)	3.46 (0.04)	3.75 (0.03)	4.09 (0.02)	4.89 (0.02)	6.17 (0.02)	0.45	0.56	0.41	0.93	1.08	79
15	2.64 (0.05)	3.41 (0.04)	3.71 (0.02)	4.09 (0.03)	4.77 (0.02)	6.14 (0.02)	1.05	0.61	0.46	1.17	1.69	100
16	2.63 (0.04)	3.41 (0.03)	3.68 (0.03)	4.06 (0.03)	4.72 (0.02)	6.06 (0.02)	0.50	0.47	0.45	0.50	0.67	918
17	2.59 (0.04)	3.41 (0.06)	3.70 (0.03)	3.93 (0.01)	4.74 (0.01)	6.11 (0.01)	0.27	0.48	0.81	1.96	1.29	117
18	2.67 (0.16)	3.35 (0.07)	3.58 (0.08)	3.92 (0.10)	4.53 (0.14)	5.70 (0.05)	0.38	1.87	0.92	1.36	1.92	75
19	2.76 (0.06)	3.47 (0.03)	3.79 (0.01)	4.16 (0.04)	4.82 (0.02)	6.22 (0.02)	0.38	0.81	1.91	1.78	1.45	151
Mean	2.68	3.41	3.71	4.05	4.77	6.12						
SD	0.11	0.09	0.11	0.10	0.10	0.15						

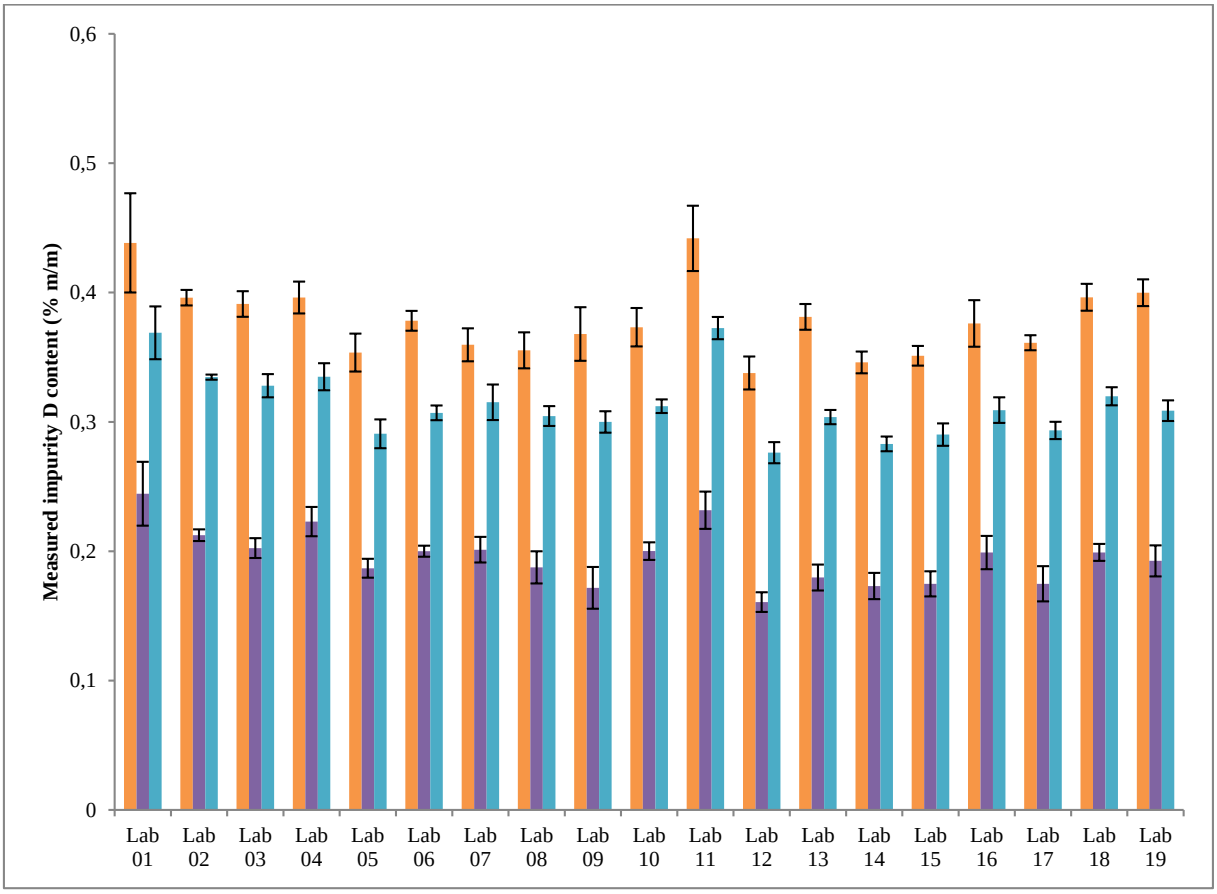
Supplementary table 2. Results of impurity D determination in salbutamol sulfate (% m/m)

		Series 1			Series 2			Series 3			Mean
	Replicate / Sample	1	2	3	1	2	3	1	2	3	
Lab 01	A	0.4516	0.4144	0.4257	0.4827	0.5146	0.4545	0.3977	0.3978	0.4064	0.4384
	B	0.2236	0.2168	0.2125	0.2700	0.2597	0.2588	0.2600	0.2580	0.2405	0.2444
	C	0.3535	0.3306	0.3603	0.4095	0.3974	0.3927	0.3587	0.3720	0.3451	0.3689
Lab 02	A	0.4003	0.4051	0.3984	0.3974	0.3988	0.3991	0.3852	0.3885	0.3913	0.3960
	B	0.2104	0.2091	0.2106	0.2133	0.2147	0.2111	0.2146	0.2142	0.2136	0.2124
	C	0.3396	0.3402	0.3348	0.3384	0.3359	0.3364	0.3301	0.3272	0.3289	0.3346
Lab 03	A	0.4078	0.3949	0.4026	0.3855	0.3843	0.3961	0.3914	0.3734	0.3847	0.3912
	B	0.2122	0.2157	0.1923	0.1890	0.1936	0.2040	0.2071	0.1985	0.2093	0.2024
	C	0.3197	0.3397	0.3389	0.3298	0.3295	0.3237	0.3286	0.3146	0.3272	0.3280
Lab 04	A	0.3934	0.4019	0.3935	0.4083	0.4045	0.4148	0.3735	0.3806	0.3952	0.3962
	B	0.2323	0.2334	0.2278	0.2300	0.2300	0.2244	0.2181	0.2036	0.2069	0.2229
	C	0.3486	0.3444	0.3386	0.3351	0.3516	0.3260	0.3216	0.3313	0.3173	0.3349
Lab 05	A	0.3712	0.3685	0.3635	0.3668	0.3306	0.3357	0.3591	0.3479	0.3388	0.3536
	B	0.1890	0.1881	0.1895	0.1977	0.2059	0.1915	0.1754	0.1743	0.1695	0.1868
	C	0.2954	0.2949	0.3007	0.3014	0.2905	0.2868	0.2847	0.2796	0.2835	0.2908
Lab 06	A	0.3963	0.3749	0.3784	0.3711	0.3666	0.3803	0.3787	0.3790	0.3783	0.3782
	B	0.2042	0.1999	0.2045	0.1887	0.1979	0.1924	0.2044	0.2065	0.2013	0.2000
	C	0.3049	0.3088	0.3029	0.3099	0.3024	0.3003	0.3142	0.3092	0.3101	0.3070
Lab 07	A	0.3429	0.3653	0.3431	0.3569	0.3646	0.3868	0.3643	0.3619	0.3509	0.3596
	B	0.2115	0.2264	0.2109	0.2077	0.1942	0.1792	0.1976	0.1986	0.1848	0.2012
	C	0.3212	0.3103	0.3317	0.3153	0.3093	0.3167	0.2940	0.3156	0.3229	0.3152
Lab 08	A	0.3467	0.3623	0.3530	0.3726	0.3742	0.3637	0.3275	0.3447	0.3534	0.3553
	B	0.1835	0.1881	0.1864	0.2027	0.1970	0.1829	0.1886	0.1829	0.1755	0.1875
	C	0.3093	0.3275	0.3079	0.3057	0.3072	0.3148	0.2869	0.2931	0.2879	0.3045
Lab 09	A	0.3601	0.3355	0.3575	0.3822	0.3858	0.3727	0.3767	0.4024	0.3391	0.3680
	B	0.1614	0.1683	0.1672	0.1707	0.1792	0.1902	0.1628	0.1724	0.1730	0.1717
	C	0.3095	0.2880	0.2771	0.3083	0.3112	0.2957	0.3332	0.2907	0.2861	0.3000
Lab 10	A	0.3738	0.4018	0.3846	0.3676	0.3716	0.3583	0.3671	0.3486	0.3855	0.3732
	B	0.1983	0.1954	0.2021	0.1968	0.2039	0.1959	0.1929	0.2081	0.2079	0.2001
	C	0.3212	0.3074	0.3149	0.3052	0.3129	0.3137	0.3001	0.3119	0.3223	0.3122
Lab 11	A	0.4119	0.4139	0.4213	0.4601	0.4766	0.4569	0.4768	0.4428	0.4167	0.4419
	B	0.2259	0.2146	0.2302	0.2393	0.2311	0.2374	0.2471	0.2309	0.2289	0.2317
	C	0.3612	0.3522	0.3500	0.3828	0.3855	0.3929	0.3817	0.3784	0.3677	0.3725
Lab 12	A	0.3203	0.3289	0.3274	0.3630	0.3419	0.3431	0.3505	0.3389	0.3263	0.3378
	B	0.1622	0.1637	0.1656	0.1508	0.1496	0.1498	0.1739	0.1636	0.1668	0.1607
	C	0.2784	0.2613	0.2762	0.2847	0.2759	0.2673	0.2846	0.2730	0.2843	0.2762
Lab 13	A	0.3792	0.3749	0.3776	0.3950	0.3869	0.3995	0.3762	0.3672	0.3739	0.3812
	B	0.1785	0.1733	0.1696	0.1753	0.1871	0.1830	0.1852	0.1833	0.1816	0.1797
	C	0.2945	0.2992	0.2866	0.3108	0.3170	0.3036	0.3172	0.3089	0.2951	0.3037
Lab 14	A	0.3410	0.3409	0.3654	0.3388	0.3443	0.3536	0.3502	0.3407	0.3389	0.3460
	B	0.1715	0.1858	0.1787	0.1667	0.1677	0.1698	0.1760	0.1711	0.1708	0.1731
	C	0.3017	0.2792	0.2911	0.2713	0.2760	0.2911	0.2749	0.2714	0.2900	0.2830

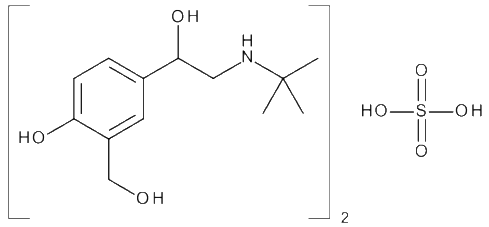
	A	0.3618	0.3400	0.3435	0.3531	0.3504	0.3623	0.3473	0.3444	0.3574	0.3511
Lab 15	B	0.1683	0.1591	0.1868	0.1805	0.1734	0.1864	0.1685	0.1714	0.1792	0.1748
	C	0.2921	0.2709	0.3045	0.3002	0.2914	0.2973	0.2900	0.2827	0.2829	0.2902
	A	0.3696	0.3912	0.3922	0.3672	0.3716	0.4040	0.3403	0.3851	0.3635	0.3761
Lab 16	B	0.2043	0.2133	0.1988	0.2068	0.1908	0.2095	0.1824	0.1974	0.1875	0.1990
	C	0.3086	0.3117	0.2991	0.3284	0.3129	0.3287	0.3066	0.2862	0.2999	0.3091
	A	0.3598	0.3586	0.3590	0.3570	0.3522	0.3578	0.3659	0.3689	0.3714	0.3612
Lab 17	B	0.1825	0.1710	0.1807	0.1789	0.1846	0.1681	0.1642	0.1720	0.1713	0.1748
	C	0.2895	0.3026	0.2998	0.3176	0.2985	0.3019	0.2750	0.2752	0.2803	0.2934
	A	0.4109	0.4131	0.4022	0.3811	0.3843	0.3933	0.3887	0.3969	0.3963	0.3963
Lab 18	B	0.2059	0.2128	0.2038	0.1877	0.1941	0.1977	0.1993	0.1951	0.1960	0.1991
	C	0.3142	0.3137	0.3177	0.3126	0.3160	0.3197	0.3231	0.3289	0.3320	0.3198
	A	0.4069	0.4095	0.3947	0.4099	0.4085	0.4085	0.3846	0.3932	0.3835	0.3999
Lab 19	B	0.2039	0.2050	0.1959	0.1845	0.1878	0.1977	0.1811	0.1898	0.1875	0.1926
	C	0.3237	0.3233	0.3253	0.2983	0.3047	0.3110	0.3035	0.2938	0.2944	0.3087

Supplementary table 3. Z-scores using labs mean as assigned value

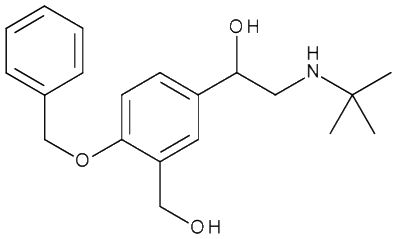
	Z-score		
Lab number	Sample A	Sample B	Sample C
01	**3.46**	*2.77*	*2.60*
02	0.92	1.05	1.08
03	0.74	0.52	0.79
04	0.93	1.62	1.10
05	-0.67	-0.32	-0.86
06	0.25	0.38	-0.14
07	-0.44	0.45	0.22
08	-0.60	-0.28	-0.25
09	0.07	-1.13	-0.45
10	0.07	0.39	0.09
11	-0.44	*2.09*	*2.76*
12	-1.26	-1.72	-1.51
13	0.37	-0.71	-0.29
14	-0.96	-1.06	-1.21
15	-0.76	-0.96	-0.88
16	0.17	0.33	-0.05
17	-0.38	-0.97	-0.74
18	0.93	0.34	0.42
19	1.07	-0.01	-0.07



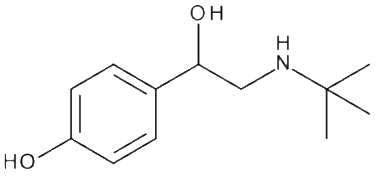
Supplementary figure 1. Measured impurity D content (% m/m) for salbutamol sulfate samples A, B, C and respective standard deviation per lab.



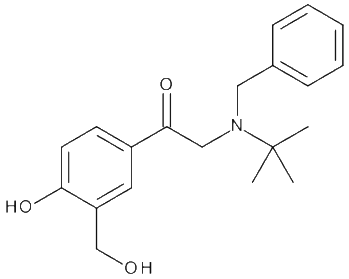
Salbutamol sulfate



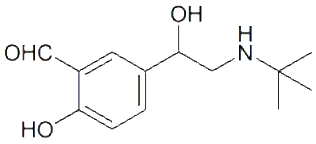
Impurity I



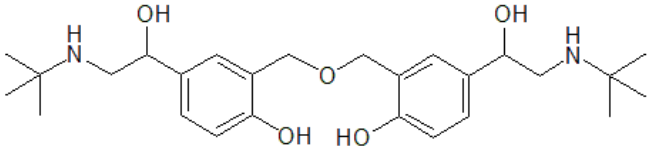
Impurity B



Impurity G



Impurity D



Impurity F

Supplementary figure 2. Salbutamol sulfate and related impurities chemical structures.