# Impurity Profiling of Challenging Active Pharmaceutical Ingredients without Chromophore

## DISSERTATION

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naturwissenschaftlichen Doktorgrades

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Adoramos a perfeição, porque não a podemos ter;
repugna-la-íamos, se a tivéssemos. O perfeito é o desumano,
porque o humano é imperfeito.

Fernando Pessoa (1888-1935)

Für meine Familie!

Die vorliegende Arbeit wurde auf Anregung und unter Anleitung von

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# A. Introduction

# 1. Separation techniques in impurity profiling

The three most important separation techniques used in the European Pharmacopoeia for the assessment of related substances are high performance liquid chromatography, gas chromatography and capillary electrophoresis. The techniques used in this work, HPLC and CE, are introduced in the following part.

## 1.1. High performance liquid chromatography

Chromatography is a process of separation using adsorption and distribution processes between a stationary and a mobile phase moving along the stationary phase. In HPLC, the mobile phase is pumped with moderately high pressure (typically 50 – 350 bar) through a steel column packed with small uniform beads or irregular formed particles of stationary phase. The analytes are detected after the column using a suitable detection device. A chromatogram based on the analog data provided by the detector is yielded by means of an integrator or an appropriate digital interface (see Fig. 1) [1, 2].

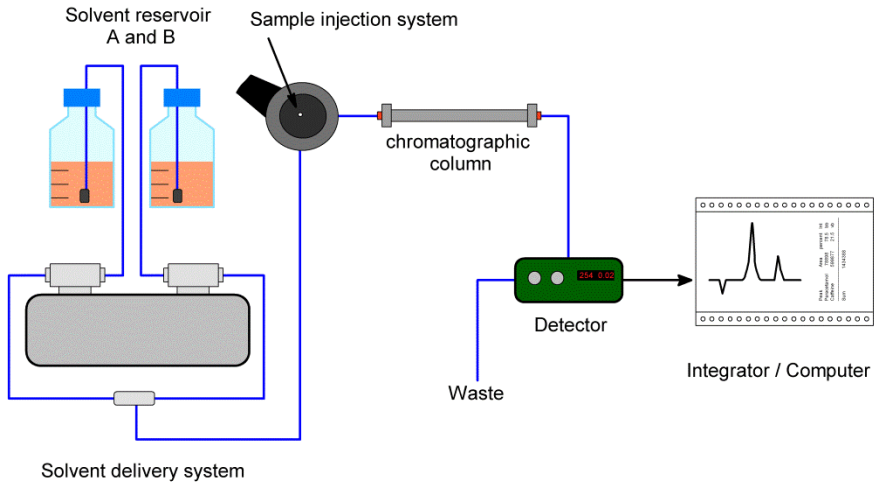



**Fig. 1.** Schematic layout of a HPLC system.

In liquid chromatography several separation modes can be distinguished: normal phase, reversed phase, size exclusion, ion exchange, ion-pair, hydrophilic interaction liquid chromatography, and chromatography using chiral modifications [3]. A stationary phase consists of a polymeric support (e.g. silica gel, poly acrylate, etc.) carrying a chemical modifications to introduce the desired characteristics. Popular modifications are listed in Table 1.

**Table 1**
Types of stationary phases applied in HPLC analytics [1, 2, 4].

| Chemistry | separation mode | main application |
|---|---|---|
| C-1 | reversed phase | large biomolecules |
| C-4 | reversed phase | |
| C-8 | reversed phase | very lipophilic SM, peptides |
| C-18 | reversed phase | lipophilic SM, small peptides |
| Phenyl | reversed phase | alternative to C-18 with modified selectivity |
| PFP | reversed phase | |
| CN | reversed phase or NP | hydrophilic SM |
| Diol | HILIC | very hydrophilic SM |
| Amide | HILIC | very hydrophilic SM |
| plain silica gel | HILIC or NP | very hydrophilic SM |
| $NH_2$ | HILIC or WAX | very hydrophilic SM, carbohydrates |
| sulfonate | SCX | cationic SM, inorganic cations |
| quaternary amine | SAX | anionic SM, inorganic anions |
| methacrylate gel | size exclusion | large biomolecules, polymers |

HILIC, hydrophilic interaction liquid chromatography; NP, normal phase; SAX, strong anion exchange; SCX, strong cation exchange; SM, small molecules

Today, most of the separations described in the Ph. Eur. [4] for the determination of related substances are based on reversed phase chromatography. The stationary phase is chemically modified silica gel with a more or less hydrophobic group at the surface of the particles. Mobile phases are usually a mixture of water or aqueous buffer and acetonitrile, tetrahydrofuran or methanol as organic modifier. The selectivity of the separation can be adjusted by the type of stationary phase, column temperature, the choice and proportion of organic modifier, buffer type, buffer concentration and pH [2].

The composition of the mobile phase has to consider both the separation and the applied detection principle. For instance the UV cutoff of a solvent or buffer salt has to be considered in UV detection and the volatility of the mobile phase is an issue when using LC-MS or other techniques that involve the evaporation of the mobile phase (e.g. CAD, ELSD, NQAD). Common mobile phase additives are summarized in Table 2 and Table 3. Selected physical properties of organic modifier common in HPLC analysis are displayed in Table 4. The most common detection technique applied in the Ph. Eur. is UV detection because it is straightforward and applicable for most of the monographed substances.

**Table 2**
Selection of buffer salts used in HPLC and their properties [2].

| Buffer | usable range | volatile | UV cutoff[a] |
|---|---|---|---|
| dihydrogen phosphate | 1.1 – 3.1 | no | < 200nm |
| hydrogen phosphate | 6.2 – 8.2 | no | < 200 nm |
| phosphate | 11.3 – 13.3 | no | < 200 nm |
| dihydrogen citrate | 2.1 – 4.1 | no | 230 nm (10 mM) |
| hydrogen citrate | 3.7 – 5.7 | no | 230 nm (10 mM) |
| citrate | 5.4 – 7.4 | no | 230 nm (10 mM) |
| acetate | 3.8 – 5.8 | yes[b] | 210 nm (10 mM) |
| formate | 2.7 – 4.7 | yes[b] | 210 nm (10 mM) |
| bicarbonate | 5.1 – 7.1 | yes[b] | < 200 nm |
| borate | 8.3 – 10.3 | yes[b] | - |

[a] Wavelength at which aqueous solution absorbs > 0.5 AU;
[b] only volatile when used as ammonium salt

**Table 3**
Selection of acids and bases used in HPLC and their properties [2].

| $pK_a$ (25 °C) | compound | volatile | UV cutoff[a] |
|---|---|---|---|
| 0.3 | trifluoroacetic acid | yes | 210 nm (0.1%) |
| 2.15, 7.20 and 12.33 | phosphoric acid | no | < 200 nm |
| 3.13, 4.76 and 6.40 | citric acid | no | 230 nm (10 mM) |
| 3.75 | formic acid | yes | 210 nm (0.1%) |
| 4.76 | acetic acid | yes | 210 nm (0.1%) |
| 4.76 | citric acid | no | 230 nm (10 mM) |
| 4.86 | propionic acid | yes | 210 nm (0.1%) |
| 6.10 | carbonic acid | yes | < 200 nm |
| 9.23 | boric acid | yes | - |
| 9.25 | ammonia | yes | 200 nm (10 mM) |
| 10.72 | triethylamine | yes | 200 nm (10 mM) |
| 11.27 | pyrrolidine | yes | - |

[a] Wavelength at which aqueous solution absorbs > 0.5 AU

**Table 4**
Selection of mobile phase components used in HPLC and their properties [2].

| solvent | separation mode | polarity index | dielectric constant | UV cutoff[a] |
|---|---|---|---|---|
| Water | RP | 10.2 | 80 | - |
| Acetonitrile | RP or HILIC | 5.8 | 37.5 | < 190 nm |
| Methanol | RP or HILIC | 5.1 | 32.7 | 205 nm |
| THF | RP or NP | 4.0 | 7.6 | 212 nm[b] |
| Acetone | RP or NP | 5.1 | 20.7 | 330 nm |
| Ethanol | RP or NP | 4.3 | 24.6 | 210 nm |
| Isopropanol | RP or NP | 3.9 | 19.9 | 205 nm |
| Hexane | NP | 0.1 | 1.9 | 195 nm |
| Methylene chloride | NP | 3.1 | 8.9 | 233 nm |
| Ethyl acetate | NP | 4.4 | 6.0 | 256 nm |

[a] wavelength at which solvent absorbs 1.0 AU;
[b] without stabilizer, with stabilizer (e.g. BHT) only usable with RID

## 1.2. Capillary electrophoresis

In capillary electrophoresis (CE) analytes are separated inside a fused silica capillary filled with background electrolyte (BGE) under influence of a high electric field due to their different migration velocities and eventually detected by an appropriate mean of detection (see Fig. 2). The migration velocity of an ion in an electric field is the product of electric field strength $E$ and electrophoretic mobility $\mu_e$. The mobility on the other hand is proportional to the charge of the ion and inversely proportional to the decelerating friction produced by the moving ion in solution. The friction again depends on the size and the spatial arrangement of the ion. Two compounds that differ either in charge or produced friction can be separated by capillary electrophoresis [1].

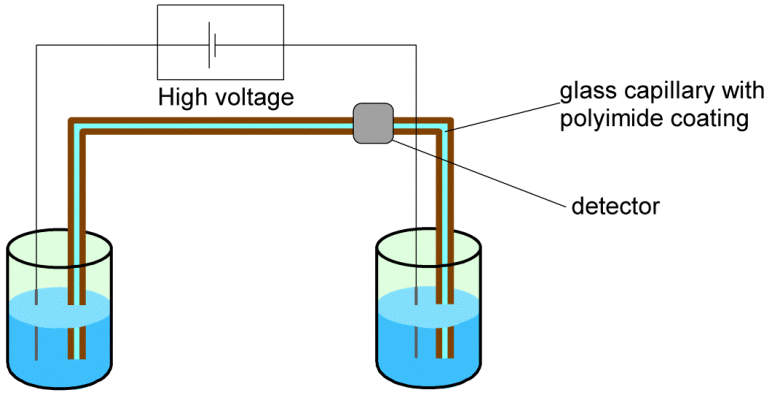



**Fig. 2.** Schematic layout of a capillary electrophoretic apparatus [1].

A special feature of capillary electrophoresis is the electroosmotic flow (EOF). If the pH of the BGE is > 3 the silanol groups on the surface of the capillary are partially deprotonated and attract positively charged counter-ions of the electrolyte thus forming a double layer of immobile negative and mobile positive charge. The positive ions move towards the cathode dragging solvent molecules (due to their hydration layer) with them. The whole BGE starts to flow towards the cathode in a characteristic flat flow profile. The EOF is the reason why positively and negatively charged ions eventually migrate towards one side of the capillary where they are detected [1]. They are separated due to their net migration speed which is the sum of electrophoretic and electroosmotic velocity. Sample injection in CE is carried out differently compared to HPLC, where a well-defined volume of sample is injected using special valves. The sample can be transferred to the capillary by hydrostatic, hydrodynamic and electrokinetic injection [1]. No matter which kind of injection is used, the amount of sample loaded to the capillary can only be estimated and fluctuates from injection to injection. To compensate for these fluctuations, the use of an internal standard is obligatory.

The main advantages of CE are the extremely high separation efficiency of about 20- to 100-times the usual plate count obtained in HPLC, easy separation of ionic species, comparatively cheap and straightforward optical resolution of small molecules using cyclodextrins or other modifiers in the BGE. A very small sample volume needed (usually only a few nL) and CE is a versatile tool due to the different applications such as MEKC and MEEKC enabling the separation of neutral species [1].

## 1.3. Amino acid analysis

Amino acid analysis (AAA) was intended to characterize peptide hydrolysates and amino acid mixtures by their content of individual amino acids [4, 5]. The principle is based on HPLC separation of amino acids using cation-exchange stationary phases with an appropriate mobile phase (sodium- or lithium based) [6, 7] (see Fig. 3). The separated amino acids are derivatized (often with OPA or ninhydrin) after the column and detected by UV- or fluorescence detection [8-11]. The main problems with amino acid analyzers are their high specificity for a class of derivatizable compounds and the need for a comparatively expensive dedicated instrument.

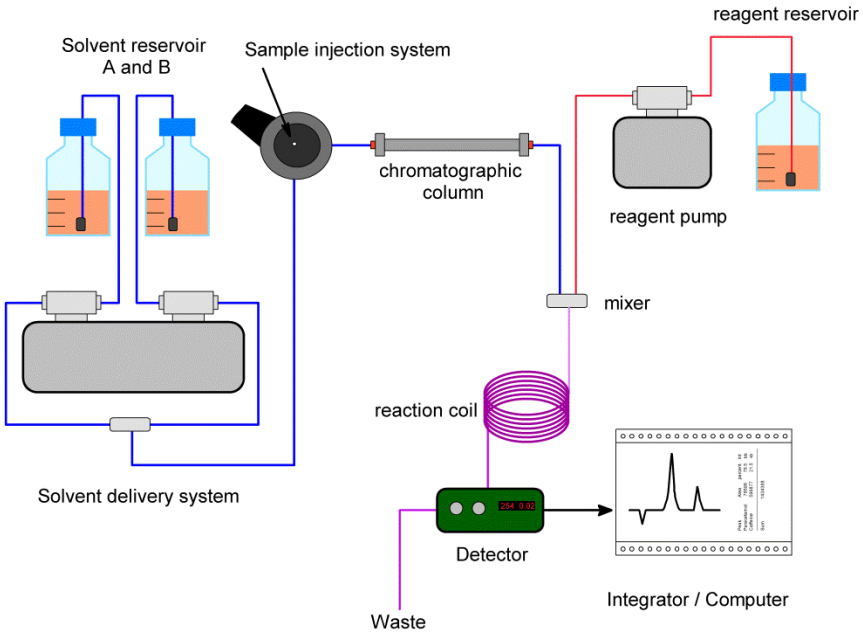



**Fig. 3.** Schematic layout of an AAA instrument [11].

# 2. Detectors used in HPLC and CE

With a few exceptions HPLC and CE use the same detection principles. The main difference for most detectors is the location of detection: In CE the detection usually takes place inside of the system (capillary) whereas in HPLC analytics the column eluate is analyzed outside. This is very important when using concentration sensitive detectors because the peak area is strongly dependent on the dwell time in the detector. To compensate for different migration speed and therefore detector dwell time, the corrected peak area (peak area divided by migration time) is usually used in CE.

## 2.1. UV/Vis-Detector

The UV/Vis detector is the most popular detector in HPLC and CE analytics due to the straightforwardness and the low price.

The UV light is usually produced by deuterium lamps which yields a continuous spectrum of light whereas special applications utilize metal lamps in order to use a single wavelength from the line spectrum of the metal (e.g. mercury or zinc). Visible light is usually produced by a tungsten halide lamp. Light of the desired wavelength (selected by a combination of a prism or more frequently a grating and a slit) is diverted through the detector cell, in CE the capillary (polyimide coating removed) and the light intensity on the other side is measured. If an analyte with an adequate chromophore is inside the detector cell, some of the light is absorbed and the intensity drops on the sample diode thus producing a signal (see Fig. 4). The relationship between analyte concentration and absorbed light is described by the Beer-Lambert-Bouguer law [1, 2]:

$$E_\lambda = log_{10}\left(\frac{I_{inc}}{I_{trans}}\right) = \varepsilon_\lambda \cdot c \cdot l \qquad \textbf{(Eq. 1)}$$

The extinction $E_\lambda$ (or absorbance) of a substance in solution is the logarithm to base 10 of the incident light intensity ($I_{inc}$) divided by the transmitted light intensity ($I_{trans}$). It is proportional to the absorbing species concentration and the path length ($l$) crossed by the light beam through the solution. As can be seen from the equation, the relationship is strictly linear for dilute solutions of the analyte. The UV detector is obviously a concentration dependent detector, where the detector dwell time and therefore the flow rate have strong impact on the peak area response. Efficient detectors use long pathways with very low internal volumes to reduce extra-column peak broadening due to diffusion.

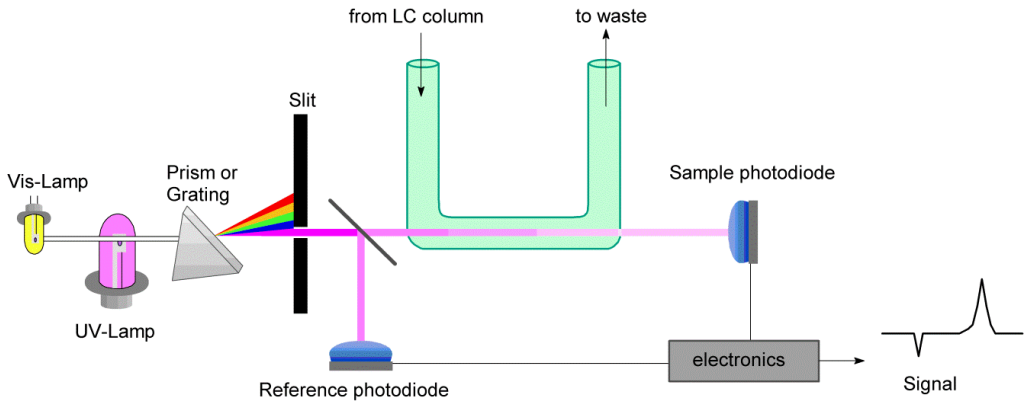



**Fig. 4.** Principle of an UV/Vis detector [1, 2].

## 2.2. Fluorescence detection

The fluorescence detector (FLD) is almost as straightforward as the UV/Vis detector, but the necessity for a fluorophore limits the number of detectable compounds. In many cases when the molecule does not contain of a suitable fluorophore, derivatizations can turn the analyte into a fluorescent molecule. For fluorescence detection, a higher light intensity is needed compared to UV/Vis detection. The light source is usually a xenon arc lamp, an argon or neon laser or strong light emitting diodes (LEDs) [1, 2].

The analyte is excited by the light beam and immediately drops back to the ground state under light emission (see Fig. 5). The intensity of the emitted light is measured and gives the signal. The emitted light is usually detected at a 90° angle towards the incident light to reduce stray light from the exciting light source (see Fig. 6). The emitted light is always of longer wavelength than the absorbed light (Stokes shift) and the fluorescence spectrum is usually the mirror image of the absorbance spectrum of the molecule (Mirror image rule) and it normally does not depend on the excitation wavelength (Kasha-Vavilov rule) [1].

Fluorescence detectors belong to the most sensitive detectors with detection limits in in the pg/mL level, but the response is usually not linear over a wide range (In extreme cases only 1 order of magnitude) [2]. These properties make the detector useful in trace analysis. The mobile phase needs special attention with regards to molecules that quench fluorescence, such as dissolved oxygen. Thorough degassing is crucial to obtain sensitive detection [12].

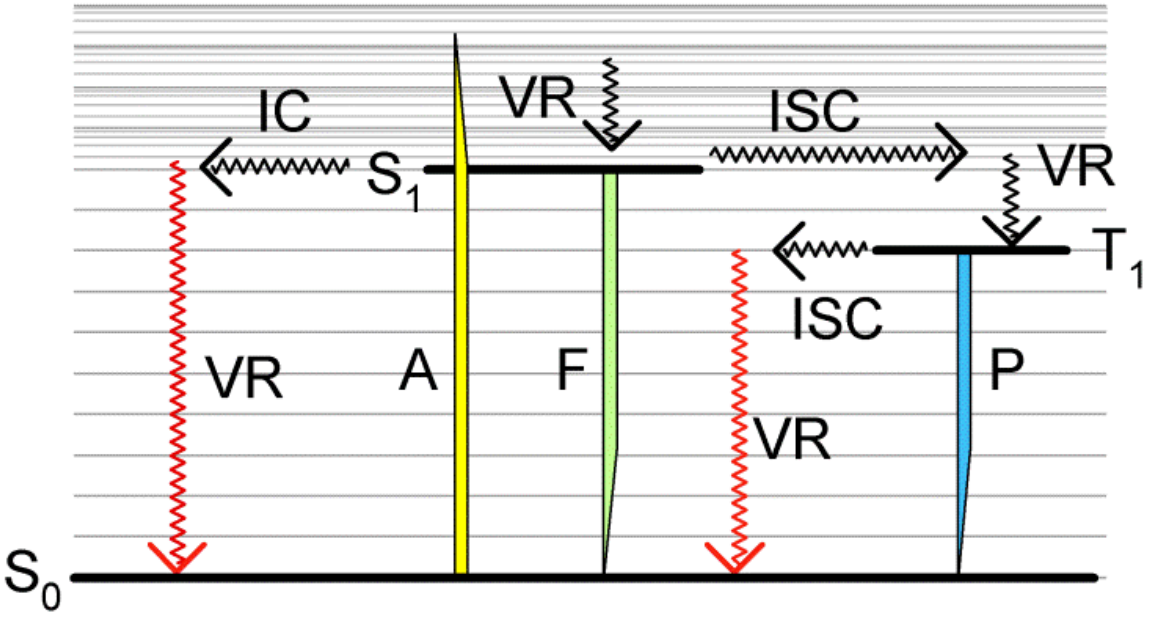



**Fig. 5.** Jablonski diagram for radiation free decay (red), fluorescence (green) and phosphorescence (blue) of a molecule after photon absorption (yellow); A, absorption; F, fluorescence; IC, internal conversion; ISC, inter-system crossing; P, phosphorescence; VR, vibrational relaxation; $S_0$, singlet ground state; $S_1$, excited singlet; $T_1$, excited triplet [1].

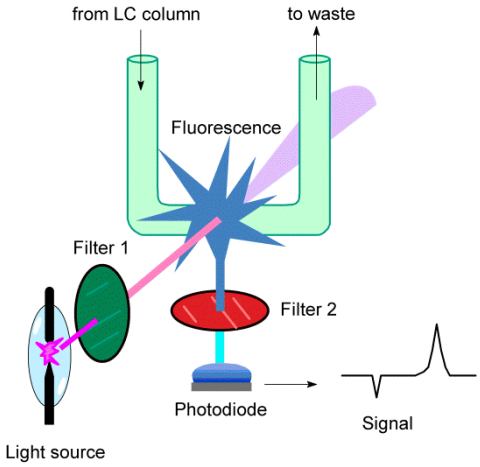



**Fig. 6.** Principle of a fluorescence detector; filters are used to select a single wavelength of excitation and to block the exciting wavelength to limit the noise due to stray light [1, 2].

## 2.3. Refractive index detector

The refractive index detector (RID) detects peaks based on the difference in refractive indices between the analyte and the mobile phase. It is known to be a kind of universal detector which means that in theory any compound can be detected, as long as its refractive index is different from that of the mobile phase. The RID uses mostly light in the visible range from 660 to 880 nm because light of higher wavelength refracts more than that of shorter wavelengths. The light is usually produced by tungsten halide lamps or LEDs [1, 2].

A beam of light refracts when it passes from one medium into another. The relationship between angle of incidence and the angle of refraction is expressed in Snell's Law of refraction [1, 2]:

$$n = \frac{n_2}{n_1} = \frac{\sin \alpha_1}{\sin \alpha_2} \qquad \textbf{(Eq. 2)}$$

where:

$n$ = Refractive index of medium 1 relative to medium 2

$n_2$ = Refractive index of medium 2

$n_1$ = Refractive index of medium 1

$\alpha_1$ = Angle of incident light in medium 1

$\alpha_2$ = Angle of refraction in medium 2

For small angles of external deflection ($\gamma$), the difference between the refractive indices of medium 1 and medium 2 is proportional to the angle of external deflection according to:

$$\tan \gamma = \frac{n_1 - n_2}{n_1} \qquad \textbf{(Eq. 3)}$$

The refractive index is affected by the wavelength of the light source and the optical density. The density however depends on the composition, the temperature and the pressure. A substance eluting from the column will change the composition and therefore change the refractive index of medium 1 (see Fig. 7). The external deflection angle ($\gamma$) will change and one of the photodiodes will be exposed to a higher intensity of light than the other and cause a signal [2].

Although any substance is detectable using this kind of detector it is not very sensitive due to the small differences in refractive indices. Small shifts in temperature, mobile phase composition and pressure lead to baseline drift and noise. This means that the RID can only be used in isocratic elution. To maintain the temperature of the cell, the column effluent and the reference mobile phase constant, most RIDs are equipped with heat exchangers between column and detection cell. The heat exchanger increases dead volume after the column thus increasing peak width. Increased peak width results in lower chromatographic performance and therefore higher detection limit. These major drawbacks make the RID only second choice for impurity profiling.

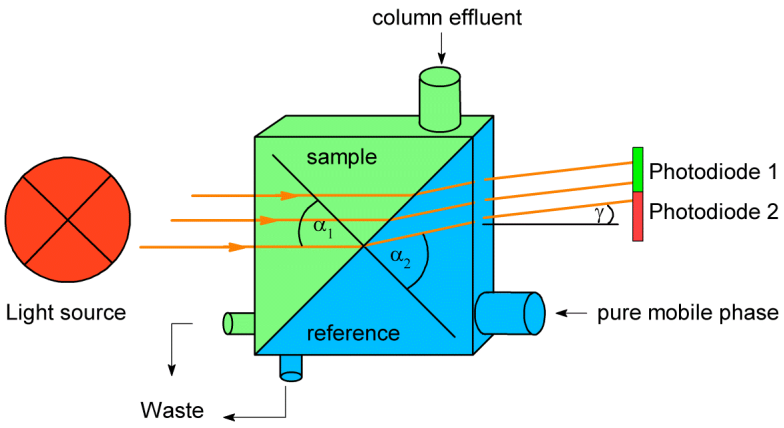



**Fig. 7.** Principle of a refractive index detector [2].

## 2.4. Evaporative light scattering detector

The evaporative light scattering detector (ELSD) is used to detect unselectively non-volatile analytes. The detection principle comprises nebulization and evaporation of the mobile phase leading to an analyte containing aerosol which is diverted through a light beam (see Fig. 8). The light scattering due to the aerosol is measured by means of a photomultiplier to give a signal [1, 2].

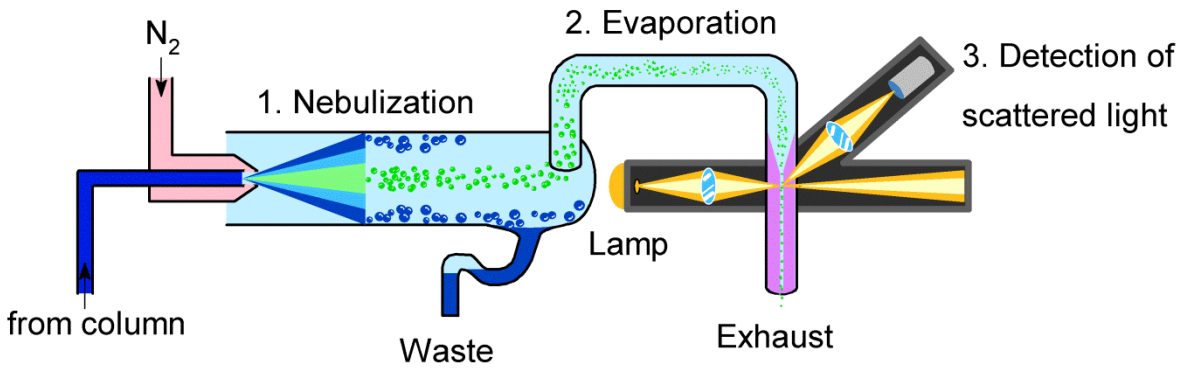



**Fig. 8.** Principle of an ELSD [2].

Light scattering is the diffuse reflection of light on a solid surface. In contrast to specular reflection, where the incident angle is equal to the reflection angle (like in a mirror) the light is reflected in many directions. Depending on the type of particle considered, there exist several types of light scattering:

*Rayleigh scattering* is the elastic scattering of light occurring on molecules and particles much smaller than the wavelength of the incident light. The intensity of Rayleigh scattering is proportional to the sixth power of the particle diameter and inversely proportional to the fourth power of the wavelength. *Mie scattering* describes the light scattering on

spherical particles if the particle size is in between 0.1 to 1.0 times λ. The intensity is not strongly dependent on the wavelength and it is proportional to the fourth power of the particle diameter. If the particle diameter is much bigger than the applied wavelength *refraction-reflection scattering* occurs. The scattered light is proportional to the second power of the particle diameter. *Tyndall scattering* is basically the same type of scattering as Mie scattering, without the limitation to spherical particles. Brillouin scattering is a type of inelastic scattering in liquids and solids. Inelastic means that the wavelength of scattered light differs from the wavelength of the incident light. The incoming light interacts with so-called acoustic phonons. These phonons correspond to vibrations of the lattice or elastic waves in liquids. Another type of inelastic scattering is *Raman scattering* where the light creates or annihilates intra-molecular vibrations and rotations, so-called optical phonons [13].

As a peak elutes from the column, the analyte concentration and therefore mean particle diameter in the detector increases from near-zero to a maximum and returns to near-zero. Since the particle diameter determines the type of light scattering, it is possible that three types of scattering occur if the concentration of the analyte is sufficiently high: Rayleigh, Mie and reflection-refraction scattering. Because the intensity of scattered light is strongly related to the particle diameter and it is different for all three types of light scattering the response can never be strictly linear over a broad range of concentration [13]. Nevertheless wide concentration ranges can be covered by using quadratic fit or log-log responses. Other types of light scattering (see above) can occur, but usually with a much lower intensity, so that their contribution to the total intensity could be neglected.

The light sources used in ELSD are usually LEDs, tungsten halide lamps, or laser light sources producing visible light. Because Raleigh scattering intensity is highly dependent on the wavelength, changes in sensitivity have to be considered when a method is transferred to another version of ELSD (with another light source) [14].

## 2.5. Condensation nucleation light scattering detector

The condensation nucleation light scattering detector (CNLSD) which is sometimes referred to as nano-quantity analyte detector (NQAD®) is the direct advancement of the ELSD. After the evaporation the aerosol is directed through a chamber with high relative humidity in order to induce condensation on the particles. The produced nebula is directed to the detection chamber and analyzed by the same principle described for the ELSD (see Fig. 9). The CNLSD shows better linearity and improved sensitivity compared to the ELSD [15].

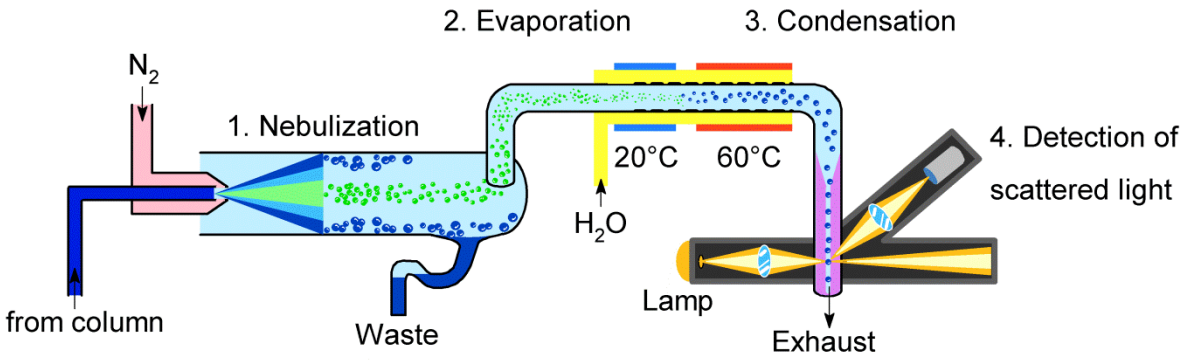



**Fig. 9.** Principle of the CNLSD [15]

## 2.6. Corona charged aerosol detector

The corona charged aerosol detector (CAD) is another kind of aerosol based detector using a completely different way of particle detection compared to ELSD and CNLSD, because it does not need optical elements.

The column effluent is nebulized with nitrogen and dried to yield an aerosol of analyte [16]. At the same time nitrogen is positively charged on a corona discharge needle and directed into a collision chamber where the aerosol is combined with the positively charged nitrogen. The charge is transferred to the aerosol particles and later detected using a sensitive amperemeter (see Fig. 11).

The CAD has been applied for a wide range of non-volatile compounds without chromophore like sugars [17], amino acids [18, 19] and bisphosphonates [20]. Detection limits are similar to CNLSD and in general superior to ELSD [15, 21, 22]. Like for all evaporative detectors, the mobile phase itself has to be completely volatile to prevent clogging of the detector.

As previously seen for the ELSD and CNLSD, the response of the CAD is also not linear over a broad range (see Fig. 12). The reason is in this case more obvious than in the case of ELSD. The amount of adsorbed charge is considered proportional to the particle surface, but the ratio of surface to volume (for spherical particles directly proportional to the particle mass) is not constant for increasing particle diameters (see Fig. 10). The mean particle diameter on the other hand depends on the analyte concentration [23]. Therefore the analyte concentration (the injected mass) cannot be strictly proportional to the surface in other words to the detector response. The logarithm of the surface/volume ratio and the logarithm of the volume however are strictly linear to the logarithm of mean particle diameter (see Fig. 10).

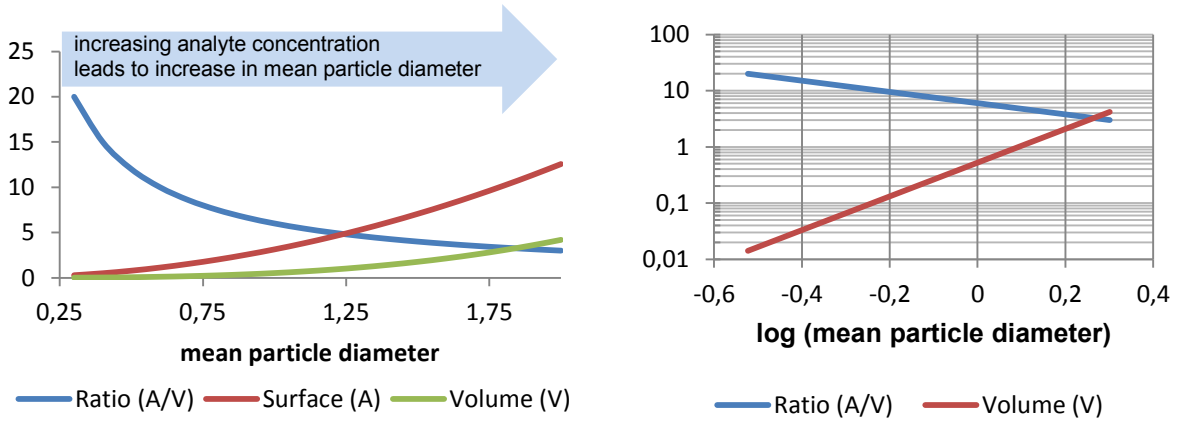



**Fig. 10.** Schematic graph showing the relationships between particle diameter, volume and surface for spherical particles V= 1/6 d³ π and A = π d²

In general models for the CAD the response is usually fit to an equation of the form

$$y = A \cdot x^b \qquad \textbf{(Eq. 4)}$$

where y is the peak height or peak area, x the concentration and b a coefficient smaller than 1. If a linear response is desired, equation (4) can be converted into a linear relationship by taking the logarithm on both sides [24]. On low concentration levels, the CAD response was found to be sufficiently linear [25].

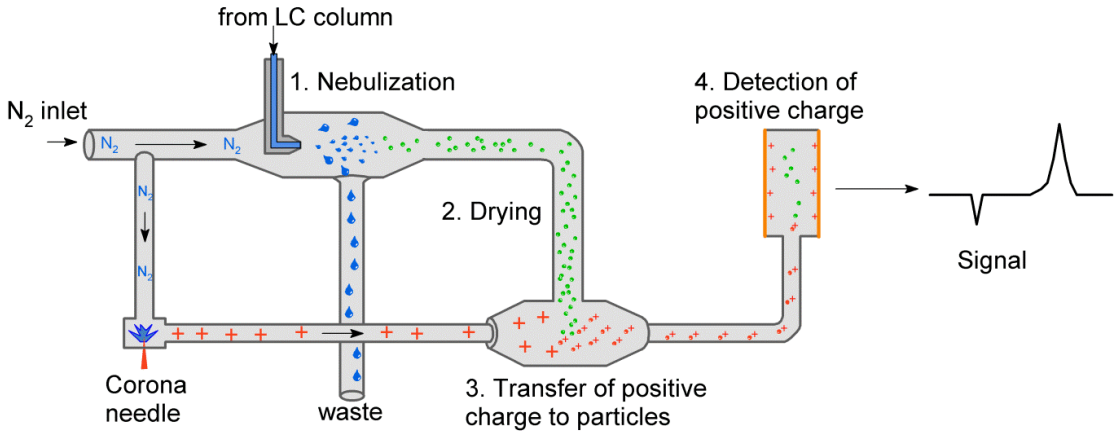



**Fig. 11.** A: Schematic layout of a CAD detector [16]

Apart from the lack of linearity, the so-called “gradient effect” also contributes to the low acceptance of this detector. The nebulization efficiency, the droplet formation and therefore response depends strongly on the percentage of organic modifier in the mobile phase. This in turn can lead to a 5-10-fold change in response of analytes when using gradient separations. Interesting approaches to solve this issue are post-column inverse gradients to give a constant mobile phase composition entering the detector (see Fig. 13) [26, 27] and three-dimensional calibration plots (see Fig. 14) [28].

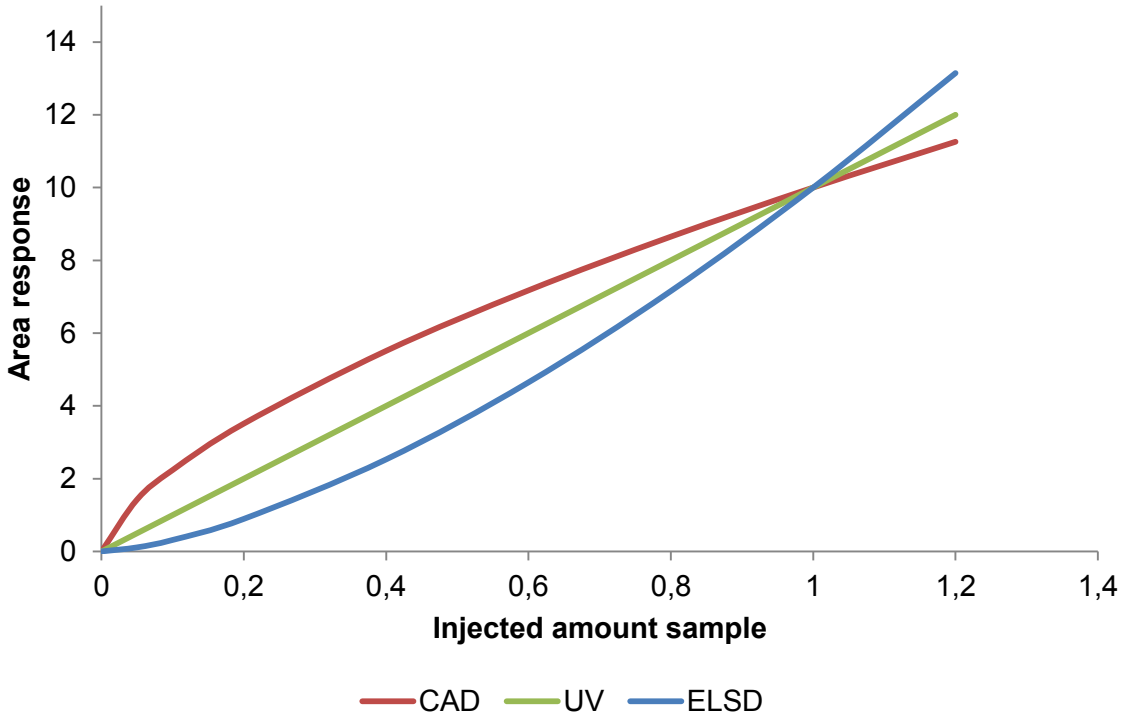



**Fig. 12.** Qualitative run of calibration curves for CAD, UV and ELSD.

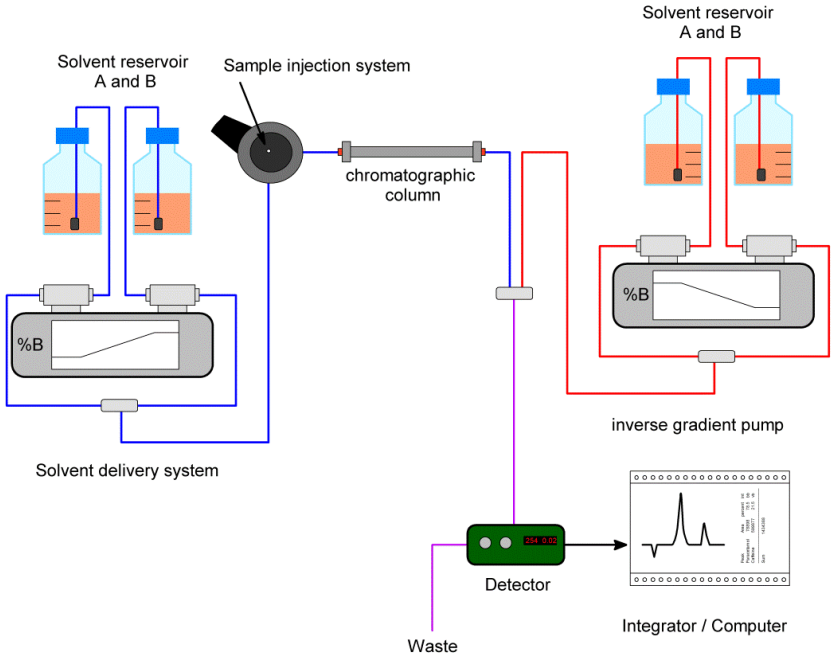



**Fig. 13.** Schematic layout of an HPLC System capable of gradient compensation [26, 27].

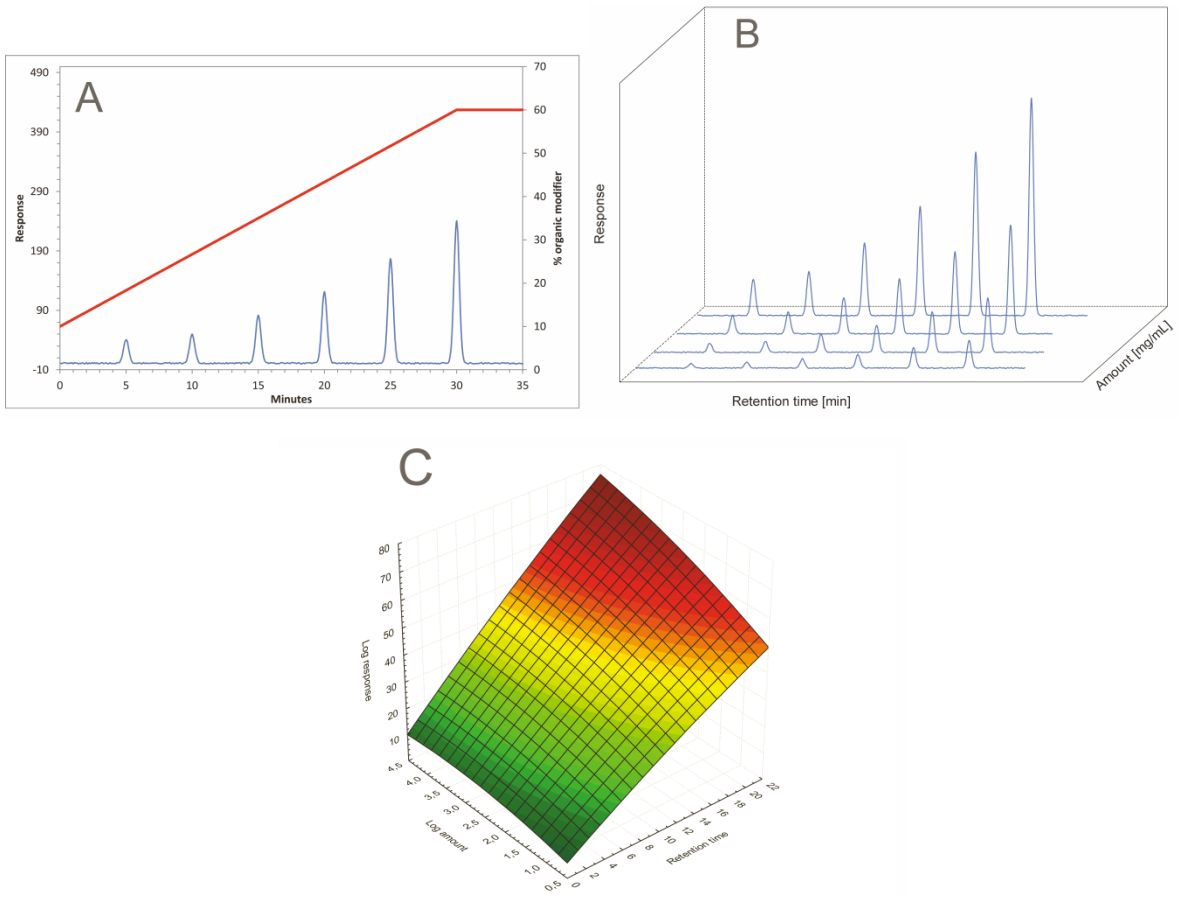



**Fig. 14.** Schematic development of 3D calibration; A: a calibrant is injected at different times during a gradient; B: this is done for different concentrations of the calibrant; C: a three-dimensional graph is created from this data and could be used to calculate the amount of an unknown substance at any time of the gradient from the response-retention time graph [28].

## 2.7. Electrochemical detector

An electrochemical detector (ECD) is used to detect oxidizable and reducible compounds with high selectivity. ECD flow cells contain usually three types of electrodes: A reference, a working and a counter electrode (see Fig. 15 A). The reference electrode is used to set a potential between working and counter electrode. The working electrode performs the electrochemical reaction and the counter electrode is used to measure the generated current (amperometric mode) or amount of charge transferred (coulometric mode) during the reaction.

Electrochemical detection can be very sensitive and specific provided that the detectors parameters are thoroughly optimized. The electrode potential has to be set for every analyte using a so called hydrodynamic voltammogram (see Fig. 15B) to avoid oxidation or reduction of mobile phase leading to increased noise. This parameter has to be optimized for every analyte which is difficult in impurity profiling because impurities are often unknown compounds. Advanced instruments are capable of coulometric electrode array

detection with multiple electrodes each operating at a different potential. This technique facilitates impurity profiling because unknown impurities are more likely to be detected. However, the ECD is a rather complicated detector needing a skilled operator and is not suitable for detecting unknown compounds due to its high specificity for oxidizable and reducible structures.

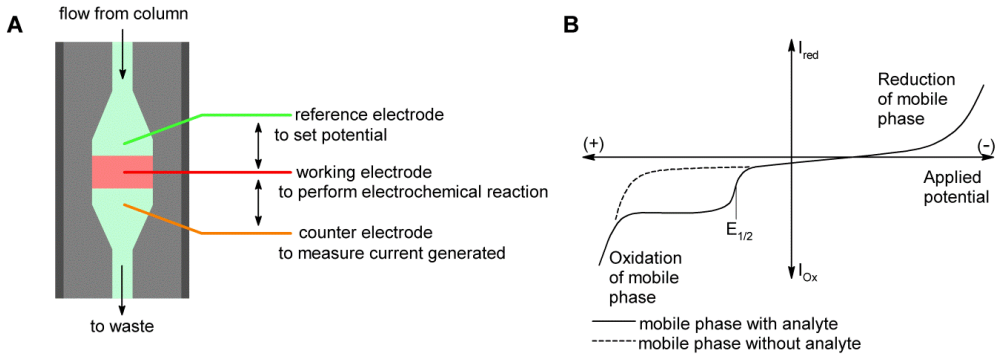



**Fig. 15.** A: Schematic layout of an ECD flowcell, B: hydrodynamic voltammogram [1, 2].

## 2.8. Capacitively coupled contactless conductivity detector

The $C^4D$ is an advancement of conventional conductivity detectors often applied in ion chromatography. The principle of this technique was first described in the beginning of the 19$^{th}$ century [29] and later used for flow-injection analysis (FIA) and ion chromatography [30, 31]. Since the advancements in 1998 [32, 33] it became a popular detector in CE analytics and is nowadays available for any kind of chromatography as well [34]. The major advantage over classical conductivity detection is the separation of eluent and electrodes (see Fig. 16) preventing electrode fouling and facilitating conductivity detection in CE, because previously the electrodes had to be shielded well from the electric field required for the separation. The technique is comparatively new and uncommon in HPLC analytics but could offer new possibilities in impurity profiling since UV inactive compounds can be detected with the $C^4D$ and like all non-destructive techniques it could be combined with other detection principles.

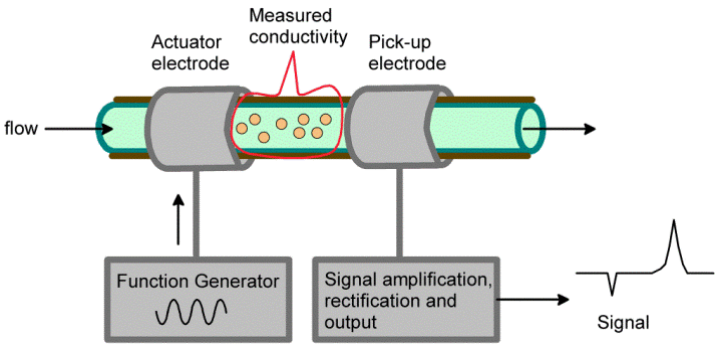



**Fig. 16.** Schematic layout of a $C^4D$ [32, 35].

## 2.9. Mass spectrometer

The combination of LC or CE and mass spectrometry is far from being simple. The removal of the mobile phase leads to a huge amount of gaseous mobile phase, which needs to be separated before the analytes can enter the mass spectrometer in order to maintain the required high vacuum inside. This task is performed by the LC-MS interface which takes care of mobile phase evaporation and analyte ion generation. Ever since suitable interfaces like electrospray ionization (ESI), atmospheric-pressure chemical ionization (APCI) and atmospheric-pressure photoionization (APPI) have been developed, the acceptance and application of LC-MS techniques has grown larger and larger. It is today one of the most powerful combinations with regards to sensitivity and selectivity. On the other hand, the acquisition and maintenance of those instruments is rather costly, because they need continuous high vacuum, nitrogen, and in some cases helium supply.

The aforementioned interfaces belong to so-called soft-ionization techniques. This means that the analyte is usually not fragmented during the ionization process in contrast to e.g. electron impact ionization (EI). During the ESI process (see Fig. 17) the analyte is charged by a high voltage applied to the nebulizer needle tip, whereas in APCI a corona discharge placed in the spray cone and in APPI UV light cause ionization of the analyte. Each of these interfaces is more or less suitable for a group of analytes. ESI e.g. is very common interface for large biomolecules, peptides and small hydrophilic compounds whereas APCI is a more efficient principle for small nonpolar molecules.

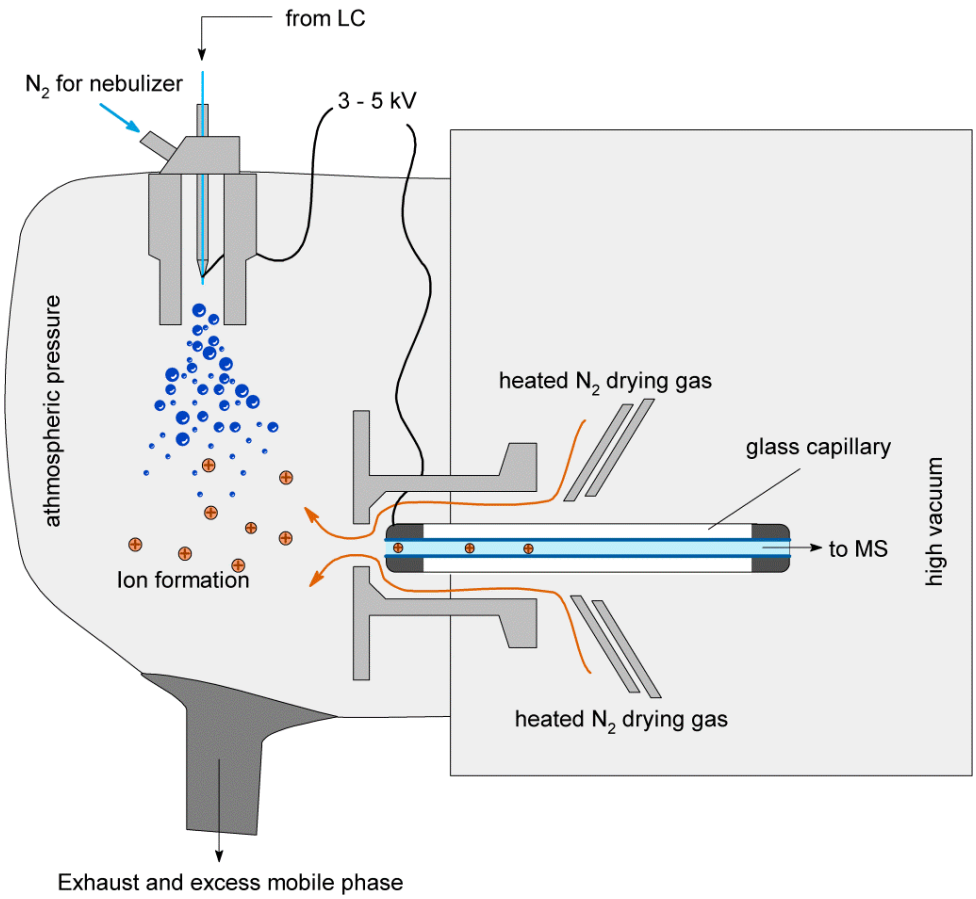



**Fig. 17.** Schematic of the electrospray interface for LC-MS detection; a charge is transferred to the analytes during the nebulization process by high voltage at the end of the nebulizer needle tip; the droplets are reduced in size until the repulsion of the equally charged analyte particles leads to a sudden burst of the droplet creating smaller droplets (coulomb explosion); This process is repeated until single ions are emitted from the droplet surface and dragged through the glass capillary into the mass spectrometer [2]

Once the analyte ion is generated, several ways for its analysis exist. There are ion-traps, single quadrupole, triple quadrupole (QqQ), time-of-flight (TOF) and quadrupole time-of-flight (qTOF) mass spectrometers. Every type of instrument is suitable for a certain application. Ion-trap is a very wide term including e.g. quadrupole ion-trap, cyclotron and orbitrap mass detectors. As its name implies, the analyte ion is trapped in an electromagnetic field e.g. created by a ring electrode and a so-called end-cap electrode on a more or less stable circular orbit. By variation of the electromagnetic field single ions with corresponding m/z value can be selected, ejected and detected (see Fig. 18 A). The electromagnetic field is also used to make the ion oscillate at its resonance frequency eventually causing its fragmentation. These instruments are very useful in structure elucidation because they can perform multiple fragmentation steps of an analyte ion in a single analysis.

The QqQ mass spectrometer is used for quantification of trace levels in difficult matrices with high specificity. Typical applications are therapeutic drug monitoring, doping control, forensics and toxicology (e.g. of food and plants). The QqQ is hereby usually used in multiple reaction monitoring (MRM) mode. The analyte ion is selected by the first quadrupole, fragmented by collision induced dissociation in the second quadrupole and the daughter ions are analyzed in the third quadrupole (see Fig. 18 B). Thus, the analyte is characterized by specific transitions (qualifier transition for identification and quantifier transition for quantification) to exclude that random matrix peaks interfere with the analysis. The use of internal standards, usually stable isotopically tagged analyte (deuterated, preferably $^{13}C$ labelled) compensate for matrix effects and analyte loss during extraction and sample preparation.

The coupling of CE and MS is possible but not very common, because the technique requires a high level of know-how and routine to yield a rugged method.

Main applications of LC-MS in impurity profiling are the identification of unknown compounds (e.g. by qTOF or ion-trap) and the sensitive and specific control of extraordinarily toxic impurities (QqQ).

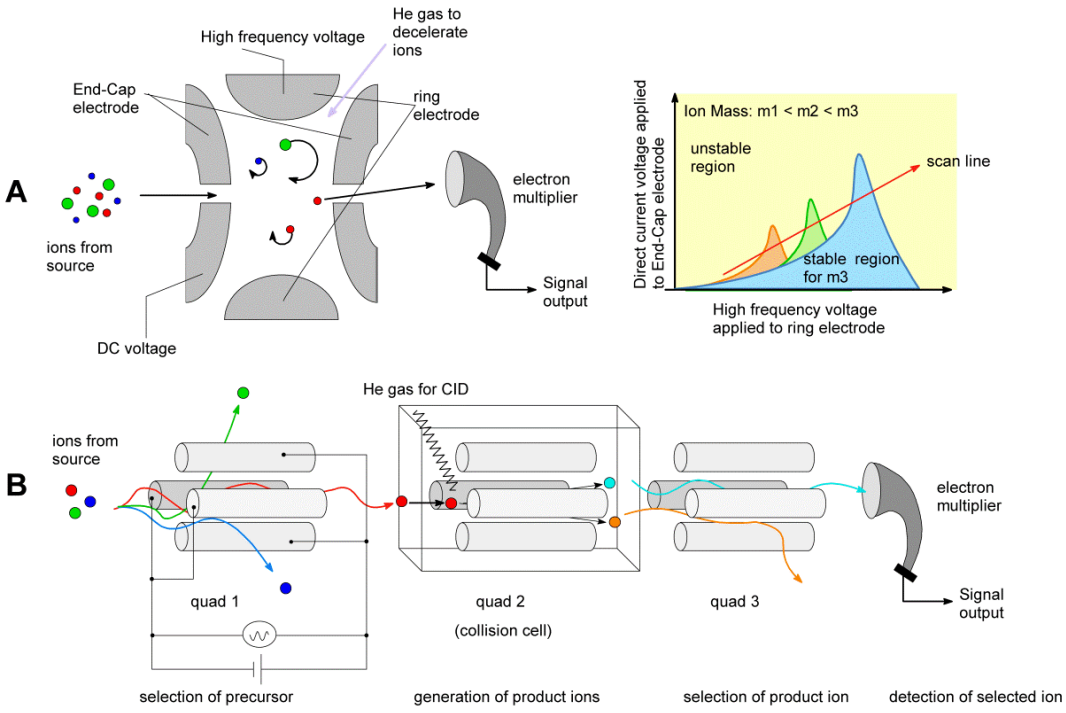



**Fig. 18.** Schematic of ion-trap (A) and triple quadrupole (B) mass analyzers; CID, collision induced dissociation; quad, quadrupole [2]

## 2.10. Chemiluminescent nitrogen detector

Like LC-MS, the chemiluminescent nitrogen detector (CLND) was first introduced in GC analysis [36-38]. It is an element specific detector able to detect nitrogen containing compounds. Because the analyte is burned during the detection process, volatile and non-volatile analytes can be detected and it is a purely mass sensitive detector (the peak area is independent of the detector dwell time and flow rate) [39, 40]. After combustion of the column effluent, substances containing nitrogen are converted to nitrogen monoxide. The reaction of NO with ozone leads to the formation of excited nitrogen dioxide which decays to the ground state under infrared light emission. The emitted light is registered by a photomultiplier tube giving a signal (see Fig. 19). Under normal circumstances the signal is directly proportional to the amount of nitrogen molecules present in the substance and analyte concentration. This means that any nitrogen containing compound can be used as an external standard.

The detector has some decisive downsides: It misses out on nitrogen free compounds like carboxylic acids, carbohydrates, alcohols etc. It is a comparatively complex instrument and rather costly due to the high gas consumption of helium (or argon), oxygen and ozone. Some substances do not give signal of expected intensity. E.g. if the substance contains two adjacent nitrogen atoms, they are converted to molecular nitrogen ($N_2$) upon combustion and do not give the reaction with ozone [41]. Another important limitation concerns the mobile phase: acetonitrile and ammonium buffers are not suitable for LC-CLND because the nitrogen present in those compounds would cause excessive baseline noise.

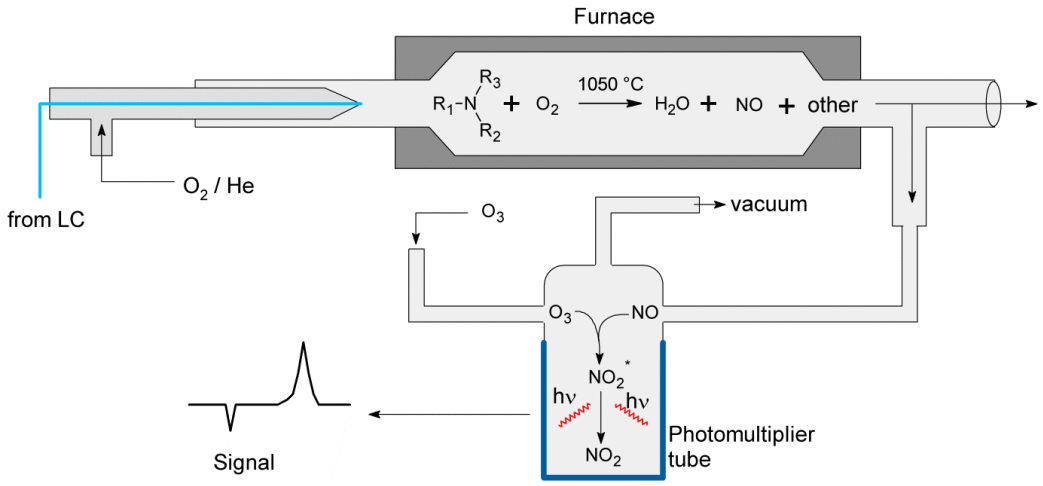



**Fig. 19.** Schematic of a chemiluminescent nitrogen detector [42, 43]

# 3. Challenges in Impurity profiling

The purity assessment of substances monographed in the Ph. Eur. is based in large part on HPLC separation followed by a suitable detection principle. Only a handful of monographs rely on capillary electrophoresis to determine related substances.

When it comes to impurity profiling using HPLC, analytes can impose several challenges: The most common detection principle is UV/vis absorbance detection, which is suitable for the greater part of analytes. On the other hand substances lacking a suitable chromophore are difficult to detect by this technique. Many of these compounds have structures also that make them difficult to separate on conventional reversed phase HPLC columns because of high hydrophilicity and/or because of their ionic character. The quantification of unknown impurities by UV detection is another important aspect. If unidentified compounds with unknown response factors are quantified using an external standard the concerned impurity might be highly over- or underestimated.

## 3.1. Strategies to overcome the separation issue

There are several approaches to address challenging separations. In HPLC analytics these approaches conclude the variation of mobile phase composition (pH, organic modifier, buffer salt type and concentration) and the change of the stationary phase. In cases where the popular reversed phase chromatography does not yield satisfactory results, the following methodologies are used to overcome a challenging separation.

### 3.1.1. Ion-pair Chromatography

A way to separate ionic species on conventional reversed stationary phases is ion-pair chromatography (IPC). In IPC the mobile phase contains of a carefully selected additive, the ion-pairing agent and the pH of the mobile phase is adjusted in a way to guarantee a near 100 % ionization of the analyte. The ion-pairing agent represents a counter ion for the analyte and has a lipophilic residue, usually alkyl or fluoroalkyl chain. Carboxylic or sulfonic acids are used for basic analytes whereas amines or ammonium salts are used for acidic compounds (see Fig. 20).

The separation principle of IPC is based on two mechanisms. On the one hand, the lipophilic residue of the ion-pairing agent is embedded in the stationary reversed phase turning it into an ion exchange stationary phase. On the other hand, the lipophilic counter ion forms an ion-pair with the analyte which is separated by reversed phase mechanisms [44, 45]. The dominating mechanism is mostly determined by the type of ion-pairing agent and by its concentration in the mobile phase.

Although difficult analytes like oligonucleotides, nucleotide phosphates, bisphosphonates and amino acids have been separated using IPC [46-50] the technique comes with some drawbacks: A RP column used with ion-pairing agents is contaminated with the reagent forever, it cannot be used for other applications. The use of extreme pH to force the ionization of the analyte lowers the life time of the column. The (re)equilibration can take very long, especially when gradient separations are used. A more serious issue is the bioaccumulation and suspected long-term toxicity of some ion-pairing agents (e.g. long chain perfluorinated carboxylic acids) [51]. Last but not least, the price for ion-pairing agents is usually a multiple of the price of simpler mobile phase additives (e.g. TFA, formic and phosphoric acid) making the technique rather costly.

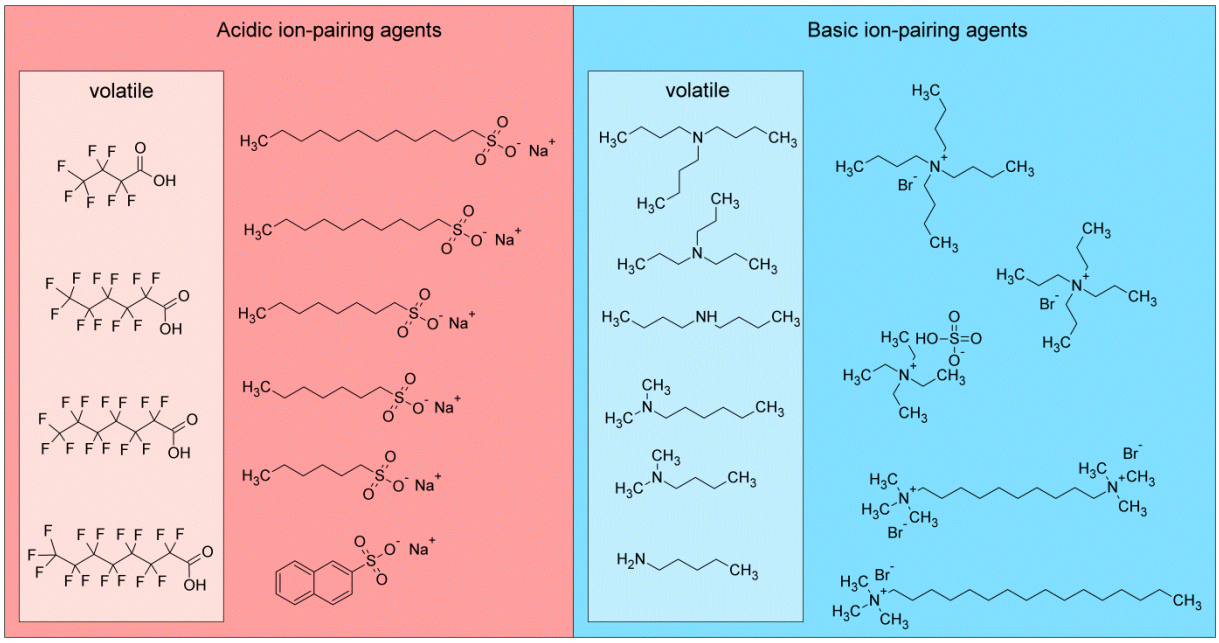



**Fig. 20.** A choice of ion-pairing agents used in IPC.

### 3.1.2. Mixed-mode Chromatography

Mixed mode chromatography (MMC) fills the gap between ion chromatography and RP chromatography, because the separation of ionizable and neutral analytes is possible in a single run [52, 53]. Mixed mode columns are especially useful for impurity profiling because it was demonstrated that the loading capacity of these columns is higher for charged analytes compared to ordinary C18 columns [54]. Separation of critical peaks is easier because the principle peaks width is reduced and the probability for unknown compounds covered by the main peak is lower. The mixture of multiple retention mechanisms enables separation of a wide variety of compounds. E.g. the combination of reversed phase and ion exchange is an intriguing alternative to reversed phase ion-pairing chromatography [55] without the downsides of IPC.

There are several ways to achieve mixed mode chromatography: Two chromatographic columns with different stationary phases could be connected in series, one chromatographic column could contain two stationary phases as a mixture or the functional groups are embedded into the stationary phase (e.g. ammonium groups carrying octadecyl residues). Some types of mixed-mode stationary phases are depicted in Fig. 21. A choice of commercially available mixed-mode columns is listed in Table 5.

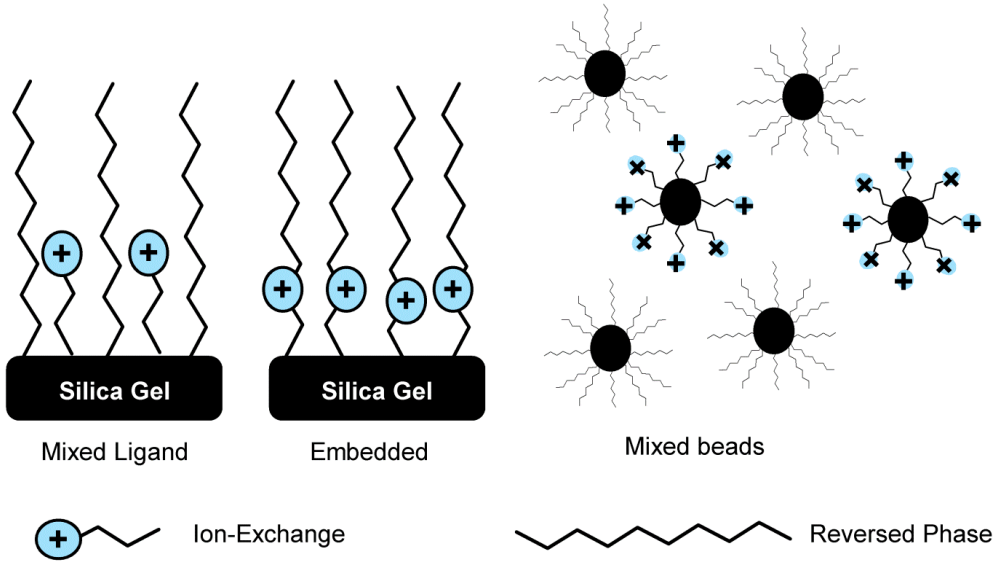



**Fig. 21.** Types of mixed mode stationary phases.

Mixed mode chromatography can solve separation problems, but is not as straightforward as common RP chromatography. The buffer ionic strength, pH and organic modifier have to be selected and tuned carefully, as their impact on the separation is considerably larger compared to RP chromatography. Another issue is the fact that one mixed mode column usually cannot be replaced by a mixed mode column with the same functionalities of a different brand and there is no standardization like USP categories for these kinds of columns. The composition of the stationary phases and the functional groups might not be identical although the column is of the same type (e.g. RP18 and SAX) [56]. This is reflected by the applications listed in Table 1 where the comparison of several mixed mode columns for one separation problem often leads to very different results regarding retention, elution order and peak shape. The column lifetime compared to common reversed phase columns seems to be reduced at least in some cases.

**Table 5**
Examples for commercially available mixed mode columns and latest applications

| Column brand name | Functional group | literature |
|---|---|---|
| Primesep 100 | RP and SCX | [57-59] |
| Primesep SB | RP and SAX | [20] |
| Coresep SB[a] | RP and SAX | [60] |
| Obelisc R | RP, HILIC and IEX | [61, 62] |
| Obelisc N | HILIC and IEX | [61, 63] |
| Primesep 200 | RP and WCX | [52] |
| Acclaim Trinity P1 | RP, SCX and WAX | [62, 64] |
| Acclaim Trinity P2 | HILIC, SAX and WCX | [65] |
| OmniPac PAX | RP and SAX | [66, 67] |
| OmniPac PCX | RP and SCX | [68] |
| Acclaim Mixed-Mode WAX-1 | RP and WAX | [69, 70] |
| Acclaim Mixed-Mode HILIC-1 | RP and HILIC | [71] |
| Acclaim Mixed-Mode WCX-1 | RP and WCX | [72] |
| Scherzo SM-C18 | RP, WAX and WCX | [62, 73] |
| Scherzo SS-C18 | RP, SAX and SCX | |
| TCI Dual ODS-CX10 | RP and SCX | |
| TCI Dual ODS-AX20 | RP and SAX | |

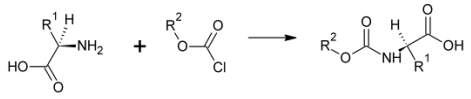
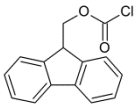
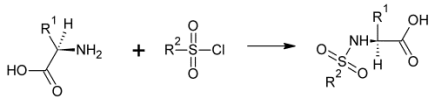
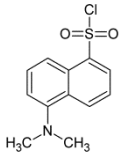
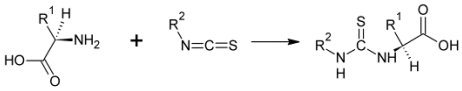
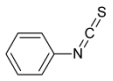
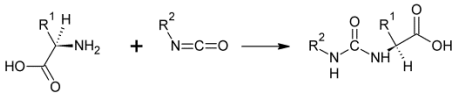
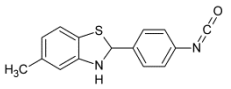
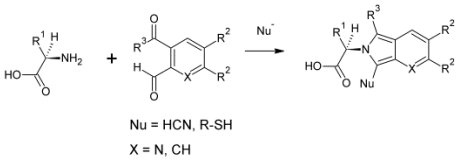
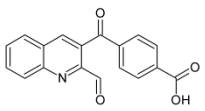
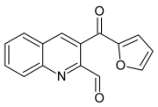
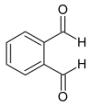
[a] solid-core particles; HILIC: hydrophilic interaction liquid chromatography, IEX: ion exchange, RP: reversed phase, SAX: strong anion exchange, SCX: strong cation exchange, WAX: weak anion exchange, WCX: weak cation exchange

### 3.1.3. Derivatization of the analyte

Derivatization procedures are used to modify the analyte structure in order to increase its retention on a stationary phase and to introduce a chromophore for UV or fluorescence detection. Suitable derivatization sites are primary or secondary amines, hydroxyl groups and carboxylic acids.

Innumerous reagents for the derivatization of all kinds of chemical compounds are available on the market. A very common reason for derivatization is the introduction of a chromo- or fluorophores in LC analysis of challenging compounds (see Table 6). Examples for challenging compounds are e.g. carbohydrates [74], fatty acids [75], amino acids [76], aliphatic amines and bisphosphonates [77]. Since the newly introduced chromophore usually represents an aromatic hydrocarbon, the retention of the derivative on reversed phase stationary phases is enhanced at the same time. Thus, derivatization is able to kill two birds with one stone.

**Table 6**
Examples for derivatization reactions used in LC-analysis of amino acids

| Reaction | derivative type | Examples | method of detection |
|---|---|---|---|
|  | urethane |  FMOC-Cl | FLD |
|  | sulfonamide |  dansyl-Cl | FLD |
|  | thiourea |  PITC | UV |
|  | urea |  4-(6-Methyl-2-benzothiazolyl)phenyl isocyanate | FLD |
|  Nu = HCN, R-SH<br>X = N, CH | isoindole and analogues |  CBQCA<br>  FQ<br>  OPA | FLD |

### 3.1.4. Capillary electrophoresis

A truly orthogonal separation compared to HPLC offers CE. Capillary electrophoresis is a versatile technique allowing for high theoretical plate counts due to the characteristic flow profile. The wide variety of available separation modes such as CZE, MEKC, MEEKC and the possibility of chiral selectors enable the separation of charged and neutral compounds as well as of enantiomers [78-81]. Disadvantages in impurity profiling are the fair sensitivity, the complexity and the lack of acceptance of CE techniques in the pharmaceutical industry. Important fields of application are the investigation of large biological molecules, like DNA and the separation of enantiomeric compounds.

## 3.2. Coping with the detection issue

The detection of substances without strong chromophore (conjugated double bonds) imposes a great challenge to analysts. Besides RID, which is very popular in carbohydrate analysis, the following detection principles can be used to detect those substances with adequate sensitivity.

### 3.2.1. Direct UV-detection

In some cases the analyte may be detected at low wavelength (< 210 nm) but the detection limit is usually insufficient for impurity profiling. However, in some cases when the mobile phase is sufficiently transparent for the low wavelength and the analytes possess moderately strong chromophores, such as amides, thiols or thioethers, the direct detection is possible [58].

### 3.2.2. Derivatization

As mentioned before, the introduction of fluoro- and chromophores can improve retention as well as detection limits, but comes with decisive downsides for impurity profiling:

1. The involvement of another substance with its own impurities and degradation products could impair the results.
2. All available derivatization reagents are more or less specific for a class of analytes, rendering the detection blind towards compounds that do not have the necessary feature to react with the reagent.
3. Some substances yield multiple or unexpected products, especially when the derivatization conditions are not precisely maintained within the specifications.
4. Degradation products of derivatives might lead to misinterpretation of the result.
5. Post-column derivatization leads to decreased chromatographic performance due to the high dead volume between column and detector resulting in extra-column band broadening.

### 3.2.3. Universal detection

So-called universal detectors can detect substances for the best part independent of their chemical structure. ELSD, CNLSD, CLND, MS, CAD and to some extent $C^4D$ belong to this group. Although called universal, the response is never completely independent of the analytes physical-chemical properties. Each detection principle comes with its own disadvantages and fields of application. In the end several detectors (universal and others) have to be assessed in order to get maximum certainty and to choose the most suitable detection principle. In many cases a combination is possible and sensible [82].

Detectors like the CAD, ELSD and CNLSD exhibit over a wide range of analytes a more or less uniform response [83]. This means that response factors of substances with comparable boiling point or vapor pressure are also similar, so that the quantification error for unknown compounds is also reduced in comparison to e.g. UV-detection where a 10-fold difference in response (e.g. due to the lack of an extended chromophore) is not uncommon (see Fig. 22). In cases where an extended chromophore (e.g. conjugated double bond in fumaric acid) is present UV detection is much more sensitive compared to CAD and ELSD. For substances with only a minimal chromophore (like carboxylic acid, or guanidine) the quantification limit (LOQ) is equal or superior using CAD and inferior using ELSD (see Table 7).

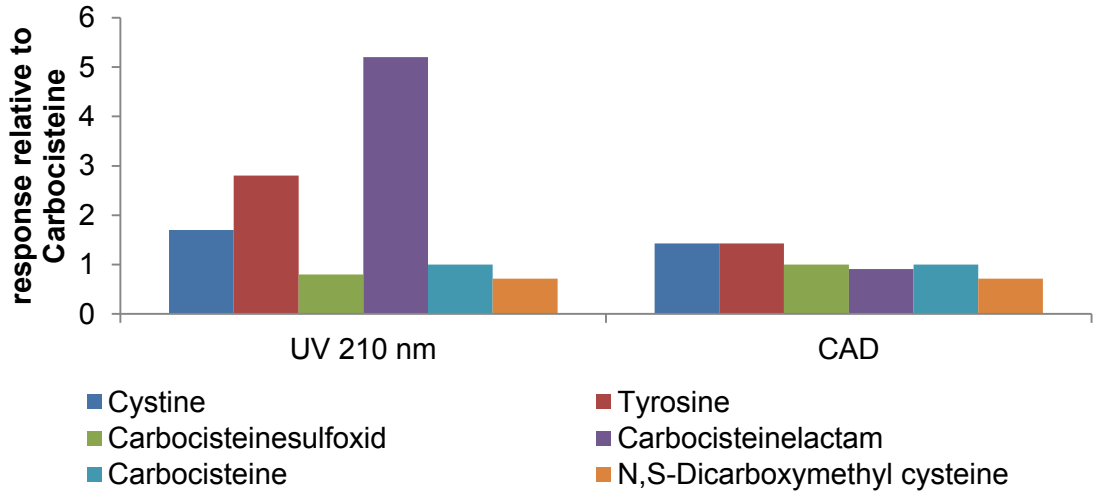



**Fig. 22.** Response variation for the related substances of Carbocisteine relative to Carbocisteine comparing UV detection at 210 nm and CAD, from own work related to [57].

**Table 7**
Comparison of obtainable LOQ or LOD for difficult analytes using different detection techniques.

| | LOQ (LOD) [ng] | | | | | | |
|---|---|---|---|---|---|---|---|
| | UV | CAD | ELSD | CNLSD | LC-MS | LC-MS/MS | CNLD |
| Citric acid | 79.9[a] | 40[b] | 800[b] | - | - | - | - |
| Succinic acid | 277[a] | 240[b] | 1333[b] | - | - | - | - |
| Fumaric acid | 1.1[a] | 40[b] | 800[b] | 80[c] | 0.3[c] | - | - |
| Malic acid | - | 40[c] | 400[c] | 92[c] | 1.5[c] | - | - |
| Aspartic acid | -<br>(30)[d] | 24[c]<br>(10)[d] | 800[c]<br>(25)[d] | 100[c]<br>- | 0.03[c]<br>(4)[d] | -<br>(2)[d] | -<br>(10)[d] |
| Glutamic acid | -<br>(30)[d] | 32[c]<br>(250)[d] | 1200[c]<br>(50)[d] | 152[c]<br>- | 0.03[c]<br>(30)[d] | -<br>(1.5)[d] | -<br>(6) |
| Streptomycin | 250[e] | 45[e] | - | - | - | - | - |

[a] λ= 210 nm, from [84]
[b] from [85]
[c] from [18]
[d] λ = 210 nm, from [86]
[e] λ = 205 nm, from [87]

## 3.3. Examples for challenging separations

According to the literature available from Table 8 a very common approach for the detection of difficult analytes is “universal detection” using CAD or ELSD. Challenging separations are frequently overcome by means of ion-pair chromatography. Some separations also rely on mixed mode chromatography to avoid expensive and fault prone ion-pairing agents.

**Table 8**
Examples for impurity profiling of challenging analytes found in the literature.

| **Main compound** | **challenge imposed by analytes** | **solution** | **Lit.** |
|---|---|---|---|
| Topiramate | no chromophore | RPC and CNLD | [43] |
| Artemisinin | no chromophore | RPC and UV detection at 210nm and LC-MS | [88] |
| Etidronate | very polar and no chromophore | MMC and CAD | [20] |
| Risedronate | very polar | IPC and UV detection 262 nm | [89, 90] |
| Alanine | very polar and no chromophore | IPC and ELSD, CNLSD, CAD and MS | [18] |
| Aspartic acid | very polar and no chromophore | IPC and CAD | [85] |
| Streptomycin | very polar and no chromphore | IPC and CAD<br>IPC with post-column derivatization and FLD | [87]<br>[91] |
| Etimicin | polar and no chromophore | RPC and ELSD and LC-MS | [92] |
| Gentamicin | very polar and no chromophore | IPC and ECD and ELSD<br>RP and LC-MS | [93]<br>[94] |

**Table 8 (continued)**

| Main compound | challenge imposed by analytes | solution | Lit. |
|---|---|---|---|
| Ibandronate | very polar and no chromophore | IEC and CD<br>IPC and ELSD<br>CE and indirect UV detection at 254 nm (chromate) | [95]<br>[48]<br>[96] |
| Lactic acid | polar and no chromophore | RPC and UV detection 210 nm | [84] |
| Amino acids | very polar and no chromophore | HILIC and CAD | [97] |
| Gabapentin | no chromophore | RPC and CAD | [19] |
| Methionin | very polar and no chromophore | MMC and UV detection 210 nm | [58] |
| Ionic liquids | no chromophore | RPC and CAD | [98] |
| Kanamycin | very polar and no chromophore | CZE and $C^4D$<br>Derivatization and CZE<br>IPC and ECD | [99]<br>[100]<br>[101] |
| Carbocisteine | very polar and no chromophore | IEC and UV detection 205 nm | [102] |
| Nucleotide phosphates | very polar | MMC and UV detection 254nm | [103] |
| Memantine | very polar and no chromophore | IPC and CAD | [104] |
| Meprobamate | no chromophore | RPC and UV detection 200 nm | [105] |
| Pipecuronium bromide | very polar and no chromophore | RPC and ECD | [106] |
| Ursodeoxycholic acid | no chromophore | RPC and RID | [107] |
| Fatty alcohol ethoxylates | no chromophore | RPC and ELSD | [108] |
| Fatty acids | no chromophore | HILIC and CAD | [109] |

# 4. Chiral separation techniques for amino acids

## 4.1. Indirect separation

The indirect separation of enantiomers comprises the reaction of the sample with an enantiomerically pure reagent to form two diastereomeric compounds. This pair of diastereomers is afterwards separated using an achiral stationary phase, usually in reversed phase mode or by CE. Crucial requirements for this method are highly pure derivatization reagents, complete derivatization, chemical and configurational stable derivatives and a suitable derivatization site, such as amines, alcohols and thiols [2].

In order to obtain reliable and reproducible results, the analyte and the reagent have to react in a selective reaction to yield well defined products. This is e.g. not the case if the analyte contains multiple functional groups that undergo the derivatization reaction or if the derivatization reagent is of poor (optical) purity. If the reagent contains a significant amount of the other enantiomer, a mixture of 4 derivatives is produced (Fig. 23). The unexpected derivatives coelute on achiral stationary phases with the desired derivatives and distort the result.

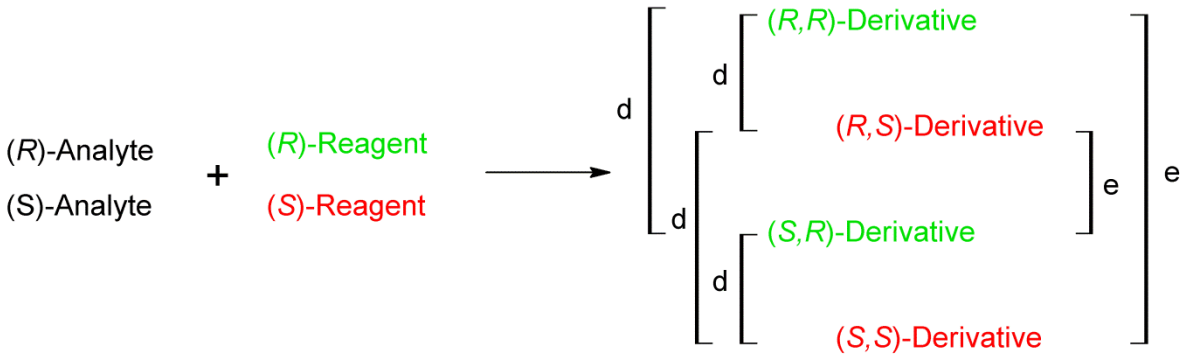



**Fig. 23.** Complex mixture of diastereomeric (d) and enantiomeric (e) derivatives if the reagent (green) is contaminated with a significant amount of the other enantiomer (red) [2].

Of all reagents listed in Table 9 and Fig. 24, FLEC and OPA are most frequently applied due to their high reactivity leading to complete derivatization. The poor stability of the OPA derivatives is often overcome by automated pre-column or in-capillary derivatization just before the separation [110].

**Table 9**
A Selection of chiral derivatization reagents.

| Reagent | Derivatization site | Literature |
|---|---|---|
| Mosher's reagent | alcohols, amines | [111] |
| DBTAAN | alcohols, amines | [112] |
| FLEC | alcohols, amines | [113] |
| OPA + chiral thiol compounds | primary amines | [114-117] |
| Marfey's reagent | primary and secondary amines, thiols | [118, 119] |
| GITC | primary and secondary amines, thiols | [120] |

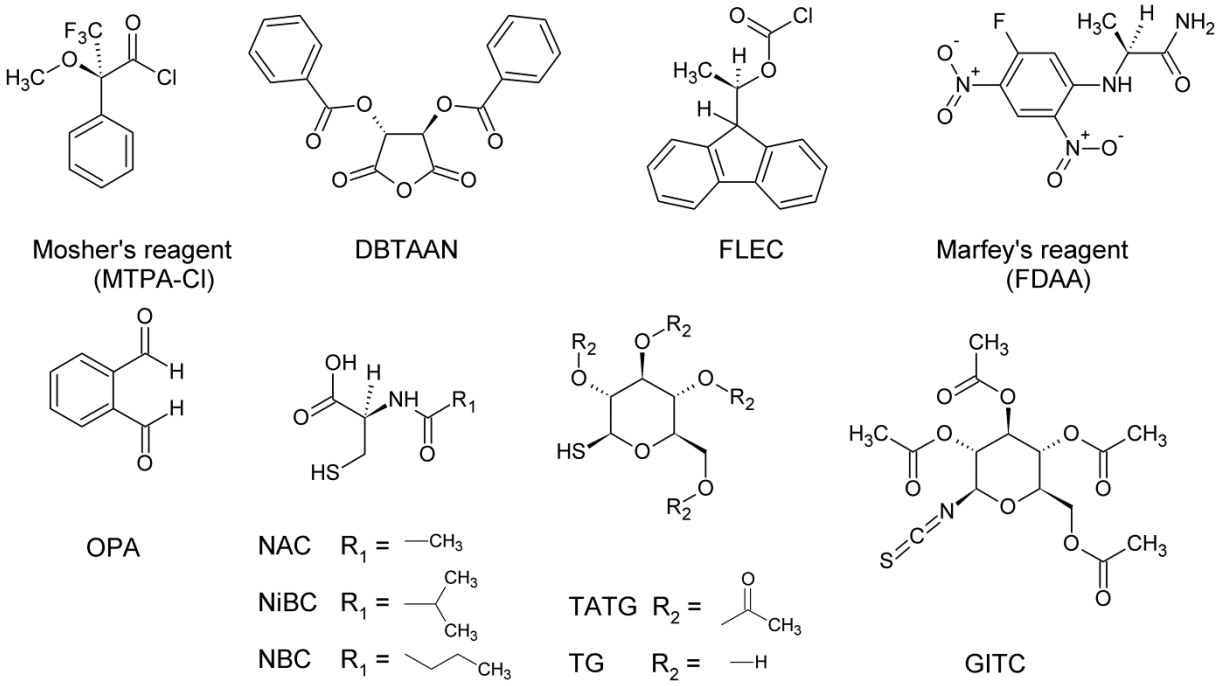



**Fig. 24.** Selection of chiral derivatization reagents and chiral thiols to use with OPA; MTPA-Cl, (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride; DBTAAN, (+)-dibenzoyl-L-tartaric anhydride; FLEC, (-)-1-(9-Fluorenyl)ethyl chloroformate; FDAA, $N_{\alpha}$-(2,4-Dinitro-5-fluorophenyl)-L-alaninamide; OPA, *o*-phthaldialdehyde; NAC, *N*-acetyl-L-cysteine; NiBC, *N*-isobutyryl-L-cysteine; NBC, *N*-n-butyryl-L-cysteine; TATG, 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranoside; TG, 1-thio-β-D-glucopyranose; GITC, 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate.

## 4.2. Direct separation using chiral stationary phases

In the direct separation of enantiomers a chiral selector (CS) is covalently linked or alternatively adsorbed to porous silica gel particles or another kind of chromatographic support to form the stationary phase. During the migration of the sample through the stationary phase, the enantiomers are retained due to the formation of diastereomeric complexes with different equilibrium constants (see Fig. 25). Several kinds of stationary phase modifications, both natural and synthetic exist for the direct separation of enantiomers.

$$[(R)\text{-CS}]_s + [(S)\text{-X}]_m \overset{k_{i,S}}{\rightleftharpoons} [(R)\text{-CS---}(S)\text{-X}]_s$$

$$[(R)\text{-CS}]_s + [(R)\text{-X}]_m \overset{k_{i,R}}{\rightleftharpoons} [(R)\text{-CS---}(R)\text{-X}]_s$$

**Fig. 25.** Equilibria of diastereomeric complex formation of (*S*)- and (*R*)-analyte with the chiral selector (CS) and corresponding equilibrium constants ($k_{i,R}$ and $k_{i,S}$).

Pirkle-phases named after a pioneer in the field of chiral recognition rules and enantiomeric separation William H. Pirkle [121] consist of silica gel modified with chiral groups able to form and accept hydrogen bonds and *π*-*π* interactions [122]. Other modifications available are based on chiral crown ethers, polysaccharides (cellulose or amylose) with *π*-*π* interactions sites bound via a carbamate group, proteins (e.g. avidin or albumin), macrocyclic antibiotics (e.g. vancomycin or teicoplanin), cyclodextrins and chiral chelating agents for chiral ligand-exchange (CLEC) [2].

The mobile phase is usually an achiral normal phase or sometimes reversed phase eluent and plays an important role as it defines the environment where the chiral recognition takes place. The selection of mobile phase and separation mode is of great importance because the formation of diastereomeric complexes can be promoted or suppressed by the eluent components.

CLEC, teicoplanin, chiral HILIC and chiral crown ethers have proven useful in the direct separation of unmodified D- and L-amino acids [123-128]. The direct separation of enantiomeric amino acids is well established and straightforward but the necessary column material is rather costly. A comprehensive overview about direct and indirect separation methods for amino acids is given by Ilisz et al. [129].

## 4.3. Direct separation using chiral CE

Capillary electrophoresis is one of the most important techniques for the separation of amino acids because they are easy to separate in an electric field due to their ionic character. The addition of chiral modifiers allows for the resolution of enantiomeric amino acids. Among all chiral modifiers applied in CE, cyclodextrins are by far the most important and most frequently used additives. Other approaches to separate D- and L- amino acids make use of diastereomeric complex formation similar to CLEC, chiral ionic liquids or a combination thereof [130-136].

Since the other techniques are still on a research stage, the application of cyclodextrins (CD) is from the analytical point of view the most reasonable approach for pharmacopoeial impurity profiling. They are commercially available in many different variations and thoroughly explored. Native cyclodextrins are cyclic oligosaccharides of α-D-glucose monomers forming a truncated cone shaped molecule consisting of a hydrophobic cavity and a hydrophilic hull (see Fig. 26). The theory of chiral recognition assumes some sort of complex formation with the enantiomeric sample, often in a way that the sample molecule is located inside the cavity [137]. However other forms of complexes with the outside of the CD or some kind of sandwich complex involving two molecules of CD are also possible [138].

Besides the native cyclodextrins (α, β and γ-CD) a high number of modified cyclodextrins is available on the market. Amongst these modifications are hydroxypropylation, acetylation or methylation to improve water solubility [139] (see Fig. 27) and the introduction of ionizable groups such as phosphate, sulfate, carboxylate and ammonium groups to induce electrophoretic mobility of the CD and to modify its chiral recognition characteristics [139-141]. The increase of water solubility is especially important for β-CD, because it seems to be the best chiral selector for many molecules, especially amino acid derivatives (due to the cavity volume) [142] but it is at the same time the CD with the lowest water solubility.

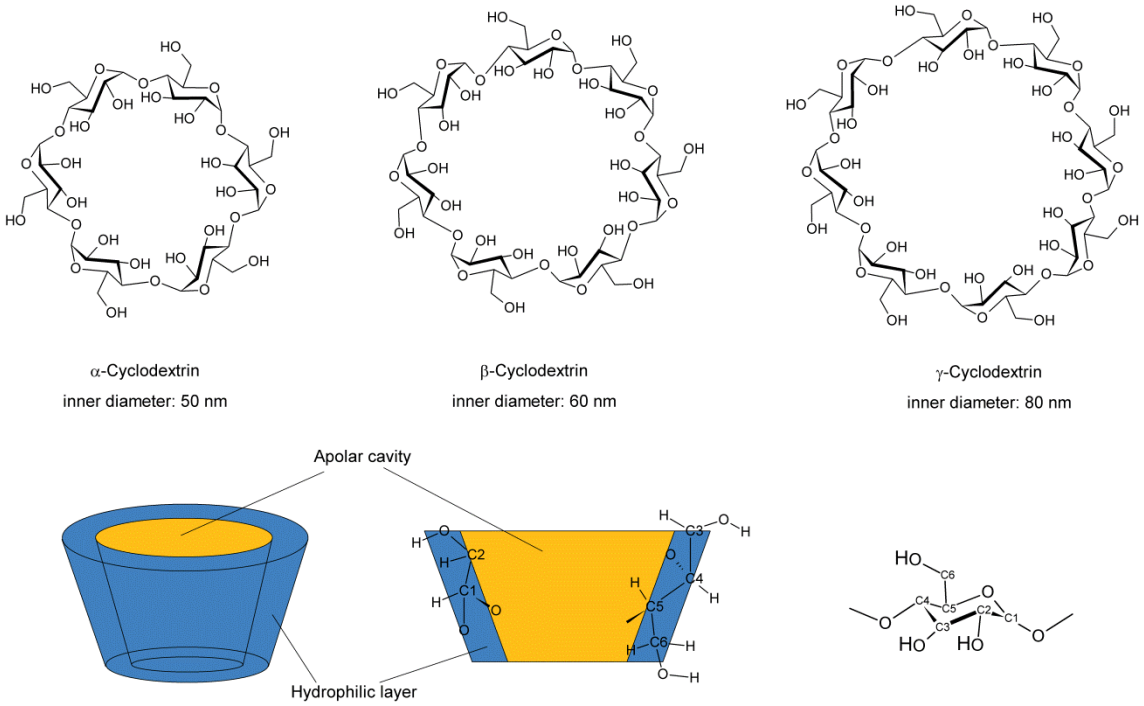



**Fig. 26.** Chemical structure and molecular shape of native cyclodextrins.

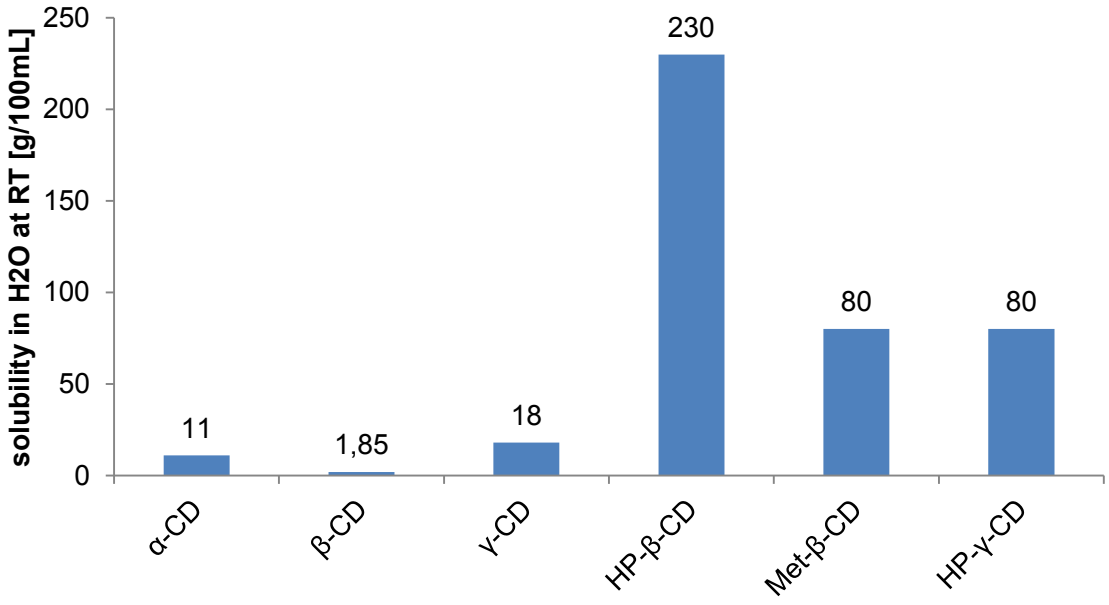



**Fig. 27.** Water solubility of native and modified cyclodextrins [143], HP-, hydroxypropyl-; Met-, methyl-

For the determination of impurities below 1% the introduction of a suitable chromophore for UV- or LIF-detection is imperative for non-aromatic amino acids because those techniques are the only routinely available means of detection compatible with the chiral BGE. The chromo- or fluorophore usually made up of aromatic hydrocarbons could also be necessary for the interaction with the cyclodextrin, because cyclic aromatic systems are known to take part in host-guest-interactions with the hydrophobic cavity [144-146]. Common derivatization reagents for D- and L-amino acids prior to CE separation with the help of cyclodextrins are: dansyl-Cl, FMOC-Cl, CBQCA, FQ, OPA, NDA (naphthalene-2,3-dicarboxaldehyde) and FITC (fluoresceine isothiocyanate) [129, 132, 147] (see also Table 6)

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# 5. References


[1] D.A. Skoog, F.J. Holler, S.R. Crouch, Instrumentelle Analytik, Springer Spektrum, Berlin, 2013.

[2] L.R. Snyder, J.J. Kirkland, J.W. Dolan, Introduction to modern liquid chromatography, 3rd ed., Wiley, Hoboken, N. J., 2010.

[3] Chromatographic separation techniques, in: European Directorate for the Quality of Medicines & HealthCare (Eds.), European Pharmacopoeia, 8th Edition, Strasbourg, France, 2014, pp.

[4] Ph. Eur. 8th edition, Council of Europe, Strasbourg, France, 2016.

[5] L.S. Ettre, C.W. Gehrke, The development of the amino acid analyzer, LC GC N Am, 24 (2006) 390-400.

[6] P. Adriaens, B. Meesschaert, W. Wuyts, H. Vaderhaeghe, H. Eyssen, Separation of ninhydrin-positive compounds on a single-column amino acid analyzer using lithium buffers, J. Chromatogr., 140 (1977) 103-108.

[7] A. Wainer, The chromatography of ninhydrin negative compounds on an amino acid analyzer column, J. Chromatogr., 26 (1967) 48-53.

[8] E. Lund, J. Thomsen, K. Brunfeldt, Use of o-phthalaldehyde for fluorescence detection in conventional amino acid analyzers - Sub-nanomole sensitivity in analysis of phenylthiohydantoin-amino acids, J. Chromatogr., 130 (1977) 51-54.

[9] R.L. Cunico, T. Schlabach, Comparison of ninhydrin and o-phthalaldehyde post-column detection techniques for high-performance liquid chromatography of free amino acids, J. Chromatogr., 266 (1983) 461-470.

[10] S. Moore, W.H. Stein, Photometric ninhydrin method for use in the chromatography of amino acids, J. Biol. Chem., 176 (1948) 367-388.

[11] D.H. Spackman, W.H. Stein, S. Moore, Automatic recording apparatus for use in the chromatography of amino acids, Anal. Chem., 30 (1958) 1190-1206.

[12] S.R. Bakalyar, M.P.T. Bradley, R. Honganen, The Role of Dissolved Gases in High-Performance Liquid Chromatography, J. Chromatogr., 158 (1978) 277-293.

[13] M. Kohler, W. Haerdi, P. Christen, J.L. Veuthey, The evaporative light scattering detector: some applications in pharmaceutical analysis, Trac.-Trend. Anal. Chem., 16 (1997) 475-484.

[14] M. Righezza, G. Guiochon, Effect of the Wavelength of the Laser-Beam on the Response of an Evaporative Light-Scattering Detector, J. Liq. Chromatogr., 11 (1988) 2709-2729.

[15] J.M. Cintrón, D.S. Risley, Hydrophilic interaction chromatography with aerosol-based detectors (ELSD, CAD, NQAD) for polar compounds lacking a UV chromophore in an intravenous formulation, J. Pharm. Biomed. Anal., 78-79 (2013) 14-18.

[16] A.D. Yim, P.H. Gamache, R.S. McCarthy (Dionex Corporation), Nebulizer for charged aerosol detection (CAD) system, US 20140352411, 2014.

[17] J.P. Hutchinson, T. Remenyi, P. Nesterenko, W. Farrell, E. Groeber, R. Szucs, G. Dicinoski, P.R. Haddad, Investigation of polar organic solvents compatible with corona charged aerosol detection and their use for the determination of sugars by hydrophilic interaction liquid chromatography, Anal. Chim. Acta, 750 (2012) 199-206.

[18] U. Holzgrabe, C.J. Nap, T. Beyer, S. Almeling, Alternatives to amino acid analysis for the purity control of pharmaceutical grade L-alanine, J. Sep. Sci., 33 (2010) 2402-2410.

[19] P.K. Ragham, K.B. Chandrasekhar, Development and validation of a stability-indicating RP-HPLC-CAD method for gabapentin and its related impurities in presence of degradation products, J. Pharm. Biomed. Anal., 125 (2016) 122-129.

[20] X.K. Liu, J.B. Fang, N. Cauchon, P.Z. Zhou, Direct stability-indicating method development and validation for analysis of etidronate disodium using a mixed-mode column and charged aerosol detector, J. Pharm. Biomed. Anal., 46 (2008) 639-644.

[21] T. Vehovec, A. Obreza, Review of operating principle and applications of the charged aerosol detector, J. Chromatogr. A, 1217 (2010) 1549-1556.

[22] N. Vervoort, D. Daemen, G. Török, Performance evaluation of evaporative light scattering detection and charged aerosol detection in reversed phase liquid chromatography, J. Chromatogr. A, 1189 (2008) 92-100.

[23] M. Righezza, G. Guiochon, Effects of the Nature of the Solvent and Solutes on the Response of a Light-Scattering Detector, J. Liq. Chromatogr., 11 (1988) 1967-2004.

[24] J.P. Hutchinson, J.F. Li, W. Farrell, E. Groeber, R. Szucs, G. Dicinoski, P.R. Haddad, Universal response model for a corona charged aerosol detector, J. Chromatogr. A, 1217 (2010) 7418-7427.

[25] R.W. Dixon, D.S. Peterson, Development and testing of a detection method for liquid chromatography based on aerosol charging, Anal. Chem., 74 (2002) 2930-2937.

[26] T. Gorecki, F. Lynen, R. Szucs, P. Sandra, Universal response in liquid chromatography using charged aerosol detection, Anal. Chem., 78 (2006) 3186-3192.

[27] A. de Villiers, T. Gorecki, F. Lynen, R. Szucs, P. Sandra, Improving the universal response of evaporative light scattering detection by mobile phase compensation, J. Chromatogr. A, 1161 (2007) 183-191.

[28] B.T. Mathews, P.D. Higginson, R. Lyons, J.C. Mitchell, N.W. Sach, M.J. Snowden, M.R. Taylor, A.G. Wright, Improving quantitative measurements for the evaporative light scattering detector, Chromatographia, 60 (2004) 625-633.

[29] H. Zahn, Über das Leitvermögen von starken Elektrolyten für Hochfrequenzströme, Physik A Hadrons and Nuclei, 51 (1928) 350-354.

[30] E. Pungor, F. Pal, K. Toth, Oscillometric Flow Cell for Measurement of Conductivity and Permittivity, Anal. Chem., 55 (1983) 1728-1731.

[31] J.F. Alder, P.R. Fielden, A.J. Clark, Simultaneous Conductivity and Permittivity Detector with a Single Cell for Liquid-Chromatography, Anal. Chem., 56 (1984) 985-988.

[32] J.A.F. da Silva, C.L. do Lago, An oscillometric detector for capillary electrophoresis, Anal. Chem., 70 (1998) 4339-4343.

[33] A.J. Zemann, E. Schnell, D. Volgger, G.K. Bonn, Contactless conductivity detection for capillary electrophoresis, Anal. Chem., 70 (1998) 563-567.

[34] P. Jankovics, S. Chopra, M.N. El-Attug, D. Cabooter, K. Wolfs, B. Noszál, A. Van Schepdael, E. Adams, Exploring the possibilities of capacitively coupled contactless conductivity detection in combination with liquid chromatography for the analysis of polar compounds using aminoglycosides as test case, J. Pharm. Biomed. Anal., 112 (2015) 155-168.

[35] P. Hauser, E.A. Villar, J. Tanyanyiwa (Peter C. Hauser), Method and apparatus for performing high-voltage contactless conductivity (HV-CCD) electrophoresis, US 20050109621, 2005.

[36] A. Robbat, N.P. Corso, P.J. Doherty, M.H. Wolf, Gas-Chromatographic Chemiluminescent Detection and Evaluation of Predictive Models for Identifying Nitrated Polycyclic Aromatic-Hydrocarbons in a Diesel Fuel Particulate Extract, Anal. Chem., 58 (1986) 2078-2084.

[37] L.O. Courthaudon, E.M. Fujinari, Nitrogen-Specific Gas-Chromatography Detection Based on Chemiluminescence, LC GC-Mag. Sep. Sci., 9 (1991) 732-734.

[38] A. Robbat, N.P. Corso, T.Y. Liu, Evaluation of a Nitrosyl-Specific Gas-Phase Chemi-Luminescent Detector with High-Performance Liquid-Chromatography, Anal. Chem., 60 (1988) 173-174.

[39] X.W. Yan, Detection by ozone-induced chemiluminescence in chromatography, J. Chromatogr. A, 842 (1999) 267-308.

[40] W.L. Fitch, A.K. Szardenings, E.M. Fujinari, Chemiluminescent nitrogen detection for HPLC: An important new tool in organic analytical chemistry, Tetrahedron Lett., 38 (1997) 1689-1692.

[41] D. Corens, M. Carpentier, M. Schroven, L. Meerpoel, Liquid chromatography-mass spectrometry with chemiluminescent nitrogen detection for on-line quantitative analysis of compound collections: advantages and limitations, J. Chromatogr. A, 1056 (2004) 67-75.

[42] S.N. Bhattachar, J.A. Wesley, C. Seadeek, Evaluation of the chemiluminescent nitrogen detector for solubility determinations to support drug discovery, J. Pharm. Biomed. Anal., 41 (2006) 152-157.

[43] M. Styslo-Zalasik, W.Y. Li, Determination of topiramate and its degradation product in liquid oral solutions by high performance liquid chromatography with a chemiluminescent nitrogen detector, J. Pharm. Biomed. Anal., 37 (2005) 529-534.

[44] T. Cecchi, Theoretical Models of Ion Pair Chromatography: A Close Up of Recent Literature Production, J. Liq. Chromatogr. R T, 38 (2015) 404-414.

[45] T. Cecchi, Ion pairing chromatography, Crit. Rev. Anal. Chem., 38 (2008) 161-213.

[46] K. Horňák, H. Schmidheiny, J. Pernthaler, High-throughput determination of dissolved free amino acids in unconcentrated freshwater by ion-pairing liquid chromatography and mass spectrometry, J. Chromatogr. A, 1440 (2016) 85-93.

[47] M. Mateos-Vivas, E. Rodríguez-Gonzalo, D. García-Gómez, R. Carabias-Martínez, Hydrophilic interaction chromatography coupled to tandem mass spectrometry in the presence of hydrophilic ion-pairing reagents for the separation of nucleosides and nucleotide mono-, di- and triphosphates, J. Chromatogr. A, 1414 (2015) 129-137.

[48] Y. Jiang, Z. Xie, Determination of ibandronate and its degradation products by ion-pair RP LC with evaporative light-scattering detection, Chromatographia, 62 (2005) 257-261.

[49] M. Biba, C.J. Welch, J.P. Foley, Investigation of a new core-shell particle column for ion-pair reversed-phase liquid chromatography analysis of oligonucleotides, J. Pharm. Biomed. Anal., 96 (2014) 54-57.

[50] Z. Xie, Y. Jiang, D.Q. Zhang, Simple analysis of four bisphosphonates simultaneously by reverse phase liquid chromatography using n-amylamine as volatile ion-pairing agent, J. Chromatogr. A, 1104 (2006) 173-178.

[51] ECHA, Adopted opinions on restriction proposals. http://echa.europa.eu/previous-consultations-on-restriction-proposals/-/substance-rev/1908/term, (accessed 26/04/2016)

[52] J.Y. Li, S. Shao, M.S. Jaworsky, P.T. Kurtulik, Simultaneous determination of cations, zwitterions and neutral compounds using mixed-mode reversed-phase and cation-exchange high-performance liquid chromatography, J. Chromatogr. A, 1185 (2008) 185-193.

[53] H. Luo, L.J. Ma, C. Paek, P.W. Carr, Application of silica-based hyper-crosslinked sulfonate-modified reversed stationary phases for separating highly hydrophilic basic compounds, J. Chromatogr. A, 1202 (2008) 8-18.

[54] N.H. Davies, M.R. Euerby, D.V. McCalley, A study of retention and overloading of basic compounds with mixed-mode reversed-phase/cation-exchange columns in high performance liquid chromatography, J. Chromatogr. A, 1138 (2007) 65-72.

[55] T. Taylor, Mixed-Mode HPLC Separations: What, Why, and How, LC GC N. Am. 32 (2014) 226-226.

[56] H. Luo, L.J. Ma, Y. Zhang, P.W. Carr, Synthesis and characterization of silica-based hyper-crosslinked sulfonate-modified reversed stationary phases, J. Chromatogr. A, 1182 (2008) 41-55.

[57] O. Wahl, U. Holzgrabe, Impurity profiling of carbocisteine by HPLC-CAD, qNMR and UV/vis spectroscopy, J. Pharm. Biomed. Anal., 95 (2014) 1-10.

[58] R. Kühnreich, U. Holzgrabe, Impurity profiling of l-methionine by HPLC on a mixed mode column, J. Pharm. Biomed. Anal., 122 (2016) 118-125.

[59] C. Lamouroux, G. Foglia, G. Le Rouzo, How to separate ionic liquids: Use of Hydrophilic Interaction Liquid Chromatography and mixed mode phases, J. Chromatogr. A, 1218 (2011) 3022-3028.

[60] O. Wahl, U. Holzgrabe, Impurity profiling of ibandronate sodium by HPLC-CAD, J. Pharm. Biomed. Anal., 114 (2015) 254-264.

[61] H. Guo, L.S. Riter, C.E. Wujcik, D.W. Armstrong, Direct and sensitive determination of glyphosate and aminomethylphosphonic acid in environmental water samples by high performance liquid chromatography coupled to electrospray tandem mass spectrometry, J. Chromatogr. A, (2016)

[62] X.D. Liu, C.A. Pohl, Comparison of reversed-phase/cation-exchange/anion-exchange trimodal stationary phases and their use in active pharmaceutical ingredient and counterion determinations, J. Chromatogr. A, 1232 (2012) 190-195.

[63] A.M. Botero-Coy, M. Ibáñez, J.V. Sancho, F. Hernández, Direct liquid chromatography-tandem mass spectrometry determination of underivatized glyphosate in rice, maize and soybean, J. Chromatogr. A, 1313 (2013) 157-165.

[64] D. Ilko, C.J. Nap, U. Holzgrabe, S. Almeling, Validation and application of an HPLC-CAD-TOF/MS method for identification and quantification of pharmaceutical counterions, Pharmeur. Bio. Sci. Notes, 2014 (2014) 81-91.

[65] P. Kubica, J. Namieśnik, A. Wasik, Comparison of hydrophilic interaction and reversed phase liquid chromatography coupled with tandem mass spectrometry for the determination of eight artificial sweeteners and common steviol glycosides in popular beverages, J. Pharm. Biomed. Anal., (2016)

[66] E.W. Tsai, D.P. Ip, M.A. Brooks, Determination of Alendronate in Pharmaceutical Dosage Formulations by Ion Chromatography with Conductivity Detection, J. Chromatogr., 596 (1992) 217-224.

[67] L. Joergensen, A. Weimann, H.F. Botte, Ion Chromatography as a Tool for Optimization and Control of Fermentation Processes, J. Chromatogr., 602 (1992) 179-188.

[68] J. Cheng, P. Jandik, N. Avdalovic, Use of disposable gold working electrodes for cation chromatography-integrated pulsed amperometric detection of sulfur-containing amino acids, J. Chromatogr. A, 997 (2003) 73-78.

[69] C.Y. Hao, D. Morse, F. Morra, X.M. Zhao, P. Yang, B. Nunn, Direct aqueous determination of glyphosate and related compounds by liquid chromatography/tandem mass spectrometry using reversed-phase and weak anion-exchange mixed-mode column, J. Chromatogr. A, 1218 (2011) 5638-5643.

[70] A.M.V. Schou-Pedersen, C. Cornett, N. Nyberg, J. Østergaard, S.H. Hansen, Structure elucidation and quantification of impurities formed between 6-aminocaproic acid and the excipients citric acid and sorbitol in an oral solution using high-resolution mass spectrometry and nuclear magnetic resonance spectroscopy, J. Pharm. Biomed. Anal., 107 (2015) 333-340.

[71] J. Han, H. Moon, Y. Hong, S. Yang, W.J. Jeong, K.S. Lee, H. Chung, Determination of glyphosate and its metabolite in emergency room in Korea, Forensic Sci. Int., 265 (2016) 41-46.

[72] Y.Q. Tian, L.M. Chen, L.H. Gao, M.L. Wu, W.A. Dick, Comparison of three methods for detection of melamine in compost and soil, Sci. Total Environ., 417 (2012) 255-262.

[73] W. Kwon, J.Y. Kim, S. Suh, M.K. In, Simultaneous determination of creatinine and uric acid in urine by liquid chromatography-tandem mass spectrometry with polarity switching electrospray ionization, Forensic Sci. Int., 221 (2012) 57-64.

[74] D.J. Harvey, Derivatization of carbohydrates for analysis by chromatography; electrophoresis and mass spectrometry, J. Chromatogr. B, 879 (2011) 1196-1225.

[75] G.L. Li, J.M. You, Y.R. Suo, C.H. Song, Z.W. Sun, L.A. Xia, X.N. Zhao, J.Y. Shi, A developed pre-column derivatization method for the determination of free fatty acids in edible oils by reversed-phase HPLC with fluorescence detection and its application to Lycium barbarum seed oil, Food Chem., 125 (2011) 1365-1372.

[76] G.H. Zheng, W.W. Jin, P. Fan, X.N. Feng, Y. Bai, T. Tao, L.J. Yu, A novel method for detecting amino acids derivatized with phenyl isothiocyanate by high-performance liquid chromatography-electrospray ionization mass spectrometry, Int J. Mass Spectrom., 392 (2015) 1-6.

[77] G. Flesch, N. Tominaga, P. Degen, Improved Determination of the Bisphosphonate Pamidronate Disodium in Plasma and Urine by Precolumn Derivatization with Fluorescamine, High-Performance Liquid-Chromatography and Fluorescence Detection, J. Chromatogr. Biomed., 568 (1991) 261-266.

[78] M. Wedig, N. Novatchev, T. Worch, S. Laug, U. Holzgrabe, Evaluation of the impurity profile of alcuronium by means of capillary electrophoresis, J. Pharm. Biomed. Anal., 28 (2002) 983-990.

[79] N. Novatchev, U. Holzgrabe, Evaluation of amino sugar, low molecular peptide and amino acid impurities of biotechnologically produced amino acids by means of CE, J. Pharm. Biomed. Anal., 28 (2002) 475-486.

[80] Y. Bitar, U. Holzgrabe, Impurity profiling of atropine sulfate by microemulsion electrokinetic chromatography, J. Pharm. Biomed. Anal., 44 (2007) 623-633.

[81] C. Borst, U. Holzgrabe, Comparison of chiral electrophoretic separation methods for phenethylamines and application on impurity analysis, J. Pharm. Biomed. Anal., 53 (2010) 1201-1209.

[82] E.W. Taylor, M.G. Qian, G.D. Dollinger, Simultaneous on-line characterization of small organic molecules derived from combinatorial libraries for identity, quantity, and purity by reversed-phase HPLC with chemiluminescent nitrogen, UV, and mass spectrometric detection, Anal. Chem., 70 (1998) 3339-3347.

[83] P.H. Gamache, R.S. McCarthy, S.M. Freeto, D.J. Asa, M.J. Woodcock, K. Laws, R.O. Cole, HPLC analysis of non-volatile analytes using charged aerosol detection, LCGC N. Am., 18 (2005) 345-354.

[84] M.S. Qureshi, S.S. Bhongale, A.K. Thorave, Determination of organic acid impurities in lactic acid obtained by fermentation of sugarcane juice, J. Chromatogr. A, 1218 (2011) 7147-7157.

[85] U. Holzgrabe, C.J. Nap, S. Almeling, Control of impurities in l-aspartic acid and l-alanine by high-performance liquid chromatography coupled with a corona charged aerosol detector, J. Chromatogr. A, 1217 (2010) 294-301.

[86] K. Petritis, C. Elfakir, M. Dreux, A comparative study of commercial liquid chromatographic detectors for the analysis of underivatized amino acids, J. Chromatogr. A, 961 (2002) 9-21.

[87] U. Holzgrabe, C.J. Nap, N. Kunz, S. Almeling, Identification and control of impurities in streptomycin sulfate by high-performance liquid chromatography coupled with mass detection and corona charged-aerosol detection, J. Pharm. Biomed. Anal., 56 (2011) 271-279.

[88] R.W. Stringham, M. Pennell, W. Cabri, G. Carzana, F. Giorgi, S. Lalli, G. Marazzi, M. Torri, Identification of impurities in artemisinin, their behavior in high performance liquid chromatography and implications for the quality of derived anti-malarial drugs, J. Chromatogr. A, 1218 (2011) 6838-6842.

[89] D. Kyriakides, I. Panderi, Development and validation of a reversed-phase ion-pair high-performance liquid chromatographic method for the determination of risedronate in pharmaceutical preparations, Anal. Chim. Acta, 584 (2007) 153-159.

[90] H.J. Jia, W. Li, K. Zhao, Determination of risedronate in rat plasma samples by ion-pair high-performance liquid chromatography with UV detector, Anal. Chim. Acta, 562 (2006) 171-175.

[91] H. Kubo, Y. Kobayashi, T. Kinoshita, Fluorescence Determination of Streptomycin in Serum by Reversed-Phase Ion-Pairing Liquid-Chromatography, Anal. Chem., 58 (1986) 2653-2655.

[92] H. Wang, Z.J. Zhang, F. Xiong, L.Y. Wu, P. Li, W.C. Ye, Isolation and structure characterization of related impurities in etimicin sulfate by LC/ESI-MSn and NMR, J. Pharm. Biomed. Anal., 55 (2011) 902-907.

[93] V. Manyanga, O. Grishina, Z. Yun, J. Hoogmartens, E. Adams, Comparison of liquid chromatographic methods with direct detection for the analysis of gentamicin, J. Pharm. Biomed. Anal., 45 (2007) 257-262.

[94] R. Grahek, L. Zupančič-Kralj, Identification of gentamicin impurities by liquid chromatography tandem mass spectrometry, J. Pharm. Biomed. Anal., 50 (2009) 1037-1043.

[95] M.N. Kumar, K.S.R.P. Kumar, V.J. Kumar, S.J. Prasanna, H.K. Sharma, V.K. Reddy, Stability indicating ion chromatography method for the simultaneous determination of ibandronate sodium drug substance and its impurities, J. Pharm. Biomed. Anal., 54 (2011) 596-601.

[96] J.A.B. Rodriguez, M.F. Desimone, S.L. Iglesias, S.A. Giorgieri, L.E. Diaz, Validation of a capillary electrophoresis method for the analysis of ibandronate related impurities, J. Pharm. Biomed. Anal., 44 (2007) 305-308.

[97] A. Socia, J.P. Foley, Direct determination of amino acids by hydrophilic interaction liquid chromatography with charged aerosol detection, J. Chromatogr. A, (2016)

[98] A. Stojanovic, M. Lämmerhofer, D. Kogelnig, S. Schiesel, M. Sturm, M. Galanski, R. Krachler, B.K. Keppler, W. Lindner, Analysis of quaternary ammonium and phosphonium ionic liquids by reversed-phase high-performance liquid chromatography with charged aerosol detection and unified calibration, J. Chromatogr. A, 1209 (2008) 179-187.

[99] M.N. El-Attug, E. Adams, J. Hoogmartens, A. Van Schepdael, Capacitively coupled contactless conductivity detection as an alternative detection mode in CE for the analysis of kanamycin sulphate and its related substances, J. Sep. Sci., 34 (2011) 2448-2454.

[100] E. Kaale, A. Van Schepdael, E. Roets, J. Hoogmartens, Development and validation of a simple capillary zone electrophoresis method for the analysis of kanamycin sulfate with UV detection after pre-capillary derivatization, J. Chromatogr. A, 924 (2001) 451-458.

[101] V. Manyanga, R.L. Dhulipalla, J. Hoogmartens, E. Adams, Improved liquid chromatographic method with pulsed electrochemical detection for the analysis of kanamycin, J. Chromatogr. A, 1217 (2010) 3748-3753.

[102] A. Fanigliulo, P. De Filippis, O. Curcuruto, P. Repeto, D. Roveda, M. Hartenstein, E. Adams, D. Cabooter, Development and validation of a stability indicating method for S-carboxymethyl-L-cysteine and related degradation products in oral syrup formulation, J. Pharm. Biomed. Anal., 115 (2015) 39-47.

[103] H. Eastwood, F. Xia, M.C. Lo, J. Zhou, J.B. Jordan, J. McCarter, W.W. Barnhart, K.H. Gahm, Development of a nucleotide sugar purification method using a mixed mode column & mass spectrometry detection, J. Pharm. Biomed. Anal., 115 (2015) 402-409.

[104] L. Rystov, R. Chadwick, K. Krock, T. Wang, Simultaneous determination of Maillard reaction impurities in memantine tablets using HPLC with charged aerosol detector, J. Pharm. Biomed. Anal., 56 (2011) 887-894.

[105] K. Karthikeyan, G.T. Arularasu, V. Murali, K.C. Pillai, Identification, isolation, characterization and response factor determination of process-related impurity in meprobamate drug substance, J. Pharm. Biomed. Anal., 54 (2011) 208-212.

[106] A. Błażewicz, Z. Fijałek, K. Samsel, Determination of pipecuronium bromide and its impurities in pharmaceutical preparation by high-performance liquid chromatography with coulometric electrode array detection, J. Chromatogr. A, 1201 (2008) 191-195.

[107] A. Peepliwal, C.G. Bonde, K.G. Bothara, A validated RP-HPLC method for quantitative determination of related impurities of ursodeoxycholic acid (API) by refractive index detection, J. Pharm. Biomed. Anal., 54 (2011) 845-849.

[108] D. Lee, S. Rumbelow, S.K.R. Williams, Identification and quantitation of trace impurities in fatty alcohol ethoxylates using HPLC and MALDI-TOF mass spectrometry, Anal. Chim. Acta, 654 (2009) 59-63.

[109] C.E. Roy, T. Kauss, S. Prevot, P. Barthelemy, K. Gaudin, Analysis of fatty acid samples by hydrophilic interaction liquid chromatography and charged aerosol detector, J. Chromatogr. A, 1383 (2015) 121-126.

[110] I. Fradi, E. Farcas, A. Ben Said, M.L. Yans, C. Lamalle, G.W. Somsen, A. Prior, G.J. de Jong, M. Kallel, J. Crommen, A.C. Servais, M. Fillet, In-capillary derivatization with (-)-1-(9-fluorenyl)ethyl chloroformate as chiral labeling agent for the electrophoretic separation of amino acids, J. Chromatogr. A, 1363 (2014) 338-347.

[111] R.J. Bopp, J.H. Kennedy, Practical Considerations for Chiral Separations of Pharmaceutical Compounds, LC GC, 6 (1988) 514-522.

[112] W. Lindner, C. Leitner, G. Uray, Liquid-Chromatographic Separation of Enantiomeric Alkanolamines Via Diastereomeric Tartaric Acid Monoesters, J. Chromatogr., 316 (1984) 605-616.

[113] F. Lai, A. Mayer, T. Sheehan, Chiral Separation and Detection Enhancement of Propranolol Using Automated Precolumn Derivatization, J. Pharm. Biomed. Anal., 11 (1993) 117-120.

[114] H. Brückner, R. Wittner, H. Godel, Automated Enantioseparation of Amino-Acids by Derivatization with Ortho-Phthaldialdehyde and N-Acylated Cysteines, J. Chromatogr., 476 (1989) 73-82.

[115] H. Brückner, T. Westhauser, H. Godel, Liquid-Chromatographic Determination of D-Amino-Acids and L-Amino-Acids by Derivatization with O-Phthaldialdehyde and N-Isobutyryl-L-Cysteine - Applications with Reference to the Analysis of Peptidic Antibiotics, Toxins, Drugs and Pharmaceutically Used Amino-Acids, J. Chromatogr. A, 711 (1995) 201-215.

[116] S. Einarsson, S. Folestad, B. Josefsson, Separation of Amino-Acid Enantiomers Using Precolumn Derivatization with Ortho-Phthalaldehyde and 2,3,4,6-Tetra-O-Acetyl-1-Thio-Beta-Glucopyranoside, J. Liq. Chromatogr., 10 (1987) 1589-1601.

[117] A. Jegorov, J. Triska, T. Trnka, M. Cerny, Separation of Alpha-Amino-Acid Enantiomers by Reversed-Phase High-Performance Liquid-Chromatography after Derivatization with Ortho-Phthaldialdehyde and a Sodium-Salt of 1-Thio-Beta-D-Glucose, J. Chromatogr. Biomed. Appl., 434 (1988) 417-422.

[118] H. Brückner, C. Gah, High-Performance Liquid-Chromatographic Separation of dl-Amino Acids Derivatized with Chiral Variants of Sanger Reagent, J. Chromatogr., 555 (1991) 81-95.

[119] R. Bhushan, H. Brückner, Use of Marfey's reagent and analogs for chiral amino acid analysis: Assessment and applications to natural products and biological systems, J. Chromatogr. B, 879 (2011) 3148-3161.

[120] N. Nimura, H. Ogura, T. Kinoshita, Reversed-Phase Liquid-Chromatographic Resolution of Amino-Acid Enantiomers by Derivatization with 2,3,4,6-Tetra-O-Acetyl-Beta-D-Glucopyranosyl Isothiocyanate, J. Chromatogr., 202 (1980) 375-379.

[121] W.H. Pirkle, T.C. Pochapsky, Chiral Stationary Phases for the Direct LC Separation of Enantiomers, Adv. Chromatogr., 27 (1987) 73-127.

[122] W.H. Pirkle, T.C. Pochapsky, Considerations of Chiral Recognition Relevant to the Liquid-Chromatographic Separation of Enantiomers, Chem. Rev., 89 (1989) 347-362.

[123] K. Petritis, A. Valleix, C. Elfakir, M. Dreux, Simultaneous analysis of underivatized chiral amino acids by liquid chromatography-ionspray tandem mass spectrometry using a teicoplanin chiral stationary phase, J. Chromatogr. A, 913 (2001) 331-340.

[124] M.H. Hyun, J.S. Jin, W.J. Lee, Liquid chromatographic resolution of racemic amino acids and their derivatives on a new chiral stationary phase based on crown ether, J. Chromatogr. A, 822 (1998) 155-161.

[125] M.H. Hyun, J.S. Jin, S.C. Han, Y.J. Cho, The effect of analyte lipophilicity on the resolution of alpha-amino acids on a HPLC chiral stationary phase based on crown ether, Microchem. J., 70 (2001) 205-209.

[126] M.H. Hyun, S.C. Han, B.H. Lipshutz, Y.J. Shin, C.J. Welch, New chiral crown ether stationary phase for the liquid chromatographic resolution of alpha-amino acid enantiomers, J. Chromatogr. A, 910 (2001) 359-365.

[127] G. Galaverna, R. Corradini, E. Demunari, A. Dossena, R. Marchelli, Chiral Separation of Unmodified Amino-Acids by Ligand-Exchange High-Performance Liquid-Chromatography Using Copper(II) Complexes of L-Amino-Acid Amides as Additives to the Eluent, J. Chromatogr. A, 657 (1993) 43-54.

[128] T. Zhang, E. Holder, P. Franco, W. Lindner, Method development and optimization on cinchona and chiral sulfonic acid-based zwitterionic stationary phases for enantiomer separations of free amino acids by high-performance liquid chromatography, J. Chromatogr. A, 1363 (2014) 191-199.

[129] I. Ilisz, A. Aranyi, Z. Pataj, A. Péter, Recent advances in the direct and indirect liquid chromatographic enantioseparation of amino acids and related compounds: A review, J. Pharm. Biomed. Anal., 69 (2012) 28-41.

[130] X.Y. Mu, L. Qi, J. Qiao, X.Z. Yang, H.M. Ma, Enantioseparation of dansyl amino acids and dipeptides by chiral ligand exchange capillary electrophoresis based on Zn(II)-L-hydroxyproline complexes coordinating with gamma-cyclodextrins, Anal. Chim. Acta, 846 (2014) 68-74.

[131] X.Y. Mu, L. Qi, H.Z. Zhang, Y. Shen, J. Qiao, H.M. Ma, Ionic liquids with amino acids as cations: Novel chiral ligands in chiral ligand-exchange capillary electrophoresis, Talanta, 97 (2012) 349-354.

[132] A. Giuffrida, G. Maccarrone, V. Cucinotta, S. Orlandini, A. Contino, Recent advances in chiral separation of amino acids using capillary electromigration techniques, J. Chromatogr. A, 1363 (2014) 41-50.

[133] S. Terabe, K. Otsuka, H. Nishi, Separation of Enantiomers by Capillary Electrophoretic Techniques, J. Chromatogr. A, 666 (1994) 295-319.

[134] B.D. Wu, Q.Q. Wang, L. Guo, R. Shen, J.W. Xie, L.H. Yun, B.H. Zhong, Amino-substituted beta-cyclodextrin copper(II) complexes for the electrophoretic enantioseparation of dansyl amino acids: Role of dual chelate-inclusion interaction and mechanism, Anal. Chim. Acta, 558 (2006) 80-85.

[135] X.N. Lu, Y. Chen, L. Guo, Y.F. Yang, Chiral separation of underivatized amino acids by ligand-exchange capillary electrophoresis using a copper(II)-l-lysine complex as selector, J. Chromatogr. A, 945 (2002) 249-255.

[136] V. Cucinotta, A. Giuffrida, G. Grasso, G. Maccarrone, G. Vecchio, Ligand exchange chiral separations by cyclodextrin derivatives in capillary electrophoresis, Analyst., 128 (2003) 134-136.

[137] M.V. Rekharsky, Y. Inoue, Complexation thermodynamics of cyclodextrins, Chem. Rev., 98 (1998) 1875-1917.

[138] J.L. Clark, J.J. Stezowski, Molecular recognition in cyclodextrin complexes of amino acid derivatives. 1. Crystallographic studies of beta-cyclodextrin complexes with N-acetyl-L-phenylalanine methyl ester and N-acetyl-L-phenylalanine amide pseudopeptides, J. Am. Chem. Soc., 123 (2001) 9880-9888.

[139] B. Chankvetadze, G. Endresz, G. Blaschke, Charged cyclodextrin derivatives as chiral selectors in capillary electrophoresis, Chem. Soc. Rev., 25 (1996) 141-&.

[140] C. Schonbeck, P. Westh, R. Holm, Complexation Thermodynamics of Modified Cyclodextrins: Extended Cavities and Distorted Structures, J. Phys. Chem. B, 118 (2014) 10120-10129.

[141] P. Zakaria, M. Macka, P.R. Haddad, Selectivity control in the separation of aromatic amino acid enantiomers with sulphated beta-cyclodextrin, J. Chromatogr. A, 1031 (2004) 179-186.

[142] F. Kitagawa, K. Otsuka, Recent progress in capillary electrophoretic analysis of amino acid enantiomers, J. Chromatogr. B, 879 (2011) 3078-3095.

[143] Product information available from Wacker Chemie AG. http://www.wacker.com/cms/de/products/ (accessed 12/04/2016)

[144] B.D. Wagner, G.J. McManus, Enhancement of the fluorescence and stability of o-phthalaldehyde-derived isoindoles of amino acids using hydroxypropyl-beta-cyclodextrin, Anal. Biochem., 317 (2003) 233-239.

[145] C. Hellriegel, H. Händel, M. Wedig, S. Steinhauer, F. Sörgel, K. Albert, U. Holzgrabe, Study on the chiral recognition of the enantiomers of ephedrine derivatives with neutral and sulfated heptakis(2,3-O-diacetyl)-beta-cyclodextrins using capillary electrophoresis, UV, nuclear magnetic resonance spectroscopy and mass spectrometry, J. Chromatogr. A, 914 (2001) 315-324.

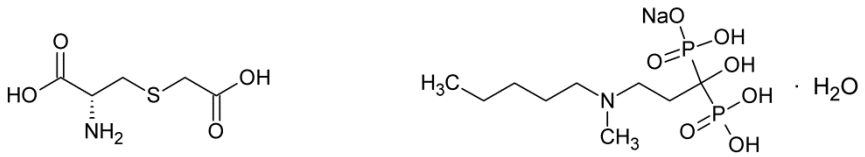
[146] M. Wedig, S. Laug, T. Christians, M. Thunhorst, U. Holzgrabe, Do we know the mechanism of chiral recognition between cyclodextrins and analytes?, J. Pharm. Biomed. Anal., 27 (2002) 531-540.

[147] X.N. Lu, Y. Chen, Chiral separation of amino acids derivatized with fluoresceine-5-isothiocyanate by capillary electrophoresis and laser-induced fluorescence detection using mixed selectors of beta-cyclodextrin and sodium taurocholate, J. Chromatogr. A, 955 (2002) 133-140.

# B. Aim of the work

The Ph. Eur. and the contained monographs are subject to constant change. New monographs are included and obsolete ones are updated or deleted. An important part of a monograph besides tests for the identification and the assay are tests for related substances and if applicable additional tests to cover other impurities. A test for related substances comprises usually a state of the art separation followed by an appropriate mean of detection. The separation power of the system has to be high enough to separate all relevant impurities. The sensitivity of the detection has to be sufficient to quantify impurities according to ICH guideline Q3A(R2) for small molecules. The limit is usually determined by the daily intake of the substance. The impurity profile of a substance has to be assessed prior to inclusion of new monographs and during the update of existing monographs preferably involving all relevant suppliers. The profile of related substances consists usually of process related impurities like starting material and by-products as well as degradation products.

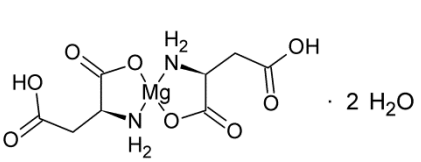
The revision of the Ph. Eur. monograph “Carbocisteine” and the introduction of the new monograph “Ibandronate sodium” demand for methods appropriate for the pharmacopoeial impurity profiling covering all process and degradation related impurities. Both substances and their respective related substances are rather simple polar molecules that do not contain a suitable chromophore for UV detection. Due to their zwitterionic character, the analytes as well as the related substances are charged all the time independent from pH and cannot be separated using reversed phase chromatography. The suitability of the CAD for this purpose should be demonstrated and the separation conditions like column and mobile phase were to be investigated, if possible avoiding ion-pairing chromatography. Both methods should eventually be validated and proposed to the expert groups dealing with the monograph revision of Carbocisteine and the creation of the new monograph for Ibandronate.



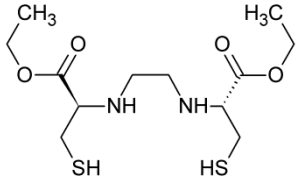
Carbocisteine



Ibandronate sodium



Magnesium aspartate



Bicisate

**Fig. 1.** Chemical structures of the compounds of interest

A method for the analysis of related substances of the $^{99m}$Tc chelating amino acid derivative Bicisate should be developed and validated. The substance is a rather lipophilic ester without strong chromophores. The related substances (precursors, by-products and degradants) are very hydrophilic, ionizable and some are semi-volatile. A suitable stationary phase should be combined with UV-CAD detection to cover all possible impurities.

The enantiomeric purity of magnesium-L-aspartate dihydrate was to be investigated. The reason for partial racemization during the synthesis should be studied and analytical methods appropriate for the purity assessment in the Ph. Eur. were to be developed and validated.

# C. Results

# 1. Impurity profiling of carbocisteine by HPLC-CAD, qNMR and UV/vis spectroscopy

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**ABSTRACT**

For the impurity profiling of the mucolytic and anti-inflammatory drug carbocisteine a high performance liquid chromatographic (HPLC) method using corona charged aerosol detection (CAD) was developed and fully validated following the ICH guideline Q2(R1). The response was linear ($R^2$> 0.995) over a small concentration range (0.05–0.25 or 0.10–0.60 % respectively) and a detection limit of at least 0.03% was registered. The separation was achieved on a mixed mode column combining hydrophobic C18 and strong cation exchange retention mechanisms using a mass spectrometer compatible volatile mobile phase consisting of trifluoroacetic acid 10 mM and acetonitrile 12 % (V/V). Impurities, not assessable by HPLC-CAD such as the volatile chloroacetic acid and the unstable cysteine, were determined by quantitative NMR (qNMR) with maleic acid as internal standard and UV/vis spectroscopy after reaction with Ellman's reagent, respectively. Six batches of three different manufacturers were tested by means of those methods. The purity varied from below 99.0 to higher than 99.8 per cent. The major impurities of all batches were the starting material cystine and *N,S*-dicarboxymethylcysteine being a synthesis by-product

*Abbreviations:* CAD, corona charged aerosol detector; COPD, chronic obstructive lung disease; Ph. Eur., European Pharmacopoeia; AAs, amino acids; OPA, ortho-phthalaldehyde; FMOC, fluorenylmethyloxycarbonyl chloride; DABS-Cl, dimethylaminoazobenzene-4-sulfonyl chloride; PITC, phenyl isothiocyanate; CBQCA, 3-(4-carboxybenzoyl)quinolone-2-carboxaldehyde; API, active pharmaceutical ingredient; AAA, amino acid analyzer; ELSD, evaporative light scattering detector; CLND, chemiluminescent nitrogen detector; qNMR, quantitative NMR; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); TFA, trifluoroacetic acid; ICH, International Conference on Harmonisation; $R^2$, coefficient of determination; S/N, signal-to-noise ratio.

## 1. Introduction

The non-proteinogenic amino acid carbocisteine is used as an anti-inflammatory mucolytic agent for the treatment of chronic obstructive lung disease (COPD) and asthma. Unlike *N*-acetylcysteine, the substance seems to interfere with the metabolism of mucus producing cells [1–3] and not with the phlegm itself.

Carbocisteine is synthesized by alkylation of *L*-cysteine with chloroacetic acid in aqueous sodium hydroxide solution [4, 5]. Until today the impurity profile of carbocisteine is assessed by means of a thin layer chromatography (TLC) test on ninhydrin-positive substances in the European Pharmacopoeia (Ph. Eur.) detecting amino acids (AAs) only. Not all related substances (see Fig. 1) originated by synthesis or caused by degradation are amino acids and therefore they are either not detected or the detection limit is unsatisfyingly high. Hence a state-of-the-art HPLC method for the separation and detection of carbocisteine and its impurities is urgently needed for the monograph.

The analysis of amino acids and their derivatives by means of HPLC has two challenges: the majority of those highly hydrophilic compounds are hardly retained on classical reversed phase columns and most of them lack an UV/vis light absorbing chromophore. One strategy to overcome these problems is the derivatization resulting in hydrophobic, UV-light absorbing or fluorescing compounds. Innumerous methods for the derivatization and separation of amino acids are known. The most common derivatizing agents used for AAs are ortho-phthalaldehyde (OPA) [6, 7], fluorenylmethyloxycarbonyl chloride (FMOC) [7–10], dimethylaminoazobenzene-4-sulfonyl chloride (DABS-Cl) [11, 12], phenyl isothiocyanate (PITC) [13], 3-(4-carboxybenzoyl)quinolone-2-carboxaldehyde (CBQCA) [10] and ninhydrin [14, 15]. Each has individual advantages and disadvantages. They all share one drawback: not all related substances contain the essential reactive amino moiety. In addition, pre-column derivatization often does not work quantitatively in the presence of a high excess of one AA, the active pharmaceutical ingredient (API), and may yield more than one product or unstable derivatives. Last but not least, the pre-column derivatization is a time consuming and therefore an expensive procedure. On the other hand the void volume of post-column derivatization loops often leads to band-broadening [16, 17] and the application requires dedicated instrumentation such as amino acid analyzers (AAAs).

Alternatives to UV or fluorescence detection are aerosol based detectors like the evaporative light scattering detector (ELSD), chemiluminescent nitrogen detector (CLND), corona charged aerosol detector (CAD) or any kind of mass spectrometer coupled to the HPLC system [18]. All techniques have in common that volatile mobile phase additives have to be used.

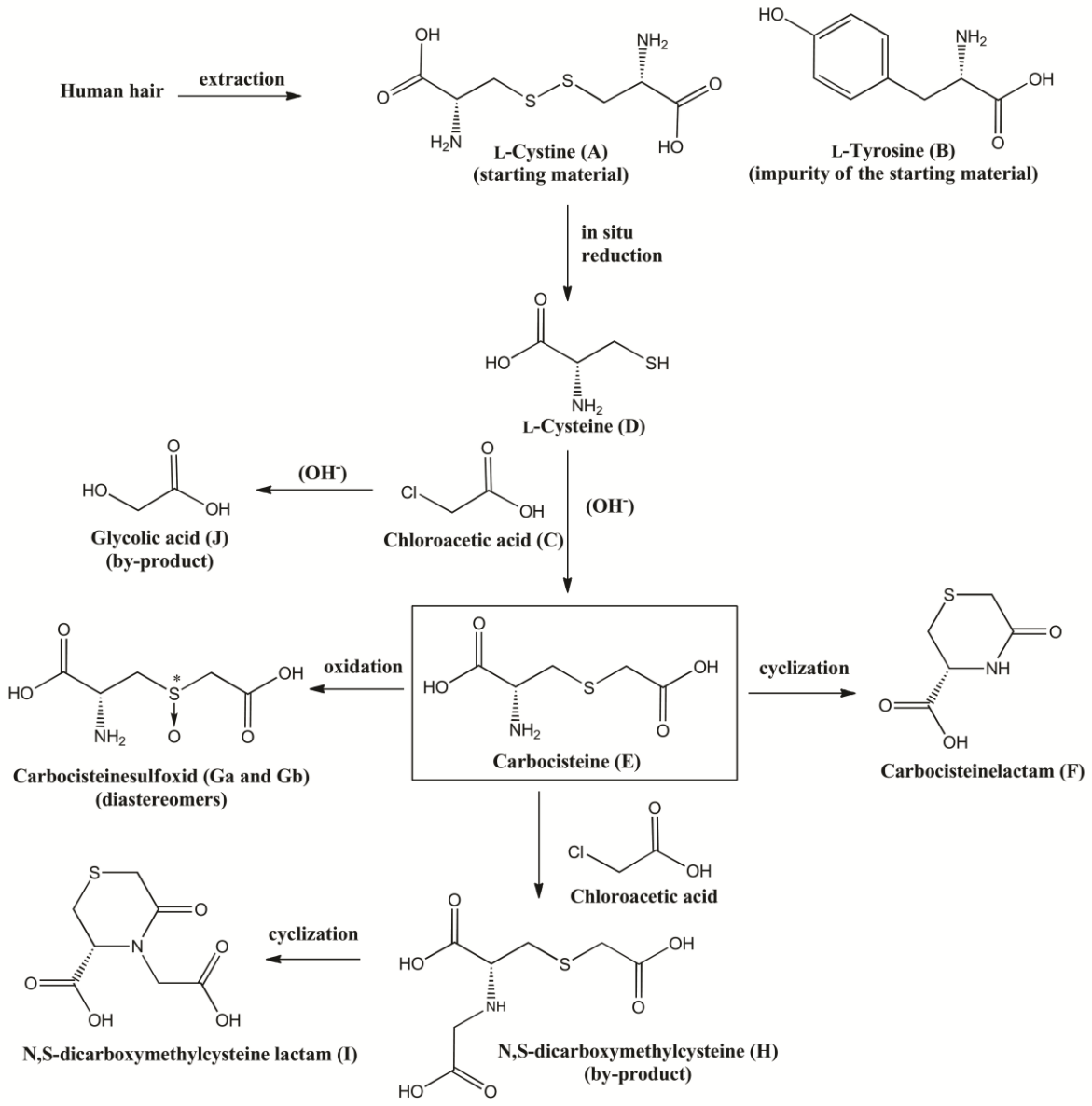


**Fig. 1.** Molecular structures of carbocisteine and its impurities.

The principle of the ELSD is spraying and drying the mobile phase, followed by detection of the light scattered by the resulting aerosol. The separation and detection of underivatized amino acids using this technique has been shown [19, 20]. However, the major disadvantages of the detector are the comparatively low sensitivity, non-linear response and spike peaks on the tail of the main peak [20] when it comes to impurity profiling.

When using CLND, the eluent is evaporated with oxygen and an inert gas (argon or helium), and pyrolyzed at high temperatures. Any nitrogen containing compound is converted to nitrogen monoxide, which reacts with ozone in the gas phase to excited nitrogen dioxide. The excited molecule drops to the ground state under infrared light emission. The response of this detector is linear over a broad concentration range and directly proportional to the

number of nitrogen atoms in the molecule [21, 22]. The CLND was successfully applied to the separation and detection of free amino acids when using a reversed phase chromatography with pentadecafluorooctanoic acid as an ion pair reagent [23].

The detection mechanism of the CAD comprises the formation of an aerosol of the column effluent and the transfer of positively charged nitrogen molecules to the aerosol particles with subsequent amperometric detection of those charged particles. The detector response is more or less independent of the molecular structure but highly dependent on the molecules' physical properties like vapor pressure so that only non-volatile substances can be detected [24]. Unlike the CLND, the CAD is able to detect a wider spectrum of substances, like organic acids or aminoglycosides [25], which makes it a good choice for a test for related substances [26]. In contrast to the ELSD, the CAD has an almost linear response in a small dedicated concentration range of about two orders of magnitude [27, 28] which is usually sufficient for impurity assessment when an external standard at an appropriate concentration is used. For both detectors, the response depends on the concentration of organic modifier in the mobile phase. This leads to a loss of the universal response, when running a gradient separation. A counter gradient can compensate for this loss of sensitivity [29].Alternatively an isocratic elution protocol has to be applied.

The aim of this study was to determine the content of carbocisteine and to assess the amount of its related substances, i.e. cystine, tyrosine, cysteine, chloroacetic acid, carbocisteinelactam, carbocisteinesulfoxid and N,S-dicarboxymethylcysteine (see Fig. 1). Since the CAD allows for volatile mobile phases only and volatile ion pairing agents create problems when it comes to validation, a mixed mode column with embedded strong cation exchanger, which made ion pair chromatography unnecessary, was used. The pros and cons of this method will be discussed.

## 2. Experimental

### *2.1. Chemicals and reagents*

The carbocisteine reference standard, all impurities (except C and J see Fig. 1), and batch samples were obtained from the European Directorate for the Quality of Medicines & Health-Care (EDQM) (Strasbourg, France). HPLC grade acetonitrile and 0.1 M hydrochloric acid were purchased from VWR International S.A.S. (Fontenay-sous-Bois, France), glycolic acid, trifluoroacetic acid, ammonium hydroxide solution (28–30%), 5,5'-dithiobis(2-nitrobenzoic acid), sodium deuteroxide (NaOD) 40 wt% in $D_2O$ (99.5% D-atom) and maleic acid standard for quantitative NMR (qNMR) (TraceCERT®) from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Deuterium oxide (99.9% D-atom) was obtained from Deutero GmbH (Kastellaun, Germany). Potassium dihydrogen phosphate was acquired from Grüssing GmbH (Filsum, Germany). We purchased sodium hydroxide solution 50% and chloroacetic acid from Merck KGaA (Darmstadt, Germany). All chemicals used for quantification were of analytical grade or even better. Ultra-pure water was produced by a water purification system from Merck Millipore (Schwalbach, Germany). All solutions were filtered through a 0.22 µm PTFE filter supplied by Machery-Nagel GmbH & Co. KG (Düren, Germany) prior to use.

### *2.2. Apparatus*

The HPLC was performed on an Agilent 1200 modular chromatographic system consisting of online vacuum degasser, binary pump, auto sampler, thermostatted column compartment and a photo-diode array detector (Agilent Technologies, Waldbronn, Germany).The Corona CAD detector (Thermo Fisher, Courtaboeuf, France) was linked to the HPLC system by a 0.25 mm internal diameter PEEK capillary and a 0.22 µm stainless steel inlet-frit. Highly pure nitrogen for the detector was produced by a Nitrogen Generator (Thermo Fisher, Courtaboeuf, France). The inlet pressure (nitrogen) was 35.0 psi. The peak areas were integrated automatically using the Agilent ChemStation® Rev B.03.02 software program.

The experiment with post-column addition of acetonitrile was performed on a Dionex UltiMate®3000 X2 chromatographic system (Dionex, Courtaboeuf, France) equipped with a ternary pump, an online degasser, a thermostatted autosampler, a thermostatted column compartment and a single wavelength UV/vis detector. Acetonitrile was added via a mixing-tee installed between detector and column. Detection was performed with a Corona CAD ultra RS (Thermo Fisher, Courtaboeuf, France). Gas inlet pressure (nitrogen) was 35.0 psi. The detection range was set to 100 pA and filter to "none". Nebulizer was set to 35 °C. The chromatograms were processed using Waters Empower®2 build 2154 software program.

Mass spectrometry experiments were performed on a LC/MSD Trap G2445D ESI ion trap (Agilent Technologies, Waldbronn, Germany) with an syringe pump KDS100 (KD Scientific, Holliston MA, USA) coupled to the electro spray ionization (ESI) interface. The flow rate was 40 µL/h. Nebulizer pressure: 15 psi, dry gas flow: 5 L/min, dry temperature: 325 °C, capillary voltage 3500 V, collision gas: helium. All NMR experiments were carried out on a Bruker Avance® (Karlsruhe, Germany; $^{1}$H 400.132 MHz $^{13}$C 100.613 MHz). The spectra were processed using Bruker TopSpin v3.0 software program. $^{1}$H NMR experiments were performed with 64 scans at a sample spinning frequency of 20 Hz, 300 K and a flip angle of 30 °, whereas $^{13}$C NMR was measured with 1024 scans with $^{1}$H decoupling and rotation (20 Hz). The experiments were carried out with broad band observer (BBO BB-H 5 mm) probe. The content of chloroacetic and glycolic acid (C and J) was determined with inverted broad band observer probe (BBI BB-H 5 mm) at 300 K using 32 scans without rotation and a flip angle of 30 ° observing the $^{1}$H nuclei. The relaxation delay between two scans was set to 60 s. Spectral width of 20.55 ppm and transmitter offset at 6.175 ppm was applied.

UV/vis absorption spectroscopy experiments were performed on a Shimadzu UVmini-1240 UV/vis spectroscop (Shimadzu Deutschland GmbH, Duisburg, Germany).

*2.3. Chromatographic procedure*

A mixed mode column SIELC Primesep®100 (250 mm × 4.6 mm i.d., with a particle size of 5 µm and pore size of 100 Å, SIELC Technologies, Prospect Heights IL, USA) was used as stationary phase. The Agilent chromatographic system was operated isocratically at 20 °C using a mobile phase composed of aqueous TFA (1 %, V/V)–acetonitrile–water (8:12:80, V/V/V), a flow-rate of 1.3 mL/min and CAD detection with the filter set to “high”. The injection volume was 20 µL.

*2.3.1 Preparation of solutions*

For the sample solutions 50 mg of carbocisteine were dissolved in 0.3 mL of a 30 g/L ammonia solution and diluted with water to 10.0 mL, resulting in a pH of approx. 8.75. The sample solutions were prepared immediately before analysis. The reference solution is produced by dilution of the sample solution 1:1000 (0.1%) with water. The system suitability solution is prepared as follows: 10 mg of carbocisteine, cystine and tyrosine were dissolved in 0.25 ml of a 40 g/L sodium hydroxide solution and diluted to 50.0 mL with water.5.0 mL of this solution were diluted to 100.0 mL with water. For the impurity stock solutions, 2.5 mg of each impurity, except tyrosine, were individually dissolved in water and diluted to 10.0 mL with the same solvent. 2.5 mg of tyrosine were dissolved in 0.15 mL of a 30 g/L ammonia solution and diluted to 10.0 mL with water. The stock solutions were stored at 2–8 °C,

protected from light and daily diluted to an appropriate concentration with water or were used for spiking sample solutions.

*2.4. Quantitative determination of chloroacetic and glycolic acid*

80.0 mg of carbocisteine and 2.5 mg of maleic acid were dissolved in a mixture of 720 µL $D_2O$ and 80 µL sodium deuteroxide (40 %, w/V in $D_2O$). The sample solutions were immediately subjected to quantitative NMR analysis. After manual phase and automatic baseline correction, the methylene singlet of chloroacetic acid at 4.07 ppm (2H), methylene singlet of glycolic acid at 3.95 ppm (2H) and the methine protons singlet of maleic acid at 6.02 ppm (2H) were integrated and quantified using the following relationship:

$$w(C,J) = \frac{MW(C,J)}{MW(IS)} \times \frac{A(C,J)}{A(IS)} \times \frac{m(IS)}{m(E)} \times 100 \quad (1)$$

where *MW(C, J)* and *MW(IS)* are the molecular weights in g/mol and *A(C, J)* and *A(IS)* are the areas for the selected NMR signals of the examined impurity *(C, J)* and maleic acid *(IS)*, respectively. The masses (weights) in mg of maleic acid *(IS)* and carbocisteine *(E)* are *m(IS)* and *m(E)*. The examined impurity content is then expressed by *w(C, J)* in per cent.

*2.5. NMR spectra of di-sodium N,S-dicarboxymethylcysteinelactam and the free acid*

Free acid (H): 10 mg of tri-sodium-N,S-dicarboxymethylcysteine in 700 µL deuterium oxide. This solution was subjected to NMR studies immediately after preparation. $^1$H NMR ($D_2O$, δ (ppm), J (Hz)): 2.77 (d, 2H, -C<u>H</u>$_2$CH-, J=6.4), 3.07-3.18 (m, 2H, HOOC-C<u>H</u>$_2$-S-), 3.20 (s, 2H, -NH-C<u>H</u>$_2$-COOH), 3.22 (t, 1H, -CH$_2$C<u>H</u>-, J=6.4). $^{13}$C NMR ($D_2O$, δ (ppm)): 34.89 (-<u>C</u>H$_2$CH-), 37.23 (-S-<u>C</u>H$_2$-COOH), 50.74 (-NH-<u>C</u>H$_2$-COOH), 61.99 (-CH$_2$<u>C</u>H-), 178.06 (HOO<u>C</u>-CH$_2$-S-), 178.52 (-CH$_2$CH-<u>C</u>OOH), 179.60 (-NH-CH$_2$-<u>C</u>OOH). MS (neg. ESI): m/z 236

Lactam (I): 9 mg of tri-sodium-N,S-dicarboxymethylcysteine were dissolved in 10 mL 0.1 M hydrochloric acid and stirred for two weeks at room temperature. The solution was neutralized with aqueous sodium hydroxide solution 0.1 M, the pH set to 8.75 and lyophilized. The residue was dissolved in 700 µL of deuterium oxide and subjected to NMR studies immediately. $^1$H NMR ($D_2O$, δ (ppm), J (Hz)): 3.05-3.09 (m, -C<u>H</u>$_2$CH-), 3.21 (d, 1H, J=17.4, S-C<u>H</u>$_2$-C=O), 3.22 (d, 1H, J=17.0, N-C<u>H</u>$_2$-COOH), 3.26-3.31 (m, -C<u>H</u>$_2$CH-), 3.57 (d, 1H, J=17.4, S-C<u>H</u>$_2$-C=O), 4.27 (t, 1H, J=3.8, -C<u>H</u>-CH$_2$-NC=O), 4.51 (d, 1H, J=17.1, N-C<u>H</u>$_2$-COOH). $^{13}$C NMR ($D_2O$, δ (ppm)): 28.18 (-<u>C</u>H$_2$CH-), 29.35 (S-<u>C</u>H$_2$-C=O), 52.45 (N-<u>C</u>H$_2$-COOH), 65.02 (O=CN-<u>C</u>H-CH$_2$), 168.33 (-N-<u>C</u>=O), 175.80 (-CH$_2$CH-<u>C</u>OOH), 176.50 (-N-CH$_2$-<u>C</u>OOH). MS (neg. ESI): m/z 218

*2.6. Determination of cysteine*

The test is based on the redox reaction of cysteine with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) at alkaline pH [30, 31], was developed and validated at Moehs Ibérica S.L. (Rubí, Spain) (personal communication). The absorbance at 410 nm was read exactly 300 s after the addition of 0.5 mL reagent solution to the sample, reference or blank solution. The content cysteine was calculated using the following equation:

$$w(D) = \frac{c(ref) \cdot Abs(sample)}{c(E) \cdot Abs(ref)} \times 100 \quad (2)$$

where *c(ref)* and *c(E)* are the concentrations in mg/mL of cysteine in the reference solution and carbocisteine in the sample solution, respectively. *Abs(sample)* and *Abs(ref)* are the absorbances of the sample and the reference solution. The content of cysteine is then expressed by *w(D)* in per cent.

*2.6.1. Preparation of solutions*

The buffer solution pH 8.0 was prepared by mixing 500.0 mL of a 0.2 M aqueous potassium dihydrogen phosphate solution with 468.0 mL of a 0.2 M aqueous sodium hydroxide solution. The resulting solution was diluted with water to 2000.0 mL. The sample solution was prepared by dissolving 200 mg of carbocisteine in 1.0 ml of a sodium hydroxide solution (40 g/L) and subsequent dilution to 20.0 mL with buffer solution pH 8.0. For the reference solution, 58.0 mg of cysteine hydrochloride monohydrate CRS were dissolved in buffer solution pH 8.0 and diluted to 100.0 mL with the same solvent. 0.25 mL of this solution was diluted to 20.0 ml with buffer solution pH 8.0. The reagent solution was prepared by dissolving 40.0 mg of DTNB in buffer solution pH 8.0 and subsequent dilution to 10.0 mL with the same solvent.

## 3. Results and discussion discussion

*3.1. HPLC method development*

*3.1.1. Chromatographic procedure*

The mixed mode column combines reversed phase retention mechanism with strong cation exchange due to the embedded sulfonic acid entities. When operating the column with acidic mobile phases, neutral compounds or carboxylic acids, i.e. carbocisteinelactam, are retained by the reversed phase mechanism whereas hydrophilic basic substances such as carbocisteine and cystine are separated by the cation exchange mechanism. Lipophilic basic com-pounds, i.e. tyrosine, are retained by both mechanisms. The influence of temperature, acetonitrile, trifluoroacetic acid (TFA) and flow-rate was studied in order to optimize the

separation and sensitivity. The acetonitrile concentration severely affects the retention of carbocisteine lactam, but has only minor impact on the retention of the amino acids except for tyrosine; however the peaks of tyrosine and cystine are swapped in the elution order while increasing acetonitrile content in the mobile phase (see Fig. 2). At 12 % (V/V) acetonitrile, the resolution between the early and the late eluting peaks was optimal. TFA concentrations slightly influence the retention times, but may affect peak shape and therefore resolution and detection limit. The TFA concentration controls the retention time of sodium and ammonium ions. A concentration of 10 mmol/L (approx. 0.08 %, V/V) TFA was chosen, because the peak shape of *N,S*-dicarboxymethylcysteine and the resolution of carbocisteine and the sodium peak were optimal (see Fig. 3). The application of weaker acids, such as formic or acetic acid, resulted in a heavy distortion of the *N,S*-dicarboxymethylcysteine (H) peak (data not shown). The flow-rate was set to 1.3 mL/min leading to as hort analysis time and an acceptable resolution. The temperature was adjusted to 20°C, because it gave the best signal-to-noise ratio. In order to lower the limit of quantification, we studied the effect of post-column addition of acetonitrile on the detector response. A dilute mixture of carbocisteine and all impurities was prepared and investigated with and without post-column acetonitrile. As expected, the enhanced evaporation of the column effluent leads to a strongly increased response (approx. by a factor of 2.5) for all impurities (see Fig. 4). Since this technique requires more sophisticated and special instrumentation, we decided to validate the method without post column addition of acetonitrile. Nevertheless, it was possible to meet the limit of quantification (LOQ) demanded by the European Pharmacopoeia (Ph. Eur.) for all impurities.

*3.1.2. Sample preparation*

Because the API is insoluble in water and unstable at low pH the samples were dissolved in a 30 g/L ammonia solution. Inorganic bases lead to large peaks due to the corresponding cation and interfere with the analysis. Acidic media, like the mobile phase, have to be avoided for sample preparation because of the formation of carbocisteinelactam (F) (see Fig. 1) at low pH.

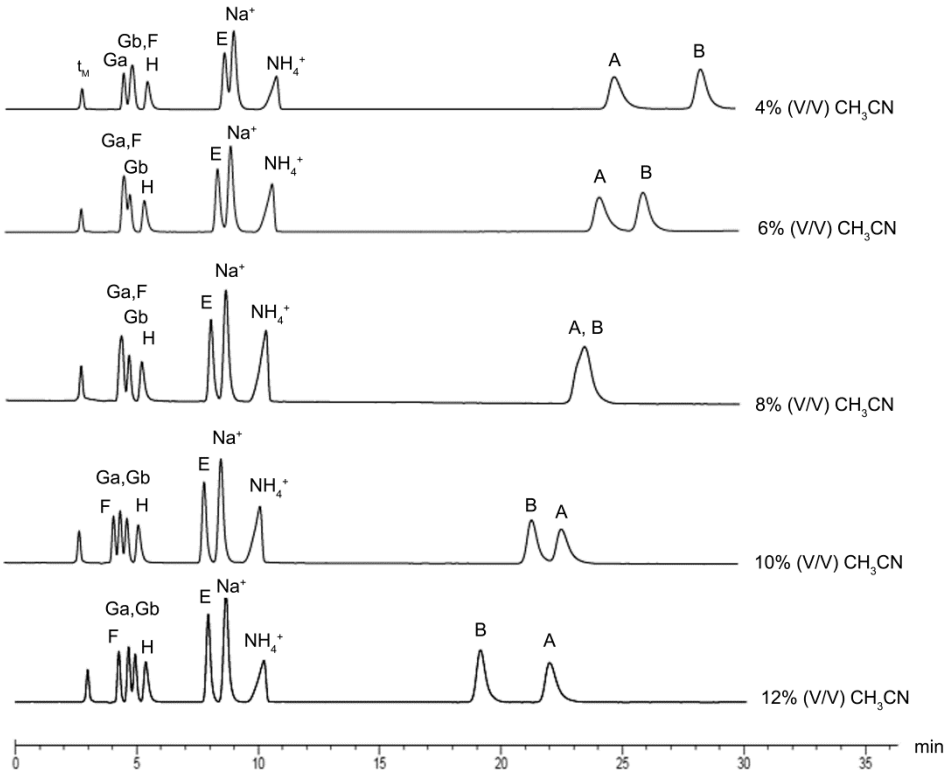


**Fig. 2.** Influence of the acetonitrile concentration in the mobile phase; TFA–acetonitrile–water (0.1:x:99.9−x, V/V/V); column temp: 30 °C; flow rate 1.0 ml/min; column: Primesep 100 250 mm × 4.6 mm 5 μm; detector: CAD; peak assignment: A: cystine, B: tyrosine, E: carbocisteine, F: carbocisteinelactam, Ga and Gb: carbocisteinesulfoxid, H: *N,S*-dicarboxymethylcysteine.

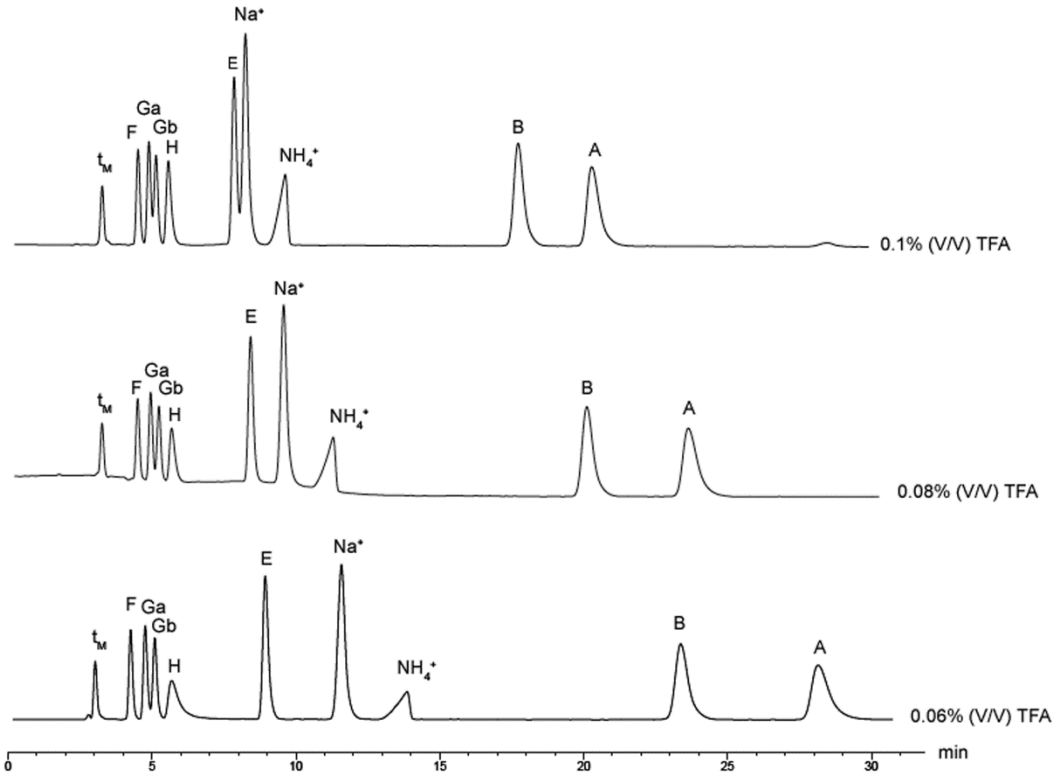


**Fig. 3.** Influence of TFA concentrations in the mobile phase; mobile phase: TFA–acetonitrile–water (x:12:88−x, V/V/V); column temp: 30 °C; flow rate 1.0 mL/min; column: Primesep 100 250 mm × 4.6 mm 5 μm; detector: CAD; peak assignment: see Fig. 2.

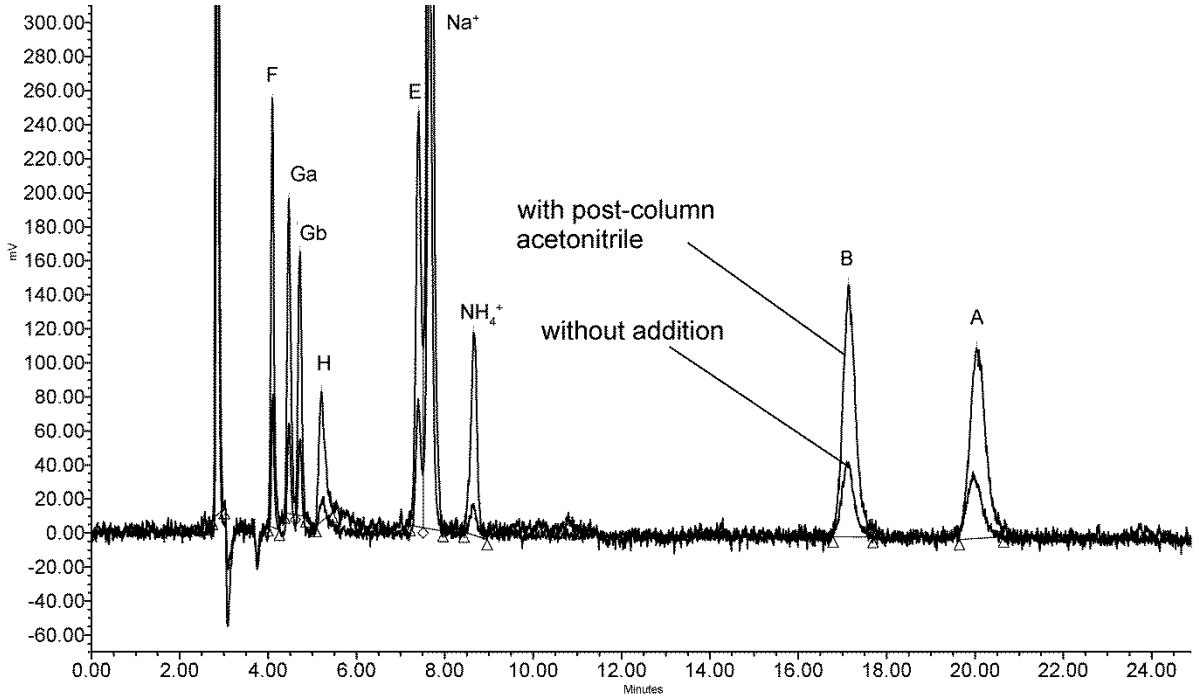


**Fig. 4.** The effect of post-column acetonitrile addition; mobile phase: TFA–acetonitrile–water (0.1:12:87.9, V/V/V); column temp: 30 °C; flow rate eluent: 1.0 mL/min; column: Primesep 100 250 mm × 4.6 mm 5 µm; detector: CAD; flow rate post column acetonitrile: 1.0 mL/min; peak assignment: see Fig. 2.

### *3.1.3. Stability of N,S-dicarboxymethylcysteine referencesubstance*

One manufacturer described stability problems with *N,S*-dicarboxymethylcysteine (H), because this substance, like carbocisteine, forms a lactam (I) under acidic conditions (see Fig. 1). The lactam of *N,S*-dicarboxymethylcysteine is usually not present in batch samples. The influence of the solutions pH on the formation of the lactam of *N,S*-dicarboxymethylcysteine were investigated by an HPLC-UV method and nuclear magnetic resonance (NMR) spectroscopy. The cyclization of *N,S*-dicarboxymethylcysteine was monitored by the appearance of the additional signals for the N-$CH_2$-COOH group resonating at δ = 4.51 and 3.22 ppm. The structure is supported by the peak of m/z 218 in the mass spectrum. In conclusion, carbocisteine and *N,S*-dicarboxymethylcysteine are rapidly cyclized in acidic solution (the peak area of the lactam doubles within 30 min) and more stable in slightly basic solutions (see Fig. 5). As a consequence the sample solution has to be prepared in dilute ammonia solution and immediately before injection. A cyclization on-column is likely, but would not cause additional peaks; the lactam is formed continuously, so that the baseline would rise slightly until the API is eluted. This was not observed and is therefore not considered to be an issue.

*3.2. Validation of the HPLC method*

The method was validated with regard to the following parameters: specificity, linearity, range, precision, accuracy, LOQ and robustness, following the International Conference on Harmonisation (ICH) guideline Q2(R1) [32]. System suitability criteria were defined and evaluated.

Specificity of the method was proven by comparing spiked samples with a blank solution. The resolution was at least 1.5 for every impurity peak (data not shown) and every impurity was separated from the main peak and from each other.

The linearity and range were determined by constructing calibration curves from 0.05 to 0.25 % for carbocisteinelactam, carbocisteinesulfoxid and tyrosine in the presence of 5 mg/mL carbocisteine with five levels equally distributed. The curves for *N,S*-dicarboxymethylcysteine and cystine ranged from 0.10 to 0.60 % because a higher amount of these impurities was expected according to previous experiments. Every level was injected in sextuple. The relative standard deviation (RSD) on every level for every impurity was below 5% and the coefficient of determination ($R^2$) for every curve was higher than 0.995. The LOQ and the LOD were calculated from the calibration curves according to ICH guideline Q2(R1) (see Table 1).

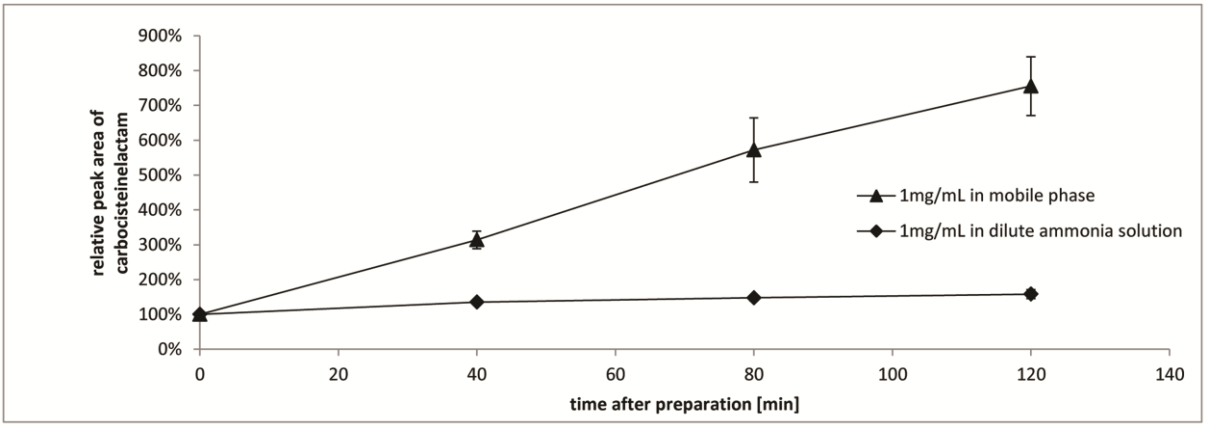


**Fig. 5.** The peak area of carbocisteinelactam in a 1 mg/ml solution of carbocisteine increases by more than 100 per cent after 30 min in the mobile phase. The error bars display the standard deviation (n = 2).

Accuracy was assessed on spiked sample solutions. The recovery rate was calculated at the lower end of the calibration curves, at the specification limit and on the upper end of the calibration curve. The recovery rates were found to be between 91 and 114 % (n = 3; RSD = 0.50–3.81 %) on every level. The quantification was done by comparing the carbocisteine peak area of the reference solution with the peak area of the impurities in the sample solution using the correction factors (see Table 1) obtained from the slopes of the calibration curves. Because the diastereomeric carbocisteinesulfoxids (Ga and Gb) are

separated with the method, it is necessary to sum up the peak areas of both peaks, if they occur concurrently.

**Table 1**
Correction factors obtained from the calibration curves and the relative retention to carbocisteine for all impurities

| Substance | $R^2$ | LOQ[a] | LOD[a] | correction factor | relative retention |
|---|---|---|---|---|---|
| Carbocisteinelactam | 0.9994 | 0.02 % | 0.006 % | 1.1 | 0.49 |
| Carbocisteinesulfoxid | 0.9996 | 0.02 % | 0.005 % | 1.0 | 0.55 and 0.58 |
| N,S-dicarboxymethylcysteine | 0.9995 | 0.04 % | 0.013 % | 1.4 | 0.65 |
| Tyrosine | 0.9983 | 0.03 % | 0.010 % | 0.7 | 2.70 |
| Cystine | 0.9970 | 0.09 % | 0.027 % | 0.7 | 3.13 |

[a] in per cent of the test solutions concentration

Repeatability and precision were determined on a real batch sample and on one spiked batch sample, because no batch contained carbocisteinelactam, carbocisteinesulfoxid and tyrosine above the LOQ. The impurity content was measured in sextuple on two different days. The RSD intra-day was between 2.3 and 5.0 % (n = 6) and inter-day determined on two consecutive days between 2.7 and 5.0 % (n = 12).

The stability of the sample solution was examined by measuring a sample once every hour for 5 h while storing it at room temperature. Using the statistical trend test of Neumann [33], no trend was detected for carbocisteinesulfoxid, *N,S*-dicarboxymethylcysteine, tyrosine and cystine. Due to the formation of carbocisteinelactam (see Fig. 1) an increase of its peak area was observed.

For checking the robustness, the operation parameters were varied in the following ranges: temperature ±5 °C, flow rate ±0.1 mL/min, CAD-filter setting: none, low, medium, high; acetonitrile content ±1 % (V/V) and TFA concentration ±2 mmol/L. An aqueous model solution containing all impurities at relevant level and carbocisteine was analyzed under either condition (see Table 2). The impurities were quantified by comparison of their peak area with the area of carbocisteine using the corresponding correction factors (see Table 1). The TFA concentration was found to be critical for good resolution between carbocisteine and sodium. This parameter is controlled in the system suitability test, because if the concentration was too high or too low, the test would fail. The relative recovery rate to the unmodified operation parameters ranged from +2 % to +48 % for *N,S*-dicarboxy-methylcysteine and from −19 % to +20 % for every other impurity. Temperature, CAD-filter setting, acetonitrile content and flow rate had no impact on recovery rate or resolution and the method is therefore considered to be robust against those influences.

**Table 2**
Method parameter robustness study – influence on critical parameters: recovery rate, resolution and signal-to-noise-ratio (S/N)

| Trifluoroacetic acid | | Acetonitrile | | CAD filter setting | | | temperature | | flow rate | | no variation | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 12mM | 8mM | 13 % | 11 % | medium | low | none | 25 °C | 15 °C | 1.4mL/min | 1.2mL/min | | | |
| 118 | 118 | 179 | 62 | 112 | 72 | 76 | 104 | 111 | 106 | 146 | 118 | S/N | Cystine 0.40 % [a] |
| 3.5 | 4.9 | 5.4 | 2.8 | 4.4 | 4.3 | 4.4 | 4.7 | 3.8 | 4.2 | 4.4 | 4.3 | Resolution | |
| 112 % | 95 % | 97 % | 104 % | 100 % | 101 % | 101 % | 102 % | 103 % | 102 % | 103 % | 100 % | recovery rate | |
| 30 | 29 | 47 | 17 | 30 | 20 | 21 | 29 | 29 | 29 | 41 | 33 | S/N | Tyrosine 0.09 % [a] |
| 18.9 | 16 | 16.7 | 17.6 | 17.2 | 17 | 23 | 15.4 | 18.8 | 16.9 | 17 | 17.2 | Resolution | |
| 102 % | 81 % | 84 % | 108 % | 101 % | 100 % | 94 % | 101 % | 97 % | 109 % | 101 % | 100 % | recovery rate | |
| 1.3 | 2.7 | 3.5 | 3.4 | 4.3 | 4.5 | 4.6 | 2.6 | 3.1 | 3.5 | 3.7 | 3.6 | S/N | Sodium |
| 54 | 70 | 87 | 32 | 65 | 45 | 48 | 51 | 59 | 53 | 78 | 62 | S/N | Carbo-cisteine 0.1 % [a] |
| 7.7 | 7.1 | 7.5 | 8.5 | 9.4 | 9.7 | 10 | 8 | 8.3 | 7.8 | 8.4 | 8.2 | Resolution | |
| 69 | 41 | 87 | 26 | 52 | 35 | 37 | 43 | 51 | 47 | 63 | 50 | S/N | N,S-dicarboxy methyl cysteine 0.13 % [a] |
| 1.6 | 1.5 | 1.4 | 1.6 | 1.9 | 2 | 2 | 1.4 | 1.7 | 1.5 | 1.6 | 1.6 | Resolution | |
| 148 % | 105 % | 128 % | 102 % | 105 % | 103 % | 108 % | 108 % | 109 % | 109 % | 105 % | 100 % | recovery rate | |
| 22 | 30 | 42 | 12 | 30 | 23 | 24 | 21 | 24 | 21 | 31 | 24 | S/N | Sulfoxid B 0.03 % [a] |
| 1.1 | 1.3 | 1.2 | 1.2 | 1.7 | 1.9 | 1.9 | 1.1 | 1.3 | 1.2 | 1.3 | 1.2 | Resolution | |
| 113 % | 113 % | 120 % | 103 % | 100 % | 103 % | 110 % | 110 % | 103 % | 103 % | 117 % | 100 % | recovery rate | |
| 25 | 34 | 47 | 14 | 34 | 26 | 29 | 24 | 27 | 24 | 35 | 27 | S/N | Sulfoxid A 0.03 % [a] |
| 1.4 | 1.6 | 1.8 | 1.2 | 2.3 | 2.7 | 2.7 | 1.6 | 1.5 | 1.5 | 1.7 | 1.6 | Resolution | |
| 115 % | 109 % | 112 % | 106 % | 100 % | 103 % | 109 % | 106 % | 103 % | 100 % | 103 % | 100 % | recovery rate | |
| 39 | 55 | 71 | 23 | 59 | 45 | 50 | 38 | 43 | 39 | 58 | 46 | S/N | Lactam 0.06 % [a] |
| - | - | - | - | - | - | - | - | - | - | - | - | Resolution | |
| 109 % | 105 % | 105 % | 105 % | 102 % | 102 % | 103 % | 107 % | 102 % | 103 % | 103 % | 100% | recovery rate | |

[a] in per cent of the test solutions concentration

For the establishment of a system suitability test, the resolution between carbocisteine and sodium as well as between tyrosine and cystine was studied. Sodium is always present because it is dis-solved from the utilized glassware and is detectable by the CAD. Thus, we had to ensure that the peak is separated from carbocisteine, since it is used for the quantification of the impurities. The resolution between the peaks due to carbocisteine and sodium is at least 3.0 controlled by the TFA concentration, whereas the acetonitrile content controls the resolution between tyrosine and cystine. It should be at least 3.5.

*3.3. Other impurities*

The alkylation reagent chloroacetic acid cannot properly be detected by the CAD because the compound is volatile. The substance is detectable with HPLC-UV in very concentrated sample solutions at a low wavelength (λ = 210 nm). The same holds true for its degradation product glycolic acid. Also, both impurities are well visible in the $^{1}$H NMR spectrum at 4.07 and 3.95 ppm (see Fig. 6) for the methylene group of chloroacetic acid and glycolic acid, respectively. By means of integration of these signals, the amount of both impurities could be calculated in comparison to maleic acid as an internal standard. The method was validated for chloroacetic acid only, because the amount of glycolic acid detected in batch samples was very low and thus of minor interest. The linearity and range were determined from 0.05 to 1.0 per cent ($R^2$= 0.9996). The method was found to be precise [RSD = 3.7 % (n = 6) measured in a batch sample containing 0.09 per cent chloroacetic acid] and accurate (proportional systematic error 1.5 % and constant systematic error 0.1 ppm). The accuracy was determined on a spiked batch sample. The spiked concentrations of 0.05, 0.10 and 0.15 per cent were measured in triplicate; the recovery rates ranged from 99 to 101 %. The LOQ determined from the slope of the calibration curve according to ICH guideline Q2(R1) is 0.03 per cent (S/N was 165 at this level). However, the sample solution is unstable. The integral of the chloroacetic acid signal decreases by almost 40% within 2 h after sample preparation while the glycolic acid signal increases. Cysteine on the other hand could selectively be determined by UV/vis spectroscopy at 410 nm after the reaction with Ellman's reagent (DTNB) at alkaline pH. This method was validated at Moehs Ibérica S.L. (Spain). The linearity was determined over the range of 0.025–0.2 per cent ($R^2$= 0.9985). The inter- and intra-day precision was demonstrated by RSDs of 8.6 and 1.8 %. (n = 14 and n = 6). The accuracy was determined in between 0.05 and 0.2 percent (recovery rates ranging from 98 to 102 %). The LOQ calculated from the regression line is 0.04 per cent. The results of the batch tests are displayed in Table 3 together with those of the HPLC-CAD experiments.

### *3.4. Batch results*

Several batch samples from different manufacturers were tested using this HPLC method, qNMR and UV/vis absorption spectroscopy. The results (Table 3) indicate that there is a typical impurity profile for each manufacturer. Whereas the batches of manufacturer a contain substantial amounts of *N,S*-dicarboxymethylcysteine and cystine, the batch from manufacturer c contains only N,S-dicarboxymethylcysteine and chloroacetic acid above the qualification limit of 0.1 %, no impurity was found to be above 0.1 % in the samples of manufacturer b.

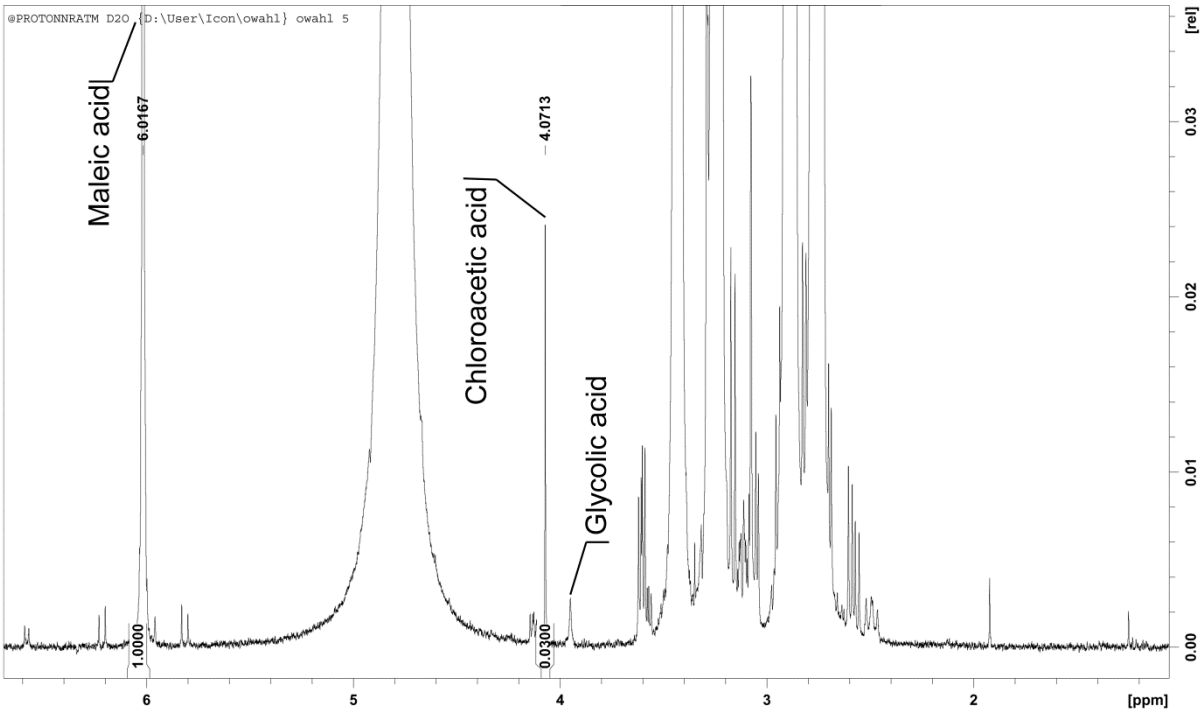


**Fig. 6.** $^{1}$H NMR spectrum of the quantitative determination of chloroacetic and glycolic acid in carbocisteine. Concentration: 100 mg/mL carbocisteine in 1 M NaOD in $D_2O$.

### *3.5. Method transfer to cystine*

The HPLC method could also be applied to cystine as a test for related substances. Cystine is technically produced from hair and horn [34] and is contaminated with tyrosine if purification fails. To achieve satisfactory resolution we had to slightly adapt the mobile phase. The TFA concentration was raised from 10 mmol/L to 15 mmol/L whereas the acetonitrile content was elevated from 12 to 25 % (V/V). The sample solvent was switched to hydrochloric acid since this solvent is volatile and cystine is stable under acidic conditions. The method is not only suitable for CAD detection, but also for UV detection at 275 nm (see Fig. 7).

**Table 3**
Results of batch testing using the HPLC method described under 2.3.

| **Manufacturer** | **a** | | **b** | | | **c** |
|---|---|---|---|---|---|---|
| **Batch** | **1** | **2** | **1** | **2** | **3** | **1** |
| **Cystine (A)** | 0.37% | 0.41% | n.d. | n.d. | n.d. | 0.09% |
| **Tyrosine (B)** | 0.03% | <0.03% | <0.03% | n.d. | <0.03% | <0.03% |
| **Chloroacetic acid (C)**[a] | 0.08% | 0.07% | 0.04% | 0.04% | 0.03% | 0.17% |
| **Cysteine (D)**[b] | < 0.04% | < 0.04% | < 0.04% | < 0.04% | < 0.04% | < 0.04% |
| **Lactam (F)** | 0.02% | 0.03% | <0.02% | <0.02% | <0.02% | 0.02% |
| **Sulfoxid (Ga, Gb)** | <0.02% | <0.02% | <0.02% | <0.02% | <0.02% | <0.02% |
| **N,S-dicarboxymethylcysteine (H)** | 0.53% | 0.55% | 0.06% | 0.05% | 0.06% | 0.61% |
| **Glycolic acid (J)**[a] | <0.05% | <0.05% | n.d. | n.d. | n.d. | <0.05% |
| **Unspecified impurity at rel. RT 2.6** | n.d. | n.d. | n.d. | n.d. | n.d. | 0.05% |
| **Sum** | 1.03% | 1.06% | 0.12% | 0.11% | 0.11% | 0.94% |

[a] determined by qNMR as described under 2.4
[b] determined by UV absorption spectroscopy after reaction with Ellman's reagent (see 2.6)

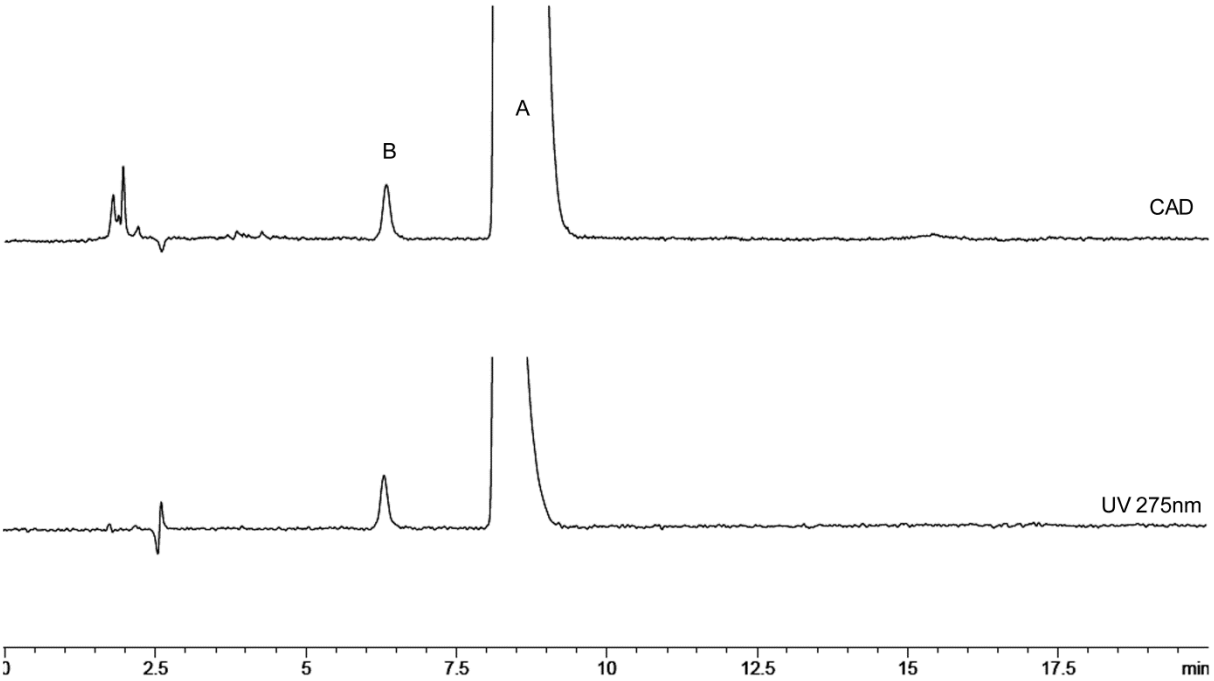


**Fig. 6.** Chromatogram of a 5 mg/mL cystine sample solution spiked with 0.05 % tyrosine in 0.1 N hydrochloric acid; mobile phase: TFA–acetonitrile–water (0.12:25:74.88,V/V/V); column temp: 20 °C; flow rate 1.5 mL/min; column: Primesep 100 250 mm × 4.6 mm 5 µm; detector: Dionex esa CAD filter: none inline after Agilent PDA detector; peak assignment: A: cystine, B: tyrosine.

## 4. Conclusion

In this study a C18 reversed phase cation exchange mixed mode HPLC method using 10 mmol/L of TFA, acetonitrile and corona charged aerosol detection for the purity control of carbocisteine was developed and validated. The method was capable of separating and quantifying the main related compounds of carbocisteine. We furthermore studied the stability of the impurity *N,S*-dicarboxymethylcysteine and confirmed the cyclization product of this impurity. The HPLC-CAD method described in this study represents a suitable

alternative to amino acid analyses and HPLC-UV detection. It is not only controlling related amino acids but also other related process and degradation impurities like carbocisteinelactam. The universal response allows for quantification without the need for an external standard. The method is considered suitable for the related substances evaluation to replace the currently described TLC test.

## Conflict of interest

None of the authors of this paper does have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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## References

[1] M. Inoue, Y. Ishibashi, H. Nogawa, T. Yasue, Carbocisteine promotes phagocytosis of apoptotic cells by alveolar macrophages, Eur. J. Pharmacol. 677 (2012)173–179.

[2] J.P. Zheng, J. Kang, S.G. Huang, P. Chen, W.Z. Yao, L. Yang, C.X. Bai, C.Z. Wang,C. Wang, B.Y. Chen, Y. Shi, C.T. Liu, P. Chen, Q. Li, Z.S. Wang, Y.J. Huang, Z.Y.Luo, F.P. Chen, J.Z. Yuan, B.T. Yuan, H.P. Qian, R.C. Zhi, N.S. Zhong, Effect of carbocisteine on acute exacerbation of chronic obstructive pulmonary disease (PEACE Study): a randomised placebo-controlled study, Lancet 371 (2008) 2013–2018.

[3] E. Puchelle, F. Aug, J.M. Polu, Effect of mucoregulator S-carboxy-methyl-cysteine in patients with chronic-bronchitis, Eur. J. Clin. Pharmacol. 14 (1978) 177–184.

[4] M.D. Armstrong, J.D. Lewis, Thioether derivatives of cysteine and homocysteine, J. Org. Chem. 16 (1951) 749–753.

[5] L. Goodman, L.O. Ross, B.R. Baker, Potential anticancer agents. 5. Some sulfur-substituted derivatives of cysteine, J. Org. Chem. 23 (1958) 1251–1257.

[6] J.R. Benson, P.E. Hare, *o*-phthalaldehyde: fluorogenic detection of primaryamines in the picomole range. Comparison with fluorescamine and ninhydrin, Proc. Natl. Acad. Sci. U. S. A. 72 (1975) 619–622.

[7] I. Molnar-Perl, Advancement in the derivatizations of the amino groups with the *o*-phthaldehyde-thiol and with the 9-fluorenylmethyloxycarbonyl chloride reagents, J. Chromatogr. B 879 (2011) 1241–1269.

[8] A. Jambor, I. Molnar-Perl, Amino acid analysis by high-performance liquid chromatography after derivatization with 9-fluorenylmethyloxycarbonyl chloride Literature overview and further study, J. Chromatogr. A 1216 (2009) 3064–3077.

[9] N. Novatchev, U. Holzgrabe, Evaluation of the impurity profile of amino acids by means of CE, J. Pharm. Biomed. Anal. 26 (2001) 779–789.

[10] S. Kopec, U. Holzgrabe, Amino acids: aspects of impurity profiling by means of CE, Electrophoresis 28 (2007) 2153–2167.

[11] E.H.J.M. Jansen, R.H. Vandenberg, R. Bothmiedema, L. Doorn, Advantages and limitations of precolumn derivatization of amino-acids with dabsyl chloride, J.Chromatogr. A 553 (1991) 123–133.

[12] S. Hernandez-Cassou, J. Saurina, Derivatization strategies for the determination of biogenic amines in wines by chromatographic and electrophoretic techniques, J. Chromatogr. B 879 (2011) 1270–1281.

[13] B.A. Bidlingmeyer, S.A. Cohen, T.L. Tarvin, Rapid analysis of amino acids using pre-column derivatization, J. Chromatogr. B 336 (1984) 93–104.

[14] S.W. Sun, Y.C. Lin, Y.M. Weng, M.J. Chen, Efficiency improvements on ninhydrin method for amino acid quantification, J. Food Compos. Anal. 19 (2006) 112–117.

[15] S. Yokoyama, J. Hiramatsu, A modified ninhydrin reagent using ascorbic acid instead of potassium cyanide, J. Biosci. Bioeng. 95 (2003) 204–205.

[16] C.K. Zacharis, P.D. Tzanavaras, Liquid chromatography coupled to on-line postcolumn derivatization for the determination of organic compounds: a review on instrumentation and chemistries, Anal. Chim. Acta 798 (2013) 1–24.

[17] R.W. Frei, L. Michel, W. Santi, New aspects of post-column derivatization in high-performance liquid-chromatography, J. Chromatogr. A 142 (1977) 261–270.

[18] U. Holzgrabe, C.J. Nap, T. Beyer, S. Almeling, Alternatives to amino acid analysis for the purity control of pharmaceutical grade L-alanine, J. Sep. Sci. 33 (2010) 2402–2410.

[19] H.J.C. das Neves, Z.B. Morais, HPLC assay of underivatized free amino acids with column switching and evaporative light-scattering detection, J. High Resolut. Chromatogr. 20 (1997) 115–118.

[20] S. Almeling, U. Holzgrabe, Use of evaporative light scattering detection for the quality control of drug substances: influence of different liquid chromatographic and evaporative light scattering detector parameters on the appearance of spike peaks, J. Chromatogr. A 1217 (2010) 2163–2170.

[21] X.W. Yan, Detection by ozone-induced chemiluminescence in chromatography, J. Chromatogr. A 842 (1999) 267–308.

[22] W.L. Fitch, A.K. Szardenings, E.M. Fujinari, Chemiluminescent nitrogen detection for HPLC: an important new tool in organic analytical chemistry, Tetrahedron Lett. 38 (1997) 1689–1692.

[23] K. Petritis, C. Elfakir, M. Dreux, A comparative study of commercial liquid chromatographic detectors for the analysis of underivatized amino acids, J. Chromatogr. A 961 (2002) 9–21.

[24] B. Zhang, X. Li, B. Yan, Advances in HPLC detection – towards universal detection, Anal. Bioanal. Chem. 390 (2008) 299–301.

[25] U. Holzgrabe, C.J. Nap, N. Kunz, S. Almeling, Identification and control of impurities in streptomycin sulfate by high-performance liquid chromatography coupled with mass detection and corona charged-aerosol detection, J. Pharm.Biomed. Anal. 56 (2011) 271–279.

[26] U. Holzgrabe, C.J. Nap, S. Almeling, Control of impurities in L-aspartic acid and L-alanine by high-performance liquid chromatography coupled with a corona charged aerosol detector, J. Chromatogr. A 1217 (2010) 294–301.

[27] T.H. Mourey, L.E. Oppenheimer, Principles of operation of an evaporative light-scattering detector for liquid-chromatography, Anal. Chem. 56 (1984) 2427–2434.

[28] S. Almeling, D. Ilko, U. Holzgrabe, Charged aerosol detection in pharmaceutical analysis, J. Pharm. Biomed. Anal. 69 (2012) 50–63.

[29] T. Gorecki, F. Lynen, R. Szucs, P. Sandra, Universal response in liquid chromatography using charged aerosol detection, Anal. Chem. 78 (2006) 3186–3192.

[30] G.L. Ellman, A colorimetric method for determining low concentrations of mercaptans, Arch. Biochem. Biophys. 74 (1958) 443–450.

[31] G.L. Ellman, Tissue sulfhydryl groups, Arch. Biochem. Biophys. 82 (1959) 70–77.

[32] ICH Guideline, Q2(R1), Validation of Analytical Procedures: Text and Methodology, 2013.

[33] S. Kromidas, Handbuch Validierung in der Analytik, 1st ed., WILEY-VCH Verlag GmbH, Weinheim, 2000, pp. 64–65.

[34] O. Folin, On the preparation of cystine, J. Biol. Chem. 8 (1910) 9–10.

# 2. Evaluation of enantiomeric purity of magnesium-L-aspartate dihydrate

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**ABSTRACT**

Magnesium supplementation in form of organic magnesium salts is a very popular practice. We examined the enantiomeric purity of "Magnesium aspartate dihydrate" monographed in the European Pharmacopeia. A chiral capillary zone electrophoresis using (2-hydroxypropyl)-β-cyclodextrin coupled to laser induced fluorescence detection and a HPLC-fluorescence method with chiral derivatization using *o*-phthaldialdehyde and *N*-acetyl-L-cysteine as an orthogonal method were developed and validated. Two batch samples of this substance and three drug products containing the salt were analyzed by means of both methods. The concentration of the D-enantiomer of aspartic acid ranged from 0.03 to 0.12%. Simulations of the synthesis revealed that the D-aspartic acid content is elevated if the dissolution of L-aspartic acid was performed at acidic pH values.

*Abbreviations:* API, active pharmaceutical ingredient; CBQCA, 3-(4-carboxybenzoyl) quinolone-2-carboxaldehyde; DMSO, dimethyl sulfoxide; DOE, design of experiments; EDQM, European Directorate for the Quality of Medicines& HealthCare; FLD, fluorescence detection; HP-β-CD, (2-hydroxypropyl)-β-cyclodextrin; ICH, International Conference on Harmonisation; IEP, isoelectric point; IS, internal standard; KF, Karl-Fischer; LIF, laser induced fluorescence; MeCN, acetonitrile; NAC, *N*-acetyl-L-cysteine; NMDA, *N*-methyl-D-aspartate; OPA, *o*-phthaldialdehyde; PFP, pentafluorophenyl; Ph. Eur., European Pharmacopeia; PH, phenyl-hexyl; PVDF, polyvinylidene fluoride.

## 1. Introduction

Innumerous magnesium salts for the sometimes controversially discussed treatment of hypomagnesaemia and cramp prophylaxis are currently available on the market [1, 2]. The salts are classified according to the anion into organic and inorganic magnesium salts. Among the inorganic magnesium salts as drug products are magnesium oxide (MgO), magnesium carbonate ($MgCO_3$), magnesium hydroxide ($Mg(OH)_2$), magnesium hydrogen phosphate ($MgHPO_4$) and magnesium chloride ($MgCl_2$). The organic salts, mostly salts of carboxylic acids, possess a higher solubility in water and are therefore supposed to be better bioavailable [3, 4]. Nevertheless, according to latest work, there is no special benefit of the organic salts for the reconstitution of the normal magnesium status compared to the inorganic salts [5, 6]. At least from the pharmaceutical point of view, the better soluble organic salts seem to be more suitable for the production of effervescent tablets and powders because the resulting liquid dosage form is a solution and not a suspension. The organic salts are salts of citric, glucuronic, acetic, glutamic or aspartic acid. In the case of aspartic acid there are three relevant salts: magnesium aspartate hydrochloride trihydrate, magnesium bis(hydrogen-DL-aspartate) tetrahydrate and magnesium bis(L-hydrogen-aspartate) dihydrate (Fig. 1). The latter one is in the focus of this study. The substance is monographed in the European Pharmacopeia (Ph. Eur.) with the title “magnesium aspartate dihydrate” [7] and should contain the L-amino acid only. Among the amino acids, aspartic acid exhibits the highest racemization rate in peptide or protein structures [8]. That is why the D-aspartic acid content in aged tissue, fossil, tooth or bone samples is widely utilized for the age determination of those samples [9–13]. The mechanism of racemization in peptides, however, is different to that of the free amino acid because the amide moiety is required in the proposed mechanisms for peptides [14–16]. Because of its neuroexcitatory effect on NMDA (N-methyl-D-asparatate) receptors [17–20] and interference with several endocrinal and hormonal systems [21–25] the D-aspartic acid concentration has to be restricted and controlled by means of a sensitive CE or HPLC method.

Due to the highest daily dose of approx. 1.8 g magnesium bis(hydrogenaspartate) dihydrate, any impurity (in our case D-aspartic acid) above 0.06 % (1 mg daily intake) has to be specified and qualified according to ICH guideline Q3A(R2) [26]. However, two challenges have to be faced: on the one hand the separation of the enantiomers and on the other hand the fact that aspartic acid lacks a chromophore for UV detection. The enantiomeric resolution of amino acids can be achieved by many techniques. The chromatographic separation of D- and L-amino acids has been shown e.g. on chiral stationary crown ether phases [27–29], by chiral ligand exchange chromatography [30–32] or by chiral gas chromatography after derivatization [33]. However, the detection problem is not overcome by most of those

techniques and in addition those stationary phases are relatively expensive. The key step therefore was to find an appropriate derivatization reaction that allowed for the detection and separation of the D- and the L-isomer of aspartic acid. A suitable derivatizing reagent with excellent characteristics for capillary electrophoretic separation coupled to laser induced fluorescence (LIF) detection is 3-(4-carboxybenzoyl)quinolone-2-carboxaldehyde (CBQCA) [34–37] (see Fig. 1) or naphthalene-2,3-dicarboxaldehyde [38]. The reactions lead to highly fluorescent derivatives easy to separate by means of CE.

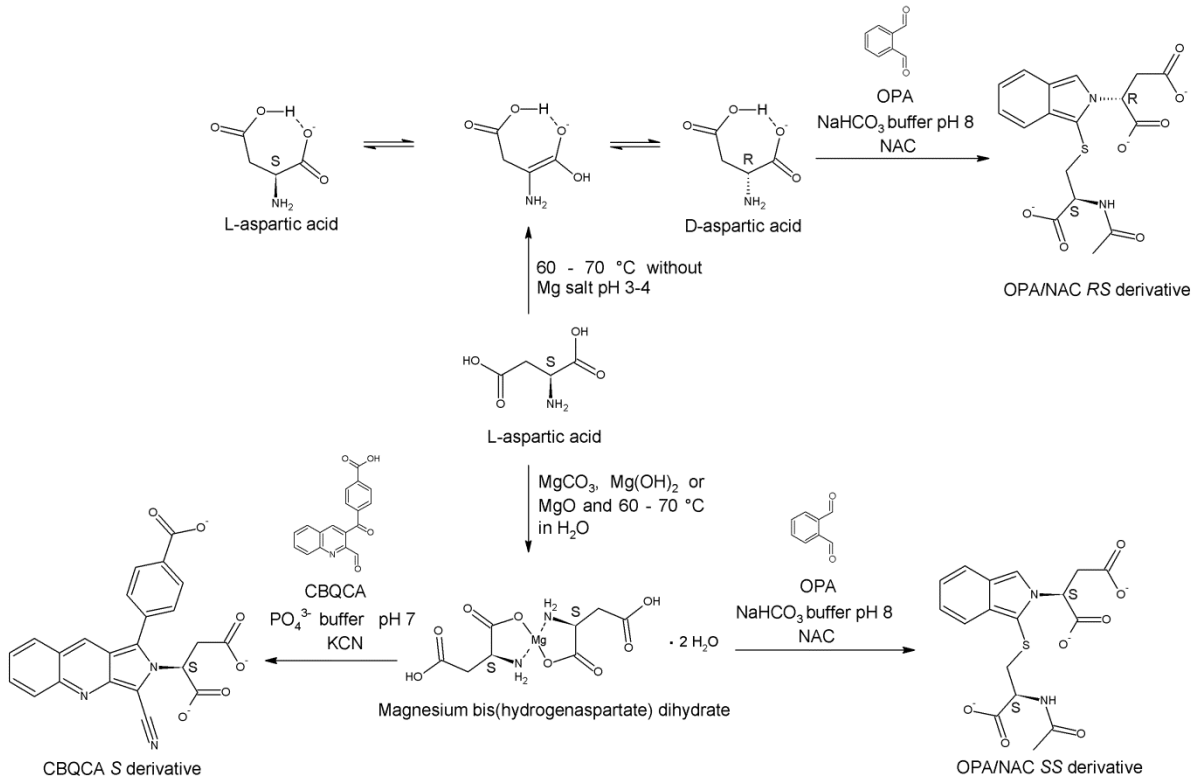


**Fig. 1.** Overview of magnesium bis(hydrogenaspartate) dihydrate synthesis, related substances and derivatization reactions; CBQCA, 3-(4-carboxybenzoyl)quinolone-2-carboxaldehyde; NAC, *N*-acetyl-L-cysteine; OPA, *o*-phthaldialdehyde.

The aims of this study were to evaluate the D-aspartic acid content in two active pharmaceutical ingredient (API) batch samples and three drug products obtained from the local market. Additionally, the reasons for elevated D-aspartic acid concentration were elucidated. For those purposes a robust CE separation of CBQCA derivatives using (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) as chiral selector was developed. The separation of diastereomeric *o*-phthaldialdehyde (OPA)/*N*-acetyl-L-cysteine (NAC) derivatives of aspartic acid (see Fig. 1) by means of an achiral HPLC separation [39] was elaborated as an orthogonal method in order to verify the CE results. The highly fluorescent isoindoles were detected by fluorescence detection (FLD) with excellent sensitivity (LOQ = 0.006 %) and repeatability.

## 2. Experimental

### *2.1. Chemicals and materials*

The magnesium aspartate dihydrate batch samples and L-aspartic acid were obtained from the European Directorate for the Quality of Medicines & HealthCare (EDQM) (Strasbourg, France). Magnaspart® 20 powder, Magnerot® A 500 granulate and Magnesium Verla® effervescent tablets were bought from a local pharmacy. HPLC grade acetonitrile, methanol, ammonium acetate and sodium hydrogen carbonate were purchased from VWR International S.A.S. (Fontenay-sous-Bois, France), hydrochloric acid 37 %, (2-hydroxypropyl)- β-cyclodextrin with average MW = 1380 g/mol (HP-β-CD), sodium tetraborate decahydrate, acetic acid ≥ 99.9 %,ammonium formate, hydrochloric acid 37 %, HYDRANAL®-Titrant 5, HYDRANAL®-Solvent, *N*-acetyl-L-cysteine (NAC) 99 %, L-glutamic acid > 99.5 %, magnesium oxide, magnesium carbonate, magnesium hydroxide and *o*-phthaldialdehyde (OPA) from Sigma–Aldrich Chemie GmbH (Steinheim, Germany), D-aspartic acid from Alfa Aesar (Karlsruhe, Germany), L-aspartic acid, disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate, formamide, Acilit®, Neutralit® and Alkalit® indicator paper for pH measurement (catalog number: 109565) from Merck KGaA (Darmstadt, Germany), dimethyl sulfoxide 99.9 % from Acros Organics (Geel, Beglium), CBQCA and potassium cyanide from Molecular Probes (Eugene, Oregon, USA), 2-propanol ≥ 99.7 % from Bernd Kraft GmbH (Duisburg, Germany). All chemicals used were of analytical grade or even better. Ultrapure water was produced using the “MilliQ Synthesis” water purification system by Merck Millipore (Schwalbach, Germany). HPLC columns tested during method development are summarized in Table 1.

**Table 1**
Overview of stationary phases screened during method development

| phase | brand name | manufacturer | dimensions |
|---|---|---|---|
| PH | Nucleodur® Phenyl-Hexyl | Machery-Nagel GmbH & Co. KG, Düren, Germany | 150 x 4.6 mm i.d., 3 µm |
| C18 | Luna® C18 | Phenomenex, Aschaffenburg, Germany | 100 x 4.6 mm i.d., 3 µm |
| C12 | Synergi® MAX-RP | Phenomenex, Aschaffenburg, Germany | 150 x 4.6 mm i.d., 4 µm |
| C8 | Nucleodur® C8ec | Machery-Nagel GmbH & Co. KG, Düren, Germany | 150 x 4.6 mm i.d., 3 µm |
| C4 | Beta-Basic-4® | Thermo Fisher Scientific Inc. Waltham MA, USA | 250 x 4.6 mm i.d., 5 µm |
| C1 | YMC-Pack® TMS | YMC Europe GmbH, Dinslaken, Germany | 100 x 4.6 mm i.d., 5 µm |

*2.2. Instrumentation*

All CE separations were performed by means of a Beckman Coulter P/ACE System MDQ (Fullerton CA, USA), equipped with a LIF-detector using an argon-ion-laser The fused-silica capillaries (BGB Analytik, Schloßböckelheim, Germany) employed for the analysis of magnesium aspartate samples after derivatization with CBQCA were of 75 µm internal diameter and effective length of 40.0 cm (total length of 50.2 cm). All HPLC separations were carried out using an Agilent 1200 modular chromatographic system consisting of online vacuum degasser, binary pump, thermostatted auto sampler, thermostatted column compartment, a photodiode array detector and a fluorescence detector (Agilent Technologies, Waldbronn, Germany). Water content was determined by means of a TitroLine® KF (Schott Instruments, Mainz, Germany) automatic titration system. The optical rotation was determined using a PerkinElmer 241 Polarimeter equipped with a thermostatted cuvette, 1 dm in length (PerkinElmer, Waltham MA, USA).

*2.3. Capillary electrophoretic procedure*

For the first method development attempts with borate buffer, the derivatized samples were loaded by pressure injection (6.89 kPa) for 5 s on the anode side. Separation was carried out with the following running buffers: 500 µM HP-β-CD were dissolved in 0.5, 1.0, 1.5, 2.0 or 2.5 mL DMSO and 5.0 mL of a 70 mM disodium tetraborate decahydrate solution, previously adjusted to pH 9.3 with 0.1 M sodium hydroxide solution. The resulting solution was diluted to 10.0 mL with water and filtrated (0.22 µm PVDF-filter) prior to use. The capillary was kept at 20 °C. Separation was achieved at a voltage of +30 kV (current approximately 60 µA) with LIF detection at $\lambda_{exc}$= 488 nm and $\lambda_{em}$= 520 nm (fix band pass filter).

The separation method with phosphate buffer used pressure injection (6.89 kPa) for 10 s on the anode side, the running buffers consisted of 100, 150, 180 and 200 µM of HP-β-CD, respectively,1.8 mL of DMSO and 3.0, 4.0 or 5.0 mL of a 0.1 M phosphate buffer pH 7.0, 7.5 or 8.0 subsequently diluted to 10.0 mL with water. The final separation buffer was composed of 180 µM of HP-β-CD, 1.8 mL of DMSO and 5 mL 0.1 M phosphate buffer pH 7 subsequently diluted to 10.0 mL. The capillary was kept at 25 °C and a voltage of +20 kV applied (current approximately 80 µA) with LIF detection at $\lambda_{exc}$= 488 nm and $\lambda_{em}$= 520 nm (fix band pass filter). New capillaries were conditioned by rinsing with 1 M NaOH (10 min, 206 kPa) followed by water (10 min, 206 kPa) and running buffer (180 s, 138 kPa). Between runs the capillaries were rinsed with 0.1 M NaOH (60 s, 206 kPa) followed by water, 2-propanol, water and running buffer (each 120 s, 206 kPa). Constant migration times were observed throughout the studies.

*2.3.1. Preparation of solutions*

0.1 M phosphate buffer pH 7.0/7.5/8.0: A 17.8 g/L solution of disodium hydrogen phosphate dihydrate was added to a 13.8 g/L solution of sodium dihydrogen phosphate monohydrate until the pH was 7.0, 7.5 and 8.0, respectively. Preparation of the CBQCA reagent: 5 mg of CBQCA were dissolved in 1.64 mL of DMSO. The internal standard solution was prepared by dissolving 40.0 mg of L-glutamic acid in 0.3 mL sodium hydroxide solution (1 M) and dilution to 10.0 mL with water. For the spiking solution, 40.0 mg of D-aspartic acid were dissolved in 0.3 mL sodium hydroxide solution (1 M) and diluted to 10.0 mL with water. Preparation of the reference solution for the correction factor: 6.4 mg of L-aspartic were dissolved in 0.1 M phosphate buffer solution pH 7.0 and spiked with 10 µL of internal standard solution, 10 µL of spiking solution and eventually diluted to 5.0 mL with 0.1 M phosphate buffer solution pH 7.0. The resolution due to the peaks of L- and D-aspartic acid was determined from a resolution reference solution containing 4 mg L- and 4 mg of D-aspartic acid in 10.0 mL of 0.1 M phosphate buffer pH 7.0. Preparation of test solutions: 8.0 mg of the substance to be examined and 10 µL of the internal standard solution were dissolved in and diluted to 5.0 mL with 0.1 M phosphate buffer solution pH 7.0.

*2.3.2. Derivatization procedure*

0.20 mL of the test or the reference solution were diluted to 10.0 mL with 0.1 M phosphate buffer pH 7.0. 20 µL of the dilutions were added to 40 µL of a 0.65 g/L solution of potassium cyanide and thoroughly mixed after addition of 20 µL of the CBQCA reagent. After 1, 2, 3, 4, 5, 6 or 24 h reaction time at room temperature protected from light, 120 µL of water were added to the mixture which was then mixed and subjected to CE analysis.

*2.4. Chromatographic procedure*

A column with pentafluorophenyl modification: Kinetex® PFP (150 × 4.6 mm i.d.; particle size: 2.6 µm; pore size: 100 Å, Phenomenex, Aschaffenburg, Germany) was used as a stationary phase. The chromatographic system was operated isocratically at 10 °C using a mobile phase composed of an aqueous ammonium acetate solution (50 mM, pH adjusted to 5.0 with glacial acetic acid) and acetonitrile (1000:50, V/V) applying a flow-rate of 1.0 mL/min. The column was flushed after every run with a mixture of acetonitrile and water (70:30 V/V) for 10 min at 1.0 mL/min. The fluorescence detector was set to an excitation wavelength of 345 nm and the emission was recorded at 445 nm. The injection and derivatization was done by an injector program described under Section 2.4.2.

*2.4.1. Preparation of solutions*

OPA reagent solution: 15 mg of *o*-phthaldialdehyde were dissolved in 2.0 mL of a 4.2 g/L solution of $NaHCO_3$ and stored at 8 °C in the auto sampler within an amber glass vial. The NAC buffer was a solution containing 18 g/L NAC and 26 g/L $NaHCO_3$, degassed by sonication and stored at 4–8 °C. Reference solution: 5.0 mg of L-aspartic, 5.0 mg of L-glutamic and 5.0 mg D-aspartic acid were dissolved in and diluted to 10.0 mL with a 4.2 g/L solution of $NaHCO_3$ and diluted again 1:100 with the same solvent. 0.1 mL of this solution was diluted to 1.0 mL with NAC buffer.

The internal standard (IS) solution was a 0.1 mg/mL solution of L-glutamic acid in a 4.2 g/L solution of $NaHCO_3$. For the API sample test solution 10.0 mg of magnesium aspartate dihydrate or 8.0 mg of L-aspartic acid and 0.1 mL of IS solution were dissolved in a 4.2 g/L solution of $NaHCO_3$ and diluted to 10.0 mL with the same solvent. This stock solution was subsequently diluted 1:4 with NAC buffer before injection. The drug product test solution was prepared by dissolution of an amount of the drug product equal to 10.0 mg of magnesium aspartate dihydrate and 0.1 mL of IS solution in a 4.2 g/L solution of $NaHCO_3$ and subsequent dilution to 10.0 mL with the same solvent. This stock solution was finally filtrated (0.22 µm cellulose acetate) and diluted 1:4 with NAC buffer before injection.

*2.4.1. Derivatization procedure*

The auto sampler drew 2 µL of OPA reagent solution, then test or reference solution, and subsequently OPA reagent solution again, mixed the 6 µL by up- and down-movement of the syringe cylinder with a speed of 100 µL/min and a volume of 2 µL. The derivatives were injected after 3, 4, 5, 6, 7, 8, 9 and 10 min reaction time, respectively.

*2.5. Determination of water by Karl-Fischer (KF) titration*

125 mg of magnesium aspartate dihydrate were dissolved in 10.0 mL of formamide by sonication and diluted to 25.0 mL with anhydrous methanol. 20.0 mL of this solution were diluted with the HYDRANAL®-solvent for KF and titrated with methanolic iodine solution (HYDRANAL®-Titrant 5) using biamperometric endpoint detection. A blank titration was performed.

*2.6. Determination of the specific rotation*

0.5 g of magnesium aspartate dihydrate were dissolved in 5 mol/L hydrochloric acid, spiked with 0, 250, 500, 750, 1000 and 1250 µL of a 20 mg/mL solution of D-aspartic acid in 5 M hydrochloric acid and diluted to 25.0 mL with the same acid. The optical rotation of this

solution was determined using the D-line of the sodium-vapor lamp spectrum (589.3 nm) at 20 °C. The specific rotation was calculated for the anhydrous substance.

*2.7. Racemization studies*

*2.7.1. Synthesis variation using different magnesium species*

The magnesium salt of aspartic acid is produced by neutralization of aspartic acid from different sources (one batch already contaminated with D-asp) with MgO, $MgCO_3$ or $Mg(OH)_2$ [40]. 37.6 mmol of L-aspartic acid and 18.8 mmol of either magnesium salt were dispersed in 20 mL of water (pH of the cold suspension approx. 7–8). The mixture was heated until the solids were dissolved (approx. 60–70 °C), the pH of the solution adjusted to 6 with L-aspartic acid at room temperature using indicator paper and immediately cooled down in an ice/sodium chloride mixture. After addition of 100 ml of cold 2-propanol, a syrup-like precipitate was formed and separated from the solution. The syrup was dried under reduced pressure (11 mbar) at 50 °C for 2 h yielding a white substance that was ground in a mortar and dried for another 24 h.

*2.7.2. Synthesis variation exposing aspartic acid to acidic pH*

37.6 mmol of L-aspartic acid (already contaminated with D-aspartic acid) were dispersed in 20 mL of water and heated (pH of the cold suspension approx. 3; 100 °C) for 30 and 60 min, respectively, before 18.8 mmol of $Mg(OH)_2$ were added. The mixture was then further heated until the solids were dissolved and treated as described in Section 2.7.1.

*2.7.3. Synthesis variation exposing aspartic acid to alkaline pH*

18.8 mmol of L-aspartic acid and 18.8 mmol of $Mg(OH)_2$ were dispersed in 20 mL of water and heated (pH of the cold suspension approx. 9; 100 °C) for 30 and 60 min, respectively, before another 18.8 mmol of L-aspartic acid were added. The mixture was then further heated until the solids were dissolved, and treated as described in Section 2.7.1.

*2.8. Data processing*

Electropherograms were processed using Beckman 32 Karat® Software 8.0 by Beckman Coulter, Inc. (Fullerton, CA, USA). Chromatograms were analyzed by Agilent ChemStation® Rev. B.03.02 (Agilent Technologies, Waldbronn, Germany). All experimental results were evaluated using STATISTICA® 8.0 by StatSoft Inc. (Tulsa OK, USA) and Microsoft Excel®2010 by Microsoft Corp. (Redmont WA, USA).

## 3. Results and discussion

### *3.1. Capillary electrophoresis*

#### *3.1.1. Method development*

For a sensitive detection the aspartic acid enantiomers were derivatized with CBQCA according to a standard protocol elaborated by Novatchev et al. [34, 36]. The conversion with CBQCA was done at pH 7 with a 50 fold molar excess in order to gain quantitative derivatization in a comparatively short time. The D-aspartic acid corrected peak area and peak height after 1, 2, 3, 4, 5 and 6 h were determined. As can be seen from Fig. 2, the reaction was complete after 3 h and the derivatives were stable for at least 24 h (stored at 8 °C). The separation method was developed using a common borate buffer showing a low current at high voltage. The resolution between the enantiomeric derivatives was not sufficient with a standard 37.5 mM disodium tetraborate decahydrate solution (pH 9.3) containing 10 mM β-cyclodextrin as running buffer. Thus, HP-β-CD at a concentration of 50 mM was chosen, because this chiral selector exhibits an improved solubility and an increased possibility of interactions with the derivatives. By means of HP-β-CD the peaks of the enantiomers were resolved. Further increase in resolution was possible by the addition of an organic modifier cf. [41]. Since the test solution contained 10% V/V of DMSO, this solvent was added to the running buffer as an organic modifier in order to increase the viscosity [42], and to decrease the relative permittivity of the buffer [43] and the zeta potential of the capillary wall, thus enhancing the selectivity [44]. Reducing the electroosmotic flow and along with that favoring the electrophoretic mobility of the negatively charged derivatives resulted in a higher number of theoretical plates and better resolution: At 25% V/V DMSO a very high resolution (typically greater than 9) was achieved in a comparatively long run time (see Fig. 3).

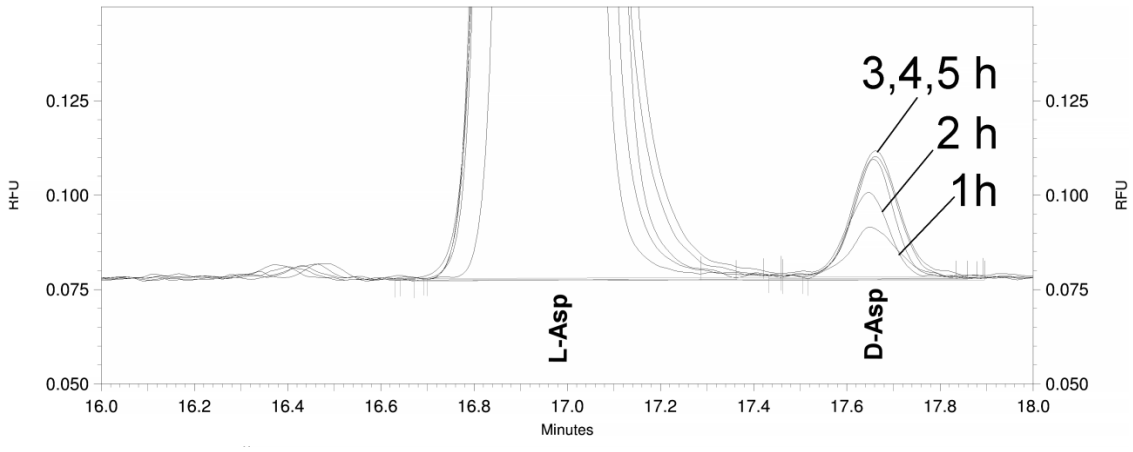


**Fig. 2.** Overlay of electropherograms of a sample containing 0.1 % D-aspartic acid after 1, 2, 3, 4 and 5 h derivatization time. The peak height due to the D-aspartate derivative reaches the maximum after 3 h.

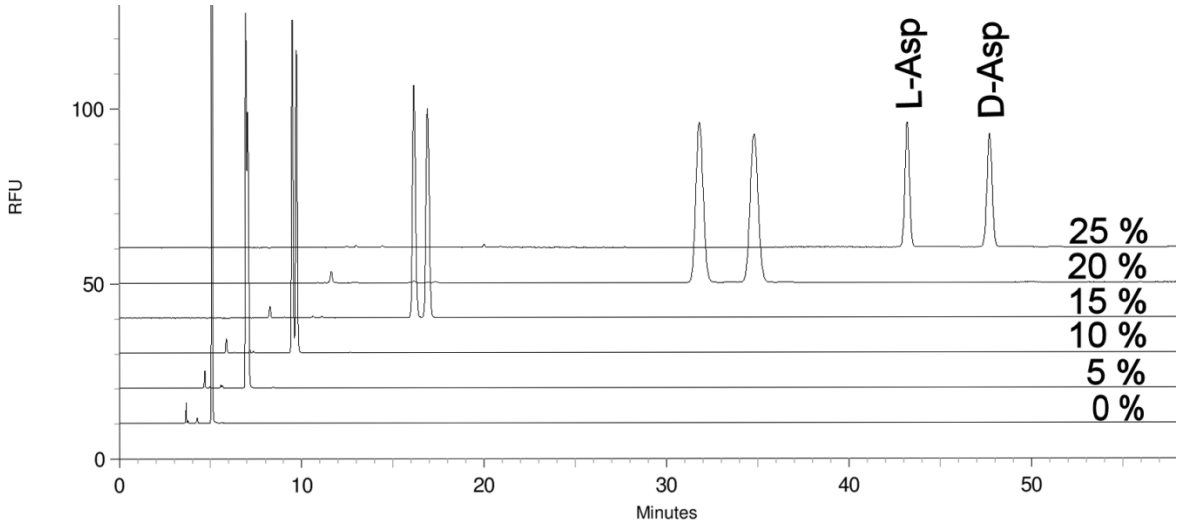


**Fig. 3.** The effect of the DMSO concentration on the capillary electrophoretic separation of the enantiomeric derivatives of D- and L-aspartic acid; conditions: BGE: 50 mM, HP-β-CD, 37.5 mM, sodium tetraborate decahydrate pH 9.3 and the displayed percentage V/V of dimethyl sulfoxide, fused silica capillary, untreated 50.2 cm × 75 µm i.d.; voltage: +30 kV; temperature: 20 °C; detection: LIF with $\lambda_{ex}$= 488 nm and $\lambda_{em}$= 520 nm at 40 cm.

In order to avoid the genotoxic borate additive, a CE separation using a sodium phosphate buffer and L-glutamic acid as an internal standard was developed based on the aforementioned findings. A design of experiments (DOE) based approach was used to determine the optimal separation parameters. By means of a three-level full factorial design [45] investigating buffer pH (7.0 and 8.0), buffer salt concentration (30 and 50 mM) and cyclodextrin concentration (10 and 20 mM) (n = 8) using the resolution between L- and D-aspartic acid as response, the optimal electrophoretic conditions were identified: A high resolving (resolution > 4) separation was achieved with 18 mM HP-β-CD, 50 mM sodium phosphate buffer pH 7.0 and 18 % V/V DMSO (see Fig. 4). The predicted resolution for those parameters (4.6) in this model was close to the real resolution (4.5). The robustness of the separation is given by the experiment design: The influence of the method parameters on the separation is displayed in the contour plots and the pareto chart derived from those experiments (Fig. 5). The dark red areas of the contour plots mark the regions where a variation in method parameters always yields a resolution of more than 4. The arrows indicate the optimized method parameters. As expected, the buffer and cyclodextrin concentration had the greatest impact on the resolution whereas pH 7 was advantageous compared to higher pH, because the resulting current and joule heating, leading to band broadening, was minimal.

The quantification was done by means of an internal standard (L-glutamic acid) using the corrected area. The corrected area of D-aspartic acid had to be multiplied by the correction factor of 1.8 because we found different fluorescence yields for the derivatives due

to D-aspartic acid and L-glutamic acid at 520 nm, possibly due to the proximity of the β-carboxylate to the fluorophore in the aspartic acid derivative resulting in partial fluorescence quenching. The correction factor was determined in the presence and in the absence of L-aspartic acid (50- and more than 16,000-fold molar excess of CBQCA compared to the total amino acid concentration) excluding the possibility of an incomplete derivatization.

### *3.1.2. Method validation*

The linearity was determined over the range of 0.1–0.5 % D-aspartic acid spiked to a magnesium aspartate batch sample containing approx. 0.1 % D-aspartic acid. The coefficient of determination was found to be higher than 0.995 ($R^2$= 0.9994). The accuracy was determined using another batch sample spiked from 0.1 to 0.5 %. The recovery rates ranged from 87 to 90 % (RSD < 1.5 % n = 2). The intermediate precision was tested on an API batch sample containing 0.11 % D-aspartic acid (RSD < 10 %, n = 6). The LOQ (S/N of 10) was found to be 0.03 % determined via a successive addition of D-aspartic acid to a blank solution until the S/N ratio reached 10.

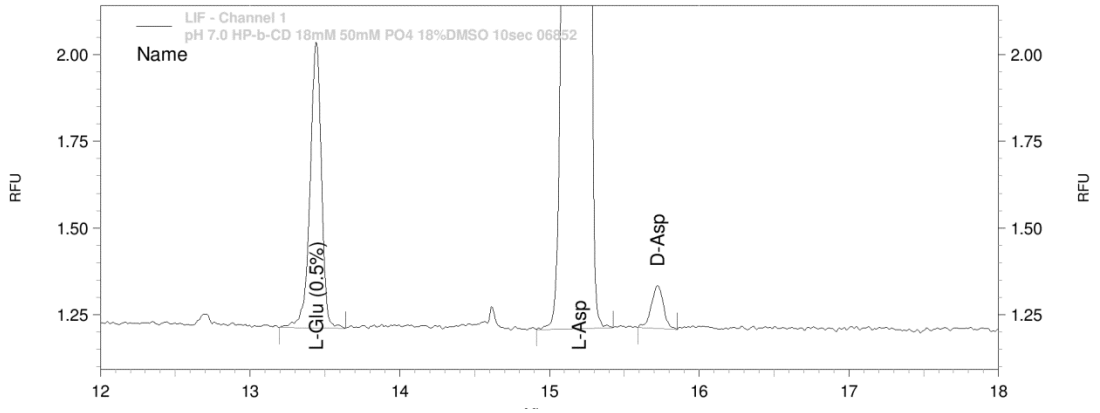


**Fig. 4.** Representative electropherogram obtained from a test solution (CBQCA derivatives) – migration order: L-glutamic acid (IS), L-aspartic acid, D-aspartic acid (D-Asp = 0.11 %); electrophoretic conditions: BGE: 18 mM, HP-β-CD 18 % V/V DMSO and 50 mM phosphate buffer pH 7.0, fused silica capillary, untreated 50.2 cm × 75 µm i.d.; voltage: +20 kV; temperature: 25 °C; detection: LIF with $\lambda_{ex}$= 488 nm and $\lambda_{em}$= 520 nm at 40 cm.

## *3.2. Chromatographic method*

### *3.2.1. Method development and optimization*

The separation method development was based on previous work by Aswad [39] and optimized regarding resolution and limit of detection (LOD). They used an isocratic elution protocol with 50 mM sodium acetate pH 5.9 and 8 % V/V methanol in combination with an octadecyl-modified silica gel column.

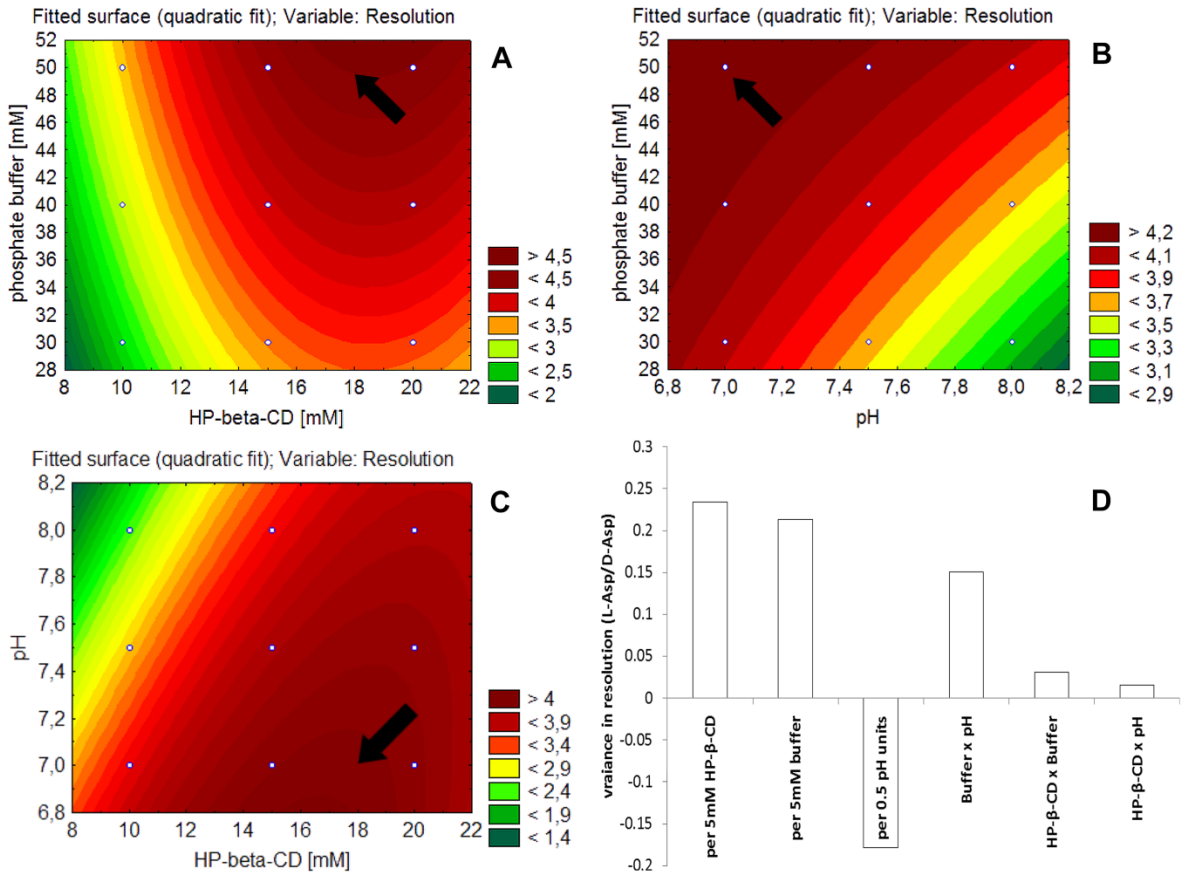


**Fig. 5.** Contour plots for the resolution between the peaks due to D-asp and L-asp as a function of pH, buffer salt and cyclodextrin concentration – A: pH is constant at 7.5; B:cyclodextrin concentration is constant at 15 mM; C: buffer salt concentration is constant at 40 mM; D: Effects on the nominal resolution; the buffer pH had to be reduced to increase the resolution, whereas cyclodextrin and buffer concentration need to be increased for a higher resolution; a combined effect for buffer pH and salt concentration was observed.

Seven available reversed phase columns with different polarities (see Table 1) were screened for method development using two mobile phases of approximately equal elution power and the same pH: 50 mM ammonium formate pH 5.0 with 5 % V/V acetonitrile and with 8 % V/V methanol, respectively (see Table 2). The highest selectivity (α) was obtained with an endcapped C8 and a pentafluorophenyl (PFP) stationary phase whereas the phenyl-hexyl phase yielded the highest capacity factors (k') for both peaks. The PFP phase was chosen because of its high selectivity, reasonable capacity factor and sharp peak form (compared to the C8 column) resulting in a lower LOD. Acetonitrile (MeCN) as organic modifier was chosen to lower the backpressure (still approx. 300 bars) due to the lower viscosity in comparison to methanol–water mixtures [46].

The method performance was explored by a DOE based approach: Column temperature (10 ± 2 °C), acetonitrile concentration (5 ± 2 % V/V), and buffer pH (5.0 ± 0.5) were modified (two at the same time) and the resolution between the diastereomeric

compounds was observed. The optimal conditions were found to be: 50 mM ammonium acetate buffer pH 5.0 and 5 % V/V acetonitrile (see Fig. 6). In fact, the resolution and the design space regarding pH robustness were greater with only 3 % V/V acetonitrile, but also increased peak width caused a decrease in S/N ratio. This deficit of robustness was deliberately chosen in favor of a higher sensitivity. Critical for the separation were pH (±0.2 pH units tolerated) and acetonitrile concentration (±1 % V/V tolerated). The impact of the temperature was negligible. As long as the method parameters only vary within the red to orange areas in the contour plots (see Fig. 7A–C), the resolution is sufficient for the quantitative determination of D-aspartic acid. The final separation conditions are marked by arrows.

**Table 2**
Results of the stationary phase screening experiments

| phase | 50 mM $NH_4HCOO$ pH 5.0 - MeOH (92:8 V/V) | | | 50 mM $NH_4HCOO$ pH 5.0 - MeCN (95:5 V/V) | | |
|---|---|---|---|---|---|---|
| | $k'_{D\text{-}Asp}$ | $k'_{L\text{-}Asp}$ | α | $k'_{D\text{-}Asp}$ | $k'_{L\text{-}Asp}$ | α |
| C1 | 1.25 | 1.40 | 1.12 | 1.16 | 1.33 | 1.14 |
| C4 | 3.23 | 3.94 | 1.22 | 7.31 | 8.55 | 1.17 |
| C8 | 3.92 | 5.27 | 1.35 | 2.89 | 3.82 | 1.32 |
| C12 | 9.52 | 12.51 | 1.31 | 6.86 | 8.52 | 1.24 |
| C18 | 12.02 | 15.39 | 1.28 | 5.42 | 6.74 | 1.24 |
| PH | 12.80 | 17.01 | 1.33 | 8.67 | 10.69 | 1.23 |
| PFP | 3.86 | 5.23 | 1.36 | 2.06 | 2.70 | 1.31 |

In order to avoid the sodium tetraborate buffer applied by Aswad [39] in the derivatization step, a hydrogen carbonate buffer was used. Phosphate buffer was no option, because OPA derivatives are known to be unstable in the presence of phosphate ions [47]. For reproducibility reasons the derivatization was automated and took place in the auto sampler which drew sample and reagent from prepositioned vials, mixed the components in the needle, let the components react for 9 min (see Fig. 7D) and injected the derivatives. Besides the formation of diastereomeric derivatives, the use of NAC as thiol component also led to a higher fluorescence yield and improved stability of the derivatives compared to 2-mercaptoethanol [48, 49] which is very commonly used for this purpose. Again L-glutamic acid was chosen as an internal standard. The peak area of D-aspartic acid had to be multiplied by the correction factor 1.1 since different response factors for the derivative due to D-aspartic and L-glutamic acid (calculated from the ratio of the calibration curve slopes) were found in the experiments.

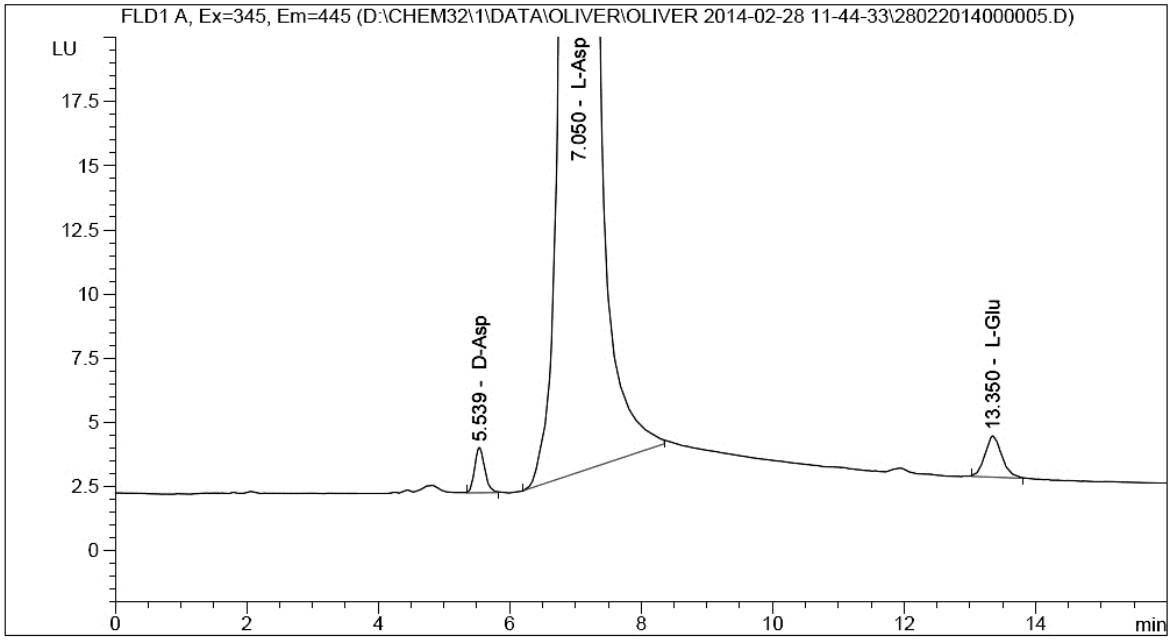


**Fig. 6.** Representative chromatogram obtained from an API test solution (OPA derivatives) – elution order: D-aspartic acid (0.08 %), L-aspartic acid, L-glutamic acid; chromatographic conditions: Mobile phase 50 mM ammonium acetate pH 5.0: acetonitrile (1000:50, V/V); flow rate = 1.0 mL/min; column: Phenomenex Kinetex® PFP 150 × 4.6 mm 2.6 µm; temperature: 10 °C; detection: FLD with $\lambda_{ex}$= 345 nm and $\lambda_{em}$= 445 nm.

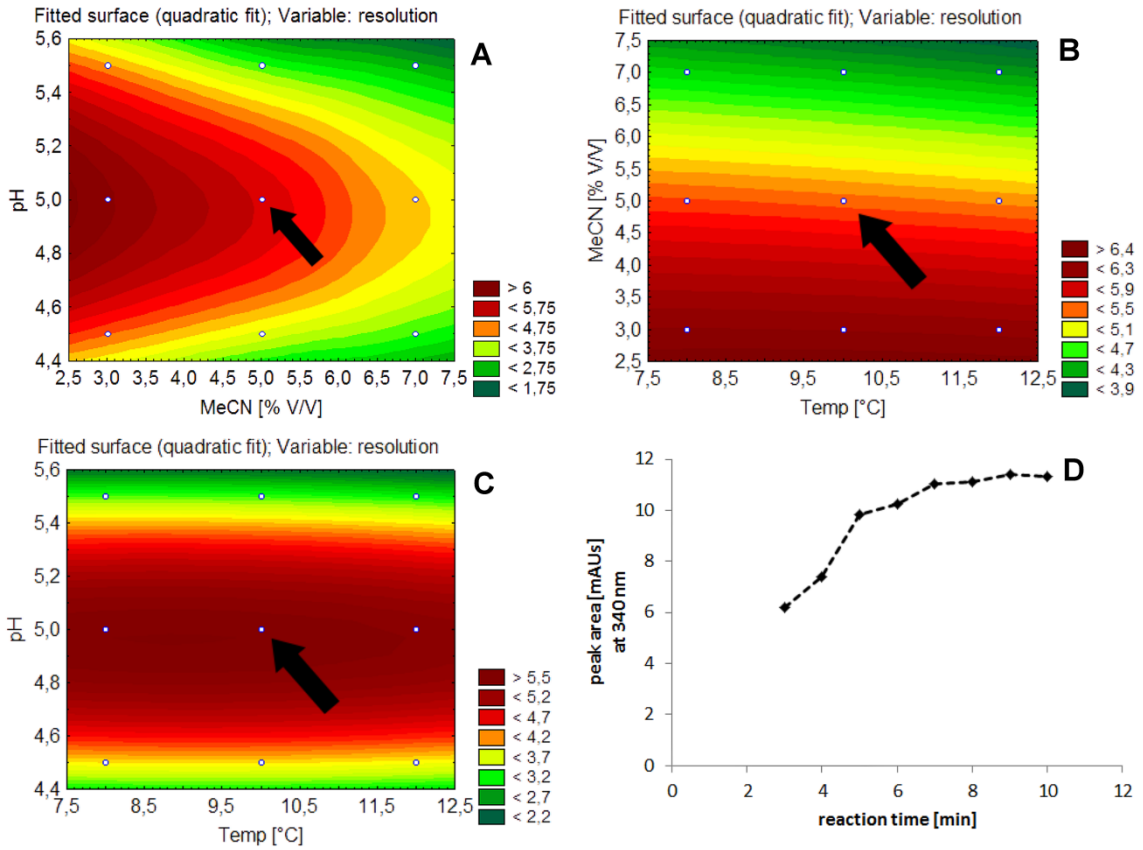


**Fig. 7.** Contour plots for the resolution between the peaks due to D-asp and L-asp as a function of pH, temperature and acetonitrile concentration; A: temperature is constant at 10 °C; B: pH is constant at 5.0; C: acetonitrile concentration is constant at 5 % V/V; D: The derivatization reaction time plotted vs. the resulting peak area; The optimal response was observed after 9 min.

*3.2.1. Method validation*

The method was linear ($R^2$= 0.9992 over 0.1–0.5 % D-asp spiked to a batch sample), accurate over the same range (recovery rate ranging from 98 to 114 % with max. RSD < 2 %, n = 3) and precise (RSD = 1.8 %, n = 6). LOQ was determined at the S/N ratio of 10 as described in Section 3.1.2 and found to be 60 ng/ml (equal to 0.006 %).

*3.3. Impurity profiling*

Two API batch samples and three drug products (one batch each) obtained from a local pharmacy were analyzed by means of those methods. The D-asp concentration found in the drug product samples ranged from 0.03 to 0.12 % whereas the API batch samples contained 0.08 and 0.11 % of D-aspartic acid. The D-aspartic acid concentration in two L-aspartic acid samples (obtained from the EDQM) was below or equal to 0.03 % (see Table 3).

*3.4. Specific rotation*

The specific optical rotation (2.6) of the magnesium aspartate dihydrate received from the EDQM and synthesized within this study (see below) was determined in hydrochloric acid (5 M). The same samples were spiked with D-aspartic acid followed by the determination of the specific optical rotation. The specific rotation of all unspiked batch samples is within the monograph limits (+22.0 to +24.0) although they contain up to 0.7 % D-aspartic acid. Thus, the test is unable to discover concentrations of the D-enantiomer below 1 % (see Fig. 8 and Table 3).

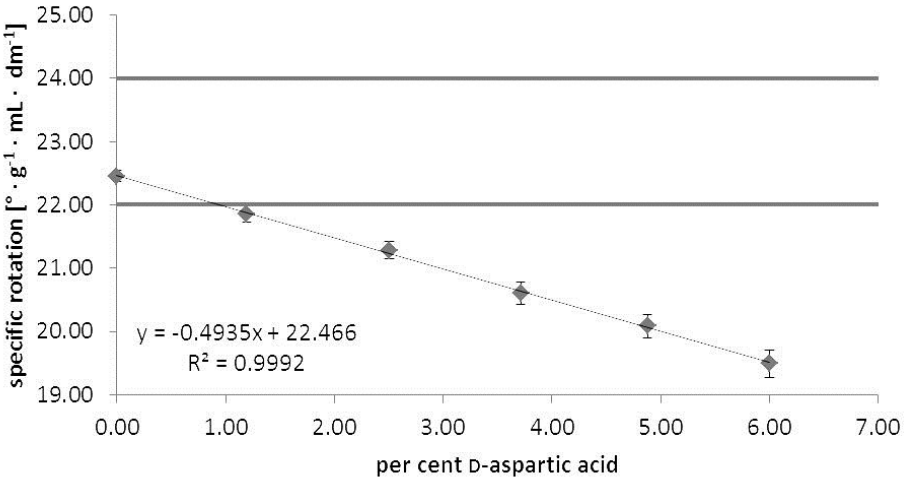


**Fig. 8.** Mg aspartate (0.03 % D-asp) was spiked with five different concentrations of D-aspartic acid and the specific optical rotation was determined (n = 3); the horizontal lines indicate the specification limits of the monograph.

*3.5. Racemization studies*

In order to find out the reason for the possible elevated D-aspartic acid levels in the magnesium aspartate dihydrate samples, the salt was synthesized using $MgCO_3$, $Mg(OH)_2$, and MgO, respectively, and two different batches of L-aspartic acid (Section 2.7.1). In no case elevated D-aspartic acid levels were found (see Fig. 9). The amount of D-aspartic acid found in the salt mirrored the level of the D-enantiomer in the starting material. However, the concentration of D-aspartic acid in the product was even lower than in the starting material due to dilution with magnesium and water of crystallization.

Erbe and Bruckner [50] previously described the racemization of aspartic acid in vinegar and dilute acetic acid. They explained this behavior with the formation of a cyclic enolate at a pH close to the isoelectric point (IEP) of 2.8 (see Fig. 1). To prove this hypothesis various batches of L-aspartic acid already containing variable amounts of D-enantiomer were dissolved in boiling water resulting in a pH of approx. 3. After dissolution a magnesium salt, $Mg(OH)_2$, was added in order to increase the pH to 6 (Section 2.7.2). The later $Mg(OH)_2$ was added to the 100 °C hot solution, the higher was the increase in the D-enantiomer content (Fig. 9). Since amino acids are known to undergo racemization at alkaline pH, L-aspartic acid was exposed to a pH of 9 for 30 and 60 min (Section 2.7.3); here a very slight increase in the D-enantiomer concentration was found only (Fig. 9). However, the ratio of $Mg(OH)_2$ and L-aspartic acid is here 1:1 instead of 1:2 so that these conditions do not lead to magnesium aspartate with the correct stoichiometry. Thus, alkaline pH is very unlikely to occur during the synthesis. All these experiments confirm the findings of Steinberg et al. [51] that the racemization rate of free aspartic acid at intermediate acidic pH is by far higher than at neutral or alkaline pH. Thus, high levels of D-aspartic acid in the API magnesium-L-aspartate are caused by the wrong synthesis procedure. Consequently, the European Pharmacopeia plans to evaluate and to limit the content of the D-enantiomer.

*3.6. Comparison of the analytical methods*

*3.6.1. Sensitivity*

HPLC-FLD with a LOD of 0.003 and a LOQ of 0.006 % D-aspartic acid is a very sensitive method. The sensitivity was realized at the cost of robustness and the necessity for special HPLC instrumentation capable of pre-column derivatization. Nonetheless, the LOQ of 0.03 % achieved with the CE method is sufficient for an adequate control (below 0.1 %) of the enantiomeric purity.

**Table 3**
Average results of the performed determinations (n = 2) and specific rotation (n = 3) ± SD

| Substance | Source | Specific optical rotation [° mL $g^{-1}dm^{-1}$] | D-Asp by HPLC-FLD[a] | D-Asp by CE-LIF[a] |
|---|---|---|---|---|
| Mg L-aspartate | EDQM | +22.60 ± 0.10 | 0.10 ± 0.00 | 0.11 ± 0.01 |
| Mg L-aspartate | EDQM | +23.11 ± 0.09 | 0.08 ± 0.00 | 0.10 ± 0.01 |
| Mg L-aspartate | own synthesis (2.7.1) | +22.46 ± 0.09 | 0.02 ± 0.00 | 0.03 ± 0.00 |
| Mg L-aspartate | own synthesis (2.7.1) | +22.29 ± 0.18 | 0.16 ± 0.02 | 0.17 ± 0.02 |
| Mg L-aspartate | own synthesis (2.7.1) | +22.97 ± 0.15 | 0.16 ± 0.00 | 0.19 ± 0.02 |
| Mg L-aspartate | own synthesis (2.7.2) | +22.49 ± 0.14 | 0.39 ± 0.02 | 0.40 ± 0.04 |
| Mg L-aspartate | own synthesis (2.7.2) | +23.37± 0.21 | 0.41 ± 0.02 | 0.42 ± 0.04 |
| Mg L-aspartate | own synthesis (2.7.2) | +22.86 ± 0.20 | 0.69 ± 0.03 | 0.64 ± 0.06 |
| Mg L-aspartate | own synthesis (2.7.2) | +22.54 ± 0.21 | 0.69 ± 0.03 | 0.67 ± 0.05 |
| Mg L-aspartate | own synthesis (2.7.3) | - | 0.03 ± 0.00 | - |
| Mg L-aspartate | own synthesis (2.7.3) | - | 0.03 ± 0.00 | - |
| Mg L-aspartate | own synthesis (2.7.3) | - | 0.04 ± 0.00 | - |
| Mg L-aspartate | own synthesis (2.7.3) | - | 0.03 ± 0.00 | - |
| Magnaspart® 20 | local pharmacy | - | 0.12 ± 0.00[b] | - |
| Magnerot® A 500 | local pharmacy | - | 0.10 ± 0.00[b] | - |
| Magnesium Verla® | local pharmacy | - | 0.03± 0.00[b] | - |
| L-aspartic acid | EDQM | - | 0.03± 0.00 | < LOQ |
| L-aspartic acid | EDQM | - | 0.02± 0.00 | < LOQ |
| L-aspartic acid | Merck KGaA | - | 0.21± 0.00 | - |

[a] in % of the test solutions concentration
[b] in % of the declared content

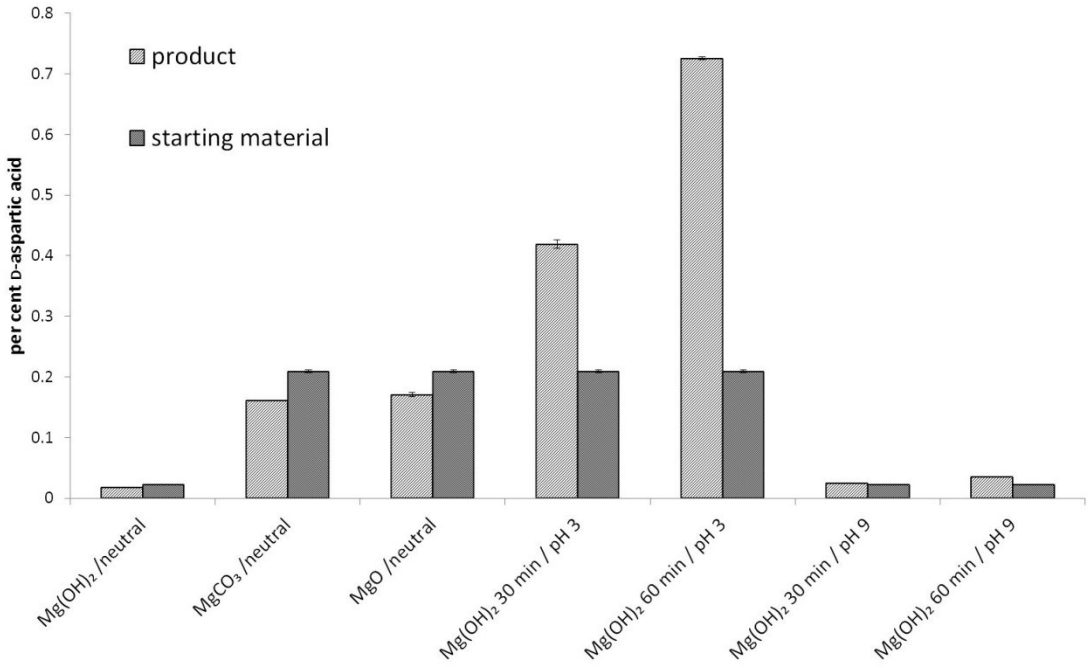


**Fig. 9.** The content of D-aspartic acid in the synthesized magnesium aspartate dihydrate samples is compared to the concentration of the D-enantiomer in the corresponding starting material. The salt was produced from $Mg(OH)_2$, $MgCO_3$, MgO and L-aspartic acid at neutral, acidic and alkaline pH (n = 2).

*3.6.2. Repeatability*

The HPLC method is highly repeatable because of the automated process of derivatization and precise sampling procedure. Both methods benefit from the use of an internal standard which equalizes dosing, reaction time and, in the case of the CE method, migration time imprecision. However, the use of an internal standard is almost obligatory since area normalization would not lead to an adequate result because of the poor linearity of the fluorescence response over such a wide range. The derivatization step in the CE method takes much longer (3 h vs. 9 min) but is easily transferable to any laboratory making it the ideal method for a pharmacopeia.

*3.6.3. Deduction*

The quick HPLC method is mostly suitable as a screening procedure with high throughput whereas the time consuming CE method is ideal for the Ph. Eur., because it is feasible in almost every laboratory able to perform CE-LIF analysis. Nevertheless, most of today's available HPLC instruments are capable of automated pre-column derivatization so that many laboratories could perform this test as well. In conclusion both methods have proven suitable for the control of the enantiomeric purity of magnesium L-aspartate dihydrate.

## 4. Conclusion

In summary there are two reasons for a high D-aspartic acid concentration in Mg aspartate dihydrate: The starting material for the synthesis was already contaminated with the D-enantiomer and/or the amino acid was exposed to heat and simultaneously to a pH close to its IEP (2.8). Avoiding acidic pH the synthesis of batches with less than 0.03 % of the D-enantiomer is possible. Against the background of D-aspartic acid mimicking the action of glutamate by activating NMDA receptors [17–20] and interfering with several endocrinal and hormonal systems [21–25], the use of racemic magnesium bis(hydrogen-DL-aspartate) tetrahydrate appears to be out of date and the chiral purity of magnesium bis(hydrogen-aspartate)dihydrate should be controlled by a sensitive method to guarantee the quality and the safety of the API. The current employed test "specific optical rotation" of the Ph. Eur. is not suitable to effectively control the enantiomeric purity of the substance. Either one of the herein presented methods is accomplishing this task. The HPLC-FLD method combines high sensitivity and a quick determination with very good precision, whereas the CE-LIF method is easily transferable to other CE systems because it does not need special instrumentation capable of online derivatization, but uses a time consuming manual sample preparation step prior to analysis instead.

## Conflict of interest statement

None of the authors of this paper does have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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## References

[1] S.R. Garrison, G.M. Allan, R.K. Sekhon, V.M. Musini, K.M. Khan, Magnesium forskeletal muscle cramps, Cochrane Database Syst. Rev. (2012).

[2] L.O. Dahle, G. Berg, M. Hammar, M. Hurtig, L. Larsson, The effect of oral mag-nesium substitution on pregnancy-induced leg cramps, Am. J. Obstet. Gynecol.173 (1995) 175–180.

[3] M. Firoz, M. Graber, Bioavailability of US commercial magnesium preparations, Magnes. Res. 14 (2001) 257–262.

[4] J.S. Lindberg, M.M. Zobitz, J.R. Poindexter, C.Y.C. Pak, Magnesium bioavailability from magnesium citrate and magnesium-oxide, J. Am. Coll. Nutr. 9 (1990) 48–55.

[5] C. Coudray, M. Rambeau, C. Feillet-Coudray, E. Gueux, J.C. Tressol, A. Mazur, Y. Rayssiguier, Study of magnesium bioavailability from ten organic and inorganic Mg salts in Mg-depleted rats using a stable isotope approach, Magnes. Res. 18 (2005) 215–223.

[6] S.W. Golf, Zur biologischen Vergleichbarkeit und biochemischen Wirksamkeit pharmazeutischer Magnesiumverbindungen, J. Miner. Stoffwechs. 6 (1999) 11–21.

[7] Magnesium aspartate dihydrate, European Pharmacopoeia, 8th edition, European Directorate for the Quality of Medicines & HealthCare, Strasbourg, France, 2014.

[8] J.L. Bada, In vivo racemization in mammalian proteins, Method. Enzymol. 106 (1984) 98–115.

[9] O.A. Sherwood, D.B. Scott, M.J. Risk, Late holocene radiocarbon and aspartic acid racemization dating of deep-sea octocorals, Geochim. Cosmochim. Acta 70 (2006) 2806–2814.

[10] C.R. Sloss, C.V. Murray-Wallace, B.G. Jones, T. Wallin, Aspartic acid racemization dating of mid-Holocene to recent estuarine sedimentation in New South Wales, Australia: a pilot study, Mar. Geol. 212 (2004) 45–59.

[11] R.E. Taylor, P.J. Ennis, P.J. Slota, L.A. Payen, Non-age-related variations in aspartic-acid racemization in bone from a radiocarbon-dated late Holocene archaeological site, Radiocarbon 31 (1989) 1048–1056.

[12] S. Arany, S. Ohtani, T. Yamamoto, T. Sugiyama, Comparison of aspartic acid racemization between mammoth and human dentinal tissues, Arch. Oral Biol. 52 (2007) 20–25.

[13] S. Arany, S. Ohtani, Age estimation of bloodstains: a preliminary report based on aspartic acid racemization rate, Forensic Sci. Int. 212 (2011) E36–E39.

[14] O. Takahashi, K. Kobayashi, A. Oda, Computational modeling of the enolization in a direct mechanism of racemization of the aspartic acid residue, Chem. Biodivers. 7 (2010) 1630–1633.

[15] O. Takahashi, K. Kobayashi, A. Oda, Modeling the enolization of succinimide derivatives, a key step of racemization of aspartic acid residues: importance of a two-$H_2O$ mechanism, Chem. Biodivers. 7 (2010) 1349–1356.

[16] J.L. Radkiewicz, H. Zipse, S. Clarke, K.N. Houk, Accelerated racemization of aspartic acid and asparagine residues via succinimide intermediates: an ab initio theoretical exploration of mechanism, J. Am. Chem. Soc. 118 (1996) 9148–9155.

[17] M.J. Schell, O.B. Cooper, S.H. Snyder, D-aspartate localizations imply neuronal and neuroendocrine roles, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 2013–2018.

[18] H. Wang, H. Wolosker, J.F. Morris, J. Pevsner, S.H. Snyder, D.J. Selkoe, Naturally occurring free D-aspartate is a nuclear component of cells in the mammalian hypothalamo-neurohypophyseal system, Neuroscience 109 (2002) 1–4.

[19] H. Wang, H. Wolosker, J. Pevsner, S.H. Snyder, D.J. Selkoe, Regulation of rat magnocellular neurosecretory system by d-aspartate: evidence for biological role(s) of a naturally occurring free D-amino acid in mammals, J. Endocrinol.167 (2000) 247–252.

[20] H. Wolosker, A. D'Aniello, S.H. Snyder, D-Aspartate disposition in neuronal and endocrine tissues: ontogeny, biosynthesis and release, Neuroscience 100 (2000) 183–189.

[21] N. Ota, T. Shi, J.V. Sweedler, D-Aspartate acts as a signaling molecule in nervous and neuroendocrine systems, Amino Acids 43 (2012) 1873–1886.

[22] M. Pampillo, T. Scimonelli, M.C. Bottino, B.H. Duvilanski, V. Rettori, A. Seilicovich, M. Lasaga, The effect of D-aspartate on luteinizing hormone-releasing hormone, alpha-melanocyte-stimulating hormone, GABA and dopamine release, Neuroreport 13 (2002) 2341–2344.

[23] E. Topo, A. Soricelli, A. D'Aniello, S. Ronsini, G. D'Aniello, The role and molecular mechanism of D-aspartic acid in the release and synthesis of LH and testosterone in humans and rats, Reprod. Biol. Endocrin. 7 (2009) 120.

[24] C. Lamanna, L. Assisi, V. Botte, M.M. Di Fiore, Involvement of D-Asp in P450 aromatase activity and estrogen receptors in boar testis, Amino Acids 32 (2007) 45–51.

[25] H. Homma, Biochemistry of d-aspartate in mammalian cells, Amino Acids 32 (2007) 3–11.

[26] Impurities in new drug substances, ICH, Q3A(R2), 2006.

[27] M.H. Hyun, J.S. Jin, W.J. Lee, Liquid chromatographic resolution of racemic amino acids and their derivatives on a new chiral stationary phase based on crown ether, J. Chromatogr. A 822 (1998) 155–161.

[28] M.H. Hyun, J.S. Jin, S.C. Han, Y.J. Cho, The effect of analyte lipophilicity on the resolution of alpha-amino acids on a HPLC chiral stationary phase based on crown ether, Microchem. J. 70 (2001) 205–209.

[29] M.H. Hyun, S.C. Han, B.H. Lipshutz, Y.J. Shin, C.J. Welch, New chiral crown ether stationary phase for the liquid chromatographic resolution of alpha-amino acid enantiomers, J. Chromatogr. A 910 (2001) 359–365.

[30] G. Galaverna, R. Corradini, E. Demunari, A. Dossena, R. Marchelli, Chiral separation of unmodified amino-acids by ligand-exchange high-performance liquid-chromatography using copper(II) complexes of l-amino-acid amides as additives to the eluent, J. Chromatogr. A 657 (1993) 43–54.

[31] N. Sanaie, C.A. Haynes, Interpreting the effects of temperature and solvent composition on separation of amino-acid racemates by chiral ligand-exchange chromatography, J. Chromatogr. A 1104 (2006) 164–172.

[32] B. Natalini, R. Sardella, G. Carbone, A. Macchiarulo, R. Pellicciari, The effect of the copper(II) salt anion in the chiral ligand-exchange chromatography of amino acids, Anal. Chim. Acta 638 (2009) 225–233.

[33] H. Ali, R. Patzold, H. Bruckner, Determination of L- and D-amino acids in smoke-less tobacco products and tobacco, Food Chem. 99 (2006) 803–812.

[34] E.A. Arriaga, Y.N. Zhang, N.J. Dovichi, Use of 3-(*p*-Carboxybenzoyl)quinoline-2-carboxaldehyde to label amino-acids for high-sensitivity fluorescence detection in capillary electrophoresis, Anal. Chim. Acta 299 (1995) 319–326.

[35] S. Kopec, U. Holzgrabe, Amino acids: aspects of impurity profiling by means of CE, Electrophoresis 28 (2007) 2153–2167.

[36] N. Novatchev, U. Holzgrabe, Evaluation of amino sugar, low molecular peptide and amino acid impurities of biotechnologically produced amino acids by means of CE, J. Pharm. Biomed. Anal. 28 (2002) 475–486.

[37] M. Ummadi, B.C. Weimer, Use of capillary electrophoresis and laser-induced fluorescence for attomole detection of amino acids, J. Chromatogr. A 964 (2002) 243–253.

[38] K.C. Lin, M.M. Hsieh, C.W. Chang, E.P. Lin, T.H. Wu, Stacking and separation of aspartic acid enantiomers under discontinuous system by capillary electrophoresis with light-emitting diode-induced fluorescence detection, Talanta 82 (2010) 1912–1918.

[39] D.W. Aswad, Determination of D-aspartate and L-aspartate in amino-acid mixtures by high-performance liquid-chromatography after derivatization with achiral adduct of *o*-phthaldialdehyde, Anal. Biochem. 137 (1984) 405–409.

[40] H.J. Shaolin Huang, F. Liu, F. Wang, Y. Zhang, Method for Preparing Magnesium Aspartate, CN 101239925 (A), Beijing Jingwei Xinkang Pharma, China, 2008.

[41] T.J. Ward, M. Nichols, L. Sturdivant, C.C. King, Use of organic modifiers to enhance chiral selectivity in capillary electrophoresis, Amino Acids 8 (1995) 337–344.

[42] J. Catalan, C. Diaz, F. Garcia-Blanco, Characterization of binary solvent mixtures of DMSO with water and other cosolvents, J. Org. Chem. 66 (2001) 5846–5852.

[43] I. Plowas, J. Swiergiel, J. Jadzyn, Relative static permittivity of dimethyl sulfoxide plus water mixtures, J. Chem. Eng. Data 58 (2013) 1741–1746.

[44] S. Orlandini, R. Gotti, S. Furlanetto, Multivariate optimization of capillary electrophoresis methods: a critical review, J. Pharm. Biomed. Anal. 87 (2014) 290–307.

[45] D. Montgomery, Design and Analysis of Experiments, 4th edition, John Wiley& Sons, New York, NY, 1996.

[46] J.W. Thompson, T.J. Kaiser, J.W. Jorgenson, Viscosity measurements of methanol-water and acetonitrile-water mixtures at pressures up to 3500 bar using a novel capillary time-of-flight viscometer, J. Chromatogr. A 1134 (2006) 201–209.

[47] P. Campins-Falco, C. Molins-Legua, A. Sevillano-Cabeza, L.A.T. Genaro, *o*-phthalaldehyde-*N*-acetylcysteine polyamine derivatives: formation and stability in solution and in C-18 supports, J. Chromatogr. B 759 (2001) 285–297.

[48] I. Molnar-Perl, A. Vasanits, Stability and characteristics of the *o*-phthaldialdehyde/ 3-mercaptopropionic acid and *o*-phthaldialdehyde/*N*-acetyl-L-cysteine reagents and their amino acid derivatives measured by high-performance liquid chromatography, J. Chromatogr. A 835 (1999) 73–91.

[49] R. Hanczko, I. Molnar-Perl, Derivatization, stability and chromatographic behavior of *o*-phthaldialdehyde amino acid and amine derivatives: *o*-phthaldialdehyde/2-mercaptoethanol reagent, Chromatographia 57 (2003) S103–S113.

[50] T. Erbe, H. Bruckner, Studies on the optical isomerization of dietary amino acids in vinegar and aqueous acetic acid, Eur. Food Res. Technol. 211 (2000) 6–12.

[51] S.M. Steinberg, P.M. Masters, J.L. Bada, The racemization of free and peptide-bound serine and aspartic-acid at 100-degrees-c as a function of pH—implications for in vivo racemization, Bioorg. Chem. 12 (1984) 349–355.

# 3. Impurity profiling of ibandronate sodium by HPLC-CAD

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**ABSTRACT**

The modern bisphosphonate drug ibandronate sodium, a challenging candidate for impurity profiling, was analyzed using high performance liquid chromatography (HPLC) combined with corona charged aerosol detection (CAD). Separation was achieved on a mixed mode column combining hydrophobic C18 and strong anion exchange retention mechanisms using a mass spectrometer compatible volatile mobile phase consisting of trifluoroacetic acid and acetonitrile while gradient elution was applied. The method was validated following the ICH guideline Q2(R1) and found suitable for the assessment of ibandronate's related substances. The observed CAD-response for all identified impurities was linear ($R^2$ > 0.995) over a small concentration range (0.05–0.25) and a quantification limit of at least 0.03 % was found. Four batches of two different manufacturers were tested by means of the method. None of the batches contained a single impurity above 0.05 %. The major impurities of all batches were the synthesis by-products *N*-desmethyl- and *N*-despentyl ibandronate as well as *N*,*N*-dimethyl pamidronate.

*Abbreviations:* CAD, corona charged aerosol detector; Ph. Eur., European Pharmacopoeia; AA, amino acids; API, active pharmaceutical ingredient; EDQM, European Directorate for the Quality of Medicines & HealthCare; EIC, extracted ion chromatogram; ELSD, evaporative light scattering detector; CLND, chemiluminescent nitrogen detector; TFA, trifluoroacetic acid; ICH, International Conference on Harmonisation; LOQ, limit of quantitation; MeCN, acetonitrile; $R^2$, coefficient of determination; S/N, signal-to-noise ratio; qNMR, quantitative nuclear magnetic resonance spectroscopy.

## 1. Introduction

First analogs of diphosphate, the so called bisphosphonates, have been developed in the middle of the 19th century [1] and were used in the industry as metal chelating agents and water softeners [2]. Since their biological effect on bone tissue and osteoclasts was discovered in the middle of the 20th century [1, 3, 4] many new structures have been developed. Among them are bisphosphonates containing a basic nitrogen atom in the side chain and those which do not (see Fig. 1), like the older derivatives: etidronate, clodronate and tiludronate. With the introduction of a basic side chain, the potency of the drugs increased tremendously because of a modified pharmacology [5, 6]. Important representatives are zoledronate, risedronate, alendronate, pamidronate, olpadronate, neridronate, incadronate and ibandronate [7–9].

For a better understanding of the impurity profile, the ibandronate synthesis had to be considered. Ibandronate sodium is synthesized by conversion of *N*-methyl-*N*-pentyl-β-alanine (1) with phosphorus oxychloride, phosphorus pentachloride and phosphorus trichloride, respectively, and phosphorous acid, and subsequent neutralization with sodium hydroxide (Fig. 2) [10–16] resulting in the α-hydroxy bisphosphonic acid sodium salt. The excess of phosphorus oxychloride and phosphorous acid used is transformed to sodium phosphate (3), sodium phosphonate (4) and sodium chloride (5) in the neutralization step. The potential impurities of the precursor (6, 8, 10) (Fig. 2) are converted to the desmethyl, the despentyl and the dimethyl derivative (7, 9, 11) of ibandronate (2), respectively. However, the occurrence of the dimethyl derivative (11) was not expected because this impurity's precursor *N*,*N*-dimethyl-β-alanine (10) is no possible side product in any known synthesis pathway (see Fig. 3) leading to the key intermediate (1) [10–12, 17]. Nevertheless low amounts of this compound were detected in all three batches of one manufacturer, maybe due to an impure starting material (e.g. methylamine contaminated with dimethylamine).

Since most of the bisphosphonate compounds and hence their potential impurities are lacking a chromophore for UV detection, other detection methods for impurity control are required. Those bisphosphonates containing primary or secondary amines in the side chain can be detected after suitable derivatization of this moiety [18–20]. However, the tertiary amine in ibandronate does not allow for such a reaction. Instead, the non-volatile impurities 3 and 4, the related substances 7, 9 and 11 as well as the amino acid precursor 1 of ibandronate (Fig. 2) can be subjected to particle based detectors like ELSD, CNLSD or CAD [21–27]. Due to the known disadvantages of the ELSD when it comes to impurity profiling, like non reproducible spike peaks and poor sensitivity [28–30], the CAD was chosen as detector because it does not show artefacts and provides better sensitivity.

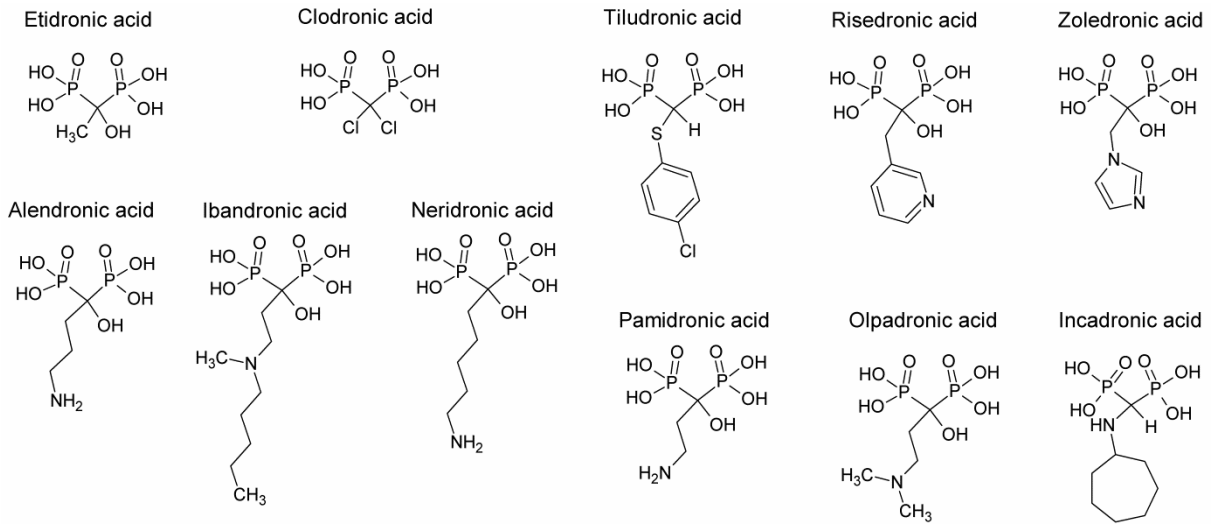


**Fig. 1.** Bisphosphonate drug substances.

Another challenge in the analysis of the strong acidic very hydrophilic compounds was the choice of a suitable stationary phase to achieve acceptable retention. A combination of reversed phase HPLC with ELSD using volatile ion-pairing reagents [21] or ion-exchange chromatography [24] are known. In order to avoid strong and mostly toxic volatile ion-pairing agents like n-amylamine, tributylamine or tripropylamine, a mixed mode column combining strong anion exchange with reversed phase properties, which was already applied successfully in the analysis of etidronate [22], was chosen. For identification of the impurities in the chromatogram, they were synthesized according to Fig. 4.

## 2. Experimental

### *2.1. Chemicals and reagents*

Ibandronate sodium monohydrate, pamidronate disodium pentahydrate, etidronate disodium and clodronate disodium tetrahydrate were obtained from the European Directorate for the Quality of Medicines & HealthCare (EDQM) (Strasbourg, France). Diethylcarbonate and chloroform were obtained from Fisher Scientific GmbH (Nidderau, Germany) whereas HPLC gradient grade acetonitrile was purchased from VWR International S.A.S. (Fontenay-sous-Bois, France), acetone, ethanol, hydrochloric acid 37 %, *N*-methyl-*N*-pentyl-amine, methyl acrylate, methylamine solution in ethanol 33 %, dimethylamine solution in ethanol 33 % and maleic acid standard for quantitative NMR (qNMR) (TraceCERT®), methanol, monosodium phosphate anhydrous, phosphorous acid, sulfolane 99 %, trifluoroacetic acid were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Deuterium oxide (99.9 % d-atom) was obtained from Deutero GmbH (Kastellaun, Germany), phosphorous oxychloride, hydrogen peroxide 30% from Merck Millipore (Schwalbach, Germany) and 2-propanol ≥ 99.7 % was purchased from Bernd Kraft GmbH (Duisburg, Germany). All

chemicals used for quantification were of analytical grade or even better. Ultrapure water was produced by a water purification system from Merck Millipore (Schwalbach, Germany). All solutions were filtered through a 0.22 µm PVDF filter supplied by Machery-Nagel GmbH & Co., KG (Düren, Germany) prior to use.

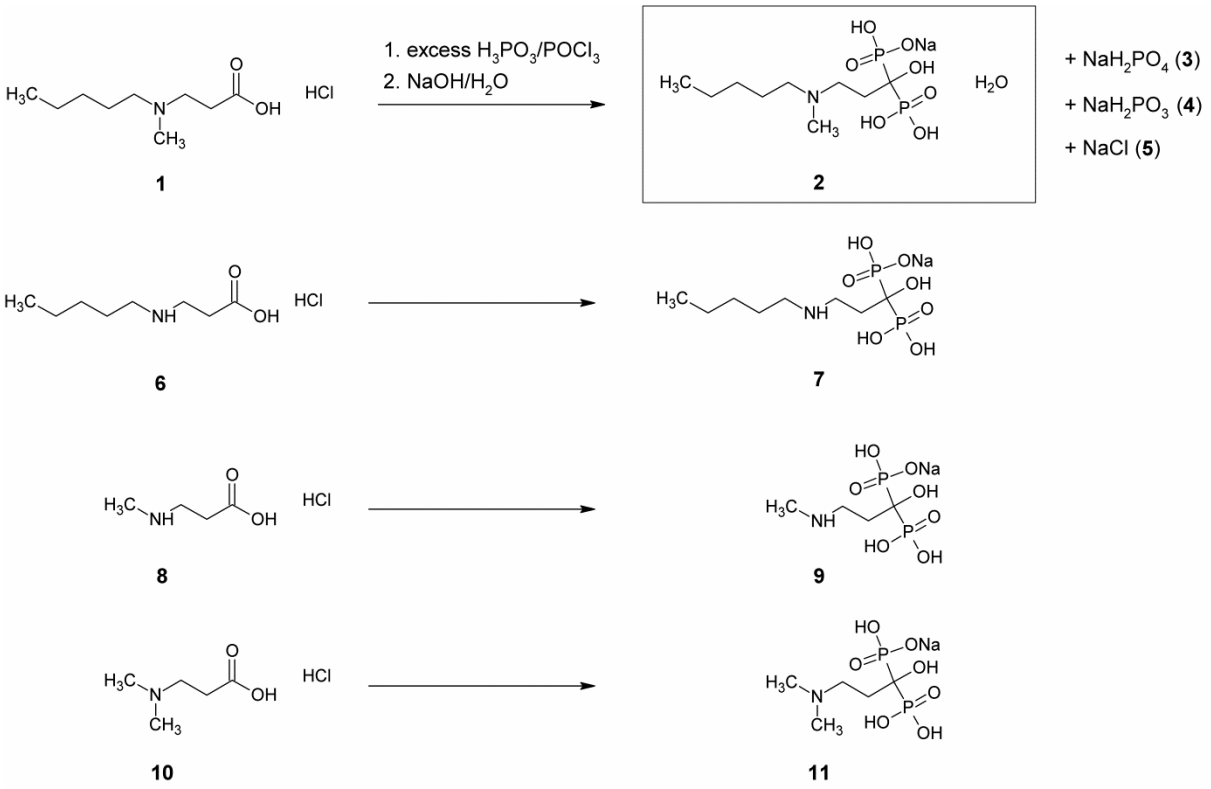


**Fig. 2.** Synthesis of ibandronate sodium (**2**) from *N*-methyl-*N*-pentyl-β-alanine HCl (**1**); the potential impurities in the precursor (**6**, **8** and **10**) lead to the related substances **7**, **9** and **11** in the last synthesis step, whereas sodium phosphate (**3**), sodium phosphite (**4**) and sodium chloride (**5**) are by-products of the reaction. [10–12, 14].

### *2.2. Apparatus*

The HPLC-CAD experiments were performed on an Agilent 1100 modular chromatographic system consisting of online vacuum degasser, binary pump, auto sampler, thermostatted column compartment and a photodiode array detector (Agilent Technologies, Waldbronn, Germany). The Corona CAD detector (Thermo Fisher, Courtaboeuf, France) was linked to the HPLC system by a 0.25 mm internal diameter PEEK capillary and a 0.22 µm stainless steel inlet frit. Highly pure nitrogen (99.9 %) for the detector was produced by an ESA Nitrogen Generator (Thermo Fisher, Courtaboeuf, France). The inlet pressure (nitrogen) was 35.0 psi. The peak areas were integrated automatically using the Agilent ChemStation® Rev B.03.02 software program.

LC–MS experiments were performed on a LC/MSD Trap G2445D ion trap (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 1100 modular chromatographic system consisting of online vacuum degasser, binary pump, thermostatted auto sampler, thermostatted column compartment and a photodiode array detector (Agilent Technologies, Waldbronn, Germany) via electro spray ionization (ESI) interface. Nebulizer pressure: 50 psi, dry gas flow: 12 L/min, dry temperature: 350 °C, capillary voltage 3500 V. IR spectra were recorded using a Jasco FT/IR-6100 Fourier transformation infrared spectrometer (Jasco Germany GmbH, Gross-Umstadt, Germany) equipped with MIRacleTM single reflection horizontal ATR accessory holding a diamond/ZnSe crystal (PIKE Technologies, Madison WI, USA). All NMR experiments were carried out on a Bruker Avance® spectrometer (Karlsruhe, Germany; $^{1}$H 400.132 MHz, $^{13}$C 100.613 MHz, $^{31}$P 161.977 MHz). The spectra were processed using Bruker TopSpin v3.0 software program. $^{1}$H NMR experiments were performed with 16 scans at a sample spinning frequency of 20 Hz, 300 K and a flip angle of 30°, whereas $^{13}$C nuclei were measured with 1024 scans, $^{1}$H broadband decoupling and rotation (20 Hz) at 300 K. $^{31}$P NMR spectra were recorded with 32 scans at 300 K, a flip angle of 30° and 20 Hz sample spinning frequency, 200.44 ppm spectral width, transmitter offset at 10 ppm with and without $^{1}$H decoupling and the ppm scale calibrated to phosphoric acid 85 %. All experiments were carried out with broad band observer (BBO BB-H 5mm) probe. The $^{1}$H-qNMR experiments were performed using a relaxation delay between two scans of 60 s applying a spectral width of 20.55 ppm, the transmitter offset at 6.175 ppm and the time domain at 64k.

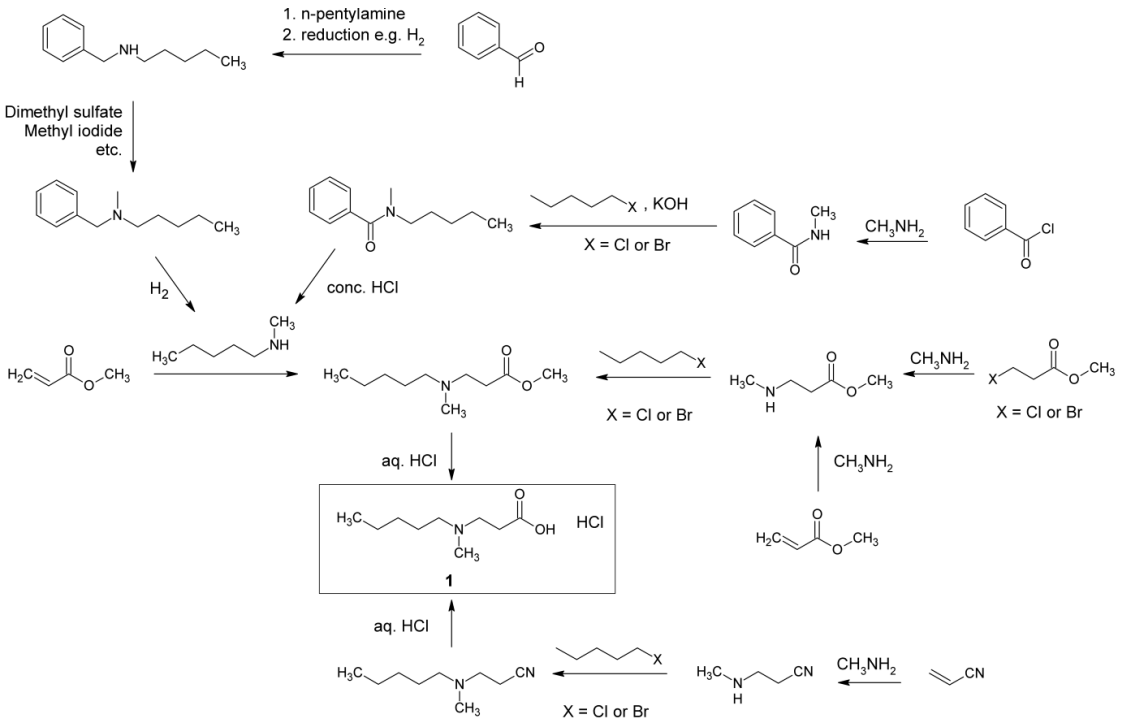


**Fig. 3.** Synthesis routes to the key intermediate *N*-methyl-*N*-pentyl-β-alanine HCl (**1**) [10–12, 15, 17].

*2.3. Chromatographic procedure*

A mixed mode column SIELC Coresep® SB (150×4.6mm i.d., with a particle size of 2.7 µm and pore size of 100 Å, SIELC Technologies, Prospect Heights IL, USA) was used as stationary phase. The chromatographic system was operated using gradient elution at 25 °C. Mobile phase A was ultrapure water while mobile phase B was composed of 15 % v/v MeCN containing 15 mmol/L TFA. The flow-rate was set to 1.2 mL/min and CAD detection with the filter set to “none” was applied. The injection volume was 10 µL. Gradient: 0–3 min: 30 % B; 3–10 min: 30–100 % B; 10–17 min: 100 % B. The last isocratic step of the gradient was modified for the analysis of etidronate and clodronate: 0–3 min: 30 % B; 3–10 min: 30–100 % B; 10–20 min: 100 % B and 0–3 min: 30 % B; 3–10 min: 30–100 % B; 10–30 min: 100 % B, respectively.

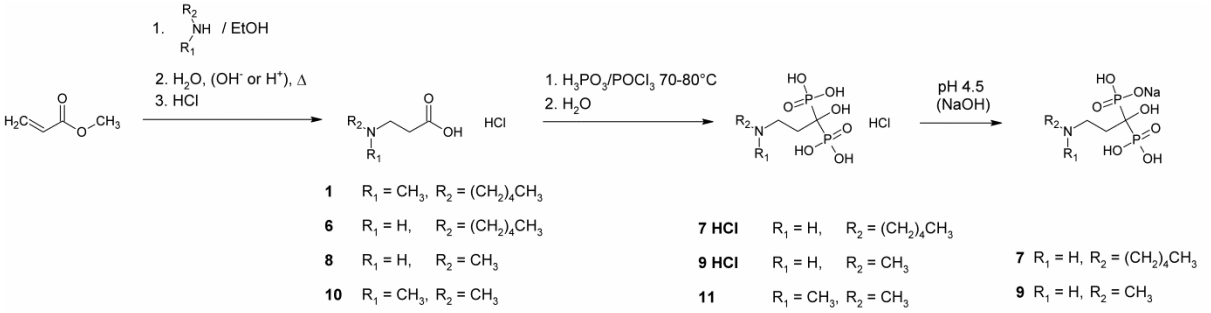


**Fig. 4.** Synthesis route to the impurities **1**, **7**, **9** and **11** according to the general procedure for the preparation of α-hydroxy bisphosphonic acids [16].

The chromatographic conditions for LC–MS were the same as for HPLC–CAD except for the flow rate which was set to 1.0 mL/min to match the requirements of the ESI-source. The column effluent was subjected to the ESI-source from 2 to 11 min only when running sample solutions in order to protect the instrument from high amounts of sodium (eluting at 1.0 min) and ibandronic acid (at 13.0 min).

*2.3.1. Preparations of solutions*

The test solutions for HPLC–CAD and LC–MS were 20 mg/mL solutions of ibandronate sodium monohydrate in water. 1 mg/mL solutions of the impurities (calculated as free base or acid, respectively) in water were prepared for spiking the test solutions. For the test solutions for linearity 20 mg ibandronate sodium monohydrate were spiked with 10, 20, 30, 40 and 50 µL of each 1 mg/mL impurity stock solution and diluted to 1.0 mL. The reference solutions for quantification contained 0.02 mg/mL of phenylalanine, phosphorous acid, sodium dihydrogen phosphate and ibandronate sodium monohydrate, respectively. The system suitability solution was composed of 5 mg phenylalanine, 5 mg sodium dihydrogen phosphate and 5 mg pamidronate sodium pentahydrate dissolved in 100.0 mL water. All

solutions were stored at 4 to 8 °C. A 20 mg/mL solution of ibandronate sodium monohydrate in 3 % hydrogen peroxide solution was prepared for the forced degradation experiment and kept at room temperature for 5 days.

*2.4. Quantitative NMR spectroscopy*

5 mg of the substance to be examined and 5 mg of maleic acid were dissolved in 700 µL $D_2O$ and immediately subjected to quantitative NMR analysis. After manual phase and automatic baseline correction, a suitable signal of the substance to be examined and the methine protons singlet of maleic acid as an internal standard at 6.02 ppm (2H) were integrated and quantified using the following relationship:

$$w(X) = \frac{MW(X)}{MW(IS)} \cdot \frac{A(X)}{A(IS)} \cdot \frac{m(IS)}{m(X)} \cdot \frac{N(IS)}{N(X)} \quad (1)$$

where MW(X) and MW(IS) are the molecular weights in g/mol, N(IS) and N(X) the numbers of protons, A(X) and A(IS) are the areas for the selected NMR signals of the examined substance (X) and maleic acid (IS), respectively. The masses (weights) in mg of maleic acid (IS) and the substance (X) are m(IS) and m(X). The purity of the tested compound is then expressed by w(X) [31].

*2.5. Synthesis of the impurities*

*2.5.1. N-Methyl-N-pentyl-β-alanine HCl (**1**)*

The substance was prepared from *N*-methyl-*N*-pentyl-amine and methyl acrylate according to Junghans and Baetz [11] (105 mg, yield: 9.5 %, purity 99 % qNMR). $^1$H-NMR ($D_2O$) $\delta_H$ (ppm) 0.84 (m, 3H, $\underline{H_3C}$-$(CH_2)_4$N-), 1.3 (m, 4H, $H_3C$-$(\underline{CH_2})_2$-$(CH_2)_2$-N-), 1.7 (m, 2H, $H_3C$-$(CH_2)_2$-$\underline{CH_2}$-$CH_2$-N-), 2.82 (s, 3H, $H_3C$-$(CH_2)$4-N-$\underline{CH_3}$) , 2.84 (t, $^3J_{HH}$ = 6.8 Hz, 2H, -N-$CH_2$-$\underline{CH_2}$-COOH), 3.04 – 3.18 (m, 2H, $H_3C$-$(CH_2)_3$-$\underline{CH_2}$-N-), 3.25 – 3.54 (m, 2H, -N-$\underline{CH_2}$-$CH_2$-COOH) ;$^{13}$C-NMR ($D_2O$) $\delta_C$ (ppm) 12.98 ($H_3\underline{C}$-$(CH_2)_4$-N-), 21.44 ($H_3C$-$\underline{C}H_2$-$(CH_2)_3$-N-), 23.10 ($H_3C$-$(CH_2)_2$-$\underline{C}H_2$-$CH_2$-N-), 27.72 ($H_3C$-$CH_2$-$\underline{C}H_2$-$(CH_2)_2$-N-), 28.64 (-N-$CH_2$-$\underline{C}H_2$-COOH) , 39.99 ($H_3C$-$(CH_2)_4$-N-$\underline{C}H_3$), 51.23 (-N-$\underline{C}H_2$-$CH_2$-COOH), 56.63 ($H_3C$-$(CH_2)_4$-$\underline{C}H_2$-N-), 174.08 (-N-$(CH_2)_2$-$\underline{C}$OOH); ESI-MS (m/z): 174.0 ($[M+H]^+$, found), 174.3 ($[M+H]^+$, calculated); IR (FT): v ($cm^{-1}$) = 2954 (m), 2644 (m), 1718 (s, C=O), 1468 (m), 1433 (m), 1420 (m), 1405 (m), 1368 (m), 1296 (m), 1198 (s, C-O), 1153 (m), 992 (m), 850 (m) , 799 (w) , 732 (w) , 653 (m)

*2.5.2. N-pentyl-β-alanine HCl (**6**)*

23.18 g (0.2 mol) of pentylamine were dissolved in 20 mL ethanol. 8.6 g (0.1 mol) Methylacrylate were dissolved in 75 mL methanol and added drop wise to the solution of pentylamine over 1 hour at room temperature. Afterwards, the solvent was removed in vacuo, 200 mL of 0.5 M sodium hydroxide were added and refluxed for 1 hour. The aqueous phase was extracted 4 times with 25 mL of chloroform to remove the residual pentylamine. The chloroform extract was discarded and the pH of the aqueous phase was set to 4.5 with hydrochloric acid. Sodium chloride was then precipitated by the addition of methanol. Most of the solvent was then removed in vacuo and the hydrochloride **6** precipitated from the solution after the addition of acetone upon storage at –20 °C for several days. The compound was obtained after recrystallization from acetone/water as white powder (2.0g, yield: 10.2%). $^{1}$H-NMR ($D_2O$) $\delta_H$ (ppm) 0.82 (m, 3H, $\underline{H_3C}$-$(CH_2)_4$N-), 1.29 (m, 4H, $H_3C$-$(\underline{CH_2})_2$-$(CH_2)_2$-N-), 1.64 (m, 2H, $H_3C$-$(CH_2)_2$-$\underline{CH_2}$-$CH_2$-N-), 2.77 (t, $^{3}J_{HH}$ = 6.6 Hz, 2H, -N-$CH_2$-$\underline{CH_2}$-COOH), 3.02 (m, 2H, $H_3C$-$(CH_2)_3$-$\underline{CH_2}$-N-), 3.27 (t, $^{3}J_{HH}$= 6.6 Hz, 2H, -N-$\underline{CH_2}$-$CH_2$-COOH); $^{13}$C-NMR ($D_2O$) $\delta_C$ (ppm) 13.01 ($H_3\underline{C}$-$(CH_2)_4$-N-), 21.43 ($H_3C$-$\underline{C}H_2$-$(CH_2)_3$-N-), 24.98 ($H_3C$-$(CH_2)_2$-$\underline{C}H_2$-$CH_2$-N-), 27.77 ($H_3C$-$CH_2$-$\underline{C}H_2$-$(CH_2)_2$-N-), 30.14 (-N-$CH_2$-$\underline{C}H_2$-COOH) , 42.76 (-N-$\underline{C}H_2$-$CH_2$-COOH), 47.82 ($H_3C$-$(CH_2)_4$-$\underline{C}H_2$-N-), 174.30 (-N-$(CH_2)_2$-$\underline{C}$OOH); IR (FT): ν ($cm^{-1}$) = 2930 (sb), 2765 (sb), 2474 (m), 1696 (s, st, C=O), 1438 (m), 1400 (m), 1303 (m), 1226 (m, st, C-O), 1050 (w), 907 (wb), 799 (w), 731 (w), 680 (w)

*2.5.3. 1-Hydroxy-3-[amino]propane-1,1-diyl bis(phosphonic acid) sodium (**7**) (pentyl)*

The substance was synthesized from **6**, phosphorous acid and phosphorus oxychloride following the general procedure for the preparation of α-hydroxy bisphosphonic acids published by Kieczykowski et al. [16]. (100 mg, purity 91.4% by qNMR). $^{1}$H-NMR ($D_2O$) $\delta_H$ (ppm) 0.81 (m, 3H, $\underline{H_3C}$-$(CH_2)_4$N-), 1.28 (m, 4H, $H_3C$-$(\underline{CH_2})_2$-$(CH_2)_2$-N-), 1.61 (m, 2H, $H_3C$-$(CH_2)_2$-$\underline{CH_2}$-$CH_2$-N-), 2.27 (tt, $^{3}J_{HH}$ = 6.9 Hz, $^{3}J_{HP}$ = 13.0 Hz, 2H, -N-$CH_2$-$\underline{CH_2}$- COH$(PO_3H)_2$), 2.97 (m, 2H, $H_3C$-$(CH_2)_3$-$\underline{CH_2}$-N-), 3.31 (t, $^{3}J_{HH}$ = 6.9 Hz, 2H, -N-$\underline{CH_2}$-$CH_2$-COH$(PO_3H)_2$); $^{13}$C-NMR ($D_2O$) $\delta_C$ (ppm) 13.01 ($H_3\underline{C}$-$(CH_2)_4$-N-), 21.46 ($H_3C$-$\underline{C}H_2$-$(CH_2)_3$-N-), 25.30 ($H_3C$-$CH_2$)$_2$-$\underline{C}H_2$-$CH_2$-N-), 27.81 ($H_3C$-$CH_2$-$\underline{C}H_2$-$(CH_2)_2$-N-), 29.58 (-N-$\underline{C}H_2$-$CH_2$-COH$(PO_3H)_2$), 44.08 (t, $^{2}J_{CP}$ = 7.7 Hz, -N-$CH_2$-$\underline{C}H_2$-COH$(PO_3H)_2$), 47.70 ($H_3C$-$(CH_2)_4$-$\underline{C}H_2$-N-), 72.43 (t, $^{1}J_{CP}$ = 134.4 Hz, -N-$(CH_2)_2$-$\underline{C}$OH$(PO_3H)_2$); $^{31}$P-NMR ($D_2O$) $\delta_H$ (ppm) 17.04 (t, $^{3}J_{HP}$ = 13.0 Hz); ESI-MS (m/z): 306.1 ($[M+H]^+$, found), 306.1 ($[M+H]^+$, calculated) 304.8 ($[M-H]^-$, found), 304.1 ($[M-H]^-$, calculated); IR (FT): ν ($cm^{-1}$) = 2956 (m), 1603 (w), 1455 (w, δ, C-H), 1391 (w), 1331 (w), 1153 (s, st, P=O), 1080 (m), 1012 (sb, st, P=O), 926 (sb, st, P–O), 900 (s, st, P–O), 768 (m), 676 (m)

*2.5.4. N-Methyl-β-alanine HCl (**8**)*

The methyl ester of **8** was prepared from methylamine and methyl acrylate according to Lv et al. [32]. The methyl ester was then directly converted into **8** without further purification according to Blower et al. [33] (4.4g, yield: 31.5%). $^{1}$H-NMR ($D_2O$) $\delta_H$ (ppm) 2.79 (s, 3H, -HN-$\underline{CH_3}$) , 2.87 (t, J = 6.4 Hz, 2H, -N-$CH_2$-$\underline{CH_2}$-COOH), 3.35 (t, J = 6.44 Hz, 2H, -N-$\underline{CH_2}$-$CH_2$-COOH);$^{13}$C-NMR ($D_2O$) $\delta_C$ (ppm) 29.99 (-N-$CH_2$-$\underline{C}H_2$-COOH) , 33.02 (-HN-$\underline{C}H_3$), 44.48 (-N-$\underline{C}H_2$-$CH_2$-COOH), 174.28 (-N-$CH_2$-$CH_2$-$\underline{C}$OOH); IR (FT): v ($cm^{-1}$) = 3073 (sb), 2832 (sb), 1731 (s, st, C=O), 1556 (m, δ, N-H), 1465 (m), 1454 (m), 1428 (m), 1402 (m), 1350 (m), 1289 (m), 1188 (m, st, C-O), 1157 (m), 1074 (w) , 1025 (m) , 952 (w) , 886 (m), 757 (m)

*2.5.5. 1-Hydroxy-3-[amino]propane-1,1-diyl bis(phosphonic acid) sodium (**9**) (methyl)*

The substance was prepared from **8**, phosphorous acid and phosphorus oxychloride according to Blower and Rafael [33] and the general procedure for the preparation of α-hydroxy bisphosphonic acids (Kieczykowski et al. [16]) (340 mg, purity: 92.5% free acid by qNMR). $^{1}$H-NMR ($D_2O$) $\delta_H$ (ppm) 2.27 (tt, $^{3}J_{HH}$ = 6.8 Hz, $^{3}J_{HP}$ = 12.6 Hz, 2H, -N-$CH_2$-$\underline{CH_2}$-$COH(PO_3H)_2$), 2.66 (s, 3H, -HN-$\underline{CH_3}$), 3.3 (t, $^{3}J_{HH}$ = 6.8 Hz, -N-$\underline{CH_2}$-$CH_2$-$COH(PO_3H)_2$);$^{13}$C-NMR ($D_2O$) $\delta_C$ (ppm) 29.41 (-N-($\underline{C}H_2$)$_2$-$COH(PO_3H)_2$), 32.81 ($H_3\underline{C}$-N-), 45.92 (t, $^{2}J_{CP}$ = 7.5 Hz, -N-$CH_2$-$\underline{C}H_2$-$COH(PO_3H)_2$), 72.54 (t, $^{1}J_{CP}$ = 134.6 Hz -N-$(CH_2)_2$-$\underline{C}OH(PO_3H)_2$); $^{31}$P-NMR ($D_2O$) $\delta_H$ (ppm) 16.95 (t, $^{3}J_{HP}$ = 12.6 Hz); ESI-MS (m/z): 250.0 ($[M+H]^+$, found), 250.1 ($[M+H]^+$, calculated), 248.0 ($[M-H]^-$, found), 248.1 ($[M-H]^-$, calculated); IR (FT): v ($cm^{-1}$) = 3141 (s), 2716 (m), 1607 (w), 1486 (w), 1466 (w), 1400 (w), 1314 (wb), 1153 (s, st, P=O), 1082 (m), 1053 (s, st, P=O), 1015 (s, st, P–O), 934 (sb), 901 (s, st, P–O), 867 (m), 775 (w), 671 (m)

*2.5.6. N,N-Dimethyl-β-alanine HCl (**10**)*

The methyl ester of **10** was prepared from dimethylamine and methyl acrylate according to Annenkov et al. [34]. The methyl ester was then treated without further purification as described by Devarajan et al. [35] to obtain the compound **10**. (2.1g, yield: 65 %). $^{1}$H-NMR ($D_2O$) $\delta_H$ (ppm) 2.84 (t, J = 6.7 Hz, 2H, -N-$CH_2$-$\underline{CH_2}$-COOH), 2.87 (s, 6H, -HN-($\underline{CH_3}$)$_2$), 3.38 (t, J = 6.7 Hz, 2H, -N-$\underline{CH_2}$-$CH_2$-COOH);$^{13}$C-NMR ($D_2O$) $\delta_C$ (ppm) 28.86 (-N-$CH_2$-$\underline{C}H_2$-COOH) , 42.97 (-HN-($\underline{C}H_3$)$_2$), 53.15 (-N-$\underline{C}H_2$-$CH_2$-COOH), 173.95 (-N-$(CH_2)_2$-$\underline{C}$OOH); IR (FT): v ($cm^{-1}$) = 2964 (sb), 2696 (sb), 1718 (s, st, C=O), 1468 (m), 1421 (m), 1402 (m), 1370 (m), 1298 (m), 1240 (m), 1197 (s, st, C-O), 1162 (m), 1149 (m), 1010 (w) , 970 (m) , 909 (w) , 855 (m), 798 (m)

*2.5.7. 1-Hydroxy-3-[amino]propane-1,1-diyl bis(phosphonic acid) HCl (**11**) (dimethyl)*

The compound was prepared from **10**, phosphorous acid and phosphorus oxychloride according to Martin et al. [36] and treated as described by Blower and Rafael [33] to yield the hydrochloride (**11**) instead of the sodium salt of **11**. (300 mg, yield 15%, purity: 94.5% by qNMR). $^{1}$H-NMR ($D_2O$) $\delta_H$ (ppm) 2.32 (tt, $^{3}J_{HH}$ = 6.9 Hz, $^{3}J_{HP}$ = 13.0, 2H, -N-$CH_2$-$\underline{CH_2}$-$COH(PO_3H)_2$), 2.84 (s, 6H, -HN-($\underline{CH_3}$)$_2$), 3.41 (t, $^{3}J_{HH}$ = 6.9 Hz, -N-$\underline{CH_2}$-$CH_2$-$COH(PO_3H)_2$);$^{13}$C-NMR ($D_2O$) $\delta_C$ (ppm) 28.03 (-N-$\underline{C}H_2$-$CH_2$-$COH(PO_3H)_2$), 42.85 (($H_3\underline{C}$)$_2$-N-), 54.47 (t, $^{2}J_{CP}$ = 7.0 Hz, -N-$CH_2$-$\underline{C}H_2$-$COH(PO_3H)_2$), 72.08 (t, $^{1}J_{CP}$ = 138.5 Hz -N-$(CH_2)_2$-$\underline{C}OH(PO_3H)_2$); $^{31}$P-NMR ($D_2O$) $\delta_H$ (ppm) 16.87 (t, $^{3}J_{HP}$ = 13.0); ESI-MS (m/z): 264.0 ($[M+H]^+$, found), 264.04 ($[M+H]^+$, calculated), 262.0 ($[M-H]^-$, found), 262.02 ($[M-H]^-$, calculated); IR (FT): v ($cm^{-1}$) = 3128 (m), 2770 (m), 1472 (w), 1380 (w), 1280 (wb), 1154 (s, st, P=O), 1095 (m), 1059 (s, st, P=O), 1028 (s, st, P–O), 978 (m), 901 (s, st, P–O), 951 (m), 927 (m), 898 (m), 712 (w), 670 (w)

## 3. Results and discussion

### *3.1. HPLC method development*

#### *3.1.1. Chromatographic procedure*

Ibandronic acid and its impurities are strong acids hardly retained on conventional reversed phases. Moreover, the only difference between the API and the related substances is the chain length of the *N*-alkyl residue demanding for lipophilic interactions for the separation. Avoiding the conventional solution, the application of ion-pairing agents, a mixed mode column combining reversed phase retention mechanism with strong anion exchange moieties was successfully applied for the separation. When operating the column with acidic mobile phases, neutral compounds or weak carboxylic acids are retained by the reversed phase mechanism whereas hydrophilic strong acidic substances such as olpadronic acid (**11**) are separated by the anion exchange mechanism. Strong acidic compounds also containing lipophilic entities, i.e. ibandronic acid, are retained by both mechanisms. The method was developed using a column length of 50 mm allowing for short run times and high throughput. In order to improve the separation of the precursor (**1**) from the sodium peak, a longer sized column (150 mm) was applied successfully and eventually used for validation and batch testing. Different kinds of mobile phase additives and buffers were checked. Acidified ammonium formate and acetate buffers within the allowed pH range (1.5–5) of the column led to poor peak shapes and thus decreased sensitivity. Only trifluoroacetic acid proved strong enough to suppress the dissociation of the strong acidic bisphosphonates.

The optimized method comprised a gradient elution (4.5 mmol/L TFA and 4.5 % v/v MeCN to 15 mmol/L TFA and 15 % v/v MeCN) after a short isocratic step eluting all impurities and the API in less than 20 min using 1.2 mL/min flow rate at 25 °C column temperature (see Fig. 5 chromatogram A).

*3.1.2. Sample preparation*

Since the sodium salt of ibandronic acid is highly soluble in water, a concentrated sample solution (20 mg/mL) could be prepared allowing for low detection and quantification limits. The sample solution was considered stable since no change in peak areas or additional peaks were observed after storage of 24 h at room temperature.

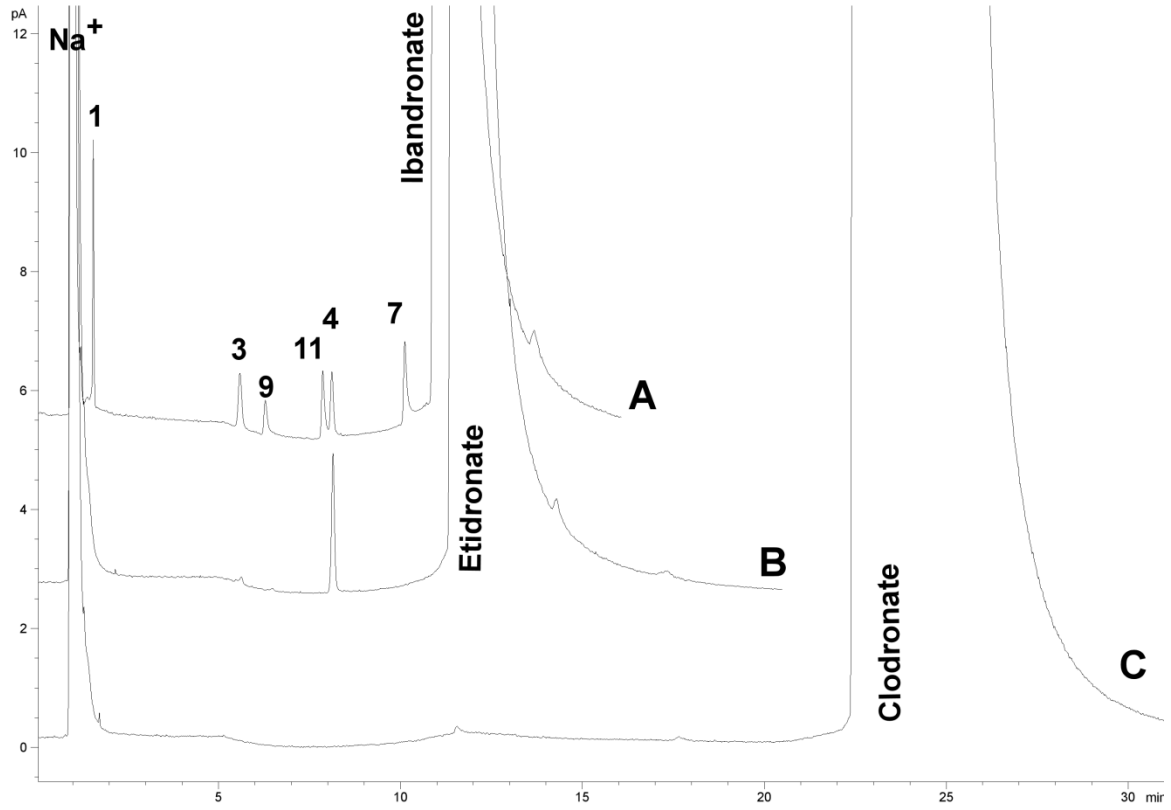


**Fig. 5.** A: Chromatogram obtained from a spiked test solution (0.05 %) of ibandronate; B: 20 mg/mL etidronate test solution in $H_2O$; C: 20 mg/mL clodronate test solution in $H_2O$; chromatographic conditions see Section 2.3; elution order A: sodium, *N*-pentyl-*N*-methyl-β-alanine (**1**), phosphate (**3**); despentylibandronate (**9**), olpadronate (**11**), phosphite (**4**), desmethylibandronate (**7**); B: sodium, phosphate, etidronate; C: sodium, clodronate.

*3.1.3. Peak assignment*

The expected related substances **1**, **7** and **9** were synthesized in order to identify the peaks in the sample solutions and for the validation. The compound **11** was prepared after the batch and LC/MS results were available to confirm the identity of the substance.

*3.2. Validation of the HPLC method*

The method was validated for impurity profiling with regards to the following parameters: specificity, linearity, range, precision, accuracy, LOQ and robustness, following the International Conference on Harmonisation (ICH) guideline Q2(R1) [37]. System suitability criteria were defined and evaluated. The purpose of this work was to develop a method applicable for the Ph. Eur. Since the drug substance is administered in doses of 150 mg p.o. once a month, 50 mg once a day or 3 mg i.v. once in three months a reporting threshold of 0.05 % was applied according to ICH guideline Q3A(R2) [38].

Specificity of the method was proven by comparing spiked samples with a blank solution and by a forced degradation experiment. The resolution was at least 1.5 for every identified impurity peak and every impurity was separated from the main peak, the sodium peak, from oxidative degradation products and from each other (see Fig. 5 chromatogram A and Fig. 7).

The linearity and range for all impurities (**1**, **3**, **4**, **7**, **9**, **11**) and phenylalanine (external reference) were determined by constructing calibration curves from 10 to 50 µg/mL (equal to 0.05–0.25 %) in spiked test solutions to account for possible interactions with the matrix (e.g. sodium or the peak caused by ibandronic acid). The coefficient of determination was at least 0.997 (see Table 1).

The LOQ for each identified impurity (**1**, **3**, **4**, **7**, **9**, **11**) was calculated from the standard deviation and the slope of the calibration curve according to ICH guideline Q2(R1). The LOQs calculated were 0.03 % or even less (see Table 1) and the S/Ns at the corresponding LOQ was 20 or higher.

Accuracy was assessed on spiked sample solutions. The recovery rate was calculated at 0.05, 0.10 and 0.15 %. The recovery rates were found to be between 81 and 120 % (n = 3, RSD = 0.30–4.81 %) on each level. The quantification of impurities eluting in front of phosphate (3) was done by comparing the phenylalanine peak area as an external standard in the reference solution for quantification with the peak area of the impurities detected in the sample solution whereas later eluting substances were quantified using the phosphite (**4**) peak in the same reference solution using the correction factors obtained from the calibration curve slopes (see Table 1). This procedure was necessary due to the rising concentration of organic modifier enhancing the evaporation of mobile phase and thus increased response of late eluting compounds.

Repeatability and precision were determined on a real batch sample and on one spiked batch sample, because no batch contained all impurities above the LOQ. The impurity

content was measured in sextuple on two different days. The RSD intra-day was between 0.8 and 1.6 % (n = 6) and inter-day determined on two consecutive days between 0.13 and 0.49 % (n = 2).

The sample solutions were stable for at least a day since no additional peaks or changes in peak areas after 24 h were noticed in any batch sample. Additional peaks between phosphite (**4**) and desmethylibandronate (**7**) were detected in forced degradation experiments exposing a 20 mg/mL solution of the substance to 3 % $H_2O_2$ for 1, 3, and 5 days. The oxidation products did not interfere with the identified impurities (see Fig. 7). Ibandronate was proven stable under other than oxidizing conditions like UV light, acidic and basic media (c. ref. [24]).

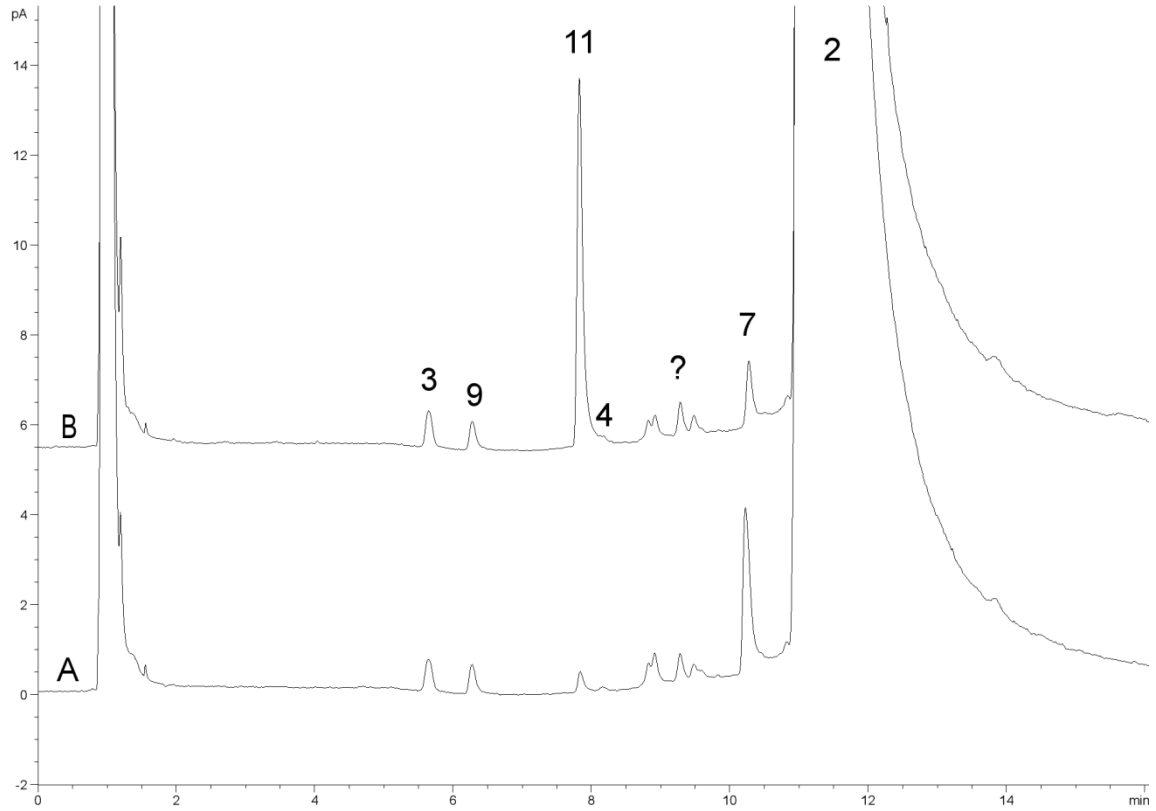


**Fig. 6.** A: Chromatogram obtained the test solutions of batch III; A: spiked with **7**; B: spiked with **11**; chromatographic conditions see Section 2.3.

For checking the robustness, the operation parameters were varied in the following ranges: temperature ±5 °C, flow rate ±0.1 mL/min, gradient time ±1 min, isocratic part ±1 min and the composition during isocratic part ±3 % mobile phase B. A spiked sample solution containing all known impurities at 0.05 % and ibandronate sodium at 20 mg/mL was analyzed under either condition. None of the selected variations did impair the separation of the related compounds (see Fig. 8).

For the establishment of a system suitability test, the resolutions between sodium and *N*-methyl-*N*-pentyl-*β*-alanine (**1**) as well as between phosphate (**3**) and despentylibandronate (**9**) were studied. Sodium is always present in huge amounts, as the API is the sodium salt. Thus, the separation of the sodium peak from the precursor (**1**) had to be ensured. Phenylalanine instead of *N*-methyl-*N*-pentyl-β-alanine and pamidronic acid instead of despentylibandronate were used for the system suitability test, because these substances behave very similar on the column and are commercially available at low cost. A resolution of at least 10 between sodium and phenylalanine and 4 between pamidronate and phosphate, respectively, was found (data not shown).

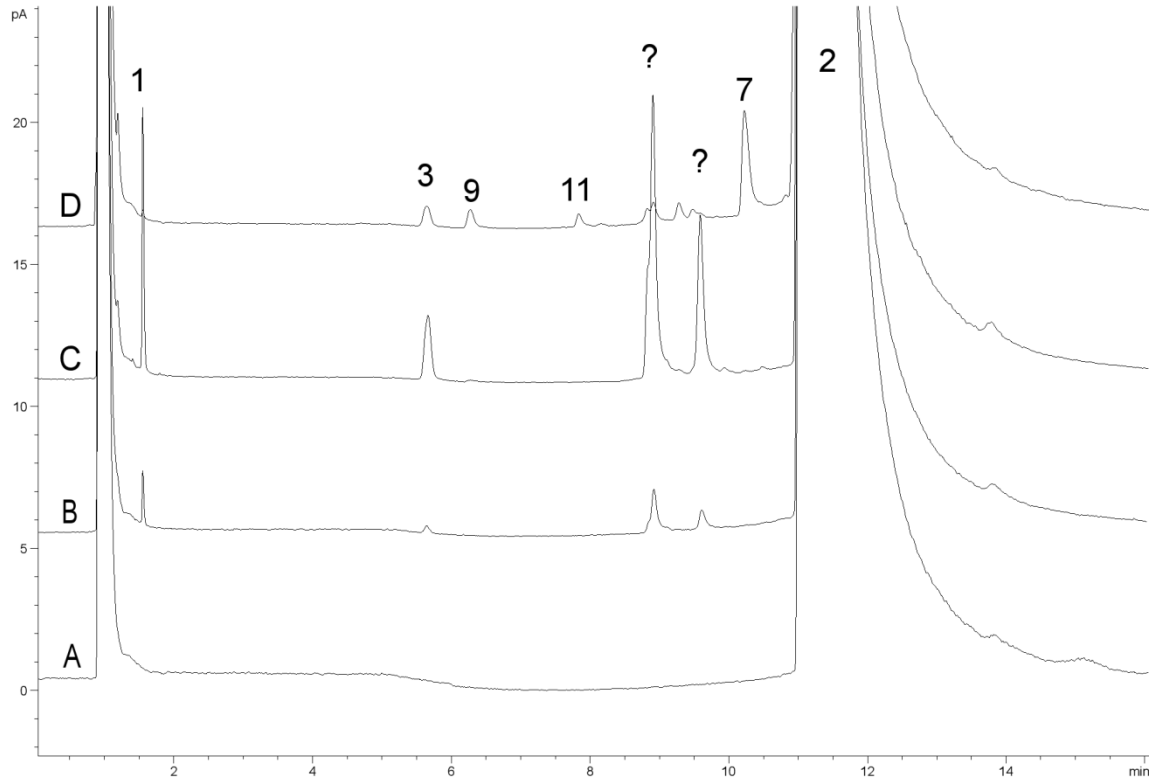


**Fig. 7.** A-C Chromatograms obtained from the test solutions of batch I in 3 % H2O2 after 0, 1 and 5 d; D: batch III – at least some of the substances eluting between **11** and **7** seem to be degradation products; chromatographic conditions see Section 2.3.

*3.3. Batch results*

One batch (I) of one manufacturer and three batches (II–IV) of a second supplier were screened using the above mentioned method (see Table 1 for results). No impurity could be detected in batch I whereas all kinds of impurities, except the key intermediate (**1**), were found in the latter three batches at very low levels (<0.05 %). A tiny unknown peak between sodium and phosphate (**3**) at about 3.0 min, a medium sized unidentified peak in front of phosphite (**4**) at 7.9 min and various other peaks eluting after phosphite were

detected (see Fig. 9). LC–MS experiments in ESI-positive and ESI-negative mode were conducted in order to identify those substances.

The compound at 3.0 min in batch II (m/z = 268) was, unlike the bisphosphonates, detected by LC–MS in positive mode very sensitively and the fragmentation pattern was distinct from them. The substance could be an intermediate of the precursor synthesis bound to some kind of protecting group because it also absorbed UV-light at 254 nm making it detectable via PDA. Since it was only found in one batch and in a very small amount, this issue was not further investigated.

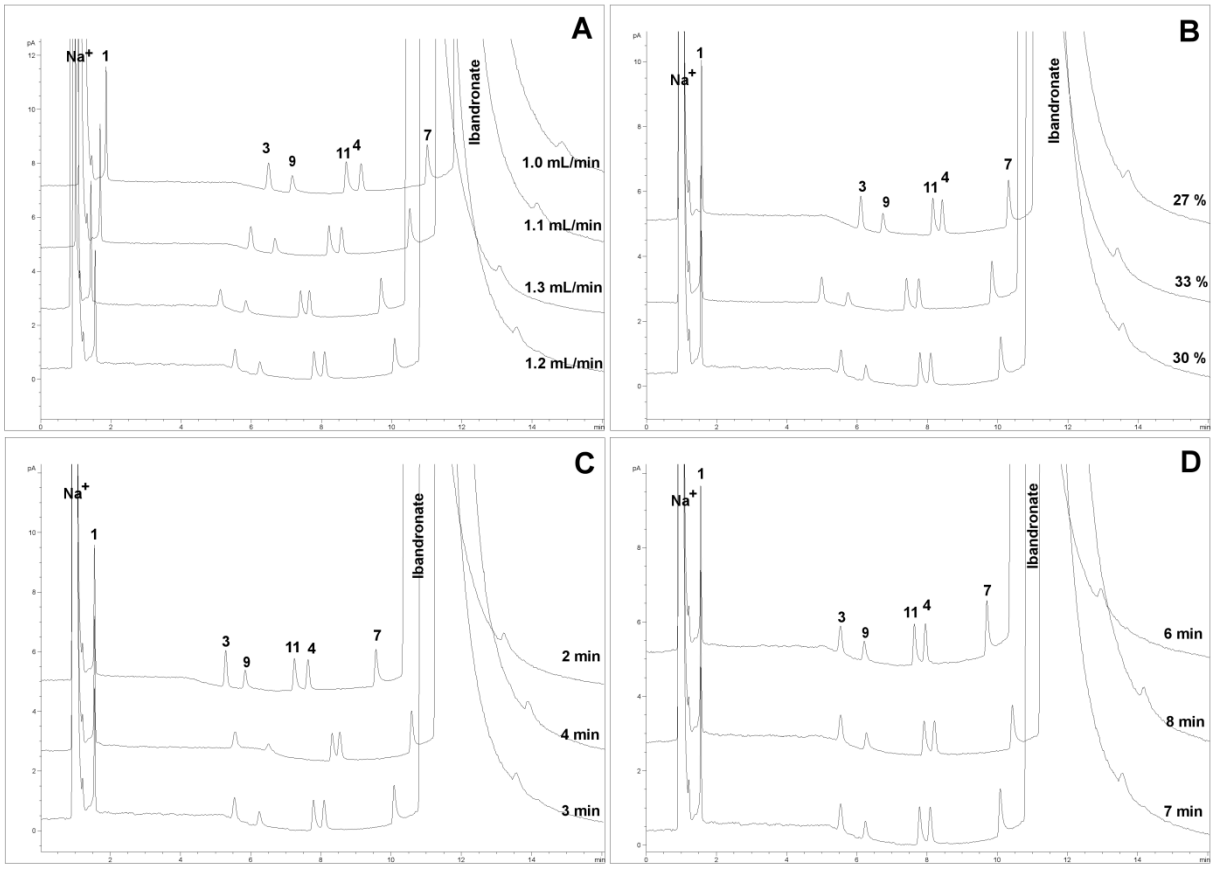


**Fig. 8.** A: Impact of the flow rate on the separation; B: influence of the mobile phase composition during the isocratic step; C: influence of the isocratic step duration; D: impact of the gradient time; chromatographic conditions see Section 2.3.

The identification of the unknown substances eluting in all batches of one manufacturer (Batches II–IV) by LC–MS revealed at least one other bisphosphonate which was not expected: olpadronate (see Fig. 1), also known as *N*,*N*-dimethyl pamidronate (**11**) elutes at 7.9 min just in front of phosphite (**4**). The corresponding m/z values 264 in ESI-positive mode and 262 in ESI-negative mode were consistent with substance **11** (see Fig. 6). The forced degradation of ibandronate in 3 % hydrogen peroxide solution indicated the formation of oxidation products, like *N*-oxide, peroxide compounds, rearranged oxidation products, combinations hereof, phosphate (**3**) and the precursor molecule *N*-methyl-*N*-pentyl-

β-alanine (**1**) (see Fig. 7). The oxidation products elute between phosphite (**4**) and **7** showing m/z values in ESI-negative mode of 334, 332 and 350, respectively, and thus indicating the introduction of one or two oxygen atoms (m/z for ibandronate is 318) and reactions such as the elimination of water from the α-hydroxy group. Some of these substances can also be found in the batches II–IV in small amounts (see Fig. 10). After 2 weeks in 3 % $H_2O_2$, a new peak arose at about 2 min. with the m/z value of 190 in ESI-positive mode. It is most likely the N-oxide of the precursor (1, m/z 174). This substance was never detected in any batch tested.

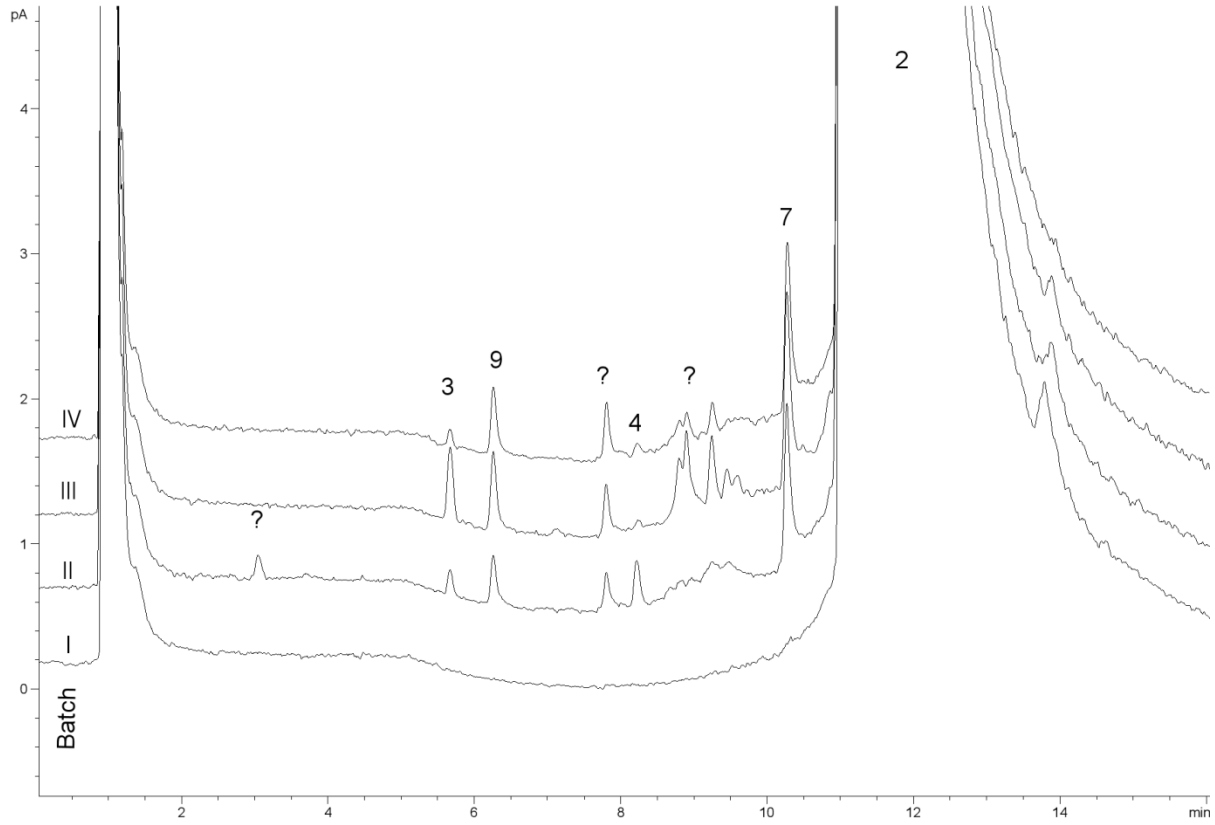


**Fig. 9.** Chromatograms obtained from the test solutions of 4 different batches – no impurity was found above 0.05 %; chromatographic conditions see Section 2.3.

*3.4. Other bisphosphonates*

The method was also found suitable to determine phosphate and phosphite in etidronic and clodronic acid if the methods run time was extended to 30 min (see Fig. 5 chromatogram B and C).

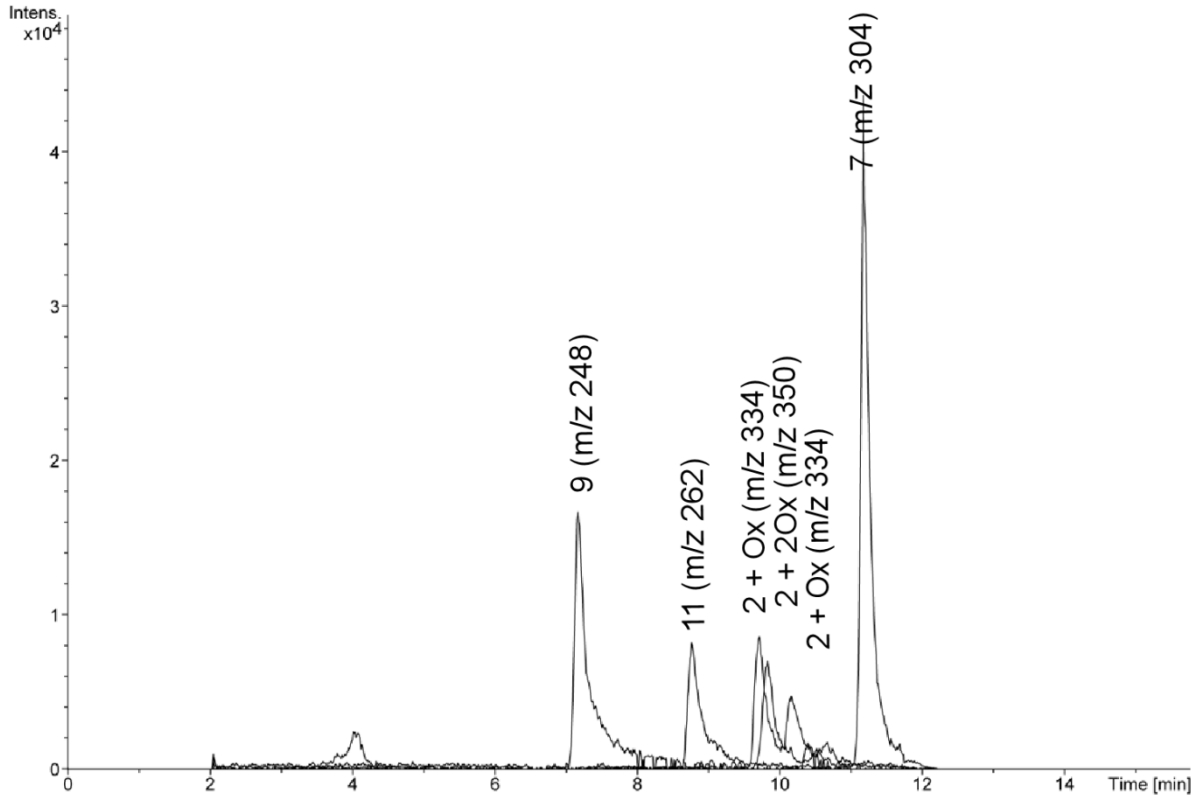


**Fig. 10.** Overlaid EIC of batch III in ESI-negative mode; the substances eluting between **11** and **7** are most likely oxidation products of ibandronate (**2**, m/z 318); chromatographic conditions see Section 2.3.

## 4. Conclusion

A new and robust method for the determination of the related compounds in ibandronate sodium monohydrate was developed and validated and new knowledge of possible impurities and degradation products of ibandronate was gained during the method development and validation. The LC–MS compatibility of the method facilitates identification of unknown structures that might be found in future batches produced by new suppliers.

## Conflict of interest

None of the authors of this paper does have a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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## References

[1] H. Fleisch, Development of bisphosphonates, Breast Cancer Res. 4 (2002) 30–34.

[2] R.G.G. Russell, M.J. Rogers, Bisphosphonates: from the laboratory to the clinic and back again, Bone 25 (1999) 97–106.

[3] M.J. Rogers, S. Gordon, H.L. Benford, F.P. Coxon, S.P. Luckman, J. Monkkonen, J.C. Frith, Cellular and molecular mechanisms of action of bisphosphonates, Cancer 88 (2000) 2961–2978.

[4] R.G.G. Russell, P.I. Croucher, M.J. Rogers, Bisphosphonates: pharmacology, mechanisms of action and clinical uses, Osteoporosis Int. 9 (1999) 66–80.

[5] M.T. Drake, B.L. Clarke, S. Khosla, Bisphosphonates: mechanism of action and role in clinical practice, Mayo Clin. Proc. 83 (2008) 1032–1045.

[6] A.A. Reszka, G.A. Rodan, Nitrogen-containing bisphosphonate mechanism of action, Mini-rev. Med. Chem. 4 (2004) 711–719.

[7] J. Barrett, E. Worth, F. Bauss, S. Epstein, Ibandronate: a clinical pharmacological and pharmacokinetic update, J. Clin. Pharmacol. 44 (2004) 951–965.

[8] J.H. Lin, Bisphosphonates: a review of their pharmacokinetic properties, Bone 18 (1996) 75–85.

[9] F. Bauss, R.C. Schimmer, Ibandronate: the first once-monthly oral bisphosphonate for treatment of postmenopausal osteoporosis, Ther. Clin. Risk Manage. 2 (2006) 3–18.

[10] Z. Jun, Z., Zaiwei, L. Xie, Preparation method of sodium ibandronate, CN20111215751 20110729, China (2013).

[11] B. Junghans, F. Baetz, Process for the Preparation of Ibandronate, MX20070005114 20070427, Switzerland (2007).

[12] X. Kui, C. Zhenghao, W. Wei, Y. Jiajiu, Method for preparing sodium Ibandronate monohydrate, CN20131150772 20130426, China. 02/05/2014.

[13] R.D. Ramchandra, K.R. Narayanrao, G.M. Ganpati, Process for the Synthesis of Ibandronate sodium, NZ20070573137 20070622 (2011).

[14] M. Egorov, S. Aoun, M. Padrines, F. Redini, D. Heymann, J. Lebreton, M. Mathe-Allainmat, A one-pot synthesis of 1-hydroxy-1,1-bis(phosphonic acid)s starting from the corresponding carboxylic acids, Eur. J. Org. Chem. (2011) 7148–7154.

[15] R.M. Pulla, V. Usharani, C.N. Venkaiah, Improved Process for the Preparation of Ibandronate Sodium,WO2006IN00251 20060717, India (2007).

[16] G.R. Kieczykowski, R.B. Jobson, D.G. Melillo, D.F. Reinhold, V.J. Grenda, I. Shinkai, Preparation of (4-amino-1-hydroxybutylidene)bisphosphonic acid sodium salt, MK-217 (alendronate sodium). An improved procedure for the preparation of 1-hydroxy-1,1-bisphosphonic acids, J. Org. Chem. 60 (1995) 8310–8312.

[17] V.V.R.M.K. Reddy, U.S. Chintamani, D. Udaykiran, C. Madhusudhan, K. Mukkanti, A facile preparation of N-methylpentan-1-amine: a key intermediate for ibandronate sodium, Indian J. Chem. B 49 (2010) 1257–1260.

[18] G. Flesch, S.A. Hauffe, Determination of the bisphosphonate pamidronate disodium in urine by pre-column derivatization with fluorescamine, high-performance liquid-chromatography and fluorescence detection, J. Chromatogr. Biomed. 489 (1989) 446–451.

[19] G. Flesch, N. Tominaga, P. Degen, Improved determination of the bisphosphonate pamidronate disodium in plasma and urine by precolumn derivatization with fluorescamine, high-performance liquid-chromatography and fluorescence detection, J. Chromatogr. Biomed. 568 (1991) 261–266.

[20] J.A. Wong, K.W. Renton, J.F.S. Crocker, P.A. O'Regan, P.D. Acott, Determination of pamidronate in human whole blood and urine by reversed-phase HPLC with fluorescence detection, Biomed. Chromatogr. 18 (2004) 98–101.

[21] Y. Jiang, Z. Xie, Determination of ibandronate and its degradation products by ion-pair RP LC with evaporative light-scattering detection, Chromatographia 62 (2005) 257–261.

[22] X.K. Liu, J.B. Fang, N. Cauchon, P.Z. Zhou, Direct stability-indicating method development and validation for analysis of etidronate disodium using a mixed-mode column and charged aerosol detector, J. Pharm. Biomed. Anal. 46 (2008) 639–644.

[23] C.K. Zacharis, P.D. Tzanavaras, Determination of bisphosphonate active pharmaceutical ingredients in pharmaceuticals and biological material: a review of analytical methods, J. Pharm. Biomed. Anal. 48 (2008) 483–496.

[24] M.N. Kumar, K.S.R.P. Kumar, V.J. Kumar, S.J. Prasanna, H.K. Sharma, V.K. Reddy, Stability indicating ion chromatography method for the simultaneous determination of ibandronate sodium drug substance and its impurities, J. Pharm. Biomed. Anal. 54 (2011) 596–601.

[25] L.B. Allen, J.A. Koropchak, Condensation nucleation light-scattering - a new approach to development of high-sensitivity, universal detectors for separations, Anal. Chem. 65 (1993) 841–844.

[26] T. Vehovec, A. Obreza, Review of operating principle applications of the charged aerosol detector, J. Chromatogr. A 1217 (2010) 1549–1556.

[27] U. Holzgrabe, C.J. Nap, T. Beyer, S. Almeling, Alternatives to amino acid analysis for the purity control of pharmaceutical grade L-alanine, J. Sep. Sci. 33 (2010) 2402–2410.

[28] S. Almeling, U. Holzgrabe, Use of evaporative light scattering detection for the quality control of drug substances: influence of different liquid chromatographic and evaporative light scattering detector parameters on the appearance of spike peaks, J. Chromatogr. A 1217 (2010) 2163–2170.

[29] S. Almeling, D. Ilko, U. Holzgrabe, Charged aerosol detection in pharmaceutical analysis, J. Pharm. Biomed. Anal. 69 (2012) 50–63.

[30] R.W. Dixon, D.S. Peterson, Development and testing of a detection method for liquid chromatography based on aerosol charging, Anal. Chem. 74 (2002) 2930–2937.

[31] S. Mahajan, I.P. Singh, Determining and reporting purity of organic molecules: why qNMR? Magn. Reson. Chem. 51 (2012) 76–81.

[32] J.L. Lv, J. Li, D. Zhang-Negrerie, S.Y. Shang, Q.Z. Gao, Y.F. Du, K. Zhao, Constructions of tetrahydro-gamma-carboline skeletons via intramolecular oxidative carbon–carbon bond formation of enamines, Org. Biomol. Chem. 11 (2013) 1929–1932.

[33] P.J.T. Blower, Martin de Rosales Rafael, Bisphosphonate Compounds for Chelating Radionuclides, WO2010GB00692 20100407, Great Britain (2010).

[34] V.V. Annenkov, S.N. Zelinskiy, E.N. Danilovtseva, C.C. Perry, Synthesis of biomimetic polyamines, Arkivoc (2009) 116–130.

[35] H. Devarajan-Ketha, K.B. Sloan, *N*,*N*'-Dialkylaminoalkylcarbonyl (DAAC) prodrugs and aminoalkylcarbonyl (AAC) prodrugs of 4-hydroxyacetanilide and naltrexone with improved skin permeation properties, Bioorg. Med. Chem. Lett. 21 (2011) 4078–4082.

[36] M.B. Martin, J.S. Grimley, J.C. Lewis, H.T. Heath, B.N. Bailey, H. Kendrick, V. Yardley, A. Caldera, R. Lira, J.A. Urbina, S.N.J. Moreno, R. Docampo, S.L. Croft, E. Oldfield, Bisphosphonates inhibit the growth of Trypanosoma brucei, Trypanosoma cruzi, Leishmania donovani, Toxoplasma gondii, and Plasmodium falciparum: a potential route to chemotherapy, J. Med. Chem. 44 (2001) 909–916.

[37] ICH Guideline, Q2(R1) Validation of Analytical Procedures, Text and Methodology, 2005.

[38] ICH Guideline, Q3A(R2), Impurities in new drug substances, 2006.

# 4. Amino acid analysis for pharmacopoeial purposes

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**ABSTRACT**

The impurity profile of amino acids depends strongly on the production process. Since there are many different production methods (e.g. fermentation, protein hydrolysis or chemical synthesis) universal, state of the art methods are required to determine the impurity profile of amino acids produced by all relevant competitors. At the moment TLC tests provided by the Ph. Eur. are being replaced by a very specific amino acid analysis procedure possibly missing out on currently unknown process related impurities. Production methods and possible impurities as well as separation and detection methods suitable for said impurities are subject to this review.

*Abbreviations*: AA, amino acid; AAA, amino acid analysis; APCI, atmospheric pressure chemical ionization; APPI, atmospheric pressure photo ionization; C4D, capacitively coupled contactless conductivity detector; CAD, corona charged aerosol detector; CBQCA, 3-(4-carboxybenzoyl)quinolone-2-carboxaldehyde; CE, capillary electrophoresis; CLND, chemiluminescent nitrogen detector; CZE, capillary zone electrophoresis; CNLSD, condensation nucleation light scattering detector; ECD, electrochemical detector; ELSD, evaporative light scattering detector; ESI, electrospray ionization; FID, flame ionization detector; FLD, fluorescence detector; FMOC-Cl, fluorenylmethoxycarbonyl chloride; FQ, 3-(furan-2-carbonyl)quinolone-2-carbaldehyde; HILIC, hydrophilic interaction liquid chromatography; HPLC, high performance liquid chromatography; IEC, ion-exchange chromatography; IPC, ion-pairing chromatography; LED, light emitting diode; LIF, laser induced fluorescence; LOD, limit of detection; MEKC, micellar electrokinetic chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid; NAC, N-acetylcystein; NQAD, Nano quantity analyte detector (see CNLSD); OPA, o-phtalaldehyde; PGC, porous graphitic carbon; Ph. Eur., European Pharmacopoeia; PITC, phenylisothiocyanate; QqQ, triple quadrupole; RID, refractive index detector; TLC, Thin-layer chromatography; TOF, time-of-flight; UHPLC, ultra-high performance liquid chromatography;

## 1. Introduction

Amino acids (AA) belong to the most important chemical structures on earth. They are building blocks of vital proteins and peptides, neurotransmitters, nourishment, poison, antibiotics, and transporters [1]. Thus, they are subject to great scientific interest [2]. In the pharmaceutical industry AA are used as starting material for therapeutic proteins, peptides and small molecules (e.g. Methotrexate, *N*-acetylcystein, pemetrexed, captopril, etc), as components of total parenteral nutrition, excipients [3-6], and active pharmaceutical ingredients (API), e.g. L-DOPA, Carbocisteine, Gabapentin, etc.

The quality and the safety of the applied AA for the intended purpose have to be assured by adequate analytical procedures and tests. The challenging part in purity assessment is in most cases the separation and detection of related substances. Since amino acids (natural and synthetic) are rather hydrophilic compounds and are lacking a chromophore, separation as well as detection by HPLC is very difficult. The nature of the impurities present in AA depends highly on their origin (e.g. recombinant, synthetic or other) and on possible modification reactions such as acylation or alkylation. Often, the related compounds are other AA, degraded AA, and precursors, causing the same challenges regarding separation and detection. Since the second half of the 20$^{th}$ century [7, 8] the detection of AA was facilitated by numerous derivatization reactions each coming along with particular advantages and disadvantages (see Fig. 1 for some examples). During the last decades, great effort has been made to find ways to separate and detect amino acids without time-consuming and fault-prone derivatization steps. Recent trends in AA analytics use HILIC or mixed-mode columns combined with CNLSD, CAD, C4D or LC-MS, respectively. The application of those comparatively new techniques enjoys increasing popularity in the field of research and discovery, whereas official regulatory authorities are more reluctant in the adoption of new ways to analyze AA and rather rely on comparatively old techniques like TLC and amino acid analysis (AAA) with post-column derivatization. Thus, this review focuses on content determination and impurity profiling of AA by liquid chromatography without using derivatization reactions.

## 2. Preparation of amino acids

Nowadays AAs are produced via various ways. Since the production pathway determines the impurity profile the manufacturing is summarized below. For the monographed AA the most common production pathways are summarized in Table 1.

*2.1. Chemical synthesis*

Since Adolph Strecker developed the first chemical synthesis of α-amino acids in the middle of the 19th century [9] many different variants of this reaction and other ways of producing amino acids have been established in order to obtain enantiomerically pure products and/or to improve yields. There are also specific syntheses e.g. for Lys: Toray and DSM procedure [10]. The question rises whether all impurities from all commercially used synthesis variants using different precursors and/or reagents are covered by a given test [11-17]. Besides Strecker synthesis another way to obtain racemic α-amino acids is the amination of α-hydroxy carboxylic acids [18].

*2.1.1. Chiral resolution*

Although asymmetric syntheses are available, they often do not provide enantiomerically pure D- and L-forms, respectively, are expensive, elaborate and thus often not profitable. Nevertheless, some approaches of racemate separation found their way into the commercial production of amino acids. E.g. the conversion of racemic methionine comprising acetylation and specific enzymatic deacetylation of the L-form is today an important and elegant way to obtain L-methionine [19]. Chiral resolution using additional steps like fractional crystallization of diastereomeric precursors [20] is essential to convert racemic AAs into one enantiomer after chemical synthesis.

*2.2. Recombinant synthesis by microorganisms and fermentation*

The biochemical synthesis of AA using microorganisms involves several advantages over chemical total synthesis, e.g. the enantiomeric selectivity. On the other hand it is a very sensitive process needing a high level of know-how. Frequently applied bacteria in those processes belong inter alia to *Corynebacterium*, *Bacillus, Brevibacterium* and *Escherichia coli* strains [21-23]. The production of one amino acid is usually up-regulated in these microbes whereas the growth inhibitory effect of the AA in question is removed by means of genetic engineering. The produced AA is eventually excreted and accumulated in the liquid phase. After separation of the biomass from the cell culture medium, the amino acid(s) are extracted from the solution and purified [24-27]. The removal of carbon and nitrogen sources or nutrients e.g. sugars, sugar alcohols, starch, amino acids, urea [28], “crude glycerol” [29] and organic acids like malic, fumaric or succinic acid needs to be assured. Residual components from the broth like antibiotics (e.g. penicillin, tetracycline, kanamycin, streptomycin or chloramphenicol [26, 27, 29-31]), co-substrates (e.g. biotin, thiamin [32, 33]), buffers like MOPS [25, 28] and growth inhibitors like β-(2-thienyl)-DL-alanine [34] need to be removed. Strategies to purify the AA include centrifugation and membrane filtration to

remove cell mass followed by ion-exchange chromatography, concentration and crystallization [24, 32, 33, 35-39].

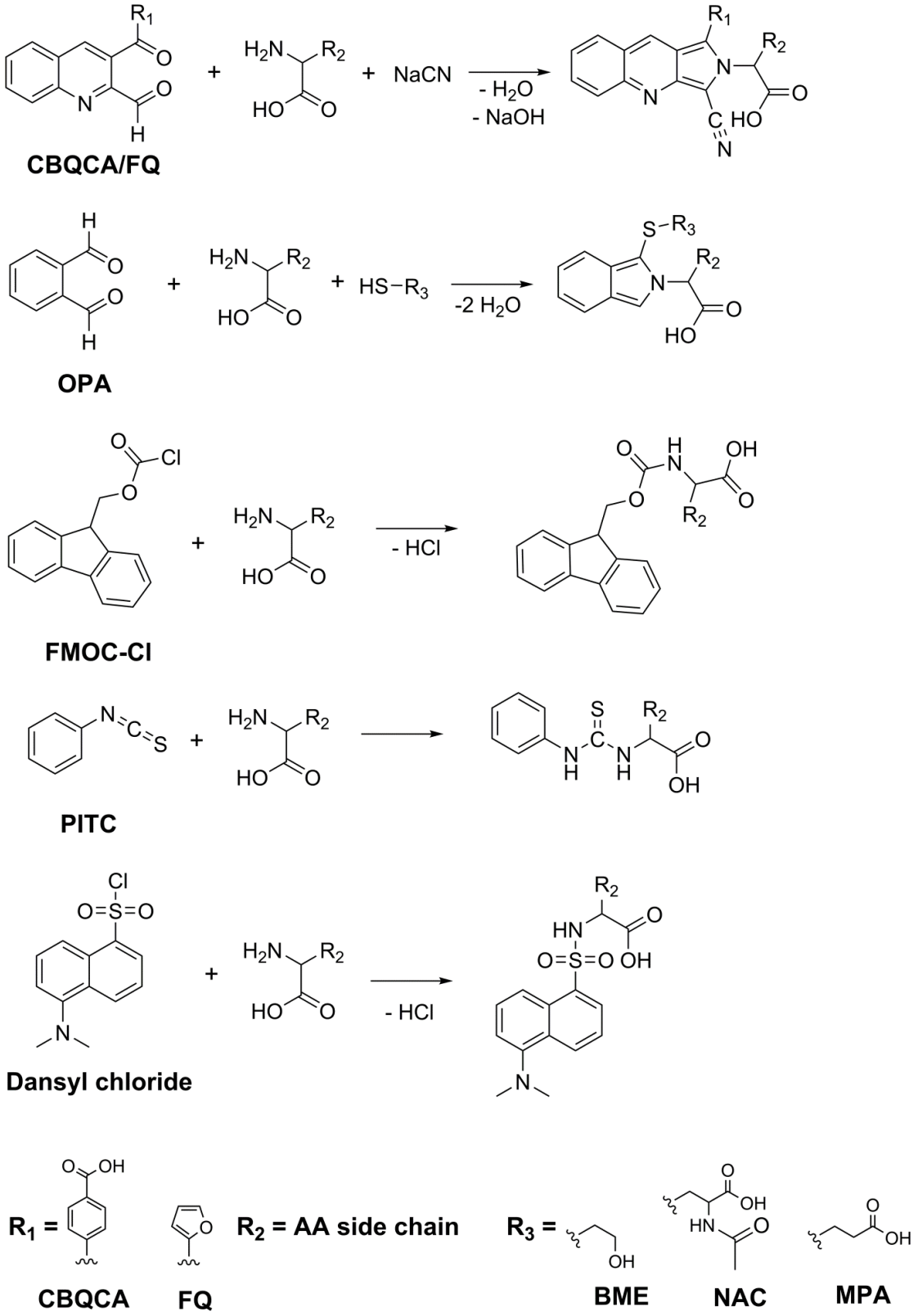


**Fig. 1.** Some derivatization reactions used in AAA [70, 72, 155-160]; BME, β-mercaptoethanol; NAC, N acetylcysteine; MPA, 3-mercaptopropionic acid

**Table 1**
Common ways to synthesize amino acids monographed in the Ph. Eur. [43, 164]

| Monograph name | separation technique currently applied by Ph. Eur. | production method | potential impurities |
|---|---|---|---|
| L-Alanine | AAA method 1 | chemical synthesis from D-chloropropionic acid [165] | starting material, by-products and intermediates |
| | | Strecker- [9] or Bucherer-Bergs-synthesis [166] of DL-Ala starting from acetaldehyde followed by L-amino acylase catalyzed asymmetric hydrolysis of *N*-acetyl-DL-Ala [167, 168] | starting material, intermediates and by-products of synthesis, by-products of stereospecific deacetylation |
| | | enzymatic decarboxylation of L-Asp [169, 170] | L-Asp and co-substrates from fermentation process e.g. pyridoxal phosphate, fumaric acid |
| | | fermentation [171, 172] | components of fermentation broth and by-products of biosynthesis |
| Aminocaproic acid | TLC + Ninhydrin | hydrogenation of cyanovaleric acid [173] | starting material and intermediates |
| L-Arginine | AAA method 1 | protein hydrolysis (gelatin) [174] | other amino acids |
| | | chemical conversion of L-ornithine [175] | starting material, intermediates and by-products like S-methylthiourea, O-methylthiourea |
| | | fermentative using *Bacillus subtilis* [176], *Corynebacterium glutamicum* [177-180] or *E. coli* [181] | components of fermentation broth and by-products of biosynthesis |
| L-Arginine L-Aspartate | TLC + Ninhydrin | by ion-exchange from calcium L -aspartate and L-arginine HCl [182] | see L-Arg and L-Asp |
| L-Arginine HCl | AAA method 1 | see L-Arg | see L-Arg |
| L-Asparagine $H_2O$ | TLC + Ninhydrin | fermentation using recombinant *E. coli* [183] | other amino acids |
| | | reaction of ammonia with L-Asp-β-methylester [184] | starting material |
| L-Aspartic acid | TLC + Ninhydrin | amination of fumaric acid using *Brevibacterium flavum* [185] | starting material |
| Carbocisteine | TLC + Ninhydrin | alkylation of L-Cys (in situ produced from L-Cis) with chloroacetic acid [186, 187] | starting material, by-products, degradation products (e.g. lactam, sulfoxide), L-Tyr [115] |
| L-Cysteine HCl $H_2O$ | AAA method 1 | reduction of L-Cis [188] | see L-Cis, ammonia, inorganic ions (cathode material) |
| | | enzymatic conversion of β-chloro-L-Ala with acetylserine desulfhydrase in presence of NaSH [189] or cysteine desulfhydrase in the presence of $H_2S$ [190] | starting material, by-products, co-substrates |
| | | synthesis from methyl α-chloroacrylate and thiourea followed by enzymatic purification [191] | starting materials, by-products, co-subsstrates |
| | | fermentation [192] | components of nutrient solution and by-products of biosynthesis |
| L-Cystine | TLC + Ninhydrin | protein hydrolysis [42] | other amino acids in particular L-Tyr |
| Gabapentin | HPLC UV 215nm | chemical synthesis [193-196] | |
| L-Glutamic acid | TLC + Ninhydrin | fermentation [24, 26, 27] | components of nutrient solution and by-products of biosynthesis |

**Table 1** (continued)

| Monograph name | separation technique currently applied by Ph. Eur. | production method | potential impurities |
|---|---|---|---|
| Glycine[a] | AAA method 1 | amination of chloroacetic acid [197] | starting material and by-products |
| | | Strecker-synthesis, hydrolysis of aminoacetonitrile [198] | starting material, intermediates and by-products |
| L-Histidine | AAA method 1 | protein hydrolysis (blood meal, casein or keratin) [174, 199] | other amino acids |
| | | fermentation [30, 33] | components of nutrient solution and by-products of biosynthesis |
| L-Histidine HCl $H_2O$ | AAA method 1 | hydrolysis of hemoglobin [200] or casein [174] | other amino acids |
| L-Leucine | AAA method 1 | protein hydrolysis [42] | other amino acids |
| | | fermentation using *Corynebacterium glutamicum* [201, 202] | components of nutrient solution and by-products of biosynthesis |
| Levodopa[b] | HPLC UV 280nm | chemical synthesis and chiral resolution using L-camphoric acid [203] or enzymatically using *E. coli* acylase [204] | starting material, intermediates and by-products |
| | | fermentation starting from 3,4-dihydroxyphenyl pyruvic acid [205] | components of nutrient solution and by-products of biosynthesis |
| Levothyroxine | HPLC UV 225nm | chemical synthesis [206, 207] | starting material, intermediates and by-products |
| Liothyronine sodium | HPLC UV 225nm | chemical synthesis [175, 208, 209] | starting material, intermediates and by-products |
| L-Lysine acetate | TLC + Ninhydrin | from L-Lys HCl [210] | see L-Lys |
| L-Lysine HCl | AAA method 1 | toray-procedure: synthetically starting with cyclohexanone via DL-α-amino-ε-caprolactam (ACL) followed by enzymatic chiral resolution with L-ACL-hydrolase [10, 175] or D-camphoric acid [211] | starting material, intermediates and by-products |
| | | protein hydrolysates [174, 212] | other (basic) amino acids |
| | | Bergs-Bucherer synthesis [175] via acrylonitrile, γ-cyanobutyraldehyde, 5-(γ-cyanopropyl)-hydantoin, chiral resolution by fractional crystallization | starting material, intermediates and by-products |
| | | fermentation by recombinant *Corynebacterium glutamicum* [213, 214] | components of nutrient solution and by-products of biosynthesis |
| L-Methionine | HPLC UV 205nm | fermentation of α-hydroxy-γ-methylmercaptobutyric acid using *Pseudomonas spec.* [215] | starting material, intermediates, by-products and components of the broth |
| | | fermentation of *O*-acetylhomoserine in methylmercaptane athomosphere [216] | starting material, intermediates, by-products and components of the broth |
| | | from DL-Met by chiral resolution [19, 217, 218] | see DL-Met, intermediates like *N*-acetyl-methionyl-methionine (3 isomers), *N*-acetyl-DL-methionine [219] |

**Table 1** (continued)

| Monograph name | separation technique currently applied by Ph. Eur. | production method | potential impurities |
|---|---|---|---|
| DL-Methionine | TLC + Ninhydrin | synthesis starting from acrolein via 3-methylmercaptopropionaldehyde and 5-(2-methylmercaptoethyl)-hydantoin [220, 221] | starting material, intermediates and by-products |
| | | fermentation [222] | components of nutrient solution and by-products of biosynthesis |
| (*S*)-Methyldopa | HPLC UV 280nm | asymmetric synthesis starting from (3,4-dimethoxyphenyl)-2-propanone [223] or (3-methoxy-4-hydroxyphenyl)-2-propanone [224] or starting from 2-phenyl-4-methyl-*S*-oxazolinone [225] | starting material, intermediates and by-products, D-tartaric acid or L-camphor sulfonic acid (chiral resolution) |
| L-Phenylalanine | AAA method 1 | reduction of L-tyrosine derivatives [175, 226] | starting material, intermediates and by-products |
| | | fermentation of DL-m-fluorophenylalanine [227]; fermentation of phenylpyruvic acid [228] | starting material, intermediates, by-products and components of the broth |
| | | fermentation using *E. coli* [229] or *Brevibacterium flavum* [175, 230] | components of nutrient solution and by-products of biosynthesis |
| | | protein hydrolysis [42] | other amino acids |
| L-Serine | AAA method 1 | fermentation of glycine using *E. coli* [231, 232] or *Corynebacterium glycinophilum* [233] | components of nutrient solution and by-products of biosynthesis |
| | | fermentation using *Corynebacterium spec.* [34] | components of nutrient solution and by-products of biosynthesis |
| | | enzymatic conversion of glycine and formaldehyde [234] | starting material, intermediates, by-products and components of the broth |
| L-Threonine | AAA method 1 | fermentation using *E. coli* or *Serratia spec.* [28, 31, 235-238] | components of nutrient solution and by-products of biosynthesis |
| | | enzymatic conversion of DL-threonine with D-threonine-aldolase [239]; DL-threonine obtained from chemical synthesis [240, 241] | starting material, intermediates, by-products and components of the broth; impurities of chemical synthesis |
| Tranexamic acid | HPLC UV 220nm | chemical synthesis starting from 4-methylbenzonitrile [242, 243], from acetamidomethylbenzoic acid [244] or from 4-aminomethylbenzoic acid [245] | starting material, intermediates and by-products |

**Table 1** (continued)

| Monograph name | separation technique currently applied by Ph. Eur. | production method | potential impurities |
|---|---|---|---|
| | | fermentation of indole, indolepyruvic or anthranilic acid using *Brevibacterium spec.* [246-248], *Bacillus subtilis* [249] or *Micrococcus luteus* [250] | starting material, intermediates, by-products and components of the broth |
| | | recombinant using *Corynebacterium glutamicum* [251] or *Brevibacterium flavum* [230, 252, 253] | components of nutrient solution and by-products of biosynthesis |
| L-Tryptophan[a] | AAA method 1 | enzymatic conversion of indole and L-serin [254] | starting material, intermediates and by-products |
| | | enzymatic chiral resolution of *N*-acetyl-DL-tryptophan [175, 255] | starting material, intermediates and by-products 1,1'-ethyliden-bis(tryptophan)[a] |
| | | Chiral resolution by precipitating diastereomeric salt from *N*-acetyl-DL-tryptophan and L(+)-threo-(1-p-nitro phenyl)-2-amino propane-1,3-diol [256] | starting material, intermediates and by-products 1,1'-ethyliden-bis(tryptophan)[a] |
| *N*-Acetyl-DL-tryptophan | HPLC UV 220nm | chemical synthesis [175, 257, 258] followed by acetylation in glacial acetic acid or acetic acid anhydride [175] | starting material, intermediates and by-products 1,1'-ethyliden-bis(tryptophan) |
| | | antarctic krill hydrolysate (enzymatic) [259]; other protein hydrolysates [41, 42, 260] | other amino acids, residues from the broth |
| L-Tyrosine | AAA method I | fermentation using *Brevibacterium*, *Serratia* or *Corynebacterium*[230, 261-263] | components of nutrient solution and by-products of biosynthesis |
| *N*-Acetyl-L-tyrosine | HPLC UV 219nm | acetylation of L-Tyrosine using acetic anhydride [264, 265] | starting material, intermediates and by-products |
| | | fermentation using *E. coli* [266], *Corynebacterium* [267] or *Candida tropicalis* [268] | components of nutrient solution and by-products of biosynthesis |
| L-Valine | AAA method I | from DL-Val by enzymatic chiral resolution after acetylation [269, 270] | starting material, intermediates and by-products |
| | | stereo selective synthesis [271-273] | starting material, intermediates and by-products |
| (*RS*)-Vigabatrin[a] | HPLC UV 210nm | Chemical synthesis starting from L-glutamic acid [274] or from 5-hexynoic 4-*N*-acetylamino-5-hexynoic acid methyl ester [275] | starting material, intermediates and by-products |

[a] Additional method for single impurities required,

[b] Starting material not detectable

**Table 2**
Possible impurities in amino acids produced by fermentation and detection methods.

| Origin of impurities | Potential impurities | Separation and detection method | |
|---|---|---|---|
| Enzymatic chiral resolution | *N*-acetyl amino acids | HPLC direct UV detection at 210nm | [219] |
| | D-amino acids | chiral MEKC or CZE of underivatized AA or dansyl derivatives | [276, 277] |
| | | chiral LC of underivatized AA using teicoplanin stationary phase and MS detection | [278] |
| Fermentation | | | |
| carbon sources | low molecular alcohols and sugar alcohols | CZE with direct UV detection | [279] |
| | | GC with FID | [280] |
| | organic acids like pyruvic, acetic, fumaric, malic or lactic acid | IEC with suppressed conductivity detection | [281] |
| | | Mixed mode Chromatography with CAD | [282] |
| | | CZE with C4D | [283] |
| | | IEC with RID | [85] |
| | carbohydrates | HILIC with CAD | [284, 285] |
| | | PGC-LC with CAD | [286] |
| | | HPLC with RID | [82-85] |
| | | IEC with pulsed amperometric detection | [287] |
| | | CZE with direct UV detection | [279] |
| | | CZE with RID | [86] |
| | | CZE with C4D after derivatization with AETMA | [288] |
| antibiotics | gentamicin | IPC with CAD | [289] |
| | | IPC with RID | [290] |
| | | IPC and ECD | [291] |
| | | IPC with ELSD | [292, 293] |
| | | CZE with UV detection at 195nm | [294] |
| | | IPC with LC-MS detection | [295] |
| | streptomycin | IPC using CAD or IPC with UV detection at 205nm | [114] |
| | | IPC with C4D | [296] |
| | tetracycline | RP HPLC with UV detection at 360 nm | [297] |
| | | RP HPLC with FLD $\lambda_{ex}$ = 374 $\lambda_{em}$ = 495 nm | [298] |
| | | RP UHPLC MS detection | [299] |
| | kanamycin | IPC with pulsed amperometric detection | [300] |
| | | CZE after pre-capillary derivatization (OPA) | [301] |
| | | IPC with ELSD | [293] |
| | | IPC with C4D | [296, 302] |

**Table 2** (continued)

| Origin of impurities | Potential impurities | Separation and detection method | |
|---|---|---|---|
| antibiotics | chloramphenicol | RP HPLC with tandem MS | [303] |
| | | RP HPLC with UV detection at 280 nm or 275 nm | [304, 305] |
| | | RP HPLC with electrochemical detection | [305] |
| | | RP HPLC with CNLSD detection | [306] |
| other components in the broth | amino acids | IPC with tandem MS | [307] |
| | | AAA with post-column ninhydrin derivatization | [44, 46, 47] |
| | | UHPLC with CAD | [118] |
| | | AAA with post-column OPA derivatization | [63] |
| | | IPC with post-column 1,2-naphthoquinone-4-sulfonate derivatization | [67] |
| | | RP-HPLC with pre-column FMOC-Cl derivatization | [70] |
| | | CE after pre-capillary derivatization (FMOC or CBQCA) using LIF or UV detection | [308, 309] |
| | | CZE with RID | [86] |
| | | IPC with ELSD detection | [310] |
| | | IEC with ECD (amperometric) | [311] |
| | | IPC with CLND | [88, 96] |
| | | IEC with ECD (pulsed amperometric) | [312] |
| | biotin | RP-HPLC with ECD (coulometric) | [313] |
| | | RP-HPLC with CAD | [314] |
| | | RP-HPLC with UV detection | [315] |
| | | RP-HPLC with FLD | [315] |
| | | CZE with UV detection at 210 nm | [316] |
| | | MEKC with UV detection at 214 nm | [317] |
| | | CZE with MS detection | [318] |
| | thiamin | RP-HPLC with CAD | [314] |
| | | RP-HPLC with UV detection at 275 nm | [319] |
| | | MEKC with UV detection at 214 nm | [317] |
| | | CZE with MS detection | [318] |
| | pyridoxal phosphate | RP-HPLC with FLD after post column derivatization | [320] |

AAA: amino acid analysis, AETMA: (2-aminoethyl)trimethylammonium chloride hydrochloride, C4D: capacitively coupled contactless conductivity detector, CAD: corona charged aerosol detector, CLND: chemiluminescent nitrogen detector, CZE: capillary zone electrophoresis, ECD: electrochemical detection, ELSD: evaporative light scattering detector, FID: flame ionization detector, FLD: fluorescence detection, GC: gas chromatography, HPLC: high performance liquid chromatography, IEC: ion-exchange chromatography, IPC: ion-pairing chromatography, MEKC: micellar electrokinetic chromatography, PGC: porous graphitic carbon, RP: reversed phase, UHPLC: ultra-high performance liquid chromatography

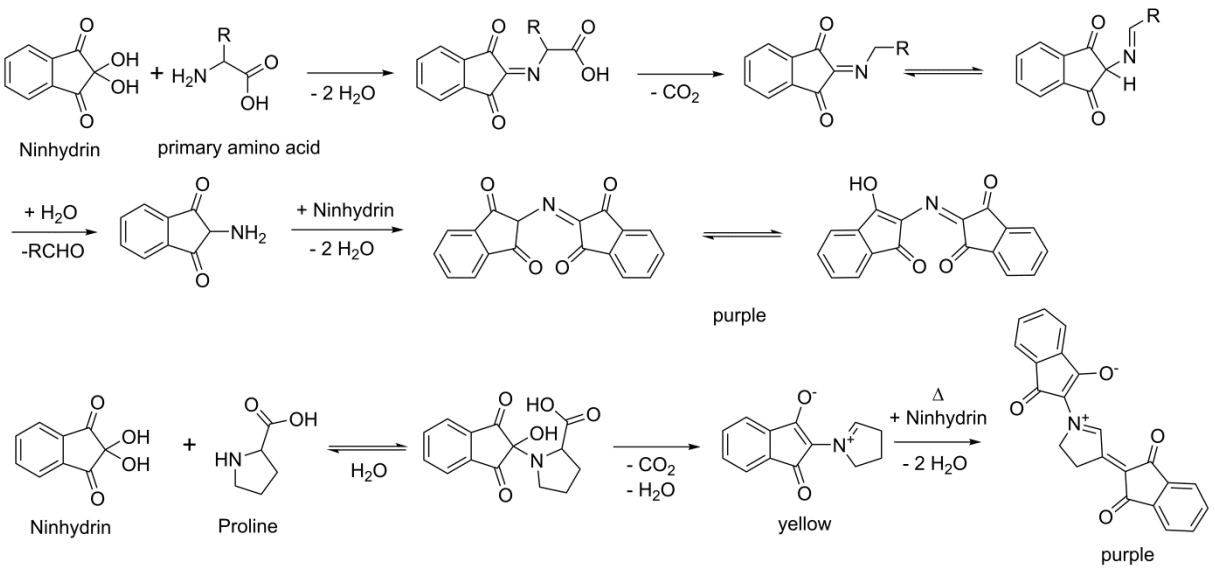


**Fig. 2.** Reaction of primary α-amino acids and proline with ninhydrin [161, 162]

*2.3. Protein hydrolysis*

Acidic hydrolysis of peptides and proteins yields solutions of the amino acids present in the chosen precursor. The crude hydrolysate is purified by fractionated crystallization and chromatographic procedures [21, 40-42]. The amino acid composition of the hydrolysate is controlled by the chosen protein starting material and the method of hydrolysis. Obviously, mainly enantiomerically pure AAs are yielded by this procedure. In turn, only natural amino acids are accessible by this method. The starting material and method of hydrolyzation have to be chosen carefully to make sure that posttranslational modifications or degraded amino acids do not find their way into the final product. AAs prone to degradation are Met, Cys and Trp. They are converted to methionine sulfoxide, methionine sulfone, cysteic acid and oxidation products of Trp. Other amino acids such as Asn and Gln e.g. are usually converted to their corresponding acid (D- and L-form due to partial racemization) and are therefore not easily accessible by protein hydrolysis.

## 3. Analytical methods for amino acids

Fermentation is today the most common way to produce AA. Beside antibiotics and carbohydrates possible impurities due to the fermentation process are ketones and acids occurring as starting material and intermediates in the biosynthesis. Methods to determine those impurities are listed in Table 2. The most frequently used techniques are summarized in the following part.

### *3.1. Pharmacopoeial methods*

#### *3.1.1. Thin-layer chromatography*

Thin-layer chromatography (TLC) is an appropriate mean of identification in combination with additional tests. For impurity profiling, the method is obsolete due to the lack of sensitivity and resolving power. Some AA monographed in the Ph. Eur. still rely on a TLC test combined with ninhydrin detection as a test for related amino acids and great effort is made to replace those TLC tests by more sensitive and specific tests. In most cases it is replaced by amino acid analysis (AAA).

#### *3.1.2. Amino acid analysis (2.2.56)*

Chapter 2.2.56 of the Ph. Eur. [43] defines AAA as a “methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations”. It is discriminated between methods of “protein hydrolysis” and “methodologies of amino acid analysis”. In the current version 8.8 of the Ph. Eur. there are 11 methods for hydrolysis and 8 methods for AAA described. Although AAA is not intended for this purpose, some of the currently monographed amino acids use analysis method 1 to determine related “ninhydrin-positive substances”. This method comprises the separation of the test solution on an cation-exchange resin using lithium- [44] or sodium-based [45] mobile phases and the post-column derivatization of the analytes with ninhydrin [46, 47]. Since the ninhydrin derivatization is well-known and characterized (see Fig. 2), it is also known that some amino acids yield multiple products and thus variation in response if parameters such as temperature, time and pH are not precisely maintained (e.g. in the case of Arg, Asn, Trp, Cys and Lys [48-56], see Fig. 3). The post-column derivatization comes with high specificity, because all possible derivatization products of one AA are covered by a single peak, whereas the resolving power of the chromatographic system suffers from post-column dead volume leading to peak broadening [57-59]. Hence the results produced by AAA might be reliable provided that reaction conditions are strictly constant for both sample and AA standard. However, the procedure is not only time consuming and costly due to the fact that a special instrument has to be used but it is also completely blind towards compounds that

do not react with ninhydrin [45]. Artefacts (e.g. substances that react unexpectedly with ninhydrin) might lead to misinterpretation of the result [60-62].

There are other ways of AAA which rely on pre- and also post-column derivatization steps. In contrast to the ninhydrin reaction, which is rather specific for α-amino acids, a chromo- or fluorophore is attached (see Fig. 1) to any amino moiety (e.g. via fluorenyl-methoxycarbonyl chloride (FMOC-Cl), phenylisothiocyanate (PITC) or dansyl chloride) or a new one is formed in situ (e.g. by ortho-phtalaldehyde (OPA), 3-(4-carboxybenzoyl)quinolone-2-carboxaldehyde (CBQCA) or 3-(furan-2-carbonyl)quinolone-2-carbaldehyde (FQ)). The question whether other derivatization reagents were more suitable for impurity profiling by AAA remains unanswered [57, 63-76]. All derivatization methods share the same drawbacks: due to the essential high reactivity of the reagents, side reactions, degradation products of the reagent as well as of the derivatives and unexpected products could impair the result. In some cases not all amino acids are readily derivatized or some amino acids yield multiple products (e.g. lysine and cysteine).

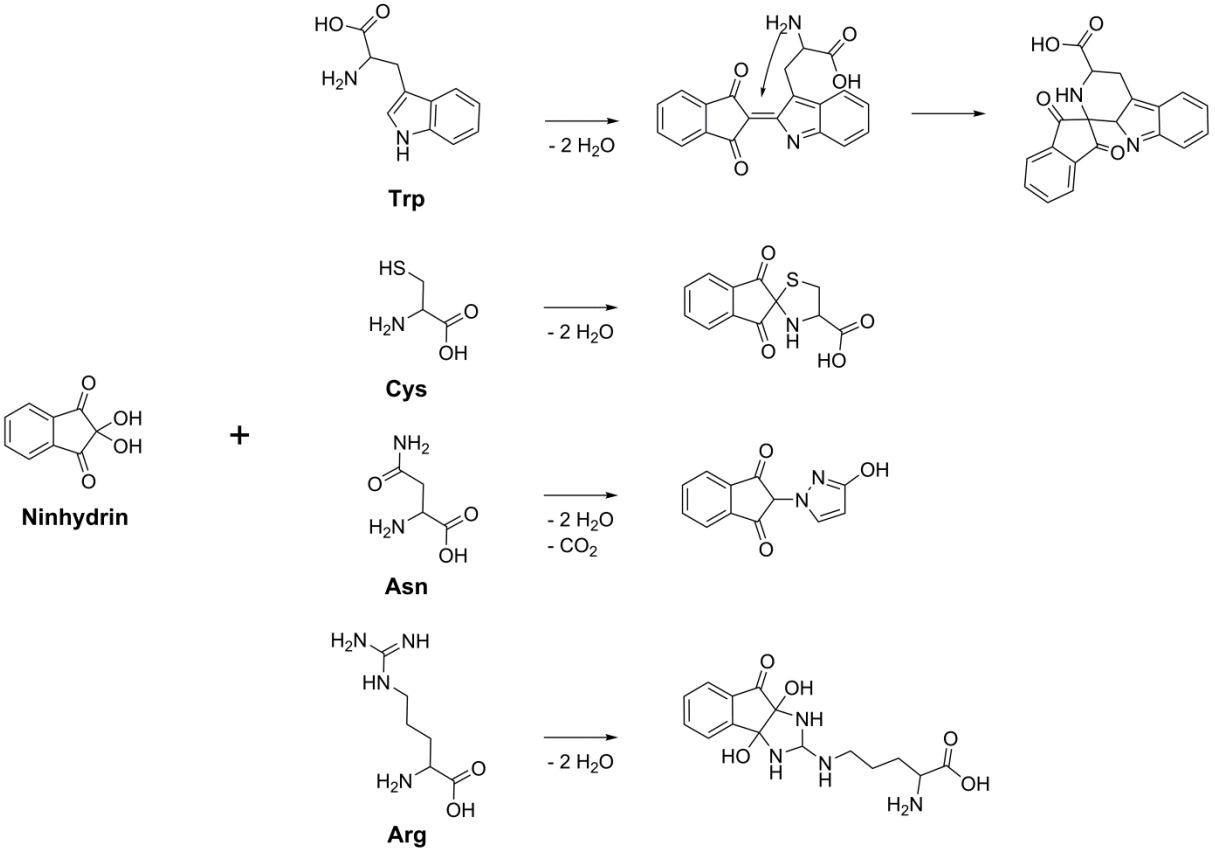


**Fig. 3.** Possible side reactions of ninhydrin with amino acids that do not lead to Ruhemann's purple [48, 50-53, 55, 56, 163]

*3.1.3. UV detection*

Most amino acids lack a suitable chromophore, like conjugated double bonds, for UV detection. However, the absorbance due to the n → π* transition of the carboxylic acid at about 210 nm enables the direct detection of underivatized amino acids at low wavelength (< 220 nm) [77-79]. Nevertheless, the poor sensitivity is one of the major drawbacks of the direct UV-detection. Improved sensitivity is provided by modern detectors with high sensitivity flow cells, high energy lamps and improved optics. Modern high efficiency columns minimize band broadening and highly pure acetonitrile shows reduced absorption at low wavelength. Other organic solvents are usually useless due to UV cutoff at about 220 nm or higher. The preparation of a highly concentrated sample solution for the determination of related substances can also compensate for the low sensitivity.

*3.2. Methods used in research and discovery*

*3.2.1. Electrochemical detection (ECD)*

The electrochemical detection after ion chromatographic separation was for a long time the most common method of detection for underivatized AA. In general there are three types of electrochemical detectors, amperometric and coulometric detectors, and a combination of both. This technique needs a skilled operator, is fault-prone and not suitable for impurity assessment because the most important advantage of these detection techniques is their high specificity for oxidizable or reducible compounds which, at the same time, limits the amount of detectable substances [80].

*3.2.2. Refractive Index (RI) detection*

The principle of the RID comprises the comparison of the RI of the column effluent or separation buffer (in CE) with the RI of pure mobile phase. Any substance eluting from the column changes the RI and therefore causes a signal in the detector. Although it is considered a universal detector, there are some decisive downsides: Due to the comparatively small differences in RIs, the detection limit is usually higher than that of other detectors (UV, CAD, CNLSD), it is not compatible to gradient elution and extremely sensible to temperature shifts. The latter aspect is dealt with in modern instruments, but the lack of sensitivity and the restriction to isocratic elution makes this detector only second choice for the determination of related substances [81].

The RID is a common detector in carbohydrate analysis [82-85] whereas the detection of AA by means of RID is rarely described in capillary zone electrophoresis (CZE) [86, 87] or HPLC [88].

*3.2.3. Evaporative and condensation nucleation light scattering detection (ELSD and CNLSD)*

In the late 1980s the ELSD was the next milestone to universal detection [89-92] after the RID. The CNLSD (also referred to as NQAD) is considered the direct advancement of the ELSD [93, 94]. Both principles are based on the evaporation of the column effluent at elevated temperatures producing an aerosol which contains the analyte. The mobile phase has to be completely volatile to get a sufficiently low background noise. In the ELSD, the aerosol is directed through a chamber equipped with a light source and the amount of light scattered by the aerosol is measured whereas in the CNLSD the aerosol is combined with water (n-butanol at the research stage) vapor before the light scattering is measured. The vapor condenses on the particles increasing their diameter and therefore augments light scattering and response. Both detectors use light of different wavelengths in the visible range, in some cases emitted from laser light emitting diodes (LEDs) [95]. Possible interactions with the analyte, if it absorbs at the applied wavelength, have to be considered, especially if substances are not quantified against themselves as external reference but against other substances with different structure (e.g. if the analyte is unknown). Although this might be rarely the case, as ELSD and CNLSD are usually applied for substances lacking a chromophore, some attention should be paid to this matter when using one of these techniques. ELSD and CNLSD have proven useful in the analysis of underivatized amino acids [96-98] although the LOD is in general equal or higher compared to low wavelength UV detection [88]. A major problem of the ELSD in impurity analysis is due to spike peaks on the tail of principle peaks leading to misinterpretation of the chromatogram. These spike peaks are completely random and unavoidable because of the necessary high sample concentration for impurity profiling [97].

*3.2.4. Corona charged aerosol detector (CAD)*

The corona charged aerosol detector is another aerosol based technique [99-103]. Instead of measuring the scattered light, the particles are diverted through a stream of positively charged nitrogen gas and the adsorbed charge is determined by a sensitive amperemeter [104]. The detector response is considered proportional to the total particle surface. This explains one important characteristic of the CAD: Since the surface is not directly proportional to the particle volume (ideally referred to as spherical) and therefore not proportional to the analyte mass, the response is not strictly linear [100]. Nevertheless, several applications have demonstrated that the response is sufficiently linear over two orders of magnitude which is adequate for determinations of related substances in most cases. However, logarithmic regression is suitable if an extended calibration range is required [105]. Furthermore, the same limitations (at the same time advantages) apply as for

the ELSD and CNLSD: The need for an LC-MS compatible mobile phase facilitates the transfer to LC-MS analysis if required (e.g. when the occurrence of unknown impurities demands for structure elucidation).

Studies comparing these three particle based detectors found approximately equal response of CNLSD and CAD and in general superior response and linearity compared to the ELSD [88, 106-110]. Compared to the ELSD and CNLSD, the CAD is a rather simple to use instrument with only a few parameters to adjust. The use of the CAD in impurity profiling increased over the years since its invention [58, 97, 111-116] and the technique could be considered as reliable [117]. Coupling to novel techniques like UHPLC are able to replace AAA [118]. Decisive downsides of all three detectors are the limitation to non-volatile analytes and the variation in response due to partial volatility or interaction with mobile phase additives e.g. salt or ion-pair formation observed for acids, bases and inorganic ions. The CAD has been successfully applied in the impurity profiling of carbocisteine [116], alanine [107], aspartic acid [113] other amino acids, carbohydrates, lipids etc. [119].

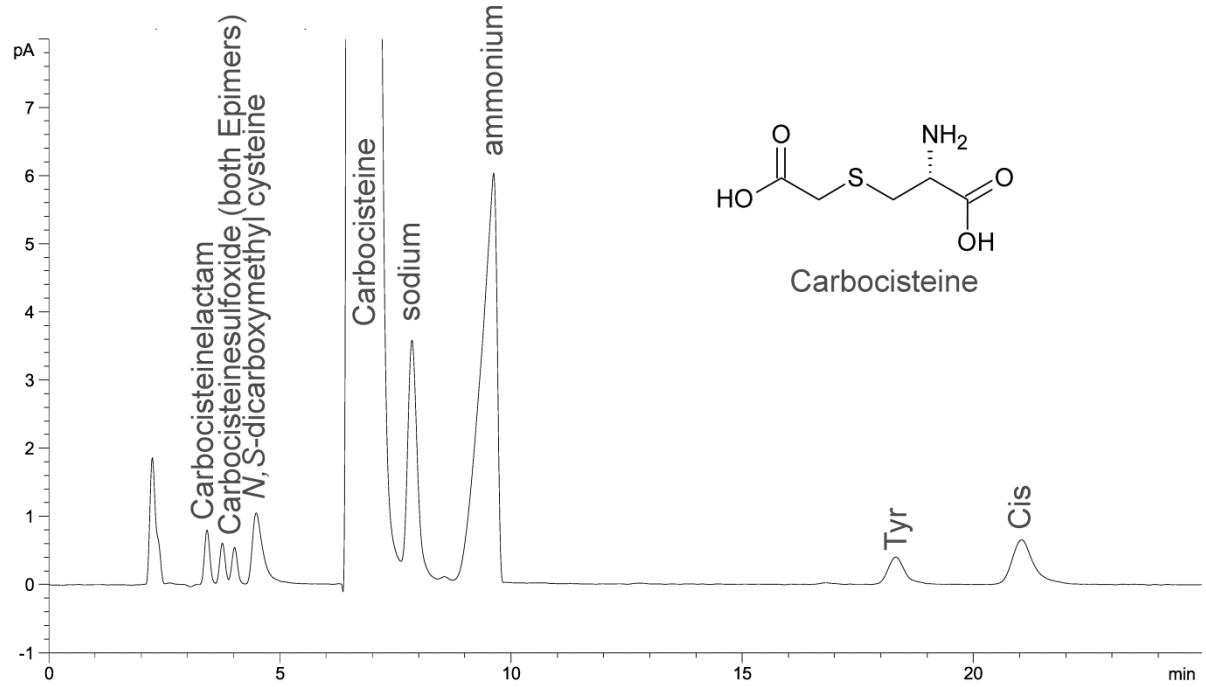


**Fig. 4.** HPLC-CAD chromatogram obtained during impurity profiling of Carbocisteine from our own work [115], chromatographic conditions: mobile phase: TFA–acetonitrile–water (0.1:12:87.9, V/V/V); column temp: 30 °C; flow rate: 1.0 mL/min; column: Primesep 100 250 × 4.6 mm 5 µm; detector: CAD

*3.2.5. Chemiluminescent nitrogen detection (CLND)*

In the CLND the column effluent is vaporized with oxygen and an inert gas (argon or helium), and pyrolyzed at high temperatures. Any nitrogen containing compound is converted to nitrogen monoxide, which reacts with ozone in the gas phase to excited nitrogen dioxide. The excited molecule drops to the ground state under infrared light emission. The emitted light intensity is linear over a broad concentration range and directly proportional to the number of nitrogen atoms in the molecule [120, 121]. Major drawback of this detector is the specificity for nitrogen containing compounds which also limits the available components for the mobile phase. Acetonitrile, ammonium buffers and amine ion-pairing agents cannot be used with the CLND due to the baseline noise caused by the contained nitrogen. On the other hand, it is possible to quantify volatile and semi volatile analytes because the reaction takes place in the gas phase. Despite of the limitations regarding mobile phase composition, amino acid analysis is possible using methanol and perfluorinated carboxylic acids as ion-pairing agents [88, 96] but the specificity for nitrogen containing compounds limits the number of detectable substances in impurity profiling.

*3.2.6. LC-MS*

Since the introduction of soft ionization techniques such as ESI, APCI and APPI the coupling of liquid chromatographic systems to almost any type of mass spectrometer has been possible. Very common types of instruments coupled to one of these ionization interfaces are triple quadrupole (QqQ), time of flight (TOF), quadrupole TOF, and ion-trap mass spectrometers, respectively. Each instrument/interface combination holds its own advantages and fields of application. Triple quadrupole mass spectrometers are mainly applied in trace analytics from difficult matrices (e.g. blood and other body fluids or plant extracts) [122-125] because of their high sensitivity and specificity (robust method design assumed) whereas TOF, qTOF and ion-traps (e.g. orbitrap) are very powerful tools of structure elucidation, because the high m/z precision permits calculation of sum formulae. This information combined with fragment spectra obtainable from MS/MS experiments (e.g. using qTOF or ion trap) even allows for amino acid sequencing of small peptides [126, 127]. Impurity profiling is usually not a domain of LC-MS analysis due to the poor linearity and the need of internal standards (ideally stable isotope tagged analyte). Last but not least, the costliness of these instruments is a limitation for most laboratories dealing with routine quality analyses.

Nevertheless, if an impurity has to be detected very sensitively and specifically, e.g. because it is extraordinarily toxic, the application of LC-MS techniques in the Ph. Eur. has to be considered on behalf of safety and innocuousness like it has been the case for meldonium

dihydrate, oseltamivir phosphate, imatinib mesilate and the confirmatory test for aristolochic acids in herbal drugs (2.8.21) method C. Impurities found in these substances are not AA but issue the same challenges regarding detection and separation. During the investigations related to the tryptophan incident [128] this powerful technique [129] was unfortunately only applied *after* the disaster had happened [130-132].

*3.2.7. Capacitively coupled contactless conductivity detection (C4D)*

C4D is a relatively new so called universal detector applied in CE and HPLC analytics of underivatized amino acids [133-145]. The detection principle is based on the measurement of the eluents or running buffers conductivity by means of electromagnetic signals send through the detector cell [146-148]. Analytes passing the detector change the conductivity and produce a signal. Like UV detection, it is a non-destructive detection principle showing sensitivity equal or superior to UV detection [139]. Like all non-destructive detectors this technique could be combined with UV and MS detectors in order to obtain more information about the analyte like UV-spectra and/or mass spectra in a single analysis.

*3.2.8. Combining detection techniques*

Combination of destructive and non-destructive detection techniques by hyphenation and by means of flow splitting can be very useful to acquire information about unknown structures. Some extreme applications combining 3 or more detection principles [149-151] are certainly not routinely available in pharmaceutical laboratories, but the combination of UV and CAD is as simple as it is powerful to obtain information about unknown impurities [152]. The determination of non-volatile, but UV inactive compounds as well as the detection of volatile, but UV active contaminants is possible using this combination. The UV-detector effluent that is usually diverted to the waste is simply directed to the CAD. This is feasible by any laboratory without further equipment or instrumentation and the additional detection dimension introduces higher certainty regarding the substance purity. The combination of UV-FLD-ECD already proved capable of replacing AAA in certain cases [153].

*3.3. Other monographed substances that could profit from modern detection principles.*

There is basically no substance relying on a test for related substances applying LC-UV detection that would not profit from orthogonal detection techniques like LC-MS or LC-CAD. Those tests applying a detection wavelength above 220 nm could benefit from aerosol based techniques like CAD, CNLSD or ELSD or C4D because unknown impurities without chromophore might be present but undetected whereas the detection limit of methods using very low wavelength could be lowered by using alternative detection principles. The impurity profiling of the unnatural AAs gabapentin, tranexamic acid and vigabatrin could benefit from

alternative techniques also [154]. The application of C4D in HPLC analysis is a comparatively new and unexplored area but could prove useful in the purity assessment of substances lacking an adequate chromophore for UV-detection.

## 4. Conclusion

Due to the many ways of producing amino acids and the diversity of suppliers, more general, state of the art methods for the quality control of amino acids should be used. Methods meeting these criteria are available but seem to be disregarded in favor of the very specific, to some extend “blind”, amino acid analysis. In some cases the Ph. Eur. deals with this issue by additional tests for single impurities complicating the test procedure instead of providing a single method covering all impurities. It is at least necessary to check extensively for ninhydrin-negative impurities before transferring an old TLC method to AAA. A substance that has been monitored by TLC for decades could have been contaminated with ninhydrin-negative compounds for years that are still not visible using the AAA; changes in the synthetic process or in the supply chain could cause new contaminants that are not detected after derivatization, e.g. the tryptophan incident [128]. Currently available AAs are from high quality and purity, but as more and more competitors expand on the market, the quality provided by future manufacturers has to be assured by using less specific methods for impurity profiling.

## Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper

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## References


[1] A.B. Hughes, amino acids, peptides and proteins in organic chemistry, Wiley-VCH, Weinheim, 2009.

[2] J.S. Ma, Unnatural amino acids in drug discovery, Chim. Oggi, 21 (2003) 65-68.

[3] R. Laitinen, K. Löbmann, H. Grohganz, C. Strachan, T. Rades, Amino acids as co-amorphous excipients for simvastatin and glibenclamide: Physical properties and stability, Mol. Pharmaceut., 11 (2014) 2381-2389.

[4] B.J. Mishra, A. Kaul, P. Trivedi, L-Cysteine conjugated poly L-lactide nanoparticles containing 5-fluorouracil: formulation, characterization, release and uptake by tissues in vivo, Drug Deliv., 22 (2015) 214-222.

[5] U. Bürger, H. Wolf, Investigations on utilization of parenterally administered amino acids by premature and hypotrophic neonates. III. Composition of an amino acid solution according to pharmacokinetic principles, Eur. J. Pediatr., 122 (1976) 169-175.

[6] A.V. Katdare, C. Lui, S.N. Kleinhart (Merck & Co., Inc.), High-content ibuprofen lysinate pharmaceutical formulation, EP 0505180B1, 1996.

[7] D.A. MacFadyen, On the mechanism of the reaction of ninhydrin with α-amino acids. I. Absorption spectra of ninhydrin and certain derivatives, J. Biol. Chem., 186 (1950) 1-12.

[8] D.A. MacFadyen, N. Fowler, On the mechanism of the reaction of ninhydrin with α-amino acids. II. A spectrophotometric study of hydrindantin reactions, J. Biol. Chem., 186 (1950) 13-22.

[9] A. Strecker, Über die künstliche Bildung der Milchsäure und einen neuen, dem Glycocoll homologen Körper, Liebigs Ann. Chem., (1850) 27-45.

[10] T. Fukumura (Toray Industries, Inc.), Process for preparing L-lysine, US 3770585, 1973.

[11] K. Yasukawa, R. Hasemi, Y. Asano, Dynamic kinetic resolution of α-aminonitriles to form chiral α-amino acids, Adv. Synth. Catal., 353 (2011) 2328-2332.

[12] A.A. Sathe, D.R. Hartline, A.T. Radosevich, A synthesis of α-amino acids via direct reductive carboxylation of imines with carbon dioxide, Chem. Commun., 49 (2013) 5040-5042.

[13] H. Yan, J.S. Oh, J.W. Lee, C.E. Song, Scalable organocatalytic asymmetric Strecker reactions catalysed by a chiral cyanide generator, Nat. Commun., 3 (2012)

[14] T. Mita, J.Y. Chen, M. Sugawara, Y. Sato, One-pot synthesis of α-amino acids from imines through CO2 incorporation: An alternative method for Strecker synthesis, Angew. Chem. Int. Edit., 50 (2011) 1393-1396.

[15] M. Gomes, R. Hernández-Valdés, C.E.S.J. Marques, M.L. Bastos, D.A.G. Aranda, O.A.C. Antunes, Enantioselective heterogeneous catalytic production of α-amino acids, React. Kinet. Catal. Lett., 87 (2005) 19-24.

[16] M.S. Iyer, K.M. Gigstad, N.D. Namdev, M. Lipton, Asymmetric catalysis of the Strecker amino acid synthesis by a cyclic dipeptide, J Am Chem Soc, 118 (1996) 4910-4911.

[17] S.J. Zuend, M.P. Coughlin, M.P. Lalonde, E.N. Jacobsen, Scaleable catalytic asymmetric Strecker syntheses of unnatural α-amino acids, Nature, 461 (2009) 968-970.

[18] R. Bou Chedid, A. Oftring, W. Staffel, M. Biel, J.-P. Melder, C. Grünanger (BASF), Process for preparing racemic α-amino acids, EP 2794552, 2012.

[19] S. Tokuyama, K. Hatano, Overexpression of the gene for *N*-acylamino acid racemase from Amycolatopsis sp TS-1-60 in Escherichia coli and continuous production of optically active methionine by a bioreactor, Appl. Microbiol. Biot., 44 (1996) 774-777.

[20] P. Allegrini, G. Barreca, A. Rosi, G. Soriato (Zambon), Process for the preparation of optically pure phenylalanine, WO 9958493, 1999.

[21] A. Kleemann, W. Leuchtenberger, B. Hoppe, H. Tanner, Amino acids., in: W. Gerhartz (Eds.), Ullmanns Encyclopedia of Industrial Chemistry, VCH Verlagsgesellschaft mbH,Weinheim, 1985, pp. 57-97.

[22] W. Ladner, U. Pressler, W. Siegel (BASF), Verfahren zur herstellung von L-lysin produzierenden mikroorganismen, WO 1992001785, 1992.

[23] K. Yamada, H.I.T.N.S. Tsutsui, K. Yotsumoto, M. Shirai (Toray Industries, Inc.), Verfahren zur Herstellung von L-Threonin durch Fermentation, EP 0205849, 1991.

[24] S. Samsonova, V. Samsonov, Y. Rostova, M. Gusyatiner (Ajinomoto Co., Inc.), A method for producing an L-amino acid belonging to the glutamate family, using a coryneform bacterium, WO 2012147989, 2012.

[25] A. Marx, B. Moeckel, W. Pfefferle, H. Sahm, A. de Graaf, L. Eggeling (Degussa AG), Process for the fermentative production of L-amino acids using coryneform bacteria, EP 1029919, 2000.

[26] L. Eggeling, K. Krumbach, H. Sahm, G. Thierbach Process for the fermentative preparation of L-glutamic acid using coryneform bacteria, US 2002127662, 2002.

[27] M. Sato, N. Akiyoshi (Ajinomoto Co., Inc.), Method for producing L-glutamic acid, US 6653110, 2003.

[28] M. Rieping (Degussa AG), Process for the fermentative preparation of L-threonine, WO 2001092545, 2001.

[29] Y. Nagai, Y. Masumitsu (Ajinomoto Co., Inc.), A method for producing L-lysine, EP 2202299, 2010.

[30] T. Mizukami, R. Katsumata, T. Oka (Kyowa Hakko Kogyo Co., Ltd.), Process for producing histidine, US 4927758 A, 1990.

[31] H. Thomas, R. Mechthild (Degussa AG), Fermentation process for the preparation of L-threonine, WO 2002018543, 2002.

[32] T. Nakanishi, H. Hagino (Kyowa Hakko Kogyo Kabushiki Kaisha), Process for producing L-proline by fermentation, US 4444885, 1984.

[33] Y. Kotani, K. Kino (Kyowa Hakko Kogyo Co., Ltd.), Process for producing L-histidine by fermentation, US 4725541 A, 1988.

[34] M. Suga, M. Sugimoto, T. Osumi, T. Nakamatsu, W. Hibino, M. Ito (Ajinomoto Co., Inc.), Method of producing L-serine by fermentation, US 20030175912, 2003.

[35] A. Yuan, C. Zhang, D. Chen, X. Xing (Jiahe Biolog Technology Co.), Method for producing lysine acetate for injection, CN 102229540, 2013.

[36] S. Miyahara, T. Matsumoto, T. Miyahara, A. Sakaguchi, K. Nitta (Mitsui Toatsu Chemicals Inc.), Method for separating glycine and L-serine from a solution containing same, US 4733009, 1988.

[37] B. Hegedus, A. Krasso (Hoffmann La Roche Inc.), Process for the purification of L-serine, US 3742034, 1973.

[38] J.S. Choi, T.H. Kim, S.H. Kim (BASF Aktiengesellschaft Patents, Trademarks and Licenses), Method for recovering a basic amino acid form a fermentation liquor II, US 20080193985, 2008.

[39] H. Ito, A. Nishi, M. Miyazawa, M. Naruse (Ajinomoto Co., Inc.), Process for isolating and purifying amino acids, US 4956471, 1990.

[40] H.J. Almquist, D.J. Gorton (F E Booth Company Inc), Amino acid extraction, US 2471053, 1949.

[41] F.A. Hoglan, J.P.A. Pugh (International Mineral & Chemical Corp.), Treatment of protein hydrolysates, US 2751408, 1956.

[42] W. Steinmetzer (Societé d'assistance technique pour produits Nestlé S.A.), Process for recovering amino acids from protein hydrolysates, US 4496703, 1985.

[43] Ph. Eur. 8th edition, Council of Europe, Strasbourg, France, 2016.

[44] P. Adriaens, B. Meesschaert, W. Wuyts, H. Vaderhaeghe, H. Eyssen, Separation of ninhydrin-positive compounds on a single-column amino acid analyzer using lithium buffers, J. Chromatogr., 140 (1977) 103-108.

[45] A. Wainer, The chromatography of ninhydrin negative compounds on an amino acid analyzer column, J. Chromatogr., 26 (1967) 48-53.

[46] S. Moore, W.H. Stein, Photometric ninhydrin method for use in the chromatography of amino acids, J. Biol. Chem., 176 (1948) 367-388.

[47] D.H. Spackman, W.H. Stein, S. Moore, Automatic recording apparatus for use in the chromatography of amino acids, Anal. Chem., 30 (1958) 1190-1206.

[48] M. Friedman, J.W. Finley, Methods of tryptophan analysis, J. Agr. Food Chem., 19 (1971) 626-631.

[49] S. Sotgia, A. Zinellu, G.A. Pinna, L. Deiana, C. Carru, Application of an unusual ninhydrin-based reaction for the indirect chiral resolution of D,L-penicillamine, Talanta, 85 (2011) 1783-1785.

[50] S. Sotgia, A. Zinellu, E. Pisanu, G.A. Pinna, L. Deiana, C. Carru, Enantiomeric reversed-phase high-performance liquid chromatography resolution of D-/L-penicillamine after spirocyclization with ninhydrin and by using copper(II)-L-proline complex as a chiral selector in the mobile phase, J. Chromatogr. A, 1205 (2008) 90-93.

[51] G. Prota, E. Ponsiglione, Reaction of ninhydrin with cysteine and its analogs: A revision, Tetrahedron, 29 (1973) 4271-4274.

[52] K. Takahashi, Specific modification of arginine residues in proteins with ninhydrin, J. Biochem., 80 (1976) 1173-1176.

[53] S.J. Sheng, J.J. Kraft, S.M. Schuster, A specific quantitative colorimetric assay for L-asparagine, Anal. Biochem., 211 (1993) 242-249.

[54] P.J. Lamothe, P.G. McCormic, Influence of acidity on reaction of ninhydrin with amino acids, Anal. Chem., 44 (1972) 821-825.

[55] A. Schönberg, E. Singer, Chemistry of ninhydrin and other cyclic 1,2,3-tricarbonyl compounds, Tetrahedron, 34 (1978) 1285-1300.

[56] A. Heesing, R. Muller-Matthesius, H. Rose, Reaction of ninhydrin with tryptophan, Liebigs Ann. Chem., 735 (1970) 72-76.

[57] M. Roth, Fluorescence reaction for amino acids, Anal. Chem., 43 (1971) 880-882.

[58] J.S. Sun, H.X. Guo, D. Semin, J. Cheetham, Direct separation and detection of biogenic amines by ion-pair liquid chromatography with chemiluminescent nitrogen detector, J. Chromatogr. A, 1218 (2011) 4689-4697.

[59] L.R. Snyder, Reaction colorimeters as detectors in high-performance liquid chromatography - Extra-column band broadening with segmented flow through reaction coil, J. Chromatogr., 125 (1976) 287-306.

[60] I.E.P. Taylor, Artefacts in amino acid analysis - Ninhydrin-positive products of carbohydrate hydrolysis, J. Chromatogr., 50 (1970) 331-333.

[61] R.M. Zacharius, W.L. Porter, Further studies on behavior of some non-nitrogenous compounds with ninhydrin during automatic ion exchange chromatography, J. Chromatogr., 30 (1967) 190-197.

[62] L.B. James, Amino acid analysis - a fall-off in performance, J. Chromatogr., 408 (1987) 291-295.

[63] R.L. Cunico, T. Schlabach, Comparison of ninhydrin and *o*-phthalaldehyde post-column detection techniques for high-performance liquid chromatography of free amino acids, J. Chromatogr., 266 (1983) 461-470.

[64] E. Lund, J. Thomsen, K. Brunfeldt, Use of *o*-phthalaldehyde for fluorescence detection in conventional amino acid analyzers - Sub-nanomole sensitivity in analysis of phenylthiohydantoin-amino acids, J. Chromatogr., 130 (1977) 51-54.

[65] M.I. Azilawati, D.M. Hashim, B. Jamilah, I. Amin, Validation of a reverse-phase high-performance liquid chromatography method for the determination of amino acids in gelatins by application of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate reagent, J. Chromatogr. A, 1353 (2014) 49-56.

[66] J. Harmeyer, H.P. Sallmann, L. Ayoub, Use of 2,4,6-trinitrobenzene sulfonic acid for demonstration of amino acids in automatic amino acid analysers, J. Chromatogr., 32 (1968) 258-268.

[67] J. Saurina, S. Hernández-Cassou, Determination of amino acids by ion-pair liquid chromatography with postcolumn derivatization using 1,2-naphthoquinone-4-sulfonate, J. Chromatogr. A, 676 (1994) 311-319.

[68] S. Hernandéz-Cassou, J. Saurina, Derivatization strategies for the determination of biogenic amines in wines by chromatographic and electrophoretic techniques, J. Chromatogr. B, 879 (2011) 1270-81.

[69] R. Hanczkó, I. Molnár-Perl, Derivatization, stability and chromatographic behavior of *o*-phthaldialdehyde amino acid and amine derivatives: *o*-phthaldialdehyde/2-mercaptoethanol reagent, Chromatographia, 57 (2003) S103-S113.

[70] A. Jambor, I. Molnár-Perl, Amino acid analysis by high-performance liquid chromatography after derivatization with 9-fluorenylmethyloxycarbonyl chloride Literature overview and further study, J. Chromatogr. A, 1216 (2009) 3064-77.

[71] I. Molnár-Perl, Advancement in the derivatizations of the amino groups with the *o*-phthaldehyde-thiol and with the 9-fluorenylmethyloxycarbonyl chloride reagents, J. Chromatogr. B, 879 (2011) 1241-1269.

[72] I. Molnár-Perl, A. Vasanits, Stability and characteristics of the *o*-phthaldialdehyde/3-mercaptopropionic acid and o-phthaldialdehyde/*N*-acetyl-L-cysteine reagents and their amino acid derivatives measured by high-performance liquid chromatography, J. Chromatogr. A, 835 (1999) 73-91.

[73] G. Sarwar, H.G. Botting, Evaluation of liquid chromatographic analysis of nutritionally important amino acids in food and physiological samples, J. Chromatogr. Biomed. Appl., 615 (1993) 1-22.

[74] D. Fekkes, State-of-the-art of high-performance liquid chromatographic analysis of amino acids in physiological samples, J. Chromatogr. B Biomed. Appl., 682 (1996) 3-22.

[75] S.A. Cohen, B.A. Bidlingmeyer, T.L. Tarvin, PITC derivatives in amino acid analysis, Nature, 320 (1986) 769-770.

[76] P. Lindroth, K. Mopper, High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with o-phthaldialdehyde, Anal. Chem., 51 (1979) 1667-1674.

[77] R. Schuster, Determination of free amino acids by high performance liquid chromatography, Anal. Chem., 52 (1980) 617-620.

[78] A. Fialaire, E. Postaire, R. Prognon, D. Pradeau, Bioavailability prediction of amino acids and peptides in nutritive mixtures by separation on non-polar stationary phases by high-performance liquid chromatography and direct UV Detection at 210 nm, J. Liq. Chromatogr., 16 (1993) 3003-3015.

[79] Y. Yokoyama, S. Tsuji, H. Sato, Simultaneous determination of creatinine, creatine, and UV-absorbing amino acids using dual-mode gradient low-capacity cation-exchange chromatography, J. Chromatogr. A, 1085 (2005) 110-116.

[80] I.G. Casella, M. Contursi, Isocratic ion chromatographic determination of underivatized amino acids by electrochemical detection, Anal. Chim. Acta, 478 (2003) 179-189.

[81] A.J. Collins, Comparison of refractive index, low-wavelength UV and UV visualization detection methods for the high-performance liquid chromatographic determination of hexamethonium bromide in an injection formulation, J. Chromatogr., 354 (1986) 459-462.

[82] T. Buttler, L. Gorton, G. Markovarga, Characterization of a sampling unit based on tangential flow filtration for online bioprocess monitoring, Anal. Chim. Acta, 279 (1993) 27-37.

[83] T.A. Buttler, K.A.J. Johansson, L.G.O. Gorton, G.A. Markovarga, Online fermentation process monitoring of carbohydrates and ethanol using tangential flow filtration and column liquid chromatography, Anal. Chem., 65 (1993) 2628-2636.

[84] N.C. van de Merbel, H. Lingeman, U.A.T. Brinkman, A. Kolhorn, L.C. de Rijke, Automated monitoring of biotechnological processes using on-line ultrafiltration and column liquid chromatography, Anal. Chim. Acta, 279 (1993) 39-50.

[85] F. Weigang, M. Reiter, A. Jungbauer, H. Katinger, High-performance liquid chromatographic determination of metabolic products for fermentation control of mammalian cell culture: Analysis of carbohydrates, organic-acids and orthophosphate using refractive index and ultraviolet detectors, J. Chromatogr. Biomed. Appl., 497 (1989) 59-68.

[86] A.R. Ivanov, I.V. Nazimov, A.P. Lobazov, G.B. Popkovich, Direct determination of amino acids and carbohydrates by high-performance capillary electrophoresis with refractometric detection, J. Chromatogr. A, 894 (2000) 253-257.

[87] A.G. Mosina, I.O. Mel'nikov, I.V. Nazimov, Y.M. Glubokov, Capillary electrophoresis on unmodified genetically coded amino acids, J. Anal. Chem., 64 (2009) 637-641.

[88] K. Petritis, C. Elfakir, M. Dreux, A comparative study of commercial liquid chromatographic detectors for the analysis of underivatized amino acids, J. Chromatogr. A, 961 (2002) 9-21.

[89] G. Guiochon, A. Moysan, C. Holley, Influence of various parameters on the response factors of the evaporative light scattering detector for a number of non-volatile compounds, J. Liq. Chromatogr., 11 (1988) 2547-2570.

[90] J.M. Charlesworth, Evaporative analyzer as a mass detector for liquid chromatography, Anal. Chem., 50 (1978) 1414-1420.

[91] R. Macrae, J. Dick, Analysis of carbohydrates using the mass detector, J. Chromatogr., 210 (1981) 138-145.

[92] T.H. Mourey, L.E. Oppenheimer, Principles of operation of an evaporative light-scattering detector for liquid-chromatography, Anal. Chem., 56 (1984) 2427-2434.

[93] L.B. Allen, J.A. Koropchak, Condensation nucleation light-scattering: A new approach to development of high-sensitivity, universal detectors for separations, Anal. Chem., 65 (1993) 841-844.

[94] D.R. Oberreit, F.R. Quant, D.B. Blackford Measuring analyte concentrations in liquids, US 20080137065, 2008.

[95] K. Gaudin, A. Baillet, P. Chaminade, Application of a xenon arc lamp as a light source for evaporative light scattering detection, Anal. Bioanal. Chem., 384 (2006) 1302-1307.

[96] K.N. Petritis, P. Chaimbault, C. Elfakir, M. Dreux, Ion-pair reversed-phase liquid chromatography for determination of polar underivatized amino acids using perfluorinated carboxylic acids as ion pairing agent, J. Chromatogr. A, 833 (1999) 147-155.

[97] S. Almeling, U. Holzgrabe, Use of evaporative light scattering detection for the quality control of drug substances: Influence of different liquid chromatographic and evaporative light scattering detector parameters on the appearance of spike peaks, J. Chromatogr. A, 1217 (2010) 2163-2170.

[98] D. Yan, G. Li, X.H. Xiao, X.P. Dong, Z.L. Li, Direct determination of fourteen underivatized amino acids from Whitmania pigra by using liquid chromatography-evaporative light scattering detection, J. Chromatogr. A, 1138 (2007) 301-304.

[99] T. Gorecki, F. Lynen, R. Szucs, P. Sandra, Universal response in liquid chromatography using charged aerosol detection, Anal. Chem., 78 (2006) 3186-3192.

[100] P.H. Gamache, R.S. McCarthy, S.M. Freeto, D.J. Asa, M.J. Woodcock, K. Laws, R.O. Cole, HPLC analysis of non-volatile analytes using charged aerosol detection, LCGC N. Am., 18 (2005) 345-354.

[101] R.W. Dixon, D.S. Peterson, Development and testing of a detection method for liquid chromatography based on aerosol charging, Anal. Chem., 74 (2002) 2930-2937.

[102] S.L. Kaufman (TSI incorporated), Evaporative electrical detector, US 6568245, 2003.

[103] A.D. Yim, P.H. Gamache, R.S. McCarthy (Dionex Corporation), Nebulizer for charged aerosol detection (CAD) system, US 20140352411, 2014.

[104] L.E. Magnusson, D.S. Risley, J.A. Koropchak, Aerosol-based detectors for liquid chromatography, J. Chromatogr. A, 1421 (2015) 68-81.

[105] D. Ilko, A. Braun, O. Germershaus, L. Meinel, U. Holzgrabe, Fatty acid composition analysis in polysorbate 80 with high performance liquid chromatography coupled to charged aerosol detection, Eur. J. Pharm. Biopharm., 94 (2015) 569-574.

[106] N. Vervoort, D. Daemen, G. Török, Performance evaluation of evaporative light scattering detection and charged aerosol detection in reversed phase liquid chromatography, J. Chromatogr. A, 1189 (2008) 92-100.

[107] U. Holzgrabe, C.J. Nap, T. Beyer, S. Almeling, Alternatives to amino acid analysis for the purity control of pharmaceutical grade L-alanine, J. Sep. Sci., 33 (2010) 2402-2410.

[108] R.D. Cohen, Y. Liu, X.Y. Gong, Analysis of volatile bases by high performance liquid chromatography with aerosol-based detection, J. Chromatogr. A, 1229 (2012) 172-179.

[109] J.P. Hutchinson, J.F. Li, W. Farrell, E. Groeber, R. Szucs, G. Dicinoski, P.R. Haddad, Comparison of the response of four aerosol detectors used with ultra high pressure liquid chromatography, J. Chromatogr. A, 1218 (2011) 1646-1655.

[110] T. Vehovec, A. Obreza, Review of operating principle and applications of the charged aerosol detector, J. Chromatogr. A, 1217 (2010) 1549-1556.

[111] Z. Long, Z.M. Guo, X.D. Liu, Q. Zhang, X.G. Liu, Y. Jin, L.N. Liang, H.S. Li, J. Wei, N.P. Wu, A sensitive non-derivatization method for apramycin and impurities analysis using hydrophilic interaction liquid chromatography and charged aerosol detection, Talanta, 146 (2016) 423-429.

[112] S. Almeling, D. Ilko, U. Holzgrabe, Charged aerosol detection in pharmaceutical analysis, J. Pharm. Biomed. Anal., 69 (2012) 50-63.

[113] U. Holzgrabe, C.J. Nap, S. Almeling, Control of impurities in L-aspartic acid and L-alanine by high-performance liquid chromatography coupled with a corona charged aerosol detector, J. Chromatogr. A, 1217 (2010) 294-301.

[114] U. Holzgrabe, C.J. Nap, N. Kunz, S. Almeling, Identification and control of impurities in streptomycin sulfate by high-performance liquid chromatography coupled with mass detection and corona charged-aerosol detection, J. Pharm. Biomed. Anal., 56 (2011) 271-279.

[115] O. Wahl, U. Holzgrabe, Impurity profiling of carbocisteine by HPLC-CAD, qNMR and UV/vis spectroscopy, J. Pharm. Biomed. Anal., 95 (2014) 1-10.

[116] O. Wahl, U. Holzgrabe, Impurity profiling of ibandronate sodium by HPLC-CAD, J. Pharm. Biomed. Anal., 114 (2015) 254-264.

[117] J. Reilly, B. Everatt, C. Aldcroft, Implementation of charged aerosol detection in routine reversed phase liquid chromatography methods, J. Liq. Chromatogr. Rel. Technol., 31 (2008) 3132-3142.

[118] C. Crafts, B. Bailey, I. Acworth, UHPLC analysis of underivatized amino acids. http://www.chromatographyonline.com/uhplc-analysis-underivatized-amino-acids, 2011 (accessed 23.02.2016)

[119] M. Ligor, S. Studzinska, A. Horna, B. Buszewski, Corona charged aerosol detection: An analytical approach, Crit. Rev. Anal. Chem., 43 (2013) 64-78.

[120] X.W. Yan, Detection by ozone-induced chemiluminescence in chromatography, J. Chromatogr. A, 842 (1999) 267-308.

[121] W.L. Fitch, A.K. Szardenings, E.M. Fujinari, Chemiluminescent nitrogen detection for HPLC: An important new tool in organic analytical chemistry, Tetrahedron Lett., 38 (1997) 1689-1692.

[122] R.N.X. Xu, L.M. Fan, M.J. Rieser, T.A. El-Shourbagy, Recent advances in high-throughput quantitative bioanalysis by LC-MS/MS, J. Pharm. Biomed. Anal., 44 (2007) 342-355.

[123] J.L. Wolfender, HPLC in natural product analysis: The detection issue, Planta Med., 75 (2009) 719-734.

[124] M. Vogeser, C. Seger, A decade of HPLC-MS/MS in the routine clinical laboratory - Goals for further developments, Clin. Biochem., 41 (2008) 649-662.

[125] W.A.H. Waterval, J.L.J.M. Scheijen, M.M.J.C. Ortmans-Ploemen, C.D. Habets-van der Poel, J. Bierau, Quantitative UPLC-MS/MS analysis of underivatised amino acids in body fluids is a reliable tool for the diagnosis and follow-up of patients with inborn errors of metabolism, Clin. Chim. Acta, 407 (2009) 36-42.

[126] E. de Hoffmann, Tandem mass spectrometry: A primer, J. Mass Spectrom., 31 (1996) 129-137.

[127] W.J. Griffiths, A.P. Jonsson, S.Y. Liu, D.K. Rai, Y.Q. Wang, Electrospray and tandem mass spectrometry in biochemistry, Biochem. J., 355 (2001) 545-561.

[128] M.L. Kamb, J.J. Murphy, J.L. Jones, J.C. Caston, K. Nederlof, L.F. Horney, L.A. Swygert, H. Falk, E.M. Kilbourne, Eosinophilia-myalgia syndrome in L-tryptophan-exposed patients, Jama, 267 (1992) 77-82.

[129] B.L. Williamson, L.M. Benson, A.J. Tomlinson, A.N. Mayeno, G.J. Gleich, S. Naylor, On-line HPLC-tandem mass spectrometry analysis of contaminants of L-tryptophan associated with the onset of the eosinophilia-myalgia syndrome, Toxicol Lett., 92 (1997) 139-148.

[130] L.A. Swygert, E.F. Maes, L.E. Sewell, L. Miller, H. Falk, E.M. Kilbourne, Eosinophilia-myalgia syndrome. Results of national surveillance, Jama, 264 (1990) 1698-1703.

[131] J. Varga, S.A. Jimenez, J. Uitto, L-tryptophan and the eosinophilia-myalgia syndrome: current understanding of the etiology and pathogenesis, J. Invest. Dermatol., 100 (1993) 97S-105S.

[132] E.A. Belongia, C.W. Hedberg, G.J. Gleich, K.E. White, A.N. Mayeno, D.A. Loegering, S.L. Dunnette, P.L. Pirie, K.L. MacDonald, M.T. Osterholm, An investigation of the cause of the eosinophilia-myalgia syndrome associated with tryptophan use, N. Engl. J. Med., 323 (1990) 357-65.

[133] P. Kubáň, P.C. Hauser, Application of gradient programs for the determination of underivatized amino acids and small peptides in reversed-phase high-performance liquid chromatography with contactless conductivity detection, J. Chromatogr. A, 1128 (2006) 97-104.

[134] M. Pumera, J. Wang, F. Opekar, I. Jelínek, J. Feldman, H. Löwe, S. Hardt, Contactless conductivity detector for microchip capillary electrophoresis, Anal. Chem., 74 (2002) 1968-1971.

[135] P. Kubáň, P.C. Hauser, Contactless conductivity detection in capillary electrophoresis: A review, Electroanal., 16 (2004) 2009-2021.

[136] P. Coufal, J. Zuska, T. van de Goor, V. Smith, B. Gaš, Separation of twenty underivatized essential amino acids by capillary zone electrophoresis with contactless conductivity detection, Electrophoresis, 24 (2003) 671-677.

[137] J. Tanyanyiwa, K. Schweizer, P.C. Hauser, High-voltage contactless conductivity detection of underivatized amino acids in capillary electrophoresis, Electrophoresis, 24 (2003) 2119-2124.

[138] K. Včeláková, I. Zusková, E. Kenndler, B. Gaš, Determination of cationic mobilities and pKa values of 22 amino acids by capillary zone electrophoresis, Electrophoresis, 25 (2004) 309-317.

[139] P. Kubáň, E.M. Abad-Villar, P.C. Hauser, Evaluation of contactless conductivity detection for the determination of UV absorbing and non-UV absorbing species in reversed-phase high-performance liquid chromatography, J. Chromatogr. A, 1107 (2006) 159-164.

[140] P. Kubáň, P.C. Hauser, Contactless conductivity detection for analytical techniques-Developments from 2012 to 2014, Electrophoresis, 36 (2015) 195-211.

[141] T.A. Sullivan (Fresenius Medical Care Holdings, Inc.), Conductivity detector for fluids, US 8525533, 2013.

[142] S.M. Harrison, I. Kaml, V. Prokoratova, M. Mazanek, E. Kenndler, Animal glues in mixtures of natural binding media used in artistic and historic objects: identification by capillary zone electrophoresis, Anal. Bioanal. Chem., 382 (2005) 1520-1526.

[143] E. Samcová, P. Tůma, Determination of proteinogenic amino acids in human plasma by capillary electrophoresis with contactless conductivity detection, Electroanal., 18 (2006) 152-157.

[144] P. Tůma, E. Samcová, K. Andělová, Determination of free amino acids and related compounds in amniotic fluid by capillary electrophoresis with contactless conductivity detection, J. Chromatogr. B, 839 (2006) 12-18.

[145] E.M. Abad-Villar, P. Kubáň, P.C. Hauser, Determination of biochemical species on electrophoresis chips with an external contactless conductivity detector, Electrophoresis, 26 (2005) 3609-3614.

[146] J.A.F. da Silva, C.L. do Lago, An oscillometric detector for capillary electrophoresis, Anal. Chem., 70 (1998) 4339-4343.

[147] A.J. Zemann, E. Schnell, D. Volgger, G.K. Bonn, Contactless conductivity detection for capillary electrophoresis, Anal. Chem., 70 (1998) 563-567.

[148] P. Hauser, E.A. Villar, J. Tanyanyiwa (Peter C. Hauser), Method and apparatus for performing high-voltage contactless conductivity (HV-CCD) electrophoresis, US 20050109621, 2005.

[149] Y.T. Jiang, D. Hascall, D.L. Li, J.H. Pease, A charged aerosol detector/chemiluminescent nitrogen detector/liquid chromatography/mass spectrometry system for regular and fragment compound analysis in drug discovery, J. Chromatogr. A, 1411 (2015) 63-68.

[150] Y. Takano, Y. Chikaraishi, N. Ohkouchi, Isolation of underivatized amino acids by ion-pair high performance liquid chromatography for precise measurement of nitrogen isotopic composition of amino acids: Development of comprehensive LC x GC/C/IRMS method, Int. J. Mass. Spectrom., 379 (2015) 16-25.

[151] K. Zhang, Y. Li, M. Tsang, N.P. Chetwyn, Analysis of pharmaceutical impurities using multi-heartcutting 2D LC coupled with UV-charged aerosol MS detection, J. Sep. Sci., 36 (2013) 2986-2992.

[152] P. Sun, X.D. Wang, L. Alquier, C.A. Maryanoff, Determination of relative response factors of impurities in paclitaxel with high performance liquid chromatography equipped with ultraviolet and charged aerosol detectors, J. Chromatogr. A, 1177 (2008) 87-91.

[153] P. Agrafiotou, S. Sotiropoulos, A. Pappa-Louisi, Direct RP-HPLC determination of underivatized amino acids with online dual UV absorbance, fluorescence, and multiple electrochemical detection, J. Sep. Sci., 32 (2009) 949-954.

[154] S. Jia, J.H. Park, J. Lee, S.W. Kwon, Comparison of two aerosol-based detectors for the analysis of gabapentin in pharmaceutical formulations by hydrophilic interaction chromatography, Talanta, 85 (2011) 2301-2306.

[155] B.N. Jones, J.P. Gilligan, *o*-Phthaldialdehyde precolumn derivatization and reversed-phase high-performance liquid chromatography of polypeptide hydrolysates and physiological fluids, J. Chromatogr., 266 (1983) 471-82.

[156] B.N. Jones, S. Pääbo, S. Stein, Amino acid analysis and enzymatic sequence determination of peptides by an improved *o*-phthaldialdehyde precolumn labeling procedure, J. Liq. Chromatogr., 4 (1981) 565-586.

[157] H.A. Moye, A.J. Boning, A versatile fluorogenic labeling reagent for primary and secondary amines: 9-fluorenylmethyl chloroformate, Anal. Lett., 12 (1979) 25-35.

[158] S.S. Simons, D.F. Johnson, Structure of fluorescent adduct formed in reaction of *o*-phthalaldehyde and thiols with amines, J. Am. Chem. Soc., 98 (1976) 7098-7099.

[159] P. Edman, Preparation of phenyl thiohydantoins from some natural amino acids, Acta Chem. Scand., 4 (1950) 277-282.

[160] B.A. Bidlingmeyer, S.A. Cohen, T.L. Tarvin, Rapid analysis of amino acids using pre-column derivatization, J. Chromatogr., 336 (1984) 93-104.

[161] D.J. McCaldin, The chemistry of ninhydrin, Chem. Rev., 60 (1960) 39-51.

[162] M. Friedman, L.D. Williams, Stoichiometry of formation of ruhemann's purple in ninhydrin reaction, Bioorg. Chem., 3 (1974) 267-280.

[163] M. Friedman, Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences, J. Agr. Food Chem., 52 (2004) 385-406.

[164] S. Kopec, U. Holzgrabe, Impurity profile of amino acids?, Pharmeur. Sci. Notes, 2005 (2005) 39-45.

[165] A. Paul, P. Tonne, E. Roske, H. Siegel (BASF AG), Preparation of D- or L-alanine or high enantiomeric purity, US 4962231, 1990.

[166] H. Bergs Verfahren zur Darstellung von Hydantoinen, DE 566094, 1932.

[167] A.B. Santoyo, J.B. Rodriguez, J.L.G. Carrasco, E.G. Gomez, I.A. Rojo, M.L.A. Teruel, Production of optically pure l-alanine by immobilized *Pseudomonas sp.* BA2 cells, J. Chem. Technol. Biot., 73 (1998) 197-202.

[168] A.S. Bommarius, K. Drauz, K. Gunther, G. Knaup, M. Schwarm, L-methionine related L-amino acids by acylase cleavage of their corresponding *N*-acetyl-DL-derivatives, Tetrahedron-Asymmetr., 8 (1997) 3197-3200.

[169] H. Jianpo, Z. Xiaobin, J. Guangyu, T. Siqing (Anhui Huaheng Bioengineering), Method for producing optically pure L-alanine by one *Pseudomonas* and two enzymes, CN 101580864, 2009.

[170] I. Chibata, T. Tosa, T. Sato, K. Yamamoto (Tanabe Seiyaku Co., Ltd.), Process for preparing L-alanine, US 3898128, 1975.

[171] R. Katsumata, S. Hashimoto, I. Kawamoto, M. Suzuki, H. Yoshida, H. Hagino, K. Nakayama (Kyowa Hakko Kogyo Co., Ltd.), Process for producing L-alanine by fermentation with *Arthrobacter*, US 5478733, 1995.

[172] A.G. Azizian, A.A. Ambartsumian, M.A. Ananikian, S.M. Kocharian Method for preparing L-alanine, US 5124257, 1992.

[173] E. Bunel, T. Koch, R. Ozer, S. Sengupta (Du Pont), Production of 6-aminocaproic acid, WO 0240440, 2002.

[174] E.E. Howe, T. Max (Merck & Co.), Recovery of arginine, histidine, and lysine from protein hydrolysates, US 2480654, 1949.

[175] W. Blaschek, S. Ebel, U. Hilgenfeldt, U. Holzgrabe, J. Reichling, V. Schulz, W. Barthlott, H.-D. Höltje, Hagers Enzyklopädie der Arzneistoffe und Drogen. http://www.drugbase.de/de/datenbanken/hagers-enzyklopaedie.html, 2015 (accessed 25.02.2016)

[176] I. Chibata, M. Kisumi, J. Kato (Tanabe Seiyaku Co., Ltd.), Process for producing L-arginine by fermentation, US 3902967, 1975.

[177] Y.H. Park, H.W. Kim, J.H. Lee, S.Y. Hwang (CJ Cheiljedang Co.), Method for producing L-arginine using *Corynebacterium glutamicum*, US 20090311757, 2009.

[178] M. Ikeda, T. Nakano, S. Mitsuhashi, M. Hayashi, K. Tanaka (Kyowa Hakko Kogyo Co., Ltd.), Process for producing L-arginine, L-ornithine or L-citrulline, US 20090123980, 2009.

[179] M. Suga, Y. Kuwabara, K. Hashiguchi, H. Ito, T. Nakamatsu, O. Kurahashi (Ajinomoto Co., Inc.), Method for producing L-arginine, US 20030124686, 2003.

[180] R. Katsumata, H. Yokoi (Kyowa Hakko Kogyo Co., Inc.), Process for producing L-arginine, US 5017482, 1991.

[181] M. Lunts, S. Fomina, T. Leonova, M. Gusyatiner (Ajinomoto Co., Inc.), Bacterium having ability to produce L-glutamic acid, L-proline or L-arginine and method for producing L-glutamic acid, L-proline or L-arginine, US 20040191876, 2004.

[182] M.D. Sackler Method of manufacture of arginine aspartate, US 3487103, 1969.

[183] S. Mitsuhashi, T. Nishino (Kyowa Hakko Kogyo Co., Ltd.), Production of L-asparagine by using microorganism transformants, JP 2013106588, 2011.

[184] J. Li, J. Zha, Y. Wan (Anhui Application Technology Institute), Improved technology for chemical preparation of L-asparagine, CN 1149577, 1997.

[185] M. Terasawa, H. Yukawa, Y. Takayama, Production of L-aspartic acid from *Brevibacterium* by the cell re-using process, Process Biochem., 20 (1985) 124-128.

[186] M.D. Armstrong, J.D. Lewis, Thioether derivatives of cysteine and homocysteine, J. Org. Chem., 16 (1951) 749-753.

[187] L. Goodman, L.O. Ross, B.R. Baker, Potential anticancer agents. V. some sulfur-substituted derivatives of cysteine, J. Org. Chem., 23 (1958) 1251-1257.

[188] J.D. Genders, N.L. Weinberg, D.J. Mazur (The Electrosynthesis Co., Inc.), High yield methods for electrochemical preparation of cysteine and analogues, US 5106463, 1992.

[189] T. Nagasawa, G.S. Dhillon, T. Ishii, H. Yamada, Enzymatic-synthesis of L-cysteine by O-acetylserine sulfhydrylase of 3-Chloro-L-alanine resistant *Bacillus sphaericus* L-118, J. Biotechnol., 2 (1985) 365-377.

[190] H. Kumagai, H. Tanaka, S. Sejima, H. Yamada, Elimination and replacement reactions of β-chloro-L-alanine by cysteine desulfhydrase from *Aerobacter aerogenes*, Agr. Biol. Chem., 41 (1977) 2071-2075.

[191] H. Behringer, P. Zillikens, Die synthese der 2-amino-thiazolin-carbonsäure-4 und über eine neue cystin-synthese, Liebigs Ann. Chem., 574 (1951) 140-156.

[192] A. Reutter-Maier, M. Brunner, T. Dassler (Wacker Chemie AG), Method for production of natural L-cysteine by fermentation, US 20140141474, 2014.

[193] Z. Peng, W. Zhu (NHWA Pharma Corporation), Process for the preparation of gabapentin hydrochloride, US 7667071, 2010.

[194] A. Kumar, S.R. Soudagar, A.M. Nijasure, N.B. Panda, P. Gautam, G.R. Thakur (IPCA Laboratories Ltd.), Process for synthesis of gabapentin, US 20080103334, 2008.

[195] F. Tenconi, C. Giordani, N. Caraccia (Bioindustria Laboratorio Italiano Medicinali S.P.A.), Process for the preparation of gabapentin, US 6576790, 2003.

[196] R. Giovanetti, A. Nicoli, M. Verzini, G. Soriato, L. Cotarca (Zach System S.P.A.), Process for the preparation of gabapentin, US 7417166, 2008.

[197] L. Yang, N. Jiang, Z. Zhao, Synthesis of glycine using two-step reaction approach in alcohol and its purification, Adv. Mat. Res., 396-398 (2012) 1711-1715.

[198] T. Mizuno, N. Tabei, H. Okamura, H. Sato, M. Oosu, Y. Too (Sumitomo Chemical Co.), Preparation of α-amino acids, JP 03093757A, 1991.

[199] W. Xianbing, Z. Ping, X. Guan, Z. Qingqun, L. Dexiang (Shine Star Hubei Biolog Engineering), Method for producing L-histidine, CN 101125831B, 2010.

[200] X. Jiang Histidine hydrochloride production method, CN 104744373A, 2015.

[201] M.H. Updike, G.J. Calton (W. R. Grace & Co.), Fermentative preparation of L-leucine, US 4421854, 1983.

[202] S. Kurihara, K. Araki, H. Ueda, M. Ikumo (Kyowa Hakko Kogyo Kabushiki Kaisha), Preparation of L-leucine by fermentation, US 3668073, 1972.

[203] J.M. Chemerda, S. Meyer, D.F. Reinhold (Merck & Co. Inc.), Method of preparing optically active L-α-methyl-3,4-dihydroxy phenylalanine, US 3366679, 1968.

[204] P. Bamberg, B. Sjoberg (Astra Laekemedel), Stereoselective preparation of L-dopa and L-*m*-tyrosine and novel compounds, US 3838008, 1974.

[205] R.L. Dyer, D.J. Lewis (G.D. Searle & Co.), Process for the preparation of L-DOPA, EP 0189938A2, 1986.

[206] C.A.P.R. Rao (Azico Pharmaceuticals Private Ltd.), Novel process for the preparation of levothyroxine sodium, WO 2015011573, 2015.

[207] B.M. Khamar, R. Gurusamy, M.N. Ravi, V.M. Reddy, B. Edde, R. Ponnaiah, I.A. Modi (Cadila Pharmaceuticals Ltd.), An improved process for the preparation of levothyroxine sodium with reduced levels of impurities, WO 2009136249, 2009.

[208] R.R. Kumar, W.L. Aubrey (Glaxo Lab Ltd.), Preparation of triiodothyronine, US 2993928, 1961.

[209] S. Sanganabhatla, S. Srivastava, S.B. Narayanan, M.A. Khan (Glenmark Generics Ltd.), A process for the preparation of liothyronine and its pharmaceutically acceptable salts thereof, IN 2009MU01400, 2009.

[210] J. Ning, L. Cai (Wuxi Jinghai Amino Acid Co.), Preparation of bulk drug of lysine acetate, CN 104829477, 2015.

[211] H.C. White (Dow Chemical Co.), Resolution of DL-lysine with D-camphoric acid, US 2859244, 1958.

[212] E.V. McCollum, A.A. Rider, The preparation of lysine from protein hydrolysates, J. Biol. Chem., 190 (1951) 451-453.

[213] P. Fürst, Dietary L-lysine supplementation: a promising nutritional tool in the prophylaxis and treatment of osteoporosis, Nutrition, 9 (1993) 71-72.

[214] O. Zelder, C. Klopprogge, H. Schroder, S. Haefner, B. Kröger, P. Kiefer, E. Heinzle, C. Wittmann (BASF), Methods for the preparation of lysine by fermentation of *Corynebacterium glutamicum*, US 8048651B2, 2011.

[215] Sumitomo Chemical Co., Process for producing L-methionine, GB 970203, 1964.

[216] G. Fremy, P. Barre, Y. Kim So, K. Son Sung, M. Lee Sang (Arkema, CJ Cheiljedang Corp.), Preparation of L-methionine, WO 2013029690, 2013.

[217] A. Kleemann, K. Klostermann, W. Leuchtenberger, R.E. Moerck, M. Karrenbauer (Degussa AG), Process for the isolation of L-amino acids, US 4827029, 1989.

[218] A. Bommarius, K. Drauz, S. Verseck (Degussa AG), Process for the production of amino acids using racemase and acylase, US 6656710, 2003.

[219] R. Kühnreich, U. Holzgrabe, Impurity profiling of L-methionine by HPLC on a mixed mode column, J. Pharm. Biomed. Anal., 122 (2016) 118-125.

[220] F. Geiger, B. Halsberghe, H.J. Hasselbach, K. Hentschel, K. Huthmacher, M. Korfer, S.P. Mannsfeld, H. Tanner, F. Theissen, J. Vanrobaeys (Degussa AG), Process for the preparation of D,L-methionine or the salt thereof, US 5990349, 1999.

[221] C. Casse (Adisseo France SAS, Claude Casse), Process for preparing 2-hydroxy-4-(methylthio)butyronitrile and methionine, WO 2008006977, 2008.

[222] K. Nakayama, K. Araki (Kyowa Hakko Kogyo Co., Ltd.), Process for producing L-methionine, US 3729381, 1973.

[223] Y. Huang, W. Hu, W. Sun, T. Liu, Y. Wang, J. Tu (Zhejiang Chiral Medicine Chemicals Co.), Process for preparation of L-methyldopa, CN 102531939, 2012.

[224] D.F. Reinhold, Fireston.Ra, W.A. Gaines, J.M. Chemerda, Sletzing.M, Synthesis of L-α-methyldopa from asymmetric intermediates, J. Org. Chem., 33 (1968) 1209-1213.

[225] E. Biekert, R. Kallischnigg (Knoll AG), Preparation of L-α-methyldopa, US 3440278, 1969.

[226] M. Tanaka, T. Kishi, Y. Kato (Kyowa Hakko Kogyo Co., Ltd.), Process for the preparation of phenylalanine, US 3410896, 1968.

[227] O. Kurahashi, T. Tsuchida, H. Kawashima, H. Enei (Ajinomoto Co., Inc.), Method for producing L-phenylalanine by fermentation, US 4591562, 1986.

[228] H. Voelskow, R. Keller, M. Schlingmann, M. Platen, J. Then, G. Wöhner (Hoechst AG), Process for the preparation of L-phenylalanine, US 4745059, 1988.

[229] H.Y. Kim, H. Rhym, D.J. Lee, C.H. Won, B.L. Lim, H.G. Choi (Miwon Co., Ltd.), Method for production of L-phenylalanine by recombinant *E. coli*, US 5304475, 1994.

[230] R. Katsumata, M. Ikeda (Kyowa Hakko Kogyo Co., Ltd.), Process for producing L-tryptophan, L-tyrosine or L-phenylalanine, US 5605818, 1997.

[231] D. Ura, T. Hashimukai, T. Matsumoto, N. Fukuhara (Mitsui Toatsu Chemicals), Process for the preparation of L-serine by an enzymatic method, US 5382517, 1995.

[232] K. Ishiwata, N. Makiguchi, H. Kawashima, T. Suzuki, M. Imadegawa (Mitsui Toatsu Chemicals Inc.), Process for the preparation of L-serine, DE 3505353, 1985.

[233] G.G. Lovinger, S.A. Whitehead (W. R. Grace & Co.), Microorganism strains for the fermentative preparation of L-serine, US 4528273, 1985.

[234] H. Yamade (Kyowa Hakko Kogyo Kabushiki Kaisha), Process for preparing L-serine, US 3755081, 1973.

[235] T. Hermann, M. Rieping (Degussa AG), Fermentation process for the preparation of L-threonine, US 20030190712, 2003.

[236] M. Rieping (Degussa AG), Processes for the fermentative preparation of L-threonine using strains of *Escherichia* in which the yjgF gene is inactivated, US 7638313, 2009.

[237] T. Hermann, D. Kruse, M. Rieping, G. Thierbach (Degussa AG), A process for preparing L-threonine, WO 2005014843, 2005.

[238] S.Y. Lee, K.H. Lee, J.H. Park, T.Y. KIM (Korea Advanced Institute of Science and Technology), L-threonine overproducing microorganism and method for preparing L-threonine using the same, EP 2121918, 2011.

[239] M. Kato, T. Miyoshi, I. Kibayashi, M. Ikemi, H. Gomi, Y. Ishimatsu, N. Koizumi, H. Yamada (Denki Kagaku Kogyo Kabushiki Kaisha), Process for preparing L-threonine, US 4492757, 1985.

[240] K. Pfister, C.A. Robinson, A.C. Shabica, M. Tishler, The synthesis of DL-threonine. II. Interconversion of DL-threonine and DL-allothreonine, J. Am. Chem. Soc., 71 (1949) 1101-1105.

[241] A.C. Shabica (Merck & Co. Inc.), Process for the preparation of DL-threonine, US 2461847, 1949.

[242] M. Levine, R. Sedlecky, Preparation of *p*-aminomethylcyclohexylcarboxylic and *m*-aminomethylcyclohexylcarboxylic acid, J. Org. Chem., 24 (1959) 115-116.

[243] P. Ravi, P.K. Neela, N.R. Nimmakayala, T.R. Ampolu, R. Turka, M. Venkateswarlu (Davuluri Ramamohan Rao), An improved process for the preparation of tranexamic acid, WO 2015104721, 2015.

[244] T. Miki, T. Naito, A. Okano (Daiichi Seiyaku Co, Mitsubishi Chem. Ind.), Process for the production of trans-4-aminomethylcyclohexane-1-carboxylic acid, US 3449411, 1969.

[245] H. Itoh, K. Itoh, C. Shibuya, Y. Usubuchi (Asahi Kasei Kogyo Kabushiki Kaisha), Process for preparing 4-aminomethyl cyclohexane carboxylic acid-1, US 3923879, 1975.

[246] T. Tsuchida, O. Kurahashi, H. Kawashima, S. Nakamori, H. Enei (Ajinomoto Co., Inc.), Method for producing L-tryptophan by fermentation, US 4588687, 1986.

[247] S.V. Gatenbeck, P.O. Hedman (AB Bofors), Fermentative process for the production of L-tryptophan and its derivatives, US 3963572, 1976.

[248] I. Shiio, S. Sugimoto, K. Kawamura (Ajinomoto Co., Inc.), Process for the production of L-tryptophan by a fermentation process, EP 0128637 B1, 1987.

[249] J.E. Thiemann, H. Pagani (Lepetit Spa), Production of L-tryptophan by fermentation, US 3700558, 1972.

[250] O. Hiroshi (Chugai Pharmaceutical), Process for the production of L-tryptophan by fermentation, US 3385762, 1968.

[251] K. Nakayama, H. Hagino (Kyowa Hakko Kogyo Co., Ltd.), Process for producing L-tryptophan, US 3849251, 1974.

[252] I. Shiio, S. Sugimoto, K. Kawamura (Ajinomoto Co., Inc.), Process for the production of L-tryptophan using sulfaguanidine-resistant microorganisms, US 4618580, 1986.

[253] K. Arima, I. Nugami, M. Yoneda (Takedo Chemical Industries Ltd.), Method for producing L-tryptophan, US 3801457, 1974.

[254] S.M.T.I.A. Ogawa, S. Iguchi, S. Morita, H. Kuwamoto (Mitsui Toatsu Chemicals Inc.), Process for producing L-tryptophane, EP 0438591B1, 1994.

[255] T. Uzuki, M. Takahashi, M. Noda, Y. Komachiya, H. Wakamatsu (Ajinomoto Co., Inc.), Selective deacylation of *N*-acyl-DL-amino acids, US 3907638, 1975.

[256] A. Gaston, V. Leon, H. Rene (Roussel Uclaf), Process of resolving DL-acyl tryptophan compounds and products obtained thereby, US 2797226, 1957.

[257] D.T. Warner, O.A. Moe, Amino acids. I. New syntheses of DL-tryptophan, DL-ornithine and DL-glutamic Acid, J. Am. Chem. Soc., 70 (1948) 2765-2767.

[258] H.R. Snyder, J.A. MacDonald, A synthesis of tryptophan and tryptophan analogs, J. Am. Chem. Soc., 77 (1955) 1257-1259.

[259] C. Xue, Y. Wang, Y. Chang, J. Xu, J. Wang, Q. Tang (Ocean Univ China), Method for preparing L-tyrosine from antarctic krill, CN 104178534, 2014.

[260] B.B. Dowling (International Minerals & Chemicals Corp.), Purification of tyrosine, US 2661371, 1953.

[261] L.J. Templeton, D.T.K. Van (Du Pont), Method of enhancing L-tyrosine production in recombinant bacteria, WO 2008060387, 2008.

[262] T. Oka, R. Katsumata, A. Ozaki, T. Mizukami, H. Yokoi, M. Hara (Kyowa Hakko Kogyo Co., Ltd.), Process for preparing L-tyrosine, EP 0332234B1, 1993.

[263] H. Hagino, K. Nakayama, H. Yoshida (Kyowa Hakko Kogyo Co., Ltd.), Process for the production of L-tyrosine, US 3787287, 1974.

[264] (Xinyi Hanling Bio Engineering), Preparation method of *N*-acetyl-L-tyrosine, CN 102827018, 2012.

[265] A. Liu (Wuhan University Hoyo Pharmace), Process for preparing *N*-acetyl-L-tyrosine, CN 1262534, 2006.

[266] H. Matsui, T. Tsuchida, S. Nakamori (Ajinomoto), Method for producing L-valine by fermentation, US 4391907, 1983.

[267] S. Abe, K. Takayama (Kyowa Hakko Kogyo Co., Ltd.), Verfahren zur biotechnischen Herstellung von L-Valin, DE 1517825B1, 1970.

[268] C. Zhang, J. Pu, Y. Yu, L. Chen, M. Chen, G. Zu (Dalian Polytechnic University), Candida tropicalis and method for preparing L-valine through microbiological method, CN 103695325, 2014.

[269] S.M. Birnbaum, L. Levintow, R.B. Kingsley, J.P. Greenstein, Specificity of amino acid acylases, J. Biol. Chem., 194 (1952) 455-470.

[270] R. Marshall, S.M. Birnbaum, J.P. Greenstein, Cobalt ion activation of renal acylase-I, J. Am. Chem. Soc., 78 (1956) 4636-4642.

[271] H. Kunz, W. Pfrengle, Carbohydrates as chiral templates - Asymmetric Ugi-synthesis of α-amino acids using galactosylamines as the chiral matrices, Tetrahedron, 44 (1988) 5487-5494.

[272] H. Kunz, W. Sager, W. Pfrengle, D. Schanzenbach, Reversal of asymmetric induction in stereoselective Strecker synthesis on galactosyl amine as the chiral Matrix, Tetrahedron Lett., 29 (1988) 4397-4400.

[273] D.S. Matteson, E.C. Beedle, A directed chiral synthesis of amino acids from boronic esters, Tetrahedron Lett., 28 (1987) 4499-4502.

[274] W. Frieben, F. Gerhart (Merrel Toraude & Co.), Process for preparing 4-amino-5-hexanoic acid, GB 2133002, 1984.

[275] B.W. Metcalf, M. Jung (Richardson-Merrell Inc.), Olefinic derivatives of amino acids, US 3960927, 1976.

[276] Z.X. Zheng, J.M. Lin, F. Qu, Chiral separation of underivatized and dansyl amino acids by ligand-exchange micellar electrokinetic capillary chromatography using a copper(II)-L-valine complex as selector, J. Chromatogr. A, 1007 (2003) 189-196.

[277] X.N. Lu, Y. Chen, L. Guo, Y.F. Yang, Chiral separation of underivatized amino acids by ligand-exchange capillary electrophoresis using a copper(II)-L-lysine complex as selector, J. Chromatogr. A, 945 (2002) 249-255.

[278] K. Petritis, A. Valleix, C. Elfakir, M. Dreux, Simultaneous analysis of underivatized chiral amino acids by liquid chromatography-ionspray tandem mass spectrometry using a teicoplanin chiral stationary phase, J. Chromatogr. A, 913 (2001) 331-340.

[279] J.D. Oliver, A.T. Sutton, N. Karu, M. Phillips, J. Markham, P. Peiris, E.F. Hilder, P. Castignolles, Simple and robust monitoring of ethanol fermentations by capillary electrophoresis, Biotechnol. Appl. Bioc., 62 (2015) 329-342.

[280] T. Hložek, M. Bursová, R. Čabala, Fast determination of ethylene glycol, 1,2-propylene glycol and glycolic acid in blood serum and urine for emergency and clinical toxicology by GC-FID, Talanta, 130 (2014) 470-474.

[281] Dionex Corporation, the determination of inorganic anions and organic acids in fermentation broth. Application note 123. http://www.dionex.com/en-us/webdocs/4082-AN123_LPN1030_2.pdf, (accessed 11.02.2016)

[282] D. Ilko, C.J. Nap, U. Holzgrabe, S. Almeling, Validation and application of an HPLC-CAD-TOF/MS method for identification and quantification of pharmaceutical counterions, Pharmeur. Bio. Sci. Notes, 2014 (2014) 81-91.

[283] P. Kubáň, P. Ďurč, M. Bittová, F. Foret, Separation of oxalate, formate and glycolate in human body fluid samples by capillary electrophoresis with contactless conductometric detection, J. Chromatogr. A, 1325 (2014) 241-246.

[284] J.P. Hutchinson, T. Remenyi, P. Nesterenko, W. Farrell, E. Groeber, R. Szucs, G. Dicinoski, P.R. Haddad, Investigation of polar organic solvents compatible with corona charged aerosol detection and their use for the determination of sugars by hydrophilic interaction liquid chromatography, Anal. Chim. Acta, 750 (2012) 199-206.

[285] S. Inagaki, J.Z. Min, T. Toyo'oka, Direct detection method of oligosaccharides by high-performance liquid chromatography with charged aerosol detection, Biomed. Chromatogr., 21 (2007) 338-342.

[286] M. Melmer, T. Stangler, A. Premstaller, W. Lindner, Solvent effects on the retention of oligosaccharides in porous graphitic carbon liquid chromatography, J. Chromatogr. A, 1217 (2010) 6092-6096.

[287] V.P. Hanko, J.S. Rohrer, Determination of carbohydrates, sugar alcohols, and glycols in cell cultures and fermentation broths using high-performance anion-exchange chromatography with pulsed amperometric detection, Anal. Biochem., 283 (2000) 192-199.

[288] J. Partyka, F. Foret, Cationic labeling of oligosaccharides for electrophoretic preconcentration and separation with contactless conductivity detection, J. Chromatogr. A, 1267 (2012) 116-120.

[289] A. Joseph, A. Rustum, Development and validation of a RP-HPLC method for the determination of gentamicin sulfate and its related substances in a pharmaceutical cream using a short pentafluorophenyl column and a charged aerosol detector, J. Pharm. Biomed. Anal., 51 (2010) 521-531.

[290] D. Samain, P. Dupin, P. Delrieu, G. Inchauspe, Multidimensional ion-pair HPLC for the purification of aminoglycoside antibiotics with refractive index detection, Chromatographia, 24 (1987) 748-752.

[291] V. Manyanga, K. Kreft, B. Divjak, J. Hoogmartens, E. Adams, Improved liquid chromatographic method with pulsed electrochemical detection for the analysis of gentamicin, J. Chromatogr. A, 1189 (2008) 347-354.

[292] V. Manyanga, O. Grishina, Z. Yun, J. Hoogmartens, E. Adams, Comparison of liquid chromatographic methods with direct detection for the analysis of gentamicin, J. Pharm. Biomed. Anal., 45 (2007) 257-262.

[293] N.C. Megoulas, M.A. Koupparis, Development and validation of a novel LC/ELSD method for the quantitation of gentamicin sulfate components in pharmaceuticals, J. Pharm. Biomed. Anal., 36 (2004) 73-79.

[294] H. Curiel, W. Vanderaerden, H. Velez, J. Hoogmartens, A. Van Schepdael, Analysis of underivatized gentamicin by capillary electrophoresis with UV detection, J. Pharm. Biomed. Anal., 44 (2007) 49-56.

[295] R. Grahek, L. Zupančič-Kralj, Identification of gentamicin impurities by liquid chromatography tandem mass spectrometry, J. Pharm. Biomed. Anal., 50 (2009) 1037-1043.

[296] P. Jankovics, S. Chopra, M.N. El-Attug, D. Cabooter, K. Wolfs, B. Noszál, A. Van Schepdael, E. Adams, Exploring the possibilities of capacitively coupled contactless conductivity detection in combination with liquid chromatography for the analysis of polar compounds using aminoglycosides as test case, J. Pharm. Biomed. Anal., 112 (2015) 155-168.

[297] W.H. Tsai, T.C. Huang, H.H. Chen, J.J. Huang, M.H. Hsue, H.Y. Chuang, Y.W. Wu, Determination of tetracyclines in surface water and milk by the magnesium hydroxide coprecipitation method, J. Chromatogr. A, 1217 (2010) 415-418.

[298] J. Chico, S. Meca, C. R., M.D. Prat, M. Granados, Restricted access materials for sample clean-up in the analysis of trace levels of tetracyclines by liquid chromatography - Application to food and environmental analysis, J. Chromatogr. A, 1181 (2008) 1-8.

[299] A. Jia, Y. Xiao, J.Y. Hu, M. Asami, S. Kunikane, Simultaneous determination of tetracyclines and their degradation products in environmental waters by liquid chromatography-electrospray tandem mass spectrometry, J. Chromatogr. A, 1216 (2009) 4655-4662.

[300] V. Manyanga, R.L. Dhulipalla, J. Hoogmartens, E. Adams, Improved liquid chromatographic method with pulsed electrochemical detection for the analysis of kanamycin, J. Chromatogr. A, 1217 (2010) 3748-3753.

[301] E. Kaale, A. Van Schepdael, E. Roets, J. Hoogmartens, Development and validation of a simple capillary zone electrophoresis method for the analysis of kanamycin sulfate with UV detection after pre-capillary derivatization, J. Chromatogr. A, 924 (2001) 451-458.

[302] M.N. El-Attug, E. Adams, J. Hoogmartens, A. Van Schepdael, Capacitively coupled contactless conductivity detection as an alternative detection mode in CE for the analysis of kanamycin sulphate and its related substances, J. Sep. Sci., 34 (2011) 2448-2454.

[303] M. Gaugain, M.P. Chotard, D. Hurtaud-Pessel, E. Verdon, Comprehensive validation of a liquid chromatography-tandem mass spectrometry method for the confirmation of chloramphenicol in urine including stability of the glucuronide conjugate and efficiency of deconjugation, J. Chromatogr. B Anal. Technol. Biomed Life Sci., 1011 (2016) 145-150.

[304] G.S. Sadana, A.B. Ghogare, Simultaneous determination of chloramphenicol and benzocaine in topical formulations by high-performance liquid chromatography, J. Chromatogr., 542 (1991) 515-520.

[305] S. Aboukhalil, W.H. Aboukhalil, A.N. Masoud, A.A. Yunis, High-performance liquid chromatographic determination of chloramphenicol and four analogs using reductive and oxidative electrochemical and ultraviolet detection, J. Chromatogr. Biomed. Appl., 417 (1987) 111-119.

[306] J. Olšovská, Z. Kameník, T. Cajthaml, Hyphenated ultra high-performance liquid chromatography-Nano quantity analyte detector technique for determination of compounds with low UV absorption, J. Chromatogr. A, 1216 (2009) 5774-5778.

[307] A. Le, A. Ng, T. Kwan, K. Cusmano-Ozog, T.M. Cowan, A rapid, sensitive method for quantitative analysis of underivatized amino acids by liquid chromatography-tandem mass spectrometry (LC-MS/MS), J. Chromatogr. B Anal. Technol. Biomed. Life Sci., 944 (2014) 166-174.

[308] N. Novatchev, U. Holzgrabe, Evaluation of amino sugar, low molecular peptide and amino acid impurities of biotechnologically produced amino acids by means of CE, J. Pharm. Biomed. Anal., 28 (2002) 475-86.

[309] N. Novatchev, U. Holzgrabe, Evaluation of the impurity profile of amino acids by means of CE, J. Pharm. Biomed. Anal., 26 (2001) 779-789.

[310] P. Chaimbault, K. Petritis, C. Elfakir, M. Dreux, Ion-pair chromatography on a porous graphitic carbon stationary phase for the analysis of twenty underivatized protein amino acids, J. Chromatogr. A, 870 (2000) 245-254.

[311] I.C. Casella, M. Gatta, T.R.I. Cataldi, Amperometric determination of underivatized amino acids at a nickel-modified gold electrode by anion-exchange chromatography, J. Chromatogr. A, 878 (2000) 57-67.

[312] V.P. Hanko, J.S. Rohrer, Determination of amino acids in cell culture and fermentation broth media using anion-exchange chromatography with integrated pulsed amperometric detection, Anal. Biochem., 324 (2004) 29-38.

[313] A. Zerzaňová, V. Žižkovský, R. Kučera, J. Klimeš, I. Jesenský, J. Dohnal, D. Barrón, Using of HPLC coupled with coulometric detector for the determination of biotin in pharmaceuticals, J. Pharm. Biomed. Anal., 45 (2007) 730-735.

[314] I. Márquez-Sillero, S. Cárdenas, M. Valcárcel, Determination of water-soluble vitamins in infant milk and dietary supplement using a liquid chromatography on-line coupled to a corona-charged aerosol detector, J. Chromatogr. A, 1313 (2013) 253-258.

[315] E. Livaniou, D. Costopoulou, I. Vassiliadou, L. Leondiadis, J.O. Nyalala, D.S. Ithakissios, G.P. Evangelatos, Analytical techniques for determining biotin, J. Chromatogr. A, 881 (2000) 331-343.

[316] T. Peréz-Ruiz, C. Martínez-Lozano, A. Sanz, E. Bravo, Electrophoretic behaviour of biotin and biocytin in capillary electrophoresis. Determination of biotin in pharmaceutical formulations, Chromatographia, 58 (2003) 757-762.

[317] D.C. da Silva, J.V. Visentainer, N.E. de Souza, C.C. Oliveira, Micellar electrokinetic chromatography method for determination of the ten water-soluble vitamins in food supplements, Food Anal. Method., 6 (2013) 1592-1606.

[318] K. Marakova, J. Piestansky, E. Havranek, P. Mikus, Simultaneous analysis of vitamins B in pharmaceuticals and dietary supplements by capillary electrophoresis hyphenated with triple quadrupole mass spectrometry, Pharmazie, 69 (2014) 663-668.

[319] P.F. Jin, L.F. Xia, Z. Li, N. Che, D. Zou, X. Hu, Rapid determination of thiamine, riboflavin, niacinamide, pantothenic acid, pyridoxine, folic acid and ascorbic acid in Vitamins with Minerals Tablets by high-performance liquid chromatography with diode array detector, J. Pharm. Biomed. Anal., 70 (2012) 151-157.

[320] C.J. Argoudelis, Simple high-performance liquid chromatographic method for the determination of all seven vitamin $B_6$-related compounds, J. Chromatogr. A, 790 (1997) 83-91.

# 5. Impurity profiling of *N,N'*-ethylenebis-L-cysteine diethyl ester (Bicisate)

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Unpublished manuscript

**ABSTRACT**

A HILIC HPLC-UV-CAD method for the impurity profiling of the $^{99m}$Tc chelating agent bicisate has been developed and evaluated. Bicisate and its impurities were separated by means of isocratic elution on a zwitterionic stationary phase using 7.5 mmol/L trifluoroacetic acid and acetonitrile (47.5:52.5 % V/V) as mobile phase. Five different batches of a manufacturer were tested using the method and LC-MS experiments were conducted in order to identify the impurities. The predominant impurities found were the oxidation product (disulfide), the monoester of ethylene dicysteine and an unknown compound with an m/z of 293 in ESI positive mode. A new degradation product of bicisate, bicisate lactam, was identified during sample solution stability assessment.

*Abbreviations:* ECD, *N,N'*-ethylenebis-L-cysteine diethyl ester; ENMC, *N*-methyl-cysteine ethyl ester; ESI, electro spray ionization; SPECT, single-photon emission computed tomography; EC, *N,N'*-ethylene-L-biscysteine; ECM, ethylene cysteine monoethyl ester; ETCA, L-thiazolidine-4-carboxylic acid ethyl ester; NMC, *N*-methyl-cysteine; TCA, L-thiazolidine-4-carboxylic acid;

## 1. Introduction

*N,N'*-Ethylenebis-L-cysteine diethyl ester (ECD) is a chelating ligand for radioactive $^{99m}$Tc applied in brain perfusion studies by single-photon emission computed tomography (SPECT) and for scintigraphy [1, 2]. The sulfhydryl groups are essential for the complexation of $^{99m}$Tc whereas the ester functions are essential for the distribution and the enrichment of the complex in the brain [3, 4]. ECD is distributed in so-called kits for the preparation of $^{99m}$Tc labeled ECD and there are only few methods to control the purity of the radio-labeled compound [5-7], but no method for the unlabeled precursor (ECD), one of the ingredients of those preparation kits is described in the literature.

The intermediate *N,N'*-ethylene-L-biscysteine (EC) is synthesized by reduction of L-thiazolidine-4-carboxylic acid (TCA) with sodium in liquid ammonia. Then the dicarboxylic acid (EC) is esterified in ethanol saturated with dry gaseous hydrochloric acid yielding ECD dihydrochloride [8-10] (see Fig. 1). The substance is sensitive to oxidation of the sulfhydryl groups and hydrolysis of the ester moieties. The oxidation product, the disulfide of ECD (ECDSS), is not able to form the $^{99m}$Tc complex whereas partial (ECM) and complete hydrolysis (EC) of the molecule lead to complexation agents with different biodistribution [3, 11, 12]. Hence, it was aimed to find a method to control the content of those impurities and impurities from the synthesis, like TCA, *N*-methyl-cysteine (NMC), TCA ethyl ester (ETCA) and NMC ethyl ester (ENMC).

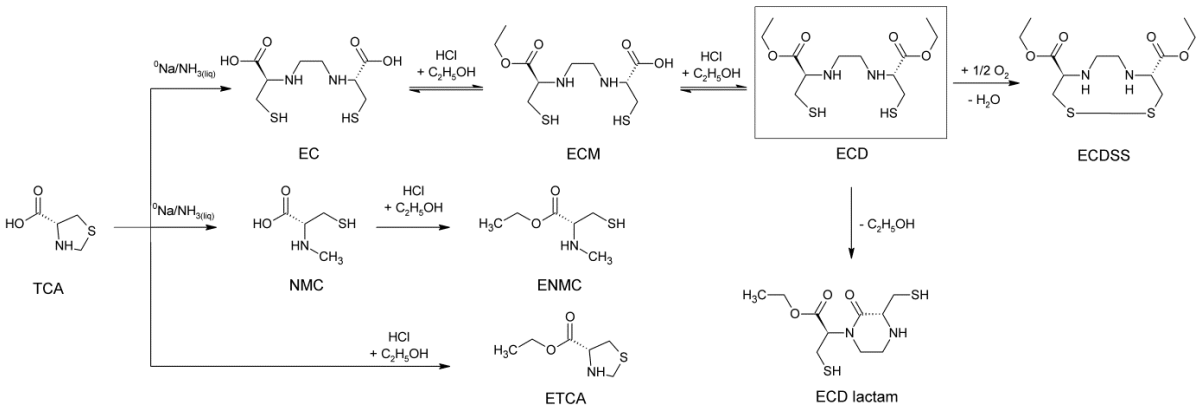


**Fig. 1.** Synthesis pathway and origin of impurities of ECD [8-10]

Since all impurities and degradation products lack a chromophore for UV detection, a particle based detector, the corona charged aerosol detector (CAD) seemed suitable. In order to discover volatile impurities that are not detected by the CAD, the method was designed as tandem UV-CAD method. A betaine HILIC column was used to separate the mostly polar

analytes with detection at 215 nm followed by CAD detection. Because the substance is administered only once, the thresholds according to ICH guideline Q3A(R2) for a substance with a daily dose lower or equal to 2 g per day were applied. Thus an identification threshold of 0.1 % and a reporting threshold of 0.05 % were applied.

## 2. Experimental

### *2.1. Chemicals and reagents*

Samples of ECD and the process related impurities were obtained from the group of Prof. Alfons Verbruggen (Laboratory for Radiopharmacy, K.U. Leuven, Belgium). HPLC gradient grade acetonitrile was purchased from VWR International S.A.S. (Fontenay-sous-Bois, France) and trifluoroacetic acid from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All chemicals used for quantification were of analytical grade or even better. Ultrapure water was produced by a water purification system from Merck Millipore (Schwalbach, Germany). All solutions were filtered through a 0.22 µm PVDF filter supplied by Machery-Nagel GmbH & Co. KG (Düren, Germany) prior to use.

### *2.2. Apparatus*

The HPLC-CAD experiments were performed on an Agilent 1100 modular chromatographic system consisting of online vacuum degasser, binary pump, auto sampler, thermostatted column compartment and a photodiode array detector (Agilent Technologies, Waldbronn, Germany). The Corona CAD detector (Thermo Fisher, Courtaboeuf, France) was linked to the HPLC system by a 0.25 mm internal diameter PEEK capillary and a 0.22 µm stainless steel inlet-frit. Highly pure nitrogen (99.9 %) for the detector was produced by an ESA Nitrogen Generator (Thermo Fisher, Courtaboeuf, France). The inlet pressure (nitrogen) was 35.0 psi. The peak areas were integrated automatically using the Agilent ChemStation® Rev B.03.02 software program.

LC-MS experiments were performed on a LC/MSD Trap G2445D ion trap (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 1100 modular chromatographic system consisting of online vacuum degasser, binary pump, thermostatted auto sampler, thermostatted column compartment and a photodiode array detector (Agilent Technologies, Waldbronn, Germany) via electro spray ionization (ESI) interface. Nebulizer pressure: 50 psi, dry gas flow: 12 L/min, dry temperature: 350 °C, capillary voltage 3500 V. Trap parameters: ion polarity: positive, source type: ESI, trap drive: 24.2, octopole RF amplitude: 156.6 Vpp, capillary exit: 115 V, skimmer: 40 V, oct 1 DC: 12 V, oct 2 DC 1.70 V, scan begin: 50 m/z,

scan end: 700 m/z, averages: 7 spectra, max accumulation time: 200 ms, ICC target: 30000, charge control: on.

*2.2. Chromatographic procedure*

A betaine HILIC column SIELC Obelisc® N (150 x 4.6 mm i.d., with a particle size of 5 µm and pore size of 100 Å, SIELC Technologies, Prospect Heights IL, USA) was used as stationary phase. The chromatographic system was operated isocratically at 25 °C. The mobile phase was composed of 7.5 mM trifluoroacetic acid-acetonitrile (47.5:52.5 % V/V) and the flow-rate was set to 1.0 mL/min. The column effluent was subjected to UV detection at 215 nm followed by CAD detection with the filter set to “none”. The injection volume was 10 µL.

The chromatographic conditions for LC/MS were the same as for HPLC-CAD. When running sample solutions the column effluent was subjected to the ESI-source from 0 to 15 and from 35 to 60 min only in order to protect the instrument from high amounts of ECD (eluting at about 15 min).

*2.2.1. Preparation of solutions*

The test solutions for HPLC-CAD and LC/MS were 5 mg/mL solutions of ECD dihydrochloride in water. 1 mg/mL solutions of the impurities TCA, ETCA, EC and NMC (calculated as free base or acid, respectively) in 0.1 mol/L hydrochloric acid were prepared for spiking the test solutions. For the test solutions for accuracy 5 mg ECD dihydrochloride were spiked with 2.5, 5.0 and 7.5 µL of each 1 mg/mL impurity stock solution and diluted to 1.0 mL. All solutions were stored at -20 °C.

**3. Results and discussion**

*3.1. Method development*

Since most of the compounds are extremely hydrophilic (e.g. TCA, EC and NMC) and not retained on conventional reversed phase columns, the separation mechanism of hydrophilic interaction liquid chromatography (HILIC) seemed suitable for an acceptable retention. Since the studied compounds contain basic, acidic or either moiety, the choice fell on a betaine HILIC stationary phase in order to use the strong electrostatic interactions between the charged analytes and the stationary phase.

The method was designed using isocratic elution in order to maintain the universal response provided by the CAD and to minimize baseline noise in UV-detection. Trifluoroacetic acid proved most suitable as volatile modifier because a pH of about 2 was required to attain acceptable peak shapes. Mobile phases containing different amounts of TFA and acetonitrile were examined. Interestingly ECD elutes earlier when the acetonitrile content was raised whereas other substances were retained longer as expected for a HILIC mechanism (see Fig. 2). The retention time of ETCA was not influenced by the acetonitrile concentration. This untypical behavior could be due to the absence of free carboxylic acids in ECD and ETCA. The optimized method used a mobile phase composed of an aqueous solution containing 7.5 mmol/L TFA and acetonitrile (47.5:52.5 % V/V) at a flow rate of 1.0 mL/min with UV (215 nm) followed by CAD detection. All impurities were separated from each other and from the ECD peak and could be detected in this way (see Fig. 3). ETCA was only detected by the UV detector because of its high vapor pressure. CAD detection was by far more sensitive for the other compounds.

*3.1.1. Sample preparation*

ECD dihydrochloride is very well soluble in water; thus, it was possible to prepare concentrated sample solutions (5 mg/mL) of the substance to achieve high sensitivity. Since the substance is considered to be unstable and especially sensitive to oxidation, the sample solutions were prepared in degassed ultrapure water purged with nitrogen and injected immediately after preparation.

*3.2. Method validation*

The method was validated for impurity profiling with regards to the following parameters: specificity, linearity, range, precision, accuracy, LOQ and robustness, following the International Conference on Harmonisation (ICH) guideline Q2(R1) [13]. *Specificity* of the method was proven by comparing spiked samples with a blank solution. Neither peaks from the blank solution nor peaks caused by degradation products of ECD did interfere with impurity peaks. The LC/MS results confirmed the identity and purity of the substance peaks. The *linearity* and *range* for all available impurities (TCA, NMC, EC and ETCA) were evaluated from 0.05 to 0.5 %. The coefficient of variation of the regression lines (method of least squares) was at least 0.9993. The *LOQ* for TCA, ETCA, NMC and EC was calculated from the slope of the regression lines and the standard error according to ICH guideline Q2(R1). The LOQ was at least 0.05 per cent compared to the main peak or even lower. *Accuracy* was assessed on spiked sample solutions 0.05, 0.10 and 0.15 % TCA, ETCA,

NMC and EC, respectively. Each level was reproduced in triplicate. The mean recovery rates ranged from 85 to 109 % (RSD between 0.0 and 4.7 %, n = 3). *Precision* was determined on a batch sample containing 0.12 % ECM spiked with 0.10 % TCA, ETCA, NMC and EC, respectively. The RSD were between 1.4 and 4.7 % (n = 6).

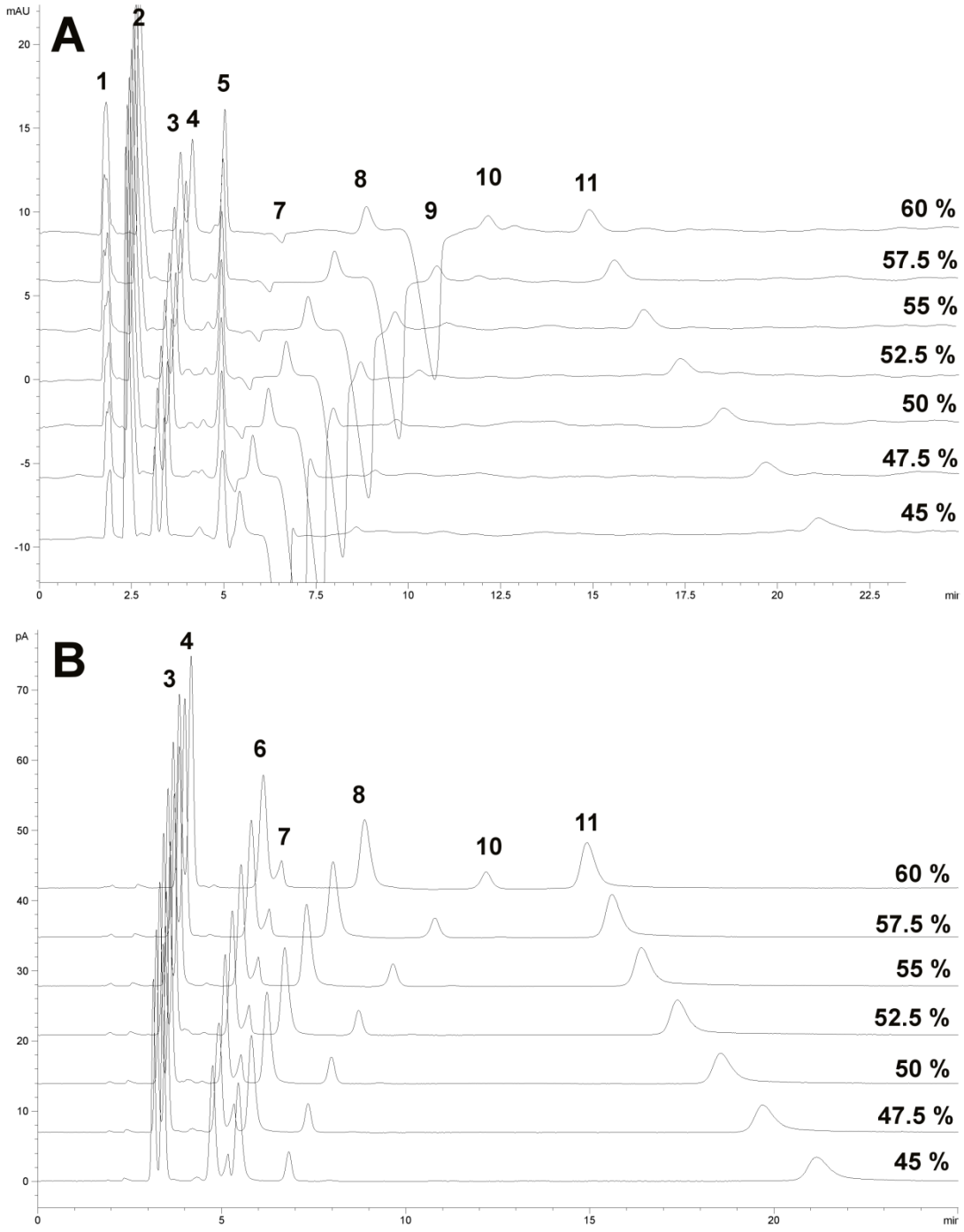


**Fig. 2.** Influence of the acetonitrile concentration on the separation of the impurities (each 0.05 mg/mL); A: UV 215 nm; B: CAD; mobile phase: 7.5 mmol/L TFA-MeCN(100-x:x % v/v); flow rate: 1.0 mL/min; 25 °C ;elution order: 1: injection peak, 2: system peak, 3: TCA, 4: NMC, 5: ETCA, 6: sodium, 7: ammonium, 8: EC, 9: chloride, 10: *N,N'*-dimethyl cystine (oxidation product of NMC), 11: ECD

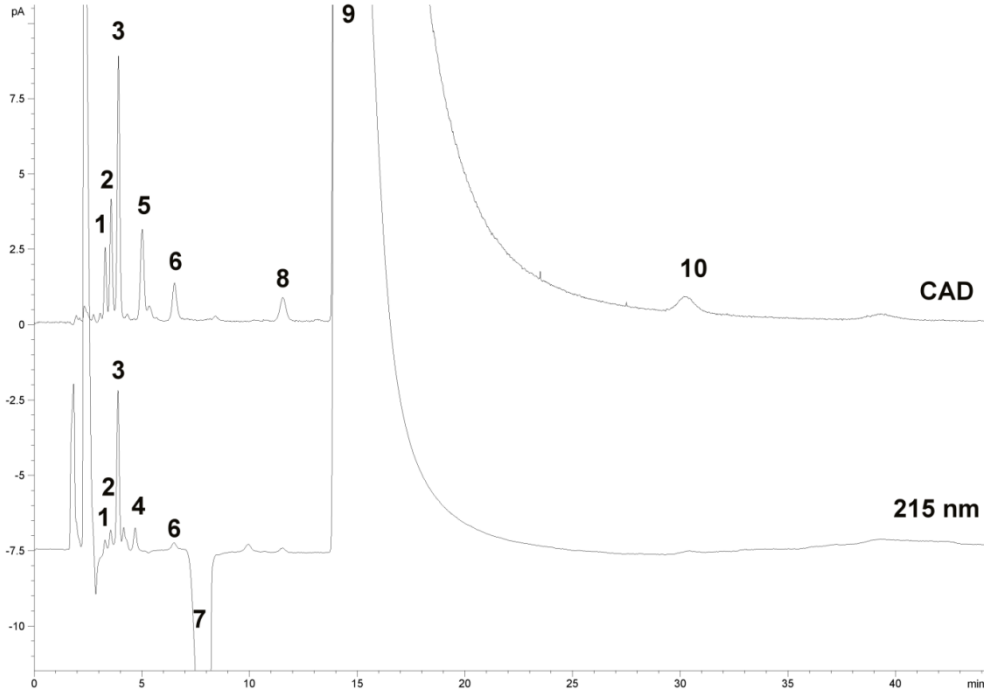


**Fig. 3.** Chromatogram obtained from a 5.0 mg/mL solution of ECD dihydrochloride in water spiked with 0.1 % TCA, ETCA, NMC and EC; elution order: injection peak, system peak, 1: TCA, 2: NMC, 3: ECDSS, 4: ETCA, 5: sodium/ammonium, 6: EC; 7: chloride; 8: ECM, 9: ECD, 10: unknown compound with m/z ($ESI^+$) 293; chromatographic conditions: see section 2.3.

The sample solutions were not stable. A drastic increase in ECDSS was observed after 1 hour at room temperature. Other degradation products like ECM and ECD lactam were formed successively (see Fig. 4). The formation of ECDSS seemed to slow down after 1 hour, maybe due to oxygen depletion of the sample solution. Only nitrogen purged and degassed water should be used to prepare the samples in order to decelerate the oxidation. The test solution had to be subjected to analysis immediately after preparation.

For checking the *robustness,* the operation parameters were varied in the following ranges: Flow rate 1.0 ± 0.1 mL/min, TFA concentration 7.5 ± 0.5 mmol/L, MeCN concentration 52.5 ± 1.0 % and temperature 25 ± 5 °C. None of the variations did impair the separation of the impurities.

*3.3. Batch results*

Five batches of one manufacturer were studied using this method. None of the available impurities TCA, ETCA, NMC nor EC were detected in any of the samples. However traces of ENMC were found by the very sensitive LC-MS method. Unavailable and unidentified impurities could be found in the CAD chromatogram and the content was

calculated using the calibration curve of EC because of the uniform response provided by this detection principle (see Table 1 and Fig. 5).

**Table 1.** HPLC-UV-CAD results of the available batches

| retention time [min] | impurity | Batch | | | | |
|---|---|---|---|---|---|---|
| | | A | B | C | D | E |
| 3.5 | TCA | n.d. | n.d. | n.d. | n.d. | n.d. |
| 3.7 | NMC | n.d. | n.d. | n.d. | n.d. | n.d. |
| 4.1 | ECDSS | 0.06 % | < 0.05 % | < 0.05 % | 0.10 % | 0.05 % |
| 4.3 | ECD lactam | n.d. | n.d. | n.d. | n.d. | < 0.05 % |
| 4.9 | ETCA | n.d. | n.d. | n.d. | n.d. | n.d. |
| 6.7 | EC | n.d. | n.d. | n.d. | n.d. | n.d. |
| 12.1 | ECM | 0.07 % | 0.11 % | 0.12 % | < 0.05 % | 0.06 % |
| 32.0 | unknown a (m/z 293 in $ESI^+$) | 0.45 % | 0.39 % | 0.12 % | 0.18 % | 0.11 % |
| 42.6 | unknown b (no signal in $ESI^+$) | n.d. | n.d. | n.d. | 0.14 % | n.d. |
| sum of impurities | | 0.58 % | 0.50 % | 0.24 % | 0.42 % | 0.22 % |

Two degradation products (ECD lactam and ECM) were identified using LC-MS. The lactam is formed by an intramolecular nucleophilic substitution of an ethyl group by one of the amino moieties whereas ECM either originates from partial hydrolysis or is present due to incomplete esterification during the synthesis. The found m/z ratios $[M+H]^+$ of 279 (ECD lactam) and 297 (ECM) and the fragmentation patterns were in concordance with the proposed structures. The late eluting unknown impurities could not be identified.

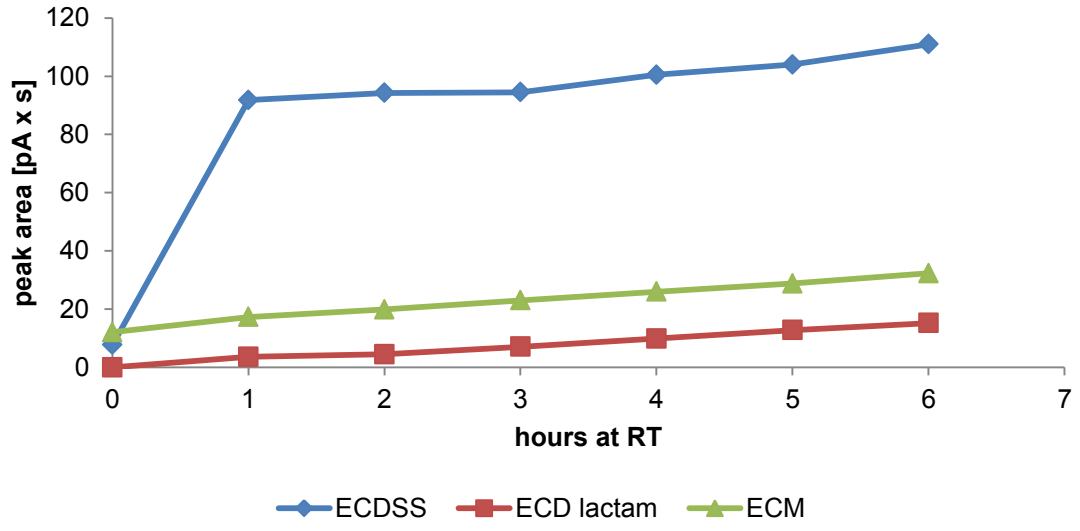


**Fig. 4.** Formation of degradation products in a 5.0 mg/mL solution of ECD dihydrochloride vs. time

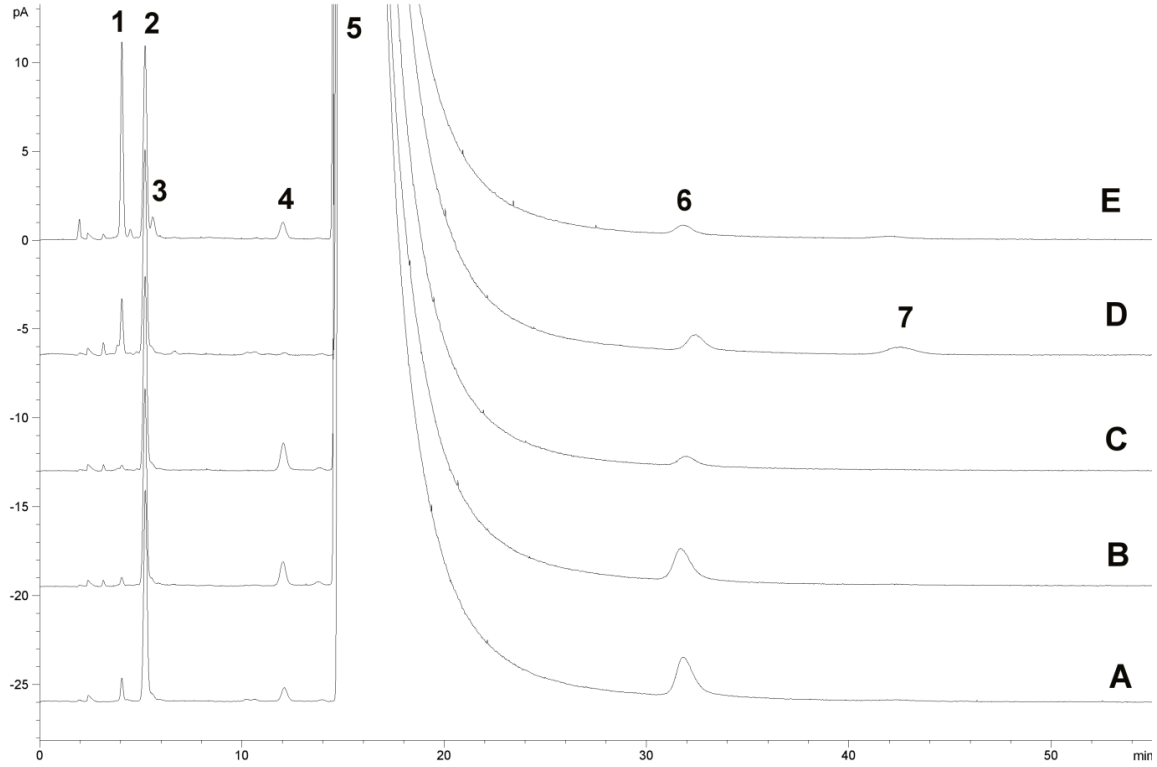


**Fig. 5.** Chromatogram obtained from 5.0 mg/mL solution of ECD dihydrochloride batches A to E; CAD detection; elution order: 1: ECDSS, 2: sodium, 3: ammonium, 4: ECM, 5: ECD, 6: unknown compound with m/z ($ESI^+$) 293; 7: unknown compound, no peak in LC-MS ($ESI^+$); chromatographic conditions: see section 2.3.

## 4. Conclusion

A robust and versatile method for the impurity control of ethylene dicysteine diethyl ester dihydrochloride was developed and evaluated. The stability of the substance in aqueous solution was investigated and one new degradation product (ECD lactam) was observed. The method is suitable to control synthesis related impurities and degradation products. A major unknown impurity was detected over all batches using CAD and needs to be identified.

## Conflict of interest statement

None of the authors of this work has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper

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## References

[1] J. Leveille, G. Demonceau, M. Deroo, P. Rigo, R. Taillefer, R.A. Morgan, D. Kupranick, R.C. Walovitch, Characterization of Technetium-99m L,L-Ecd for Brain Perfusion Imaging .2. Biodistribution and Brain Imaging in Humans, J. Nucl. Med., 30 (1989) 1902-1910.

[2] R.C. Walovitch, T.C. Hill, S.T. Garrity, E.H. Cheesman, B.A. Burgess, D.H. Oleary, A.D. Watson, M.V. Ganey, R.A. Morgan, S.J. Williams, Characterization of Technetium-99m L,L-Ecd for Brain Perfusion Imaging .1. Pharmacology of Technetium-99m Ecd in Nonhuman-Primates, J. Nucl. Med., 30 (1989) 1892-1901.

[3] H.P. Vanbilloen, B.J. Cleynhens, A.M. Verbruggen, Importance of the two ester functions for the brain retention of Tc-99m-labelled ethylene dicysteine diethyl ester (Tc-99m-ECD), Nucl. Med. Biol., 25 (1998) 569-575.

[4] R.C. Walovitch, E.H. Cheesman, L.J. Maheu, K.M. Hall, Studies of the Retention Mechanism of the Brain Perfusion Imaging Agent Tc-99m-Bicisate (Tc-99m-Ecd), J. Cerebr. Blood. F. Met., 14 (1994) S4-S11.

[5] T. Verduyckt, D. Kieffer, D. Huyghe, B. Cleynhens, K. Verbeke, A. Verbruggen, G. Bormans, Identity confirmation of 99mTc-MAG3, 99mTc-sestamibi and 99mTc-ECD using radio-LC-MS, J. Pharm. Biomed. Anal., 32 (2003) 669-78.

[6] K.C. Amin, G.B. Saha, R.T. Go, A rapid chromatographic method for quality control of technetium-99m-bicisate, J. Nucl. Med. Tech., 25 (1997) 49-51.

[7] C. Tsopelas, D. Smyth, Characterization and quality control analysis of 99mTc-bicisate, J. Nucl. Med. Tech., 33 (2005) 89-93.

[8] T. Assaad, Synthesis and characterization of novel benzovesamicol analogs, Turk. J. Chem., 35 (2011) 189-200.

[9] M. Yanagi, T. Uehara, Y. Uchida, S. Kiyota, M. Kinoshita, Y. Higaki, H. Akizawa, H. Hanaoka, Y. Arano, Chemical Design of Tc-99m-Labeled Probes for Targeting Osteogenic Bone Region, Bioconjug. Chem., 24 (2013) 1248-1255.

[10] P. Blondeau, C. Berse, D. Gravel, Dimerization of an Intermediate during Sodium in Liquid Ammonia Reduction of L-Thiazolidine-4-Carboxylic Acid, Can. J. Chem., 45 (1967) 49-52.

[11] E. Durand, P. Chaumet-Riffaud, N. Grenier, Functional Renal Imaging: New Trends in Radiology and Nuclear Medicine, Semin. Nucl. Med., 41 (2011) 61-72.

[12] L. Kabasakal, Technetium-99m ethylene dicysteine: a new renal tubular function agent, Eur. J. Nucl. Med., 27 (2000) 351-357.

[13] ICH, Validation of Analytical Procedures: Text and Methodology, ICH, Q2(R1). http://www.ich.org, 1996 (accessed November 2014)

# D. Final discussion

The aim of the studies performed here was to find appropriate means of analysis to determine related substances of challenging analytes. These challenges in particular comprise the lack of a chromophore in the analyte molecule and physical-chemical properties such as high hydrophilicity or amphoterism impeding both detection and separation by HPLC. The benefit of mixed-mode chromatography, a promising approach to address the separation and the potential of the corona charged aerosol detector for pharmacopoeial purposes were evaluated.

## 1. The CAD in impurity profiling

The CAD turned out to be a valuable alternative to commonly applied detection principles. The greater part of related substances could be detected with sufficient sensitivity and linearity. However, the impurity profiling of Carbocisteine illustrates one of the major drawbacks of CAD detection: volatile and semi-volatile analytes cannot be detected. In this special case the most toxic impurity, chloroacetic acid, was not detectable using this technique. The combination of UV and CAD applied for Bicisate was suitable to overcome this problem for a semi-volatile ester. However, for chloroacetic acid UV detection was also not suitable due to the low response of the substance. The content of chloroacetic acid was determined by qNMR to get an idea about how much of this compound could be expected in the API. Because qNMR, especially for a single impurity, is not a desirable method for the Ph. Eur., additional methods were evaluated for the assessment of chloroacetic acid. A GC method with derivatization (ethyl ester) proposed by a manufacturer of carbocisteine and a new ion-chromatography method with conductivity detection were tested. All three methods yielded similar results (data not included in this work).

Overall, the CAD was a useful detector for all non-volatile compounds and enabled the detection of several unexpected und unknown impurities. The direct transfer of a CAD method to LC-MS proved very helpful in the identification of those unknown impurities in Ibandronate and Bicisate. It is a valuable additional technique and should be considered more frequently in the impurity profiling to find unexpected substances. The combination of UV and CAD is easily feasible and covers a higher number of detectable analytes when mobile phases with a low UV-cut off are used. However, the only available volatile modifiers with low UV cut-off are TFA, analogue fluorinated acids and TEA.

## 2. Enantiomeric purity of magnesium aspartate

The direct separation of underivatized non-aromatic amino acids is rarely described in the literature. However, special stationary phases are available for the separation of D- and L-amino acids. These columns are rather expensive and are dedicated to a single separation problem. It could be demonstrated that the indirect separation on a conventional PFP reversed phase and the direct separation of CBQCA derivatives by CZE yield sufficiently precise results with excellent sensitivity.

It was shown that aspartic acids racemizes rather quickly compared to other amino acids in moderately acidic solution. Optimal parameters for the synthesis process could be deducted from these findings. Furthermore, it was outlined, that the test for optical rotation in the Ph. Eur. is unable to detect D-aspartic acid below 1 % and additional tests are necessary to ensure the optical purity of the salt.

## 3. Mixed-mode chromatography in impurity profiling

Three different kinds of mixed-mode columns were used during this work: A RP-18 with embedded strong cation exchanger (Carbocisteine), a RP-18 with embedded strong anion exchanger (Ibandronate) and a betaine HILIC (Bicisate), which includes HILIC and ion-exchange mechanisms in this case.

The principle of mixed-mode chromatography was clearly outlined during the development of the method for Carbocisteine and Bicisate. The retention of neutral impurities or compounds where one mechanism of retention dominates (e.g. RP for Tyrosine) is mainly influenced by the amount of organic modifier, whereas ionic species are mostly affected by ionic strength of the mobile phases (e.g. TFA concentration). This characteristic behavior allows for the fine tuning of a separation in a different way compared to IPC where the increase of organic modifier usually leads to a decreased retention of all analytes.

An important objection to the use of mixed-mode chromatography for pharmacopoeial purposes is the limitation to only a few manufacturers and the comparatively short lifetime of the columns. On the other hand, they are useful in preliminary screening experiments in order to discover potential and new impurities. “Conventional” more generic methods, e.g. using IPC could be developed based on these findings.

## 4. Other applications of the CAD

The CAD has not only proven useful in impurity profiling, but is also becoming more and more popular for other very interesting potential applications. Comparatively new is the application in blood sample analysis and metabolomics. The detector is here not used for the quantification of the compounds of interest but as a screening tool to determine the sample clean up efficiency. Many matrix compounds like amino acids, derivatives and physiological metabolites which could interfere with the actual analysis (suppression of MS-signal) are well detectable by CAD [1]. In another study the ratio of DAD to CAD response as a compound specific property is used for peak identification in the blood sample [2].

Another field of application where the detector has been used before but still holds interesting possibilities is the analysis of excipients. Excipients are often very similar to what is herein referred to as a challenging analyte. Inorganic salts, polymers for gel formation, triglycerides, surfactants, starch, sugars, and sugar alcohols belong to the most frequently applied excipients in pharmaceutical technology. Many of these substances e.g. polysorbates are mixtures of many similar compounds and the quality is usually verified by a variety of inconvenient methods such as saponification value, acid and peroxide value. Using HPLC-CAD it was possible to characterize these surfactants and a relationship between the composition and their functionality related characteristics like critical micelle concentration, cloud point and hydrophilic-lipophilic balance was discovered [3]. The analysis of polidocanol [4], polyethylene glycol [5] and lipids [6] is also described in the literature.

The ability to detect excipients could be used for a future application of the CAD: fingerprinting of whole formulations (after suitable sample preparation) in order to assign an unknown sample to a manufacturer or to discover counterfeit drug products in a similar way like the detector has been used before to verify the authenticity of food products (e.g. olive oil) [7]. Additional information like MS, UV or DAD chromatograms could be combined with the CAD chromatogram to retrieve more information from the sample if necessary.

## 5. Conclusion

In all cases were the CAD could be used for impurity profiling the calibration curves were sufficiently linear for single point calibration over a small range and quantification limits were always acceptable for impurity analysis (see Table 1). It could be demonstrated, that the CAD was capable of detection of difficult analytes and the possibilities of mixed-mode chromatography in the separation of hydrophilic compounds were outlined. The CAD is a useful additional detector in impurity profiling increasing the amount of detectable substances. In cases were the expected impurities are not known it is of particular interest because the response is for a great part independent of the structure, as long as the analyte

is not volatile or semi-volatile. Due to the limitation to non-volatile impurities the CAD can never completely replace other detection techniques but it is a valuable tool for screening purposes in order to find new impurities. The easy transfer to LC-MS in order to identify unknown structures is one of the main advantages of CAD detection. A major disadvantage is the limitation to volatile mobile phase additives leaving only little scope for optimization (e.g. even the combination of TFA and $NH_3$ leads to excessive baseline noise).

In conclusion no detection technique can guarantee a 100 % certainty about the purity of an analyte, but the chance to get more information about the composition of a sample has to be used. Mixed-mode chromatography is an interesting approach to solve difficult separations, especially in combination with “universal” detectors like the CAD because the assessment of inorganic impurities is possible at the same time. Furthermore CAD detection has the potential to reduce the error made when quantifying unknown impurities because of the universal response.

**Table 1**
Overview of impurities determined by HPLC-CAD, the corresponding LOQ and linearity

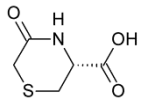
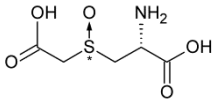
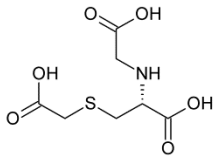
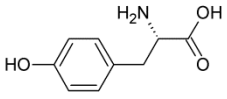
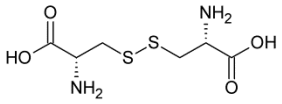
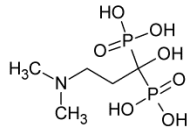
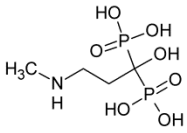
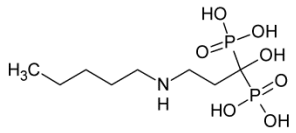
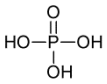
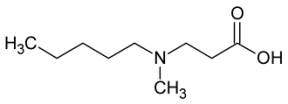
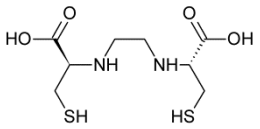
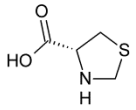
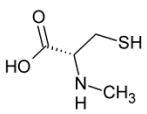
| Analyte | LOQ[a] | $R^2$ | Amount on column |
|---|---|---|---|
|  Carbocisteinelactam | 0.02 % | 0.9994 | 50-250 ng |
|  Carbocisteinesulfoxid | 0.02 % | 0.9996 | 50 -250 ng |
|  *N,S*-dicarboxymethylcysteine | 0.04 % | 0.9995 | 100 – 600 ng |
|  Tyrosine | 0.03 % | 0.9983 | 50 – 250 ng |
|  Cystine | 0.10 % | 0.9984 | 100 – 600 ng |

**Table 1 (continued)**

| Analyte | LOQ[a] | $R^2$ | Amount on column |
|---|---|---|---|
|  Dimethylpamidronate | 0.03 % | 0.9972 | 80 – 400 ng |
|  Despentylibandronate | 0.02 % | 0.9990 | 80 – 400 ng |
|  Desmethylibandronate | 0.02 % | 0.9990 | 80 – 400 ng |
|  Phosphorous acid | 0.02 % | 0.9989 | 80 – 400 ng |
|  Phosphoric acid | 0.02 % | 0.9994 | 80 – 400 ng |
|  *N*-methyl-*N*-pentyl-β-alanine | 0.03 % | 0.9973 | 80 – 400 ng |
|  *N,N'*-ethylene-biscysteine | 0.03 % | 0.9998 | 25 – 250 ng |
|  Thiazolidine-4-carboxylic acid | 0.02 % | 0.9999 | 25 – 250 ng |
|  *N*-methyl-cysteine | 0.05 % | 0.9993 | 25 – 250 ng |

[a] calculated from the linearity plots according to ICH guideline Q2(R1) with regards to the test solution concentration

## References

[1] D. Schimek, K.A. Francesconi, A. Mautner, G. Libiseller, R. Raml, C. Magnes, Matrix removal in state of the art sample preparation methods for serum by charged aerosol detection and metabolomics-based LC-MS, Anal. Chim. Acta, 915 (2016) 56-63.

[2] J. Viinamäki, I. Ojanperä, Photodiode array to charged aerosol detector response ratio enables comprehensive quantitative monitoring of basic drugs in blood by ultra-high performance liquid chromatography, Anal. Chim. Acta, 865 (2015) 1-7.

[3] A.C. Braun, D. Ilko, B. Merget, H. Gieseler, O. Germershaus, U. Holzgrabe, L. Meinel, Predicting critical micelle concentration and micelle molecular weight of polysorbate 80 using compendial methods, Eur. J. Pharm. Biopharm., 94 (2015) 559-568.

[4] D. Ilko, S. Puhl, L. Meinel, O. Germershaus, U. Holzgrabe, Simple and rapid high performance liquid chromatography method for the determination of polidocanol as bulk product and in pharmaceutical polymer matrices using charged aerosol detection, J. Pharm. Biomed. Anal., 104 (2015) 17-20.

[5] D.W. Kou, G. Manius, S.D. Zhan, H.P. Chokshi, Size exclusion chromatography with Corona charged aerosol detector for the analysis of polyethylene glycol polymer, J. Chromatogr. A, 1216 (2009) 5424-5428.

[6] C. Schönherr, S. Touchene, G. Wilser, R. Peschka-Süss, G. Francese, Simple and precise detection of lipid compounds present within liposomal formulations using a charged aerosol detector, J. Chromatogr. A, 1216 (2009) 781-786.

[7] J.M. Bosque-Sendra, L. Cuadros-Rodríguez, C. Ruiz-Samblás, A.P. de la Mata, Combining chromatography and chemometrics for the characterization and authentication of fats and oils from triacylglycerol compositional data, Anal. Chim. Acta, 724 (2012) 1-11.

# E. Summary

The impurity profiling of pharmaceutical ingredients can oppose many challenges. The best part of active pharmaceutical ingredients (APIs) and the related substances are detectable by UV detection, a very common detection principle. However, if an API lacks a suitable chromophore other means of detection are necessary. The corona charged aerosol detector (CAD) is a detector capable of detecting substances independent of their chemical structure. This “universal” detector has only one limitation: The analyte has to have a sufficiently low vapor pressure. Another important challenge that comes often together with the lack of a chromophore concerns the separation. These substances (e.g. most amino acids and derivatives) often contain structures that make them difficult to retain on conventional reversed phase columns.

Possible solutions to overcome these challenges, like the application of the CAD and the benefit of so-called mixed-mode stationary phases in impurity profiling for pharmacopoeial purposes were explored in this work. The related substances analyzed in this thesis comprise amino acids, inorganic ions, bisphosphonic acids, basic and acidic derivatives of amino acids (esters and amides).

The successful development and validation of mixed-mode liquid chromatography methods with CAD detection for carbocisteine and ibandronate sodium might help to increase the acceptance of this versatile detector in the pharmaceutical industry and in official authorities dealing with the determination of related substances.

The combination of UV and CAD detection proved very useful during the analysis of Bicisate. Most of the related substances and some unidentified impurities were detectable by CAD whereas a synthesis by-product, a semi-volatile ester, was only detectable in the UV trace. The simple combination covers all relevant impurities in a single analysis.

Two truly orthogonal methods regarding separation and detection for the enantiomeric purity of magnesium-L-aspartate helped to find the reason for elevated D-aspartic acid content in the drug substance. A very quick and sensitive indirect separation using the OPA derivatization with NAC was developed as a powerful screening tool, whereas the direct separation of D- and L-CBQCA-Asp derivatives confirmed the results. Both methods were optimized in order to do without substances mentioned on the REACH list, like sodium tetraborate which is very frequently applied in standard derivatization protocols and CE separations.

The importance of orthogonal detection principles in the determination of related substances of amino acids was discussed in a review article dealing with the revision of amino acid monographs in the Ph. Eur..

# F. Zusammenfassung

Die Reinheitsprüfung pharmazeutischer Wirkstoffe kann den Analytiker vor verschiedene Hürden stellen. So gilt für den größten Teil pharmazeutischer Wirkstoffe und deren verwandte Substanzen, dass sie mit Hilfe des weit verbreiteten UV-Detektors nachweisbar sind. Verfügt ein Wirkstoff hingegen nicht über ein geeignetes Chromophor, so benötigt man andere Möglichkeiten der Detektion. Der corona charged aerosol detector (CAD) ist in der Lage Substanzen unabhängig von ihrer chemischen Struktur zu detektieren, vorausgesetzt, sie sind schwerflüchtig. Eine weitere Herausforderung, die häufig mit dem Fehlen eines Chromophors einhergeht betrifft die Trennung. Verbindungen dieser Art (z.B. die meisten Aminosäuren und deren Derivate) enthalten häufig Strukturen, die eine Trennung auf konventionellen Umkehrphasen erschweren.

Mögliche Ansätze um die genannten Herausforderungen zu meistern, wie zum Beispiel die Verwendung des CAD und sogenannter mixed-mode Phasen in der pharmazeutischen Reinheitsanalytik wurden erarbeitet und an konkreten Anwendungen erprobt. Die in dieser Arbeit bestimmten verwandten Substanzen sind vor allem Aminosäuren, anorganische Ionen, Bisphosphonate sowie basische und saure Derivate von Aminosäuren (Ester und Amide).

Die erfolgreiche Entwicklung und Validierung von mixed-mode flüssig-chromatographischer Methoden kombiniert mit CAD für Carbocistein und Ibandronat Natrium könnte dabei helfen die Akzeptanz in der Pharmazeutischen Industrie und bei den für Reinheitsprüfungen zuständigen Behörden für diesen vielseitigen Detektor zu verbessern.

Die Kombination von UV-Detektion und CAD erwies sich bei der Analyse von Bicisate als sehr nützlich. Die meisten verwandten Substanzen und einige unbekannte Verunreinigungen konnten mittels CAD detektiert werden, während ein Nebenprodukt der Synthese, ein halb-flüchtiger Ester, nur mit Hilfe des UV Detektors sichtbar war. Die Kombination zweier Detektionstechniken ermöglichte die Bestimmung aller relevanten Verunreinigungen in einer einzigen Analyse.

Die Bestimmung der optischen Reinheit von Magnesium-L-Aspartat gelang mittels zweier orthogonaler Methoden und der Grund für das Auftreten von erhöhten Konzentrationen an D-Aspartat wurde gefunden. Eine schnelle indirekte Bestimmung der OPA/NAC-Derivate eignete sich als Screening-tool, während die direkte Trennung der enantiomeren CBQCA-Derivate die Ergebnisse bestätigte. Beide Methoden wurden im Hinblick darauf optimiert, dass sie ohne Substanzen wie Natriumtetraborat, eine Substanz auf der REACH Liste für besonders besorgniserregende Substanzen, sowie gebräuchlicher Puffer bei Derivatisierungsreaktionen und CZE Trennungen, auskamen.

Die Bedeutung von orthogonalen Detektionstechniken bei der Bestimmung der verwandten Substanzen von Aminosäuren wurde in einem Übersichtsartikel, der in Zusammenhang mit der Revision von Aminosäuren Monographien des Europäischen Arzneibuches steht, diskutiert.

# G. Appendix

# 1. List of Publications and Documentation of Authorship

***1*** ***Impurity profiling of carbocisteine by HPLC-CAD, qNMR and UV/vis spectroscopy***

*Wahl, O., Holzgrabe, U.*

J Pharm Biomed Anal, Volume 95 (2014), Pages 1 – 10

doi:10.1016/j.jpba.2014.02.012

***2*** ***Evaluation of enantiomeric purity of magnesium-L-aspartate dihydrate***

*Wahl, O., Holzgrabe, U.*

J Pharm Biomed Anal, Volume 102 (2015), Pages 100 – 109

doi:10.1016/j.jpba.2014.08.013

***3*** ***Impurity profiling of ibandronate sodium by HPLC-CAD***

*Wahl, O., Holzgrabe, U.*

J Pharm Biomed Anal, Volume 114 (2015), Pages 254 – 264

doi:10.1016/j.jpba.2015.06.002

***4*** ***Amino acid analysis for pharmacopoeial purposes***

*Wahl, O., Holzgrabe, U.*

Talanta, Volume 154 (2016), Pages 150 – 163

doi:10.1016/j.talanta.2016.03.071

***5*** ***Impurity profiling of N,N'-ethylenebis-L-cysteine diethyl ester (Bicisate)***

*Wahl, O., Holzgrabe, U.*

unpublished manuscript

This section contains a list of individual contribution for each author to the publications reprinted in this thesis.

**P1** **Wahl O, Holzgrabe U,** Impurity profiling of carbocisteine by HPLC-CAD, qNMR and UV/vis spectroscopy. J Pharm Biomed Anal 2014, 95, 1-10.

| Author | 1 | 2 |
|---|---|---|
| Study design | x | x |
| Experimental work | x | |
| Data analysis and interpretation | x | x |
| Manuscript planning | x | x |
| Manuscript writing | x | |
| Correction of manuscript | x | x |
| Supervision of Oliver Wahl | | x |

**P2** **Wahl O, Holzgrabe U,** Evaluation of enantiomeric purity of magnesium-L-aspartate dihydrate. J Pharm Biomed Anal 2015, 102, 100-109.

| Author | 1 | 2 |
|---|---|---|
| Study design | x | x |
| Experimental work | x | |
| Data analysis and interpretation | x | x |
| Manuscript planning | x | x |
| Manuscript writing | x | |
| Correction of manuscript | x | x |
| Supervision of Oliver Wahl | | x |

**P3** **Wahl O, Holzgrabe U,** Impurity profiling of ibandronate sodium by HPLC-CAD. J Pharm Biomed Anal 2015, 114, 254-264.

| Author | 1 | 2 |
|---|---|---|
| Study design | x | x |
| Experimental work | x | |
| Data analysis and interpretation | x | x |
| Manuscript planning | x | x |
| Manuscript writing | x | |
| Correction of manuscript | x | x |
| Supervision of Oliver Wahl | | x |

**P4** **Wahl O, Holzgrabe U,** Amino acid analysis for pharmacopoeial purposes. Talanta 2016, 154, 150-163.

| Author | 1 | 2 |
|---|---|---|
| Manuscript planning | x | x |
| Manuscript writing | x | |
| Correction of manuscript | x | x |
| Supervision of Oliver Wahl | | x |

**P5** **Wahl O, Holzgrabe U,** Impurity profiling of N,N'-ethylenebis-L-cysteine diethyl ester (Bicisate), unpublished manuscript

| Author | 1 | 2 |
|---|---|---|
| Study design | x | x |
| Experimental work | x | |
| Data analysis and interpretation | x | x |
| Manuscript planning | x | x |
| Manuscript writing | x | |
| Correction of manuscript | x | x |
| Supervision of Oliver Wahl | | x |

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Die Beiträge der Mitautorin an den Publikationen sind in den vorausgehenden Tabellen aufgeführt.

| | | |
|---|---|---|
| Prof. Dr. Ulrike Holzgrabe | _____ | _____ |
| | Ort, Datum | Unterschrift |
| Oliver Wahl | _____ | _____ |
| | Ort, Datum | Unterschrift |

## 2. Conference contributions

Wahl, O., Holzgrabe, U.
*Tests for related substances of amino acids by HPLC-CAD*
Pharmaceutical and Biomedical Analysis 2013, Bologna

Wahl, O., Holzgrabe, U.
*Enantiomeric purity of Magnesium bis(L-hydrogenaspartate) dihydrate*
DPhG Jahrestagung 2014, Frankfurt a. M.

# 3. Abbreviations

| | |
|---|---|
| AAA | amino acid analysis |
| APCI | atmospheric-pressure chemical ionization |
| APPI | atmospheric-pressure photoionization |
| AU | absorbance unit |
| BGE | background electrolyte |
| BHT | butylated hydroxytoluene |
| $C^4D$ | capacitively coupled contactless conductivity detector |
| CAD | corona charged aerosol detector |
| CBQCA | 3-(4-carboxybenzoyl)quinolone-2-carboxaldehyde |
| CE | capillary electrophoresis |
| CD | cyclodextrin |
| CLEC | chiral ligand-exchange |
| CNLD | chemiluminescent nitrogen detector |
| CNLSD | condensation nucleation light scattering detector |
| CS | chiral selector |
| CZE | capillary zone electrophoresis |
| DBTAAN | (+)-dibenzoyl-L-tartaric anhydride |
| ECD | electrochemical detector |
| EI | electron impact ionization |
| ELSD | evaporative light scattering detector |
| EOF | electroosmotic flow |
| ESI | electrospray ionization |
| FDAA | $N_\alpha$-(2,4-Dinitro-5-fluorophenyl)-L-alaninamide |
| FIA | flow-injection analysis |
| FITC | fluoresceine isothiocyanate |
| FLD | fluorescence detector |
| FLEC | (-)-1-(9-Fluorenyl)ethyl chloroformate |
| FMOC-Cl | fluorenylmethoxycarbonyl chloride |
| FQ | 3-(2-furoyl) quinoline-2-carboxaldehyde |
| GITC | 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate |
| HILIC | hydrophilic interaction liquid chromatography |
| IEX | ion exchange |
| IPC | ion-pair chromatography |
| LED | light emitting diode |
| LOD | limit of detection |
| LOQ | limit of quantification |
| MEEKC | micro emulsion electrokinetik chromatography |
| MEKC | micellar electrokinetik chromatography |
| MMC | mixed mode chromatography |
| MRM | multiple reaction monitoring |

| | |
|---|---|
| MTPA-Cl | (*S*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride |
| NAC | *N*-acetyl-L-cysteine |
| NBC | *N*-n-butyryl-L-cysteine |
| NDA | naphthalene-2,3-dicarboxaldehyde |
| NiBC | *N*-isobutyryl-L-cysteine |
| NQAD | nano-quantity analyte detector |
| OPA | o-phthaldialehyde |
| Ph. Eur. | European pharmacopoeia |
| PITC | phenylisothiocyanate |
| QqQ | triple quadrupole mass spectrometer |
| qTOF | quadrupole time-of-flight mass spectrometer |
| RID | refractive index detector |
| RP | reversed phase |
| SAX | strong anion exchange |
| SCX | strong cation exchange |
| TATG | 2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranoside |
| TFA | trifluoroacetic acid |
| TG | 1-thio-β-D-glucopyranose |
| TOF | time-of-flight mass spectrometer |
| USP | United States pharmacopoeia |
| WAX | weak anion exchange |
| WCX | weak cation exchange |