Simultaneous quantification of commonly used counter ions in peptides and active pharmaceutical ingredients by mixed mode chromatography and evaporative light scattering detection

Simultaneous quantification of commonly used counter ions in peptides and active pharmaceutical ingredients by mixed mode chromatography and evaporative light scattering detection

Alessandro Streuli[1], Christopher R. Coxon[2], Christian Steuer[1]*

[1] ETH Zurich, Institute of Pharmaceutical Sciences, Vladimir-Prelog-Weg1-5/10, CH-8093 Zurich, Switzerland
[2] Institute of Chemical Sciences, School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh, UK, EH14 4AS

* corresponding author: christian.steuer@pharma.ethz.ch

Abstract:

In academia and industry, the analysis of counter ions in small molecules and synthetic peptides represents a great challenge. Due to the frequent use of salt forms and the application of a wider range of counter ions in pharmaceutically used substances, simple and generic methods for quantification are required. Especially, the analysis of trifluoracetic acid (TFA) in synthetic peptides is of high interest. Quantification of TFA is needed to assess the content and safety of synthetic peptides and for the interpretation of functional assay results, respectively. In here, a full quantitative mixed mode high performance liquid chromatography based method coupled to evaporative light scattering detection is presented. Finally, 14 positively and negatively charged counter ions were simultaneously quantified within 30 minutes. The method was validated in terms of specificity, accuracy, precision, limit of quantification, sample stability and carry over as proposed by the International Council of Harmonization. In order to prove the applicability of the procedure, small molecules reference substances and synthetic peptides were analyzed, respectively. The obtained results indicated a successful determination of counter ions in small molecules and differences to expected concentrations of prepared peptide solutions. Furthermore, an unexpectedly high content of sodium was observed for synthetic peptides.

Keywords:

Mixed-mode chromatography, evaporative light scattering detection, counter ion analysis, solid phase peptide synthesis, trifluoracetic acid, method development

Abbreviations:

API, active pharmaceutical ingredient; ACN, acetonitrile; CAD, charged aerosol detector; DAD, diode array detector; DIC, diisopropylcarbodiimide; DMF, N,N'-dimethylformamide; ELSD, evaporative light scattering detector; HPLC, high-performance liquid chromatography; LoQ, limit of quantification; MMC, mixed mode chromatography; QC, quality control; RP, reversed phase; Rs, resolution; RSD, relative standard deviation; SPPS, solid phase peptide synthesis TFA, trifluoroacetic acid, TIS, triisopropylsilane;

Introduction:

A well-known technique to improve physical stability, solubility and dissolution, absorption and polymorphism as well as the processing properties of active pharmaceutical ingredients (API) without altering chemical structures is the development of salts with appropriate properties. Beside the improvement of physical properties, salt formation shows a high impact on biological properties, such as bioavailability and toxicology. Based on the orange book database published in 2007 by the U.S. Food and Drug Administration, 47.3% of all APIs were marketed as basic or acid salt forms [1, 2]. In addition to the intentional salt formation to improve the physicochemical properties of the APIs, undesired salt formation may also occur during the synthesis, purification or processing of the APIs. An example of corresponding salt formation is the occurrence of trifluoracetic acid (TFA) salts during solid phase peptide synthesis (SPPS). As a final step, TFA is often used to cleave synthetic peptides from the solid support and to remove side chain protecting groups [3, 4]. Furthermore, TFA is implemented in the peptide purification step via high-performance liquid chromatography (HPLC) as an ion pairing reagent to improve the separation of polar compounds on reversed-phase (RP) columns [5-8]. As shown previously, TFA shows a tremendous impact on the molecular weight and solubility of peptides. Additionally, TFA can influence secondary structure elements of peptides and shows cell-toxicity, which results in interference in bioassays [6]. Therefore, the identity and amount of counter ion(s) in test solutions must be determined correctly to reduce sources of error in concentration-dependent protein and cell-based assays [5]. In general, the analysis of counter ions and toxic inorganic impurities from synthesis play an essential role in drug development, quality control and batch release. Thus, the analysis of ions plays a major role to ensure drug efficacy and safety compliance. Furthermore, appropriate analyses are required to detect and quantify potential contaminations with salts or heavy metals ensuring patient safety. Two different methods are preferably used as analytical techniques: 1) the detection of ions by means of ion chromatography with conductivity detection; 2) ultra high performance liquid chromatography with an appropriate detector for example a charged aerosol detector (CAD) [9, 10]. Besides the described use in pharmaceutical fields, the application of the method in soil and water analysis would also be feasible. Hereby, the focus is set on the detection of degradation products of herbicides, disinfectants, fertilizers and other as for example phosphate, bromide and chloride [11, 12].

The use of the evaporative light scattering detector (ELSD) offers the possibility of ion analysis, but also increases the detection window to analytes such as amino acids, excipients such as sugars and species not containing any charge [13, 14], and therefore, are not susceptible to CAD analysis. In general, ELSD can be considered as quasi-universal detection mode since it is able to detect any component less volatile than the mobile phase. In contrast to refractive index detection - commonly used for sugar analysis - ELSD based procedures are suitable for chromatographic gradient mode analysis [15, 16]. At the same time, ELSD offers a more affordable and a less complex alternative to mass spectrometric devices and inductive coupled plasma analysis. In regard to the mode of operation, the procedure consists of three coherent processes: nebulisation of the mobile phase, evaporation of the mobile phase and detection of the non-

volatile residual particles. In contrast to other spectroscopic detectors, the ELSD shows a non-linear response over the intended concentration range and simultaneously quantification limits below 0.1 µg are hard to reach.[17-19]

Herein, we present a new method based on mixed mode chromatography (MMC) method coupled to ELSD for the detection of anions and cations in synthetic peptides and active pharmaceutical ingredients. To the best of our knowledge, there is no validated procedure for the identification and quantification of counter ions, including TFA, using MMC and an ELSD. The presented method is universally applicable even in less equipped laboratories. Our results clearly demonstrate the need of an additional analytical tool for the purity control of synthetic peptides.

Experimental

Material

All chemicals used exceeded a purity of 98% or are of the highest available analytical grade, as indicated. Therefore, all reagents fulfil the American Chemical Society standard of a purity of 95%, otherwise indicated[20].

Ammonium acetate, ammonium formate, toluene-4-sulfonic acid, citrate, potassium disulfate, sodium chloride, sodium tartrate and TFA were obtained from Fluka AG and Thermo Fisher Scientific AG (Reinach, BL, Switzerland/ Sunnyvale, CA, USA), respectively. HPLC-grade acetonitrile (ACN), ammonium dihydrogen phosphate, calcium chloride, acetic acid, L-aspartate and sodium sulfate were from Merck (Darmstadt, HE, Germany). Ammonium bromide, L-lysine acetate and sodium phosphate were from Sigma Aldrich (St.Louis, MO, USA). Potassium bromide was obtained from ROTH (Karlsruhe, BW, Germany), formic acid was obtained from VWR chemicals (Radnor, PA, USA). The producer of sodium nitrate was unknown. Lysine acetylsalicylate, chloroquine phosphate, hydroxychloroquine sulfate and ketamine hydrochloride were used in CRS quality. Fmoc-L-amino acids, Rink Amide ProTide resin and Oxyma pure were purchased from CEM UK. Solvents, including peptide synthesis grade N,N'-dimethylformamide (DMF) and triisopropylsilane (TIPS) were purchased from Fisher Scientific, UK. Diisopropylcarbodiimide (DIC) and TFA intended for deprotection were sourced from Fluorochem UK. Pure water was obtained from an in-house water purification system from Labtec (Villmergen, AG, Switzerland).

Methods

Analytical procedure

Sample measurements were executed using HPLC separation with online UV-detection and online evaporative light scattering detection. Chromatographic separations were carried out on a VWR ELITE Lachrome Series LC modular system consisting of a L-2130 gradient pump, L-2200 autosampler, L-2350

column oven, L2455 diode array detector and a VWR Sedex Model 90 LT evaporative light scattering detector. OpenLab software (Version A. 04.08 - Agilent Technologies, Santa Clara, CA, USA) was used for system operation as well as peak interpretation. For chromatographic separation, an Acclaim Trinity P1 (3 µm × 100 mm, SN: 002536, Thermo Fischer Scientific, Sunnyvale, CA, USA) was used without guard columns. The final gradient is shown in table 1 and consisted of three different solvents. Mobile phase A, B and C consisted of ACN, 150 mM ammonium formate in water (pH 4.0) and of water, respectively. The flow rate and column oven temperature were set to 0.5 mL/min and 30 °C. The autosampler was cooled to 5 °C. Injection volume was 5 µL. All samples were dissolved and subsequent dilution was always performed in mobile phase B, respectively. Authentic samples were tested accordingly and analyzed in triplicate. The theoretical amount of TFA in synthesized peptides was calculated under the assumption, that every positively charged binding site is occupied. Scan settings of the diode array detector (DAD) were as follows: recording from 200 to 400 nm with individual scans at 220 and 254 nm with a scan rate of 400 ms, respectively. The ELSD temperature was set to 40 °C. The gas pressure of the nebulizer was set to 3.5 bar. Nitrogen was used as nebulizer gas. The gain of the detector was set to ten with a filter setting set to a medium level. The recording rate was set to 15 Hz.

Validation

Validation was performed according to international guidelines [21, 22]. Calibrators with eight different concentrations were prepared by combining and diluting the two stock solutions and aliquoted for eight validation days. Stock solution 1 consisted of 0.96 mg/mL ammonium bromide, 0.37 mg/mL ammonium phosphate, 0.96 mg/mL calcium chloride, 0.96 mg/mL L-lysine acetate, 0.6 mg/mL potassium disulfate, 1.2 mg/mL toluene-4-sulfonic acid and 15 mg/mL TFA. Stock solution 2 consisted of 0.6 mg/mL L-aspartate, 0.24 mg/mL L-asparagine, 0.8 mg/mL sodium tartrate and 2.1 mg/mL citrate. As solvent 150 mM ammonium formate buffer with pH of 4.0, was used. For the quality control (QC) samples, independent stock solutions were prepared. The stock solutions were diluted, QC Low, QC Med and QC High prepared, and aliquoted for eight validation days. All aliquots were stored at 5 °C in the refrigerator. Over a time span of two weeks, daily regression curves were estimated applying the generalized least square regression model. Linear, potential and polynomial models were considered as models [15, 16]. The determined model was retained for each analyte over the period of the validation. The calculated regression curves (n = 8) of the sampling day were used for the back-calculation of the concentration for each calibrator. The accuracy, including the bias of the method was reported at each concentration as percent deviation of the mean calculated concentration compared to the theoretical established concentration. The intra- and inter-day precision of the method was calculated as absolute and relative standard deviation according to Peters et al. [14]. The set acceptance values for the accuracy, intra- and inter-day precision were ≤ 10% relative standard deviation (RSD) and ≤15% RSD for the lowest QC level, respectively. Limit of detection (LoD) and limit of quantification (LoQ) were determined based on the standard deviation of the detector response and the slope of the calibration curve [22]. For the stability of the regression line, the measured values of the eight

evaluated QC sample sets was inserted into the calibration equation of the first day and the accuracy was calculated. The stability of the determined calibration curve was given as bias from the theoretical value. The stability of the analyte mix was assessed at -21 °C and 25 °C. Further, stability tests in the autosampler at 5 °C were performed. Samples were analysed after 0, 4, 8, 12, 24, 48, 72 and 168 hours. The stability was evaluated via the content respectively the corresponding peak area of the samples. To achieve better comparability, the values were further normalized to t = 0h. To test the stability at -21 °C, also called freeze and thaw stability, a QC Med aliquot was frozen at -21 °C, thawed and injected at the time points 0, 4, 8, 12, 24, 48, 72 and 168 hours (eight freeze-thaw cycles). The sample was removed from the freezer 30 minutes before the injection and was re-frozen 60 minutes after the injection. To test the stability at room temperature, QC Med samples were stored in brown glass vials on the bench and analysed seven times in duplicates at different time points 0, 24, 96, 132, 180, 276 and 348 hours. Carry over was tested by injecting blank samples after highest calibrator and QC High samples, respectively.

For data analysis, RStudio (Version 1.2.5033 - RStudio Inc., Boston, Massachusetts, USA), GraphPad Prism (Version 8.4.3 - GraphPad Software, San Diego, CA, USA) and Microsoft Excel (Version 1908 - Microsoft Corporation, Redmond, WA, USA) were used.

Peptide synthesis and purification

Peptides were synthesized via automated solid-phase peptide synthesis on Rink amide-ProTide resin (100–200 mesh, 0.56 mmol/g loading) using a CEM Liberty Blue peptide synthesiser. The peptide chain was elongated in consecutive cycles of standard Fmoc-deprotection and coupling. Piperidine in DMF (20% v/v) was used for deprotection and N, N′-diisopropylcarbodiimide, Oxyma pure and amino acids (0.1 M, solutions in DMF) for coupling with microwave heating (5 min, 90 °C). The resin was cleaved with a mixture of TFA:TIPS:H_2O (90:5:5) for 3 h. After the allocated time, the resulting solution was filtered to remove resin beads and evaporated before being resuspended and washed with cold diethyl ether following centrifugation. The crude peptides were purified using a high-performance liquid chromatography system (RP-HPLC) (Shimadzu, Kyoto, Japan). Separation was performed on a NucleodurTMC18 HTec column (150 × 21 mm, 5 µm, 110 Å, Macherey-Nagel, Düren, Germany) using a linear gradient from 5-40% acetonitrile in water containing 0.1% TFA (Sigma-Aldrich, St. Louis, USA) within 20 min. The flow rate was set to 24.5 mL/min and detection was performed at a wavelength of 214 nm. The purified products were flash frozen (liquid N_2) and lyophilised.

Results and discussion

Method development

The development of a mixed mode HPLC-ELSD method for the simultaneous separation of 14 ionic species, including TFA and three amino acids, is described. The final method successfully separated L-asparagine, L-aspartate, bromide, calcium, chloride, citrate, L-lysine, tartrate, phosphate, potassium,

sodium, sulphate, toluene-4-sulfonic acid and TFA. A corresponding chromatogram of an analyzed QC Med sample is shown in figure 1.

At low and medium concentrations, up to calibrator 4, a baseline separation with a resolution (Rs) ≥ 1.5 was obtained for all species except chloride and bromide. At higher concentrations, the resolution of the TFA and L-aspartate peaks decreased to a value of 1.30 – 1.35. This is partly due to an increasing tailing of the L-aspartate peak. The other species remained baseline separated even at high concentrations. The separation of chloride and bromide was considered acceptable even without baseline separation, as the peaks were easily distinguishable. Starting conditions of ACN showed a tremendous effect on the retention behavior of TFA. With increasing water:ACN ratio at starting conditions, TFA peak vanished in the chromatogram. Retention behavior of all other target analytes was not affected significantly. Several commonly used buffer systems were tested in detail. Buffer constitution and concentrations are of high importance for chromatographic mixed mode separation and signal intensity of the ELSD, respectively. Using ammonium acetate buffer, no separation and detection of calcium, citrate, phosphate, sulfate tartrate and toluene-4-sulfonic acid was achieved. Variations in the concentration of the buffer led to changes in the retention times of the detectable species (data not shown). In general, doubly-charged species were not detected at all. The use of buffer at a concentration of 200 mM or higher was not possible as this resulted in significant baseline fluctuation as a result of incomplete buffer evaporation. On the contrary, the use of ammonium formate buffer, at a concentration of 100 mM and 150 mM, enabled the detection of single and double charged species. Below the concentration of 150 mM ammonium formate, a detection of calcium and sulphate was not feasible. These results are consistent with reports published by Zhang et al. [23]. It can be hypothesized that ammonium formate acts as an ion exchanger in MMC. The effect of column oven temperature (30 °C, 35 °C, 40 °C) on the separation varied between ionic species. While phosphate and TFA eluted earlier with increasing temperature, retention time of L-aspartate remained constant. Accordingly, the R_s of TFA and L-aspartate decreased (R_s: 1.37; R_s: 0.93, R_s: 0.0). Similar trends were observed for the chloride and bromide analytes. Temperature showed a stronger effect on the elution of bromide than on chloride. This resulted also in lower resolution of both analytes. The reequilibration time showed a significant effect on the retention behavior of all analytes and the quality of separation. In particular, the retention times of L-aspartate, bromide, chloride, L-lysine, potassium, sodium, TFA and toluene-4-sulfonic acid were enormously influenced by the reequilibration time. Figure 2A shows the dependence of the retention time and reequilibration time for all target analytes. In general, the elution time of the aforementioned analytes increased proportionally with the reequilibration time. For the remaining analytes only small to no changes were observed. The separation of the analytes TFA & L-aspartate and chloride & bromide worsened with increasing reequilibration time. In contrast, the resolution between bromide & L-lysine increased with higher reequilibration times. Reequilibration times of 10 and 15 minutes delivered satisfying results regarding the quality of separation. In view of the dwell volume of the system (1.8 mL) and to avoid potential carry overs in the further analysis the reequilibration time was set to 15

minutes. Column oven temperature showed no influence on the other analytes or the peak quality and was, therefore, it was kept at 30 °C.

The signal intensity of analytes is dependent on drift tube temperature. Elevated temperature showed a decreasing effect on TFA signal intensity whereas other analytes were not affected. Detection of TFA was only possible below a temperature of 45 °C. If the temperature was further reduced to 40 °C, the peak area of TFA increased strongly. As a consequence, the limit of detection and the LoQ of the substance was lowered. A comparison of the TFA peak area for an injection of a concentration of 1.0 mg/mL at 35 °C, 37.5 °C, 40 °C, 42.5 °C and 45 °C is shown in figure 2B. However, baseline stability was not given at lower temperature (data not shown). It is obvious, that the temperature effect seems to be more relevant for analytes with low boiling points Therefore, the operating temperature of the ELSD was set to 40 °C for all further experiments.

Method validation

Selectivity

Blank samples were analyzed for chromatographic interferences. A representive blank run is shown as overlay in figure 1. No co-eluting or interfering peaks were observed for TFA nor any other target analyte.

Calibration model

A chromatographic mixed mode procedure was developed showing high accuracy and low imprecision. The regression lines were determined, using non-weighted models. A calibration curve using eight different concentrations were analyzed to determine the calibration model. For all analytes except calcium, citrate and TFA, the power model showed better results. For citrate and TFA the polynomial second order model was applied. With regard to calcium, the linear model showed the best fit. A comparison of different calibration models for TFA is shown in figure 3. These results are consistent with the literature in which the response of the ELSD is mainly described as exponential. The calculated R^2 were adequate for all analytes and indicated a strong correlation (R^2 >0.99). The calibration model, calibration range, the range of R^2 and median of R^2 for all analytes can be found in the supporting information (S1). The back calculated concentrations of all analytes except TFA were in a range of ± 15% of the theoretical value. Only calibrant 1 showed a deviation of 23%. All other calibration levels were determined within ± 6% of the theoretical value (table 2). The calibration for TFA was stable for at least 14 days. Although deviation were higher at QC Med and QC High level, deviation was always lower than 20%. Also, calibration curves for all other analytes were found to be stable for the whole validation time. (S2, supporting information).

Accuracy and precision

A chromatographic mixed mode procedure was developed showing high accuracy and low imprecision. The regression lines were determined, using aforementioned calibration models. Method validation data for TFA are summarized in table 2. The accuracy was determined as bias from the theoretical concentration. The intra- and inter-day precision is given as RSD_R and RSD_T , respectively. In terms of accuracy, the determined bias was smaller than 5% for QC High and QC Med. Additionally, bias was smaller than 8% at QC Low level. Precision data for all analytes fulfilled the pre-set criteria. Only for TFA, the inter-day precision of the QC Low fell outside of the criteria. In comparison, the intra-day precision was with 1.1% in line with the criteria and very satisfying. In authentic samples, TFA content is rather in the range of QC Med level. Accuracy and intra-day precision for QC Med is -3.4% and 2.1%, respectively. Therefore, authentic samples were quantified accordingly. Validation data for all other target analytes, can be found in the supporting information (S3).

Limit of quantification

For TFA, LoD and LoQ was determined at 119.1 µg/mL and 361.0 µg/mL, respectively (table 2). LoD and LoQ of all other analytes were shown in table S3 (supporting information).Only for phosphate, the lowest calibrator was slightly below the value determined for the LoQ. However, quantification of the aforementioned target analyte at low level was acceptable and fulfilled pre-set accuracy and precision thresholds.

Stability

The calibrators and QC samples were found stable up to eight freeze-thaw (FT) cycles at -20 °C, up to seven days in the autosampler (5 °C) and up to four days at bench conditions (25 °C). Data of stability assessment can be found in the supporting information (S4).

Carry over

For carry over testing, blank samples were injected after highest calibrators and QC samples. Minor peaks corresponding to sodium, calcium and the buffer were detected. However, similar observations were made in blank samples submitted prior to any measurements. It is assumed, that these analytes originate from the solvent. Subsequently, blanks are subtracted from all prepared samples.

Authentic sample analysis

The counter ion content of four APIs was successfully assessed with the developed and validated method. For all samples, the bias of the found concentration compared to the expected concentration was in a range of -1.4% and 3.7%. Corresponding LC-ELSD chromatograms are given in the supporting information (S5) Interestingly, at the chosen settings the organic main compounds are not detectable rendering the method

specific for selected target analytes. Further, the TFA content of five synthetic peptides was assessed. The sequence of selected peptides can be found in table 3. Taking possible ionizable residues of peptides into account, maximal TFA content of each peptide was calculated accordingly. The deviation between theoretical and actual concentration was between -9.6% and 10.6%. Besides the expected peaks of the peptide and TFA, unexpectedly high amounts of sodium were found in every peptide sample and could be derived from the aqueous solvent used in the HPLC purification step. Subsequently, sodium is enriched by lyophylization. An exemplary chromatogram for a peptide 1 is shown in figure 4. The theoretical concentration, actual concentration as well as the bias for each authentic sample is depicted in table 3.

Conclusion

A robust and accurate mixed mode HPLC-ELSD method for the detection and quantification of 14 commonly used common pharmaceutical counter ions was successfully developed and validated. The developed method was applied to several small molecule APIs and to six synthetic peptides. Counter ions in APIs were determined with an accuracy of 97% or higher. Additionally, the method allowed the determination of TFA ions in peptide solutions and discovered the occurrence of high sodium levels in synthetic peptides after purification by preparative HPLC.

Acknowledgements The authors are grateful to Prof. Gisbert Schneider for providing access to the peptide purification system in the Schneider Lab at ETH Zurich

Funding CS was financially supported by the Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung (IZSEZO_1686615).

Conflict of interest The authors declare that they have no conflict of interest.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

References

1.U.S. Food and Drug Administration (FDA). Approved Drug Products with Therapeutic Equivalence Evaluations (Orange Book). Silver Spring, Maryland2020.
2.Paulekuhn GS, Dressman JB, Saal C. Trends in active pharmaceutical ingredient salt selection based on analysis of the Orange Book database. J Med Chem. 2007;50(26):6665-72.
3.Verhoork SJM, Jennings CE, Rozatian N, Reeks J, Meng J, Corlett EK, et al. Tuning the Binding Affinity and Selectivity of Perfluoroaryl-Stapled Peptides by Cysteine-Editing. Chemistry. 2019;25(1):177-82.
4.Neuhaus CS, Gabernet G, Steuer C, Root K, Hiss JA, Zenobi R, et al. Simulated Molecular Evolution for Anticancer Peptide Design. Angew Chem Int Ed Engl. 2019;58(6):1674-8.
5.Allenspach MD, Fuchs JA, Doriot N, Hiss JA, Schneider G, Steuer C. Quantification of hydrolyzed peptides and proteins by amino acid fluorescence. J Pept Sci. 2018;24(8-9):e3113.

6.Roux S, Zekri E, Rousseau B, Paternostre M, Cintrat JC, Fay N. Elimination and exchange of trifluoroacetate counter-ion from cationic peptides: a critical evaluation of different approaches. J Pept Sci. 2008;14(3):354-9.
7.Little MJ, Aubry N, Beaudoin ME, Goudreau N, LaPlante SR. Quantifying trifluoroacetic acid as a counterion in drug discovery by 19F NMR and capillary electrophoresis. J Pharm Biomed Anal. 2007;43(4):1324-30.
8.Kaiser E, Rohrer J. Determination of residual trifluoroacetate in protein purification buffers and peptide preparations by ion chromatography. J Chromatogr A. 2004;1039(1-2):113-7.
9.Ismail OH, Antonelli M, Ciogli A, De Martino M, Catani M, Villani C, et al. Direct analysis of chiral active pharmaceutical ingredients and their counterions by ultra high performance liquid chromatography with macrocyclic glycopeptide-based chiral stationary phases. J Chromatogr A. 2018;1576:42-50.
10.Hadda PR, Jackson PE, Shaw MJ. Developments in suppressor technology for inorganic ion analysis by ion chromatography using conductivity detection. J Chromatogr A. 2003;1000(1-2):725-42.
11.Binghui Z, Zhixiong Z, Jing Y. Ion chromatographic determination of trace iodate, chlorite, chlorate, bromide, bromate and nitrite in drinking water using suppressed conductivity detection and visible detection. J Chromatogr A. 2006;1118(1):106-10.
12.Guo ZX, Cai Q, Yang Z. Determination of glyphosate and phosphate in water by ion chromatography--inductively coupled plasma mass spectrometry detection. J Chromatogr A. 2005;1100(2):160-7.
13.Zhang T, Nguyen D, Franco P. Use of evaporative light scattering detector in the detection and quantification of enantiomeric mixtures by HPLC. J Sep Sci. 2006;29(10):1517-24.
14.Stolyhwo A, Colin H, Guiochon G. Use of light scattering as a detector principle in liquid chromatography. J Chromatogr A. 1983;265:1-18.
15.Montesano D, Cossignani L, Giua L, Urbani E, Simonetti MS, Blasi F. A Simple HPLC-ELSD Method for Sugar Analysis in Goji Berry. Journal of Chemistry. 2016;2016:6271808.
16.Clement A, Yong D, Brechet C. Simultaneous Identification of Sugars by HPLC Using Evaporative Light Scattering Detection (ELSD) and Refractive Index Detection (RI). Application to Plant Tissues. J Liq Chromatogr. 1992;15(5):805-17.
17.Megoulas NC, Koupparis MA. Twenty Years of Evaporative Light Scattering Detection. Crit Rev Anal Chem. 2005;35(4):301-16.
18.Deschamps FS, Baillet A, Chaminade P. Mechanism of response enhancement in evaporative light scattering detection with the addition of triethylamine and formic acid. Analyst. 2002;127(1):35-41.
19.Mourey TH, Oppenheimer LE. Principles of operation of an evaporative light-scattering detector for liquid chromatography. Anal Chem. 1984;56(13):2427-34.
20.Tom Tyner, Francis J. Overview. Tyner T, Francis J, editors: American Chemical Society; 2017.
21.Peters FT, Drummer OH, Musshoff F. Validation of new methods. Forensic Sci Int. 2007;165(2-3):216-24.
22.(ICH) ICoH. Validation of Analytical Procedures: Text and Methodology Q2 (R1). London International Council on Harmonisation; 2005.
23.Zhang K, Dai L, Chetwyn NP. Simultaneous determination of positive and negative pharmaceutical counterions using mixed-mode chromatography coupled with charged aerosol detector. J Chromatogr A. 2010;1217(37):5776-84.

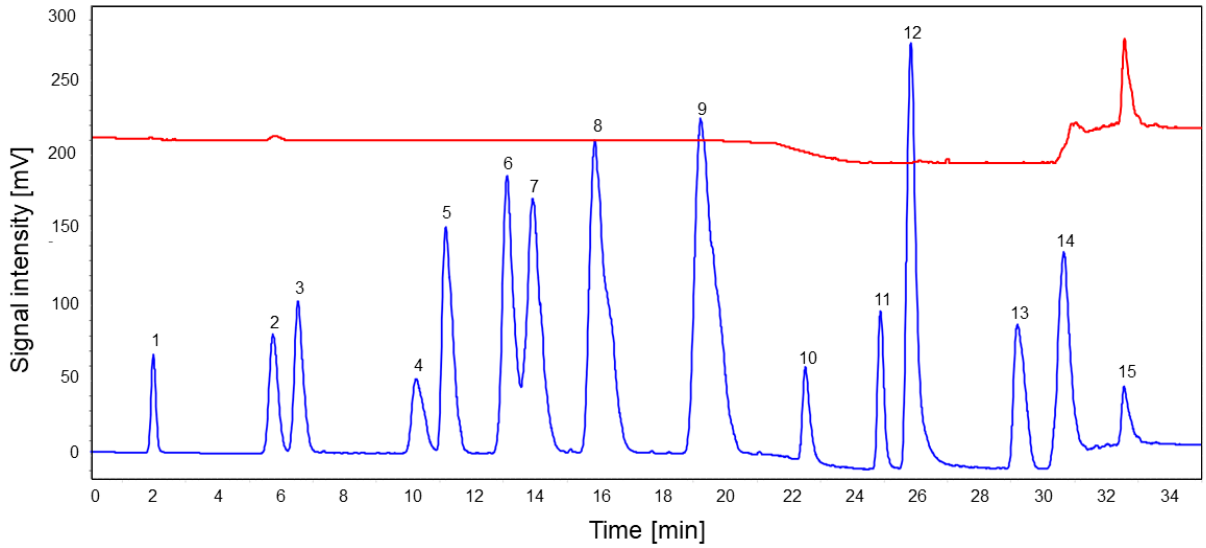

Figure 1: Example chromatogram of a QC Med sample. The peaks correspond as follows: 1: L-asparagine, 2: sodium, 3: potassium, 4: L-aspartate, 5: TFA, 6: chloride, 7: bromide, 8: L-lysine, 9: toluene-4-sulfonic acid, 10: phosphate, 11: tartrate, 12: citrate, 13: sulphate, 14: calcium, 15: buffer peak. Additionally, overlay with blank run is shown in red.

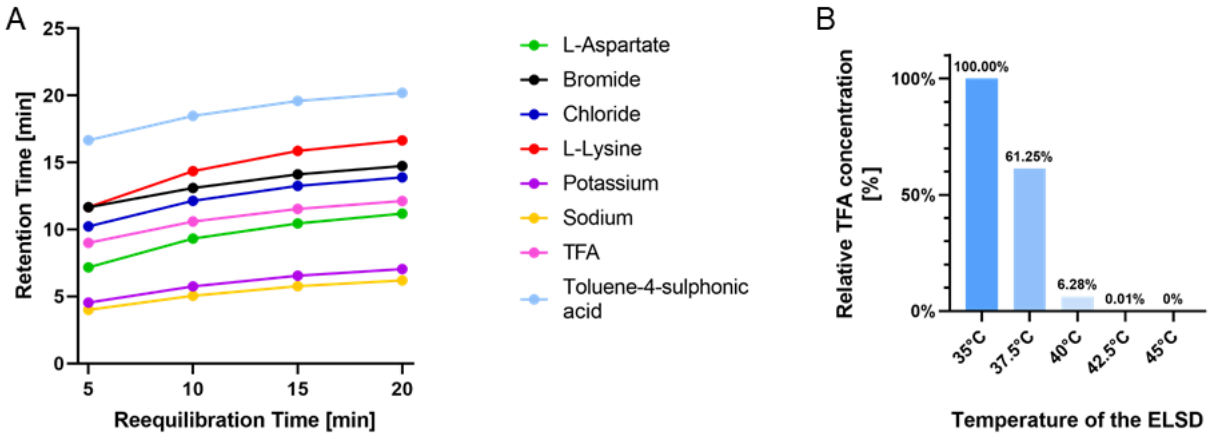

Figure 2: (A) Plot of the difference in retention time of the analytes L-aspartate, bromide, chloride, L-lysine, potassium, sodium, TFA and toluene-4-sulphonic acid against the reequilibration time. Measurements were performed in duplicate (n=2) with the reequilibration time of 5, 10, 15 and 20 minutes; (B) Comparison of the peak areas of TFA analyses at different ELSD temperatures. Measurements were performed in triplicate (n=3). Values are normalized to the peak area at 35 °C.

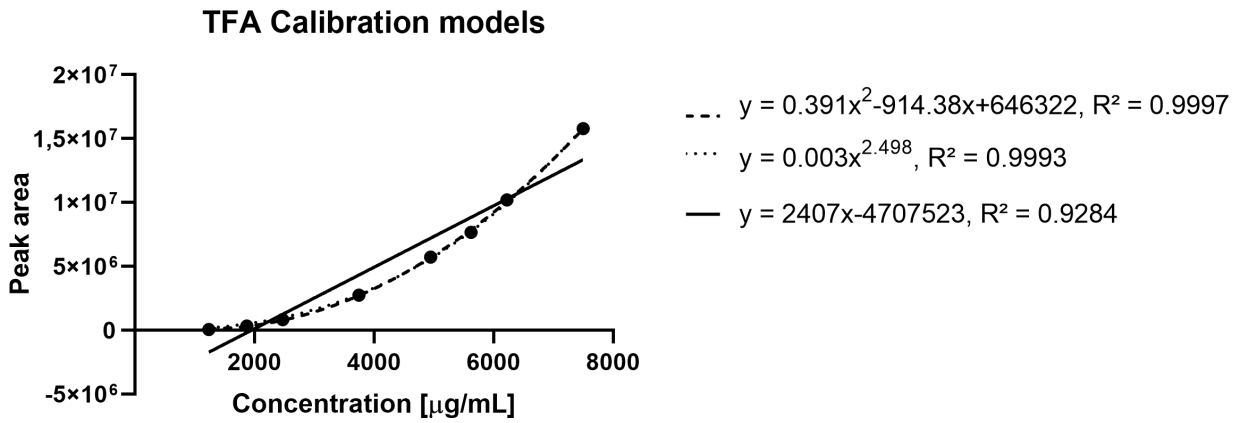

Figure 3: Plot of different calibration models evaluated for TFA

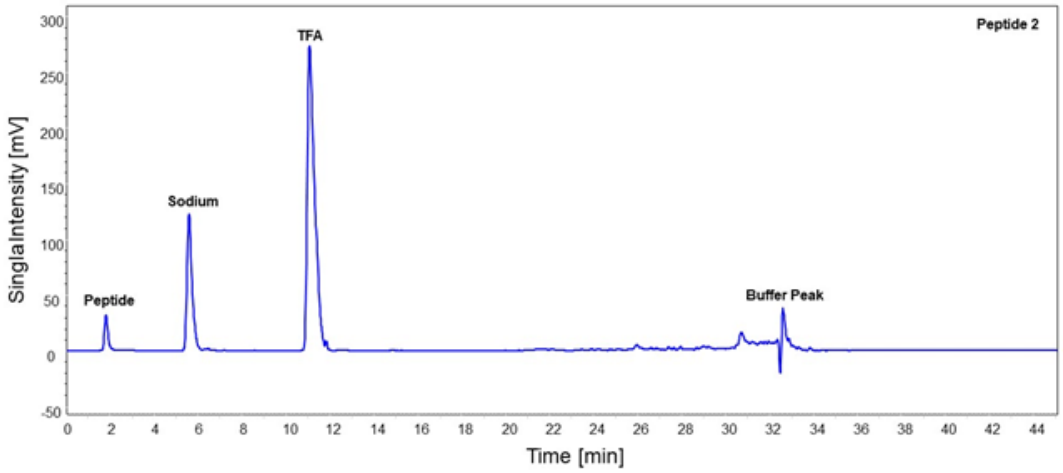

Figure 4: HPLC-ELSD chromatogram of Peptide 1

Table 1: Final gradient for the separation of target analytes.

Time [%]	Solvent A [%]	Solvent B [%]	Solvent C [%]	Flow [mL/min]
0.0	58.0	4.0	38.0	0.50
10.0	57.0	8.0	35.0	0.50
15.0	57.0	8.0	35.0	0.50
17.5	5.0	90.0	5.0	0.50
30.0	5.0	90.0	5.0	0.50
30.5	58.0	4.0	38.0	0.50
30.6	58.0	4.0	38.0	1.00
45.0	58.0	4.0	38.0	1.00
45.1	58.0	4.0	38.0	0.50

Table 2: Method validation data for TFA. Limits, Calibration model and back-calculated values for all calibration and QC levels.

LoD	LoQ	Calibration model		Calibration level [µg/mL]								QC level [µg/mL]		
[µg/mL]	[µg/mL]	Second Order Polynomial		1	2	3	4	5	6	7	8	Low	Med	High
119.1	361.0	$y = 0.3906x^2 - 914.38x + 646322$ $R^2 = 0.9997$	Theoretical values	1237.5	1875	2475	3750	4950	5625	6225	7500	1560.0	4350.0	6862.5
			Found values	1474	1956	2475	3673	4878	5491	6157	7433	1676.2	4203.2	6548.4
			Bias [%]	22.9	5.8	0.8	-1.8	-1.4	-2.3	-1.0	-0.8	7.4	-3.4	-4.6
			RSD_R [%]									1.1	2.1	2.1
			RSD_T [%]									*23.3*	9.3	4.5

Table 3: Theoretical (calculated) and actual (measured) concentration of counter ions in authentic samples

Sample	Primary sequence	Sum formula	calculated [µg/mL]	measured [µg/mL]	Bias [%]
Chloroquine Phosphate	---	$C_{18}H_{26}ClN_3$, 2 H_3PO_4	147.8	153.3	3.7
Hydroxychloroquine Sulfate	---	$C_{18}H_{26}ClN_3O$, H_2SO_4	126.1	129.7	2.9
Ketamine Hydrochloride	---	$C_{13}H_{16}ClNO$, HCl	66.5	67.3	1.2
Lysine Acetylsalicylate	---	$C_9H_8O_4$, $C_6H_{14}N_2O_2$	211.0	208.1	-1.4
Peptide 1	H_2N-VTHRLAGALSR-$CONH_2$	$C_{50}H_{90}N_{20}O_{13}$	4821.0	5331.7	10.6
Peptide 2	H_2N-VTHRAAGLLSR-$CONH_2$	$C_{50}H_{90}N_{20}O_{13}$	4209.2	3804.7	-9.6
Peptide 3	H_2N-ATHRLAGLLSR-$CONH_2$	$C_{51}H_{92}N_{20}O_{13}$	4868.5	5220.5	7.2
Peptide 4	H_2N-VTHRLAGLLSRSG-$CONH_2$	$C_{58}H_{104}N_{22}O_{16}$	4127.9	4147.5	0.5
Peptide 5	Ac-NH-VTHRLAGALSR-$CONH_2$	$C_{52}H_{92}N_{20}O_{14}$	3624.7	3939.8	8.7