Universidade de Aveiro Departamento de Química
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Mário Manuel Quialheiro Simões, Professor Auxiliar do Departamento de Química da Universidade de Aveiro e do Dr. Pedro Caetano Maia Serôdio, Químico Analítico Sénior do Departamento de Investigação e Desenvolvimento (I&D) – Grupo de Desenvolvimento de Química Analítica da Hovione FarmaCiência SA.

“It is in your moments of decision that your destiny is shaped.”

Anthony Robbins

o júri

presidente

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Palavras-chave

Solventes residuais; Amostras farmacêuticas; Cromatografia Gasosa com amostragem estática por *headspace*; Qualidade Analítica por Design; Desenvolvimento analítico.

Resumo

A presença de solventes residuais em produtos farmacêuticos representa uma das principais preocupações da indústria farmacêutica, devido ao seu impacto nas propriedades dos produtos e na saúde dos pacientes. Por estes motivos, torna-se necessário desenvolver estratégias analíticas que suportem decisões em cada fase do desenvolvimento farmacêutico e simultaneamente maximizem a eficiência e produtividade do laboratório de controlo de qualidade farmacêutico.

Nesta tese foi desenvolvido um método geral de Cromatografia Gasosa com amostragem por *Headspace* estático aplicando a abordagem Qualidade Analítica por Design (AQbD), para a determinação de solventes residuais na Hovione FarmaCiencia SA. O fluxo da coluna, o programa de temperatura do forno, o *split ratio*, o *split at vent*, a pressão do vial e a temperatura e o tempo de equilíbrio do *headspace* foram identificados como parâmetros críticos do método. De forma a otimizar a seletividade, a sensibilidade e o tempo de análise, estes parâmetros foram estudados através de planos de desenho experimental e de análises estatísticas. A partir do espaço de *design* obtido, foram selecionadas as condições operatórias finais do método. A robustez do método foi verificada e confirmada. Os resultados de validação demonstraram especificidade, seletividade, sensibilidade, linearidade, exatidão e precisão, pelo que o método desenvolvido é adequado à determinação de 29 solventes residuais diferentes em amostras farmacêuticas. A aplicação da abordagem AQbD permitiu adquirir o máximo conhecimento acerca do método e minimizar o impacto das fontes de variabilidade na sua performance.

Keywords

Residual solvents; Pharmaceutical samples; Static Headspace Gas Chromatography; Analytical Quality by Design; Analytical development.

Abstract

The presence of residual solvents in pharmaceuticals represents one of the main concerns of the pharmaceutical industry due to its impact on both products' properties and patients' health. For these reasons, it is necessary to develop analytical strategies that support the decision making process in each stage of the pharmaceutical development and simultaneously maximize the efficiency and productivity of the laboratory responsible for pharmaceutical quality control.

In this thesis, a general Static Headspace-Gas Chromatography method was developed applying the Analytical Quality by Design (AQbD) approach for determination of residual solvents in Hovione FarmaCiencia SA. The column flow, oven temperature program, split ratio, split at vent, vial pressure and headspace equilibration temperature and time were identified as critical method parameters. In order to optimize the selectivity, sensibility and run time, these parameters were studied through experimental design plans and statistical analysis. From the obtained design space, the final operating conditions of the method were selected. The robustness of the method was verified and confirmed. The validation results demonstrated specificity, selectivity, sensitivity, linearity, accuracy and precision, so the developed method is suitable for the determination of 29 different residual solvents in pharmaceutical samples. The application of the AQbD approach allowed to acquire the maximum understanding about the method and to minimize the impact of the sources of variability in its performance.

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Abbreviations

AQbD – Analytical Quality by Design

ATP – Analytical Target Profile

BA – Benzyl alcohol

C – Controlled variable

CMA – Critical Method Attribute

CMP – Critical Method Parameter

DIPEA – *N*,*N*-diisopropylethylamine

DMA – *N*,*N*-dimethylacetamide

DMF – *N*,*N*-dimethylformamide

DMI – 1,3-dimethylimidazolidin-2-one

DMSO – Dimethyl sulfoxide

DoE – Design of experiments

FID – Flame Ionization Detector

FT-IR – Fourier Transform Infrared Spectroscopy

GC – Gas chromatography

HS-GC – Static Headspace – Gas Chromatography

HS-GC-FID – Static Headspace – Gas Chromatography with Flame Ionization Detector

ICH – International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use

LOD – Limit of detection

LOQ – Limit of quantitation

MODR – Method Operable Design Region

N – Noise variable

NMP – 1-methylpyrrolidin-2-one

NMR – Nuclear Magnetic Resonance

NOC – Normal Operable Conditions

OFAT – One-factor-at-a-time

QbD – Quality by Design

RSD – Relative standard deviation

S/N – Signal-to-noise ratio

SPME – Solid-phase microextraction

USP – United States Pharmacopoeia

VICH – International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products

X – Experimental variable

Chapter 1. Introduction

The presence of residual solvents in pharmaceuticals, resulting from the use of solvents in certain steps of the manufacturing process, is one of the main concerns of the quality laboratories in the context of the pharmaceutical industry. Issues such as the potential undesired alteration of physicochemical properties of the drug substances and drug products and, above all, the toxic effects on human and animal health are associated with the presence of these volatile organic impurities in pharmaceuticals.

Although each pharmaceutical company presents its own strategies in the development and production of pharmaceuticals,[1] the use of solvents is universal and widely applied in this industrial sector.[2,3] These chemical substances may act as reaction medium, forming a solution in which the reactants of interest are dissolved; may participate in chemical reactions as reagents, providing molecules to synthesize new compounds; and can be used in extraction, crystallization and filtration steps. Due to their ability to perform these various operations, solvents are often applied in the pharmaceutical manufacturing of active drug substances, excipients and drug products[3,4] and in the cleaning and sterilization of process equipment.[5] However, their use may potentially influence the purity, stability, safety and therapeutic efficacy of the pharmaceuticals.

Ideally, final pharmaceutical products completely devoid of solvents should be obtained but the complete removal of the solvents used during the manufacturing process is, in practical terms, a difficult task to perform.[4] Additionally, it should be noted that the solvents applied in the pharmaceutical activities are not totally pure, containing related impurities.[3] These impurities may arise from the production process of solvents, their storage and/or transport, the stabilizers used to maintain their properties, the denaturing agents added, or from the chemical transformation of the solvents during the manufacturing of pharmaceuticals, resulting in the formation of undesirable side products.[3] Consequently, the final products are expected to contain remaining amounts of solvents and other organic impurities that may be associated with them. It is then imperative to increase and improve the knowledge about the composition of the obtained products, in particular their content in volatile impurities.

The interest of the scientific community regarding the presence of residual solvents in pharmaceuticals, and its potential impact on their quality, has been demonstrated in numerous research papers about this topic and in the attention dedicated by international regulatory entities in the pharmaceutical sector. On 17 July 1997, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) established the official limit values for specific residual solvents in pharmaceuticals in

Q3C Guideline. This guideline has been adopted by the European Pharmacopoeia, the United States Pharmacopoeia (USP) and the Japanese Pharmacopoeia,[6] to harmonize the international regulatory information on pharmaceutical quality based requirements. The acceptable concentration level for each solvent has been defined on the basis of available toxicological data and the solvents were grouped into three classes according to their level of toxicity, as shown in Table 1. Class 1 refers to the most toxic solvents, whose use in the production of pharmaceuticals should be avoided due to their carcinogenic and genotoxic effects and their deleterious effects on the environment.[6] Class 2 includes solvents with significant toxic effects, including non-genotoxic carcinogenicity, neurotoxicity and teratogenicity, and their use in the pharmaceutical industry should be limited.[6] Finally, class 3 corresponds to the solvents that represent a lower risk to human health, since they have a reduced toxic potential.[6] During the pharmaceutical manufacturing, preference should be given to the use of solvents belonging to this class. Similarly, to what happened to the pharmaceuticals for human use, in April 1996 an international entity was created to regulate the veterinary products. Adapting the ICH Q3C guideline, the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) established a guideline to recommend the acceptable amounts for residual solvents in veterinary pharmaceutical products.[7] The solvents included in this guideline and their limits are the same as those mentioned in the ICH Q3C Guideline (Table 1).

To ensure the maintenance of the quality standards intended for pharmaceuticals, analytical chemistry assumes the great responsibility to provide analytical tools capable of correctly detecting, identifying and quantifying the residual solvents present in drug substances and drug products.

A number of different methods using a variety of analytical techniques have been proposed in the United States[8] and European[9] Pharmacopoeias for the control of residual solvents in pharmaceutical samples. These methods are mainly based on Static Headspace – Gas Chromatography (HS-GC). Although, the HS-GC methods recommended by the pharmacopoeias present different procedures, which makes it difficult to use them routinely, and they are not directly applicable to new pharmaceutical samples, which implies the re-development of the method and its validation. In addition, these methods are time-consuming, since they use a long vial equilibration time (45-60 min) and present a run time higher than 60 min. As an alternative, HS-GC methods that are specific for each pharmaceutical product and for the residual solvents to be analyzed in that sample have been reported in the literature[10–23]. However, this approach implies that a significant number of different HS-GC methods need to

be developed, validated and managed on a daily basis in a quality control laboratory in the pharmaceutical industry, which compromises its efficiency and productivity. In order to avoid these problems, the implementation of a general HS-GC method is suggested. Several authors[24–29] have successfully developed and validated general HS-GC methods for determination of residual solvents in pharmaceuticals. The use of the same HS-GC operating conditions for a wide variety of residual solvents, the simultaneous determination of residual solvents from a single injection of a sample solution and the application of the method for the analysis of different pharmaceutical samples are some of the advantages associated to the development of a general HS-GC method.

Table 1. Solvent classes and their acceptable concentration limits in pharmaceutical products, according to the ICH Q3C and VICH 18(R) Guidelines.[6,7]

Class 1		Class 2		Class 3	
Solvent	**Concentration limit (ppm)**	**Solvent**	**Concentration limit (ppm)**	**Solvent**	**Concentration limit (ppm)**
Benzene	2	Acetonitrile	410	Acetic acid	5000
Tetrachloromethane	4	Chlorobenzene	360	Propan-2-one	5000
1,2-dichloroethane	5	Chloroform	60	Anisole	5000
1,1-dichloroethene	8	Cumene	70	Butan-1-ol	5000
1,1,1-trichloroethane	1500	Cyclohexane	3880	Butan-2-ol	5000
		1,2-dichloroethene	1870	Butyl acetate	5000
		Dichloromethane	600	2-methoxy-2-methylpropane	5000
		1,2-dimethoxyethane	100	Methylsulfinylmethane	5000
		N,*N*-dimethylacetamide	1090	Ethanol	5000
		N,*N*-dimethylformamide	880	Ethyl acetate	5000
		1,4-dioxane	380	Ethoxyethane	5000
		2-ethoxyethanol	160	Ethyl formate	5000
		Ethane-1,2-diol	620	Formic acid	5000
		Formamide	220	Heptane	5000
		Hexane	290	2-methylpropyl acetate	5000
		Methanol	3000	Propan-2-yl acetate	5000
		2-methoxyethanol	50	Methyl acetate	5000
		Hexan-2-one	50	3-methylbutan-1-ol	5000
		Methylcyclohexane	1180	Butan-2-one	5000
		4-methylpentan-2-one	4500	2-methylpropan-1-ol	5000
		1-methylpyrrolidin-2-one	530	Pentane	5000
		Nitromethane	50	Pentan-1-ol	5000
		Pyridine	200	Propan-1-ol	5000
		Sulfolane	160	Propan-2-ol	5000
		Tetrahydrofuran	720	Propyl acetate	5000
		Tetralin	100	*N*,*N*-diethylethanamine	5000
		Toluene	890		
		1,1,2-trichloroethene	80		
		Xylene (1,3-xylene; 1,4-xylene; 1,2-xylene)	2170		

Another problem related to analytical strategies in the context of the pharmaceutical industry is the difficulty in guaranteeing the reliability and robustness of the methods developed through the traditional approach. This method development approach provides limited methodology understanding, limited variability control and limited flexibility to change throughout analytical and pharmaceutical process lifecycle.[30–33] These limitations lead to a higher probability to generate analytical data that are not meaningful, which results in additional and time-consuming investigations and material resources and money expenditure. In order to overcome these issues, the pharmaceutical industry has been exploring the application of Quality by Design (QbD) principles in the development of the analytical methods, an approach called Analytical Quality by Design (AQbD).

Considering all of the above, the objective of the present work is the development of a general Static Headspace – Gas Chromatography with Flame Ionization Detector (HS-GC-FID) method applying the AQbD approach for determination of residual solvents and related volatile organic impurities in the pharmaceutical industry.

1.1. Analytical determination of residual solvents in the pharmaceutical industry

The pharmaceutical industry aims to obtain pure, stable, safe and therapeutically effective pharmaceutical products, with the goal of satisfying the therapeutic needs of patients and thus improving their quality of life and their well-being. To achieve the quality standards required by the regulatory authorities, the pharmaceutical development and production processes are subject to rigorous quality control. As purity is one of the basic parameters defining the quality of pharmaceuticals,[34] the content of residual solvents should be monitored to ensure that both drug substances and drug products meet the established specifications.

The quantitation of residual solvents in pharmaceutical samples is an analytical task that presents inherent experimental difficulties. Factors such as the complexity of the matrices of the pharmaceutical samples and the presence of the residual solvents at trace levels may compromise the correct determination of these compounds. It is therefore natural that this is an area of continuous improvement and that, over the years, new analytical strategies capable of improving criteria such as sensitivity, selectivity and time of analysis are proposed and implemented.

Concern about the presence of residual solvents in pharmaceuticals dates back to the late 1970s and especially to the 1980s,[35] when the importance of detecting and quantifying these chemical compounds was recognized. The need to have the tools to give an effective answer to this question motivated the analytical development in this area of study. The first analytical methodology proposed for the determination of residual solvents in pharmaceuticals were the loss-on-drying,[35–38] technique based on the weight loss of the sample when heated under isothermal conditions during a certain period of time.[39] Although simple and easily applicable, loss-on-drying methods are neither specific nor precise.[40] Since these methods are not able to unambiguously identify the residual solvents (lack of specificity), analysis of samples containing different solvents only provides information about the overall amount of residual solvents. In addition, the difficulty in ensuring that the total residual solvents content has been removed from the pharmaceutical matrix and the potential formation of undesirable volatile compounds,[39] resulting from sample degradation during heating, may compromise the reliability of the analytical results. Other disadvantages relate to the high detection limits presented, which implies the use of high sample amounts (about 1-2 g),[35,37] and the extended sampling time.[39] The mentioned limitations demonstrate that this methodology is not the most appropriate for monitoring residual solvents in pharmaceuticals.

Loss-on-drying technique evolved towards thermogravimetric analysis, differential thermal analysis and differential scanning calorimetry, techniques that allowed to reduce the

required amount of sample (2-80 mg),[41,42] increasing the sensitivity of the analysis. However, like the loss-on-drying methodology, these methods are non-specific and present a long analysis time.[39,41,42]

To achieve results with higher levels of reliability and speed, the pharmaceutical industry has begun to explore other analytical strategies. Some spectroscopic methods have occasionally been used to determine residual solvents in the pharmaceutical industry. Vachon and Nairn[43] used Fourier Transform Infrared Spectroscopy (FT-IR) to determine the presence of dichloromethane residues in acetylsalicylic acid-Eudragit® microspheres with pharmaceutical application by measuring the characteristic solvent band in the spectra. Generally, the limitations of this spectroscopic technique are the potential interferences of target analytes and matrix bands and the limits of detection above 100 ppm, which compromises the correct detection of class 1 solvents and some of the class 2 solvents.[35]

Nuclear Magnetic Resonance (NMR) is another technique used for the analysis of residual solvents.[35] Avdovich *et al.*[44] applied proton nuclear magnetic resonance (^{1}H-NMR) to monitor residual benzene, toluene, acetone, methyl ethyl ketone and ethyl ether in cocaine samples. In some samples, dichloromethane and ethyl acetate were also detected. The following limits of detection were obtained: benzene (100 ppm), toluene (150 ppm), acetone (100 ppm), methyl ethyl ketone (200 ppm) and ethyl ether (250 ppm). These limits of detection are too high to ensure the correct detection of class 1 solvents and class 2 solvents with concentration limits below 100 ppm. Moreover, this technique is not able to quantify the exact amount of the residual solvent, allowing only to estimate this value.

The limitations presented by FT-IR and NMR, especially regarding sensitivity of the method, are the main reasons why these techniques are not applied in the routine analysis of a pharmaceutical industry's quality laboratory.

Among the various analytical techniques proposed over the years, Gas Chromatography (GC) has emerged as the methodology of election for determination of residual solvents in the pharmaceutical industry. This chromatographic technique is applied to the study of volatile compounds and is capable of both providing identification of individual components and quantifying their exact concentration. Considering the volatile nature of the residual solvents and associated impurities, the GC is the most specific analytical methodology to perform the monitoring of these compounds. Additionally, GC presents the characteristic ability of chromatographic methods to separate components, sensitivity sufficient for the detection of classes 1 and 2 solvents, and the possibility of analyzing liquid or solid samples of a complex nature.[35] Thus, capable of overcoming the previously mentioned difficulties associated with the

determination of residual solvents in pharmaceutical samples, GC is the most appropriate technique to perform this analysis.

In the GC technique, the introduction of the sample into the gas chromatograph can be performed through different procedures, namely by direct injection,[24] headspace analysis[38,45,46] or solid-phase microextraction (SPME).[47] Direct injection has the advantages of being simple and does not require specialized equipment beyond the standard GC system.[24,48] However, the effect of the sample matrix can be a potential limitation when applying this procedure, which may compromise the correct determination of the compounds. Interactions between the analytes of interest and other components in the sample matrices can occur utilizing direct inject.[49] These unwanted interactions may result in decreased chromatographic response of the target residual solvents and promote the formation of volatile products in injection port, leading to the presence of peaks in the obtained chromatographic profile that may be mistakenly identified as peaks of the target analytes. Foust and Bergren[50] developed a direct injection method for the determination of solvent residues in pharmaceutical samples. In the mentioned work, the unexpected detection of benzene, a class 1 solvent, was reported. The authors suggested that the formation of benzene resulted from the interaction between the salts of the drug substances and the diluent of the method, benzyl alcohol. Additionally, the non-volatile components of the samples may be retained in the column, which causes column degradation and impairment of the subsequent injections, or may degrade thermally causing interfering peaks.[51,52]

The problems presented by the direct injection system, mainly with respect to the matrix effect of the drug substances or drug products, can be minimized by the previous separation of the volatile portion of the samples to be analyzed. In this regard, headspace analysis and SPME techniques emerged as an important alternatives to the direct injection in the study of samples that present complex matrices, as is the case of pharmaceuticals.

The SPME techniques are characterized by being highly sensitive, rapid and solvent-free. However, to apply these methods it is necessary to optimize several parameters with an impact on the efficiency of the extraction of the analytes, which is a difficult and time-consuming task. In addition, both the selectivity and the repeatability of the analysis can be compromised. The mentioned disadvantages limit the use of SPME in routine analysis of pharmaceuticals. These techniques were pertinently reviewed by different authors.[36,37,53]

Used since 1980s,[38,45,46] headspace – gas chromatography is commonly applied for determination of residual solvents in pharmaceuticals. The main advantage of this procedure is that only the volatile compounds of the sample are injected into the GC system and submitted to the chromatographic analysis. This allows to increase the lifetime of the chromatographic

column and to prevent the occurrence of interfering peaks resulting from both the thermal degradation of the non-volatile matrix and their interaction with the analytes of interest. Headspace technique also improves the chromatographic response for the target analytes and increases the efficiency of the analysis. In the headspace analysis, volatile sample compounds can usually be extracted through two distinct approaches: static and dynamic.

Currently, static headspace is the most widely used sample preparation technique in the determination of residual solvents in pharmaceuticals. In this technique, the sample is placed, in liquid or sometimes solid form, inside a sealed vial where the extraction of the compound volatiles will occur. Thereafter, the vial is heated to a given temperature for a specific time, with or without vial agitation. This operation, illustrated in Figure 1, promotes the partial vaporization of the volatile molecules and their diffusion of the sample (liquid phase) into the gas phase, called headspace, and of the gas phase into the liquid phase, until a thermodynamic equilibrium state between the two phases is achieved.[51]

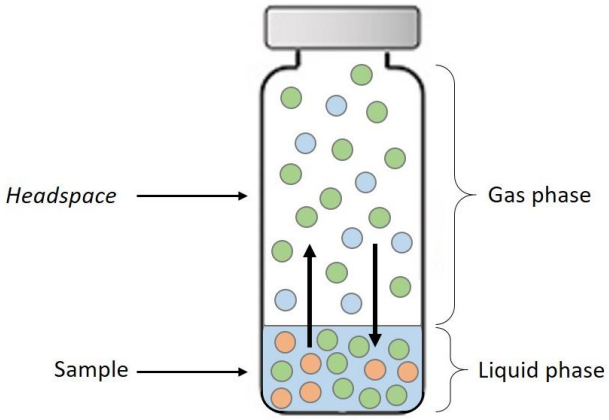


Figure 1. Diffusion of the volatile components of a sample, between the liquid phase and the gas phase, in a headspace vial heated to a given temperature during a specific time, until the system achieves the thermodynamic equilibrium. Legend: **Orange** – Non-volatile (or less volatile) component; **Green** – Volatile component; **Blue** – Diluent.

At the equilibrium, the concentration of volatile analytes remains constant in the gas phase and the sampling process must be as efficient as possible to ensure that the volatile content is completely extracted from the sample matrix. After the equilibrium between the two phases of the vial is reached, the gas phase is collected and injected into the GC system to perform the chromatographic separation.

In addition to preventing problems of direct injection, the static headspace is a universal, easily operated and highly automated technique that is appropriate for the analysis of pharmaceutical samples soluble in water or other organic solvents and provides good precision,

accuracy, reproducibility and robustness.[23,19,54,55] The static headspace has adequate detection limits for the determination of residual solvents in pharmaceuticals but the dynamic headspace provides higher levels of sensitivity.[51] However, in the routine analysis preference is given to the use of the static headspace due to its greater ease of application and its superior precision and reproducibility, which increases both the efficiency and the productivity of the pharmaceutical quality control laboratory.

Finally, regarding to the system of detection, it is verified that in GC methods developed for the determination of residual solvents, the use of the Flame Ionization Detector (FID) is predominant. The FID has adequate sensitivity and selectivity for trace organic volatile analysis. In addition, mass spectrometry can be used to support the identification of the compounds when unexpected peaks are obtained in the chromatogram or when unknown solvent determinations are performed, such as in forensic examinations of pharmaceuticals.[35, 37,54,56]

1.2. The Analytical Quality by Design (AQbD) approach in Analytical Method Development

In the pharmaceutical industry, the quality control plays a decisive role in the development and production of pharmaceuticals by ensuring that the specifications imposed are met.[34,57] Pharmaceutical quality monitoring relies on analytical chemistry and accompanies and evaluates the pharmaceutical process from the discovery of a molecule with therapeutic effect, through pharmaceutical development, to the exit of the batch to the market.[34,57] The obtained analytical results allow the acquisition of important information that reveals the impact of the process conditions on the quality of the product and which supports the acceptance or rejection of the pharmaceutical sample in question.[34,57,58] Therefore, considering the relevance of their contribution, the applied analytical methods must satisfy, during their development, validation, transfer and routine use, certain criteria for obtaining valid analytical results.[34,58]

In the traditional approach for analytical methods lifecycle, the development, validation, transfer and routine use are considered as isolated and independent steps of the other. This approach is not focus on the end purpose of the method nor on having a deep method understanding. The analytical development is, usually, based on one-factor-at-a-time (OFAT) and trial-and-error experiments and the method performance quality is only determined at the validation stage, through the evaluation of the validity of the obtained experimental results. In the OFAT and trial-and-error experiments, one parameter is studied and optimized individually while the others are remained constant.[57,59] The study of the factors one by one do not allows to investigate the effects of the interaction between variables and, therefore, to adequately evaluate and control the variability of the method performance. This, typically, leads to a narrow knowledge space and higher risk of a method failures through its lifecycle, mainly verified during validation or transfer steps. When a method fails it becomes necessary to re-develop the method or revalidate the developed method after its transfer, which results in a high expense of time and resources and a low responsiveness of the quality control laboratory with a significant impact on the pharmaceutical development and production. To overcome these questions, the pharmaceutical industry introduced the concept of QbD, initially applied only to pharmaceutical development and production, in the development of analytical methods.

For a pharmaceutical product, the principles QbD are defined as a systematic and structured approach that, outlining the final objective(s), seeks to guarantee the quality of the pharmaceuticals by identifying the potential sources of the pharmaceutical process variability and understanding how its properties are affected by this variability.[1] Thus, the deep knowledge acquired through previous scientific information, statistical concepts and the experimental

evaluation of the risk parameters, is determinant for the control and mitigation of the sources of the process variability. Similarly, the extension of these principles to the field of analytical chemistry allows to deepen the knowledge of the relationship between the analytical method inputs (attributes and operating conditions) and outputs (analytical performance throughout the lifecycle of the method), to control and reduce the sources of variability and, finally, to satisfy the end of purpose of the method.

The application of the principles QbD in the development of analytical methods, called the AQbD approach, is a continuous and scientific based strategy in which the steps of development, validation, transfer and routine use of a method are closely related to each other. This provides a holistic approach to control the risk of analytical method failure throughout its lifecycle, where knowledge and risk management are key concepts.[57] Thus, the analytical method performance is designed and understood during the method development. The proposed stages of the implementation of this methodology are presented in Figure 2.

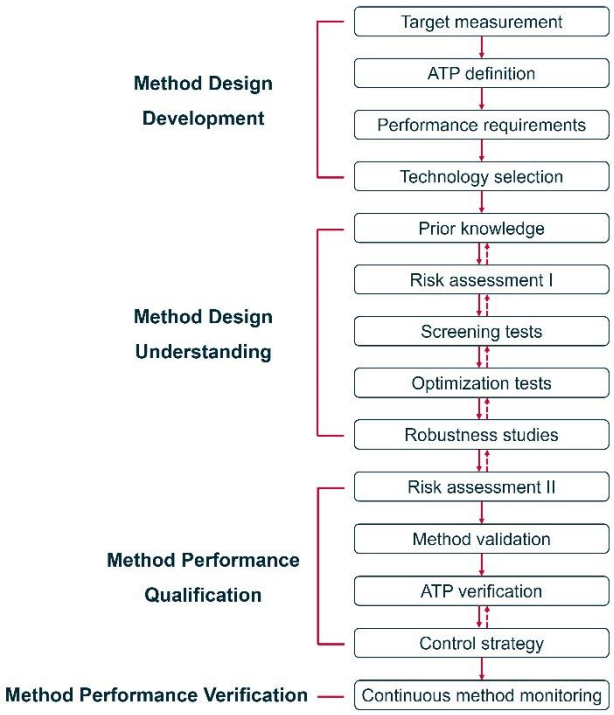


Figure 2. Workflow of Analytical Quality by Design (AQbD) approach applied in the analytical method development.

The AQbD workflow starts with the definition of the target measurement and the establishment of the Analytical Target Profile (ATP). The ATP defines the goals of the method, i.e., define the maximum acceptable risk of the method to fail, determining the quality requirements that method performance must meet to achieve the intended objective. Thereafter,

the most appropriate analytical technique is selected considering the method requirements stated in the ATP.[33,57,58]

In the second stage of the AQbD workflow, denominated by Method Design Understanding, it is intended to acquire relevant scientific information about the method, namely to identify the sources of variability, designated by critical method parameters (CMPs) and to understand its impact on the critical method attributes (CMAs) of the method and on the analytical performance. The tasks included in this stage consult knowledge gained in previous experiences, tools for risk assessment and perform the experimental study of CMPs. Contrary to the traditional approach, in the AQbD methodology the experimental studies are mainly performed through Design of experiments (DoE). In these tests, the factors are tested simultaneously, which allows to evaluate the interaction between the factors in study and to determine the relation of these factors with the performance of the method. The experimental data are analyzed statistically through a modelling software and the design space, designated by Method Operable Design Region (MODR), is defined. This region establishes all possible combinations of operating conditions, for which the performance of the method is not compromised, and the goal stated in the ATP is achieved. If the method is robust it will present flexibility in the face of any change within the design space. Thus, its analytical performance will not be affected nor is it necessary to revalidate the method.[57–59] In addition, the optimum conditions of the method can be selected, which are used in the routine analysis and called Normal Operating Conditions (NOC).[57,59]

Finally, the developed method is submitted to validation to verify if it provides analytical results that meet the criteria defined in the ATP and a control strategy is established to monitor the potential failures of the method, eliminating them and thus ensuring the desired method performance.[57–59] In addition, following the implementation of the method in the routine, continuous management is performed throughout its lifecycle as a way of ensuring that the obtained analytical results are consistently maintained according to ATP and that the method is in a state of control.[59–61]

Given the importance of analytical methods throughout the pharmaceutical development lifecycle, the application of the AQbD strategy to analytical development is a promising tool for obtaining quality analytical results to support the decision-making process. Thus, through a continuous and scientifically structured process, analytical methods with high levels of reliability and robustness can be implemented in quality control laboratories for use in routine and which simultaneously allows to save time and resources.

In recent years, the implementation of QbD principles in the development of analytical methodologies, mainly based on liquid chromatography techniques,[30–33,60–64] has been increasing in pharmaceutical industry. The authors reported the development and validation of robust and well-controlled methods with a well-understood MODR. Additionally, the authors highlight the time and cost effectiveness achieved by the application of AQbD methodology in the analytical development. However, based on the literature, there is a gap in the development of GC or HS-GC methods applying the AQbD strategy. In this sense, the present work intends to constitute an innovative and relevant contribution in this scientific area.

Chapter 2. Materials and Methods

2.1. Chemicals and reagents

All solvents used in the standard solutions' preparation were of analytical grade or similar. Hexane (≥ 98.0 %), diisopropyl ether (≥ 99.0 %), methyl isobutyl ketone (≥ 99.0 %), heptane (≥ 99.3 %), propan-1-ol (≥ 99.5 %), *tert*-butanol (≥ 99.5 %), butan-1-ol (≥ 99.8 %), methyl *tert*-butyl ether (≥ 99.8 %), acetonitrile (≥ 99.9 %), propan-2-ol (≥ 99.9 %) and methanol (≥ 99.9 %) were acquired from Merck (Darmstadt, Germany). Acetone (≥ 99.5 %), methyl ethyl ketone (≥ 99.5 %), ethyl acetate (≥ 99.7 %) and dichloromethane (≥ 99.9 %) were obtained from Honeywell Riedel-de-Haën (Germany). Mesityl oxide (89.6%), diethyl ether (≥ 97.5 %), 4-hydroxy-4-methylpentan-2-one (99%), propyl acetate (99 %), 2-methylbut-2-ene (≥ 99.0 %), *N*,*N*-diisopropylethylamine (DIPEA) (≥ 99 %) and 2-methylbut-1-ene (≥ 99.5 %) were purchased from Sigma-Aldrich (Darmstadt, Germany). Butyl acetate (≥ 99.0 %), isopropyl acetate (≥ 99.0 %) and tetrahydrofuran (≥ 99.8 %) were obtained from Acros Organics (Geel, Belgium). Cyclohexane (≥ 99.8 %) and toluene (≥ 99.8 %) were acquired from Carlo Erba Reagents (Val de Reuil, France). Ethanol (≥ 99.9 %) was purchased from PanReac AppliChem (Darmstadt, Germany) and 1,4-dioxane (≥ 99 %) was obtained from Alfa Aesar (Karlsruhe, Germany). Method's diluent (≥ 99.9 %) was obtained from Scharlau (Barcelona, Spain).

2.2. Gases

Helium, nitrogen, hydrogen and oxygen were used in the developed method. Helium, with 99.9999 % of purity, was used as carrier gas. Nitrogen, hydrogen and oxygen were associated with the FID system. The four gases were supplied by Air Liquide (Portugal).

2.3. Instrumentation

In this work two different HS-GC systems were used, namely a Hewlett-Packard (HP6890) gas chromatograph coupled to an Agilent Headspace G1888 autosampler and an Agilent 7890B gas chromatograph coupled to an Agilent Headspace 7697A autosampler. Both headspace models were equipped with a 1.0 mL sample loop and connected to the inlet through a transfer line. Both gas chromatography systems were coupled to a FID system and controlled by a data acquisition software, Empower 3® (Waters Corporation®). The chromatographic separations were performed on an USP G43 phase column. The injector was equipped with a 2.0 mm I.D. deactivated liner (Part Number 5181-8818) and 1 µL of each standard/sample solution was injected in GC system.

In the preparation of stock standard solutions, the target analytes were pipetted using a 100 µL, a 1000 µL or a 5000 µL pipette from Eppendorf Research plus. In the preparation of working, intermediate and sensitivity standard solutions, aliquots were pipetted using Class A glass pipettes. In the preparation of sample solutions, a calibrated analytical balance was used to weigh samples. Headspace vials with screw top and caps from Agilent Technologies were used.

Some of the experimental steps were based on DoE strategy using a data modelling software, Fusion QbD® (S-Matrix Corporation®).[65]

2.4. Analytical procedure

2.4.1. Preparation of standard solutions

Stock standard solutions were prepared at a concentration higher than the ICH/VICH limits and controlled levels by adding the appropriate volume of each target analyte into a 100 mL volumetric flask containing the method's diluent. The flasks were then brought up to volume with method's diluent and mixed well.

A working standard solution at 100% of the ICH/VICH limits and controlled levels was prepared by adequate dilution from stock standard solutions into a 100 mL volumetric flask containing method's diluent. The flask was then brought up to volume with method's diluent and mixed well.

Due to differences in the sensitivity of the target analytes, an intermediate standard solution was prepared by suitable dilutions from stock standard solutions into a 100 mL volumetric flask containing method's diluent. The flask was then brought up to volume with method's diluent and mixed well.

A sensitivity standard solution combining all analytes of interest was prepared at appropriate practical LOQs by pipetting an adequate volume of intermediate standard solution into a 100 mL volumetric flask and diluting to volume with method's diluent. The solution was then mixed well before injection.

For linearity studies, the standard solutions containing the 29 target analytes at 20%, 120%, 140% and 200% of the ICH/VICH limits and controlled levels were prepared by appropriate dilutions from stock standard solutions with method's diluent.

2.4.2. Preparation of sample solutions

Five pharmaceutical samples were tested (Sample 1: Drug product A, Sample 2: Drug product B, Sample 3: Drug substance A, Sample 4: Drug product C and Sample 5: Drug substance B). The sample solutions were prepared at 100 mg/mL concentration in method's diluent. The spiked sample solutions for accuracy and repeatability studies were prepared at 100 mg/mL sample concentration using standard solutions containing the 29 target analytes at LOQ level, 100% or 140% of the ICH/VICH limits and controlled levels as respective diluents.

Chapter 3. Results and Discussion

3.1. Method Design Development

3.1.1. Establishment of the Target Measurement and definition of the Analytical Target Profile (ATP)

The implementation of AQbD pretends, in a structured and scientific manner, to design the analytical method performance characteristics to satisfy the intended purpose of the method. Therefore, the starting point is to state the analysis objective and the acceptance criteria for the results generated by the analytical method. The present study proposed to develop an analytical method to determine residual solvents and its volatile impurities or stabilizers in pharmaceuticals to support decisions, in each step of the manufacturing process, about whether a drug substance or drug product meets the specification criteria. Once the target measurement is established, method performance requirements are defined as Analytical Target Profile (ATP), which is a key concept of AQbD strategy. The method must be able to quantify residual solvents and its volatile impurities or stabilizers in drug substances and drug products with the following requirements for the reportable results: at limit of quantitation (LOQ) concentrations, Accuracy = 100% ± 30% and Precision ≤ 25%, and at ICH/VICH limits and defined control levels, Accuracy = 100% ± 10% and Precision ≤ 10%.

3.1.2. Analytical Method Performance Requirements

The ATP establishes a combined criterion for accuracy (systematic variability) and precision (random variability), which are critical method performance characteristics directly associated with the target measurement uncertainty, that is the maximum acceptable error of the reportable result without compromising the method purpose. Although not mentioned in ATP, relevant analytical performance characteristics such as linearity and range, selectivity, sensitivity, quantitation limit, detection limit and robustness, described in ICH Q2(R1) Guideline, should be considered to ensure the intended level of quality of the analytical method and to allow the achievement of ATP. Therefore, requirements for these performance characteristics were defined in the present work. The method should be robust, linear over the study range for each analyte (from LOQ to 200% of the ICH/VICH limits and defined control levels), sensitive with a quantitation limit equal to or below 50 ppm for each analyte and be able to separate all analytes, in a run time less than 30 minutes.

3.1.3. Selection of the analytical technique

Based on literature review about analytical determination of residual solvents in the pharmaceutical industry (Chapter 1), the HS-GC-FID was chosen due to its capability to separate the target compounds, its chemical specificity for the target analytes, its capability to provide highly sensitive and robust analysis and the high level of automation. Therefore, this analytical methodology is the most appropriate to achieve the requirements defined in ATP for the quality of the reportable results in detection, identification and quantification of residual solvents and its organic volatile impurities or stabilizers in pharmaceutical samples.

3.2. Method Design Understanding

3.2.1. Prior knowledge

An extensive and comprehensive review of Hovione's HS-GC-FID methods for determination of residual solvents in drug substances or drug products was performed to collect relevant and representative information in order to increase and improve understanding of the proposed analytical method. The knowledge acquired regarding the monitored analytes and the diluent and the chromatographic column used in these methods as well as brief reviews of literature were used to guide and support decisions on analytical parameters overall procedure for the present work of method's development.

Selection of the target analytes

In the implemented methods used for drug substances and drug products, the number of analytes determined was usually restricted to between one and eleven components. After reviewing these methods, the most commonly monitored analytes were listed in Table 2.

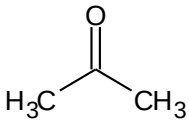
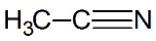
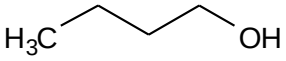
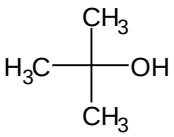
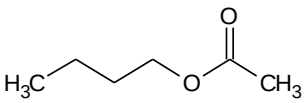
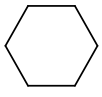
Table 2. List of the most commonly monitored analytes in HS-GC methods assessed.

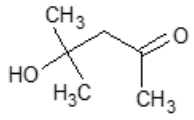
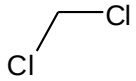
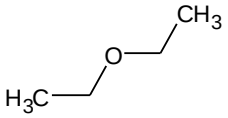
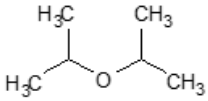
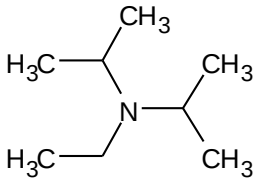
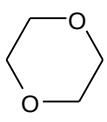
Analytes
Methanol
Acetone
Dichloromethane
Ethanol
Ethyl acetate
Tetrahydrofuran
Toluene
Heptane
Acetonitrile
Butan-1-ol
Isopropyl acetate
Benzene
4-hydroxy-4-methylpentan-2-one
Mesityl oxide
Propan-2-ol
2-methylbut-1-ene
2-methylbut-2-ene
Acetaldehyde
Methyl *tert*-butyl ether
Propan-1-ol
Tert-butanol
Triethylamine
Butyl acetate
Butane-2,3-dione
Chlorobenzene
Chloroform
Diethyl ether
Diisopropyl ether
2-Methyltetrahydrofuran
N,*N*-dimethylformamide
Dimethyl sulfoxide
1,4-dioxane
Ethyl benzene
Methyl ethyl ketone
Methyl isobutyl ketone
Propionaldehyde
Propyl acetate

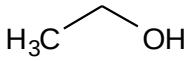
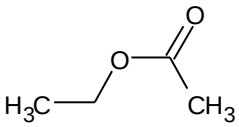
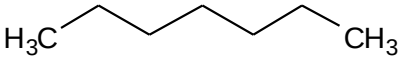
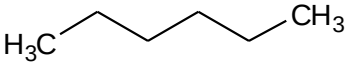
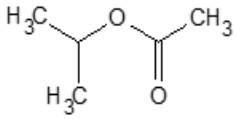
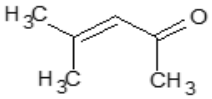
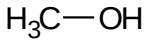
Among the analytes listed in Table 2, 29 compounds were selected to be controlled by the general HS-GC-FID method under development. The target compounds are listed in Table

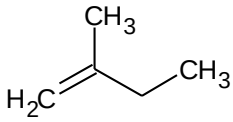
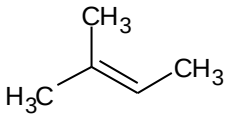
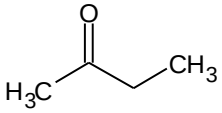
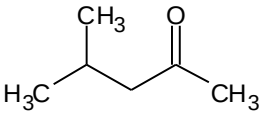
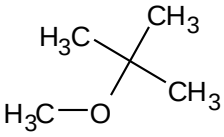
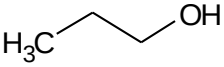
3 together with their structure, chemical class, molecular formula, concentration limit in pharmaceutical products (according to ICH/VICH guidelines) and physical properties (dielectric constant, as a measure of its polarity, and boiling point).

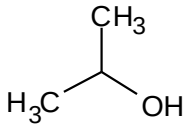
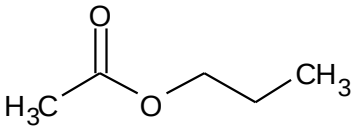
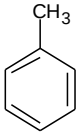
Table 3. List of the 29 selected target analytes to be controlled by the general HS-GC method and their relevant properties (structure, chemical class and molecular formula, concentration limit in pharmaceuticals, boiling point and dielectric constant).

Compound	Concentration limit (ppm)	Boiling point (ºC)[66]	Dielectric constant (ε)[67]
 **Acetone** Ketone C_3H_6O	5000	56.1	21.01
 **Acetonitrile** Nitrile C_2H_3N	410	81.6	36.64
 **Butan-1-ol** Alcohol $C_4H_{10}O$	5000	117.6	17.84
 ***tert*-butanol** Alcohol $C_4H_{10}O$	------	82.3	12.47
 **Butyl acetate** Ester $C_6H_{12}O_2$	5000	126.0	5.07
 **Cyclohexane** Aliphatic hydrocarbon C_6H_{12}	3880	80.7	2.02

Compound	Concentration limit (ppm)	Boiling point (°C)[66]	Dielectric constant (ε)[67]
 **4-hydroxy-4-methylpentan-2-one** Alcohol $C_6H_{12}O_2$	------	167.9	18.20
 **Dichloromethane** Halogenated hydrocarbon CH_2Cl_2	600	39.8	8.93
 **Diethyl ether** Ether $C_4H_{10}O$	5000	34.4	4.27
 **Diisopropyl ether** Ether $C_6H_{14}O$	------	68.4	3.81
 ***N,N*-diisopropylethylamine (DIPEA)** Amine $C_8H_{19}N$	------	114.0	------
 **1,4-dioxane** Ether $C_4H_8O_2$	380	101.2	2.22

Compound	Concentration limit (ppm)	Boiling point (°C)[66]	Dielectric constant (ε)[67]
 **Ethanol** Alcohol C_2H_6O	5000	78.2	25.30
 **Ethyl acetate** Ester $C_4H_8O_2$	5000	77.1	6.08
 **Heptane** Aliphatic hydrocarbon C_7H_{16}	5000	98.4	1.92
 **Hexane** Aliphatic hydrocarbon C_6H_{14}	290	68.7	1.89
 **Isopropyl acetate** Ester $C_5H_{10}O_2$	5000	88.6	------
 **Mesityl oxide** Ketone $C_6H_{10}O$	------	129.7	15.60
 **Methanol** Alcohol CH_4O	3000	64.5	33.00

Compound	Concentration limit (ppm)	Boiling point (°C)[66]	Dielectric constant (ε)[67]
 **2-methylbut-1-ene** Aliphatic hydrocarbon C_5H_{10}	------	31.1	2.18
 **2-methylbut-2-ene** Aliphatic hydrocarbon C_5H_{10}	------	38.5	1.98
 **Methyl ethyl ketone** Ketone C_4H_8O	3000	79.6	18.56
 **Methyl isobutyl ketone** Ketone $C_6H_{12}O$	4500	115.7	13.11
 **Methyl *tert*-butyl ether** Ether $C_5H_{12}O$	5000	55.1	------
 **Propan-1-ol** Alcohol C_3H_8O	5000	97.0	20.80

Compound	Concentration limit (ppm)	Boiling point (°C)[66]	Dielectric constant (ε)[67]
 **Propan-2-ol** Alcohol C_3H_8O	5000	82.2	20.18
 **Propyl acetate** Ester $C_5H_{10}O_2$	5000	101.0	5.62
 **Tetrahydrofuran** Ether C_4H_8O	720	66.0	7.52
 **Toluene** Aromatic hydrocarbon C_7H_8	890	110.6	2.38

Some of the solvents, although included in the Hovione's list of monitored residual solvents, were excluded due to poor headspace properties or poor chromatographic properties.[25] The list includes 9 ICH/VICH class 2 solvents (acetonitrile, cyclohexane, dichloromethane, 1,4-dioxane, hexane, methanol, methyl isobutyl ketone, tetrahydrofuran and toluene), 13 ICH/VICH class 3 solvents (acetone, butan-1-ol, butyl acetate, methyl *tert*-butyl ether, ethanol, ethyl acetate, diethyl ether, heptane, isopropyl acetate, methyl ethyl ketone, propan-1-ol, propan-2-ol and propyl acetate), 2 organic volatile impurities associated with acetone (4-hydroxy-4-methylpentan-2-one and mesityl oxide), 2 solvent stabilizers (2-methylbut-1-ene and 2-methylbut-2-ene) and 3 other solvents of interest that can be used in raw materials (diisopropyl ether, DIPEA and *tert*-butanol).

Compounds of different chemical classes and polarities are present, namely aliphatic, aromatic and halogenated hydrocarbons (non-polar compounds), alcohols, amines, nitriles,

ethers, esters and ketones (compounds with intermediate polarity).[68] This group of 29 compounds also presents a wide range of boiling points (from 2-methylbut-1-ene at 31.1 ºC to 4-hydroxy-4-methylpentan-2-one at 167.9 ºC) and toxicities. Regarding the concentration limits in pharmaceuticals, during the development of the general HS-GC method the values defined by the ICH/VICH Guidelines were considered for the solvents of classes 2 and 3. For the remaining target analytes, which are not listed in the ICH/VICH Guidelines, a suitable control limit was defined based on literature.[69–73]

The investigation of the chemical and physical properties of the target compounds allows the choice of the appropriate column stationary phase for an efficient separation. The chromatographic separation is based on the selectivity of the stationary phase relatively to the analytes to be analyzed, which is determined by the strength of the intermolecular interactions that are established between the stationary phase and the analytes, according to their physical and chemical properties. Depending on its chemical nature (type and amount of functional groups), the polarity of the stationary phase influences the strength of these interactions and, consequently, the ability to separate the target compounds. Retention is stronger when the polarity of the stationary phase and the analytes is similar, increasing the separation of the compounds to be analyzed. Considering this information and knowing the polarities of the selected target compounds, the stationary phase of the chromatographic column to be used in the developing HS-GC method should have a low to medium polarity.

In addition, the fact that the group of target analytes is heterogeneous in terms of boiling points indicates that, in this case, the application of a gradient temperature program will be more appropriate for the chromatographic separation than an isothermal temperature program.[74–76]

Selection of the chromatographic column

The chromatographic column is considered the heart of the gas chromatography system[74,75] because it is the sector where the separation of the sample components takes place. Therefore, the selection of the column is a critical factor during the analytical development. In this complex task, the length, the inner diameter, the film thickness and the stationary phase of the column are the main parameters to be considered in order to optimize the analysis for both resolution and speed.

Different columns are used in the most representative HS-GC methods implemented at Hovione. The USP G43 is the most frequently used column in Hovione's methods. According to the literature,[13] these columns are considered the most suitable in the determination of

volatile organic impurities in pharmaceuticals by HS-GC. G43 phase is the one whose use is recommended by the United States and the European Pharmacopoeias.[8,9] In addition, other studies on the determination of residual solvents in pharmaceuticals[27,28,76,77] has reported that, because of their intermediate polarity, this phase is suitable for separating both apolar and polar compounds. Thus, this column has selectivity for most of the ICH/VICH classes 2 and 3 solvents and other volatile organic impurities.

In the present work, the target analytes present a wide range of polarities so, based on the information collected from HS-GC methods implemented at Hovione and literature, a G43 phase was selected as the column to be used in the development of the general HS-GC method.

Selection of the method's diluent

With respect to the method's diluent, previous investigations[12,14,27,28,76,78] refer the importance of making an adequate choice. This parameter is critical since it directly impacts on the sensitivity of the HS-GC method, on the selection of the HS equilibration temperature and time and on the acquisition of blank chromatograms free of interfering peaks. Therefore, the main requirements that a solvent must meet to be considered as an appropriate diluent for the determination of residual solvents in drug substances and drug products are the ability to dissolve a wide range of pharmaceutical samples, thermostability, high boiling point (so the peak of diluent elutes after the analytes peaks, not interfering with the analysis of residual solvents by coelution) and high purity and inertness.

The most used diluents for HS-GC methods are summarized in Table 4.

Table 4. Solvents used as diluents in HS-GC methods.

Diluent
N,*N*-dimethylacetamide (DMA)
Dimethyl sulfoxide (DMSO)
1-methylpyrrolidin-2-one (NMP)
Benzyl alcohol (BA)
1,3-dimethylimidazolidin-2-one (DMI)
Water
DMA + DMSO

Table S.1. (Supplementary Data) summarizes the physical properties (dielectric constant, as a measure of its polarity, and boiling point) of water and organic solvents as well as the respective structure and molecular formula. All solvents have relatively low volatility and are polar. Water is considered the ideal diluent for hydrophilic samples because it has no

toxic effects, it is not expensive and because it does not respond when using FID. However, this solvent is not suitable for dissolving hydrophobic samples. *N*,*N*-dimethylacetamide (DMA), dimethyl sulfoxide (DMSO), 1-methylpyrrolidin-2-one (NMP), benzyl alcohol (BA) and 1,3-dimethylimidazolidin-2-one (DMI) show a more global capability of dissolving drug substances and drug products than water and have also higher boiling points, so their use as diluent is suggested.

Urakami *et al.*[78] studied the influence of the matrix medium used for the determination of residual solvents in pharmaceuticals by HS-GC. DMSO, *N*,*N*-dimethylformamide (DMF), DMA, BA, DMI and water were the investigated solvents. The authors verified the degradation of DMSO, DMF, DMA and BA at headspace oven temperatures above 100 °C or under ultrasonic action. For BA, the generated artifacts were identified as benzene and toluene. Benzene is a class 1 solvent, whose concentration level should be lower than the limits described in the ICH guideline (2 ppm). Toluene is one of the target analytes of the present work of analytical development so the presence of the toluene peak in the diluent blank (BA) will affect the correct quantification of this solvent. For these reasons, the BA will not be used as diluent in the general HS-GC method.

In a study conducted by Somuramasami *et al.*[16], DMI was selected as method diluent because allowed to work at a high headspace oven temperature (120 ºC) to improve the sensitivity of the HS-GC method, with no interfering peaks. Despite this positive aspect, DMI is an expensive solvent when compared to DMA, DMSO, NMP and BA and therefore its use is limited in laboratories. Therefore, DMI will not be used as diluent in the general HS-GC method.

Dai *et al.*[28] studied DMSO, DMF, DMA, BA and DMI in terms of the presence of impurities in blank chromatograms. DMSO and DMA showed cleanest chromatograms with insignificant or no interfering peaks in the retention time window of the target analytes. Evidence of degradation of these solvents was found after prolonged heating at 120 ºC or sonication. Although DMSO and DMA have similar physical properties and exhibit identical sensitivities for the target analytes, DMA has a lower freezing point which is more compatible with refrigerated storage of standard solutions[56] and, on the other hand, is more toxic than DMSO. In addition, DMA can contain trace amounts of DMF impurity.[28,56] Regarding DMSO, it easily degrades when used with chloride salt samples to form dimethylsulfide, which elutes closely to acetone; is nucleophilic and might react with some functional group of the pharmaceutical samples.[28]

Method's diluent was selected based on its polarity, boiling and freezing points, global solubilizing and dissolving power for the wide polarity range of samples under study, purity grade and inertness.

Headspace sampling conditions

The sensitivity, precision and accuracy of the HS-GC method depend on static headspace sampler parameters related to temperature (oven, loop and transfer line temperatures), time (GC cycle time, vial equilibration, vial pressurization, loop fill, loop equilibration and sample injection), pressure (vial and carrier gas pressures), and shake conditions.[13,27] Prior knowledge for headspace temperature, time and shake conditions was obtained after a survey among published studies using HS-GC-FID technique and supplier's technical guides. Regarding temperature conditions, it is known that the increase of vial equilibration temperature promotes the migration of the volatile compounds to the gas phase providing a more accurate analysis.[51] However, it is also necessary to consider the susceptibility of thermal degradation of the pharmaceutical sample and the method's diluent with increasing temperature.[79] Previous works[27,80] and Agilent Headspace samplers' technical guides[81,82] has indicated that the value of vial equilibration temperature should be about 10-20 ºC below the boiling point of the selected diluent. To prevent condensation of the sample, it is recommended that the temperature of the loop is about 5-10 ºC higher than the equilibration temperature's value and that the temperature of the transfer line is about 5-10 ºC higher than the loop temperature's value.[81,82] Regarding time conditions of the headspace, the default values according to the Agilent's technical guides are: pressurization time, 0.20 min; loop fill time, 0.20 min; loop equilibration time, 0.05 min; sample injection time, 1.0 min and loop fill ramp rate, 20 mL/min.[81,82] These recommended values were used as a support for method development. Finally, the mechanical agitation of the vial during heating improves the transfer of volatile analytes from the sample into the gas phase, which allow to achieve the equilibrium state more rapidly.[13,14,16,19,54,83]

3.2.2. Risk assessment

A general Fishbone (or Cause & Effect) diagram, presented in Figure 3, was designed to identify all the HS-GC-FID method variables (causes) that can significantly reduce the desired chromatographic performance quality (effect), previously defined in the ATP. In the suggested diagram, the identified potential risk factors were divided into six different categories of the analytical method, namely Gas Chromatography (GC) system, Headspace sampler, Diluent, Sample preparation, Material/Solvents quality and last, but not the least, the Human factors.

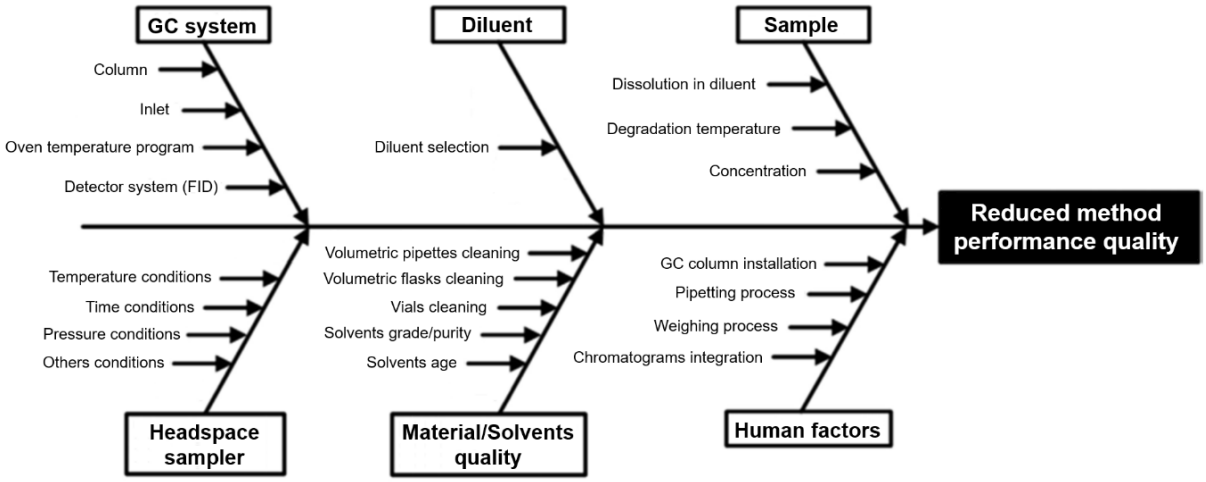


Figure 3. General Fishbone (Cause & Effect) diagram for risk analysis in the development of a HS-GC-FID method. The identified potential risk factors are divided into six different categories of the analytical method.

In addition, the critical method attributes (CMAs) of the method were identified and the respective goals were defined, as shown in Table 5. CMAs are chromatographic responses that should be within an appropriate limit or range to ensure the desired quality of the method. Resolution between adjacent peaks, peak area, peak tailing factor, signal-to-noise ratio (S/N), last peak retention time and presence of extra or interfering peaks were considered the most important method attributes, since these parameters are related with the selectivity, sensitivity and total run time of the HS-GC-FID method.

Table 5. Identification of the Critical Method Attributes (CMAs) and definition of their goals.

Critical Method Attributes (CMAs)	Goal
Resolution	≥ 1.5 between all peaks
Peak area	Maximize
Peak asymmetry factor	0.9 – 2.0
Signal-to-noise ratio (S/N)	Maximize
Last peak retention time	≤ 26 minutes
Extra or interfering peaks	Minimize

The resolution between adjacent peaks should be not less than 1.5, to achieve a complete separation and an accurate measurement of the analytes. As shown in Figure 4, the overlapping of peaks is found for values of resolution lower than 1.5.[84]

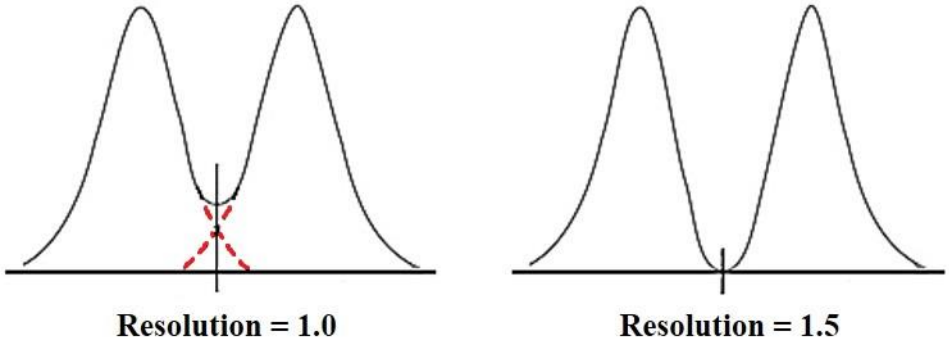


Figure 4. Relationship between the resolution of two peaks and their chromatographic separation.

In relation to the peak shape, ideally, it is intended to obtain symmetric peaks with a Gaussian profile. However, in practice, asymmetric peaks are often obtained. The deviations from the ideal Gaussian shape can be expressed by asymmetry factor, A_S. A symmetric peak assumes a value of A_S equal to 1. When a peak exhibits a shallow frontal slope, there is the phenomenon called peak fronting and the A_S is less than 1. On the contrary, the peak tailing results from a shallow rear slope and the A_S is greater than 1. In the chromatographic analysis, values of A_S between 0.9 and 2.0 are considered acceptable.[84]

To reduce the total run time of the method and to ensure that the analysis time is not more than 30 minutes, the retention time of the last eluting peak should not exceed 26 minutes. Additionally, both the peak area and signal-to-noise should be maximized to improve the sensitivity of the method. The presence of the extra or interfering peaks should be minimized in order to not compromise the correct identification and quantification of the target analytes.

Several factors have a potential effect on CMAs. To rank these factors and to define which ones should be monitored or controlled to ensure the desired performance quality of the general HS-GC method under development, a risk assessment was performed. In this regard, each method parameter included in the Fishbone diagram was scored according to their level of impact on each relevant method attribute, using a scale of 1 to 3, where 1 corresponds to the lowest risk variables and 3 to the highest risk variables. Additionally, based on CNX approach, the method parameters were classified as Controlled (C), if they can be controlled and fixed; as Noise (N), if they cannot be controlled or predicted; and as Experimental (X), if their impact on method attributes needs to be further investigated by experimentation. Ranking of CMAs was done by four experts from Hovione's analytical development team to collect their knowledge and experience. The average of the risk (calculated from the values assigned by each expert) that each method parameter represents for each response was obtained. The results of the risk assessment are presented in Table 6.

As expected, the N variables were related to the age of the chromatographic column and to Material/Solvents Quality and Human factors, since the variability of the parameters of these categories is difficult to predict or control. The C variables were related to the categories of GC system, Headspace sampler, Diluent and Sample. The classification of the variables as C was based on the available prior knowledge and experience from previous works of analytical development.

The variables of the HS-GC-FID method presenting highest scores were GC oven gradient temperature program (total score of 15) and injection volume (total score of 13), both related to GC system. However, from these two factors, only the temperature program was categorized as X. Carrier gas flow rate (column flow), vial equilibration temperature, time and pressure and carrier gas pressure (split at vent) were also classified as X. These six variables were considered as critical parameters and will be studied experimentally.

Table 6. Classification of each potential Critical Method Parameter according to their level of impact on each Critical Method Attribute, using a scale of 1 to 3, and their categorization as Controlled (C), Noise (N) and Experimental (X) variable by the CNX approach. The risk assessment exercise involved the participation of four experts from Hovione's analytical development team.

1 - Low risk	Method parameter that have little or no impact
2 - Moderate risk	Method parameter that have moderate/indirect impact
3 - High risk	Method parameter that have significant/severe/direct impact

Potential Critical Method Parameters (pCMPs)				Critical Method Attributes (CMAs)							Control, Noise, Experimental (C, N, X)
				Resolution	Last Peak Retention Time	Signal-to-noise ratio (S/N)	Peak Area	Peak Tailing	Extra or interfering peaks	Total Score	
				Average	Average	Average	Average	Average	Average		
GC system	Column		Length	3	3	1	1	1	1	10	Controlled (C)
			Internal diameter	2	2	2	1	2	1	10	Controlled (C)
			Film thickness	2	1	2	1	2	1	9	Controlled (C)
			Stationary phase	2	2	2	2	3	1	12	Controlled (C)
			Carrier gas type	1	2	2	1	2	1	9	Controlled (C)
			Carrier gas flow rate	2	2	1	1	2	1	9	Experimental (X)
			Age	2	2	2	2	2	1	11	Noise (N)
	Inlet		Type	2	1	2	2	1	2	10	Controlled (C)
			Injection mode	2	1	2	2	2	1	10	Controlled (C)
			Temperature	1	1	2	2	2	1	9	Controlled (C)
			Injection volume	2	1	3	3	2	2	13	Controlled (C)
		Liner	Type	1	1	2	2	2	1	9	Controlled (C)
			Internal diameter	1	1	2	2	2	1	9	Controlled (C)
			With/without glass wool	1	1	1	1	1	2	7	Controlled (C)
			Activation/deactivation	1	1	2	2	2	1	9	Controlled (C)
	Oven	Temperature program	Isocratic	2	3	2	2	1	1	11	Not applicable
			Gradient	3	3	2	2	3	2	15	Experimental (X)
	Detector		Type	2	1	2	2	1	2	10	Controlled (C)
			Temperature	1	2	2	2	1	1	9	Controlled (C)
			Gases Flow	1	1	2	2	1	1	8	Controlled (C)
Headspace sampler	Temperature conditions		Vial equilibration temperature	1	1	2	2	2	2	10	Experimental (X)
			Loop temperature	1	1	2	2	2	1	9	Controlled (C)
			Transfer line temperature	1	1	2	2	2	1	9	Controlled (C)
	Time conditions		GC cycle time	1	1	1	1	1	1	6	Controlled (C)
			Vial equilibration time	1	1	2	2	1	1	8	Experimental (X)
			Pressurization time	1	1	2	2	1	1	8	Controlled (C)
			Loop fill time	1	1	2	2	1	1	8	Controlled (C)
			Loop equilibration time	1	1	2	2	1	1	8	Controlled (C)
			Injection time	1	1	2	2	1	1	8	Controlled (C)
	Pressure conditions		Vial pressure	1	1	2	2	1	1	8	Experimental (X)
			Carrier gas pressure	1	1	2	2	1	1	8	Experimental (X)
	Others conditions		Shake level	1	1	2	2	1	1	8	Controlled (C)
			Phase ratio (diluent volume / vial volume)	1	1	2	2	1	1	8	Controlled (C)
			Loop size	1	2	1	1	1	1	7	Controlled (C)
Diluent			Diluent selection	2	1	2	2	2	3	12	Controlled (C)
Sample			Complete sample dissolution	2	1	2	2	1	2	10	Controlled (C)
			Sample degradation temperature	2	2	2	1	1	3	11	Controlled (C)
			Sample concentration	2	1	2	3	1	2	11	Controlled (C)
Material/Solvents Quality			Volumetric flasks cleaning	1	1	1	1	1	3	8	Noise (N)
			Volumetric pipettes cleaning	1	1	1	1	1	3	8	Noise (N)
			Vials cleaning	1	1	1	1	1	3	8	Noise (N)
			Solvents grade / purity	1	1	2	1	1	3	9	Noise (N)
			Solvents age	1	1	2	1	1	3	9	Noise (N)
Human factors			GC column installation	2	2	2	2	2	1	11	Noise (N)
			Pipetting process	1	1	2	2	1	2	9	Noise (N)
			Weighing process	1	1	2	2	1	2	9	Noise (N)
			Chromatograms integration	1	1	1	2	1	1	7	Noise (N)

3.2.3. Experimental strategy: establishment of the operating conditions of the general HS-GC method

3.2.3.1. Optimization of the general HS-GC method selectivity and total run time

Based on ATP, selectivity is one of the performance characteristics of the method to be optimized. Additionally, it is intended that the chromatographic analysis to be performed in the shortest possible time. In this work, the resolution between all peaks and the retention time of the last eluting compound were evaluated as a selectivity requirement and as an indicator of analysis time, respectively. As previously mentioned, the resolution between adjacent peaks should be not less than 1.5, to achieve a complete separation and an accurate measurement of the analytes. To reduce the total run time of the method and to ensure that the analysis time is not more than 30 minutes, the retention time of the last eluting peak should not exceed 26 minutes. Then, the critical parameters, whose variability have an impact on these CMAs, were selected to be studied as well as its range. The column flow and GC oven gradient temperature program (initial oven temperature, initial hold time, initial ramp rate, second oven temperature, second hold time and second ramp rate) were considered as variables in this experimental plan.

Screening tests

To investigate the impact that column flow and GC oven gradient temperature program, identified as critical factors during the risk assessment exercise, may have on resolution and on retention time of the last eluting peak, and to verify method performance, a screening test was, initially, performed. A DoE approach was decided to be used in order to understand the effects of column flow, initial oven temperature, initial hold time, initial ramp rate, second oven temperature, second hold time and second ramp rate and its interactions (multivariate analysis). The low and high limits of these seven factors were defined: column flow (1.0 mL/min – 2.0 mL/min), initial oven temperature (30.0 ºC – 45.0 º C), initial hold time (0.0 min – 20.0 min), initial ramp rate (2.0 ºC/min – 30.0 ºC/min), second oven temperature (60.0 ºC – 85.0 ºC), second hold time (0.0 min – 5.0 min) and second ramp rate (10.0 ºC/min – 30.0 ºC/min). For each variable, the range was defined based on available information from previous experiments. Besides that, the selected range represents a large interval of values so that the evaluation of the chromatographic performance is sufficiently comprehensive. The variables and its respective range are presented in Table 7.

Table 7. GC variables considered in the screening tests for the optimization of the general HS-GC method selectivity and total run time, and range in which these factors were studied during DoE-I.

Variables	Type	Units	Range
Column Flow	Continuous	mL/min	1.0 – 2.0
Initial oven temperature	Continuous	ºC	30.0 – 45.0
Initial hold time	Continuous	Min	0.0 – 20.0
Initial ramp rate	Continuous	ºC/min	2.0 – 30.0
Second oven temperature	Continuous	ºC	60.0 – 85.0
Second hold time	Continuous	Min	0.0 – 5.0
Second ramp rate	Continuous	ºC/min	10.0 – 30.0

In order to assess the impact of these variables on method performance, the responses in the screening study and its acceptance criteria were defined, as shown in Table 8.

Table 8. Method responses considered in the screening tests for the optimization of the general HS-GC method selectivity and total run time, and their acceptance criteria, which must be fulfilled.

Method responses	Acceptance criteria
Resolution	≥ 1.5 between all peaks
Last peak retention time	≤ 26 minutes

A 2^{7-3} Resolution IV design with nineteen experiments including three center points was applied to determine the variables and interactions between variables that have significant effects on selected method attributes. This experimental plan, DoE-I, was automatically designed by Fusion QbD® software[65] and is presented in Table 9. The DoE design was translated into a sample set for GC analysis and for each experiment indicated in the Table 9, a different method was created in Empower software. During the screening study, a standard solution containing the 29 analytes at the 100% of the ICH/VICH limits and control levels was injected to evaluate the method performance for each target analyte. For each experimental run, the following sequence was injected: Blank (method's diluent) and standard solution, both injected once.

For the chromatogram obtained in each experimental run, the resolution of all target analytes and the retention time of the last eluting peak were evaluated. According to the chromatographic profiles obtained for each experimental run, it was possible to verify that the resolution between adjacent peaks was ≥ 1.5, except for resolution of acetonitrile, dichloromethane, propan-1-ol and 1,4-dioxane. Thus, these resolutions were considered as

critical and were, for that reason, the only ones to be studied. The obtained results are presented in Table 9.

Based on the results (Table 9), it was noticed that it was not possible to obtain a resolution ≥ 1.5 for all these analytes in any of the experimental runs. Therefore, none of the tested combinations of factors reached a reasonable compromise between the resolutions of acetonitrile, dichloromethane, propan-1-ol and 1,4-dioxane nor, consequently, an acceptable method performance. However, it is to be noted that the operating conditions defined for run 18 were those that were closest to provide experimental results that meet the target goals established in Table 8.

Table 9. Screening tests for the optimization of the general HS-GC method selectivity and total run time: Experimental design of experiments (DoE) run sequence and the respective chromatographic results.

	Critical Method Parameters (CMPs) – input							Critical Method Attributes (CMAs) - output				
Run No.	**Column flow (A)**	**Initial oven temp. (B)**	**Initial hold time (C)**	**Initial ramp rate (D)**	**Second oven temp. (E)**	**Second hold time (F)**	**Second ramp rate (G)**	**Resolution acetonitrile**	**Resolution dichloromethane**	**Resolution propan-1-ol**	**Resolution 1,4-dioxane**	**Last Peak Retention Time**
1	1.0	30.0	20.0	30.0	85.0	0.0	10.0	0.0	0.0	2.7	0.9	27.468
2	1.0	45.0	20.0	30.0	60.0	5.0	10.0	2.3	2.8	0.0	0.0	32.062
3	1.0	30.0	0.0	30.0	60.0	5.0	30.0	2.6	2.5	0.0	1.9	10.333
4	2.0	30.0	20.0	2.0	60.0	5.0	10.0	0.0	0.0	2.4	1.8	42.852
5	2.0	30.0	0.0	30.0	85.0	5.0	10.0	0.0	2.2	0.0	1.4	8.976
6	2.0	30.0	0.0	2.0	85.0	0.0	30.0	1.3	3.2	1.6	0.0	24.712
7	2.0	45.0	0.0	30.0	60.0	0.0	10.0	0.0	2.3	0.0	1.4	6.874
8	2.0	45.0	20.0	2.0	85.0	0.0	10.0	2.2	2.9	0.0	0.0	33.172
9	1.5	37.5	10.0	16.0	72.5	2.5	20.0	1.8	3.2	1.5	0.0	18.199
10	1.5	37.5	10.0	16.0	72.5	2.5	20.0	1.8	3.2	1.5	0.0	18.197
11	2.0	45.0	20.0	30.0	85.0	5.0	30.0	2.2	2.9	0.0	0.0	25.550
12	1.0	45.0	20.0	2.0	60.0	0.0	30.0	2.3	2.8	0.0	0.0	31.158
13	2.0	45.0	0.0	2.0	60.0	5.0	30.0	2.3	2.8	0.0	3.3	15.182
14	1.0	45.0	0.0	2.0	85.0	5.0	10.0	2.5	2.7	0.0	3.1	24.900
15	1.0	45.0	0.0	30.0	85.0	0.0	30.0	2.3	1.6	0.0	3.7	5.160
16	1.5	37.5	10.0	16.0	72.5	2.5	20.0	1.8	3.2	1.5	0.0	18.196
17	1.0	30.0	20.0	2.0	85.0	5.0	30.0	0.0	0.0	2.6	1.6	49.702
18	1.0	30.0	0.0	2.0	60.0	0.0	10.0	1.7	3.0	1.4	3.6	22.482
19	2.0	30.0	20.0	30.0	60.0	0.0	30.0	0.0	0.0	2.3	1.7	24.240

Using the Fusion QbD® software,[65] a statistical analysis of the results from DoE was performed, as shown in Table 10. For each evaluated critical method attribute, a regression model was obtained.

Table 10. Statistical analysis results (outcome-based observed relationships) of the DoE study, DoE-I, applied in the screening test for the optimization of the general HS-GC method selectivity and total run time.

Critical Method Attribute (CMA)	**Model**		**Critical Method Parameters (CMPs)**				
	Transformation	**R^2**	Statistical significant CMP or interaction	Coefficient value	*p*-value ($p<0.05$)	Lower 95% conf. Limit	Upper 95% conf. Limit
Resolution (acetonitrile)	N/AP*	0.9996	Column flow (A)	- 0.356	<+/- 0.0001	-0.380	-0.332
			Initial oven temp. (B)	0.656	<+/- 0.0001	0.632	0.680
			Initial hold time (C)	-0.231	<+/- 0.0001	-0.255	-0.207
			Initial ramp rate (D)	-0.181	<+/- 0.0001	-0.205	-0.157
			Second oven temp. (E)	-0.044	0.0052	-0.068	-0.020
			Second hold time (F)	0.131	<+/- 0.0001	0.107	0.155
			Second ramp rate (G)	0.269	<+/- 0.0001	0.245	0.293
			A*C	0.331	<+/- 0.0001	0.307	0.355
			A*D	-0.269	<+/- 0.0001	-0.293	-0.245
			A*E	0.469	<+/- 0.0001	0.445	0.493
			A*G	0.181	<+/- 0.0001	0.157	0.205
			B*D	-0.131	<+/- 0.0001	-0.155	-0.107
Resolution (dichloromethane)	Reciprocal Square	1.0000	Initial oven temp. (B)	-244.107	<+/- 0.0001	-244.120	-244.095
			Initial hold time (C)	244.098	<+/- 0.0001	244.085	244.110
			Initial ramp rate (D)	0.015	0.0180	0.003	0.028
			A*E	-244.114	<+/- 0.0001	-244.126	-244.101
Resolution (propan-1-ol)	N/AP*	0.9995	Column flow (A)	-0.025	0.0756	-0.054	0.004
			Initial oven temp. (B)	-0.813	<+/- 0.0001	-0.841	-0.784
			Initial hold time (C)	0.438	<+/- 0.0001	0.409	0.466
			Initial ramp rate (D)	-0.188	<+/- 0.0001	-0.216	-0.159
			Second oven temp. (E)	0.050	0.066	0.021	0.079
			Second hold time (F)	-0.188	<+/- 0.0001	-0.216	-0.159
			A*B	0.025	0.0756	-0.004	0.054
			A*C	-0.050	0.0066	-0.079	-0.021
			A*D	-0.025	0.0756	-0.054	0.004
			A*E	-0.438	<+/- 0.0001	-0.466	-0.409
			A*G	0.188	<+/- 0.0001	0.159	0.216
			B*D	0.188	<+/- 0.0001	0.159	0.216
Resolution (1,4-dioxane)	Natural Log	0.9916	Initial oven temp. (B)	-0.864	<+/- 0.0001	-0.999	-0.729
			Initial hold time (C)	-1.169	<+/- 0.0001	-1.304	-1.034
			Initial ramp rate (D)	-0.144	0.0382	-0.279	-0.009
			A*C	0.154	0.0292	0.019	0.288
			A*E	-0.983	<+/- 0.0001	-1.118	-0.848
Last Peak Retention Time	N/AP*	0.9996	Column flow (A)	-1.09803	0.0003	-1.40583	-0.79023
			Initial oven temp. (B)	-2.55284	<+/- 0.0001	-2.86064	-2.24504
			Initial hold time (C)	8.96541	<+/- 0.0001	8.65761	9.27321
			Initial ramp rate (D)	-6.20991	<+/- 0.0001	-6.51771	-5.90211
			Second oven temp. (E)	1.16222	0.0002	0.85442	1.47002
			Second hold time (F)	2.40184	<+/- 0.0001	2.09404	2.70964
			Second ramp rate (G)	-1.05547	0.0003	-1.36327	-0.74767
			A*E	-0.75447	0.0015	-1.06227	-0.44667
			A*F	-1.95659	<+/- 0.0001	-2.26439	-1.64879
			A*G	0.78172	0.0013	0.47392	1.08952
			B*D	2.38147	<+/- 0.0001	2.07367	2.68927

*N/AP: Not applicable

The graphical representation of the effects of the seven studied factors on each attribute are presented in Figure 5. The graphs are based on the statistical data presented in Table 10, and present each model term effect on response scale. The height of a given bar is equal to the magnitude of the corresponding model term's effect on the response. A blue bar corresponds to a positive effect, while a gray bar corresponds to a negative effect.[65]

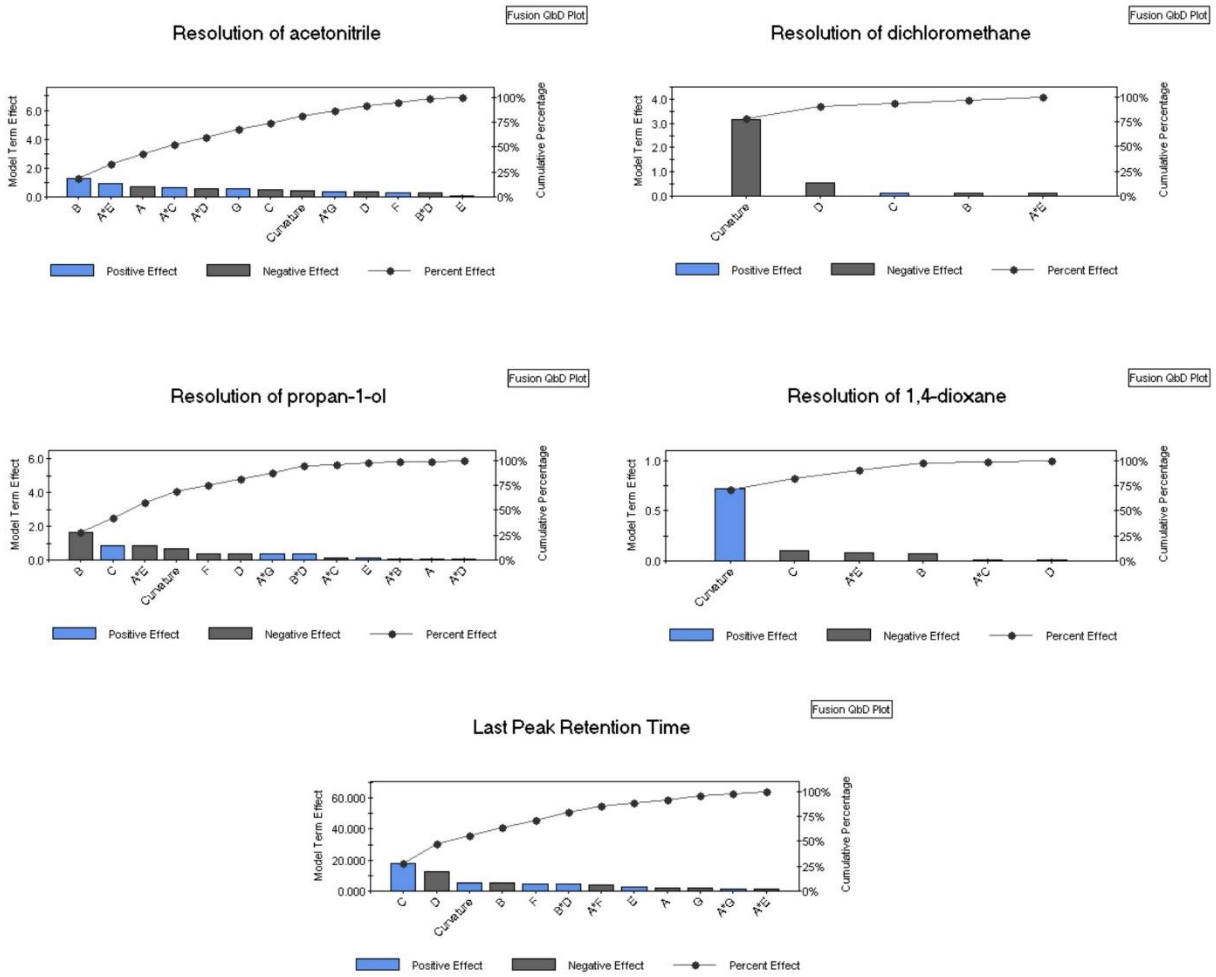


Figure 5. Evaluation of the statistical significance of model terms and their impact (positive or negative) on each considered response in the screening study (DoE-I) for the optimization of the general HS-GC method selectivity and total run time. Legend: Blue bar – positive effect; Gray bar – negative effect.

The regression model obtained for resolution of acetonitrile presented a $R^2 = 0.9996$, so it is valid. This value means that, at least, 99% of the variation of these response can be explained by this model. Regarding the statistical significance of model terms (factor or interaction between factors) on resolution of acetonitrile, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05. Additionally, from effect plot (Figure 5), it was possible to verify that initial oven temperature (B), interaction between column flow and second oven temperature (A*E), column flow (A), interaction between column flow and initial hold time (A*C), interaction between column flow and initial ramp rate (A*D), second ramp rate (G) and initial hold time (C) are the method parameters that present greater impact (positive or negative) on resolution of acetonitrile, taking into consideration a cumulative percentage of, approximately, 75%. Initial oven temperature (B) is the main critical factor and it has a positive impact. This means that when the initial oven

temperature (B) increases, the resolution of acetonitrile increases. Interaction between column flow and second oven temperature (A*E), interaction between column flow and initial hold time (A*C) and second ramp rate (G) have also a positive effect on resolution of acetonitrile. On the contrary, these method attribute is negatively affected by column flow (A), interaction between column flow and initial ramp rate (A*D) and initial hold time (C). This means that when the value of these factors increases, the resolution of acetonitrile decreases.

For resolution of dichloromethane, the obtained regression model showed a $R^2 = 1.0000$. From this value it is clear that the model adequately describes the relationship between the model terms and the considered CMA. Regarding the statistical significance of model terms (factor or interaction between factors) on resolution of dichloromethane, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05. From effect plot (Figure 5), it was possible to verify that this response is positively affected by initial hold time (C) and is negatively affected by initial ramp rate (D), initial oven temperature (B) and interaction between column flow and second hold time (A*E). This means that when the initial hold time (C) increases, the resolution of dichloromethane increases. Conversely, the resolution of dichloromethane decreases when the initial ramp rate (D) and initial oven temperature (B) increase. Initial ramp rate (D) is the tested variable with the most significant impact on this response.

Relatively to resolution of propan-1-ol, a regression model with a $R^2 = 0.9995$ was obtained, so it is valid. This value means that, at least, 99% of the variation of these response can be explained by this model. Regarding the statistical significance of model terms on resolution of propan-1-ol, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05, except for column flow, second hold time, interaction between column flow and initial oven temperature and interaction between column flow and initial ramp rate, which presented a *p*-value > 0.05. Considering effect plot (Figure 5), initial oven temperature (B), initial hold time (C), interaction between column flow and second oven temperature (A*E) and initial ramp rate (D) are the factors with the main impact (positive or negative) on resolution of propan-1-ol, taking into consideration a cumulative percentage of, approximately, 75%. Initial oven temperature (B) is the main critical factor and it has a negative impact. This means that when the initial oven temperature (B) increases, the resolution of propan-1-ol decreases. Interaction between column flow and second oven temperature (A*E) and initial ramp rate (D) have also a negative effect on resolution of propan-1-ol. On the contrary, these method attribute is positively affected by initial hold time (C). This means that when the value of this factor increases, the resolution of propan-1-ol increases.

The regression model obtained for resolution of 1,4-dioxane presented a $R^2 = 0.9916$, so it is valid. This value means that, at least, 99% of the variation of these response can be explained by this model. Regarding the statistical significance of model terms (factor or interaction between factors) on resolution of 1,4-dioxane, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05. From effect plot (Figure 5), it was possible to verify that this response is positively affected by interaction between column flow and initial hold time (A*C) and is negatively affected by initial hold time (C), interaction between column flow and second oven temperature (A*E), initial oven temperature (B) and initial ramp rate (D). This means that the resolution of 1,4-dioxane decreases when the initial hold time (C), initial oven temperature (B) and initial ramp rate (D) increase.

Finally, for last peak retention time, the obtained regression model showed a $R^2 = 0.9996$. From this value it is clear that the model adequately describes the relationship between the model terms and the considered CMA. Regarding the statistical significance of model terms (factor or interaction between factors) on retention time of the last eluting peak, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05. Additionally, from effect plot (Figure 5), it was possible to verify that initial hold time (C), initial ramp rate (D), initial oven temperature (B) and second hold time (F) are the method parameters that present greater impact (positive or negative) on retention time of the last eluting peak, taking into consideration a cumulative percentage of, approximately, 75%. Initial hold time (C) is the main critical factor and it has a positive impact. This means that when the initial hold time (C) increases, the retention time of the last eluting peak increases. Second hold time (F) has also a positive effect on last peak retention time. On the contrary, these method attribute is negatively affected by initial ramp rate (D) and initial oven temperature (B). This means that when the value of these factors increases, the retention time of the last eluting peak decreases. Initial hold time (C) is the tested variable with the most significant impact on this response.

The main objective of the screening test was to identify, within the seven factors in study, which were the ones with the most significant effects and to select the appropriate ranges of these CMPs for further development work. From the statistical analysis, it is verified that all fitting models are valid, since the R^2 are ≥ 0.99. This value means that, at least, 99% of the variation of each response can be explained by the respective model. Since the models are statistical valid, it was possible to evaluate the statistical significance of model terms (individual factors or interaction between factors) and their impact (positive or negative) on each response.

Overall, it can be concluded that, individually, initial oven temperature (B), initial hold time (C) and initial ramp rate (D) are the CMPs that present the major impact in the models obtained for the studied attributes. Regarding initial oven temperature (B), it was verified that this factor has a negative effect on the resolution of dichloromethane, propan-1-ol and 1,4-dioxane, and on retention time of the last eluting peak. The goal is to maximize the considered resolutions and to minimize the last peak retention time, so the strategy will be to reduce the initial oven temperature (B), without compromising the achievement of the acceptance criteria of the resolution of acetonitrile and the last peak retention time. The improvement of the resolution of the target analytes promoted by the decrease of the initial oven temperature had previously been reported by Liu *et al.*[85], in a work in which the authors intended to determine volatile residual solvents in traditional Chinese medicines applying the HS-SPME-GC technique.

Regarding initial hold time (C), it was verified that this factor has a negative effect on resolutions of acetonitrile and 1,4-dioxane. Thus, the referred resolutions could be increased by reducing the initial hold time. However, it is necessary to establish a reasonable compromise so that the resolution of dichloromethane and the resolution of propan-1-ol do not decrease with the decrease of initial hold time value.

At last, regarding initial ramp rate (D), it was verified that this factor has a negative effect on all considered responses. In this way, the resolutions of acetonitrile, dichloromethane, propan-1-ol and 1,4-dioxane could be increased by setting a slower initial ramp rate. However, it is necessary to establish a reasonable compromise so that the retention time of the last eluting peak do not increase with the decrease of initial ramp rate value. In addition, column flow (A) presents also a significant impact, mainly when combined with other parameters. Based on the outcome of the screening tests, it was possible to limit the number of critical variables before optimization design. Therefore, column flow, initial oven temperature, initial hold time and initial ramp rate were further studied and optimized. Additionally, it was possible to narrow the range of study for these variables based on the prediction of the best combinations. These predicted combinations were reported considering the acceptance criteria, presented in Table 11, for the method responses identified as critical during the screening tests.

Table 11. Method performance goals considered in the prediction of the best combinations of the column flow, initial oven temperature, initial hold time and initial ramp rate conditions.

Method responses	Goal	Lower bound	Upper bound
Resolution – acetonitrile	Maximize	1.5	N/AP*
Resolution – dichloromethane	Maximize	1.5	N/AP*
Resolution – propan-1-ol	Maximize	1.5	N/AP*
Resolution – 1,4-dioxane	Maximize	1.5	N/AP*
Last peak retention time	Minimize	N/AP*	26 minutes

*N/AP: Not applicable

Fourteen answers were generated by the Fusion QbD® software[65] and ordered, in Table 12, from global best to worst in terms of meeting the specified method performance goals.

Table 12. Combinations of the critical variables conditions and the respective results for critical method attributes, predicted by Fusion QbD® software based on the obtained statistical models.

	Variable Settings				Predicted Responses				
Prediction	**Column flow (mL/min)**	**Initial oven temperature (ºC)**	**Initial hold time (min)**	**Initial ramp rate (ºC/min)**	**Resolution (acetonitrile)**	**Resolution (dichloromethane)**	**Resolution (propan-1-ol)**	**Resolution (1,4-dioxane)**	**Last Peak Retention Time**
1	1.0	30.0	0.0	2.0	1.7	0.0	1.6	2.0	21.891
2	1.3	30.0	0.0	2.0	1.5	0.1	1.5	2.8	19.332
3	1.8	30.0	1.7	2.0	1.5	0.1	1.5	1.6	26.978
4	1.6	34.5	5.2	2.0	1.5	0.0	1.5	1.5	23.138
5	1.0	30.0	0.0	10.0	1.5	0.0	1.5	6.2	26.743
6	2.0	37.4	6.0	2.0	1.5	0.0	1.5	2.1	23.747
7	1.9	35.8	8.3	2.0	1.6	0.0	1.5	1.6	27.517
8	1.0	34.4	0.0	2.0	1.5	0.0	1.5	7.1	30.964
9	1.4	30.0	2.6	2.0	1.5	0.0	1.5	2.8	28.726
10	1.0	34.5	0.0	2.1	1.5	0.0	1.5	7.1	27.329
11	1.0	30.0	0.0	3.7	1.4	0.0	1.5	7.6	28.647
12	1.0	30.0	6.0	30.0	1.2	0.0	1.5	4.2	18.872
13	1.0	33.6	17.6	2.0	1.4	0.0	1.5	0.1	43.476
14	1.3	41.4	20.0	2.0	1.2	0.0	1.5	0.1	34.907

Globally, from the results provided by the software predictions, it was possible to conclude that the column flow should be between 1.0 and 2.0 mL/min, the initial oven temperature should be less than 45 ºC, the initial hold time should be less than 10 minutes and the initial ramp rate should be equal to or slower than 2.0 ºC/min. Then, an optimization study will be performed.

Optimization tests

In order to increase the critical resolutions (acetonitrile, dichloromethane, propan-1-ol and 1,4-dioxane), it was decided to optimize the column flow, initial oven temperature, initial hold time and initial ramp rate, which were the most significant method variables identified in the previous screening test. A DoE was the experimental strategy selected and performed during this optimization test. Table 13 presents the factors studied during DoE-II and their ranges. For each variable, the range was defined taking into account the conclusions of the screening study.

Table 13. GC variables considered in the optimization tests for the optimization of the general HS-GC method selectivity and total run time, and range in which these factors were studied during DoE-II. Legend: CF_0 – reference value of the column flow factor; OT_0 – reference value of the initial oven temperature factor; IHt_0 – reference value of the initial hold time factor and IRR_0 – reference value of the initial ramp rate factor.

Variables	Type	Units	-1 level	0 level	1 level
Column Flow	Continuous	mL/min	$CF_0 - 0.3$	CF_0	$CF_0 + 0.2$
Initial oven temperature	Discrete numeric	ºC	$OT_0 - 2$	OT_0	$OT_0 + 3$
Initial hold time	Discrete numeric	min	$IHt_0 - 2.5$	IHt_0	$IHt_0 + 2.5$
Initial ramp rate	Continuous	ºC/min	$IRR_0 - 2.0$	IRR_0	$IRR_0 + 2.5$

The attributes considered during this DoE and their acceptance criteria are presented in Table 14.

Table 14. Method responses considered in the optimization tests for the optimization of the general HS-GC method selectivity and total run time, and their goals, which must be fulfilled.

Method responses	Acceptance criteria
Resolution – acetonitrile	≥ 1.5
Resolution – dichloromethane	≥ 1.5
Resolution – propan-1-ol	≥ 1.5
Resolution – 1,4-dioxane	≥ 1.5
Last peak retention time	≤ 26 minutes

A 2^{4-1} Resolution IV design with ten experiments including two center points was applied to determine the optimum operating conditions that allow the optimization of the selected chromatographic responses. This experimental plan, DoE-II, was automatically designed by Fusion QbD® software[65] and is presented in Table 15. During the optimization study, a standard solution containing the 29 analytes at 100% of the ICH/VICH limits and control levels was injected to evaluate the method performance for each target analyte. For each experimental run, the following sequence was injected: Blank (diluent) and working standard solution, both injected once.

For the chromatogram obtained in each experimental run, the resolution of all target analytes and the retention time of the last eluting peak were evaluated. The results obtained for the method attributes considered as critical are presented in Table 15.

Table 15. Optimization tests for the optimization of the general HS-GC method selectivity and total run time: Experimental design of experiments (DoE) run sequence and the respective chromatographic results.

	Critical Method Parameters - input				**Critical Method Attributes – output**				
Run No.	**Column flow (A)**	**Initial oven temp. (B)**	**Initial hold time (C)**	**Initial ramp rate (D)**	**Resolution acetonitrile**	**Resolution Dichloromethane**	**Resolution propan-1-ol**	**Resolution 1,4-dioxane**	**Last Peak Retention Time**
1	$CF_0 - 0.3$	$OT_0 + 3$	$IHt_0 + 2.5$	$IRR_0 - 2.0$	1.8	1.5	3.2	3.5	25.974
2	$CF_0 + 0.2$	$OT_0 + 3$	$IHt_0 + 2.5$	$IRR_0 + 2.5$	1.7	1.4	3.1	4.0	16.730
3	$CF_0 - 0.3$	$OT_0 - 2$	$IHt_0 + 2.5$	$IRR_0 + 2.5$	1.3	1.8	3.3	3.9	18.280
4	$CF_0 - 0.3$	$OT_0 + 3$	$IHt_0 - 2.5$	$IRR_0 + 2.5$	2.3	0.0	2.9	2.7	12.390
5	$CF_0 - 0.3$	$OT_0 - 2$	$IHt_0 - 2.5$	$IRR_0 - 2.0$	1.4	1.9	3.3	1.1	30.864
6	$CF_0 + 0.2$	$OT_0 + 3$	$IHt_0 - 2.5$	$IRR_0 - 2.0$	1.7	1.3	3.1	3.5	20.544
7	CF_0	OT_0	IHt_0	IRR_0	1.5	1.5	3.3	3.9	16.645
8	CF_0	OT_0	IHt_0	IRR_0	1.6	1.5	3.3	3.9	16.637
9	$CF_0 + 0.2$	$OT_0 - 2$	$IHt_0 - 2.5$	$IRR_0 + 2.5$	1.9	0.0	3.1	3.2	12.861
10	$CF_0 + 0.2$	$OT_0 - 2$	$IHt_0 + 2.5$	$IRR_0 - 2.0$	1.2	2.1	3.2	1.2	35.022

According to the chromatographic profiles obtained for each of the tested combination of the factors, it was possible to verify that in some experimental runs, acceptable results were achieved both for the resolutions considered as critical and for the resolutions of the remaining target analytes, namely in Runs 1, 2, 3, 6, 7 and 8.

Using the Fusion QbD® software,[65] a statistical analysis of the results from DoE was performed, as shown in Table 16. A regression model was obtained for each evaluated critical method attribute.

Table 16. Statistical analysis results (outcome-based observed relationships) of the DoE study, DoE-II, applied in the optimization tests for the optimization of the general HS-GC method selectivity and total run time.

Critical Method Attribute (CMA)	Model		Critical Method Parameters (CMPs)				
	Transformation	R^2	Statistical significant CMP or interaction	Coefficient value	*p*-value ($p<0.05$)	Lower 95% conf. Limit	Upper 95% conf. Limit
Resolution (acetonitrile)	N/AP*	0.9627	Initial oven temp. (B)	0.215	0.0007	0.140	0.290
			Initial hold time (C)	-0.163	0.0026	-0.238	-0.087
			Initial ramp rate (D)	0.139	0.0052	0.063	0.214
			A*B	-0.137	0.0055	-0.212	-0.062
Resolution (dichloromethane)	N/AP*	0.9759	Initial oven temp. (B)	-0.106	0.0009	-0.140	-0.073
			Initial hold time (C)	0.081	0.0026	0.048	0.115
			Initial ramp rate (D)	-0.081	0.0026	-0.115	-0.048
Resolution (propan-1-ol)	N/AP*	0.9904	Initial oven temp. (B)	-0.277	0.0011	-0.369	-0.185
			Initial hold time (C)	0.377	0.0004	0.283	0.470
			Initial ramp rate (D)	-0.379	0.0004	-0.472	-0.285
			A*B	0.276	0.0012	0.183	0.369
Resolution (1,4-dioxane)	N/AP*	0.9498	Initial oven temp. (B)	0.538	0.0146	0.176	0.899
			Initial hold time (C)	0.263	0.1143	-0.099	0.624
			Initial ramp rate (D)	0.563	0.0125	0.201	0.924
			A*C	-0.638	0.0081	-0.999	-0.276
Last Peak Retention Time	N/AP*	0.9987	Initial oven temp. (B)	-2.67363	0.0001	-3.08404	-2.26321
			Initial hold time (C)	2.41838	0.0001	2.00796	2.82879
			Initial ramp rate (D)	-6.51788	<+/- 0.0001	-6.92829	-6.10746
			A*C	6.20106	0.0001	1.75796	2.57879

*N/AP: Not applicable

The graphical representation of the effects of the four studied factors on each attribute are presented in Figure 6. The graphs are based on the statistical data presented in Table 16, and present each model term effect on response scale. The height of a given bar is equal to the magnitude of the corresponding model term's effect on the response. A blue bar corresponds to a positive effect, while a gray bar corresponds to a negative effect.[66]

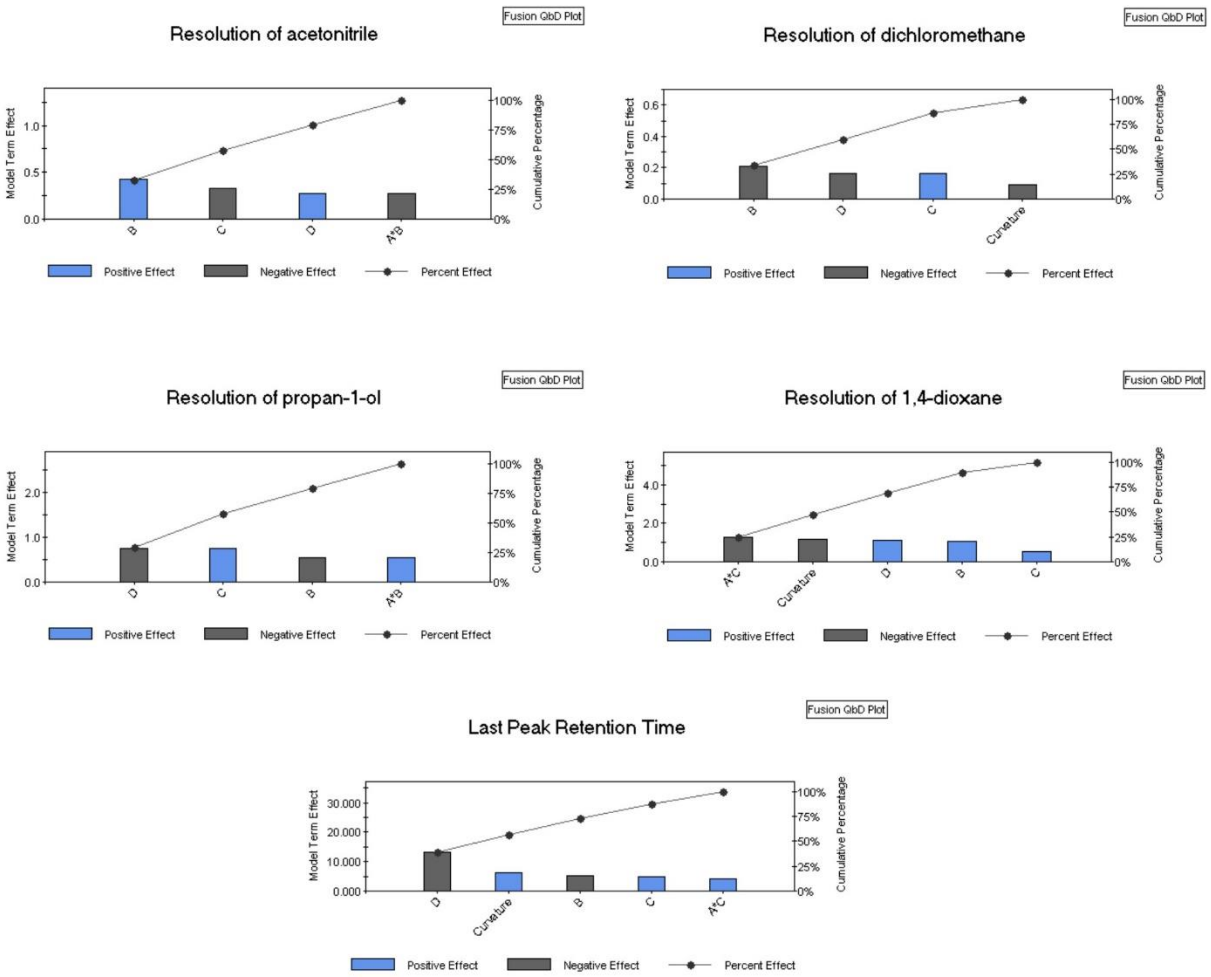


Figure 6. Evaluation of the statistical significance of model terms and their impact (positive or negative) on each considered response in the optimization study (DoE-II) for the optimization of the general HS-GC method selectivity and total run time. Legend: Blue bar – positive effect; Gray bar – negative effect.

The regression model obtained for the resolution of acetonitrile presented a $R^2 = 0.9627$, so it is valid. This value means that, at least, 96 % of the variation of these response can be explained by this model. Regarding the statistical significance of model terms (factor or interaction between factors) on resolution of acetonitrile, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05. Additionally, from effect plot (Figure 6), it was possible to verify that initial oven temperature (B), initial hold time (C), initial ramp rate (D) and interaction between column flow and initial oven temperature (A*B) are the method parameters that present greater impact (positive or negative) on the resolution of acetonitrile. Initial oven temperature (B) is the main critical factor and it has a positive impact, such as in the screening tests. This means that when the initial oven temperature (B) increases, the resolution of acetonitrile increases. Initial ramp rate (D) has also a positive effect on resolution of acetonitrile. On the contrary, these method attribute is negatively affected

by initial hold time (C), as analyzed in the screening studies, and interaction between column flow and initial oven temperature (A*B). This means that this response decreases when the initial hold time (C) increases.

For the resolution of dichloromethane, the obtained regression model showed a R^2 = 0.9759. From this value it is clear that the model adequately describes the relationship between the model terms and the considered CMA. Regarding the statistical significance of model terms (factor or interaction between factors) on resolution of dichloromethane, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05. From effect plot (Figure 6), it was possible to verify that this response is positively affected by initial hold time (C), as analyzed in screening studies. This means that when the initial hold time increases, the resolution of dichloromethane increases. Conversely, as analyzed in screening studies, these response is negatively affected by initial oven temperature (B) and initial ramp rate (D). This means that when the initial oven temperature and the initial ramp rate increase, the resolution of dichloromethane decreases. Initial oven temperature (B) is the tested variable with the most significant impact on this response.

Relatively to the resolution of propan-1-ol, a regression model with a R^2 = 0.9904 was obtained, so it is valid. This value means that, at least, 99 % of the variation of these response can be explained by this model. Regarding the statistical significance of model terms on resolution of propan-1-ol, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05. Considering effect plot (Figure 6), initial ramp rate (D), initial hold time (C), initial oven temperature (B) and interaction between column flow and initial oven temperature (A*B) are the factors with the main impact (positive or negative) on the resolution of propan-1-ol. As seen in screening studies, initial ramp rate (D) and initial oven temperature (B) have a negative impact on resolution of propan-1-ol. This means that when the initial ramp rate (D) and initial oven temperature (B) increase, the resolution of propan-1-ol decreases. On the contrary, as analyzed in screening studies, these method attribute is positively affected by initial hold time (C). This means that these response increases when the initial hold time (C) increases. Additionally, interaction between column flow and initial oven temperature (A*B) has also a positive effect on resolution of propan-1-ol.

The regression model obtained for resolution of 1,4-dioxane presented a R^2 = 0.9498, so it is valid. This value means that, at least, 94 % of the variation of these response can be explained by this model. Regarding the statistical significance of model terms (factor or interaction between factors) on resolution of 1,4-dioxane, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05, except for initial

hold time (C), which presents a *p*-value > 0.05. From effect plot (Figure 6), it was possible to verify that this response is positively affected by initial ramp rate (D) and initial oven temperature (B). This means that the increase of these factors promotes the increase of the resolution of 1,4-dioxane. Conversely, these response is negatively affected by interaction between column flow and initial hold time (A*C).

Finally, for the last peak retention time, the obtained regression model showed a R^2 = 0.9987. From this value it is clear that the model adequately describes the relationship between the model terms and the considered CMA. Regarding the statistical significance of model terms (factor or interaction between factors) on resolution of dichloromethane, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05. Additionally, from effect plot (Figure 6), it was possible to verify that initial ramp rate (D), initial oven temperature (B), initial hold time (C) and interaction between column flow and initial oven temperature (A*B) are the method parameters that present greater impact (positive or negative) on retention time of the last eluting peak. As seen in screening studies, initial ramp rate (D) and initial oven temperature (B) have a negative impact on retention time of the last eluting peak. This means that when the initial ramp rate (D) and initial oven temperature (B) increase, the retention time of the last eluting peak decreases. On the contrary, as analyzed in screening studies, these method attribute is positively affected by initial hold time (C). Additionally, interaction between column flow and initial hold time (A*C) has also a positive effect on the retention time of the last eluting peak. Initial ramp rate (D) is the tested variable with the most significant impact on this response.

From the statistical analysis, it is verified that all fitting models are valid, since the R^2 are ≥ 0.95. This value means that, at least, 95% of the variation of each response can be explained by the respective model. Since the models are statistical valid, it was possible to evaluate the statistical significance of model terms (individual factors or interaction between factors) and their impact (positive or negative) on each response. Based on this information and considering that the main objective of the optimization tests was to define which operating conditions will result in the best method performance, a Method Operable Design Region (MODR) and the Normal Operable Conditions (NOC) were established, considering the method response goals previously presented in Table 11.

Based on the model obtained for each response, it is possible to generate a 2D contour plot for each of the five responses as a function of two experiment variables: initial oven temperature (X-axis) and initial ramp rate (Y-axis). During the statistical analysis, it was possible to conclude that, in general, initial oven temperature and initial ramp rate were more

critical than column flow and initial hold time. For that reason, in the construction of contour plots, it was decided to vary initial oven temperature and initial ramp rate within their experiment ranges and to fix the value of column flow and initial hold time. Column flow was considered the horizontal trellis variable and the -1, 0 and 1 levels, defined in Table 13, were the constant level settings used in the left, center and right graphs, respectively in horizontal series. Initial hold time was considered the vertical trellis variable and the 1, 0 and -1 levels, defined in Table 13, were the constant level settings used in the first, second and third rows graphs, respectively in vertical series.

In these plots, the graph region where the obtained results for the considered response are unacceptable is represented by a single color. In this work, red, light blue, dark blue, gray and green correspond, respectively, to the shaded region where the obtained results for the resolution of acetonitrile, resolution of dichloromethane, resolution of propan-1-ol, resolution of 1,4-dioxane and last peak retention time are unacceptable, considering the acceptance criteria defined in Table 11. It was intended to identify the unshaded region, where the graphed variable settings will allow the achievement of acceptable results for all considered responses simultaneously, and it is possible to assure the desired method performance. So, as multiple responses were involved, to obtain the combined design space (MODR) the contour plot obtained for each analyzed response were overlaid in a single plot. Figure 7 shows the different overlay graphics (knowledge space) obtained for this system.

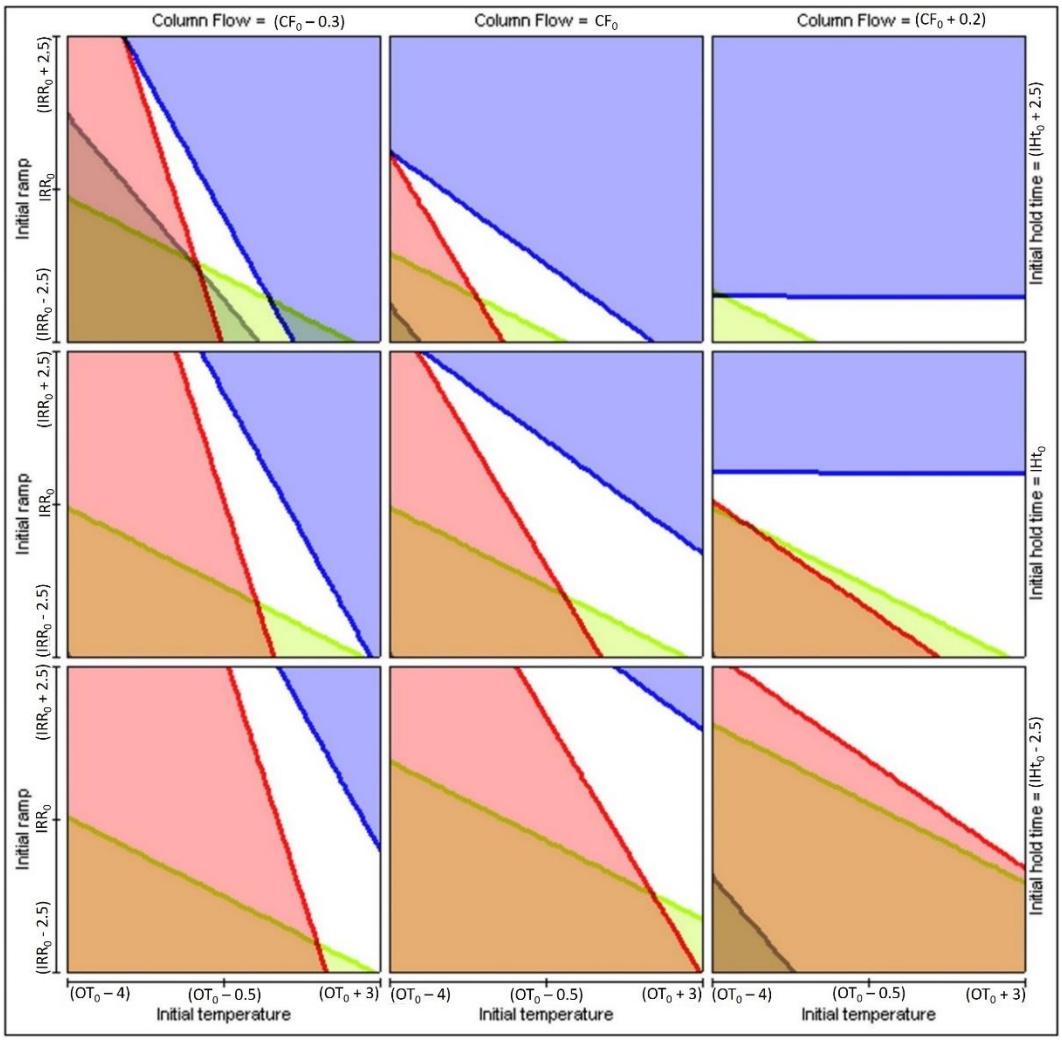


Figure 7. Establishment of the knowledge space.

The knowledge space consists in nine overlay graphs, which represents all the different possible combinations for the four variables in study. From the analysis of Figure 7, it was possible to verify that the overlay plots in which the column flow was set at (CF_0 + 0.2) mL/min, were those that allow obtaining more robust unshaded/white region (MODR). In the screening studies it was concluded that, in general, the decrease of the initial oven temperature, initial hold time and initial ramp rate resulted in better method performances. Among the three graphs with the column flow equal to (CF_0 + 0.2) mL/min (Figure 8), the one whose MODR allowed to establish the best compromise between these working requirements was the overlay graph in which the initial hold time was set at (IHt_0 + 2.5) min.

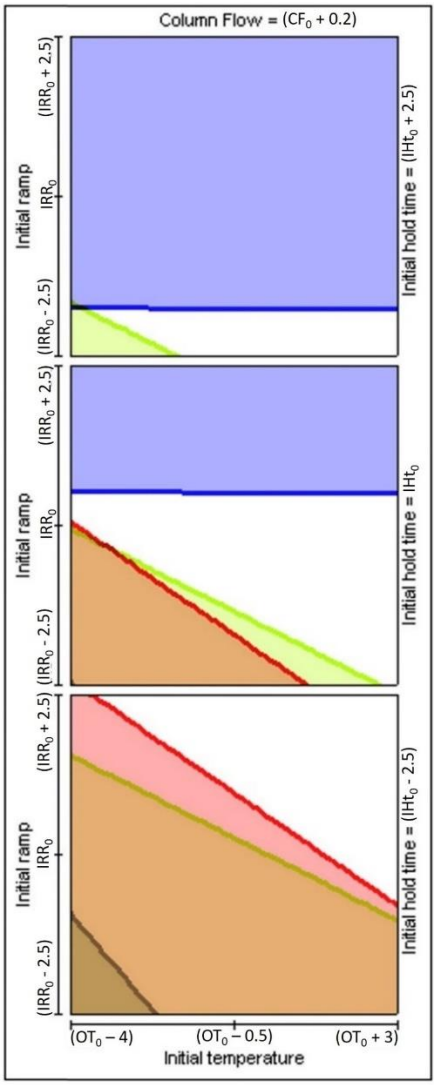


Figure 8. Overlaid graphs, among the knowledge space, with a more robust unshaded/white region (MODR).

After the best design space was selected, it was intended to define the NOC. Each combination of the CMPs inside the MODR is a potential working point. Among the potential working points, the one that represents the optimum experimental conditions should be selected to obtain better response values and, consequently, the highest global method performance quality. Therefore, as shown in Figure 9, the selected NOC (point T) corresponds to the following experimental values: column flow, (CF_0 + 0.2) mL/min; initial oven temperature, OT_0 ºC; initial hold time, (IHt_0 + 2.5) min; initial ramp rate, (IRR_0 – 2.0) ºC/min.

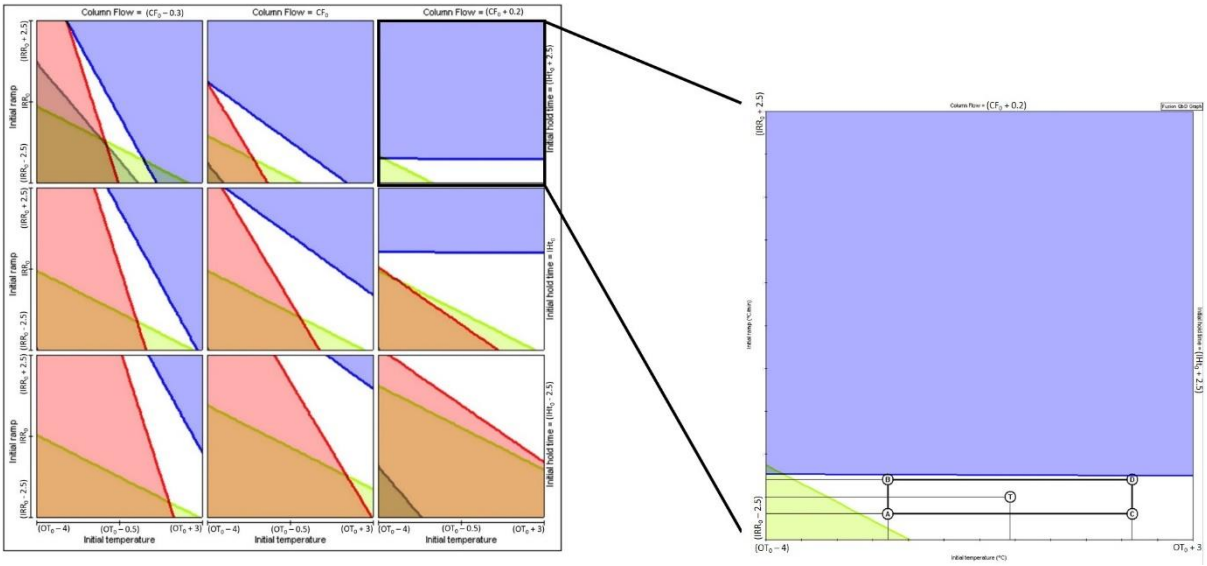


Figure 9. Selection of the Normal Operable Conditions (NOC) of the general HS-GC method.

After NOC establishment, it is necessary to verify if there is good agreement between the response model predictions generated by the Fusion QbD® software,[65] shown in Table 17, and the real results obtained for optimal conditions (NOC).

Table 17. Model predictions generated by the Fusion QbD® software for Normal Operable Conditions (NOC).

Critical Method Attribute (CMA)	Normal Operable Conditions (NOC)		
	Prediction	**Lower 95% conf. Limit**	**Upper 95% conf. Limit**
Resolution – acetonitrile	1.7	1.4	1.9
Resolution – dichloromethane	3.2	3.1	3.3
Resolution – propan-1-ol	1.3	1.1	1.6
Resolution – 1,4-dioxane	2.8	1.6	4.1
Last peak retention time	22.822	21.413	24.230

Additionally, this verification was performed for other operating conditions along MODR, in order to indicate the robustness of the method. Four different points (A, B, C and D) were selected to be verified. The operating conditions for these four points and the response model predictions generated by the software were presented in Tables 18 and 19, respectively.

Table 18. Operating conditions of the four experimental points (A, B, C and D) along Method Operable Design Region (MODR) considered in the verification of the validity of the model predictions generated by the Fusion QbD® software.

Point	Column Flow (ml/min)	Initial oven temperature (ºC)	Initial hold time (min)	Initial ramp rate (ºC/min)
A	NOC	NOC - 2	NOC	NOC - 0.2
B	NOC	NOC - 2	NOC	NOC + 0.2
C	NOC	NOC + 2	NOC	NOC - 0.2
D	NOC	NOC + 2	NOC	NOC + 0.2

Table 19. Model predictions generated by the Fusion QbD® software for four experimental points (A, B, C and D) along Method Operable Design Region (MODR).

Critical Method Attribute (CMA)	A			B			C			D		
	Prediction	**Lower 95% conf. Limit**	**Upper 95% conf. Limit**	**Prediction**	**Lower 95% conf. Limit**	**Upper 95% conf. Limit**	**Prediction**	**Lower 95% conf. Limit**	**Upper 95% conf. Limit**	**Prediction**	**Lower 95% conf. Limit**	**Upper 95% conf. Limit**
Resolution (acetonitrile)	1.6	1.3	1.9	1.6	1.3	1.9	1.7	1.5	2.0	1.7	1.5	2.0
Resolution (dichloromethane)	3.3	3.2	3.4	3.3	3.2	3.4	3.1	3.0	3.2	3.1	3.0	3.2
Resolution (propan-1-ol)	1.4	1.1	1.7	1.3	1.0	1.6	1.4	1.1	1.7	1.3	1.0	1.6
Resolution (1,4-dioxane)	2.3	1.1	3.6	2.4	1.2	3.7	3.2	1.9	4.5	3.3	2.1	4.6
Last peak retention time	25.540	24.069	27.011	24.381	22.930	25.832	21.262	19.820	22.704	20.103	18.682	21.525

Note that this verification was performed only after the optimization of the operating conditions related to the sensitivity of the method.

3.2.3.2. Optimization of the general HS-GC method sensitivity

In addition to the selectivity, the sensitivity is a relevant analytical characteristic that must be improved in order to ensure the desired method performance, previously defined in the ATP. In this work, the signal-to-noise ratio (S/N) of each target analyte was evaluated as a sensitivity requirement. It is intended to maximize the S/N without increasing the required sample concentration and the injection volume nor compromising the shape of peaks and the efficiency of the chromatographic separation. Thus, it is necessary to guarantee that the general HS-GC method has adequate sensitivity to detect and quantify, with accuracy and precision, these low concentration levels. Then, to increase the S/N of each target analyte and, consequently, to improve the method's sensitivity, the critical parameters, whose variability have an impact on the S/N, were selected to be studied and optimized. Parameters related to headspace sampler, such as vial equilibration temperature, vial equilibration time, vial pressure were considered in this experimental plan. Split at vent and split ratio were also evaluated.

Screening tests

During the development of this general HS-GC method, a screening study was performed to investigate the potential impact of vial equilibration temperature, vial equilibration time, vial pressure, split ratio and split at vent, parameters identified as critical factors during the risk assessment exercise, on the S/N values of the target analytes and to verify the method performance. In order to understand the effects of these variables and its interactions (multivariate analysis), a DoE approach was applied. Five factors were studied in within defined ranges: vial equilibration temperature [(T_0-20) ºC – (T_0+20) ºC], vial equilibration time [(t_0-10) min – (t_0+10) min], vial pressure [(vp_0-5) psi – (vp_0+5) psi], split ratio [(sr_0-1) – (sr_0+2)] and split at vent [(s_0-20) mL/min – (s_0+40) mL/min]. For each variable, the range was defined based on available information from previous experiments. The variables and their respective ranges are presented in Table 20.

Table 20. Variables considered in the screening tests for the optimization of the general HS-GC method sensitivity, and range in which these factors were studied during DoE-III. Legend: T_0 – reference value of the vial equilibration temperature factor; t_0 – reference value of the vial equilibration time; vp_0 – reference value of the vial pressure; sr_0 – reference value of the split ratio factor and s_0 – reference value of the split at vent factor.

Variables	Type	Units	Range
Vial equilibration temperature	Continuous	ºC	$(T_0-20) – (T_0+20)$
Vial equilibration time	Continuous	min	$(t_0-10) – (t_0+10)$
Vial pressure	Continuous	psi	$(vp_0-5) – (vp_0+5)$
Split ratio	Continuous	-	$(sr_0-1) – (sr_0+2)$
Split at vent	Continuous	mL/min	$(s_0-20) – (s_0+40)$

In order to assess the impact of these variables on method performance, the analytical responses and its goals were defined, as shown in Table 21.

Table 21. Method response considered in the screening tests for the optimization of the general HS-GC method sensitivity, and its goal, which must be fulfilled.

Method response	Goal
Signal-to-noise ratio (S/N)	Maximize for all target analytes

A two-level Plackett-Burman design with fourteen experiments including two center points was applied to determine the variables and interactions between variables that have significant effects on selected method attributes. This experimental plan, DoE-III, was automatically designed by Fusion QbD® software[65] and is presented in Table 22. The DoE design was translated into a sample set for GC analysis and, for each experiment indicated in the Table 22, a different method was created in Empower software. During the screening study, a standard solution containing the 29 analytes at 100% of the ICH/VICH limits and control levels was injected to evaluate the method performance for each target analyte. For each experimental run, the following sequence was injected: Blank (diluent) and working standard solution, both injected once.

For each experimental run, the S/N values of all target analytes were monitored. According to the chromatographic profiles obtained for each experimental run, it was possible to verify that all tested combinations demonstrated enough sensitivity to determine the 29 target analytes in the working standard solution. However, some analytes, such as acetonitrile, dichloromethane, propan-1-ol, tetrahydrofuran, 1,4-dioxane, DIPEA and 4-hydroxy-4-methylpentan-2-one, had low sensitivities. Thus, the S/N values of these analytes were

considered as critical and for that reason, operating conditions were optimized to provide better sensitivity to these analytes. The obtained results are presented in Table 22.

Based on the obtained results (Table 22), it was noticed that the operating conditions defined for run 13 were the ones that allowed to obtain the higher S/N values for these analytes, with exception of DIPEA and 4-hydroxy-4-methylpentan-2-one. This combination of factors corresponds to: vial equilibration temperature, (T_0+20) ºC; vial equilibration time, (t_0+10) min, split ratio, (sr_0-1); vial pressure, (vp_0-5) psi and split at vent, (s_0-20) mL/min.

Table 22. Screening tests for the optimization of the general HS-GC method sensitivity: Experimental design of experiments (DoE) run sequence and the respective chromatographic results in terms of critical S/N values.

	Critical Method Parameters (CMPs) – input					Critical Method Attributes (CMAs) – output						
Run No.	**Equilibration temperature (A)**	**Equilibration time (B)**	**Split ratio (C)**	**Vial pressure (D)**	**Split at vent (E)**	**S/N acetonitrile**	**S/N dichloromethane**	**S/N propan-1-ol**	**S/N tetrahydrofuran**	**S/N 1,4-dioxane**	**S/N DIPEA**	**S/N 4-hydroxy-4-methylpentan-2-one**
1	$T_0 - 20$	$t_0 + 10$	$sr_0 + 2$	$vp_0 + 5$	$s_0 - 20$	943	886	4 807	3 296	207	317	771
2	$T_0 + 20$	$t_0 - 10$	$sr_0 + 2$	$vp_0 + 5$	$s_0 + 40$	1 872	1 541	12 189	4 997	404	670	1 454
3	$T_0 + 20$	$t_0 - 10$	$sr_0 - 1$	$vp_0 - 5$	$s_0 + 40$	2 675	2 182	16 485	7 052	570	1 430	1 115
4	$T_0 - 20$	$t_0 - 10$	$sr_0 + 2$	$vp_0 - 5$	$s_0 + 40$	681	683	3 382	2 537	147	128	458
5	$T_0 + 20$	$t_0 + 10$	$sr_0 - 1$	$vp_0 + 5$	$s_0 + 40$	1 879	1 561	11 766	5 100	406	84	1 510
6	$T_0 + 20$	$t_0 + 10$	$sr_0 + 2$	$vp_0 - 5$	$s_0 - 20$	3 262	2 595	17 948	8 634	683	368	1 090
7	$T_0 - 20$	$t_0 - 10$	$sr_0 - 1$	$vp_0 + 5$	$s_0 - 20$	935	876	4 467	3 313	204	447	875
8	T_0	t_0	sr_0	vp_0	s_0	1 386	1 213	7 103	4 241	289	702	409
9	$T_0 - 20$	$t_0 - 10$	$sr_0 - 1$	$vp_0 - 5$	$s_0 - 20$	1 391	1 263	6 482	4 846	299	800	857
10	$T_0 + 20$	$t_0 - 10$	$sr_0 + 2$	$vp_0 + 5$	$s_0 - 20$	2 733	2 117	17 382	6 857	591	1 225	3 400
11	$T_0 - 20$	$t_0 + 10$	$sr_0 + 2$	$vp_0 - 5$	$s_0 + 40$	678	679	3 397	2 510	148	124	464
12	T_0	t_0	sr_0	vp_0	s_0	2 003	1 775	10 294	6 195	425	1 043	658
13	$T_0 + 20$	$t_0 + 10$	$sr_0 - 1$	$vp_0 - 5$	$s_0 - 20$	3 940	3 048	21 558	10 149	821	573	1 329
14	$T_0 - 20$	$t_0 + 10$	$sr_0 - 1$	$vp_0 + 5$	$s_0 + 40$	634	635	3 336	2 357	137	103	569

Using the Fusion QbD® software,[65] a statistical analysis of the results from DoE was performed, as shown in Table 23. For each evaluated critical method attribute, a regression model was obtained.

Table 23. Statistical analysis results (outcome-based observed relationships) of the DoE study, DoE-III, applied in the screening tests for the optimization of the general HS-GC method sensitivity.

Critical Method Attribute (CMA)	Model		Critical Method Parameters (CMPs)				
	Transformation	R^2	Statistical significant CMP or interaction	Coefficient value	p-value (p<0.05)	Lower 95% conf. Limit	Upper 95% conf. Limit
S/N (acetonitrile)	Natural Log	0.9783	Equilibration temperature (A)	0.57	< 0.0001	0.50	0.64
			Split ratio (C)	-0.07	0.0555	-0.14	0.00
			Vial pressure (D)	-0.12	0.0044	-0.19	-0.05
			Split at vent (E)	-0.23	< 0.0001	-0.30	-0.16
S/N (dichloromethane)	Natural Log	0.9714	Equilibration temperature (A)	0.48	< 0.0001	0.41	0.55
			Split ratio (C)	-0.06	0.0675	-0.13	0.01
			Vial pressure (D)	-0.12	0.0042	-0.19	-0.05
			Split at vent (E)	-0.20	0.0001	-0.27	-0.13
S/N (propan-1-ol)	Natural Log	0.9722	Equilibration temperature (A)	0.67	< 0.0001	0.58	0.75
			Vial pressure (D)	-0.08	0.0544	-0.16	0.00
			Split at vent (E)	-0.19	0.0004	-0.28	-0.11
S/N (tetrahydrofuran)	Natural Log	0.9655	Equilibration temperature (A)	0.41	< 0.0001	0.34	0.48
			Split ratio (C)	-0.07	0.0509	-0.14	0.00
			Vial pressure (D)	-0.12	0.0032	-0.19	-0.05
			Split at vent (E)	-0.21	0.0001	-0.27	-0.14
S/N (1,4-dioxane)	Natural Log	0.9756	Equilibration temperature (A)	0.56	< 0.0001	0.49	0.63
			Split ratio (C)	-0.06	0.0788	-0.14	0.01
			Vial pressure (D)	-0.11	0.0084	-0.18	-0.04
			Split at vent (E)	-0.23	0.0001	-0.30	-0.15
S/N (DIPEA)	Natural Log	0.7729	Equilibration temperature (A)	0.39	0.0353	0.03	0.75
			Equilibration time (B)	-0.55	0.0070	-0.91	-0.19
			Split at vent (E)	-0.45	0.0196	-0.81	-0.09
S/N (4-hydroxy-4-methylpentan-2-one)	N/AP*	0.8652	Equilibration temperature (A)	327.76	0.0001	216.09	439.43
			Vial pressure (D)	107.93	0.0566	-3.74	219.59

*N/AP: Not applicable

The graphical representation of the effects of the seven studied factors on each attribute are presented in Figure 10. The graphs are based on the statistical data presented in Table 23, and present each model term effect on response scale. The height of a given bar is equal to the magnitude of the corresponding model term's effect on the response. A blue bar corresponds to a positive effect, while a gray bar corresponds to a negative effect.[65]

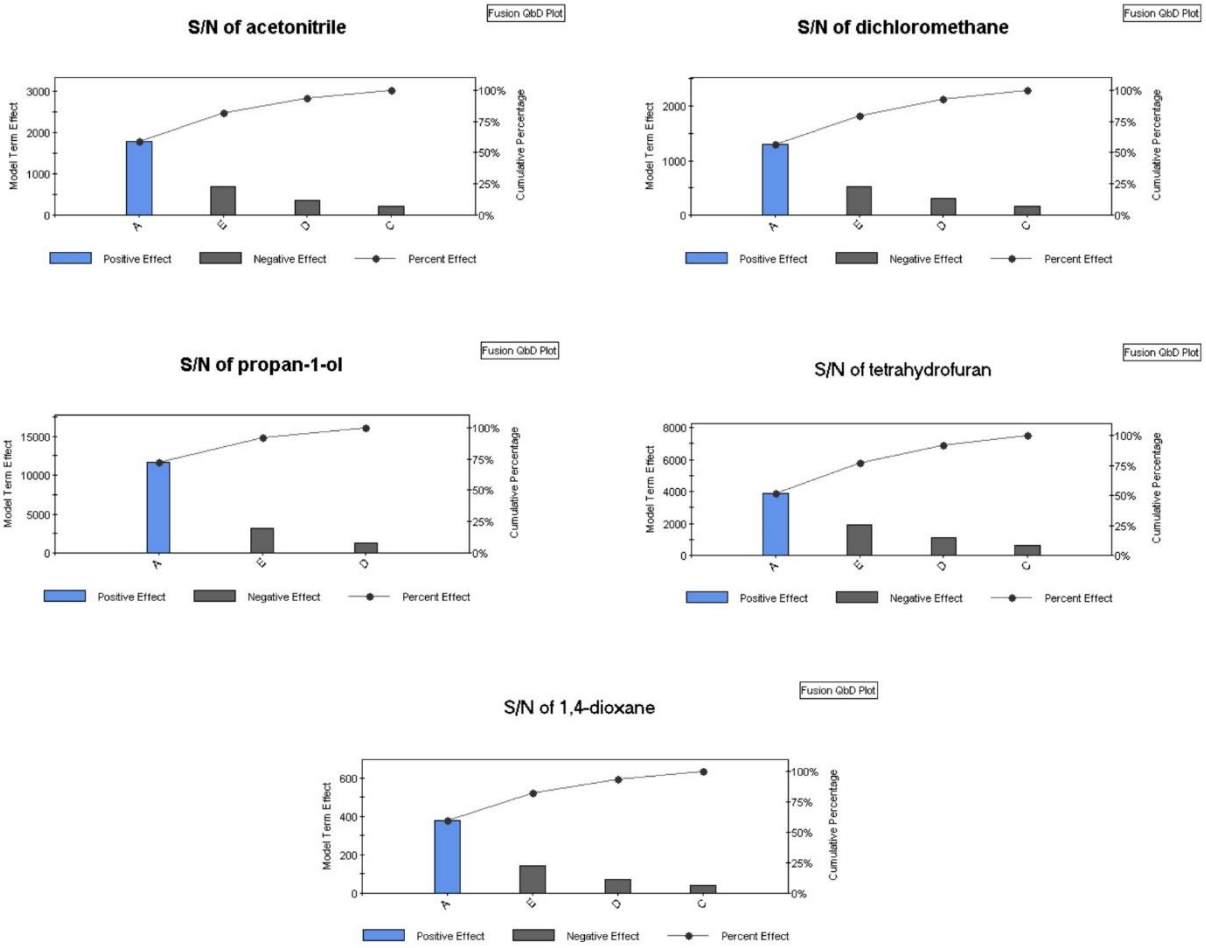


Figure 10. Evaluation of the statistical significance of model terms and their impact (positive or negative) on each considered response in the screening study (DoE-III) for the optimization of the general HS-GC method sensitivity. Legend: Blue bar – positive effect; Gray bar – negative effect.

The regression model obtained for S/N value of acetonitrile presented a $R^2 = 0.9783$, so it is valid. This value means that, at least, 98 % of the variation of these response can be explained by this model. Regarding the statistical significance of model terms (factor or interaction between factors) on S/N value of acetonitrile, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05, with exception of split ratio (C), which presents a *p*-value > 0.05. Additionally, from effect plot (Figure 10), it was possible to verify that equilibration temperature (A), split at vent (E) and vial pressure (D) are the method parameters that present impact (positive or negative) on S/N value of acetonitrile. Vial equilibration temperature (A) is the main critical factor and it has a positive impact. This means that when the vial equilibration temperature increases, the S/N value of acetonitrile increases. On the contrary, these method attribute is negatively affected by split at vent (E) and vial pressure (D). This means that when the value of these factors increases, the S/N value of acetonitrile decreases.

For S/N value of dichloromethane, the obtained regression model showed a $R^2 = 0.9714$. From this value it is clear that the model adequately describes the relationship between the model terms and the considered CMA. Regarding the statistical significance of model terms (factor or interaction between factors) on S/N value of dichloromethane, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05, with exception of split ratio (C), which presents a *p*-value > 0.05. From effect plot (Figure 10), it was possible to verify that this response is positively affected by equilibration temperature (A) and is negatively affected by split at vent (E) and vial pressure (D). This means that when the equilibration temperature (A) increases, the S/N value of dichloromethane increases. Conversely, these response decreases when the split at vent (E) and vial pressure (D) increase. Vial equilibration temperature (A) is the tested variable with the most significant impact on this response.

Relatively to S/N value of propan-1-ol, a regression model with a $R^2 = 0.9722$ was obtained, so it is valid. This value means that, at least, 97 % of the variation of these response can be explained by this model. Regarding the statistical significance of model terms on S/N value of propan-1-ol, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05. Considering effect plot (Figure 10), equilibration temperature (A), vial pressure (D) and split at vent (E) are the factors with impact (positive or negative) on S/N value of propan-1-ol. Vial equilibration temperature (A) is the main critical factor and it has a positive impact. This means that when the vial equilibration temperature increases, the S/N value of propan-1-ol increases. On the contrary, these method attribute is negatively affected by vial pressure (D) and split at vent (E). This means that when the value of these factors increases, the S/N value of propan-1-ol decreases.

The regression model obtained for S/N value of tetrahydrofuran presented a $R^2 = 0.9655$, so it is valid. This value means that, at least, 97% of the variation of these response can be explained by this model. Regarding the statistical significance of model terms (factor or interaction between factors) on S/N value of tetrahydrofuran, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05. From effect plot (Figure 10), it was possible to verify that this response is positively affected by equilibration temperature (A) and is negatively affected by split at vent (E), vial pressure (D) and split ratio (C). This means that the increase of equilibration temperature promotes the increase of the S/N value of tetrahydrofuran. Conversely, this response decreases when the values of split at vent, vial pressure and split ratio increase. Vial equilibration temperature (A) is the tested variable with the most significant impact on this response.

For S/N value of 1,4-dioxane, the obtained regression model showed a $R^2 = 0.9756$. From this value it is clear that the model adequately describes the relationship between the model terms and the considered CMA. Regarding the statistical significance of model terms (factor or interaction between factors) on S/N value of 1,4-dioxane, it was verified that all model parameters are statistically significant, since they presented a *p*-value ≤ 0.05, with exception of split ratio (C), which presents a *p*-value > 0.05. From effect plot (Figure 10), it was possible to verify that this response is positively affected by equilibration temperature (A) and is negatively affected by split at vent (E) and vial pressure (D). This means that when the equilibration temperature (A) increases, the S/N value of dichloromethane increases. Conversely, these response decreases when the split at vent (E) and vial pressure (D) increase. Vial equilibration temperature (A) is the tested variable with the most significant impact on this response.

Relatively to S/N value of DIPEA, a regression model with a $R^2 = 0.7729$ was obtained and it is considered not valid. This means that the variation of these response cannot be explained by this model. Since the generated model is statistical invalid, it was not possible to evaluate the statistical significance of model terms on S/N value of DIPEA.

Finally, to S/N value of 4-hydroxy-4-methylpentan-2-one, the obtained regression model showed a $R^2 = 0.8652$ and it is considered not valid. This means that the variation of these response cannot be explained by this model. Since the generated model is statistical invalid, it was not possible to evaluate the statistical significance of model terms on S/N value of 4-hydroxy-4-methylpentan-2-one.

The main objective of this screening tests was to identify, within the five factors in study, which were the ones with the most significant effects on S/N values of the target analytes and to select the appropriate operating conditions of these CMPs. From the statistical analysis, it is verified that the models generated for S/N of acetonitrile, dichloromethane, propan-1-ol, tetrahydrofuran and 1,4-dioxane are valid, and the variation of each response can be explained by the respective model. Since the models are statistical valid, it was possible to evaluate the statistical significance of model terms (individual factors or interaction between factors) and their impact (positive or negative) on each response. On the contrary, the models generated for S/N of DIPEA and 4-hydroxy-4-methylpentan-2-one are invalid and it is not possible to evaluate the effects of the studied factors on these responses.

For the valid models, it can be concluded that vial equilibration temperature (A) has statistical significance and presents a positive impact in all considered method attributes. This means that when the vial equilibration temperature (A) increases, the S/N values of analytes increases and, therefore, their sensitivities. So, the considered method responses could be

increased by increasing the vial equilibration temperature. These results agree with prior knowledge about Headspace parameters (3.2.1. Prior knowledge – Headspace sampling conditions). Thus, in this development work, the optimal value for the equilibration temperature was (T_0 + 20) °C, which corresponds to the upper limit of the range of values tested for this factor. Although, based on literature,[28,78,79] the possible degradation of the pharmaceutical samples and the thermal instability of the solvents used as diluents are some of the problems that can arise if high vial equilibration temperatures are applied. These issues may potentiate the occurrence of unwanted peaks in the chromatogram. To prevent this type of problems, it was decided to set the vial equilibration temperature at T_0 ºC, a slight lower temperature than the upper limit tested. Furthermore, based on the chromatographic results (Table 22), it was verified that operating at this target temperature did not compromise the achievement of adequate sensitivity for the determination of the 29 target analytes, in particular for acetonitrile, dichloromethane, propan-1-ol, tetrahydrofuran, 1,4-dioxane, DIPEA and 4-hydroxy-4-methylpentan-2-one.

Regarding vial equilibration time, it was verified that this factor has not statistical significance and impact on S/N of acetonitrile, dichloromethane, propan-1-ol, tetrahydrofuran and 1,4-dioxane. In a previous investigation, Panovska *et al.*[54] optimized a HS-GC-FID-MS method for profiling of residual solvents in active pharmaceutical ingredients implementing DoEs studies. The authors verified that the equilibration time does not have as significant effect as the equilibration temperature on the sensitivity of the method. Additionally, in this work, no significant increase in the chromatographic responses was observed when vial equilibration time was significantly varied. On the other side, Dai *et al.*[28] suggested that an equilibration time of 10 minutes is sufficient to achieve the equilibrium state between the liquid and gas phases. In the present work, it was decided to establish a sufficient vial equilibration time to ensure a complete equilibration and a high level of headspace precision for pharmaceutical matrices with different complexities.

Regarding split ratio, it was verified that this factor only has statistical significance and impact (negative effect) on S/N value of tetrahydrofuran. Thus, as anticipated by prior knowledge, the method response could be increased by reducing the split ratio. In this development work, split ratio was optimized at (sr_0 – 1), which corresponds to the lower limit of the range of values tested for this factor.

Regarding vial pressure, it was verified that this factor has statistical significance for all considered method attributes and presents a negative impact on the considered method responses. This means that when the vial pressure increases, the S/N values of acetonitrile,

dichloromethane, propan-1-ol, tetrahydrofuran and 1,4-dioxane decreases. Previously, Fliszar *et al.*[13] verified that the application of high vial pressures resulted in lower values of sensitivity, suggesting that the increase of the pressure inside the vial cause the dilution of the volatile analytes in the gas phase. The goal is to maximize the S/N values, so the strategy will be to reduce the vial pressure. In this development work, it was decided to establish the vial pressure at vp_0 psi, a typical value recommended by headspace sampler's supplier.

At last, regarding split at vent, it was verified that this factor has statistical significance and presents a negative impact in all considered method attributes. This means that when the split at vent increases, the S/N values of analytes decrease and, therefore, their sensitivities. The goal is to maximize the S/N values, so the strategy will be to reduce the split at vent. In this development work, the optimal value for split at vent was (s_0 – 20) mL/min, which corresponds to the lower limit of the range of values tested for this factor. Although, this value of split at vent resulted in low resolution between the last eluting peak (4-hydroxy-4-methylpentan-2-one) and the diluent peak. For that reason, it was decided to slightly increase the split at vent and establish it at s_0 mL/min. Furthermore, based on the chromatographic results (Table 22), it was verified that operating at a higher split at vent value did not compromise the achievement of adequate sensitivity for acetonitrile, dichloromethane, propan-1-ol, tetrahydrofuran, 1,4-dioxane, DIPEA and 4-hydroxy-4-methylpentan-2-one.

Based on the outcome of these screening tests, it was possible to define the combination of values for the five tested variables that allow the achievement of an appropriate sensitivity for the determination of the 29 target analytes. So, the best operating conditions were: vial equilibration temperature, T_0 ºC; vial equilibration time, t_0 min; split ratio, (s_0 – 1); vial pressure, vp_0 psi and split at vent, s_0 mL/min.

Combining the information of the optimization of the general HS-GC method selectivity, run time and sensitivity, it was possible to obtain the combination of all studied factors that result in the high method performance quality. For the critical method parameters tested in the development of the method, the final operating conditions were: column flow, (CF_0 + 0.2) mL/min; initial oven temperature, OT_0 ºC; initial hold time, IHt_0 + 2.5 min; initial ramp rate, IRR_0 – 2.0 ºC/min; vial equilibration temperature, T_0 ºC; vial equilibration time, t_0 min; split ratio, (s_0 – 1); vial pressure, vp_0 psi and split at vent, s_0 mL/min. Therefore, NOC corresponds to the final optimized operating headspace and GC conditions that includes several parameters as specified in Table 24.

Table 24. Final operating GC system and headspace parameters of the general HS-GC method.

GC conditions		Headspace conditions
Oven	Initial temperature	Vial equilibration temperature
	Initial hold time	Loop temperature
	Initial ramp rate	Transfer Line temperature
	Final temperature	GC cycle time
	Hold time 1	Vial equilibration time
	Ramp rate	Pressurization time
	Final temperature	Loop fill time
	Hold time 2	Loop equilibration time
	Ramp rate	Sample injection time
	Final temperature	Fill mode
	Hold time 3	Fill pressure/vial pressure
	Run time	Fill flow
Column	Carrier gas	Loop fill mode
	Mode	Loop fill ramp rate
	Flow	Loop final pressure
	Length	Vent after extraction
	Internal diameter	Vial size
	Film thickness	Shake
Injector	Mode	Split flow at vent
	Temperature	
	Split ratio	
	Injection volume	
	Inlet liner	
FID Detector	Temperature	
	Mode	
	Makeup flow	
	Hydrogen flow	
	Air flow	

The NOC was evaluated by injecting two standard solutions containing the 29 target analytes at LOQ level and 100% of the ICH/VICH limits and control levels. Both standard solutions were injected three times. The obtained chromatographic profiles for NOC at these two different concentration levels are presented in Figures 11 and 12, respectively.

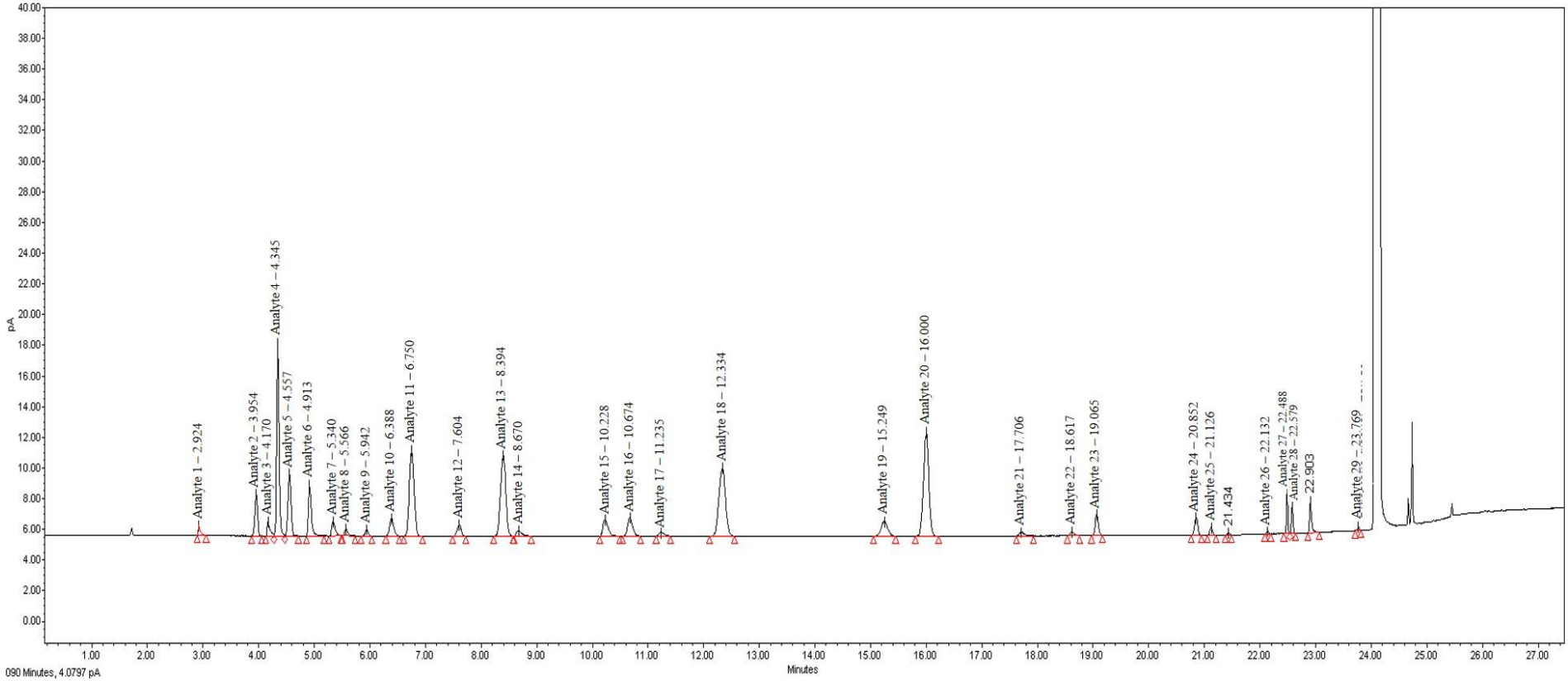


Figure 11. Chromatographic profile obtained for Normal Operable Conditions (NOC) by injecting a standard solution containing the 29 target analytes at LOQ level.

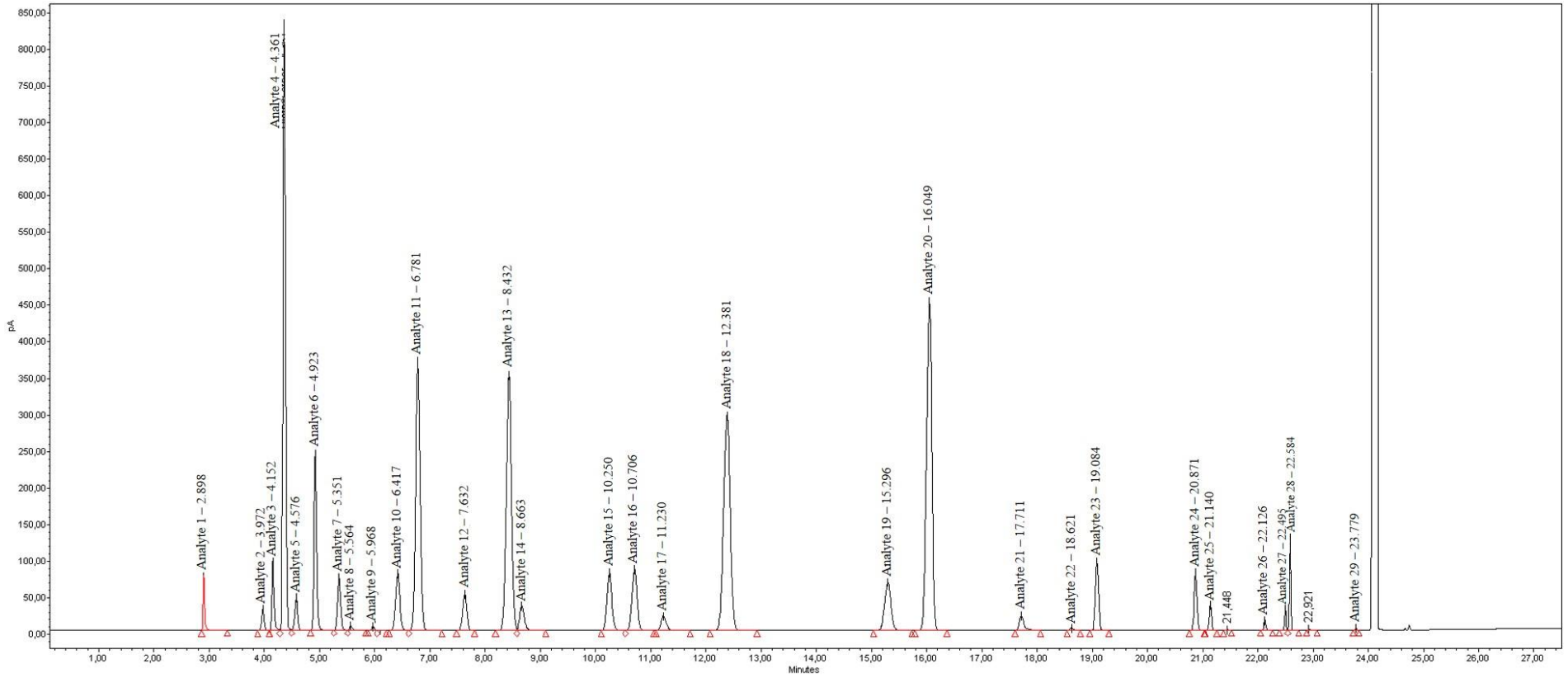


Figure 12. Chromatographic profile obtained for Normal Operable Conditions (NOC) by injecting a standard solution containing the 29 target analytes at 100% of the ICH/VICH limits and defined control levels.

At LOQ level, the results were evaluated in terms of S/N values and are presented in Table 25.

Table 25. Results of S/N values obtained for Normal Operable Conditions (NOC) by injecting a standard solution containing the 29 target analytes at LOQ level.

	S/N	Criteria	Conformity
Analyte 1	162	S/N ≥ 10	Pass
Analyte 2	422		Pass
Analyte 3	192		Pass
Analyte 4	2317		Pass
Analyte 5	578		Pass
Analyte 6	519		Pass
Analyte 7	145		Pass
Analyte 8	60		Pass
Analyte 9	57		Pass
Analyte 10	162		Pass
Analyte 11	845		Pass
Analyte 12	123		Pass
Analyte 13	783		Pass
Analyte 14	56		Pass
Analyte 15	175		Pass
Analyte 16	210		Pass
Analyte 17	32		Pass
Analyte 18	640		Pass
Analyte 19	95		Pass
Analyte 20	667		Pass
Analyte 21	27		Pass
Analyte 22	38		Pass
Analyte 23	238		Pass
Analyte 24	150		Pass
Analyte 25	92		Pass
Analyte 26	26		Pass
Analyte 27	600		Pass
Analyte 28	380		Pass
Analyte 29	35		Pass

According to the results, the acceptance criteria defined for S/N values was met for all the target analytes, since the obtained S/N was above 10. Thus, it was possible to conclude that the NOC can ensure an appropriate sensitivity to detect and quantify the target analytes at required LOQ levels.

At 100% of the ICH/VICH limits and control levels, the chromatographic results were analyzed in terms of meeting the method performance goals, defined in Table 11, for the resolution of the 29 target analytes and for the retention time of the last eluting peak, and were presented in Table 26. The average of peak area and the relative standard deviation (%RSD) of

the three injections were determined for each target analyte and were also presented in Table 26.

Table 26. Results of retention time, resolution, average of peak area and %RSD obtained for Normal Operable Conditions (NOC) by injecting a standard solution containing the 29 target analytes at 100% of the ICH/VICH limits and defined control levels.

	Retention time (min)	Resolution	Peak area	Average of peak area	% RSD (n=3)
Analyte 1	2.898	-	158.436 159.030 159.285	158.917	0.3
Analyte 2	3.972	15.4	96.372 95.992 96.411	96.258	0.2
Analyte 3	4.152	2.2	279.663 279.707 280.771	280.047	0.2
Analyte 4	4.361	2.6	2600.534 2592.987 2607.107	2600.209	0.3
Analyte 5	4.576	2.4	159.805 159.431 160.249	159.828	0.3
Analyte 6	4.923	3.7	838.393 837.102 841.129	838.875	0.2
Analyte 7	5.351	4.3	290.616 290.742 292.734	291.364	0.4
Analyte 8	5.564	2.0	24.787 24.871 25.108	24.922	1
Analyte 9	5.968	4.0	19.456 19.434 19.472	19.454	0.1
Analyte 10	6.417	3.6	419.696 419.766 422.098	420.520	0.3
Analyte 11	6.781	2.4	2126.916 2123.230 2137.470	2129.205	0.3
Analyte 12	7.632	5.7	264.826 264.007 265.878	264.904	0.4
Analyte 13	8.432	5.0	2284.216 2280.304 2297.737	2287.419	0.4
Analyte 14	8.663	1.5	189.392 189.645 190.734	189.924	0.4
Analyte 15	10.250	10.1	489.412 488.870 490.870	489.717	0.2

	Retention time (min)	Resolution	Peak area	Average of peak area	% RSD (n=3)
Analyte 16	10.706	2.7	529.815 529.280 531.778	530.291	0.2
Analyte 17	11.230	3.0	134.806 134.811 135.458	135.025	0.3
Analyte 18	12.381	5.7	2432.252 2428.629 2444.987	2435.289	0.4
Analyte 19	15.296	13.2	529.432 528.929 531.778	530.046	0.3
Analyte 20	16.049	3.8	2990.110 2986.679 3009.807	2995.532	0.4
Analyte 21	17.711	10.3	115.725 115.830 117.147	116.234	1
Analyte 22	18.621	7.0	14.350 14.320 14.398	14.356	0.3
Analyte 23	19.084	4.1	375.614 375.719 377.508	376.280	0.3
Analyte 24	20.871	17.4	285.743 285.737 287.500	286.327	0.4
Analyte 25	21.140	3.0	109.001 109.026 109.618	109.215	0.3
Analyte 26	22.126	9.5	33.538 33.819 34.694	34.017	2
Analyte 27	22.495	6.3	54.236 54.173 54.889	54.433	1
Analyte 28	22.584	1.9	221.377 221.150 222.948	221.825	0.4
Analyte 29	23.779	15.5	6.793 6.723 6.765	6.760	1

According to the obtained results, all target analytes met the acceptance criterion for the % RSD of the analytes peak areas, since the values were equal to or less than 10%. In addition, the acceptance criteria defined for resolution and retention time of the last eluting peak were met for all the target analytes, since the obtained resolutions were equal to or greater than 1.5 and the obtained retention time was less than 26 minutes. Thus, it was possible to conclude that

the NOC can ensure an appropriate selectivity and run time in the determination of the 29 target analytes. As shown in Table 27, the results of critical resolutions and retention time of the last eluting peak were also compared with the model predictions generated by the Fusion QbD® software,[65] previously presented in Table 17.

Table 27. Comparison between prediction model and experimental results for critical resolutions and retention time of the last eluting peak obtained in the Normal Operable Conditions (NOC) verification.

Critical Method Attribute (CMA)	Normal Operable Conditions (NOC)	
	Prediction	Experimental value
Resolution – acetonitrile	1.7	2.0
Resolution – dichloromethane	3.2	4.0
Resolution – propan-1-ol	1.3	1.5
Resolution – 1,4-dioxane	2.8	7.0
Last peak retention time	22.822	23.779

According to the Table 27, a good compromise between model predictions and experimental results was obtained for critical resolutions and retention time of the last eluting peak in NOC verification.

In conclusion, the acceptance criteria defined for S/N values, resolution and retention time of the last eluting peak were met for all the target analytes. Thus, the NOC of the developed HS-GC method can ensure an appropriate sensitivity, selectivity and run time for the determination of the 29 target analytes. Once the NOC is verified, it is necessary to evaluate if the method is robust. In this sense, four different points were tested in robustness studies.

3.2.4. Robustness studies

The robustness of the analytical method consists of its capacity to remain unaffected by small, but deliberate variations in the method parameters. Based on the knowledge gathered during the experimental studies, for the optimization of selectivity, run time and sensitivity of the HS-GC method, the method parameters to be studied during robustness exercise were selected. Robustness was studied for small and deliberate variations in GC system conditions, such as initial oven temperature (± 2 ºC of target temperature at NOC) and initial ramp rate (± 0.2 ºC/min of target initial ramp rate at NOC), and headspace conditions, such as vial equilibration temperature (± 10ºC of target equilibration temperature at NOC) and vial equilibration time (± 5 minutes of target equilibration time at NOC). To perform this study four different points (A, B, C and D) were selected. Regarding the initial oven temperature and initial ramp rate, the operating conditions of points A, B, C and D were within the MODR region. The operating conditions for these four points are presented in Table 28.

Table 28. Operating conditions of the four experimental points (A, B, C and D) along Method Operable Design Region (MODR) considered in the robustness studies.

Point	Initial oven temperature (ºC)	Initial ramp rate (ºC/min)	Equilibration temperature (ºC)	Equilibration time (min)
A	NOC – 2	NOC – 0.2	NOC – 10	NOC – 5
B	NOC – 2	NOC + 0.2	NOC - 10	NOC + 5
C	NOC + 2	NOC – 0.2	NOC + 10	NOC – 5
D	NOC + 2	NOC + 0.2	NOC + 10	NOC + 5

The robustness conditions A, B, C and D were evaluated by injecting a standard solution containing the 29 target analytes at 100% of the ICH/VICH limits and control levels and by spiking the samples 2 (Drug product B) and 3 (drug substance A) with the 29 target analytes at 100% of the ICH/VICH limits and control levels (see Sections 2.4.1. Preparation of standard solutions and 2.4.2. Preparation of sample solutions). For each of the four combinations of operating conditions, both standard and sample solutions were injected three times.

For standard solution, resolution and retention time of the last eluting peak were considered in the study of the robustness. The obtained chromatographic results were analyzed against the method performance goals, defined in Table 11, for the resolution of the 29 target analytes and for the retention time of the last eluting peak at each robustness condition, and were presented in Table 29.

Table 29. Results of retention time and resolution obtained for the four experimental points (A, B, C and D) along Method Operable Design Region (MODR) by injecting a standard solution containing the 29 target analytes at 100% of the ICH/VICH limits and defined control levels.

	A		B		C		D	
	Resolution	**Last Peak RT**	**Resolution**	**Last Peak RT**	**Resolution**	**Last Peak RT**	**Resolution**	**Last Peak RT**
Analyte 1	-		-		-		-	
Analyte 2	15.8		15.8		15.2		15.0	
Analyte 3	2.4		2.4		1.8		1.8	
Analyte 4	2.6		2.6		2.7		2.7	
Analyte 5	2.4		2.4		2.4		2.4	
Analyte 6	3.8		3.8		3.5		3.5	
Analyte 7	4.6		4.6		3.9		3.8	
Analyte 8	1.9		1.9		2.2		2.2	
Analyte 9	4.1		4.1		4.0		4.0	
Analyte 10	2.4		2.5		2.4		2.4	
Analyte 11	2.3		2.3		2.6		2.6	
Analyte 12	5.9		5.9		5.6		5.6	
Analyte 13	5.2		5.2		4.7		4.7	
Analyte 14	1.6		1.5		1.5		1.5	
Analyte 15	10.1	36.766	10.1	23.749	9.7	23.791	9.5	18.207
Analyte 16	2.9		2.9		2.6		2.5	
Analyte 17	2.7		2.8		3.1		3.1	
Analyte 18	5.8		5.8		5.7		5.7	
Analyte 19	13.5		14.0		12.1		10.8	
Analyte 20	3.8		3.8		4.0		4.0	
Analyte 21	11.6		10.5		9.8		8.1	
Analyte 22	6.4		5.2		5.7		7.4	
Analyte 23	5.6		4.1		4.2		2.9	
Analyte 24	22.2		17.4		17.5		17.0	
Analyte 25	2.0		2.9		3.0		3.3	
Analyte 26	11.2		9.5		9.6		8.8	
Analyte 27	8.0		6.2		6.4		5.5	
Analyte 28	3.6		1.8		2.0		1.5	
Analyte 29	16.8		15.2		17.0		16.2	

According to the generated results, the acceptance criterion defined for resolution was met for all the target analytes in the different combinations of operating conditions, since the obtained resolution was equal to or greater than 1.5. Regarding the retention time of the last eluting peak, the acceptance criterion was met for all combinations of operating conditions, with exception of point A since the obtained value was greater than 26 minutes.

Thus, it was possible to conclude that the operating conditions of the points B, C and D can ensure appropriate selectivity and run time in the determination of the 29 target analytes. The operating conditions of the point A only can ensure the selectivity required in the analysis.

As shown in Table 30, the results of critical resolutions and retention time of the last eluting peak were also compared with the model predictions generated by the Fusion QbD® software,[65] previously presented in Table 19.

Table 30. Comparison between prediction model and experimental results for critical resolutions and retention time of the last eluting peak obtained in the verification of the operating conditions of the four experimental points (A, B, C and D) along Method Operable Design Region (MODR).

Critical Method Attribute (CMA)	A		B		C		D	
	Prediction	Experimental value	Prediction	Experimental value	Prediction	Experimental value	Prediction	Experimental value
Resolution acetonitrile	1.6	1.9	1.6	1.9	1.7	2.2	1.7	2.2
Resolution dichloromethane	3.3	4.1	3.3	4.1	3.1	4.0	3.1	4.0
Resolution propan-1-ol	1.4	1.6	1.3	1.5	1.4	1.5	1.3	1.5
Resolution 1,4-dioxane	2.3	6.4	2.4	5.2	3.2	5.7	3.3	7.4
Last peak retention time	25.540	36.766	24.381	23.749	21.262	23.791	20.103	18.207

According to the Table 30, a good compromise between model predictions and experimental results was obtained for all critical resolutions in the robustness conditions A, B, C and D. Regarding to the values of last peak retention time, a good compromise was also obtained for all robustness conditions, except for robustness condition A.

For sample solutions, the %RSD of the obtained analyte concentrations were determined at each robustness condition. The results were presented in Supplementary Data (S.3. Robustness studies – Tables S.2. to S.5.). Based on these results and considering that the defined acceptance criterion for the %RSD at 100% of the ICH/VICH limits and defined control levels. is ≤ 10%, it was demonstrated that the sample 2 and 3 met the acceptance criteria for all target analytes in the evaluation of the robustness conditions A, B, C and D.

In conclusion, the changes in the analytical conditions did not significantly influence the selectivity nor the precision of the analysis. Thus, the developed HS-GC method was considered robust for the determination of the 29 target analytes.

3.3. Method Performance Qualification

3.3.1. Method Validation/ATP Verification

3.3.1.1. Specificity and selectivity

The method specificity was evaluated to demonstrate that the method is capable to assess unequivocally each of the 29 compounds of interest. This study was performed by injecting each of the following solutions once: Blank (diluent) and individual standard solution for each target analyte at working level (see Section 2.4.1. Preparation of standard solutions). The obtained chromatograms are presented in Supplementary Data (S.4. Specificity and selectivity – Figures S.1. to S.29.). Based on chromatographic data, no significant interference between blank and any of the peaks of interest was observed. However, the blank chromatogram presented two injection artifact peaks, one with retention time of approximately 1.8 minutes and the other with retention time of approximately 22.9 minutes, which was identified as an impurity of method's diluent.

The method selectivity was demonstrated by injecting a blank solution and a standard solution containing the 29 target analytes at working level (see Section 2.4.1. Preparation of standard solutions). The retention times of the target analytes and the resolution between peaks were obtained from the typical chromatogram of the working standard solution (see Figure 10 above), and were presented in Table 34 (see above). The resolution between each two adjacent chromatographic peaks was found to be equal to or greater than 1.5. All target analytes were resolved from each other and from method's diluent.

3.3.1.2. Limit of Detection (LOD)

The limit of detection (LOD) corresponds to the minimum concentration of analyte in a sample (or standard solution) that the analytical method is capable of detecting, but not necessarily quantifying. Different approaches can be applied to determine LOD. In chromatographic techniques, the determination of LOD is often based on the S/N, as these analytical procedures exhibit a baseline noise. The LOD is calculated by comparing measured signals from samples or standard solutions with known low concentrations of analyte with those of samples without the compound of interest or blanks. A S/N between 3:1 or 2:1 is generally considered acceptable for estimating the LOD.

In the present work, the LOD was evaluated by preparing two independent standard solutions (LOD 1 and LOD 2) containing the 29 analytes at the defined LOD level (see Section

2.4.1. Preparation of standard solutions), as shown in Table 31. Each preparation was injected three times. The obtained results are also presented in Table 31. A representative LOD chromatogram is shown in Supplementary Data (S.5. Limit of Detection – Figures S.30.). According to the results, all the analytes fulfilled the acceptance criterion (S/N ≥ 3) for LOD.

Table 31. Limit of detection (LOD) results obtained by the analysis of two independent standard solutions containing the 29 target analytes at LOD level.

	Limit of detection (LOD)		
	S/N		
	min – max	Criteria	Conformity
Analyte 1	70 – 73	S/N ≥ 3	Pass
Analyte 2	203 – 214		Pass
Analyte 3	80 – 83		Pass
Analyte 4	1114 – 1157		Pass
Analyte 5	279 – 292		Pass
Analyte 6	241 – 247		Pass
Analyte 7	69 – 70		Pass
Analyte 8	27 – 28		Pass
Analyte 9	28 – 29		Pass
Analyte 10	79 – 81		Pass
Analyte 11	420 – 433		Pass
Analyte 12	59 – 64		Pass
Analyte 13	388 – 399		Pass
Analyte 14	25 – 32		Pass
Analyte 15	81 – 83		Pass
Analyte 16	89 – 90		Pass
Analyte 17	14 – 15		Pass
Analyte 18	315 – 325		Pass
Analyte 19	47		Pass
Analyte 20	331 – 341		Pass
Analyte 21	11 – 13		Pass
Analyte 22	17 – 19		Pass
Analyte 23	116 – 130		Pass
Analyte 24	74 – 77		Pass
Analyte 25	46 – 48		Pass
Analyte 26	11 – 13		Pass
Analyte 27	291 – 308		Pass
Analyte 28	190 – 240		Pass
Analyte 29	15 – 18		Pass

3.3.1.3. Limit of Quantitation (LOQ)

The minimum concentration at which the analyte can be quantified with acceptable accuracy and precision is designated by LOQ. Similar to LOD, the determination of LOQ, in chromatographic techniques, is often based on the S/N. The LOQ is the lower end of the linear range of the analytical method and is calculated by comparing measured signals from samples or standard solutions with known low concentrations of analyte with those of samples without the compound of interest or blanks. A S/N of 10:1 is generally considered acceptable for estimating the LOQ.

In the present work, the LOQ was evaluated by injecting three times, two independent standard solutions (LOQ 1 and LOQ 2) containing the 29 analytes at the defined LOQ level (see Section 2.4.1. Preparation of standard solutions), as shown in Table 32. Table 32 also presents the obtained results. A representative LOQ chromatogram is shown in Figure 9 above. According to the results, all the target analytes met the defined acceptance criteria, since the obtained S/N was above 10 and % RSD were less than 25%.

Table 32. Limit of quantitation (LOQ) results obtained by the analysis of two independent standard solutions containing the 29 target analytes at LOQ level.

	Limit of quantitation (LOQ)					
	S/N			% RSD (areas) n=6	Criteria	Conformity
	min – max	Criteria	Conformity			
Analyte 1	162 – 169	S/N ≥ 10	Pass	3	RSD ≤ 25%	Pass
Analyte 2	420 – 438		Pass	2		Pass
Analyte 3	192 – 197		Pass	1		Pass
Analyte 4	2308 – 2365		Pass	1		Pass
Analyte 5	573 – 596		Pass	1		Pass
Analyte 6	517 – 533		Pass	1		Pass
Analyte 7	144 – 150		Pass	2		Pass
Analyte 8	58 – 64		Pass	6		Pass
Analyte 9	57 – 58		Pass	1		Pass
Analyte 10	162 – 168		Pass	2		Pass
Analyte 11	845 – 876		Pass	1		Pass
Analyte 12	119 – 128		Pass	2		Pass
Analyte 13	783 – 807		Pass	1		Pass
Analyte 14	56 – 60		Pass	6		Pass
Analyte 15	175 – 181		Pass	1		Pass
Analyte 16	182 – 212		Pass	1		Pass
Analyte 17	32 – 34		Pass	6		Pass
Analyte 18	639 – 656		Pass	1		Pass
Analyte 19	95 – 99		Pass	1		Pass
Analyte 20	666 – 687		Pass	1		Pass
Analyte 21	26 – 27		Pass	4		Pass
Analyte 22	38 – 40		Pass	3		Pass
Analyte 23	237 – 244		Pass	1		Pass
Analyte 24	150 – 156		Pass	2		Pass
Analyte 25	92 – 95		Pass	2		Pass
Analyte 26	25 – 27		Pass	7		Pass
Analyte 27	590 – 619		Pass	2		Pass
Analyte 28	378 – 473		Pass	2		Pass
Analyte 29	34 – 38		Pass	2		Pass

3.3.1.4. Linearity and Range

Linearity is defined as the ability to obtain a proportional relationship between the analytical results and the concentration of the analyte within a given range.[86] The relationship is determined by the construction of calibration curves of signals as a function of analyte concentration or content and by the respective regression line. The correlation coefficient (R^2) is, generally, the parameter of the regression line used to indicate the linearity of the analytical method. The smaller the difference between the unit and the value of the correlation coefficient, the greater the linearity of the method.[87] The interval between the upper and lower levels of analyte, for which the linear relationship is valid, is denominated by range of the analytical method.

The linearity studies were based on the preparation and triplicate injections of standard solutions containing the 29 target analytes at 6 different concentration levels: LOQ, 20%, 100%, 120%, 140% and 200% of the ICH/VICH limits and defined control levels (see Section 2.4.1. Preparation of standard solutions). As shown in Table 33, the linearity range for each analyte is sufficiently wide considering the intended purpose of the method. Results for linearity are also presented in Table 40 together with linear regression equation and correlation coefficient (R^2). The calibration curves (based on peak area) obtained for each analyte, including the respective linear regression equations and the value of R^2, are presented in Supplementary Data (S.6. Linearity and range – Figures S.31. to S.59.). The values of R^2 of these 29 analytes were within 0.9981 – 1.0000. Therefore, it was demonstrated that the developed HS-GC method is linear for all target analytes between LOQ and 200% of the maximum limit defined for each analyte.

Table 33. Linearity results obtained by the analysis of six standard solutions containing the 29 target analytes between LOQ level and 200% of the ICH/VICH limits and defined control levels.

	Linearity (6 concentration levels)		
	Linear range (ppm) LOQ – 200% maximum limit	Linear regression equation	Correlation coefficient (R^2)
Analyte 1	27 – 6007	y = 0.063x - 2.8318	0.9997
Analyte 2	9 – 351	y = 1.0693x - 3.7097	0.9981
Analyte 3	45 – 10024	y = 0.0631x - 5.1791	0.9996
Analyte 4	45 – 10029	y = 0.5694x - 27.965	0.9997
Analyte 5	9 – 351	y = 1.0103x - 2.3408	0.9988
Analyte 6	45 – 10002	y = 0.1864x - 8.1309	0.9998
Analyte 7	45 – 10035	y = 0.0656x - 3.3679	0.9998
Analyte 8	22 – 825	y = 0.0678x - 0.279	0.9997
Analyte 9	33 – 1207	y = 0.0373x - 0.1515	0.9999
Analyte 10	45 – 10016	y = 0.0937x - 3.8681	0.9999
Analyte 11	45 – 10000	y = 0.4727x - 14.123	0.9999
Analyte 12	3 – 581	y = 1.03x - 2.9018	0.9996
Analyte 13	45 – 10033	y = 0.5086x - 10.96	0.9999
Analyte 14	45 – 10036	y = 0.0422x - 2.8448	0.9997
Analyte 15	45 – 10039	y = 0.1363x - 5.1486	0.9999
Analyte 16	45 – 10038	y = 0.1182x - 3.9788	0.9999
Analyte 17	6 – 1440	y = 0.3006x - 1.106	0.9999
Analyte 18	35 – 7778	y = 0.7007x - 12.648	0.9999
Analyte 19	45 - 10005	y = 0.1183x - 2.2704	0.9999
Analyte 20	45 - 10023	y = 0.6565x - 4.6487	1.0000
Analyte 21	45 - 10038	y = 0.0254x - 1.4339	0.9998
Analyte 22	21 – 765	y = 0.0432x - 0.3468	0.9997
Analyte 23	45 - 10011	y = 0.0836x - 2.0785	0.9999
Analyte 24	41 – 9000	y = 0.0706x - 1.64	0.9999
Analyte 25	8 – 1785	y = 0.1338x - 0.5611	0.9999
Analyte 26	2 – 356	y = 0.1935x + 0.2604	0.9999
Analyte 27	90 – 3340	y = 0.0371x - 0.3284	0.9999
Analyte 28	45 - 10016	y = 0.0489x - 1.0358	0.9999
Analyte 29	90 – 3342	y = 0.0037x - 0.0735	0.9991

3.3.1.5. Accuracy

The accuracy of the method was determined by preparing each of the five pharmaceutical samples (Drug product A, Drug product B, Drug substance A, Drug product C and Drug substance B), spiked with the 29 target analytes at three different concentration levels: LOQ, 100% and 140% of the ICH/VICH limits and defined control levels (see Section 2.4.2. Preparation of sample solutions). For each concentration level, three preparations were performed and injected once. Three independent “as is” sample solutions of each sample were prepared and injected once, to be used in recovery calculations. Accuracy was expressed by the

recovery percentage for each target analyte in each of the five samples and the results were presented in Supplementary Data (S.7. Accuracy – Tables S.6. to S.10.).

For Drug product A, the mean recovery percentages of the target analytes were within 89% - 111% at LOQ level. At 100% of the ICH/VICH limits and defined control levels, the values were within 99% - 106%, except for Analyte 28 with a mean recovery percentage of 68%. Finally, at 140% of the ICH/VICH limits and defined control levels, the values were within 96% - 107%, except for Analyte 28 with a mean recovery percentage of 68%.

For Drug product B, the mean recovery percentages of the target analytes were within 84% - 121% at LOQ level, except for Analyte 28 with a mean recovery percentage of 40%. At 100% of the ICH/VICH limits and defined control levels, the values were within 95% - 103%, except for Analyte 28 with a mean recovery percentage of 73%. Finally, at 140% of the ICH/VICH limits and defined control levels, the vales were within 99% - 105%, except for Analyte 28 with a mean recovery percentage of 75%.

For Drug substance A, the mean recovery percentages of the target analytes were within 77% - 100% at LOQ level. At 100% of the ICH/VICH limits and defined control levels, the values were within 90% - 100%. Finally, at 140% of the ICH/VICH limits and defined control levels, the vales were within 89% - 102%.

For Drug product C, the mean recovery percentages of the target analytes were within 86% - 117% at LOQ level, except for Analyte 28 with a mean recovery percentage of 47%. At 100% of the ICH/VICH limits and defined control levels, the values were within 92% - 110%. Finally, at 140% of the ICH/VICH limits and defined control levels, the values were within 101% - 114%.

For Drug substance B, the mean recovery percentages of the target analytes were within 71% - 119% at LOQ level, except for Analyte 17 with a mean recovery percentage of 163%. At 100% of the ICH/VICH limits and defined control levels, the values were within 88% - 101%. Finally, at 140% of the ICH/VICH limits and defined control levels, the vales were within 92% - 103%.

Based on these results and considering that the defined acceptance criterion for the recovery percentage at LOQ level is 100 ± 30% and at 100% and 140% of the ICH/VICH limits and defined control levels is 100 ± 10%, it was demonstrated that all target analytes met the defined acceptance criteria in the five samples, with exception of Analyte 28. The unsatisfactory results obtained for Analyte 28 in Drug products A, B and C may have been due to the strong affinity of these samples for this solvent. Camarasu[12] documented a similar interaction between DMF and the matrix of a drug product during the validation of a HS-GC method for

determination of class 1, 2 and 3 residual solvents. Therefore, the developed HS-GC method is adequate to determine accurately the target analytes, except Analyte 28, in the five studied pharmaceutical samples, within the analytical range of determinations.

3.3.1.6. Precision

The precision of the general HS-GC method was assessed by evaluating both repeatability (intraday precision) and intermediate precision (interday precision).

Repeatability was assessed by the %RSD of the obtained analyte concentrations for the injections performed to evaluate the accuracy at LOQ level, 100% and 140% of the ICH/VICH limits and defined control levels. The results were presented in Supplementary Data (S.8. Repeatability – Table S.11.). The obtained values of %RSD at LOQ level were equal to or less than 15% for all target analytes in the five studied samples. At 100% and 140% of the ICH/VICH limits and defined control levels, the values of %RSD were equal to or less than 5% for all target analytes in the five studied samples. Based on these results and considering that the defined acceptance criterion for the %RSD at LOQ level is ≤ 25% and at 100% and 140% of the ICH/VICH limits and defined control levels is ≤ 10%, it was demonstrated that all target analytes met the defined acceptance criteria in the five samples. Therefore, the repeatability of the developed HS-GC method was demonstrated for all target analytes within the analytical range of determinations.

Intermediate precision was assessed by injecting consecutively six times a standard solution containing the 29 target analytes at 100% of the ICH/VICH limits and defined control levels (see Section 2.4.1. Preparation of standard solutions). The analysis was performed on a different day and using a different equipment and column. The average of peak area and the %RSD of the six injections were determined for each target analyte. The results obtained were compared with the average results from NOC verification at same concentration level and expressed by percentage of difference (% difference). The results were presented in Supplementary Data (S.9. Intermediate Precision – Table S.12.). The obtained values of %RSD were equal to or less than 3% for all target analytes, whereas the values of % difference were equal to or less than 13% for all target analytes, except for Analyte 29 with 183%. Considering that the defined acceptance criterion for the % difference is ≤ 20%, it was demonstrated that the obtained results were within the defined acceptance criterion for all target analytes, with exception of Analyte 29. Therefore, the developed HS-GC method presented reasonable intermediate precision within the analytical range of determinations for all target analytes, except for Analyte 29.

3.3.2. Control Strategy

Finally, an analytical control strategy is required to maintain the high level of quality performance of the method and to meet the requirements of the ATP throughout the lifecycle. So, to ensure the method performance quality, the CMAs and CMPs should be within their acceptable ranges. According to the robustness studies, the acceptable ranges of the critical method parameters are the following: column flow, (CF_0 + 0.2) mL/min; initial hold time, IHt_0 + 2.5 min; initial oven temperature, (OT_0 ± 2) ºC; initial ramp rate, (IRR_0 ± 0.2) ºC/min; vial equilibration temperature, (T_0 ± 10) ºC and vial equilibration time, (t_0 ± 10) minutes. The control strategy for general HS-GC method performances is based on the evaluation of the system suitability, which includes the verification of the absence of significant interfering peaks in the diluent's chromatogram and the fulfillment of the acceptance criteria for sensitivity at LOQ level and selectivity and precision at 100% of the ICH/VICH limits and defined control levels.

Chapter 4. Final conclusions

The main objective of this work was the development of a general HS-GC-FID method for determination of residual solvents and other related volatile organic impurities in drug substances and drug products. In this regard, the relevance of AQbD methodology, based on knowledge and risk management, was demonstrated in the analytical development of this method. The definition of the ATP, the acquisition of knowledge from previous studies, the risk assessment exercise, the use of DoEs to perform the screening and optimization tests and the verification of the ATP were some of the investigated key steps of the AQbD workflow.

The implementation of the QbD principles to the development of this method allowed to manage the knowledge related to the method performance, to control all sources of method variability and to ensure that the method is flexible to support changes throughout its lifecycle and pharmaceutical development cycle. The critical GC and headspace conditions were optimized and the combination of the operating conditions that result in the high quality method performance was selected.

The developed and validated general method was demonstrated adequate performance characteristics for the correct and simultaneous determination, in a run time less than 30 minutes, of the 29 target analytes in matrices with high complexity, such as pharmaceuticals. This method presents robustness, specificity and selectivity for all target analytes, capability to detect and quantify all compounds at lower concentration, excellent linearity for all target analytes over the studied range (LOQ - 200 % of the ICH/VICH limits and defined control levels), accuracy for all target analytes between LOQ and 140% of the ICH/VICH limits and defined control levels, except for Analyte 28, and repeatability for all compounds between LOQ and 140% of the ICH/VICH limits and defined control levels. The operating conditions selected during the method development work complies with the defined ATP criteria and, therefore, the developed HS-GC method satisfy its intended purpose.

With respect to Hovione's quality control laboratories, this work could be applied for routine analysis. The present work provides analytical method understanding to solve future method challenges, reducing the time spent in the analytical development process. The developed general HS-GC-FID method establishes a universal and well-known process related residual solvent and volatile organic impurities chromatographic profile. The knowledge of standard elution order and chromatographic profile is helpful to support the faster and easier identification of extra/unknown peaks, avoiding additional and time-consuming investigations and help and guide the identification of root causes. Other contribution of this work is the increase of the laboratories' productivity and efficiency, since one or several HS-GC-FID

instruments will be always ready to receive pharmaceutical samples from different processes/projects and to run this method's conditions. In addition, significant cost and time savings are possible, as it promotes the reduction of the reagents consumption and there is no need to purchase chromatographic columns with different characteristics or to spend time in setting up the equipment, changing column or in HS-GC-FID system stabilization.

Regarding to the method validation, the developed general method allows the reduction of the time spent in this process and the number of generated protocols. The matrix independent parameters, i.e., the method performance characteristics such as specificity, selectivity, LOD, LOQ, linearity and precision of standard solutions are assessed only once for the 29 target analytes and no re-validation is needed for the study of new pharmaceutical samples by this HS-GC-FID method. For each new pharmaceutical sample is only necessary to validate the matrix dependent parameters such as accuracy (recoveries), repeatability and robustness.

Ultimately, the successful application of the QbD principles to the development of this general HS-GC-FID method will allow correct and reliable analytical results to be reported on the content of the residual solvents and volatile organic impurities in pharmaceutical samples. This information will support important decisions during the pharmaceutical process, in order to obtain final pharmaceutical products with high level of quality and that not represent any risk to patients.

The mentioned considerations demonstrate that the present work assumes a relevant progress for the quality control in the pharmaceutical industry.

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Supplementary Data

S.1. Calculations

1. Theoretical weight of each analyte in Stock Standard solutions A and B

$$\boldsymbol{Analyte\ weight\ (mg) = \rho \times V} \qquad \textbf{(Equation 1)}$$

Where,

ρ is analyte density (mg/mL);

V is analyte volume (mL).

2. Content (mg/mL) of each analyte in Stock Standard solutions

$$\boldsymbol{Analyte\ concetration\ (mg/mL) = \frac{m}{V}} \qquad \textbf{(Equation 2)}$$

Where,

m is theoretical analyte weight in Stock Standard solution (mg);

V is Stock Standard solution volume (mL).

3. Content (ppm) of each analyte expressed on a weight basis relative to a 100 mg sample weight

$$\boldsymbol{Analyte\ concentration\ (ppm) = \frac{C}{W_t} \times 10^6} \qquad \textbf{(Equation 3)}$$

Where,

C is analyte concentration in Stock Standard solution (mg/mL);

W_t is theoretical sample weight (mg) (100 mg).

4. Content (mg/mL and ppm) of each analyte in Working Standard solution

$$\boldsymbol{Analyte\ concentration\ (mg/mL\ and\ ppm) = \frac{C_1 \times V_1}{V_2}} \qquad \textbf{(Equation 4)}$$

Where,

C_1 is analyte concentration in Stock Standard solution (mg/mL or ppm);

V_1 is Stock Standard Solution aliquot (mL);

V_2 is Working Standard solution volume (mL) (100 mL).

5. Content (mg/mL and ppm) of each analyte in Intermediate Standard solution

$$\textbf{\textit{Analyte concentration}}\ (\textbf{mg/mL and ppm}) = \frac{C_1 \times V_1}{V_2} \qquad \textbf{(Equation 5)}$$

Where,

C_1 is analyte concentration in Working Standard solution (mg/mL or ppm);

V_1 is Working Standard solution aliquot (mL);

V_2 is Intermediate Standard solution volume (mL) (100 mL).

6. Content (mg/mL and ppm) of each analyte in Sensitivity Standard solution

$$\textbf{\textit{Analyte concentration}}\ (\textbf{mg/mL and ppm}) = \frac{C_1 \times V_1}{V_2} \qquad \textbf{(Equation 6)}$$

Where,

C_1 is analyte concentration in Intermediate Standard solution (mg/mL or ppm);

V_1 is Intermediate Standard solution aliquot (mL);

V_2 is Sensitivity Standard solution volume (mL) (100 mL).

7. Content (ppm) of each analyte in the "as is" sample solution

$$\textbf{\textit{Initial conc.}}\ (\textbf{\textit{ppm}}) = \frac{A_2 \times C_1}{A_1} \times \frac{W_t}{W_2} \qquad \textbf{(Equation 7)}$$

Where,

A_1 is average analyte peak area from the 3 working standard solution injections;

A_2 is analyte peak area from sample solution injection;

C_1 is standard analyte concentration (ppm)*;

W_t is theoretical sample weight (mg) (100 mg);

W_2 is sample weight (mg).

8. Content (ppm) of each analyte in the sample spiked at LOQ level

$$\boldsymbol{Conc.(ppm)} = \frac{\boldsymbol{A_2 \times C_1}}{\boldsymbol{A_1}} \times \frac{\boldsymbol{W_t}}{\boldsymbol{W_2}} \qquad \textbf{(Equation 8)}$$

Where,

A_1 is analyte peak area from the sensitivity standard solution injection;

A_2 is analyte peak area from sample solution injection;

C_1 is standard analyte concentration (ppm)*;

W_t is theoretical sample weight (mg) (100 mg);

W_2 is sample weight (mg).

9. Content (ppm) of each analyte in the sample spiked at 100% and 140% of the ICH/VICH limits and defined control levels

$$\boldsymbol{Initial\ conc.(ppm)} = \frac{\boldsymbol{A_2 \times C_1}}{\boldsymbol{A_1}} \times \frac{\boldsymbol{W_t}}{\boldsymbol{W_2}} \qquad \textbf{(Equation 9)}$$

Where,

A_1 is average analyte peak area from the 3 injections of working standard solution;

A_2 is analyte peak area from sample solution injection;

C_1 is standard analyte concentration (ppm)*;

W_t is theoretical sample weight (mg) (100 mg);

W_2 is sample weight (mg).

10. Recovery calculation

$$\%\ \boldsymbol{Recovery\ (analyte)} = \frac{\boldsymbol{C_1 - C_2}}{\boldsymbol{C_3}} \times \mathbf{100} \qquad \textbf{(Equation 10)}$$

Where,

C_1 is analyte concentration in the sample spiked at LOQ level, 100% or 140% of the ICH/VICH limits and defined control levels;

C_2 is analyte concentration in the "as is" sample solution;

C_3 is standard analyte concentration (ppm) at LOQ level, 100% or 140% of the ICH/VICH limits and defined control levels.

11. Repeatability calculation

$$\% \boldsymbol{RSD} = \frac{\boldsymbol{\sigma}}{\boldsymbol{\mu}} \times \mathbf{100} \qquad \textbf{(Equation 11)}$$

Where,

σ is absolute standard deviation of analyte concentration from the 3 injections of the sample spiked at LOQ level, 100% or 140% of the ICH/VICH limits and defined control levels;

μ is average analyte concentration from the 3 injections of the sample spiked at LOQ level, 100% or 140% of the ICH/VICH limits and defined control levels.

12. Intermediate precision calculation

$$\% \boldsymbol{RSD} = \frac{\boldsymbol{\sigma}}{\boldsymbol{\mu}} \times \mathbf{100} \qquad \textbf{(Equation 12)}$$

Where,

σ is absolute standard deviation of analyte peak area from the 6 injections of working standard solution;

μ is average analyte peak area from the 6 injections of working standard solution.

$$\% \boldsymbol{Difference} = \frac{|\boldsymbol{A_1} - \boldsymbol{A_2}|}{\boldsymbol{A_2}} \times \mathbf{100} \qquad \textbf{(Equation 13)}$$

Where,

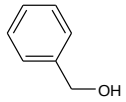
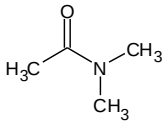
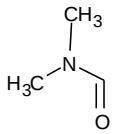
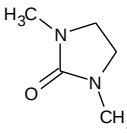
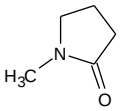
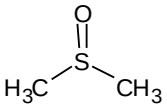
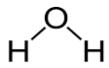
A_1 is average analyte peak area from the 6 injections of working standard solution (intermediate precision results);

A_2 is average analyte peak area from the 3 injections of working standard solution (NOC verification results).

S.2. Prior knowledge

In Table S.1., it is presented the list of the most common method's diluent used in HS-GC-FID methods, together with their relevant properties such as boiling point and dielectric constant.

Table S.1. Relevant properties (boiling point and dielectric constant) of the most frequently used diluents in residual solvents analysis.

Diluent	Boiling point (ºC)[67]	Dielectric constant (ε)[68]
 Benzyl alcohol C_7H_8O	205.3	11.92
 *N*,*N*-dimethylacetamide (DMA) C_4H_9NO	165.9	38.85
 *N*,*N*-dimethylformamide (DMF) C_3H_7NO	152.8	38.25
 1,3-dimethylimidazolidin-2-one (DMI) $C_5H_{10}N_2O$	225.0	------
 1-methylpyrrolidin-2-one (NMP) C_5H_9NO	204.2	32.55
 Dimethyl sulfoxide (DMSO) C_2H_6OS	191.9	47.24
 Water H_2O	100.0	80.10

S.3. Robustness studies

Table S.2. Results of the analyte concentrations and its %RSD obtained for the evaluation of the robustness condition A by spiking the samples 2 and 3 with a standard solution containing the 29 target analytes at 100% of the defined maximum limit, considering a sample concentration at 100 mg/mL.

CONDITION A	Sample 2 (Drug product B)	Sample 3 (Drug substance A)
	% RSD	
Analyte 1	1	2
Analyte 2	1	5
Analyte 3	1	2
Analyte 4	1	1
Analyte 5	1	1
Analyte 6	1	2
Analyte 7	1	2
Analyte 8	1	3
Analyte 9	1	2
Analyte 10	1	2
Analyte 11	1	1
Analyte 12	1	1
Analyte 13	1	1
Analyte 14	1	2
Analyte 15	1	2
Analyte 16	1	2
Analyte 17	1	2
Analyte 18	1	1
Analyte 19	1	2
Analyte 20	1	1
Analyte 21	1	3
Analyte 22	1	1
Analyte 23	1	2
Analyte 24	1	2
Analyte 25	1	2
Analyte 26	1	2
Analyte 27	1	2
Analyte 28	1	2
Analyte 29	5	7

Table S.3. Results of the analyte concentrations and its %RSD obtained for the evaluation of the robustness condition B by spiking the samples 2 and 3 with a standard solution containing the 29 target analytes at 100% of the defined maximum limit, considering a sample concentration at 100 mg/mL.

CONDITION B	Sample 2 (Drug product B)	Sample 3 (Drug substance A)
	% RSD	
Analyte 1	2	1
Analyte 2	7	2
Analyte 3	2	2
Analyte 4	5	1
Analyte 5	6	2
Analyte 6	2	1
Analyte 7	2	2
Analyte 8	2	1
Analyte 9	2	1
Analyte 10	2	2
Analyte 11	3	0.3
Analyte 12	5	1
Analyte 13	2	0.2
Analyte 14	3	3
Analyte 15	2	1
Analyte 16	2	1
Analyte 17	2	1
Analyte 18	3	0.3
Analyte 19	2	1
Analyte 20	2	0.2
Analyte 21	5	4
Analyte 22	2	2
Analyte 23	2	2
Analyte 24	3	2
Analyte 25	2	2
Analyte 26	3	1
Analyte 27	3	3
Analyte 28	3	2
Analyte 29	8	2

Table S.4. Results of the analyte concentrations and its %RSD obtained for the evaluation of the robustness condition C by spiking the samples 2 and 3 with a standard solution containing the 29 target analytes at 100% of the defined maximum level, considering a sample concentration at 100 mg/mL.

CONDITION C	Sample 2 (Drug product B)	Sample 3 (Drug substance A)
	% RSD	
Analyte 1	0.03	1
Analyte 2	0.2	2
Analyte 3	0.05	1
Analyte 4	0.1	2
Analyte 5	0.2	2
Analyte 6	0.04	1
Analyte 7	0.04	1
Analyte 8	0.1	1
Analyte 9	0.03	1
Analyte 10	0.1	1
Analyte 11	0.1	1
Analyte 12	0.2	2
Analyte 13	0.1	1
Analyte 14	0.1	1
Analyte 15	0.2	1
Analyte 16	0.02	1
Analyte 17	0.04	1
Analyte 18	0.1	1
Analyte 19	0.04	1
Analyte 20	0.1	1
Analyte 21	0.2	2
Analyte 22	0.2	1
Analyte 23	0.01	1
Analyte 24	0.1	1
Analyte 25	0.02	1
Analyte 26	0.3	1
Analyte 27	0.1	1
Analyte 28	0.03	1
Analyte 29	1	3

Table S.5. Results of the analyte concentrations and its %RSD obtained for the evaluation of the robustness condition D by spiking the samples 2 and 3 with a standard solution containing the 29 target analytes at 100% of the defined maximum level, considering a sample concentration at 100 mg/mL.

CONDITION D	Sample 2 (Drug product B)	Sample 3 (Drug substance A)
	% RSD	
Analyte 1	0.3	1
Analyte 2	1	1
Analyte 3	0.4	1
Analyte 4	1	1
Analyte 5	1	1
Analyte 6	0.4	1
Analyte 7	1	1
Analyte 8	1	1
Analyte 9	2	1
Analyte 10	1	1
Analyte 11	1	1
Analyte 12	2	1
Analyte 13	1	1
Analyte 14	1	1
Analyte 15	1	1
Analyte 16	1	1
Analyte 17	1	1
Analyte 18	2	1
Analyte 19	2	1
Analyte 20	3	1
Analyte 21	1	1
Analyte 22	1	1
Analyte 23	2	1
Analyte 24	2	1
Analyte 25	2	1
Analyte 26	3	2
Analyte 27	3	1
Analyte 28	2	1
Analyte 29	1	1

S.4. Specificity and selectivity

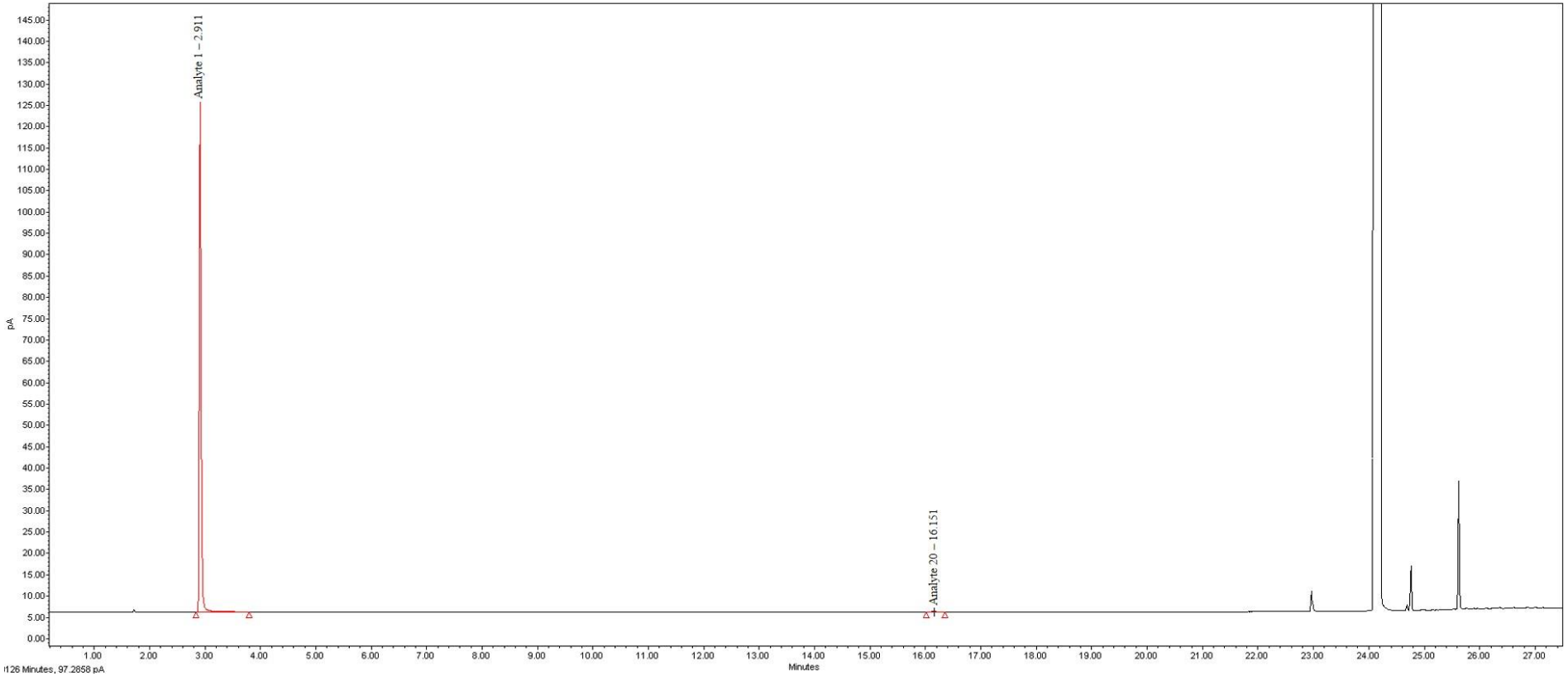


Figure S.1. Chromatogram of Analyte 1 at ICH/VICH limit.

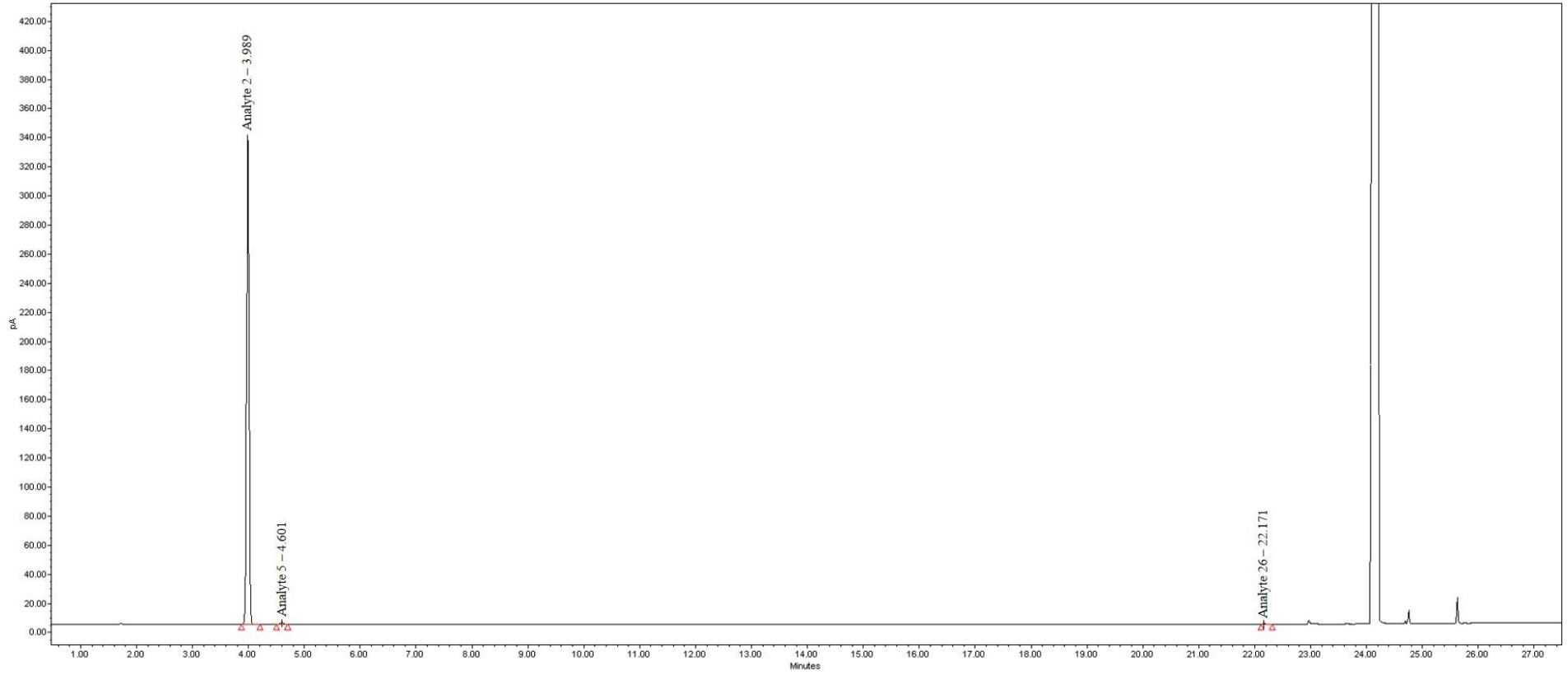


Figure S.2. Chromatogram of Analyte 2 at defined control level.

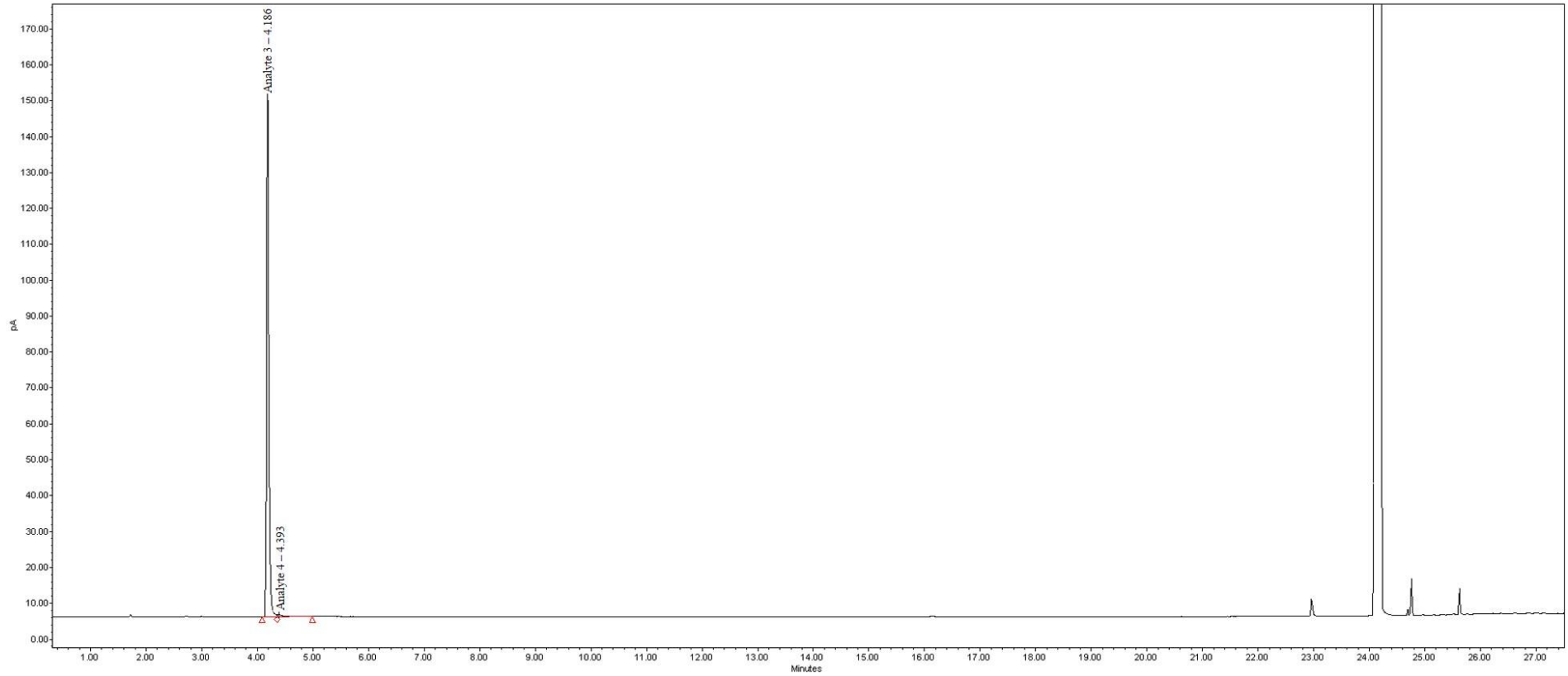


Figure S.3. Chromatogram of Analyte 3 at ICH/VICH limit.

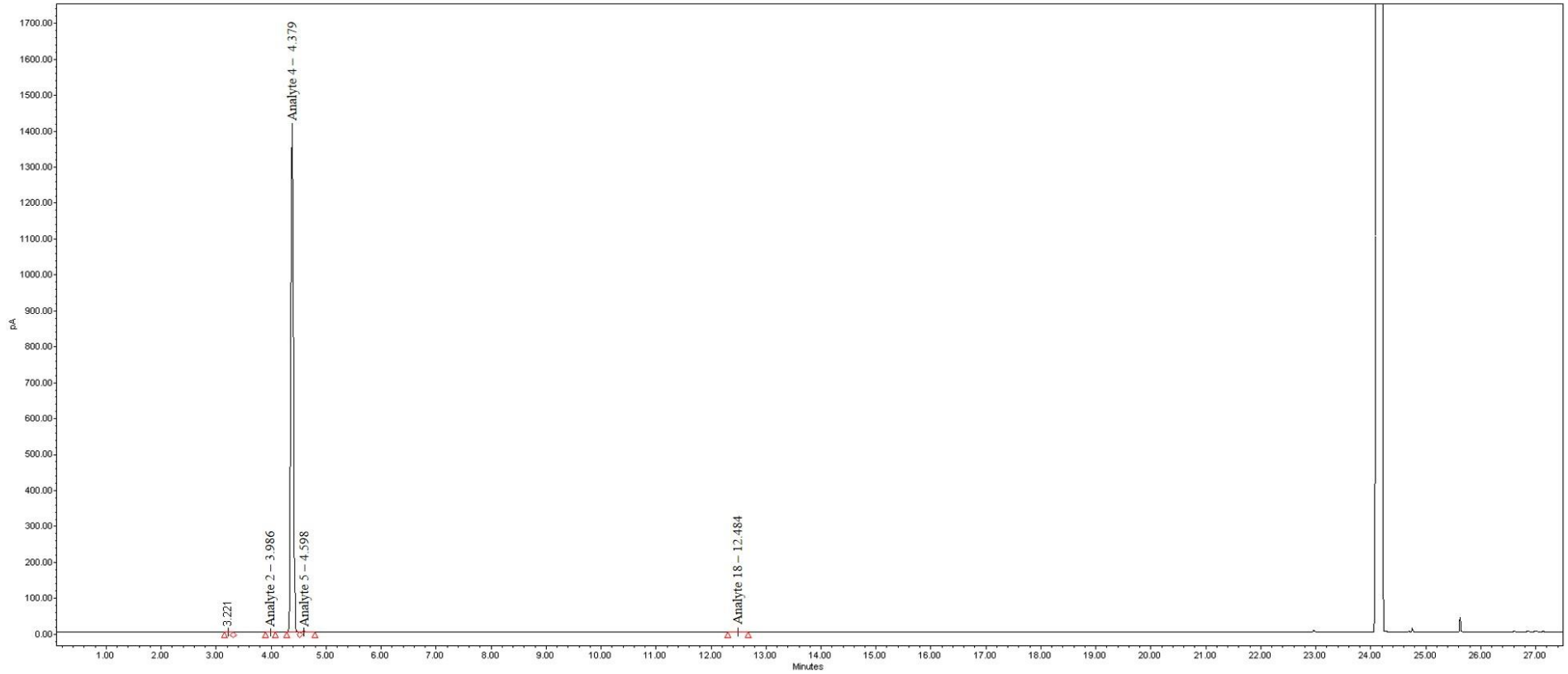


Figure S.4. Chromatogram of Analyte 4 at ICH/VICH limit.

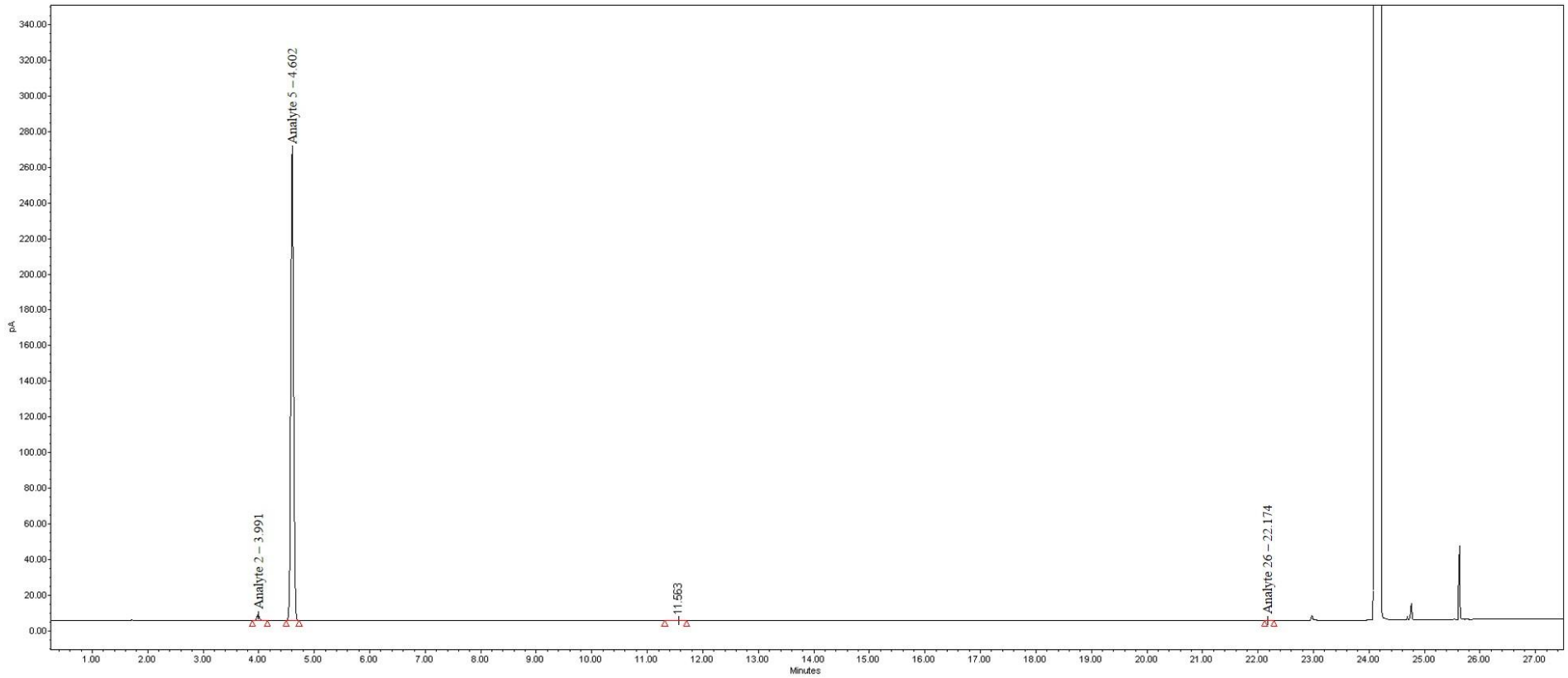


Figure S.5. Chromatogram of Analyte 5 at defined control level.

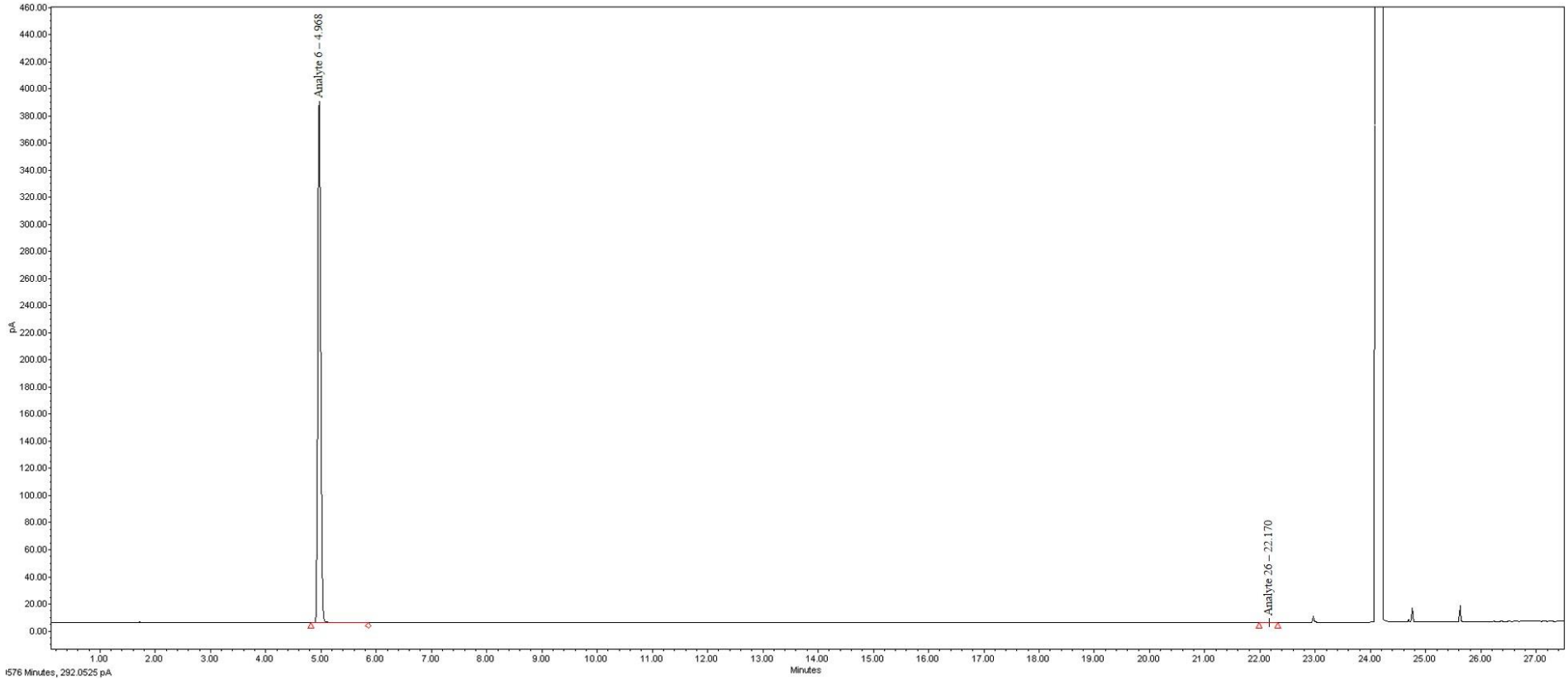


Figure S.6. Chromatogram of Analyte 6 at ICH/VICH limit.

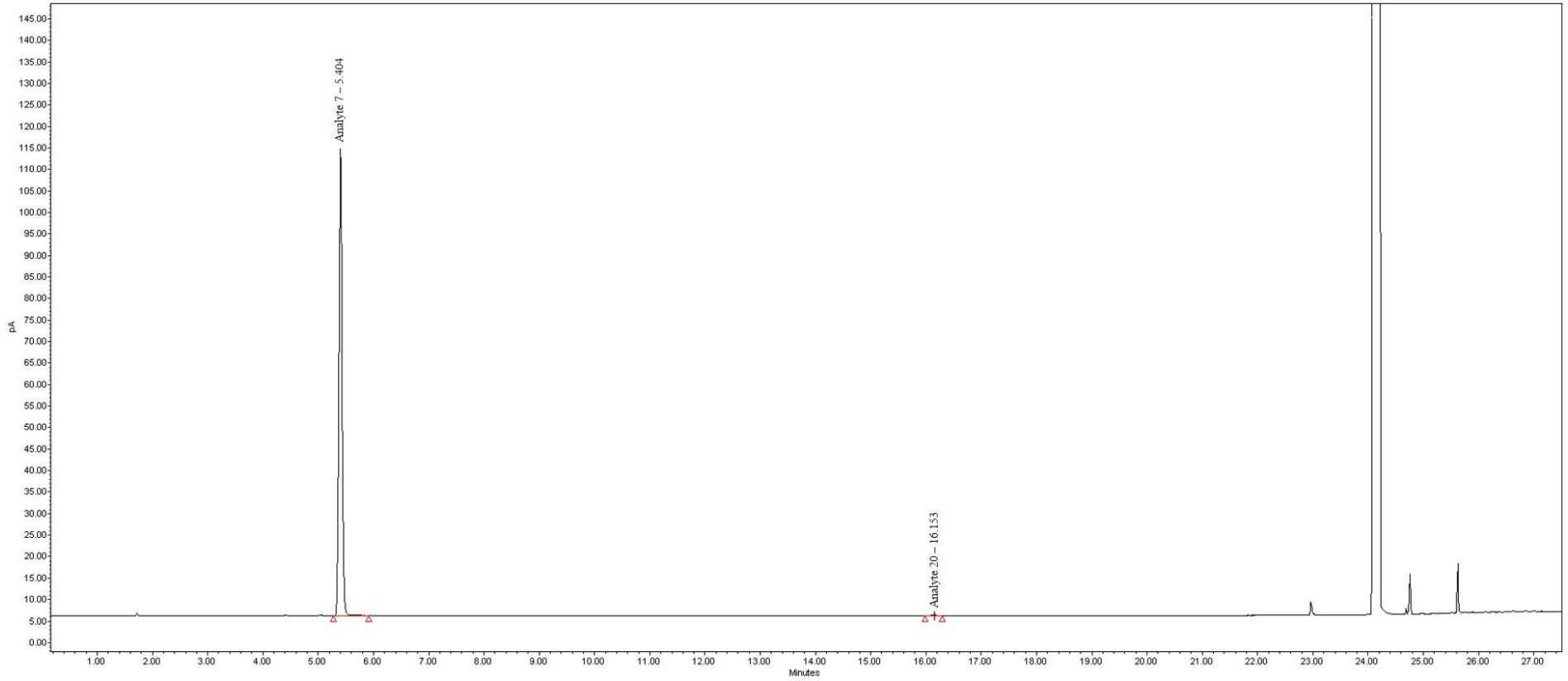


Figure S.7. Chromatogram of Analyte 7 at ICH/VICH limit.

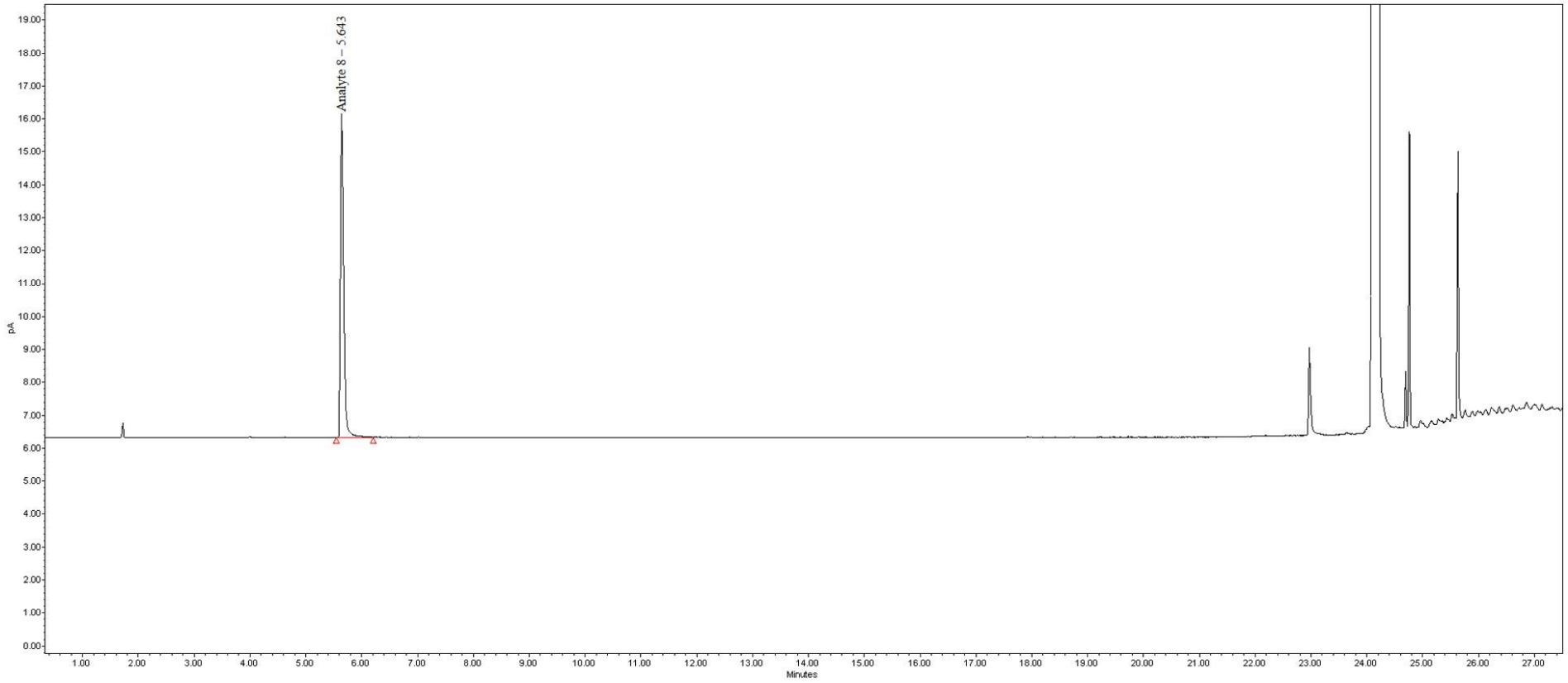


Figure S.8. Chromatogram of Analyte 8 at ICH/VICH limit.

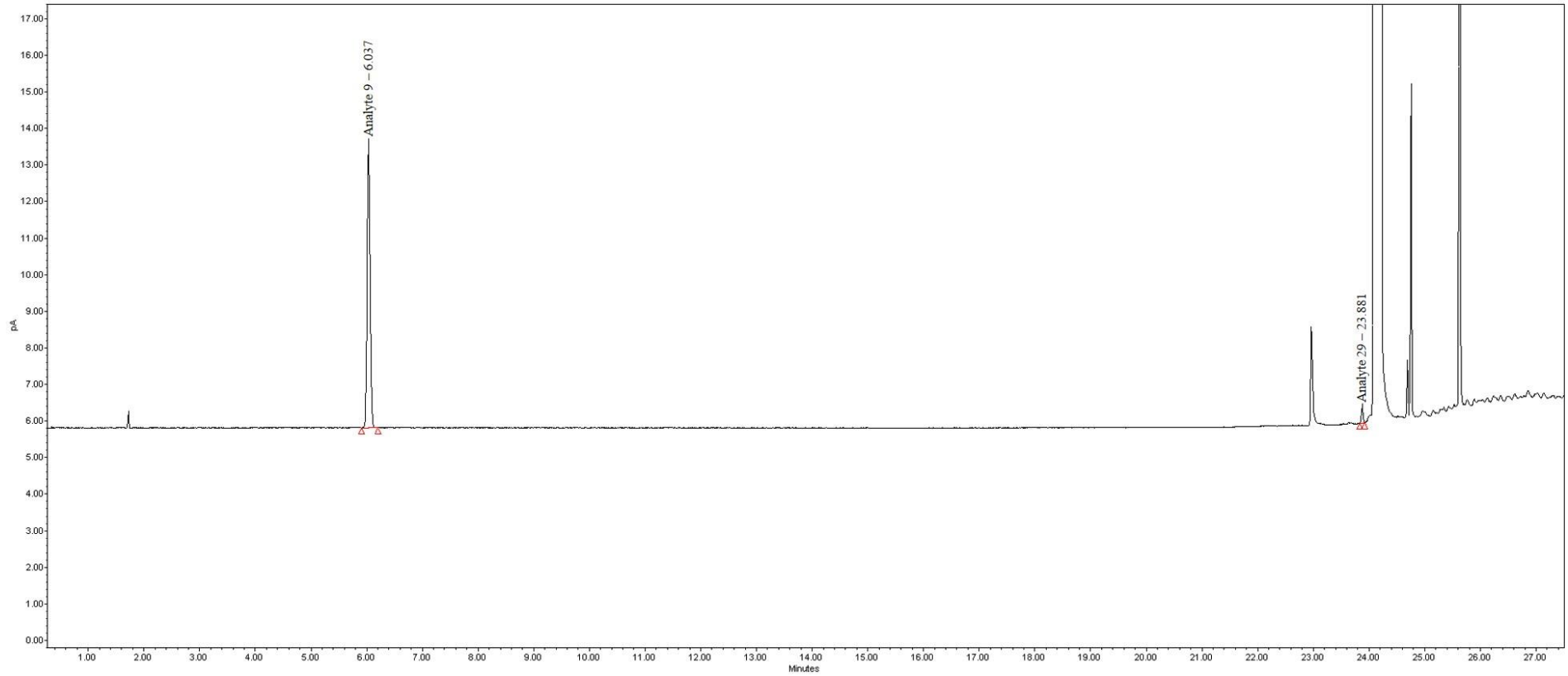


Figure S.9. Chromatogram of Analyte 9 at ICH/VICH limit.

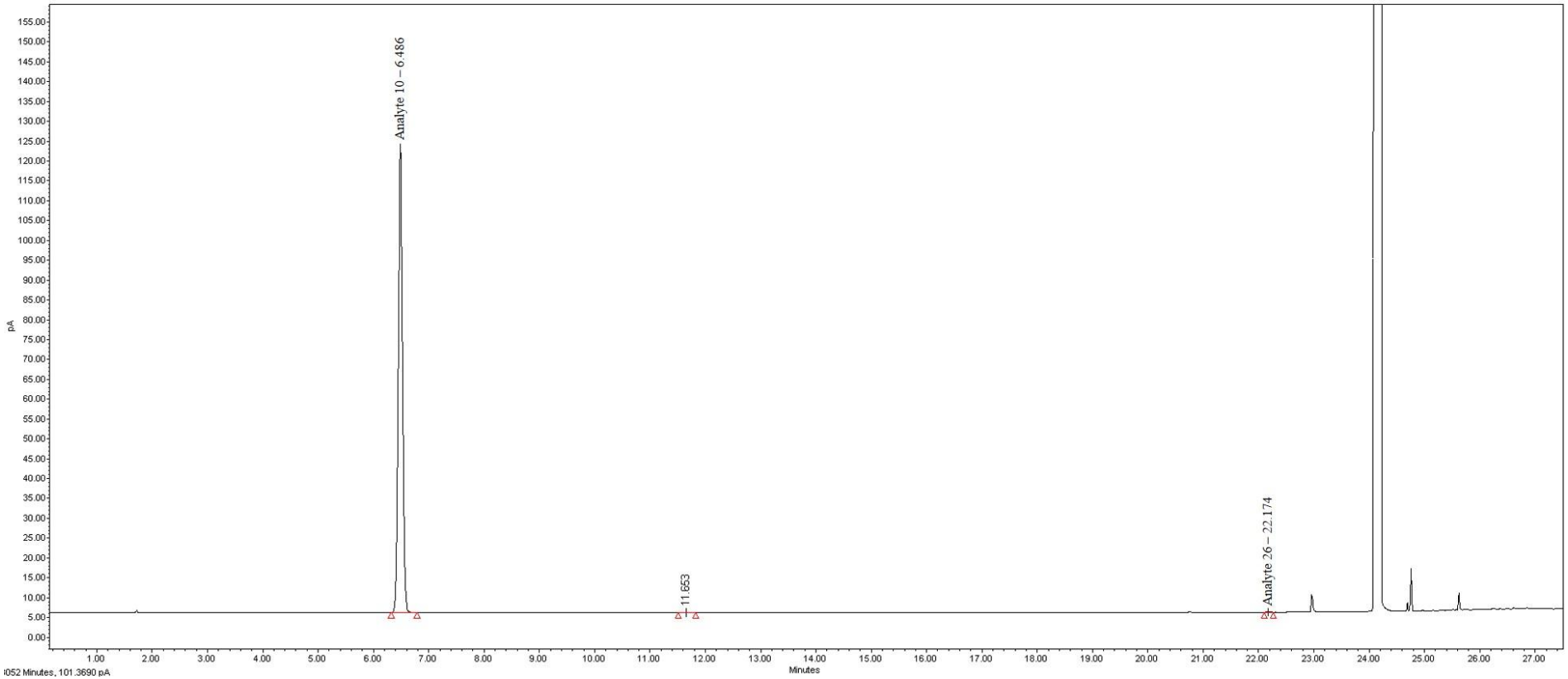


Figure S.10. Chromatogram of Analyte 10 at defined control level.

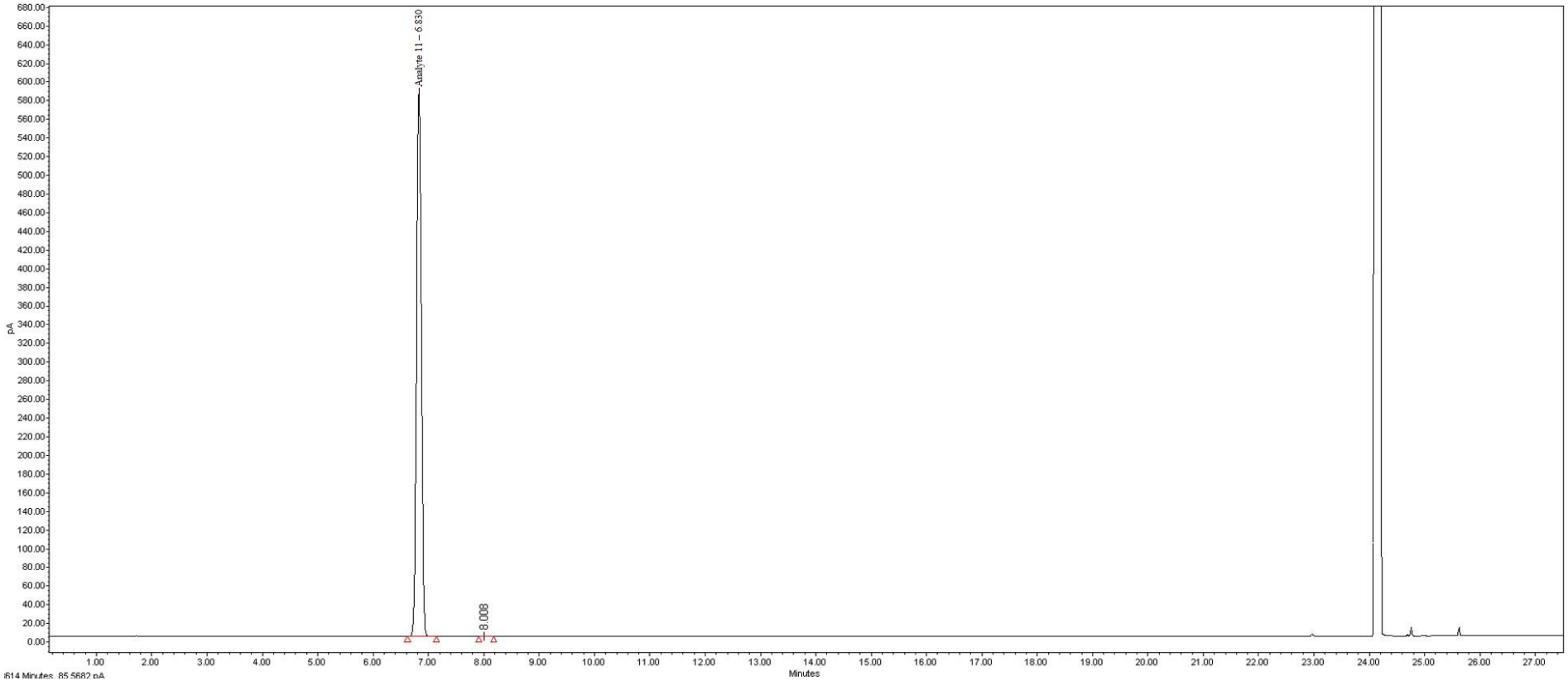


Figure S.11. Chromatogram of Analyte 11 at ICH/VICH limit.

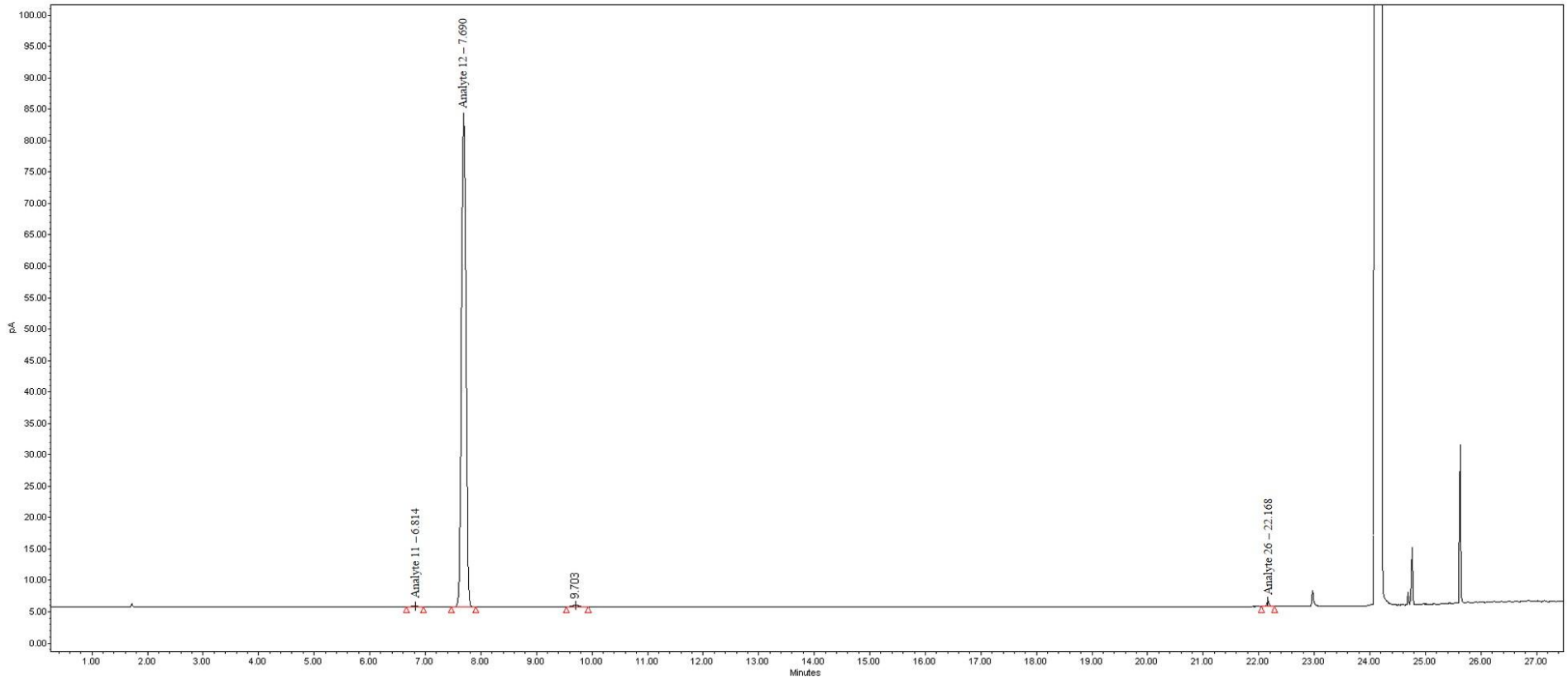


Figure S.12. Chromatogram of Analyte 12 at ICH/VICH limit.

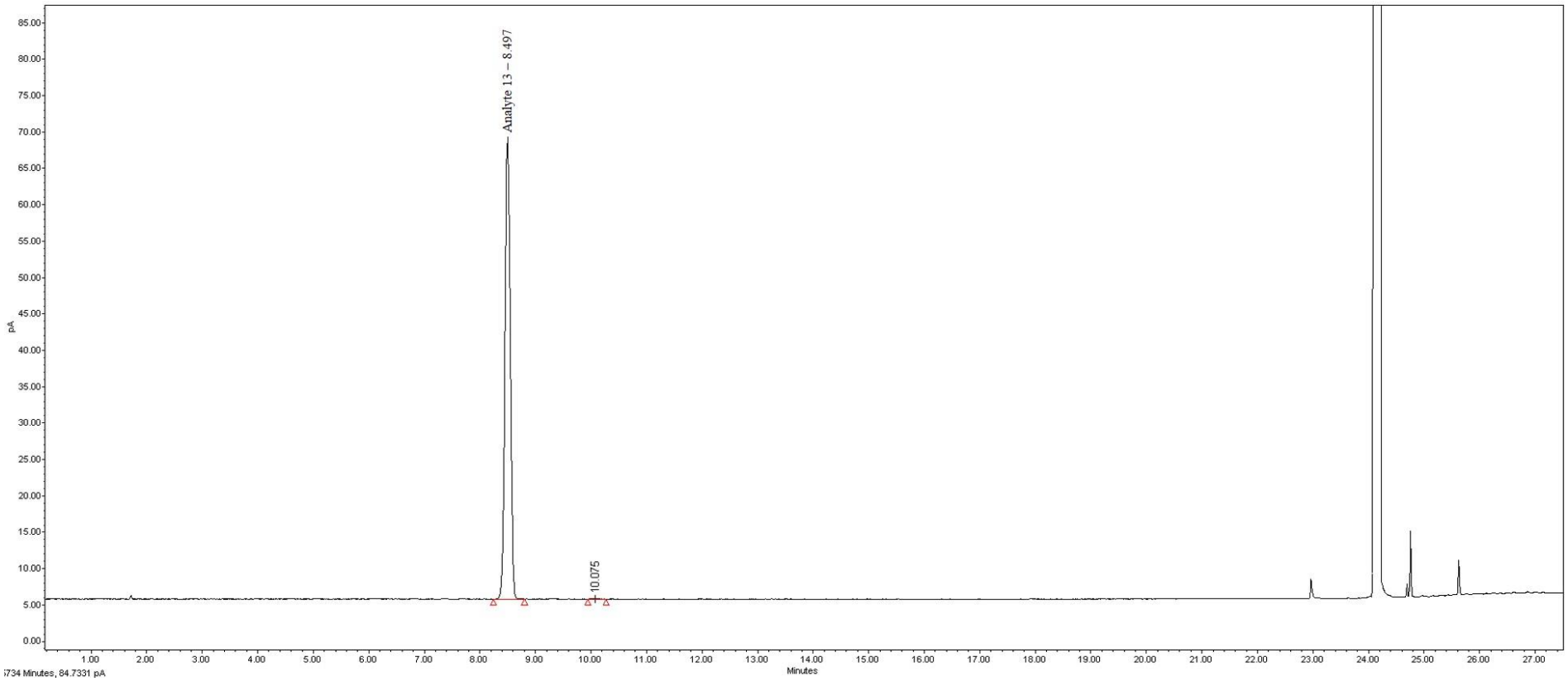


Figure S.13. Chromatogram of Analyte 13 at defined control level.

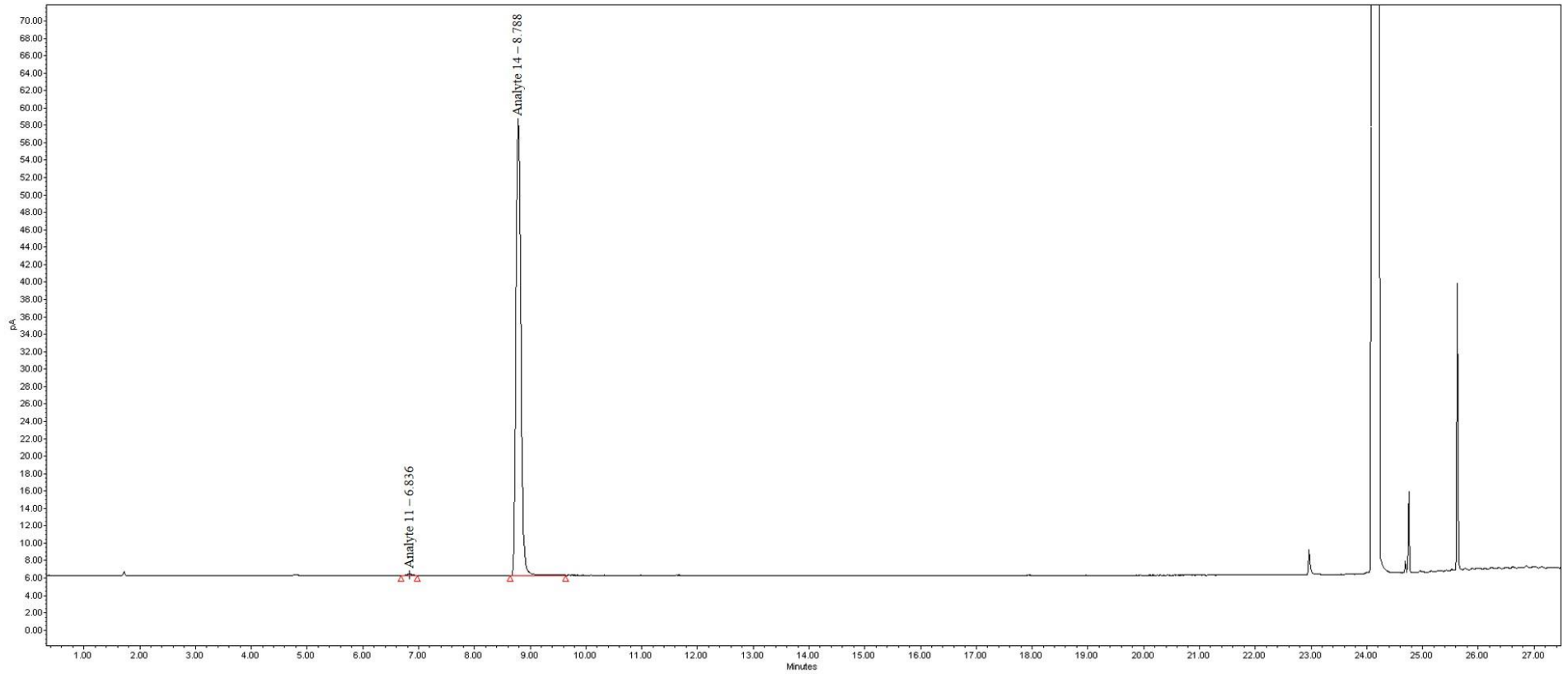


Figure S.14. Chromatogram of Analyte 14 at ICH/VICH limit.

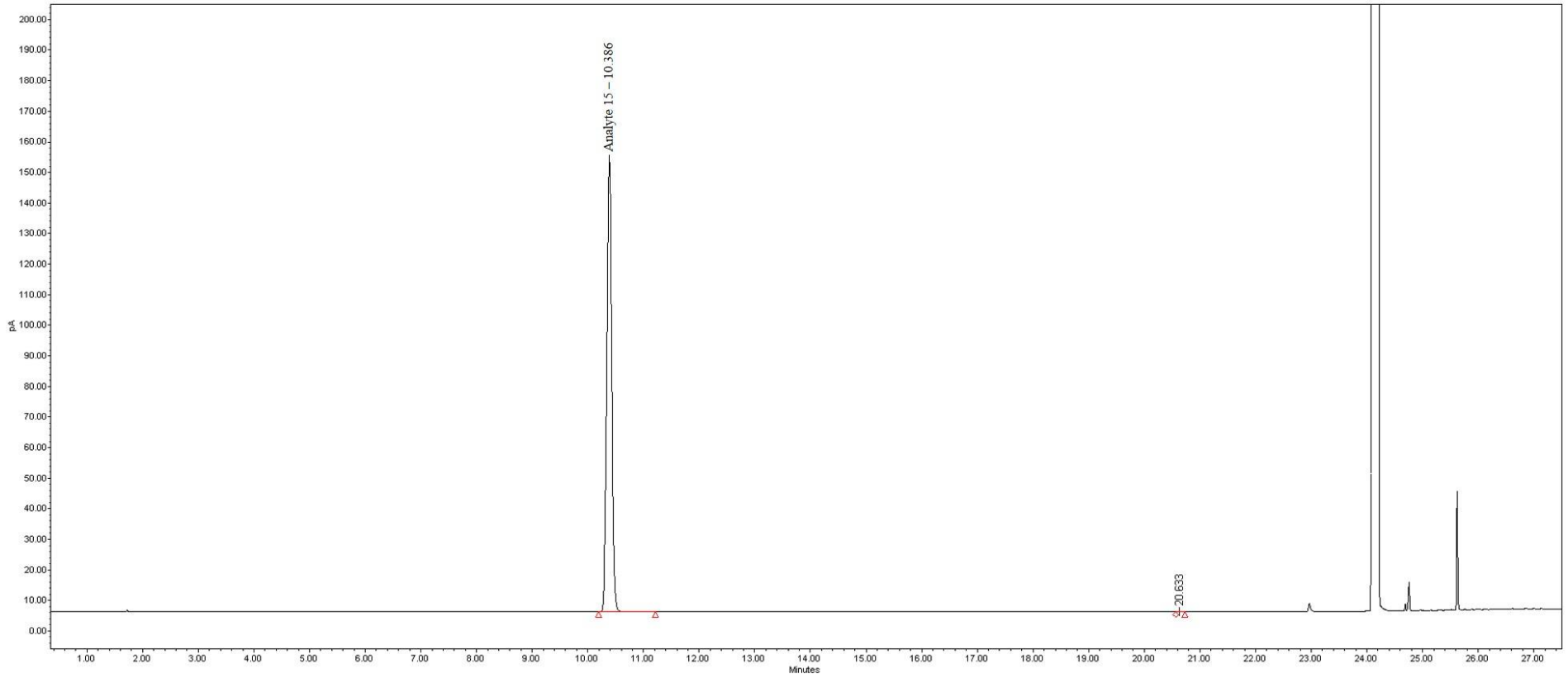


Figure S.15. Chromatogram of Analyte 15 at ICH/VICH limit.

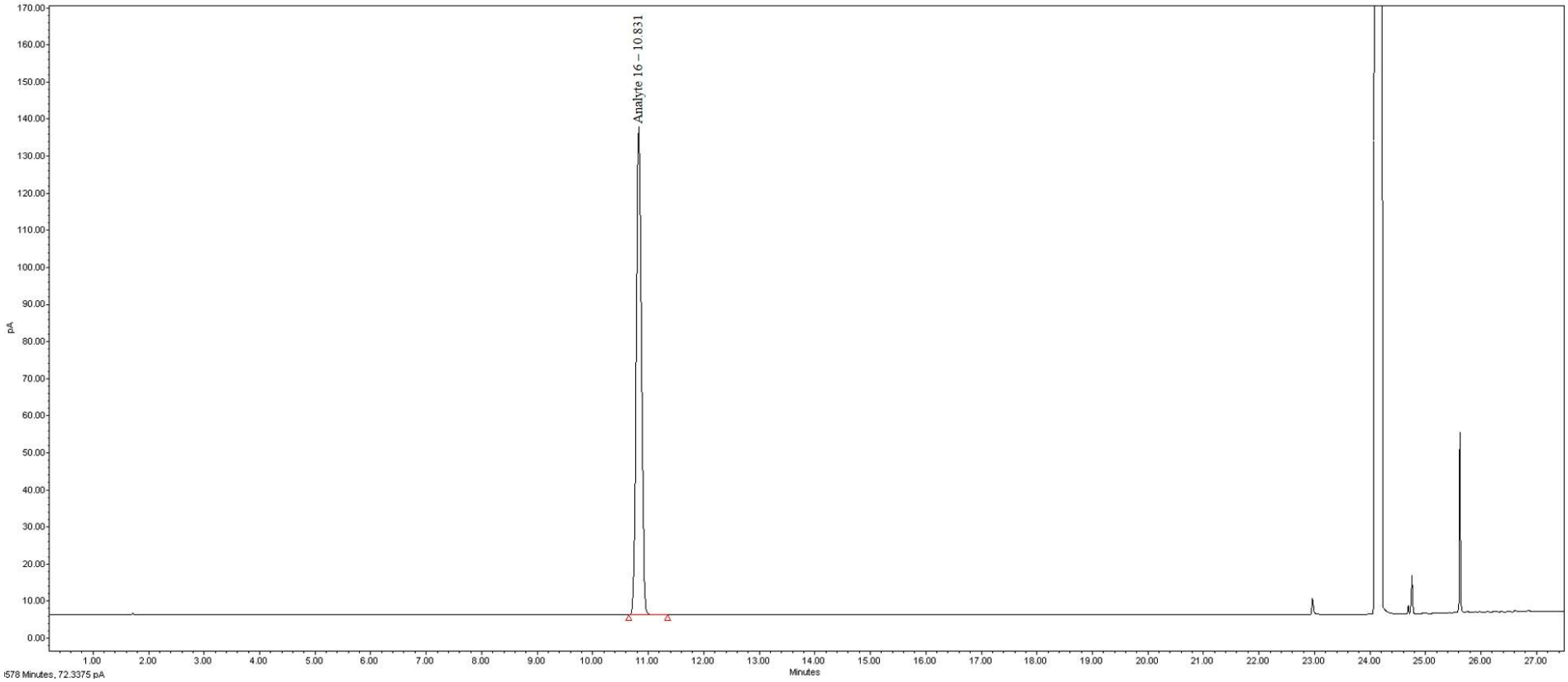


Figure S.16. Chromatogram of Analyte 16 at ICH/VICH limit.

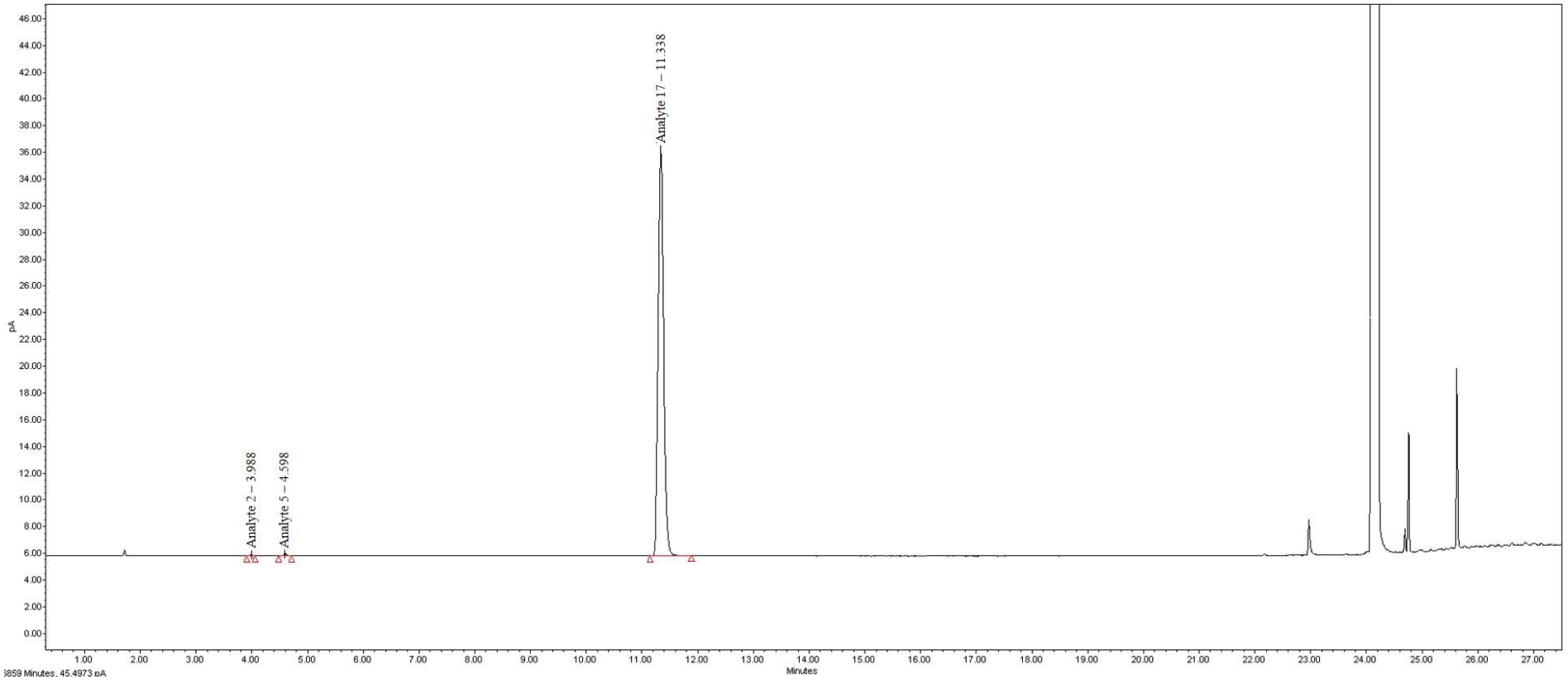


Figure S.17. Chromatogram of Analyte 17 at ICH/VICH limit.

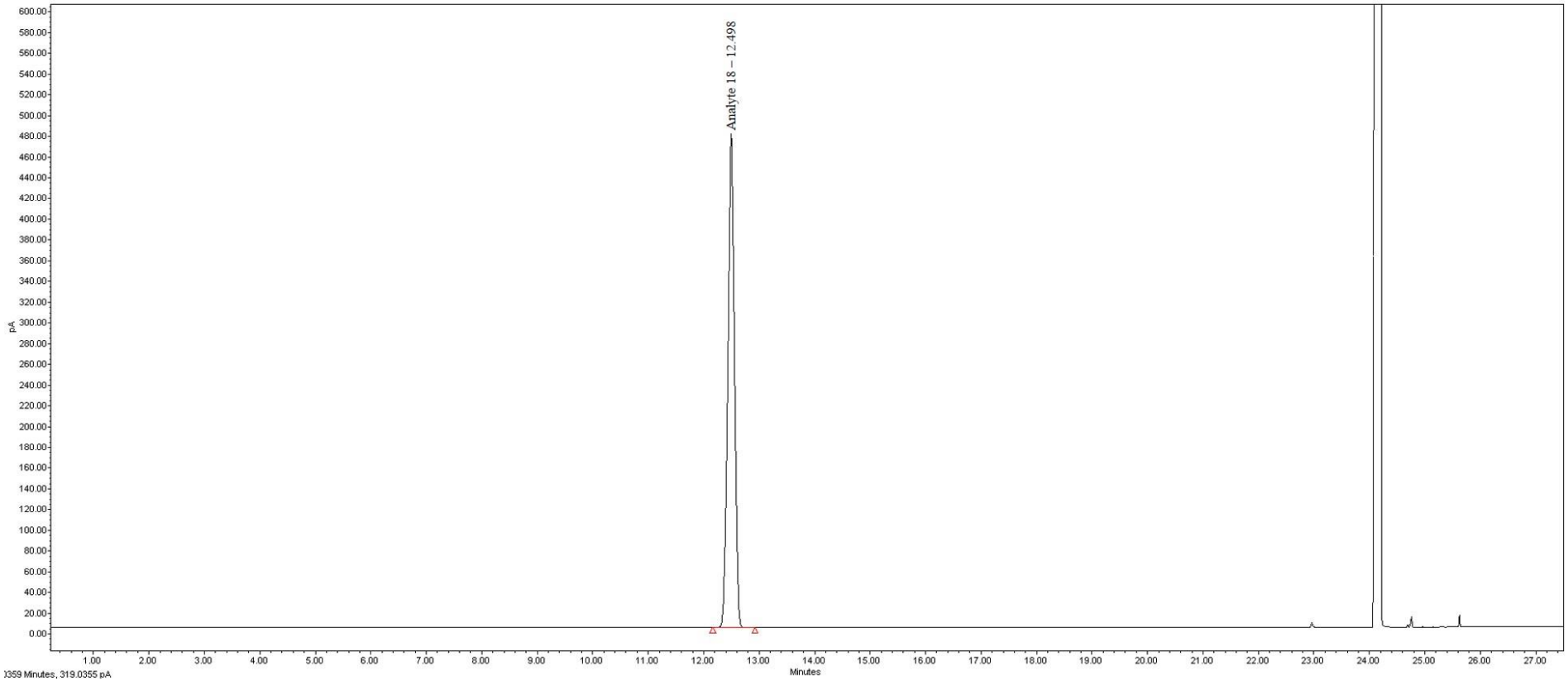


Figure S.18. Chromatogram of Analyte 18 at ICH/VICH limit.

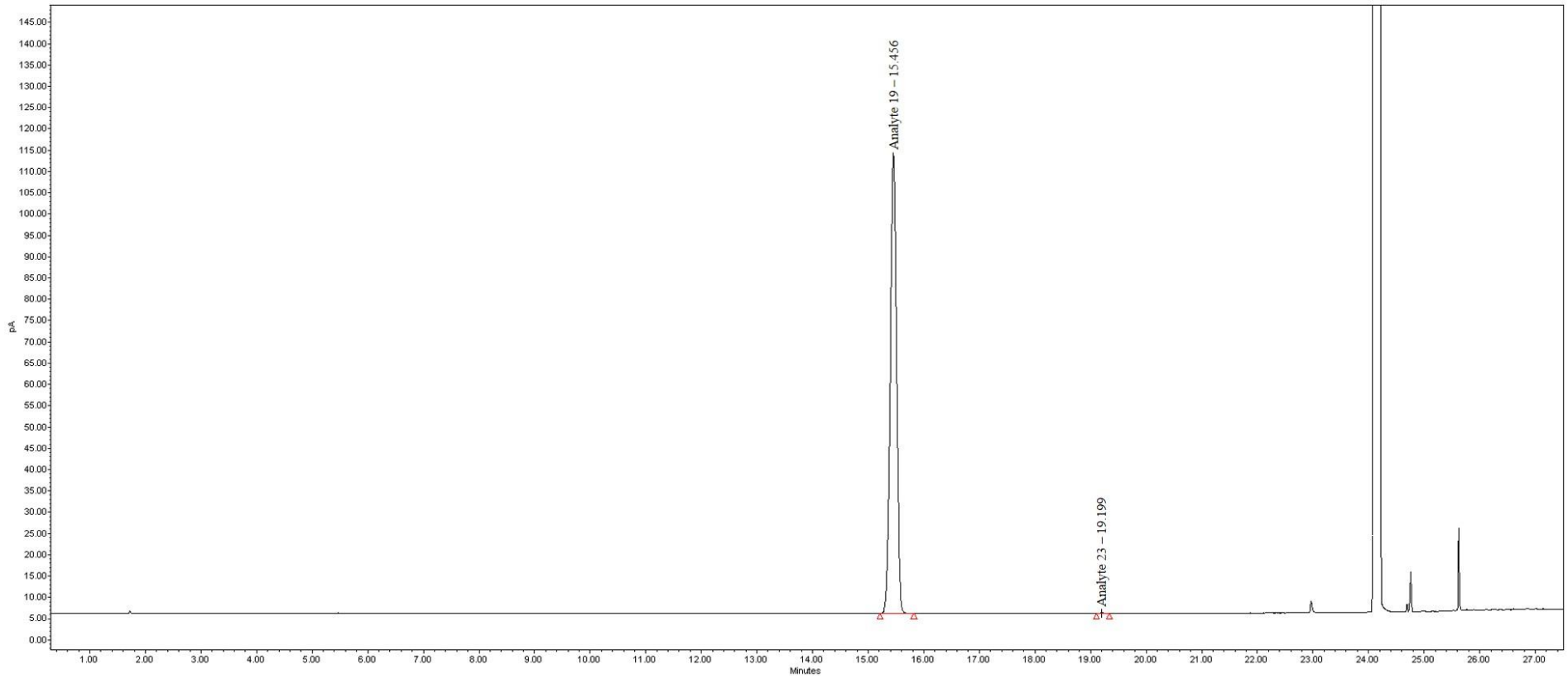


Figure S.19. Chromatogram of Analyte 19 at ICH/VICH limit.

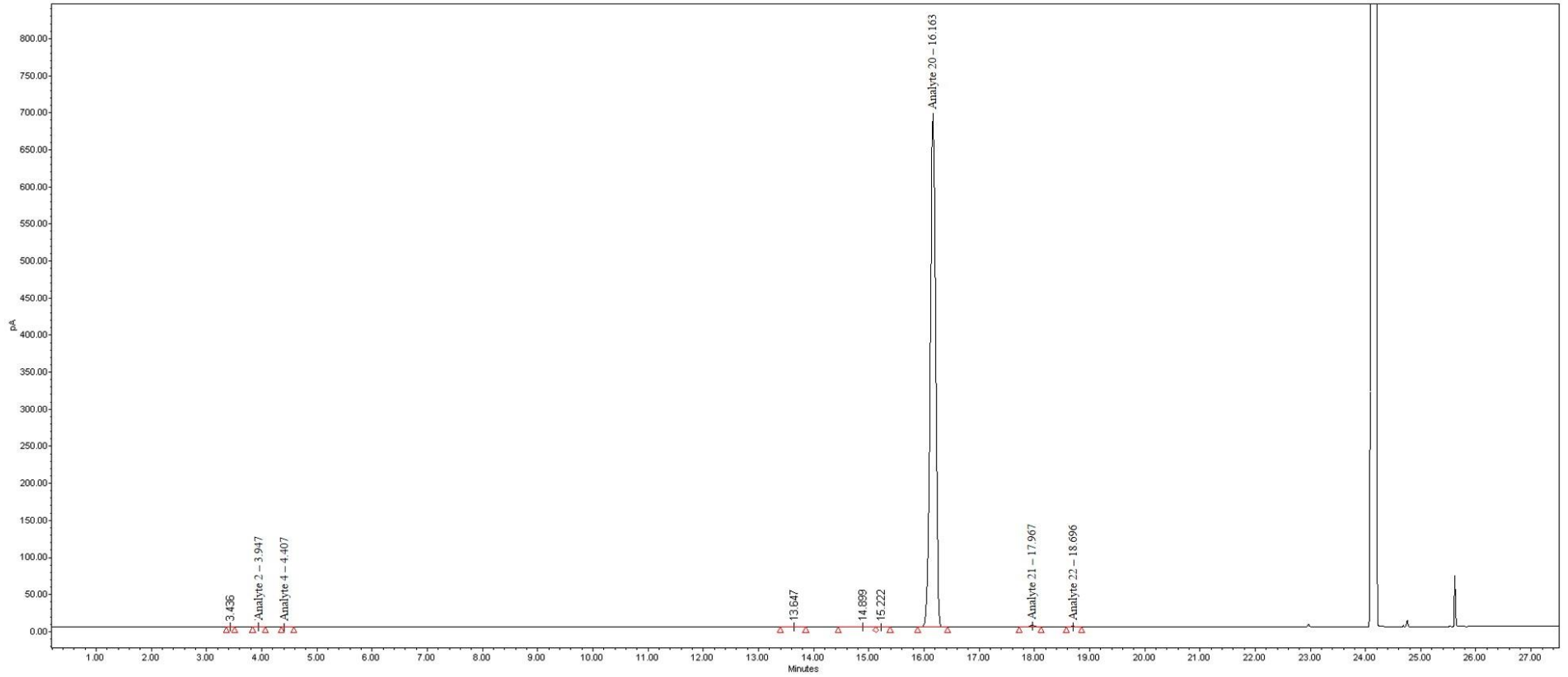


Figure S.20. Chromatogram of Analyte 20 at ICH/VICH limit.

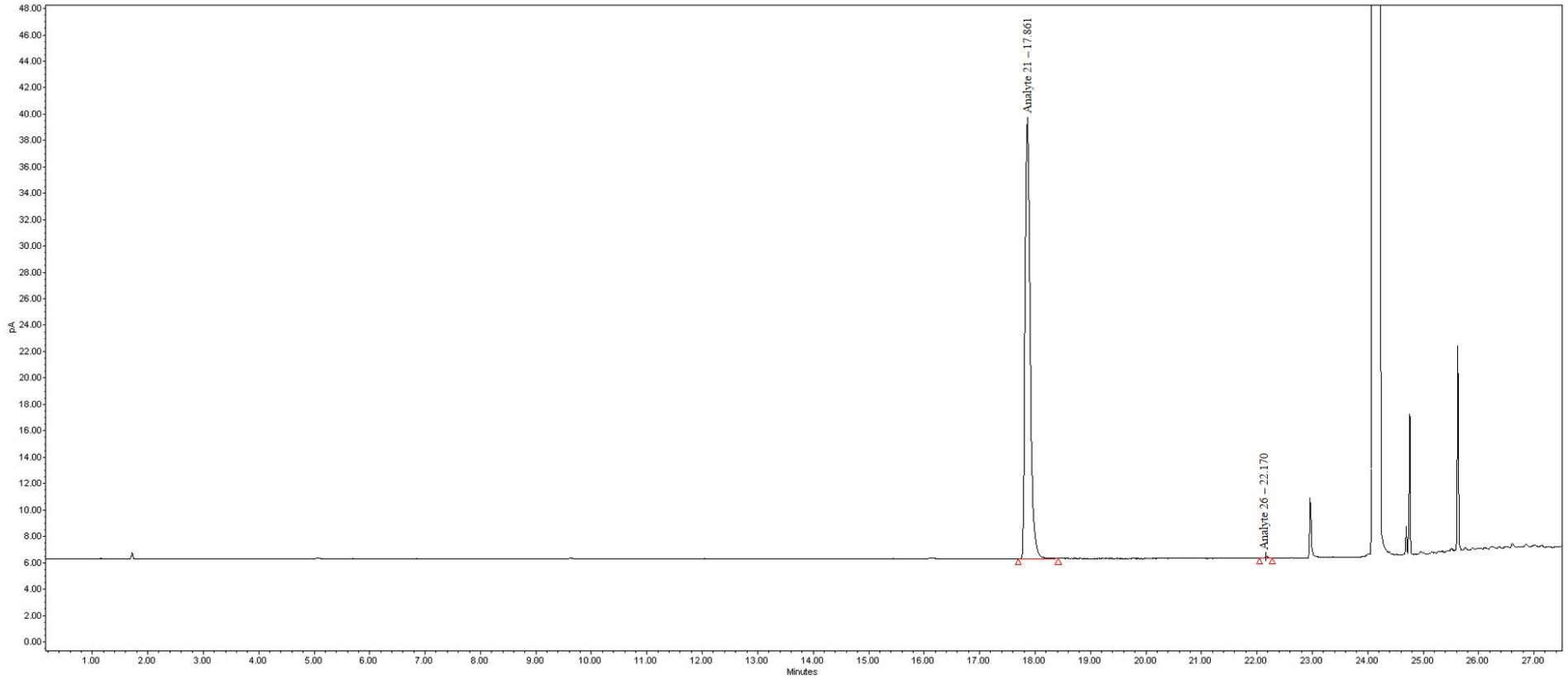


Figure S.21. Chromatogram of Analyte 21 at ICH/VICH limit.

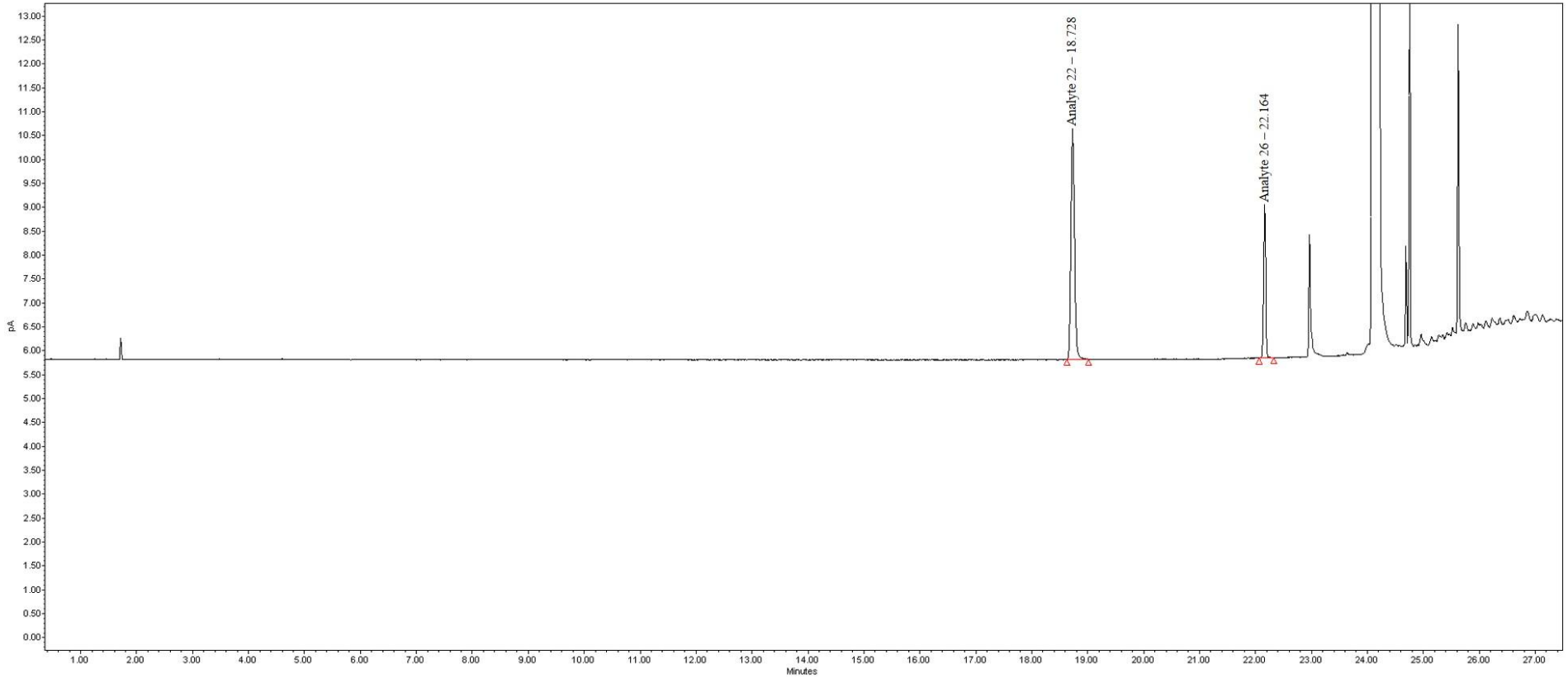


Figure S.22. Chromatogram of Analyte 22 at ICH/VICH limit.

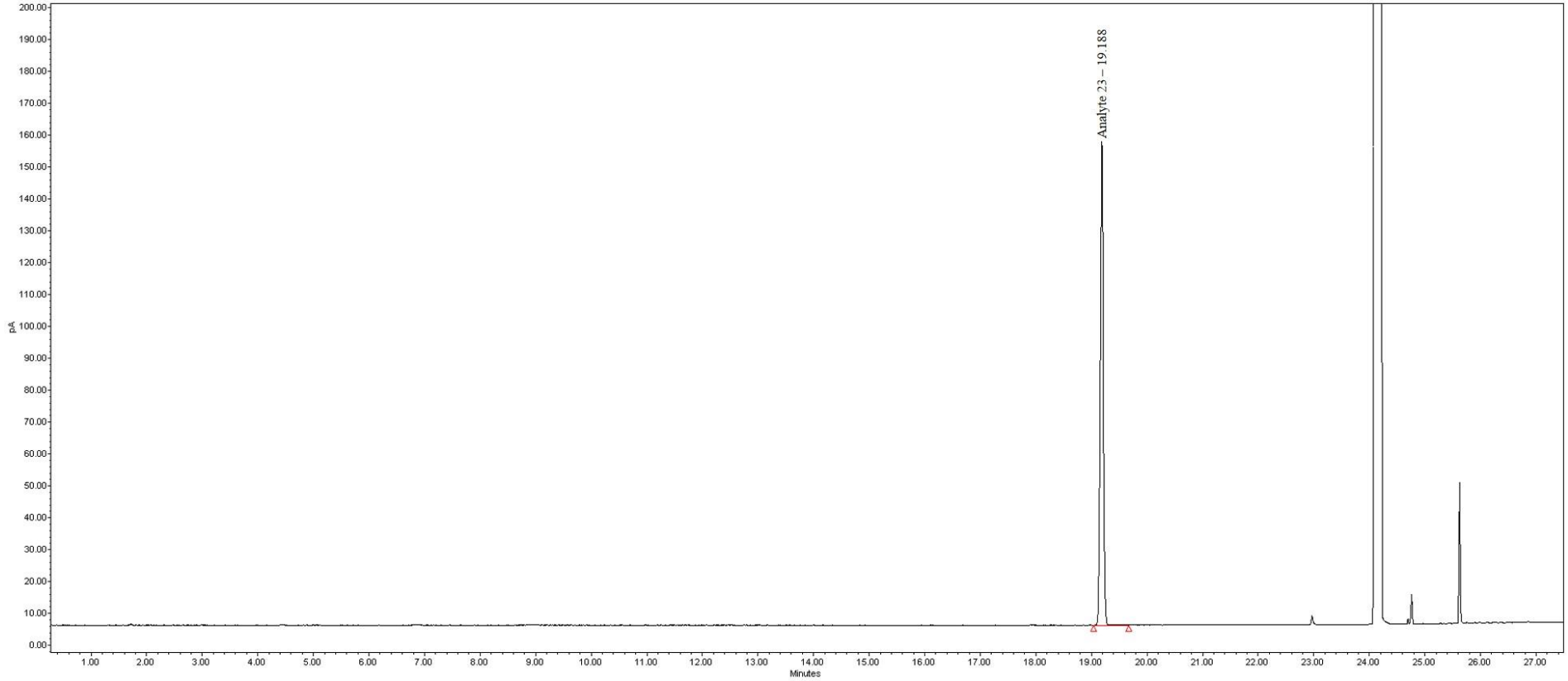


Figure S.23. Chromatogram of Analyte 23 at ICH/VICH limit.

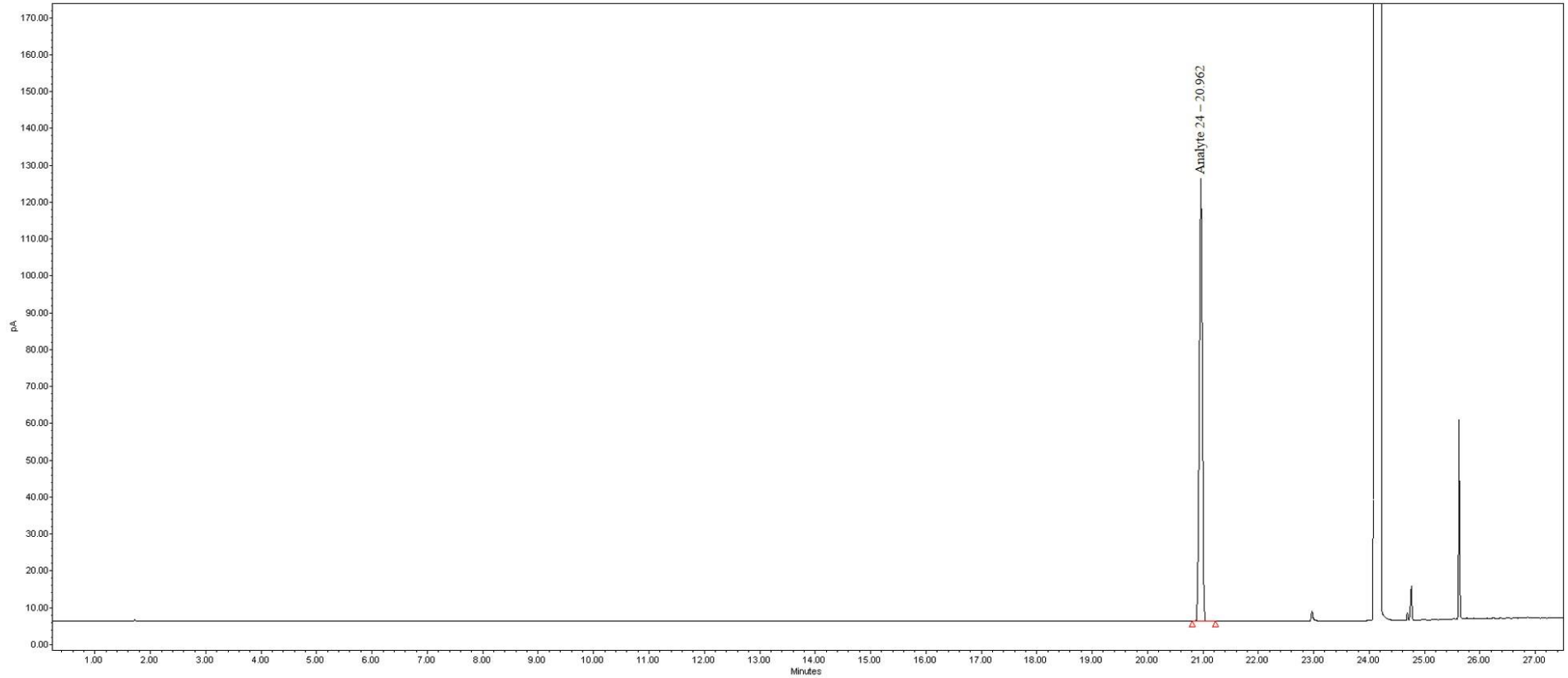


Figure S.24. Chromatogram of Analyte 24 at ICH/VICH limit.

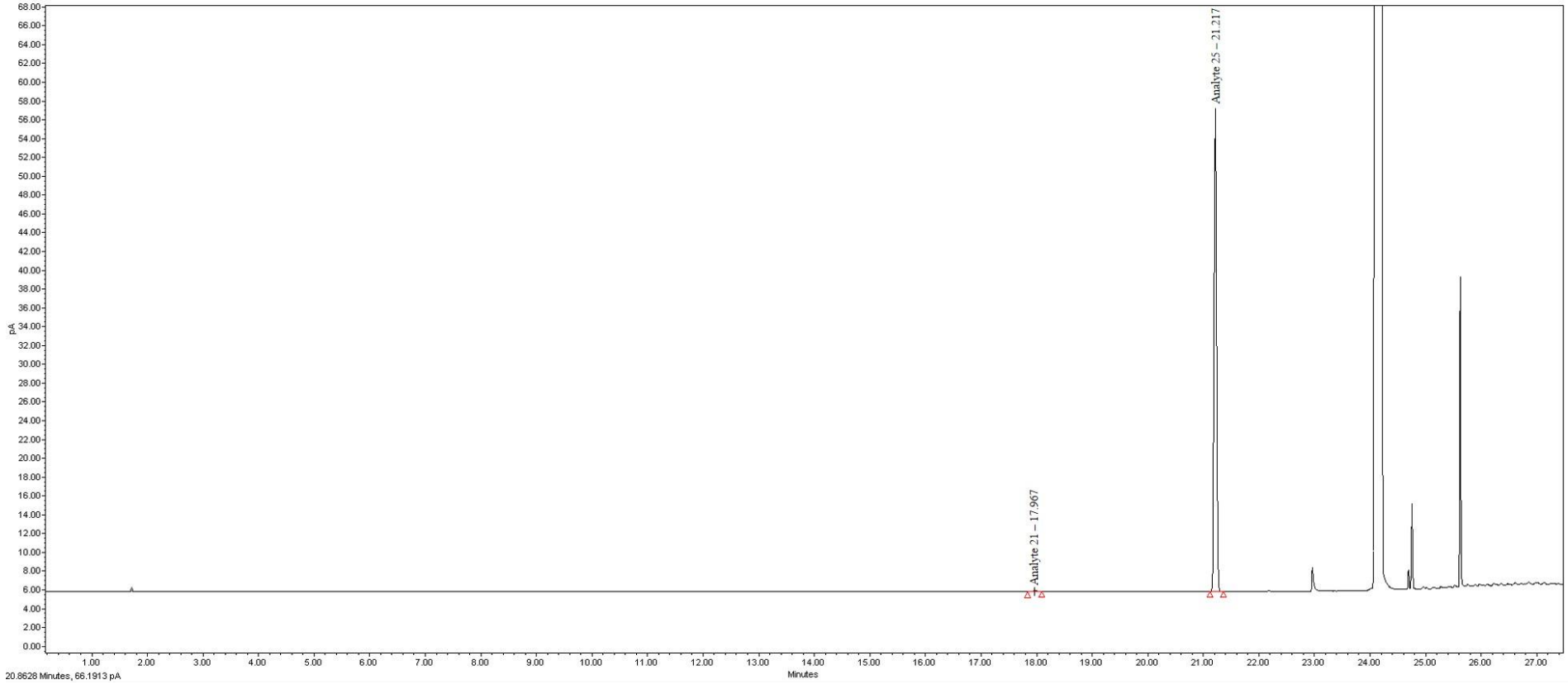


Figure S.25. Chromatogram of Analyte 25 at ICH/VICH limit.

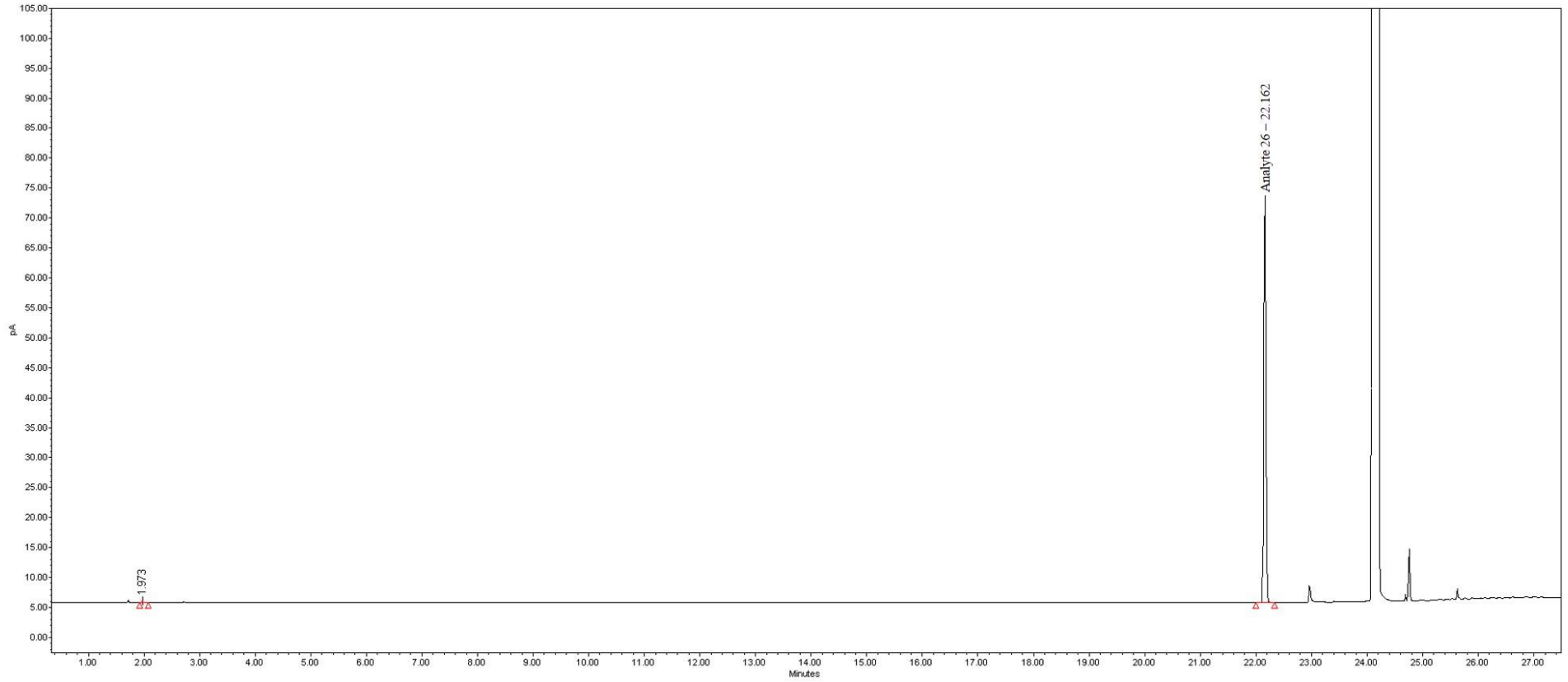


Figure S.26. Chromatogram of Analyte 26 at defined control level.

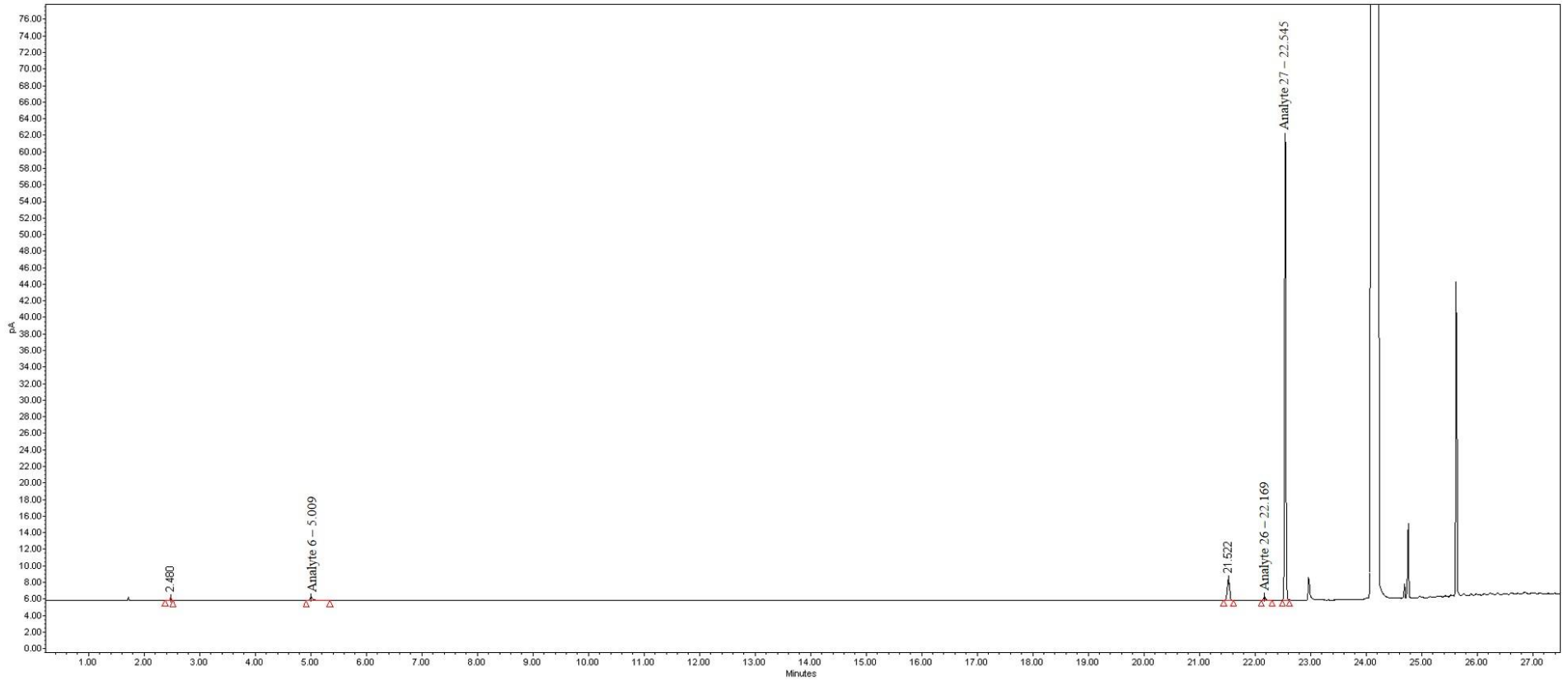


Figure S.27. Chromatogram of Analyte 27 at defined control level.

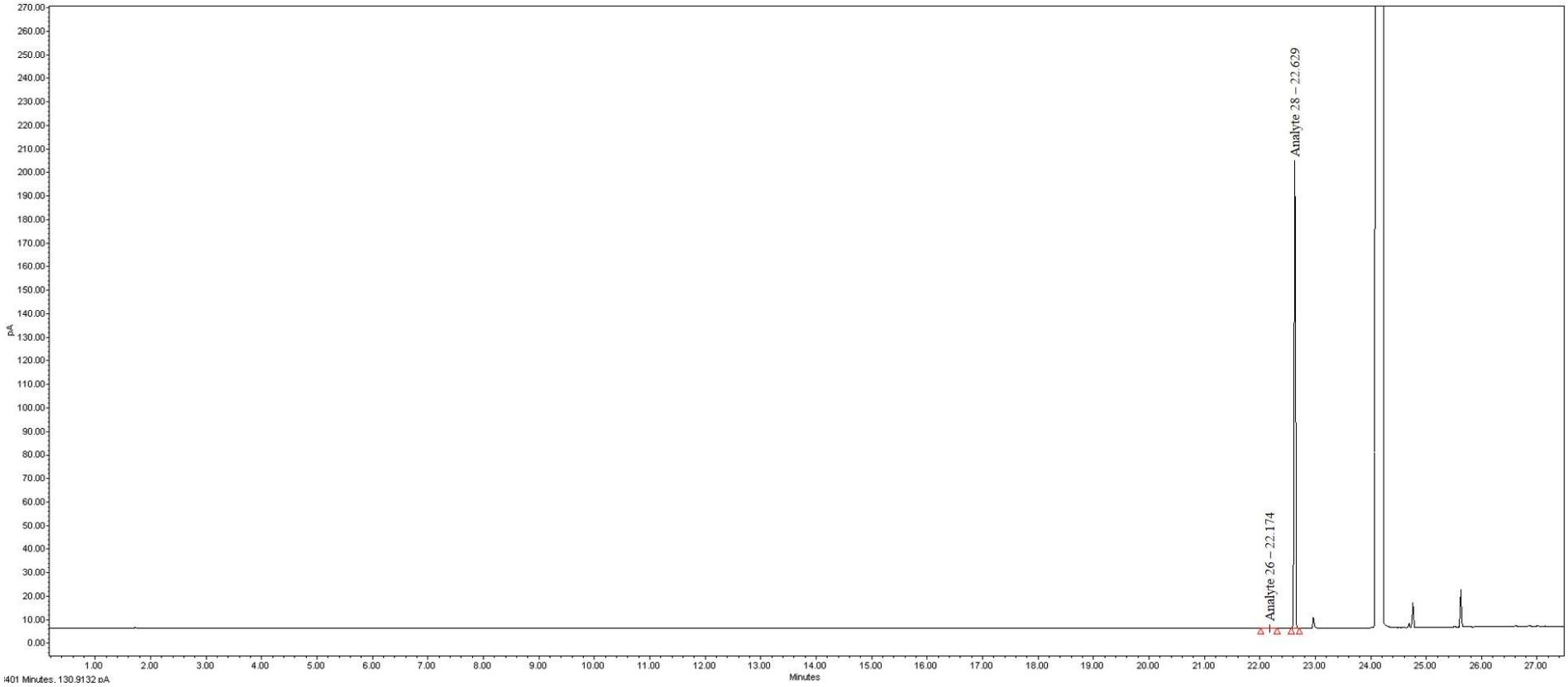


Figure S.28. Chromatogram of Analyte 28 at ICH/VICH limit.

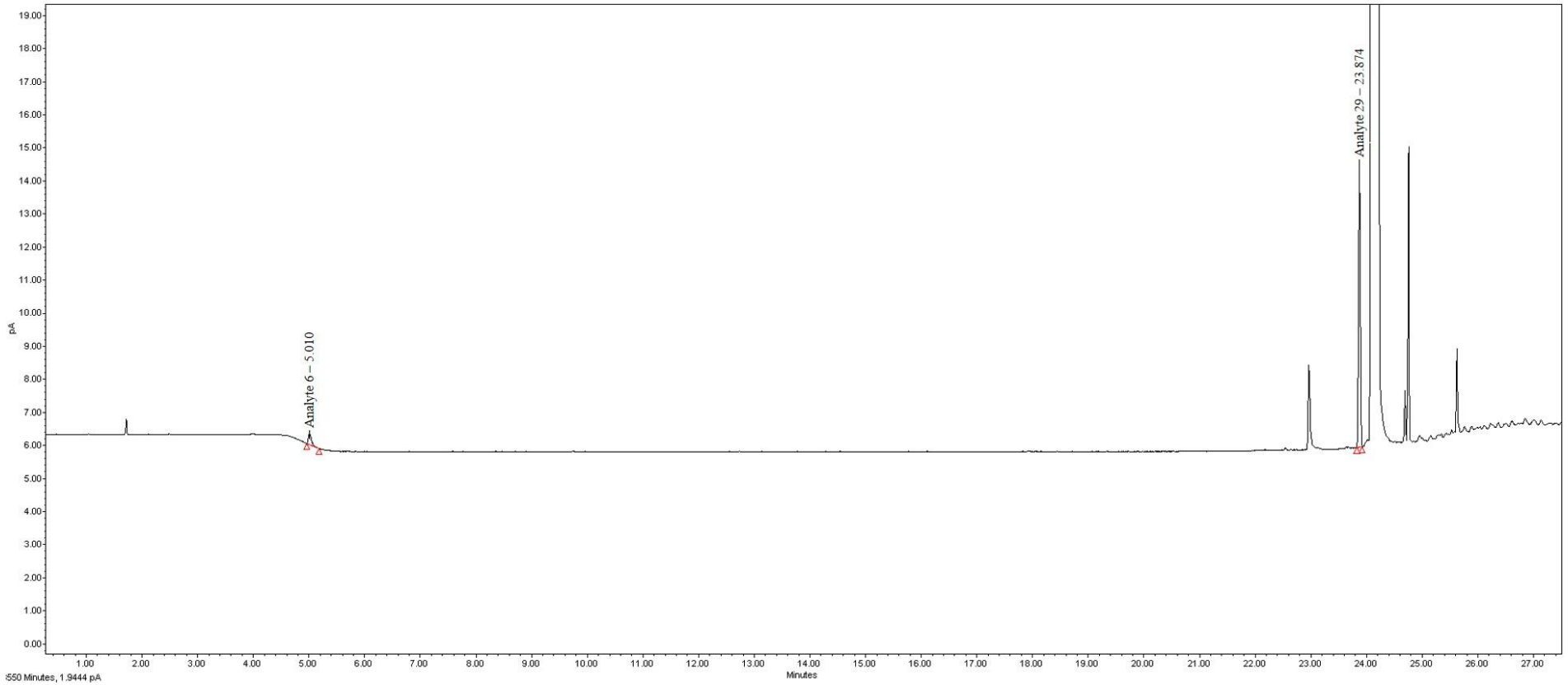


Figure S.29. Chromatogram of Analyte 29 at defined control level.

S.5. Limit of detection

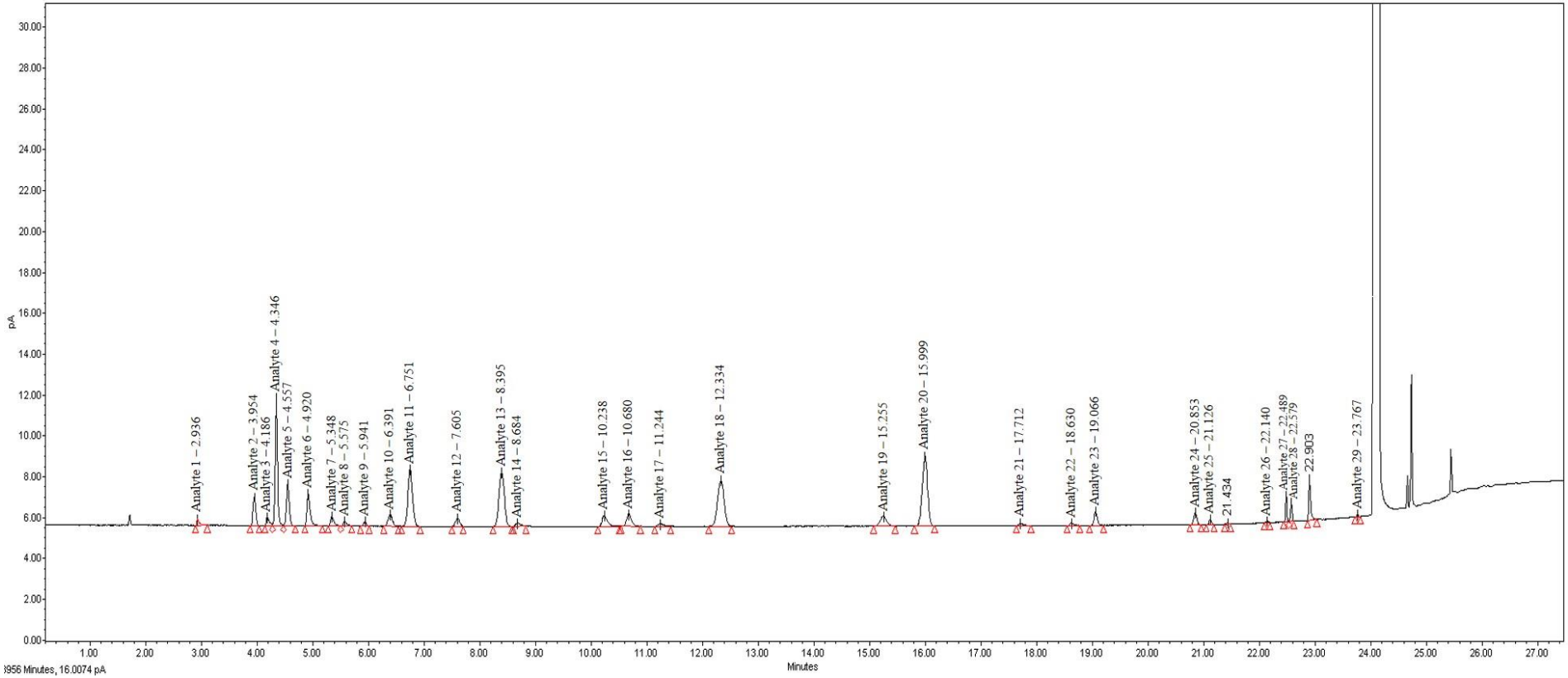


Figure S.30. Chromatographic profile obtained for Normal Operable Conditions (NOC) by injecting a standard solution containing the 29 target analytes at LOD level.

S.6. Linearity and range

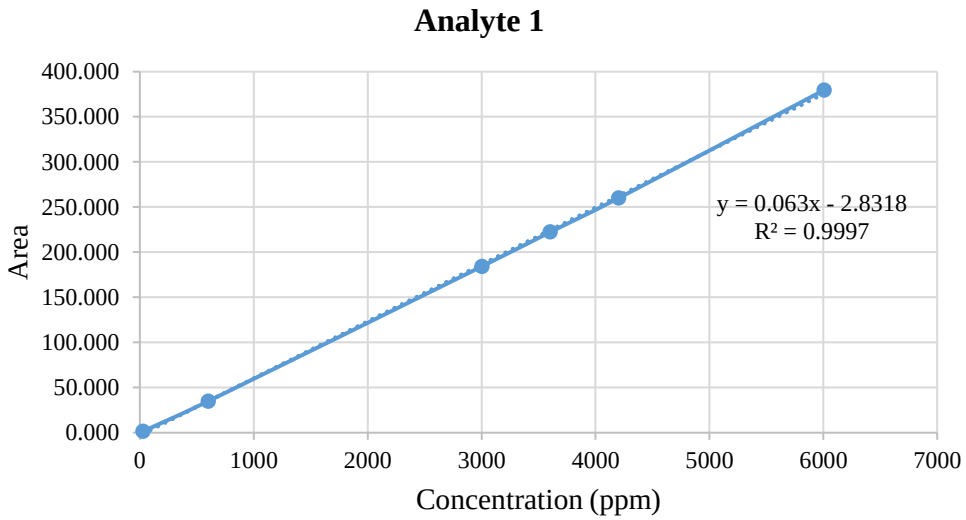


Figure S.31. Calibration curve obtained by the injection of Analyte 1 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

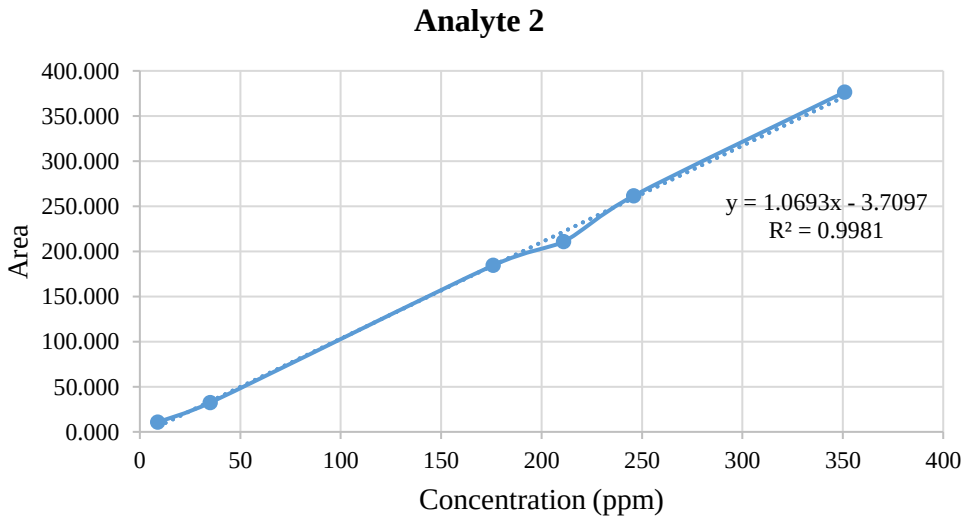


Figure S.32. Calibration curve obtained by the injection of Analyte 2 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

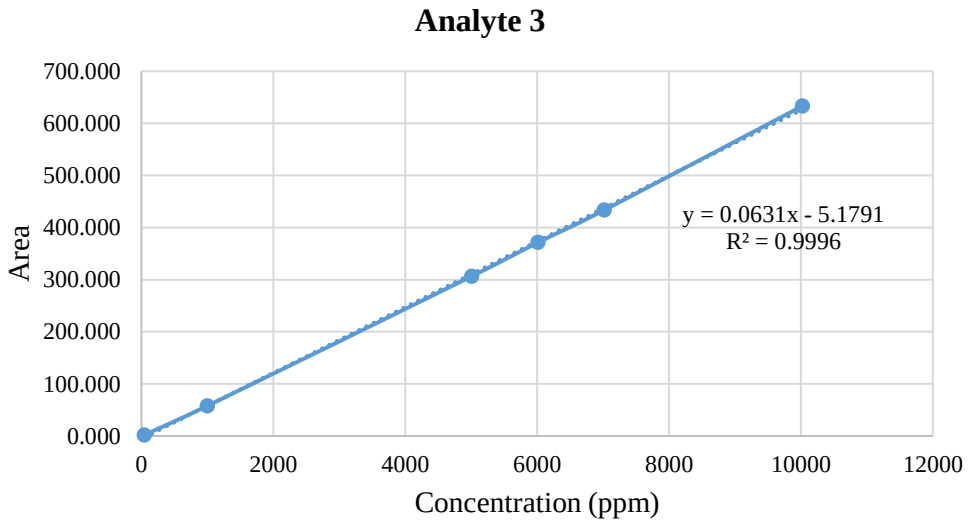


Figure S.33. Calibration curve obtained by the injection of Analyte 3 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

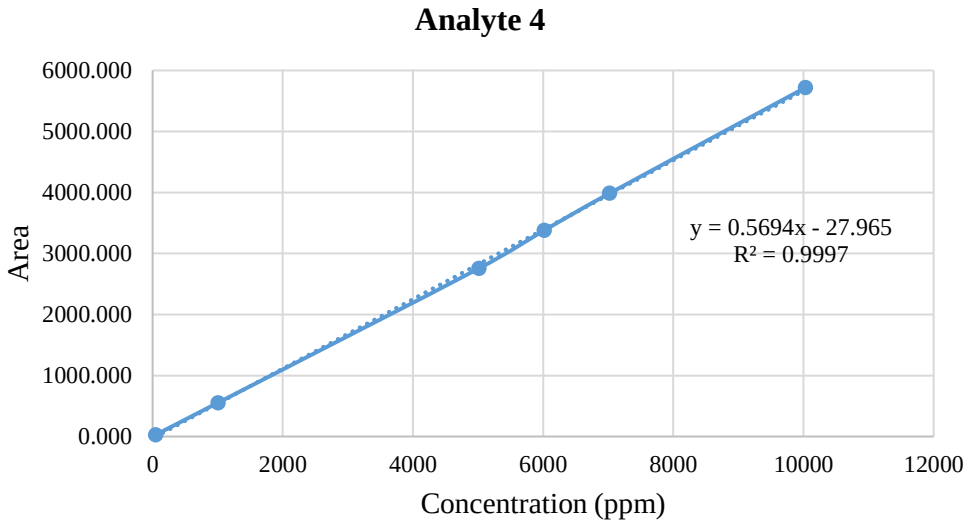


Figure S.34. Calibration curve obtained by the injection of Analyte 4 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

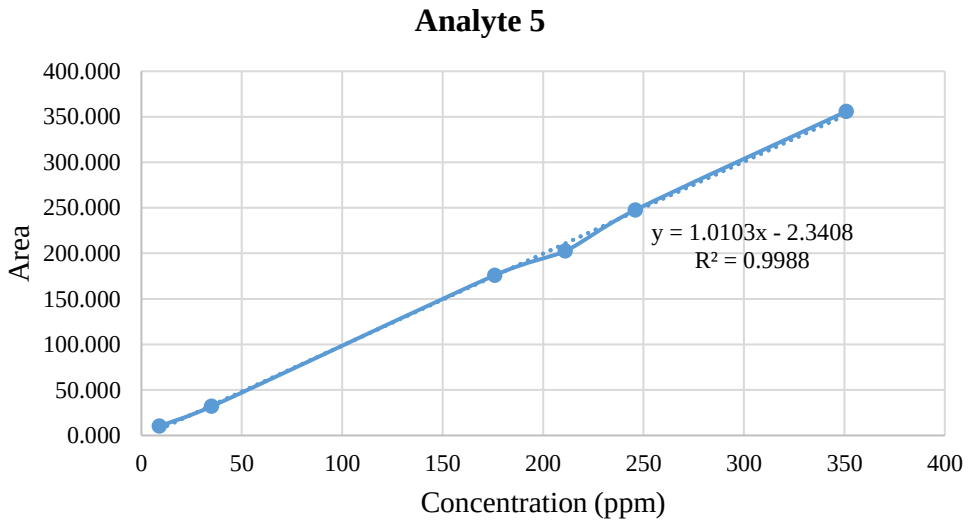


Figure S.35. Calibration curve obtained by the injection of Analyte 5 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

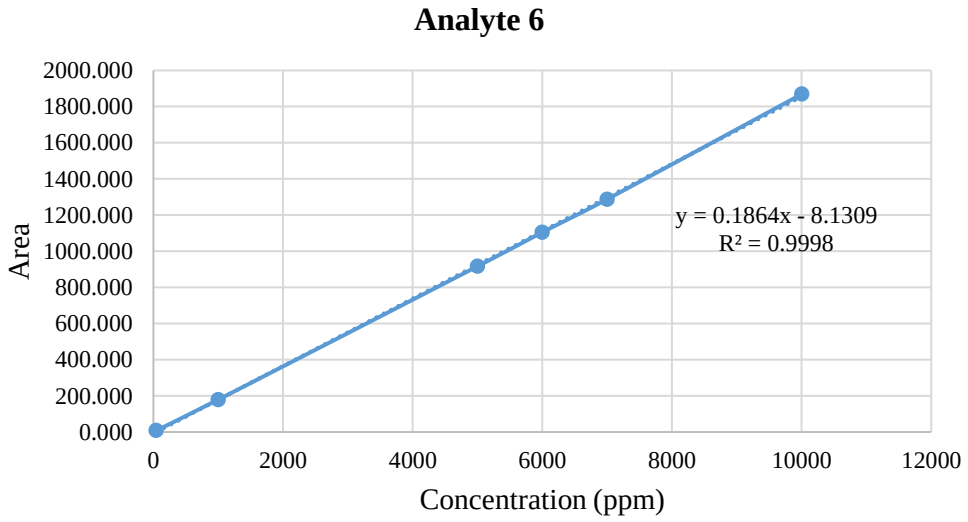


Figure S.36. Calibration curve obtained by the injection of Analyte 6 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

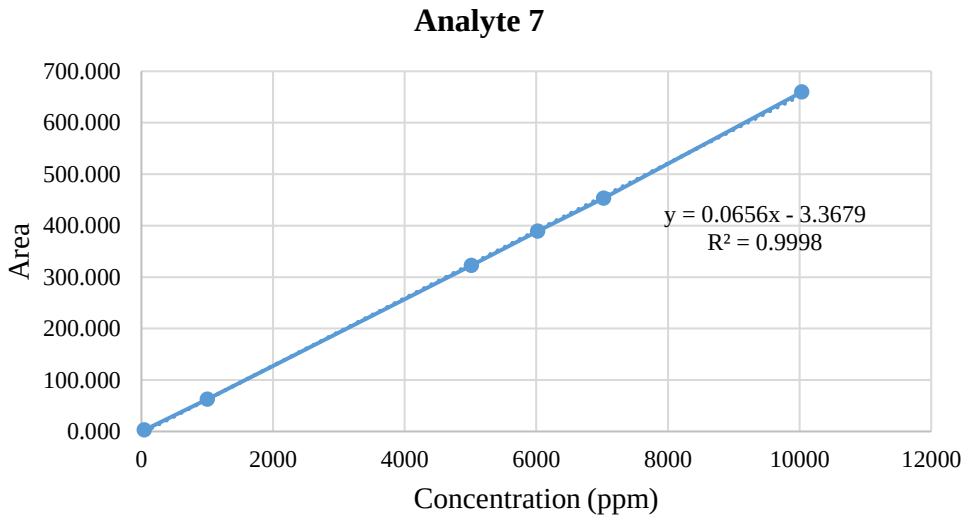


Figure S.37. Calibration curve obtained by the injection of Analyte 7 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

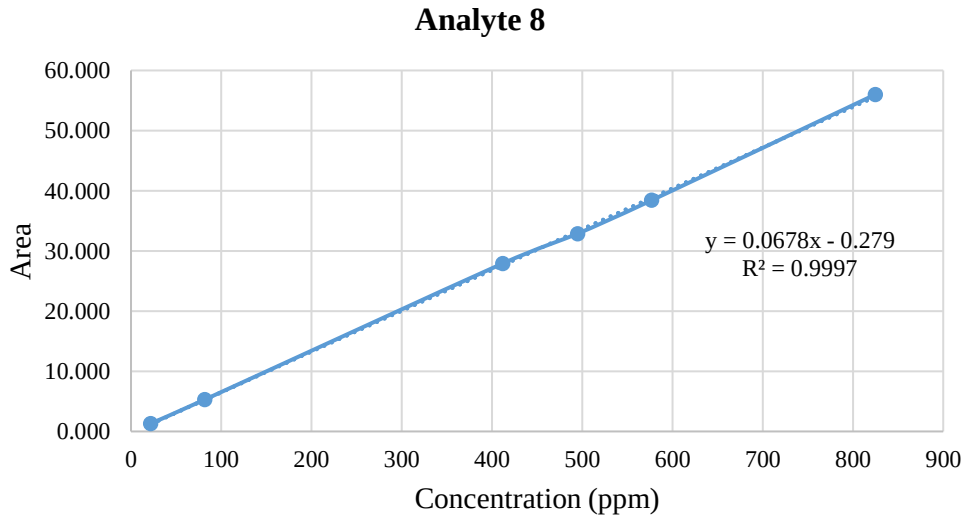


Figure S.38. Calibration curve obtained by the injection of Analyte 8 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

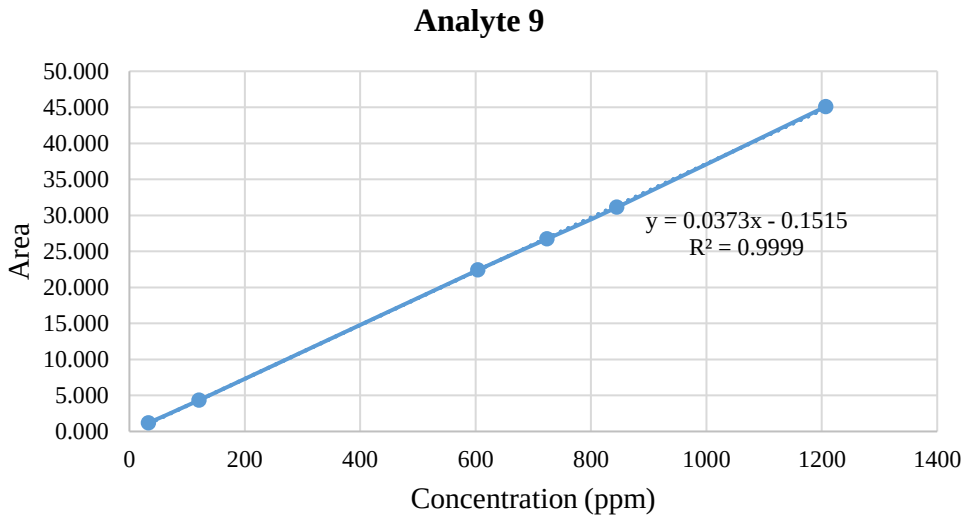


Figure S.39. Calibration curve obtained by the injection of Analyte 9 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

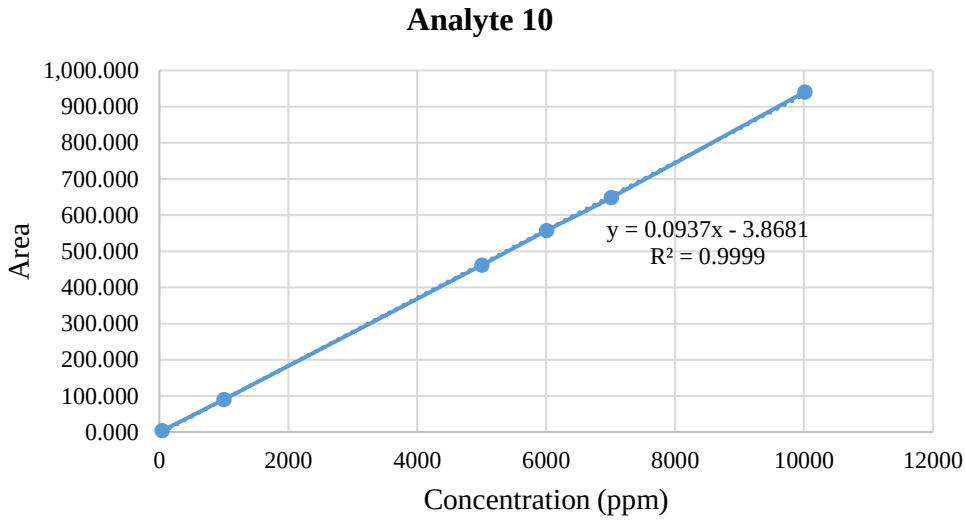


Figure S.40. Calibration curve obtained by the injection of Analyte 10 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

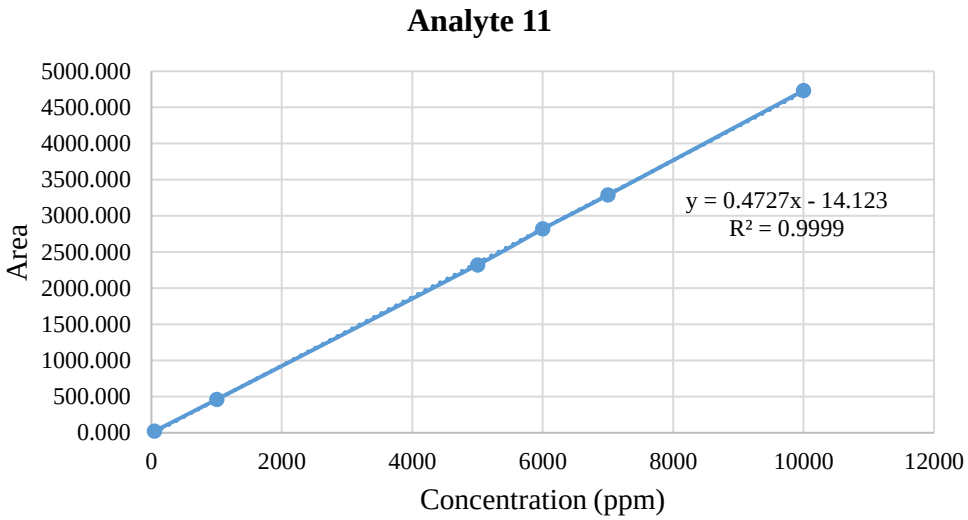


Figure S.41. Calibration curve obtained by the injection of Analyte 11 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

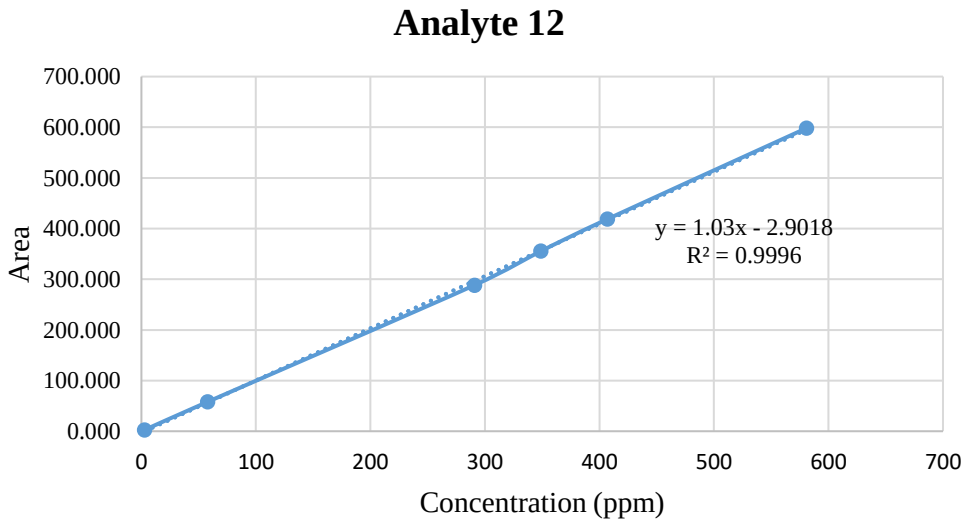


Figure S.42. Calibration curve obtained by the injection of Analyte 12 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

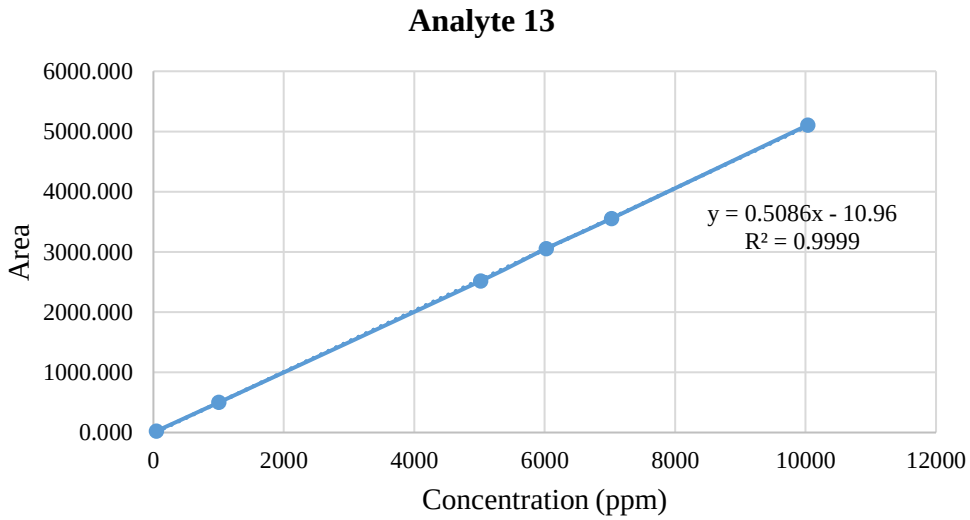


Figure S.43. Calibration curve obtained by the injection of Analyte 13 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

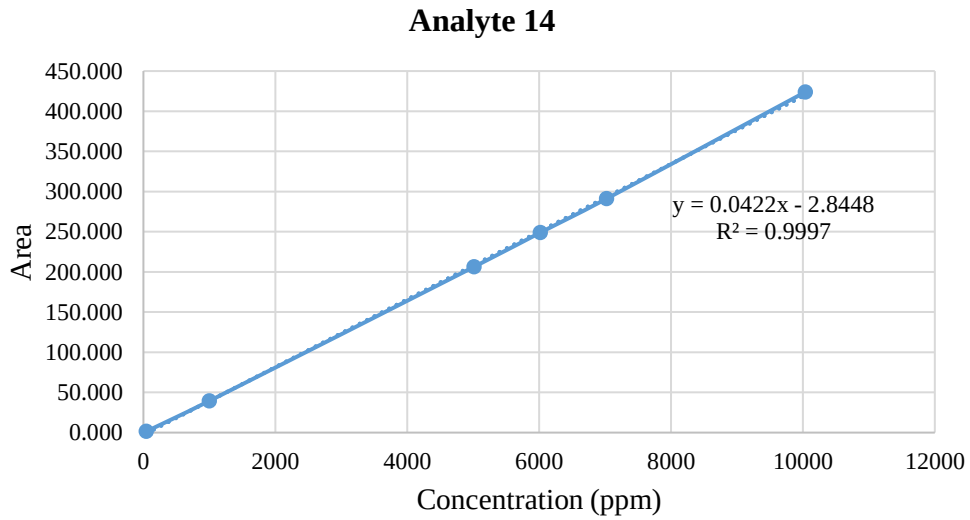


Figure S.44. Calibration curve obtained by the injection of Analyte 14 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

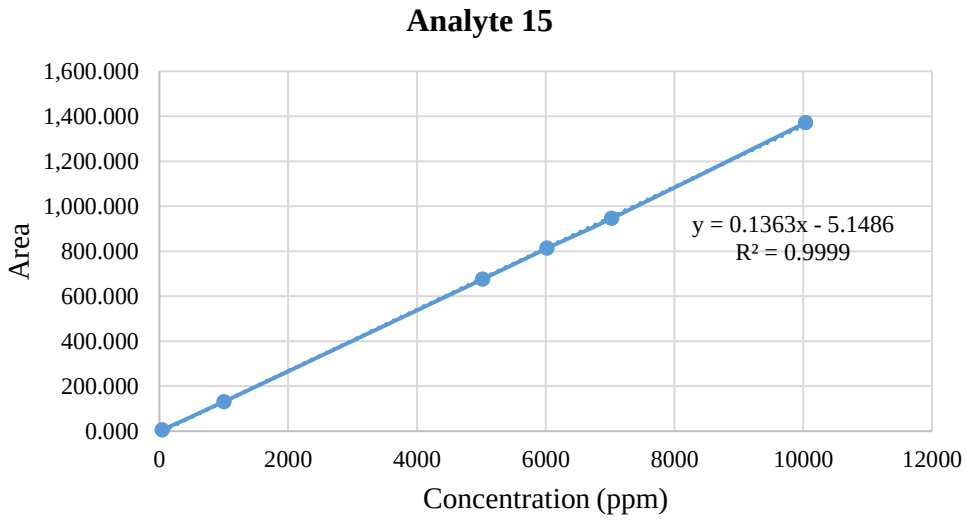


Figure S.45. Calibration curve obtained by the injection of Analyte 15 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

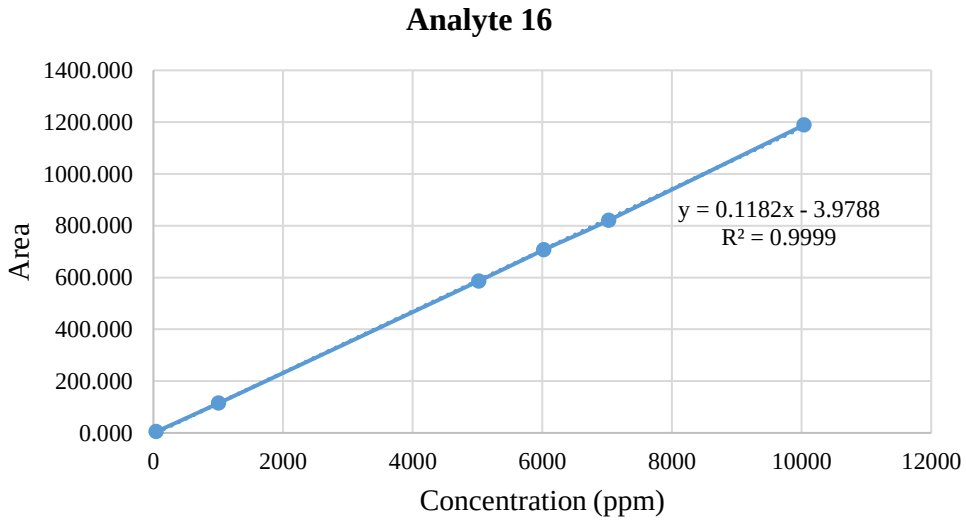


Figure S.46. Calibration curve obtained by the injection of Analyte 16 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

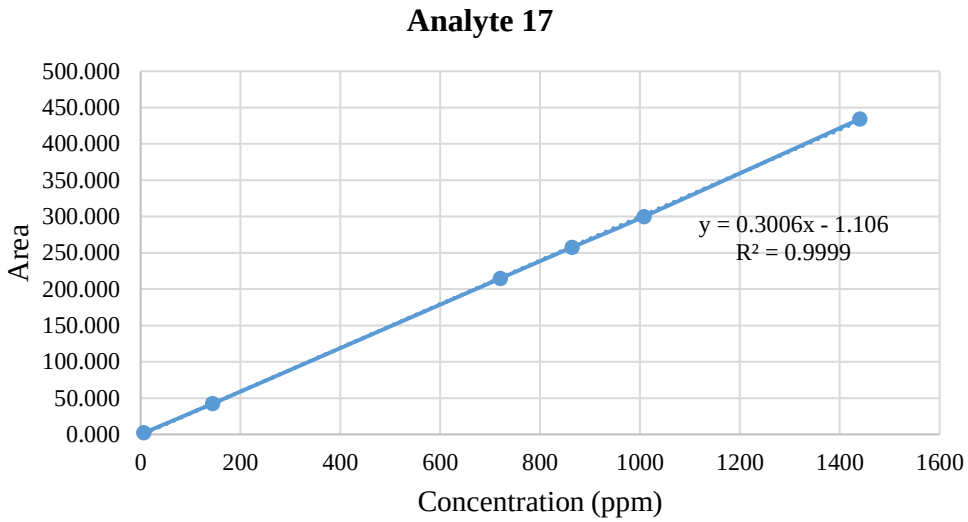


Figure S.47. Calibration curve obtained by the injection of Analyte 17 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

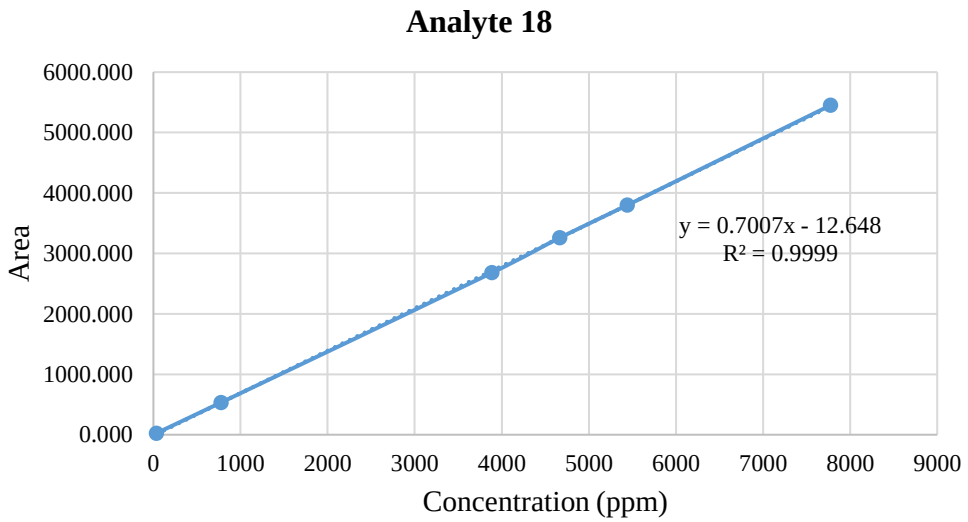


Figure S.48. Calibration curve obtained by the injection of Analyte 18 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

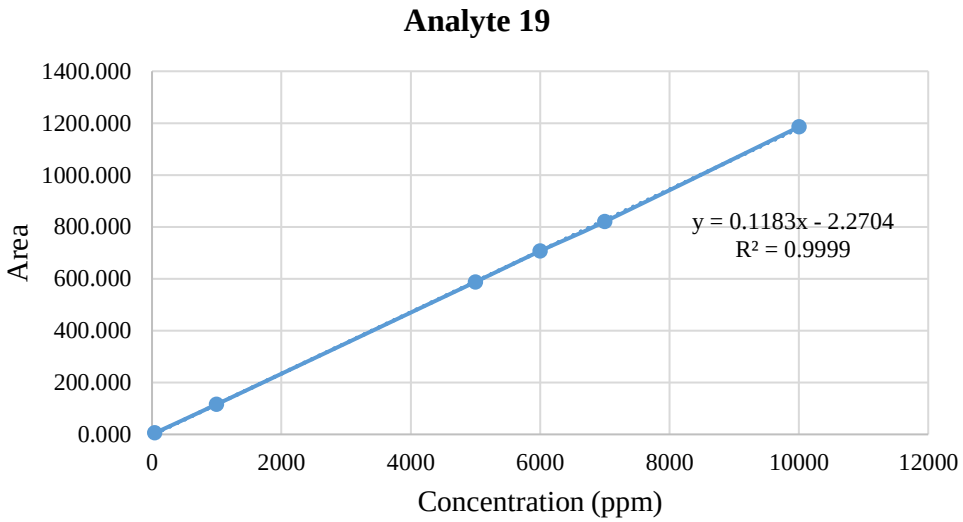


Figure S.49. Calibration curve obtained by the injection of Analyte 19 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

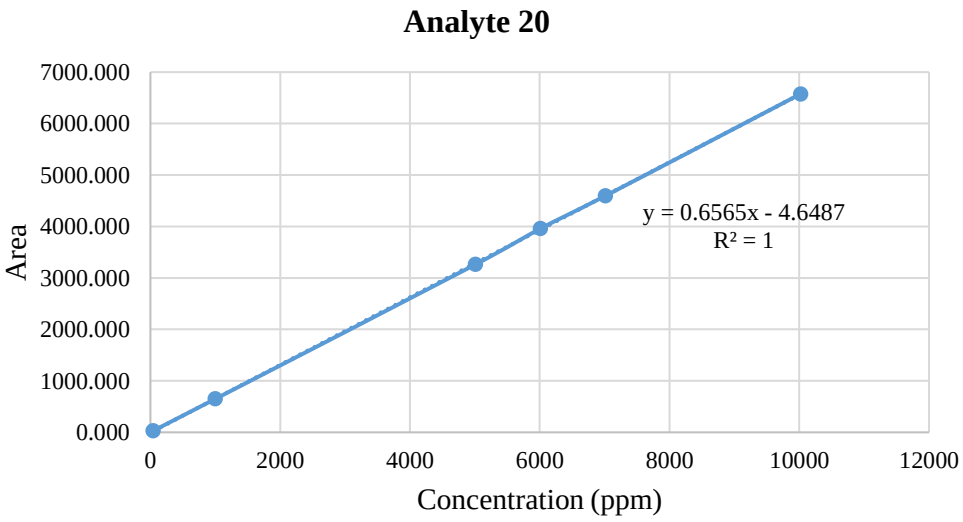


Figure S.50. Calibration curve obtained by the injection of Analyte 20 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

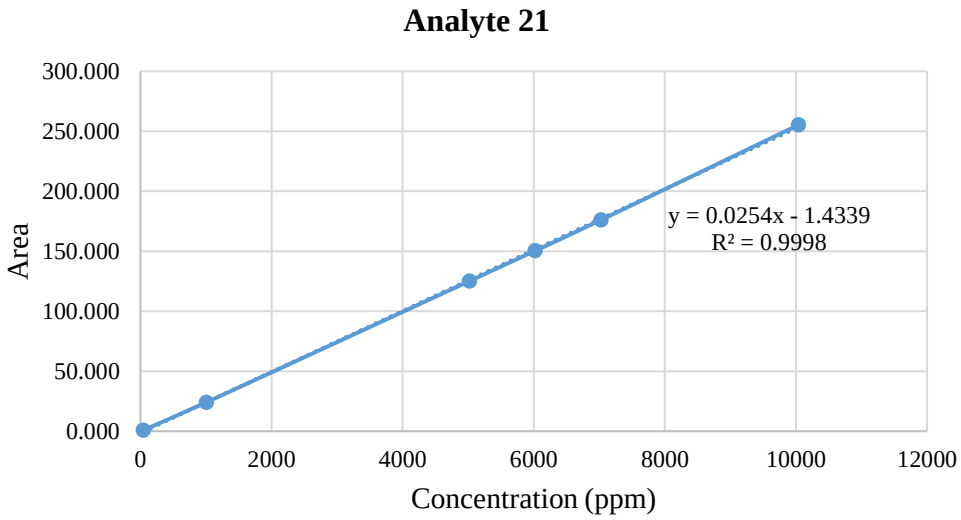


Figure S.51. Calibration curve obtained by the injection of Analyte 21 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

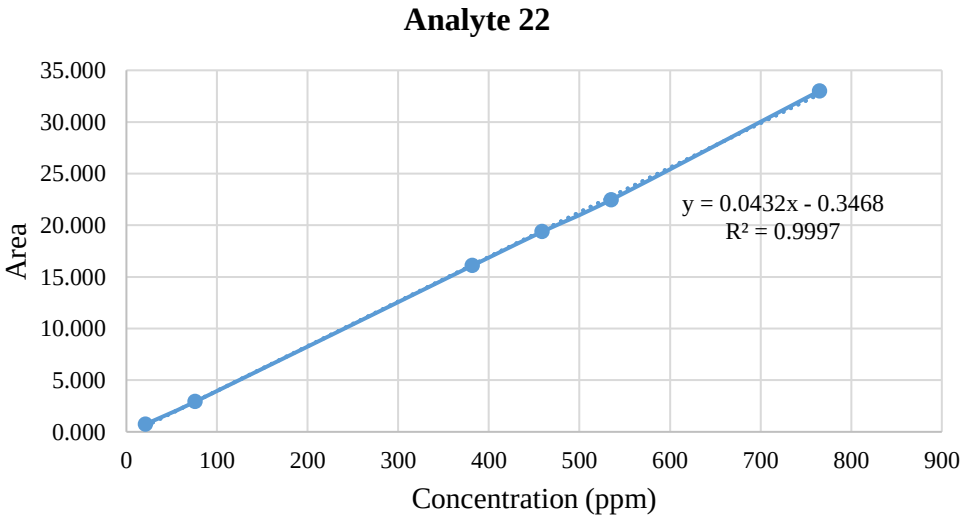


Figure S.52. Calibration curve obtained by the injection of Analyte 22 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

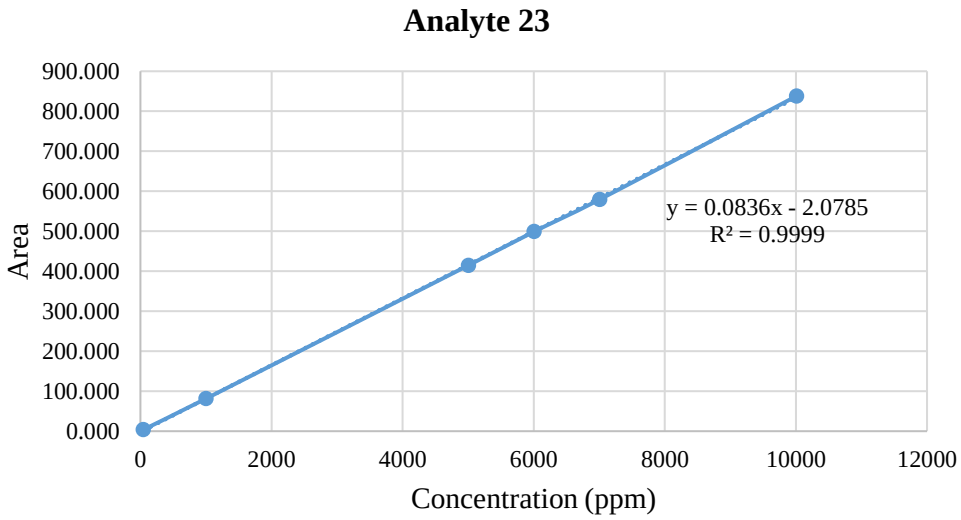


Figure S.53. Calibration curve obtained by the injection of Analyte 23 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

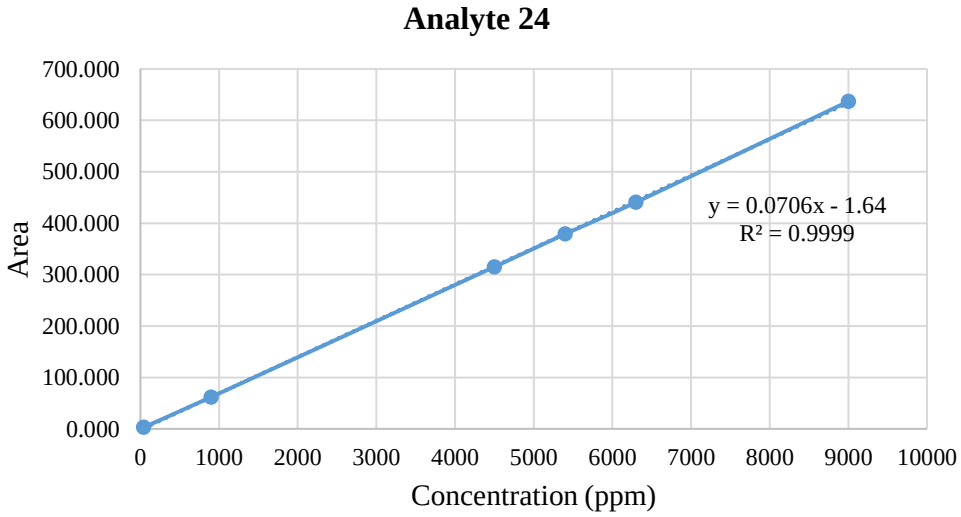


Figure S.54. Calibration curve obtained by the injection of Analyte 24 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

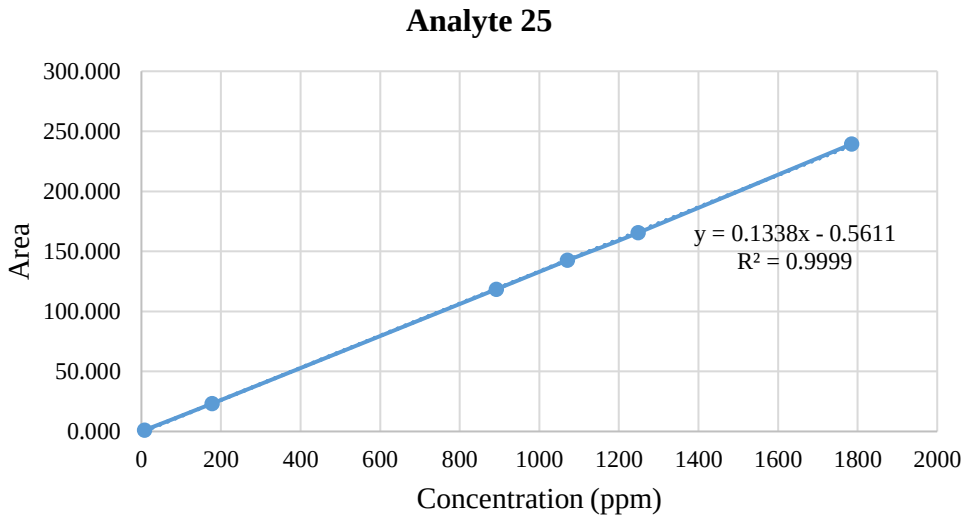


Figure S.55. Calibration curve obtained by the injection of Analyte 25 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

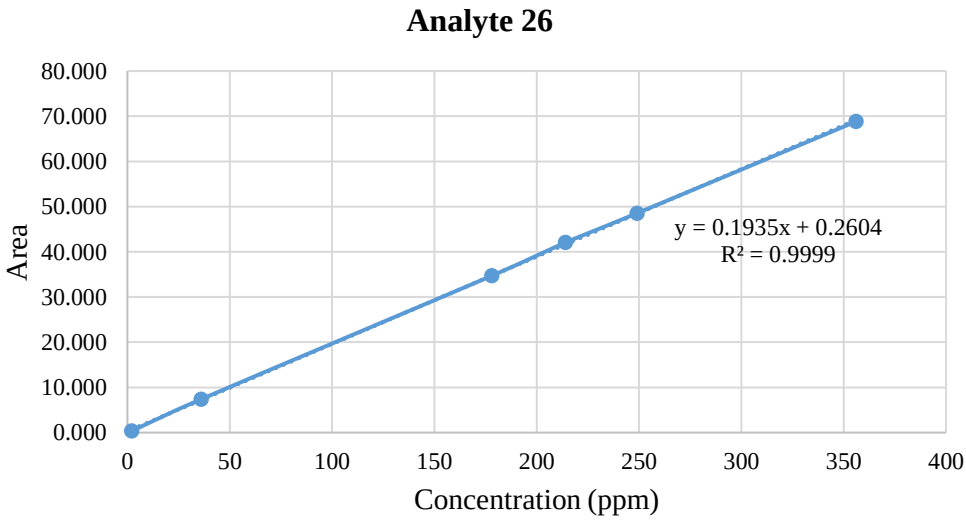


Figure S.56. Calibration curve obtained by the injection of Analyte 26 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

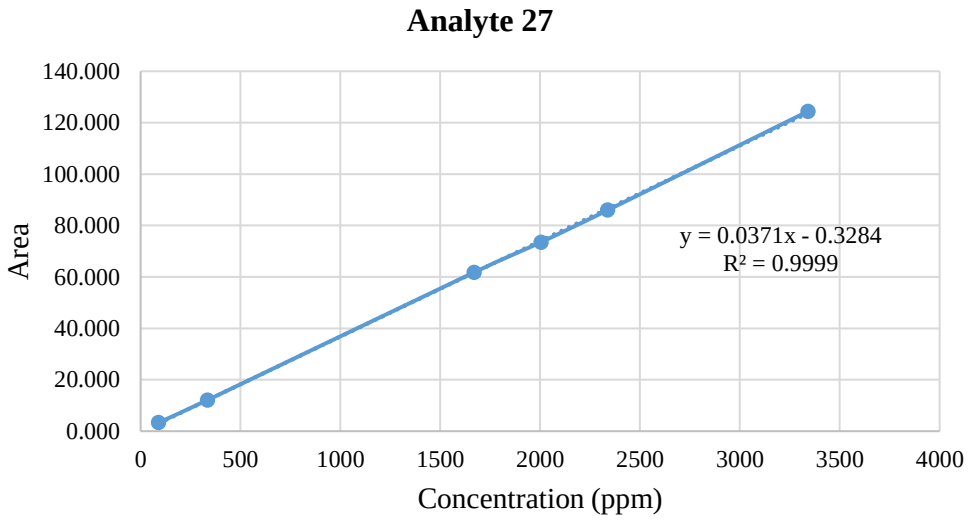


Figure S.57. Calibration curve obtained by the injection of Analyte 27 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

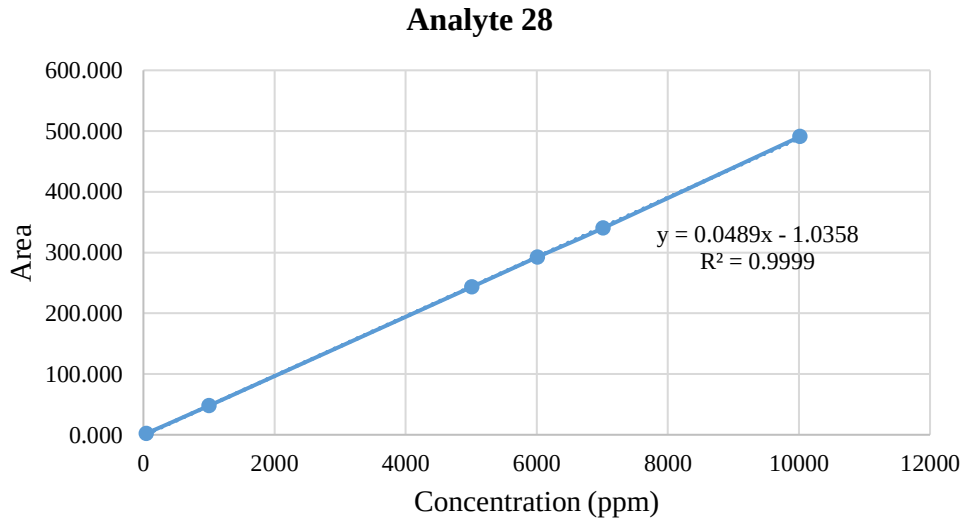


Figure S.58. Calibration curve obtained by the injection of Analyte 28 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

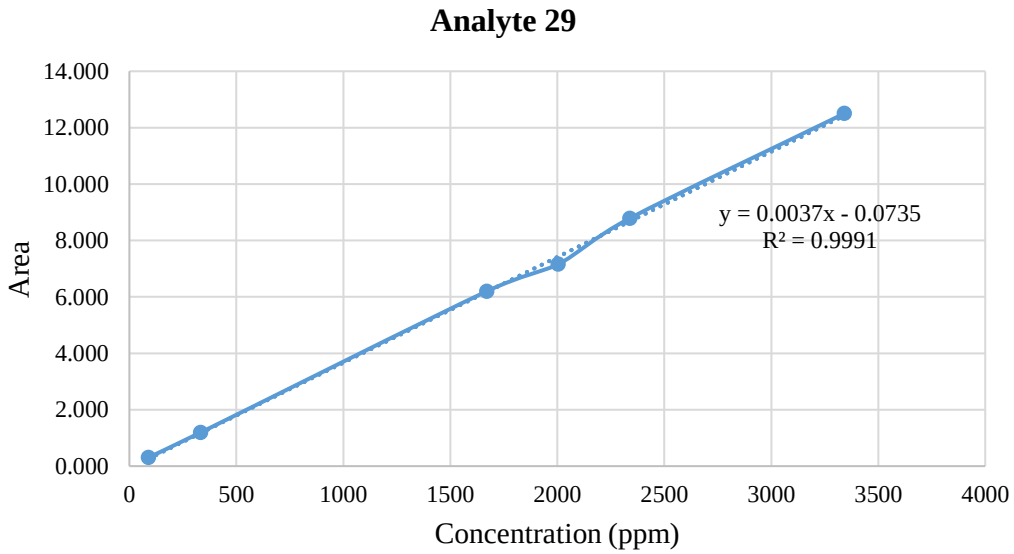


Figure S.59. Calibration curve obtained by the injection of Analyte 29 and the remaining target analytes at 6 different concentration levels (LOQ, 20%, 100%, 120%, 140% and 200% of the defined maximum limit).

S.7. Accuracy

Table S.6. Results obtained from recovery tests performed in Drug Product A (Sample 1), considering a sample concentration of 100 mg/mL.

	Sample 1 (Drug product A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 1	LOQ	100	101
		102	
		103	
	ICH/VICH limit	102	103
		102	
		105	
	140% ICH/VICH limit	102	102
		102	
		103	
Analyte 2	LOQ	99	98
		98	
		96	
	ICH/VICH limit	102	103
		103	
		103	
	140% ICH/VICH limit	96	96
		94	
		97	
Analyte 3	LOQ	111	110
		110	
		109	
	ICH/VICH limit	102	104
		103	
		105	
	140% ICH/VICH limit	103	103
		102	
		104	
Analyte 4	LOQ	100	99
		100	
		97	
	ICH/VICH limit	101	102
		102	
		102	
	140% ICH/VICH limit	98	98
		97	
		99	
Analyte 5	LOQ	100	99
		100	
		97	
	ICH/VICH limit	102	102
		102	
		102	
	140% ICH/VICH limit	97	96
		95	
		97	

	Sample 1 (Drug product A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 6	LOQ	114	89
		76	
		79	
	ICH/VICH limit	100	101
		100	
		102	
	140% ICH/VICH limit	99	99
		98	
		100	
Analyte 7	LOQ	106	105
		105	
		103	
	ICH/VICH limit	103	104
		103	
		106	
	140% ICH/VICH limit	103	103
		103	
		104	
Analyte 8	LOQ	101	99
		100	
		97	
	ICH/VICH limit	99	102
		102	
		104	
	140% ICH/VICH limit	102	102
		101	
		103	
Analyte 9	LOQ	104	102
		101	
		100	
	ICH/VICH limit	102	103
		103	
		104	
	140% ICH/VICH limit	102	102
		101	
		103	
Analyte 10	LOQ	106	105
		105	
		102	
	ICH/VICH limit	103	104
		104	
		106	
	140% ICH/VICH limit	103	104
		103	
		105	

	Sample 1 (Drug product A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 11	LOQ	100	100
		100	
		98	
	ICH/VICH limit	101	101
		101	
		102	
	140% ICH/VICH limit	99	99
		98	
		100	
Analyte 12	LOQ	98	98
		99	
		97	
	ICH/VICH limit	102	102
		102	
		103	
	140% ICH/VICH limit	99	98
		97	
		99	
Analyte 13	LOQ	101	101
		101	
		99	
	ICH/VICH limit	102	102
		102	
		103	
	140% ICH/VICH limit	100	100
		99	
		101	
Analyte 14	LOQ	114	111
		106	
		113	
	ICH/VICH limit	103	104
		103	
		107	
	140% ICH/VICH limit	103	104
		104	
		105	
Analyte 15	LOQ	103	101
		100	
		99	
	ICH/VICH limit	100	101
		101	
		103	
	140% ICH/VICH limit	100	100
		100	
		101	

	Sample 1 (Drug product A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 16	LOQ	106	103
		102	
		101	
	ICH/VICH limit	101	101
		101	
		103	
	140% ICH/VICH limit	100	100
		99	
		101	
Analyte 17	LOQ	94	96
		93	
		101	
	ICH/VICH limit	99	100
		100	
		101	
	140% ICH/VICH limit	98	99
		98	
		100	
Analyte 18	LOQ	99	99
		99	
		97	
	ICH/VICH limit	100	101
		101	
		101	
	140% ICH/VICH limit	98	98
		97	
		99	
Analyte 19	LOQ	105	102
		102	
		100	
	ICH/VICH limit	101	102
		101	
		103	
	140% ICH/VICH limit	101	101
		100	
		102	
Analyte 20	LOQ	101	100
		101	
		99	
	ICH/VICH limit	102	102
		102	
		103	
	140% ICH/VICH limit	100	100
		99	
		101	

	Sample 1 (Drug product A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 21	LOQ	103	102
		102	
		100	
	ICH/VICH limit	104	105
		103	
		109	
	140% ICH/VICH limit	104	105
		105	
		105	
Analyte 22	LOQ	103	107
		97	
		120	
	ICH/VICH limit	99	99
		99	
		100	
	140% ICH/VICH limit	100	101
		102	
		101	
Analyte 23	LOQ	101	100
		101	
		99	
	ICH/VICH limit	101	102
		101	
		104	
	140% ICH/VICH limit	101	101
		100	
		102	
Analyte 24	LOQ	105	102
		102	
		99	
	ICH/VICH limit	101	102
		101	
		105	
	140% ICH/VICH limit	101	102
		101	
		103	
Analyte 25	LOQ	98	98
		98	
		98	
	ICH/VICH limit	100	101
		100	
		103	
	140% ICH/VICH limit	100	100
		100	
		101	

	Sample 1 (Drug product A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 26	LOQ	96	97
		99	
		96	
	ICH/VICH limit	67	68
		68	
		70	
	140% ICH/VICH limit	68	68
		67	
		70	
Analyte 27	LOQ	99	98
		100	
		97	
	ICH/VICH limit	100	101
		100	
		104	
	140% ICH/VICH limit	101	101
		101	
		102	
Analyte 28	LOQ	104	104
		105	
		102	
	ICH/VICH limit	101	103
		101	
		105	
	140% ICH/VICH limit	101	102
		101	
		103	
Analyte 29	LOQ	106	109
		116	
		105	
	ICH/VICH limit	109	106
		103	
		106	
	140% ICH/VICH limit	105	107
		110	
		106	

Table S.7. Results obtained from recovery tests performed in Drug Product B (Sample 2), spiked at LOQ level, 100% and 140% of the ICH/VICH limits and defined control levels, considering a sample concentration of 100 mg/mL.

	Sample 2 (Drug product B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 1	LOQ	95	101
		106	
		102	
	ICH/VICH limit	102	102
		103	
		102	
	140% ICH/VICH limit	104	104
		103	
		104	
Analyte 2	LOQ	105	101
		100	
		98	
	ICH/VICH limit	102	102
		103	
		100	
	140% ICH/VICH limit	102	103
		104	
		102	
Analyte 3	LOQ	110	110
		110	
		111	
	ICH/VICH limit	103	103
		103	
		102	
	140% ICH/VICH limit	104	104
		103	
		105	
Analyte 4	LOQ	101	99
		99	
		97	
	ICH/VICH limit	101	101
		102	
		99	
	140% ICH/VICH limit	102	102
		103	
		102	
Analyte 5	LOQ	102	99
		98	
		97	
	ICH/VICH limit	101	101
		102	
		99	
	140% ICH/VICH limit	102	102
		103	
		102	

	Sample 2 (Drug product B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 6	LOQ	85	84
		83	
		84	
	ICH/VICH limit	101	101
		101	
		100	
	140% ICH/VICH limit	101	101
		101	
		100	
Analyte 7	LOQ	104	102
		98	
		103	
	ICH/VICH limit	103	103
		103	
		102	
	140% ICH/VICH limit	104	104
		103	
		106	
Analyte 8	LOQ	107	103
		93	
		110	
	ICH/VICH limit	102	102
		103	
		102	
	140% ICH/VICH limit	104	104
		103	
		105	
Analyte 9	LOQ	114	111
		109	
		108	
	ICH/VICH limit	102	102
		103	
		102	
	140% ICH/VICH limit	104	104
		104	
		104	
Analyte 10	LOQ	103	105
		102	
		110	
	ICH/VICH limit	103	103
		104	
		103	
	140% ICH/VICH limit	104	105
		104	
		106	

	Sample 2 (Drug product B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 11	LOQ	101	100
		99	
		99	
	ICH/VICH limit	100	100
		101	
		100	
	140% ICH/VICH limit	101	102
		102	
		102	
Analyte 12	LOQ	107	105
		105	
		103	
	ICH/VICH limit	101	101
		102	
		99	
	140% ICH/VICH limit	102	103
		103	
		103	
Analyte 13	LOQ	102	100
		100	
		100	
	ICH/VICH limit	101	101
		102	
		100	
	140% ICH/VICH limit	102	102
		102	
		103	
Analyte 14	LOQ	107	110
		106	
		116	
	ICH/VICH limit	103	103
		103	
		102	
	140% ICH/VICH limit	104	105
		103	
		106	
Analyte 15	LOQ	123	121
		111	
		130	
	ICH/VICH limit	101	101
		101	
		100	
	140% ICH/VICH limit	101	102
		102	
		103	

	Sample 2 (Drug product B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 16	LOQ	98	97
		97	
		97	
	ICH/VICH limit	101	101
		102	
		101	
	140% ICH/VICH limit	102	102
		102	
		103	
Analyte 17	LOQ	87	91
		90	
		96	
	ICH/VICH limit	98	98
		98	
		97	
	140% ICH/VICH limit	99	99
		99	
		100	
Analyte 18	LOQ	98	97
		97	
		96	
	ICH/VICH limit	98	98
		99	
		98	
	140% ICH/VICH limit	99	100
		100	
		100	
Analyte 19	LOQ	102	101
		101	
		101	
	ICH/VICH limit	102	102
		102	
		101	
	140% ICH/VICH limit	102	103
		102	
		104	
Analyte 20	LOQ	102	101
		101	
		100	
	ICH/VICH limit	100	100
		101	
		100	
	140% ICH/VICH limit	101	102
		101	
		102	

	Sample 2 (Drug product B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 21	LOQ	95	100
		101	
		104	
	ICH/VICH limit	102	102
		102	
		101	
	140% ICH/VICH limit	103	105
		103	
		107	
Analyte 22	LOQ	93	89
		94	
		81	
	ICH/VICH limit	98	98
		98	
		97	
	140% ICH/VICH limit	100	101
		100	
		102	
Analyte 23	LOQ	99	98
		98	
		98	
	ICH/VICH limit	101	101
		102	
		101	
	140% ICH/VICH limit	102	102
		102	
		104	
Analyte 24	LOQ	110	109
		110	
		106	
	ICH/VICH limit	101	101
		102	
		100	
	140% ICH/VICH limit	102	103
		102	
		104	
Analyte 25	LOQ	101	101
		101	
		100	
	ICH/VICH limit	99	99
		100	
		99	
	140% ICH/VICH limit	100	101
		100	
		102	

	Sample 2 (Drug product B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 26	LOQ	46	40
		37	
		38	
	ICH/VICH limit	72	73
		73	
		73	
	140% ICH/VICH limit	74	75
		74	
		76	
Analyte 27	LOQ	102	102
		102	
		102	
	ICH/VICH limit	99	99
		100	
		99	
	140% ICH/VICH limit	101	102
		101	
		104	
Analyte 28	LOQ	103	101
		101	
		100	
	ICH/VICH limit	101	101
		102	
		100	
	140% ICH/VICH limit	102	103
		102	
		105	
Analyte 29	LOQ	87	92
		98	
		91	
	ICH/VICH limit	94	95
		97	
		93	
	140% ICH/VICH limit	97	99
		99	
		100	

Table S.8. Results obtained from recovery tests performed in Drug Substance A (Sample 3), spiked at LOQ level, 100% and 140% of the ICH/VICH limits and defined control levels, considering a sample concentration of 100 mg/mL.

	Sample 3 (Drug substance A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 1	LOQ	102	94
		94	
		85	
	ICH/VICH limit	102	100
		100	
		99	
	140% ICH/VICH limit	101	102
		103	
		101	
Analyte 2	LOQ	99	96
		93	
		97	
	ICH/VICH limit	96	94
		93	
		93	
	140% ICH/VICH limit	97	97
		100	
		95	
Analyte 3	LOQ	98	97
		92	
		99	
	ICH/VICH limit	102	100
		99	
		99	
	140% ICH/VICH limit	101	101
		103	
		100	
Analyte 4	LOQ	97	95
		92	
		96	
	ICH/VICH limit	97	95
		94	
		94	
	140% ICH/VICH limit	97	98
		99	
		96	
Analyte 5	LOQ	98	95
		91	
		94	
	ICH/VICH limit	97	95
		94	
		94	
	140% ICH/VICH limit	98	98
		100	
		96	

	Sample 3 (Drug substance A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 6	LOQ	85	85
		83	
		86	
	ICH/VICH limit	98	97
		96	
		96	
	140% ICH/VICH limit	98	98
		99	
		97	
Analyte 7	LOQ	99	97
		95	
		97	
	ICH/VICH limit	102	100
		99	
		99	
	140% ICH/VICH limit	100	101
		103	
		100	
Analyte 8	LOQ	91	95
		97	
		98	
	ICH/VICH limit	100	99
		98	
		98	
	140% ICH/VICH limit	99	99
		101	
		99	
Analyte 9	LOQ	101	100
		100	
		99	
	ICH/VICH limit	101	99
		99	
		98	
	140% ICH/VICH limit	100	101
		102	
		100	
Analyte 10	LOQ	98	96
		94	
		98	
	ICH/VICH limit	102	100
		99	
		99	
	140% ICH/VICH limit	100	101
		103	
		100	

	Sample 3 (Drug substance A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 11	LOQ	93	92
		86	
		96	
	ICH/VICH limit	98	97
		96	
		96	
	140% ICH/VICH limit	98	98
		100	
		97	
Analyte 12	LOQ	102	99
		94	
		102	
	ICH/VICH limit	97	95
		94	
		94	
	140% ICH/VICH limit	98	98
		100	
		96	
Analyte 13	LOQ	98	96
		94	
		97	
	ICH/VICH limit	99	97
		96	
		97	
	140% ICH/VICH limit	98	99
		100	
		97	
Analyte 14	LOQ	93	90
		85	
		93	
	ICH/VICH limit	102	99
		98	
		98	
	140% ICH/VICH limit	99	101
		103	
		99	
Analyte 15	LOQ	92	91
		90	
		93	
	ICH/VICH limit	99	97
		96	
		97	
	140% ICH/VICH limit	97	98
		99	
		97	

	Sample 3 (Drug substance A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 16	LOQ	96	94
		92	
		93	
	ICH/VICH limit	99	97
		96	
		97	
	140% ICH/VICH limit	97	98
		99	
		97	
Analyte 17	LOQ	87	88
		88	
		90	
	ICH/VICH limit	97	96
		95	
		95	
	140% ICH/VICH limit	97	97
		98	
		96	
Analyte 18	LOQ	97	95
		92	
		96	
	ICH/VICH limit	97	96
		95	
		95	
	140% ICH/VICH limit	97	97
		99	
		96	
Analyte 19	LOQ	97	96
		92	
		98	
	ICH/VICH limit	99	98
		97	
		97	
	140% ICH/VICH limit	98	98
		100	
		97	
Analyte 20	LOQ	96	94
		91	
		95	
	ICH/VICH limit	98	96
		95	
		96	
	140% ICH/VICH limit	97	98
		99	
		96	

	Sample 3 (Drug substance A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 21	LOQ	103	97
		92	
		95	
	ICH/VICH limit	101	98
		97	
		97	
	140% ICH/VICH limit	97	99
		103	
		98	
Analyte 22	LOQ	74	77
		76	
		79	
	ICH/VICH limit	98	96
		95	
		95	
	140% ICH/VICH limit	97	98
		100	
		96	
Analyte 23	LOQ	96	95
		91	
		97	
	ICH/VICH limit	99	97
		96	
		97	
	140% ICH/VICH limit	97	98
		100	
		97	
Analyte 24	LOQ	97	95
		95	
		94	
	ICH/VICH limit	99	97
		96	
		96	
	140% ICH/VICH limit	97	98
		100	
		96	
Analyte 25	LOQ	99	96
		94	
		96	
	ICH/VICH limit	98	96
		95	
		96	
	140% ICH/VICH limit	96	97
		99	
		96	

	Sample 3 (Drug substance A)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 26	LOQ	80	77
		76	
		77	
	ICH/VICH limit	96	98
		98	
		99	
	140% ICH/VICH limit	97	98
		99	
		97	
Analyte 27	LOQ	93	91
		88	
		91	
	ICH/VICH limit	96	94
		92	
		92	
	140% ICH/VICH limit	94	95
		98	
		94	
Analyte 28	LOQ	97	94
		91	
		95	
	ICH/VICH limit	99	97
		96	
		96	
	140% ICH/VICH limit	96	97
		100	
		96	
Analyte 29	LOQ	91	87
		82	
		88	
	ICH/VICH limit	92	90
		91	
		88	
	140% ICH/VICH limit	85	89
		94	
		89	

Table S.9. Results obtained from recovery tests performed in Drug Product C (Sample 4), spiked at LOQ level, 100% and 140% of the ICH/VICH limits and defined control levels, considering a sample concentration of 100 mg/mL.

	Sample 4 (Drug product C)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 1	LOQ	99	100
		104	
		97	
	ICH/VICH limit	102	102
		102	
		102	
	140% ICH/VICH limit	104	104
		104	
		105	
Analyte 2	LOQ	94	86
		85	
		80	
	ICH/VICH limit	95	92
		93	
		89	
	140% ICH/VICH limit	108	106
		104	
		105	
Analyte 3	LOQ	100	101
		101	
		100	
	ICH/VICH limit	103	103
		103	
		103	
	140% ICH/VICH limit	105	105
		105	
		106	
Analyte 4	LOQ	96	92
		91	
		88	
	ICH/VICH limit	97	95
		96	
		93	
	140% ICH/VICH limit	106	104
		103	
		104	
Analyte 5	LOQ	95	89
		88	
		84	
	ICH/VICH limit	95	94
		95	
		91	
	140% ICH/VICH limit	107	105
		104	
		105	

	Sample 4 (Drug product C)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 6	LOQ	106	110
		110	
		114	
	ICH/VICH limit	100	100
		100	
		100	
	140% ICH/VICH limit	102	102
		101	
		102	
Analyte 7	LOQ	110	107
		109	
		101	
	ICH/VICH limit	104	104
		104	
		104	
	140% ICH/VICH limit	106	106
		105	
		106	
Analyte 8	LOQ	111	101
		105	
		87	
	ICH/VICH limit	100	101
		100	
		102	
	140% ICH/VICH limit	105	105
		105	
		106	
Analyte 9	LOQ	102	101
		104	
		98	
	ICH/VICH limit	102	101
		101	
		101	
	140% ICH/VICH limit	104	104
		103	
		105	
Analyte 10	LOQ	105	105
		106	
		104	
	ICH/VICH limit	105	105
		105	
		105	
	140% ICH/VICH limit	106	106
		105	
		106	

	Sample 4 (Drug product C)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 11	LOQ	98	96
		96	
		95	
	ICH/VICH limit	100	99
		99	
		97	
	140% ICH/VICH limit	104	103
		102	
		103	
Analyte 12	LOQ	98	93
		91	
		89	
	ICH/VICH limit	97	96
		97	
		93	
	140% ICH/VICH limit	107	106
		104	
		105	
Analyte 13	LOQ	100	98
		98	
		97	
	ICH/VICH limit	101	100
		100	
		99	
	140% ICH/VICH limit	105	104
		103	
		105	
Analyte 14	LOQ	95	101
		110	
		98	
	ICH/VICH limit	104	105
		105	
		105	
	140% ICH/VICH limit	106	106
		105	
		107	
Analyte 15	LOQ	98	101
		101	
		103	
	ICH/VICH limit	102	101
		101	
		101	
	140% ICH/VICH limit	102	102
		102	
		103	

	Sample 4 (Drug product C)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 16	LOQ	107	106
		106	
		105	
	ICH/VICH limit	102	101
		101	
		101	
	140% ICH/VICH limit	103	102
		102	
		103	
Analyte 17	LOQ	87	89
		91	
		89	
	ICH/VICH limit	99	99
		99	
		98	
	140% ICH/VICH limit	102	101
		100	
		102	
Analyte 18	LOQ	98	96
		96	
		93	
	ICH/VICH limit	99	98
		98	
		97	
	140% ICH/VICH limit	103	102
		101	
		103	
Analyte 19	LOQ	103	102
		103	
		101	
	ICH/VICH limit	103	103
		102	
		103	
	140% ICH/VICH limit	103	103
		103	
		104	
Analyte 20	LOQ	100	99
		99	
		98	
	ICH/VICH limit	102	101
		101	
		100	
	140% ICH/VICH limit	105	104
		103	
		105	

	Sample 4 (Drug product C)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 21	LOQ	118	117
		120	
		114	
	ICH/VICH limit	105	106
		106	
		106	
	140% ICH/VICH limit	108	108
		106	
		108	
Analyte 22	LOQ	94	90
		91	
		86	
	ICH/VICH limit	99	99
		99	
		100	
	140% ICH/VICH limit	102	101
		100	
		102	
Analyte 23	LOQ	101	101
		103	
		99	
	ICH/VICH limit	103	103
		103	
		103	
	140% ICH/VICH limit	103	103
		103	
		104	
Analyte 24	LOQ	95	97
		98	
		98	
	ICH/VICH limit	104	104
		104	
		104	
	140% ICH/VICH limit	105	105
		104	
		106	
Analyte 25	LOQ	103	104
		107	
		103	
	ICH/VICH limit	102	102
		102	
		102	
	140% ICH/VICH limit	103	103
		102	
		103	

	Sample 4 (Drug product C)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 26	LOQ	47	47
		52	
		42	
	ICH/VICH limit	97	100
		101	
		103	
	140% ICH/VICH limit	100	101
		101	
		103	
Analyte 27	LOQ	98	99
		102	
		98	
	ICH/VICH limit	101	102
		102	
		102	
	140% ICH/VICH limit	103	103
		103	
		104	
Analyte 28	LOQ	99	101
		104	
		101	
	ICH/VICH limit	104	104
		104	
		104	
	140% ICH/VICH limit	105	105
		104	
		105	
Analyte 29	LOQ	102	111
		113	
		118	
	ICH/VICH limit	107	110
		112	
		111	
	140% ICH/VICH limit	114	114
		114	
		114	

Table S.10. Results obtained from recovery tests performed in Drug Substance B (Sample 5), spiked at LOQ level, 100% and 140% of the ICH/VICH limits and defined control levels, considering a sample concentration of 100 mg/mL.

	Sample 5 (Drug substance B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 1	LOQ	100	103
		107	
		104	
	ICH/VICH limit	101	100
		102	
		98	
	140% ICH/VICH limit	103	103
		103	
		102	
Analyte 2	LOQ	92	94
		94	
		96	
	ICH/VICH limit	99	95
		97	
		90	
	140% ICH/VICH limit	101	99
		95	
		99	
Analyte 3	LOQ	98	99
		98	
		101	
	ICH/VICH limit	102	100
		101	
		97	
	140% ICH/VICH limit	103	103
		104	
		102	
Analyte 4	LOQ	94	95
		96	
		97	
	ICH/VICH limit	98	96
		97	
		91	
	140% ICH/VICH limit	100	98
		96	
		99	
Analyte 5	LOQ	92	94
		95	
		96	
	ICH/VICH limit	98	96
		97	
		91	
	140% ICH/VICH limit	101	99
		96	
		99	

	Sample 5 (Drug substance B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 6	LOQ	109	119
		123	
		125	
	ICH/VICH limit	99	98
		99	
		94	
	140% ICH/VICH limit	100	99
		99	
		98	
Analyte 7	LOQ	101	98
		97	
		97	
	ICH/VICH limit	102	101
		102	
		98	
	140% ICH/VICH limit	103	103
		104	
		102	
Analyte 8	LOQ	93	93
		89	
		97	
	ICH/VICH limit	103	101
		102	
		98	
	140% ICH/VICH limit	103	103
		104	
		102	
Analyte 9	LOQ	99	97
		99	
		95	
	ICH/VICH limit	100	99
		100	
		95	
	140% ICH/VICH limit	101	101
		101	
		100	
Analyte 10	LOQ	97	99
		98	
		101	
	ICH/VICH limit	102	101
		102	
		98	
	140% ICH/VICH limit	103	103
		104	
		103	

	Sample 5 (Drug substance B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 11	LOQ	94	96
		96	
		97	
	ICH/VICH limit	99	96
		98	
		93	
	140% ICH/VICH limit	99	98
		98	
		98	
Analyte 12	LOQ	89	93
		95	
		95	
	ICH/VICH limit	98	95
		97	
		91	
	140% ICH/VICH limit	101	99
		97	
		99	
Analyte 13	LOQ	96	97
		97	
		99	
	ICH/VICH limit	99	97
		98	
		94	
	140% ICH/VICH limit	100	99
		98	
		98	
Analyte 14	LOQ	96	95
		86	
		102	
	ICH/VICH limit	101	100
		101	
		97	
	140% ICH/VICH limit	102	103
		104	
		103	
Analyte 15	LOQ	96	96
		97	
		96	
	ICH/VICH limit	98	97
		98	
		94	
	140% ICH/VICH limit	99	98
		99	
		97	

	Sample 5 (Drug substance B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 16	LOQ	95	96
		95	
		97	
	ICH/VICH limit	99	97
		98	
		94	
	140% ICH/VICH limit	99	99
		99	
		98	
Analyte 17	LOQ	142	163
		147	
		189	
	ICH/VICH limit	96	95
		96	
		92	
	140% ICH/VICH limit	97	96
		97	
		95	
Analyte 18	LOQ	94	95
		95	
		96	
	ICH/VICH limit	97	95
		96	
		91	
	140% ICH/VICH limit	98	96
		96	
		96	
Analyte 19	LOQ	96	98
		100	
		98	
	ICH/VICH limit	99	98
		99	
		95	
	140% ICH/VICH limit	99	99
		100	
		99	
Analyte 20	LOQ	95	96
		96	
		98	
	ICH/VICH limit	98	96
		97	
		92	
	140% ICH/VICH limit	99	98
		97	
		97	

	Sample 5 (Drug substance B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 21	LOQ	103	99
		105	
		89	
	ICH/VICH limit	100	98
		100	
		95	
	140% ICH/VICH limit	101	103
		105	
		104	
Analyte 22	LOQ	91	87
		98	
		73	
	ICH/VICH limit	96	94
		96	
		92	
	140% ICH/VICH limit	97	97
		98	
		96	
Analyte 23	LOQ	95	96
		96	
		97	
	ICH/VICH limit	98	97
		98	
		94	
	140% ICH/VICH limit	99	99
		100	
		98	
Analyte 24	LOQ	98	98
		96	
		100	
	ICH/VICH limit	98	97
		98	
		94	
	140% ICH/VICH limit	99	100
		101	
		99	
Analyte 25	LOQ	93	94
		93	
		95	
	ICH/VICH limit	96	95
		96	
		92	
	140% ICH/VICH limit	97	97
		98	
		96	

	Sample 5 (Drug substance B)		
	Concentration level	**Recovery (%)**	**Average of recovery (%)**
Analyte 26	LOQ	67	71
		67	
		77	
	ICH/VICH limit	86	89
		94	
		89	
	140% ICH/VICH limit	87	92
		94	
		94	
Analyte 27	LOQ	91	92
		92	
		94	
	ICH/VICH limit	95	93
		94	
		90	
	140% ICH/VICH limit	96	97
		99	
		97	
Analyte 28	LOQ	96	97
		96	
		99	
	ICH/VICH limit	98	96
		98	
		94	
	140% ICH/VICH limit	98	99
		101	
		99	
Analyte 29	LOQ	89	87
		80	
		91	
	ICH/VICH limit	90	88
		89	
		86	
	140% ICH/VICH limit	93	97
		100	
		99	

S.8. Repeatability

Table S.11. Results obtained from repeatability tests performed in samples 1, 2, 3, 4 and 5, spiked at (1) LOQ level, (2) 100% and (3) 140% of the ICH/VICH limits and defined control levels, considering a sample concentration of 100 mg/mL.

	RSD (%)					
	Conc. level	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
Analyte 1	(1)	2	6	9	4	3
	(2)	1	1	1	0.2	2
	(3)	1	1	1	0.4	1
Analyte 2	(1)	1	4	4	8	2
	(2)	0.3	2	2	3	5
	(3)	1	1	2	2	3
Analyte 3	(1)	1	1	4	1	2
	(2)	2	1	1	0.2	2
	(3)	1	1	1	1	1
Analyte 4	(1)	2	2	3	4	2
	(2)	0.5	1	2	2	4
	(3)	1	0.4	2	1	2
Analyte 5	(1)	2	3	4	6	2
	(2)	0.4	1	2	2	4
	(3)	1	1	2	2	3
Analyte 6	(1)	2	1	1	1	2
	(2)	1	1	1	0.5	3
	(3)	1	1	1	1	1
Analyte 7	(1)	1	3	2	4	2
	(2)	2	1	1	0.2	2
	(3)	1	1	2	1	1
Analyte 8	(1)	2	9	4	13	4
	(2)	2	1	1	1	3
	(3)	1	1	1	1	1
Analyte 9	(1)	2	2	1	3	2
	(2)	1	1	1	0.4	3
	(3)	1	0.4	1	1	1
Analyte 10	(1)	2	1	2	1	2
	(2)	2	1	2	0.2	2
	(3)	1	1	2	1	1
Analyte 11	(1)	1	1	3	2	2
	(2)	1	1	1	1	3
	(3)	1	0.3	2	1	1
Analyte 12	(1)	1	2	5	5	4
	(2)	0.4	1	2	2	4
	(3)	1	0.4	2	1	2

	RSD (%)					
	Conc. level	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
Analyte 13	(1)	1	1	2	2	1
	(2)	1	1	1	1	3
	(3)	1	0.4	2	1	1
Analyte 14	(1)	4	5	5	8	8
	(2)	2	1	2	0.5	2
	(3)	1	2	2	1	1
Analyte 15	(1)	2	2	1	3	1
	(2)	1	0.4	1	0.1	3
	(3)	1	1	1	1	1
Analyte 16	(1)	2	1	2	1	1
	(2)	1	1	1	0.2	3
	(3)	1	1	1	1	1
Analyte 17	(1)	4	5	2	2	2
	(2)	1	1	1	0.3	3
	(3)	1	1	1	1	1
Analyte 18	(1)	1	1	3	2	1
	(2)	1	1	1	1	3
	(3)	1	0.3	2	1	1
Analyte 19	(1)	2	1	3	1	2
	(2)	1	0.5	2	0.2	2
	(3)	1	1	1	1	1
Analyte 20	(1)	1	1	2	1	2
	(2)	1	1	2	1	3
	(3)	1	1	2	1	1
Analyte 21	(1)	1	5	6	3	9
	(2)	3	1	3	1	3
	(3)	1	2	3	1	2
Analyte 22	(1)	11	8	3	5	15
	(2)	1	1	1	0.4	3
	(3)	1	1	2	1	1
Analyte 23	(1)	1	1	3	2	1
	(2)	1	1	2	0.2	2
	(3)	1	1	2	1	1
Analyte 24	(1)	2	2	1	2	2
	(2)	2	1	2	0.2	2
	(3)	1	1	2	1	1
Analyte 25	(1)	0.3	1	2	2	1
	(2)	2	1	2	0.2	3
	(3)	1	1	2	1	1
Analyte 26	(1)	1	13	8	11	8
	(2)	2	1	1	3	4
	(3)	2	1	1	1	5

	RSD (%)					
	Conc. level	**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**	**Sample 5**
Analyte 27	(1)	1	0.2	3	2	2
	(2)	2	1	2	0.3	2
	(3)	1	2	3	1	2
Analyte 28	(1)	2	0.5	4	1	2
	(2)	2	1	2	0.3	2
	(3)	1	2	2	1	1
Analyte 29	(1)	4	2	5	5	7
	(2)	3	2	2	2	3
	(3)	2	2	5	0.1	4

S.9. Intermediate precision

Table S.12. Results obtained from intermediate precision tests performed by injecting six times a standard solution containing the 29 target analytes at 100% of the ICH/VICH limit and defined controlled levels.

	% RSD (n=6)	% difference
Analyte 1	1	1
Analyte 2	3	1
Analyte 3	1	2
Analyte 4	2	1
Analyte 5	3	3
Analyte 6	1	1
Analyte 7	2	3
Analyte 8	1	1
Analyte 9	2	13
Analyte 10	2	3
Analyte 11	2	1
Analyte 12	3	1
Analyte 13	2	1
Analyte 14	2	1
Analyte 15	1	2
Analyte 16	1	3
Analyte 17	1	3
Analyte 18	2	1
Analyte 19	2	3
Analyte 20	3	2
Analyte 21	2	8
Analyte 22	2	3
Analyte 23	2	3
Analyte 24	2	2
Analyte 25	3	3
Analyte 26	3	2
Analyte 27	3	1
Analyte 28	3	1
Analyte 29	3	183