Towards a full integration of optimization and validation phases: An Analytical-Quality-by-Design approach

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Abstract

When using an analytical method, defining an Analytical Target Profile (ATP) focused on quantitative performance represents a key input, and this will drive the method development process. In this context, two case studies were selected in order to demonstrate the potential of a Quality-by-Design (QbD) strategy when applied to two specific phases of the method lifecycle: the pre-validation study and the validation step. The first case study focused on the improvement of a Liquid Chromatography (LC) coupled to Mass Spectrometry (MS) stability-indicating method by the means of the QbD concept. The Design of Experiments (DoE) conducted during the optimization step (i.e. determination of the qualitative Design Space (DS)) was performed *a posteriori*. Additional experiments were performed in order to simultaneously conduct the pre-validation study to assist in defining the DoE to be conducted during the formal validation step. This predicted protocol was compared to the one used during the formal validation. A second case study based on the LC/MS-MS determination of glucosamine and galactosamine in human plasma was considered in order to illustrate an innovative strategy allowing the QbD methodology to be incorporated during the validation phase. An operational space, defined by the qualitative DS, was considered during the validation process rather than a specific set of working conditions as conventionally performed. Results of all the validation parameters conventionally studied were compared to those obtained with this innovative approach for glucosamine and galactosamine. Using this strategy, qualitative and quantitative information were obtained. Consequently, an analyst using this approach would be able to select with great confidence several working conditions within the operational space rather than a given condition for the routine use of the method. This innovative strategy combines both a learning process and a thorough assessment of the risk involved.

Keywords: Quality-by-Design; Quantitative Design Space; Validation; Risk management; Method lifecycle

1 Introduction

Numerous reference documents such as the International Conference on Harmonisation (ICH) guidelines [1-4] and the U.S. Pharmacopeia (USP) recommendations [5-7] deal with the method development process and the topic of validation. All of these documents emphasize the need to manage risk during the entire method lifecycle. As already widely discussed in the scientific literature [8-13], applying the Quality-by-Design (QbD) concept to analytical methods ensures a controlled risk-based development of a method where quality assurance will be guaranteed [1]. Nowadays, the QbD concept is mainly applied to the development step of the method as an alternative approach to the Quality-by-Testing methodology, as discussed by Hubert et al. [14]. However, the QbD strategy encompasses more than this single step of the method lifecycle. For instance, the control strategy forms part of this strategy, since this is recommended to ensure optimal method performance [4], although the robustness of the method is assessed separately by the determination of the analytical method Design Space (DS). This control strategy needs to be implemented in order to consolidate the understanding of the method and to allow its continuous improvement [15]. In the same way, the validation step must be part of the continuous evaluation of the analytical method rather than being an isolated activity. A similar approach is recommended by the FDA for process validation, and this has been illustrated by Katz [16] et al.

In this context, defining the objectives of the method by means of an Analytical Target Profile (ATP) [17] is the major first step of the QbD methodology. As established by a stimuli article of the USP Statistics Expert Committee [18], an ATP for an analytical procedure may be defined, for example, as follows: "*The procedure should be able to quantify [analytes] in the presence of [X, Y, Z] over a range of A% to B% of the nominal concentration with an*

accuracy and uncertainty ensuring the reportable results fall within +/-C% of the true value with quantified guarantees". Taking this definition into account, it therefore seems essential that the ATP be established before starting to develop the procedure. This ensures the definition in advance of the required level of performance given the user requirements. Consequently, the ATP should remain the reference concept throughout the method lifecycle. The goals of the present study are set within this context. The capability of the procedure to meet the specifications needs to be continuously reconsidered throughout the method lifecycle. As a first stage towards a full integration of the optimization and validation phases, the power of the QbD step using the Design of Experiments (DoE) was enhanced by performing additional experiments in order to obtain quantitative data leading to the gathering of valuable pre-validation information. In order to illustrate the feasibility of this innovative approach, a case study already presented elsewhere [14] is selected. This research was centered on the optimization of a Liquid Chromatography (LC) coupled to Mass Spectrometry (MS) stability-indicating method using a QbD methodology. The study was undertaken in order to identify the operational conditions, i.e. the Design Space (DS), that would ensure good results in the future in terms of the separation of the two analytes as well as protection from interfering peaks caused by the presence of impurities and/or co-extracted pharmaceutical matrix compounds. Using an *a posteriori* study, conducted as part of the DoE implemented during the QbD optimization step, the demonstration is made that this particular step of the method lifecycle could also be used to estimate the calibration model, the accuracy, and the limits of detection/quantification, as well as assisting in defining the DoE to be applied during the formal validation step.

From this quantitative information regarding the overall studied domain, a formal validation of a single set of working conditions could be considered. However, when considering the whole lifecycle of an analytical method, two major factors favor the continuous improvement

of the method. First, in-study results often highlight surprising discoveries (whether “good” or “bad”) about the procedure. Second, the product itself is generally subject to modification or alteration (i.e. minor modifications in the formulation of the product, testing of the product following a new type of stress test, specification changes, etc.). In these cases, a return to the procedure development stage should be encouraged, as facilitated by the implementation of a QbD approach. However, any time that the procedure changes, the need to partially or completely validate the adapted method should be considered [7]. Otherwise, a statistical demonstration of the method equivalence should be implemented. [19,20]. Taking this into account, the benefits of extending the QbD concept to the validation stage of the method would seem to be highly relevant. Indeed, this new strategy could allow the evaluation of the quantitative performance of the method within the qualitative DS. Within this high quality operational space, the quantitative robustness of the method would be evaluated for multiple operational conditions rather than for one single set of conditions, as usually occurs during the validation step. The evaluation of the proposed strategy forms the second part of the present study. For this purpose, a case study involving a method previously developed by the Quality-by-Testing approach is selected. An optimization of this method was required for two reasons. First, an improvement of the separation and detection conditions was required in order to eliminate the on-column mutarotation phenomenon observed with amino sugars [21]. Second, a change in the biological matrix used (i.e. from dog plasma to human plasma) as well as a change of equipment was needed [22].

2 Experimental

2.1 Chemicals and reagents

Methanol (MeOH; HPLC gradient grade) was purchased from J.T. Baker (Deventer, the Netherlands). Water (ULC/MS grade), acetonitrile (ACN; HPLC supra-gradient grade) and formic acid (ULC/MS grade) were provided by Biosolve B.V. (Valkenswaard, the Netherlands). Ammonia solution (32%, extra pure), Ammonium acetate (AnalaR, Normapur) and Ammonium bicarbonate (Rectapur) were acquired from VWR International (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q Plus 185 water purification system from Millipore (Billerica, MA, USA).

Chemicals (under confidential agreement) and reagents involved in the pre-validation study (i.e. Part I of the present study) were described in a previous study [14].

D-(+)-Glucosamine hydrochloride (99%+) and D-(+)-Galactosamine hydrochloride (99%+) were purchased from Sigma (St. Louis, MO, USA). D-(13C6)-glucosamine hydrochloride (99 atom-% 13C), used as the internal standard, was provided by Omicron Biochemicals INC. (South Bend, IN, USA).

Pooled human plasma of mixed gender origin (50% male donors / 50% female donors) was obtained from Sera Laboratories International Ltd. (Haywards Heath, United Kingdom).

2.2 Sample preparation

Within the framework of a new predictive approach applied to the pre-validation study phase of the method lifecycle (i.e. Part I of the present study), an *a posteriori* study was conducted based on previous research where the qualitative performance of this method had already been demonstrated [14]. Since the quantitative performance of the method can be affected only by the presence of unexpected compounds from aged placebo tablets (the pharmaceutical form

involved in the study), which interfere with both major impurities (P4NX99, P4FX98 and P4NX99-D, see [14] for details), samples were prepared using only these compounds. Stock solutions were prepared by dissolving an appropriate quantity of analytes in a mixture of formic acid 0.1% and MeOH in the proportions 80/20 (v/v). Two kinds of standard were then prepared by making suitable simultaneous dilutions of both stock solutions in the presence of the extracted placebo pharmaceutical form, in order to mimic real samples. The first standard contained a high concentration of P4NX99 and a low concentration of P4FX98 (100 ng mL^{-1} and 50 ng mL^{-1} of injected concentrations, respectively), while the second standard was prepared with the opposite levels of concentration (25 ng mL^{-1} and 200 ng mL^{-1} of injected concentrations, respectively). These solutions were prepared independently and in triplicate. Another previous study [22] was also selected as a case study in order to illustrate the applicability of the QbD methodology throughout the method lifecycle and, in particular, during the validation phase (i.e. Part II of the present study). The screening part of the QbD development was conducted on a mixture of pure glucosamine and galactosamine chemicals at a concentration of 1000 ng mL-1 in order to ensure detection despite the use of a multiplex interface. During the subsequent phases of the QbD development, stock solutions were prepared and mixed together in plasma at appropriate concentration levels (see below). Prepared plasma samples were vortex-mixed for several seconds in order to achieve homogenization. A 100 µL aliquot of the plasma sample was loaded onto a Phree phospholipid removal cartridge acquired from Phenomenex (Torrance, CA, USA). 300 µL of a mixture of ACN with 1% formic acid were then added. Finally, vacuum was applied at 2-7 inches Hg until the filtrate could be collected.

2.3 Experiments

Experiments were performed on two kinds of liquid chromatography (LC) coupled to mass spectrometer (MS) systems. The first system involved a High Performance Liquid

Chromatography (HPLC) system composed as follows: a Waters (Milford, MA, USA) sample manager 2777, a CTC Analytics AG (Zwingen, Switzerland) Stack Cooler DW with a CTC Analytics AG Peltier thermostat allowing samples to be cooled at 10 °C, four Waters binary HPLC pumps 1525µ and a Waters temperature control module controlling a column oven. This HPLC system was coupled to a Waters MicroMass single quadrupole mass spectrometer (Quattro, Ultima/ZQ) equipped, when necessary, with a MicroMass 4-way multiplex interface (MXI). The second system was composed of a Waters "I-Class" Ultra high Performance Liquid Chromatograph (UPLC) coupled with a Waters XEVO TQ-S tandem mass spectrometer (MS-MS). The HPLC/MS system was involved in the study of the new predictive approach for the pre-validation study (Part I of the present study) and during the screening phase of the QbD method development for the determination of glucosamine and galactosamine in human plasma (Part II of the present study). The UPLC/MS-MS system, on the other hand, was used during the optimization phase of Part II and during the quantitative Design Space determination study.

The LC/MS conditions used during the experiments of the pre-validation study, described in the first part of this study, were fixed as defined under the QbD optimization study described in [14]. Four columns were simultaneously tested, using LC/MXI-MS equipment [14], throughout the screening study that took place during the second part of the present study. These were:

- Grace Alltech (Columbia, MD, USA) Alltima HP HILIC 2.1x150 mm (3.0 µm)
- Waters XBridge Amide 2.1x150 mm (3.5 µm)
- Waters XBridge BEH HILIC 2.1x150 mm (3.5 µm)
- ThermoFisher Scientific (Waltham, MA, USA) Syncronis HILIC 2.1x150 mm (5.0 µm)

These columns were tested in order to select the best one for improving the chromatographic performance for a selective determination of glucosamine and galactosamine, in the shortest possible time, without causing the on-column motarotation of each epimer. Each column was also available in a UPLC geometry in order to conduct the optimization phase of the QbD development with the selected column using the UPLC/MS-MS equipment. The Liquid Chromatography and Mass Spectrometry conditions for the experiments, either fixed *a priori* based on scientific knowledge or investigated during the screening design as well as during the optimization phase and the quantitative Design Space determination, are described in Table 1.

2.4 A predictive approach developed for the pre-validation study

The responses obtained from the performance of the adapted DoE for P4FX98 and P4NX99 were modeled in relation to the experimental factors of methanol, acetonitrile, and the buffer, as well as the concentrations of P4FX98 and of P4NX99, resulting in a multivariate calibration function:

$$\mathbf{Y} = \boldsymbol{\beta_0} + \boldsymbol{\beta_1}\times\mathrm{MeOH} + \boldsymbol{\beta_2}\times\mathrm{ACN} + \boldsymbol{\beta_3}\times\mathrm{Buffer} + \boldsymbol{\beta_4}\times\mathrm{Concentration} + \boldsymbol{\beta_5}\times\mathrm{MeOH}\times\mathrm{ACN} + \boldsymbol{\beta_6}\times\mathrm{Concentration} + \boldsymbol{\beta_7}\times\mathrm{Concentration}\times\mathrm{ACN} + \boldsymbol{\beta_8}\times\mathrm{Concentration}\times\mathrm{Buffer} + \varepsilon$$

From this model, on a fine grid covering the experimental domain, responses for P4FX98 and P4NX99 were then simulated a large number of times (i.e. 10,000), for both the simulated calibration standards and the simulated validation standards. Different numbers of series (or runs) and replicates per series were tested to assess the predictive ability of the analytical procedure to be validated. Simulated results were then computed over the grid of the experimental domain for each combination of series and replicates per series. The probability of obtaining results within +/-15% of the nominal concentration was also computed.

2.5 Optimization study for the selective determination of glucosamine and galactosamine in human plasma

The determination of the qualitative performance required for the selective determination of glucosamine and galactosamine (i.e. epimeric compounds) in human plasma was performed following the QbD approach. This approach, which has been well described in the scientific literature [23-25], was implemented, taking into account the separation of glucosamine and galactosamine, as well as some resulting extracted compounds from the human plasma matrix. The selected responses were the retention times of these compounds recorded in the Multiple Reaction Monitoring mode at a mass transition (*m/z* ratio) of 180/162. The mass transition (*m/z*) used for the internal standard was 186/168. In the present Analytical Quality-by-Design study, the separation criterion (S) was considered as the most relevant Critical Quality Attribute (CQA). It should be noted that, from ICHQ8 reference document point of view, this definition of the CQA is slightly different. However, from an analytical point of view, all the characteristics involved in the optimization of the method can be considered as a CQA. This is the case of the separation criterion S in the present paper that must be within an appropriate limit to ensure the desired method quality. Indeed, without an appropriate separation any quantitative analysis could be performed for that quality purpose. A first screening DoE was performed for the selection of the column and the influent Critical Method Parameters (CMPs), allowing the determination of both amino sugars while avoiding the on-column mutarotation phenomenon. Following this, a central composite design, with the ACN percentage in the mobile phase (*X.ACN*) and pH (*pH*) as factors, was conducted. Based on the current scientific knowledge of the influence of the temperature factor (*T*), this parameter was manually added to the optimization DoE, and was extended as far as possible within the capabilities of the equipment being used, leading to a custom central composite design with a total of 13 experimental conditions ($n = 15$).

238 The responses measured on each chromatogram were the retention times at the beginning,
239 apex and end. The methodology, applied in order to calculate the DS based on the predictive
240 responses and their associated prediction errors, was the same as that explained in previous
241 papers [14,25,26]. In the present case, the following model was applied:

$$\mathbf{Y} = \boldsymbol{\beta_0} + \boldsymbol{\beta_1}\times\mathrm{ACN} + \boldsymbol{\beta_2}\times\mathrm{ACN}^2 + \boldsymbol{\beta_3}\times\mathrm{ACN}^3 + \boldsymbol{\beta_4}\times\mathrm{pH}^2 + \boldsymbol{\beta_5}\times\mathrm{pH}^3 + \boldsymbol{\beta_6}\times\mathrm{pH}^4 + \boldsymbol{\beta_7}\times\mathrm{Temp} + \boldsymbol{\beta_8}\times\mathrm{ACN}\times\mathrm{Temp} + \boldsymbol{\beta_9}\times\mathrm{ACN}\times\mathrm{Temp}\times\mathrm{pH} + \varepsilon$$

242

243 $$\mathbf{Y} = \mathbf{XB} + \mathbf{E}, \quad (1)$$

244 with ε_n, the n[th] line of $\mathbf{E}$, assumed to follow a multivariate Normal distribution,
245 $\varepsilon_n \sim N(\mathrm{O},\Sigma)$, $n = 1,\ldots,N$, with N representing the number of experiments. $\mathbf{X}$ is therefore the
246 (N x F) centered and reduced design matrix and $\mathbf{B}$ is the (F x M) matrix containing the F
247 effects for each of the M = 3 x P responses. Σ is the covariance matrix of the residuals.

248 *2.6 A Quality-by-Design approach for a quantitative Design Space*
249 *determination*

250 The responses obtained from implementing the DoE for glucosamine and galactosamine were
251 modeled in relation to the experimental factors pH and acetonitrile, as well as the
252 concentrations of glucosamine and galactosamine, resulting in a multivariate calibration
253 function:

$$\mathbf{Y} = \boldsymbol{\beta_0} + \boldsymbol{\beta_1}\times\mathrm{pH} + \boldsymbol{\beta_2}\times\mathrm{ACN} + \boldsymbol{\beta_3}\times\mathrm{Concentration} + \boldsymbol{\beta_4}\times\mathrm{pH}\times\mathrm{ACN} + \boldsymbol{\beta_5}\times\mathrm{Concentration}\times\mathrm{pH} + \boldsymbol{\beta_6}\times\mathrm{Concentration}\times\mathrm{ACN} + \varepsilon$$

254

255 From this model, on a fine grid covering the experimental domain, responses for glucosamine
256 and galactosamine were then simulated a large number of times (i.e. 10,000) for both the
257 simulated calibration standards and the simulated validation standards. Simulated results were
258 then computed over the grid of the experimental domain and the predictive probability of
259 obtaining results within +/-15% of the nominal concentration was computed.

2.7 Software

Coding was carried out with the R 2.15.1 software. The e.noval software v3.0 (Arlenda, Liège, Belgium) was used to compute the validation results of the analytical method and to obtain the accuracy profiles for the conventional approach to the validation step in order to quantify glucosamine and galactosamine in human plasma.

3 Results and discussion

Scientists traditionally consider all the steps of the method lifecycle as a series of stand-alone steps. Although the QbD approach is increasingly being applied nowadays, the pre-validation and validation studies are usually performed separately from this strategy. In this way, knowledge obtained during these particular steps of the lifecycle is only informative for one single set of work conditions. The QbD approach, on the other hand, allows a much broader outlook: working within an operational space while managing the risk.

3.1 Part I: Pre-validation study during the QbD optimization step

The optimization step of a method development considered by a QbD strategy allows the qualitative performance of the step to be determined within an operational space through the use of a DoE (i.e. the qualitative DS). If the optimization is successful, this step occurs immediately before the pre-validation study. Therefore, it seems conceivable that the DoE, performed during this particular step, could be elaborated further in order to simultaneously conduct both the optimization step and the pre-validation study phase. In this way, it would be possible to carry out an evaluation of the Design of Experiment to be implemented during the formal validation step. A recently developed stability-indicating method was selected to illustrate the use of this new approach as part of the pre-validation study. This method allows the selective determination of two major degradation products (i.e. P4NX99 and P4FX98, under confidential agreement) of the active principal ingredient of a commonly used medicine [14].

In order to simultaneously perform the optimization step of the method as well as the pre-validation study, the DoE used here needed to be adapted. In particular, each condition of the DoE was reproduced in triplicate, while at the same time, two different concentrations of

290 P4NX99 and P4FX98 were alternatively tested. These concentrations were selected to
291 estimate the limit of quantification of the method (i.e. upper and lower). The DoE
292 implemented during the optimization phase was then conducted once again taking into
293 account these modifications. The DS obtained during the method development is illustrated in
294 Fig. 1. This figure also presents the tested conditions of the DoE (red circles). The odd points
295 were tested with a high concentration of P4NX99 (i.e. 100 ng mL^{-1}) and a low concentration
296 of P4FX98 (i.e. 50 ng mL^{-1}) and inversely for the even points (25 ng mL^{-1} and 200 ng mL^{-1},
297 respectively). This figure also shows a representative chromatogram for each condition of the
298 DoE as well as a reminder both of the compounds investigated in each Selected Ion
299 Monitoring chromatogram and of the Critical Quality Attributes selected. The joint predicted
300 probabilities of meeting all of these CQAs with their acceptance limit (λ) for each specific
301 point of the DoE as well as for the selected working point (i.e. the set of conditions selected
302 for the formal validation [14], the blue spot) were also indicated. It should be noted that, in
303 comparison with the usual optimization DoE, the adaptations did not increase the working
304 time independently of the repetitions of the DoE points.
305 From the 9 tested conditions, only 8 and 6 chromatograms were exploitable for P4FX98 and
306 P4NX99, respectively. Indeed, chromatograms obtained at “P1” could not be used for either
307 of the two compounds due to the fact that selectivity was made impossible by the presence of
308 interfering compounds. For the same reason, data from conditions “P2” and “P3” were also
309 rejected but only in the case of P4NX99. Moreover, a deconvolution process was required for
310 some conditions, introducing additional uncertainty for these quantitative data. Based on the
311 exploitable quantitative data, calibration and validation sets were simulated, as explained in
312 the Experimental section above. In this way, the probability of each point of the DoE being
313 within the acceptance limits, *a priori* fixed at +/- 15%, was calculated and the results are
314 presented in Table 2. These experiments have also allowed simulating the probability

315 throughout the area defined by the DoE for each concentration. Unfortunately, with only 5
316 usable conditions for P4NX99, this simulation was unsuccessful. Figure 2 shows the
317 distribution of the probability being within the acceptance limits calculated with all the
318 available quantitative data by concentration level for P4FX98. Fig. 2A shows the results
319 obtained for a concentration level of 50 ng mL^{-1}, while Fig. 2B presents the concentration
320 level at 200 ng mL^{-1}. Following this analysis, several Designs of Experiments, to be
321 conducted during the validation step, were tested. The different designs evaluated were a
322 combination of validation series and repetitions of validation standards during each series. A
323 minimum of three validation series and a minimum of two repetitions for each series were
324 considered since their combination led to the smallest Design of Experiments that would need
325 to be implemented during a formal validation in order to attain sufficient statistical power.
326 Ten thousand simulations were then computed for each combination in order to assess their
327 probability of producing a successful validation. A validation was considered successful if the
328 calculated tolerance interval at 95% was included within the acceptance limits fixed at +/-
329 15% for each concentration level. Table 3 shows the probability of a successful validation
330 according to the designs tested for each experimental condition of the optimization DoE.
331 These results show a high probability of attaining a successful validation even in the case of a
332 validation DoE considering three series and three repetitions per series throughout the
333 optimization DoE. This probability approached 100% when a 4 by 4 DoE was considered.
334 The line highlighted in bold in Table 3 presents the experimental condition of the
335 optimization DoE that comes nearest to the validated working condition (i.e. the blue dot in
336 Fig. 1). As demonstrated by the formal validation performed during the previous study [14],
337 the tolerance interval at 95% was included within the acceptance limits fixed at +/- 15% for
338 the concentration range between 50 ng mL^{-1} and 200 ng mL^{-1}. In other words, the present
339 study, performed *a posteriori*, predicts the success of the validation step as was actually

demonstrated during the formal validation. Quantified guarantees of achieving good levels of total error definitely represent a movement towards the next steps of the analytical method lifecycle, i.e. robust assessments and routines that can be used in the laboratory [27].

3.2 Part II: Validation of an operational space

As a key concept of the method lifecycle, the Analytical Target Profile must firstly, be selected at an early stage of the QbD methodology and secondly, be exclusively directed by the final requirements of the user. In this context, the principal objective of a quantitative method is to quantify with confidence while assessing the risk. A qualitative DS obtained by applying a QbD strategy represents only a preliminary step in the implementation of an efficient quantitative method. Indeed, nowadays, the application of this methodology stops at this point. The quantitative performance of the method is then assessed for a single set of conditions within this operational space. The second part of the present study focuses on a similar application of the QbD strategy during the validation step. In order to illustrate this innovative methodology, a previously developed method, using the Quality-by-Testing approach, was selected [22]. This method needed to be optimized in order to allow the selective determination of glucosamine from galactosamine while avoiding the on-column mutarotation phenomenon observed with the initial method. The biological matrix considered was human plasma, while, simultaneously, the equipment being employed was a triple quadrupole mass detector. In a scenario such as this, where polar drug substances are analyzed at very low concentration levels in bioanalytical applications, hydrophilic interaction chromatography (HILIC) plays an important role due to its larger retention possibilities for this kind of compound, which occurs very widely in bioanalysis [26]. The use of large proportions of highly volatile organic components (e.g., acetonitrile, methanol, etc.) in the mobile phase provides excellent ionization efficiency with the commonly used MS sources such as electrospray ionization, and this leads to enhanced sensitivity [28,29].

3.2.1 Qualitative DS: an operational space for the validation

The screening part of this study has allowed selecting influential Critical Method Parameters (CMPs) as well as the column showing the greatest separation efficiency for glucosamine and galactosamine. This enabled to consider the following CMPs: ACN percentage (*X.ACN* varied between 80% and 90%), pH (*pH* varied between 5 and 10) and temperature (*T* varied between 25 and 75 °C). UPLC rather than HPLC was used during the optimization DoE in order to enhance the selective capabilities and reduce the total run time of the method. Consequently, a geometric transfer was implemented for the selected column in order to move it towards the corresponding UPLC geometry. The selected column was a Waters Acquity UPLC BEH Amide 2.1x100 mm (1.7 µm). Enhanced separative capabilities are essential when considering the biological matrix during the optimization step. Indeed, a major concern regarding HILIC-MS(/MS) bioassays and even reversed-phase LC-MS(/MS) bioassays is the impact of the matrix effect (ME) [30-32]. As recommended by the FDA [33], the ME should be assessed during the development of the method. The specific methodology implemented during this study is detailed in the "Supplementary Data" document.

Once the CMPs had been identified (i.e. *X.ACN*, *pH* and *T*) during the screening study, a custom central composite design (T was added manually) with a total of 13 experimental conditions (n = 15, central point tested in triplicate) was conducted. Human plasma spiked with glucosamine, galactosamine and the internal standard as well as non-spiked plasma (for the ME assessment, as explained in the "Supplementary Data" document) were tested. This experimental domain was carefully selected on the basis of the preliminary results obtained with pure chemicals in order to allow the separation of both the epimeric compounds at a transition of *m/z*: 180/162. The concentration of the internal standard was fixed at 250 ng mL^{-1} for all the solutions. Finally, as is now widely discussed in the scientific literature [23-25], a

389 qualitative DS was computed using Monte-Carlo simulations from the prediction errors of a
390 set of CQAs for which the acceptance limits (λ) were fixed, as described below:
391 - Separation between glucosamine, galactosamine and endogenous plasma compounds
392 eluted just before epimeric compounds > 0.2 min.
393 - Total run time < 30 min.
394 A three-dimensional (*X.ACN*, *pH*, *T*) probability surface was then obtained. Three
395 representative slices of this multi-dimensional surface are presented in Fig. 3. The two-
396 dimensional representations were obtained by fixing one parameter at its optimal value in the
397 case of Fig. 3A and Fig. 3B, while *T* was fixed at 50 °C in the case of Fig. 3C, since this was
398 the selected working temperature for the validation of the operational space. Fig. 3A, where
399 the fixed parameter was *X.ACN* at 88.5%, shows that the interaction *pH – T* was barely
400 significant. However, a DS with a quality level (π) of more than 0.81 was defined for a *pH*
401 ranging from 5.2 to 6.4 and a *T* ranging from 35 °C to 75 °C. The slice where the *pH*
402 parameter was fixed at 5.75 is presented in Fig. 3B. At 50 °C and above, the level of quality
403 obtained when considering all the constraints (i.e. the CQAs) was found to be acceptable and
404 relatively constant for the parameter *X.ACN* ranging from 83% to 89%. This finding is
405 confirmed by Fig. 3C, which presents the computed probability surface at a fixed temperature
406 of 50 °C, in particular for the range of *pH* between 5 and 6.8. Within this area, dark lines
407 highlight two DS, with a π of 0.825. These DS represent the sets of conditions where the
408 chromatographic performance, with regard to the separation of glucosamine, galactosamine
409 and endogenous plasma compounds within a maximum run time of 30 minutes, presented a
410 very acceptable level of quality.

411 ### 3.2.2 Strategy for the validation of an operational space

412 Once the qualitative DS has been obtained, the next step of the method lifecycle is the
413 validation of the method. As with the conventional approach to the validation, a unique set of

414 conditions within the qualitative DS is chosen. A validation DoE is then applied to the
415 selected working conditions, considering an approach using accuracy profiles based on
416 statistical tolerance intervals. Nowadays, this approach is fully approved by the authorities
417 [18], as well as being widely discussed and applied by scientists [34-35]. Within the
418 qualitative DS centered around the parameter X.ACN at 86% in Fig. 3C, the working
419 conditions defined by the red dot (i.e. $X.ACN$ = 86%, pH = 6, T = 50 °C) could have been
420 appropriate and therefore suitable to be subjected to a formal validation. However, this
421 conventional approach only allows the assessment of the quantitative performance of the
422 method for the selected working conditions, which represents a break from the QbD process.
423 Indeed, the qualitative performance of the method is evaluated throughout a defined domain,
424 but the quantitative performance is only assessed for one single set of conditions. The
425 qualitative DS guarantees an area of robustness for the studied CMPs in terms of the selected
426 CQAs. Consequently, the analyst is able to find alternative working conditions, where the
427 qualitative performance is already demonstrated. This allows him/her to be able to respond to
428 an unexpected or a scheduled change in the method that originates from a separation issue.
429 Nevertheless, this learning process is only applicable for the qualitative part of the method,
430 not for its quantitative performance. How can the quantitative performance of the newly
431 selected working conditions be assessed? Without any further information, the analyst could
432 be placed in in the position of selecting, within the operational space, a working condition
433 with a poor probability of validation success. In order to provide a remedy for this scenario,
434 an innovative validation approach, based on the QbD concept, was applied to the case study
435 addressed in this section.
436 Within the probability surface presented in Fig. 3C, a qualitative DS was selected that was as
437 large as possible, and with a minimum quality level (π) of 0.5. This is outlined in the figure
438 by the blue lines. Both the blue dots and the red dot represent the experimental conditions

tested during the validation DoE for this operational space. The red dot represents the central condition of this custom DoE but also the condition selected as a reference to compare with a conventional approach to the validation step (i.e. a validation of a unique set of conditions within the operational space). The parameter *T* was fixed at 50 °C since the separative performance compared to the optimal temperature (i.e. 62.5 °C) was similar and the lower temperature exerted less of a strain on the equipment. The design of experiments for the validation was developed for a period of 3 series throughout the operational space, as can be seen in Fig. 4. For each series, a minimum of 2 repetitions of the calibration standard for each concentration level was tested. In the meantime, a minimum of 5 repetitions of the validation standard for each concentration level was also tested. Three repetitions were always performed in the case of the validation standards for the reference points, as flagged with the red color in Fig. 4. Three concentration levels were tested for glucosamine and galactosamine, covering a range from 25 ng mL^{-1} to 500 ng mL^{-1} and from 200 ng mL^{-1} to 1000 ng mL^{-1} (injected concentrations) in all the standards, respectively. Each sample was spiked with the internal standard in order to obtain a concentration of 500 ng mL^{-1} (injected concentration).

3.2.3 Validation results

Computation of data gathered via this validation DoE throughout the operational space was performed as explained in the Experimental section above. A probability surface was calculated for each concentration level of glucosamine and galactosamine, and this is presented in Fig. 5A and Fig. 5B, respectively. Each probability surface represents the probability that each future result, for the concentration level tested and throughout the operational space, will be between +/- 15% of the true value. This predictive methodology is similar to the one used during the conventional approach to the validation for the "β-expectation tolerance interval" and allows the assessment of a key feature of the validation study within an operational space: the uncertainty regarding the performance of the method.

As can be seen in Fig. 5A, probability surfaces obtained for the validation of the determination of glucosamine in human plasma were extremely homogenous throughout the operational space and for the entire considered dosing range. The probability of being within +/- 15% of the true value was always higher than 97%, whatever the concentration level. Based on this evaluation, taking into account all the validation results with their associated uncertainty, the quantitative performance of the method was guaranteed across the operational space for glucosamine. The validation data gathered for galactosamine on the other hand led to a distinctive situation. In this case, the computed probability surfaces, also taking into account all the validation results with their associated uncertainty, showed a probability of being within +/- 15% of the true value ranging between 68% and 72% for concentration levels of 500 ng mL^{-1} and 1000 ng mL^{-1}. This probability fell between 45% and 72% for the lower concentration level (i.e. 200 ng mL^{-1}). From the perspective of a formal validation, these results could not be considered as acceptable. However, these probability surfaces led to the discovery of some very useful information. As can be seen in Fig. 5B, a high percentage of ACN and pH resulted in a greater chance of achieving a successful validation. With this information, it would be possible to influence positively the selection of different working conditions, sometimes necessary during the life cycle of the method (i.e. continuous improvement process). In addition to computing these probability surfaces, a probability profile could be computed from those first results for a specific set of working conditions within the operational space. These probability profiles could then be compared to the risk (α) profiles obtained during a formal validation (i.e. *the risk α = 1 – the probability of being within +/- 15% of the true value*). Furthermore, accuracy profiles could also be computed from these probability surfaces for a specific set of working conditions within the operational space.

3.2.4 Comparison with a conventional approach to the validation step

The DoE of this innovative strategy was wisely elaborated. Indeed, experiments on the central working conditions were repeated as for a formal validation, testing 3 repetitions of the validation standards over 3 working days (see Fig. 4). In this context, these experiments could be independently computed in order to obtain results in the same way as from a formal validation of the method. As suggested in the section above, an accuracy profile could be calculated for each working condition within the operational space and for each analyte. These profiles could thus be compared to those obtained from the formal validation of the central point, as can be seen in Fig. 6A and Fig. 6B for glucosamine and galactosamine, respectively. As can be seen on the right hand side of Fig. 6A, the formal validation of the selected working conditions (i.e. the central point of the validation Design of Experiments) was successful for glucosamine. The quantitative performance for this molecule throughout the operational space was very good and homogenous, as highlighted in Fig. 5A. Consequently, very accurate predictions for each set of working conditions within the operational space were obtained when considering all the validation results with their associated uncertainty. Unlike the glucosamine results, those obtained for galactosamine were less favorable. Indeed, the quantitative performance throughout the operational space was found to be less homogeneous. However, as can be seen in Fig. 6B, analysis of the validation results for the central condition of the validation DoE led to a successful validation when considering a formal validation process. As the strategy developed for the validation of the operational space considered the uncertainty throughout that entire operational space, the predictive validation results were less optimistic than when only a single set of working conditions was considered. These poorer predictive results may thus have been the result of a lack of statistical power. As with a formal validation process, an additional validation series (i.e. an additional working day) could be considered here. The validation DoE presented in

513 Fig. 4 would thus need to be adapted in order that these additional experiments cover the
514 operational space. Computation of the results of the validation DoE has also allowed to
515 calculate the probability profiles for any working conditions within the operational space, as
516 explained in the previous section. In the case of glucosamine, the risk (α) throughout the
517 dosing range for the central experimental condition of the validation DoE was calculated and
518 was shown to vary between 0.1% and 0.4%. The result regarding the same parameter obtained
519 via the formal validation varied between 0.1% and 0.5%. This comparison demonstrates the
520 high quality of prediction across the entire operational space in the case of glucosamine.
521 Moreover, the linearity of the method throughout the operational space was calculated from
522 all the results of the validation DoE and compared to the results obtained via the formal
523 validation. In the case of glucosamine, the slope, the intercept and the coefficient of
524 determination (R^2) were equal to 1.00, 0.26 and 0.99, respectively. In the case of the formal
525 validation, the calculations of these results were equal to 1.00, -0.05 and 0.99, respectively.
526 These results demonstrate once more the high quality of the prediction obtained by this
527 validation Design of Experiment. This validation of the operational space was performed over
528 three working days as for a formal validation, confirming the quantitative performance of the
529 method across an area rather than for a single set of conditions during the same time period as
530 used in a conventional validation process.
531

4 Conclusion

Defining the objectives of the method using an Analytical Target Profile (ATP) should be the first step of the QbD methodology. In the case of the development of a quantitative method, this ATP should also be focused on the quantitative performance of the method. By integrating the pre-validation study alongside the QbD optimization phase, some parameters of the validation can be evaluated. As described in the first part of this study, the Design of Experiments for use during a formal validation can be developed simultaneously with the selection of the qualitative Design Space, without increasing the working time usually dedicated to this step of the method lifecycle. Following a similar approach, an estimation of the calibration model, the accuracy, and the limits of detection/quantification may also be considered during this step. Consequently, the methodology to be implemented would require further improvement. In particular, repetitions considered at each experimental point would need to be made with more than one concentration level in order to improve the quality of the prediction.

As specified in the latest USP [18] and FDA [36] documents, the validation step of the method lifecycle must not be an isolated activity but should be part of the continuous improvement of the method. The routine use of the method allows the continuous acquisition of information via quality control samples, for instance. However, without a deep understanding of the method (i.e. qualitative and quantitative knowledge), it is rather difficult to take advantage of the information gained. For example, with the case study presented here for glucosamine determination, and even for galactosamine, the quantitative and qualitative information obtained, would allow the analyst to consider selecting other working conditions within the operational space with great confidence. Indeed, using this strategy, it would thus

be possible to evaluate the quantitative performance of the method before the selection of different working conditions. This would allow a corrective action to be implemented or a preventive action to be initiated following, for instance, a problem encountered during the routine use of the method. This methodology is not restricted to overcoming routine issues. It could also be employed when a change of the applicability of the method needs to be considered, for instance, a change in the biological matrix (gender, species, etc.). It is for this reason that this innovative strategy combines both a learning process and a thorough assessment of the risk. However, even though this did not happen with the presented case study, this innovative approach could lead to the use of a validation protocol that is more expensive in terms of analytical time. This concern would need to be addressed with further development of the strategy. Nonetheless, from our point of view, this potential additional analytical cost should be set against the benefits provided by this approach during the whole analytical lifecycle.

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References

[1] ICH, Q8(R2), Pharmaceutical development (2009).

[2] ICH, Q2(R1), Validation of analytical procedures (1996).

[3] ICH, Q9, Quality risk management (2006).

[4] ICH, Q10, Pharmaceutical quality system (2008).

[5] U.S. Pharmacopeial Convention, chapters 1224, 1225, 1226, USP panel expert.

[6] U.S. Pharmacopeial Convention, chapters 1032, 1033, 1034, USP panel expert.

[7] U.S. Pharmacopeial Convention, chapter 1210, USP panel expert.

[8] J.J. Peterson, A Bayesian approach to the ICH Q8 definition of design space, J. Biopharm. Stat. 18 (2008) 959.

[9] J.J. Peterson, M. Yahyah, A Bayesian Design Space approach to robustness and System Suitability for pharmaceutical assays and other processes, Statistics in Biopharmaceutical Research 1 (2009) 441.

[10] P. Nethercote, J. Ermer, Quality by Design for Analytical Methods: Implications for Method Validation and Transfer, Pharm. Tech. 36 (10) (2012) 52.

[11] S. Orlandini, S. Pinzauti, S. Furlanetto, Application of quality by design to the development of analytical separation methods, Anal. Bioanal. Chem. 405 (2013) 443.

[12] B. Debrus, D. Guillarme, S. Rudaz, Improved Quality-by-Design compliant methodology for method development in reversed-phase liquid chromatography, J. Pharm. Biomed. Anal. 84 (2013) 215.

[13] E. Rozet, P. Lebrun, P. Hubert, B. Debrus, B. Boulanger, Design Spaces for analytical methods, Trends Anal. Chem. 42 (2013) 157.

[14] C. Hubert, P. Lebrun, S. Houari, E. Ziemons, E. Rozet, Ph. Hubert, Improvement of a stability-indicating method by Quality-by-Design versus Quality-by-Testing: A case of a learning process, J. Pharm. Biomed. Anal. 88 (2014) 401.

[15] P. Borman, P. Nethercote, M. Chatfield, D. Thompson, K. Truman, The Application of Quality by Design to Analytical Methods, Pharm. Technol. 31 (2007) 142.

[16] P. Katz, C. Campbell, FDA 2011 Process Validation Guidance: Process Validation Revisited, Journal of GXP Compliance 16 (2012) 18.

[17] M. Pohl, M. Schweitzer, G. Hansen, M. Hanna-Brown, P. Borman, K. Smith, J. Larew, P. Nethercote, Implications and Opportunities of Applying QbD Principles to Analytical Measurements, Pharm. Technol. Eur. 22 (2010) 29.

[18] R.K. Burdick, D.J. LeBlond, D. Sandell, H. Yang, H. Pappa, Statistical Methods for Validation of Procedure Accuracy and Precision, Pharmacopeial Forum 39 (2013).

[19] P. Borman, M. Chatfield, I. Damjanov, P. Jackson, Design and analysis of method equivalence studies, Anal. Chem. 81 (2009) 9849.

[20] W. Dewé, Review of statistical methodologies used to compare (bio)assays, J. Chromatogr. B 877 (2009) 2208.

[21] A.M. Skelley, R.A. Mathies, Rapid on-column analysis of glucosamine and its mutarotation by microchip capillary electrophoresis, J. Chromatogr. A 1132 (2006) 304.

[22] C. Hubert, S. Houari, F. Lecomte, V. Houbart, C. De Bleye, M. Fillet, G. Piel, E. Rozet, Ph. Hubert, Development and validation of a sensitive solid phase extraction/hydrophilic interaction liquid chromatography/mass spectrometry method for the accurate determination of glucosamine in dog plasma, J. Chromatogr. A 1217 (2010) 3275.

[23] P. Lebrun, B. Govaerts, B. Debrus, A. Ceccato, G. Caliaro, P. Hubert, B. Boulanger, Development of a new predictive modelling technique to find with confidence equivalence zone and design space of chromatographic analytical methods, Chemometr. Intell. Lab. Syst. 91 (2008) 4.

626 [24] B. Debrus, P. Lebrun, A. Ceccato, G. Caliaro, E. Rozet, I. Nistor, R. Oprean, F.J.
627 Rupérez, C. Barbas, B. Boulanger, P. Hubert, Application of new methodologies based
628 on design of experiments, independent component analysis and design space for robust
629 optimization in liquid chromatography, Anal. Chim. Acta 691 (2011) 33.
630 [25] P. Lebrun, B. Boulanger, B. Debrus, Ph. Lambert, Ph. Hubert, A Bayesian Design
631 Space for analytical methods based on multivariate models and predictions, J.
632 Biopharm. Stat. (accepted July 2012, http://hdl.handle.net/2268/128222).
633 [26] P. Hemstrom, K. Irgum, Hydrophilic interaction chromatography, J. Sep. Sci. 29
634 (2006) 1784.
635 [27] P. Borman, M. Chatfield, I. Damjanov, P. Jackson, Method ruggedness studies
636 incorporating a risk based approach: A tutorial, Anal. Chim. Acta 703 (2011) 101.
637 [28] Y. Hsieh, Potential of HILIC-MS in quantitative bioanalysis of drugs and drug
638 metabolites, J. Sep. Sci. 31 (2008) 1481.
639 [29] M.R. Gama, R.G. da Costa Silva, C.H. Collins, C.B.G. Bottoli, Hydrophilic interaction
640 chromatography, Trends Anal. Chem. 37 (2012) 48.
641 [30] E. Chambers, D.M. Wagrowski-Diehl, Z. Lu, J.R. Mazzeo, Systematic and
642 comprehensive strategy for reducing matrix effects in LC/MS/MS analysis, J.
643 Chromatogr. B 852 (2007) 22.
644 [31] J.-N. Mess, C. Côté, A. Bergeron, M. Furtado, F. Garofolo, Selection of HILIC
645 columns to handle matrix effect due to phospholipids, Bioanalysis 1 (2009) 57.
646 [32] B. Tan, A. Negahban, T. McDonald, Y. Zhang, C. Holliman, Utilization of
647 hydrophilic-interaction LC to minimize matrix effects caused by phospholipids,
648 Bioanalysis 4 (2012) 2049.
649 [33] U.S. Department of Health and Human Services, Food and Drug Administration
650 (FDA), Center for Drug Evaluation and Research (CDER), Center for Veterinary

651 Medicine (CVM), Guidance for industry: Bioanalytical Method Validation (Rockville,
652 MD, May 2001).
653 [34] E. Rozet, A. Ceccato, C. Hubert, E. Ziemons, R. Oprean, S. Rudaz, B. Boulanger, Ph.
654 Hubert, Analysis of recent pharmaceutical regulatory documents on analytical method
655 validation, J. Chromatogr. A 1158 (2007) 111.
656 [35] E. Rozet, V. Wascotte, N. Lecouturier, V. Preat, W. Dewé, B. Boulanger, Ph. Hubert,
657 Improvement of the decision efficiency of the accuracy profile by means of a
658 desirability function for analytical methods validation. Application to a diacetyl-
659 monoxime colorimetric assay used for the determination of urea in transdermal
660 iontophoretic extracts, Anal. Chim. Acta 591 (2007) 239.
661 [36] U.S. Department of Health and Human Services, Food and Drug Administration
662 (FDA), Center for Drug Evaluation and Research (CDER), Center for Biologics
663 Evaluation and Research (CBER), Guidance for Industry Analytical Procedures and
664 Methods Validation for Drugs and Biologics (Rockville, MD, February 2014).
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List of figures

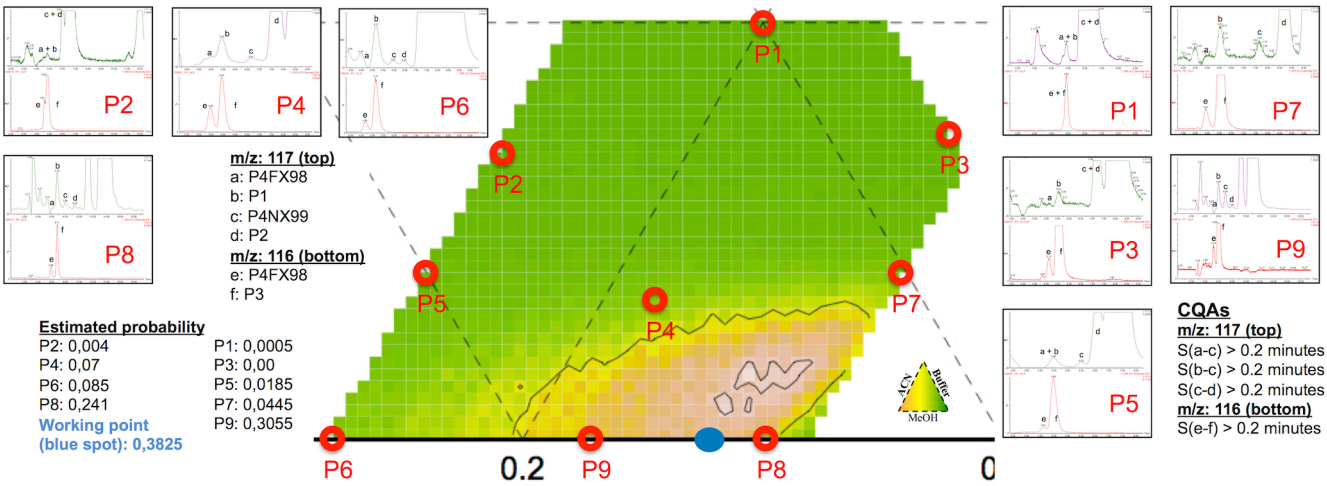
Figure 1: Qualitative DS obtained during the method development. The red circles represent the tested conditions with their representative chromatogram and estimated probability. Peaks obtained in each chromatogram for both channels are labeled from “a” to “f”. A summary of the CQAs selected with their acceptance limit (ƛ) for obtaining the qualitative DS are also specified. The blue spot represents the set of working conditions selected for the formal validation of the method (see [14]).

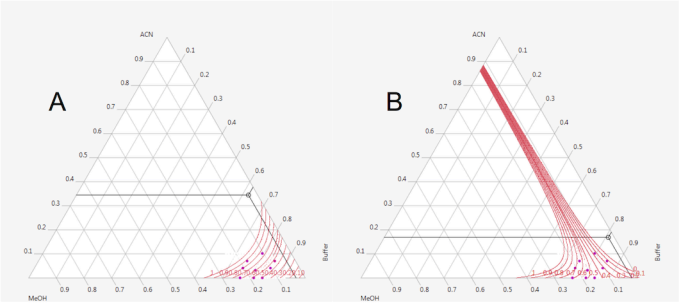
Figure 2: Simulation of the distribution of the probability of being within the acceptance limits of +/- 15% for P4FX98. (A) Concentration level at 50 ng mL^{-1}. (B) Concentration level at 200 ng mL^{-1}.

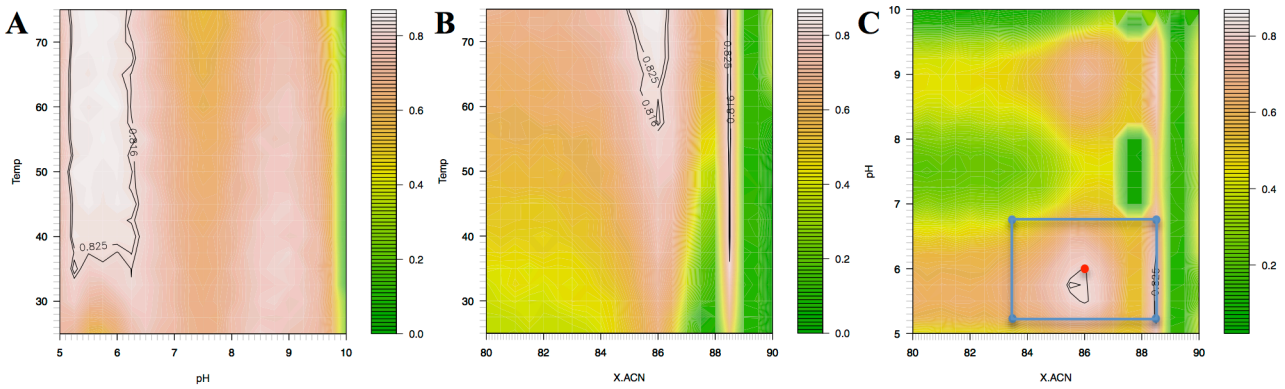
Figure 3: Two-dimensional qualitative probability surfaces (i.e. P(CQAs > ƛ)) with their DS defined by a dark line. (A) *X.ACN* was fixed at 88.5%, *pH* varied between 5 and 10 and *T* varied between 25 °C and 75 °C. (B) *pH* was fixed at 5.75, *X.ACN* varied between 80% and 90% and *T* varied between 25 °C and 75 °C. (C) *T* was fixed at 50 °C, *pH* varied between 5 and 10 and *X.ACN* varied between 80% and 90%. The area surrounded by blue dots and blue lines represents the qualitative DS selected as the operational space. The red dot corresponds to the reference point selected for the formal validation.

Figure 4: Design of Experiments for the validation of the operational space. The flags over the experimental conditions represent the number of repetitions using the color coding indicated on the top right hand side of the figure.

690 **Figure 5**: Probability of the surface being within +/- 15% of the true value by concentration
691 level for (A) glucosamine and (B) galactosamine
692
693 **Figure 6**: Accuracy profile of the validation of the working conditions of the reference point
694 obtained for (A) glucosamine and (B) galactosamine. On the left hand side of the figure,
695 accuracy profiles are obtained from the validation DoE. The accuracy profiles presented on
696 the right hand side of the figure, were obtained by performing a formal validation of the
697 selected set of conditions. The plain red lines represent the relative bias, the dashed lines
698 represent the 95% β-expectation tolerance limits and the dotted curves represent the
699 acceptance limits (30% at LLOQ and 15% elsewhere).
700


P2
P4
P6
P8
P1
P7
P3
P9
P5
P1
P2
P3
P4
P5
P6
P7
P8
P9
0.2
0
ACN
Buffer
MeOH
m/z: 117 (top)
a: P4FX98
b: P1
c: P4NX99
d: P2
m/z: 116 (bottom)
e: P4FX98
f: P3
Estimated probability
P2: 0,004
P4: 0,07
P6: 0,085
P8: 0,241
Working point
(blue spot): 0,3825
P1: 0,0005
P3: 0,00
P5: 0,0185
P7: 0,0445
P9: 0,3055
CQAs
m/z: 117 (top)
S(a-c) > 0.2 minutes
S(b-c) > 0.2 minutes
S(c-d) > 0.2 minutes
m/z: 116 (bottom)
S(e-f) > 0.2 minutes


A
ACN
MeOH
Buffer
B
ACN
MeOH
Buffer


A
B
C
Temp
pH
X.ACN
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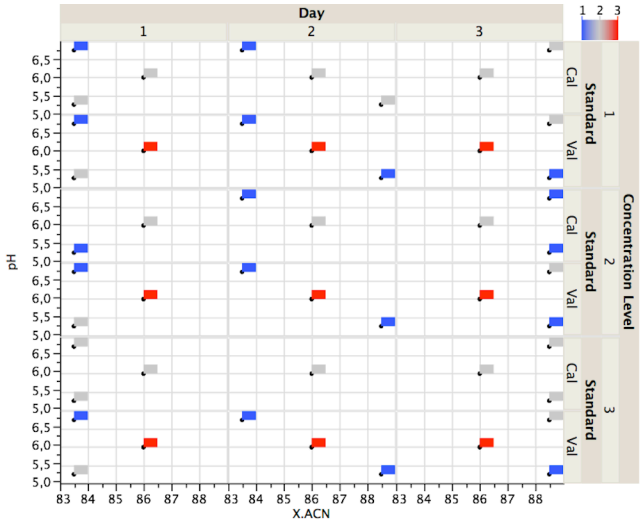
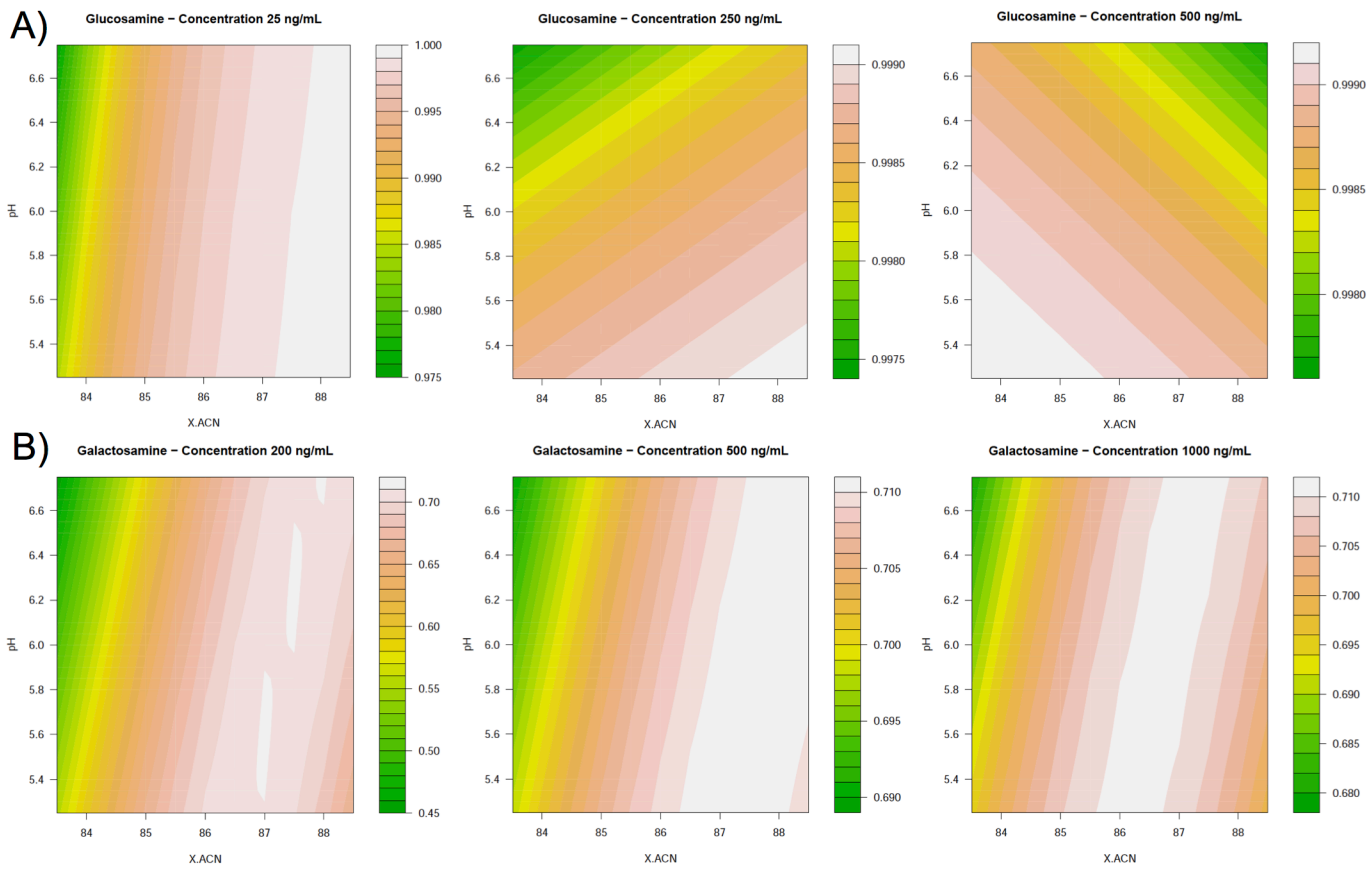
0.85
0.875
0.9
0.925


Day
1
2
3
1 2 3
Concentration Level
Standard
Cal
Val
pH
6,5
6,0
5,5
5,0
83 84 85 86 87 88
X.ACN


A)
Glucosamine – Concentration 25 ng/mL
Glucosamine – Concentration 250 ng/mL
Glucosamine – Concentration 500 ng/mL
B)
Galactosamine – Concentration 200 ng/mL
Galactosamine – Concentration 500 ng/mL
Galactosamine – Concentration 1000 ng/mL
pH
X.ACN

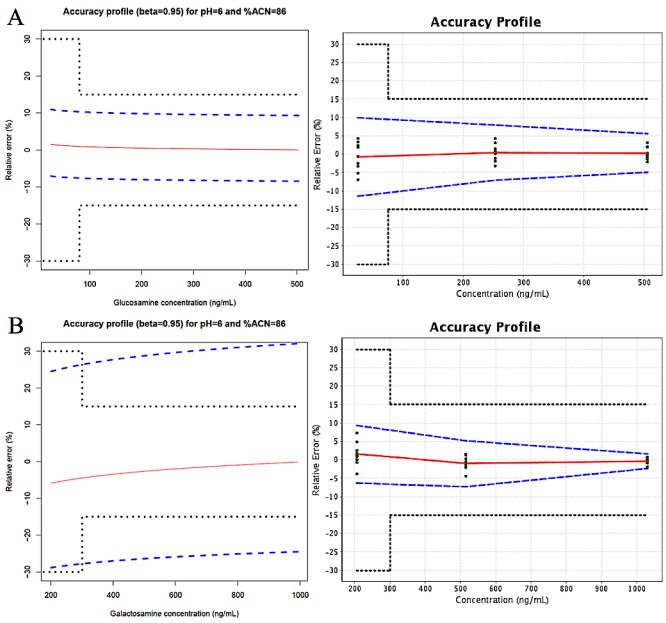

A
Accuracy profile (beta=0.95) for pH=6 and %ACN=86
Relative error (%)
Glucosamine concentration (ng/mL)
Accuracy Profile
Relative Error (%)
Concentration (ng/mL)
B
Accuracy profile (beta=0.95) for pH=6 and %ACN=86
Relative error (%)
Galactosamine concentration (ng/mL)
Accuracy Profile
Relative Error (%)
Concentration (ng/mL)

Table 1: LC and MS conditions, *a priori* fixed or investigated, during the screening design as well as during the optimization phase and quantitative Design Space determination

	Screening Design (HPLC/MS)	Optimization design (UPLC/MS-MS)	Quantitative DS determination (UPLC/MS-MS)
Type of DoE conducted	Fractional Factorial Design	Central Composite Design	Custom Central Composite Design
ACN percentage (%) (binary mixture with buffer)	65 – 90	80 – 90	83.5 – 88.5
Buffer concentration (mM)	10 – 50	150	150
pH of mobile phase	3 – 7.5	5 – 10	5.25 – 6.75
Column temperature (°C)	25	25 – 75	50
GluN concentration (ng mL^{-1})	1000	50 – 500	25 – 500
GalN concentration (ng mL^{-1})	2000	200 – 1000	200 – 1000
GluN-13C6 concentration (ng mL^{-1})	NA	250	500
Flow rate (µL min^{-1})	250	300	300
Injection volume (µL)	10	10	10
MS or MS-MS mode GluN/GalN (*m/z*)	180	180.2 → 162.2	
MS or MS-MS mode GluN-13C6 (*m/z*)	NA	186.2 → 168.2	
MS source and mode	ESI+		
Cone temperature (°C)	100	150	
Capillary temperature (°C)	400	500	
Nebulizer gas (L h^{-1})	100	150	
Desolvation gas (L h^{-1})	500	1000	
Cone voltage (V)	18	25	
Capillary voltage (kV)	3.00	3.50	
Source offset (V)	NA	60.0	
Collision gas flow rate (mL min^{-1})	NA	0.25	
Nebulizer gas flow (bar)	NA	7.00	
MS-MS mode collision energy (eV)	NA	7.00	
Dwell time for GluN and GalN (ms)	125	250	
Dwell time for GluN-13C6 (ms)	NA	30	

NA: Not Applicable

Table 2: Probability of obtaining results within the acceptance limits of +/- 15% for each experimental point of the DoE

Experimental point of the DoE	MeOH	ACN	Buffer	P4FX98 concentration	Probability
P2	0.171	0.069	0.76	200.6	0.6927
P3	0.0752	0.0752	0.8496	50.15	0.2001
P4	0.162925	0.033075	0.804	200.6	0.5512
P5	0.1995	0.0405	0.76	50.15	0.9458
P6	0.24	0	0.76	200.6	0.4996
P7	0.10716	0.04324	0.8496	50.15	0.2486
P8	0.1504	0	0.8496	200.6	0.4707
P9	0.1864	0	0.8136	50.15	0.1651

Table 3: Probability (%) of attaining a successful validation according to the tested designs (day x repetition) for the acceptance limits of +/- 15% and a probability of 95% of attaining future results within these limits

				Probability of validation success (%)			
Experimental point of the DoE	MeOH	ACN	Buffer	3x3	4x2	4x3	4x4
P2	0.171	0.069	0.76	98.9	98.9	99.6	100.0
P3	0.0752	0.0752	0.8496	99.5	98.9	99.8	99.9
P4	0.162925	0.033075	0.804	99.5	99.1	100.0	100.0
P5	0.1995	0.0405	0.76	99.3	98.2	99.9	100.0
P6	0.24	0	0.76	99.5	98.5	99.8	99.9
P7	0.10716	0.04324	0.8496	99.5	99.3	99.7	99.9
P8	**0.1504**	**0**	**0.8496**	**99.4**	**99.2**	**99.7**	**99.9**
P9	0.1864	0	0.8136	99.1	98.8	99.7	99.9

Supplementary data

Towards a full integration of optimization and validation phases: An Analytical-Quality-by-Design approach.

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Matrix effect assessment

Enhanced separative capabilities were essential when considering the biological matrix during the optimization step. Indeed, A major concern about HILIC/MS(-MS) bioassays or even reversed-phase LC/MS(-MS) bioassays is the impact of the matrix effect (ME). The ME refers to the ionization suppression or enhancement caused by unobserved substances co-eluted from biological matrix. This kind of co-elution competition takes place between compounds during the ionization process, and especially when considering ESI mode [25-27]. Therefore, minimizing this phenomenon is crucial. In the case of this study, a generic protocol combining acidified organic protein precipitation (i.e. ACN with 1% formic acid) and specific extraction of phospholipids was conducted using Phree Phospholipid Removal Plates from Phenomenex by the mean of a vacuum manifold. However, as recommended by FDA [28], the lack of ME has to be assessing during the development of the method. This can be done by the monitoring of the variability of the MS response for the analyte using a post-column infusion scheme during the analysis of an extracted blank matrix sample. This methodology allows identifying the chromatographic region where compounds responsible of the ME are eluted for the tested experimental condition. As the ME could be due to many endogenous compounds, it is difficult to manage this response such as a unique compound. Consequently, this methodology only leads to a categorical response: the lack or not of a matrix effect at the retention time of target analyte. This king of response is difficult to model throughout the entire experimental space. Therefore, a more specific methodology was envisaged in order to assess this problematic. In the case of HILIC method, ME is largely encountered due to a co-elution between early-eluted analytes and endogenous phospholipids or formulation vehicles [27]. Other endogenous compounds typically responsible of the ME in reversed-phase mode are potentially present but are directly eluted in HILIC mode. In this framework, precursor ion scans with the product ion of *m/z*: 184 (i.e. the specific daughter ion from the hydrophilic

45 head of the phospholipids) were performed with the scan range from 490 to 890 m/z for each
46 experimental condition with none spiked plasma. A combination of all recorded spectra
47 during the run time for each experimental condition were performed in order to identify
48 potential phospholipids remaining after plasma preparation. Specific transition of identified
49 phospholipids (i.e. *m/z*: 496-184, 760-184 and 786-184, principally) were then extracted from
50 each chromatogram in order to compare the retention time of this particular phospholipids
51 with the retention time of glucosamine and galactosamine obtained from the analysis of
52 spiked plasmas. This methodology allowed modeling the chromatographic behavior of
53 remaining phospholipids in order to introduce a separation criteria of remaining
54 phospholipids, glucosamine and galactosamine as a CQA if necessary (i.e. if remaining
55 phospholipids were eluted within the retention windows of glucosamine or galactosamine).
56 Thanks this methodology, the separative DS could manage the ME throughout the envisaged
57 experimental domain as requested by FDA recommendations. In the case of the present study,
58 no remaining phospholipids were found to elute within the retention time windows of
59 glucosamine or galactosamine for any experimental condition.
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