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Optimization of a mass spectrometric analytical method for the quality assessment of insulin and its analogs

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Received April 10, 2024, accepted June 25, 2024

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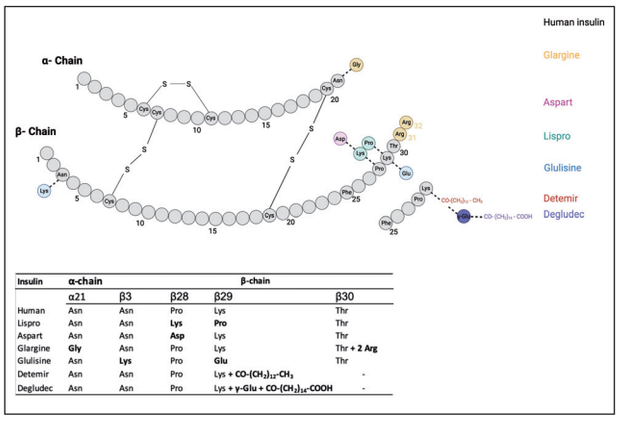
Pharmazie 79: 146-150 (2024) *doi: 10.1691/ph. 2024.4013*

The principal aim of this study was to optimize analytical methodology based on mass spectrometry for the evaluation of the quality of recombinant human insulin and its analogs. In this study ESI-MS was used to assess the quality of human insulin, short acting insulin analogs, insulin lispro, insulin aspart and insulin glulisine and long acting analogs including insulin glargine, insulin degludec, and insulin detemir, in respective pharmaceutical formulations. In this study, with the aimed to optimize analytical conditions, different factors influencing the analytical performance such as pH, ionic strength, sample dilution, organic solvent addition were addressed. The study results demonstrated that MS is a suitable technique for the analysis of biotechnological compounds like insulin and its analogs. Although the obtained results provide an important information regarding this methodology, further studies are needed to validate this analytical approach and check for its suitability to be used in the regulatory environment.

1. Introduction

Insulin, made up of a total of 51 amino acids, is structured as a dimer with two distinct polypeptide chains: chain A, comprising of 21 amino acids, and chain B, 30 amino acids. Interchain disulfide bonds connect cysteine residues in both chains (A7 with B7 and A20 with B19), whereas an intrachain bond within the chain A connects cysteine residues in A6 with A11 (Liu et al. 2018). Insulin analogs are insulin derivatives produced by recombinant DNA technology designed to modify the pharmacokinetics of native insulin. Specific changes in the amino acid sequence of native insulin have been introduced to improve pharmacokinetic parameters, with the aim to shorten the time of the onset of effect and to increase the duration of effect, to reduce hypoglycemia and improve the quality of life of diabetic patients (Berenson et al. 2011; Roach 2008; Fig. 1) .

Insulin is a peptide hormone produced in the beta cells of the islets of Langerhans in the pancreas and its principal role in the organism is to regulate the glucose levels in the bloodstream (Joshi et al. 2007). Pharmaceutical formulations containing insulin or its analogs are used for the treatment of diabetes mellitus. Human insulin is administered parenterally; it is produced using rDNA technology in different expression platforms including *E. coli* and *S. cerevisiae*, and enzymatically from porcine insulin whereupon the alanyl residue at position B30 is removed and replaced with an esterified residue of threonine by a trypsin-catalyzed reaction. (Rose et al. 1991)



Insulin	α-chain	β-chain			
	α21	β3	β28	β29	β30
Human	Asn	Asn	Pro	Lys	Thr
Lispro	Asn	Asn	**Lys**	**Pro**	Thr
Aspart	Asn	Asn	**Asp**	Lys	Thr
Glargine	**Gly**	Asn	Pro	Lys	Thr + **2 Arg**
Glulisine	Asn	**Lys**	Pro	**Glu**	Thr
Detemir	Asn	Asn	Pro	Lys + **CO-$(CH_2)_{12}$-CH_3**	-
Degludec	Asn	Asn	Pro	Lys + **γ-Glu + CO-$(CH_2)_{16}$-COOH**	-

Fig. 1: Structure of human insulin and modifications at insulin analogs (Slightly modified from Hamidli et al. 2022)

Insulin, like many other protein molecules, shows instability in aqueous solutions. Literature data reveals that insulin is subjected to different degradation pathways leading to the production of degradation products which could compromise the potency of the insulin and lead to immunogenicity. Differentiating insulin from its related compounds poses a challenge due to their highly similar chemical structures. Common techniques for the quality study of insulin and its analogs in pharmaceutical formulations include radioimmunoassay (Sakamoto et al. 2018; Shen et al. 2019), enzyme immunoassay (Andersen et al. 1993; Webster et al. 1990), luminescent immunoassay (Carslake et al. 2017), high-performance liquid chromatography (HPLC) (Abdelwaly et al. 2022; Von Zuben et al. 2020), and capillary electrophoresis (CE) (Schipper and Rudaz 2016; Šolínová et al. 2019). The intricate similarity in chemical structures complicates the differentiation of insulin and its related molecules. As a biological drug substance, insulin possesses a complexly folded protein structure that is influenced by factors such as pH, zinc ions, and the amino acid sequence. Standard analytical methodologies recommended by the European Pharmacopeia include RP-HPLC and Size-exclusion Chromatography (SEC) (European Directorate for the Quality of Medicines and Healthcare 2023). On the other hand, for the quality assessment of insulin, methods such as peptide mapping, amino acid composition, molecular weight determination, or size estimation using HPLC, SDS-PAGE, capillary electrophoresis, and mass spectrometry for drug product analysis are used [www.ich.org]. HPLC remains the conventional analytical method for insulin analysis. Recent research efforts have been directed towards the integration of Capillary Zone Electrophoresis (CZE) coupled with MS which could be used as a routine regulatory approach

for biopharmaceutical analysis, particularly in the quality study of insulin (Andrasi et al. 2020; Hamidli et al. 2022; Haunschmidt et al. 2010; Orlandini et al. 2007). Despite yielding satisfactory outcomes for the assessment of complex drugs, HPLC, due to its sensitivity and robustness, proved to show advantages comparing to CZE (Kowalski and Plenis 2007). On the other hand, MS coupled with various separation techniques (i.e HPLC, CZE) has been widely used for the quality assessment including identification and purity study of proteins of therapeutic relevance (Noor et al. 2021). Therefore, the principal aim of this work consists on optimization of MS technique for the assessment of quality of insulin and its analogs. This research was principally focused on the optimization of experimental parameters for the analysis.

2. Investigations, results and discussion

2.1. Development of an MS method for the determination of insulin analogs

2.1.1. Optimization of insulin concentration and addition of organic solvents

In the first step, insulin lispro was analyzed at pH 7 across three different concentrations: undiluted, seven-fold dilution, and ten-fold dilution. At these three concentration levels, differences in the corresponding peak areas of the insulin samples were observed. The undiluted sample showed noticeable formation of various adducts. Diluting the samples proved beneficial for improving selectivity.

Comparison of experimental results revealed that there was a significant 50% decrease in peak intensity, even with a tenfold difference in concentrations. At the same time, the *m/z* values between the two peaks differed, with first peak appearing at *m/z* = 1452.6620 and second peak at *m/z* = 1485.6398. These observations indicated a gradual decrease in signal intensity with higher concentrations of sample constituents, attributed to suppression from elements like sodium ions. Consequently, the ten-fold diluted samples were found to be more suitable for further measurements.

Additionally, optimization of organic solvent additives for insulin analysis was explored. Under conditions of pH 2.1, insulin lispro and insulin glulisine were investigated using different organic solvent additives, such as 2-propanol and methanol, comparing with a sample without any organic solvents added. Interestingly, for insulin lispro, the addition of isopropanol notably reduced the signal intensity, while the effect of methanol on suppressing the sample signal intensity was less pronounced (results not shown). For insulin glulisine, the addition of isopropanol significantly increased the peak intensity, while methanol addition resulted in its suppression.

Opposite to previous findings, the addition of organic solvents in the analysis of insulin lispro samples resulted in a decrease in peak intensity and a significant increase in the number of observed peaks. The use of organic solvents might have contributed to the rise in smaller peaks, spreading the overall peak intensity across these smaller ones.

Other insulin analogs showed similar results to the results of insulin lispro. In all cases, measurements without the addition of organic solvents exhibited higher peak intensity. Therefore, in further measurements the addition of organic solvents was precluded.

2.2. Optimization of buffer pH and polarity

Each insulin analog sample was examined at six different pH levels (1.7, 2.1, 2.6, 4, 7, and 9) using positive polarity, and one sample was tested at pH 9 with negative polarity. Comparing insulin lispro at pH 1.7 and pH 7 (results not shown) revealed a shift in charge distribution: the 5+ charge was more prevalent under acidic conditions (pH 1.7), while the 4+ charge was more dominant at neutral pH (pH 7). This shift highlights the influence of pH on the charge states of insulin lispro, with higher protonation levels observed in more acidic environments.

Another notable difference observed between the samples at pH 1.7 and pH 7 was the limited formation of adducts under acidic conditions. This contrasts sharply with conditions at neutral pH, making pH 1.7 more suitable for MS measurements. Additionally, the spectrum at lower pH levels exhibited higher intensities compared to the neutral conditions, further supporting the preference for lower pH values in conducting MS-based measurements of insulin analogs. However, this doesn't necessarily indicate the definitive optimum pH for these specific measurements.

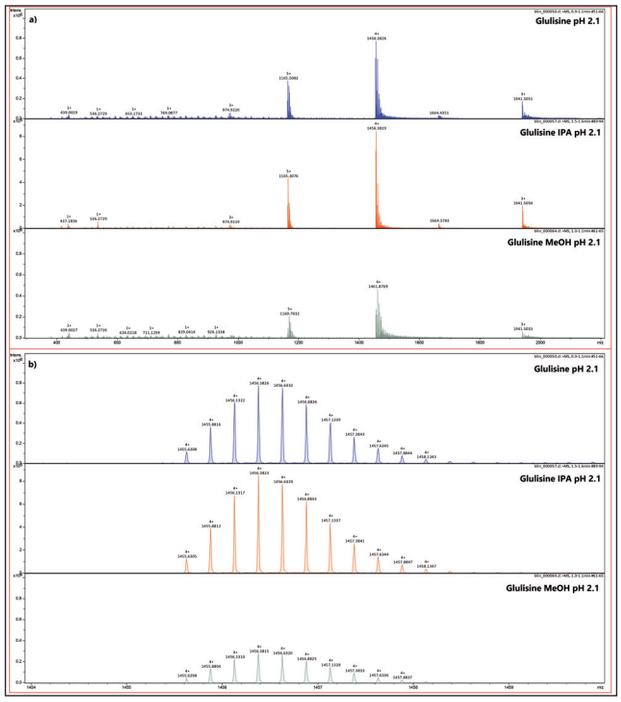


Fig. 2: Optimization of insulin glulisine sample with the addition of organic solvents. pH=2.1; a) representation of highest peaks in spectra, b) enhanced view of the highest intensity peaks.

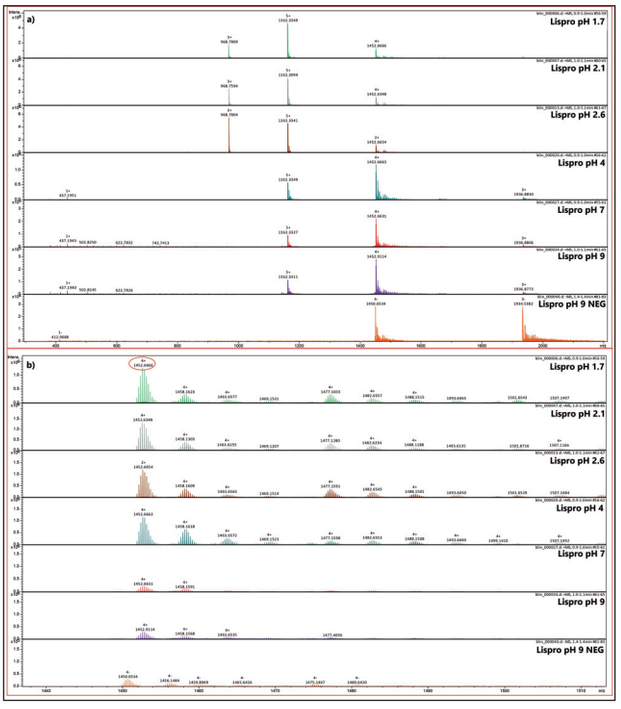


Fig. 3: Measurement of insulin lispro in six pH values in positive polarity mode and one in negative polarity mode; a) MS spectrums of different pH value, b) magnified MS spectrums.

Both spectra displayed three charges (at pH 1.7 = 6^+, 5^+, and 4^+; at pH 7 = 5^+, 4^+, and 3^+). Ideally, a lower number of charges within a spectrum tends to correspond with higher peak intensity.
Figure 3 illustrates the results of measurements of insulin lispro under seven different pH conditions. Similar to the earlier findings, acidic conditions proved more effective for measuring insulin analogs. Particularly, when examining the spectra at pH 1.7, 2.1, and 2.6, these conditions notably show the highest peak intensity, along with minimal adduct formation.

2.2.1. Comparison of human insulin and its analogs at identical pH environments

Human insulin and its analogs were studied under similar pH conditions, covering six specific pH levels (1.7, 2.1, 2.6, 4, 7, and 9) in positive polarity mode, with an assessment at pH 9 in negative polarity mode. Each insulin analog demonstrated unique charge patterns and exact charge numbers. However, in the case of human insulin and insulin lispro, the differences observed are minimal. This similarity is attributed to their nearly identical chemical structures, differing only in the amino acid sequence within chain B.
In contrast, insulin glargine stands out as the insulin analog with the highest charge number. Two main factors contribute to this difference. Initially, its amino acid sequence includes two amino acids, arginine in B31 and B32, which enhance protonation due to these additional elements in the chain. Secondly, while most insulins have an isoelectric point around 5.5, insulin glargine has an isoelectric point of 6.7, resulting in five distinct charges.
A consistent pattern was observed in both human insulin and insulin analog samples. As the pH gradually transitions from more acidic to more basic conditions, there was a noticeable rightward shift in the charge number of the highest peak, typically progressing from 5^+ to 4^+.
As the pH values approach neutral levels, the detection of dimers becomes noticeable. When the pH gets close to 4, there is a significant rise in the quantity of dimers, getting closer to the isoelectric point of insulin (around 5.5). Consequently, at pH 7, the occurrence of dimers is reduced, which can be attributed to the considerable deviation from the isoelectric point.

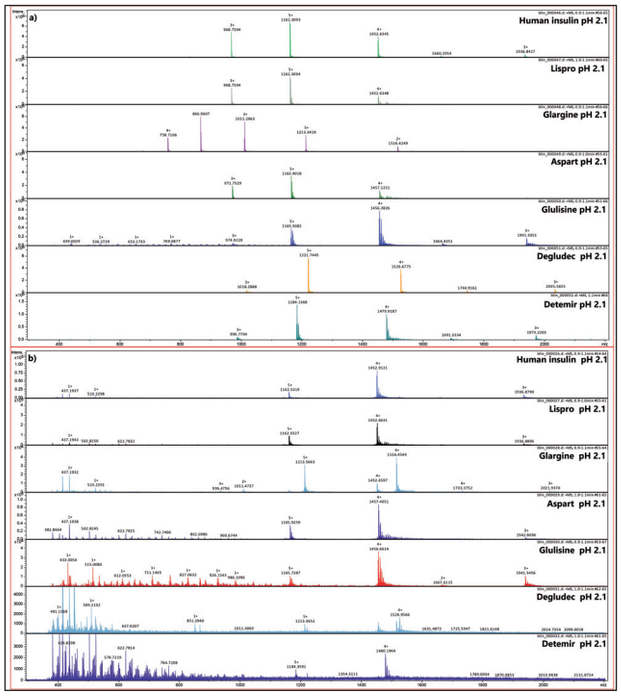


Fig. 4: Measurement of human insulin and insulin analogs in pH 2.1 and pH 7 with calculated average molecular mass.

2.3. Optimization of inorganic buffers concentration

Various concentrations of ammonium acetate were applied to explore its impact as a buffer in assessing insulin lispro at pH 7. Examining the spectra in Fig. 5 revealed interesting outcomes. The sample lacking added buffer notably exhibited higher peak intensity, accompanied by a significant presence of adducts. Similarly, the sample with the highest concentration of ammonium acetate displayed the lowest peak intensity. Interestingly, the buffer-free sample had a higher occurrence of Na^+ adducts than its actual charge number (5^+ instead of 4^+).
This difference could be attributed to the pH condition of the sample under evaluation. In neutral to slightly alkaline pH conditions, there is often a higher prevalence of negatively charged ions, favoring the presence of Na^+ over H^+ ions. Another potential contributing factor could be the existence of ammonia within the ESI chamber.
To ensure the reliability and consistency of these observed patterns, the measurement process was repeated over multiple days.

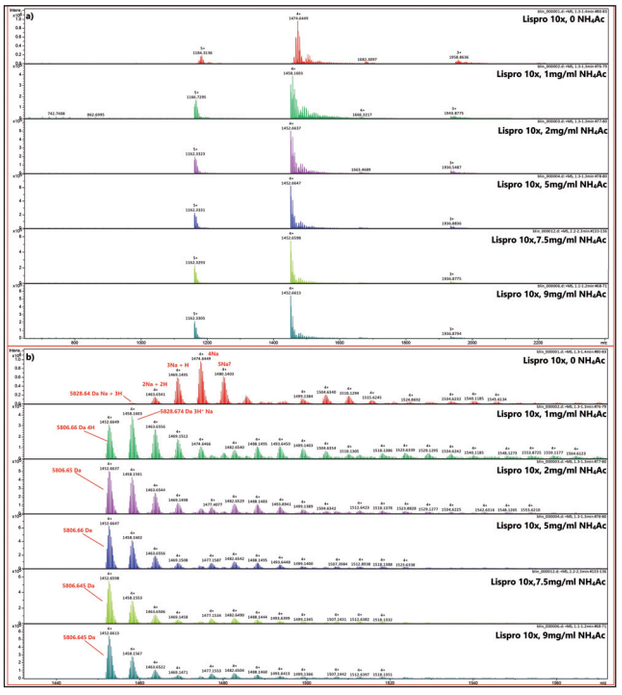


Fig. 5: Measurement of insulin lispro (pH 7) with added ammonium acetate; a) various concentrations of ammonium acetate, b) magnified MS spectrum.

At pH 7, the application of an ammonium acetate buffer facilitated the identification of dimers, trimers, and tetramers (results not shown).
Insulin lispro was analyzed at pH 7 using ammonium bicarbonate, with a concentration change similarly to that of ammonium acetate. Samples using both added buffers were compared, showing that ammonium bicarbonate significantly enhanced the resolution of insulin sample peaks, providing a clearer result compared to ammonium acetate (results not shown).

2.4. Robustness of MS measurement

The initial results, illustrated in Fig. 6, were repeated to verify the robustness of the analytical method. Figure 6 shows similar differences as seen in the earlier figures, indicating a higher prevalence of adducts. As previously mentioned, the slightly neutral pH conditions and the presence of ammonia in the ESI chamber could contribute to an increased formation of adducts, particularly involving Na+ ions. This is because neutral or slightly basic conditions can enhance the likelihood of sodium ions forming adducts with the analyte. However, in the subsequent measurement, the sample showed a reduction in both the intensity and the number of adducts.

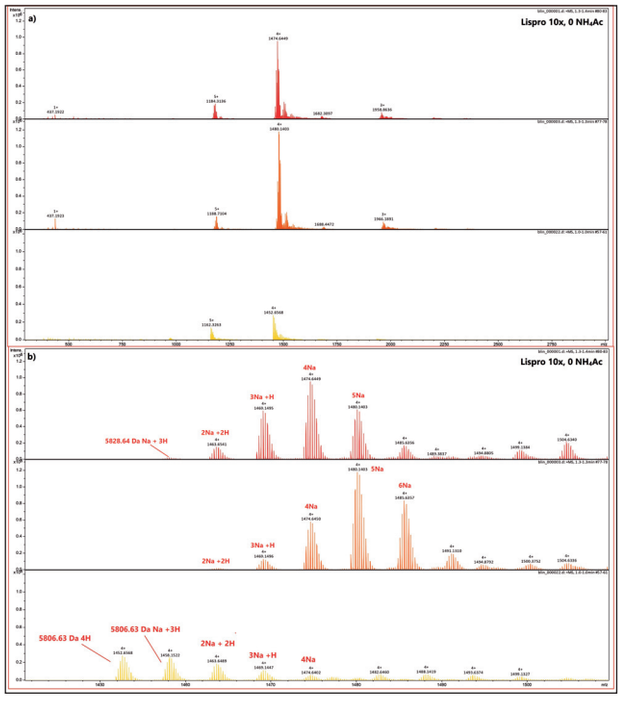


Fig. 6: Test comparison of similar measurements on different days.

In this study, various analytical conditions including pH, buffer type and concentration, sample dilution, and the use of organic solvent additives were explored to optimize the mass spectrometry (MS) conditions for evaluating the quality of insulin and its different analogs. The experimental data revealed that sample dilution significantly improved peak intensities. For insulin and its analogs, except for the glulisine sample, the absence of organic solvent additives led to reduced ion suppression and consequently better selectivity. Testing under different pH conditions demonstrated that in acidic environments, due to enhanced protonation, insulin samples exhibited higher charge states. Additionally, data for insulin lispro at pH 7, without additives and at varying concentrations, showed extra peaks for dimers, trimers, and tetramers, although with lower intensity. In conclusion, MS provides a rapid and optimized method for analyzing insulin and its analogs with enhanced selectivity.

3. Experimental

3.1. Chemicals and reagents

All reagents utilized in this study adhered to analytical standards. Ammonium acetate, ammonia, isopropanol, acetic acid, hydrochloric acid, and sodium hydroxide were supplied by Sigma-Aldrich (St. Louis, MO, USA). A syringe membrane filter was employed to filter all samples. The insulin and analogue formulations, including Tresiba®, Novorapid®, Levemir® (from Novo Nordisk), Humulin R®, Humalog® (from Lilly), and Lantus®, Apidra® (from Sanofi), were used in this investigation.

3.2. Instrumentation

An electrospray mass spectrometer (maXis II UHR ESI-QTOF MS instrument, Bruker, Karlsruhe, Germany) served as the analytical tool. The coupling was achieved through a CE-ESI Sprayer interface (G1607B, Agilent). Sheath liquid was delivered using a 1260 Infinity II isocratic pump (Agilent). The CE instrument's operations were controlled by OpenLAB CDS ChemStation software.

The study employed two distinct MS methodologies, tailored to a mass range of 300-2200 m/z, with adjustments made for positive and negative ion modes, each calibrated to their respective mass ranges. Both modes operated at a spectral rate of 1 Hz. Key parameters for both approaches included capillary voltage set at 4500 V, an end plate offset of -500 V, a nebulizer pressure of 0.6 bar, dry gas temperature maintained at 200 °C, and a dry flow rate of 4 L/min. Critical parameters for the positive ion polarity method encompassed a collision energy of 10 eV, collision RF set at 1800 Vpp, a transfer time of 120 seconds, and a pre-pulse storage duration of 10 seconds. Conversely, notable parameters for the negative ion polarity approach differed in collision energy (-8 eV) and transfer time (110 s).

The mass spectra obtained from it of Control version 4.1 (build: 3.5, Bruker) (build: 200.55.2969) were analyzed using Compass Data Analysis version 4.4 for data processing purposes.

3.3. Buffers and BGE

The initial phase of experimentation involved the utilization of buffers with varying pH levels. These buffers encompassed: 1 M formic acid (pH 1.7), 0.5 M formic acid (pH 2.1), 1 M acetic acid (pH 2.6), 50 mM ammonium acetate (pH 4.0), 50 mM ammonium acetate adjusted with ammonia (pH 7.0), and 1 M Ammonia (pH 9).

In the subsequent phase, buffers with an identical pH (pH 7) were employed, differing in concentration. Ammonium acetate was used at concentrations of 1 mg/mL, 2 mg/mL, 5 mg/mL, 7.5 mg/mL, and 9 mg/mL, while simultaneously, Ammonium Bicarbonate was used at 1 mg/mL, 2 mg/mL, 5 mg/mL, 7.5 mg/mL, and 9 mg/mL.

Additionally, organic solvent additives, namely isopropyl alcohol (IPA) and methanol (MeOH), were introduced alongside the buffers.

3.4. Sample preparation and calibration

The insulin formulations were subjected to dilution with the prepared buffers, generally at a 10-fold dilution; however, in the case of insulin detemir, the dilution was increased to 20-fold.

3.4.1. Sample calibration

The sample calibration process involved the use of both distilled water and a calibration solution. Initial pre-washing with water occurred under a pressure of 4 bars for a duration of 7-10 min. Subsequently, sample injection was carried out at 2 bars pressure, which extended for up to 60 min, dependent on the nature of the samples being measured. The process concluded with an injection of the calibration solution, executed at 4 bars pressure, lasting for 10 min, although in certain instances, the addition of calibration solution was unnecessary. Prior to presenting any specific measurements, the mass spectrum displayed various peaks, primarily consisting of low mass components presumed to be impurities.

Conflicts of interest: none declared

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