HILIC UPLC/ QTof MS method development for the quantification of AGEs inhibitors : trouble shooting protocol

RESEARCH ARTICLE

HILIC UPLC/ QTof MS Method Development for the Quantification of AGEs Inhibitors – Trouble Shooting Protocol

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Abstract: Objective. The paper reports an attempt to develop and validate a HILIC UPLC/ QTof MS method for quantifying N-ε-carboxymethyl-L-lysine (CML) *in vitro*, testing N-ε-carboxy[D_2]methyl-L-lysine (d_2-CML) and N-ε-carboxy[4,4,5,5-D_4]methyl-L-lysine (d_4-CML) as internal standards. **Results.** During the method development, several challenging questions occurred that hindered the successful completion of the method. The study emphasizes the impact of issues, generally overlooked in the development of similar analytical protocols. For instance, the use of glassware and plasticware was critical for the accurate quantification of CML. Moreover, the origin of atypical variation in the response of the deuterated internal standards, though widely used in other experimental procedures, was investigated. **Method.** A narrative description of the systematic approach used to address the various drawbacks during the analytical method development and validation is presented. **Conclusion.** Reporting those findings can be considered beneficial while bringing an insightful notion about critical factors and potential interferences. Therefore, some conclusions and ideas can be drawn from these trouble-shooting questions, which might help other researchers to develop more reliable bioanalytical methods, or to raise their awareness of stumbling blocks along the way.

Keywords: Advanced glycation endproducts (AGEs); carboxymethyl-lysine (CML); method development; HILIC; UPLC/QTof MS.

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1. INTRODUCTION

Advanced glycation endproducts (AGEs) are complex, heterogeneous, sugar-derived protein modifications that are implicated in the pathogenesis of diabetic complications, Alzheimer's disease, and the process of normal aging [1–3]. On a biochemical level, glycation is defined as the spontaneous, non-enzymatic reaction of glucose or other reducing sugars with an amino group of proteins, lipids, and nucleic acids [4]. As one would expect, any inhibitors of glycation can be beneficial for the prevention and treatment of different pathological conditions, and to increase lifespan. Therefore, a critical aspect in identifying potent AGEs inhibitors is the presence of validated analytical methods for unambiguous determination of their anti-AGEs properties. In the beginning, the early assessment of AGEs was limited to detecting and quantifying those with intense intrinsic fluorescence like pentosidine and argpyrimidine [5-6]. Nevertheless, fluorescence properties do not definitely correlate to a high functional or pathogenic role in the human body. As a result, a lot of efforts were made for establishing a more universal and reliable technique covering a broad range of detectable AGEs. Quantification of AGEs with instrumental methods, such as liquid chromatography (LC), requires a completely different sample preparation and performance of the analysis compared to the non-selective colorimetric/fluorimetric techniques [7]. For instance, the sample preparation in LC methods can minimize interferences generated from chemical characteristics of compounds in the incubation mixture (e.g., autofluorescence of impurities), or the potential binding ability of some small molecules to the proteins in the reaction mixture resulting in quenching of the signal.

In the current project, an attempt to develop and validate a method for the quantification of N-ε-carboxymethyl-L-lysine (CML) in *in vitro* samples has been conducted. Consequently, many factors had to be taken into consideration while establishing each step of the analytical protocol. The initial choice that defines the direction of the analytical procedure was selecting the AGE of interest. CML is a well-characterized and extensively studied product in the field of advanced glycation. It has been analyzed as an indicator of the Maillard reaction in food products, and also in biological systems as a lysine residue in proteins formed by both glycation and lipid peroxidation pathways [8–13]. Several analytical techniques for the quantification of CML in biological matrixes have been proposed based on instrumental

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methods like isotope dilution LC-MS/MS analysis, and HPLC with a derivatization reaction with ortho-phthalaldehyde (OPA) or 6-aminoquinolyl-N-hydroxy-succinimidyl-carbamate (AQC), or immunochemical methods [9,14–17]. CML was firstly isolated and described from glycated proteins *in vivo* and is considered to be the most abundant AGE in the human body; e.g. it was detected in tissues of lens crystallin, skin collagen, and urine [18]. Increases in the concentration of CML have been observed in the blood and tissues of patients with diabetes mellitus, in the intracellular neurofibrillary deposits in Alzheimer's disease, and in atherosclerotic plaques [19–21]. Moreover, high serum levels of CML have been discovered in patients on dialysis and with diabetic nephropathy [22]. As a result, investigating the concentration of CML *in vitro*, and inhibition of its formation was considered a promising goal for the analytical procedure.

Another major question was which CML fraction was going to be used for the analysis. In biological systems, AGEs exist in two forms: free AGEs (glycated amino acids) and protein-bound AGEs (protein glycation adducts) [23]. CML, specifically, exists in a free form or bound to lysine residues within peptides and proteins [12]. The choice of which fraction to be measured has a crucial impact on the further sample preparation steps. To facilitate the quantification of free CML, proteins and the protein bound CML should be removed from the reaction mixture. On the other hand, a standard approach in the sample preparation for analysis of protein-bound AGEs involves their enzymatic or chemical release from proteins in the mixture. Therefore, a hydrolysis step is needed in the characterization of protein bound CML in complex matrices. Several methods have been described: The enzymatic hydrolysis is recommended for analyzing acid unstable AGEs, e.g., pyrraline and N-ε-fructosyllysine. It requires a cocktail of enzymes like pepsin, pronase E, aminopeptidase, and prolidase [24,25]. Alternatively, other methods were described based on the use of proteinase K or trypsin [26,27]. Acid hydrolysis, on the other hand, is performed by heating the sample with 6 M hydrochloric acid for 24 h at a temperature of 100 ºC [28]. In contrast to several other AGEs, CML is an acid-stable compound which facilitates its analysis by using hydrolysis with 6 M hydrochloric acid [9].

To obtain accurate data for the level of CML in the hydrolyzed sample, a suitable clean-up step is essential to remove interferences and matrix effects, and to maximize the sensitivity of the final LC/MS analysis. Various types of cartridges for solid-phase extraction can be used. For example, Supelco C18 cartridges, Oasis HLB 6cc, MCX cartridges or P3 plates (protein precipitation plates) [9,25,28,32–34]. Finally, the analytical instrument used to perform the measurements plays a key role. Although CML has hydrophilic characteristics, reverse phase chromatography coupled to triple quadrupole detection is widely used [9,28,30,35]. The measurement is facilitated by the addition of an ion-pairing reagent like nonafluoropentanoic acid (NFPA) during the sample preparation and as a modifier of the mobile phase. Consequently, the CML affinity to the column, the purity of the spectrum and the MS signal vastly increase [9]. However, despite its good compatibility with LC-MS, the high concentration of NFPA results in a mobile phase with a very low pH (approximately 2) which can cause rapid deterioration of the reverse phase column [36]. Another approach that enables the quantification of CML by reverse phase chromatography is a preliminary derivatization step. During the method development presented in the paper, a derivatization with hydrochloric acid and *n*-butanol (1:3) was tested [37]. The derivatization reaction involves butyl esterification of the amino acid adding 56 mass units (Figure 1).

Additionally, since CML is a highly polar compound, reversed-phase chromatography (such as C_{18}) can be replaced by hydrophilic interaction liquid chromatography (HILIC). When HILIC mode is used, the non-derivatized CML is retained without any mobile phase modifier and the drawbacks of using ion-pairing reagents can be avoided [38].

In general, when employing LC-MS quantification of an analyte, the use of an internal standard is advisable [39]. Particularly, for CML the internal standard could be its stable-isotope labelled form like N-ε-carboxy[D_2]methyl-L-lysine (d_2-CML) or N-ε-carboxy[4,4,5,5-D]methyl-L-lysine (d_4-CML), or a structural analogue: (S-(4-pyridylethyl)- L-cysteine (PEC) or α-aminobutyric acid [16,40].

Although all discussed factors were delicately balanced during the analytical method development, some challenges occurred that required a troubleshooting approach.

2. MATERIALS and INSTRUMENTS

2.1. Solvents and Reagents

Acetonitrile, isopropanol, *n*-butanol (99%), dimethyl sulfoxide (DMSO), and sodium hydroxide were analytical grade and were purchased from Fisher Scientific (Hampton, NH, USA) or Acros Organics (Geel, Belgium). Formic acid (99+%) and trifluoroacetic acid (p.a. 99%) were obtained from Acros Organics. Bovine serum albumin (98%), sodium azide (99.5%), D-glucose (>99.5%), and D-ribose (>99%) were ordered from Sigma Aldrich. Sodium dihydrogen phosphate was from Merck (Darmstadt, Germany). The Ac-Gly-Lys-OMe acetate salt (Gk-peptide) was provided by Bachem (California, USA). For the UPLC/MS experiments on AGEs, the standard N-ε-carboxymethyl-L-lysine (CML) was purchased from Polypeptide Group (Strasbourg, France); the internal standards S-β(4-pyridylethyl)-L-cysteine (PEC) was from Santa Cruz Biotechnology (Dallas, Texas); and d_2-CML and d_4-CML were from Iris Biotech GMBH (Marktredwitz, Germany). Water was obtained by a Milli-Q system from Millipore (Bedford, USA) and was filtered through a 0.22-µm membrane filter.

2.2. General Apparatus and Instruments

Ultrasonication was performed with a Branson 3510 ultrasound bath. Also, a small centrifuge Sigma 1-15 PK (Fisher Bioblock Scientific, Merelbeke, Belgium), a vacuum centrifuge Savant equipped with Refrigerator Vapor Trap (RVT) 400 and SpeedVac Concentrator (SPD) 121P (Thermo Scientific, Massachusetts, USA) were used. Measurements of the pH were achieved with SensION (Hach, Mechelen, Belgium). Incubation at 37 °C was done in a REV SCI (Incufridge) machine and the heating plate (dry bath FB 15101) used for the hydrolysis step was from Fisher

Scientific. The removal of proteins in the *in vitro* samples was achieved on P3 (Protein Precipitation Plates) cartridges equipped with 96 deep-well collection plates (2 mL) (Porvair Sciences, Wrexham, United Kingdom), Oasis® PRiME HLB cartridges (186008057, Waters) or iSPE®-HILIC cartridges (200.001.0025, Hilicon). Ultra-Performance Liquid Chromatography (UPLC) was performed using a Waters Acquity® system (Waters, Milford, MA) that comprised a binary solvent manager, sample manager, column manager, and UV detector. The chromatographic conditions were optimized on different columns: an Acquity UPLC BEH C18 column (100 mm x 2.1 mm, 1.7 µm) (Waters) and an Acquity UPLC BEH Amide column (100 x 2.1 mm, 1.7 µm) (Waters). The UPLC system was coupled to a Xevo G2-XS QTof mass spectrometer (Waters). MassLynx 4.1 software was employed.

3. RESULTS and DISCUSSION

3.1. Troubleshooting – method development

3.1.1. To set the scene

In the beginning, the method aimed to measure the free CML. The incubated samples and the calibration curve samples were prepared using a bovine serum albumin (BSA) / glucose incubation mixture under the following conditions: In brief, sodium phosphate buffer (500 mL, 50 mM) was prepared (pH 7.4) containing 0.02% (m/V) sodium azide; a BSA solution with a final concentration of 22.2 mg/mL and a glucose solution (1111.1 mM final concentration) were made in the phosphate buffer [41,42]. The incubation mixture equivalent to maximum CML formation contained 135 µL BSA solution, 135 µL glucose solution and 30 µL DMSO incubated at 37 °C for 7 days. All sample preparation steps are presented in Table 1.

1 mM stock solutions of the standards CML and PEC were prepared in Milli-Q water [43]. Each stock was further diluted with water to obtain a concentration between 50 nM and 2500 nM. The concentration range of the assay and the calibration standards were established based on the amount of CML formed in the *in vitro* model.

The starting point of the method development was based on ultraperformance liquid chromatography (UPLC) coupled to a quadrupole time-of-flight mass spectrometer (QTof), using a derivatization reaction with hydrochloric acid and *n*-butanol (1:3) [11,44]. Additionally, *S*-β(4-pyridylethyl)-L-cysteine (PEC) was selected, derivatized using the same reaction, and used as an internal standard for precise quantification of CML (Figure 1) [39]. The analysis was performed on an Acquity UPLC BEH C18 column (50 x 2.1 mm, 1.7 µm) and the mobile phase consisted of water with 0.1% formic acid and acetonitrile with 0.1% formic acid. The gradient elution started with 1% B for 2.5 min, then rising to 40% B by the 11th minute. At 11.10 min the percentage of B was 55%, which was kept till the 15th minute. Next, for 3 min the starting percentages were restored. The flow rate was 0.6 mL/min, and the column temperature was 40 °C. The injection volume was 10 µL. Electrospray in positive ionization mode (ESI) was applied with a sample cone set at 40 V, and capillary 1.01 kV. A solution of sodium formate was used to calibrate the mass spectrometer. Leucine enkephalin was used as a lock mass. All samples were analyzed in glass vials.

After the derivatization, the following signals were observed: *m/z* 317.2440 $[M + H]^+$ and *m/z* 283.1480 $[M + H]^+$ for the CML dibutyl ester and PEC monobutylester, respectively. Measurements were performed in a full scan mode. Despite that generating product ions will increase the specificity of the measurement, the sensitivity remained poor after fragmentation. In general, the derivatization is utterly important when CML is analyzed with LC-UV due to the lack of chromophores in its structure. In the QTof measurement, the derivatization with hydrochloric acid and *n*-butanol (1:3) was used to increase the ESI sensitivity by improving the ionization, and reducing the polarity of CML [45]. However, the results obtained by this method showed no consistency after repeated injection, and no concentration-dependent results could be obtained for the different standards. Also, changes in mass spectrometric parameters could not resolve this issue and reproducible results were not obtained. A probable explanation was incomplete derivatization by the hydrochloric acid and *n*-butanol (1:3) reaction since no pattern was found for the results.

3.1.2. The dilution mixture

To avoid the derivatization reaction, but to sustain an optimal chromatographic separation and mass spectrometric detection, the possibility of hydrophilic interaction chromatography (HILIC) was investigated. This is a potent and favorable technique to resolve highly polar molecules, e.g., amino acids and amino acid-like compounds such as CML. Therefore, the Acquity UPLC BEH Amide column (100 x 2.1 mm, 1.7 µm) (Waters) was selected instead of the BEH C18 column. A mobile phase of 10 mM ammonium formate pH 3 (eluent A) and 85% acetonitrile in 10 mM ammonium formate (eluent B) were used for gradient elution: starting percentage of 85% B for 6.0 min, then the percentage of B was reduced to 67.4% till 10.0 minute. From 10.0 to 12.0 min the percentage B was further decreased to 55.6%. Next, between 12.1 min and 18.0 min, the percentage was kept at 85% B [46]. Since no derivatization was performed the following signals could be observed after mass spectrometric detection *m/z* 205.119 $[M+H]^+$ and *m/z* 227.085 $[M + H]^+$ for CML and PEC, respectively.

Previously, the stock solutions of CML and PEC, and their further dilutions were prepared in Milli-Q water. However, one of the most important parameters to obtain suitable peak shapes in HILIC mode is the nature of the solvent. Therefore, a crucial aspect in the further development of applying the HILIC mode was how those standard dilutions were made. Generally, samples that contain a high percentage of water would elute rapidly on a HILIC column and peak splitting could occur [47]. As a result, finding the right composition and proportion for the dilution mixture was critical for the analysis. To begin with, a high content of acetonitrile in water was tested [48]. Although, with 95% acetonitrile, peak splitting was avoided the highest concentration of CML standard (2500 nM) gave a relatively low response, possibly due to the decreased solubility of the compound of interest in the 95% acetonitrile solution. Therefore, isopropanol was also included in the dilution mixture [49]. In the end, a composition of 45% isopropanol, 45% acetonitrile, and 10%

water resulted in a nice peak shape and good intensity of the signal (Figure 2). Despite complete solubility of the standards and good chromatographic output, no linear trend between the concentration and the signal was observed after injecting the different standard concentrations.

3.1.3. The vials

A probable explanation was the attachment of CML to the glasswork. This can be related to a study by Kalasin and Santore (2009), who demonstrated that a small amount of fibrinogen could be adsorbed onto a negatively charged silica surface [50]. Therefore, the adhesion properties of peptides and proteins could be a reason for inaccurate results and wrong conclusions in certain experiments [51]. To reduce the surface binding of proteins several approaches have been described, such as adding Tween-20 or salt in a high concentration, addition of BSA, using coated tubes with polyethylene glycol (PEG) or siliconizing agents, or designing a solvent system that reduces the tendency of proteins to interact with the surface [52–54]. Although including BSA in the solvents or using it to coat surfaces seems to be the easiest approach, it is not feasible in some experiments such as the purification of peptides / proteins for mass spectrometric analyses [51]. In conclusion, the phenomenon of glass adhesion required the glass vials to be replaced by polypropylene vials (Varian 0.1 ml screw-top vial kit, 99788790). This step was of great importance and remained crucial for the positive outcome of the whole experimental procedure. In Figure 3 a comparison is shown between the response for 2500 nM and 1000 nM CML in polypropylene vials and glass vials. The intensity for both concentrations measured in the polypropylene vials was much higher compared to the same dilutions prepared in the glass vials. After selecting the polypropylene vials for the following experiments, standard dilutions of CML in isopropanol, acetonitrile, and water (45:45:10) mixture were analyzed, and the calibration curve was generated. A linear response was observed for the standard dilutions of CML. However, using the same conditions a peak splitting and tailing were observed for the PEC standard. Consequently, several other chromatographic systems were tested as an alternative, unfortunately, no satisfactory results were obtained (Figure 4). Finally, a mobile phase containing water with 0.1% formic acid (eluent A) and acetonitrile with 0.1% formic acid (eluent B), was used previously with the BEH C18 column, improved the results for the PEC standard. Figure 5 represents the chromatographic results from the standard solutions of 2500 nM CML and PEC in the isopropanol, acetonitrile, and water (45:45:10) mixture with the new chromatographic conditions. Both CML and PEC showed optimal results and a calibration curve including several standard dilutions could be generated.

3.1.4. The sample preparation

At this point of the research, a decision was taken to focus on quantifying the "total" CML since the amount of free fraction was considerably lower than the bound fraction due to the simplicity of the applied incubation model. Taking into consideration the protein bound CML in the further experiment, an acid hydrolysis step was included in the protocol. To be certain that CML is not produced from Amadori rearrangement products (ARPs) during acid hydrolysis a sodium borohydride reduction step is normally performed [9,29]. In the current research, the reduction step was not included because the composition of the reaction mixture to generate CML was considered relatively simple compared to those reported in the literature [8,30,31]. In the latter cases, the analysis was conducted on food or biological samples, in contrast to the *in vitro* model used in this method. The resulted hydrolysates required an extra clean-up step before submitting them for LC/MS analysis. To facilitate the sample clean-up the composition of the incubation mixture was changed. The previously used mixture contained BSA (66.5 kDa) as the protein source and glucose as the sugar source. It was speculated that sample incubation using a peptide instead of a protein with high molecular weight like BSA would require less sample clean-up preparation. Therefore, Gk-peptide (N-acetyl-glycyl-L-lysine methyl ester, 259.31 g/mol) was introduced into the incubation mixture together with ribose as the sugar component. However, after the complete acid hydrolysis of the peptide, a hydrolysate clean-up procedure was to be optimized.

Based on preliminary research, several solid-phase extraction models were tested: protein precipitation plates P3, Oasis® PRiME HLB cartridges, and iSPE®-HILIC cartridges. The results from the P3 plates suggested the best recovery of the eluted CML / PEC standard dilutions. The steps for sample preparation used to evaluate the total CML level are summarized in Table 1. The necessity to reconstitute the acid hydrolysate in water and the use of acetonitrile for the elution through the P3 cartridges was based on the guidelines for the protein precipitation plates from the manufacturer [55].

3.1.5. The internal standard – a friend or foe

To avoid variability in the quantification data using the internal standard PEC, the validation of the method continued using N-ε-carboxy[D_2]methyl-L-lysine (d_2-CML) instead. In general, analytes and their stable isotope labeled internal standard have identical physicochemical properties, therefore the latter can compensate better for any variation in the signal of the ion during the analysis [56]. In preliminary tests, the calibration curves of dilutions of the standard CML and d_2-CML were successfully obtained (Figure 6). However, after analyzing incubated samples using the described conditions, the amount of CML formed was excessively high, causing suppression of the d_2-CML molecular ion signal (Figure 7A).

3.1.6. The stock solution concentrations and incubation time

The next step in the method development was to investigate the optimal conditions for CML formation causing no interference with the signal of the internal standard d_2-CML. Two factors were examined, different concentrations of the Gk- and ribose solutions and the duration of the incubation time. The concentrations tested for both the peptide and the sugar in the reaction mixture are included in Table 2. 1.7 mg/mL Gk-peptide and 1 mg/mL ribose were selected for the optimal composition of the incubation mixture and the time was set to just 24h. In this case, the area of CML was more than four times lower than the area generated with the higher concentrations (2.5 mg/mL Gk-peptide and 1.5 mg/mL ribose, see Table 2), which enabled the detection of the internal standard response without any interference (Figure 7B). With

a higher concentration of the peptide and the sugar, CML quickly reached an amount where suppression of the intensity of d_2-CML occurs. Therefore, a balance between the generation of sufficient CML allowing the possibility of testing inhibition of CML formation, and the intensity of the internal standard had to be established.

After completion of this analytical protocol, method validation was approached following the FDA Guidelines of Bioanalytical Method Validation regarding linearity, precision, and quality control [57].

3.2 Troubleshooting - Method validation

The method was linear between 75 nM and 5000 nM CML. For evaluating the between-day variation samples were prepared and analyzed on 3 different days in triplicate [57]. These included eight different concentrations of CML standard to obtain a calibration curve; three quality control samples; and one sample representing the maximum amount of CML formed after incubation of the GK-peptide with ribose. All samples were prepared in triplicate. The quality control samples contained 75, 2500, and 5000 nM CML and were prepared from a different stock solution than the one used for the calibration curve samples. The summary of the protocol used in the method validation is included in Table 3. The results for the calibration curves used for the determination of the between-day precision of CML in *in vitro* samples with d_2-CML as an internal standard are presented in Figure 8. The samples representing the CML formation on day 3 had a lower value than normally expected in comparison to the previous two measurements (data not presented). Therefore, the last experiment had to be performed again.

However, at a particular moment, the company producing the "Varian" polypropylene vials was out of business, and there was an urgent need to find them a suitable replacement. Consequently, exhausting testing of different polyproline vials was approached: LCGC Certified Clear Glass 12x32 mm Screw Neck Vial, Total Recovery, with Cap and Pre-slit, PTFE/Silicone Septum, 1 ml volume (186000385C, Waters), TruView LCMS Certified Clear Glass 12x32 mm Screw Neck Vial, with Cap and Pre-slit, PTFE/Silicone Septum, 2ml volume (186005666CV, Waters), polypropylene Screw neck vials 0.300 ml (702226, Macherey-Nagel GmbH & Co.KG), Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and Preslit PTFE/Silicone Septum, 0.700 ml volume (186005221, Waters) and Screw top 12x32 mm polypropylene vials, certified, 0.250 ml (5190-2242, Agilent). The linearity experiment was performed for incubated samples using different test vials. Based on the calibration curves from the CML and d_2-CML standards, the polypropylene vials from Agilent were considered the best choice about performance.

At the same time, the change from d_2-CML to N-ε-carboxy[4,4,5,5-D]methyl-L-lysine (d_4-CML) was considered to improve the quantification of the results and the precision of the method. Since a compound and its stable isotope labeled internal standard will theoretically co-elute, it is important that the mass difference between them is at least 3 mass units in order to be separated in the mass analyzer. When the difference is less than 3 as it was the case with d_2-CML and CML (Figure 9), the isotope peak of the analyte might interfere with the signal of the internal standard [56].

Similar to d_2-CML, the optimal conditions for CML formation and the subsequent effect of the formed CML on the d_4-CML response were investigated by monitoring two incubation conditions for 36 h. Condition 1 included the use of 1.7 mg/ml for the Gk-peptide and 1 mg/mL for the ribose (Figure 10A); and condition 2: 0.85 mg/mL of the protein and 0.50 mg/mL for the sugar, respectively (Figure 10B). The first graphs from Figure 10A and B represent the ratio of the signal of CML and d_4-CML (2000 nM) internal standard in terms of the incubation time used, while the second and third graph show the individual signal over time for CML and d_4-CML (2000 nM) respectively. Finally, the following conditions were selected: 1.7 mg/mL for the peptide and 1 mg/mL for the sugar, with an incubation time of 14 h. These conditions provide sufficient CML formation without affecting the internal standard response. Longer incubation times result in a higher concentration of CML, but also in a negative effect on the response of the deuterated internal standard.

Recently, there has been increased attention to the adequate monitoring of internal standard response for LC-MS/MS analysis. The variable response of the internal standard in the current method was similar to the one previously reported by Aimin Tan *et al.* [57]. The gradual decrease and randomly scattered low internal standard values have been explained by autosampler needle blockage. However, in our case the response for CML in the calibration curve samples was not affected, ruling out the possibility of needle blockage and leading to the assumption that a matrix effect was present. According to the literature, one of the parameters that are often overlooked when working with HILIC mode is the composition of the needle wash solvent set up in the instrument (i.e., strong/ weak needle wash, purge solvent, etc.) [47]. Therefore, the weak wash was changed from water: methanol: acetonitrile: isopropanol (25:25:25:25) with 0.02% formic acid to acetonitrile 90% only which was close to the starting conditions of the mobile phase. However, despite the implemented changes, the response to the internal standard did not improve. To exclude the matrix effect, several injection volumes were tested. The initial injection volume was set at 10 µL (Figure 11). However, smaller sample volumes like 5 µL and 2 µL were also investigated. Finally, 5 µL of the sample was selected for further tests. The data from the experiment with a 2 µL injection volume is not shown.

Unfortunately, after applying all the changes, the results still showed inconsistency for the internal standard. Based on these results a clear evaluation of the samples can only be performed if the calibration curve is based only on the area of CML instead of the ratio of CML/ d_4-CML.

4. CONCLUSION

There is a famous saying: "It is good to have an end to a journey towards, but it is the journey that matters, in the end". This project was undertaken to develop an analytical procedure that can address the demand for reliable quantification of AGEs in biological samples. By implementation of mass spectrometry, the lysine derivative - N-ε-carboxymethyl-L-lysine (CML), was analyzed in *in vitro* samples. CML, one of the most abundant AGEs *in vivo* as well as *in vitro*, was the logical choice for the developed method. The current study tried to address all the limitations during the

method development of the analytical protocol. The latter can be summarized as follows: The quantification of CML was challenged with a UPLC/ QTof MS detection system connected to a BEH Amide column, and gradient elution with water with 0.1% formic acid (eluent A) and acetonitrile with 0.1% formic acid (eluent B), using 2000 nM d_4-CML as an internal standard. The samples were generated by incubating Gk-peptide (1.7 mg/mL) and ribose (1 mg/mL) for 14 h at 37 ºC; then the sample preparation required acid hydrolysis with 6 M hydrochloric acid at 100 ºC for 24 h, and a clean-up step through P3 cartridges. Before analysis, the samples were reconstituted in isopropanol: acetonitrile: water (45:45:10) and placed in polypropylene vials ready for analysis. Among the most significant findings, emerging from this study, were the type of vials and the dissolution mixture used to prepare the samples which significantly affected the quantification results. Polypropylene vials were proved advantageous to the common glass vials; and the application of a dissolution mixture containing isopropanol: acetonitrile: water in proportion 45:45:10 influenced to a greater extent the peak shape and the intensity of the signal. Notwithstanding the numerous attempts to finalize the method, further work is needed to understand the peculiar results with the, though generally applied, deuterated internal standard d_4-CML. As part of the future perspectives, some aspects can be investigated to tackle the factors that hinder the positive outcome of the analytical procedure. For example, the application of enzyme hydrolysis instead of acid hydrolysis. The process is labor-intensive and involves several enzymes and antibiotics over 4 days. However, it can be fully automated and performed in control conditions through a robotic processor (e.g., CTC PAL sample auto processor with sterile reagent addition of argon) [58]. This protocol can avoid oxidative degradation of protein adduct residues and subsequently, overestimation of oxidation adducts. Moreover, additional possibilities can be tested in the context of sample clean-up, for example, the use of centrifugal filters (Millipore Amicon Ultra) instead of the protein precipitation plates [59]. Additional testing of other anti-adhesive vials deserve further attention. However, despite its exploratory nature, this study offers insight into some underestimated aspects like the vials for analysis and speculates about the critical role of matrix effects or probable interactions in the reaction mixture on the internal standard response. Further research needs to examine more closely the factors and the connections that determined the results of the current study.

5. ACKNOWLEDGEMENTS

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6. CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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Table **1**. Developed procedures for sample preparation in the analytical protocols for measuring free and total CML.

Measuring free CML	Measuring total CML
• 75 µL sample - BSA/Glu mixture, DMSO or standard CML solution for the calibration curve incubated at 37 ºC for 7 days • addition of 15 µL internal standard PEC • protein precipitation with methanol: acetonitrile (1:3) • vortex (1 min) • centrifuging for 20 min at 20 °C, 14 000 rpm • the supernatant is taken • dried under nitrogen at 70 °C • derivatization for 90 min at 70 °C with hydrochloric acid: *n*-butanol (1:3) • dried under nitrogen at 70 °C • dissolve in 150 µL water • transfer in glass vials • injecting 10 µL for analysis	• 75 µl sample - Gk-peptide (5 mg/mL) and Rib (3 mg/mL), and the standard CML solution for the calibration curve incubated at 37 ºC for 24 h • addition of 15 µL internal standard d_2-/d_4-CML • hydrolysis of the sample with 6 M hydrochloric acid at 100 ºC for 1 h • drying the hydrolysate in a vacuum centrifuge • reconstitution in Milli-Q water • cleaning through P3 cartridges, elution with acetonitrile • drying the collected sample in a vacuum centrifuge • reconstitution in isopropanol: acetonitrile: water (45:45:10) mixture • injecting 10 µL for analysis

Table **2**. Different test concentrations for Gk-peptide and ribose stock solutions used in the preparation of the incubation mixture.

	Initial concentration	New concentration 1	New concentration 2
Gk-peptide	111.1 mg / 10 mL (final concentration 5 mg/mL)	55.55 mg / 10 mL (final concentration 2.5 mg/mL)	37.04 mg / 10mL (final concentration 1.7 mg/mL)
D-ribose	66.7 mg/ 10 mL (final concentration 3 mg/mL)	33.35 mg/ 10 mL (final concentration 1.5 mg/mL)	22.24 mg/ 10 mL (final concentration 1 mg/mL)

Table **3**. Summarized conditions used for method validation.

Standard preparation	• Prepare stock solutions of CML and d_2/ d_4-CML in Milli-Q water (1 mM) • Obtain standard dilutions between 75 and 5000 nM in a mixture of acetonitrile: isopropanol: water (45:45:10)
Sample preparation	• 75 µl incubated sample – **standard curve samples**: Gk-peptide (1.7 mg/mL), Rib (1 mg/mL) and standard solutions CML; **real samples**: Gk-peptide (1.7 mg/mL), Rib (1 mg/mL) and test compound in DMSO*; **maximum amount of CML formed**: Gk-peptide (1.7 mg/mL), Rib (1 mg/mL) and DMSO*; **QC samples**: Gk-peptide (1.7 mg/mL), Rib (1 mg/mL) and standard solutions CML from different stock solution. • addition of 15 µl internal standard d_2/ d_4-CML • hydrolysis of the sample with 1ml 6 M hydrochloric acid at 100 ºC for 1 h • drying the hydrolysate in a vacuum centrifuge • reconstitution in 200 µl Milli-Q water • cleaning 100 µl hydrolysate through P3 cartridges with addition of 300 µl acetonitrile • drying of the collected sample in a vacuum centrifuge • reconstitution in 200 µl isopropanol: acetonitrile: water (45:45:10) mixture *10% final concentration of DMSO
UPLC conditions	• Instrument: ACQUITY UPLC system coupled to a Xevo G2-XS QTof MS system, operating in negative ion mode • Gradient elution: **min** **% A** **% B** 0.00 15.0 85.0 6.00 15.0 85.0 6.10 20.9 79.1 10.00 32.6 67.4 12.00 44.4 55.6 12.10 15.0 85.0 18.00 15.0 85.0 • Injection volume: 10 µl • Mobile phase A: water and 0.1% formic acid • Mobile phase B: acetonitrile and 0.1% formic acid • Column: Acquity UPLC BEH Amide column (100 x 2.1 mm, 1.7 µm) • Column temperature: 40 ºC • Flow: 0.4 ml/min • Tune parameters: sample cone 40 V, capillary 1.01 kV; Temperature: source 120 °C, desolvation 550 °C; Gas flow: gas cone 50 L/h, desolvation gas 1000 L/h