UNIVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Programa de Pós-Graduação em Fármaco e Medicamentos
Área de Produção e Controle Farmacêuticos

Aldo Renato Couto

O impacto da incerteza de medição na definição do prazo de validade de medicamentos

Dissertação para obtenção do Título de Mestre
Orientador: Prof. Dr. Felipe Rebello Lourenço

São Paulo – 2023

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Aldo Renato Couto

O impacto da incerteza de medição na definição do prazo de validade de medicamentos

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Dissertação para Obtenção do Título de Mestre

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Prof. Dr. Felipe Rebello Lourenço

Orientador/Presidente

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“Entenda os seus medos, mas jamais deixe que eles sufoquem os seus sonhos.”

“Quando acordei hoje de manhã, eu sabia quem eu era, mas acho que já mudei muitas vezes desde então.”

Alice no País das Maravilhas

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RESUMO

COUTO A.R. **O impacto da incerteza de medição na definição do prazo de validade de medicamentos.** 2023. Dissertação (Mestrado) – Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo.

Para definir o prazo de validade de um medicamento ou insumo farmacêutico ativo é necessário realizar estudos de estabilidade, em que diversos parâmetros são testados ao longo do tempo. Os resultados dos testes são comparados a limites de especificação definidos para cada parâmetro. Porém, a legislação brasileira e as diretrizes de qualidade do ICH que estabelecem critérios para avaliação dos estudos de estabilidade não consideram a incerteza de medição, além disso, avaliam individualmente a conformidade dos parâmetros. Desta forma, os objetivos deste trabalho foram avaliar o impacto da incerteza de medição na definição do prazo de validade de um medicamento, propor procedimento para definição do prazo de validade com base no risco total do consumidor. Adicionalmente, desenvolve-se metodologia para estimar a incerteza de medição para procedimentos espectrofotométricos multivariados. Os riscos particulares do consumidor de cada parâmetro e o risco total do consumidor foram calculados utilizando o método de Monte Carlo (MCM), considerando a incerteza de medição. Foi desenvolvida uma abordagem para definição do prazo de validade baseado no risco total do consumidor máximo admissível, com a avaliação simultânea de todos os parâmetros. Além do impacto da incerteza de medição, foram avaliados os impactos da abordagem multiparâmetro, a abordagem do ICH Q1E, o modelo de regressão (simples, parcial ou completo), a correlação experimental e os limites de especificação no risco total do consumidor do prazo de validade definido. Por fim, foi proposta metodologia para a avaliação da incerteza de medição de AAS e AS, quantificados por um procedimento espectrofotométrico multivariado, calculadas pelas abordagens *bottom-up* (utilizando MCM) e *top-down* (Nordtest e VAM Project). Este trabalho demonstrou o impacto e a importância da incerteza de medição e dos riscos nas avaliações de conformidade em estudos de estabilidade para a definição de um prazo de validade. A abordagem multiparâmetro desenvolvida permitiu definir um prazo de validade considerando o risco da avaliação de conformidade de todos os parâmetros garantindo a qualidade, segurança e eficácia do produto farmacêutico.

PALAVRAS CHAVES: Incerteza de medição; Estudo de Estabilidade; Avaliação de Conformidade; Método de Monte Carlo; Método Multivariado

ABSTRACT

COUTO A.R. **The impact of measurement uncertainty on the definition of drug shelf life.** 2023. Master's Degree - Pharmacy Department, Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo.

In order to define the shelf life for a medicinal or active pharmaceutical ingredient, it is necessary to perform stability studies, in which several parameters are tested over time. Test results are compared to defined specification limits for each parameter. However, the Brazilian legislation and the ICH quality guidelines that establish criteria for the evaluation of stability studies do not consider measurement uncertainty, in addition, they individually assess the compliance of the parameters. Thus, the objectives of this work were to evaluate the impact of measurement uncertainty on defining the shelf life of a medicine, to propose a procedure for defining the shelf life based on the total consumer's risk. Additionally, a methodology to estimate measurement uncertainty for multivariate spectrophotometric procedures is developed. The particular consumer's risks of each parameter and the total consumer's risk were calculated using the Monte Carlo method (MCM), considering the measurement uncertainty. An approach for shelf life definition based on the maximum admissible total consumer's risk was developed, with the simultaneous evaluation of all parameters. In addition to the impact of measurement uncertainty, the impacts of the multiparameter approach, the ICH Q1E approach, the regression model (simple, partial or full), the experimental correlation and the specification limits on the total consumer's risk of the defined shelf life were evaluated. Finally, a methodology was proposed for assessing the measurement uncertainty of AAS and AS, quantified by a multivariate spectrophotometric procedure, calculated by bottom-up (using MCM) and top-down (Nordtest and VAM Project) approaches. This work demonstrated the impact and importance of measurement uncertainty and risks in conformity assessments in stability studies for the definition of a shelf life.The multiparameter approach developed allowed to define a shelf life considering the risk of conformity assessment of all parameters, thus ensuring the quality, safety and efficacy of the pharmaceutical product.

KEYWORDS: Measurement Uncertainty; Stability Study; Conformity Assessment; Monte Carlo Method; Multivariate Method

SUMÁRIO

INTRODUÇÃO

1. INTRODUÇÃO

O prazo de validade de um medicamento indica seu período máximo de uso, desde que armazenado em condições definidas pelo fabricante, em que suas características físico-químicas e microbiológicas são garantidas, assegurando assim, a eficácia e a qualidade do produto farmacêutico. Desta forma, é imprescindível realizar um estudo de estabilidade para obter conhecimento sobre a estabilidade química dos insumos farmacêuticos ativos (IFA) ou produtos farmacêuticos [1,2].

O estudo de estabilidade visa testar a variação de determinados parâmetros do IFA ou do produto farmacêutico em função do tempo, considerando condições ambientais como a temperatura, umidade e a luz, com o intuito de definir o prazo de validade. Para isso, é realizado o estudo de estabilidade acelerado, onde parâmetros do IFA ou do produto farmacêutico são avaliados em condições forçadas de armazenamento com a finalidade de definir um prazo de validade provisório; e o estudo de estabilidade de longa duração, em que os parâmetros são avaliados em condições ideais de armazenamento sendo o estudo que determina o prazo de validade definitivo [1–3]. Os parâmetros testados nos estudos de estabilidade acelerado e de longa duração são: aspecto, teor do IFA, quantificação dos produtos de degradação, carga microbiana, entre outros parâmetros relacionados com a forma farmacêutica [1,4]. Quando um ou mais parâmetros, após um determinado período, não cumprirem com as especificações, então, o estudo é interrompido e o prazo de validade pode ser definido [1,2]. Em situações onde parâmetros quantitativos apresentam mudança em relação ao tempo, o prazo de validade pode ser definido a partir de modelos de regressão adequados, que explicam a variação dos parâmetros estudados em função do tempo [5].

Para avaliar os mecanismos de degradação do IFA, os produtos de degradação que podem ser formados, a compatibilidade do IFA com excipientes e o impacto da embalagem na degradação, são realizados os estudos de degradação forçada. Nos estudos de degradação forçada, o produto farmacêutico ou o IFA é submetido a condições de estresse como luz, temperatura, umidade, hidrólise, oxidação, entre outros, com o objetivo de avaliar a formação dos possíveis produtos de degradação [6,7]. Paralelamente aos estudos de degradação forçada, ocorre o desenvolvimento de procedimentos analíticos indicativos de estabilidade, que serão

utilizados nos estudos de estabilidade acelerado e/ou longa duração, com o objetivo de quantificar o IFA e/ou produtos de degradação na presença de interferentes [7,8].

Os resultados analíticos relativos aos parâmetros do IFA ou do produto avaliados durante os estudos de estabilidade devem ser comparados aos limites de especificação, estabelecidos por farmacopeias, regulamentações ou legislações. Portanto, os procedimentos analíticos utilizados para quantificar tais parâmetros devem ser confiáveis, uma vez que os resultados analíticos obtidos serão usados na tomada de decisão sobre a conformidade/não-conformidade do produto. O desempenho de procedimentos analíticos utilizados nas análises de medicamentos é baseado em parâmetros de validação definidos em guias e diretrizes; porém, em alguns casos, não consideraram a avaliação da incerteza de medição [9–11]. Desta forma, mesmo um procedimento analítico validado, realizado por analistas treinados e que utilize equipamentos e vidrarias calibradas; ainda assim, existe algum grau de incerteza associado ao valor medido. Assim, os resultados analíticos devem ser fornecidos juntamente com as respectivas incertezas de medição, uma vez que a informação da incerteza deve ser considerada na tomada de decisão em avaliações de conformidade.

A incerteza de medição é caracterizada como uma faixa de valores que podem ser atribuídos ao resultado do mensurando com um dado nível de confiança, usualmente 95% [12,13]. Existem duas abordagens para estimar a incerteza de medição: *top-down* e *bottom-up*. A abordagem *top-down* estima a incerteza de medição por meio da combinação pragmática dos parâmetros de validação de um procedimento analítico, a exatidão e a precisão, não necessitando de um amplo estudo para identificar as fontes de incerteza [14–17]. Por outro lado, a abordagem *bottom-up* abrange a identificação fontes de incerteza nas etapas do procedimento analítico, quantificação e propagação das incertezas padrão das fontes de incertezas para estimar a incerteza combinada e expandida [12,13,18–20].

A estimação da incerteza pela abordagem *top-down* é mais prática, pois podem ser usados dados de estudos de precisão intermediária/reprodutibilidade, de recuperação, materiais de referência certificados e de testes de proficiência [14,15,21]. Todas as fontes de incerteza impactam nos parâmetros de validação do procedimento analítico, portanto a incerteza de medição obtida por este meio representa toda a variabilidade do resultado. Esta abordagem é mais interessante quando não há necessidade de um estudo aprofundado sobre as fontes de incerteza

que impactam nas análises, economizando tempo e recursos [19]. Há diversos guias e diretrizes sobre a avaliação da incerteza de medição pela abordagem *top-down*, como: ISO 11352, Nordtest NT TR 537 handbook e VAM Project 3.2.1 - Part (d): Protocol for uncertainty evaluation from validation data [22–24]. Entretanto, a incerteza de medição obtida pela abordagem *top-down* fornece informações limitadas sobre as contribuições das fontes de incerteza na incerteza de medição calculada [19].

Na avaliação da incerteza de medição pela abordagem *bottom-up*, deve-se definir a equação que relaciona os fatores de entrada (*inputs*) com o resultado da medição (*output*), identificar todas as possíveis fontes de incerteza, calcular e propagar suas incertezas padrão por meio da equação para obter a incerteza combinada. As fontes de incerteza podem ser oriundas da calibração dos equipamentos e vidrarias, das etapas de amostragem, da pureza das substâncias, do efeito e interferência da matriz, entre outros. A avaliação da incerteza pela abordagem *bottom-up* permite determinar a contribuição de cada fonte de incerteza na incerteza combinada, sendo um conhecimento útil quando é necessário reduzir a incerteza final [12,13,18,19,25]. Os métodos para propagar as fontes de incerteza e calcular a incerteza combinada são: a lei da propagação da incerteza, em que as fontes são combinadas através de um método algébrico [13,18]; a planilha de Kragten, que é um método numérico para a propagação e cálculo da incerteza combinada [26,27]; e o método de Monte Carlo (MCM), que também é um método numérico que propaga e calcula a incerteza de medição a partir da simulação de milhares de valores de resultado da medição (*output*) obtidos considerando os fatores de entrada (*inputs*), suas respectivas incertezas padrão e suas distribuições de probabilidade [18,19,25,28].

Quando é considerada a incerteza de medição, o resultado analítico pode ser expresso como uma função de densidade de probabilidade. No contexto da avaliação de conformidade, a utilização da incerteza de medição permite calcular os riscos na tomada de decisão, especialmente quando a faixa de valores atribuíveis ao resultado está tanto dentro quanto fora dos limites de especificação [29]. Dependendo do resultado da medição, os riscos são denominados risco do consumidor ou do produtor. O risco do consumidor ocorre quando o valor medido está dentro dos limites de especificação, mas, devido à incerteza de medição, uma parte da faixa de valores atribuíveis ao mensurando está fora limites de

especificação. Desta forma, há necessidade de calcular o risco de aceitar o produto, que na verdade está fora dos limites de especificação, e corresponde ao risco do consumidor utilizar um produto inapropriado ao uso. O risco do produtor ocorre quando o valor medido está fora dos limites de especificação, porém uma parte da faixa de valores atribuídos ao resultado está dentro dos limites de especificação. Assim, deve-se calcular o risco de rejeitar um produto, que na verdade está dentro dos limites de especificação, portanto corresponde ao risco do produtor inutilizar um produto apto ao uso [14,16,29–33].

Os estudos de estabilidade acelerado e de longa duração avaliam a conformidade de um conjunto de parâmetros ao longo do tempo, assim a conformidade do produto farmacêutico ocorre apenas quando todos os seus parâmetros estão dentro dos limites de especificação. Neste contexto, o risco decorrente da avaliação de conformidade de um único parâmetro é denominado risco particular e, dependendo do resultado da medicação e do valor da incerteza de medição, pode ser nomeado risco particular do consumidor ou do produtor. Com a combinação dos riscos particulares de todos os parâmetros é obtido o risco total, que representa o risco de continuar o estudo de estabilidade e, consequentemente, definir um prazo de validade maior, quando na verdade um ou mais parâmetros estão fora dos limites de especificação (risco total do consumidor). Por outro lado, o risco de interromper o estudo e definir o prazo de validade, quando na verdade todos os parâmetros estão dentro dos limites de especificação, corresponde ao risco total do produtor [29,32,33]. Mesmo quando todos os riscos particulares estão dentro de um nível aceitável, o risco total ainda pode ser significativo [29].

No capítulo I será avaliado o impacto da incerteza de medição na definição do prazo de validade de uma solução de ácido acetilsalicílico (AAS), com base no risco total (considerando todos os parâmetros simultaneamente). Os parâmetros testados no estudo de estabilidade foram o teor do AAS e do seu principal produto de degradação, ácido salicílico (AS).

A diretriz do ICH Q1E “Evaluation of Stability Data” trata da avaliação dos dados dos estudos de estabilidade para a definição do prazo de validade de um medicamento ou IFA. Vale salientar que a RDC 318/2019, que estabelece critérios para realização de estudos de estabilidade de medicamentos e IFA’s, é baseada nesta diretriz. Porém, o ICH Q1E e a RDC 318/2019 não consideram a informação

da incerteza de medição, o que impede o cálculo do risco particular na avaliação de conformidade, e propõem a avaliação individual dos parâmetros.

Conforme o exposto anteriormente, o presente capítulo propõe uma abordagem multiparâmetro para a definição do prazo de validade, baseado na avaliação simultânea da conformidade de todos os parâmetros considerando a incerteza de medição dos resultados, os riscos particulares do consumidor dos parâmetros e o risco total consumidor no prazo de validade definido. Assim, é possível definir um prazo de validade mais seguro considerando a incerteza de medição e um risco total máximo admissível.

No capítulo II é estimada a incerteza de medição de um procedimento espectrofotométrico multivariado para quantificação simultânea de AAS e AS. A incerteza combinada do AAS e AS foram estimadas por duas metodologias da abordagem *top-down* (Nordtest e VAM Project) e pela abordagem *bottom-up* utilizando MCM.

Procedimentos analíticos multivariados são amplamente utilizados na análise de produtos farmacêuticos e são capazes de quantificar uma ou mais substâncias em uma matriz da amostra contendo interferentes. Em março de 2022, foi lançado o draft do ICH Q2(R2) “Validation of Analytical Procedures”, que traz requisitos para a validação de procedimentos multivariados. Porém, com base em buscas na literatura, não há artigos sobre a avaliação da incerteza de medição para quantificações realizadas empregando procedimentos espectrofotométricos multivariados.

Assim, foi desenvolvida uma metodologia de estimação da incerteza combinada utilizando MCM que considera as fontes de incerteza da preparação das amostras e das soluções padrão utilizadas na curva de calibração, a estimação da incerteza padrão dos espectros das amostras pelo método *bootstrap* e o impacto das incertezas das soluções padrão na obtenção da equação por mínimos quadrados parciais (PLS) que quantificam o AAS e AS. Adicionalmente, foram calculadas as incertezas de medição do AAS e AS por duas metodologias da abordagem *top-down* (Nordtest e VAM Project).

OBJETIVOS

2. OBJETIVOS

O objetivo deste trabalho foi avaliar o impacto da incerteza de medição associada aos resultados analíticos obtidos durante estudos de estabilidade na avaliação de conformidade e na determinação do prazo de validade de medicamento, considerando os riscos particulares dos parâmetros e o risco total do consumidor. São objetivos específicos:

- Identificar e quantificar as fontes de incerteza relacionadas ao preparado das amostras e das soluções padrão de um procedimento espectrofotométrico multivariado utilizado para quantificar simultaneamente AAS e AS.
- Estimar a incerteza de medição das quantificações do AAS e AS obtidos por um procedimento espectrofotométrico multivariado pelas abordagens *top-down* (utilizando os guias Nordtest/ISO 11352 e VAM Project) e *bottom-up* (por meio do método de Monte Carlo).
- Avaliar o impacto da incerteza de medição na definição do prazo de validade através da estimação do risco particular do consumidor de cada parâmetro (AAS e AS) na avaliação de conformidade do estudo de estabilidade e do risco total do consumidor para o prazo de validade definido.
- Desenvolver uma planilha para determinação do prazo de validade baseado na avaliação multiparâmetro, onde todos os parâmetros são avaliados simultaneamente. Além de considerar um risco total do consumidor máximo admissível para definição do prazo de validade

CAPÍTULO I - Definition of medicine shelf-life based on the assessment of the total risk of false conformity decisions due to measurement uncertainty – A multiparameter approach

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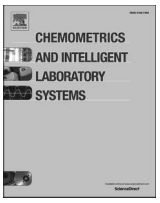

Definition of medicine shelf-life based on the assessment of the total risk of false conformity decisions due to measurement uncertainty – A multiparameter approach

Aldo Renato Couto, Felipe Rebello Lourenço *

Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580 – Bloco 15, CEP, 05508-000, São Paulo, SP, Brazil

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ABSTRACT

The ICH quality guidelines define how drug stability study should be accomplished in order to obtain a safe shelf life. To assess the risks of decision making one must consider the measurement uncertainty information. The stability study must consider the conformity assessment of all parameters (content, degradation product, among others), but the ICH approach does not consider the use of uncertainty information and considers the evaluation of only the most critical parameter to define the shelf life time. In this way, the current approach does not make it possible to assess the total consumer's risk, which can lead to the risk of obtaining a longer shelf life than the drug supports. The present study proposes to consider the uncertainty information in the definition of the shelf life, simultaneously considering all the parameters tested in the stability study. A MS-Excel spreadsheet was developed and made available as supplementary material (*Stability_4Risk*) for risk values calculation and shelf life definition, considering a stability study data and simultaneous parameters evaluation. The measurement uncertainty information, regression model types, metrological correlation of measured results, specification limits and the simultaneous parameters evaluation impacted on the risks and, consequently, on the definition of the shelf life. Thus, it was possible to establish a new approach for the definition of a safe shelf life, based on total consumer's risk, considering the measurement uncertainty and the simultaneous parameters evaluation, which guarantees the quality, efficacy and safety of medicines until its shelf life.

1. Introduction

Stability study of a drug has as one of its objectives the shelf life definition, that is, the safe period of time for drug use. For this, periodic conformity tests are accomplish on various parameters (content, degradation product, microbial limits, among others) until one or more parameters are outside the specification limits [1,2]. In this way, stability study provides data that allow establishing regression models that will be used to define the shelf life [3].

The International Council for Harmonization (ICH) quality guidelines provide guidance on several aspects of the stability study, such as the duration and environmental conditions of the tests, the decision tree, statistical analysis, among others. The National Health Surveillance Agency (ANVISA - Brazil) [4], the Food and Drugs Administration (FDA - USA) [5] and European Medicines Agency (EMA - European Union) [6] are some regulatory institution that use ICH quality guidelines as a reference for stability studies and, consequently, used to estimate shelf life a new molecules or a new pharmaceutical products.

Analytical procedures used in the stability study must guarantee the quality of analytical results, because through them, the compliance decisions will be taken. Measurement uncertainty is useful to demonstrate the quality of analytical results, being an attribute associated with measured value that guarantees, with a certain probability degree, that the true value will be within a range of values, that is, the result is represented as a probability distribution function considering the measurement variability [7]. In this way, measurement uncertainty should be assessed, because, even with all attention has been taken to ensure results quality, such as analytical procedure validation, use of certified reference materials (CRM), use of calibrated/qualified equipment, laboratory accreditation according to ISO 17025, and participation in proficiency testing, there will always some level of uncertainty associated with measurement.

* Corresponding author.
E-mail address: feliperl@usp.br (F.R. Lourenço).

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However, no ICH quality guides require the use of measurement uncertainty information in stability study and there are no studies in literature that assess its impact on shelf life definition.

When considering measurement uncertainty in compliance decisions, it is possible to calculate different types of risks related to false decisions considering the number of parameters used in the calculation. The risks considered in the present study are: 1) Particular consumer's risk – assesses the probability of a batch used in the stability study being erroneously accepted, considering a certain shelf life, when in fact it is outside the specification limits; 2) Particular combined consumer's risk – simultaneously evaluates all batches of a parameter corresponding to the probability of one or more batches of a parameter being erroneously accepted, considering a certain shelf life, when in fact they are outside the specification limits; 3) Total consumer's risk - simultaneously evaluates all batches of all parameters studied, corresponding to the probability of the drug being wrongly accepted, considering the time elapsed from the study, when in fact the study should be interrupted and a shelf life estimated [8–11]. Although the scope of this article is to determine a safe shelf life for use through consumers' risks, particular producer's risk, particular combined producer's risk and total producer's risk can also be calculated.

The importance of measurement uncertainty has already been demonstrated in pH determination [12,13], dissolution test [14], and active pharmaceutical ingredient (API) quantification [15,16]. There are several studies that show measurement uncertainty impact on the conformity assessment of medicines and drugs [16–19], it has also been studied how the choice of batches number and replicates used in a stability study can decrease measurement uncertainty [20].

In addition to not considering measurement uncertainty, ICH guides propose the evaluation of individual parameters, that is, a uniparameter approach. With the data generated by the stability study, a regression model is adjusted for each batch that serves as a degradation model for a drug [21,22]. The evaluation considers the 95% confidence limit for the mean of the regression models of each lot against the specification limits. In this way, periodic conformity tests on the parameter under study are performed, to find out if the drug will be within the proposed shelf life or to define its shelf life considering the time elapsed until the parameter is outside the specification limit [3]. However, the risk assessment of an individual parameter does not represent the total consumer's risk [23]. Total consumer's risk combines particular consumers' risks of all studied parameters, considering the correlation between them [8,24,25].

Thus, this study aims to simultaneously evaluate all parameters compliance, considering measurement uncertainties associated with each parameter and errors from stability study regression models, in order to calculate consumers' risks for an initially proposed shelf life time or, alternatively, to define a shelf life time based on maximum acceptable total consumer's risk.

2. Theory

2.1. The ICH Q1E approach to shelf life estimation

As the result of quantitative data regression analysis obtained from stability study, each batch of each parameter has an equation that relates change in output quantity (e.g. concentration) as a function of time. The linear or non-linear relationship can be adopted, using on an arithmetic or logarithmic scale [3]. The linear relationship was used in the present work (eq. (1)).

$$y_{ij} = \alpha_{ij} + x\beta_{ij} + e_{ij} \quad \text{eq. 1}$$

where, y and x are the output quantity (dependent variable) and the time (independent variable), respectively; α and β are the intercept and slope, respectively; e is the error; and i and j correspond to the parameter and batch studied, respectively.

Batches conformity is examined individually. Attributes that only have lower or upper specification limits are evaluated unilaterally, while attributes with upper and lower specification limits are evaluated bilaterally, considering the 95% confidence level. Then, the time in which a batch exceeds specification limit is stipulated, which is the batch shelf life time [3].

If all shelf life times of batches is longer than the initially proposed time and the particular consumers' risks of all batches are acceptable, then shelf life time can be defined as the initially proposed time. However, even if particular risks are low, total risk can be significantly high (greater than maximum permissible risk). When one or more batches have shelf life shorter than the proposed shelf life, ICH Q1E recommends to adopt a poolability test in order to compare the intercepts and slopes of regression models obtained for the batches. The poolability test may lead to three types of regression models: 1) Simple model – when the slope and intercept values obtained for all batches are similar, all batches are combined to obtained an unique regression equation with common intercept and slope values; 2) partial model - when the slope values are similar, but the intercept values are different, batches are combined to obtained a common slope value, however the intercept values are obtained for each batch individually; and 3) full model - when both intercept and slope values are different, then a regression equation will be obtained for each batch individually. In simple model case, shelf life time is based on regression model of the most critical parameter. In this way, particular combined risk assessment of the most critical parameter is assumed as the total risk, without considering the risk of other parameters. In the case of partial or full models, shelf life time definition is based on most critical batch of the most critical parameter. In this way, most critical batch particular risk assessment is assumed as the total risk, without considering the risk of other batches and parameters.

Therefore, two limitations are noted in ICH approach: not considering measurement uncertainty in conformity assessment with a view to determining shelf life; and considering only the most critical batch/parameter (uniparameter approach), without considering how other batches/parameters affect shelf life (multiparameter approach).

2.2. The importance of measurement uncertainty in the stability study

When a measured value is very close to specification limit and there is a partial overlap between the uncertainty and the specification limit, there is false acceptance risk (consumer's risk), considering a certain shelf life time. Considering that, during stability study, it is necessary to consider measurement uncertainty information in order to evaluate the batch/product conformity against specification limits. Measurement uncertainty allows one to estimate consumer's risk, which can be useful in determining product shelf life time. Several studies demonstrate how to use measurement uncertainty information in the assessment of particular and total risks [23,24], as well as how the experimental correlation impacts on risk estimation [18].

To illustrate measurement uncertainty impact on stability study, a hypothetical drug batch was simulated with a labeled percent concentration of 100% that decreases over time, with lower and upper specification limits (LSL and USL, respectively) of 90 and 110%, respectively, and relative measurement uncertainty of $\mu = 0.87$. Using ICH Q1E approach, which does not consider uncertainty information in conformity assessment, shelf life time was 4.142 min; however, consumer's risk cannot be calculated (Fig. 1 A1). In Fig. 1 A2, it is demonstrated how measurement uncertainty can be used to calculate the consumer's risk (19.4%). Thus, to ensure an acceptable consumer's risk, it is necessary to consider uncertainty information in defining appropriate shelf life time.

2.3. Monte Carlo method in calculating consumer's risk

Monte Carlo method was used to calculate consumers' risks considering the initially proposed shelf life time (for example, 2 years) or to

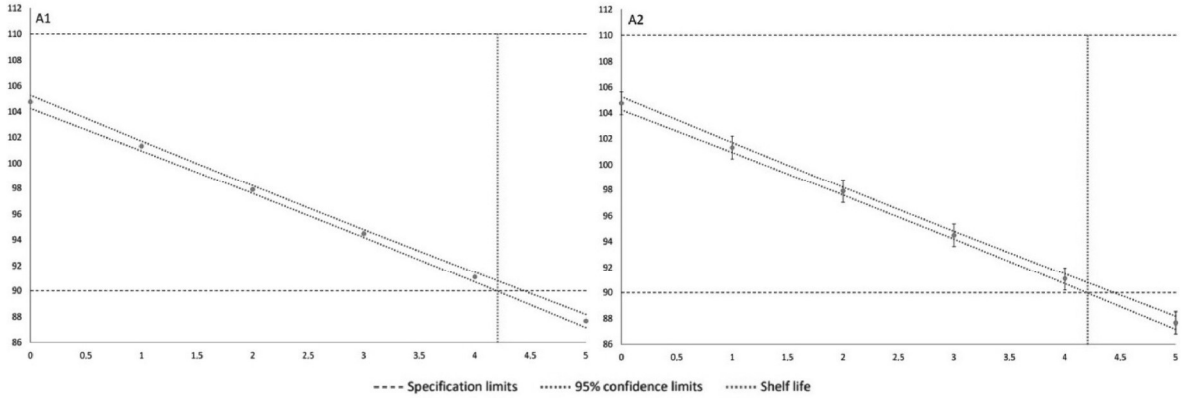

Fig. 1. (A1) Batch with a shelf life time of 4.142 min, but without considering the measurement uncertainty information, it is not possible to calculate consumer's risk. (A2) Batch with a shelf life of 4.142 min; however, considering measurement uncertainty information, the consumer's risk is 19.4%.

estimate shelf life time considering a maximum admissible total consumer's risk (typically, 5%). Simulations are generated through variations of initial concentrations, considering the measured values and their respectives measurement uncertainty, as well as the regression equation models variation, considering coefficient (slopes and intercepts values) errors. Regression models for each batch are used (eq. (1)), according to poolability test result, but corrected by simulated the initial concentration (eq. (2)).

$$\mathbf{y_{ijk}} = \boldsymbol{\alpha_{ik}} + (\mathbf{c_{ik}} - \boldsymbol{\alpha_{ik}}) + \boldsymbol{\beta_k}\ \mathbf{x_{ijk}} + \mathbf{e_{ijk}} \qquad \text{eq. 2}$$

where, i is parameter (from 1 to 4 parameters); j is batch (from 2 to 3 batches); k is simulation number (from 1 to 50,000); and c is simulated initial concentration.

In this way, 50,000 final concentrations (y_{ijk}), are obtained, considering an initially idealized shelf life (x).

Simulations are performed in MS-Excel software. To perform simulations, the formula NORM.INV(RAND(), c_i, u_{c_i}) is used, with RAND() being a random number between 0 and 1, in order to generate values from a normal distribution with mean c_i and standard deviation u_{c_i}. Other authors used Monte Carlo method to calculate false compliance decisions risk applied to active pharmaceutical ingredients quantification in different dosage forms [8,18,19,23].

2.4. The multiparameter approach

In stability study, several drug parameters may be evaluated and, consequently, the conformity assessment must consider all parameters simultaneously. For this, 50,000 final concentration values, for each batch, are simulated using Monte Carlo method, which are compared against conformity assessment specification limits. Then, particular consumers' risks are calculated, for each batch, as the ratio between the number of simulated values outside specification limits (o) and total number of simulations (n) (eq. (3)).

$$p(Y_{ij}) = \frac{o}{n} \qquad \text{eq. 3}$$

where, $p(\mathrm{Y_{ij}})$ corresponds to the probability of the $\mathrm{Y_{ij}}$ generated value is outside the specification limits, for the i^{th} parameter (e.g. AAS and AS) and j^{th} batch (e.g. batches A, B and C); o and n represent the number of simulated values that are out-of-specification and the total number of simulated values, respectively.

It is worth noting that even if particular consumers' risks are acceptable (e.g. less than 5%), total consumer's risk can be significantly high (e.g. greater than 5%). This situation is more relevant when a large number of parameters must be evaluated during the stability study [23,24].

The combined particular consumer's risk for the i^{th} parameter can be obtained by combining the particular risks evaluated for each batch in the stability study (eq. (4)).

$$R^*_{Ci} = p(Y_{i1}) + p(Y_{i2}) + p(Y_{i3}) - p(Y_{i1} \cap Y_{i2}) - p(Y_{i1} \cap Y_{i3}) - p(Y_{i2} \cap Y_{i3}) + p(Y_{i1} \cap Y_{i2} \cap Y_{i3}) \qquad \text{eq. 4}$$

where R^*_i is the combined particular consumer's risk for the i^{th} parameter.

In a situation where stability study is performed with only 2 batches, equation (4) can be simplified (**eq. 5**).

$$R^*_{Ci} = p(Y_{i1}) + p(Y_{i2}) - p(Y_{i1} \cap Y_{i2}) \qquad \text{eq.5}$$

Then, total consumer's risk can be obtained by combining the particular consumers' risks for the parameter evaluated in the stability study. Considering the maximum of 4 parameters calculated by the *Stability_4Risk* (eq. (6)), we have that:

$$R^{T^*}_C = p(R^*_1) + p(R^*_2) + p(R^*_3) + p(R^*_4) - p(R^*_1 \cap R^*_2) - p(R^*_1 \cap R^*_3) - p(R^*_1 \cap R^*_4) - p(R^*_2 \cap R^*_3) - p(R^*_2 \cap R^*_4) - p(R^*_3 \cap R^*_4) + p(R^*_1 \cap R^*_2 \cap R^*_3 \cap R^*_4) \qquad \text{eq. 6}$$

where R^{T^*} is total consumer's risk.

When 3 parameters are studied simultaneously, total consumer's risk can be obtained as follows (eq. (7)).

$$R^{T^*}_C = p(R^*_1) + p(R^*_2) + p(R^*_3) - p(R^*_1 \cap R^*_2) - p(R^*_1 \cap R^*_3) - p(R^*_2 \cap R^*_3) + p(R^*_1 \cap R^*_2 \cap R^*_3) \qquad \text{eq. 7}$$

Considering the stability study performed in this work, 2 parameters (AAS and AS) were analyzed and total consumer's risk was obtained as follows (eq. (8)).

$$\boldsymbol{R}^{T^*} = \boldsymbol{P}\left(\boldsymbol{R}_1^*\right) + \boldsymbol{P}\left(\boldsymbol{R}_2^*\right) - \boldsymbol{P}\left(\boldsymbol{R}_1^* \cap \boldsymbol{R}_2^*\right) \quad \text{eq. 8}$$

when stability study evaluates a single parameter, total consumer's risk ($R_C^{T^*}$) is equal to particular risk ($\boldsymbol{p}(\boldsymbol{R}_1^*)$).

Stability_4Risk does not calculate producer risks, but these can be estimated based on the consumers' risks values. Particular producer's risk (eq. (9)), particular combined producer's risk (eq. (10)), and total producer's risk (eq. (11)), respectively, can be calculated as presented below:

$$\mathbf{Rp}\left(\mathbf{Y_{ij}}\right) = 1 - \mathbf{P}\left(\mathbf{Y_{ij}}\right) \quad \text{eq. 9}$$

$$\mathbf{PR_i^*} = 1 - \mathbf{R_i^*} \quad \text{eq. 10}$$

$$\mathbf{PR^{T^*}} = 1 - \mathbf{R^{T^*}} \quad \text{eq. 11}$$

To simulate batches simultaneous evaluation relevance obtained by multiparameter approach, the drug content of 500 hypothetical batches, with lower and upper specification limits (LSL and USL, respectively) of 90 and 110%, respectively, and relative measurement uncertainty μ = 0.87, were simulated. Simultaneously, the degradation product formation of the 500 batches, with an upper specification limit (USL) of 15%, and relative measurement uncertainty of μ = 2.29 (Fig. 2), were also simulated. Although drug content exceeds specification limit in a period time lower than those obtained for the degradation product formation (4.20 and 4.71 min, respectively, both with a confidence level of 95%), when considering total consumer's risk of 5%, degradation product formation is the most critical parameter (shelf life time of 3.6 and 3.8 min, respectively).

3. Material and method

3.1. Chemicals

The following products were used in stability study: acetylsalicylic acid USP, salicylic acid USP and sodium hydroxide PA (Synth, Shandong, China), anhydrous monobasic potassium phosphate (Inlab, São Paulo, Brazil), and HPLC grade methanol (Honeywell Riedel - from Haen, Germany).

3.2. Instruments

Spectrometric analyzes were performed using a Genesys 50 UV–Vis spectrophotometer (Thermo Fischer Scientific, USA). Standard and sample preparation were done using an analytical balance (Shimadzu, Japan) and volumetric apparatus. Quantification of AAS and AS was performed by scanning UV–Vis spectroscopy in range from 252 to 308 nm. The pH measurement was performed by the pH meter PG2000 (Gehaka).

Statistical analyzes were performed using MS-Excel (Microsoft) and Minitab 18 software (Minitab, LLC).

3.3. Estimation of measurement uncertainties for AAS and AS parameters

Measurement uncertainty associated with the AAS and AS quantification using spectrophotometer was calculated by Monte Carlo method, from 50,000 sample spectra simulated. Spectra simulations were obtained by using the formula NORM.INV(RAND(), A_λ s_{A_λ}), where A_λ e s_{A_λ} correspond to the mean and the pooled standard deviation of the absorbance values, respectively, obtained for each wavelength (λ). Correlation between absorbance values obtained at different wavelengths (λ) was propagated to simulations by multiplying random generators normally distributed by Cholesky decomposition matrix. Through regression model obtained by partial least squares (PLS), 50,000 concentrations of AAS and AS were quantified from 50,000 simulated spectra, which allowed determining combined standard uncertainty associated with AAS and AS measurements. Uncertainties associated with weighing and sample dilution steps were disregarded, as their contribution to final combined uncertainty was not relevant [26]. Finally, the measurement uncertainty values association with AAS and AS concentrations were found to be u_{AAS} = 1.7% u_{AS} = 0.27%, respectively.

3.4. Stability study

Stability study was accomplished for obtaining data objective to establish degradation model that allows analyzing measurement uncertainty impact on the total consumer's risk and on definition of shelf life time. Therefore, only drug content (AAS) and degradation product (AS) parameters were considered. Acetylsalicylic acid (AAS) was chosen as active pharmaceutical ingredient (API) and salicylic acid (AS) as its degradation product. AAS choice is justified due to extensive knowledge about its physicochemical properties and vast literature on AAS and AS quantification analytical procedures. Study conditions choice (pH 7.2 and temperature of 50 °C) aimed to obtain, in a short period, a degradation model that would provide an adequate data set for determination of shelf life time, considering the approach proposed in this work.

3.4.1. Preparation of the batches

250 mL of a 0.2 M potassium phosphate monobasic solution and 175 mL of 0.2 M sodium hydroxide solution were prepared. The solutions were mixed, the volume was made up to 1000 mL and mixed manually [27]. For pH measurement, the pHmeter was calibrated with solutions

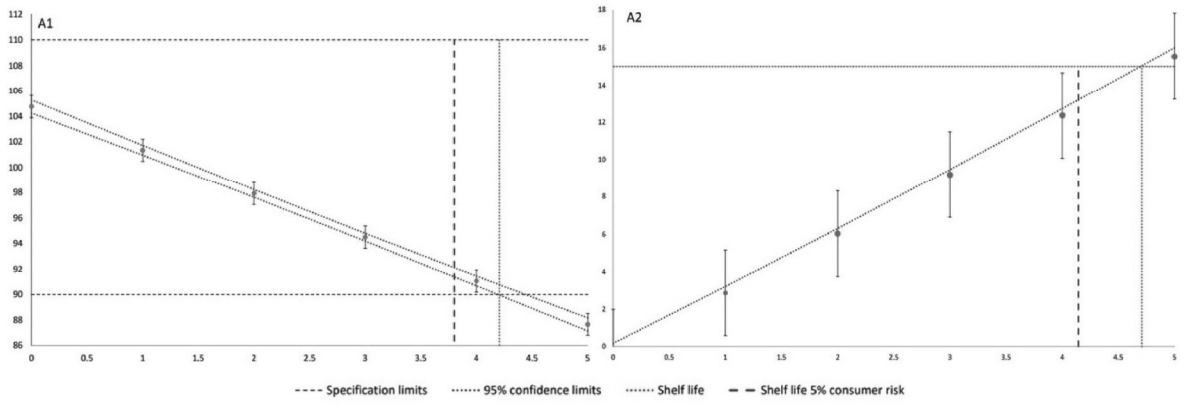

Fig. 2. (A1) Decreasing drug content in relation to time, with 4.20 (without considering uncertainty) (dotted red line) and 3.8 min (considering uncertainty and consumer's risk of 5%) (dashed black line) shelf life time. (A2) Degradation product formation increasing over time, with 4.71 (not considering uncertainty) (red dotted line) and 3.6 min (considering 5% uncertainty and consumer's risk) (black dashed line) shelf life time.

with pH 4.0 and 7.0, the measurement was performed in duplicate. The pH of the solution was 7.2. The solution was kept at 50 °C.

3.4.2. Preparation of the batches

Three AAS 100 mg aliquots were weighed into three 100 mL volumetric flasks (batches A, B and C) and diluted with 2 mL of methanol. Buffer solution at 50 °C was added to flasks, filling in volume to 100 mL, followed by manual shaking. The batches were kept in water bath at 50 °C.

3.4.3. Calibration curve

Partial least squares (PLS) were used to obtain a calibration models. The absorbances obtained from 252 to 308 nm were used to quantify AAS and AS in the samples subject to stability study.

3.4.4. Spectrophotometric analysis and stability study time points

To obtain initial time concentration (t_0), 0.5 mL of each batch was added separately into 10 mL conical tubes containing deionized water 7 mL (dilution 1:14) and shaken. Then, about each solution 2 mL was added to quartz cuvette for reading in UV–Vis spectrophotometer, in range of 252 nm–308 nm. Batches analyzes were performed in triplicate. Every 30 min, new aliquots of 0.5 mL were removed from the batches in a water bath for analysis in a UV–Vis spectrophotometer until the final time of 120 min (t_{30}, t_{60}, t_{90}, and t_{120}).

3.4.5. Specification limits

For conformity assessment, we considered lower and upper 90 and 110% specification limits (AAS_{LSL} and AAS_{USL}, respectively) for AAS, and the upper 4% limit (AS_{USL}) for AS, as limits based on the Aspirin Effervescent Tablets for Oral Solution monograph from the United States Pharmacopeia [28].

3.5. Spreadsheet development (Stability_4Risk.xlsx)

An MS-Excel spreadsheet was developed to simultaneously evaluate up to four quantitative parameters tested in a stability study, considering two or three batches per parameter. The Monte Carlo simulation method was used to vary the initial concentration values based on the measured values and their respective measurement uncertainties, as well as to vary the stability regression equations based on the coefficient values (slopes and intercepts values) and their respective errors (with a confidence level of 95%). In this way, 50,000 final concentration values are simulated, which are considered in the conformity assessment (estimate of the risk of false decisions). Simultaneous assessment of the simulated results for each batch and/or parameter makes it possible to obtain the combined particular consumers' risks and the total consumer's risk.

Initially, the stability study settings must be informed on the "Stability Regression Model" worksheet, such as: the number of parameters (cell L7, from 1 to 4 parameters), the name of each parameter (cells L10:L13), the number of batches (cells F6, from 2 to 3 batches), the batch identification (cells D10:D12), number of replicates (cell L6, from 1 to 3 replicates), the number of defined times (cells F7, 4 to 9 times), the times of the study (cells H10:H18), and the α used for the poolability test (cells C16:D16). After the study was properly configured, the fields referring to the results of the measured values must be filled in, according to their respective replicas and times, for each batch of each parameter (cells C25:N33 for batch 1; cells C39:N47 for batch 2; and cells C53:N61 for batch 3). If filled in correctly, the results of the analysis of covariance (ANCOVA) will be presented for each parameter (cells B63:N91 for parameter 1; cells B93:N121 for parameter 2; cells B123:N151 for parameter 3; and cells B153:N181 parameter 4), where the results of the poolability test, the regression models and their adjusted R^2 values are presented.

Then, in the "Shelf Life Determination" worksheet, one must inform the initial values of the parameters (cells F7, H7, J7 and L7), their respective measurement uncertainties (cells F8, H8, J8 and L8), the types of limits of specification ("interval", "minimum" or "maximum") of each parameter (cells F10, H10, J10 and L10), the values of the specification limits according to the type of the parameter (lower specification limits in cells F11, H11, J11 and L11, and upper specification limits in cells F12, H12, J12 and L12), and the maximum admissible value of the total consumer's risk (cells L14, 1, 5 or 10%). The "Increase cells" button simulates 50,000 initial values (considering F7, H7, J7 and L7), varying according to the uncertainty of the parameters (considering F10, H10, J10 and L10), and simulates 50,000 regression models varying according to the coefficient errors with a confidence level of 95%, for all batches. After the "Increase cells" button is activated, any change in the values and/or information in the worksheet, the simulations must be updated by pressing the "Actualize random generator" button. The spreadsheet also allows entering the experimental correlation matrix between values (light purple cells C37:M47) [15]. The shelf life time initially proposed (cell F14) can be entered, thus calculating the particular combined consumers' risks for each parameter (cells F55, H55, J55 and L55) and the total consumer's risk (cell L57). If the proposed shelf life time results in a total consumer's risk above the maximum admissible risk or if there is no proposed shelf life, one can use the "Run simulation" button, which automatically defines a shelf life time (cells F14 and F52), considering the total consumer's risk selected (cell L14). The particular combined consumers' risks for each parameter (cells F55, H55, J55 and L55) and the total consumer's risk (cell L57) are also provided.

The spreadsheet generates a scatter plot for each parameter, indicating the shelf life time. The choice of the parameter can be made in the "Stability Regression Model" worksheet (cells K16:L16). The graph is shown on the "Dispersion Plot" worksheet, showing the specification limits, the regression confidence interval, the shortest shelf life time of the batches of the chosen parameter, the shortest shelf life time considering all parameters (most critical parameter) and the shelf life time considering the multiparameter approach (approach proposed in this work), which considers the total consumer's risk admitted. The spreadsheet also provides a scatter plot of the simulated values, as well as the conformity assessment ("IN" or "OUT") obtained for the parameter and batches selected in the "Shelf Life Determination" worksheet (cell F62 for the parameter, and cells K62 and M62 for the batches). This graph is presented in the "Multivariate plot" worksheet.

4. Results and discussion

Partial least squares (PLS) were used to obtain a calibration model. The quantification of AAS and AS parameters by the spectrophotometric method was linear in the range from 40 to 93.42 μg/mL and 2.69–6.72 μg/mL, with R2 values of 0.9995 and 0.9998 for AAS and AS, respectively. The analysis of variance (ANOVA) results obtained for AAS and AS quantification are provided in Table 1 and Table 2, respectively.

To evaluate the impact of using measurement uncertainty information in determining the shelf life, and consequently in evaluation the risk of false decisions, five different scenarios were studied: Scenario A) effect of choosing the ICH approach *vs.* the multiparameter approach; Scenario B) effect of choosing the regression models (simple, partial or full model); Scenario C) impact of measurement uncertainty values associated with the studied parameters; Scenario D) effect of the correlation between the parameters studied; and Scenario E) impact of specification limits on shelf life determination.

Table 1
Analysis of Variance of AAS quantification.

Source	DF	SS	MS	F	P
Regression	2	12080.7	6040.37	28103.75	0.000
Residual Error	23	4.9	0.21		
Total	25	12085.7			

Table 2
Analysis of Variance of AS quantification.

Source	DF	SS	MS	F	P
Regression	2	192.553	96.2766	73876.68	0.000
Residual Error	23	0.030	0.0013		
Total	25	192.583			

4.1. Effect of choosing the ICH approach vs. the multiparameter approach in determining the shelf life (scenario A)

In scenario A, we studied the impact of choosing the ICH approach *vs.* the multivariate approach (proposed in this work) on consumer's risk, that is, the risk of erroneously accepting a batch that has one or more parameters out of specification at the end of the shelf life. When the consumer's risk evaluation considers only the analysis of the most critical parameter/batch (ICH approach), part of the information obtained during the stability study is ignored and the risk of false decisions due to the other parameters (in the case of this work, the AAS parameter) is neglected. In this way, the consumer's risk corresponds to the particular risk of the critical parameter (in the case of this work, the AS parameter). However, AAS can contribute to the total consumer's risk though it is not the most critical parameter. Thus, the total risk assessment must consider all parameters analyzed in the stability study.

For this scenario, shelf life, particular consumers' risks (for AS and AAS) and total consumers' risks were evaluated, considering the ICH approach (maximum particular consumer's risk of 5%, of the most critical parameter/batch - in this case, batch A of the AS parameter) and the multi-parameter approach (maximum total risk of 5% considering all parameters analyzed in the stability study - in this case, AS and AAS).

Six shelf life were simulated for each approach (ICH or multiparameter) with the measurement uncertainty of the parameters, u_{AAS} = 1.7% u_{AS} = 0.27%, and $\alpha = 0.25$. In each simulation, the random generators were kept the same for the estimated risk calculations for the ICH and multiparameter approaches. Therefore, the difference between the values of risks and shelf life derives exclusively from the difference between the approaches. That is, the fact that the ICH approach considers only the most critical batch/parameter (in this case, only AS batch A), while the multiparameter approach considers all parameters simultaneously. ANOVA indicated that there is a significant difference between the estimated shelf life values, as well as between the values of combined particular risks (for AAS and AS) and total consumers' risks, considering the different approaches.

When using the ICH approach to calculate the shelf life, considering that the risk of false decisions was determined based on the most critical batch/parameter (in this case, AS batch A), the information from the other batches/parameters is not considered, which may underestimate the total consumer's risk. That is, using the ICH approach, the particular consumer's risk for batch A is 5%; however, the combined particular risk and the total consumer's risk are 13.998% and 14.016%, respectively (Table 3).

Table 3
Shelf lifes, particular risks and total consumer's risk considering ICH and multiparameter approaches.

Response	ICH approach (batch A of AS) (n = 6)	Multiparameter approach (n = 6)
Shelf life	17.564 ± 0.019 min a	16.859 ± 0.010 min b
Particular risk for AAS	0.021 ± 0.005% a	0.011 ± 0.004% b
Particular risk for AS	13.998 ± 0.332% a	4.990 ± 0.003% b
Total risk	14.016 ± 0.334% a	5.000 ± 0.00% b

The means with different letters are statistically different, p-value < 0.01. The results correspond to the mean ± standard deviation obtained from 6 simulations.

On the other hand, adopting the multiparameter approach, all batches/parameters are considered in the definition of the shelf life, being possible to ensure the total consumer's risk. The difference between the shelf life obtained by the ICH and multiparameter approaches was 4.01% (17.564 and 16.859 min for ICH and multiparameter, respectively). This difference results in a 64.33% lower total consumer's risk in the multi-parameter approach (5.000%) compared to the ICH approach (14.016%). The differences between the observed total consumer's risk values may be even greater as the number of parameters analyzed during the stability study increases (e.g., considering other parameters such as the determination of pH and microbial limits). The increase in the number of batches/parameters studied affects the total combined risk, because the conformity assessment must consider the combination of results obtained for all batches and parameters studied, which consequently affects the determination of the shelf life to be adopted to ensure a total consumer's risk of 5%.

4.2. Effect of regression models in determining shelf life (scenario B)

In scenario B, we evaluated the effect of regression models in defining the shelf life time. Different levels of significance ($\alpha = 0.25$ and $\alpha = 0.50$) were adopted in the poolability test in order to obtain different regression models for the stability of AAS and AS. The resulting models can be seen in Table 4. The shelf life time per batch (for AAS and AS) obtained through the approach proposed in this work and those calculated according to the ICH are shown in Table 5.

Shelf life time is affected by the choice of the regression model (simple, partial or full model), since the slope and intercept values will be considered when calculating the shelf life time. In addition, there is an increase in the width of the confidence interval for the estimated shelf life time, since the amount of data used in the full model and partial model is smaller compared to the simple model. In cases where a full model or partial model is used, the shelf life time is defined by the batch with the shortest shelf life (in the case of this study, batch A, considering the AS parameter). In the case of this study, there was an increase in the shelf life time (from 20.071 min to 20.084 min, corresponding to approximately 0.06% per increase) when the α value was changed from 0.25 to 0.50 (Table 5).

To determine the shelf life through the multiparameter approach, six shelf life times were obtained using the *Stability_4risk* (available as a supplementary material), considering the total consumer's risk of 5%, the measurement uncertainty values of the AAS parameters and AS (u_{AAS} = 1.7% and u_{AS} = 0.27%, respectively), and $\alpha = 0.25$ and 0.50. The mean shelf life times and their respective standard deviations are shown in Table 6. ANOVA indicated that there is a significant difference between the estimated shelf life times, as well as between the particular risks for AAS and AS, considering the regression models obtained with α values of 0.25 and 0.50.

When comparing the shelf life times obtained through the approach proposed in this work (multiparameter approach considering the measurement uncertainties of AS and AAS, with a total consumer's risk of 5%) with the ICH approach, it is noted that these were 15.94%

Table 4
Regression models related to the stability studies of AAS and AS, using the significance levels $\alpha = 0.25$ and $\alpha = 0.50$.

Parameter	$\alpha = 0.25$	$\alpha = 0.5$
AAS	Batch A = 101.555 -0.227t*	Batch A = 100.839–0.227t**
	Batch B = 101.555 -0.227t	Batch B = 102.188–0.227t
	Batch C = 101.555 -0.227t	Batch C = 101.639–0.227t
AS	Batch A = 0.154 + 0.18t**	Batch A = 0.083 + 0.181t***
	Batch B = −0.049 + 0.18t	Batch B = −0.163 + 0.182t
	Batch C = −0.257 + 0.18t	Batch C = −0.073 + 0.177t

Legend: *Simple model (common slope and common intercept); **Partial model (common slope and individual intercept); and ***Full model (Individual slope and individual intercept).

Table 5
Shelf life calculated according to the ICH, considering different regression models (obtained with α values of 0.25 and 0.50).

Parameter	α = 0.25	α = 0.50
AAS	Batch A = 47.284 min.	Batch A = 41.366 min.
	Batch B = 47.284 min.	Batch B = 47.571 min.
	Batch C = 47.284 min.	Batch C = 45.118 min.
AS	Batch A = 20.071 min.	Batch A = 20.084 min.
	Batch B = 21.228 min.	Batch B = 21.385 min.
	Batch C = 22.385 min.	Batch C = 21.479 min.

Table 6
Shelf life obtained by the multiparameter approach, particular risks for the AAS and AS parameters, and total consumer's risk, considering different regression models (obtained with α values of 0.25 and 0.50).

Response	α = 0.25 (n = 6)	α = 0.50 (n = 6)
Shelf life	16.871 ± 0.009 min a	16.629 min ± 0.009 b
Particular Risk for AAS	0.01 ± 0.001% b	0.17 ± 0.01% a
Particular Risk for AS	4.98 ± 0.08% a	4.83 ± 0.02% b
Total Risk	5.00 ± 0.00%[a]	5.00 ± 0.00%[a]

Means with different letters are statistically different, p-value < 0.01. The results correspond to the mean ± standard deviation obtained from 6 simulations.
[a] There is no difference between the averages.

(20.071–16.871 min) and 17.20% (from 20.084 to 16.629 min) lower, considering the regression models obtained with α values of 0.25 and 0.50, respectively. This can be explained due to two factors: a) the Monte Carlo simulation method considers the mean as the intercept of the most restrictive model (with shorter shelf life) and the standard deviation as the measurement uncertainty. The intercept value of the AAS model obtained with α = 0.50 (100.839) decrease when compared to the intercept value of the AAS model obtained with α = 0.25 (101.555) (see Table 4); and b) the multiparameter approach considers the variability of the initial concentrations (through the measurement uncertainty of the AAS and the AS) and the variability of the batch regression models (through the slope and intercept errors), in order to consider for conformity assessment the combination of results obtained for all batches and parameters studied. The change in the AAS model (from simple model to partial model) led to increased variability (see Table 4). The combination of the aforementioned factors led to an increase in the contribution of the AAS parameter to the total risk and, consequently, decreased the shelf life. The effect of these two factors can be seen in Table 6. Considering the regression model obtained with α = 0.25, the contribution of the AAS parameter to the total consumer's risk (5%) is around 1.97%, while its contribution is of 4.83% for the regression model with α = 0.50. Thus, considering the regression models obtained with α = 0.50, the AAS parameter was the most critical for defining the shelf life time, despite the AS parameter having a shorter shelf life than AAS when considered the ICH criteria. The total consumer's risk values for the shelf life calculated according to the ICH, evaluated by the multiparameter approach (proposed in this work) and considering the measurement uncertainty values (u_{AAS} = 1.7% and u_{AS} = 0.27%), were of 87% and 85%, considering α values of 0.25 and 0.50, respectively.

Particular consumers' risks, considering u_{AAS} = 1.7% and u_{AS} = 0.27% and regression models obtained with α = 0.25, considering the ICH approaches (A1 and A2, for AAS and AS, respectively) and the approach proposed in this work (B1 and B2, for AAS and AS, respectively), respectively, were showed in Fig. 3. Considering the ICH approach, the particular consumers' risks were 0.1% and 87.4% for AAS and AS, respectively, adopting a shelf life of 20.071 min. On the other hand, considering the approach proposed in this work, particular consumers' risks were 0.0% and 5.0% for AAS and AS, respectively, adopting a shelf life of 16.871 min.

4.3. *Effect of measurement uncertainty in determining shelf life (scenario C)*

In scenario C, we estimate the impact of measurement uncertainty values associated to of AAS and AS quantities on the definition of shelf

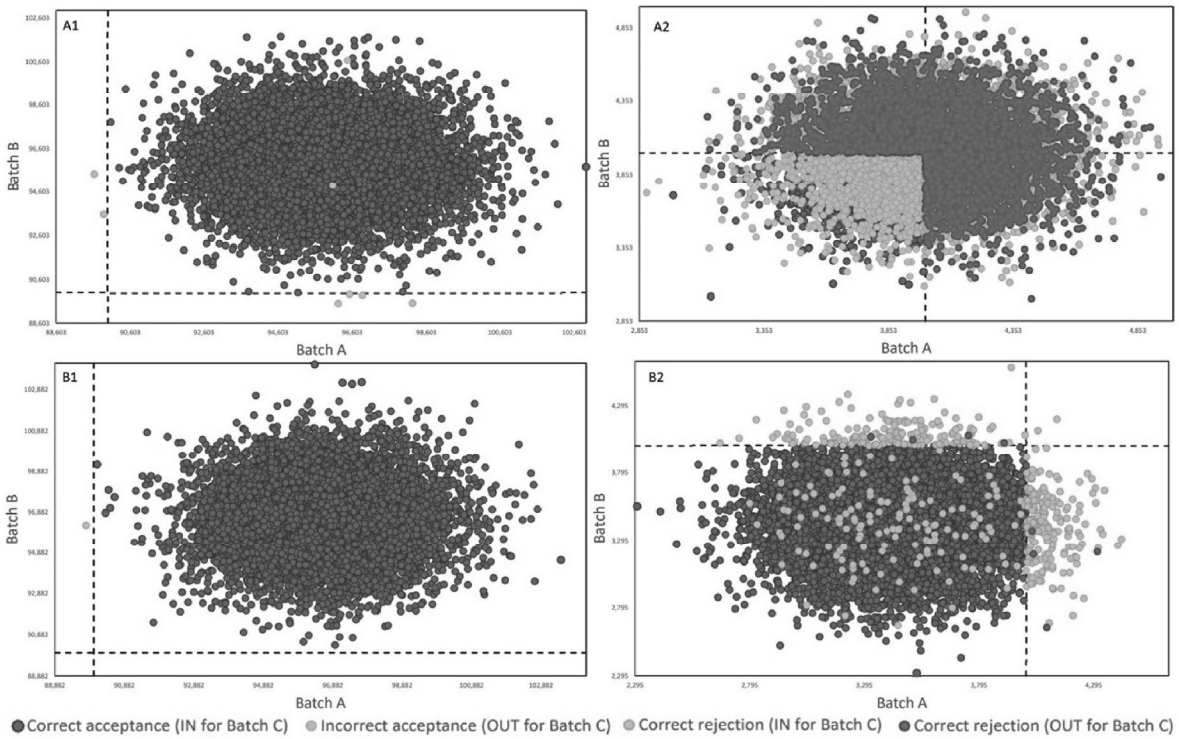

Fig. 3. Particular consumers' risks, considering u_{AAS} = 1.7% and u_{AS} = 0.27% and regression models obtained with α = 0.25, considering the ICH approaches (A1 and A2, for AAs and AS, respectively) and the approach proposed in this work (B1 and B2, for AAs and AS, respectively).

Table 7
Shelf-life, particular consumers' risks and total consumer's risk considering the low, medium and high levels of AAS and AS measurement uncertainties.

Response	$u_{AAS} = 1.0e^{-20}$% e $u_{AS} = 1.0e^{-20}$% (n = 6)	$u_{AAS} = 1.7$% e $u_{AS} = 0.27$% (n = 6)	$u_{AAS} = 2.5$% e $u_{AS} = 0.5$% (n = 6)
Shelf life	19.338 ± 0.004 min a	16.873 ± 0.005 min b	13.995 ± 0.019 min. C
Particular Risk for AAS	0.000 ± 0.000% c	0.015 ± 0.003% b	0.615 ± 0.047% a
Particular Risk for AS	5.000 ± 0.000%a	4.986 ± 0.003% b	4.409 ± 0.049% c
Total Risk	5.00 ± 0.00%[a]	5.00 ± 0.00%[a]	5.00 ± 0.00%[a]

Means with different letters are statistically different, p-value < 0.01. The results correspond to the mean ± standard deviation obtained from 6 simulations.
[a] There is no difference between the averages.

life, as well as their contributions to the total consumer's risk. Three AAS and AS uncertainties values were used: low level, with negligible uncertainty values ($u_{AAS} = 1.0e^{-20}$% and $u_{AS} = 1.0e^{-20}$%); medium level, with the uncertainty values obtained experimentally ($u_{AAS} = 1.7$% and $u_{AS} = 0.27$%); and high level, with uncertainty values equal to the target uncertainty based on the specification limits of each parameter ($u_{AAS} = 2.5$% and $u_{AS} = 0.5$%).

To determine a shelf life through the multiparameter approach, six shelf life times were obtained by the *Stability_4risk*, considering the total consumer's risk of 5%, for each level of uncertainty (low, medium and high). The shelf life times were defined as the average of the 6 values obtained for each level, and the results are presented in Table 7. The ANOVA indicated that there is a significant difference between the estimated shelf life times, as well as between the particular risks for AAS and for the AS, considering the three levels of measurement uncertainties.

To generate the simulations, the Monte Carlo method needs data variability, so it is not possible to use a measurement uncertainty value equal to 0. In this case, to calculate the risks by the multiparameter approach when the uncertainty information is neglected, a value close to 0 was established in order to represent the non-consideration of uncertainty in the stability study (ICH approach). When using a very low uncertainty value ($1.0e^{-20}$) for both parameters, the variation of the initial concentration remains practically constant, therefore does not impact the total consumer's risk and, consequently, the definition of the shelf life. The shelf life time defined with a total consumer's risk of 5% and negligible measurement uncertainty values ($u_{AAS} = 1.0e^{-20}$% and $u_{AS} = 1.0e^{-20}$%) was 19.338 min, and represents the situation in which the shelf life was determined by the multiparameter approach without taking into account the uncertainty information. Thus, the shelf life time defined by multiparameter approach (19.338 min) is shorter than the shelf life time generated by the ICH approach (20.071 min). This is due to the combination of the results obtained for all batches and parameters studied in the conformity assessment. In addition, the variability of the batches obtained through the measurement uncertainty and of the regression models coefficient errors, result in the possibility that the generated values of AS for batches B and/or C were out of specification and those for batch A are within the limits, even if the batch (A) of the parameter (AS) is the most critical (Table 8). Given that, at this level of uncertainty, AS was the only parameter that contributed to the total consumer's risk (see Table 7), it is worth noting that the risk value obtained is the result of the contribution of the values generated for the 3 batches (A, B and C) analyzed in the stability study, as can be seen in Table 8.

Table 8
Risk values of AS parameter batches, considering $u_{AS} = 1.0e^{-20}$%.

Batch	"IN"	"OUT"	Risk
Particular risk for batch A	49098	902	1.80%
Particular risk for batch B	49196	804	1.61%
Particular risk for batch C	49160	840	1.68%
Particular combined risk	47501	2499	5.00%

When there are specification limits for an analysis in a specific context, there must be a law or regulation that establishes the target uncertainty. Since there is no obligation to quantify the measurement uncertainty of the analytical methods used in stability studies, there are also no rules for the target uncertainty. In cases like this, the target uncertainty is determined as a function of the specification limits [29, 30]. The upper specification limit of the AS parameter is 4% being its target uncertainty value of 0.5%, while the lower and upper limits of the AAS parameter are from 90% to 110%, which leds to a target uncertainty value of 2.5%.

The shelf life time considering the target uncertainties of the AAS and AS parameters as a function of the total consumer's risk of 5% was 13.995 min. Therefore, using measurement uncertainty values considered adequate to the specification limits of the stability study, the shelf life time obtained is 30.3% shorter (13.995 min) than the shelf life time obtained using the ICH approach (20.071 min).

When comparing the shelf life obtained with negligible uncertainties ($u_{AAS} = 1.0e^{-20}$% and $u_{AS} = 1.0e^{-20}$%) with those obtained with target uncertainties ($u_{AAS} = 2.5$% and $u_{AS} = 0.5$%), the impact of measurement uncertainty becomes evident in defining the shelf life time. That is, the shelf life time obtained using the target uncertainty values (13.995 min) was 27.6% smaller than obtained with the negligible uncertainty (19.338 min). Fig. 4A shows the particular combined consumers' risks (0% and 5%, respectively) for the AAS and AS parameters (A1 and A2, respectively), considering the shelf life time obtained without the uncertainty information (19.338 min). For comparison purposes, Fig. 4B shows the particular combined consumers' risks (2.4% and 77.9%, respectively) for the AAS and AS parameters (B1 and B2, respectively), considering the same shelf life (19.338 min), but considering the impact of measurement uncertainties ($u_{AAS} = 2.5$% and $u_{AS} = 0.5$%) on risk values. Additionally, the total consumer's risk obtained with a shelf life time of 19.338 min, considering the target uncertainty values, was 78.3%. Risks and shelf life times were calculated using the multiparameter approach.

When not considering the measurement uncertainty in the stability studies, it can be incurred that, due to the variation of the initial values of the batch of the analyzed parameters, the defined shelf life time is not adequate to guarantee that all parameters are within specification at the end of the term.

4.4. Effect of experimental correlation in determining shelf life (scenario D)

In scenario D, we evaluated how the experimental correlation between the values of AS and AAS affects the total consumer's risk and, consequently, the definition of the shelf life. The experimental correlation can be divided into two terms: the intrinsic (or natural) correlation and the metrological (or artificial) correlation.

The intrinsic (or natural) correlation corresponds to the correlation between two parameters as a result of the product's profile over the shelf life (for example, the correlation between the amount of AAS - which degrades over time - and the amount of AS - which is formed from the degradation of AAS). On the other hand, the metrological correlation rises from the sharing of analytical steps relevant to two parameters under study (for example, the steps of sampling, dilutions and shared instrumental readings for the quantification of AAS and AS) [23]. Shared analytical steps suggest that metrological correlation should be positive ($r \geq 0.00$).

In this scenario, the impact of the correlation between the AAS and AS values on the total consumer's risk and, consequently, on the determination of the shelf life (Table 9), was evaluated, considering three situations: a) independent values of AAS and AS ($r = 0.00$); b) AAS and AS values with moderate metrological correlation ($r = 0.50$); and,

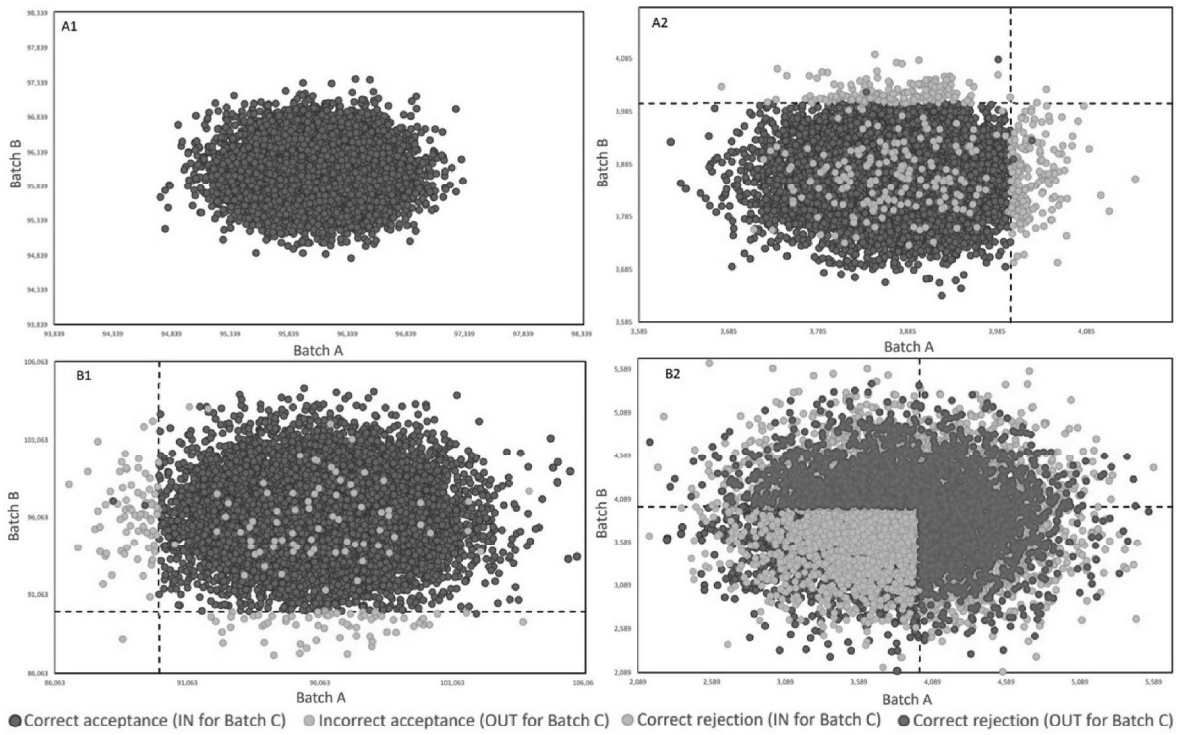

Fig. 4. Particular combined risk values adopting a shelf life time of 19,338 min, obtained without the uncertainty information for the parameters AAS (A1, combined particular risk of 0%) and AS (A2, combined particular risk of 5%) and using the uncertainty target values for AAS (B1, 2.4% combined particular risk) and AS (B2, 77.9% combined particular risk) parameters.

Table 9
Shelf life, particular consumers' risks and total consumer's risk considering that AAS and AS values are independent ($r = 0.00$), moderately correlated ($r = 0.50$) or strongly correlated ($r = 0.99$).

Response	$r = 0.00$ (n = 6)	$r = 0.50$ (n = 6)	$r = 0.99$ (n = 6)
Shelf life	13.987 ± 0.017 min c	14.157 ± 0.025 min b	15.044 ± 0.025 min a
Particular Risk for AAS	0.59 ± 0.022% a	0.587 ± 0.019% a	0.369 ± 0.033% b
Particular Risk for AS	4.434 ± 0.024% b	4.413 ± 0.02% b	4.631 ± 0.033% a
Total Risk	5.00 ± 0.00%[a]	5.00 ± 0.00%[a]	5.00 ± 0.00%[a]

Means with different letters are statistically different, p-value < 0.01. The results correspond to the mean ± standard deviation obtained from 6 simulations.
[a] There is no difference between the averages.

c) correlated values of AAS and AS ($r \geq 0.99$).

Normally distributed random generators represent the dispersion of initial concentrations of AAS and AS parameters for each batch as a function of the measured values and their respective measurement uncertainty values. To assess the effect of the correlation on the consumer's total risk, the matrix containing the random generators was multiplied by the Cholesky matrix obtained from the correlation matrix of the AAS and AS values, in order to obtain correlated random generators. The correlated random generators are multiplied by the uncertainty values and added to the initial concentrations (measured values). Therefore, 50,000 initial correlated concentrations are generated based on measured values and their respective uncertainty values. Finally, the total consumer's risk is calculated, and the shelf life is defined according to the multiparameter approach.

Six shelf life times were simulated, considering a total consumer's risk of 5%, with the target uncertainty values of the parameters, $u_{AAS} = 2.5\%$ and $u_{AS} = 0.5\%$, and $\alpha = 0.25$. ANOVA indicated that there is a significant difference between the values of estimated shelf life, as well as between the values of particular risks (for AAS and AS), considering that the values of AAS and AS are independent ($r = 0.00$), moderately correlated ($r = 0.50$) or strongly correlated ($r = 0.99$).

Positive correlation indicates that the initial concentration values vary together in the same direction, with the magnitude of the variation depending on the correlation value ($r = 0.00$, for independent values, to $r = 1.00$, for fully correlated values). As the shelf life is defined considering a total risk value of 5%, the correlation between the values of AAS and AS will have an impact on the value of the shelf life and, consequently, on the contribution of each parameter (AAS and AS) for the total risk of 5%.

The shelf life values determined by the multiparameter approach considering a total consumer's risk of 5%, using independent ($r = 0.00$) and strongly correlated ($r = 0.99$) generators, show a difference of 7% (13.987 and 15.044 min, respectively). The particular consumers' risks of 0.8% and 10.6% of the AAS and AS parameters (A1 and A2, respectively), and total consumer's risk of 11.3% for a shelf life of 15.044 min, with independent generators ($r = 0.00$) are showed in Fig. 5. On the other hand, the particular consumers' risks of 0.4% and 4.5% of the AAS and AS parameters (B1 and B2, respectively), and total consumer's risk of 5.0% for a shelf life of 15.044 min, with strongly correlated generators ($r = 0.99$) (Fig. 5).

4.5. *Effect of change in specification limits in determining shelf life (scenario E)*

Considering that specification limits are different depending on the characteristics of the active pharmaceutical ingredient (API), its degradation products and the dosage form, it is important to understand how the definition of these limits affect the determination of the shelf life, when considering the uncertainty of measurement and consumer's risk.

In scenario E, we study how the definition of specification limits affect the shelf life and, consequently, the total consumer's risk, considering measurement uncertainty and the multiparameter

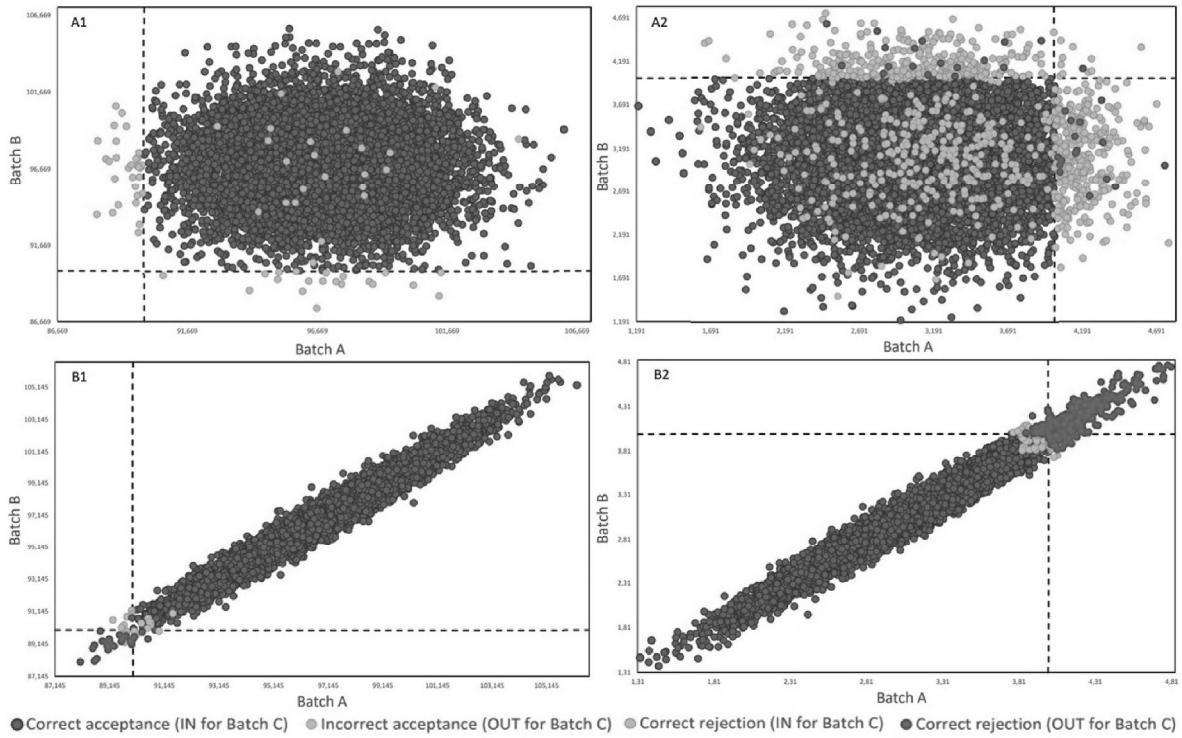

Fig. 5. Particular combined risk values, adopting a shelf life time of 15.044 min, obtained with independent generators ($r = 0.00$) for the parameters AAS (A1, particular combined risk of 0.8%) and AS (A2, particular combined risk of 10.5%) with total consumer's risk 11.3%, and with strongly correlated generators ($r = 0.99$) for the parameters AAS (B1, particular combined risk of 0.4%) and AS (B2, particular combined risk of 4.5%) with total consumer's risk 5.0%,.

approach. A pragmatic approach to ensuring reduced risk involves adopting limits that are more restrictive than specification limits. Usually, acceptance limits are defined in order to ensure a reduced risk of false acceptance, by adding or subtracting a guard band (multiple of the standard uncertainty) to the specification limits [29]. Alternatively, control limits can be adopted, in order to define a more restrictive control interval than the specification interval.

In the view of this concept, we determined the shelf life time when control limits ($AAS_{LCL} = 92.5\%$ and $AAS_{UCL} = 107.5\%$, and $AS_{UCL} = 3.5\%$) are more restrictive than the specification limits ($AAS_{LSL} = 90\%$ and $AAS_{USL} = 110\%$, and $AS_{USL} = 4\%$), with $u_{AAS} = 1.7\%$ and $u_{AS} = 0.27\%$. The values of the control limits are hypothetical and were established in order to show the effect of the limits of the shelf life definition.

Six shelf life time determinations were obtained by the multiparameter approach, considering a total consumer's risk of 5%, for both the control limits and specification limits. The shelf life times were defined as the average values obtained for each limit, as shown in Table 10. ANOVA indicated that there is a significant difference between the estimated shelf life times, as well as between the particular risks for the AAS and for the AS, considering control limits and specification limits.

Table 10
Shelf life, particular consumers' risks and total consumer's risk considering control limits and specification limits for AAS and AS.

Response	$AAS_{LSL} = 90\%$ - $AAS_{USL} = 110\%$ $AS_{USL} = 4\%$ (n = 6)	$AAS_{LCL} = 92.5\%$ - $AAS_{UCL} = 107.5\%$ $AS_{UCL} = 3.5\%$ (n = 6)
Shelf life	16.878 ± 0.018 min a	13.996 ± 0.007 min b
Particular Risk for AAS	0.017 ± 0.006% b	0.742 ± 0.023% a
Particular Risk for AS	4.984 ± 0.005% a	4.292 ± 0.025% b
Total Risk	5.00 ± 0.00%	5.00 ± 0.00%

Means with different letters are statistically different, p-value < 0.01. The results correspond to the mean ± standard deviation obtained from 6 simulations.
*There is no difference between the averages.

The effect of changing specification limits to control limits was substantial. The shelf life time obtained with the control limits was 17.1% shorter than the shelf life time obtained with the specification limits. An important detail was the 43-fold increase in the contribution of AAS to total consumer's risk (Table 10). This was due to the uncertainty of the AAS, $u_{AAS} = 1.7\%$, being close to the target uncertainty when considering the control interval (target uncertainty of 1.875%), consequently impacting the shelf life.

On the other hand, there was a 13.9% reduction in the particular risk of AS when adopting the control limits when compared to the risk value obtained with the specification limits. Despite the reduction in the range when the control limits were adopted, the uncertainty of the AS, $u_{AS} = 0.27\%$, is still below the target uncertainty (target uncertainty of 0.438%, considering the control limits). Therefore, with the uncertainty of the AAS close to its target uncertainty, the AS is no longer the only factor contributing to the total consumer's risk. Another factor that resulted in a decrease in the risk of AS was the decrease in the shelf life due to the new control limits.

The scatter plots of the AAS and AS parameters, with a shelf life of 18,941 min (A1 and A2, respectively), considering the specification limits ($AAS_{LSL} = 90\%$ and $AAS_{USL} = 110\%$, and $AS_{USL} = 4\%$), as well as the scatter plots of the AAS and AS parameters, with an shelf life of 13.996 min (AAS_{LCL}=92.5% and $AAS_{UCL} = 107.5\%$, and $AS_{UCL} = 3.5\%$) are showed in Fig. 6.

4.6. Comparison of the scenarios

The simultaneous parameters evaluation (scenario A), regression model types (scenario B), the measurement uncertainty information (scenario C), metrological correlation of measured results (scenario D) and specification limits (scenario E) impacted on the risks, consequently, on the definition of the shelf life. The number of parameters that should

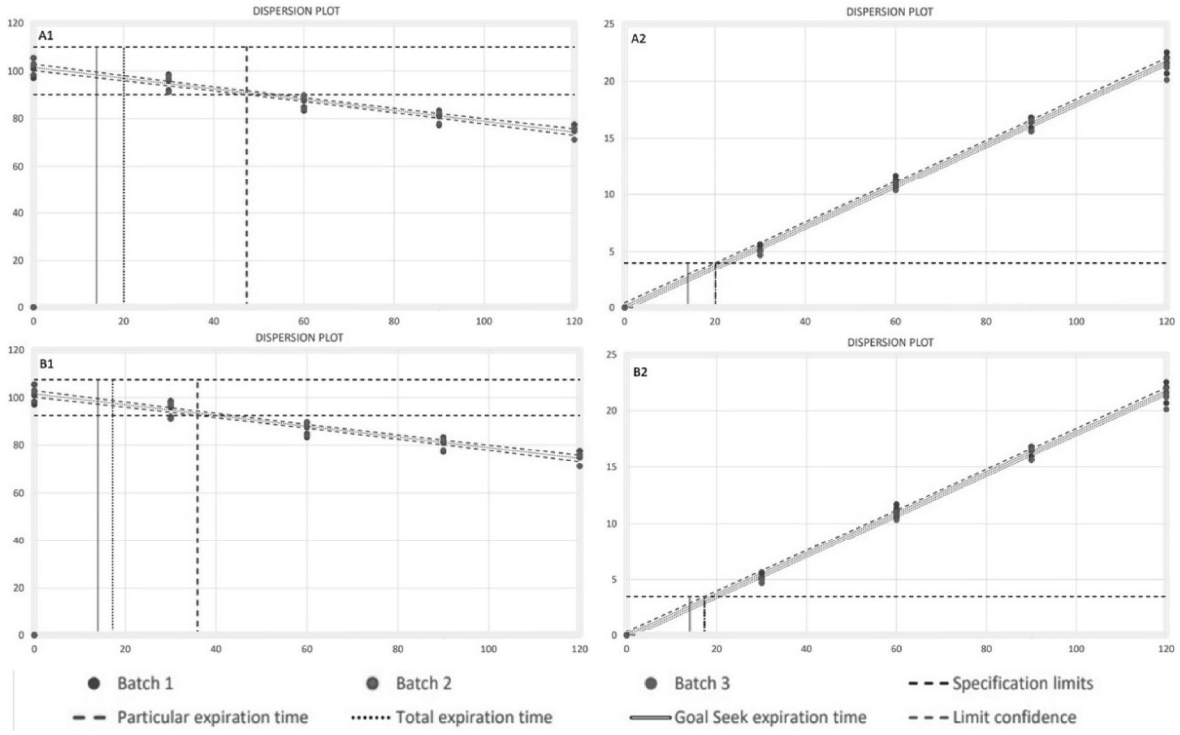

Fig. 6. (A1) Scatter plot of AAS with shelf life time of 16.878 min established by the Multiparameter approach using the specification limits (AAS_{LSL} = 90% and AAS_{USL} = 110%). (A2) Scatter plot of AS with shelf life time of 16.878 min established by the Multiparameter approach using the upper specification limit (AS_{USL} = 4%). (B1) Scatter plot of AAS with shelf life time of 13.996 min established by the Multiparameter approach using the control limits (AAS_{LCL}=92.5% and AAS_{UCL} = 107.5%). (B2) AS scatter plot with shelf life time of 13.996 min established by the multiparameter approach using the upper control limit (AS_{UCL} = 3.5%).

be evaluated, the choice regression model type, and specification limits are usually previously defined, according to the current regulatory requirements. On the other hand, the analytical procedures adopted in the stability study may be select in order to provide an acceptable shelf life time. It is worth to mention that analytical procedure should provide results with reduced measurement uncertainty values. In addition, correlation between measured values due to relevant analytical steps sharing may be considered to reduce total risk of false decisions.

5. Conclusion

In the present study, considering measurement uncertainty information in stability study and simultaneous conformity assessment of all studied parameters were demonstrated in different scenarios by using the *Stability_4Risk* spreadsheet, made available as supplementary material. Multiparameter approach considering measurement uncertainty information guaranteed a safe shelf life definition, considering a maximum admitted total consumer's risk value, regardless measurement uncertainty values, tested number of batches (2 or 3 batches), and studied parameters (1–4 parameters). In this way, by defining a more assertive shelf life time, which considers results variability and coefficients of the regression models error generated by data acquired in stability study, quality, efficacy and safety of drugs and medicines are guaranteed until their expiration shelf life.

Author statement

Aldo Renato Couto was responsible for Formal analysis; Investigation; Methodology; Validation; and Roles/Writing - original draft.Felipe Rebello Lourenço was responsible for Conceptualization; Funding acquisition; Project administration; Resources; Supervision; and Writing - review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Felipe Rebello Lourenco reports financial support was provided by State of Sao Paulo Research Foundation (FAPESP - Fundação de Amparo à Pesquisa do Estado de São Paulo). Aldo Renato Couto reports financial support was provided by National Council for Scientific and Technological Development (CNPq - Conselho Nacional de Desenvolvimento Científico Tecnológico).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemolab.2022.104649.

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CAPÍTULO II - Bottom-up and top-down measurement uncertainty evaluation for multivariate spectrophotometric procedures

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Bottom-up and top-down measurement uncertainty evaluation for multivariate spectrophotometric procedures

Aldo Renato Couto, Felipe Rebello Lourenço *

Departamento de Farmácia, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 580 – CEP 05508-000, Cidade Universitária, São Paulo, Brasil

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ABSTRACT

Multivariate spectrophotometric procedures are widely used in drug analysis to quantify one or more substances in a sample. Even if the analytical procedure is validated, there is always some degree of uncertainty related to the analytical results. No studies were found in the literature that evaluated the measurement uncertainty for multivariate spectrophotometric procedures. Thus, this paper aims to estimate the measurement uncertainty of the quantification of acetylsalicylic acid (ASA) and salicylic acid (SA), in an ASA samples, by a stability-indicating multivariate spectrophotometric procedure. The measurement uncertainties of ASA and SA were estimated by the top-down approach, using the Nordtest/ISO 11352 and VAM Project methodologies, and by the bottom-up approach, using the Monte Carlo method (MCM). The calculation of measurement uncertainty considered uncertainty sources related to the preparation of sample and standard solutions and the impact of these sources on the model obtained by partial least squares (PLS). The uncertainty of the spectra was estimated by bootstrap resampling method. The expanded uncertainties estimated for ASA were 3.7%, 4.1% and 3.7% calculated by MCM, Nordtest/ISO 11352 and VAM Project, respectively. The expanded uncertainties estimated for SA considering the concentration range from 2.67 to 6.67 μg/mL were 0.25, 0.36 and 0.35 μg/mL and for the concentration range from 6.67 to 18.67 μg/mL were 5.3%, 5.3% and 6.0% calculated by MCM, Nordtest/ISO 11352 respectively. It was concluded that the developed methodology allows to estimate the measurement uncertainty of a multivariate spectrophotometric analytical procedure by bottom-up and top-down approaches.

1. Introduction

Degradation products are substances formed from the degradation of active pharmaceutical ingredient (API) during storage period that affect the safety, efficacy and quality of the pharmaceutical product. In order to evaluate the possible degradation products of a pharmaceutical product, or API's, and their formation pathways, a stress degradation study is performed where the product is evaluated at elevated temperature and humidity, which may include alkaline and/or acid hydrolysis and oxidation studies, with the aim of obtained the possible products of degradation. Additionally, using the information and samples obtained from stress degradation study, it is possible to develop and validate stability-indicating analytical procedures that identify and quantify the API and/or degradation product [1,2]. These procedures should be able to identify changes in the quality of the pharmaceutical product according to storage time, in the presence of interferents, in degraded and real samples in accelerated storage conditions of temperature and humidity or in adequate storage conditions [2,3].

Spectrophotometric analytical procedures are widely used in drug analysis, in addition to being simpler and more economical than chromatographic procedures [4–6]. However, API analysis together with degradation products can be challenging when there is overlapping absorption spectra. Multivariate spectrophotometric methods are used to resolve the spectral overlap between a substance in the presence of interferents or between two or more substances present in samples, without the need for their separation [4,5,7,8]. Multiple wavelengths are utilized to enhance the accuracy and inferential capability of the substances [4,7].

Among the multivariate methods, partial least squares regression (PLS) is the most used. PLS uses spectral data matrix and substance concentrations to obtain the latent variables, in parallel, maximizing the covariance between spectral and concentration data [9]. There is

* Corresponding author.
E-mail address: feliperl@usp.br (F. Rebello Lourenço).

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extensive literature on using PLS to quantify one or more than one API in a test sample [5,7,9–11] or API's with their degradation products [4,6,8,12–14].

In 2022, ICH Q2(R2) draft on the validation of pharmaceutical analytical procedures was published. It is the first guideline for validation of pharmaceutical analytical procedures that includes validation of multivariate analytical procedures [15].

Even if validated, an analytical procedure will still have an uncertainty related to the result. Measurement uncertainty is a parameter related to a measurement, representing a range of values that can be attributed to the analysis result with a certain level of confidence. [16,17]. Uncertainty sources such as sampling, weighing, volumetric apparatus, environmental conditions, equipment and instruments, purity of reagents and reference standards, among others, may contribute to measurement uncertainty. Sources of uncertainty can be comprised of random errors, which result in unpredictable variations of the result in repeated analyzes and which can be estimated by the standard deviation of certain quantities of results; and systematic errors, which are constant variations or that vary in a predictable way in the course of the analyzes and which can be estimated as bias calculated from recovery studies [16–18]. Measurement uncertainty can be estimated by two approaches: top-down and bottom-up [16,19–22].

The top-down approach is a practical method for calculating the measurement uncertainty, as data from method validation, total error/tolerance intervals, quality control, interlaboratory study or proficiency testing are used to estimate measurement uncertainty [16,19,20,23–27]. In the top-down approach, uncertainties related to precision, trueness and additional uncertainty sources (e.g. uncertainty from sampling), which are not included in the first two, are quantified and combined to obtain the overall measurement uncertainty [16,19,20,25,28].

The bottom-up approach considers all possible uncertainty sources that affect the result of an analytical procedure to estimate the measurement uncertainty. Studies are conducted to quantify the standard uncertainties of the uncertainty sources, and in other cases, there is prior knowledge of the values of the standard uncertainties (e.g. uncertainty values from calibration certificates). An equation is modeled to relate the sources of uncertainty, treated as variables in the equation, to the measured result. In this way, the measurement uncertainty can be estimated by propagating the standard uncertainties of the sources through the equation [16,21]. Despite of being more laborious, bottom-up approach provides a detailed evaluation of measurement uncertainty. In this way, it is possible to evaluate the impact of each uncertainty source on the combined uncertainty [16–18,22,29]. Three methods are widely used to propagate standard uncertainties and estimate the measurement uncertainty: law of propagation of uncertainty [16,18,30], Kragten spreadsheet [16,18,29,31] and Monte Carlo Method (MCM) [16,18,22,29,31,32].

The law of propagation of uncertainty is an algebraic method for combining standard uncertainties. This method involves calculating the sensitivity factor obtained through the partial derivatives of the measurement result in relation to the input variables and, if applicable, considers the covariance between the input variables [16,18,21,29,31,32]. The calculations can be challenging, particularly for complex analytical procedures, especially for nonlinear models. Additionally, in the case of nonlinear models, appropriate higher-order terms need to be added, which can further complicate the calculation [22,29]. The Kragten spreadsheet is a numerical approach that uses spreadsheet software to combine sources of uncertainty without the need to calculate the sensitivity factor. This makes the estimation of measurement uncertainty less complex [29,31]. In addition, in cases of non-linear models, a quadratic approximation can be used [33]. However, the uncertainty propagation law and the Kragten spreadsheet require that the dominant uncertainty sources have normal distribution, scaled t-distribution or shifted t-distribution [18,22,29,31].

The MCM is a numerical method that samples new values of the input variables, considering their standard uncertainties and the determined probability density functions (PDF). These values are then propagated through the equation of the analytical procedure to calculate the final measurement result.Unlike other methods, there are no restrictions on the types of PDFs (e.g., normal, rectangular, triangular, t-student, log-normal, Poisson, among others) of the input variables in the MCM. If there is dependence between some variables, they can use the same PDF for value simulation. MCM is readily valid regardless of the linearity or non-linearity of the model, which makes it simpler to implement [22,29,31].

The disadvantages to the MCM include the need for coding and implementation in appropriate software, as well as the computational cost required to perform a sufficient number of iterations for stochastic convergence of the uncertainty value [16,22,31]. Despite the disadvantages, MCM can be implemented using software such as R, Python, or even using in an MS-Excel spreadsheet [16,32,34–39].

Several papers evaluate measurement uncertainty of univariate spectrophotometric procedures for quantification of APIs in pharmaceutical products [40–42]. Soovälli et al [43] investigated the main uncertainty sources in measurements by UV–Vis spectrophotometric procedures. However, to the best of our knowledge, there are no works that provide detailed estimation of the measurement uncertainty of multivariate spectrophotometric procedures.

This paper aims to estimate the measurement uncertainty of a multivariate spectrophotometric analytical procedure using bottom-up and top-down approaches. For this, a stability-indicating procedure of an acetylsalicylic acid (ASA) solution used in a forced degradation study was used, where ASA and its main degradation product, salicylic acid (SA) were jointly quantified [12]. Considering the complexity of the procedure and the limitation of PDF types in the uncertainty propagation law and the Kragten spreadsheet, MCM was used to propagate the uncertainty sources and calculate the combined uncertainty. Additionally, the impact of uncertainty sources of the standard solutions used in the calibration curve on the PLS model was considered to obtain the PLS model and the bootstrap resampling method was used to obtain the uncertainty related to the spectra. As a result, it was possible to evaluate the impact of each uncertainty source on the combined uncertainty. To calculate the measurement uncertainty using the top-down approach, method validation data were used, and the calculations were based on two different methodologies: Nordtest NT TR 537 handbook/ ISO 11352 (both use the same methodology - in this paper we adopt the term Nordtest to refer to both documents) and VAM Project 3.2.1 - Part (d): Protocol for uncertainty evaluation from validation data (VAM Project guide was published as part of the National Measurement Systems Valid Analytical Measurement (VAM) Programme).

2. Theory

2.1. Calculation of uncertainty by top-down approach

One of the ways of estimating measurement uncertainty by the top-down approach is through the performance parameters resulting from analytical procedure validation. The uncertainty components of precision, trueness and, if necessary, other uncertainty components (e.g. uncertainty from sampling), are combined to obtain the combined uncertainty of the measurand (u_c) [16,19,20,25,28].

The estimation of the standard uncertainty of the precision and trueness uncertainty components depends on how the analytical procedure validation is performed. Calculations consider whether or not the sample matrix used in the validation is the same as the matrix of the test sample, whether the procedure will quantify to analyte in a narrow or in a wide concentration range, whether a certified reference material (CRM) is used, whether spiking will be added to a sample with or

without a native analyte, whether the result is compared to a reference method, or whether data from a proficiency testing is used [16,19,20,25].

The calculations of measurement uncertainty presented in this paper were performed considering a sample matrix identical to the test sample matrix, covering a wide concentration range. The spiking was added to the matrix without the native analyte.

2.1.1. Precision uncertainty estimation (u_p)

To quantify the precision uncertainty considering that the sample matrix used in the validation is identical to the test sample matrix and that the analyte will be analyzed within a wide concentration range, replicates must be taken at different concentration levels covering the reportable range and in intermediate precision condition (different days, if possible, different analyst and equipment) [16,19,20,25,44].

Relative standard deviations (s') of the replicated samples are calculated for each concentration level studied. It must be evaluated whether the s' of the concentration levels are not significantly different [16,19,20,25,44]. An F-test to evaluate equality of variances can be performed if there are sufficient degrees of freedom (10 or more) [19]. Alternatively, homoscedasticity may be checked using a Cochran, Bartlett or Levene test when comparing more than two variances. Moreover, it is up to the analyst to determine whether there is a difference between the values [19]. If the standard deviations are not significantly different, the pooled relative standard deviation (s'_p) is estimated using Eq. (1). The precision uncertainty is $u'_p = s'_p$ [19,20,44].

$$s'_p = \sqrt{\frac{(n_1-1)s'^{2}_{1} + (n_2-1)s'^{2}_{2} + \cdots + (n_i-1)s'^{2}_{i}}{(n_1-1)+(n_2-1)+\cdots+(n_i-1)}} \quad (1)$$

Where, n is the number of replicates used to calculate the standard deviation of the i^{th} concentration and s' is the relative standard deviation of the i^{th} concentration.

When five or more analyte concentrations are evaluated, graphs can be made relating s' (Fig. 1a) and standard deviations (s) (Fig. 1b) of the replicated samples (under repeatability condition) for each concentration level against the concentrations [16,19,20,45].

The behavior of de s and s' can be explained by the dominant contributions of the independent and proportional to the concentration uncertainty sources, respectively, in the overall uncertainty. Thus, three different situations occur considering the types of the predominant sources in the global uncertainty, exemplified in Fig. 1: uncertainty sources proportional to the concentration are dominant between 10 mg and 30 mg, with s' practically constant in this range (Fig. 1a), therefore s' can be combined to obtain u'_p (Eq. (1)); sources of uncertainty independent of concentration are dominant between 1 mg and 10 mg, being s practically constant in this range (Fig. 1b), thus the s are combined to obtain pooled standard deviation (s_p), being $s_p = u_p$ (Eq. (2)); and both types of uncertainty sources contribute significantly to the global uncertainty at 10 mg (Fig. 1a e b), coinciding with the point where s' values start to stabilize and s values begin to increase as the concentration rises, in this case, the uncertainty can be expressed either as an absolute value (u_p) or as a percentage (u'_p) [16,19,20,25,46].

$$s_p = \sqrt{\frac{(n_1-1)s_1^2 + (n_2-1)s_2^2 + \cdots + (n_i-1)s_i^2}{(n_1-1)+(n_2-1)+\cdots+(n_i-1)}} \quad (2)$$

Where, s is the standard deviation of the i^{th} concentration.

Usually, the interval where s' values are not constant coincides with the concentration range between the detection limit (LoD) and twice the quantification limit (LoQ). Within this range, the s values of the samples for each concentration level have approximately constant values [20,44,46].

The calculation of the measurement uncertainty in the concentration below 10 mg and in the concentration range 10 to 30 mg must only use the data obtained from the concentrations present within their respective ranges.

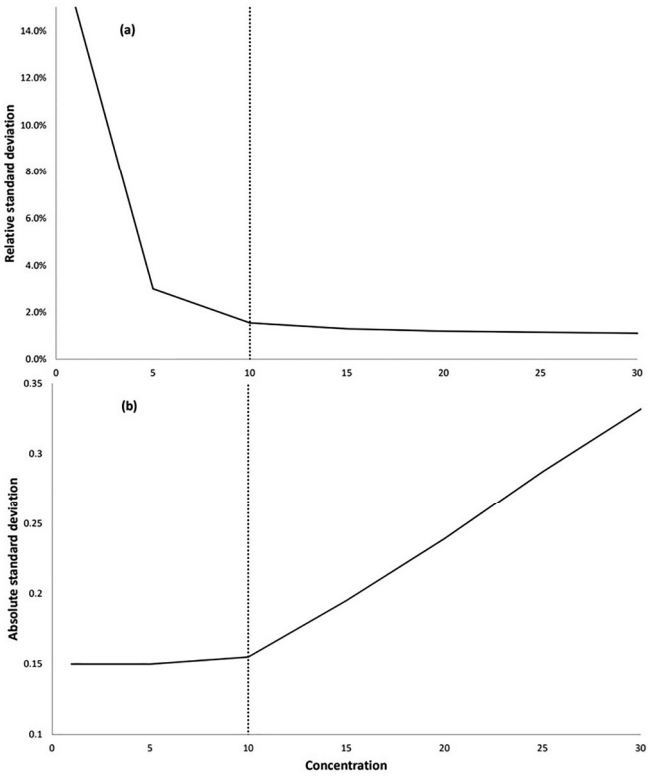

Fig. 1. (a) Relative standard deviation (s') and (b) standard deviation (s) and their respective concentration levels.

The definition of the concentration that divides the two concentration ranges can be defined by selecting the concentration at which s' is practically constant. Another approach to determining the two concentration ranges is to divide the expanded absolute measurement uncertainty at low concentration (U) by the expanded relative measurement uncertainty at high concentration (U') (Eq. (3)). This calculation can provide a concentration value at which the division can be based [20].

$$C_d = \frac{U}{U'} \quad (3)$$

Where, C_d is the concentration on which the division of the two concentration ranges will be based.

2.1.2. Trueness uncertainty estimation (u_t)

The standard uncertainty of the trueness component was estimated considering a spiking performed on a sample matrix identical to the test sample matrix, without native analyte, and each sample was prepared individually. Two approaches were used to quantify trueness uncertainty: Nordtest NT TR 537 handbook/ ISO 11352 and VAM Project 3.2.1 - Part (d): Protocol for uncertainty evaluation from validation data [19,20,25].

2.1.2.1. Trueness uncertainty calculation by Nordtest methodology. The trueness uncertainty calculated using the Nordtest/ISO 11352 approach consists of two components of uncertainty: root mean square of the mean bias ($\overline{b}^*$) (Eq. (5)) and the uncertainty of the concentration of the spiking added to the sample matrix (u_+), both considering the evaluated concentrations. For this, initially the mean bias is calculated for each concentration level:

$$\overline{b} = \frac{1}{n}\sum_{n=1}^{n}\frac{(\overline{C} - C_+)}{C_+} \quad (4)$$

Where, $\overline{C}$ is the mean concentration of the samples quantified by the procedure for each concentration level,C_+ is the spiking concentration to the sample matrix for each concentration level and n represents the number of samples analyzed for each concentration level.

Then, the average square root considering the average biases ($\overline{b}^*$) of all concentrations used in the measurement uncertainty calculation is calculated as Eq. (5):

$$\overline{b}^* = \sqrt{\frac{1}{i}\sum_{i=1}^{i}\overline{b}_i^2} \quad (5)$$

Where, $\overline{b}$ is the i^{th} concentration bias.

The recovery bias depends on two components of uncertainty,u_t and u_p. To avoid overestimating the measurement uncertainty due to the contribution of u_p, the equation Eq. (5) uses the mean biases of each concentration level.

The uncertainty of spike preparation and transfer of its volumes to the samples (u_+) is composed of the standard uncertainties of the uncertainty sources related to sample preparation for each studied concentration level (according to Eq. (6)).

$$u_+ = \sqrt{\sum_{j=1}^{j} u_j^2} \quad (6)$$

Where, j are the standard uncertainties of the uncertainty sources.

The preparation uncertainty is dependent on the concentration level, so a u_+ is estimated for each concentration level. The sample preparation uncertainty considers all concentration levels studied (u_+^*). To calculate u_+^*, the u_+ values from each concentration level are combined using Eq. (7).

$$u_+^* = \sqrt{\frac{1}{i}\sum_{i=1}^{i} u_+^2 i} \quad (7)$$

Finally, the uncertainty components $\overline{b}^*$ e u_+^* are combined to obtain the trueness uncertainty (u_t) of the analytical procedure considering the concentration levels using Eq. (8).

$$u_t = \sqrt{(\overline{b}^*)^2 + (u_+^*)^2} \quad (8)$$

2.1.2.2. Trueness uncertainty calculation by VAM Project methodology. To calculate trueness uncertainty using the VAM Project methodology for a validation study with samples at various concentration levels, the mean recovery uncertainty of the results of the samples analyzed for each concentration level is calculated ($u_{\overline{r}_i}$). For this, first the average recovery $\overline{r}_i$ per concentration is calculated using Eq. (9):

$$\overline{r}_i = \frac{1}{n}\sum_{1}^{n}\frac{\overline{C}}{C_+} \quad (9)$$

Where, $\overline{r}_i$ is the mean recovery of the samples at the i^{th} concentration, $\overline{C}$ is the mean concentration of the samples analyzed at a concentration, C_+ is the theoretical concentration of the spiking added in the sample matrix and n is the number of samples at a concentration.

Next, $u_{\overline{r}_i}$ is calculated as Eq. (10):

$$u_{\overline{r}_i} = \overline{r}_i \times \sqrt{\left(\frac{s^2}{(n \times \overline{C}^2)}\right) + \left(\frac{u_+}{C_+}\right)^2} \quad (10)$$

Where s^2 is the variance of the results from the analyzed samples at a concentration level, u_+ is the uncertainty associated with the concentration of the spiking at a concentration level (calculated according to Eq. (6)).

The statistical test to evaluate whether the average recoveries ($\overline{r}_i$) for each concentration level are significantly different from 1 (100%) is performed as described in Eq. (11) [19,28,44,46]:

$$\frac{(1 - \overline{r}_i)}{u_{\overline{r}_i}} < t_{df}^{95\%} \quad (11)$$

Where, $t_{df}^{95\%}$ is the value from the t-distribution with 95% confidence for the degrees of freedom (df) used to calculate $\overline{r}_i$.

If $\overline{r}_i$ is not significantly different from 1 (i.e. 100% recovery), there will still be an uncertainty associated with $\overline{r}_i$ that needs to be corrected considering $t_{df}^{95\%}$ to obtain u_i^c (Eq. (12)), which is the corrected uncertainty of the average recovery at a concentration level.

$$u_{\overline{r}}^c i = \frac{\left(t_{df}^{95\%} \times u_{\overline{r}_i}\right)}{1.96} \quad (12)$$

If $\overline{r}_i$ is significantly different from 1 (i.e. 100% recovery), the measurement results of the samples should be corrected by multiplying them by the inverse of the average recovery ($1/\overline{r}_i$) [19,28,44,46].

To obtain the uncertainty of the means of the corrected sample results considering all concentration levels ($u_{\overline{r}}$), the root mean square of all $u_{\overline{r}}^c i$ values is calculated using Eq. (13).

$$u_{\overline{r}} = \sqrt{\frac{1}{i}\sum_{i=1}^{i} u_{\overline{r}}^c i} \quad (13)$$

Where $u_{\overline{r}}$ is an uncertainty component of u_t, representing the uncertainty related to the dispersion of results between samples at each concentration level.

If the analytical procedure is used to quantify an analyte in a wide range of concentrations and/or in different sample matrices, it is necessary to consider the component of uncertainty related to the dispersion of the mean recoveries across all concentrations/matrices ($u_{\overline{R}}$). The component $u_{\overline{R}}$ is calculated as the standard deviation of the mean recoveries of all studied concentrations/matrices.

The components of uncertainty u_r and $u_{\overline{R}}$ are then combined to obtain u_t (Eq. (15)), considering the mean recovery across all concentrations (Eq. (14)).

$$\overline{r} = \frac{1}{i}\sum\nolimits_{i=1}^{i}\overline{r}_i \quad (14)$$

$$u_t = \overline{r} \times \sqrt{u_{\overline{r}}^2 + u_{\overline{R}}^2} \quad (15)$$

2.1.3. Calculation of combined uncertainty (u_c) and expanded uncertainty (U)

After calculating u_t and u_p, the next step is to calculate the combined uncertainty using the precision and trueness uncertainty components. The combined uncertainty can be reported either as an absolute or relative uncertainty, as described in section 2.1.1. In absolute combined uncertainty, the uncertainty values for precision and trueness are expressed in the same units as the analyte (Eq. (16)):

$$u_c = \sqrt{u_t^2 + u_p^2} \quad (16)$$

In relative combined uncertainty, the uncertainty values of precision and trueness are expressed as percentages, u'_p and u'_t respectively (Eq. (17)):

$$u'_c = \sqrt{u'^2_t + u'^2_p} \quad (17)$$

The combined uncertainty, u_c or u'_c, should be multiplied by a coverage factor (k) to obtain the expanded measurement uncertainty (U

or $U^{'}$). In most cases, $k = 2$ is used, as it covers 95% of the distribution of values in a normal distribution [16,19,20,25,28]. A coverage factor of 3 can be used to cover approximately 99% of the distribution of values. However, these coverage factors may be insufficient if the precision or trueness studies have a small number of degrees of freedom, less than 10 [19,28] or less than 6 [16]. In such cases, the *t*-value for the chosen confidence level and the degrees of freedom of the component with the highest contribution to the combined uncertainty should be used as the coverage factor [16].

2.2. Estimation of measurement uncertainty by bottom-up approach by MCM

MCM is a numerical and iterative method that can be used to estimate the measurement uncertainty through the generation of random numbers considering the values of the uncertainty sources, their standard uncertainties and their PDF's. After a total of *M* iterations, a set of measurement results (y_m) is obtained, which can be considered the numerical representation of the probability distribution of the measurand (*Y*) [16,18,22,31,32]. Through this representation, information characterizing the measurand can be obtained, such as the mean of the simulated values (*Y*) and the standard deviation (measurement uncertainty, u_Y). The application of MCM follows the example presented in Fig. 2.

Since the MCM calculations are stochastic, it is necessary to choose a sufficiently large value for *M* to obtain accurate estimates of sample uncertainties that vary by up to 1% between different simulations. To determine the minimum value of *M*, one must perform repeated simulations on several quantities (for example, 10^3, 10^4, 10^5, 10^6), and the variation in sample uncertainty (u_{Y_n}) should be evaluated [32]. Considering the measurement uncertainty value obtained in this article, this is a simple way to define *M*, alternatively to the method proposed in JCGM 101:2008 [22] which is based on a numerical tolerance.

For analytical procedures that quantify samples at a wide concentration range, measurement uncertainties must be estimated at different concentrations that include the entire reportable range (u_{Y_i}, given *i* studied concentration levels). For this, *M* results are simulated for each sample used in the study (Y_n, where *n* is the number of samples studied), the standard deviation of these simulated results are the measurement uncertainties of the respective samples (u_{Y_n}).

The measurement uncertainty per concentration level is obtained by pooled standard deviation of the simulated measurement uncertainties of each sample (u_{Y_n}), calculated according to Eq. (18).

$$u_{Y_i} = \sqrt{\frac{1}{n}\sum_{1}^{n} {u_{Y_n}}^2} \tag{18}$$

Where, *n* is the total number of sample solutions analyzed at the $i^{\text{-th}}$ concentration level.

The expression of the measurement uncertainty calculated by MCM follows the rule in section 2.1.1, where Fig. 1 can be constructed with the values of relative and absolute measurement uncertainties per concentration level obtained by MCM plotted against the concentration levels of the samples. Thus, one should consider dividing the graph into two concentration ranges, as exemplified in Fig. 1. Where the relative uncertainty is approximately constant, the uncertainty will be expressed as a percentage; where the relative uncertainty is not constant, the uncertainty is expressed as an absolute value. Therefore, the final calculation to obtain the combined uncertainty of the procedure (u_Y) is the pooled standard deviation of u_{Y_i} (Eq. (19)). Similarly, the combined relative uncertainty u'_Y is calculated according to Eq. (20). The expanded uncertainty is obtained by multiplying the combined uncertainty by the coverage factor *k*.

$$u_Y = \sqrt{\frac{1}{i}\sum_{i=1}^{i} {u_{Y_i}}^2} \tag{19}$$

$$u'_Y = \sqrt{\frac{1}{i}\sum_{i=1}^{i} {u'_{Y_i}}^2} \tag{20}$$

One important information that can be obtained from MCM is the relative contribution of each source of uncertainty to the combined measurement uncertainty, also called sensitivity analysis. For this, simulations are conducted by varying only one input variable while keeping the others fixed, resulting in a measurement uncertainty considering only one source of uncertainty (u_{x_j}). This process is repeated for all variables. Then the relative contribution of each of the variables is determined $\left({u_{x_j}}^2/{u_Y}^2\right)$ [31].

2.2.1. Bootstrap for estimation of measurement uncertainty

The quantification of uncertainty arising from spectra data is a challenge. These uncertainty components can be combined using MCM from analytical procedure data. However, the sources of uncertainty resulting from intermediate precision, such as analyzes performed on several days performed by different analysts, in addition to the

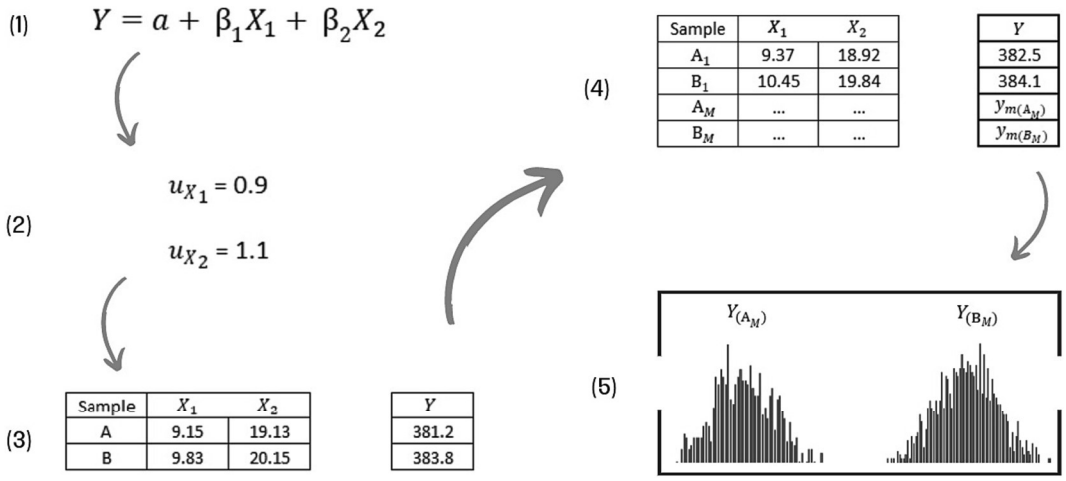

Fig. 2. MCM for estimating the combined measurement uncertainty of two samples, A and B, considering the standard uncertainty of two uncertainty components. (1) Definition of the function of the measurand. (2) Definition and quantification of uncertainty sources,u_{x_1} and u_{x_2}. (3) Experimental data for the two samples (A and B). (4) Sampling of the input values (X1 and X2) in the function of the components considering the initial value and the PDF defined for them resulting in simulated measurand values. (5) In the end, it results in *M* values of the measurand for each sample where information can be extracted from their probability distributions.

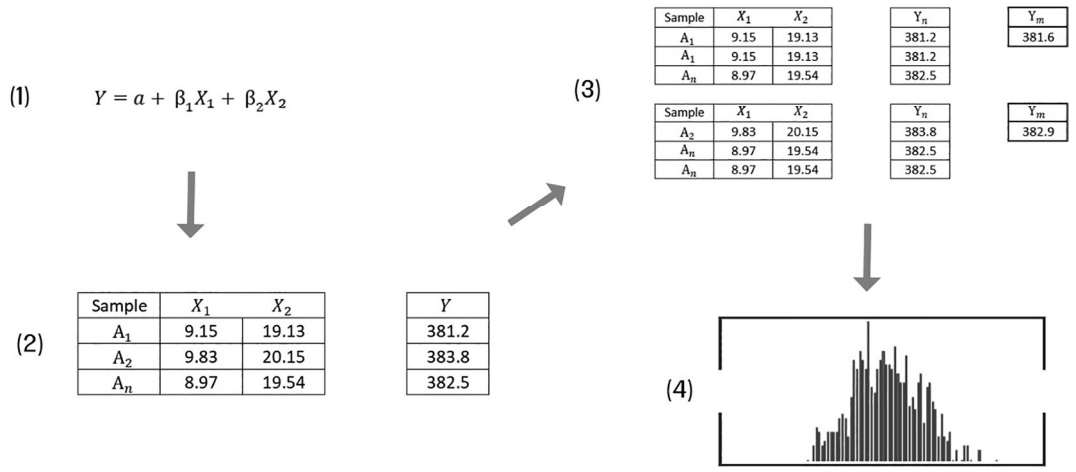

Fig. 3. Bootstrap for estimating the measurement uncertainty of a sample matrix, $A_{[nX2]}$, where n is the number of samples. (1) Definition of the measurand function. (2) The structure of the matrix $A_{[n\times 2]}$. (3) New data sets $A_{[n\times 2]}$ are formed through random resampling with replacement. (4) From the set of values Y_m, it is possible to obtain information about the measurand.

variability of the measurand value due to variations in the performance of sample preparation steps on different days or even its instability in the matrix of the sample, among other sources of uncertainty, are not easily identified and quantified. In such cases, when reliable information about the uncertainty value and the PDF of the variables is not available, the application of MCM is impaired [47–49]. Thus, there may be sources of uncertainty that are not evaluated and not propagated, which impact in the response of the analyzed analyte and, consequently, in the generated data.

One way to solve these problems is to use the bootstrap resampling method. Bootstrap is a computational method that does not consider any assumptions of the PDF. It involves resampling the original dataset to obtain new datasets. The data corresponding to the samples are randomly selected with replacement to compose a new dataset of the same size as the original dataset [49–54]. Due to the randomness of the selection, in a resampling, some samples may not be present while others may be selected two or more times [49,52–54]. The information of interest is obtained at each new resampling (e.g. measurand value), thus generating a large number of values, which allows obtaining information from its probability distribution [49–52,54,55].

The measurement uncertainty can be estimated by bootstrapping in situations where the MCM is difficult to implement. The confidence interval obtained by the bootstrap method can be understood as the interval around the measured value attributed to the measurand with a desired confidence level, that is, the expanded measurement uncertainty [21,52,55]. Fig. 3 exemplifies the application of bootstrap to obtain information about the measurand.

Bootstrap is used in several areas to estimate and propagate uncertainty, for example, in noise analysis due to the intrinsic variability of the environmental noise and the lack of knowledge about the PDF of input variables [48,50,51]; binding constant in spectrophotometric titrations [54]; responses from oscillometric methods of devices used to measure blood pressure where it is not possible to guarantee the repeatability of measurement conditions [52]; hematological analytes without information about the PDFs to obtain more reliable values of the standard uncertainty components obtained through the top-down method following the Nordtest guide [49]; and different quantities by mobile devices in a crowdsensing system, analogous to a distributed measuring instrument [47].

The bootstrap method is useful for quantifying measurement uncertainty when the distribution of the data is unknown. Additionally, it can be used to quantify a source of uncertainty and propagate it through MCM [54].

3. Materials and methods

3.1. Description of the method

A spectrophotometric analytical procedure was used to indicate the stability of a solution containing acetylsalicylic acid (ASA) in a forced degradation study. The procedure is capable of quantifying ASA and its main degradation product, salicylic acid (SA) [12]. The matrix was composed by starch and microcrystalline cellulose.

For the preparation of the ASA stock solution, 50 mg of ASA USP (Synth, China) was weighed on an analytical balance (Shimadzu, Japan) and added to a 50 mL volumetric flask (Brand, USA). Likewise, 10 mg of SA USP (Synth, China) was weighed and added to a 100 mL volumetric flask (Brand, USA) to obtain the SA stock solution. Then, 1 mL of HPLC grade methanol (Honeywell Riedel - from Haen, Germany) was added to each of the flasks to solubilize the respective substances. The volume of the flasks was completed with pH 7.2 buffer solution (prepared using anhydrous monobasic potassium phosphate (Inlab, Brazil) and sodium hydroxide PA (Synth, Shandong, China) in order to obtain a final concentration of 0.05 M and 0.035 M, respectively). The pH of the buffer solution was determined using a PG2000 pHmeter (Gehaka).

Samples and standard solutions were prepared individually in 14 mL tubes. Predetermined aliquots of the ASA and SA solutions were added to the tubes using a 1 mL automatic pipette (BrandTech). Aliquots of SA larger than 1 mL (e.g., 1.1 mL and 1.4 mL) needed to be added in two aliquots with half volumes (e.g., 0.55 mL and 0.7 mL). Then, with the aid of a 10 mL automatic pipette (Kasvi), ultrapure water was added to reach a final volume of 7.5 mL. Finally, the standard solutions and sample solutions were added into a 1 cm quartz cuvette and read on a Genesys 50 UV–Vis scanning spectrophotometer (Thermo Fischer Scientific, USA). The absorbances of standard solutions and sample solutions were determined between 254 nm and 308 nm (with 2 nm resolution).

3.2. Software

MCM simulations, bootstrap resampling, and the quantification of combined uncertainty were performed using Python 3.9.7 with Integrated Development Environment Anaconda. The following packages were used: Numpy version 1.32.4, Pandas version 1.4.3, and the PLS model was generated using the PLS Regression from the Scikit Learn package version 1.1.1. Minitab 18 software (Minitab, LLC) was used to define the PLS model. Microsoft Excel software was used for the top-

Table 1
All concentration levels of ASA and SA from the training set with their combinations used in the calibration curve per experiment day.

Sample	ASA (μg/ml)	SA (μg/ml)	Sample	ASA (μg/ml)	SA (μg/ml)
S1	26.67	0.00	S16	26.67	10.67
S2	40.00	0.00	S17	40.00	10.67
S3	53.33	0.00	S18	53.33	10.67
S4	66.67	0.00	S19	66.67	10.67
S5	80.00	0.00	S20	80.00	10.67
S6	26.67	2.67	S21	26.67	14.67
S7	40.00	2.67	S22	40.00	14.67
S8	53.33	2.67	S23	53.33	14.67
S9	66.67	2.67	S24	66.67	14.67
S10	80.00	2.67	S25	80.00	14.67
S11	26.67	6.67	S26	26.67	18.67
S12	40.00	6.67	S27	40.00	18.67
S13	53.33	6.67	S28	53.33	18.67
S14	66.67	6.67	S29	66.67	18.67
S15	80.00	6.67	S30	80.00	18.67

down uncertainty calculation. The Python program codes and the Excel spreadsheet with the top-down uncertainty calculation are available in the supplementary material.

3.3. Calibration curve and analysis of sample solutions

Standard solutions were prepared as described in section 3.1. The concentration levels of ASA and SA used in the calibration curve are presented in Table 1. The standard solutions shown in Table 1 were prepared in triplicates, under intermediate precision conditions (three different days with the same operator and equipment), totaling 90 standard solutions used for the PLS model development (training set). It was adopted multilevel factorial design, with five levels of ASA and six levels of SA concentrations (totalizing 30 solutions – S1 to S30).

The data were evaluated for the occurrence of outliers, having excluded the values of two standard solutions (P13 and P17). To determine the optimal number of latent variables for the PLS model, leave-one-out and k-fold (k = 10) cross validation method was used, choosing the number of latent variables that resulted in the PLS model with the highest R^2 prediction value.

For the validation of the PLS model, six samples were prepared in duplicate under repeatability conditions, resulting in a total of twelve sample solutions as shown in Table 2. The sample solutions were prepared according to section 3.1. The twelve sample solutions (Table 2) were prepared in triplicate under intermediate precision conditions (on three different days), resulting in a total of 36 sample solutions used for the validation of the PLS model (test set). The sample solutions were prepared using stock solutions different from those used in the standard solutions of the calibration curve.

Table 2
The ASA and SA concentration levels of the test set per experiment day.

Sample	Duplicate	ASA (μm/mL)	SA (μm/mL)
T1	1	26.67	0.00
T1	2	26.67	0.00
T2	1	40.00	18.29
T2	2	40.00	18.29
T3	1	53.33	10.45
T3	2	53.33	10.45
T4	1	66.67	2.61
T4	2	66.67	2.61
T5	1	80.00	6.53
T5	2	80.00	6.53
T6	1	53.33	14.37
T6	2	53.33	14.37

3.4. Bottom-up evaluation of measurement uncertainty using MCM and bootstrap

The evaluation of measurement uncertainty involves four stages: defining the measurand and relating it to the input variables; identifying the sources of uncertainty; quantifying the sources of uncertainty and converting them into standard uncertainties; and calculating the combined and expanded uncertainty.

Eq. (21) and Eq. (22) are used for quantifying ASA and SA, respectively, in the sample solutions and quantitatively relate the input variables to the measurands.

$$\boldsymbol{R}_{ASA} = \left(\frac{\widehat{\boldsymbol{C}}_{ASA}}{\boldsymbol{C}_{ASA}}\right) X \left(\frac{\frac{\boldsymbol{W}_{ASA}}{\boldsymbol{VF}_{50}} X \frac{\boldsymbol{P}_{ASA}}{(\boldsymbol{P}_{SA}+\boldsymbol{P}_{ASA}+\boldsymbol{P}_{wat})}}{\boldsymbol{C}_{ASA}}\right) \tag{21}$$

$$\boldsymbol{R}_{SA} = \left(\frac{\widehat{\boldsymbol{C}}_{SA}}{\boldsymbol{C}_{SA}}\right) X \left(\frac{\frac{\boldsymbol{W}_{SA}}{\boldsymbol{VF}_{100}} X \frac{\boldsymbol{P}_{SA}}{(\boldsymbol{P}_{SA}+\boldsymbol{P}_{ASA}+\boldsymbol{P}_{wat})}}{\boldsymbol{C}_{SA}}\right) \tag{22}$$

Where, $\boldsymbol{R}_{ASA}$ and $\boldsymbol{R}_{SA}$, are the vectors of ASA and SA recoveries, respectively; $\widehat{\boldsymbol{C}}_{ASA}$ and $\widehat{\boldsymbol{C}}_{SA}$ are the vectors of predicted concentrations (μg/mL) of ASA and SA, respectively; $\boldsymbol{C}_{ASA}$ and $\boldsymbol{C}_{SA}$ are the vectors of theoretical concentrations; $\boldsymbol{W}_{ASA}$ and $\boldsymbol{W}_{SA}$ are the vectors of weighed masses (mg) of ASA and SA; $\boldsymbol{VF}_{50}$ and $\boldsymbol{VF}_{100}$ are the vectors of values for 50 mL and 100 mL volumetric flasks, respectively; $\boldsymbol{P}_{ASA}$ and $\boldsymbol{P}_{SA}$ are the vectors of volumes (mL) of ASA and SA stock solutions, respectively; and e $\boldsymbol{P}_{wat}$ is the vector of volumes of ultrapure water sufficient to complete 7.5 mL.

All uncertainty sources related to sample preparation were considered, such as weighing, dilution, and aliquot transfer of solutions. Taking into account that the sample solutions (test set) and the standard solutions (training set) were obtained using the same preparation method, it was possible to evaluate the uncertainty considering its impact on the generation of the PLS model. For this, simulated concentrations of the standard solutions (Table 1) were obtained by MCM, considering the uncertainty related to their preparation, generating a different PLS model at each iteration. It is important to note that the number of 10 components was kept fixed. The terms $\widehat{\boldsymbol{C}}_{ASA}$ and $\widehat{\boldsymbol{C}}_{SA}$ in Eq. (21) and Eq. (22) represent the quantification of the sample solutions, through their spectra, by the PLS model. Thus, the uncertainty of the PLS is propagated to the recoveries of the sample solutions, $\boldsymbol{R}_{ASA}$ and $\boldsymbol{R}_{SA}$.

The Ishikawa diagram is a way to present the relationship between sources of uncertainty and the steps of the analytical procedure. It is important to avoid considering the uncertainty sources more than once, which can lead to overestimation of the measurement uncertainty. Fig. 4 shows the diagrams for the sources of uncertainty in the quantification of ASA and SA.

To implement the MCM it is necessary to define the PDFs for each source of uncertainty. The standard uncertainties of ASA and SA purities were defined with the rectangular distribution, where R(0, u) generates values following a rectangular distribution with mean 0 and width u. For the other standard uncertainties, the normal distribution was defined, given that N(0, u) generates normally distributed values with mean 0 and standard deviation u. The values generated by the distributions in each iteration of the MCM were added to the values of the input variables.

The distributions and uncertainty values that were used to sample each input variable are shown in Table 3. The values were obtained through the source calibration certificates.

Sample preparation should be taken into account when generating simulated values for input variables and their relationships to ascertain which sources of uncertainty affect each sample or group of samples.

Each stock solution was used to obtain the concentrations of standard solutions (Table 1) or sample solutions (Table 2). Therefore, a single value was sampled for the sources of uncertainty related to the purity of

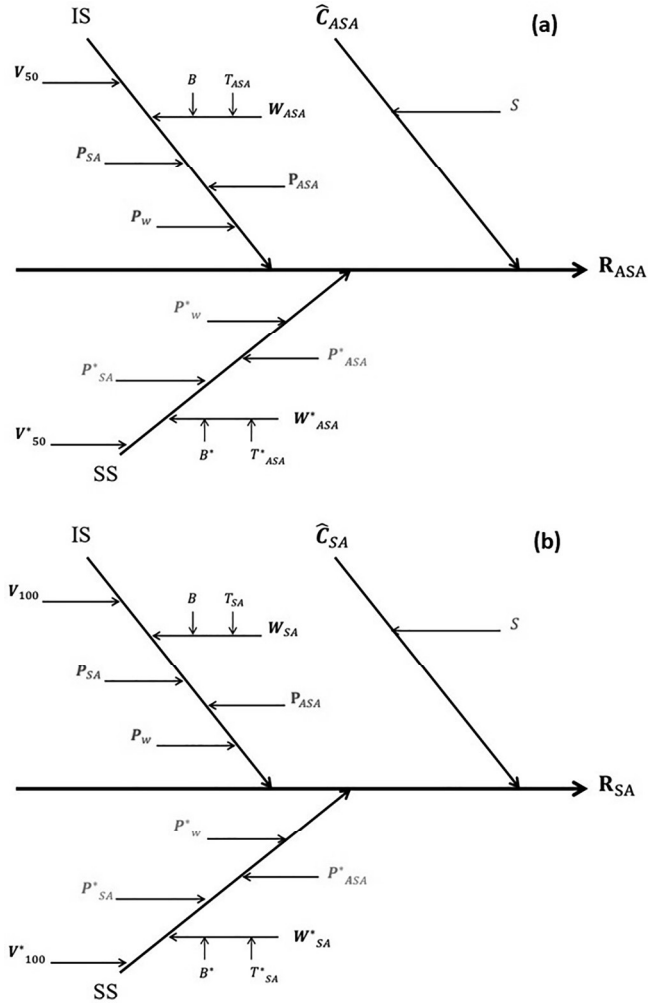

Fig. 4. Ishikawa diagrams indicative of the relationship between the sources of uncertainty and the outcome of the recovery of ASA (a) and SA (b). The sources of uncertainty for the sample solutions in red and the sources of uncertainty for the standard solutions in blue are the same in the two diagrams. Uncertainty sources marked with (*) refer to the standard solution. Where: IS, are the sample solutions; SS, are the standard solutions; $\widehat{C}_{ASA}$ and $\widehat{C}_{SA}$, are the standard uncertainties of the PLS models in the quantification of ASA and SA, respectively; S is the standard uncertainty of the spectrum obtained by bootstrap;$\boldsymbol{R}_{ASA}$ and $\boldsymbol{R}_{SA}$ are the recoveries of ASA and SA; $\boldsymbol{W}_{ASA}$ and $\boldsymbol{W}^*_{ASA}$ are the standard uncertainties of the mass of ASA added in the stock solution considering the standard uncertainty of the balance (B) and the standard uncertainty of the purity of the ASA ($\boldsymbol{T}_{ASA}$); $\boldsymbol{W}_{SA}$ and $\boldsymbol{W}^*_{SA}$ are the standard uncertainties of the SA mass added to the stock solution considering the standard uncertainty of the balance (B) and the standard uncertainty of the SA purity ($\boldsymbol{T}_{SA}$); $\boldsymbol{V}_{50}$ and $\boldsymbol{V}^*_{50}$ are the standard uncertainties of the 50 mL flask used for the ASA stock solution; $\boldsymbol{V}_{100}$ and $\boldsymbol{V}^*_{100}$ are the standard uncertainties of the 100 mL flask used for the SA stock solution and; $\boldsymbol{P}_{ASA}$ and $\boldsymbol{P}^*_{ASA}$ are the standard uncertainties of transfer volumes from the ASA stock solution to the sample solutions; $\boldsymbol{P}_{SA}$ and $\boldsymbol{P}^*_{SA}$ are the standard uncertainties of transfer volumes from the stock solution of SA to the sample solutions; $\boldsymbol{P}_w$ and $\boldsymbol{P}^*_w$ are the standard uncertainties of transfer volumes from ultrapure water to sample solutions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

substances, weighing, and volumetric flasks for each stock solution. In total, three stock solutions were simulated for the sample solutions and three for the standard solutions for each of the analyzed substances. The variables referring to the stock solution are: $\boldsymbol{W}_{ASA}$ and $\boldsymbol{VF}_{50}$ for ASA stock solution; and, $\boldsymbol{W}_{SA}$ and $\boldsymbol{VF}_{100}$ for SA stock solution.

Table 3
Distributions of the input variables considering the standard uncertainty values of the uncertainty sources.

Input variables	Standard uncertainty
$\boldsymbol{W}_{ASA}$	R_{purity} (0, 0.29%); N_{balance} (0, 0.065 mg)
$\boldsymbol{W}_{SA}$	R_{purity} (0, 58%); N_{balance} (0, 0.065 mg)
$\boldsymbol{VF}_{50}$	N_{vf50} (0, 0.065 mL)
$\boldsymbol{VF}_{100}$	N_{vf100} (0, 0.065 mL)
$\boldsymbol{P}_{ASA}$	N_{pASA} (0, 0.49 %)
$\boldsymbol{P}_{SA}$	N_{pSA} (0, 0.49 %)
$\boldsymbol{P}_{wat}$	N_{pwat} (0, 0.49 %)

On the other hand, the volumes of aliquots transferred by pipettes from the stock solutions to the sample solutions are independent. Thus, volumes of each aliquot (stock solutions of ASA and SA, and water) were simulated for each of the samples.$\boldsymbol{P}_{ASA}$, $\boldsymbol{P}_{SA}$ and $\boldsymbol{P}_{wat}$ are variables referring to aliquot transfers. It is worth noting that the simulated values and PDF's of the variables $\boldsymbol{P}_{ASA}$, $\boldsymbol{P}_{SA}$ and $\boldsymbol{P}_{wat}$ are the same in both equations (Eq. (21) and Eq. (22)).

The terms $\widehat{C}_{AAS}$ and $\widehat{C}_{AS}$ refer to the quantification of ASA and SA by the spectra of sample solutions. It was not possible to identify and quantify the uncertainty sources individually and define a PDF for the spectrum that represents all the systematic and random effects. This occurred because the spectra contain sources of uncertainty that are not easily identified and/or quantified. In addition, experiment time and ambient temperature can influence the degradation of the ASA solution [12], so these are also sources of uncertainty that were not evaluated.

To solve these problems, the bootstrap method was used to resample the spectra of the sample solutions considering the concentration levels of ASA and SA. As a result, spectra uncertainties were obtained for each sample solution. In this way, spectrum uncertainty was quantified and propagated; without, however, having carried out an individual assessment of the uncertainty components related to the spectrum [43].

With the values of the uncertainty sources of the sample preparation, the information about their PDF's, in addition to the uncertainty of the spectra obtained by bootstrap, it was possible to propagate them by MCM through Eq. (21) and Eq. (22). Propagation concerned both the sample solutions and the standard solutions. Simulations were performed using 50,000 iterations.

Therefore, in each iteration, standard solutions are simulated using MCM, generating a new PLS model. The model quantifies the spectra of the resampled sample solutions by bootstrapping. These quantifications are divided by their respective theoretical concentrations resulting in recovery values. The concentrations of the sample solutions are simulated by MCM and divided by their theoretical values to generate percentage values, which are corrections to the recoveries, obtained by PLS quantification of the spectra. Thus, 50,000 recovery values of ASA and SA are obtained after a full MCM simulation. With these values the average recoveries for each sample and their combined uncertainties were calculated.

4. Results and discussion

The prediction R^2 values obtained for PLS model were found to be above 0.999 for both ASA and SA. The residuals were homoscedastic and normally distributed. The analytical procedure was linear for the studied concentration ranges of ASA and SA. The LoQ of SA, obtained by the standard deviation of the quantification of 15 replicas of "blank" solutions were calculated according to ICH Q2(R2), was 2.17 μg/mL [15]. More detailed information about the PLS model has been made available in the supplementary material.

To evaluate the uncertainties obtained by the top-down and bottom-up approaches, the behavior of the absolute uncertainties and relative uncertainties in relation to the concentration level was analyzed (Fig. 5). This analysis aimed to assess the need to express the combined uncertainty as an absolute or relative value, and if necessary, to divide the

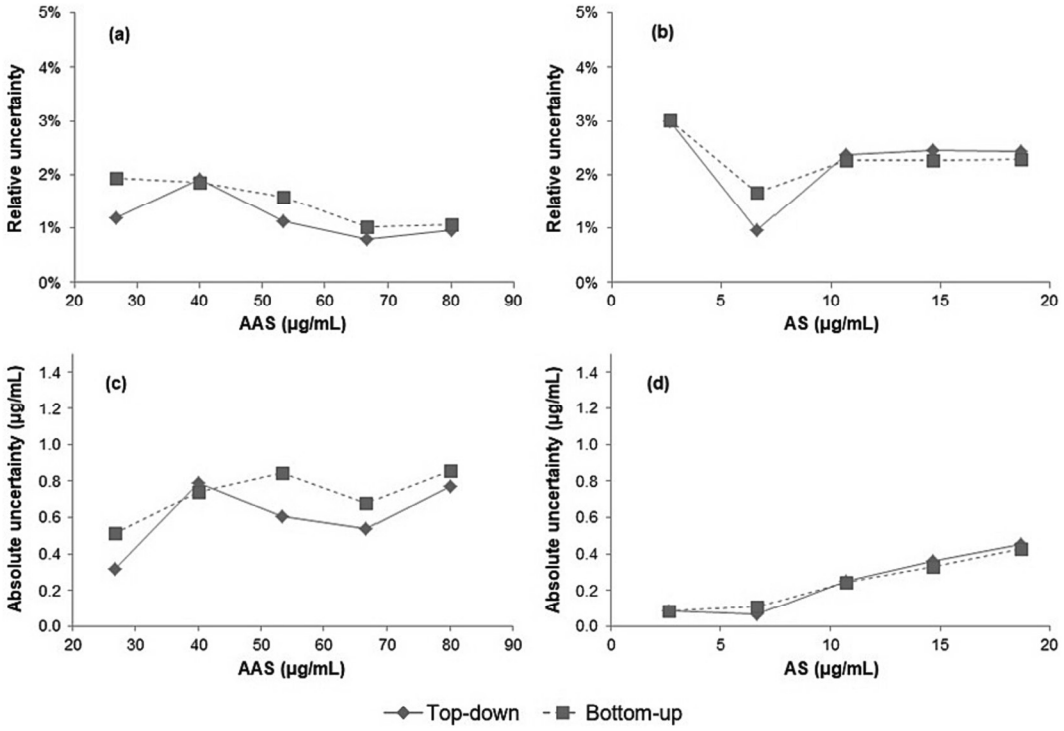

Fig. 5. Precision uncertainties (top-down) and combined uncertainties (bottom-up) relative (a) and absolute (c) of ASA with their respective concentration levels. Precision uncertainties (top-down) and combined uncertainties (bottom-up) relative (b) and absolute (d) of SA with their respective concentration levels.

concentration range into two ranges and calculate combined uncertainties for each range. For this, the top-down approach considered the absolute or relative uncertainties of the precision components associated with each concentration level, while by the bottom-up approach the combined absolute and relative uncertainties for each concentration level were considered.

Fig. 5 shows the practically constant behavior of the precision and combined relative uncertainties of the ASA (Fig. 5) considering all the studied concentrations. Based on this behavior, a single relative precision and relative combined uncertainty was calculated for the bottom-up and top-down approaches, respectively, considering all ASA concentration levels.

For SA, there was a steep decline in the relative precision uncertainties and combined uncertainties at concentrations of 2.67 and 6.67 µg/mL, followed by a constant behavior at concentrations starting from 10.67 µg/mL (Fig. 5b). Considering the practically constant behavior of the absolute uncertainties between 2.67 and 6.67 µg/mL and of the relative uncertainties from the concentration of 6.67 µg/mL, the SA concentration levels were divided into two ranges. Sources of the uncertainty were estimated and combined with absolute values considering the concentration range from 2.67 to 6.67 µg/mL. While for concentration range from 6.67 µg/mL to 18.67 µg/mL, sources of the uncertainty were estimated and combined with relative values. The calculation of the uncertainties of the two concentration ranges considered only the data from their respective samples.

It is worth noting that although the precision and combined uncertainties used in the graphs and calculated respectively by the bottom-up and top-down approaches have different values and calculations, they showed similar behavior.

With the defined concentration ranges for SA, their combined absolute and relative uncertainties were calculated using the top-down approach and the bottom-up approach using the MCM, considering the data obtained from the respective concentration ranges. While for ASA it was possible to calculate a single combined relative uncertainty that was obtained with all the validation data. Two different methodologies were considered to calculate the trueness uncertainty through the top-down approach (VAM Project and Nordtest). For the calculation of the combined uncertainty by the bottom-up approach using the MCM, the number of iterations required for stochastic convergence of uncertainty values was evaluated based on the maximum variation of 1% between multiple simulations. For this, 10 simulations with 50,000 iterations were performed and the maximum variation of combined uncertainty was calculated for each ASA and SA sample. The combined uncertainties for ASA and SA samples varied by less than 1%, and therefore, the number of 50,000 iterations are adequate for uncertainty evaluation. Then, the combined uncertainties of ASA and SA for difference concentration level were obtained.

Table 4 presents the combined uncertainties of ASA and SA using the bottom-up and top-down approaches.

When evaluating the ASA recoveries by Eq. (12), the recovery at a concentration of 40 µg/mL is statistically different from 1 (100%). However, it was considered that the trueness uncertainty of this concentration level contributes 36% (VAM Project) and 40% (Nordtest) to the combined uncertainty. When applying the correction to the recovery at this concentration, the combined uncertainties calculated by both methodologies decreased to approximately 2.2%. Thus, the correction

Table 4
Combined uncertainties for different concentration levels of ASA and SA calculated by MCM and by the two top-down methodologies.

Analyte	Concentration (µg/mL)	u_c(MCM)	u_c (Nordtest)	u_c (VAM)
ASA	26.67	2.3 %	2.0 %	2.0 %
	40.00	2.2 %	2.5 %	2.5 %
	53.33	1.8 %	1.9 %	1.6 %
	66.67	1.3 %	1.8 %	1.4 %
	80.00	1.3 %	1.9 %	1.5 %
SA	2.67	0.098 µg/mL	0.18 µg/mL	0.18 µg/mL
	6.67	0.15 µg/mL	0.18 µg/mL	0.17 µg/mL
	10.67	2.6 %	2.6 %	3.1 %
	14.67	2.6 %	2.7 %	3.0 %
	18.67	2.7 %	2.6 %	2.9 %

did not have a significant impact on the combined uncertainty considering all concentration levels, and therefore, the recoveries at this concentration were not corrected.

AS recoveries are not statistically different from 1 (100%). Considering that Eq. (12) uses recovery uncertainty to evaluate recovery, the recovery uncertainty of the 2.67 μg/mL concentration level was high enough that the recovery of 106.1% was considered equal to 100%.

With the uncertainty component values with absolute values of the concentrations of 2.67 μg/mL and 6.67 μg/mL of the SA, it was possible to confirm that the definition of the concentration ranges was correct. The uncertainty sources not affect by concentration level have a greater impact on the combined uncertainty at low concentrations; and thus, the absolute uncertainty is constant in this concentration range [16,20]. This is reflected in the practically equal values of the combined uncertainties of the concentration levels of 2.67 μg/mL and 6.67 μg/mL obtained by the two methodologies of the top-down approach (Table 4).

The combined uncertainty values obtained for the concentrations of 40.00, 66.67 and 80.00 μg/mL of ASA by MCM were 12%, 13% and 13% lower than those values obtained by the VAM Project methodology, respectively, and 14%, 29%, and 28% lower than those values obtained using the Nordtest, respectively (Table 4). While the combined uncertainty values for the concentrations 26.67 and 53.33 μg/mL obtained using MCM were 15% and 13% bigger than those values obtained using the VAM Project methodology, and 12% bigger and 5% lower than those values obtained using the Nordtest, respectively (Table 4).

The combined uncertainty values of SA for the concentrations of 10.67, 14.67, and 18.67 μg/mL obtained by MCM were 13%, 15%, and 9% smaller than those values obtained by the VAM Project methodology, respectively, and 0.1% and 3% lower, and 3% bigger than those values obtained by the Nordtest, respectively (Table 4). While the combined uncertainty values of the concentrations 2.67 and 6.67 μg/mL obtained by MCM were 45% and 9% smaller than those values obtained by VAM Project, respectively, and 47% and 15% smaller than those values obtained by Nordtest, respectively (Table 4).

Statistical comparisons indicate that there are not difference among the uncertainty values obtained using MCM, Nordtest and VAM Project approaches for both ASA (p-valor = 0.331) and SA (p-valor = 0.670).

It is worth to mention that the relative uncertainty values obtained for ASA are approximately constant in the range from 26.67 to 80.00 μg/mL. In the case of SA, the absolute uncertainty values are approximately constant below 6.67 μg/mL, while the relative uncertainty values are approximately constant in the range from 6.67 to 18.67 μg/mL. Thus, the uncertainty evaluation for may be simplified and divided into one (from 26.67 to 80.00 μg/mL for ASA) and two concentration ranges (below 6.67 μg/mL and from 6.67 to 18.67 μg/mL for SA), as presented in Table 5. Table 5 presents the combined uncertainty values of the analytical procedure obtained by MCM and by the two top-down methodologies. In addition, the expanded uncertainty values are presented, considering $k = 2$ for an approximately 95% confidence level.

The combined uncertainties values for ASA and SA obtained by VAM Project methodology were similar those values obtained by Nordtest methodology (Table 5). The contribution of trueness uncertainty to combined uncertainty for the concentration range of 2.67 μg/mL to 6.67 μg/mL of SA was 82% and 83% for VAM and Nordtest, respectively. For the range of 6.67 μg/mL to 18.67 μg/mL the main uncertainty source was the precision uncertainty contributing with 65% (VAM) and 85% (Nordtest).

For ASA, there was a difference in the contributions of precision and trueness uncertainties to the combined uncertainty. Uncertainty contributions of precision and trueness were 47% and 53%, respectively, for VAM Project methodology; while it was 39% and 61%, respectively, for Nordtest methodology.

The combined uncertainty value for ASA obtained by MCM was 2% and 11% lower than the values obtained by the VAM Project and Nordtest methodologies, respectively.

The value of the combined uncertainty for SA in the concentration range of 2.67 to 6.67 μg/mL obtained by the MCM was 28% and 30% lower than the values obtained by the VAM Project and Nordtest methodologies, respectively. The combined uncertainty value of SA in the concentration range of 10.67 to 18.67 μg/mL obtained by MCM was 13% lower than the values obtained by the VAM Project and practically equal to those obtained by Nordtest methodologies, respectively.

Differences in the combined uncertainty values between top-down and bottom-up approaches are not atypical. This occurs due to the bottom-up approach considering each uncertainty source individually and preventing sources from being considered more than once in the uncertainty calculation [29]. On the other hand, the pragmatic combination of precision and trueness uncertainties by the top-down approach can consider repeated sources of uncertainty, leading to overestimation of the combined uncertainty [29,44,46]. This statement impacts on all combined uncertainties obtained by the VAM Project and Nordtest methodologies and becomes more evident when evaluating the impact of the trueness uncertainty components on the combined uncertainty of SA for the concentration range from 2.67 to 6.67 μg/mL. The absolute value of $u_{\bar{r}}$ for this concentration range (Eq. (13)), obtained by the VAM Project methodology (0.18 μg/mL), considers the standard deviation of the concentrations predicted by the PLS model (Eq. (10)). While for the Nordtest methodology, u_t had an absolute value of 0.18 μg/mL, which considers the root mean square of the mean biases for this concentration range (Eq. (5)). In both cases, random and systematic effects can overestimate the uncertainty assessments of trueness and precision, respectively. This fact does not occur in bottom-up evaluation. Thus, higher uncertainty values are expected for the top-down approach compared to the bottom-up approach.

The variability of 20 to 30% of the uncertainty value is expected, due to the degrees of freedom of the standard deviation of the random errors obtained with few experimental results [21,45]. Considering this, only the combined uncertainty value of SA for a concentration of 2.67 μg/mL obtained by the MCM had a difference greater than 30% when compared to the values obtained by the two top-down methodologies. It should be noted that this concentration is close to the LoQ of SA (2.17 μg/mL).

One advantage of uncertainty evaluation using bottom-up approach is the possibility of performing a sensitivity analysis, which allows one obtaining the percentage contribution of each source of uncertainty to the combined uncertainty. The contributions of the uncertainty sources of sample preparation and spectrum for the combined uncertainty of ASA and SA are presented in Table 6.

The uncertainty related to the spectra data was the source that most contributed to the final uncertainty of the ASA and SA. Bootstrap of the spectra of the sample solutions was responsible for quantifying, in a combined way, systematic and random components that were not accounted for by MCM.

The contribution of the standard uncertainty of the 10 mL pipette is related to the aliquot volume of the ASA and SA stock solutions (Eq. (21)

Table 5
Combined and expanded uncertainties of ASA and SA estimated by MCM and by the two methodologies of the top-down approach.

Analyte	Range (μg/mL)	MCM		Nordtest		VAM Project	
		u_c	U	u_c	U	u_c	U
ASA	26.67 – 80	1.8 %	3.7 %	2.0%	4.1 %	1.9 %	3.7 %
SA	2.67—6.67	0.13 μg/mL	0.25 μg/mL	0.18 μg/mL	0.36 μg/mL	0.18 μg/mL	0.35 μg/mL
	6.67 – 18.67	2.6 %	5.3 %	2.6 %	5.3 %	3.0 %	6.0 %

Table 6
Sensitivity analysis of ASA and SA uncertainty sources.

Analyte	Range (μg/mL)	Balance	Volumetric flask	Purity	Pippete 10 mL	Pippete 1 mL	Spectra
ASA	26.67 – 80	1.7 %	1.7 %	14.4 %	17.5 %	8.2 %	56.7 %
SA	2.67—6.67	16.5 %	0.2 %	13.4 %	11.4 %	13.2 %	45.4 %
	6.67 – 18.67	18.4 %	0.2 %	3.6 %	2.7 %	14.4 %	60.7 %

and Eq. (22), respectively) added into the samples using the 1 mL pipette. This is because the larger the volume of the aliquots of ASA and SA added to the samples, the smaller the volume of ultrapure water needed to reach the total sample volume of 7.5 mL. The largest volumes of aliquots transferred by the 1 mL pipette were for the concentration levels of 10.67, 14.67, and 18.67 μg/mL of SA (0.8 mL, 1.1 mL, and 1.4 mL, respectively, with the last two aliquots transferred in smaller aliquots of 0.55 mL and 0.7 mL). While the aliquots transferred in the concentration range of 2.67 and 6.67 μg/mL of SA (0.2 mL and 0.5 mL, respectively) and all concentration levels of ASA (0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL, and 0.6 mL) were similar volumes, as well as the contribution of the standard uncertainty of the 10 mL pipette (11.4% and 17.5%, respectively).

The contribution of the balance standard uncertainty was greater in SA than in ASA, because the amount of weighed mass of SA (10 mg) used in the stock solutions was 20% of the amount of weighed mass of ASA (50 mg). Considering that the balance is a source of uncertainty independent of the analyte concentration, its impact is greater when smaller mass quantities are weighed.

Like the balance, the volumetric flask is also a source of uncertainty independent of the analyte concentration. Therefore, its contribution to the combined uncertainty of SA was identical in both concentration ranges, as both use a 100 mL volumetric flask. On the other hand, it had a greater contribution to the combined uncertainty of ASA, since a 50 mL volumetric flask was used.

The purity uncertainty is proportional to the concentration, which is why there was a difference in its contribution in the two concentration ranges of SA. In the higher concentration ranges, there was a lower impact of purity uncertainty due to the higher overall concentration of the analyte.

Based on the sensitivity analysis, it was evaluated which modifications can be made in the sources of uncertainty to reduce the combined uncertainty. It is possible to decrease the uncertainty of the analytical procedure by proportionally increasing the masses of ASA and SA in the stock solutions in order to reduce the contribution of the standard uncertainty of the balance in SA.

To reduce the contribution of the standard uncertainty of the 10 mL pipette, a volumetric flask that would be completed with water can be used instead of inserting the aliquot of water with the 10 mL pipette to complete the sample volume (7.5 mL).

Although the uncertainty arising from spectra data contributes approximately 1/2 to 2/3 of the combined uncertainty, it is not possible to suggest changes that reduce this uncertainty. For this, detailed studies are required to identify and quantify the sources of uncertainty that directly impact the spectra data and other random and systematic effects quantified in the bootstrap step.

5. Conclusion

Measurement uncertainties of ASA and SA in ASA samples subjected to forced degradation study were calculated. ASA and SA were quantified by a stability-indicating multivariate spectrophotometric procedure. The analytical procedure has particularities such as the overlapping of ASA and SA wavelengths, which was modeled by PLS, and the measurands were analyzed over a wide concentration range.

Measurement uncertainties were estimated using the top-down approach based on VAM Project and Nordtest/ISO 11352 methodologies, as well the bottom-up approach using MCM. Data from the sample solutions used in method validation were used to calculate the combined uncertainties of ASA and SA by MCM, and the method validation parameters (precision and trueness) were used to calculate the uncertainty using both top-down methodologies.

To quantify the measurement uncertainties of ASA and SA by MCM, the standard uncertainties of uncertainty sources related to the preparation of sample solutions and standard solutions used to fit the PLS model were considered. Thus, the estimated combined uncertainties also considered the quantification uncertainty of the sample solutions' spectra by the PLS model. In addition, bootstrapping the sample solutions from the spectra inserted systematic and random components that were not identified and accounted by the MCM.

Due to SA being quantified from concentrations close to the LoQ, the behavior of the measurement uncertainty varied according to the SA concentration level. Therefore, it was necessary to calculate an uncertainty expressed in absolute value for concentrations lower than approximately 3LoQ and a relative measurement uncertainty for concentrations higher than approximately 3LoQ using both the top-down approach methodologies and MCM.

The estimated expanded uncertainties obtained by the top-down approach were greater than the expanded uncertainties estimated using MCM. When considering the uncertainty evaluation by the bottom-up approach, the sensitivity analysis allowed concluding that the uncertainty associated to the spectra constituted the most relevant source of uncertainty (contributing with about 1/2 to 2/3 of the total uncertainty). Thus, the procedure can be simplified considering only the uncertainty source of the spectrum (in this work, evaluated by bootstrap), however the differences between the uncertainty values obtained by the top-down and bottom-up approaches can be even greater.

Thus, a methodology for uncertainty evaluation using MCM for multivariate spectrophotometric analytical procedures was developed, where wavelength overlap is resolved using PLS. In order to facilitate the application of the methodology developed in the paper to other spectrophotometric procedures, the Python codes used to propagate standard uncertainties and estimate combined uncertainties using MCM were made available.

CRediT authorship contribution statement

Aldo Renato Couto: Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Felipe Rebello Lourenço:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Felipe Rebello Lourenco reports financial support was provided by State of Sao Paulo Research Foundation.

Data availability

Data are avaliable as supplementary data

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2023.109194.

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CONCLUSÕES

3. CONCLUSÕES

No capitulo I deste trabalho foi possível avaliar a importância da incerteza de medição nos estudos de estabilidade ao permitir o cálculo do risco particular na avaliação de conformidade dos parâmetros estudados e do risco total do consumidor do prazo de validade definido. Também foi demonstrado que a avaliação multiparâmetro permitiu calcular o risco total do consumidor do prazo de validade definido, considerando todos os parâmetros simultaneamente, enquanto que a avaliação uniparâmetro, vigente na diretriz ICH Q1E "Evaluation for stability data", calcula somente o risco particular de cada parâmetro.

Além disso, foi desenvolvida e disponibilizada uma planilha em Excel para a definição do prazo de validade considerando um risco total máximo admissível. Os riscos particulares do AAS e AS, e o risco total do consumidor foram obtidos por MCM. A planilha suporta até quatro parâmetros avaliados em um estudo de estabilidade, considerando até três lotes.

Através da planilha, foi possível avaliar o impacto de diferentes cenários no risco total do consumidor e, consequentemente, na definição do prazo de validade, tais como: a abordagem multiparâmetro, a abordagem do ICH Q1E, o modelo de regressão utilizado (simples, parcial ou completo), o impacto da incerteza de medição, o efeito da correlação experimental e o efeito dos limites de especificação. Desta forma, foi adquirido um conhecimento mais amplo do impacto da incerteza de medição nos estudos de estabilidade e a importância da avaliação multiparâmetro na definição do prazo de validade.

No capítulo II foi desenvolvida uma metodologia para a estimação da incerteza de medição pela abordagem *bottom-up*, utilizando MCM, da quantificação do AAS e AS por um procedimento espectrofotométrico multivariado. O cálculo da incerteza por MCM considerou as fontes de incerteza da preparação das soluções padrão e amostra, a incerteza do modelo PLS devido o impacto da incerteza padrão da solução padrão na curva de calibração e a incerteza dos espectros das amostras obtida por *bootstrap*. Para fins de comparação da incerteza de medição obtida pela abordagem *bottom-up*, também foram calculadas as incertezas de medição do AAS e AS pela abordagem *top-down*, utilizando os guias da Nordtest/ ISO 11352 e VAM Project.

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ANEXOS

1) Artigos publicados:

a) L. Separovic, R.S. Simabukuro, A.R. Couto, M.L.G. Bertanha, F.R.S. Dias, A.Y. Sano, A.M. Caffaro, F.R. Lourenço. Measurement uncertainty and conformity assessment applied to drug and medicine analyses – A review. Crit. Rev. Anal. Chem. 1–16 (2021). Link: https://doi.org/10.1080/10408347.2021.1940086

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c) A.R. Couto, P.B. Silva Passarin, F.R. Lourenço, Risk of false conformity decisions in LAL gel-clot test due to false-negative results, Chemom. Intell. Lab. Syst. 234 (2023) 104760. https://doi.org/10.1016/J.CHEMOLAB.2023.104760

d) A. Renato Couto, F. Rebello Lourenço, Bottom-up and top-down measurement uncertainty evaluation for multivariate spectrophotometric procedures, Microchem. J. 193 (2023) 109194. https://doi.org/10.1016/J.MICROC.2023.109194