THE USE OF AEROSOL-BASED DETECTION SYSTEMS
IN THE
QUALITY CONTROL OF DRUG SUBSTANCES

Dissertation zur Erlangung des naturwissenschaftlichen Doktorgrades
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Gelingt es dir nicht

in die Quelle der Dinge einzudringen

wird dein Geist

vergeblich nach Ruhe suchen.

Kanchi Sōsan (? – 606 B.C.)

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Use of evaporative light scattering detection (ELSD) for the quality control of drug substances in: T.J. Quintin (Hrsg.), Chromatography: Types, Techniques and Methods, Nova Publishers, Inc. New York, 2010, 483-496.

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LIST OF ABBREVIATIONS

AAA	Amino Acid Analyzer
ACN	Acetonitrile
Ala	Alanine
API	Active Pharmaceutical Ingredient
APCI	Atmospheric Pressure Chemical Ionization
APPI	Atmospheric Pressure Photo Ionization
Asn	Asparagine
Asp	Aspartic Acid
BRP	Biological Reference Preparation
CAD	Corona Charged Aerosol Detection
CAS	Chemical Abstracts Service
CE	Capillary Electrophoresis
CI	Chemical Ionization
CID	Collision Induced Dissociation
CNLSD	Condensation Nucleation Light Scattering Detection
CoE	Council of Europe
CRS	Chemical Reference Substance
CZE	Capillary Zone Electrophoresis
DAD	Diode-Array Detector
DC	Direct Current
DHS	Dihydrostreptomycin
DNP	Dinitrophenylhydrazone
EAA	Electrical Aerosol Analyser
EDQM	European Directorate for the Quality of Medicines & HealthCare
EIC	Extracted Ion Chromatogram
ELSD	Evaporative Light Scattering Detection
EMA/EMEA	European Medicines Agency
EMS	Eosinophilia-Myalgia Syndrome
ESI	Electrospray Ionization
EU	European Union
FAB	Fast Atom Bombardement
FIA	Flow Injection Analysis
GC	Gas Chromatography

Gln   Glutamine

Glu   Glutamic Acid

GoE   Group of Experts

HCl   Hydrochloric Acid

HFBA   Hepafluorobutyric Acid

HILIC   Hydrophilic Interaction Chromatography

HMPC   Committee on Herbal Medicinal Products

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

ICR   Ion Cyclotron Resonance

IPA   Isopropylalcohol

IPC   Ion-Pair Chromatography

JP   Japanese Pharmacopoeia

LC/HPLC   (High Performance) Liquid Chromatography

LIT   Linear Quadrupole Ion Trap

LoD   Limit of Detection

LoQ   Limit of Quantification

MeOH   Methanol

MID   Multiple Ion Detection

MRM   Multiple Reaction Monitoring

MS   Mass Spectrometry

MSD   Mass Spectrometric Detection

m/z   Mass-to-charge ratio

NMR   Nuclear Magnetic Resonance Spectroscopy

NPA   National Pharmacopoeia Authorities

NP-LC   Normal Phase Liquid Chromatography

NQAD   Nano Quantity Analyte Detection

ODS   Octadecylsilyl

OMCL   Official Medicines Control Laboratory

OT   Orbitrap

PAD   Pulsed Amperometric Detection

PFHA   Perfluoroheptanoic Acid

PFNA   Perfluorononanoic Acid

PFPA   Pentafluoropropionic acid

Ph.Eur.	European Pharmacopoeia
QIT	Quadrupole Ion Trap
QqQ-MS	Triple-Quadrupole Mass Spectrometry
RF	Radiofrequency
RP-LC	Reversed Phase Liquid Chromatography
RSD	Relative Standard Deviation
SIM	Selected Ion Monitoring
SLM	Standard Litre per Minute
SM	Streptomycin
SRM	Single Reaction Monitoring
TCM	Traditional Chinese Medicine
TFA	Trifluoroacetic Acid
THF	Tetrahydrofuran
TIC	Total Ion Current
TLC	Thin Layer Chromatography
TOF-MS	Time-of-Flight Mass Spectrometry
USP	United States Pharmacopeia
UV/Vis	Ultraviolet / Visible light
u	Unified Atomic Mass Unit
WCPC	Water-based Condensation Particle Counter
WHO	World Health Organisation

1. __INTRODUCTION__

People prescribing or being prescribed a medicine have little chance of detecting whether or not it is of appropriate quality or not. Patients trust their physicians and their pharmacists who in turn put their trust in the manufacturer who has a fundamental role in ensuring the medicine is fit for its purpose and is safe to use. Unfortunately, the achievement of the today's standard of patient´s safety, was the result of a long history of tragic incidents. Some examples of which are [1]:

- In 1901, children receiving antitoxin for diphtheria treatment died of tetanus because the horse serum that had been used to prepare the antitoxin was contaminated with tetanus.

- In the early 20th century, syrup to calm "colicky" babies and tonics for adults often contained alcohol, opium and morphine which addicted many people.

- In 1937, sulfanilamide was formulated into an elixir and marketed for use in children. The liquid formulation contained diethylene glycol [2], the same chemical used in antifreeze, and killed 107 people, most of them children.

- In 1947, nearly 300 people were killed or injured by sulfathiazole tablets containing also the sedative phenobarbital.

- In 1955, a company producing polio vaccine failed to inactivate the virus completely. About 60 individuals developed polio.

- In the 1960th, thalidomide was marketed as a sleeping pill and to treat morning sickness. When regulatory agencies gave permission to sell the drug for those indications, they knew nothing of its serious side effects. It turned out to be teratogenic. An estimate of 10.000 cases of infant deformities in Europe was linked to thalidomide use.

- In 1982, several consumers of over-the-counter Tylenol® capsules suddenly died of cyanide poisoning. An intensive investigation of the production records showed that this was not the result of a raw materials mix-up during manufacturing. Rather, tampering apparently occurred on store shelved. A new vulnerability was identified in the supply chain.

- In 1989, an outbreak of toxic reactions to the over-the counter L-tryptophan, resulted in 38 deaths and probably thousands of less severe reactions. The event was the result of a manufacturing process change that increased the level of a harmful by-product.

This long history of sad events resulted in very strict regulations regarding the production, quality control, marketing authorisation and prescription of medicines which is the today's regulatory framework covering the field of medicines.

One element of this framework is the quality control of drug substances and excipients used for the manufacture of medicines. Although it is evident that the quality control is not a means to compensate for bad manufacturing practices, it is an additional safety net to ensure patients safety. In this respect, Pharmacopoeias describing and defining the quality of medicines have always played an important role. The history of Pharmacopoeias dates back to the ancient high cultures with the Egyptian "Papyrus Ebers" (about 1550 B.C) being one of the most prominent examples [3]. Today, more than 2500 years later, Pharmacopoeias are not only still in use, but define the quality requirements for medicines marketed all over the world. Currently 3 major Pharmacopoeias, namely the European Pharmacopoeia (Ph.Eur.) [4], the United States Pharmacopoeia (USP) [5] and the Japanese Pharmacopoeia (JP) [6] can be considered as the worldwide leading standards in this domain. Moreover, the International Pharmacopoeia [7] issued by the World Health Organisation (WHO) play an important role in defining the quality standards for medicines in developing countries. Within the European Union (EU) as well as in the further member states having signed the European Pharmacopoeia Convention, the Ph.Eur. is the legally binding standard. For EU-members this requirement is specified in Annex 1 of Directive 2001/83/EC [8] which was revised and adopted under the reference Directive 2003/63/EC [9]. In turn, it is implicit that the European Pharmacopoeia is permanently updated and remains state-of-the-art. Whilst this is normally not a problem for new monographs, the interest of the parties involved in the elaboration and updating of already existing monographs, especially for old and multi-source substances is often limited. It is in this field, that the contribution of Academia is crucial to keep the European Pharmacopoeia a modern up-to-date standard.

1.1 <u>The European Pharmacopoeia</u>

1.1.1 History

The European Pharmacopoeia (Ph.Eur.), belonging to the Council of Europe (CoE), was founded on 22 July 1964 by 8 member states (Belgium, France Germany, Italy, Luxembourg, the Netherlands, Switzerland and the United Kingdom) by signing the Convention on the Elaboration of a European Pharmacopoeia [10]. This convention was prepared by the work of four international organisations from 1951 to 1964: the Brussels Treaty Organisation (1951-1952), the Western European Union (1953-1959), the European Economic Community (EEC) and eventually the Council of Europe (with the agreement of the EEC) which finalised the convention so that it could be opened for signature.

For the first time in their history these countries undertook common efforts aimed at harmonising their national pharmacopoeias and replacing their national rules by a common supranational set of regulations. For this reason, the foundation of the European Pharmacopoeia in 1964, can be considered as the starting point of the construction of a common European regulatory framework for medicines.

The first meeting of the Ph.Eur. Commission – the highest decision making body – took place on 25 April 1964, hence already slightly before the corresponding treaties were signed. It was chaired by Mr Leon Robert, the Luxembourg delegate. In September 1964, the rules of procedure of the Commission were adopted and the Head of the Technical Secretariat was appointed. During 1965 and 1966, the Secretariat was reinforced and two vice-Chairs were appointed. The preparatory phase for setting up the functional organs ended with the inauguration of the laboratory on 18 April 1967, thus making it possible for the first definitive texts on general methods for chemical, physical and physico-chemical analyses to be adopted. The 1st edition of the European Pharmacopoeia was published in 1967. Whilst it took 13 years until the 2nd Edition was published in 1980 and even 17 year to publish the 3rd Edition in 1997, a new edition of the Ph.Eur. is nowadays published every three years. In between 2 editions, it is updated by 3 supplements per year. In July 2010, the 7th edition of the European Pharmacopoeia was published.

Since the beginning in 1964, the European Pharmacopoeia Commission has held 140 sessions and has seen 15 terms of office of the Chair of Commission.

Today, it consists of 37 delegations having signed the European Pharmacopoeia Convention (36 member states plus the delegation of the European Union (EU), a representative of the Pharmaceutical Unit (Directorate General for Enterprise) and a representative of the European Medicines Agency (EMA)).

Additionally, 22 countries from all over the world and the WHO are following the work of the European Pharmacopoeia as official observer states. Hence, the European Pharmacopoeia has become a global key actor in the field of quality of medicines.

Taking into account the growing importance of the European Pharmacopoeia as well as the fact that further responsibilities regarding the protection of public health were taken over, in 1996 the institution was re-named to become the European Directorate for the Quality of Medicines (EDQM). The growth in activities and responsibilities went together with a permanent growth in terms of human resources. Currently more than 200 staff members are working for the EDQM in its new headquarter in Strasbourg (France) which was inaugurated on 20 March 2007. In 2008, further activities in the field of combating counterfeits and healthcare activities were taken over and the abbreviation EDQM stands now for European Directorate for the Quality of Medicines & HealthCare.

1.1.2 Legal Background

Nowadays, the activities of the European Pharmacopoeia are embedded in a complex framework of legal regulations, treaties and agreements. Without asserting the claim of being comprehensive, the most important ones are given below [11]:

- Directive 75/318/EEC of 20 May 1975 of the Council of the Communities making the monographs of the European Pharmacopoeia obligatory when constituting marketing authorisation (MA) applications for medicines for human use.

- Directive 81/852/EEC of 28 September 1981 of the Council of the Communities makes the monographs of the European Pharmacopoeia obligatory when constituting marketing authorisation (MA) applications for medicines for veterinary use.

- Directives 91/507/EEC and 92/18/EEC revised Directives 75/318/EEC and 81/852/EEC, respectively, so that all medicines, notably biologicals, are covered by the provisions of these directives.

- Directives 2001/82/EC and 2001/83/EC on medicines for human and veterinary use codify and supersede all previously published directives and maintain the mandatory character of European Pharmacopoeia monographs in the preparation of dossiers for MA applications.

- Annex 1 of Directive 2001/83/EC was revised and adopted under the reference Directive 2003/63/EC. This amendment maintains the mandatory character of the monographs but also the general monographs and monographs on dosage forms in marketing authorisation (MA) applications.

- Directive 2001/83 EC and Directive 2001/82/EC were amended by 2004/27/EC Article 111 and 2004/28/EC Article 80 to legally permit the EDQM to ask national inspection services to collaborate on the inspection of manufacturing and distribution sites for raw materials for pharmaceutical use, and to legally recognise the role played by the European network of Official Medicines Control Laboratories (OMCLs) in the area of independent testing.

1.1.3 Organisation

1.1.3.1 The Technical Secretariat

The EDQM consists of the Technical Secretariat of the European Pharmacopoeia Commission, long referred to as the "European Pharmacopoeia", and other more recent services. A number of which provide support activities related to the use of the European Pharmacopoeia, such as the Certification of Suitability of Monographs and the European Network of Official Medicines Control Laboratories for the medicines for human and veterinary use (set up in 1994). In the EDQM headquarters in Strasbourg (France) pharmacists, chemist, biochemists, biologists, technicians, specialised secretaries, computer engineers and programmers, specialised translators, statisticians and archivists, of about 22 different nationalities are working together. As regards the European Pharmacopoeia, they are in charge of the following key tasks:

- preparation and publication of adopted texts and distribution of the European Pharmacopoeia and other publications;

- conduct of analytical studies and organization collaborative trials for the establishment of European Pharmacopoeia chemical reference substances

(CRS) or biological reference preparations (BRP); moreover, the laboratory contributes to the experimental verification of the monographs;

- preparation, management and distribution of reference substances;

- organisation of congresses, seminars and training sessions on subjects related to the European Pharmacopoeia;

- responsible for the organisation and administration of the meetings of the European Pharmacopoeia Commission, the Groups of Experts and Working Parties charged with the elaboration and updating of monographs.

1.1.3.2 The European Pharmacopoeia Commission

The European Pharmacopoeia Commission is the highest decision making body of the European Pharmacopoeia. It determines the general principles applicable to the elaboration of the European Pharmacopoeia, sets up and decides upon the programme of work and appoints experts to specialised groups responsible for preparing monographs. Moreover, the Commission adopts new and revised monographs and recommends the fixing of the time limits for the implementation of the decisions taken. Meeting of the Commission are held in the EDQM headquarters in Strasbourg three times a year. Each national delegation has one vote. On all technical matters, decisions of the Commission are taken by unanimous vote of national delegations casting votes. The Commission works in English and French, the official languages of the Council of Europe. The Commission meetings are chaired by a president which is elected to 3-year terms and cannot be immediately re-elected.

1.1.3.3 Groups of Experts

The European Pharmacopoeia is elaborated collectively, by experts from all the member states coming from universities, the pharmaceutical and chemical industries that produce raw materials (active substances and excipients), national control laboratories for medicines, national competent authorities responsible for medicines, inspection, or any other relevant scientific profession.
The experts are proposed by their national delegation and are nominated by the Commission on the basis of their competencies. All the meetings of the expert groups are held in the EDQM headquarters in Strasbourg. Currently there are 20

permanent groups of experts (GoEs) supplemented by 46 "ad hoc" specialised working parties which meet as required by the working programme of the European Pharmacopoeia.

The draft texts prepared by the GoEs are published in their entirety in a periodical called "Pharmeuropa", and readers have three month to submit comments on these texts. The GoEs then analyse the comments, revise the text if necessary and submit it to the Commission for adoption.

1.1.4 Structure of the European Pharmacopoeia

The European Pharmacopoeia [4] is issued in 2 volumes. Currently the publication cycle follows the mandate of the European Pharmacopoeia Commission. After each Commission session, a new supplement is prepared.

1.1.4.1 Volume 1

Volume 1 is subdivided into five chapters:

1. General Notices:

    This chapter contains all basic information necessary for the use of the European Pharmacopoeia. It is of crucial importance for the correct application of the tests described in the individual monographs.

2. Methods of Analysis

    In this chapter more than 200 general analytical methods are described. The chapter is divided into several subsections dealing with apparatus, physical and physico-chemical methods, identification tests, limit tests, assays, biological tests, biological assays, methods employed in pharmacognosy and pharmaceutical technology procedures.

    Strictly speaking, the chapter is for information only. However, as soon as a general method is referred to in a monograph, the corresponding text becomes binding. Whilst this is the case for most of the methods described, some of them, like for example the method for near-infrared spectrophotometry are not referred to in any monograph and were only added to serve as a reference in case of use in marketing applications. However, it should be kept in mind that the European Pharmacopoeia is not a text book and information and explanations given are often rather brief and not necessarily comprehensive.

3. Materials for Containers and Containers

4. Reagents

5. General texts

This chapter contains a lot of rather important information and is crucial for an appropriate control of the quality of drug substances and excipients. Several of the texts described in here apply to almost all monographs and are therefore essential for a correct use of the Pharmacopoeia. Important examples are: 5.4 Residual Solvents [12] and 5.10 Control of Impurities in Substances for Pharmaceutical Use [13]. Others like chapter 5.12 on Reference Standards [14] are given for information only, but provide nevertheless information that is important for the application of the monographs.

6. Monographs

The European Pharmacopoeia does not contain monographs for specific finished products. However, some general monographs on dosage forms are included in volume 1. Moreover, this volume contains the monographs on several other classes of substances like vaccines, immunosera, radiopharmaceutical preparations and homoeopathic preparations.

Amongst the monographs given in volume 1, the following should be specifically mentioned:

-   Products of fermentation [15]
-   Substances for pharmaceutical use [16]

Both describe general requirements for substances belonging to these groups of products and are to be applied in addition to the monographs for the individual substances.

1.1.4.2 Volume 2

Volume 2 lists individual substance monographs in alphabetic order. In the 7th edition, more than 2200 monographs are published. The monographs are presented in the same structural format containing amongst others, and where applicable, the following sub-sections: title, relative atomic and molecular masses, chemical abstracts service (CAS) registry number, definition, limits of content, production (if required), characters, identification, tests and assays, storage conditions, labelling information, warnings, list of impurities, functionally related characteristics. As

mentioned above, the explanations given under the heading "Monographs" in the General Notices [17] are key for a correct understanding and application of the individual substance monographs.

1.1.5 Elaboration of Monographs

The elaboration of European Pharmacopoeia monographs follows the Guide for the Work of the European Pharmacopoeia [18]. The work programme is decided by the European Pharmacopoeia Commission. The Commission considers for addition to the work programme monographs on active substances, excipients and, for certain classes, medicinal products that are approved for use in the member states. In the interest of public health, the Commission may decide to elaborate monographs on items that do not meet these criteria. At the time of addition to the work programme, a monograph or general chapter is allocated to a procedure and to a group of experts or working party. The work in the expert groups is based on the "Technical Guide for the Elaboration of Monographs" [19] which is a guidance for the authors of monographs and also a means of communicating to the users (i.e. industry, licensing authorities and official medicines control laboratories) the principles for the elaboration of monographs of the European Pharmacopoeia. Since the principles applied and the guidance given for the elaboration of monographs should be the same as those applied by the licensing authorities, the Technical Guide may also serve as a guideline for the elaboration of specifications intended for inclusion in licensing applications. In this context it is very important to note that the procedures for the tests and assays in the individual monographs must have been validated according to the current practice at the time of elaboration.

Currently there are two different ways for the elaboration of new monographs called "Procedure 1" and "Procedure 4". Procedure 1 describes the "classical" way of monographs elaboration in the groups of experts. The different steps of this procedure are explained in Figure 1-1. Procedure 4 is considered to be a "fast track" procedure for substances which are still under patent. The innovator collaborates directly with a group of experts consisting of members of the licensing authorities, national pharmacopoeia authorities or Official Medicines Control Laboratories (OMCL) and EDQM staff. The laboratory work is done only in the EDQM laboratory

and/or national pharmacopoeia or OMCL laboratory. Figure 1-2 gives an overview of the different steps of Procedure 4.
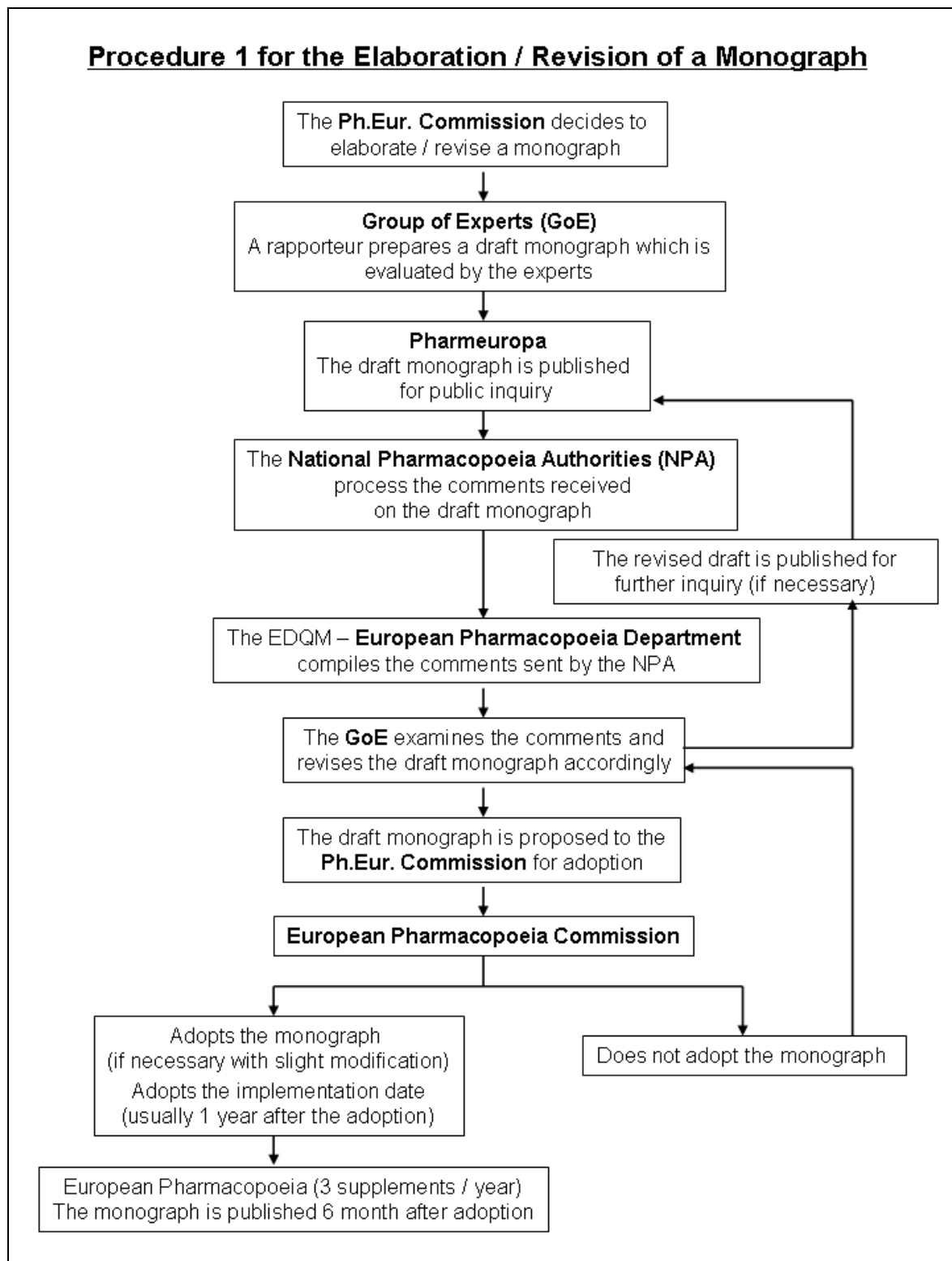
Figure 1-1. Elaboration of Ph.Eur. monographs – Procedure 1. Modified from [20].

Procedure 4 for the Elaboration of a Monograph

The **Ph.Eur. Commission** decides to elaborate a monograph

Group of Experts P4 / EDQM
A rapporteur from GoE P4 (consisting of members of the licensing authorities, NPA or Official Medicines Control Laboratories (OMCL)) and EDQM staff members prepare a draft monograph which is discussed with the manufacturer

Laboratory verification in the EDQM laboratory and/or NPA or OMCL Laboratory

Report and list of questions is sent to the **manufacturer**: the technical secretariat of the EDQM and the rapporteur evaluate the response and prepare an amended draft

GoE P4 approves draft for publication in **Pharmeuropa**
The draft monograph is published for public enquiry (lasting 3 month)

The **NPA** process the comments received on the draft monograph compiles the comments sent by the NPA

The EDQM technical secretariat compiles the comments sent by the NPA

The revised draft is published for further inquiry (if necessary)

The **rapporteur and the EDQM technical secretariat** examine the comments and revise the draft monograph accordingly. The revised draft is confirmed by the GoE P4

The draft monograph is proposed to the **Ph.Eur. Commission** for adoption

European Pharmacopoeia Commission

Adopts the monograph
(if necessary with slight modification)
Adopts the implementation date
(usually 1 year after the adoption)

Does not adopt the monograph

European Pharmacopoeia (3 supplements / year)
The monograph is published 6 month after adoption

Figure 1-2. Elaboration of Ph.Eur. monographs – Procedure 4. Modifed from [21].

1.2 <u>Impurities Control</u>

Assuming an active pharmaceutical ingredient (API) would be of absolutely purity, only an identification test of the substance would be required before use. Unfortunately, in reality this cannot be assumed and APIs may always contain impurities stemming from different sources.

Whilst the CHARACTERS section of the monographs describes the physicochemical properties of a substance and the IDENTIFICATION section lists the tests that need to be carried out to ensure that the item tested is in fact the substance in question, it is the TESTS section which is principally directed at limiting impurities in chemical substances [19]. General chapter 5.10 "Control of impurities in substances for pharmaceutical use" of the Ph.Eur. [13] describes details of the corresponding policy applied. Whilst it is essential to ensure adequate purity in the interest of public health, it is not the aim of the Pharmacopoeia to impose excessive requirements that restrict unnecessarily the ability of manufacturers to produce compliant products. A brief description of the tests possibly to be applied to ensure a sufficient purity of a substance as well as the aim of these tests is given below [19]:

1.2.1 Appearance of solution

This test ascertains the general purity of a substance by the detection of particulate matter, impurities insoluble in the solvent selected, or of coloured impurities. It is mandatory for substances intended for parenteral preparations. Apart from the latter class of substances, it is only to be applied if it yields useful information concerning the general purity of a substance, e.g. if it detects coloured impurities which are not covered by one of the other tests.

It can comprise a test for clarity and degree of opalescene and degree of coloration of liquids or of solid substances dissolved in an appropriate solvent according to the Ph.Eur.

1.2.2 pH or Acidity/Alkalinity

This test allows the limitation of acidic or alkaline impurities stemming from the method of preparation or arising from degradation of the substance. The test may also be used to verify the stoichiometric composition of certain salts.

Two types of the test for proteolytic impurities are used in the Ph.Eur.: a semi-quantitative titration experiment using indicators or electrometric methods to define the limits, the acidity-alkalinity test; or a pH measurement which is included if the material has buffering properties.

1.2.3 Enantiomeric purity

The enantiomeric purity can be controlled by different methods. Either by a simple test for optical rotation or more appropriately by chiral separation methods since the specific optical rotation is often insufficient to limit the presence of the unwanted enantiomer (distomer) in the presence of the active enantiomer (eutomer).

1.2.4 Absorption spectrophotometry

The UV/Vis-absorption may be used in the purity tests as a limit for certain impurities. This test can be particularly useful when impurities show an absorbance in a region where the substance to be examined does not absorb. The test may be performed in two different ways: a) by direct measurement on the solution; b) after carrying out a chemical reaction where the impurity forms a product that absorbs at a wavelength where the substance to be examined is transparent.

1.2.5 Heavy metals

This test is intended to detect heavy metals that precipitation at pH 3.5 in the form of dark coloured sulfides through the action of sulfide ions or reagents capable of producing them. Thioacetamide is used as the precipitating agent and sodium sulfide is allowed as an alternative, provided it has been demonstrated to be suitable. It is noted, that this test is a general test for metal contamination e.g. stemming from production equipment or contamination of raw material. However, it does not replace the specific control of metal catalysts according to the respective EMA guideline [22].

1.2.6 Loss on drying / Thermogravimetry / Water determination

These methods aim at determining the water content respectively the content of volatile matters (loss on drying / thermogravimetry). The test is on the one hand important for the determination of the content which is normally specified in a monograph on the dried or anhydrous substance; on the other hand it aims at

confirming that in case of hydrates, the appropriate hydrate is used. Whilst the determination by the Karl Fischer method or coulometric titration is specific for water, the loss on drying test co-determines the amount of residual solvents present. Given the specification limit does not exceed 0.5%, the test makes the control of class 3 residual solvents according to the corresponding ICH-guideline [23] superfluous.

1.2.7 Sulfated ash

This test is usually intended for the global determination of foreign cations present in organic substances and in those inorganic substances which themselves are volatile under the conditions of the test. The limit in a test for sulfated ash is usually set at 0.1%.

1.2.8 Residual solvents

Although not normally described as a test in the individual substance monographs, the control of residual solvents according to chapter 5.4 of the Ph.Eur [12] which applies the corresponding ICH Guideline [23], is normally required via the general monograph on "Substances for Pharmaceutical use" [16]. The standard test described in the Ph.Eur. [24] is based on a static headspace gas-chromatographic method. The method, which is intended for the identification of residual solvents, may also be used as a limit test. However, to be used for quantitative purposes, this method must be further validated. For the quantification of residual solvents, other suitable, validated methods like direct injection gas-chromatographic methods may also be used.

1.2.9 Related substances

The policy for the control of impurities – also referred to as *related substances* - is described in Ph.Eur. chapter 5.10 "Control of impurities in substances for pharmaceutical use" [13] and in the general monograph "Substances for pharmaceutical use" [16]. The provisions for related substances detailed in this general monograph apply to all active substances covered by a monograph, unless otherwise stated. The limits to be applied for the control of impurities, which are based on the ICH guideline "Impurities in New Drug Substances" [25], are given in Table 1-1.

Table 1-1. Ph.Eur. requirements for the impurities control in active substances except synthetic peptides.

Use	Maximum daily dose	Reporting threshold	Identification threshold	Qualification threshold
Veterinary only	Not applicable	> 0.1 %	0.2 %	> 0.5 %
Human or human and veterinary	> 2 g/day	> 0.03 %	> 0.05 %	> 0.05 %
Human or human and veterinary	≤ 2 g /day	> 0.05 %	> 0.10 % or daily intake > 1.0 mg (whichever lower)	> 0.15 % > 1.0 mg (whichever lower)

The following exceptions from the thresholds given in Table 1-1 are given in the general monograph: biological and biotechnological products, peptides, oligonucleotides, radiopharmaceuticals, products of fermentation and semi-synthetic products derived therefrom, crude products of animal or plant origin or herbal products.

For peptides obtained from chemical synthesis, the requirements of Table 1-2 apply:

Table 1-2. Ph.Eur. requirements for the impurities control in peptides obtained by chemical synthesis.

Reporting threshold	Identification threshold	Qualification threshold
> 0.1%	> 0.5%	> 1.0%

The general requirement of defining a reporting-, identification- and qualification threshold is also applicable to those substances exempted from the limits given in Tab. 1-1 and 1-2.

Impurities to be controlled include amongst others starting material, impurities contained in the starting material (provided they are not removed during the production process), intermediates and by products of synthesis; co-extracted substances in products of natural origin. Moreover, the methods must be capable of detecting degradation products.

Special attention must be paid to potentially genotoxic impurities which need to be controlled following the EMA "Guideline on the Limits of Genotoxic Impurities" [26].

The most common and preferred method for the control of organic impurities is liquid chromatography (LC). Another technique frequently used in monographs is gas chromatography (GC) and in some cases capillary electrophoresis (CE) is employed also. Although many existing monographs still apply thin-layer chromatography (TLC), this technique is no longer considered to be "state of the art". Therefore, the Ph.Eur. Commission has decided in 2004 to set up a work programme to systematically replace TLC methods for the control of related substances by more suitable methods. Since then, about 120 monographs have been revised and modernised.

In general, monographs cover a number of specified impurities which are listed in the impurities section. Specified impurities are those that occur in the current batches of the substances used in approved products and for which an individual acceptance criterion is provided. Wherever feasible, Ph.Eur. monographs also include an acceptance criterion for all other impurities (at the corresponding identification threshold) and a limit for the total of impurities above the reporting threshold. The acceptance criteria for specified impurities take into account both, qualification data and batch analysis data (checked during elaboration of the monograph on not less than 3 batches).

In case of products from natural sources or of semi-synthetic products derived there from such as certain antibiotics, the composition is often quite complex and a determination and limitation of impurities is not possible (e.g. monograph of gentamicin sulfate [27]. In these cases it is the aim of the European Pharmacopoeia to describe at least a composition test in order to ensure that the quality of the substances marketed in Europe is well defined and is of adequate quality.

2. **SUBJECT OF THIS DOCTORAL THESIS**

As outlined under 1.1.3.3, the main contribution to the elaboration and improvement of Ph.Eur. monographs and methods comes from the pharmaceutical and chemical industries, national control laboratories for medicines, national competent authorities and from universities. Whilst it is not normally a problem to get sufficient support for and contribution to the development of monographs for relatively new substances – especially for those still under patent protection, the situation is often different when it comes to the improvement of monographs for relatively old substances, in particular for multi sources products. In these cases the interest of industries in investing resources is rather limited or often simply non-existent.

Due to the more and more difficult budgetary situation in the public sector national control laboratories for medicines and national competent authorities cannot fill this gap. Moreover, these institutions often lack the necessary scientific research competence and structure. Therefore, the contribution of academia in this area is of crucial importance to ensure the quality of medicines and to maintain state of the art monographs.

The aim of this thesis is to contribute to the process of modernisation of monographs by exploring new possibilities and the development of new methods for the European Pharmacopoeia. The work performed is not only limited to the actual case studies but also aims at being a hint as regards the use of modern analytical techniques for the improvement of other monographs. The focus of the work performed was to explore possibilities and to find solutions for the analysis and control of substances lacking a suitable chromophor by LC. Whilst instrument manufacturers have developed several alternative detection systems and strategies to address this problem, these techniques have – apart from some rare examples – not yet found their way into the Pharmacopoeias.

Based on a number of examples taken from different classes of substances (i.e. amino acids, antibiotics, synthetic peptides, and herbal products), the possibility of using evaporative light scattering detection (ELSD), corona charged aerosol detection (CAD), nano quantity analyte detection (NQAD), mass detection (MS) and to some extent also nuclear magnetic resonance spectroscopy (NMR) was explored.

In detail, the following subjects were studied:

- The influence of different liquid chromatographic and evaporative light scattering detector parameters on the appearance of spike peaks and on the detector sensitivity.

- The development of a LC-CAD method for the control of impurities in L-aspartic acid and L-alanine.

- A comparison of the performance characteristics and the suitability of different evaporation based detectors, i.e. ELSD, CAD, NQAD, and MS, for the quantification of impurities in alanine. In addition, a quantification of the impurities by NMR was included in the study.

- The development of a LC-CAD method for the control of impurities in streptomycin sulfate.

- The application of LC-CAD for the characterization of horse chestnut standardized dry extract and for the development of a suitable reference standard strategy for the corresponding Ph.Eur. draft monograph.

- The use of collision induced dissociation MS (CID-MS) as a rapid tool for the identity verification of peptides.

3. ANALYTICAL INSTRUMENTATION AND TECHNIQUES

Below, a brief description of the different techniques considered relevant for this thesis is given. The fact that the degree of detail given in the subsequent explanations may vary substantially is taking into account that some of the techniques and instruments are well established and were already subject to many publications (i.e. LC, MS, NMR, spray drying) whilst for others (i.e. ELSD, CAD, NQAD) this is not necessarily the case. Special emphasis is put on some details if this is considered important for the work performed.

3.1 Liquid Chromatography (LC)

3.1.1 General

LC was discovered in 1903 by M.S.Tswett [28]. Over the past 100 years, it has become one of the most powerful and widespread chromatographic separation methods in pharmaceutical analysis. It is based on the difference in the distribution of species between two non-miscible phases, in which the mobile phase is a liquid which percolates through a stationary phase located in a column. LC is mainly based on mechanisms of adsorption, mass distribution, ion exchange or size exclusion [29]. LC systems consist of a pumping system, an injector, a chromatographic column (a column temperature controller may be used), a detector and a data acquisition system (or an integrator or a chart recorder). The mobile phase is continuously pumped at a defined flow rate through the system and mixed (if required) by the pump. Microprocessor controlled systems are capable of accurately delivering a mobile phase of either constant (isocratic elution) or varying composition (gradient elution), according to a defined program. In the case of gradient elution, pumping systems which deliver solvent(s) from several reservoirs are available and solvent mixing can be achieved on either the low or the high-pressure side of the pump(s). The injector is used to introduce a plug of sample into the mobile phase without having to stop the mobile phase flow and without introducing air into the system. As the injected sample reaches the column, it is slowed down by specific chemical or physical interactions with the stationary phase. How much the analyte is retained depends on the nature of the analyte and on the compositions of the stationary and mobile phases.

After having passed the column, the analyte passes the detector. In the detector a physico-chemical property of the analyte (e.g. the UV-absorbance, fluorescence, refractive index, conductivity, redox-potential, mass-to-charge ratio etc.) is used to detect the different compounds. The detector response is digitally amplified and sent to a data system where it is recorded as the "chromatogram".

Subsequently some key definitions and equations taken from chapter 2.2.46 "Chromatographic Separation Techniques" of the Ph.Eur. [30] are given as they were applied in the experiments:

Retention time (t_R)

The retention time (t_R) is the time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the corresponding peak in the chromatogram.

Symmetry factor (A_s)

The symmetry factor (A_s) (or tailing factor) of a peak is calculated as described in Equation (3-1):

$$A_S = \frac{W_{0.05}}{2d} \qquad (3\text{-}1)$$

Where:

$w_{0.05}$ = width of the peak at one-twentieth of the peak height;

d = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Column performance and apparent number of theoretical plates

The column performance (apparent efficiency) may be calculated from data obtained under either isothermal, isocratic or isodense conditions, depending on the technique, as the apparent number of theoretical plates (N) from the expression given in Equation (3-2), where the values of t_R and w_h have to be expressed in the same units (time, volume or distance).

$$N = 5.54 \times \left(\frac{t_R}{W_h}\right)^2 \qquad (3\text{-}2)$$

Where:

t_R = retention time of the peak corresponding to the component;

w_h = width of the peak at half-height.

The apparent number of theoretical plates varies with the component as well as with the column and retention time.

Resolution (R_s)

The resolution (R_s) between peaks of 2 components may be calculated using the equation given in Equation (3-3):

$$R_S = \frac{1.18 \times (t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$
(3-3)

Where:

$t_{R2} > t_{R1}$

t_{R1}, t_{R2} = retention times of the peaks;

w_{h1}, w_{h2} = peak widths at half-height.

A resolution value of 1.5 usually represents baseline separation of 2 peaks.

Peak-to-valley ratio

The peak-to-valley ratio (p/v) may be employed as a system suitability requirement in a test for related substances when baseline separation between 2 peaks is not reached. The peak-to-valley ratio is calculated as shown in Equation (3-4):

$$\frac{p}{v} = \frac{H_p}{H_v}$$
(3-4)

Where:

H_p = height above the extrapolated baseline of the minor peak;

H_v = height above the extrapolated baseline at the lowest point of the curve
       separating the minor and the major peaks.

Relative retention (r)

The relative retention of a peak is calculated as an estimate using Equation (3-5):

$$r = \frac{t_{R2} - t_M}{t_{R1} - t_M}$$
(3-5)

Where:

t_{R2} = retention time of the peak of interest;

t_{R1} = retention time of the reference peak (usually the peak corresponding to the substance to be examined);

t_M = hold-up time: time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to an unretained component.

The unadjusted relative retention (r_G) is calculated from Equation (3-6):

$$r_G = \frac{t_{R2}}{t_{R1}} \qquad\qquad (3\text{-}6)$$

Unless otherwise indicated, values for the relative retention stated in this document correspond to the unadjusted relative retention.

Signal-to-noise ratio (*S/N*)

The short-term noise influences the precision of quantification. The signal-to-noise ratio is calculated using Equation (3-7):

$$\frac{S}{N} = \frac{2H}{h} \qquad\qquad (3\text{-}7)$$

Where:

H   = height of the peak corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to at least 5 times the width at half-height;

h   = range of the noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to at least 5 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

3.1.2 Reversed Phase Liquid Chromatography (RP-LC)

Historically, LC is divided into two different sub-classes which are based on the polarity of the mobile and stationary phases. Since the development of LC was started from TLC, the first stationary phases used were silica based "normal phases". Like in TLC, non-polar mobile phases (e.g. hexane) were used. This chromatography was consequently called normal phase liquid chromatography (NP-LC).

Nowadays, reversed phase liquid chromatography (RP-LC) is the most commonly used technique. This technique results from the adsorption of more or less hydrophobic molecules onto a hydrophobic solid support in a polar mobile phase (e.g. water-methanol mixture as the mobile phase and octadecylsilyl silica gel as the stationary phase). A huge variety of different mobile and stationary phases is available for method optimization allowing efficient separation of a very wide range of molecules. In RP-LC, decreasing the mobile phase polarity by the use of organic solvents like methanol or acetonitrile reduces the hydrophobic interaction between the solute and the solid support resulting in desorption. The more hydrophobic the analyte the more avidly it will adsorb onto the solid support. This requires a higher concentration of organic solvent to promote desorption.

3.1.2.1 Stationary phases used in RP-LC

RP-LC has a non-polar stationary phase and an aqueous, moderately polar mobile phase. Several popular reversed phases are listed in Table 3-1.

Table 3-1. Examples of popular reversed phases.

Name	Formula	Abbreviation
Octyl	$Si-[CH_2]_7-CH_3$	C_8
Octadecyl	$Si-[CH_2]_{17}-CH_3$	C_{18}
Phenyl	$Si-[CH_2]_n-C_6H_6$	C_6H_6
Cyanopropyl	$Si-[CH_2]_3-CN$	CN
Aminopropyl	$Si-[CH_2]_3-NH_2$	NH_2
Diol	$Si-[CH_2]_3-O-CH(OH)-CH_2-OH$	

Amongst the PR-LC phases, octadecylsilyl (or C18) is the most commonly used one, as it is a highly robust hydrophobic phase, which produces good retention with sufficiently hydrophobic (non-polar) analyte molecules. This phase can also be used for the separation of more polar compounds when used with appropriate mobile phase additives. In general, shortening the alkyl chain will shorten the retention time. However, there are only very slight selectivity differences between e.g. C18 and C8 columns. The use of more polar phases such as cyano, phenyl or amino phases show altered selectivity compared to the alkyl phases. These phases are able to interact with polar analyte functional groups via dipole-dipole interactions, and the phenyl column can interact with analyte aromatic moieties via π-π electron interactions.

A common way to produce RP-LC stationary phase material is to treat silica beads with $Cl-Si(CH_3)_2R$, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. However, standard C18-column material is usually only stable between pH 2 to 8. Lower pH results in hydrolysis of the alky chains whilst at a pH above 8 the silica beads are dissolved [31]. A possibility to obtain higher pH-stability is to replace the methyl-groups in the alkyl chorosilane-reagent by isopropyl or isobutyl groups and therefore to protect the alkylsilane ether by steric hindrance. Another way of protecting the alkylsilane bonding can be achieved by crosslinking the alkysilyl chains using trichloroalkylsilane as starting material.

Treatment with alkyl chorosilane usually only results in derivatisation of about 50% of the free silanol functions. The remaining free silanol groups can have a negative impact on the analytical performance, especially when analyzing basic compounds. However, it is possible to avoid this negative effect by deactivating the remaining free silanol groups using short chain alkyl chorosilanes like trimethylchlorosilane. This process is usually called "endcapping" [32]. Moreover, it is possible to modify a stationary phase by "hydrophilic endcapping" which means that the deactivation of the free silanol functions is performed using polar reagents. The resulting phases are considered to be particularly suitable for the separation of polar compounds and should also tolerate high amounts of water in the mobile phase without "phase collapse" [33]. A further distinction can be made by the type of silica gel used. "Type A" silica gel is characterized by a higher content of metal impurities (e.g. Na, Ca, Mg, Al) which increases the acidity of the remaining free silanol functions and can therefore lead to chromatographic problems like peak-tailing when analyzing basic

compounds. On the other hand, "Type B" silica gel is highly purified and contains only very low amounts of metal residues [31, 34]. Finally, several other parameters like particle shape (spheric or irregular), active surface, pore-size or carbon-load can have an important influence on the column characteristics. Taken together, the technological development in the field of RP-LC columns allows the production of a huge variety of different columns bound to different inorganic or organic support material with all kinds of modifications. Unfortunately, traceable and scientifically reliable information on the production technology and on the composition of the column material is often difficult to obtain from the producers. Therefore, a lot of information can be taken from commercial manufacturers brochures only. On the one hand, this development allows the analyst to select a more or less "custom made" column for a separation problem, on the other hand it makes the selection of the most appropriate column rather complex and time consuming. Moreover, based on the studies performed, it is concluded that the commercial information concerning the suitability of a certain column material should be taken with some caution and that modifications and special developments often do not result in practically relevant improvements.

3.1.2.2 Ion-pair chromatography

Ion-pair chromatography (IPC) can be an alternative to classical RP-LC in case insufficient separation or poor chromatographic results are obtained for ionic or ionisable samples. The major difference of ion-pair chromatography compared to classical reversed phase LC is the addition of an ion-pair reagent to the mobile phase [35]. The ion pair reagent can either be positively or negatively charged. In the case of negatively charged ion pair reagents alkysulfonates, such as hexane-sulfonate or lauryl-sulfonate, are often used. In the case of acidic (anionic) samples a positively charged ion-pair reagent like tetrabutylammonium salts can be employed. The retention process of IPC, which has not yet been fully explored, is very different from RP-LC and therefore can result in large changes in separation selectivity for ionic samples.

Different IPC models are discussed in literature [35, 36]. Amongst those, the most well known are:

The ion-pair model

In an aqueous mobile phase, neutral ion pairs are formed by the analyte and the ion pair reagent. These ion pairs interact with the apolar surface of the reversed phase stationary phase. The separation of the analytes is a result of the different affinity of the ion pairs to the stationary phase.

The dynamic ion exchange model

The hydrophobic alkyl group of the ion-pair reagent is attracted by the C8 or C18 reversed-phase stationary phase. The charge carried by the reagent (e.g SO_3^-) thereby attaches to the stationary phase. This negative charge on the stationary phase is balances by positive ions (Na^+) from the reagent and/or buffer. A positively charged sample ion like a protonated base (BH^+) can now exchange with Na^+ resulting in the retention of the sample ion by an ion exchange process.

The following considerations are based on the theory of the dynamic ion exchange model:

It is important to note that the retention process can continuously be changed from reversed-phase to ion-exchange separation by modifying the amount of ion pair reagent in mobile phase and consequently the amount of reagent adsorbed to the stationary phase. For each ion-pair reagent, the column uptake increases for higher reagent concentrations in the mobile phase, but levels off upon column saturation. Because ion-pair reagents with longer alkyl chains are more hydrophobic they are stronger retained by the column resulting a saturation of the stationary phase at lower reagent concentrations. Since separation is mainly dependent on the resulting charge on the column, similar separations will be obtained with ion-pair reagents differing in the chain-length of the alkyl chain when the reagent concentration of the mobile phase is adjusted to give the same molar uptake by the column.

For an ionic hydrophilic sample compound BH^+, retention is mainly based on an ion-exchange process. Consequently, as the charge of the column increases with an increased loading of the column with ion-pair reagent (e.g. octadecylsulfate$^-$), retention of BH^+ increases until saturation of the column is obtained. A further increase in the ion-pair reagent concentration leads to an increase in the counter ion concentration (e.g. Na^+), which competes with the retention of the sample ion on the column and will therefore reduce retention of the analyte on the column.

By an appropriate adjustment of the pH and ion-pair reagent concentration, simultaneous retention of ionised and non-ionised samples by both reversed-phase and ion-exchange processes can be achieved. A further means to optimise the separation is to adjust the amount of organic modifier (e.g. methanol or acetonitrile) in the mobile phase. In these "mixed-mode" separations a change in the concentration of the organic modifier has a direct impact on the separation of non-ionised samples in the reversed-phase separation mode. For ionised samples an increase of the amount of organic modifier will reduce the adsorption of the ion-pair reagent to the stationary phase. Eventually, also changes in temperature have an impact on the separation since several equilibration processes like sample retention by ion-exchange, and/or reversed phase, ionization of the buffer and sample and sorption of the ion-pair reagent are temperature dependent.

In IPC thorough column equilibration is an important factor and may take a considerable amount of time. This is particularly true for very hydrophobic ion-pair reagents and/or in case of quaternary-ammonium reagents. The slow equilibration in IPC can pose problems in gradient elution. For this reason, IPC in gradient mode is not recommended.

3.1.2.3 Volatile mobile phases

Until the mid-1980s the main reason for the use of volatile mobile phases in LC was for purification purposes as the collected mobile phase could be removed by freeze-drying [37, 38]. Modern LC-detection techniques like electrospray mass spectrometry (ESI-MS), ELSD, CAD or other evaporation-based detectors require volatile mobile phases. One possibility to achieve this goal is to substitute non-volatile additives with volatile ones. A problem arising from this option is that the additive nature and concentration change the chromatographic retention and resolution even when the pH of the mobile phase is kept constant [39]. Early evaluations of volatile mobile-phase additives used formate, acetate, carbonate and bicarbonate ammonium salts for coupling LC with thermospray mass spectrometry [40, 41, 42]. According to Petritis et al. [39], it was only at the beginning of this millennium that perfluorinated carboxylic acids were evaluated as volatile anionic ion-pair reagents for the chromatographic separation of underivatised amino acids and small peptides as well as several aliphatic amines as volatile cationic ion-pair reagents for the separation of inorganic anions, organophosphoric acids and amino acids.

Eluent volatility of these additives was confirmed by ELSD as this detection technique requires a volatile mobile phase to avoid a high background noise. A systematic evaluation of the volatility of different anionic and cationic ion-pair reagents performed by Petritis et al. [39] showed that all acids and bases tested in the study were at least volatile at 2 mmol/L. However, at concentrations above 5 mmol/L, higher homologues of perfluorinated carboxylic acids like tridecafluoroheptanoic acid and pentadecafluorooctanoic acid were no longer sufficiently volatile and resulted in an increased ELSD background noise. Moreover, solubilisation of these compounds in water became difficult. Furthermore, adjusting the pH of mobile phases containing perfluorinated carboxylic acids by means of bases revealed that almost all combinations resulted in non-volatile salts.

3.1.3 Hydrophilic Interaction Chromatography (HILIC)

Hydrophilic interaction chromatography (HILIC), which can be considered complementary to RP-LC, consists of polar stationary phases like silica or a polar bonded phase in combination with appreciable quantities of water (usually at least 2.5% by volume) and higher portions of an organic solvent (often acetonitrile) as mobile phases [43]. Usually, separations are carried out using 5-40% water or aqueous buffers either as isocratic or as gradient separations. In HILIC, hydrophilic, polar and charged compounds are stronger retained than hydrophobic neutral species, which is opposite to RP-LC. Increasing the aqueous amount of the mobile phase normally results in less retention. The order of elution is often similar to normal phase chromatography. Compared with RP-LC, HILIC has some advantages. In MS, ELSD and CAD the detector sensitivity is enhanced since the high content of organic modifier in the mobile phase results in better nebulization and evaporation of the solvent. Moreover, higher flow-rates can be used since the viscosity of mobile phases with a high amount of organic modifier is lower which reduces the column backpressure. In addition, solute diffusion is increased in HILIC mobile phases. The separation mechanism of HILIC is very complex and may vary depending on the type of stationary phase used. A good overview of possible separation mechanisms is given by Hemström and Irgum [44].

An increasing number of different phases is nowadays available for HILIC separations including pure silica, diol, aminopropyl, and zwitterionic (e.g. sulfonylbetaine) stationary phases. These phases can be either bound to silica or to organic polymeric matrices, the latter resulting usually in columns tolerating a wider pH range.

3.2 <u>Detectors</u>

3.2.1 UV/Vis spectrophotometry

Ultraviolet/visible (UV/Vis) detectors, are the most commonly employed LC detectors. The light source is typically a deuterium lamp, which is appropriate for wavelength between 190 and 400 nm. For wavelength of visible light (400 to 700 nm) a higher energy tungsten-halide lamp is often employed. The signals due the intensity of the emitted light before passing through the flow-cell (I_0) and of the light having passed the flow-cell (I) are converted by the detector electronics into absorbance values according to the formula given in Equation (3-8).

$$A = \frac{I_0}{I} \qquad (3\text{-}8)$$

UV-Vis detectors are concentration dependent detectors in which the relation between the analyte concentration and the absorbance is given Beer´s law [35] (cf. Equation (3-9)):

$$A = C \times \varepsilon \times L_{fc} \qquad (3\text{-}9)$$

Where:

A   = absorbance;

C   = analyte concentration;

E   = analyte molar absorbtivity;

L_{fc} = light-path length of the flow-cell.

Two different types of UV/Vis detectors can be distinguished; variable-wavelength detectors and diode-array detectors (DADs). Whilst DADs allows simultaneous collection of chromatograms at different wavelength during a single run and therefore provide more information on the sample composition, variable-wavelength detectors

are usually more sensitive and are therefore better suited when it comes to the detection of low analyte concentrations.

3.2.2 Pulsed amperometry

Amperometric detection utilises a flowing current to initiate a chemical conversion of electro-active analytes, i.e. those substances which undergo a reduction or oxidation reaction according to the potential applied [45]. It can represent an alternative LC detection method for compounds lacking a suitable chromophor for UV detection. However, due to the nature of the electrode processes it is not easy to apply and strict control of several parameters is necessary to obtain reproducible results. The most important ones are: temperature, pH, and mass transfer (the eluent flow through the reactor should be continuous and without pulsation). The temperature influences the reaction at the working electrode, but also affects the background reactions producing the baseline current. Thermal stability is therefore a key requirement to obtain reproducible signal response. Similarly, pH alterations affect the current/potential curves (voltammograms) and the possible results of poor pH control include reduction of signal intensity and lower signal-to-noise ratios. Eventually, redox reactions depend on the mass transfer at the electrode/electrolyte interface. Therefore a constant eluent flow is necessary for both baseline stability and reproducible signals. Any pulsation from the pump delivering the eluent to the reactor cell must therefore be avoided.

Reaction products can adsorb onto the surface of the electrode during amperometric detection, which may drastically change the electrochemical properties of the electrode. For this reason, pulsed mode or pulsed amperometric detection (PAD), employing a measuring potential and two cleaning potentials to ensure the electrochemical regeneration of the electrode surface, is the most versatile detection method in LC [45].

Pulsed amperometric detection usually applies a multistep potential waveform and the detection potential is chosen to be appropriate for the desired detection mechanism [46, 47].

Although for the above reasons LC-PAD is not easy to apply and requires an experienced operator, several applications in the pharmaceutical domain, especially in the field of aminoglycoside antibiotics have been published [48, 49, 50, 51, 52, 53, 54].

3.2.3 Mass spectrometry

Although detection by mass spectrometry requires an evaporation of the mobile phase prior to detection of the analyte, there are substantial differences to the evaporation-based detection systems discussed in chapter 3.2.4. For this reason detection by mass spectrometry (MS) is described separately.

In mass spectrometry ions generated from inorganic or organic compounds by suitable methods are separated by their mass-to-charge ratio (m/z). Subsequently, these ions are detected either qualitatively or quantitatively. In its basic setup, a mass spectrometer consists of an ion source, a mass analyzer and a detector all of them operating under high vacuum as shown in Figure 3-1.
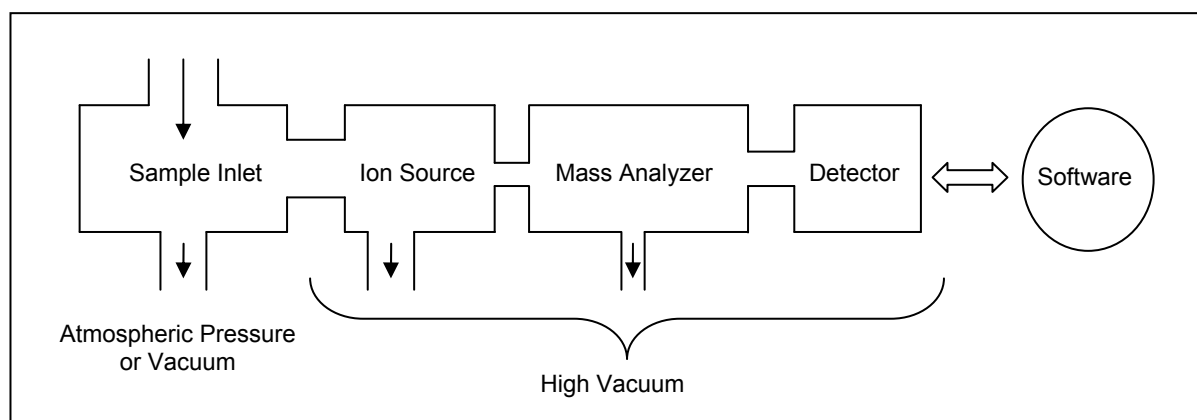
Figure 3-1. Schematic depiction of a mass spectrometer.

3.2.3.1 Ionization methods

Ionization methods being used in LC-MS include electrospray ionization (ESI), chemical ionization (CI), fast atom bombardement (FAB), thermospray, electrospray and atmospheric-pressure chemical ionization (APCI) [55]. A further technique that has been developed over the past years is atmospheric-pressure photo ionization (APPI).

Amongst these ionization techniques, ESI and APCI are the most frequently used ionization techniques in LC-MS. One big advantage of these techniques is the fact that ionization takes place at atmospheric pressure.

In the ESI source the LC capillary ends in a fine needle which is held at a voltage of typically 3-4 kV. LC-eluent exiting the capillary it transformed into an aerosol by means of electro-spraying and expands into a counter-current stream of dry nitrogen gas that serves as a heat supply for the vaporization of the solvent. It is assumed that

by this process, highly charged droplets are formed that undergo desolvation during their passage across the source of the mass spectrometer. As the size of the droplets reduces, a point is reached at which the repulsion between the charges on the surface of the droplets is strong enough to overcome the surface tension. At this point, a "Coulombic explosion" occurs, producing a number of smaller droplets with a radius of about 10% of that of the parent droplet. After a series of such explosions, a point is reached at which ions are formed that are transferred into the mass spectrometer [55]. It is however noted that there are also alternative explanations for the mechanism leading to the formation of ions, e.g. the ion-evaporation [56] and the charge-residue model [57].

According to the ion-evaporation model, the droplets become smaller and smaller until the surface charge is sufficiently high for direct ion evaporation into the gas phase. In the charge-residue model, it is presumed that repeated Coulombic explosions take place until droplets are formed that contain a single ion.

Amongst the parameters influencing the electrospray process, the LC eluent flow-rate is of great importance for the mass spectrometric performance. The flow rate affects both the size and size distribution of the droplets formed during the electrospray process and, consequently, the number of charges on each droplet. Although recent developments allow working at LC flow-rates of 1 mL/min, acceptable sensitivity is usually only achieved at eluent flow rates between 100 - 500 µL/min. However, reduction of the LC eluent flow-rate can easily be achieved by splitting the flow after the column outlet. At such flow rates, electrospray acts as a concentration sensitive detector, i.e. the signal intensity is proportional to the concentration of the analyte in the mobile phase rather than the absolute amount of analyte present. Flow-rate changes do not affect the intensity of the signal coming from the detector and splitting of the flow may therefore be carried out without an accompanying reduction in sensitivity [55]. One of the shortcomings of ESI is that non-polar analytes may not be ionized. This problem can often be overcome by using APCI.

The processes taking place in APCI are rather similar to ESI. The major difference is that the ion formation process of APCI results in a better ionization of less polar analytes. The spray formed from the column eluate is heated and dried. The neutral species thus produced are then passed through a corona discharge - which occurs when the field at the tip of an electrode is sufficiently high to ionize the gas

surrounding it but is insufficiently high to cause a spark – where ionization of the analyte is effected with the evaporated solvent acting as reagent gas. A further advantage of APCI is that it is compatible with flow rates between 0.5 and 2 mL/min. Both ESI and APCI are soft-ionization techniques producing mainly intact molecular species.

3.2.3.2 Mass analyzers

A summary of different mass analyzers, based on an overview given by Gross [58], is presented in Table 3-2.

Table 3-2. Overview of mass analyzers.

Type	Acronym	Principle
Ion cyclotron resonance	ICR	Trapped ions, separation by cyclotron frequency (Lorentz force) in magnetic field.
Linear quadrupole	Q	Continuous ion beam in linear radio frequency quadrupole field, separation due to stability of trajectories.
Linear quadrupole ion trap	LIT	Continuous ion beam and trapped ions; storage and eventually separation in linear radio frequency quadrupole field due to stability of trajectories.
Magnetic sector	B	Deflection of a continuous ion beam; separation by momentum in magnetic field due to Lorentz force.
Orbitrap	OT	Ions trapped in an electrostatic field between barrel-like electrode and a coaxial inner spindle-like electrode. Separation by oszillation frequency similar to ICR.
Quadrupole ion trap	QIT	Trapped ions; separation in three-dimensional radio frequency quadrupole field due to stability of trajectories.
Time-of-flight	TOF	Time dispersion of a pulsed beam, separation by time-of-flight.

As in many other areas of analytical chemistry, resolution is an important parameter also in MS where it means the ability to differentiate between closely related signals. In MS, these signals are the m/z-ratios of the ions and resolution is defined as shown in Equation (3-10).

$$R = m/\Delta m \qquad\qquad (3\text{-}10)$$

Where:

R   = resolution;

m   = m/z to be measured;

Δm   = is the difference (in mass units "u") between the ion of interest and the ion
           from which it can be separated.

Although there is no exact definition, "low-resolution" instruments are usually considered those obtaining a resolution up to 2000 which means that these instruments can separate and measure m/z-ratios to the nearest integer value.

The term "high resolution" is usually used for instruments having a resolution above 5000.

Since a time-of-flight (TOF) and a triple-quadrupole analyzer (QqQ) was used in the conducted studies, these two types of mass analyzers are subsequently described in more detail.

Time-of-Flight mass analyzers

The principle of TOF is rather simple: Ions of different m/z-ratios are dispersed in time during their flight along a field-free drift path of known length. Provided all the ions start their journey at the same time or at least within a sufficiently short time interval, the lighter ones will arrive earlier at the detector than the heavier ones. This demands that they emerge from a pulsed ion source which can be realized either by pulsing ion packages out of a continuous beam or more conveniently by employing a true pulsed ionization method.

Modern TOF mass analyzers are "reflector" (also called "reflectron") instruments, a technique that has been developed by Mamyrin [59]. Here, the reflector acts as an ion mirror that focuses ions of different kinetic energies in time. Although the reflector almost doubles the flight pass and therefore also the dispersion in time-of-flight, this effect is of lower importance compared to its capability of compensating for the initial energy spread.

The latter effect largely increases the resolving power of TOF instruments. Modern reflector-TOF instrument are usually high-resolution instruments having a resolution of well above R = 10.000.

According to Gross [58], the major advantages of TOF instruments are the following:

- In principle, the m/z ratio is unlimited.
- From each ionizing event, a complete spectrum is obtained within several tens of milliseconds.
- The transmission of a TOF-analyzer is very high, giving rise to high sensitivity.
- The construction of a TOF instrument is comparatively straightforward and inexpensive.
- Recent instruments allow for accurate mass measurement and tandem MS experiments.

Linear quadrupole / Triple quadrupole mass analyzers

Linear quadrupole mass analyzers consist of four parallel hyperbolic or cylindrically shaped rod electrodes: The opposite pairs are connected electrically and a voltage, consisting of both radiofrequency (RF) and direct current (DC) components, is applied, with the RF components on the two pairs of rods being 180° out-of-phase. At a specific value of these voltages, ions from a particular m/z follow a stable trajectory through the rods and reach the detector. A mass spectrum is therefore produced by varying the RF and DC voltages in a systematic way to bring ions of increasing or decreasing m/z ratios to the detector [55]. Quadrupole analyzers are usually low-resolution instruments operating at "unit resolution" which means that two signals can be distinguished from each other if their mass difference is at least 1u.

Triple quadrupole (QqQ) mass spectrometers have become standard instruments, especially when accurate quantification is required. To operate a QqQ-MS in the MS/MS mode, the first linear quadrupole (Q_1) serves as a normal mass analyser as described above. The second quadrupole (q_2) however is not used as a mass separation device but as a collision cell, where fragmentation of ions transmitted by the quadrupole is carried out, and as a device for focusing any product ion into the third quadrupole (Q_3) where the fragment ions stemming from q_2 are analyzed. Fragmentation in q_2 is usually achieved by means of a collision gas like nitrogen or argon (= Collision Induced Dissociation; CID).

QqQ-MS instruments can be operated in different modes (Table 3-3). For completeness it is noted that QqQ instruments can also be used in linear quadrupole mode.

Table 3-3 – Operation modes of triple quadrupole MS instruments.

Operation Mode*	Setting of Q1	Setting of q2	Setting of Q3
Product ion scan (m_1 to be defined)	Lock for m_1 (no scan)	CID	Scan up to m_1 to collect fragments
Precursor ion scan (m_2 to be defined)	Scan from m_2 upwards to cover potential precursors	CID	Lock for m_2 (no scan)
Constant neutral loss scan (Δm to be defined)	Scan desired range	CID	Scan range shifted by Δm to low mass

* Masses for reaction $m_1^+ \rightarrow m_2^+ + n$

3.2.3.3 Data acquisition and analysis

A mass spectrum is a two-dimensional graph of the signal intensity (ordinate) versus m/z (abszissa). The signal intensity directly reflects the abundance at the corresponding m/z ratio. The m/z ratio is by definition dimensionless. In many cases the number of charges is equal to one in which case the m/z scale is equivalent with the mass scale. However, especially for peptides and proteins it is quite common to have multi-charged molecules.

Often but not necessarily, the signal with the highest m/z-ratio can be attributed to the molecular ion. Depending on the ionization technique used, the molecular ion peak may be accompanied by peaks of lower m/z ratios resulting from fragmentation. The most intense peak in a mass spectrum is called "base peak".

Generally mass spectral data can be used qualitatively (for identification purposes) or quantitatively (to determine the amount of an analyte present in a sample).

However, the data for these two purposes are quite different. For identification purposes (**qualitative use**), a mass spectrum covering all of the m/z-ratios likely to appear is recorded. The MS is therefore set up to scan repeatedly over a selected m/z-range for an appropriate period of time. At the end of each scan, the mass spectrum obtained is stored for subsequent manipulation before a further spectrum is recorded. The signal resulting from repeated scanning is usually called total-ion current (TIC) trace.

As with many other techniques, **quantification by mass** requires a comparison of the intensity of the signal generated by the analyte with the signal obtained from of a reference standard at known concentration. In order to increase the sensitivity, quantification is usually performed in selected-ion-monitoring (SIM) or multiple-ion detection (MID) mode. This means that the mass analyzer does not scan over a broad range of m/z ratios but is locked on one or a very limited number of m/z ratios. This results in a longer measurement time per ion, and therefore in an increased sensitivity.

In QqQ-MS highly sensitive and specific quantification is possible by filtering a selected m/z ratio from the analyte with the first quadrupole, fragmentation in the collision cell and subsequent selection of one or several specific product ions with the second quadrupole. In the case the second mass filter is focussed on one product ion, this process is called "single reaction monitoring" (SRM); if it measures several product ions from the same precursor, it is called "multiple reaction monitoring" (MRM). SRM and MRM guarantee on the one hand high specificity of the determination since co-determination of compounds with the same m/z ratio as the analyte is practically excluded, and on the other hand, double selection/filtering of a target mass reduces significantly the noise level which is key for high sensitivity.

3.2.4 Evaporation based detection systems

3.2.4.1 General

Several evaporation based LC detectors have been developed in the past years. These detectors are particularly interesting to serve as an alternative for the detection of analytes lacking a suitable chromophor and therefore not eligible to UV-Vis detection. However, a prerequisite for the use of these detectors is that the solute is considerably less volatile than the mobile phase. In practise this means that the choice of mobile phase additives is limited and that volatile mobile phases (cf. 3.1.2.3) must be used. Another important constraint is that the detector response often varies with the amount of organic modifier present in the mobile phase. Although post-column mobile phase compensation is a possibility to circumvent this problem [60], this technique is analytically not easy to apply and is therefore not a straightforward option suitable for pharmaceutical quality control. Therefore, these detectors are in practise of limited suitability when it comes to gradient LC methods. Since the principles of evaporation based detectors are very similar as regards the first two steps, i.e. nebulization and evaporation of the LC eluate, these steps are detailed only under 3.2.4.2, but apply also to the subsequently described detectors.

3.2.4.2 Evaporative Light Scattering Detector (ELSD)

The ELSD is considered to be a universal detector in liquid chromatography, provided the solute is considerably less volatile than the solvent at the operating temperature [61]. It measures the amount of light scattered by particles of mobile phase that have been dried through the evaporation. In general, evaporative light scattering detectors deliver a signal for all compounds that do not evaporate during the mobile-phase evaporation stage [62].

Although design characteristics may differ from one manufacturer to another, the principle of detection is the same. It comprises the following steps: nebulization of the eluate, evaporation of the solvent and light scattering by the residual volatile particles including the analyte.

Each process contributes to the overall response of the detector [63].
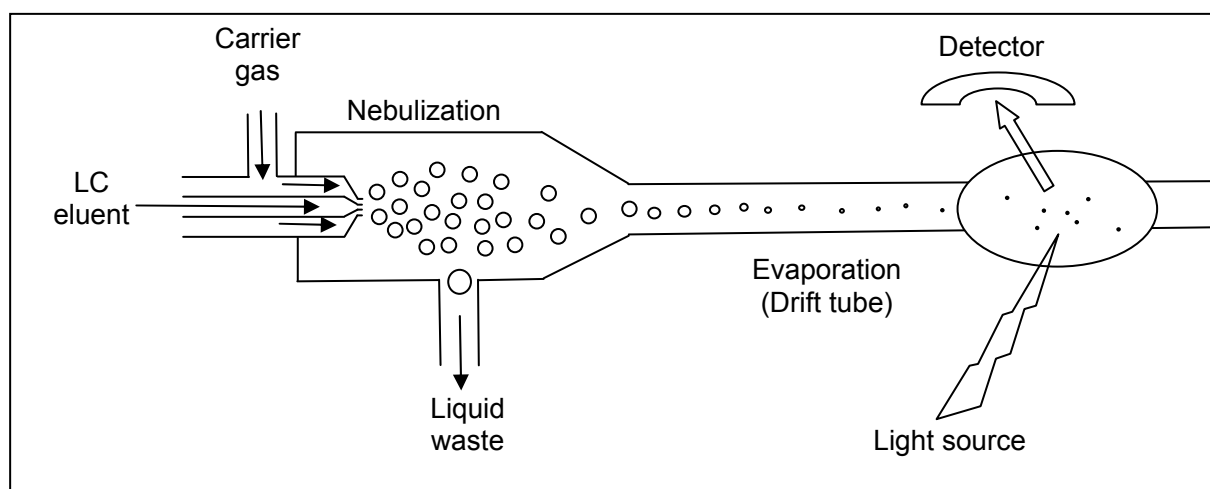
Figure 3-2. Principles of the ELSD.

Nebulization

In general, nebulizers use the Venturi effect produced by a carrier gas, typically air or nitrogen, which flows coaxially to the mobile phase eluting from the chromatographic column. This step transforms the liquid phase into small droplets [63].

The mean drop diameter (D_{SV}) produced by a Venturi type nebulizer, also known as Sauter mean diameter can be described by the empirical equation of Nukiyama-Tanasawa [64]:

$$D_{sv} = \frac{585 \times \sqrt{\sigma}}{(v_g - v_l) \times \sqrt{\rho}} + 597 \times \left(\frac{\mu}{\sqrt{\sigma\rho}} \right)^{0.45} \left(1000 \times \frac{Q_l}{Q_g} \right)^{1.5} \qquad (3\text{-}11)$$

Where:

σ   = surface tension of the mobile phase;

ρ   = density;

μ   = viscosity;

$(v_g\text{-}v_l)$ = the difference between the nebulizer gas and liquid velocities;

Q_l/Q_g = the ratio of liquid and gas volumetric flow rates.

Based on this equation, Guiochon et al. [65] conclude that:

- The average droplet size decreases with increasing gas flow rate, because both terms of the above equation will decrease and with decreasing solvent flow rate, since in both cases the relative velocity increases and both terms of Equation (3-11) decrease.

- The average droplet size depends on the nature of the solvent since both terms depend on the density, surface tension and viscosity of the nebulized liquid. For solvents commonly used in reversed phase chromatography, i.e. methanol or acetonitrile, the average particle diameter will decrease with increasing amounts of the organic solvent.

According to Dreux et al. [66] the percentage of effluent actually transferred to the drift tube varies according to the mobile phase composition. It can be as high as 90 % or more with organic solvent mixtures, but it is only 10 % with water. This is due to the fact, that the biggest droplets are eliminated by condensation on the walls of the chamber and are directed to a waste outlet or drain which continuously siphons off the condensed liquid.

Evaporation

After nebulization, the droplets are carried by the nebulizer gas into a heated drift tube where the eluent is evaporated and a cloud of the non-volatile material contained in the eluent is formed.

After complete solvent evaporation, the diameter (D) of a particle entering the detection chamber can be calculated from the corresponding wet droplet diameter (D_0) as in Equation (3-12).

$$D = D_o \times \left(\frac{C}{\rho} \right)^{\frac{1}{3}} \qquad\qquad (3\text{-}12)$$

where C is the concentration of the solute in the droplets and ρ its density.

It has to be noted that D_o is not equivalent to the mean drop diameter (D_{sv}), although both closely related. The modification of the particle size distribution may be due to a loss of the larger droplets by impaction on the surface of the drift tube or spray chamber. Some droplets may also coagulate, mainly in dense aerosol and turbulent aerosol formation stages [67].

It is important to use as low temperatures for the evaporation as possible to maintain the uniformity of the particle size. In addition, low temperature evaporation enhances solute crystallization; the larger the solute particle, the higher the intensity of the light scattering.

At higher temperatures, too rigorous solvent volatilization can cause non-uniform particle sizing, or inhibit crystal formation, adversely affecting the light scattering process.

Maintaining low temperatures in the drift tube is also a primary concern when working with moderately volatile solutes; at higher temperatures, such compounds can be completely volatilized and non-detectable [66].

Light scattering

After evaporation of the eluent, the solute particles enter into the detector cell, where the solute passes through a light beam, either produced by a polychromatic source (tungsten or halogen lamp) or by a laser-emitting diode.

The amount of light scattered by the solute particles is measured using a photomultiplier or a photodiode [66].

According to [68] there are four main processes by which the path of electromagnetic radiation can change direction when passing though a medium containing a suspended phase.

These are Rayleigh scattering, Mie scattering, reflection and refraction. The importance of each process is governed by the radius of the particles compared to the wavelength of the incident light. Raleigh scattering is most predominant when the particles are much smaller than the wavelength of the incident light ($r < 5 \times 10^{-2}$). Once the dimensions of the particles become greater than $\lambda/20$, Mie scattering becomes the most important mechanism. When the particle size approaches the wavelength of the incident light, then reflection and refraction begin to prevail.

The signal intensity may result from two different domains of the light scattering, either Rayleigh and Mie, or Mie and reflection-refraction. However, it appears that scattering is predominantly cause by reflection and refraction.

As pointed out above, the detector response provided by the ELSD is a result of various light scattering mechanisms and depends on the particle size distribution. The response is non-linear and can be described by Equation (3-13) [65]:

$$A = a \times m^b \qquad\qquad (3\text{-}13)$$

where A is the peak area, a and b are numerical coefficients depending on the experimental conditions and m is the solute mass.

Linearisation can be obtained by double logarithmic expression as shown in Equation (3-14).

$$\log A = b \times \log m + \log a \qquad\qquad (3\text{-}14)$$

3.2.4.3 Corona Charged Aerosol Detector (CAD)

Some years ago, the CAD was introduced by Dixon and Peterson [69]. CAD uses similar nebulization and evaporation steps as ELSD, but with detection of the resultant aerosol through electrical charging of the aerosol and detection of the charged particles using an electrical aerosol analyzer (EAA).
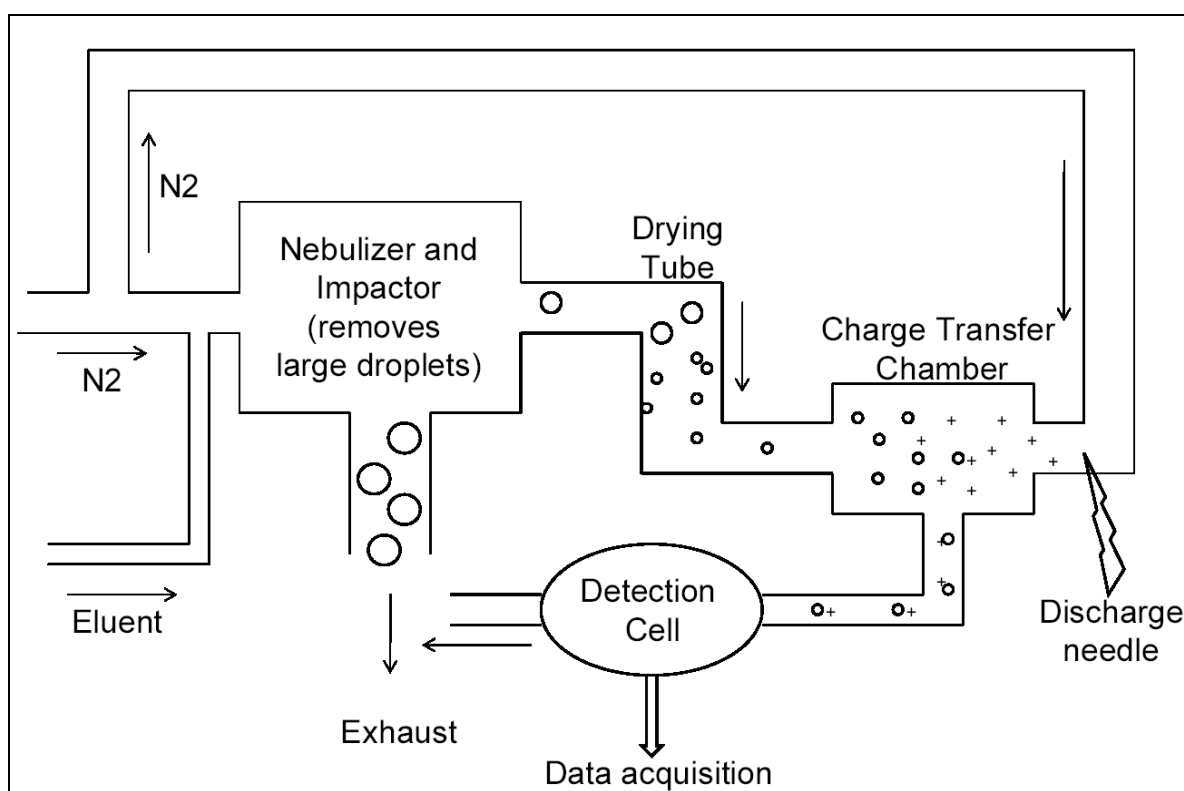
Figure 3-3. Principles of the CAD.

The EAA is a commercial instrument that was built in the 1970s for sizing aerosols and is described in detail by Liu and Pui [70]. The EAA operates by charging particles as they pass near a region of positive corona discharge, removal of particles of larger mobility (which is inversely related to particles size) through attraction to a charged rod of negative charge, and detection of the remaining charged particles with a filter / electrometer. Because aerosol charging is more sensitive, as compared to ELSD for small particles (diameter less than 100 nm), CAD offers inherent advantages over ELSD [69]. When the CAD is operated under normal conditions, the charge imparted

to particles in the corona discharge region depends primarily on the particle size whilst the particle composition does not seem to have a significant impact on the signal [70, 71]. According to Dixon and Peterson [69] the maximum sensitivity per particle mass occurs at particle diameters of about 10 nm. Although it is possible to use equations for instrument sensitivity along with equations for size distribution of droplets generated by a pneumatic nebulizer to predict the response, this is complicated by a number of facts, such as the loss of droplets to walls in the spray chamber and the possible charging of droplets from the spray electrification. Thus, the determination of the response is usually done by an empirical approach. Fortunately, operating the CAD is rather simple and just requires setting a few controllable parameters like the gas inlet pressure and the signal output range [72]. Compared with the ELSD, the CAD detector was reported to have an about 10-fold increased sensitivity [60, 69, 73, 74, 75, 76].

As it is the case for the ELSD, the response of CAD is not directly linear over a broad concentration range, and good linearity is obtained only on a logarithmic scale [60, 69, 77]. However, the response of the CAD was reported to be linear over a limited range of about 2 orders of magnitude in different studies [76, 78, 79].

3.2.4.4 Nano Quantity Analyte Detector (NQAD)

The NQAD is a relatively new aerosol based LC detector which is partly based on original techniques from early work on Condensation Nucleation Light Scattering Detection (CNLSD) developed by L.B. Allen, J.A. Koropchak et al. [80, 81]. Like ELSD and CAD the first step is a continuous nebulization of the column eluent. Thereafter, the mobile phase is evaporated from the droplets, leaving analyte particles suspended in air which are not volatile at the evaporation conditions. However, measurement of the analyte is not taken at this point. Rather, the dry aerosol moves into another chamber and the size of the aerosol distribution is measured by the increase in the number of particles counted using a Water-based Condensation Particle Counter (WCPC). The WCPC condenses water vapour onto particles and enlarges the particles to a size that they can be detected individually using a laser-based optical sensor.

The WCPC detector in the NQAD will only condense vapour onto particles that are above a certain size – particles below this size are not counted. As the size of the

particles increases due to an eluting analyte, the number that is large enough to grow due to water vapour condensation increases. The number of particles counted by the WCPC detector is then converted to an analog output signal [82].
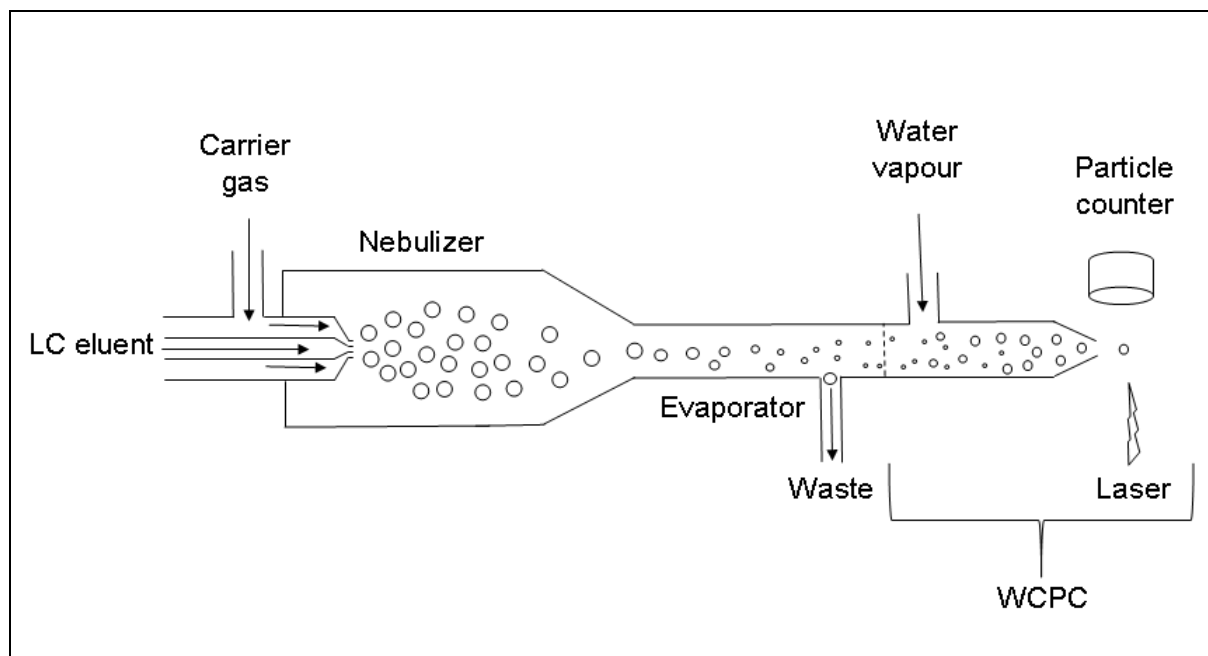
Figure 3-4. Principles of the NQAD.

As this is the case for other aerosol based LC detectors, the sensitivity of the NQAD is limited by the level of background non-volatile residue that co-elutes with the analyte. However, the NQAD is able to measure smaller changes in the size shift of the dry aerosol distribution than any other current aerosol-based detector [83].

The dynamic range of the detector spans from greater than 1 part per thousand to below 1 part per billion [84]. The residue left after evaporation of the mobile phase effectively sets the detection limit and it is therefore important to use high purity solvents and a clean LC system. According to [82], the NQAD has a linear response over several orders of magnitude.

4. RESULTS AND DISCUSSION

4.1 Suitability of ELSD for the impurities control – analysis of the performance characteristics and appearance of spike peaks

An appropriate control of the impurities, also referred to as "related substances" is one of the major objectives in the quality control of active pharmaceutical ingredients (API) and excipients. For this reason, the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) defined in guideline Q3A(R2) on the control of impurities in new drug substances [25], a reporting threshold of 0.05% for impurities in new drug substances with an average daily dose below 2 g and 0.03% above this daily dose. Any impurity exceeding this threshold must be reported and quantified. In the European Pharmacopoeia (Ph.Eur.) [13], the limits given in this guideline were extended to all substances for pharmaceutical use obtained by chemical synthesis. However, the Ph.Eur. refers to the "reporting threshold" as "disregard limit". Consequently, methods used for the control of impurities must exhibit a limit of quantification being at least at the reporting threshold or disregard limit.

Today the most common detection technique coupled to LC is the UV detection due to its wide field of applications, its high sensitivity, the wide range of linearity and the applicability for gradient elution. However, the basic requirement for this kind of detection is a chromophore. In case of absence of a chromophore, UV detection is only possible after pre- or post-column derivatization. Unfortunately, a lot of problems arise from derivatization, e.g. assurance of complete derivatization of all substances to analyze, the purity of the derivatization reagent, the appearance of by-products, the purification from by-products and the surplus of the derivatization reagent. Luckily, several alternatives for the detection of substances without a chromophor are available: fluorometry, refractometry, amperometry and mass spectrometry. However, they all have their own limitations and special requirements.

Another detection method is by ELSD. This detector was initially applied in food and petrochemistry for all kinds of carbohydrates, polymers [85, 86, 87] and sugars [88]. Subsequently the field of application was extended to e.g. triglycerides [89], (phospho-) lipids [90] and cyclodextrins [91]. In recent years the use of ELSD also entered the field of pharmaceutical analysis and quality drug control [92], e.g.

steroids [93], amino-glycosides [63], bisphosphonates [94] and amino acids [95, 96]. As demonstrated by several authors [63, 92, 94, 97] ELSD could represent a convenient alternative detection mode. This is particularly true when the amounts of the analytes to be determined are not too different from one another, as is the case e.g. for the drug gentamicin which is composed of five closely related components of 2 to 40 percent in addition to the impurities each of less than 3 percent [93, 98, 99, 100].

Efforts were made by Kopec [96] to develop a method for the control of impurities in aspartic acid (Asp) and alanine (Ala) using a LC ion-pair chromatographic method with ELSD detection. However, non-reproducible spikes were found after the main peak in the injections of concentrated test solutions of these substances as well as in concentrated solutions of other substances, e.g. fumaric acid, spectinomycin, or caffeine. Consequently, an appropriate identification and quantification of potential impurities was not possible. An influence of the LC column could be excluded, since the spikes also appeared when injecting the test solutions without a column. Interestingly, the appearance of spike peaks hindering the detection of impurities in LC with ELSD is hardly reported in literature.

Since the tolerance of the analytical system towards high analyte concentrations is a key requirement for a possible use of the ELSD for the impurities control in "substances for pharmaceutical use", experimental work was carried out to explore the dependence of the detector sensitivity as well as the appearance of spike peaks on different eluent composition, eluent flow and ELSD settings. Therefore, all tests were performed by flow injection analysis (FIA), using standard LC equipment without an analytical column. To obtain sufficient system pressure, a restriction coil was used instead.

The concentration of the test solutions was chosen in a way that a solution at a concentration of 0.05% of the concentration of the test solution would still deliver a signal above the limit of quantification (= signal-to-noise ratio of 10). In the analysis of drug substances this limit is normally referred to as "disregard limit" since impurities below this limit usually can be disregarded whilst those above must be reported. Since it was found within the first series of experiments, that this is not always guaranteed using a 20 mg/mL test solution, the concentration was increased to 40 mg/mL. Experimental details are given in chapter 7.1.

4.1.1 Influence of the ELSD parameters and of the mobile phase composition and flow-rate on the detector response

The ELSD response depends on several parameters, i. e. the ELSD scavenger gas flow-rate, the nebulizer and evaporation (drift tube) temperature, the LC mobile phase composition and the LC flow-rate. Based on the experiments conducted it can be concluded that:

a) The ELSD used in the experiments allowed the adjustment of the scavenger gas flow-rate in the nebulizer compartment of the detector. When the scavenger gas flow was increased, the detector signal was found to decrease (cf. Fig. 4-1 and 4-2). This finding is in agreement with results obtained in direct infusion studies on olive oil [65], glycerol [101] and polystyrene [102]. The increase in the gas flow-rate causes a decrease in signal response due to the reduced particle size and the reduction in residence time of the particles within the optics.
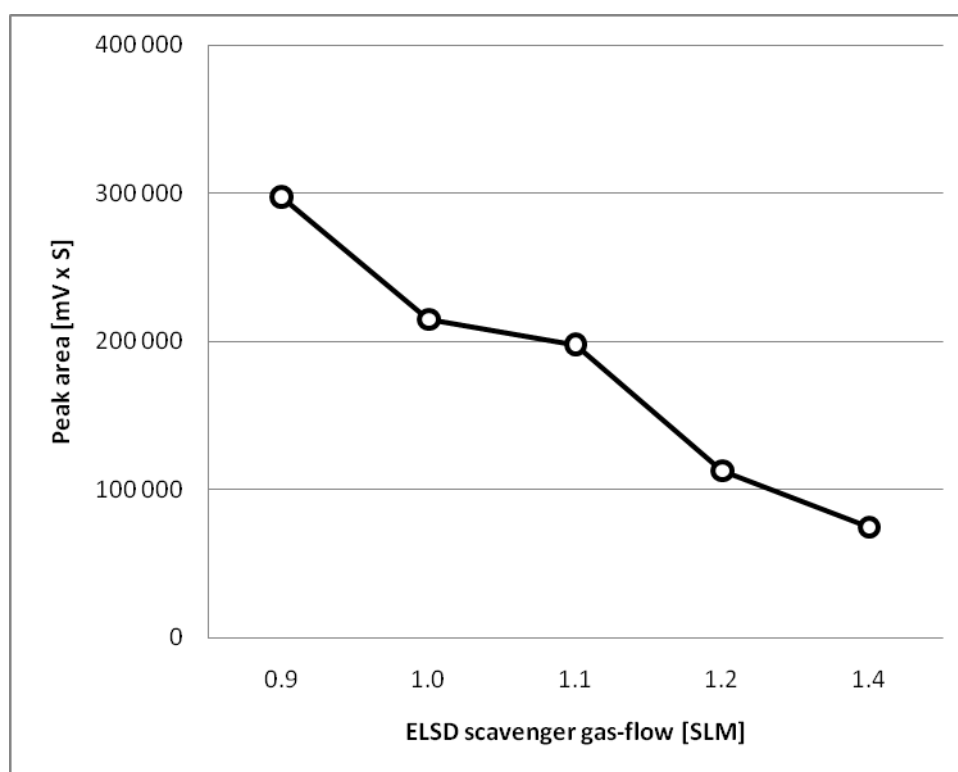
Figure 4-1. Correlation between the detector response at different scavenger gas flow-rates. Experimental conditions: Test solution: 0.02 mg/mL Asp in water; eluent flow-rate: 0.5 mL/min; eluent: water/methanol (80/20 v/v); injection volume: 40 µL; ELSD scavenger gas flow-rates of 0.9 SLM, 1.0 SLM, 1.1 SLM, 1.2 SLM and 1.4 SLM. Modified from [76].
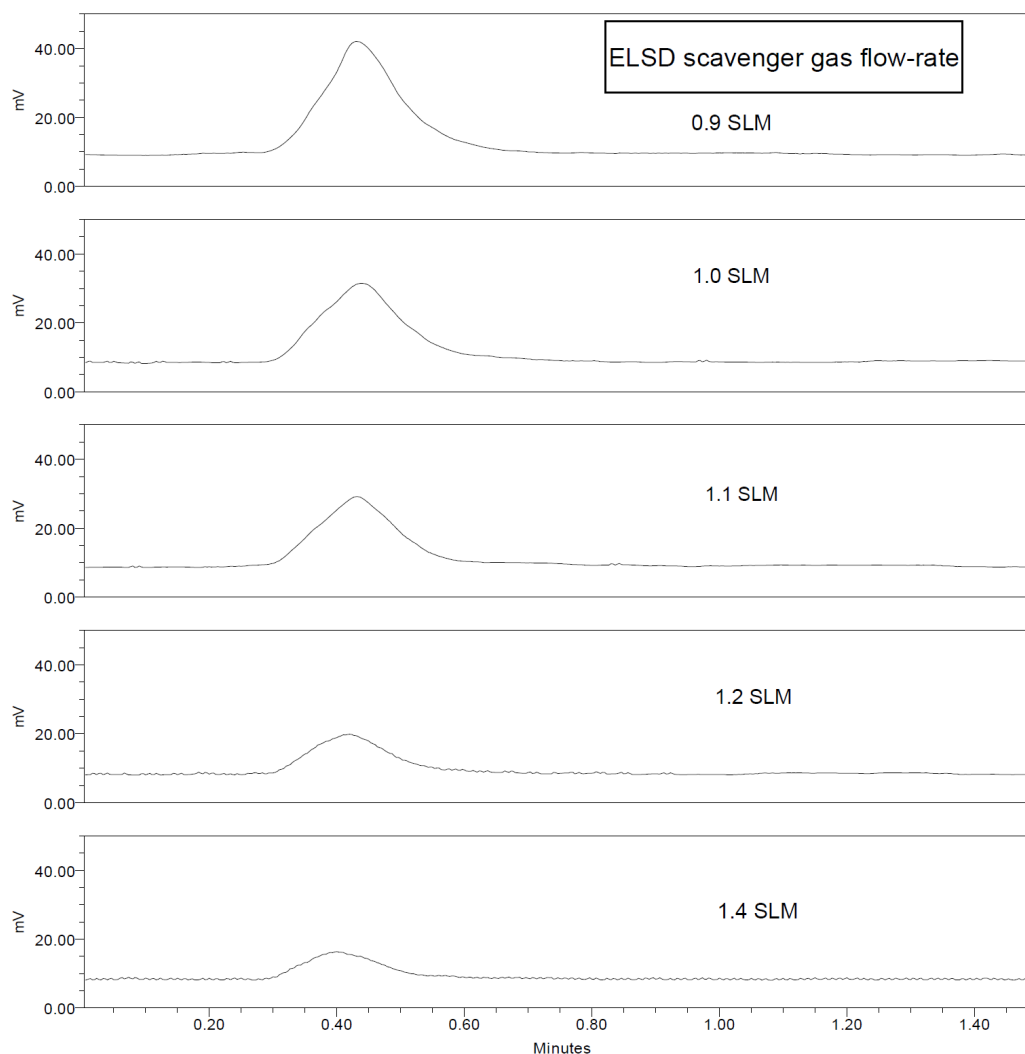
Figure 4-2. Correlation between the ELSD scavenger gas flow-rate and the peak area of Asp. Conditions as given for Fig.4-1. Signal-to-noise ratio decreases from about 200 at 0.9 SLM to about 25 at 1.4 SLM. Modified from [76].

b) As regards the impact of the drift tube temperature on the signal intensity, the highest signal was measured at 60 °C (cf. Fig. 4-3) It is assumed, that at temperatures below 60 °C the solvent evaporation is still incomplete and therefore the ELSD signal as a function of the particle size of the evaporated solid particles is low. At a temperature of about 60 °C the drying conditions seem to reach an optimum and the highest detector output is obtained. At temperatures above 60 °C the signal intensity starts to decrease again. Since aspartic acid is not volatile, evaporation of the solute can be excluded as a reason. A possible explanation for this observation is that at high drift tube temperatures too rigorous solvent volatilization causes non-uniform particle sizing or inhibits crystal formation, adversely affecting the light scattering process [101].
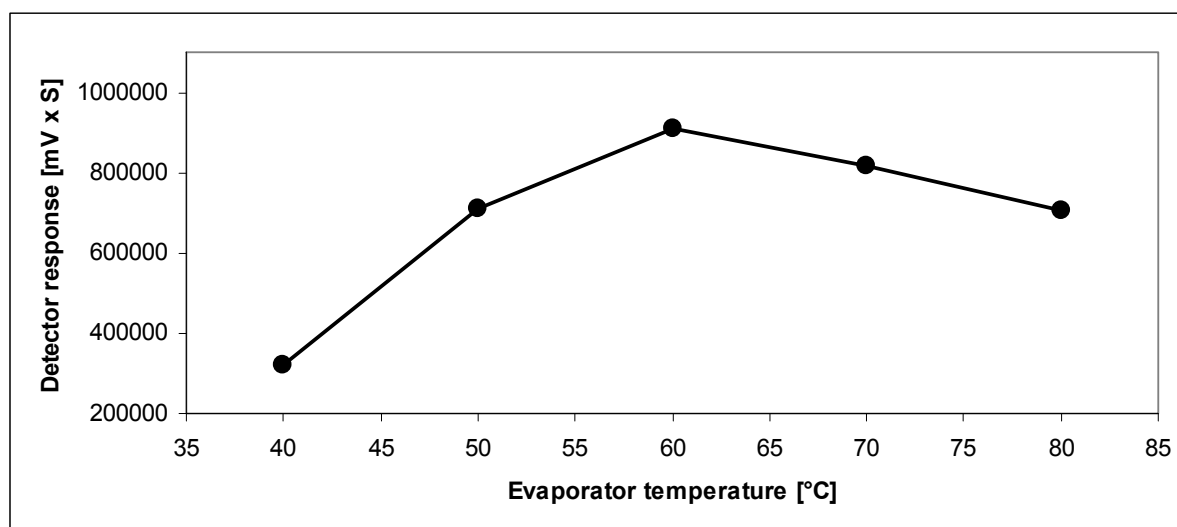
Figure 4-3. Correlation between the detector response of Asp and the evaporation (drift tube) temperature. Test solution: 0.02 mg/mL Asp in water; eluent: water/methanol (80/20 v/v); eluent flow-rate: 0.5 mL/min; injection volume: 40 µL; ELSD scavenger gas flow-rate: of 1.0 SLM: nebulizer temperature: 30 °C; evaporation temperature increased from 40 °C to 80 °C in 10 °C steps. Modified from [76].

c) The peak area decreases with increasing mobile phase flow-rate. This finding could be confirmed on two examples (cf. Fig. 4-4) a.) a 0.02 mg/mL solution of Asp employing a mixture of water/methanol (80/20 v/v) as a mobile phase and b.) a 0.02 mg/mL solution of alfadex with a mixture of water/acetonitrile (90/10 v/v) as a mobile phase. All measurements were carried out at an ELSD scavenger gas flow-rate of 1.0 SLM.

In the literature the following explanations for the reduction of the signal intensity are given: 1) incomplete solvent vaporization occurring at higher mobile phase flow-rates [101, 102], and 2) at higher mobile phase flow rates larger droplets are formed and the loss of the larger particles by impaction on the surfaces of the drift tube or spray chamber may be responsible [65].

The latter explanation is in some way contradictory to the above statement that the sensitivity is reduced when smaller droplets are formed. However, it must be kept in mind that in both cases the observed changes in the signal intensity are referred to the intensity of the signal at optimum average conditions. The reason for the signal reduction at high gas flow rates is different from the one at high mobile phase flow-rates. As explained above the possible reason in the first case is a change in the light scattering process due to reduction of the average particle size and a reduced

detector residence time whilst in the latter case the reduction of the signal intensity is caused by a loss of analyte particles in the drift tube.

d) The detector response increases as a function of the percentage of organic solvent in the mobile phase. The relation between the content of organic solvent in the mobile phase and the detector response of a 0.02 mg/mL solution of mexiletine hydrochloride is presented in Fig. 4-5. Similar results were reported for triglycerides in different organic solvents measured by FIA [68], for hydrocortisone acetate [103], and for 5-fluorocytosine [104], all using acetonitrile/water mobile phases.

An explanation of this effect is seen in the increase of the "transport efficiency" of the nebulizer with increasing amounts of organic modifier in the eluent. This means that whilst the average particle size remains constant a greater number of particles reach the detection chamber, resulting in an increased detector signal [67, 68, 103, 104, 105, 106].
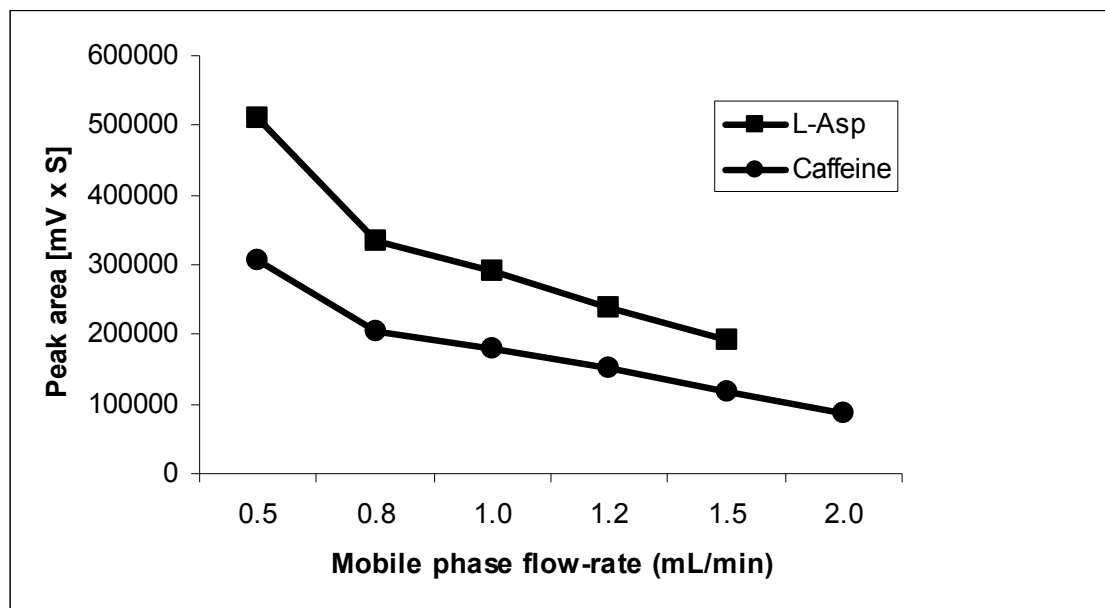
Figure 4-4. Correlation between the detector response of L-aspartic acid and alfadex and the eluent flow rate. Experimental conditions: 0.02 mg/mL solutions of Asp and alfadex both dissolved in water. ELSD settings as described in experiment 7.1.3.2 eluent composition for Asp: water/methanol (80/20 v/v); for alfadex: water/acetonitrile (90/10 v/v). The tests with water/methanol were performed injecting 40 µL at eluent flow rates of 0.5 mL/min, 0.7 mL/min, 0.8 ml/min, 1.0 mL/min, 1.2 mL/min and 1.5 mL/min; for water/acetonitrile a flow rate of 2.0 mL/ min was tested in addition. Modified from [76].
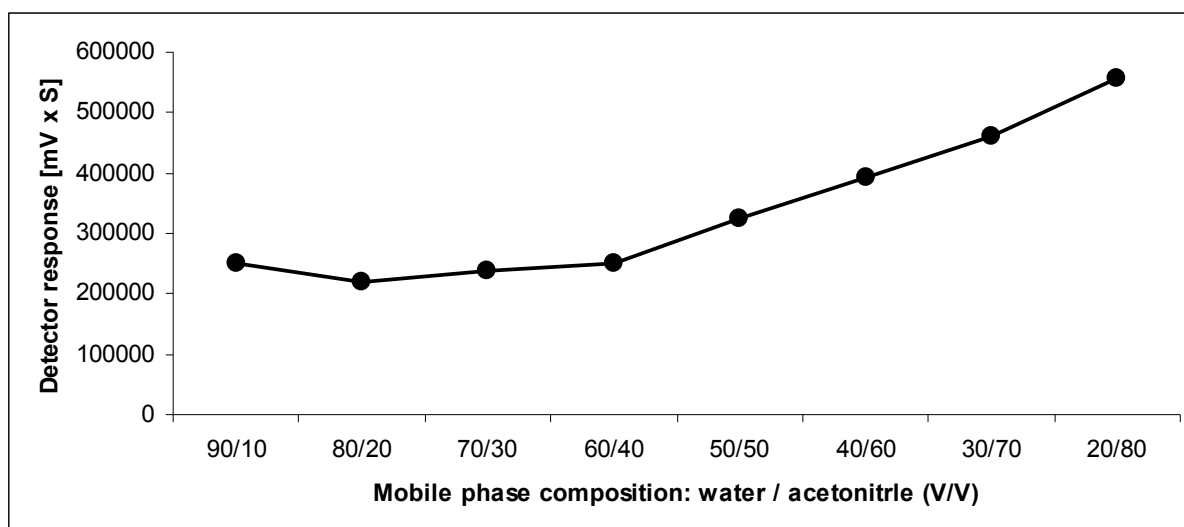
Figure 4-5. Correlation between the eluent composition and the detector response. Conditions: 0.02 mg/mL solution of mexiletine HCl in water/methanol (50/50 v/v); injection volume: 40µL, eluent flow-rate: 0.5 mL/min; eluent composition modified in 10% (v/v) steps from water/acetonitrile (90/10 v/v) to water/acetonitrile (20/80 v/v); ELSD scavenger gas flow-rate: 1.0 SLM; nebulization and evaporation temperature: 50 °C. Modified from [76].

4.1.2 Influence of the ELSD parameters and of the mobile phase composition and flow-rate on the appearance of spike peaks

As it has been demonstrated under 4.1.1, the ELSD settings as well as the mobile phase composition and mobile phase flow may have a significant impact on the detector sensitivity. Therefore, a requirement for the following experiments was that a 0.05% dilution of the concentrated test solution still delivers a peak above the limit of quantification. On the one hand this criterion was introduced to ensure that appearance/disappearance of spikes is not simply a function of the method sensitivity and on the other hand the aim was to demonstrate that the chosen sample concentration would be suitable to control impurities in a pharmaceutical substance according to Q3A(R2) [25]. In the following, the influence of different ELSD settings as well as of different mobile phase compositions and flow-rates on the appearance of spike peaks was investigated.

4.1.2.1 Influence of the mobile phase composition

A strong increase of spike peaks was found when analyzing a 40 mg/mL solutions of Asp and mexiletine hydrochloride (cf. Fig.4-6) with increasing amounts of organic modifier in the eluent (cf. 7.1.3.1). Since the experiments were performed as FIA

(without analytical column), these peaks cannot be due to separated impurities, but must be related to the substance itself.
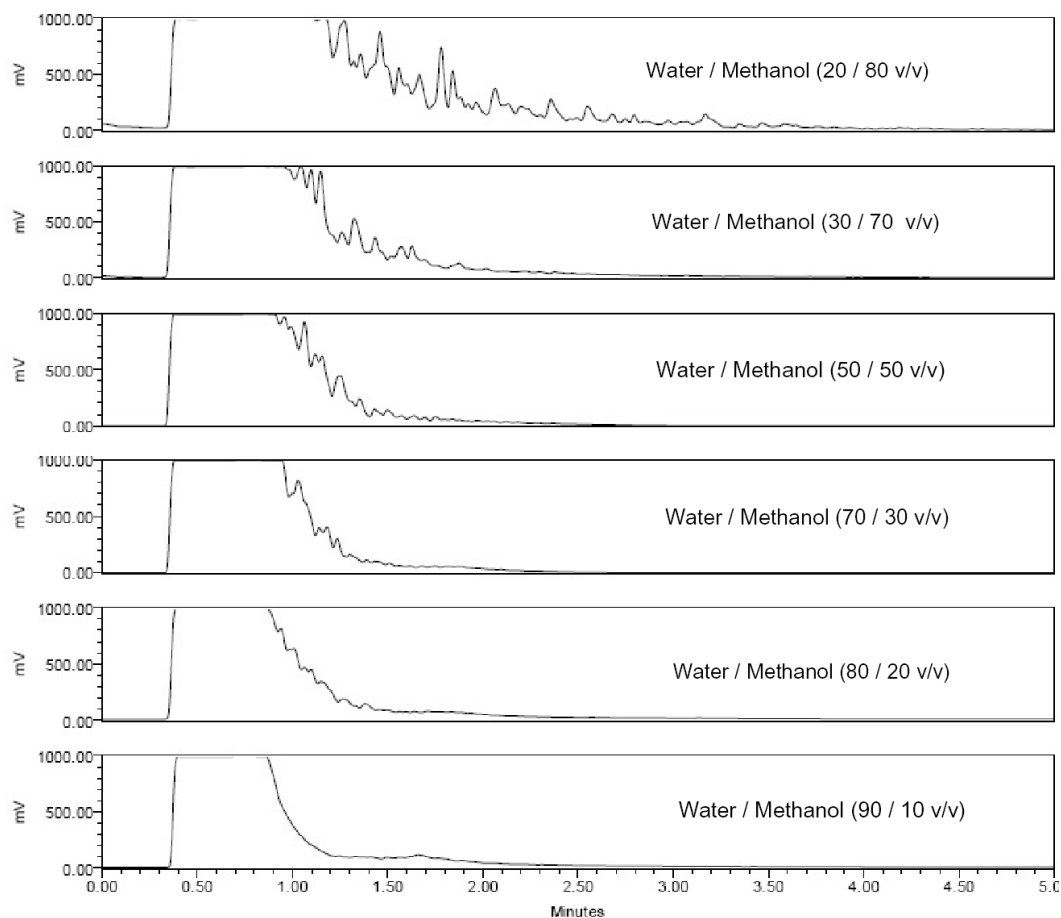
Figure 4-6. Impact of the eluent composition on the occurrence of spikes, i. e. water/methanol ratio at 40 mg/mL mexiletine HCl. Conditions: eluent flow-rate: 0.5 mL/min; eluent composition modified in 10% (v/v) steps from water/acetonitrile (90/10 v/v) to water/acetonitrile (20/80 v/v); injection volume: 40 µL,; ELSD scavenger gas flow-rate: 1.0 SLM; nebulization and evaporation temperature: 50 °C. Modified from [76].

The injections were carried out at a relatively low eluent flow-rate of 0.5 mL/min and a scavenger gas flow-rate of 1.0 SLM. It is assumed that under these experimental conditions the occurance of the spikes is caused by an improper nebulization resulting in the formation of large droplets. These droplets may not be fully evaporate in the drift tube and eventually appear as spike peaks in the detector [65,107]. Increasing amounts of organic solvent in the mobile phase improve the evaporation and less large droplets are lost due to impaction on the drift tube walls. Since more of the larger droplets reach the detector, spike peaks are much more marked at high concentrations of organic solvent than in highly aqueous eluents.

4.1.2.2 Influence of the mobile phase flow-rate

The impact of the mobile phase flow-rate on the appearance of spike peaks was investigated using 40 mg/mL test solutions of Asp, alfadex and mexiletine hydrochloride. Using either a mixture of water/methanol (80/20 v/v) or water/acetonitrile (90/10 v/v) at an eluent flow-rate of 0.5 mL/min (cf. 7.1.3.2) several spikes occurred on the tail of the main peaks in each test solution. In contrast, no spikes were found when injecting either water as a blank or a 0.05% dilution of the test solution.

To examine the influence of the mobile phase flow-rate, injections at increasing flow-rates, were conducted: 0.5 mL/min, 0.7 mL/min, 0.8 mL/min, 1.0 mL/min, 1.2 mL/min and 1.5 mL/min for water/methanol. For water/acetonitrile as a mobile phase, a flow-rate of 2.0 mL/min was tested in addition.

A significant decrease or even a disappearance of the spike peaks was found at LC flow-rates of 0.7 mL/min or higher, when the mobile phase composition was water/methanol (80/20 v/v) or water/acetonitrile (90/10 v/v). At flow-rates of 1.0 mL/min and 1.2 mL/min, spike peaks occurred in none of the injections. Using in turn, a mobile phase with a high amount of organic modifier, e.g. water/methanol (20/80 v/v), a significant reduction of the spike peaks was possible when the eluent flow-rate was increased. However, in this case the minimum flow-rate required was 1.0 mL/min (cf. Fig. 4-7).

It is believed that at the selected ELSD gas flow-rate of 1.0 SLM and low mobile phase flow-rates the nebulizer is not working properly to form an appropriate aerosol. Large droplets may occasionally be generated resulting in spikes in the detector response [108].
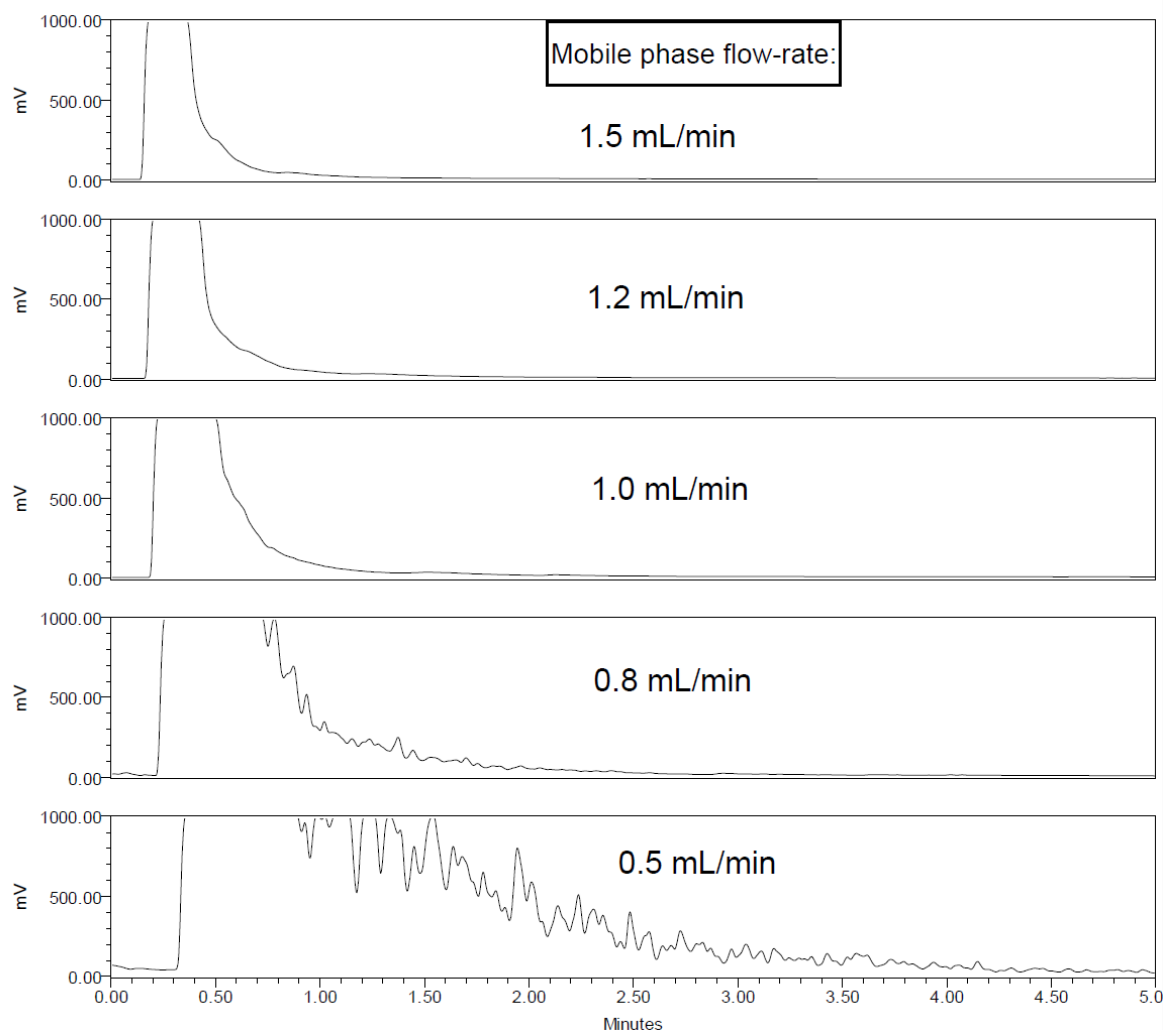
Figure 4-7. Decrease of spike peaks in a 40 mg/mL test solution of mexiletine HCl using water/methanol (20/80 v/v) as an eluent at increasing LC flow-rates. Conditions: ELSD settings as described in 7.1.3.2. Injection volume: 40 µL; mobile phase flow-rates: 0.5 mL/min, 0.8 mL/min, 1.0 mL/min, 1.2 mL/min and 1.5 mL/min. Modified from [76].

4.1.2.3 Influence of the ELSD gas flow-rate

Test solutions containing 40 mg/mL of alfadex and Asp were analysed at different combinations of mobile phase flow-rates and ELSD scavenger gas flow-rates using a mixture of water/methanol (80/20 v/v) as an eluent. (cf. 7.1.3.3). At ELSD gas flow-rates of 0.9 SLM and 1.0 SLM spikes eluting after the major peak were systematically detected. However, at a gas flow-rate of 1.1 SLM no spikes were detected in three successive injections of the Alfadex and Asp test solutions. When the gas flow-rate was further increased to 1.2 SLM spike peaks occurred in one out of three injections of Asp and to some minor spikes in two out of three injections of the Alfadex solution. At a gas flow-rate of 1.4 SLM spikes were found in the three injections of the Asp

solution. In contrast, no spike peaks were detected when the alfadex solution was injected. Furthermore, a spike was found, in one out of three injections of the Asp test solution at a combination of 1.2 mL/min of mobile phase flow-rate and 1.4 SLM scavenger gas flow-rate. Fig. 4-8 shows the occurrence of spike peaks in the Asp test solution at different ELSD gas flow-rates for a mobile phase flow-rate of 0.5 mL/min.
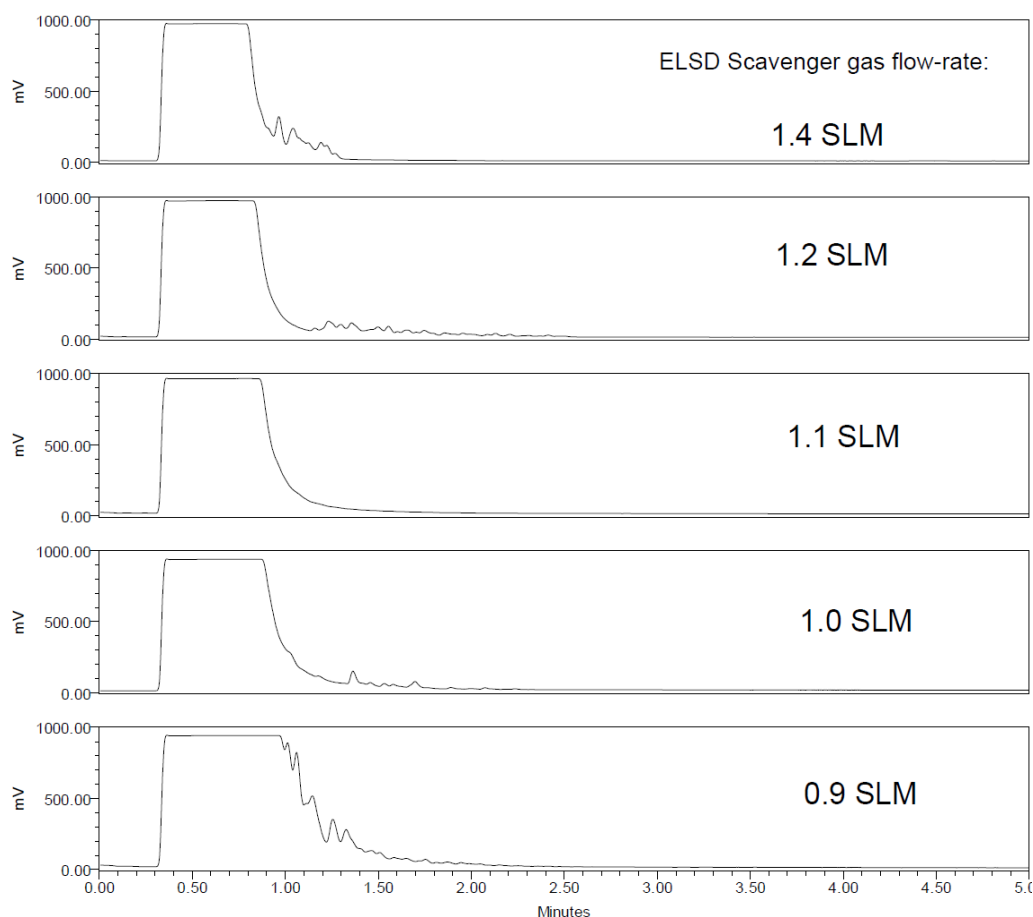
Figure 4-8. Occurrence of spike peaks at different ELSD gas flow-rates for a 40 mg/mL test solution of Asp employing an eluent with low amounts of organic modifier. Conditions: injection volume: 40 µL; eluent: water/methanol (80/20 v/v); flow-rate: 0.5 mL/min; ELSD nebulization and evaporation temperature. 50 °C; ESLD gas flow-rates: 0.9 SLM, 1.0 SLM., 1.1 SLM, 1.2 SLM and 1.4 SLM. Modified from [76].

For eluents containing high amounts of organic modifier, the reduction of spike peaks by increasing the scavenger gas flow-rate was found to be much more difficult. Using a mixture of water/methanol (20/80 v/v) as an eluent, a significant reduction of spike peaks for a 40 mg/mL solution of mexiletine HCl was only obtained when a scavenger gas flow-rate of 2.2 SLM was employed (cf. Fig. 4-9).
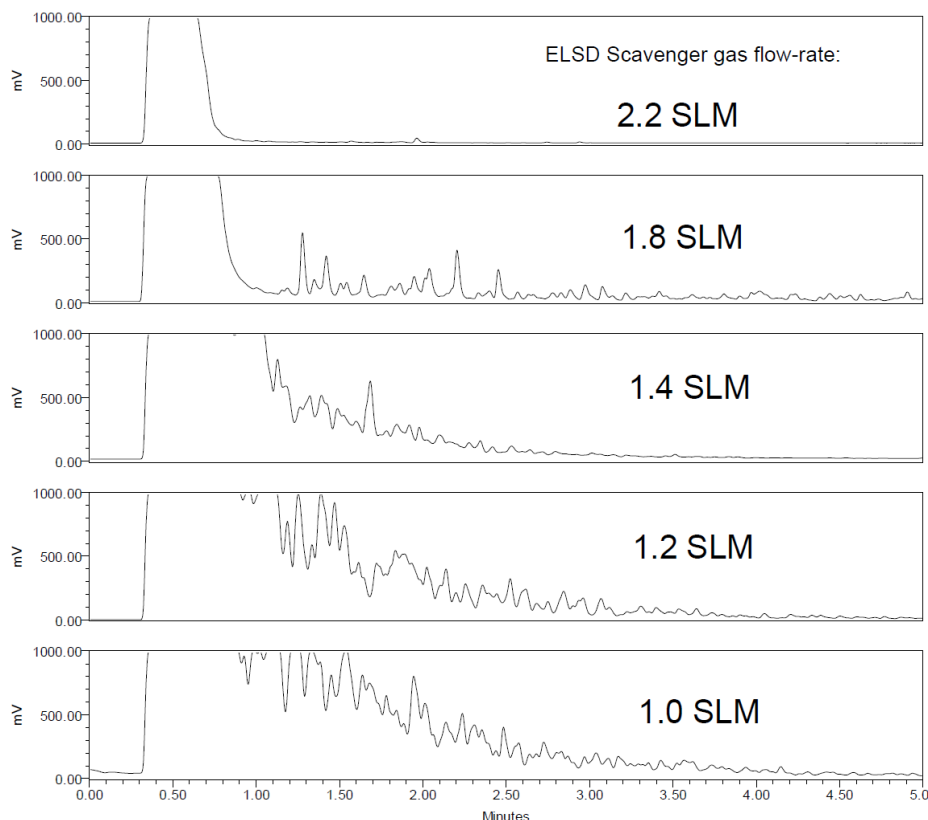
Figure 4-9. Spike peaks at different ELSD gas flow-rates for 40 mg/mL test solution of mexiletine HCl using a mobile phase with high amounts of organic modifier. Conditions: injection volume: 40 µL; eluent: water/methanol (20/80 v/v); eluent flow-rate: 0.5 mL/min; ELSD nebulization and evaporation temperature. 50 °C; ESLD scavenger gas flow-rates: 1.0 SLM, 1.2 SLM, 1.4, 1.8 SLM and 2.2 SLM. Modified from [76].

However, as pointed out above, the reduction of the spikes at high scavenger gas flow-rates goes together with a significant reduction of the sensitivity: A 0.05% dilution of the mexiletine HCl test solution was found below the detection limit.

It is assumed that the reason for the spike peaks is the same as discussed under 4.1.2.1; i.e. under the selected conditions large droplets are generated due to improper nebulization. Consequently, the evaporation of the solvent in the drift tube is incomplete, which eventually results in the occurrence of spike peaks on the tail of the main substance signal. Because the number of large droplets reaching the detector is higher at increased amounts of organic modifier, this phenomenon is much more pronounced in mobile phases containing high amounts of organic solvents compared to highly aqueous eluents.

4.1.2.4 Influence of the nebulizer and evaporation (drift tube) temperature.

The detector used in this study permitted an independent adjustment of the nebulizer temperature and the drift tube (evaporation) temperature. In the two sets of experiments described in 7.1.3.4, the impact of different temperature settings on the appearance of spike peaks was studied. In the first series of experiments both, the nebulizer and the drift tube temperatures were altered. Subsequently, the nebulizer temperature was kept at a constant low value and only the drift tube temperature was changed. However, concerning the effect on the signal intensity and on the occurrence of spikes, no differences were found.

Regarding the occurrence of spikes when injecting a 40 mg/mL solution of Asp using the conditions described under 7.1.3.4, only a limited influence of the drift tube temperature was noticeable. Slightly less spikes were found up to a temperature of 60 °C, but even when the evaporation temperature was further increased up to 80 °C, they did not completely disappear (cf. Fig. 4-10).
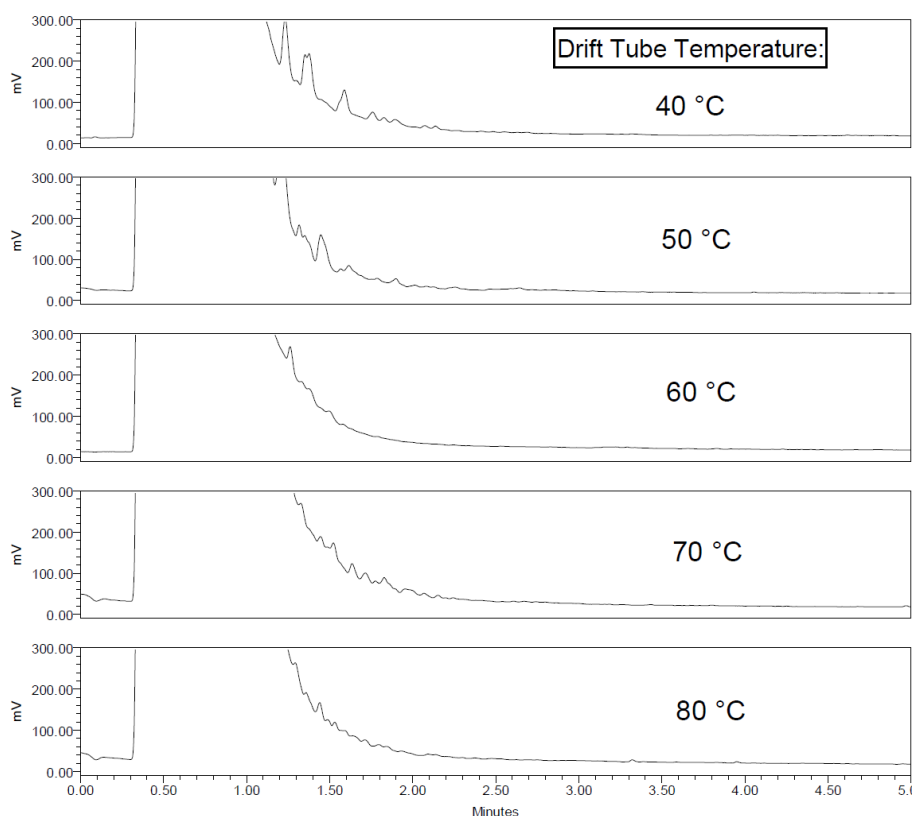
Figure 4-10. Occurrence of spike peaks at drift tube temperatures from 40 °C to 80 °C. Conditions: Test solution: 40 mg/mL Asp; eluent: water/methanol (80/20 v/v); eluent flow-rate: 0.5 mL/min; injection volume: 40 µL; ELSD gas flow-rate: 1.0 SLM; nebulizer temperature: 30 °C; evaporation temperature: increased from 40 °C to 80 °C in 10 °C steps. Modified from [76].

The slight reduction of spikes at increased temperatures may be due to an improved drying of the nebulized eluate. However, for analysis involving volatile substances or – as in the control of impurities in pharmaceutical substances – substances of unknown structure – an increase of the evaporation temperature is not an option. In these cases, the lowest possible temperature ensuring evaporation the eluent should be selected.

4.1.3 Conclusion

It was found that the selected ELSD settings as well as the mobile phase composition and flow-rate can have a significant impact on method sensitivity. A careful selection and adjustment of these parameters may therefore help to improve the quantification limit of a given method. Unfortunately, most parameters improving the method sensitivity foster at the same time the occurrence of spikes eluting on the tail of the principal peak. Based on the results of this study, it can be concluded that the occurrence of spikes is particularly a problem when mobile phases with high amounts of organic modifier are employed and when low LC flow-rates and ELSD gas flow-rates are used. In the case of mobile phases with high percentages of organic modifier, an increase of the LC flow-rate was found to be more effective to reduce spike peaks than a modification of the ELSD gas flow-rate. For the detector used in this study it can be concluded that even for mobile phases containing high amounts of organic modifier the application of eluent flow-rates at or above 1.0 mL/min together with ELSD scavenger gas flow-rates of at least 1.1 SLM were suitable measures to avoid the occurrence of spikes even for analytes in highly concentrated solutions.

Taken together, the development of a robust LC-ELSD method for the control of impurities in drug substances both ELSD and LC parameters must be carefully selected to identify the best compromise between the avoidance of spike peaks and appropriate method sensitivity.

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4.2 Control of impurities in L-aspartic acid and L-alanine by liquid chromatography coupled with a corona charged-aerosol detector

Amino acids belong to the most widely used biological compounds e.g. in the fields of nutrition, cosmetics, agriculture and medicines [109]. In the latter field amino acids are widely used in "classical" medicinal applications including the parenteral nutrition of patients with insufficient renal clearance, liver insufficiency, in the paediatric domain or the use of certain amino acids like tryptophan because of their specific pharmacological effects [110, 111] in medicines against depression and as sleep inducing substances [112]. Moreover, they are also of interest for "alternative" medicinal treatment (e.g. amino acids in whitmania pigra used in traditional Chinese medicine (TCM) [113].

Based on their significant use in the fields of nutrition and medicinal products a proper control of the quality of the amino acids is of crucial importance for the consumer or patient.

Unfortunately, due to their physico-chemical properties, i. e. the lack of a chromophor in most of the amino acids, their analysis and especially the purity control of low level impurities is a particular analytical challenge and no analytical method has yet been found which is superior to all the others [95].

This is probably one of the major reasons, why in Pharmacopoeia monographs [4, 5] amino acids are still controlled by a thin layer chromatography (TLC) test for ninhydrin-positive substances, accompanied by a limit test for ammonia instead of a high-performance liquid chromatography method (HPLC) for related substances as it is a common standard for the quality control in most other pharmacopoeial monographs of active pharmaceutical ingredients (APIs).

In industry the purity of amino acids is usually controlled using Amino-Acid-Analysers (AAA). The analysis is based on ion-exchange chromatography, normally using complex gradients, followed by post-column derivatisation with ninhydrin, dinitrophenylhydrazone (DNP) or other suitable reagents. The major disadvantage of these methods, apart from the fact that AAA-instruments are not broadly available outside some specialised laboratories, is that impurities other than amino acids are not detected. In some cases, especially for amino acids produced by enzymatic

synthesis, an additional ion exchange chromatography method is employed to control residues of organic acids used as starting materials.

However, the paramount importance of having a general test for related substances became evident in 1989 when it was hypothesized that one or more trace impurities produced during the manufacture of tryptophan might have been responsible for the outbreak of a disabling autoimmune illness called eosinophilia-myalgia syndrome (EMS) leading to the death of several patients [114, 115].

Considering the above, it was concluded that the development of tailor-made related substances test for the individual amino acids would be a step forward in the quality control of amino acids. This specific related substances test should take into account the real impurity profile including – in contrast to AAA – also impurities other than amino acids. Following this idea, Asp and Ala were selected as examples.

For an appropriate design of the corresponding methods it was important to know the possible ways of production/synthesis. In principle, four different routes are used for the industrial production of amino acids. These are chemical synthesis, hydrolysis of proteins/peptides followed by chromatographic separation, enzymatic synthesis and fermentation [116, 117, 118]. For Asp a chemical synthesis was reported [119], but does not have practical relevance for industrial production. Moreover, different fermentation methods were described [120, 121] and it is also possible to obtain Asp as a product of protein hydrolysis [118]. According to available information, enzymatic production of Asp and Ala starting from fumaric acid currently appears to be the predominant means of production [117, 122, 123].

As described above, a biological product like Asp can be obtained using rather different processes with numerous possible impurities. For this reason, the European Pharmacopoeia Commission has introduced the general monograph on products of fermentation [15]. This monograph applies general rules for the quality of a product obtained by fermentation, defined in a general manner. These include inactivation or removal of the producer micro-organism, purification processes, residues from the producer micro-organism, culture media, substrates and precursors. Therefore, in practical terms, the related substances control in the substance specific monograph can be limited to certain specific impurities.
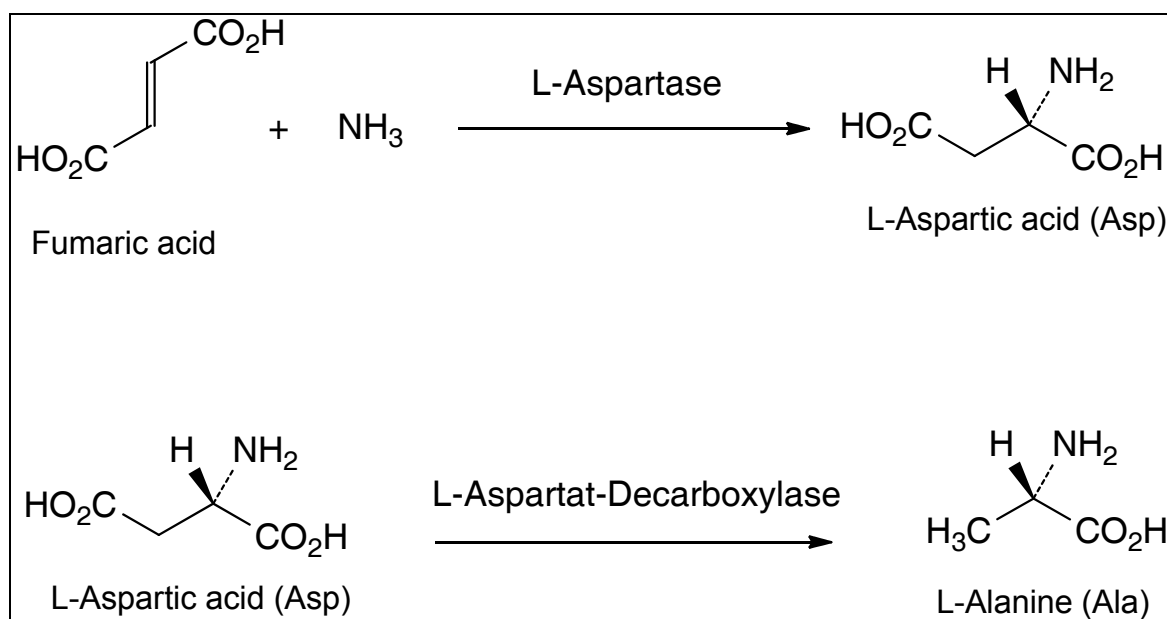
Figure 4-11. Enzymatic production of Asp and Ala starting from fumaric acid.

For Asp obtained by enzymatic production possible impurities are a) fumaric acid as a starting material, b) maleic acid as an impurity of fumaric acid, c) malic acid which may be produced from fumaric acid by enzymatic reaction, and d) alanine (Ala) as a decarboxylation product of Asp. In case of a production of Asp by protein hydrolysis glutamic acid (Glu) could possibly occur as a by-product. Since Glu and Asp are acidic amino acids, it is possible that Glu is not completely removed by a chromatographic purification step [118]. The amino acid Ala is also easily accessible by enzymatic synthesis using Asp as a starting material [123]. Therefore, the impurity profile of Ala produced in this way should be similar to the one described above, but also includes Asp as a potential impurity.

Although an increasing number of papers about the CAD are being published in literature, Nováková et al. [124] reported that pharmaceutical applications of the CAD are still rare. The aim of this study was to develop and validate a LC method using a CAD for the control of related substances in Asp and Ala. The method should ensure the appropriate control of possible impurities – often referred to as related substances – on an ICH [25] conform level for drug substances with an average daily dose above 2 g – hence, a reporting threshold of 0.03%.

Moreover, several batches of pharmaceutical grade Asp and Ala obtained by different manufacturers / suppliers together with samples of reagent grade Asp and Ala were tested using this new method.

To the best of my knowledge, this is the first time that a single related substances method simultaneously covering related amino acids as well as other process related impurities (organic acids) has been successfully employed. This method is therefore considered to be an important improvement compared with the TLC test for ninhydrin-positive substances currently published in the Pharmacopoeias. Also compared with the AAA-method used by amino acid manufacturers the described LC-CAD method is considered to be very favourable.

4.2.1 Method development

As discussed above Asp may contain the following non-volatile organic impurities:

- L-Alanine (Ala)
- L-Glutamic acid (Glu)
- Fumaric acid
- Maleic acid
- Malic acid

In addition to the above compounds the following organic acids (stemming from the citrate cycle [125] and structurally related amino acids were included in the method development and validation:

- Citric acid
- Succinic acid
- L-Glutamine (Gln)
- L-Asparagine (Asn)

For the development of a reversed phase ion chromatography method capable of separating amino acids and organic acids it must be kept in mind that different principles of separation will contribute to obtain the desired separation of the different substance.

Sufficient acidity of the mobile phase is a key requirement for such a "mixed mode" separation. The mobile phase pH must on the one hand ensure protonation of the amino acids to allow interaction with the ion pair reagent as described under 3.1.2.2. On the other hand it must also guarantee that the organic acids do not dissociate and will therefore interact with the reversed phase surface of the column.
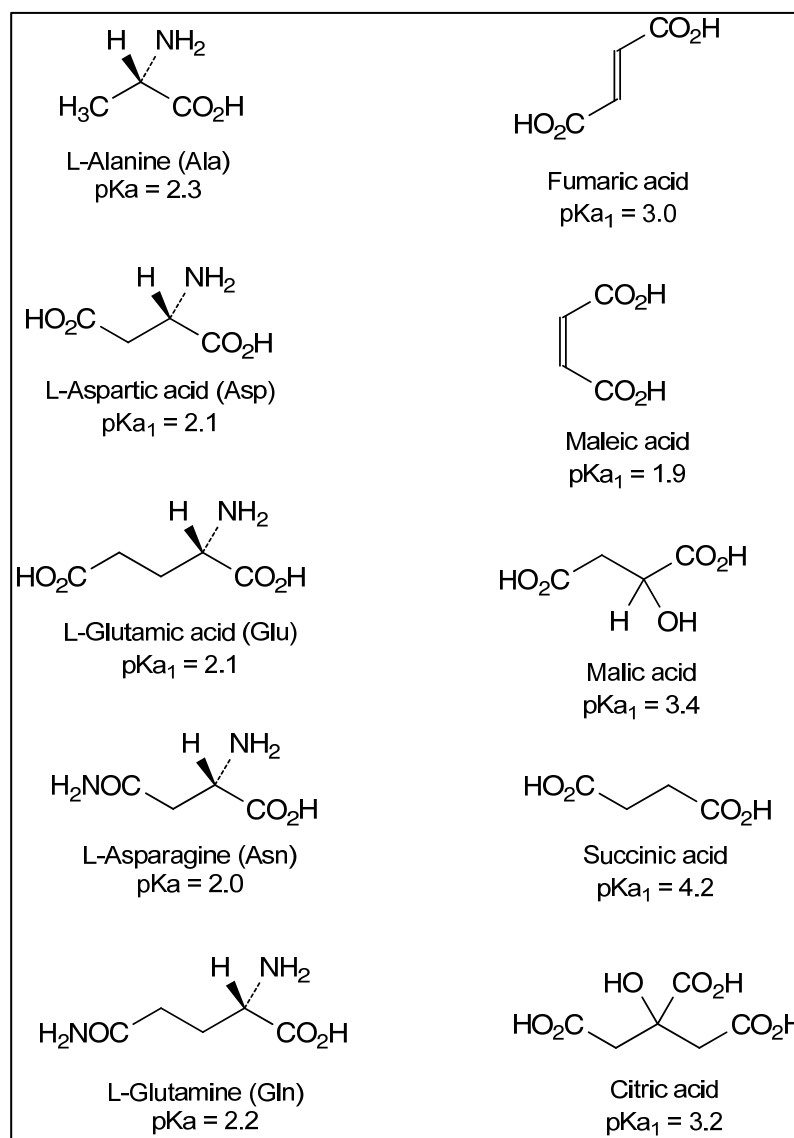
Figure 4-12. Structures and pKa values of selected amino- and organic acids.

Whilst a suitably low pH could be obtained by the addition of acidic ion-pair reagent to the mobile phase, the major challenge was to find a suitable compromise between coverage of the C18-surface of the LC-column allowing separation of the amino acids but leaving at the same time sufficient column surface uncovered to enable retention and separation of the organic acids on the reversed phase column surface.

Attempts were made by Kopec [96] to develop a LC method for the separation of amino acids and organic acids using heptafluorobutyric acid (HFBA) as ion-pair reagent. Most promising results for the separation of different organic and amino acids at 0.01 mg/mL level were reported using a mixture of 0.5% of HFBA in water and 0.3% HBFA in methanol (83/17 v/v).

A series of experiments was performed to verify if the use of HFBA could be a viable option for method development. As recommended by Kopec, a Synergi Hydro RP 18 column (250 mm x 4.6 mm, 5 µm) was used for the testing. The concentration of HFBA was varied between 0.2% and 0.8% in mobile phases containing methanol / water between 98/2 v/v and 70/30 v/v. The best separation was obtained at 0.4% HFBA in water/methanol 85/15 v/v.

However, as shown in Figure 4-13, several compounds partly co-eluted and the shape of the malic acid peak was found to be distorted.
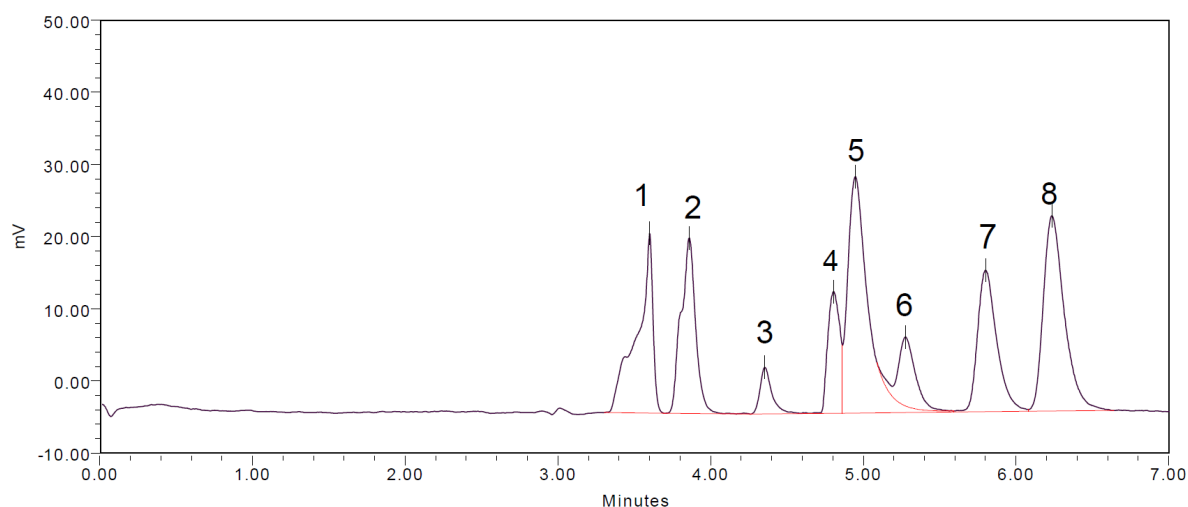
Figure 4-13. Separation of a model-mixture of 1. malic acid (0.02 mg/mL), 2. maleic acid (0.02 mg/mL), 3. citric acid (0.01 mg/mL), 4. succinic acid (0.02 mg/mL), 5. Asn (0.01 mg/mL - overlapping with a system peak), 6. Asp (0.01 mg/mL), 7. Glu (0.01 mg/mL), 8. Ala (0.01 mg/mL). Chromatographic conditions: Synergi Hydro-RP column (250 mm x 4.6 mm, 5 µm), column temperature 30 °C, flow rate 0.8 mL/min, injection volume 40 µL, mobile phase 0.4% HFBA in water / methanol 85/15 v/v. Detection by CAD.

Taking into account a relative density of HFBA of 1.65 g/mL and a molecular mass of 241 u, a concentration of 0.4% of HFBA in the mobile phase corresponds to approximately 31 mmol/L.

Likely, this amount of HFBA is too high to leave sufficient uncovered C18 column surface for a good separation of the organic acids, but still far too low to provide good separation of the amino acids based on an ion-pair mechanism. For this reason, it was decided to continue the method development with an ion-pair reagent having a longer alkyl chain. Therefore, perfluoroheptanoic acid (PFHA) was selected for further method development.

First trials were performed staying with a Synergi Hydro RP 18 column (250 mm x 4.6 mm, 5 µm) and using 10 mmo/L (3.64 g/L) of PFHA in a mobile phase containing

methanol and water. As before, different amounts of organic modifier were tested. Moreover, the column temperature was varied (cf. 7.2.3.2).

Best results were obtained using a mobile phase containing 2% of methanol as organic modifier. However, under all conditions tested the organic acids were hardly retained and basically not separated (cf. Fig. 4-14).
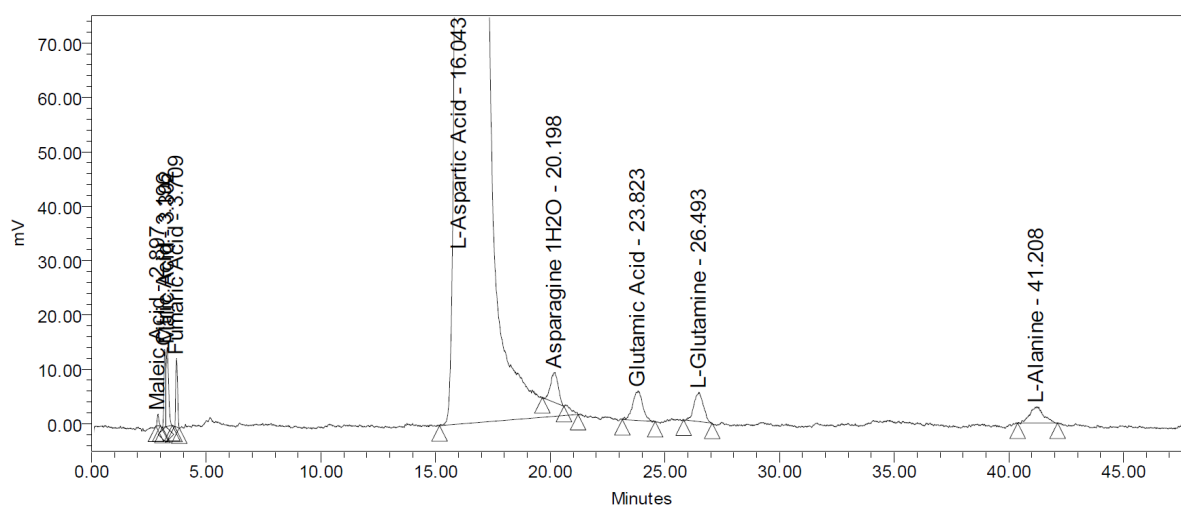
Figure 4-14. Separation of a solution containing 5 mg/mL solution of Asp spiked with 0.2% of malic acid, maleic acid and succinic acid, citric acid, fumaric acid, Asn, Ala, Glu and Gln in water: Chromatographic conditions: Synergi Hydro-RP column (250 mm x 4.6 mm, 5 µm), column temperature 20 °C, flow rate 0.8 mL/min, injection volume 40 µL, mobile phase 10 mmol/L PFHA in water / methanol 98/2 v/v. Detection by CAD.

The results found confirmed the assumption that for a good separation of both organic and amino acids an optimum equilibrium between ion-pair and reversed phase separation mechanism is required.

Since the separation of the amino acids by ion-pair chromatography was found satisfactory, the amount of ion-pair reagent was gradually reduced to allow also separation of the organic acids. Eventually, best separation of the organic acids was achieved using a mobile phase containing 1 mmol/L of PFHA in 98% water and 2% methanol (v/v). A representative chromatogram is depicted in Fig. 4-15.
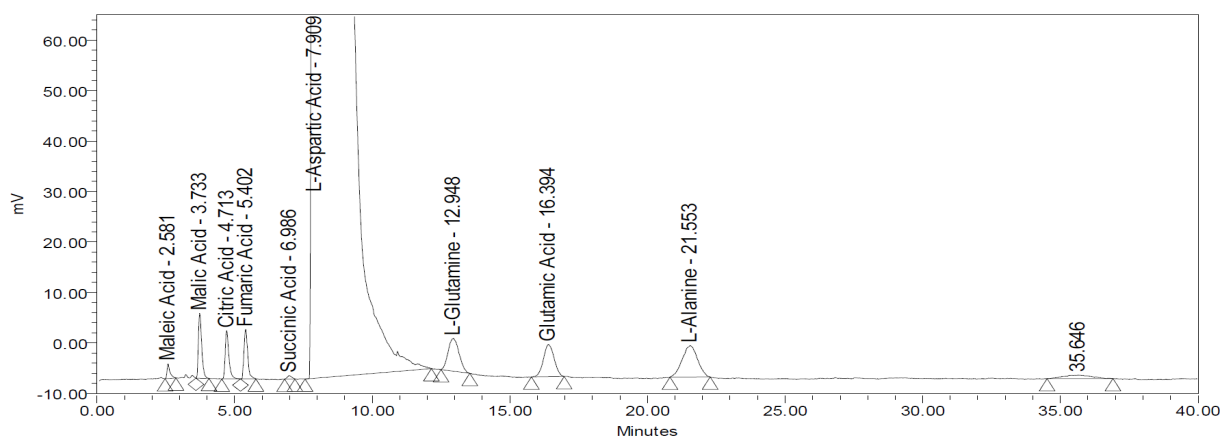
Figure 4-15. Separation using 1 mmol/L PFHA in water / methanol 98/2 v/v. Conditions and solutions as described in Fig. 4-14.

The only drawback was that Asn was no longer separated from Asp.

To overcome this problem it was tired to replace PFHA by perfluorononanoic acid (PFNA). However even at a low concentrations of 0.2 mmol/L of PFHA in water / methanol (95/5 v/v) on the one hand most of the organic acids were not separated and on the other hand the retention times of the amino acids became very long (about 35 min. for Ala). For this reason and because Asn was not an expected impurity for Asp and has only been added to verify method performance, it was decided to continue with the fine tuning and validation a method using PFHA.

Nevertheless, LC-MS/MS analysis was performed using several batches of pharmaceutical grade aspartic acid to determine the amounts of possibly present Asn (cf. 7.2.3.5). It was found that some trace amounts of Asn could be detected. However, as shown in Table 4-1, the amounts found were always clearly below the disregard limit of 0.03% according to the corresponding ICH guideline [25]. Therefore, this was not considered to be problematic for the method.

Table 4-1. Results of the LC-MS/MS quantification of Asn in 5 samples of pharmaceutical grade Asp.

Sample	Asn (in % - referred to Asp)
1	0.0150%
2	0.0099%
3	0.0089%
4	0.0087%
5	0.0098%

As pointed out before, better detector sensitivity is obtained at increased amounts of organic modifier in the mobile phase. Moreover, higher amounts of organic modifier shorten the overall method run-time, which makes the method more attractive for the use in industries. Taking these elements into account, it was eventually decided after further experiments to increase the amount of organic modifier in the mobile phase from 2% (v/v) to 4% (v/v). As can be seen in Fig. 4-16, the separation of Asp from its supposed related compounds using an Inertsil ODS 3 column (150 mm x 4.6 mm; 5 µm) at slightly modified LC conditions (LC flow rate 1.0 mL/min, column-temperature 30 °C) is still assured. However, at these conditions the last substance (Ala) elutes already at 13.2 min instead of 21.5 min. The apparently reduced resolution between Asp and Gln is mainly attributed to the fact, that the amount of Asp was increased from 5 mg/mL to 10 mg/mL.
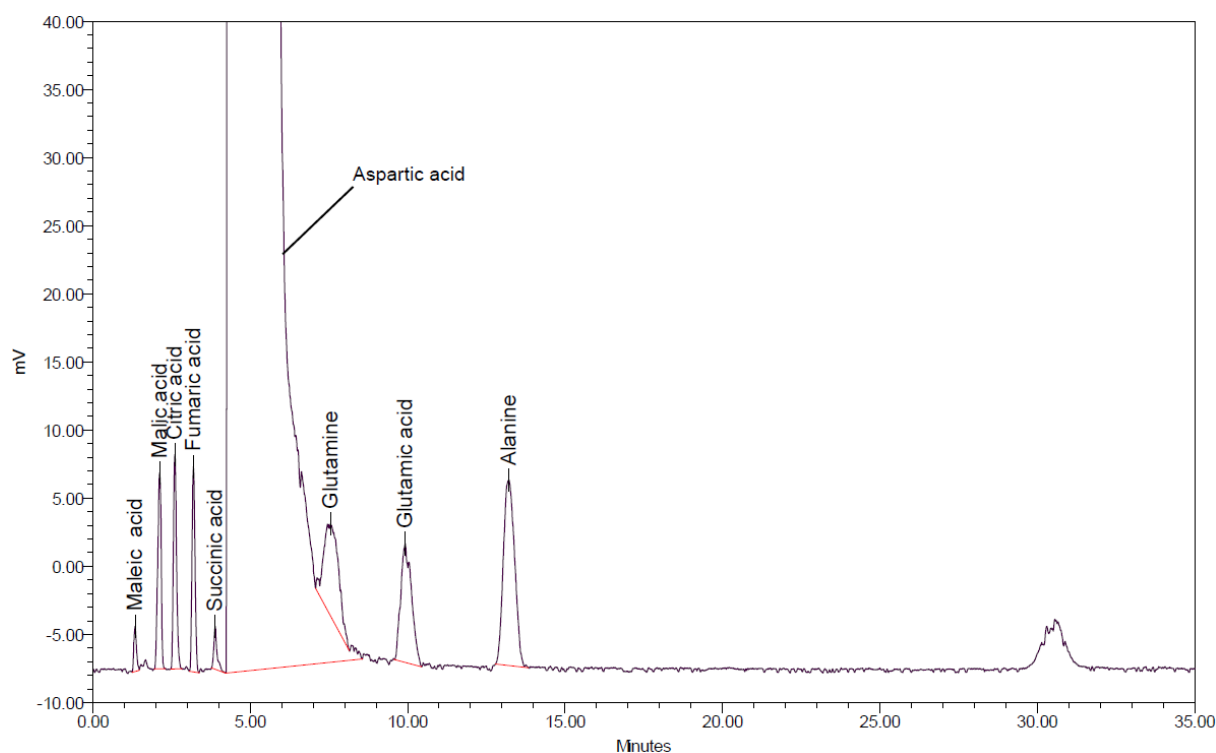
Figure 4-16. Injection of a 10 mg/ml solution of Asp spiked with about 0.1% with maleic acid, malic acid, cirtic acid, fumaric acid, succinic acid, Gln, Glu and Ala. Chromatographic conditions: Inertsil ODS 3 column (150 mm x 4.6 mm, 5 µm), column temperature 30 °C, flow rate 1.0 mL/min, injection volume 40 µL, mobile phase 10 mmol/L PFHA in water / methanol 96/4 v/v. Detection by CAD.
Elution order: maleic acid, malic acid, citric acid, fumaric acid, succinic acid, Asp, Gln, Glu, Ala. Modified from [126].

4.2.2 Investigation of the method performance and method validation

As a result of the experiments performed during the method development described in 4.2.1, a further investigation of the method performance parameters and a full validation of the final method described in 7.2.3.3 following the requirements of the "Technical Guide for the Elaboration of Monographs" [19] as well as ICH Guideline Q2(R1) [127] was carried out.

Since succinic acid and citric acid were only added to proof sufficient resolution between the organic acids, but are not considered to be impurities occurring in Asp, only a partial validation (i.e. selectivity, repeatability of injection, response factor and limit of quantification) of the method regarding these two substances was performed.

4.2.2.1 Specificity and System Suitability Testing

Specificity

Under the selected chromatographic conditions, the method was shown to be sufficiently selective to separate Asp from its possible impurities and several structurally related compounds, i.e. Ala, Glu, Gln, fumaric acid, maleic acid, malic acid, citric acid and succinic acid (cf. Fig. 4-16).

A system peak at about 31 min was detected in all injections, including the blank injections (cf. Fig. 4-17).
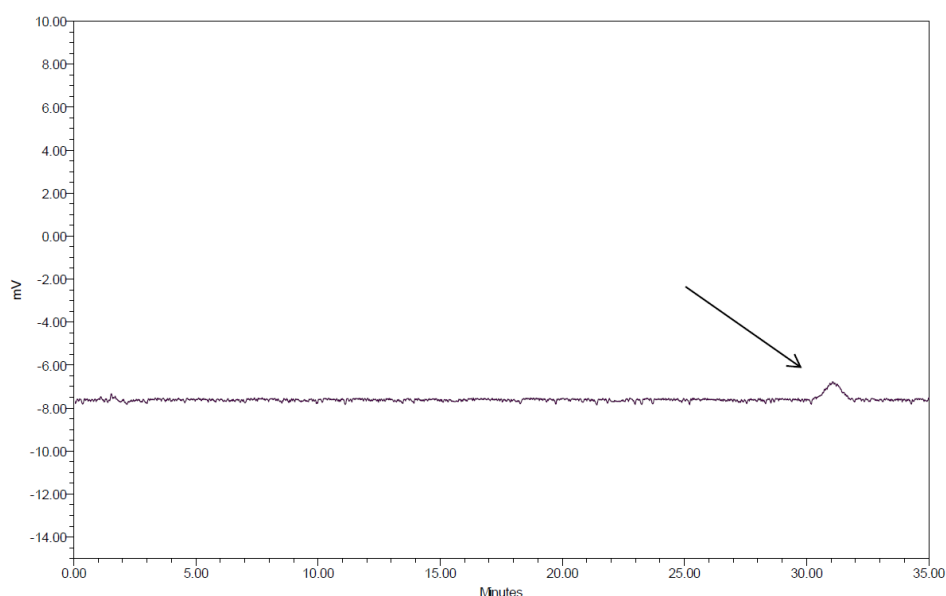
Figure 4-17. Injection of the mobile phase. System peak at about 31 min. Chromatographic conditions cf. Fig. 4-16.

An LC-TOF-MS analysis was performed to explore the nature of this system peak, but did not give any indication on its nature. However, since this peak did not interfere with any other peak, its presence was considered not to be problematic. Table 4-2 gives an overview of the retention times and unadjusted relative retentions (referred to Asp) found.

Table 4-2. Retention times and unadjusted relative retentions.

Compound	Retention time	Unadjusted relative retention (to Asp)
Maleic acid	1.35	0.31
Malic acid	2.12	0.48
Citric acid	2.61	0.59
Fumaric acid	3.20	0.73
Succinic acid	3.89	0.89
L Aspartic acid	**4.39**	**1**
Glutamine	7.56	1.72
Glutamic acid	9.92	2.26
Alanine	13.22	3.01

In conclusion, the method is considered to be sufficiently selective to separate Asp from possible impurities stemming from enzymatic synthesis as well as from several other related compounds.

System Suitability

Sufficient selectivity of the method was verified employing a mixture of 10 mg/mL of Asp spiked with 0.01 mg/mL (0.1%) of fumaric acid, succinic acid and Gln.

Based on the results obtained, the following resolution criteria are considered appropriate to ensure sufficient method selectivity:

Resolution: minimum 2.0 between the peaks due to fumaric acid and succinic acid.

Peak-to-valley ratio: minimum 1.2, where H_p = height above the baseline of the peak due to Gln and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to Asp.

Typical values found for the resolution in this study were:

Resolution fumaric acid / succinic acid: 3.0 to 4.8

Peak-to-valley ratio Asp / Gln: 2.0 to 3.9

The resolution between fumaric acid and succinic acid was selected to ensure selectivity for the compounds not interacting with the ion-pair reagent (organic acids). Moreover, this criterion ensures that succinic acid as the organic acid eluting closest to Asp is separated from the principle peak. Since succinic acid has a low response in the CAD, the test checks indirectly also sufficient method sensitivity.

The second resolution criterion was chosen to demonstrate sufficient resolution of the compounds forming ion-pairs with PFHA (amino acids). Based on the information available and on the results of the tests of several batches of Asp, Gln is not a really occurring impurity. However, Gln was selected because it is the compound eluting closest after the Asp peak.

The above resolution criteria were also applied as evaluation criteria in the verification of method robustness (cf. 4.2.2.7).

4.2.2.2 Linearity and range

A verification of the linearity using solutions of 0.025%, 0.05%, 0.10%, 0.14%, 0.20%, 0.25%, 0.50%, and 1.0% (referred to a 10 mg/mL test solution), performed on a mixture containing the above amino and organic acids,revealed that a quadratic relation describes best the relation between the concentration and the detector response (average of 3 injections per concentration):
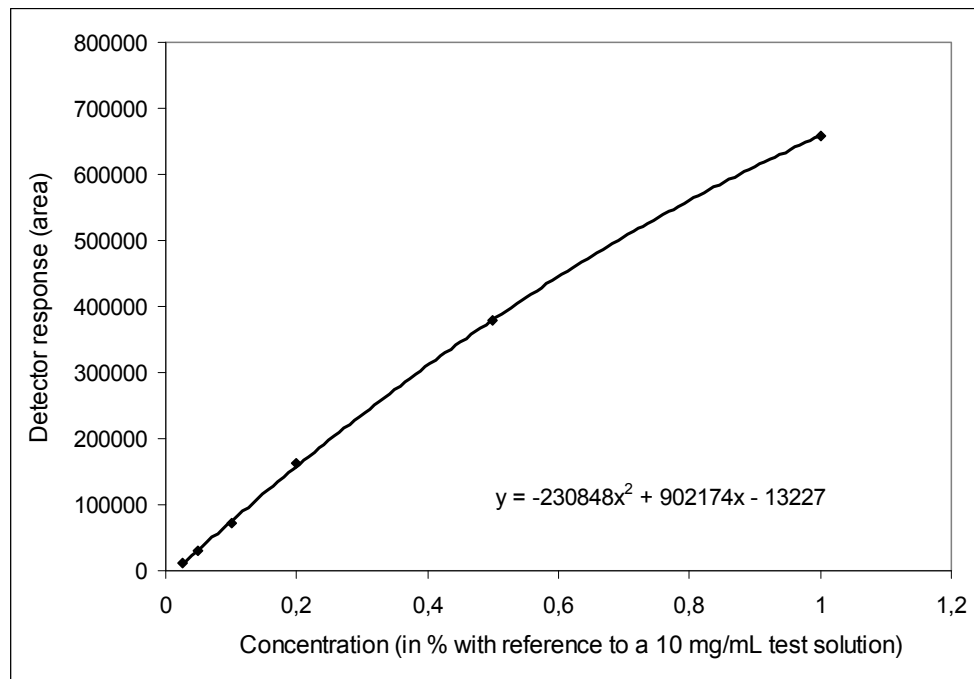
Figure 4-18. Calibration curve of malic acid from 0.025% to 1.0% (8 concentration levels). Example for the quadratic relation between the concentration and detector response. Modified from [126].

However, as can be seen in Figure 4-19, for a range from 0.025% up to 0.20% determined on the average peak area of three injections on the concentration levels 0.025%, 0.05%, 0.10%, 0.14%, and 0.20%, the relation between the concentration and the detector response was reasonably linear for all compounds (r^2 ranging between 0.996 for maleic acid and 0.999 for Ala, Glu, fumaric acid and malic acid).
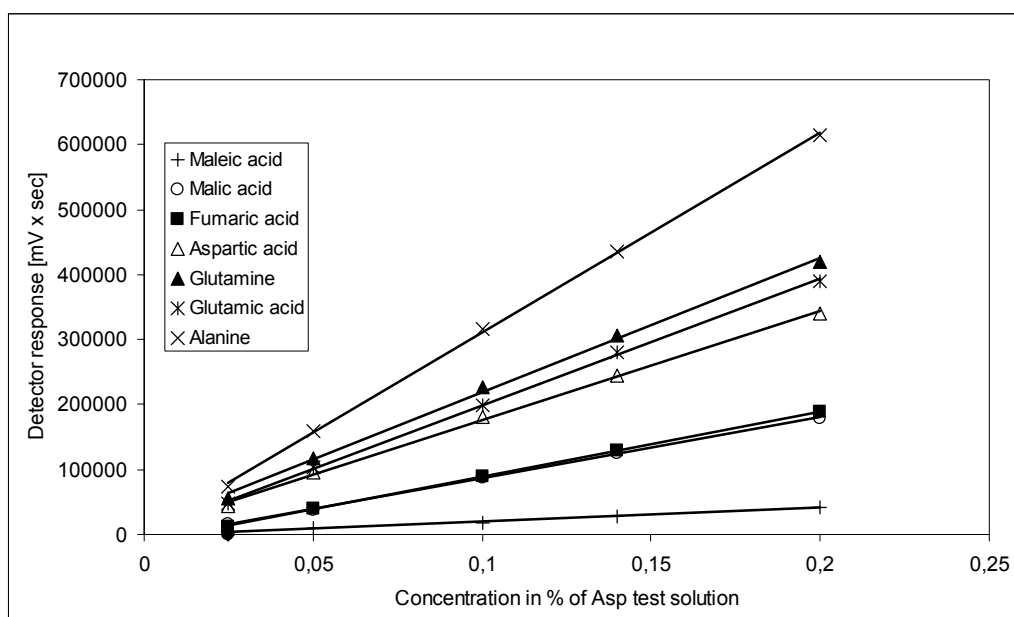
Figure 4-19. Calibration curves of maleic acid, malic acid, fumaric acid, Asp, Gln, Glu and Ala from 0.025% to 0.2% (Linear fit - 5 concentration levels).

4.2.2.3 Accuracy

The accuracy was assessed using a sample of Asp spiked with 0.05%, 0.1%, 0.15% and 0.20% of the impurities. The recovery was determined using a 0.01 mg/mL dilution of the corresponding component as external standard. An overview of the results can be found in Table 4-3.

Table 4-3. Recovery of impurities in a 10 mg/mL Asp solution spiked at different levels in (%).

Compound	Recovery at 0.05%	Recovery at 0.1%	Recovery at 0.15%	Recovery at 0.2%
Maleic acid	109.4%	98.1%	101.1%	98.9%
Malic acid	103.2%	97.0%	98.8%	94.2%
Fumaric acid	102.7%	100.5%	98.4%	94.6%
Glutamine	*51.6%*	*75.5%*	*79.4%*	*79.5%*
Glutamic acid	97.0%	100.8%	95.7%	90.5%
Alanine	91.6%	101.0%	101.9%	95.9%

The low recovery for Gln – especially at low concentration levels – is probably due to partial co-elution with the Asp peak. As Gln was not found in any of the batches tested (cf. 4.2.3), the low recovery is not detrimental to the method.

Considering that the method is intended for the determination of very low impurity levels near to the limit of quantification, a recovery of 90.0% to 110.0% as found in this study, was considered to be acceptable in terms of accuracy.

4.2.2.4 Precision

Repeatability

The repeatability was assessed using 5 concentration levels covering the specified range from 0.025% to 0.2% (referred to the concentration of the Asp test solution of 10 mg/mL). On each level 3 replicate injections were performed. A 10 mg/mL solution of Asp spiked with the different compounds was used for this determination. For the

0.0025 mg/mL level, a solution containing all compounds at this concentration level was employed. For Asp the determination was performed on 3 concentration levels – i.e. 0.0025 mg/mL, 0.01 mg/mL, and 0.02 mg/mL. Each solution was injected in triplicate.

A relative standard deviation (RSD) of maximum 5% was applied as an acceptance criterion for the repeated injections. As can be seen in Table 4-4, this criterion was met for all substances. Hence, the precision of the method is considered acceptable.

It is noted that the intermediate precision and the reproducibility was not assessed. This will have to be done in the case the method is accepted for the Ph.Eur. monograph of Asp.

Table 4-4. Repeatability of injection (average of three injections) at different concentration levels.

Compound	RSD in% (0.0025 mg/mL)	RSD in% (0.005 mg/mL)	RSD in% (0.010 mg/mL)	RSD in% (0.015 mg/mL)	RSD in% (0.02 mg/mL)
L-Asp	1.86%	-	1.56%	-	1.56%
L-Ala	1.77%	3.45%	0.49%	3.52%	0.46%
L-Glu	1.77%	3.19%	0.14%	0.59%	0.47%
L-Gln	0.59%	2.43%	2.78%	0.79%	1.63%
Fumaric acid	4.14%	0.21%	1.56%	0.84%	0.69%
Maleic acid	0.46%	1.75%	1.06%	1.53%	1.64%
Malic acid	3.40%	0.67%	0.50%	0.81%	0.32%
Citric acid	1.27%	2.01%	0.47%	0.45%	0.61%
Succinic acid	1.84%	1.84%	3.69%	0.30%	0.91%

4.2.2.5 Detection limit (LoD) / Quantification limit (LoQ)

The limit of detection (based on a signal-to-noise ratio of 3) and the limit of quantification (based on a signal-to-noise ratio of 10:1) was determined using 0.0025 mg/mL solutions of the different components (corresponding to 0.25% - average value of three injections – Fig. 4-20 and Fig. 4-21).
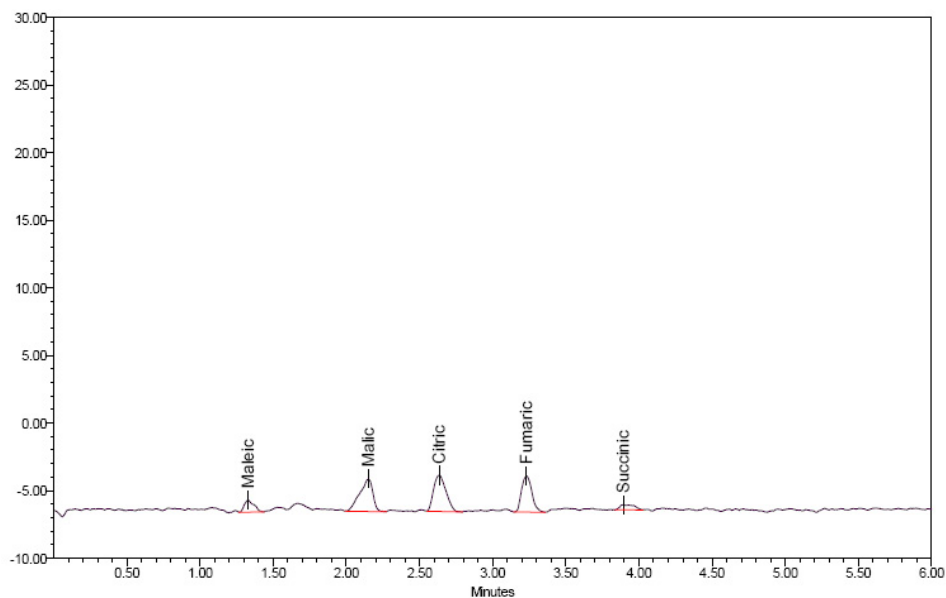
Figure 4-20. Mixture of organic acids injected at a concentration of 0.0025 mg/mL - elution order: maleic acid, malic acid, citric acid, fumaric acid, succinic acid. Chromatographic conditions cf. Fig. 4-16.
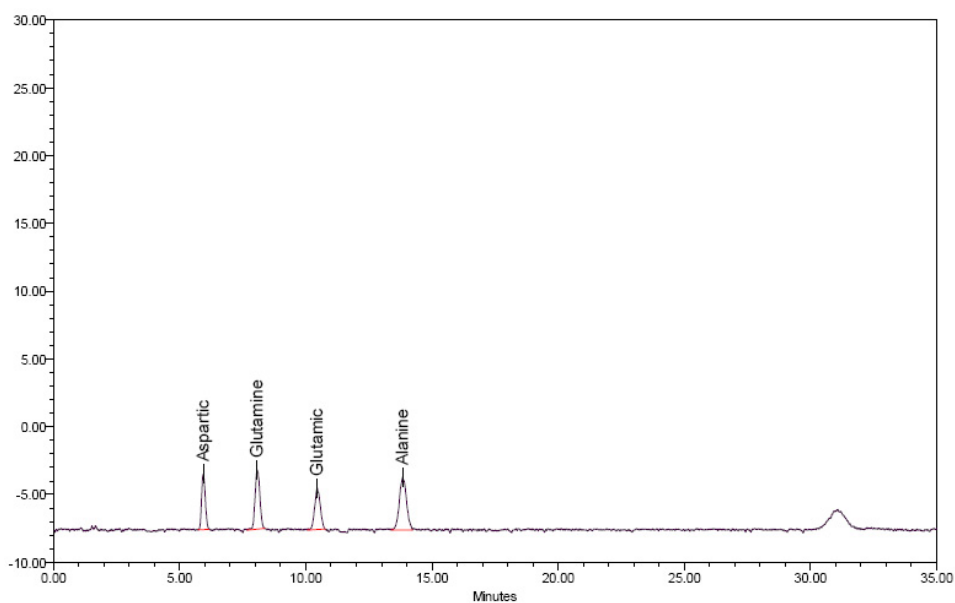
Figure 4-21. Mixture of amino acids injected at a concentration of 0.0025 mg/mL - elution order: Asp, Gln, Glu, Ala. Chromatographic conditions cf. Fig. 4-16.

The results given in Table 4-5 demonstrate that the method is sufficiently sensitive to determine the specified impurities with a disregard limit / reporting threshold of 0.03%.

Table 4-5. Limit of detection (based on a signal-to-noise ratio of 3) and limit of quantification (based on a signal-to-noise ratio of 10) determined using 0.0025 mg/mL solutions of the different components.

Compound	S/N ratio for 2.5 µg/mL solution	LoD in µg/mL	LoD in % (refered to the test solution)	LoQ in µ/mL	LoQ in % (refered to the test solution)
Asp	41	0.2	0.002%	0.6	0.006%
Ala	43	0.2	0.002%	0.6	0.006%
Glu	31	0.2	0.002%	0.8	0.008%
Gln	39	0.2	0.002%	0.6	0.006%
Fumaric acid	26	0.3	0.003%	1.0	0.01%
Maleic acid	8	0.9	0.009%	3.1	0.03%
Malic acid	24	0.3	0.003%	1.0	0.01%
Citric acid	27	0.3	0.003%	0.9	0.01%
Succinic acid	4	2.0	0.02%	6.3	0.06%

4.2.2.6 Correction factors

The relative response factors (referred to Asp) / Ph.Eur. correction factors of the different compounds were determined using the slope of the calibration curves presented under 4.2.2.3.

Since high purity reagents were used in this study, all substances were used "as is" and no correction for the purity was performed. However, since citric acid monohydrate was employed a correction for the theoretical water content was made. The relative response factors / correction factors found are presented in Table 4-6.

It is worth mentioning that the response factors of the organic acids are considerably lower compare to those of the amino acids. It is assumed that 2 effects are

responsible for this finding which are a lower tendency to form positively charged ions (lower chargeability) and – to a lesser extent – higher volatility of at least some of the organic acids.

However, as regards the applicability of the method, this is not considered to be an issue since the quantification of the specified impurities will be performed using the corresponding substances as external standards. For unspecified impurities, all compounds eluting before Asp could be quantified with reference to fumaric acid. All compounds eluting after the peak due to Asp could be quantified using Asp as external standard. This would keep the error of determination in a reasonable range.

Table 4-6. Response factors (relative to Asp) / correction factors of the different compounds determined from the slope of the calibration curve presented under 4.2.2.3.

Compound	Relative response factor	Correction factor
L-Asp	1	1
L-Ala	1.88	0.53
L-Glu	1.12	0.89
L-Gln	1.21	0.83
Fumaric acid	0.586	1.7
Maleic acid	0.129	7.8
Malic acid	0.554	1.8
Citric acid	0.665	1.5
Succinic acid	0.138	7.3

4.2.2.7 Robustness

Unless otherwise specified below, a 10 mg / mL aqueous solution of Asp spiked with 0.1% of Ala, Glu, Gln, fumaric acid, maleic acid, malic acid, citric acid and succinic acid was used for the subsequent experiments. This solution is referred to in the following paragraphs as "spiked Asp test solution".

Selection / influence of a sample solvent / stability of solution

In order to achieve the required method sensitivity, a 10 mg/mL solution of Asp is required. Unfortunately, it was found that Asp is difficult to dissolve. Various solvents and combinations of solvents were tested to prepare a 10 mg/mL solution of Asp, but to no avail:

- Acetone   - Acetone / Water (50/50 v/v)

- Acetonitrile   - Acetonitrile / Water (50/50 v/v)

- Dimethylformamide   - Dimethylformamid / Water (50/50 v/v)

- Ethanol   - Ethanol / Water (50/50 v/v)

- Ethyl acetate   - Ethyl acetate / Water (50/50 v/v)

- Methanol   - Methanol / Water (50/50 v/v)

- Tetrahydrofuran   - Tetrahydrofuran / Water (50/50 v/v)

- Water (ambient temperature)

It is know that Asp dissolves after addition of acids. Unfortunately, the addition of acids (i.e. hydrochloric acid (HCl), formic acid, trifluoroacetic acid (TFA) and acetic acid) was not found to be an option since they were either not sufficiently volatile and produced intense signals in the CAD detector (e.g. HCl) and/or deteriorated significantly the separation of the early eluting peaks (e.g. TFA).

Finally it was found that a 10 mg/mL solution of Asp in water could be produced when heating the sample to 60 °C. The solution was found to be stable for 4 - 6h before re-precipitation occurred. As discussed under 4.2.2.9 some degradation of Asp to fumaric acid and maleic acid may occur when boiling an aqueous Asp solution for several hours. However, as can be seen from the results of the testing of 8 batches of Asp in solutions prepared as proposed above (cf. 4.2.3), malic acid was not detected and fumaric acid was not found in 3 of the batches tested. 3 further batches contained only amounts of fumaric acid below 0.01%. In none of the batches further unspecified impurities were found. Hence, dissolution at 60 °C is not considered to be problematic and could be applied for the preparation of the test solution.

Furthermore, the stability of solution was verified employing a spiked Asp test solution. The solution was injected in triplicate immediately after preparation as well as after 4h, 8h, 12h, 16h, and 24h. The overall relative standard deviation of the recovery of the different components, taking into account all results at the different time points until 12h respectively 24h, was determined (cf. Table 4-7).

Table 4-7. Overall relative standard deviation of repeated injections of a 10 mg/mL solution of Asp containing about 0.1% of Ala, Glu, Gln, fumaric acid, maleic acid, malic acid, citric acid and succinic acid injected in triplicate immediately after preparation and after 4h, 8h, 12h, 16h, and 24h (48h for malic acid). For the determination of the overall rsd of Asp, a 0.01 mg/mL solution was used.

Compound	RSD after 12h (%)	RSD 24h (%)
L-Aspartic acid	2.1%	1.6%
L-Alanine	4.0%	3.6%
L-Glutaminc acid	3.6%	5.5%
L-Glutamine	7.6%	8.3%
Fumaric acid	4.3%	4.9%
Maleic acid	2.4%	1.9%
Malic acid	3.5%	**3.6%** *(after 48h)*
Citric acid	1.6%	1.3%
Succinic acid	14.3%	12.4%

Applying a relative standard deviation of maximum 5% as an acceptance criterion for the stability, it is concluded that for Ala, fumaric acid and maleic acid, malic acid and citric acid as well as for a 0.1% dilution of Asp the solutions are stable for 24h.

For Glu the result after 24h is slightly outside the above acceptance criterion. However, this is still not considered to be problematic. The results found for Gln and succinic acid are not considered to be an issue since these substances are only used to verify system suitability.

For maleic acid, the result at 24h would have led to an overall RSD of 6.4%. However, as can be seen in Figure 4-22 the results obtained after 48h showed, the value after 24h could be considered an outlier. It was therefore replaced by the result obtained after 48h.
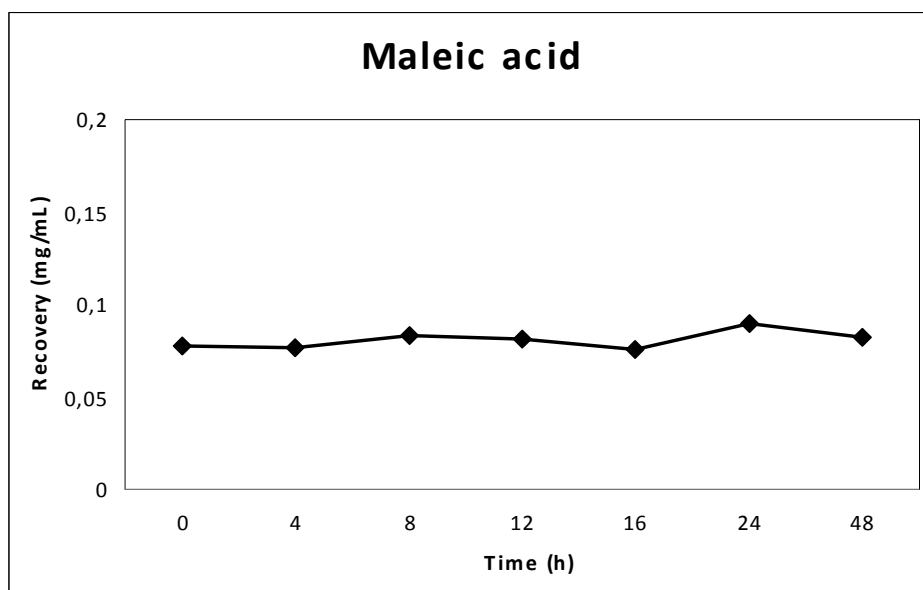
Figure 4-22. Recovery of maleic acid in a 10 mg/mL solution of Asp containing about 0.1% of maleic acid. The recovery was determined in triplicate immediately after preparation and after 4h, 8h, 12h, 16h, 24h, and 48h.

Influence of the LC flow-rate

The impact of the LC flow-rate on the system selectivity was studied for values from 0.6 to 1.2 mL/min in steps of 0.2 mL/min using a spiked Asp test solution. At all flow-rates, the compounds were separated from one another and the proposed resolution requirements were met (cf. Table 4-8).

Table 4-8. Overview of the resolution and peak-to-valley ratio obtained at different LC flow-rates.

Flow rate (mL/min)	Resolution fumaric acid / succinic acid	P/V ratio Gln
0.6 mL/min	4.5	3.5
0.8 mL/min	4.3	4.3
1.0 mL/min	3.9	4.8
1.2 mL/min	3.3	3.5

In conclusion, the method is considered to be sufficiently robust regarding the influence of the LC flow-rate on the selectivity.

Influence of the column temperature

The influence of the column temperature was examined injecting a spiked Asp test solution at 15 °C, 30 °C, 35 °C and 40 °C. The injections were conducted at a flow rate of 0.8 mL/min. However, as demonstrated above, this difference in the LC flow-rate compared to 1.0 mL/min has no significant impact on the resolution.

As can be seen in Table 4-9, at all temperatures tested the compounds were separated from one another and the proposed resolution requirements were met.

Table 4-9. Overview of the resolution and peak-to-valley ratio obtained at different column temperatures.

Column temperature	Resolution fumaric acid / succinic acid	P/V ratio Gln
15 °C	3.7	4.0
30 °C	4.4	5.0
35 °C	4.2	4.4
40 °C	4.2	4.4

In conclusion, the method is considered to be sufficiently robust regarding the influence of the column temperature on the selectivity. A column temperature of 30 °C instead of "ambient temperature" was selected for the method because it is considered preferable to work at a defined temperature that can easily controlled by conventional LC-systems.

Effect of the ion-pair reagent

The influence of different concentrations of the ion-pair reagent (PFHA) was examined injecting a spiked Asp test solution. The amount of ion-pair reagent was modified as follows: 0.5 mmol/L, 0.8 mmol/L, 1.0 mmol/L, 1.2 mmol/L, 1.5 mmol/L.

At a concentration of 0.5 mmol/L of PFHA, succinic acid was found to co-elute with Asp. Also fumaric acid was only partly separated from the peak due to Asp. For the other concentrations of ion-pair reagent, all compounds were separated from one another and the proposed resolution requirements were met. An overview of the results obtained is given in Table 4-10.

Table 4-10. Overview of the resolution and peak-to-valley ratio obtained at different concentration of PFHA.

Conc. of ion-pair reagent (mmol/L)	Resolution fumaric acid / succinic acid	P/V ratio Gln
0.5 mmol/L	Measurement not possible (co-elution of succinic acid and Asp)	1.4
0.8 mmol/L	5.9	2.0
1.0 mmol/L	4.2	4.5
1.2 mmol/L	4.0	3.2
1.5 mmol/L	2.5	5.4

As already pointed out earlier, low amounts of ion-pair reagent improve the separation of the organic acids. With increasing amounts of PFHA, the selectivity for the organic acids decreased but at the same time the separation of the amino acids which are interacting with the ion-pair reagent increased. The selected concentration of 1.0 mmol/L of PFHA is considered to be a good compromise regarding the method selectivity and total run-time. In summary, it is concluded that the method is sufficiently robust regarding small changes in the concentration of the ion-pair reagent in the mobile phase.

Modification of the mobile phase composition (organic modifier concentration)

The effect of different amounts of organic mobile phase modifier (methanol) on the method performance was verified injecting a spiked Asp test solution. The amount of organic modifier was modified from 1% (v/v) to 7% (v/v) in 1% steps.

For the mobile phases containing 1% (v/v) and 2% (v/v) of organic modifier an additional solvent peak was detected at a retention time of about 15 min – 16 min (cf. Fig. 4-23 and 4-24). This peak was no longer detected when the amount of organic modifier was increased to 3% (v/v) upwards.
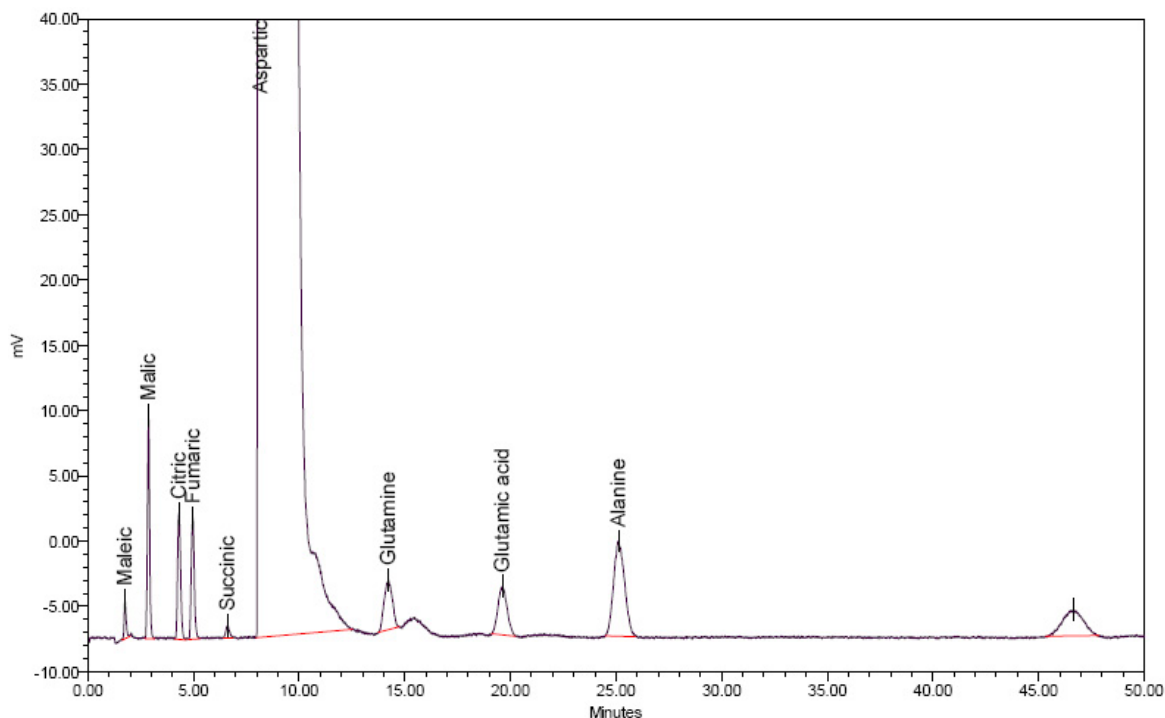
Figure 4-23. Injection of a spiked Asp test solution at 0.8 mL/min. Mobile phase containing 1% v/v of methanol. Further chromatographic conditions cf. Fig. 4-16. Elution order: maleic acid, malic acid, citric acid, succinic acid, Asp, Gln, Glu, Ala.
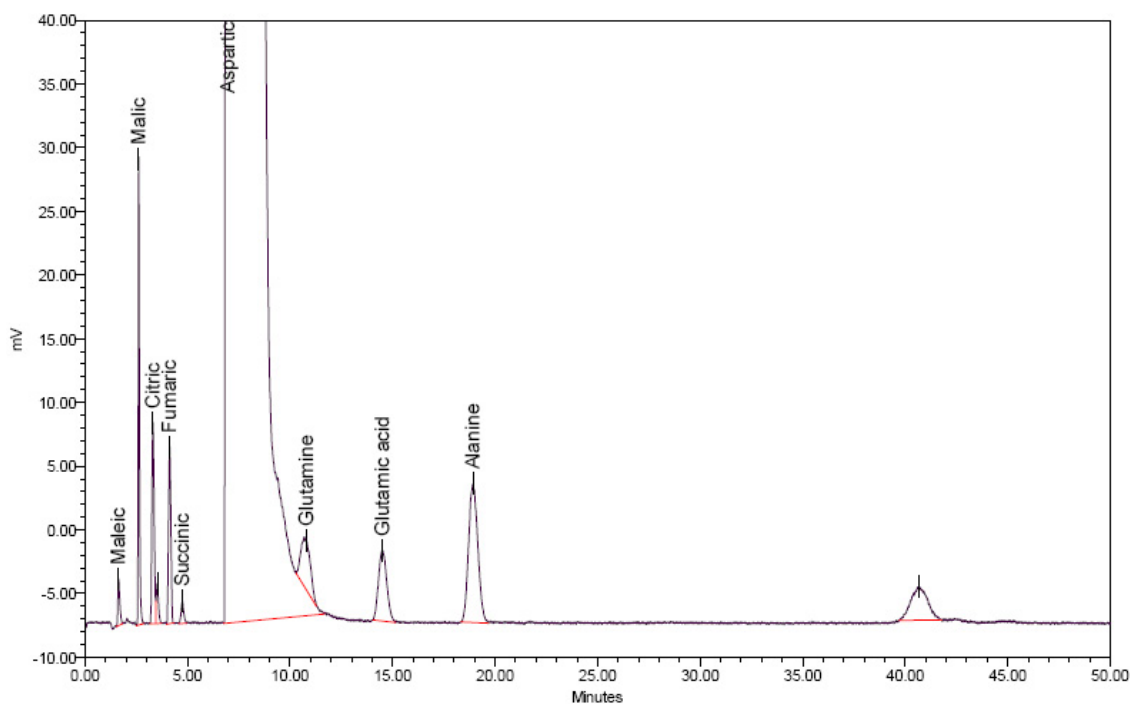
Figure 4-24. Injection of a spiked Asp test solution at 0.8 mL/min. Mobile phase containing 7% v/v of methanol. Further chromatographic conditions cf. Fig. 4-16. Elution order: maleic acid, malic acid, citric acid, succinic acid, Asp, Gln, Glu, Ala. An additional peak appears between citric acid and fumaric acid. This peak is probably due to a contamination of the solution.

The separation of the different compounds decreased with increasing amounts of organic modifier. However, the specified resolution requirements were met in all cases. On the other hand, an increasing sensitivity could be noted with increasing amounts of methanol. This is attributed to an improved volatility of the mobile phase resulting in a better nebulization and ionization of the eluate. The results are summarized in Table 4-11.

The method is considered to be sufficiently robust towards changes of the mobile phase composition from 3% v/v to 7% v/v of organic modifier (methanol).

Table 4-11. Overview of the resolution, peak-to-valley ratio and S/N-ratio obtained with different amounts of organic modifier in the mobile phase.

Organic modifier (in % v/v)	Resolution fumaric acid / succinic acid	P/V ratio Gln	S/N-ratio succinic acid	S/N-ratio Glu
1%	5.6	16	15	61
2%	5.2	8	20	66
3%	4.3	5.3	21	71
4%	4.3	3.6	21	71
5%	3.6	2.4	25	82
6%	3.2	1.7	26	90
7%	2.8	1.6	29	91

Selection of a stationary phase

Several stationary phases (reversed phase C18 and C8) were tested. As reported in the manufacturer manual [128] of the CAD detector, stationary phases may exhibit significant differences in bleeding and therefore may produce substantially different background noise. To obtain a stable level of baseline-noise, all columns were flushed with the mobile phase at a flow rate of 0.2 mL/min for 12h before use.

Since succinic acid was found to give the lowest response, the signal-to-noise ratio (S/N-ratio) obtained from a 0.01 mg/mL solution of this acid (corresponding to 0.1%,

referred to an Asp test solution of 10 mg/mL) is given for information. Table 4-12 shows that significant differences in the noise level (determined as average peak-to-peak noise) were found.

Table 4-12. Baseline noise and signal-to-noise ratio of succinic acid of different LC columns.

Column	Baseline noise (µV)	S/N ratio succinic acid
Inertsil ODS 3 (150 mm x 4.6 mm, 5 µm)	191	15
Zorbax Eclipse XDB-C18 (150 mm x 4.6 mm, 5 µm)	136	34
Symmetry C18 (150 mm x 3.9 mm, 5 µm)	295	10
Purospher RP-18e (250 mm x 4.0 mm, 5 µm)	700	4
YMC Pack PR 18 (150 mm x 4.6 mm, 5 µm)	2000	Below LoD
Synergi Hydro RP C18 (150 mm x 4.6 mm, 4 µm)	332	10
Kromasil C18 (150 mm x 4.0 mm, 5 µm)	800	Below LoD
YMC Pack **PR 8** (150 mm x 4.6 mm, 5 µm)	300	14

In terms of noise and LoD, the Inertsil ODS 3 and Zorbax Eclipse XDB C18 columns were found to be the most appropriate columns. However, since the selectivity of the Zorbax Eclipse XDB C18 column was insufficient, the Inertsil ODS 3 column was selected for further testing. For completeness it is mentioned that in terms of selectivity, the Kromasil C18, the Synergi Hydro RP C18 column and the Purospher RP-18e column would have been also suitable but delivered less good results in terms of sensitivity.

It is reported in literature [129, 130] that using normal C18 reversed phase columns problems of reproducibility and a loss of column performance could occur when the amount of organic modifier in the mobile phase drops below 5%. As described under 3.1.2.1 it is possible to modify a stationary phase by "hydrophilic endcapping" and several column manufacturer offer corresponding stationary phases specifically designed for highly aqueous mobile phases (e.g. Synergi Hydro RP C18).

However, using the Inertsil ODS 3 column no such problem was encountered. The column performance in terms of selectivity and sensitivity did not decrease during more than 400h of use.

Forced degradation experiments

To verify the stability of the substance and to check for the appearance of further impurities, several degradation experiments were performed. For all tests, a 10 mg/mL solution of Asp in water was used.

The LC profile of a freshly prepared 10 mg/mL aqueous Asp test solution under the method conditions described in 7.2.3.3 is given in Figure 4-25 for information. The batch was found to contain 0.066% of malic acid and 0.008% of fumaric acid.
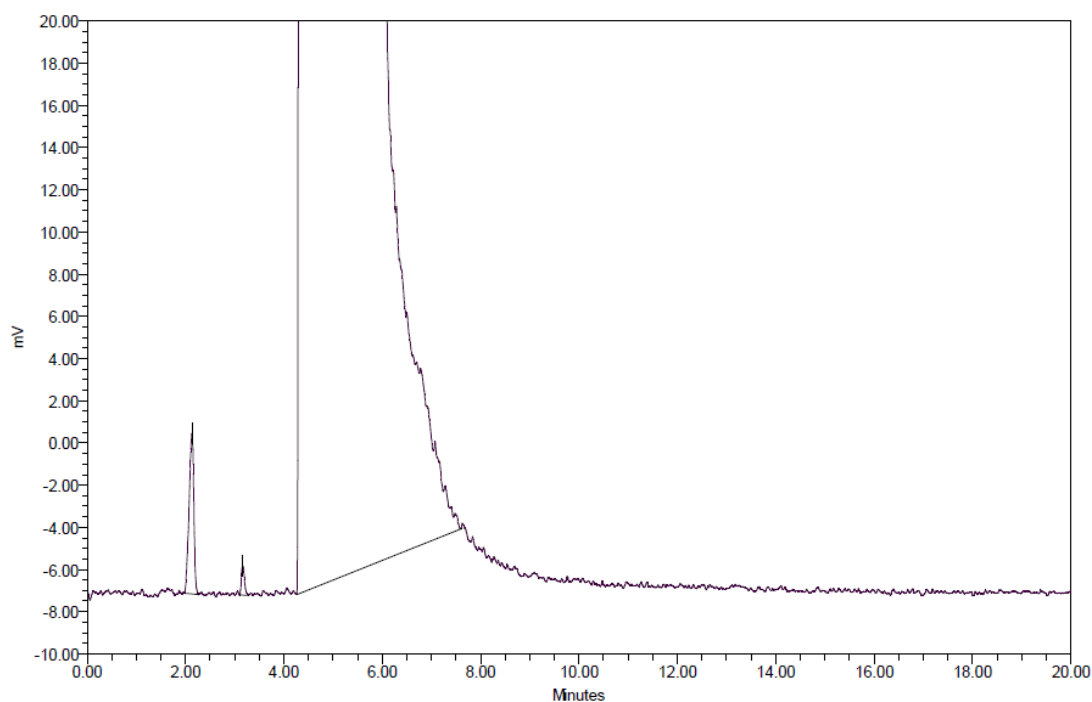
Figure 4-25. Injection of a 10 mg/mL solution of Asp using the LC method described under 7.2.3.3.

The different degradation experiments are discussed below from a) to d). Unless otherwise indicated, a 10 mg/mL aqueous solution of the above sample of Asp was employed.

a) Influence of UV light at 254 nm and 366 nm

The test solution was exposed to UV light at 254 nm and 366 nm for 18h. The peak due to fumaric acid could no longer be detected. Except from that, no noticeable degradation could be observed.

b) Influence of the temperature

Boiling of the test solution for 4 h resulted in an increase of the peak due to fumaric acid. Moreover, three additional peaks appeared with unadjusted relative retentions of 0.51 (peak 3), 2.6 (peak 5) and 4.3 (peak 6) (cf. Fig. 4-26):
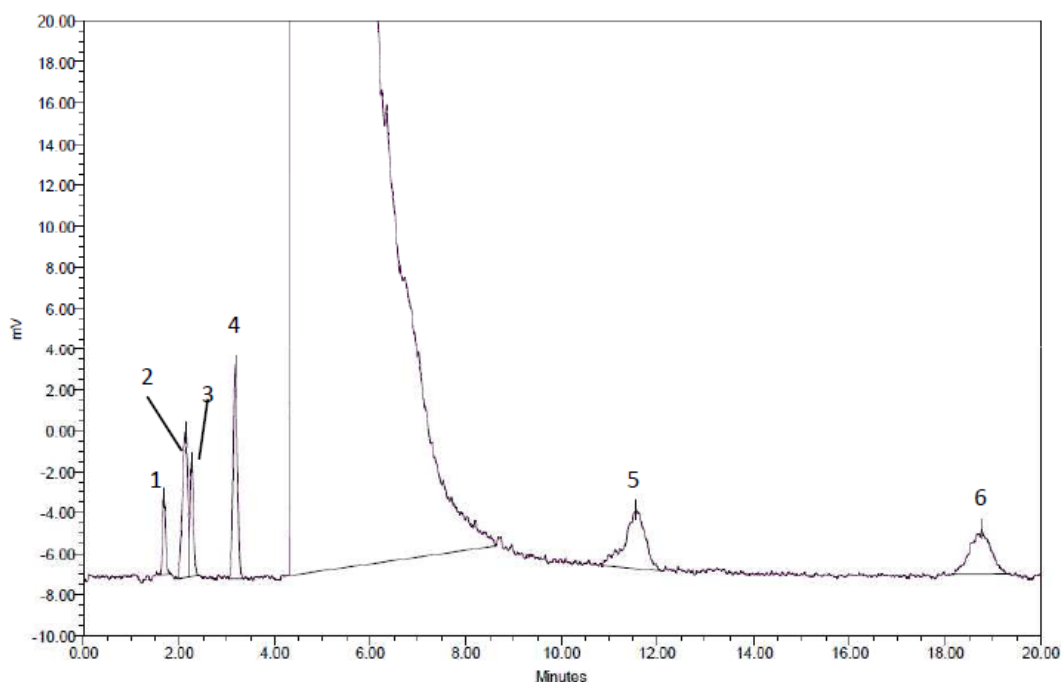
Figure 4-26. Injection of a 10 mg/mL solution of Asp boiled for 6h. Chromatographic conditions cf. 7.2.3.3.

Detailed results are presented in Table 4-13. The quantification of the unspecified impurities eluting before Asp was performed using fumaric acid as a reference whilst peaks eluting after Asp were quantified against an appropriate dilution of Asp.

The occurring degradation products were analyzed by LC/TOF-MS. Based on the m/z ratios found, the possible molecular formulas of the impurities are presented in Table 4-14.

Table 4-13. Asp impurities produced by thermal degradation

Peak	Impurity	Initial imp. content	Imp. content after boiling for 4h
1	Maleic acid	-	0.024%
2	Malic acid	0.066%	0.067%
3	Unknown – relative retention: 0.51	-	0.035%
4	Fumaric acid	0.008%	0.082%
5	Unknown –relative retention: 2.6	-	0.049%
6	Unknown –relative retention: 4.3	-	0.041%

Table 4-14. Supposed sum formulas of the degradation products of Asp produced by thermal degradation.

Impurity	m/z-ratio found (M+H⁺)	Supposed sum fomula of the (M+H⁺)-ion
Unknown (peak 3) relative retention: 0.51	231.0623	$C_8H_{10}N_2O_6$ (m/z = 231.0612)
Unknown (peak 5) relative retention: 2.6	231.0625	$C_8H_{10}N_2O_6$ (m/z = 231.0612)
Unknown (peak 6) relative retention: 4.3	249.0727	$C_8H_{12}N_2O_7$ (m/z = 249.0717)

It is assumed that the generated unknown impurities are condensation products of Asp. Possible structures are suggested in Figure 4-27.
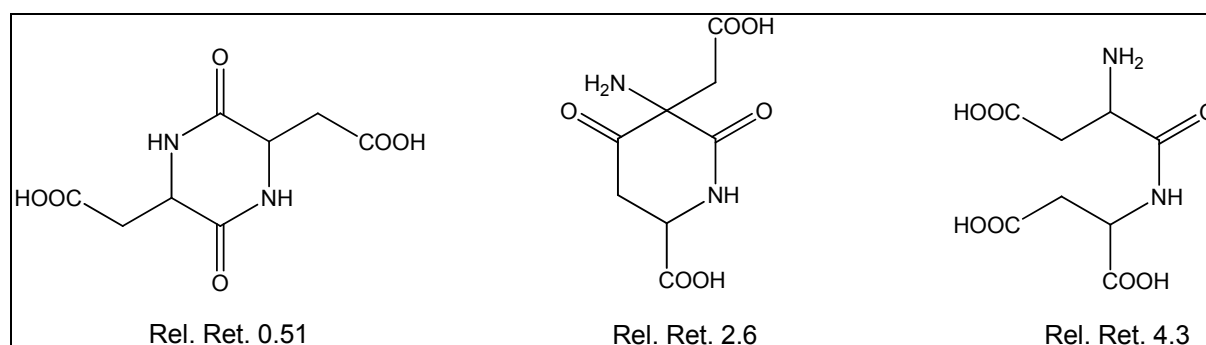
Figure 4-27. Supposed structures of the "unknown" impurities generated by thermal degradation.

c) Influence of oxidizing agents

500 mg of Asp were mixed with 40 mL of water. After addition of 5 mL of hydrogen peroxide 30% v/v the solution was stirred and heated to 60 °C for 4 h. To obtain complete dissolution, the solution was further heated to about 80 °C and injected. Compared with the impurity profile of the batch obtained under the proposed method conditions, no additional impurities were found.

d) Influence of strong acids and bases

The influence of strong acids and bases could not be verified since the CAD detector does not tolerate elevated concentrations of non-volatile acids or bases. Even a degradation experiment with formic acid was not found feasible since the high amounts of formic acid present in the injected sample significantly changed the chromatographic profile.

4.2.3 Purity determination of L-Aspartic acid

Employing the method described under 7.2.3.3 the purity of 5 samples of pharmaceutical grade Asp supplied by three different manufacturers was examined. Moreover, 3 samples of Asp purchased from reagent suppliers were tested. Since the linearity of the range was only verified starting from 0.025% upwards, a calculation of the values below this threshold is strictly speaking not correct. However, since the error is considered negligible and inclusion of quantitative figures gives a better image of the batch quality, these results were also reported.

An overview of the results found is presented in Table 4-15. A chromatogram of the test solution of Asp sample 3 is presented in Figure 4-28 for information. The peaks of the impurities occurring in the test solutions were identified by comparison of their retention times with the corresponding peaks in the reference solution.

All batches tested were found to have a purity of greater than 99.8% (w/w) with malic acid and Ala as major impurities. In some of the batches additionally small amounts of fumaric acid and Glu were observed. In one of the batches of the reagent grade material an unknown impurity was found at a relative retention of about 0.33. The amount of Ala and Glu found in sample 1 and 3 were in full agreement with the results obtained from amino acid analysis (data not shown).

Table 4-15. Results of the purity testing of 8 samples of Asp. Modified from [126].

Sample N°	Malic acid	Fumaric acid	Glu	Ala	Unspec rel.ret. 0.33	Sum
Pharmaceutical grade						
1	0.017%	0.008%	Nd	0.007%	Nd	0.031%
2	0.047%	nd	0.014%	0.052%	Nd	0.113%
3	0.051%	nd	0.019%	0.067%	Nd	0.137%
4	0.034%	nd	Nd	nd	Nd	0.034%
5	0.052%	0.005%	Nd	nd	Nd	0.057%
Reagent grade						
6	0.033%	0.046%	nd-	nd	0.037%	0.116%
7	0.066%	0.008%	Nd	nd	Nd	0.074%
8	nd	0.059%	0.036%	nd	Nd	0.095%

nd = not detected.
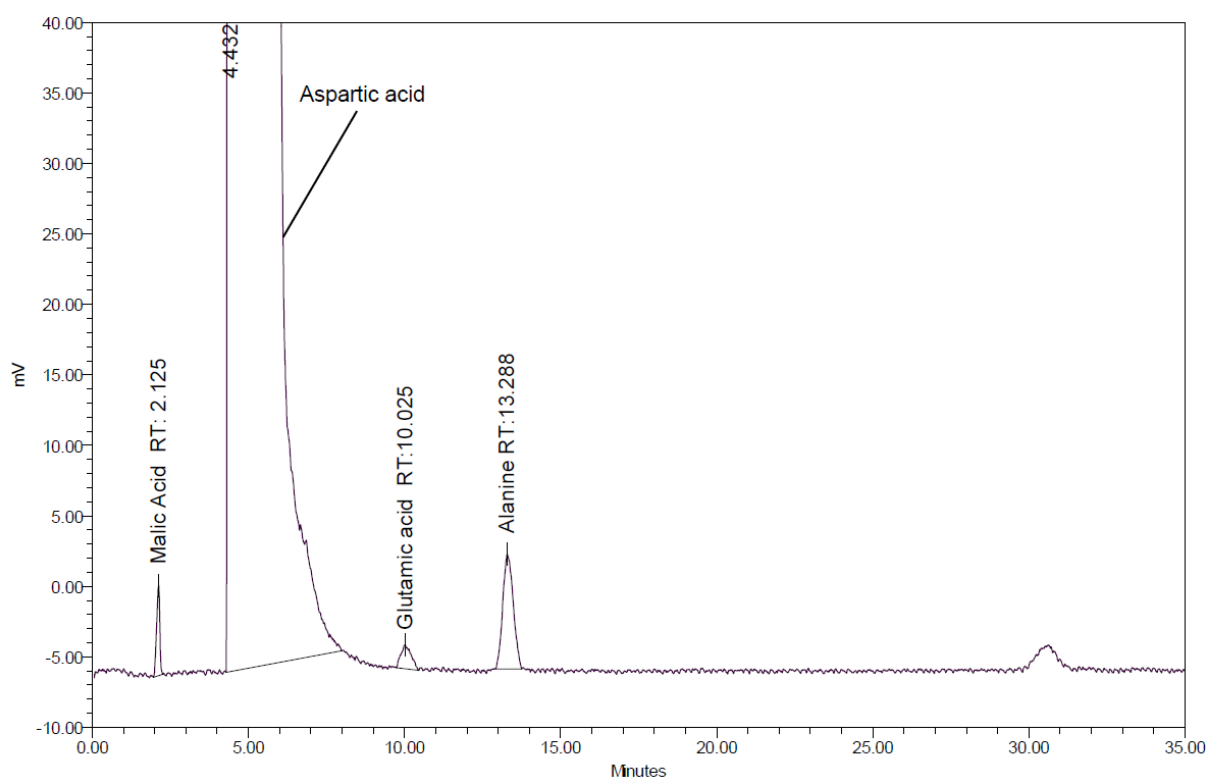
Figure 4-28. Chromatogram of a 10 mg/mL test solution of Asp sample-N° 3 in water. Chromatographic conditions cf. 7.2.3.3. Modified from [126].

4.2.4 Purity determination of L-Alanine

For the purity testing of Ala the above method was slightly modified, i.e. the concentration of PFHA was increased to 1.5 mmol/L. This was found necessary to obtain resolution of Ala and Glu at high concentrations of Ala. For the preparation of a 10 mg/mL test solution of Ala, heating was not necessary to obtain. For peak identification and quantification the reference solution described under 7.2.3.3 was employed. Moreover, the same strategy regarding the quantification of possible unspecified impurities was applied.

Using this modified method, 12 samples of pharmaceutical and reagent grade Ala supplied by 6 different suppliers were examined. An overview of the results found is presented in Table 4-16. All samples were found to have a purity of greater than 99.9% (w/w) with only small amounts of Asp and Glu as detectable impurities. A chromatogram of an Ala test solution (sample-N° 3) is given in Fig. 4-29 for information.

Table 4-16. Results of the purity testing of 12 samples of Ala. Modified from [126].

Sample N°	Asp	Glu	Sum
1	0.015%	0.044%	0.059%
2	0.005%	0.048%	0.053%
3	0.009%	0.056%	0.065%
4	nd	nd	Nd
5	nd	nd	Nd
6	0.029%	0.044%	0.073%
7	0.020%	0.038%	0.058%
8	nd	0.051%	0.051%
9	nd	nd	Nd
10	nd	nd	Nd
11	nd	nd	Nd
12	nd	nd	Nd

nd = not detected
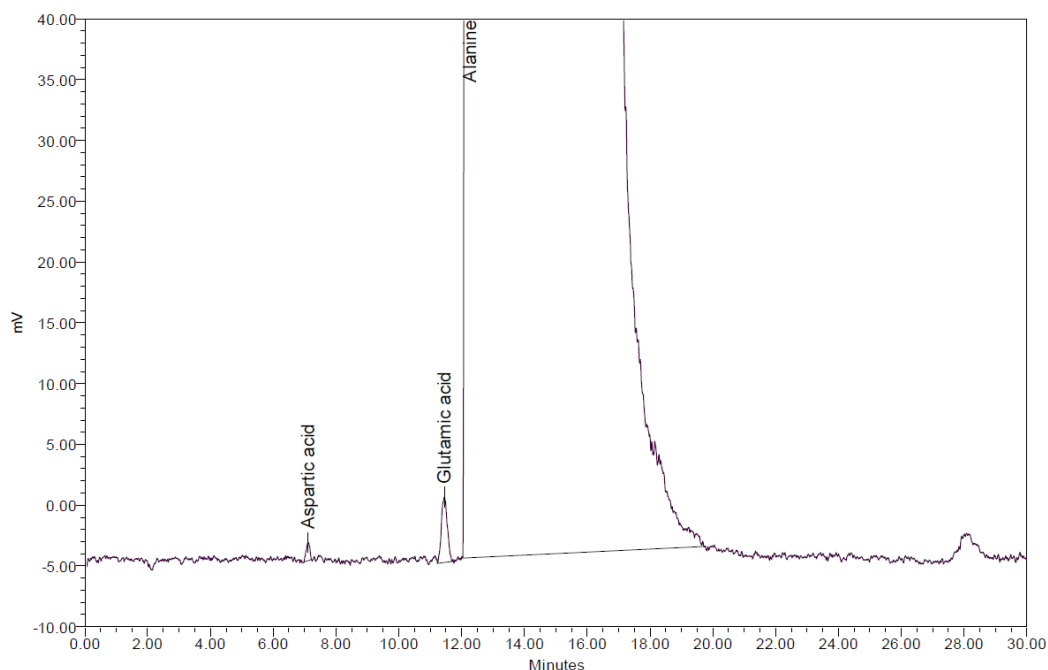
Figure 4-29. Chromatogram of a 10 mg/mL test solution of Ala sample-N° 3 in water. Chromatographic conditions as described in 7.2.3.4.

4.2.5 Conclusion

In this study a C18 reversed phase ion-pair LC method using 1 mmol/L of PFHA as an ion-pair reagent and a CAD for the purity control of Asp was developed and validated. The method was capable of separating the major organic and amino acids known to occur as process related impurities. With a slight increase of the PFHA concentration from 1.0 mmol/L to 1.5 mmol/L, the method was also found to be suitable for the purity control of Ala. The limits of quantification for the potential impurities were found between 0.006% and 0.03% (referred to the concentration of a 10 mg/mL test solution), allowing the control of impurities on an ICH [25] conform level for drug substances with an average daily dose above 2 g.

Whilst the detector response of the CAD follows an exponential function over a broader concentration range, it was found to be linear in a range from 2.5 μg/mL to 20 μg/mL. The LC method described in this study represents an easy to use alternative to amino acid analysis for the control of impurities in Asp and Ala. It has the additional benefit of controlling not only related amino acids but also other process impurities like organic acids. The method is considered suitable to be described as a pharmacopoeial related substances control method to replace the currently described TLC test and was published for public consultation in Pharmeuropa 22.4 [131].

4.3 Comparison of different detection systems for the purity control of L-Alanine

Under 4.1 the performance characteristics of the ELSD were investigated and key criteria and limitations of this detector for the impurities control were established. It was concluded that for the development of a LC-ELSD method for the control of impurities in drug substances both LC and ELSD parameters must be carefully chosen to find the best compromise between sensitivity and avoidance of spike peaks. Eventually it was decided to favour the detection by the CAD for the method development of a LC method to control impurities in Asp and Ala described under 4.2, since this detector was reported to be significantly more sensitive than ELSD [60, 61, 73, 74, 75]. Nevertheless it is tempting to examine the differences of the performance characteristics of these 2 detectors in detail. Additionally, the recently developed Nano Quantity Analyte Detector (NQAD), described in 3.2.4.4, was included to verify if this detector could represent an alternative to the detection by CAD. Another possibility to cope with the problem of non-UV responding impurities in AAs is the use of LC coupled with MS detection (MSD) [95, 132]. This technique allows a reliable and highly sensitive quantification of trace level impurities, although at the expense of a high prize, MSD using a triple quadrupole mass spectrometer was added as a 4th possibility.

Besides chromatographic methods, quantitative NMR (qNMR) nowadays offers another possibility for the impurity control in drug substances [133, 134] and is already described for corresponding purposes in pharmacopoeial monographs (e.g. [135]). Therefore, qNMR was included as an orthogonal, non-chromatographic approach for the impurity control in Ala and the results were compared to those obtained by the "classical" separation-based approach. This study is considered to be the first comparative case study amongst the above evaporation based detectors and also including an alternative non-chromatographic technique. It may be of benefit for analysts looking for the most appropriate detection method for the determination of analytes not eligible to UV detection as it is the case for most of the AAs.

For this study the LC method developed for the purity testing of Ala (cf. 4.2.4) was employed. Experimental details (e.g. detector setting, preparation of test and calibration solutions are described in 7.3.3). Some modifications to the LC-CAD method had to be applied to adapt it to MS/MS-detection.

The most important changes were the selection of a small particle size column (Zorbax Eclipse XDB C18 column - 50 mm x 4.6 mm; particle size: 1.8 µm) and a corresponding reduction of the LC flow-rate to 0.5 mL/min. Furthermore, the injection volume was reduced to 3 µL. Under these conditions, the peak due to alanine eluted at about 6.2 min (cf. 7.3.3.3). The Ala test sample was prepared by spray drying (cf. 7.3.3.1). For this purpose one of the Ala samples identified as being devoid of detectable impurities in the previously conducted experiments (cf. 4.2.4) was spiked with 2 organic acids (malic acid and fumaric acid) and 2 AAs (Asp and Glu). The amount of substance used for the spiking was chosen such that even in case of low detector sensitivity a quantifiable signal would be obtained. Eventually the homogenized spiked sample was tested for residual water. Based on the result of a triplicate coulometric determination of water and a 6 fold determination of the loss on drying, an average amount of 0.6% of water/volatile substances was found. Considering this value the produced spiked Ala sample was supposed to have the following nominal contents ("as is") of the different spiked substances: 0.46 % malic acid, 0.41 % fumaric acid, 0.39 % Asp and 0.98 % Glu.

4.3.1 Quantitative ^{1}H NMR spectroscopy[*]

Since ^{1}H NMR spectra generally contain a variety of signals compared to chromatographic techniques like LC where one component is represented by one peak, using quantitative NMR spectroscopy it is sufficient to find one baseline separated resonance for each component of interest. In Table 4-17 the chemical shifts of the corresponding NMR signals of the major component Ala and its impurities are summarized. Slight variations of the exact chemical shifts can be observed in mixtures when the quantitative composition of the contaminants is changed which is due to corresponding variation of the pH-value of the test solution. This technique provides at least one baseline separated signal for each impurity except for Asp. The methylene-resonances of Asp and malic acid overlap and the CH-resonance of Asp interferes with a ^{13}C satellite of Ala (cf. Fig. 4-30).

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[*] It is noted that the NMR work reported in this chapter was not carried out by the author. The purity determination of Ala described below was performed by Tanja Beyer, University of Wuerzburg, Institute of Pharmacy and Food Chemistry, Wuerzburg Germany.

Table 4-17. ^{1}H NMR resonances and T_1 relaxation times of Ala and its potential impurities. Modified from [136].

Component	Chemical shift [ppm]	T_1 relaxation time [s]
Ala	1.46 ppm (d, CH_3)	~ 2.2 s
	3.77 ppm (q, CH)	~ 7.2 s
Glu	2.00 ppm - 2.20 ppm (m, CH_2)	~ 1.5 s
	2.35 ppm - 2.55 ppm (m, CH_2)	~ 1.5 s
	CH-resonance interferes with CH-signal of Ala	(unknown)
Asp	2.69 ppm - 2.90 ppm (m, CH_2)	~ 1.5 s
	CH-resonance interferes with ^{13}C-satellite of Ala	~ 3.6 s
Malic acid	2.55 ppm - 2.85 ppm (m, CH_2)	~ 1.5 s
	4.36 ppm (q, CH)	~ 3.6 s
Fumaric acid	6.59 ppm (s, CH)	~ 8.7 s

However, removal of the ^{13}C satellites in the spectrum can be achieved performing ^{13}C decoupling pulse sequences. Alternatively, the Asp content can be determined by subtracting the peak area of the CH signal of the malic acid from the peak area of the combined CH_2 resonances of malic acid and Asp.
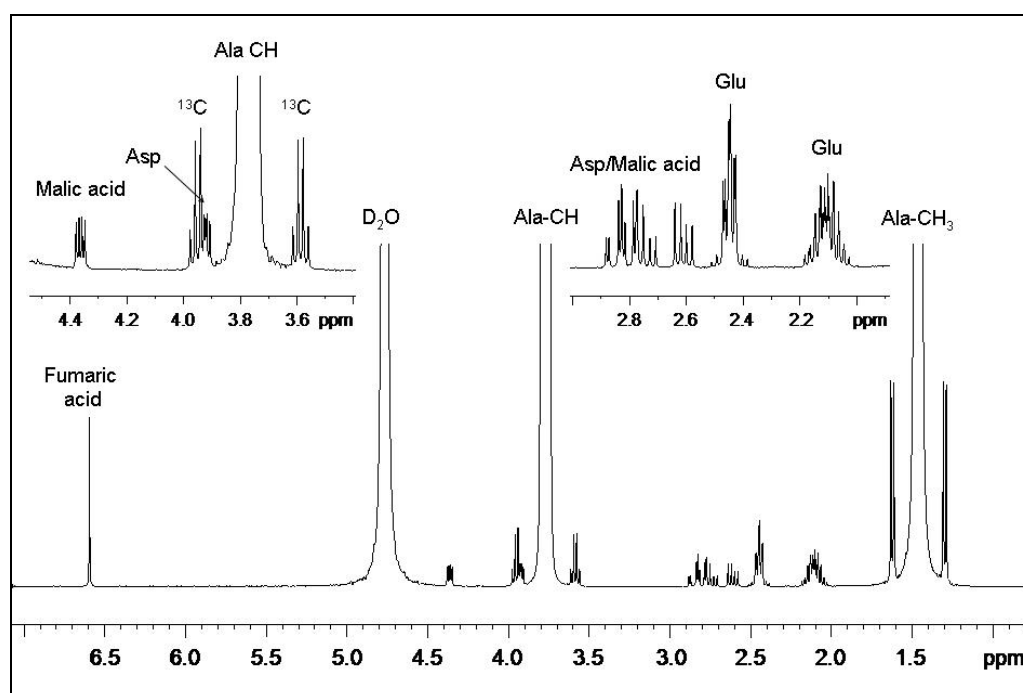
Figure 4-30. ^{1}H-NMR spectrum of the Ala sample analyzed in D2O (400 MHz). The individual components are Ala, Asp, Glu, malic acid and fumaric acid. Modified from [136].

4.3.2 Limit of Quantification (LoQ)

A low LoQ is of paramount importance for the control of impurities in drug substances. Preferably, the method should be sufficiently sensitive to enable quantification of occurring impurities below the ICH [25] level for drug substances with an average daily dose above 2 g – hence, a reporting threshold of 0.03%.

Using the aforementioned methods, the limits of quantification of the CAD, NQAD and MS detector – calculated using a signal-to-noise ratio (S/N-ratio) of 10:1 - were extrapolated from the detector response of 2.5 µg/mL solutions of the different components. For ELSD, the LoQ´s were estimated based on the S/N-ratios obtained with corresponding reference solutions containing the different components.

To determine the experimental LoQ for the impurities by means of NMR spectroscopy different predefined amounts of the related substances were spiked to pure samples of Ala with a concentration of approximately 50 mg/750 µL to define the respective amount of impurities possessing a signal-to-noise ratio of 10:1.

Although the instrument settings were not optimized for the most sensitive detection, the lowest limits of quantification were obtained by triple-quadrupole-MSD (cf. Tab. 4-18), as these instruments are designed for high sensitive quantifications. Very good sensitivity and low limits of quantification were also obtained using the CAD. Even though the instrument manufacturer of the NQAD emphasised ultra-high sensitivity and applicability for trace amounts of analytes [137], the NQAD was found to be less sensitive than the CAD. The difference in sensitivity was substance depending (cf. Tab. 4-18). For malic acid and fumaric acid the CAD was about twice more sensitive than the NQAD, and for Asp and Glu, the CAD was 4 to 5 times more sensitive than the NQAD. To the best of my knowledge, the sensitivity of ELSD may vary significantly between instruments from different manufacturers. With the instrument used, the limits of quantification were found higher than 0.1% (cf. Tab. 4-18) and therefore significantly above those obtained by MSD, CAD and NQAD.

Interestingly, a very sensitive determination of the impurities was also possible by means of the NMR spectroscopy, even though a frequently discussed disadvantage of this technique is its low sensitivity when compared to other spectroscopic methods. Note that the detection sensitivity may be improved by increasing the number of scans and/or the concentration of the test solution as well as by applying spectrometers with higher magnetic field strength or modern probes, e.g. cryoprobes [138].

The S/N ratio scales as $B_0^{3/2}$ and is proportional to the square root of the number of scans; for example, obtaining the same S/N ratio on a 400 MHz spectrometer compared to a 600 MHz spectrometer requires more than three times the number of scans. Furthermore, the sensitivity may be increased at least by a factor of four when applying a cryoprobe instead of a conventional probe. Especially for the quantification of impurities of low concentration the application of strong magnetic fields is therefore highly efficient in routine analysis.

In the present case, improving the S/N ratio by increasing the concentrations is limited by the solubility of Asp, Glu and fumaric acid in D_2O. However, LOQs between 0.004% and 0.04% of the concentration of the main component Ala could be achieved for the impurities (cf. Tab. 4-18) which is in the same range as found with the NQAD and CAD and even better than with the ELSD. Additionally, with a cryoprobe equipped 600 MHz NMR spectrometer only 16 scans were necessary to obtain a similar S/N ratio as with the 400 MHz machine and 128 scans. The data obtained by 400 MHz NMR spectroscopy reflect in a very impressing manner the suitability of qNMR for the control of low level impurities.

Table 4-18. Limits of quantification (based on a signal-to-noise ratio of 10:1) expressed in % referred the concentration of Ala in the corresponding test solution. Due to the non-linear response of the ELSD the figures given for this detector are only approximate values estimated based on the S/N-ratios obtained with corresponding reference solutions. Modified from [136].

Compound	ELSD	NMR (400 MHz)	NQAD	CAD	MS
Malic acid	0.1 %	0.04 %	0.023 %	0.010 %	0.001 %
Fumaric acid	0.2 %	0.004 %	0.020 %	0.010 %	0.005 %
Asp	0.2 %	0.03 %	0.025 %	0.006 %	0.0001 %
Glu	0.3 %	0.02 %	0.038 %	0.008 %	0.0001 %

4.3.3 Linearity of range

Acceptable linearity of the detector response is an important parameter for a convenient quantification of impurities occurring in a drug substance at different concentration levels. For ELSD, the detector response is not directly linear.

However, in a logarithmic coordinate system a linear relation between analyte concentration and detector response can be obtained over a wide range [60, 69, 77]. For CAD, the detector signal was reported to be linear over a limited range of about two orders of magnitude in different studies [78, 139]. The NQAD is claimed to have superior linearity compared to other aerosol-based detectors, covering several orders of magnitude for most analytes [137]. Quantification by HPLC/MSD is also normally known to deliver a good linear detector response [140, 141]. With regard to NMR spectroscopy linearity is given over a very broad range since the intensity of a signal is directly proportional to the number of nuclei being observed [133, 142] under well-defined experimental conditions (e.g. relaxation delay, signal-to-noise ratio, integrated region, phase etc.). As expected, the results of this study confirmed the linearity of the signal response for malic acid, fumaric acid, Asp and Glu, for qNMR as well as HPLC-MS detection. Although following a quadratic calibration function over a broader calibration range, the CAD was found to provide a reasonably linear response in a concentration range of about one order of magnitude (2.5 µg/mL to 20 µg/mL) [126].

The evaluation of the linearity of the NQAD was complicated by the fact that the repeatability of injection especially at low concentration levels was relatively poor. At 0.025% level (= 100 ng on column) the relative standard deviation (RSD) of 3 replicate injections varied between 4% and 20%. Improved repeatability of injection was found when more concentrated solutions were injected. At 0.2% (800 ng on column) a RSD between 1.4% and 6.1% was found for three replicate injections.

The results of this study showed that a linear detector response of the NQAD can reasonably be used for the quantification over a range of about one order of magnitude which is comparable to the CAD. For example, the calibration curve for glutamic acid from 2.5 µg/mL to 20 µg/mL (6 concentration levels) could be described by the equation $y = 75960\ x - 121.46$ ($r^2 = 0.9944$) with y being the detector response (peak area) and x being the concentration of the test solution (µg/mL). However, as can be seen in Figure 4-31, over a broader concentration range, as for CAD, a quadratic calibration function is probably more appropriate.
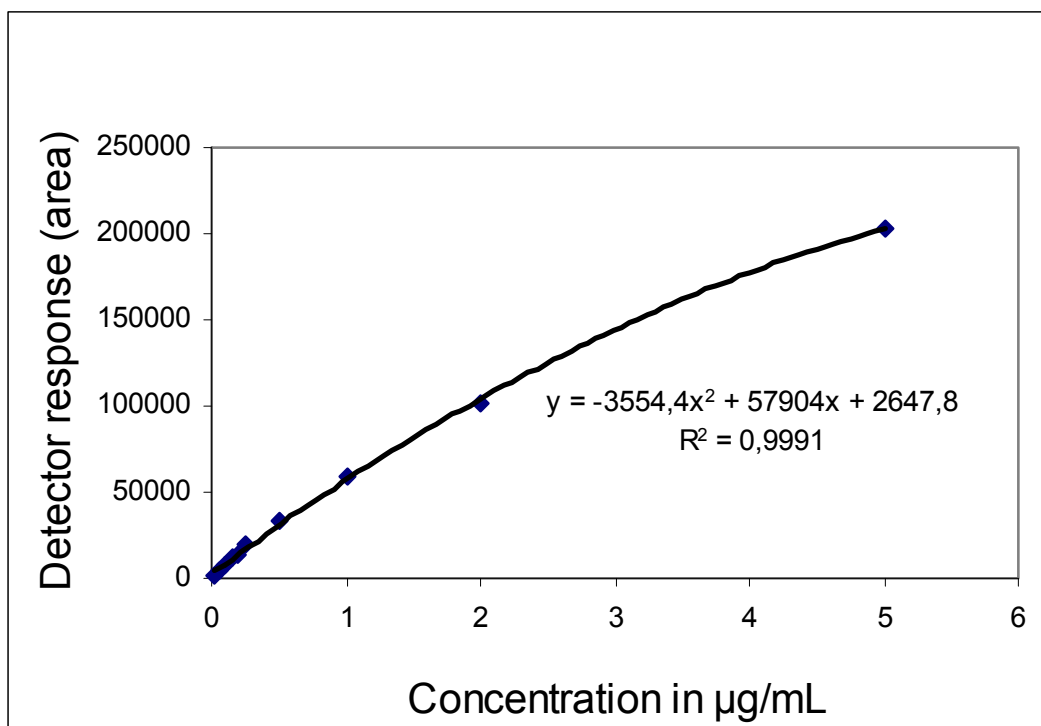
Figure 4-31. NQAD calibration curve of Glu from 2.5 to 500 µg/mL (ten concentration levels). Experimental conditions as described in 7.3.3.2. Modified from [136].

4.3.4 Purity determination of Ala

The purity of a batch of Ala spiked with known amounts of malic acid, fumaric acid, Asp and Glu (composition see above under 4.3) was determined using LC coupled to CAD, ELSD, MSD and NQAD. As described in 7.3.3.2 and 7.3.3.3 quantification of the individual impurities was performed using corresponding external standards. The quantification of each impurity by means of qNMR was carried out by employing the integrals of the CH_3-resonance of Ala and the impurity signal under consideration of the relative molecular mass and the number of contributing nuclei, respectively. The quantitative analysis of Asp was carried out by both integration of the CH-resonance after ^{13}C decoupling and subtracting the signal intensity of malic acid from the peak area of the combined CH_2-resonances of malic acid and Asp, respectively. Comparison of both procedures revealed the quantitative results for Asp to be in perfect agreement. An overview of the results obtained and a comparison with the spiked amount is given in Table 4-19 and Figure 4-32.

Table 4-19. Comparison of the impurity determination of a spiked sample of Ala.

(N.B.: Results in %-amount refer to the content of the corresponding compound in the spiked sample of Ala in % (m/m). The %-recovery values are based on the theoretical amounts of the different compounds added to the test sample of Ala.). Modified from [136].

	Amount spiked	NQAD		CAD		ELSD		MS		qNMR (400 MHz)	
		Amount (%)	Recovery	Amount (%)	Recovery	Amount (%)	Recovery	Amount (%)	Recovery	Amount (%)	Recovery
Malic acid	0.46 %	0.45 %	98 %	0.46 %	100 %	0.43 %	93 %	0.40 %	87 %	0.44 %	96 %
Fumaric acid	0.41 %	0.31 %	76 %	0.40 %	98 %	0.42 %	102 %	0.38 %	95 %	0.41 %	100 %
Asp	0.39 %	0.35 %	90 %	0.38 %	97 %	0.40 %	103 %	0.37 %	95 %	0.38 %	97 %
Glu	0.98 %	0.97 %	99 %	0.94 %	96 %	1.08 %	110 %	0.94 %	96%	0.98 %	100 %
Total	2.2 %	2.1 %		2.2 %		2.3 %		2.1 %		2.2 %	
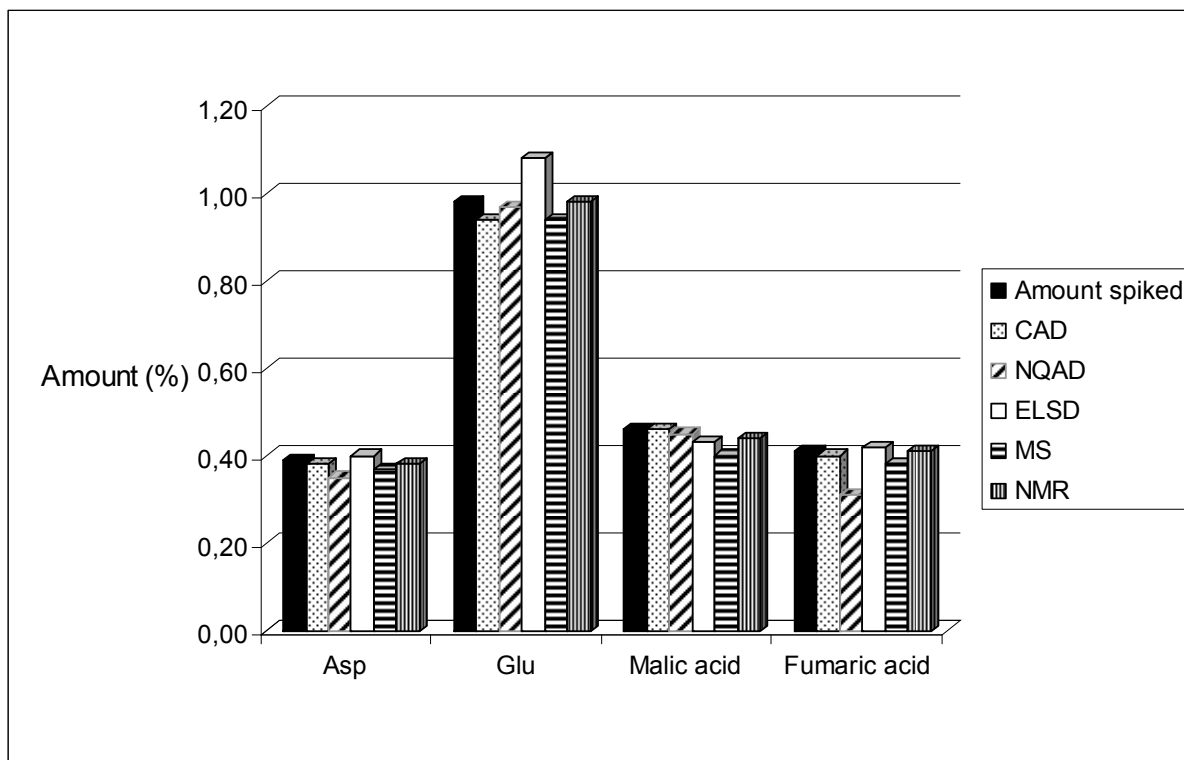
Figure 4-32. Comparison of the results for the quantification of Asp, Glu, malic acid and fumaric acid obtained by CAD, NQAD, ELSD, MS and NMR. Modified from [136].

Additionally, the chromatographic profiles obtained with the different LC-detectors as well as the ^{1}H-NMR spectrum are presented in Figure 4-30 and 4-33, respectively.

(A)
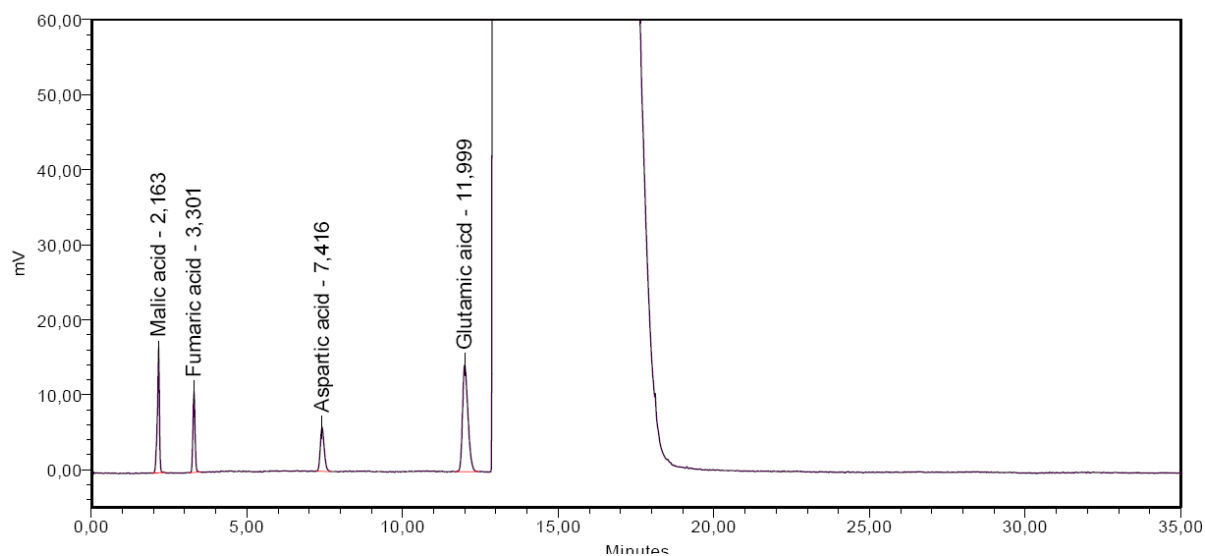
(B)
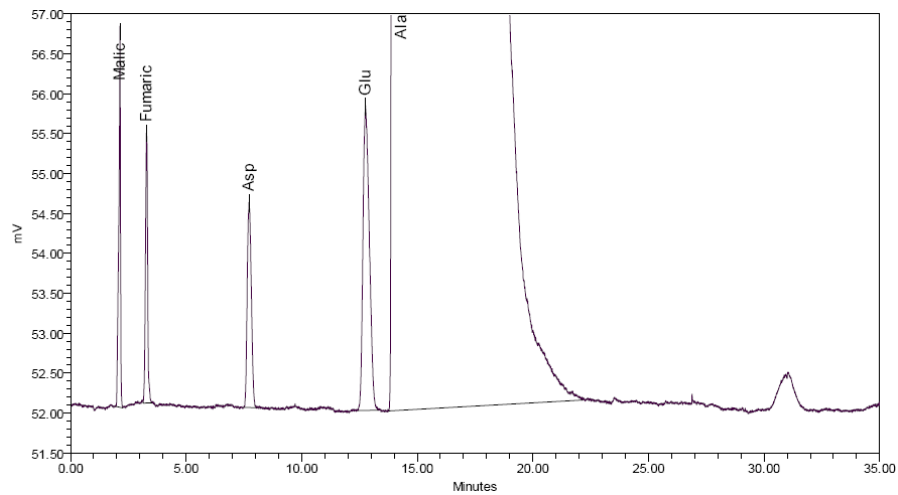
(C)
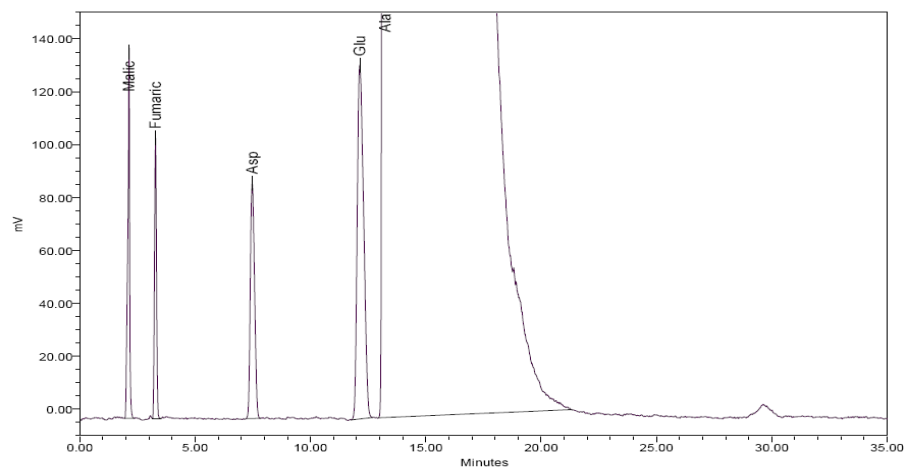
(D)
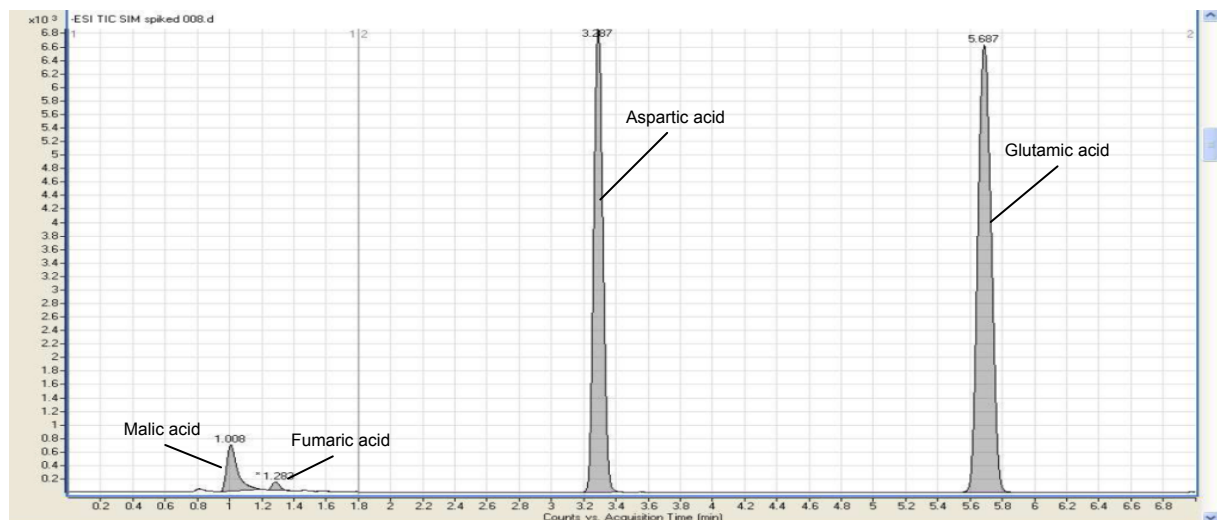
Figure 4-33. Chromatographic profile of spiked Ala (as described in 7.3.3.1) – detection by (A) ELSD,
(B) NQAD, (C) CAD, (D) MS – chromatographic conditions and detector settings as described in
7.3.3.2 and 7.3.3.3. In (B) and (C), an additional peak appeared at about 30-31 min. This peak is also
present in the blank. Modified from [136].

In general, the results obtained by the different chromatographic methods and by qNMR were rather comparable to each other. Moreover, close agreement with the theoretical values was found. Only the amount of fumaric acid determined by LC-NQAD differs substantially from the theoretical content as well as from the results obtained with the other detectors/methods. This finding could be partly explained by the relatively high variability of the individual measurements (RSD = 11.9 %, n = 11, for fumaric acid in the reference solution). Since the calibration curve for fumaric acid was found to be linear between 0.1 % and 0.5 % (r^2 = 0.9962) and an impact of a non-linear detector response on the result found could be excluded, no further factors explaining the deviating result for fumaric acid can be given.

4.3.5 Conclusion

Taken together, the control of impurities in Alanine at an ICH [25]-conform level could be ensured using LC coupled to CAD, MSD and NQAD detection as well as using qNMR. Although delivering satisfying results for the analysed spiked test sample, the ELSD detector used in this study was found to be not sufficiently sensitive for the control of impurities at very lower levels. Additionally, the non-linear response of the ELSD, requiring a double logarithmic calibration curve instead of using a single external standard – as it is common practice in this type of tests – makes the method rather cumbersome. Moreover, ELSD detection of such samples consisting of > 99 % of the main component and low amounts of impurities (< 0.2 %) is in danger to produce "ghost" peaks [76]. Comparing the remaining options, LC/MSD and NMR spectroscopy are considered to be very powerful and are surely a considerable option for specific applications. However, detection by MS is normally highly specific and problems might occur regarding the quantification of further occurring impurities. Although MS may allow the identification of these impurities, the corresponding substance must be available for the use as external standards for the quantification. Regarding efficiency aspects, qNMR allows for simultaneous qualitative and quantitative determination of major and minor components, e.g. impurities, within a single measurement, as well as additional structural elucidation of previously unknown components. In terms of performance, prize and ease of use CAD and NQAD appear to be the most suitable alternative when it comes to the sensitive determination of substance not eligible to UV-VIS detection. Based on the results of this study CAD appeared slightly superior to NQAD in terms of repeatability and sensitivity.

4.4 Development of a LC method for the control of impurities in streptomycin sulfate

Streptomycin, also called streptomycin A (cf. Fig. 4-34), is an oligosaccharide with basic properties. Similar to other members of the aminoglycoside family it consists of a monosaccharide, an amino sugar and a guanidino substituted cyclohexane with at least 3 hydroxyl functions. The three units are linked via α-glycosidic bonds. In the case of streptomycin these three units are streptidine (a diguanidino derivative of inositol), L-streptose (a rare 3-methylpentose), and N-methyl-L-glucosamine. The streptose carbonyl function is mainly present in its hydrated form [143].

Historically, streptomycin was first isolated in 1943 by Albert Schatz, a graduate student in the laboratory of Selman Abraham Waksman at the Rutgers University, New Jersey, USA [144] from S*treptomyces griseus*.

Streptomycin forms an irreversible bonding with the S12 protein of the 30S subunit of the bacterial ribosome [145], which causes an inhibition of the protein biosynthesis and therefore leads to the death of the microbial cell. The substance is an inhibitor of both, Gram-positive and Gram-negative bacteria.

Like other aminoglycoside antibiotics (e.g. gentamicin, tobramycin, amikacin, netilmicin, kanamycin, and neomycin) it is usually applied parenterally. Whilst for human use streptomycin is nowadays mainly employed as a second line therapeutic against tuberculosis, it is still a first line antibiotic in veterinary medicine used against Gram-negative bacteria in large animals (horses, cattle, sheep etc.). A major disadvantage of streptomycin as well as of all other aminoglycoside antibiotics is their pronounced ototoxic side effect [146].

The current Ph.Eur. monograph of streptomycin sulfate[147] does not describe an appropriate test for the control of the related substances. Solely a TLC limit test for the pharmacologically less active streptomycin B is given with a limit of 3.0%. This is particularly striking since streptidine was described to be a possible source for the ototoxicity of streptomycin [148].
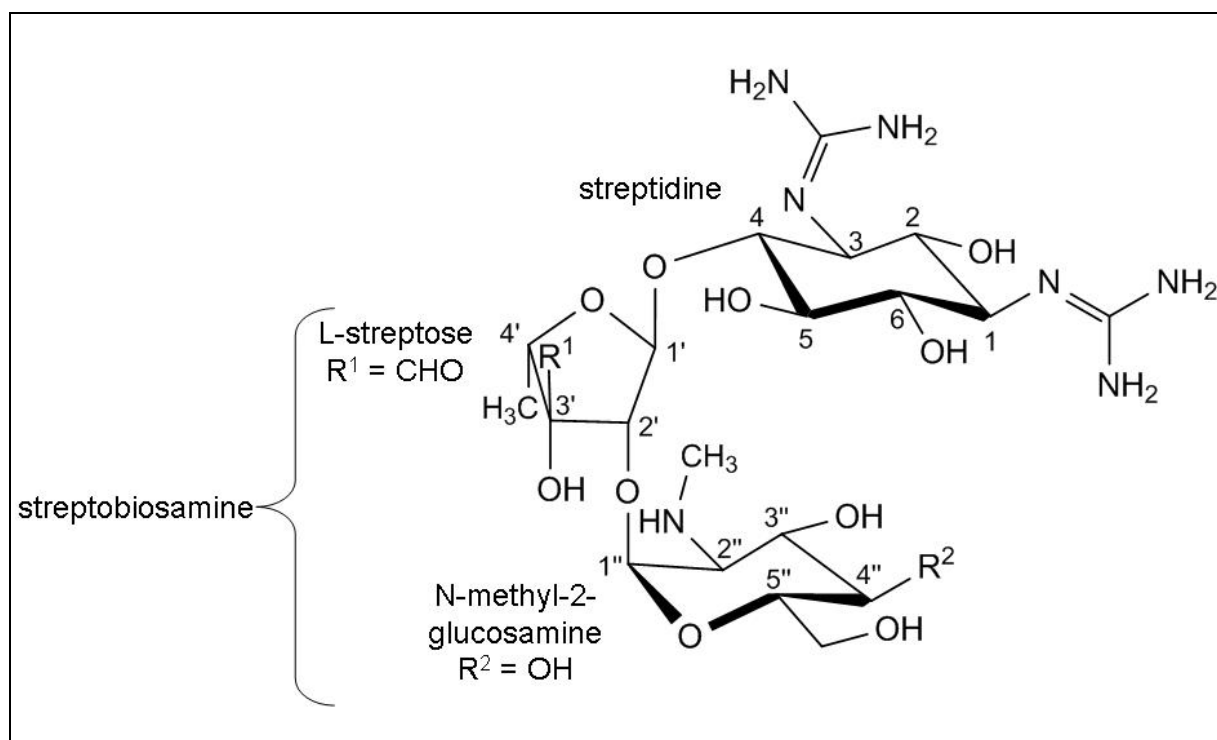
	m/z-ratio $[M+H]^+$	R^1	R^2
Streptomycin A	582.2735	$CH(OH)_2$ / CHO	OH
Dihydrostreptomycin	584.2886	CH_2OH	OH
Streptomycin B	744.3258	CHO	

Figure 4-34. Structures of streptomycin components. Modified from [149].

Several LC methods for the identification and control of the related substances in streptomycin sulfate using detection by UV-spectrophotometry, [150, 151] mass spectrometry [152] and pulsed amperometric detection (PDA) [153] have been described in the literature. Furthermore, a capillary zone electrophoresis (CZE) method was proposed for this purpose [154]. Unfortunately, each of these methods has major disadvantages. Due to a lack of a suitable chromophore, the analysis by UV-spectrophotometry requires high analyte concentrations and a low detection wavelength (about 200 nm). In addition, impurities lacking the UV-absorbing

guanidine-groups of streptidine will not be detected. The application of mass spectrometry requires suitable specific reference materials for the quantificiation of the impurities. However, these are currently not available. The application of PAD is technically very difficult and requires a skilled operator. Another disadvantage is that non-oxidizable compounds are not detected. Finally, the selectivity of most of the above methods in terms of resolution of streptomycin from its related substances is insufficient.

To overcome the aforementioned limitations, detection by CAD was considered to represent a promising alternative. Since CAD requires a volatile mobile phase and the method should ideally be isocratic in order to obtain a constant detector response, none of the methods published in literature could be employed. Therefore, a new LC method for related substances was elaborated from scratch.

The method development was notably challenging since apart from streptidine no further impurity was available to verify the selectivity of the method.

Since the streptomycin sulfate samples used in this study were kindly provided by the group of Hoogmartens and Adams, University of Leuven, Belgium, it was decided to first analyse all samples using the LC-UV method published by this group [150] and to use the results obtained as a starting point for further method development. Furthermore, the compliance of the samples with the requirements of the TLC test for streptomycin B described in the Ph.Eur. monograph of streptomycin sulfate [147] was verified.

4.4.1 TLC test for streptomycin B according to the Ph.Eur monograph.

The TLC test for related substances was performed as described in the Ph.Eur. monograph of streptomycin sulfate (cf. 7.4.3.1).

For none of the samples tested the spot due to streptomycin B was found to be more intense than the spot of the mannose reference solution corresponding to the Ph.Eur. limit of 3.0% of streptomycin B. However, it has to be noted that the test was difficult to evaluate since the spot due to mannose in the reference solution was found very faint. A chromatogram showing the current batch 3 of the Ph.Eur. reference standard as well as 2 further samples of streptomycin sulfate is depicted in Figure 4-35.
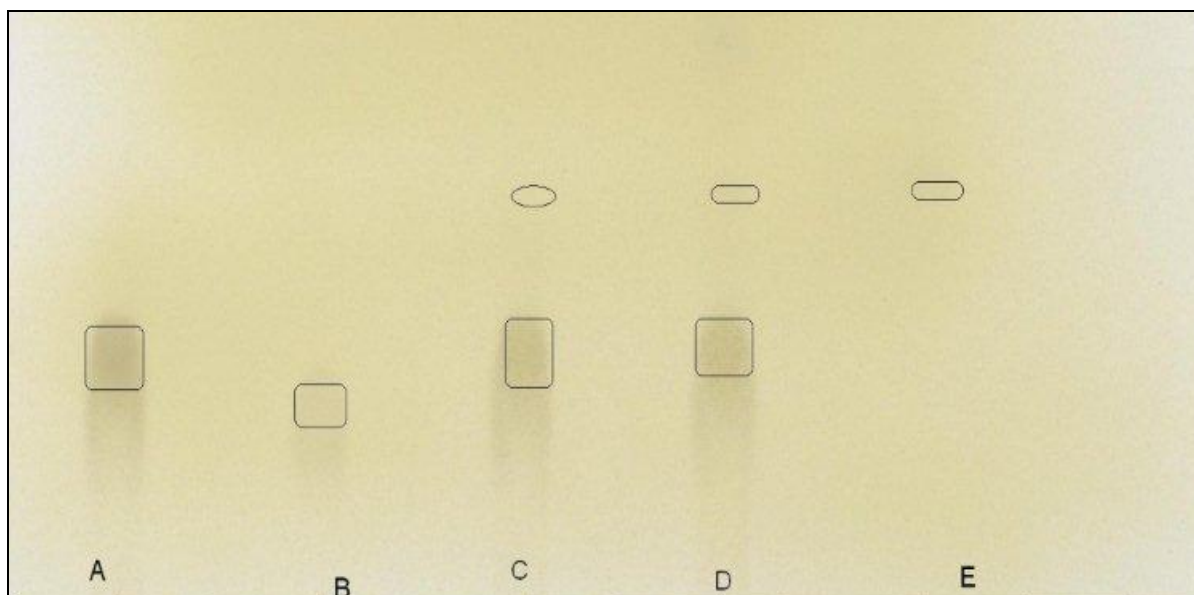
Figure 4-35. TLC limit test for streptomycin B according to the Ph.Eur. Lane A – sample 6; Lane B – sample 9; Lane C - sample 8; Lane D - CRS 3, lane E – mannose reference solutions 3% (experimental conditions cf. 7.4.3.1).

4.4.2 Analysis of streptomycin sulfate using a LC-UV literature method

The method published by the group of Hoogmartens and Adams [150] is based on reversed phase ion-pair chromatography (RP-IPC) using sodium octanesulfonate as ion-pair reagent, a Supelcosil ABZ alkylamide column and UV detection at 205 nm. The details of the method are described in 7.4.3.2.

4.4.2.1 Evaluation of the method performance

In a first step, the method performance in terms of selectivity and sensitivity was verified using the method of Hoogmartens and Adams [150].

Limit of quantification

For the injection of a 0.2% dilution of a 2.5 mg/mL streptomycin sulfate test solution (Figure 4-36), a S/N-ratio of about 5 was determined. Based on this value, the limit of quantification for streptomycin sulfate (based on an S/N-ratio of 10) was calculated to be 0.01 mg/mL, being 0.4% referred to a 2.5 mg/mL test solution.
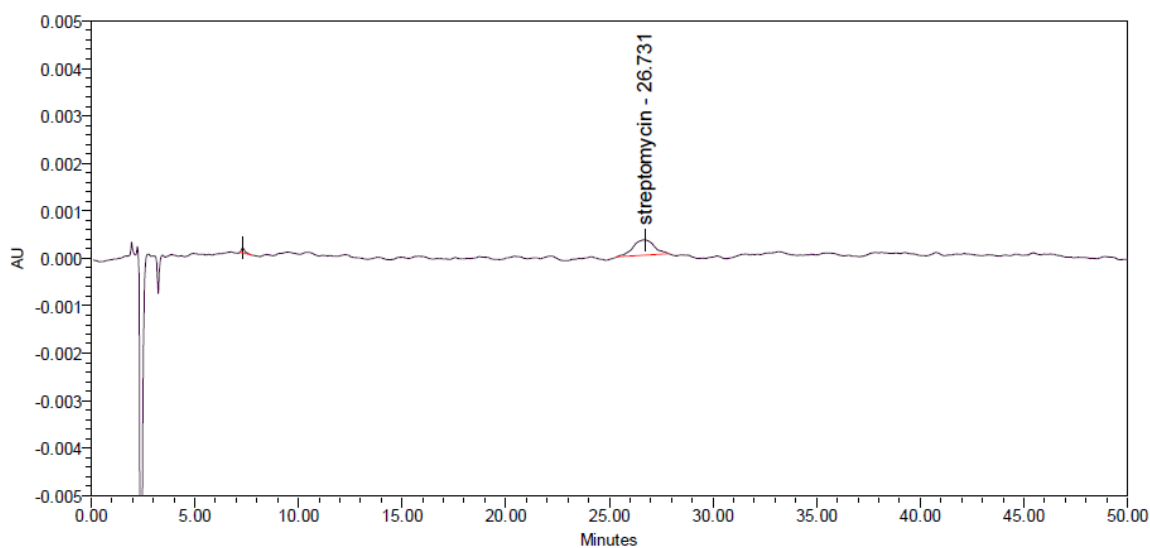
Figure 4-36. Chromatogram of a 0.005 mg/mL aqueous solution of streptomycin sulfate. Experimental conditions cf. 7.4.3.2.

Unfortunately, no LoQ was given for streptomycin sulfate in [150]. For streptomycin B and dihydrostreptomycin LoQs of 0.25 µg each (corresponding to about 0.5% referred to a concentration of the streptomycin sulfate test solution of 2.5 mg/mL) were reported, and for streptidine sulfate a LoQ of 0.02 µg (corresponding to about 0.04%). However, this is not surprising since streptidine elutes much earlier than streptomycin and as a very narrow and high peak. Moreover streptidine contains the two guanidino functions responsible for the absorbance of the streptomycin molecule. Taken together, the method is not considered sufficiently sensitive to control the impurities in streptomycin sulfate with a reporting threshold of 0.1% as it is normaly applied in the Ph.Eur. for antibiotics derived from fermentation.

Selectivity

The method selectivity was difficult to evaluate since, apart from streptidine sulfate, no impurity was available and no resolution criterion was proposed in [150]. Therefore the chromatogram of sample N° 10 (cf. Figure 4-37), which was found to be the most impure sample, was compared with the example chromatogram depicted in [150]. The two chromatographic profiles as well as the retention times of the major peaks were found rather similar.
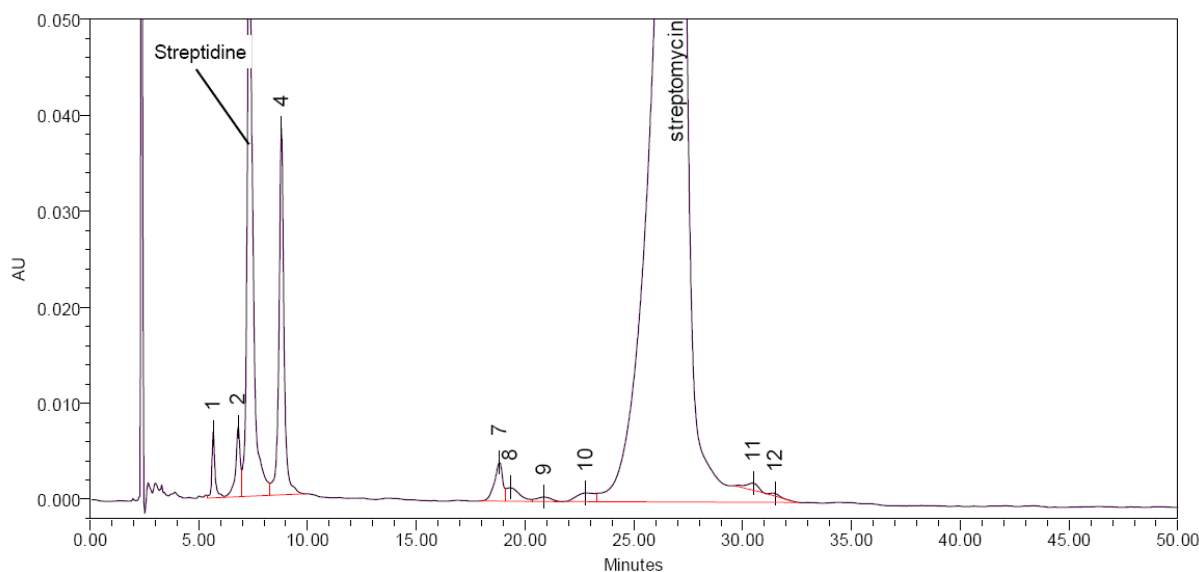
Figure 4-37. LC-chromatogram of streptomycin sulfate sample N° 10. Mobile phase consisting of 14 g sodium sulfate, 1.5 g disodium 1-octanesulfonate, 50 mL of a 0.2 M phosphate buffer at pH 3.0 and 50 mL of acetonitrile, in 1 L of water. Chromatographic separation performed using a Supelcosil ABZ alkylamide column (250 mm x 4.6 mm, 5 µm) at a column temperature of 45 °C. Injection of 20 µL of a 2.5 mg/mL solution of streptomycin sulfate in water. LC-flow-rate of 1.0 mL/min. UV-detection at 205 nm. Streptidine and peaks 1 to 12 are putative impurities of streptromycin sulfate.

Nevertheless, it appeared that several peaks were partly co-eluting with one another or with the peak due to streptomycin. The method was therefore not considered sufficienty selective to be described for the impurities control in the Ph.Eur. monograph.

4.4.2.2 Determination of the related substances in streptomycin sulfate samples

Despite the aforementioned limitations, the published batch results obtained with the above method showed that considerable amounts of impurities could be separated from streptomycin sulfate [150]. It was therefore decided to apply this LC-UV method (cf. 7.4.3.2) to 12 samples of streptomycin sulfate (including the current Ph.Eur. streptomycin sulfate CRS 3) in order to select the sample containing the most impurities for the development of a new method. In preliminary tests, the purity of streptomycin sulfate sample 7, determined by area-normalization, was found to be about 94%. It was decided to use a 0.5% dilution of the test solution of this sample as an external standard for the quantification of the impurities in all samples. The results are presented in Table 4-20.

Table 4-20. Impurities determined in commercial streptomycin sulfate samples (% m/m) expressed as streptomycin sulfate on the substance as is. LC-UV method as described in 7.4.3.2 (ND, not detected (below LoD).

Sample	P1	P2	Strepti-dine	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	Total
				Impurities found in commercial samples of streptomycin sulfate (% mm)										
1	0.15	0.14	1.8	0.10	ND	ND	0.79	ND	0.53	ND	ND	ND	0.36	**3.9**
2	1.08	0.88	5.6	3.42	ND	0.08	1.47	ND	0.65	ND	0.50	ND	0.16	**13.8**
3	0.75	0.82	4.4	1.21	0.10	0.08	0.95	ND	0.46	ND	0.84	ND	ND	**9.6**
4	1.24	0.61	5.9	1.62	ND	ND	1.37	ND	0.87	ND	0.35	ND	0.23	**11.2**
5	0.21	0.35	2.8	0.17	0.10	ND	0.9	ND	0.07	ND	ND	0.11	ND	**4.7**
6	1.07	0.94	6.1	2.79	ND	0.11	0.65	ND	0.32	ND	0.48	0.04	0.18	**12.7**
7	0.21	0.27	2.8	0.28	ND	ND	1.24	ND	0.79	ND	0.07	ND	0.13	**5.8**
8	1.09	0.81	5.5	2.81	0.41	ND	1.84	ND	0.71	ND	1.22	0.7	0.17	**15.3**
9	0.16	0.14	2.1	0.07	ND	ND	0.78	ND	0.49	ND	ND	ND	0.30	**4.0**
10	0.61	1.16	5.6	5.81	ND	ND	0.82	0.67	0.23	0.44	0.22	0.07	ND	**15.6**
11	ND	ND	0.77	ND	ND	ND	0.80	ND	0.34	ND	ND	ND	0.31	**2.2**
12 (Ph.Eur. CRS 3)	ND	0.16	0.87	ND	ND	ND	2.70	ND	0.43	ND	0.17	0.06	0.36	**4.8**

Since a correction factor of 0.51 (compared to the detector response of streptomycin sulfate) was found for streptidine sulfate, the peak area of the peak due to streptidine was corrected accordingly. However, it must be kept in mind that this correction does not deliver a precise estimate of the amount of impurities, since under the conditions of this method streptidine probably co-eluted with other impurities not necessarily possessing the same correction factor.

Moreover, the evaluation of the chromatogram was complicated by the fact that the peaks after 15 min were rather broad and partly co-eluted with the streptomycin peak (cf. Fig. 4-37 and 4-38).
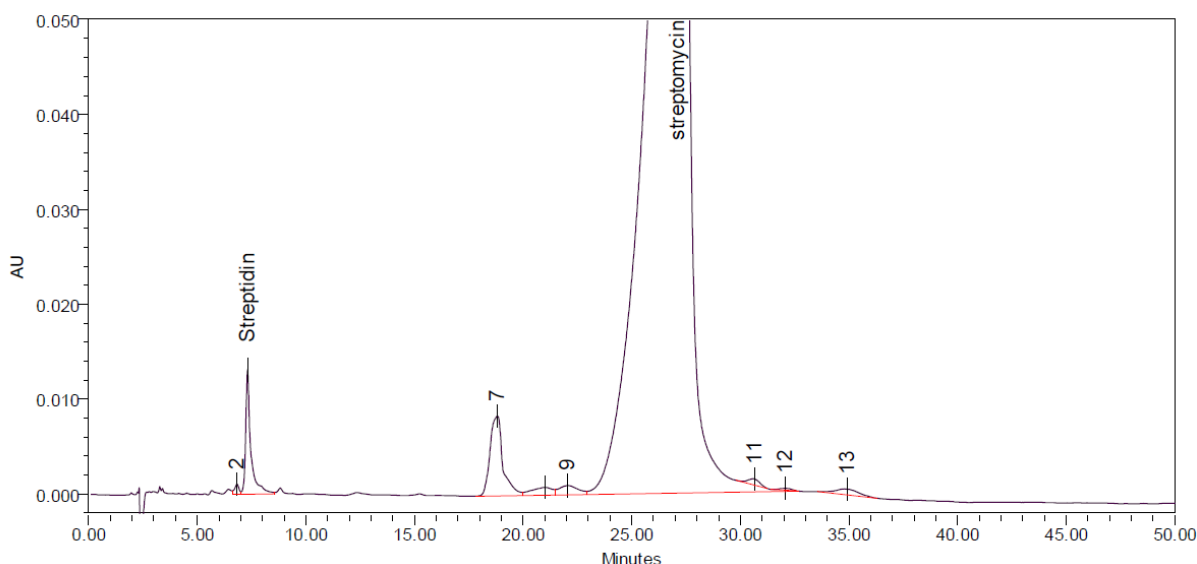
Figure 4-38. Test solution of streptomycin sulfate Ph.Eur. CRS 3. Chromatographic conditions as given in Figure 4-37. Streptidine and peaks 2, 7, 9, 11, 12 and 13 are putative impurities of streptromycin sulfate.

Taken together, the above method revealed several shortcomings, i.e. high LoQ, different UV response of the compounds, unsatisfying peak shape and separation. However, for the purpose of this study which was the development of an LC-CAD method, the results obtained with this method were considered to be a useful starting point as well as a benchmark.

The analysis performed in this study showed 13 impurities being separated. The total amount of impurities in the analyzed samples ranged between 2.2% and 15.6%.

Sample 10 (cf. Fig. 4-37) was found to contain the most impurities and was therefore selected for the further method development.

4.4.3 Development of a HILIC-CAD method

As described in chapter 3.1.3, HILIC can represent a viable option for the separation of polar, hydrophilic compounds. Furthermore, a paper has recently been published describing the analysis of streptomycin and dihydrostreptomycin by HILIC coupled to MS [152]. Therefore, the possibility of developing a HILIC-CAD method for the composition testing of streptomycin sulfate was explored using two different types of HILIC columns. In a first series of experiments an Obelisc N column was tested. As depicted in Figure 4-39, this column is described to have very polar characteristics. According to the column manufacturer it should provide good results for polar and charged analytes with both positively and negatively charged groups based on interactions with the analytes in multiple separation modes (normal phase, HILIC, ion-exchange) [155].
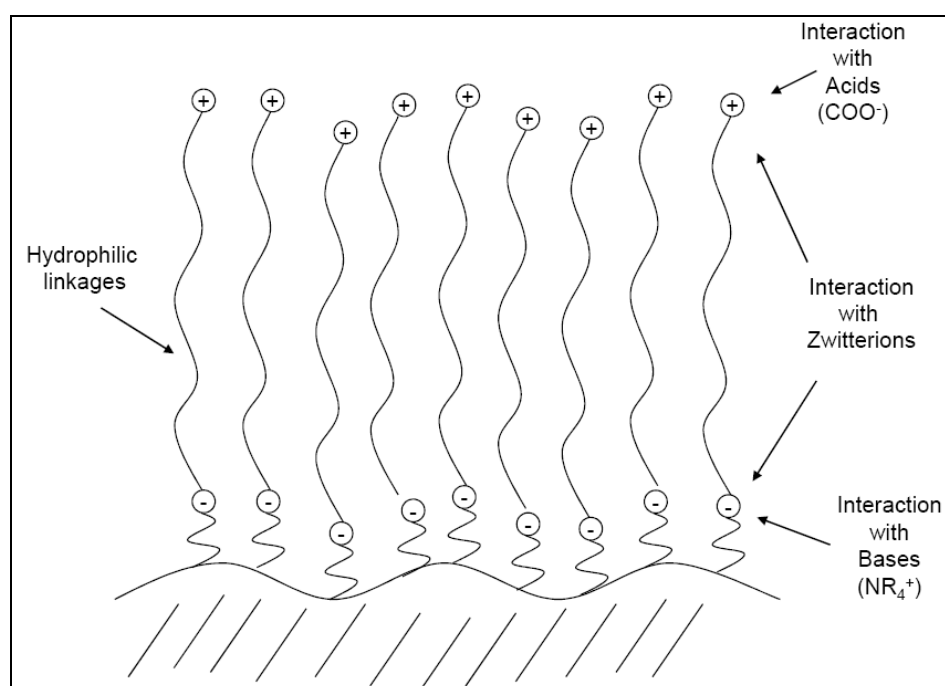
Figure 4-39. Schematic illustration of the surface of an Obelisc N column.

A second series of experiments was performed using a ZIC® HILIC column. This column is described to have characteristics similar to the Obelisc N column, but shows inversion of the charged groups (Figure 4-40). Moreover the linkage of the two charged groups is not hydrophilic.
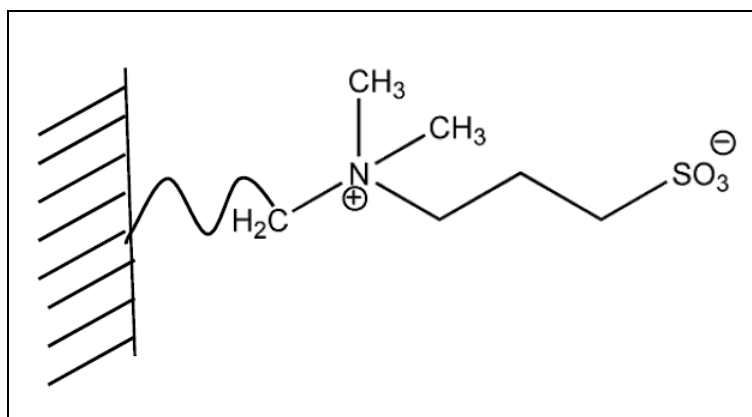
Figure 4-40. Schematic illustration of the surface of a ZIC® HILIC column.

Based on a publication from Kawano [152] it appeared that concentrations of about 200 mmol/L of volatile buffer would have a positive impact on the peak shape and separation of streptomycin and dihydrostreptomycin. Unfortunately, it was not possible to test buffers at such a high concentration in combination with the CAD. At concentrations above 20 mmol/L of ammonium formate and ammonium acetate the baseline offset caused by the mobile phase buffer was found to increase drastically, resulting in very poor method sensitivity. For further testing, it was therefore decided that 20 mmol/L represent the upper concentration limit for the buffer as regards the method development. Similar limitations were found for the addition of formic acid or acetic acid used to adjust the pH of the mobile phase. Already very small additions in a range of 0.1% (v/v) resulted in a significant increase of the background signal.

Both columns were tested using a 20 mmol/L ammonium acetate buffer solution at pH 6.8 and 4.9 in mobile phases containing varying amounts of acetonitrile. Additionally a buffer free eluent composed of water and acetonitrile was employed. A details description of the experiments performed is given in 7.4.3.3.

Results using a ZIC® HILIC column

With high amounts of organic modifiers in the mobile phase (85% v/v acetonitrile) streptomycine did not elute from the column. As can be seen in Figure 4-41, some more intense substance peaks were found when the amount of organic modifier was reduced to 80% (v/v), but at the same time the small peaks between 6 and 10 min were less good separated than before.

Further reduction of the amount of organic modifier to 75% (v/v) resulted in a reasonable retention time of 19 to 23 min for the major peaks but to a complete loss of separation of the small peaks. Moreover, a high baseline offset (between 250 and 300 mV) was noted indicating the above mentioned high background noise caused by the ammonium acetate buffer. Unfortunately, an adjustment of the mobile phase pH to 4.9 with acetic acid did not improve the separation but resulted in a further increase of the background noise.

a)
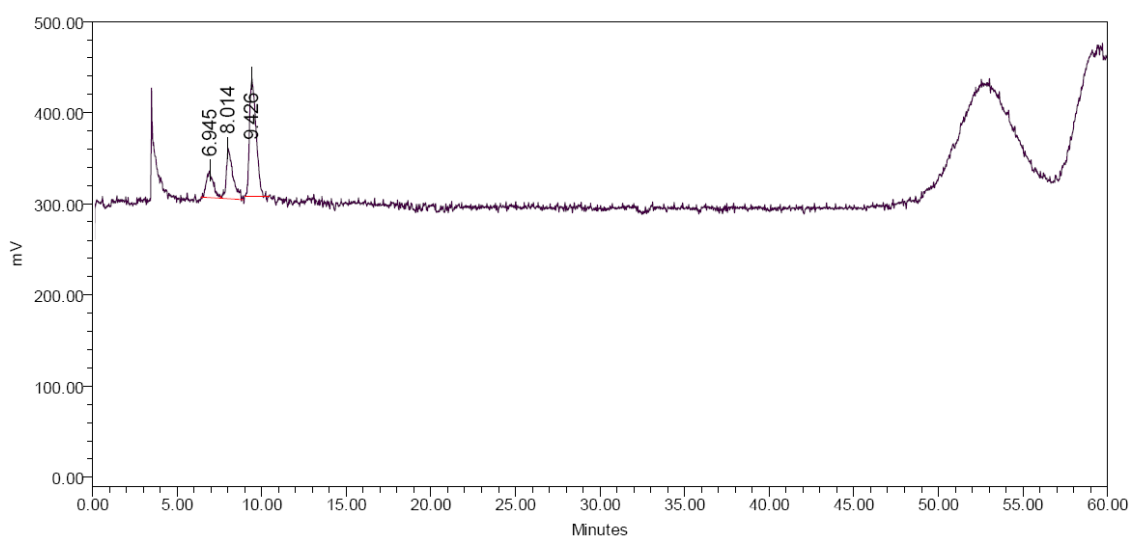
b)

Figure 4-41. LC-CAD chromatograms of a 2.5 mg/mL solution of streptomycin sulfate in water. Injection volume 20 µL; LC flow rate: 0.5 mL/min using a ZIC® HILIC column (150 mm x 4.6 mm, 3.5 µm) at ambient temperature. Mobile phase: a) 20 mmol/L ammonium acetate / acetonitrile 20/80 (v/v); b) 20 mmol/L ammonium acetate / acetonitrile 75/25 (v/v).

Results using an Obelisc N column

The results obtained with an Obelisc N column at different compositions of the mobile phase with and without addition of a buffer were even more disappointing than the ones of the ZIC® HILIC column. None of the chromatographic conditions tested provided a basis for further method development. To underline this conclusion, the chromatogram obtained with a mobile phase consisting of 20 mmol/L ammonium acetate / acetonitrile (20/80 v/v) is shown in Figure 4-42.

Figure 4-42. LC-CAD chromatograms of a sample of 2.5 mg/mL of streptomycin sulfate in water. Injection volume 20 µL; LC flow rate: 0.5 mL/min using an Obelisc N column (150 mm x 4.6 mm, 5 µm) at ambient temperature. Mobile phase composition 20 mmol/L ammonium acetate / acetonitrile (20/80 v/v).

Based on the above presented results it was concluded that a method development using zwitterionic HILIC columns is not very promising. Since it was considered highly likely that the same problems as regards the use of buffer solutions, pH adjustment and the limitation to isocratic methods would also be detrimental to a method development when using other types of HILIC columns, it was decided to employ another another type of separation mechanism for the method development.

4.4.4 Development of an ion-pair chromatograpy (IPC) - CAD method for the determination of the related substances in streptomycin sulfate

In the LC-UV method developed by Adams et al. [150] IPC using a non-volatile ion-pair reagent was successfully employed. Moreover an IPC method with TFA as an ion-pair reagent and detection by ELSD has been published for the quantification of streptomycin sulfate [156]. Furthermore, good experience with volatile ion-pair reagents was made previously for the development of the related substances method for Asp and Ala. Therefore, it was decided to try the same approach for streptomycin sulfate.

4.4.4.1 Pre-selection of the column

The first step of the method development was the selection of the most promising LC column. The three columns with the lowest baseline noise in the Asp and Ala quality assessment (i.e. a Zorbax Eclipse XDB C18 column, a Symmetry C18 column and an Inertsil ODS 3 column; cf. 4.2) were tested employing water / methanol containing 1 mmol/L of HFBA as a mobile phase. The tests were performed with 80% (v/v) and 95% (v/v) of organic modifier measuring the average peak-to-peak noise of an injection of the mobile phase. Additionally, a 5 mg/mL test solution of streptomycin sulfate sample 10 was injected to obtain an idea about the selectivity of the three columns.

The average peak-to-peak noise of all columns with both mobile phase compositions was found to be comparably low, ranging from 96 µV to 150 µV. Since the shape of the streptomycin peak exhibited a significant tailing on all columns, even when diluted solutions were injected, the column efficiency (number of theoretical plates calculated for streptomycin) was not considered to be a suitable criterion for the selection of the most promising column. Therefore, the decision was based on the retention behaviour and the chromatographic profile of the test solution. Compared with the Symmetry column (retention time about 6 min.), the Inertsil and the Zorbax Eclipse XDB column showed a longer retention of streptomycin (retention time about 14 min. to 15 min.). With regard to the chromatographic profile, the Inertsil ODS column appeared to be most promising (Figure 4-43). Since this column has been proven to be very robust and compatible with highly aqueous mobile phases before, it was selected for the method development.
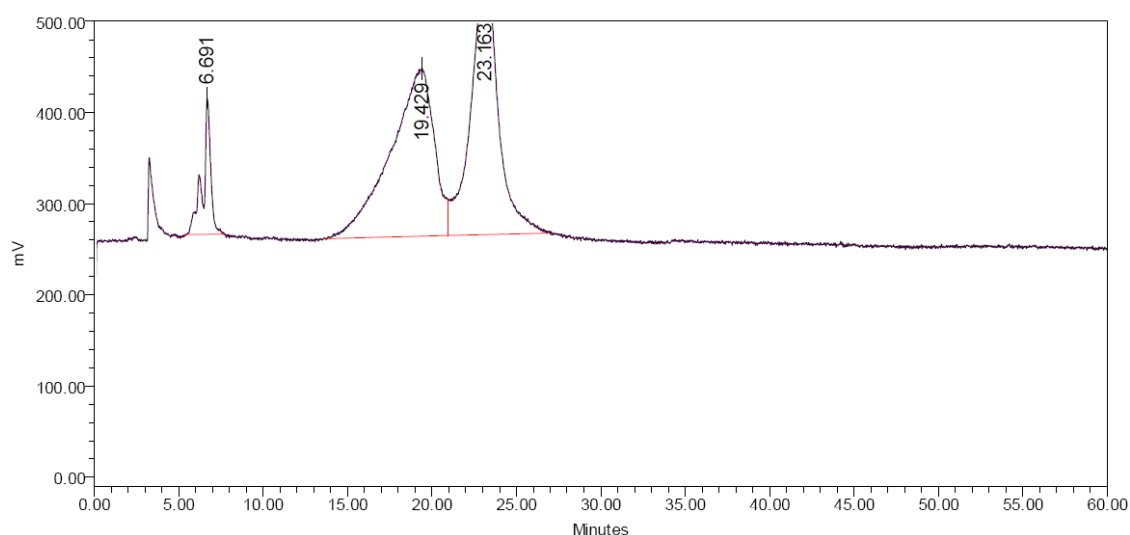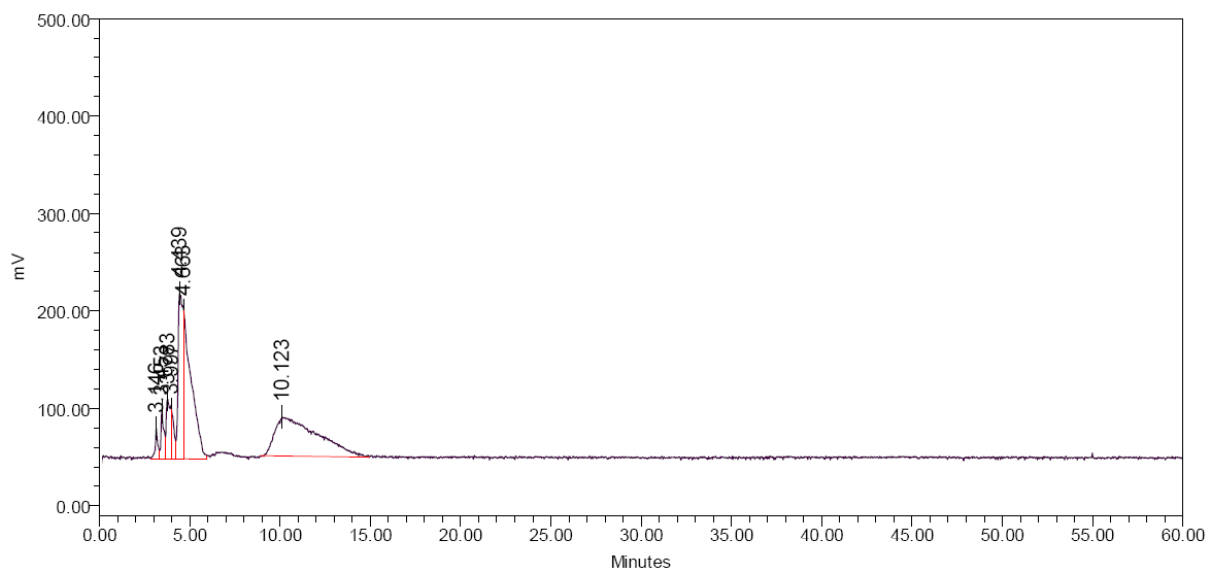
Figure 4-43. LC-CAD chromatograms of a 5 mg/mL solution of streptomycin sulfate in water. Injection volume 20 µL; LC flow rate: 1.0 mL/min using an Inertsil ODS 3 column (150 mm x 4.6 mm, 5 µm) at 30 °C. Mobile phase: 1.0 mmol/L HFBA in water / methanol (80/20 v/v).

4.4.4.2 Selection of the ion-pair reagent

Typically the LC method development starts with a gradient run to estimate the best ratio of the aqueous and the organic part of the mobile phase for subsequent isocratic runs [35]. To facilitate the choice of the most promising ion-pair reagent, this approach was followed using selected volatile ion-pair reagents of increasing chain-length and a water / methanol gradient from 100% water to 40% water during 30 min. The tests were performed injecting a 2.5 mg/mL solution of streptomycin sulfate sample 10 using different concentrations of TFA, HFBA, perfluorohepanoic acid (PFHA) and perfluorononanoic acid (cf. 7.4.3.4), respectively.

As expected, the retention time of streptomycin increased with the concentration and with the chain-length of the ion-pair reagent. However, the selection of the most suitable ion-pair reagent was not only based on the retention of streptomycin but also on the separation of occurring secondary peaks. Using 0.5 mmol/L of TFA the chromatographic profile very poor (Figure 4-44 a)). Higher concentrations of TFA resulted in a strong increase of the baseline-noise. Since TFA is a strong acid (pk_a = 0.23) it is assumed the pH of the mobile phase dropped below the lower pH-limit of the column (pH 2.0) causing dissolution of the column material.

For HFBA a concentration of 2.5 mmol/L delivered the best results and a very promising compromise between the retention time of the streptomycin peak and the separation of the secondary peaks (Figure 4-44 b)). Using PFHA, the retention time of streptomycin was very long, but the separation of the secondary peaks was

nevertheless poor (Figure 4-44 c)). Based on these results it was decided to continue the method development using HFBA as an ion-pair reagent.

a)
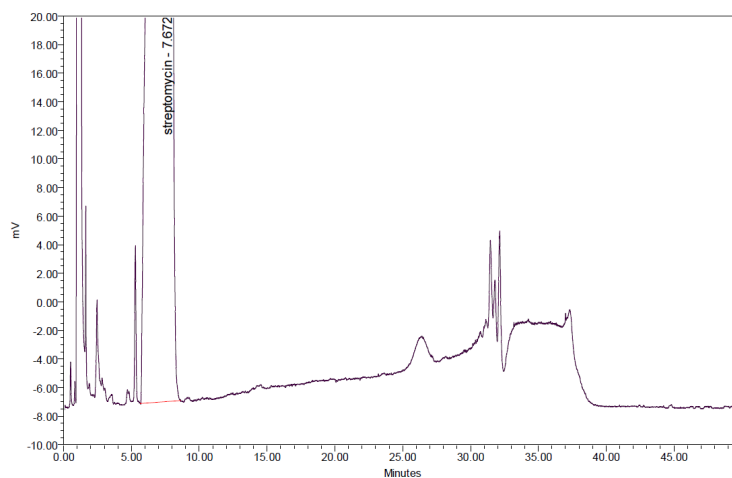
b)
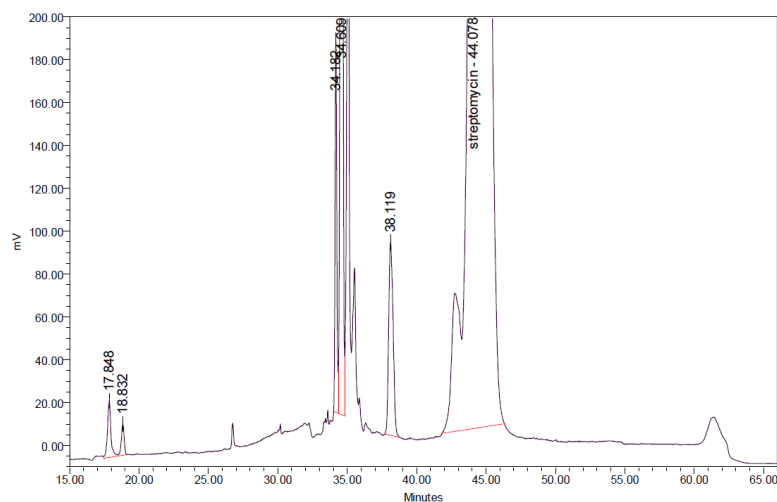
c)
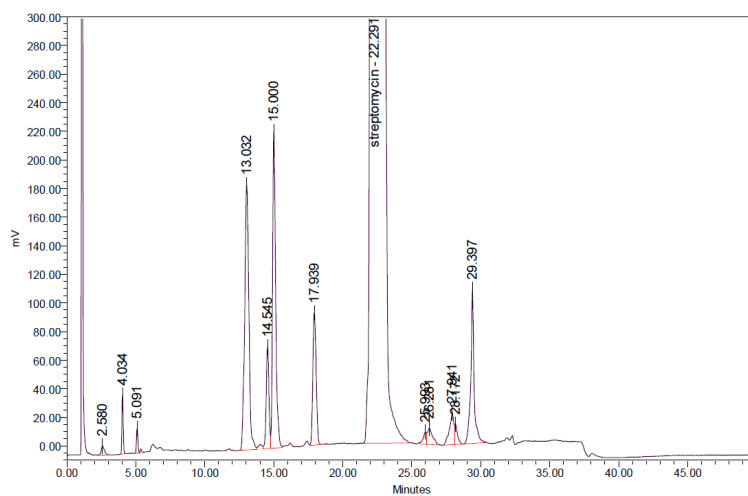
Figure 4-44. LC-CAD chromatograms of a 2.5 mg/mL solution of streptomycin sulfate in water. Injection volume 20 µL; LC flow rate: 1.0 mL/min using an Inertsil ODS 3 column (150 mm x 4.6 mm, 5 µm) at 30 °C. Gradient: 100% to 40% aqueous in 30 min. Mobile phase: a) 0.5 mmol/L TFA in water / methanol; b) 2 mmol/L PFHA in water / methanol; c) 2 mmol/L HFBA in water / methanol.

4.4.4.3 Development of isocratic method conditions using HFBA and PFPA

Extensive testing was performed varying the amount of HFBA between 0.5 mmol/L and 7 mmol/L in mobile phases containing 10% to 60% of methanol.

Unfortunately the promising chromatographic profile obtained under gradient conditions (cf. Fig. 4-44 b) could not be transferred into suitable isocratic conditions. The use of low amounts of methanol and high concentrations of ion-pair reagent resulted in an improved separation, but at the same time in unreasonably long retention times. The opposite effect was observed when the amount of methanol was increased and when the amount of ion-pair reagent was reduced. This situation is best illustrated by the chromatogram of a 2.5 mg/mL test solution of streptomycin sample 10 in a mobile phase consisting of water / methanol (80/20 v/v) with 5 mmol/L of HFBA (cf. Fig. 4-45).
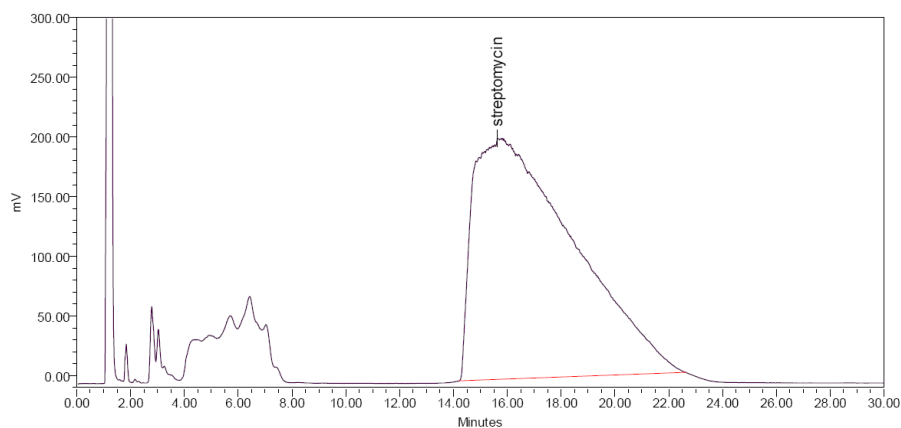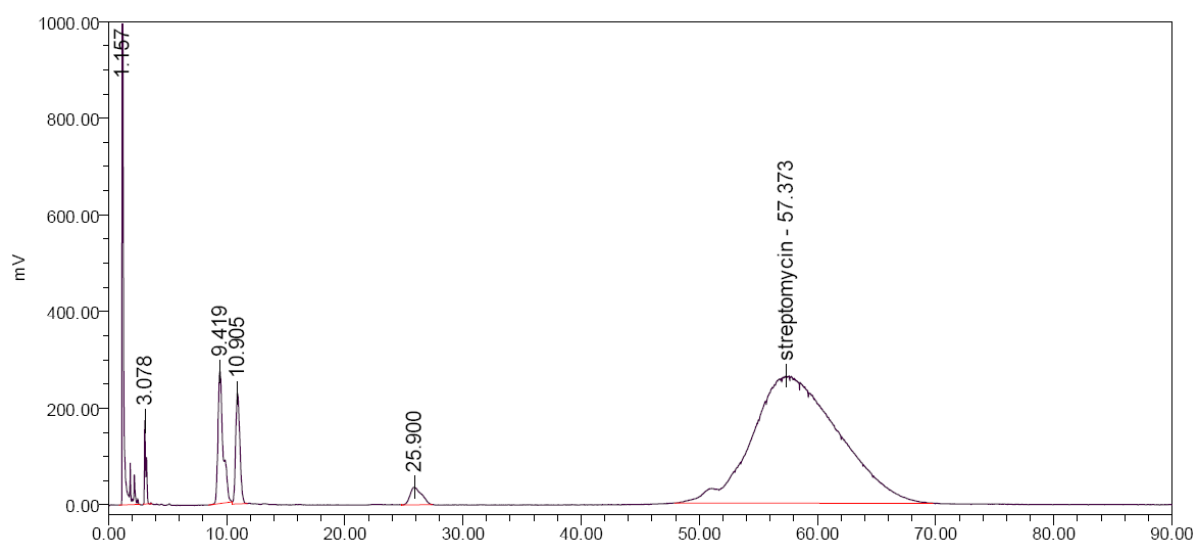

Figure 4-45. LC-CAD chromatogram of a 2.5 mg/mL solution of streptomycin sulfate in water. Injection volume 20 µL; LC flow rate: 1.0 mL/min using an Inertsil ODS 3 column (150 mm x 4.6 mm, 5 µm) at 30 °C. Mobile phase: 5.0 mmol/L HFBA in water / methanol (80/20 v/v).

In order to overcome these problems the ion-pair reagent was changed to pentafluoropropionic acid (PFPA). Numerous combinations of different PFPA concentrations and variations in the amount of the organic modifier were tested (cf. 7.4.3.4). Additionally it was tried to increase the separation of the early eluting peaks on the one hand and at the same time to assure a reasonable retention time of streptomycin by applying an "ion-pair gradient" from 10 mmol/L to 2 mmol/L PFPA in a purely aqueous mobile phase.

Since the amount of organic modifier was kept unchanged, the detector response was expected to remain constant over the gradient. Unfortunately the chromatographic profile obtained with this type of gradient was very poor showing little retention of the peaks and also a very wavy baseline.

Eventually, a mobile phase containing 6 mmol/L of PFPA in a purely aqueous mobile phase was identified to deliver a good separation of streptomycin from its impurities. Further optimisation of the chromatographic conditions was carried out by a gradual modification of the eluent flow rate and column temperature. The chromatogram depicted in Figure 4-46 represents the best chromatographic profile with this mobile phase obtained using a column temperature of 30 °C and an eluent flow rate of 0.8 mL/min.
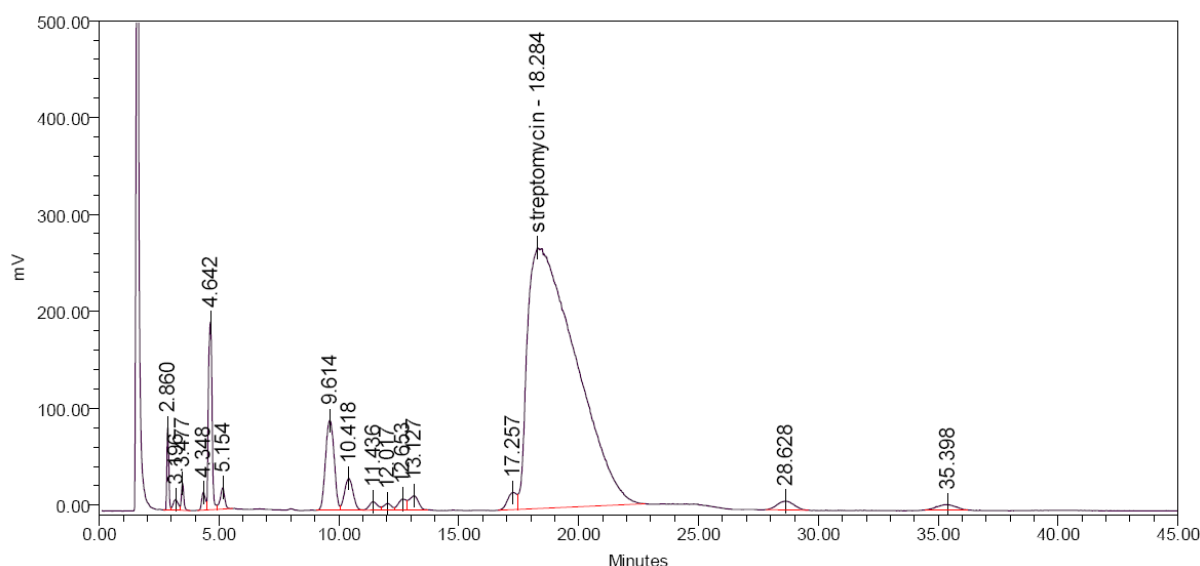
Figure 4-46. Injection of a 5 mg/mL solution of streptomycin sulfate sample 10. Mobile phase: 6.0 mmol/L PFPA in water at an eluent flow rate of 0.8 mL/min. Further conditions cf. Fig. 4-45.

Since the above mobile phase was mass compatible, the method was transferred to LC-MS in order to gather more information about the identity of the detected impurities and also to check if streptidine and streptomycine B are separated from streptomycin. Unfortunately it was found that streptomycin B, the only compound currently limited by the Ph.Eur. monograph, is not separated from streptomycin (cf. Fig. 4-47).
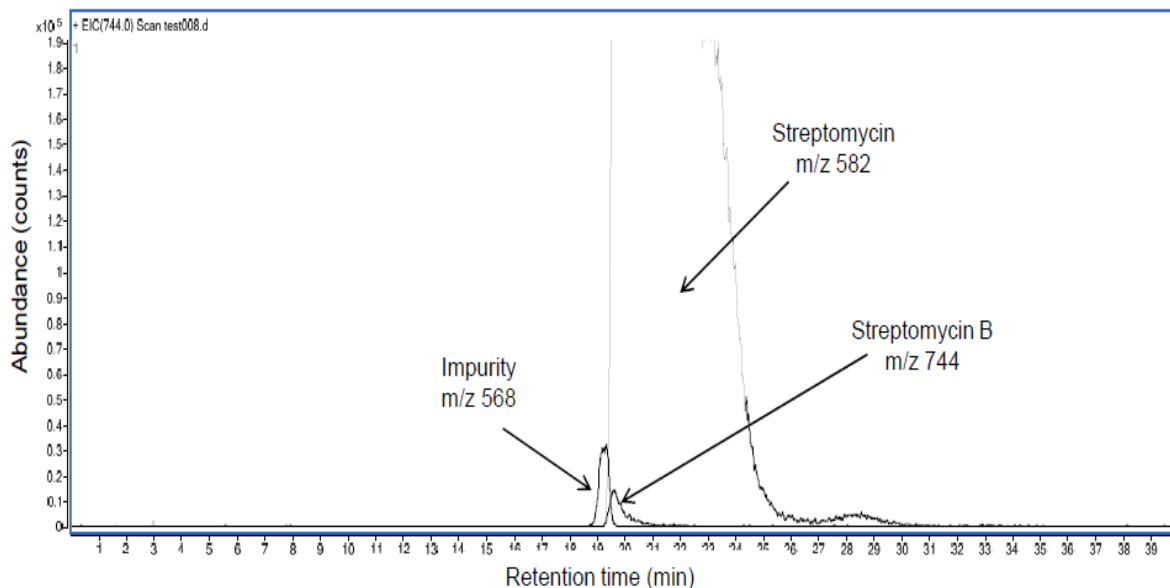
Figure 4-47. Overlay of the EIC-MS signals for streptomycin B (m/z 744), streptomycin (m/z 582), and an impurity with a m/z-ratio of 568 in a chromatogram of a 5 mg/mL solution of streptomycin sulfate in water. Injection volume 20 µL; LC flow rate: 0.8 mL/min using an Inertsil ODS 3 column (150 mm x 4.6 mm, 5 µm) at 30 °C. Mobile phase: 6.0 mmol/L PFPA in water.

Consequently, a further optimization of the method was necessary. First it was tried to improve the number of theoretical plates and therefore the resolution power of the column by reducing the particle size of the C18 stationary phase material and by using at the same time a longer column. Employing a 250 mm x 3.0 mm Inertsil ODS 3 column with a particle size of 3 µm, the chromatographic separation was improved, but still no separation of streptomycin B from streptomycin was achieved. A 250 mm x 4.6 mm YMC-Pack Pro C18 column with a particle size of 3 µm was tested in addition. Since the separation on this column was slightly better than on the Intertsil ODS 3 column, the further experiments were continued on the YMC column.

Considering all the experiments performed so far, it was deemed unlikely that the separation problem could be solved by further adjustments of the mobile phase using methanol as an organic modifier. However, it is known that a change in the organic solvent type often results in a change in selectivity and in improved resolution. According to Snyder et al. [35], the selectivity of different RP-LC solvents is based on the three solvent properties acidity, basicity and polarity. Based on their differences in these three parameters, acetonitrile, tetrahydrofuran (THF) and acetone were selected as the candidates for further testing [157].

In this respect it is noted that due to its UV cut-off of 330 nm acetone is not normally used for LC with UV detection. However, this is not an issue when the LC is coupled with a CAD or an MSD. In contrast, in this case the relatively high volatility of acetone is advantageous.

A series of experiments was performed using varying amounts of the three organic modifiers at different concentrations of PFPA. A detailed description of the tests conducted is given in 7.4.3.4.

The best separation of all related substances including streptomycin B was achieved using a YMC-Pack Pro C18 column (250 mm x 4.6 mm; 3 µm) with a mobile phase containing 20 mmol/L of PFPA and 1% (v/v) of acetone as organic modifier at a flow rate of 0.8 mL/min at a column temperature of 40 °C.

A screening of all samples revealed, that sample 4 contains most impurities (cf. Figure 4-48). Therefore, this sample was selected for the identification of the impurities by MS.
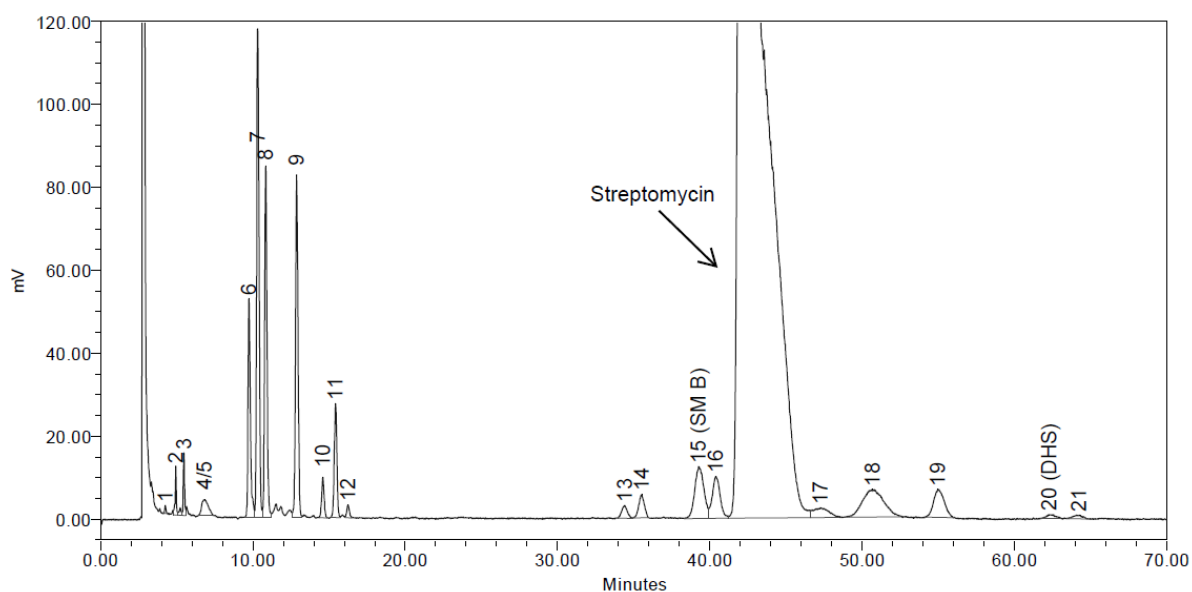
Figure 4-48. Column: YMC-Pack Pro column (250 mm x 4.6 mm; particle size 3 µm); Column temperature: 40 °C; Mobile phase: 20 mmol/L of PFPA in a mixture of water/acetone 99/1 (v/v). Flow rate: 0.8 mL/min. Injection of 10 µL of a 5 mg/mL solution of streptomycin sulfate sample 4 in water. Modified from [149].

4.4.5 Identification and quantification of the impurities in streptomycin sulfate

The identification and quantification of the impurities in the streptomycin sulfate samples was carried out using the aforementioned LC conditions (cf. 7.4.3.5).

4.4.5.1 Identification of the impurities by LC-MS

For the identification of the impurities, the exact mass of all compounds separated from streptomycin was determined coupling the above LC method with a TOF-MS. Moreover, MS/MS experiments were performed using a triple-quadrupole mass spectrometer. An overview of the m/z values of the observed impurities is given in Table 4-21. Furthermore, this table contains the neutral loss (NL) determined by LC-MS/MS where appropriate and also the suggested name of the impurity. For the impurities already reported in the literature [151, 152], the name of the compound together with a link to the corresponding literature was indicated. For the impurities not yet reported in the literature, the results are discussed below. This includes also impurities 13, 14 and 17 where the analytical data obtained in this study were not in agreement with the structures suggested in the literature. For impurities 8, 9 and 13 additional confirmation of the structure could be obtained by in-situ degradation experiments (cf. 7.4.3.5). The supposed structural formulae of the impurities are presented in Figure 4-49. More detailed explanations on the identification of the newly detected impurities are given on the subsequent pages.

Table 4-21. Results of the exact mass measurement by TOF-MS and the neutral loss by MS/MS for the impurities found in streptomycin sulfate. Modified from [149].

Peak No.[1]	m/z-ratio theoretical [M+H]+	m/z-ratio [M+H]+ observed by TOF-MS	Rel. error (ppm)	Supposed formula	MS/MS neutral loss (product m/z)	Proposed name
1	**222.0972**	**222.0975**	**-1.47**	**$C_8H_{15}NO_6$**	**N.A.***	**N-acetyl-inositolamine**
2	222.1084	222.1084	0.2	$C_7H_{15}N_3O_5$	N.A.	N-amidino-scyllo-inosamine [152]
3	**194.1023**	**194.1020**	**1.71**	**$C_7H_{15}NO_5$**	**18 (176)**	**N-methylglucosamine**
4/5	**356.1551**	**356.1542**	**2.58**	**$C_{13}H_{25}NO_{10}$**	**18 (338) / 180 (176)**	**Mannosido-N-methylglucosamine**
4/5	**370.1708**	**370.1709**	**-0.26**	**$C_{14}H_{27}NO_{10}$**	**18 (352) / 180 (190)**	**Mannosido-N,N-dimethylglucosamine**
6	221.1244	221.1240	1.96	$C_7H_{16}N_4O_4$	N.A.	3-deguanidino-3-aminostreptidine [151] / N-amidinostreptamine [152]
7	263.1462	263.1462	0.13	$C_8H_{18}N_6O_4$	N.A.	Streptidine [151, 152]
8	**664.2454**	**664.2448**	**0.93**	**$C_{21}H_{41}N_7O_{15}S$**	**82 (582)**	**Streptomycin sulfite-adduct**
9	**664.2454**	**664.2450**	**0.66**	**$C_{21}H_{41}N_7O_{15}S$**	**82 (582)**	**Streptomycin sulfite-adduct**
10	571.2570	571.2571	-0.26	$C_{20}H_{38}N_6O_{13}$	162 (409)	2´-O-de-N-methlyglucosamino-2´-O-mannosido DHS [151]
11	409.2041	409.2025	4.09	$C_{14}H_{28}N_6O_8$	146 (263)	2´-O-de-N-methlyglucosamine DHS [151] / O-L-dihydrostreptose-(1´-> 4)-streptidine [152]
12	367.1823	367.1807	4.42	$C_{13}H_{26}N_4O_8$	146 (221)	O-L-dihydrostreptose-(1 -> 4)-N-amidino-streptamine [152]
13	**598.2679**	**598.2665**	**2.31**	**$C_{21}H_{39}N_7O_{13}$**	**175 (423) / 422 (176)**	**Streptomycin acid**
14	**612.2835**	**612.2823**	**1.96**	**$C_{22}H_{41}N_7O_{13}$**	**189 (423) / 422 (190)**	**2´´-N-dimethylstreptomycin acid**
15	744.3258	744.3353	0.28	$C_{27}H_{49}N_7O_{17}$	162 (582) / 406 (338) / 568 (176)	SM B [151] / mannosido-streptomycin [152]
16	568.2573	568.2559	2.45	$C_{20}H_{37}N_7O_{12}$	161 (407) / 406 (162)	2´´-N-demethyl SM [151] / N-demethylstreptomycin [152]
17	**728.2595**	**728.2581**	**-1.97**	**$C_{25}H_{41}N_7O_{18}$**	**146 (582) / 128 (600)**	**Unknown structure**
18	596.2886	596.2875	1.86	$C_{22}H_{41}N_7O_{12}$	189 (407) / 406 (190)	N,N-dimethyl-L-glucosamine-(1´´ -> 2´)-L-streptose-(1´-> 4´)-streptidine [152]
19	570.2729	570.2727	0.38	$C_{20}H_{39}N_7O_{12}$	161 (409) / 408 (162)	N-demethyl DHS [152]
20	584.2886	584.2886	0.05	$C_{21}H_{41}N_7O_{12}$	175 (409) / 408 (176)	DHS [151, 152]
21	625.2788	625.2790	-0.35	$C_{22}H_{40}N_8O_{13}$	43 (582)	6-dehydro-6-carbamate streptomycin [151]

[1] Peak-N° as referred to in Figure 4-48.

* N.A., not available; SM, Streptomycin; DHS, Dihydrostreptomycin;

Bold face indicates impurities not yet reported in literature.

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Figure 4-49. Supposed structures of the impurities found in streptomycin sulfate. Modified from [149].
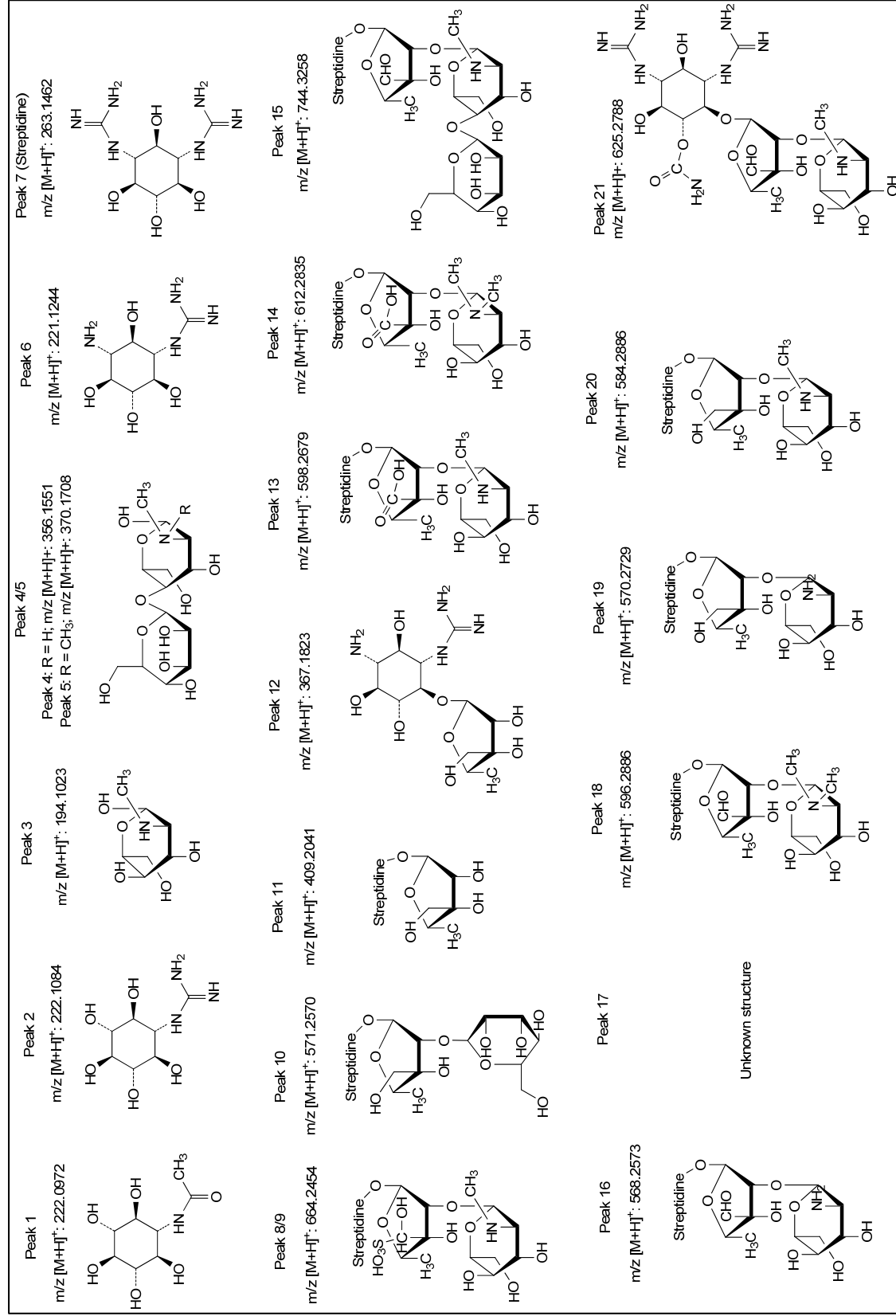
Peak 3 [M+H]$^+$ m/z 194: The only neutral loss found in the MS/MS spectrum was due to a loss of water (18 U). Apart from this, no further fragmentation could be observed. By TOF-MS, a m/z ratio of 194.1020 was measured which is in very close agreement with the m/z-ratio of N-methylglucosamine (m/z M+H$^+$: 194.1023). N-methylglucosamine can be formed by the hydrolysis of streptomycin and is also known to occur as a by-product produced by Streptomyces griseus [158, 159]. Furthermore, the neutral loss of 18 was also observed for other impurities containing N-methylglucosamine (cf. Tab. 4-21 – peak 4/5). Consequently, peak 3 was allocated to N-methlyglucosamine.

Peak 4/5 [M+H]$^+$ m/z 356: The first impurity eluting in this peak showed an exact mass corresponding to the mannosido-N-methylglucosamine moiety of streptomycin B. This was supported by a neutral loss of 180 U in the MS/MS experiment which was in agreement with the loss of the mannose sugar.

Peak 4/5 [M+H]$^+$ m/z 370: The second impurity in this peak exhibited an exact mass which is in accordance with the sum formula of mannosido-N,N-dimethylglucosamine. Like peak 4/5 m/z 356, a neutral loss of 180 U, corresponding to the mannose sugar, was found.

Peak 8 and 9 [M+H]$^+$ m/z 664: All compounds possessing a carbonyl group in position R^1 (cf. Fig.4-34) exhibited also an abundant signal of the hydrated form of this group (geminal diol) with a m/z ([M+H]$^+$+18) [160]. However, this signal was not found for peaks 8 and 9, suggesting a modification of the carbonyl group R^1. The exact mass of these peaks was found to be in good agreement with the mass of the hydrogen sulfite adducts of the streptomycin carbonyl group. Further proof for this assumption was provided by an MS/MS experiment where a neutral loss of 82 U, corresponding to H_2SO_3, was found. Furthermore, the peak area of the two peaks was very similar, pointing to the formation of a chiral C-atom upon addition of hydrogensulfite to the streptomycin carbonyl group R^1. Since hydrogensulfite is an known antioxidant in streptomycin sulfate solutions [161], a streptomycin sulfate sample solution was spiked with sodium hydrogensulfite. Subsequent analysis by LC-MS showed that both peaks were generated in-situ.

Peak 13 [M+H]$^+$ m/z 598: A neutral loss of 175 U, corresponding to N-methyl-glucosamine, was detected in the MS/MS experiment. Although a decrease of the peak size was found for all peaks having a carbonyl group in position R^1 (cf. Fig.4-34) when hydrogensulfite was added, this was not observed for peak 13. Moreover, like for peaks 8 and 9, no signal due to the geminal-diol of streptomycin was found. It was therefore assumed that peak 13 is a streptomycin analogue with a modification in position R^1 and not an isomer of hydroxyl-streptomycin as suggested by [162]. The observed m/z of 598.2665 in the ESI-positive mode was in good agreement with the sum formula of streptomycin acid, a substance described as being an oxidation product of streptomycin [163]. Eventually, a signal with a m/z-ratio of 596.2591 was also found in a sample enriched with this reaction product in the ESI-negative mode, indicating that the peak can be attributed to streptomycin acid.

Peak 14 [M+H]$^+$ m/z 612: A neutral loss of 189 U, corresponding to N,N-dimethyl-glucosamine, was found in the MS/MS spectrum. As with peaks 8, 9 and 13, the signal of the carbonyl geminal-diol was not found. Moreover, no decrease of the peak area was found upon addition of sodium hydrogensulfite. In analogy to peak 13, peak 14 was attributed to 2´´-N,N-dimethyl-streptomycin acid.

Peak 17 [M+H]$^+$ m/z 728: An impurity with an integer mass of 728 is described in the literature [151] to be 4´-(O-dihydrostreptose)-streptomycin. However, the determination of the exact mass of impurity 17 by TOF-MS (cf. Tab. 4-21) revealed a mass error of 98 ppm when compared with this structure. Therefore, it was considered unlikely that impurity 17 is 4´-(O-dihydrostreptose)-streptomycin. Based on the exact mass, the most likely calculated sum formula of impurity 17 is $C_{25}H_{41}N_7O_{18}$, having a mass error of -1.97 ppm only. In the MS spectrum of peak 17 no signal due to the hydrated form of the carbonyl group R^1 (cf. Fig. 4-34) was found at m/z 746. Therefore, it was hypothesized that a modification of the streptomycin carbonyl group has taken place. The MS/MS experiments resulted in product ions of m/z 582 and m/z 600, corresponding to streptomycin and to the geminal diol of streptomycin, respectively. Taken together, these findings suggest the assumption that impurity 17 is a streptomycin with a $C_4H_2O_6$ modification of the carbonyl group R^1. However, no sound corresponding structural formula could be derived. Consequently, impurity 17 was listed as an impurity with unknown structure.

4.4.5.1 Linearity of the detector response

Although it is known that the relation between the response of CAD and the analyte concentration is not linear over a wide range [77], a quasi-linear response over about two orders of magnitude has repeatedly been demonstrated [76, 78, 79].

In this study, 6 solutions containing 5 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL and 250 µg/mL of streptomycin sulfate, corresponding to a concentration range from 0.1% to 5% referred to a 5 mg/mL test solution, were used to verify the linearity of the detector response. It was found that a linear function can be applied to describe the correlation between the detector response and the concentration in this range (cf. Fig. 4-50).
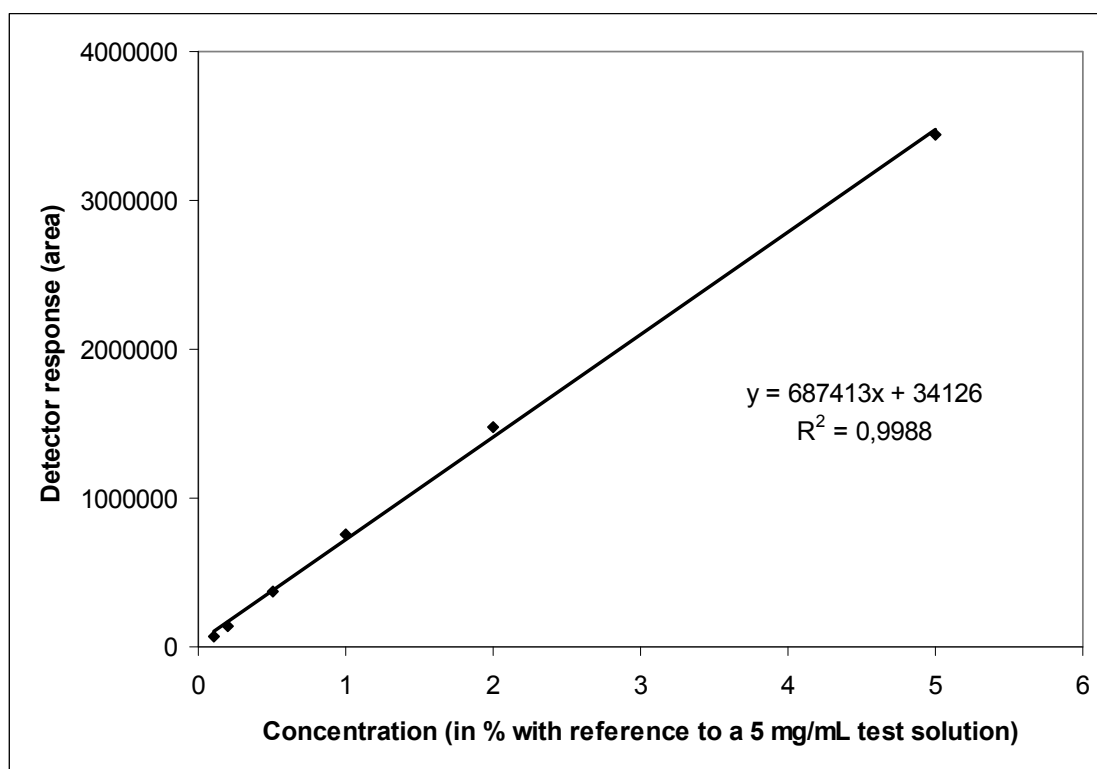
Figure 4-50. LC-CAD calibration curve of streptomycin sulfate from 5 µg/mL to 250 µg/mL (6 concentration levels). Conditions as described in Fig.4-48. Modified from [149].

4.4.5.2 Response factor of streptidine sulfate and limits of quantification

Although one of the advantages of the CAD is that the detector response is often very similar for different substances [60, 69, 77], this is not always the case and sometimes differences in the detector response are found [76]. For this reason, the relative response factor of streptidine sulfate (compared to streptomycin sulfate) was verified on 2 different concentration levels, i.e. using solutions containing 25 µg/mL and 50 µg/mL of streptomycin sulfate and streptidine sulfate. A relative response factor of 1.0 was found for streptidine sulfate.

The limits of quantification were calculated on the basis of a signal-to-noise ratio (S/N-ratio) of 10. The LoQs were extrapolated from the S/N-ratios obtained with 5 µg/mL solutions of streptomycin sulfate and streptidine sulfate, respectively. For streptomycin sulfate the LoQ was determined to be 4.5 µg/mL and for streptidine sulfate the LoQ was found to be 0.4 µg/mL, corresponding to 0.09 % and 0.008%, respectively, referred to a 5 mg/mL test solution. The significantly lower LoQ of streptidine is due to the fact that the substance elutes much earlier than streptomycin and that it is very narrow and high.

4.4.5.3 Quantitative determination of the impurities by LC-CAD

The above LC-CAD method (cf. 7.4.3.5) was applied for the purity testing of 12 commercially available samples of streptomycin sulfate from different sources, including the Ph.Eur. CRS 3. The impurities were quantified against streptomycin sulfate, using a linear calibration curve in the range of 0.1% to 3% (referred to a 5 mg/mL streptomycin sulfate test solution). The amount of streptomycin B, the only impurty currently limited by the Ph.Eur. monograph using a TLC method and a limit of 3%, was found in amounts between 0.7% and 2.9%. Streptidine was determined at levels from 0.5% to 2.9%. Moreover, concentrations of up to 2.6% (impurity 11 in sample 10) were found for impurities 6, 8, 9, 11 and 18. Impurities 2, 4/5, 10, 14, 16, 17 and 19 were present at levels between 0.5% and 1.0%. Impurities 1, 3, 12, 13, 20 (DHS) and 21 were found to be below 0.5% in all samples. An overview of the results is given in Table 4-22. Example chromatograms of samples 7 and 10 are presented in Figure 4-51.

(a)
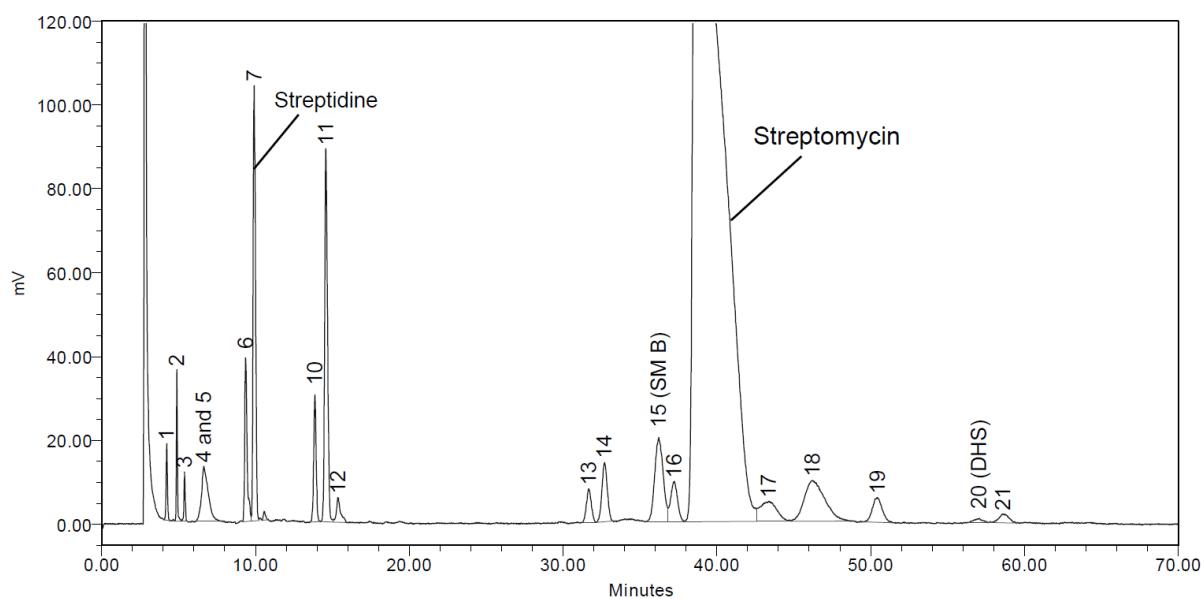
(b)
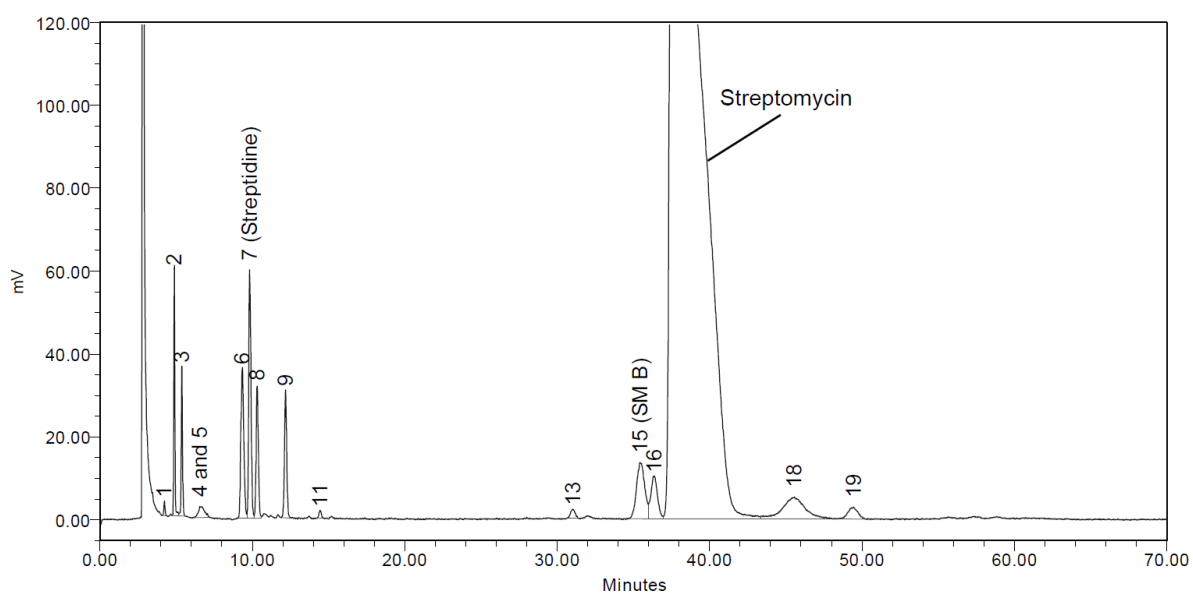
Figure 4-51. LC chromatograms of the test solutions of (a) sample 7, (b) sample 10, SMB, streptomycin B; DHS, dihydrostreptomycin; Conditions as described in Fig.4-48. Modified from [149].

The sum of impurities of 4.6% to 16.0% was found between 0.4% and 3.3% higher compared with the results of the LC-UV method (cf. 4.4.2.2, Tab. 4-20). This can on the one hand be explained by the higher selectivity of the method, i.e. better separation of the impurities from the peak of streptomycin and on the other hand by the higher method sensitivity of the LC-CAD method (LoQ streptomycin sulfate: 0.09%) compared to the LC-UV method (LoQ streptomycin sulfate: 0.4%).

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Table 4-22. Impurities found in commercial samples of streptomycin sulfate (%, m/m), expressed as streptomycin sulfate (as is). Modified from [149].

Sample N°:	Impurity-Peak N° (cf. Tab. 4-21 and Fig. 4-49 for peak identity)																					Sum of imp.
	1	2	3	4/5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
1	ND	0.05	0.21	0.31	0.80	0.95	ND	ND	ND	ND	ND	0.06	± 0.04	0.68	0.65	ND	1.2	± 0.09	ND	ND	5.1
2	0.07	0.19	0.19	0.36	0.88	2.4	1.1	1.1	0.38	1.5	0.21	0.30	0.72	1.2	0.77	0.70	2.4	0.70	0.11	0.11	15.4
3	0.04	0.24	0.39	0.09	1.3	1.8	0.69	0.67	0.19	0.49	0.11	0.23	0.39	0.69	0.68	0.24	1.4	0.93	0.11	± 0.06	10.7
4	0.03	0.14	0.19	0.26	1.3	2.9	2.1	2.2	0.25	0.74	0.08	0.18	0.32	1.2	0.82	0.37	1.5	0.74	± 0.09	± 0.08	15.5
5	0.02	0.38	0.15	0.92	0.83	1.6	0.67	0.61	0.04	0.05	0.04	0.07	ND	0.72	0.62	ND	0.99	0.15	ND	ND	7.9
6	0.06	0.16	0.19	0.33	0.91	2.6	1.1	1.0	0.32	1.3	0.24	0.30	0.65	0.22	0.76	0.72	2.3	0.70	0.11	± 0.07	14.0
7	0.04	0.67	0.46	0.17	1.1	1.4	0.71	0.71	ND	0.05	ND	0.11	ND	1.1	0.70	ND	1.2	0.28	ND	ND	8.7
8	0.04	0.21	0.28	0.11	1.2	2.6	1.2	1.1	0.54	1.4	0.05	0.27	0.78	1.7	0.96	0.33	1.8	0.98	0.11	0.12	15.8
9	ND	0.23	0.22	0.48	1.2	1.3	0.31	0.26	ND	ND	ND	± 0.05	ND	0.79	0.74	ND	1.2	0.10	ND	ND	6.9
10	0.26	0.39	0.15	0.90	0.96	2.5	ND	ND	0.77	2.6	0.25	0.39	0.77	1.7	0.68	0.81	2.0	0.59	± 0.08	0.21	16.0
11	ND	0.03	0.14	ND	0.84	0.56	ND	ND	ND	± 0.02	ND	0.06	± 0.05	0.88	0.57	ND	1.4	± 0.09	ND	ND	4.6
12 (Ph.Eur. CRS 3)	ND1	0.52	0.08	ND	0.46	0.50	0.15	0.15	0.04	0.03	ND	ND	ND	2.9	0.81	ND	1.8	0.36	0.17	± 0.06	8.0

1 ND, not detected (below LOD), ± is used for values below the LOQ, these were taken into account for the calculation of the total amount of impurities.

4.4.6 Conclusion

The quality of streptomycin sulfate is not sufficiently controlled by the current Ph.Eur. monograph because only streptomycin B is limited by a TLC test for related substances but no ICH [25] compliant test for the control of the further related substances is described. Due to unsatisfying selectivity and/or sensitivity, the methods described in literature [150, 151, 152, 153] are not considered suitable for this purpose either.

In this study, a LC-CAD method for the control of the related substances in streptomycin sulfate employing PFPA as a volatile ion-pair reagent was elaborated. With this method the separation and quantification of 21 impurities with a limit of quantification of at least 0.1%, referred to the concentration of the streptomycin sulfate test solution, was found to be possible. Due to a linear response of the CAD in the range of 0.1% to 5% (compared to then concentration of the test solution), the quantification of the impurities could be performed using a linear calibration curve of streptomycin sulfate.

The analysis of 12 commercially available samples of streptomycin sulfate from different manufacturers resulted in a total amount of impurities between 4.6% and 16.0%. Based on the results found, the following provisional specification limits for the different impurities in streptomycin sulfate are suggested: streptomycin B, max. 3.0%; streptidine, max. 3.0%; impurity 18, max. 2.0%; impurities 6, 8, 9, 11, max. 1.5%, impurities 2, 4/5, 10, 14, 16, 17, and 19, max. 1.0%; impurities 1, 3, 12, 13, 20 (DHS), and 21, max. 0.5%; unspecified impurities, max. 0.2%; sum of impurities, max. 16.0%.

Since the method had the advantage of being compatible with MS detection, the exact mass of all impurities could be determined by TOF-MS. In addition MS/MS fragmentation experiments could be carried out in order to gather further structural information. Comparing the results to data published in the literature, structural formulae could be suggested for 12 impurities. For another 8 impurities tentative structures could be derived from the results of the conducted experiments. Only for one impurity it was not found possible to propose a suitable structural formula.

4.5 Application of LC-CAD for the characterization of horse-chestnut standardised dry extract

β-Aescin is the major bioactive constituent extracted from the seeds of Aesculus hippocastanum, commonly known as horse-chestnut. Because of its anti-inflammatory, anti-edematous and capillary protective properties, it is largely used and is, at least in Germany, the most widely prescribed medication for chronic venous insufficiency and edema [164, 165, 166]. In the past years, it has also found wide application in the cosmetic field, especially for the prevention or treatment of so-called cellulites [167]. In China, the dried ripe seeds of aesculus chinensis are used to normalize stomach function and relieve distension in the abdomen [168, 169]. Clinical studies considering the effects of aescins have shown a significant reduction of both, leg volume and capillary filtration [170], resulting in a decrease of subjective patient complaints. The anti-inflammatory effects of aescins are believed to be a result of the inhibition of histamine and serotonin induced vascular permeability [171]. Horse-chestnut seed extract has also been shown to reduce the activity of lysosomal enzymes in damaged veins. This results in a lower breakdown of mucopolysaccharides around the capillary wall and therefore leads to a decreased vascular permeability [172].

Chemically, β-aescin consists of a mixture of more than 30 saponins derived from the triterpenes protoaescigenin (Figure 4-52: $R^1 = R^3 = R^4 = H$, $R^2 = OH$) and barringtogenol C (Figure 4-52: $R^1 = R^2 = R^3 = R^4 = H$) [173, 174].
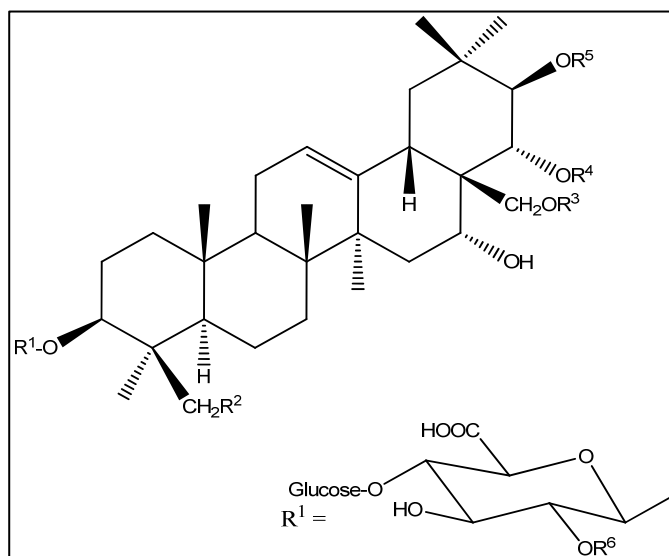
Figure 4-52. Basic molecular structure of aescin.

The proposed names and composition of the major aescin constituents is given in Table 4-23 [165].

Table 4-23. Composition of the major aescin constituents.

Name	R^2	R^3	R^4	R^5	R^6
Aescin Ia	OH	H	Ac	Tig	Glucose
Aescin Ib	OH	H	Ac	Ang	Glucose
Aescin IIa	OH	H	Ac	Tig	Xylose
Aescin IIb	OH	H	Ac	Ang	Xylose

Ac = Acetyl, Ang = Angeloyl, Tig = Tigloyl

Apart from the substituents given in Table 4-23, further substitution patterns are described with R^2 being OH or H, R^3 being Ac, Ang or Tig, R^4 being H, Ac, Ang or Tig, R^5 being H, Ac, Ang, Tig, iso-butyryl or 2-methylbutyryl, and R^6 being glucose, xylose or galactose [169, 175].

According to the current Community Herbal Monograph on Aesculus Hippocastanum L. Semen of the EMA [176] the aescin content limits of horse-chestnut standardized dry extracts are defined based on a photometric determination.

This determination is based on a color reaction of the triterpene glycosides with iron(III)chloride. Unfortunately the sample preparation procedure is rather complex and laborious, and requires a lot of experience to obtain reproducible results [177]. The chemical structure of the colored reaction products is unknown. However, based on the supposed reaction mechanism it is very likely that the reaction is not selective for β-aescin but would also cover other less or non-active aescin fractions like the water soluble α-aescin or cryptoaescin.

For this reason, a more selective LC assay determination for β-aescin was proposed for the Ph.Eur. draft monograph of horse-chestnut standardized dry extract [178].

In this method, the percentage content of triterpene glycosides, expressed as aescin, is determined based on the sum of the areas of the peaks eluting between two marker substances (methyl salicylate and ibuprofen). For the quantification, an aescin CRS (Chemical Reference Substance) was suggested as an external standard.

In 2006, the EDQM Laboratory characterized a first aescin candidate CRS [179] to be used as an external standard in the LC-UV assay determination. The proposed content value of 79.6% was assigned applying the "mass-balance approach" as given in Equation 4-1.

$$\text{Aescin} \, (\% \, m/m) = \left[(100 - LoD)x \, \frac{\text{chromatographic purity} \, (\% \, \text{area})}{100} \right]. \quad (4\text{-}1)$$

As an alternative strategy, the use of purified protoaescigenin instead of an aescin CRS was discussed. One of the advantages seen in this approach was that the use of a pure and chemically well defined substance would facilitate the establishment of a reference standard and would also make the establishment of a future replacement of the CRS less problematic. However, analyses using protoaescigenin as a reference standard [180, 181] revealed a high inter-laboratory variability (11.9% to 28.2%; n = 4) when testing different batches of horse-chestnut standardized dry extracts.

In addition to the problem of defining an appropriate reference standard strategy, further concerns on the LC assay method for horse-chestnut standardized dry extract were raised with regards to a possible interference of the internal marker peaks of methyl salicylate and ibuprofen with peaks due to aescin. Moreover, it was suggested to replace the proposed gradient LC method by a faster isocratic method [182].

The goals of this study was to elaborate the most appropriate reference standard strategy and at the same time to look into the possibility of further improving the method by utilizing an isocratic method without any marker substance. For this purpose, 7 highly purified ß-aescin extracts from 6 different suppliers were used.

4.5.1 LC-MS analysis of purified ß-aescin

The draft monograph of horse-chestnut standardized dry extract describes the following multi step gradient method using a 0.05% (v/v) solution of trifluoroacetic acid (TFA) in water as mobile phase A and acetonitrile as mobile phase B on a Grace Vydac C18 column (250 mm x 4.6 mm; particle size 5 µm): 0–15 min isocratic at 70% mobile phase A; 15-25 min linear gradient from 70% to 65% mobile phase A; 25-35 min isocratic at 65% mobile phase A; 35-65 min linear gradient step from 65% to 50% mobile phase A; 65-70 min linear gradient from 50% to 10% mobile phase A (cf. [178]).

For the analysis by LC-MS, this mobile phase had to be slightly adapted: TFA was replaced by formic acid in order to avoid ion suppression. Moreover, a Novapak C18 column (150 mm x 3.9 mm, 4 µm) was used and the LC flow rate was reduced from 1.0 mL/min to 0.7 mL/min. The following step gradient was applied: 65% mobile phase A from 0 to 15 min, 50% mobile phase A from 15.1 to 25 min and 10% mobile phase A from 25.1 to 30 min. Mobile phase A was composed of 0.1% (v/v) of formic acid in water, mobile phase B was acetonitrile (cf. 7.5.3.1).

The LC-UV chromatogram obtained under these conditions (cf. Fig. 4-53) was found to be less good resolved than the chromatogram obtained under the conditions described in the draft monograph (cf. Fig. 4-54).

Nevertheless, it was considered appropriate to gain some basic information about the composition of the substance.
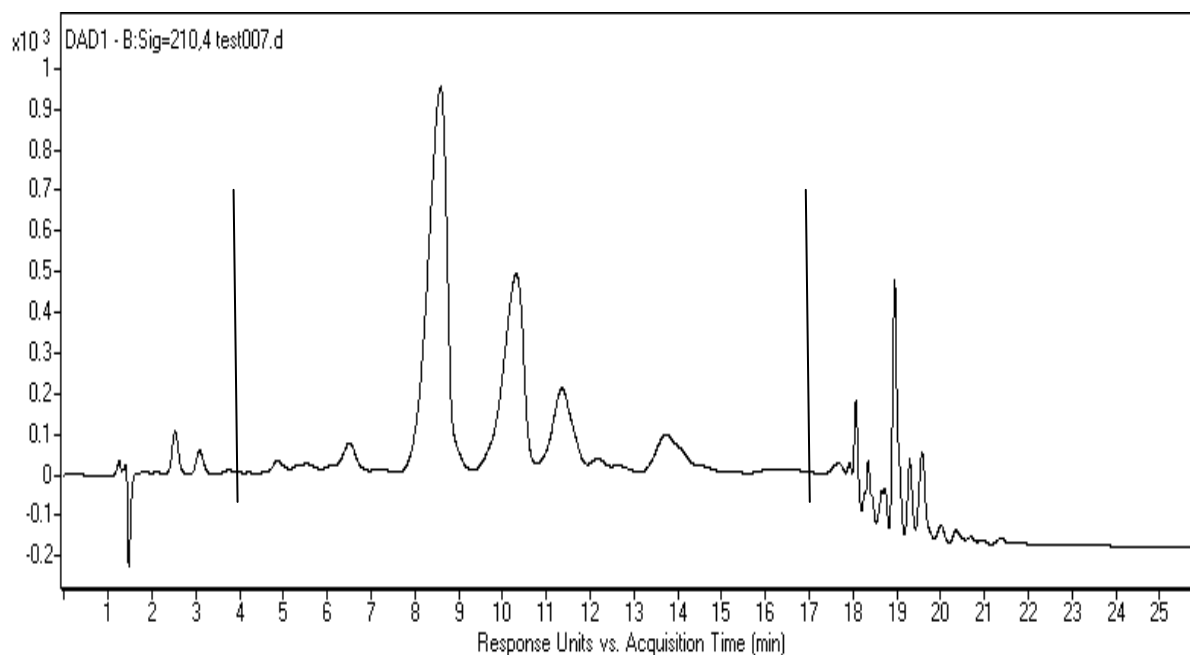
Figure 4-53. Injection of 20 µL of a 5 mg/mL solution of aescin sample 1 (proposed CRS) using the LC conditions applied for the MS detection as specified under 7.5.3.1 but using detection by UV 210 nm. The vertical lines at 4 min and 17 min mark the region where Extracted Ion Chromatograms were generated in the MS-mode (cf. Figure 4-55).
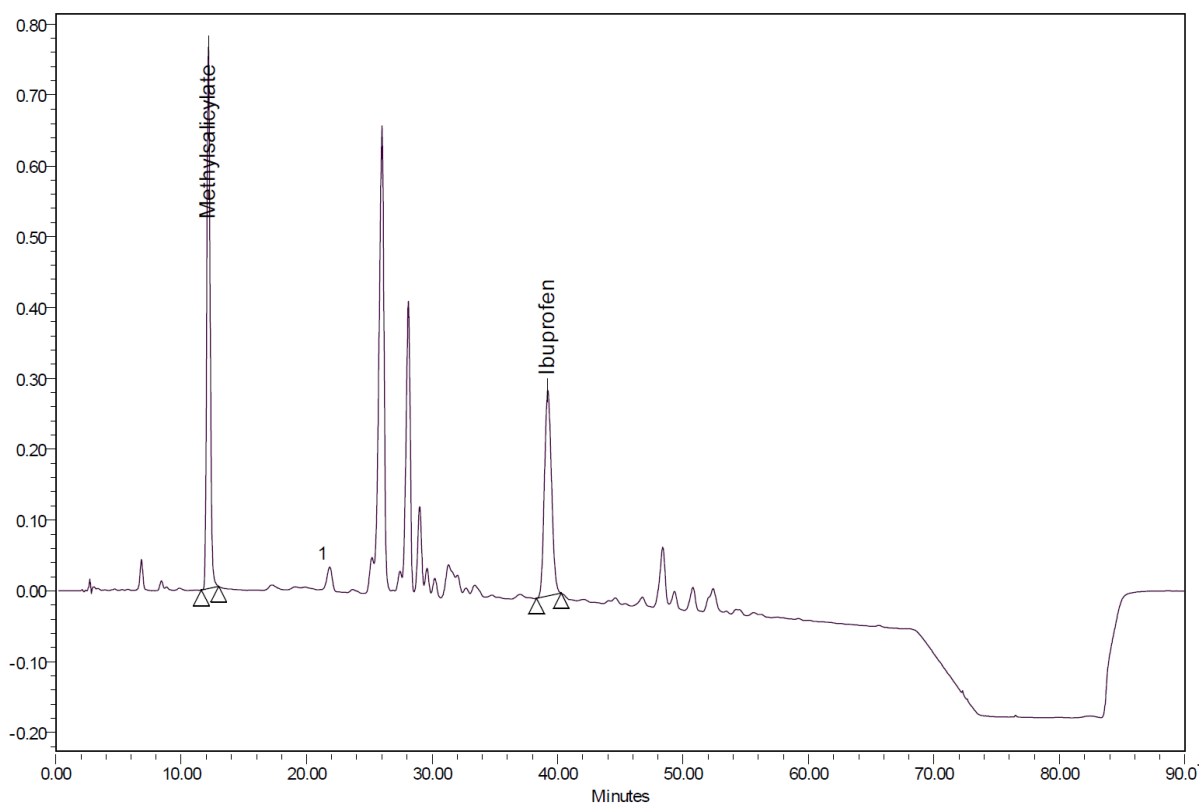
Figure 4-54. Aescin sample 1 (proposed CRS) – UV detection 210 nm. LC method as described in the Pharmeuropa draft monograph of Horse –chestnut standardized dry extract [178].

Since in the ESI-positive mode intense fragmentation was observed, the measurement was performed in the ESI-negative mode where the fragmentation was significantly reduced. The extracted ion chromatograms (EICs) for 12 different m/z-ratios determined between 4 min and 17 min are shown in Figure 4-55.

These results confirm the very complex composition of aescin. At the same time they show that even at a given retention time, the signal obtained by UV detection represents two or more co-eluting compounds rather than one individual substance.
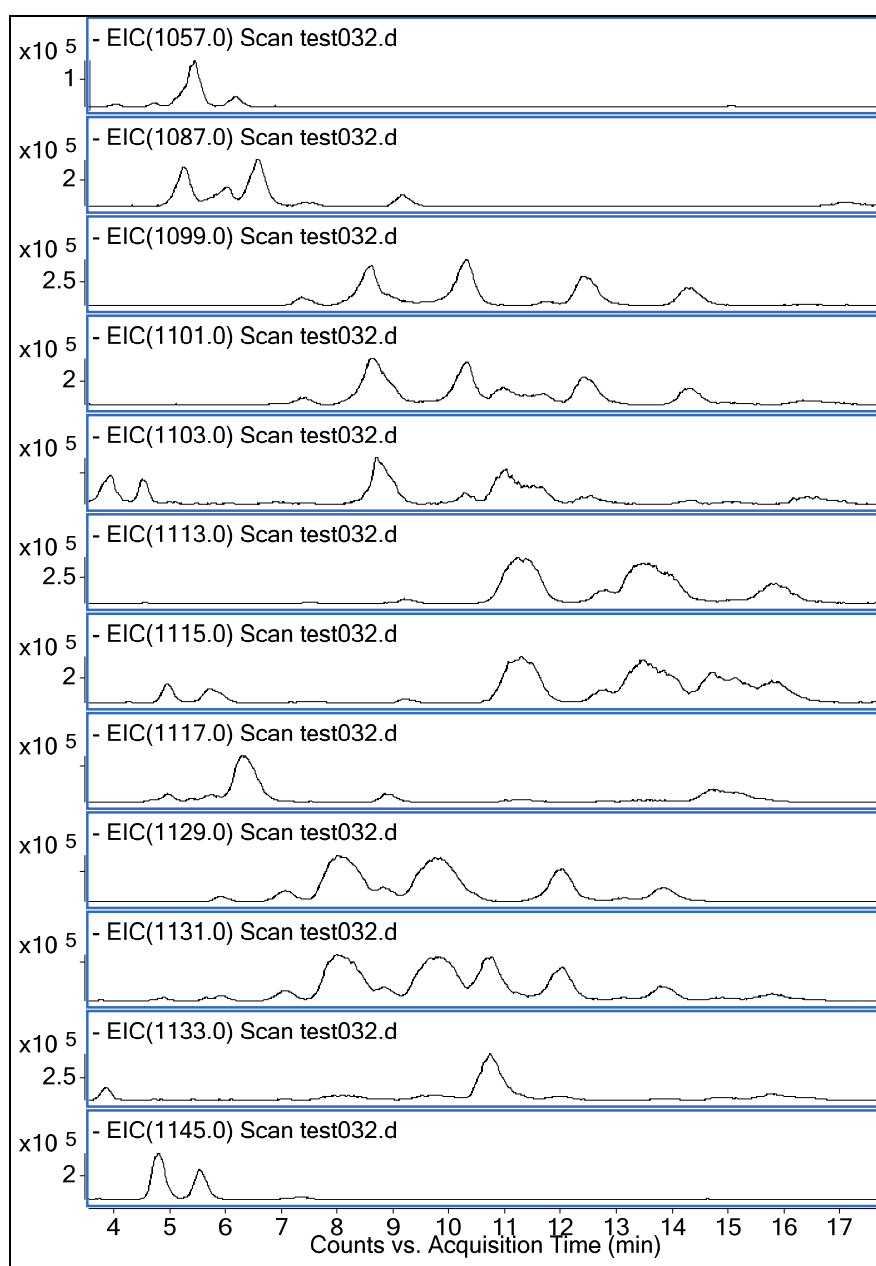
Figure 4-55. Selected LC-MS extracted ion chromatograms (EIC) of aescin sample N° 2. Chromatographic conditions as described in chapter 7.5.3.1.

4.5.2 Elaboration of a reference standard strategy for the determination of aescin in horse-chestnut standardized dry extract

4.5.2.1 Characterization of the aescin samples

To allow better comparability of the LC results, the different samples used in this study were characterized by the determination of the loss on drying and the sulfated ash. The latter was conducted to verify the absence of inorganic impurities. Moreover, the assay titration method for aescin R1 was conducted as described in the draft monograph of horse-chestnut standardized dry extract [178]. The results obtained are presented in Table 4-24.

Table 4-24. Batch results for the titration assay, loss on drying and sulfated ash of the aescin samples.

Sample	Titration result (dried substance) Ph.Eur. limit: 96.0-103.0%	Loss on drying (105°C, 4h)	Sulfated ash
1	**98.3%** (n=3; rsd=0.07%)	4.15% (n=3; sd=0.06)	0.02%
2	94.5%	2.1%	0.01%
3	**96.6%** (n=3; rsd=0.08%)	No loss (n=3)	0.05%
4	104.8% (n=2)	4.94% (n=3; sd=0.04)	Not done[1]
5	92.4% (n=3; rsd=0.18%)	3.28% (n=3; sd=0.08)	0.06%
6	108.2% (n=2)	9.35% (n=2)	No residue
7	113.9% (n=3; rsd=0.08%)	9.23% (n=3; sd=0.09)	No residue

[1] quantity available was not sufficient to perform the test.

The water content of the different samples varies substantially. Moreover only samples 2 and 3 complied with the proposed Ph.Eur. assay requirements for aescin R1.

4.5.2.2 Comparison of the aescin samples by LC-UV according to the draft monograph

The LC gradient method described in the draft monograph [178] was used for the comparison of the different aescin samples. The sum of the area of the peaks between those due to methyl salicylate and ibuprofen was determined.

The representative LC-UV chromatogram of aescin sample 1 (proposed CRS) is presented in Figure 4-54 for information.

Following the "mass balance approach" as described for the content assignment of aescin prop. CRS (cf. Equation 4-1), the %-amount of active triterpene glycosides (eluting between the two markers methyl salicylate and ibuprofen) was determined in all samples. Additionally, the %-content of the active aescin fraction was determined using aescin prop. CRS as external standard. The results are presented in Table 4-25.

Table 4-25. LC-UV quantification of 7 aescin samples.

Sample	Chromatogr. purity (mass balance) in %	%-content using Aescin prop. CRS 1	Difference (in %; [100 – (mass balance / prop. CRS x 100)]
1	79.6%*	79.6%	0%
2	80.5%	81.0%	0.6%
3	72.7%	52.3%	39.0 %
4	75.8%	54.8	38.3%
5	77.1%	59.4%	29.8%
6	73.5%	60.4%	21.7%
7	66.8%	56.9%	17.4%

* Assigned content value as proposed in [179].

The comparison of the results obtained by "mass balance" with those using aescin prop. CRS (sample 1) as external standard revealed similar values only for aescin sample 2, but huge differences for all other samples. Thus, the method was not found to be capable of giving a good estimate for the "true content" of the samples.

In consequence, the establishment of an aescin primary reference standard applying the "mass balance approach" (cf. Equation 4-1) for the content assignment was not considered to be a practicable option.

4.5.2.3 Use of protoaescigenin as a reference standard

Protoaescigenin can be obtained as a highly purified single component and can therefore be well characterize for its use as a primary reference standards. Even though a high inter-laboratory variability was observed when using this substance as a standard for the content determination of different batches of horse-chestnut standardized dry extracts [180, 181], the possibility of using protoaescigenin as a reference standard was again explored in this study.

In order to identify the possible reason for the high variability, several of the highly purified aescin samples were tested using the LC gradient method of the draft monograph [178] and the %-content results of the aescin fraction eluting between the two marker substances were compared with both, the aescin candidate CRS and protoaescigenin as an external standard for quantification. The retention time of the protoeascigenin used as external standard had previously been verified using the same LC method. The substance was found to elute at about 12 min, being within the window defined by the 2 retention time markers of the active aescin fraction (cf. Fig. 4-56).
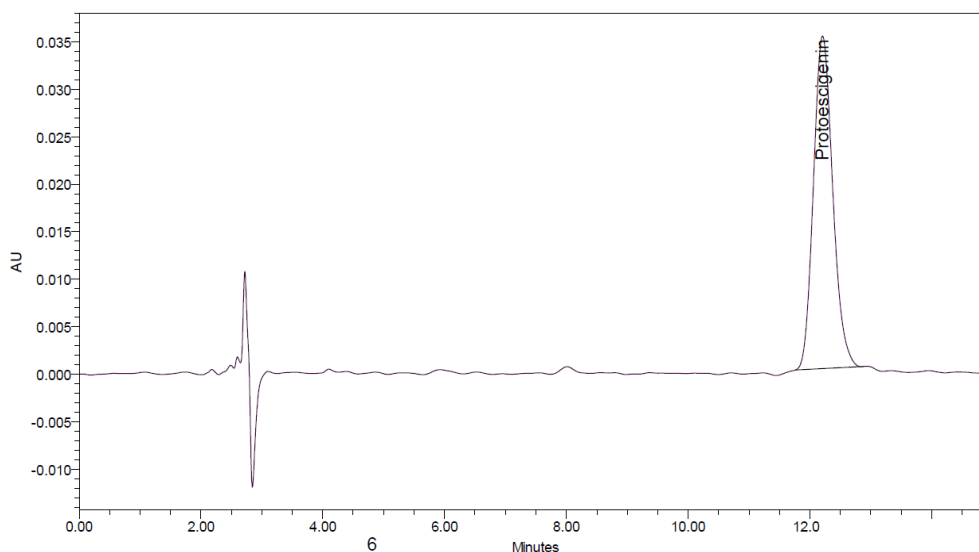
Figure 4-56. Chromatogram of a 0.1 mg/mL solution of protoaescigenin – UV detection 210 nm. LC method as described in the Pharmeuropa draft monograph of horse-chestnut standardized dry extract [178].

Whereas the absolute amounts of triterpene saponins was found to be different, when using on the one hand aescin prop. CRS and on the other hand protoaescigenin as external standard, the ratio of the two results was constant (cf. Tab. 4-26).

Table 4-26. LC-UV quantification of highly purified aescin sample using the aescin candidate CRS (sample 1) and protoaescigenin as external standards.

Sample	%-amount of triterpen saponins using aescin prop. CRS (%)	%-amount of triterpen saponins using proto-aescigenin (%)	Ratio protoaescigenin / aescin prop. CRS
3	52.3%	65.5%	1.25
4	54.8%	68.2%	1.24
5	59.4%	74.4%	1.25
6	60.4%	75.6%	1.25
7	56.9%	71.3%	1.25

Due to the absence of a suitable chromophor, the LC-UV determination of aescin was performed at a detector wavelength of 210 nm. However, the UV spectrum of protoaescigenin (cf. Fig. 4-57) clearly shows that a measurement at 210 nm is not advisable, because small variations in the wavelength have a significant impact on the absorbance and thus on the results.
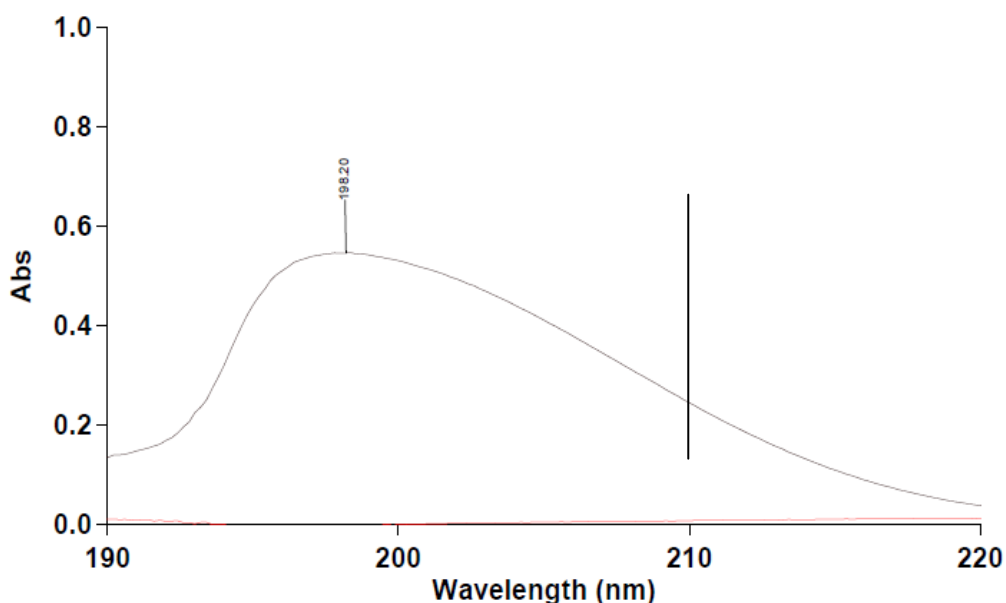
Figure 4-57. UV-spectrum of a 0.04 mg/mL solution of protoaescigenin in acetonitrile / 0.05% aqueous TFA (60/40 v/v).

This was confirmed by a simultaneous measurement of the LC-UV absorbance of aescin and protoaescigenin at 206 nm, 210 nm and 214 nm using a Diode-Array Detector (DAD). The relative standard deviation (rsd) of the mean peak area of protoaescigenin measured at these wavelengths was found to be 42%. On the other hand, the rsd of the total peak area of the active fraction of aescin samples 1 and 3 - averaging the total peak areas at 206 nm, 210 nm and 214 nm - was only about 6.4%.

Since the wavelength accuracy of standard UV detectors can vary by up to ± 2 nm [183], it is likely that the variability of results found in the protoaescigenin interlaboratory study [180, 181] was caused by the imprecision of the UV detectors used by the participants.

The expansion of the UV-spectrum of protoaescigenin (cf. Fig. 4-58) showed that the absorbance of protoaescigenin between 199 nm and 204 nm is almost constant.
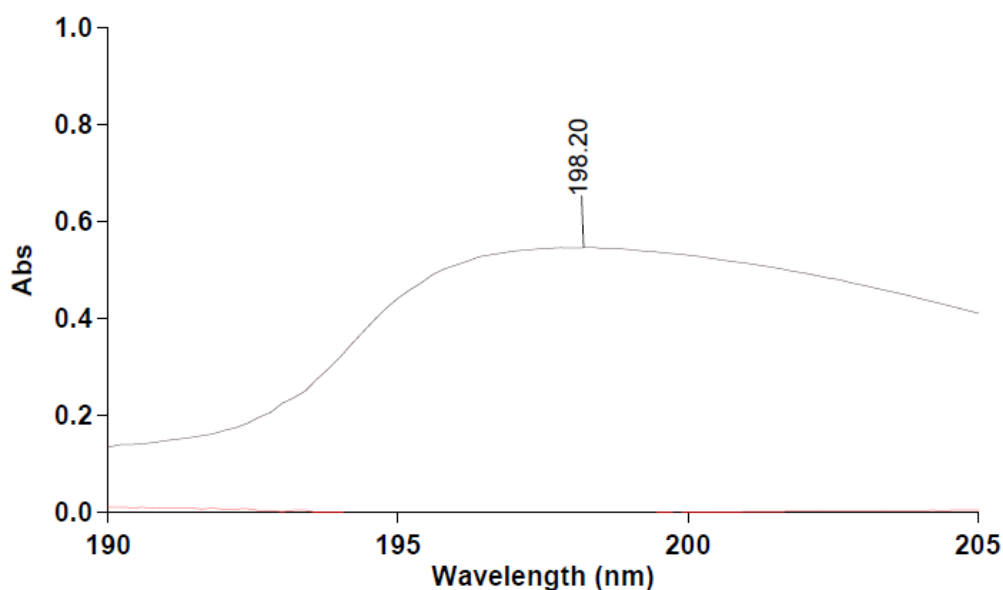
Figure 4-58. UV-spectrum of a 0.04 mg/mL solution of protoaescigenin in acetonitrile / 0.05% aqueous TFA (60/40 v/v).

Therefore, a change of the detection wavelength for protoaescigenin from 210 nm to 201 nm whilst keeping the detection wavelength for aescin at 210 nm could be a suitable measure to overcome the problem of the inter-laboratory variability of results and to allow the use of protoaescigenin instead of aescin as a reference standard.

4.5.3 Use of the CAD for the method improvement

Whilst the use of protoaescigenin as a reference standard could solve the problem of a high inter-laboratory variability when measuring the same sample, this did not provide an explanation for the important differences in the amount of active fraction when comparing the results of different samples by %-area normalization with those obtained using a reference standard.

A possible explanation for these differences could be that UV detection is not the most suitable option for the measurement of a complex mixture of related but nevertheless different substances like the compounds contained in the active aescin fraction.

Because aescin is lacking of strong UV-chromophors, the UV absorbance is not very pronounces and consequently the measurement must be performed at low wavelength. The major structural elements contributing to the absorbance are represented by the angelic acid and tiglic acid residues. However, it must be borne in mind, that the absorbance maxima as well as the specific absorbance values of these two acid are not identical. Tiglic acid shows a specific absorbance value (ε) of 12.500 in the maxium at 213 nm whilst for angelic acid $\varepsilon = 9000$ in the maximum at 216 nm [184]. Moreover, the amount of angelic acid and tiglic acid residues in the active aescin fraction can vary substantially [174].

One of the advantaged of the CAD is that it delivers a mass dependent response. Therefore, the detection by CAD might be used to confirm that the differences in response found between the aescin samples is in fact based on differences in the UV absorbance. Since the CAD is not suitable for gradient elutions the gradient method had to be transferred into an isocratic method.

Slightly modifying a method published by Wagner et al. [185] a mass and CAD-compatible isocratic LC method using acetonitrile / 0.05 % aqueous TFA (35/65, v/v) as mobile phase was tested. In order to verify the appropriateness of these conditions, the UV chromatograms of all aescin samples under gradient conditions were screened. The peak eluting earliest after the 1st retention time marker methylsalicylate in sample 1 (Peak "1" – cf. Fig. 4-54) as well as the latest peak eluting before the 2nd marker ibuprofen (Peak "2" eluting at about 34 min in sample 3 – cf. Fig. 4-59) were collected at the outlet of the UV detector.
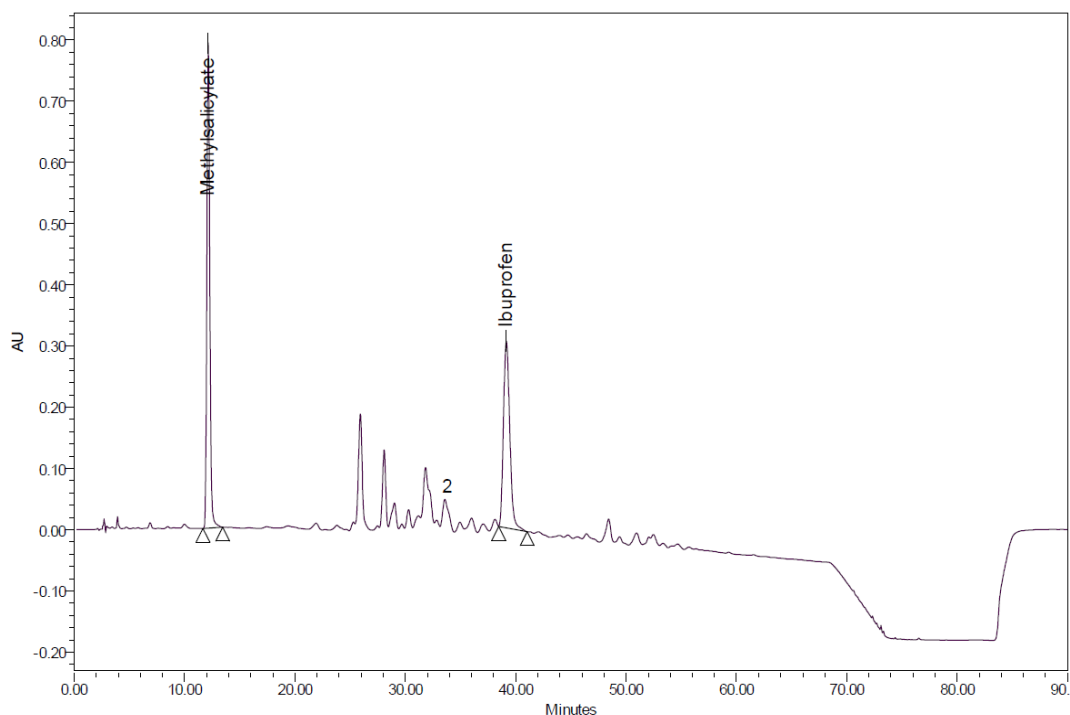
Figure 4-59. Aescin sample 3 – UV detection 210 nm. LC method as described in the Pharmeuropa draft monograph of horse–chestnut standardized dry extract [178].

The collected fractions were re-injected under the above isocratic conditions and were found to elute at 8.3 min (cf. Fig. 4-60) and 16.0 min (cf. Fig. 4-61), respectively.
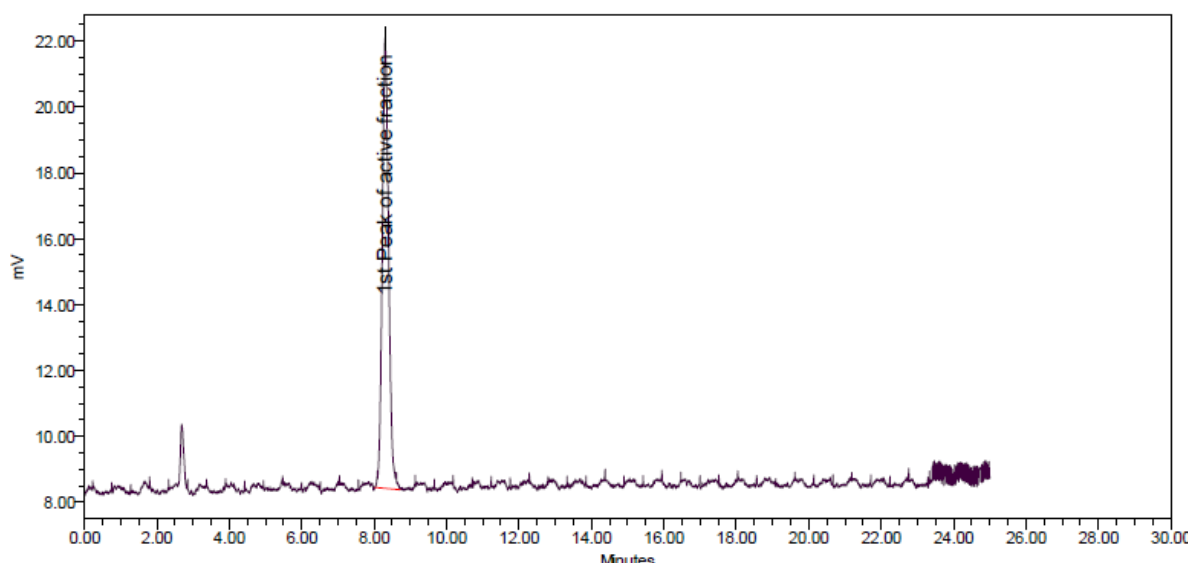
Figure 4-60. Collected fraction "1" (cf. Fig. 4-54) of aescin sample 1; 100 μL re-injected under isocratic conditions with acetonitrile / 0.05 % aqueous TFA (35/65, v/v) using a Grace Vydac C18 column (250 mm x 4.6 mm; particle size 5 μm)) at a temperature of 25 °C and a flow-rate of 1.0 mL/min. Detection by CAD.
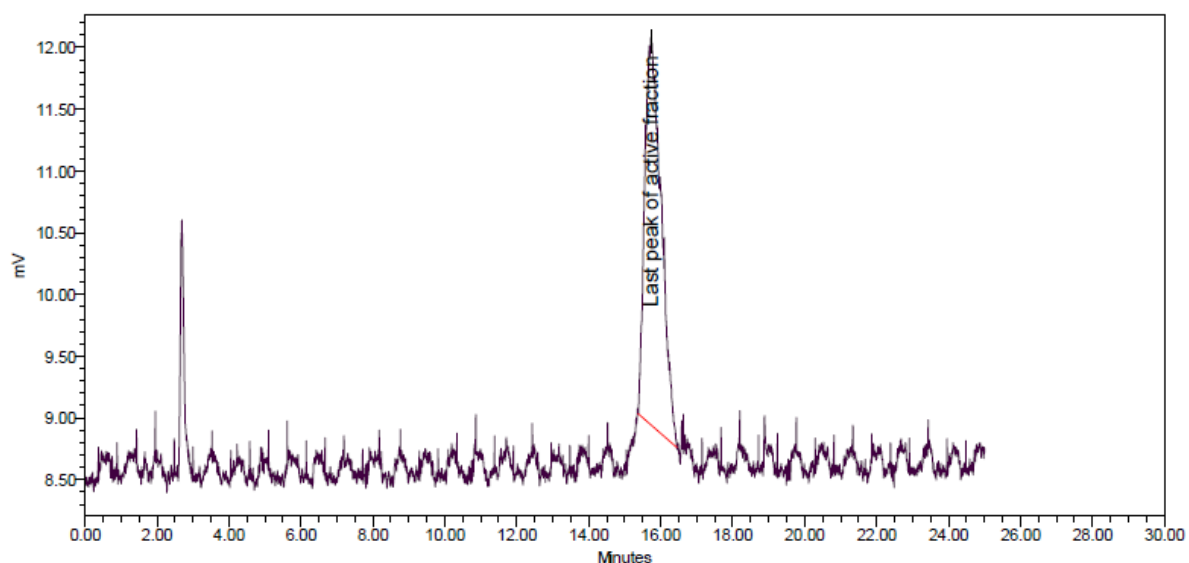
Figure 4-61. Collected fraction "2" (cf. Fig. 4-59) of aescin sample 3; Chromatographic conditions and detection as given in Fig. 4-60.

Based on these results as well as on a comparison of the chromatographic profiles obtained under gradient (cf. Fig. 4-59) and isocratic (cf. Fig. 4-62) conditions, the retention time window between 6 min and 20 min was found to be appropriate for the elution of the active aescin fraction. In order to clean the column from the components not eluted under the isocratic condition, a 10 min. "washing step" with acetonitrile / 0.05% TFA (90/10, v/v) was applied after 21 min.
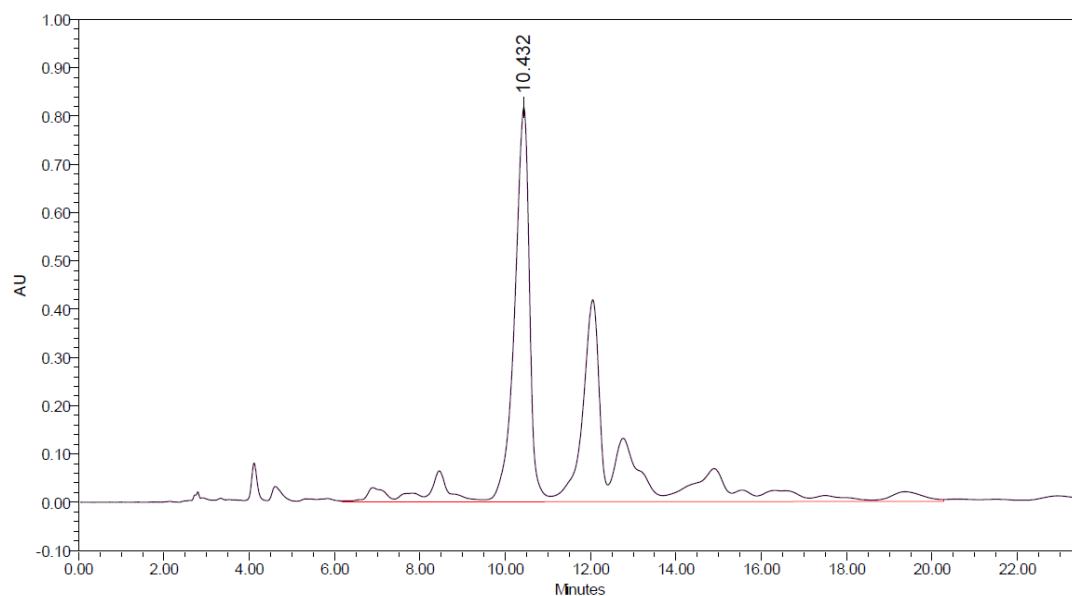
Figure 4-62. Injection of 20 µL of a 5 mg/mL solution of aescin sample 2 - Chromatographic conditions as given in Figure 4-60 – Detection by UV 210 nm.

The amount of the fraction elution between 6 min and 20 min was quantified using protoaescigenin as an external standard. Detection was performed by UV at 210 nm and by CAD. For the determinations with CAD, the concentration of the test solutions was adapted as detailed in 7.5.3.3. This was necessary to take into account the differences in the peak height of the most intense peaks and to avoid saturation of the detector signal. Since the CAD does not have a linear response over a broader range, a protoaescigenin calibration curve was recorded using 11 dilutions of a protoaescigenin stock solution covering a concentration range from 0.002 mg/mL to 0.4 mg/mL. The double logarithmic calibration curve obtained is presented in Figure 4-63.
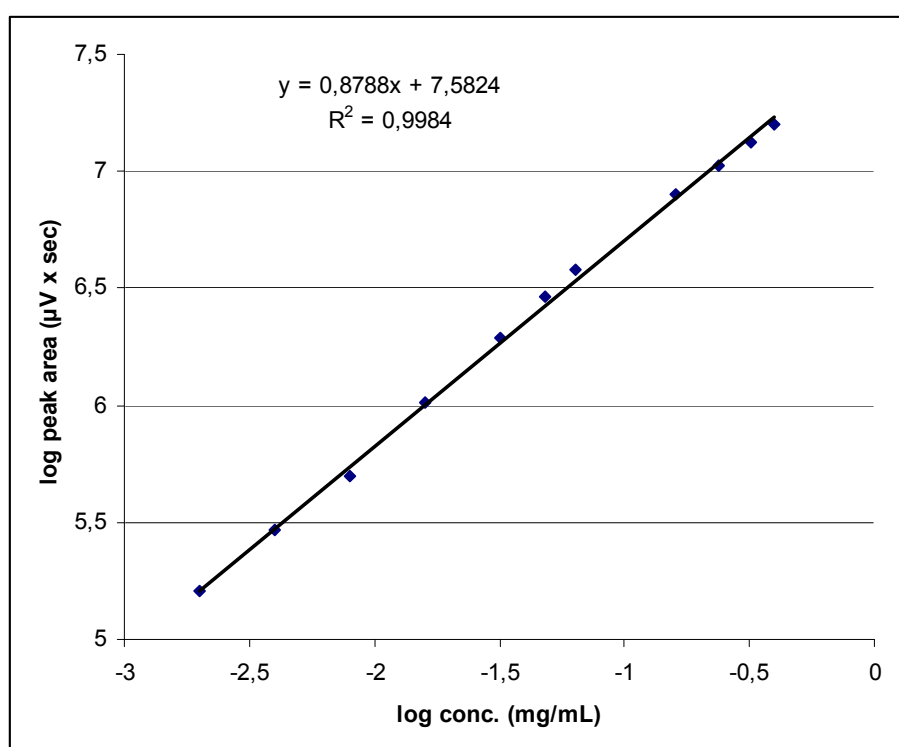
Figure 4-63. CAD-calibration curve of protoaescigenin from 2.0 µg/mL to 400 µg/mL (log/log fit - 11 concentration levels - r^2 = 0.9984). Conditions: as described in 7.5.3.3.

Since the peak due to protoaescigenin elutes at 6 min (cf. Fig. 4-64), being exactly the time defined to be the starting point for the elution of the active fraction, protoaescigenin could not only be used as an external standard for quantification, but also to define the retention time window of the active fraction. Consequently, the retention time window of the active fraction was set between the retention time of protoeascigenin and 3.5 times this time.
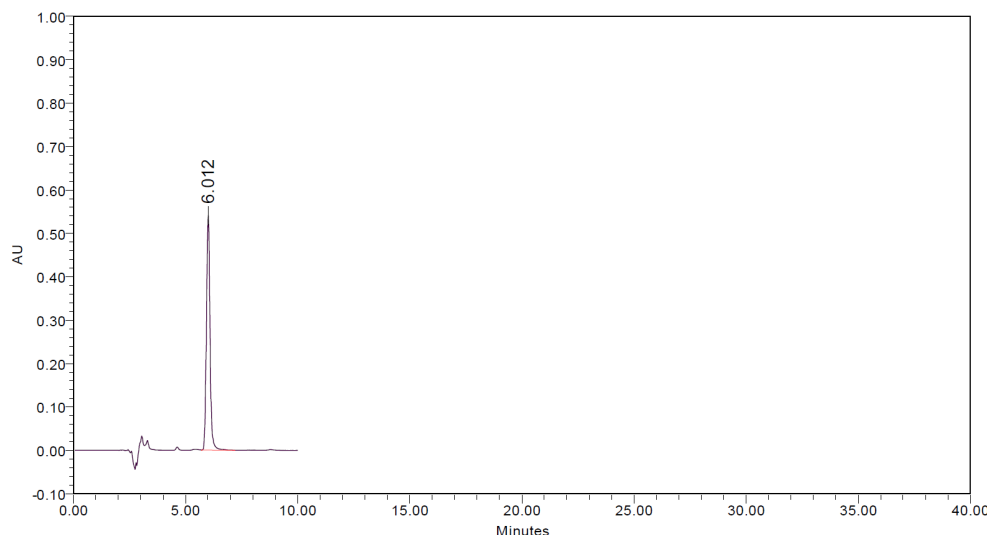
Figure 4-64. Injection of 20 µL of a 5 mg/mL solution of protoaescigenin - Chromatographic conditions as given in Fig. 4-60 – Detection by UV 210 nm.

Studying the chromatograms of the isocratic runs of the aescin samples, the definition of the integration parameters turned out to be of crucial importance. This became particularly evident for sample 3 (modified aescin), where peak integration using the "valley-to-valley" approach resulted in an important underestimation of the overall peak area (cf. Fig. 4-65 and 4-66). Therefore, the integration was performed setting the baseline at the start and the end of the active aescin fraction.
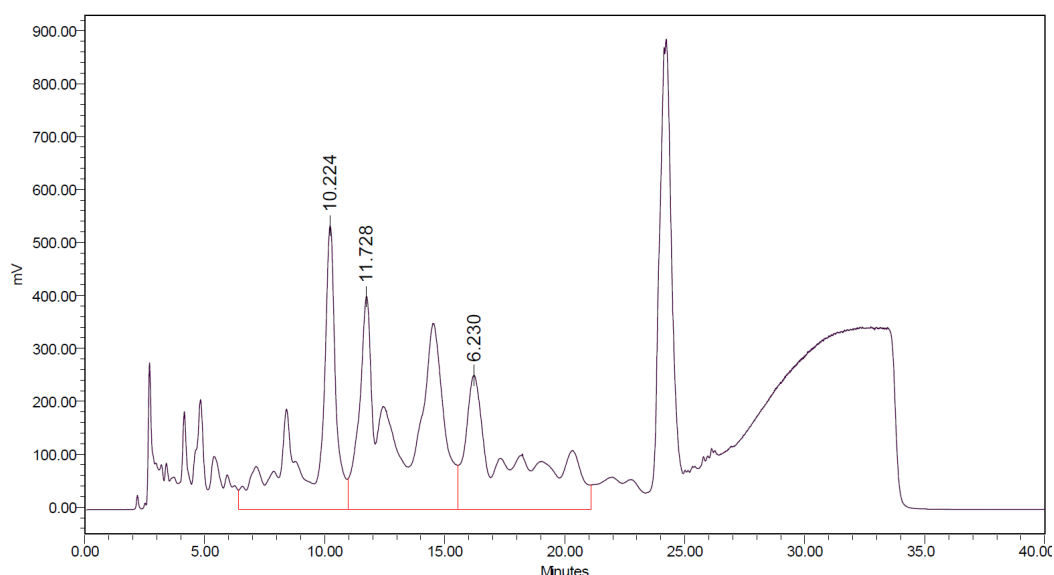
Figure 4-65. Injection of 20 µL of a 2.6 mg/mL solution of aescin sample 3 - Chromatographic conditions as given in Fig. 4-60 – Detection by CAD.
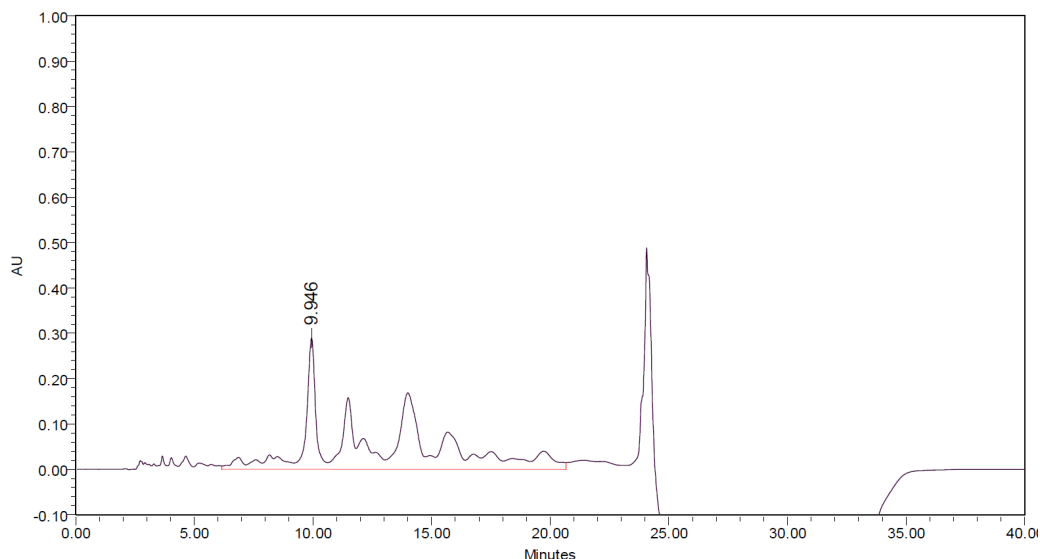
Figure 4-66. Injection of 20 µL of a 5 mg/mL solution of aescin Sample 3 - Chromatographic conditions as given in Fig. 4-60 – Detection by UV 210 nm.

For aescin sample 1 (candidate CRS) the differences due to the integration mode was less pronounced (cf. Fig. 4-67), but could nevertheless lead to different results if the analysis is performed by different operators or in different laboratories.
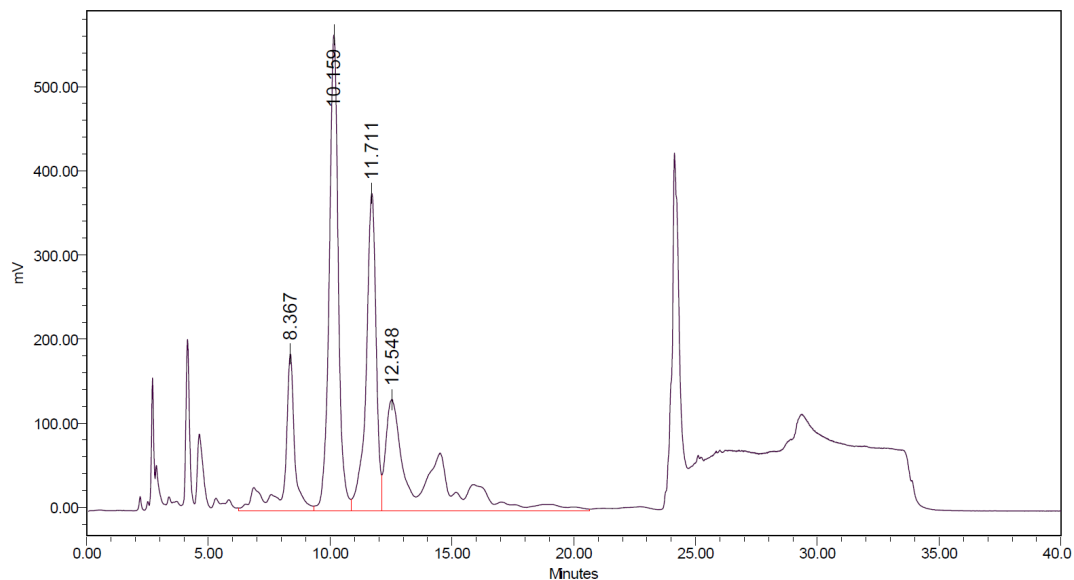
Figure 4-67. Injection of 20 µL of a 1 mg/mL solution of aescin sample 1 - Chromatographic conditions as given in Fig. 4-60 – Detection by CAD.

The results obtained using the gradient method given in the draft monograph and the aforementioned isocratic method applying UV and CAD detection are given in Table 4-27. In all cases protoaescigenin was used as an external standard.

For the sake of comparison, the results were normalized to 100% for aescin sample 1 (candidate CRS).

Table 4-27. Comparison of the different aescin samples using the gradient LC/UV method proposed in the draft monograph of horse-chestnut standardized dry extract and an isocratic LC-method with detection by UV and by CAD.

Sample	UV-detection (gradient)	UV-detection (isocratic)	CAD (isocratic)
1	100.0%	100.0%	100.0%
2	104.5%	98.9%	101.1%
3	62.0%	83.6%	105.5%
4	67.9%	81.9%	95.1%
5	72.8%	98.4%	103.4%
6	79.0%	86.1%	101.4%
7	74.4%	84.7%	100.3%
Mean	80.1%	90.5%	101.0%
Observed inter-sample variability	20.1%	9.0%	3.2%

Interestingly, the results obtained by the isocratic UV-method were mostly higher compared to those of the gradient UV-method (except sample 2). This was particularly true for the samples showing less or smaller distinct peaks (e.g. sample 3). This effect might be due to the fact that during the gradient elution a significant amount of substance eluting in the active fraction is not taken into account since it is "hidden" by the drifting baseline of the gradient.

The integration parameters selected for the chromatograms obtained under isocratic conditions took also into account the minor components as well as all compounds not forming distinct peaks. Therefore, a substantially lower inter-sample variability compared to the results of the gradient-UV method was obtained.

A comparison of the S/N-ratios obtained for different peaks in the active aescin fraction of sample 3 by CAD and UV 210 nm detection (cf. Fig. 4-65 and Fig. 4-66) revealed the detection by CAD to be between 4 and 8 times more sensitive than detection by UV.

The result obtained by LC-CAD showed that the total amount of triterpene glycosides eluting in the active fraction of samples 2 to 7 is higher than it appears when using UV detection. Moreover, compared to the results of the isocratic method with UV detection, the inter-sample variability was found to be further reduced by a factor of about 3.

4.5.4 Conclusion

It could be demonstrated that the use of an aescin reference standard for the determination of the percentage content of triterpene glycosides, expressed as aescin, as proposed in the Ph.Eur. draft monograph of horse-chestnut standardized dry extract [178], is problematic.

The use of a chemically well defined single component such as protoaescigenin is considered to be a practicable alternative for the reference standard strategy. The problems encountered with this substance in previous inter-laboratory testing were examined and possible reasons have been identified. The replacement of the currently described UV-detection wavelength of 210 nm by a wavelength of 201 nm, is seen as a possible solution to reduce the inter-laboratory variability of the results to an acceptable value.

The replacement of the proposed LC gradient method by an isocratic UV method resulted in better comparable results between the different purified ß-aescin samples. The use of the CAD instead of UV detection eliminated the problems related to the differences in the UV-response of the various components contained in the active aescin fraction and also resulted in a 4 to 8 times increased method sensitivity.

In conclusion, an isocratic LC method with detection by CAD and using protoaescigenin as an external standard for quantification is considered to be the most suitable approach for the determination of the percentage content of triterpene glycosides, expressed as aescin, in a Ph.Eur. monograph of horse-chestnut standardized dry extract.

4.6 Use of collision induced dissociation MS for the identity verification of peptides

Modern developments in the field of analytical chemistry do not only allow to improve the methods for the control of impurities in a drug substance, but offer also many further possibilities to improve the quality of other parts of a pharmacopoeial monographs. The work reported in this chapter aims at demonstrating how collision induced dissociation mass spectrometry (CID-MS) can be employed to improve the pharmacopoeial identification testing of pharmacologically active peptides.

Concerning the scope of the identification section of a monograph, it is important to note that the described tests are not aiming at a full elucidation of the structure or composition. The objective of the monograph section is rather to ensure, as far as possible, the specificity to an extent that substances exhibiting similar structures are distinguished [17, 19]. In many cases the identification of a drug substance can easily be achieved by standard techniques like infrared spectroscopy, either on its own or supplemented by further tests, e.g. an optical rotation test for chiral substances or an identification reaction for the counter-ion of the active ingredient.

However, for some substances the above test do not deliver a sufficient degree of certainty about the identity of a substance and more sophisticated or laborious tests need to be carried out. Another important element to be considered is that some pharmaceutically active substances like synthetic peptides are very costly. Therefore, the substance consumption in the quality control testing should be as low as possible.

In this study, two different classes of related peptides were selected, i. e. human insulin and 2 insulin analogues (insulin lispro and insulin aspart) and four synthetic gonadotropin releasing hormone analogues (gonadorelin, goserelin buserelin and leuprorelin) being either nona- or decapeptides. For the above substances, the pharmacopoeial identification currently requires a combination of several partly very laborious tests. The aim was to verify if CID-MS could be employed as a quick, easy to apply and resources saving stand- alone test that would guarantee the same if not an increased level of reassurance about the identity of the test item.

4.6.1 Identification of peptides using CID-MS (De Novo Peptide Sequencing)

The identification of peptides by means of tandem mass spectrometry is nowadays routinely employed for the determination of the amino acid (AA) sequence of peptides via the fragement ions produced by low energy CID, as it is the case in ion trap MS or triple quadrupole MS [186, 187, 188, 189, 190, 191]. One of the advantages of these instruments is that by using an ESI-source predominantly multiply charged peptide precursor-ions are generated. These multiple charged precursors have the advantage that they are relatively easy to fragment in low energy CID compared with singly charged precursors [192, 193, 194].

If the charge of a CID-fragment is retained on the N-terminal side, the ion is classed as *b*. Another class of ions are the a-ions which are derived from b-ions upon a loss of CO. If the charge is retained on the C-terminus, the ion type is *y*. A subscript indicates the number of residues in the fragment. Thus, for a singly protonated precursor ion as depicted in Figure 4-68, a b_2-ion together with a neutral fragment would be generated if the charge is retained within the first two amino acids of the N-terminus. The neutral fragment containing the C-terminus of the peptide, is not detectable with the mass spectrometer. Conversely, a y_2-fragment and a neutral species containing the peptide N-terminus would be generated if the charge is retained on the C-terminal side [192, 195, 196].
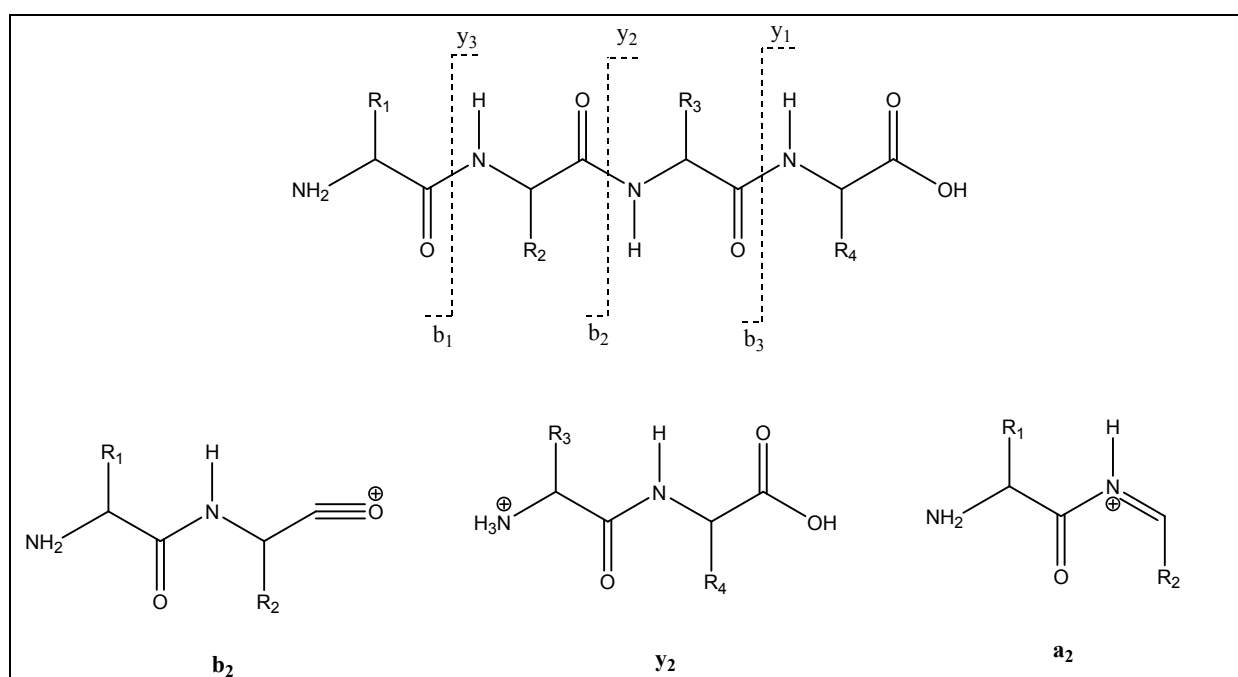
Figure 4-68. Peptide cleavage by CID-MS. Modified from [197].

Moreover, ions resulting from the loss of water, ammonia or carbon dioxide or side chains from the sequence fragment ions are also often present. Following this scheme, it is very easy to predict the masses of the expected fragments. This task can be significantly facilitated by the use of corresponding computer programs [186, 198, 199, 200].

In addition to the above fragments, internal cleavage ions may be generated by double backbone cleavage combining b- and y-type fragmentation (cf. Fig. 4-69).
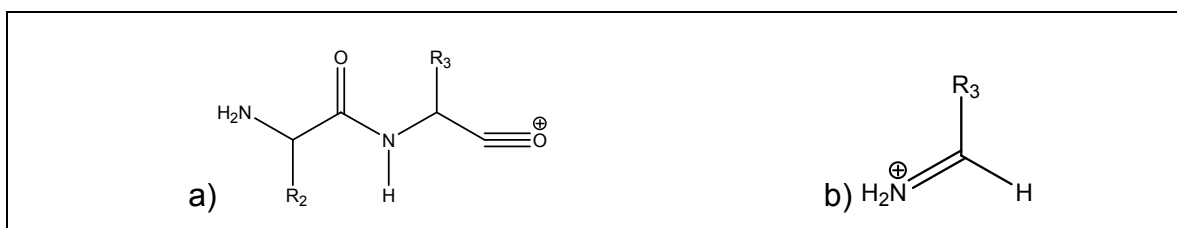
Figure 4-69. CID fragments a) produced by double backbone cleavage b) immonium ion following a combined a- and y-type cleavage.

Another occurring internal cleavage pathway results in the formation of immonium ions. These ions have the general structure $RCH=NH_2^+$ (R = the amino acid side chain – cf. Fig. 4-69) and a mass of 27 u less than the residue they are generated from [201]. The immonium ions for histidine (His) at m/z = 110, isoleucine (Ile) and leucine (Leu) at m/z = 86, phenylalanine (Phe) at m/z = 120, proline (Pro) at m/z = 70, tryptophane (Trp) at m/z = 159 and tyrosine (Try) at m/z = 136, produce often strong signals [202].

To delineate the complete sequence, fragmentation must occur at each peptide bond. Unfortunately, this is rarely the case [196, 203, 204]. Nevertheless, by combining the results obtained from C- and N-terminal fragmentation of a peptide, confirmation of the AA sequence is nevertheless possible in many cases. However, this technique has its limitations: although in principle possible [205], D and L-forms of AAs cannot easily be distinguished from each other. Moreover, the distinction of the structural isomers Leu and Ile is not possible.

4.6.2 Pharmacopoeial identification of insulin

Insulin, produced in the human pancreas, is a peptide consisting of 51 amino acids with a molecular mass of 5808 u [206]. It is organized in 2 chains, an A-chain with 21 AAs and a B-chain with 30 AAs. The two chains are linked via 2 cysteine (Cys) disulfide bridges (cf. Fig. 4-70). In the recent past, several slightly modified versions of human insulin, such as insulin lispro and insulin aspart exhibiting different absorption or duration of action characteristics obtained marketing authorization [207, 208, 209]. Alike these insulins, human insulin is nowdays mainly manufactured biosynthetically by means of recombinant DNA technology. Structurally insulin lispro differs from human insulin by an inversion of two beta-chain amino acids, i.e. reversal of B-28 proline (Pro) and B-29 lysine (Lys). Compared with human insulin, the AA Pro in position 28 of the B-chain of insulin aspart is substituted by aspartic acid (Asp) [210].
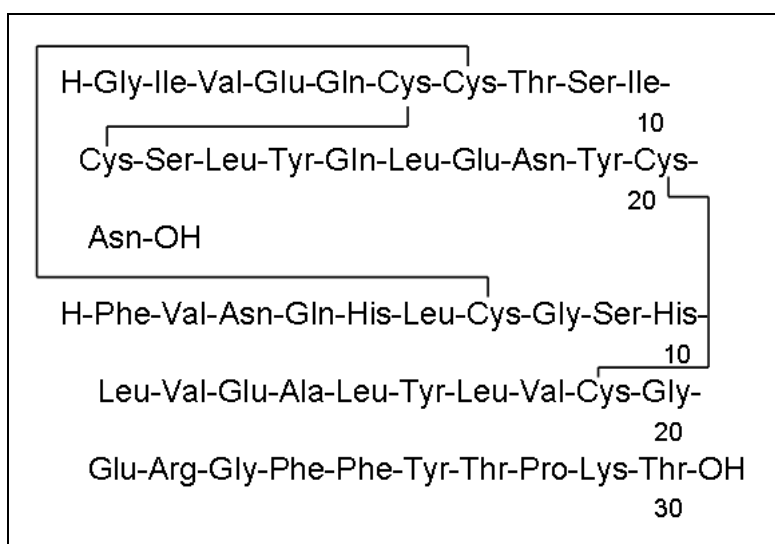
Figure 4-70. AA-sequence of human insulin.

For the identification of the three insulins, the Ph.Eur. monographs [206, 211, 212], prescribe the conduct of two different tests. In the first test the reversed phase liquid chromatographic (RP-LC) retention time of the substance – determined using a UV-detector - is compared with the retention time of a human insulin reference standard. As a second test peptide mapping is described. In this test the molecule is digested employing a Glu-C-protease. Subsequently the peptide fragments are analyzed by RP-LC and compared to those obtained using a corresponding reference standard treated in the same way. In the USP monographs of human insulin and insulin lispro [5] the same approach is used.

Unfortunately, identification by comparison of the LC retention times is not very specific. According to the "Technical Guide for the Elaboration of Monographs for the European Pharmacopoeia" [19] LC is only used for identification purposes if no suitable alternative is available. The limited applicability of LC for identification purposes is confirmed by the fact that peptide mapping LC results in identical chromatographic profiles for insulin aspart and insulin lispro. (cf. Fig. 4-71 a, b).
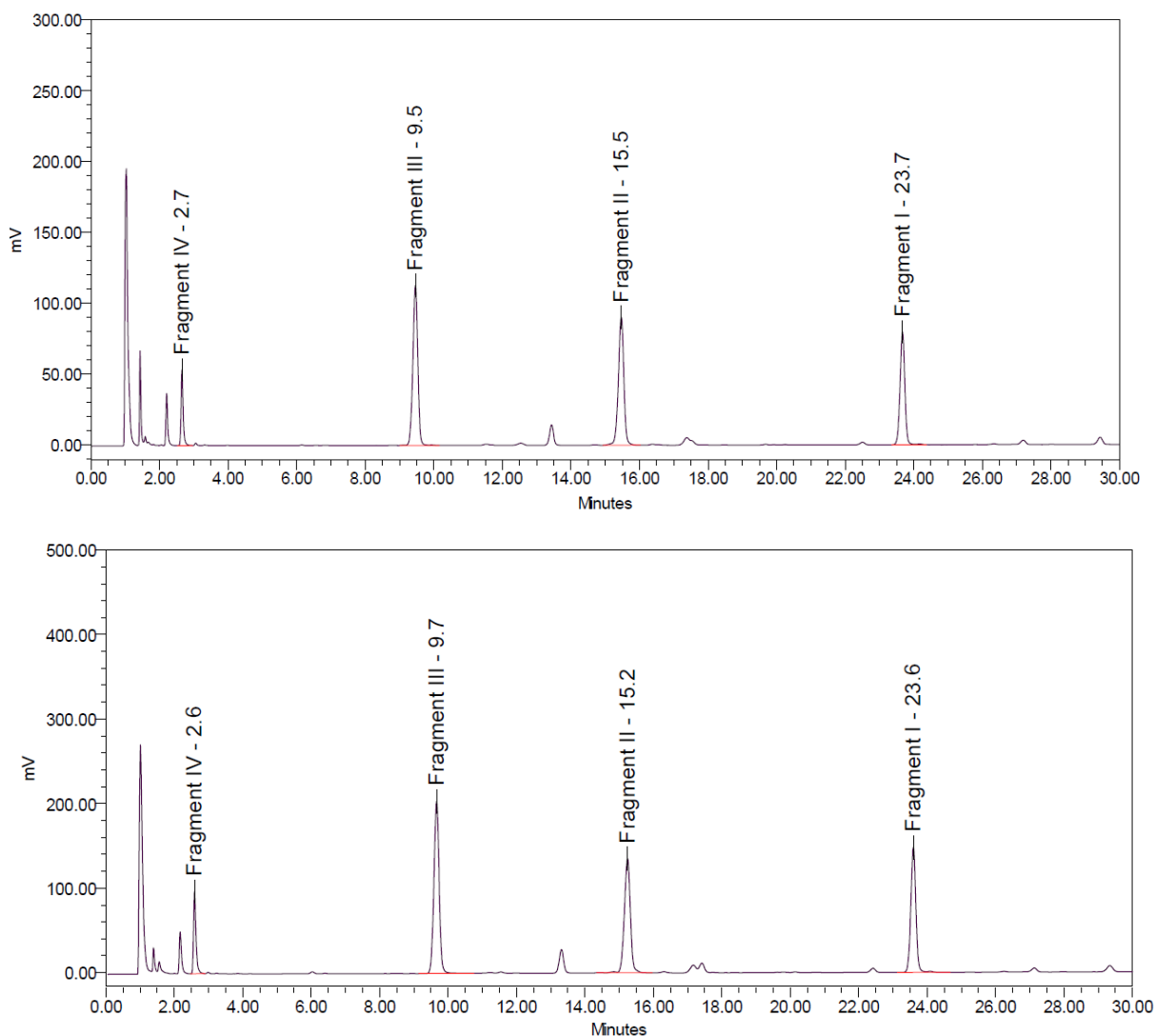
Figure 4-71. Chromatographic profile of a) insulin aspart and b) insulin lispro applying peptide mapping according to the conditions described in the corresponding Ph.Eur. monographs. Modifed from [197].

All in all, the tests described in the current pharmacopoeial monographs of the above insulins are not considered to be sufficiently specific to assure a reliable identification of the substances.

4.6.3 CID-MS identification of human insulin, insulin lispro and insulin aspart

Caused by a substitution of the AA Pro in position 28 of the insulin B-chain by Asp, the molecular mass of insulin aspart (M_r = 5826 u) is different from the molecular mass of human insulin (M_r = 5808 u). In contrast, the molecular masses of human insulin and insulin lispro are identical since the only difference between the two molecules is an inversion of the amino acids proline and lysine in position B-28/29.

Since the upper limit of the mass range of the triple-quadrupole MS used in this study was at a m/z of 2000, the molecular mass of the three peptides could only be verified focusing on multiply charged molecular ions, i.e. the $[M+4H]^{4+}$ and the $[M+5H]^{5+}$ ions. The expected molecular masses of the peptides could be confirmed and insulin aspart could be clearly distinguished from human insulin and insulin lispro. However, to differentiate human insulin from insulin lispro and to confirm the position 28 modification in insulin aspart, additional structural sequence information was required. This information could be obtained by CID-MS experiments after digestion of the insulins with Staphylococcus aureus V8 protease, an enzyme that splits the sequence of amino acids at the C-terminal side of each Glu.

As described in the literature [213], four peptide fragments were obtained. The corresponding ESI-MS scan of a digested solution of insulin lispro is shown in Figure 4-72.
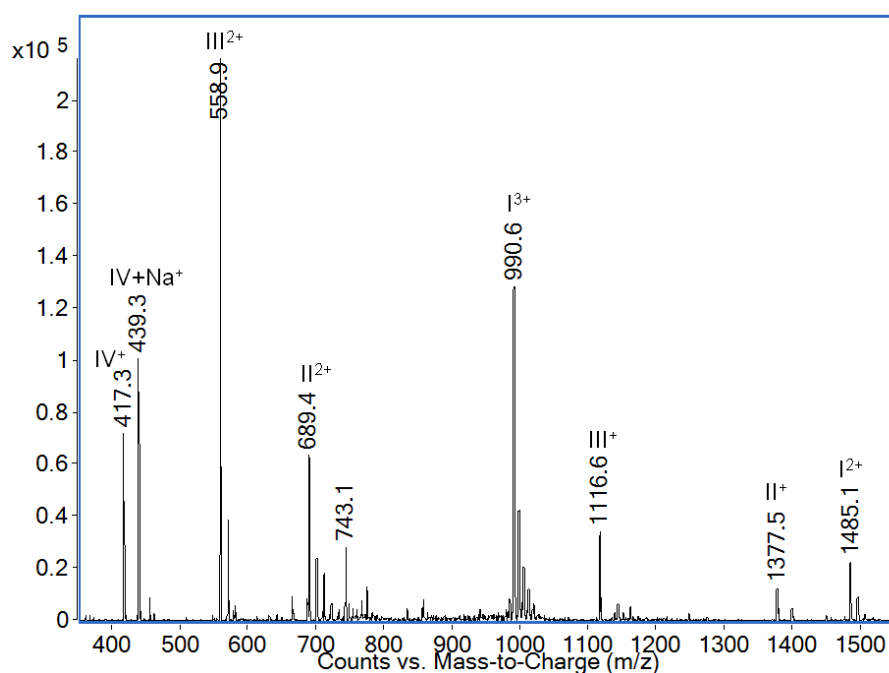
Figure 4-72. MS spectrum of the four fragments resulting from enzymatic digestion of insulin lispro. Signals marked as IV$^+$ and IV+Na$^+$ belong to fragment 4, signals III^{2+} and III$^+$ to fragment 3, signals II^{2+} and II$^+$ to fragment 2 and signals I^{3+} and I^{2+} to fragment 1.

Fragment III, a linear nonapeptide with a m/z-ratio $[M+H]^+$ of 1116.6 composed of the C-terminal AAs of the B-chain [213], was selected for CID-MS. The theoretical CID-MS fragmentation scheme of the fragment III nonapeptides of the three insulins is depicted in Figure 4-73. Ions b_1 to b_6 and y_1 are identical whilst the b_7, b_8 and the y_2 and y_3-fragments are different. Moreover, the y_4 to y_8-fragments of insulin aspart are not equal to those of human insulin and insulin lispro.
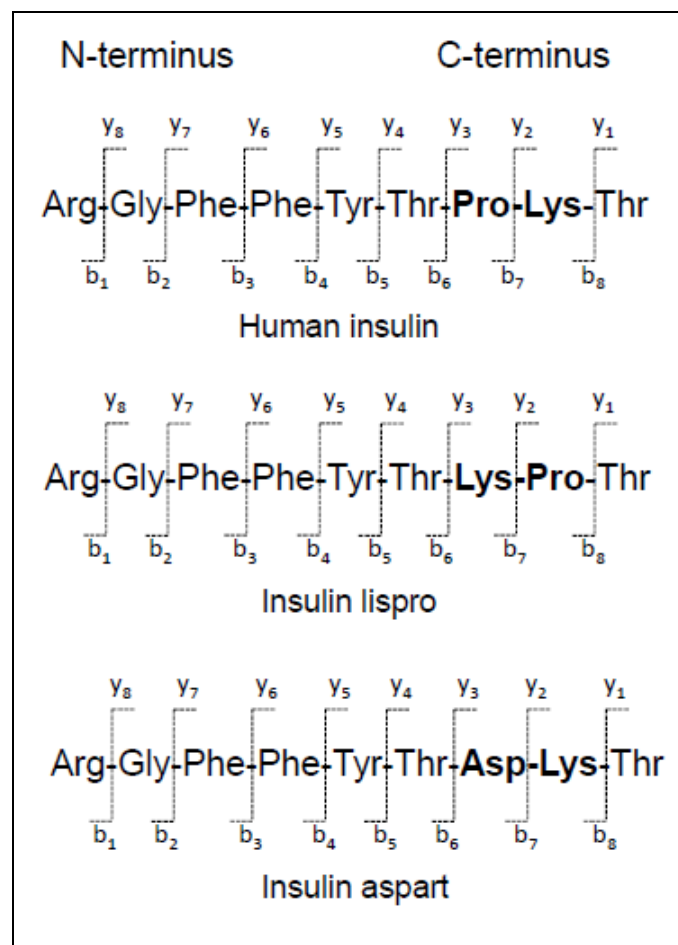
Figure 4-73. Theoretical b and y low energy CID-MS fragments of digested fragment III of a) human insulin, b) insulin lispro, c) insulin aspart. Modified from [197].

The AA-sequencing was not straightforward since several expected b-fragments and y-fragments were missing (cf. Fig. 4-74). However, with two exceptions (b_7-fragment of human insulin and y_3-fragment of insulin aspart) the essential fragments allowing a distinction of the three insulins could be detected. The results are summarized in Table 4-28.

Table 4-28. Summary of CID-MS results for the digested fragments III of human insulin, insulin lispro and insulin aspart. Modified from [197].

PRECURSORS OF DIGESTION FRAGMENT III IN MS2-SCAN

		Human insulin	Insulin lispro	Insulin aspart
precursor m/z	z=1	*1116.6*	*1116.6*	*1134.6*
precursor m/z	z=2	**558.9**	**558.9**	**567.8**

TANDEM-MS RESULTS AFTER CID OF DIGESTION FRAGMENT III *

Internal cleavage ions (m/z): [Pro-Lys]+(**226.1**) | [Thr-Lys]+(*230.1*) | [Asp-Lys]+(*244.1*)

AA Sequence (Human | Lispro | Aspart)			b-ion	N-terminal m/z Human	N-terminal m/z Lispro	N-terminal m/z Aspart	y-ion	C-terminal m/z Human	C-terminal m/z Lispro	C-terminal m/z Aspart
Arg-Gly			2	-	-	-		Precursor		
Phe			3	361.2	-	*361.2*	7	-	-	-
Phe			4	508.3	508.3	*508.3*	6	-	-	-
Tyr			5	671.3	671.3	*671.3*	5			-
Thr			6	**772.4**	772.4	*772.4*	4	-	-	-
Pro	Lys	Asp	7	-	**900.5**	**887.4**	3	345.2	345.2	-
Lys	Pro	Lys	8	997.5	997.5	*1015.5*	2	248.2	**217.1**	248.2
Thr				Precursor			1		120.1	*120.1*

*****Bold** face indicates strong signals, *italic* indicates weak signals.

(a)
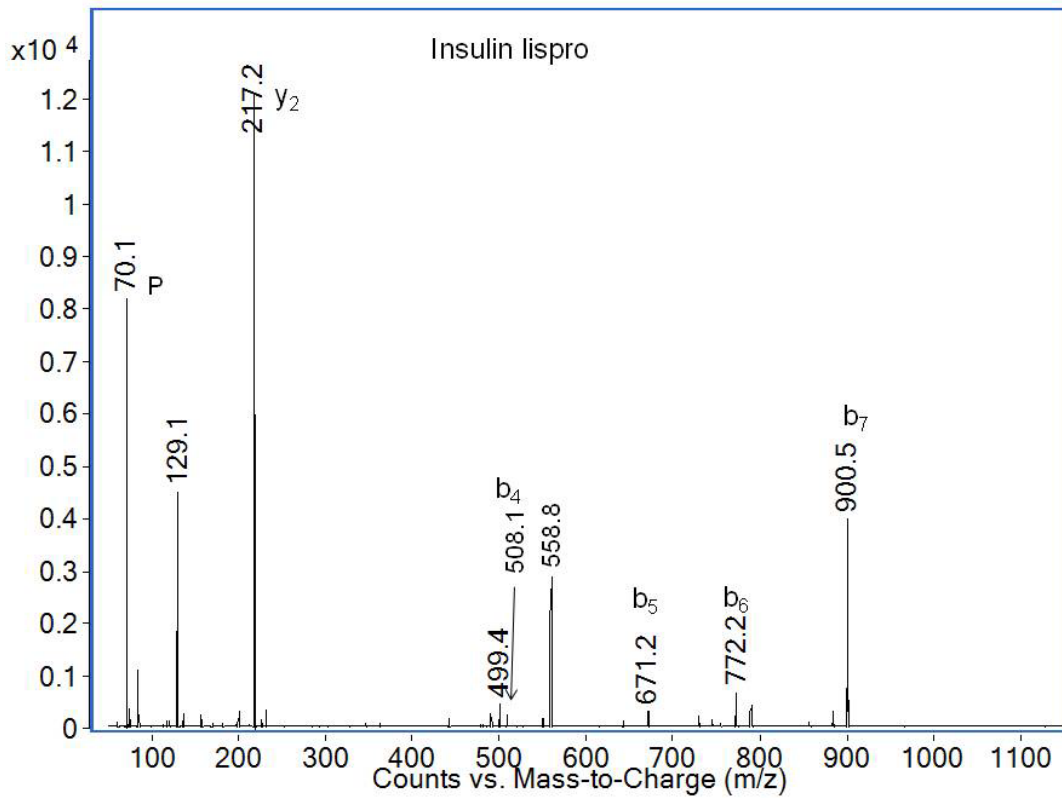
(b)
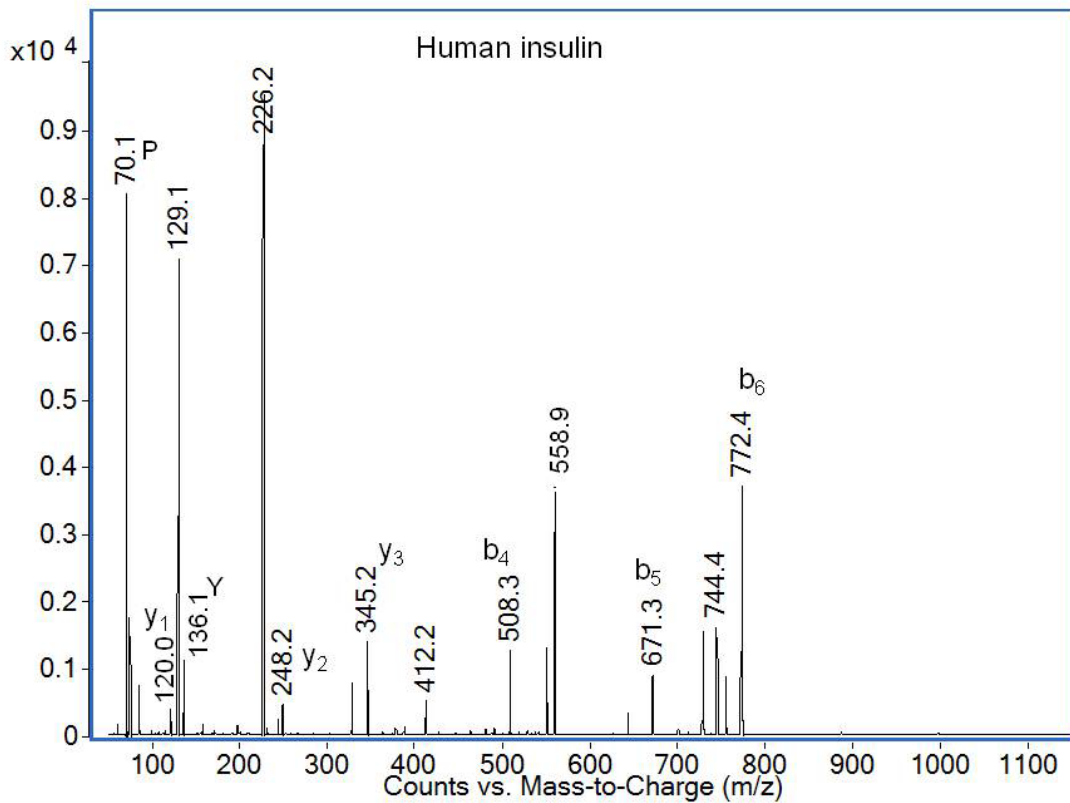
(c)
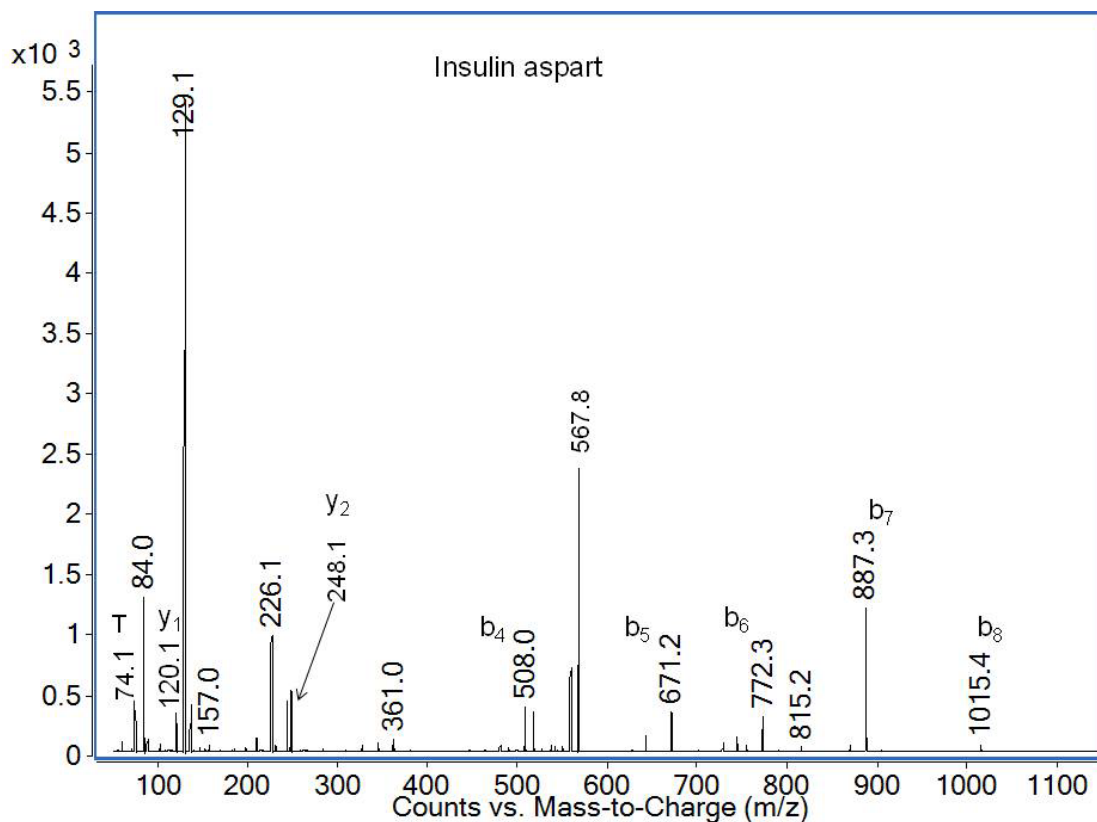
Figure 4-74. CID-MS spectra obtained from the [M+2H]$^+$ precursor ions of the digested fragment III of, a) insulin lispro, b) human insulin c) insulin aspart. A single letter code is used to indicate the AAs (P = proline, T = threonin, W = tryptophan and Y = tyrosin). Modified from [197].

The absence of the b$_7$-fragment can be explained by the fact that the AA in position 7 is Pro. For this AA it is known that the N-terminal peptide bond is particular labile whereas the bond on the C-terminal side is not. Consequently N-terminal cleavage of Pro is privileged and C-terminal fragments (in this case the b$_7$-ion) show only a very low abundance or are even absent [192, 193, 204]. Therefore, the b$_7$ fragment of human insulin was not expected to occur. The reason for the absence of the y$_3$-fragment for insulin aspart can be explained by the fact that for Asp C-terminal cleavage is privileged [192]. Nevertheless, for both, human insulin and insulin aspart, three out of four discriminating fragments were found. In the case of insulin lispro all four could be detected. Furthermore, some fragments resulting from internal cleavage could be assigned to [Pro-Lys]$^+$ (m/z = 226.1) for human insulin, to [Thr-Lys]$^+$ (m/z = 230.1) for insulin lispro and to [Asp-Lys]$^+$ (m/z = 244.1) for insulin aspart.

These internal sequence combinations can only occur in the corresponding insulin analogues and are therefore also considered to be indicative for the corresponding structures.

Taken together, the combination of the confirmation of the molecular masses of the insulins via the m/z-ratios of the $[M+4H]^{4+}$ and $[M+5H]^{5+}$ ions with the specific differences found in the AA sequence by CID-MS allowed a quick and efficient identification and distinction of human insulin, insulin lispro and aspart. The degree of certainty of this identification is considered superior to the identification tests currently described in the corresponding monographs.

4.6.4 Pharmacopoeial identification of gonadotropin releasing hormone analogues

The European Pharmacopoeia contains monographs for the synthetic nonapeptides goserelin [214], buserelin [215] and leuprorelin [216] and for the decapeptide gonadorelin acetate [217]. These peptides belong to the group of gonadotropin releasing hormone (GnRH) agonists and are therapeutically used in various applications in gynaecology, reproductive medicine, and oncology [218]. In the USP [5] monographs for gonadorelin acetate and leuprorelin acetate are described. The 4 peptides have very similar structures (cf. Fig. 4-75), differing only in the amino acid (AA) in position 6 as well as in the AA on the C-terminal side of the peptides.
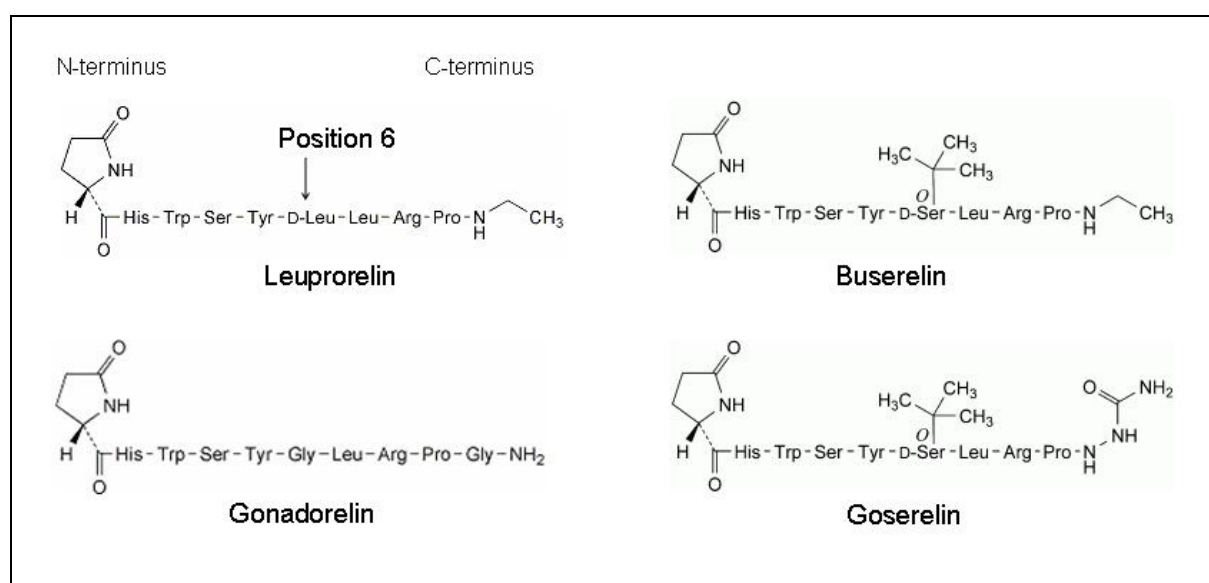
Figure 4-75. Structures of leuprorelin buserelin gonadorelin and goserelin. Modified from [197].

The fact that the identification of these peptides is not very consistent has already been highlighted by Vergote et al. [219]. In the corresponding Ph.Eur. monograph gonadorelin acetate is identified by a combination of LC and of thin layer chromatography (TLC). In contrast, the USP prescribes a combination of mass spectrometric verification of the molecular mass and LC. For leuprorelin, the USP requires only the identification by infrared spectrophotometry (IR) and LC whilst a combination of IR, LC and amino acid analysis (AAA) is prescribed in the Ph.Eur. For buserelin and goserelin, the Ph.Eur. employes a combination of three different tests, i.e. a LC, a nuclear magnetic resonance spectroscopy (NMR) and an AAA for identification purposes. Although a change to ^{1}H NMR has recently been proposed for goserelin [220] it is interesting to note that the technique currently applied in the goserelin monograph is ^{13}C NMR whilst in the monograph of buserelin ^{1}H NMR is given. Considering the limitations of the above techniques – at least in the way they are currently described, none of them can be considered sufficiently specific to provide reliable information about the AA sequence and therefore the identity. Moreover, the requirement to perform several tests in combination is very demanding in terms of time, equipment and amount of analyte to be used.

4.6.5 CID-MS identification of Gonadotropin Releasing Hormones

The molecular masses of leuprorelin, gonadorelin, leuprorelin, goserelin and buserelin were confirmed by direct mass measurement using the $[M+H]^+$ and $[M+2H]^{2+}$ ions. For CID-MS, the singly positive charged ($[M+H]^+$) and the double positive charged ($[M+2H]^{2+}$) precursors of the 4 peptides were selected. Whilst the CID-MS of the singly charged precursors mainly delivered intense b-fragments, b and y fragments were generated from the $[M+2H]^{2+}$-ions. A fragmentation scheme showing all theoretical b and y fragments of leuprorelin (as an example for the nonapeptides) and of gonadorelin is given in Figure 4-76 for information. An overview of all masses measured is presented in Table 4-29.

Table 4-29. Gonadotropin Releasing Hormones - Summary of the MS results. Modified from [197].

PRECURSORS FOUND IN MS/MS SCAN

Peptide:		Gonadorelin	Leuprorelin	Goserelin	Buserelin
Sum formula [M]		$C_{55}H_{75}N_{17}O_{13}$	$C_{59}H_{84}N_{16}O_{12}$	$C_{59}H_{84}N_{18}O_{14}$	$C_{60}H_{86}N_{16}O_{13}$
theoretical m/z-ratio	z=1	1182.6	1209.7	1269.6	1239.6
theoretical m/z-ratio	z=2	591.9	605.4	635.5	620.4
m/z-ratio found	z=1	1182.6	1209.7	1269.6	1239.6
m/z-ratio found	z=2	591.9	605.4	635.5	620.4

FRAGMENTS DETECTED IN CID-MS

b-ion	AA Seq. Gonadorelin	AA Seq. Leuprorelin	AA Seq. Goserelin	AA Seq. Buserelin	N-term Gonadorelin	N-term Leuprorelin	N-term Goserelin	N-term Buserelin	y-ion[1]	C-term Gonadorelin	C-term Leuprorelin	C-term Goserelin	C-term Buserelin
1	Pyr	Pyr	Pyr	Pyr		112.1			9/8	-	-	Precursor	-
2	His	His	His	His		249.1			8/7	934.5	961.5	965.5*	935.4*
3	Trp	Trp	Trp	Trp		435.2			7/6	748.4	775.4	835.4	805.4
4	Ser	Ser	Ser	Ser		522.2			6/5	661.4	688.4	692.4*	662.4*
5	Tyr	Tyr	Tyr	Tyr		685.3			5/4	498.3	525.3	585.3	555.3
6	Gly	D-Leu	D-Ser-C4H9	D-Ser-C4H9	742.3	798.4	772.3*		4/3	441.3	412.3	442.3	412.3
7	Leu	Leu	Leu	Leu	855.4	911.5	885.4*		3/2	328.2	299.2	329.2	299.2
8	Arg	Arg	Arg	Arg	1011.5	1067.6	1097.5		2/1	172.1	143.1	173.1	143.1
	Pro-Gly-NH2	Pro-NH-C2H5	Pro-N3H4CO	Pro-NHC2H5	Precursor								

Additionally detected immonium ions (m/z): Pro(70),His(110),Trp(159),Leu/Ile(86),Tyr(136):

[1] The first figure indicates the position in the decapeptide gonadorelin. The second figure stands for the position of the AA residue in the three nonapeptides.

* further fragmentation occurred and only the [M+H]$^+$-ion minus the tert.-butyl residue of D-Ser could be measured.

Figure 4-76. Theoretical b and y low energy CID-MS fragments of gonadorelin and leuprorelin. Modified from [197].

For the nonapeptides buserelin, goserelin and leuprorelin it was possible to confirm the complete AA sequence by the corresponding sequence of y-fragment ions (C-terminal fragments) and via the expected series of b-fragment ions (N-terminal fragmentation). The only exception was that the y_8 fragment, resulting from the splitting of the N-terminal pyroglutamic acid (Pyr) from the remaining 8 AAs of the nonapeptide, could not be detected. However, taking into account that intense b_2 fragments with an m/z-ratio of 249 were detected (cf. Fig. 4-77 a/b), it was concluded that the formation of a Pyr-His b_2-fragment is privileged and that therefore a y_8-fragment with N-terminal His does not occur [204]. The fragments marked with an asterisk in Table 4-29 correspond to the so-called "satellite-ions" (ions produced by split-off of the side chain modification; in this case the *tert.* butyl residue) [192] of the b- and y-fragments containing modified D-Ser which is present in position 6 of goserelin and buserelin (cf. Fig. 4-75).

In the case of gonadorelin, the y_1 (C-terminal Gly) and y_9 (resulting from the splitting of the N-terminal Pyr from the remaining 9 AAs of the decapeptide) fragments were not detected. The reason for the absence of the y_9 fragment, corresponding to the y_8 fragment of the nonapeptides, was already discussed above.

The absence of the y_1 fragment (C-terminal Gly) can be explained by the fact that the AA in position 9 is Pro (cf. Fig. 4-76). As already outlined under 4.6.3, the N-terminal peptide bond of Pro is particularly labile. Therefore, a cleavage at the C-terminal side is not favorable.

Consequently, the absence of the y_1-ion can even be interpreted as an indirect confirmation of the sequence since in case of an inversion of Gly and Pro at the C-terminal end, the Gly ion should have appeared as y_1-fragment.

The CID-MS spectra of the leuprorelin precursors with a double positive charge (z=2) and with a single positive charge (z=1) are given in Figure 4-77 a,b for information.

(a)
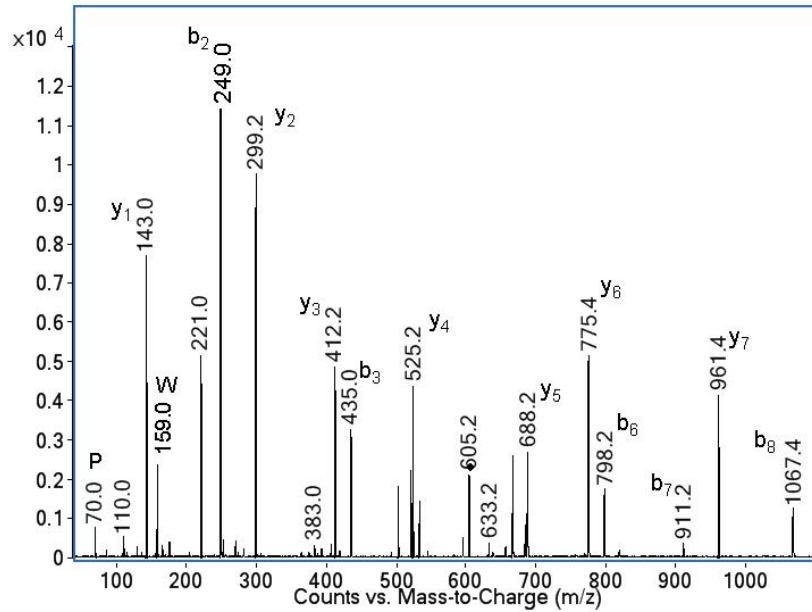
(b)
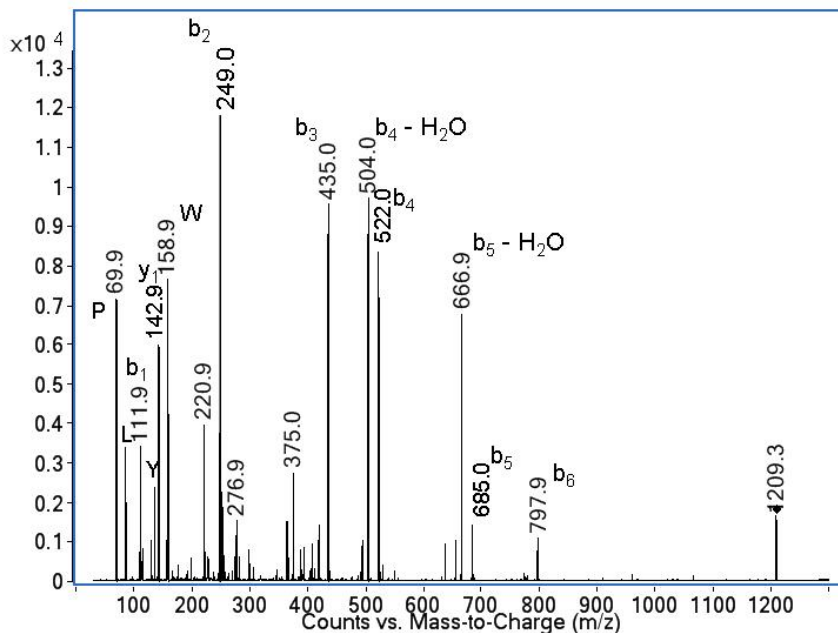
Figure 4-77. Mass spectra of leuprorelin after low energy CID of the precursor with (a) z = 2 and (b) z = 1; b and y fragments detected are indicate in the spectrum. A single letter code is used to indicate the AAs (P = proline, L = leucine, Y= tyrosin, W = tryptophan). Modified from [197].

4.6.6 Conclusion

It has been demonstrated in the above experiments on the example of two different groups of pharmacologically active peptides (GnRHs and insulins) how low energy CID-MS can successfully be employed for the identification of peptides in pharmacopoeial monographs. For this application, the combination of a direct confirmation of the molecular mass by measuring the m/z-ratio of the molecule ion with information about the peptide sequence obtained by CID-MS experiments delivered a higher degree of certainty of the identity of the examined substances than the set of tests currently described in the corresponding Ph.Eur. and USP monographs.

The advancement of the MS instruments made it possible that CID-MS spectra can nowadays easily be recorded even without being a specialized mass spectrometrist. Moreover, decreasing instrument prizes allow more and more lab access to this technique. Therefore, with an appropriate set of pre-defined acceptance criteria, the verification of the identity of the above substances can be carried out by a standard laboratory operator even without in-depth knowledge of mass spectrometry.

For example, the acceptance criteria for insulin lispro could be composed of the confirmation of the m/z ratio of the intact molecule (e.g. via the $[M+4H]^{4+}$ or the $[M+5H]^{5+}$ ion) combined with the detection of the b_7 fragment at an m/z ratio of 900 and the y_2 fragment at an m/z ratio of 217. For goserelin the verification of the m/z-ratio of the precursor ion and the detection of either the b or the corresponding y-fragment for each step of the AA sequence could be specified as criteria for a positive identification. In order to exclude any misinterpretation or unforeseen differences in the results caused by different MS settings or different types of MS instruments, the CID-MS spectra of the test sample could additionally be evaluated against those of a corresponding reference standard.

In comparision with the current situation where the pharmacopoeial Monographs prescribe the conduct of several, partly rather laborious tests for the verification of the identity, MS would offer several advantages, i.e. a significant gain in efficiency and throughput due to easy sample preparation; fast measurement and result interpretation; an important reduction of the amount of costly peptides consumed during testing; a reduction of the amount of reference standards required per test; a higher degree of certainty of the identity of the substance tested.

5. <u>SUMMARY</u>

An appropriate quality control of the active pharmaceutical ingredients (APIs) is one of the key elements for the safe application of medicines and for the protection of the consumers health. The European Pharmacopoeia (Ph.Eur.) with its general texts, general monographs and individual substance monographs represents the legally binding standard defining the minimum quality requirements for the identification, purity control and content determination of APIs and excipients used for the production of medicines marketed in Europe.

The work presented in this thesis was mainly targeted at exploring the capabilities of evaporation based LC detectors as well as further alternatives for the control of impurities in substances not exhibiting a suitable chromophore for UV-detection. In the course of the work carried out, several new methods for the identification, impurities control and composition testing of APIs were elaborated.

An evaporation based detector that entered into the field of pharmaceutical analysis in the recent years was the Evaporative Light Scattering Detector (ELSD). However, non-reproducible spikes were reported when injecting concentrated test solutions as they are usually required for the control of impurities. The reasons, for the appearance of these spikes as well as possibilities for their avoidance were explored in a systematic study. Moreover, the dependence of the detector sensitivity on different eluent composition, eluent flow-rate and ELSD settings was investigated. Unfortunately, most parameters increasing the method sensitivity also foster the appearance of spike peaks eluting on the tail of the principal peak, which make the use of the ELSD for the control of impurities problematic.

In the course of the revision of the Ph.Eur. monographs for aspartic acid and alanine, a C18 reversed phase ion-pair LC method using 1 mmol/L of perfluoroheptanoic acid as an ion-pair reagent and a charged aerosol detector (CAD) was developed and fully validated for the purity control of Asp. The method was capable of separating the organic acids and major amino acids known to occur as process related impurities. With a slight modification, the method was also applicable for the purity control of Ala. The limits of quantification for the potential impurities were found between 0.006% and 0.03% (referred to the concentration of a 10 mg/mL test solution), allowing the control of impurities on an ICH [25] conform level for drug substances with an average daily dose above 2 g. The developed method represents an easy to

use alternative to amino acid analysis for the control of impurities in Asp and Ala. It has the additional benefit of controlling not only related amino acids but also other process impurities like organic acids.

Based on the developed LC-CAD method for the impurity control of alanine, a comparative study of the performance characteristics of different evaporation based LC detectors, i.e. ELSD, CAD and the recently developed Nano Quantity Analyte Detector (NQAD) was carried out. Additionally, an MS detector and qNMR were included in this study. It was found that the control of impurities in Alanine at an ICH conform level could be ensured using LC coupled to CAD, MSD and NQAD detection as well as by the use of qNMR. In terms of performance, prize and ease of use CAD and NQAD were found to be the most suitable alternatives. In terms of repeatability and sensitivity, the CAD appeared slightly superior to the NQAD.

The quality of streptomycin sulfate is not sufficiently controlled by the current Ph.Eur. monograph in that an appropriate test for the control of the related substances is missing. A study was carried out to develop a C18 reversed phase ion-pair LC method using pentafluoropropionic acid as an ion-pair reagent and a CAD for the identification and control of the related substances. The developed method allowed the separation of 21 impurities from streptomycin. Moreover, coupling of the method to MS allowed the identification of the separated impurities. The method was shown to be sufficiently sensitive to control the related substances with a disregard limit of 0.1% as it is normally applied in the Ph.Eur. for products derived from fermentation. The analysis of 12 sample of streptomycin sulfate from different manufacturers resulted in a total amount of impurities between 4.6% and 16.0%. This method is considered to be a viable option to improve the related substances control of streptomycin sulfate.

Currently, the aescin content of horse-chestnut standardized dry extract is determined using a complex and laborious photometric determination. A more selective LC-UV assay determination for β-aescin has been proposed for the Ph.Eur. draft monograph of horse-chestnut standardized dry extract. Possibilities were explored to further improve the LC-method using detection by CAD. It was demonstrated that by the use of a modified LC-CAD method several problems related to the differences in the UV-response of the various components contained in the active aescin fraction could be eliminated. Moreover the proposed reference

standard strategy was reviewed. It could be demonstrated that the current proposal of using aescin as a reference standard is problematic. A chemically well defined single component like protoaescigenin was identified to be a more suitable alternative.

Eventually, it was demonstrated on the example of two different clusters of pharmacologically active peptides how low energy collision induced dissociation mass spectrometry (low energy CID-MS) can successfully be used for identification testing in pharmacopoeial monographs. In this respect, the combination of a direct confirmation of the molecular mass via the m/z-ratio of the molecule ions with structural sequence information obtained by low energy CID-MS experiments was found to deliver a higher degree of certainty of the identity of a given substance than the set of tests currently described in the monographs. A significant gain in efficiency and throughput and important reduction of the amount of sample consumed during testing were identified as being additional advantages of this approach.

Taken together, it could be demonstrated on various examples how recent technological advancements in the field of analytical chemistry can contribute to improve the quality control of APIs. In the work reported here, special emphasis was put on the use of the CAD and several concrete proposals for the improvement of monographs were made. Moreover, it was demonstrated how other modern detectors and analytical techniques like the NQAD, MS and qNMR can successfully be employed to maintain state-of-the-art monographs and therefore to retain the leading role of the European Pharmacopoeia in securing an appropriate quality of medicines in Europe and worldwide.

6. <u>ZUSAMMENFASSUNG</u>

Eine angemessene Qualitätskontrolle pharmazeutischer Wirkstoffe ist eines der wesentlichen Elemente für die sichere Anwendung von Arzneimitteln sowie für den Schutz der Gesundheit der Verbraucher. Das Europäische Arzneibuch mit seinen generellen Texten, allgemeinen Monografien und individuellen Wirkstoffmonografien stellt den rechtlich verbindlichen Mindeststandard bezüglich der Identifizierung, Reinheitsprüfung und Gehaltsbestimmung von pharmazeutischen Wirk- und Hilfsstoffen in Europa dar.

Die hier präsentierten Untersuchungen hatten zum Ziel, das Potential von evaporationsbasierten HPLC-Detektoren sowie weiterer moderner Techniken hinsichtlich ihrer Eignung zur Reinheitsprüfung von pharmazeutischen Wirkstoffen zu untersuchen. Im Zuge der Arbeiten wurden verschiedene neue Methoden zur Identifizierung, Verunreinigungskontrolle und zur Überprüfung der Zusammensetzung von pharmazeutischen Wirkstoffen entwickelt.

Ein evaporationsbasierter Detektor, der in den letzten Jahren Einzug in die pharmazeutische Analytik gehalten hat, ist der Lichtstreudetektor (ELSD). Allerdings wurde berichtet, dass es im Zusammenhang mit der Injektion konzentrierter Testlösungen zum Auftreten nicht reproduzierbarer Spikes kam. In einer systematischen Studie wurden die Gründe hierfür ermittelt und gleichzeitig Möglichkeiten ihrer Vermeidung aufgezeigt. Darüberhinaus wurde die Abhängigkeit der Empfindlichkeit des Detektors von der Zusammensetzung der mobilen Phase, der LC-Flußrate und den Einstellungen des ELSD untersucht. Unglücklicherweise förderten die meisten Parameter, die die Detektorempfindlichkeit steigerten, gleichzeitig auch das Auftreten von Spike-Peaks in Folge des Hauptsignals. Diesbezüglich wird der Einsatz des ELSD zur Kontrolle von Verunreinigungen als kritisch angesehen.

Im Zuge der Revision der Monografien für Asparaginsäure und Alanin wurde eine C18-Ionenpaar-HPLC-Methode unter Verwendung von 1 mmol/L Perfluorheptansäure und Detektion mittels eines geladenen Sprühnebeldetektors (Charged Aerosol Detector – CAD) für die Reinheitskontrolle von Asparaginsäure entwickelt und validiert. Mit Hilfe dieser Methode konnten sowohl organische Säuren als auch die wesentlichen Aminosäuren, die als prozessrelevante Verunreinigungen bekannt sind, abgetrennt werden. Nach geringfügiger Modifikation konnte diese Methode auch zur

Reiheitskontrolle von Alanin eingesetzt werden. Bezogen auf eine 10 mg/mL Testlösung wurden Bestimmungsgrenzen für die verschiedenen Verunreinigungen zwischen 0.006% und 0.03% erreicht. Somit war die Methode geeignet, eine Reinheitsprüfung auf einem Niveau zu gewährleisten, welches mit der ICH-Richtlinie für pharmazeutische Wirkstoffe [25] mit einer täglichen Dosis von mehr als 2 g im Einklang steht. Die entwickelten Methoden stellen hinsichtlich der Reinheitsprüfung von Asparaginsäure und Alanin eine leicht anwendbare Alternative zur klassisichen Aminosäure-Analyse dar. Ein weiterer Vorteil ist, dass sie nicht auf die Kontrolle von verwandten Aminosäuren beschränkt sind, sondern auch andere prozessrelevante Verunreinigungen wie organische Säuren erfassen.

Basierend auf der HPLC-CAD-Methode für Alanin wurde eine vergleichende Studie der Leistungsfähigkeit verschiedener evaporationsbasierter HPLC-Detektoren durchgeführt. Untersucht wurden der ELSD, der CAD und der kürzlich entwickelte „Nano Quantity Analyte Detektor" (NQAD). Weiterhin wurden ein massenspektrometrischer Detektor (MSD) sowie die Quantifizierung mittels NMR-Spektroskopie (qNMR) in die Untersuchung einbezogen. Im Ergebnis war die Reinheitskontrolle von Alanin auf einem ICH konformen Niveau unter Verwendung des CAD, NQAD und MSD sowie mittels qNMR möglich. Hinsichtlich der Kriterien Leistungsfähigkeit, Preis und Benutzerfreundlichkeit waren der CAD und der NQAD die geeignetsten Alternativen. Bezüglich Wiederholbarkeit und Empfindlichkeit war der CAD dem NQAD leicht überlegen.

Die Qualität von Streptomycinsulfat wird von der aktuellen Arzneibuchmonografie mangels eines geeigneten Reinheitstests nicht ausreichend kontrolliert. Aus diesem Grund wurde eine C18-Ionenpaar-HPLC-Methode unter Verwendung von Perfluorpropionsäure und Detektion mittels CAD zur Identifizierung und Kontrolle der Verunreinigungen entwickelt. Mit dieser Methode konnten 21 Verunreinigungen von Streptomycin abgetrennt und nach massenspektormetrischer Kopplung identifiziert werden. Die Methode erwies sich als ausreichend empfindlich, um Verunreinigungen mit einer Ausschlussgrenze von 0.1%, wie sie im Europäischen Arzneibuch für Fermentationsprodukte üblicherweise angewandt wird, zu kontrollieren. Die Analyse von 12 Streptomycinsulfat-Proben verschiedener Hersteller ergab Gehalte an Verunreinigungen zwischen 4.6 und 16.0 %. Nach hiesiger Einschätzung ist die entwickelte Methode für die Reinheitsprüfung in der Streptomycinsulfat-Monografie geeignet.

Derzeit wird der Aescingehalt in standardisiertem Rosskastanien-Trockenextrakt mittels einer komplexen photometrischen Methode bestimmt. Für den entsprechen-den Entwurf einer Monografie des Europäischen Arzneibuchs wurde eine selektivere HPLC-UV-Methode vorgeschlagen. Die Möglichkeiten einer weiteren Verbesserung dieser Methode unter Verwendung des CAD wurden untersucht. Es konnte gezeigt werden, dass eine modifizierte HPLC-CAD-Methode geeignet ist, Probleme, die sich aus der unterschiedlichen UV-Absorption der im Aescingemisch enthaltenen Substanzen ergeben, zu eliminieren. Weiterhin wurde die vorgeschlagene Referenz-standard-Strategie überprüft. Es konnte gezeigt werden, dass die Verwendung von Aescin als Referenzsubstanz problematisch ist. Eine chemisch eindeutig definierte Einzelsubstanz wie Protoaescigenin wurde als besser geeignete Alternative identifiziert.

Abschließend wurde am Beispiel von zwei Gruppen pharmakologisch wirksamer Peptide nachgewiesen, wie kollisionsinduzierte Massenspektrometrie (CID-MS) erfolgreich für die Identitätskontrolle in Arzneibuchmongrafien eingesetzt werden kann. Diesbezüglich erbrachte die Kombination der direkten Massenbestätigung mittels des m/z-Verhältnisses des Molekül-Ions mit den aus dem CID-MS-Experiment erhaltenen Informationen ein höheres Maß an Gewissheit hinsichtlich der Identitätsbestätigung als die derzeit beschriebenen Tests. Als weitere Vorteile dieses Ansatzes wurden ein wesentlicher Effizienzgewinn sowie eine erhebliche Reduktion des durch die Testung verbrauchten Probenmaterials identifiziert.

Zusammenfassend zeigen die Arbeiten an verschiedenen Beispielen, wie aktuelle Entwicklungen im Bereich der analytischen Chemie dazu beitragen können, die Qualitätskontrolle von Arzneimittelwirkstoffen zu verbessern. Das Hauptaugenmerk in dieser Arbeit wurde auf mögliche Anwendungen der CAD gerichtet. In dieser Hinsicht wurden verschiedene konkrete Vorschläge für die Ver-besserung von Arzneibuchmonografien gemacht. Darüberhinaus wurde gezeigt, wie andere moderne Detektoren und Techniken wie der NQAD, MS und qNMR erfolgreich eingesetzt werden können, um dem Stand der Technik entsprechende Monografien zu gewährleisten und somit die führende Rolle des Europäischen Arzneibuchs in der Sicherung einer angemessenen Arzneimitelqualität in Europa und weltweit aufrecht zu erhalten.

7. <u>MATERIAL AND METHODS</u>

7.1 <u>Suitability of ELSD for the impurities control – analysis of the performance characteristics and appearance of spike peaks</u>

7.1.1 Reagents and Chemicals
Water was delivered by an ELGA PureLab Ultra system (Elga Antony, France). Diluted hydrochloric acid (0.37 g/L) was prepared starting from hydrochloric acid 37% p.a. (Merck, Darmstadt, Germany). Methanol puriss. p.a., acetonitrile puriss. p.a., and L-aspartic acid (Asp) +99% were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), mexiletine hydrochloride was provided by ICN (Budapest, Hungary), and α-cyclodextrin (Alfadex) by Roquette (Lestrem, France). Nitrogen +99% was delivered by a Peak Systems NM18LA nitrogen generator (Lab Gaz Systems, Massy, France).

7.1.2 Equipment
A Waters Alliance Separation Module 2695 consisting of autosampler, injector and quarternary pump (St. Quentin-en-Yvelines, France) equipped with Waters Empower Pro data processing software was used for flow injection analysis. Detection was performed by a Polymer Laboratories PL-ELS 2100 Evaporative Light Scattering Detector (Marseille France) using nitrogen as scavenger gas. All analyses were run with a peek-tube 1/32'' restriction coil having an internal diameter of 125 µm and a length of 19 cm (Interchim, Montlucon, France).

7.1.3 Methods
In 4 sets of flow injection analysis (FIA) experiments, the influence of the subsequent eluent composition and flow and ELSD parameters on the appearance of spike peaks was examined. The experiments were carried out using as test solutions 1) 40 mg/mL Asp (prepared by dissolution of about 400 mg Asp in 2 mL of dilute hydrochloric acid (0.37 g/L) and dilution to 10.0 mL with water), 2) 40 mg/mL mexiletine HCl in water/methanol (50/50 v/v), and 3) 40 mg/mL alfadex in water. For a sensitivity check, 1.0 mL of the test solutions was diluted to 100.0 mL with water. 1.0 mL of this solution was further diluted to 20.0 mL with water (conc. 0.05%). In all experiments an injection volume of 40 µL and an ELSD detector gain of 1.0 was

employed. To ensure that all possible spike peaks elute within the run-time and do not influence the subsequent injection, a run-time of 10 min. was selected for the concentrated solutions. For the diluted solutions (0.05%) a run-time of 1.5 min was considered sufficient.

Table 7-1 gives an overview of the different eluent composition, eluent flow and ELSD parameters used in the experiments:

Table 7-1. Overview of the applied experimental conditions.

(A)

7.1.3.1) Variation of the eluent composition					
	Eluent composition	**Eluent flow rate**	**ELSD scavenger gas flow rate**	**ELSD drift tube temp.**	**ELSD nebul. temp.**
Test solutions: 1) and 2)					
a)	water/acetonitrile 90/10 v/v to 20/80 v/v modified in 10% steps	0.5 mL/min	1.0 standard litr per minute (SLM)	50 °C	50 °C
b)	water/methanol 90/10 v/v to 20/80 v/v modified in 10% steps.	0.5 mL/min	1.0 SLM	50 °C	50 °C

(B)

7.1.3.2) Variation of the eluent flow rate					
	Eluent composition	**Eluent flow rate**	**ELSD scavenger gas flow rate**	**ELSD drift tube temp.**	**ELSD nebul. temp.**
Test solutions: 1) and 3); Test solution 2 only under conditions a)					
a)	water/methanol (80/20 v/v)	0.5; 0.7; 0.8; 1.0; 1.2; 1.5 mL/min	1.0 SLM	50 °C	50 °C
b)	water/acetonitrile (90/10 v/v)	see 7.1.3.2 a) + 2.0 mL/min	1.0 SLM	50 °C	50 °C

(C)

7.1.3.3) Variation of the ELSD scavenger gas-flow rate					
Test solutions: 1) and 3)					
a)	water/methanol (80/20 v/v)	0.5; 0.7; 0.8; 1.0; 1.2; 1.5 mL/min	0.9; 1.0; 1.1; 1.2; 1.4 SLM	50 °C	50 °C
Test solution 2)					
b)	water/methanol (50/50 v/v)	see 7.1.3.3 a)	0.9; 1.0; 1.1; 1.2, 1.4; 1.8; 2.2 SLM	50 °C	50 °C
c)	water/methanol (20/80 v/v)	see 7.1.3.3 a)	see 7.1.3.3 b)	50 °C	50 °C

(D)

7.1.3.4) Variation of the nebulizer and evaporation (drift tube) temperature					
Test solution: 1)					
a)	water/methanol (80/20 v/v)	0.5 mL/min	1.0 SLM	40; 50; 60; 70 °C	
b)	water/methanol (80/20 v/v)	0.5 mL/min	1.0 SLM	40; 50; 60; 70; 80 °C	30 °C

7.2 Control of impurities in L-aspartic acid and L-alanine by high-performance liquid chromatography coupled with a corona charged-aerosol detector

7.2.1 Reagents and Chemicals

Water was delivered by an ELGA PureLab Ultra system (Elga Antony, France). Acetonitrile for chromatography, isopropanol p.a., methanol puriss. p.a., heptafluorobutyric acid (HFBA) 98% and perfluoroheptanoic acid (PFHA) 99% were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The organic acids, L-glutamic acid, and L-glutamine were of 99% purity. For citric acid and L-asparagine the monohydrates were used. The reagents were either supplied by

Sigma-Aldrich (St-Quentin Fallavier, France), Fluka (St-Quentin Fallavier, France) or Acros (Noisy le Grand, France).

Test samples of aspartic acid and alanine were kindly provided by Merck (Darmstadt, Germany), Kyowa Hakko (Tokyo, Japan), Degussa Rexim (Radebeul, Germany), Ajinomoto (Leuven, Belgium), Amino GmbH (Frellstedt, Germany), and Shanghai Kyowa (Shanghai, China). Reagent grade standards of the two amino acids were purchased from Sigma, Aldrich, and Fluka (St-Quentin Fallavier, France).

Hydrogen peroxide 30% was supplied by Merck (Darmstadt, Germany). Nitrogen +99% was delivered by a Peak Systems NM18LA nitrogen generator (Lab Gaz Systems, Massy, France).

7.2.2 Equipment

A Waters Alliance Separation Module 2695 including thermostated autosampler, quarternary pump and column oven (St. Quentin-en-Yvelines, France) equipped with Waters Empower Pro data processing software was used for liquid chromatography.

Detection was performed by a Corona CAD Detector (ESA Bioscience INC. - Vendor: Eurosep Instruments Cergy Pontoise France).

Evaporative light scattering detection was performed using a Polymer Laboratories PL-ELS 2100 Evaporative Light Scattering Detector (Marseille, France). The Inertsil ODS 3, Synergi Hydro RP 18, Kromasil C18 and YMC Pack Pro C8 columns were purchased from Interchim (Montlucon, France), the Zorbax Eclipse XDB C18 column from Agilent (Waldbronn, Germany), the Symmetry C18 colum from Waters (St. Quentin-en-Yvelines, France), and the Purospher RP 18 column from Merck (Darmstadt, Germany).

LC-MS analysis was performed using an Agilent 6400 Series Triple Quadrupole LC/MS (Waldbronn, Germany) and an Acquity UPLC BEH C8 column supplied by Waters (St. Quentin-en-Yvelines, France).

7.2.3 Methods
7.2.3.1 HFBA Method development

For the tests performed with HFBA as ion-pair reagent, a Synergi Hydro RP18 column (150 mm x 4.6 mm, particle size 4 µm) was employed at a flow rate of 0.8 mL/min and a column temperature of 30 °C. The injection volume was 40 µL. The concentration of the different compounds in the aqueous mixture injected was: malic

acid, maleic acid and succinic acid at 0.02 mg/mL, citric acid, fumaric acid, Asp, Asn, Ala, Glu and Gln at 0.01 mg/mL.

An overview of the different mobile phase tested is given in Table 7-2.

Table 7-2 - Overview of HFBA mobiles phases tested – percentage values given in %-v/v.

Mobile Phase A – HFBA in water (v/v) :	Mobile Phase B - HFBA in methanol (v/v):	Composition of eluent A/B (v/v):
0.2%	0.2%	98/2; 95/5; 90/10; 85/15; 80/20; 70/30
0.5%	0.5%	98/2; 95/5; 90/10; 85/15; 80/20
0.4%	0.4%	98/2; 95/5; 90/10; 85/15; 80/20; 70/30
0.5%	0.3%	90/10; 85/15; 80/20
0.8%	0.8%	90/10; 85/15; 80/20

7.2.3.2 PFHA Method development

A first series of trials was performed using a mobile phase containing 10 mmol/L (3.64 g/L) of PFHA in methanol / water. A Synergi Hydro RP 18 column (250 mm x 4.6 mm, particle size 5 µm) was used at a flow rate of 0.8 mL/min and detection by CAD. 40 µL of a 5 mg/mL solution of Asp spiked with 0.2% of malic acid, maleic acid and succinic acid, citric acid, fumaric acid, Asn, Ala, Glu and Gln was injected. The amount of the organic modifier and the column temperature was varied as given in Table 7.3 to find best separation conditions.

Table 7-3. Overview of 10 mmol/L PFHA mobiles phases tested – percentage values given in % (v/v).

Mobile Phase A – 10 mmol/L of PFHA in water :	Mobile Phase B – 10 mmol/L of HBFA in methanol:	Column temperatures
98%	2%	20 °C; 40 °C
95%	5%	20 °C; 40 °C
90%	10%	20 °C; 40 °C; 50 °C; 60 °C
85%	15%	20 °C; 30 °C; 40 °C; 50 °C; 60 °C
80%	20%	20 °C; 30 °C; 40 °C; 50 °C; 60 °C

Thereafter, the amount of PFHA was gradually reduced to 5 mmol/L, 3 mmol/L, 1 mmol/L and 0.2 mmol/L in a mobile phase consisting of water/methanol 95/5 v/v. Eventually, the amount of methanol in a mobile phase containing 1 mmol/L of PFHA was varied between 1% v/v and 10% v/v.

The best separation was obtained with an Inertsil ODS 3 column (150 mm x 4.6 mm; particle size 5 µm) at a column temperature of 30 °C. The mobile phase was composed of 1.0 mmol/L of PFHA in water/methanol 96/4 (v/v). Prior to use, the column was flushed over night at a flow rate of 0.2 mL/min using methanol/water (50/50 v/v). Thereafter the column was conditioned for about 3 h using the mobile phase.

7.2.3.3 Method validation

The following method was validated for its use as a related substances method for Asp produced by enzymatic synthesis:

Column: Inertsil ODS 3 (150 mm x 4.6 mm; particle size 5 µm)

Column temperature: 30 °C

Column equilibration: condition the column at a flow rate of 0.2 mL/min until a stable level of baseline noise is obtained.

Mobile phase: 1 mmol/L of PFHA in water / methanol 96/4 (v/v).

Flow-rate: 1.0 mL/min

Injection volume: 40 µL

Run-time: 6 times the retention time of the peak due to L-Asp

Detection: Charged Aerosol Detectoin (gas pressure of 35 psi in the 100 pA
                      detection range).

Unadjusted relative retention with reference to Asp (retention time = about 4.4 min): malic acid = about 0.5, fumaric acid = about 0.7, succinic acid = about 0.9, Gln = about 1.7, Glu = about 2.3, Ala = about 3.0.

Test solution: 500 mg of Asp dissolved in 50 mL of water (heat to 60 °C and stir until complete dissolution).

Inject freshly prepared solutions (the solution is stable for about 4-6h before re-precipitation occurs).

Reference solution (a): Dissolve 20 mg of malic acid, 20 mg of fumaric acid, 100 mg of succinic acid, 20 mg of Asp, 20 mg of Gln, 20 mg of Glu and 20 mg of Ala in water an dilute to 20.0 ml with the same solvent (sonicate if necessary).

Reference solution (b): Dilute 1.0 mL of reference solution (a) to 100.0 mL with water. (0.1%).

Reference solution (c): To 5 mL of the test solution add 100 µL of reference solution (a) and dilute to 10.0 mL with the test solution.

System suitability test: using reference solution (c):

> *Resolution:* minimum 2.0 between the peaks due to fumaric acid and succinic acid.

> *Peak-to-valley ratio* : minimum 1.2, where H_p = height above the baseline of the peak due to Gln and H_v = height above the baseline of the lowest point of the curve separating this peak from the peak due to Asp.

Quantification:

> *Malic acid:* using the peak due to malic acid in ref. sol. (b).

> *Fumaric acid:* using the peak due to fumaric acid in ref. sol. (b).

> *Glutamic acid:* using the peak due to glutamic acid in ref. sol. (b).

> *Alanine:* using the peak due to alanine in ref. sol. (b).

> *Unspecified impurities:*

> *Eluting before Asp:* using the peak due to malic acid in ref. sol. (b).

> *Eluting after Asp:* using the peak due to aspartic acid in ref. sol. (b).

Specificity:

The method specificity was verified using an 10 mg/mL aqueous solution of Asp spiked with about 0.1 % of maleic acid, malic acid, citric acid, fumaric acid, succinic acid, Gln, Glu and Ala under the above described chromatographic conditions.

For the investigation of the nature of the peak eluting at about 31 min. in the blank, the LC was coupled to the TOF-MS detector using a 1/10 T-split after the column. The TOF-MS was operated in the ESI positive mode.

Linearity and range:

The verification of the linearity was determined using solutions of 0.025%; 0.05%; 0.10%; 0.14%; 0.20%; 0.25%; 0.50%; and 1.0% (referred to a 10 mg/mL Asp test solution), performed on a mixture containing maleic acid, malic acid, fumaric acid, Asp, Gln, Glu and Ala (average of 3 injections per concentration).

Accuracy:

The accuracy was assessed using a 10 mg/mL aqueous solution of Asp spiked with 0.05%; 0.1%; 0.15%; and 0.20% of maleic acid, malic acid, fumaric acid, Gln, Glu and Ala.

Repeatability:

The repeatability was assessed using a 10 mg/mL aqueous solution of Asp spiked at 5 concentration levels; i.e. 0.0025 mg/mL, 0.005 mg/mL, 0.01 mg/mL, 0.015 mg/mL and 0.020 mg/mL with maleic acid, malic acid, citric acid, fumaric acid, succinic acid, Gln, Glu and Ala. For Asp the determination was performed on 3 concentration levels; i.e. 0.0025 mg/mL; 0.01 mg/mL; 0.02 mg/mL. On each level 3 replicate injections were performed.

LoD / LoQ:

The limit of detection (based on a signal-to-noise ratio of 3) and the limit of quantification (based on a signal-to-noise ratio of 10:1) was determined on the average of three injections using 0.0025 mg/mL solutions of the aforementioned compounds.

Stability of solution:

The stability of solution was verified employing a spiked Asp test solution (cf. Repeatability). The solution was injected in triplicate immediately after preparation as well as after 4h, 8h, 12h, 16h and 24h. The overall relative standard deviation of the recovery of the different components, taking into account all results at the different time points until 12h respectively 24h, was determined. For malic acid an additional measurement was performed after 48 h. For the determination of the overall rsd of Asp, a 0.01 mg/mL solution was employed.

Influence of the LC flow-rate:

The impact of the LC flow-rate was studied from 0.6 mL/min to 1.2 mL/min in steps of 0.2 mL/min using a spiked Asp test solution (cf. Repeatability).

Influence of the column temperature:

The influence of the column temperature was examined injecting a spiked Asp test solution (cf. Repeatability) at 15 °C, 30 °C, 35 °C, and 40 °C at an LC flow-rate of 0.8 mL/min.

Effect of the concentration of the ion-pair reagent:

The influence of different concentrations of the ion-pair reagent (PFHA) was analyzed injecting a spiked Asp test solution (cf. Repeatability) using mobile phases containing 0.5 mmol/L; 0.8 mmol/L; 1.0 mmol/L; 1.2 mmol/L; and 1.5 mmol/L of PFHA.

Modification of the concentration of organic modifier in the mobile phase:

The effect of different amounts of methanol in the mobile phase was studied injecting a spiked Asp test solution (cf. Repeatability) with mobile phases containing 1%, 2%, 3%, 4%, 5%, 6%, and 7% of methanol.

Selection of the stationary phase:

The following stationary phases were tested: Inertsil ODS 3 (150 mm x 4.6 mm, 5 µm); Zorbax Eclipse XDB-C18 (150 mm x 4.6 mm, 5 µm); Symmetry C18 (150 mm x 3.9 mm, 5 µm); Purospher RP-18e (250 mm x 4.0 mm, 5 µm); YMC Pack PR 18 (150 mm x 4.6 mm, 5 µm); Synergi Hydro RP C18 (150 mm x 4.6 mm, 4 µm); Kromasil C18 (150 mm x 4.0 mm, 5 µm); YMC Pack PR 8 (150 mm x 4.6 mm, 5 µm).

All columns were flushed with the mobile phase at a flow rate of 0.2 mL/min for 12h before use. The baseline noise of a blank injection (water) was determined as average peak-to-peak noise. Moreover, the signal-to-noise ratio (S/N-ratio) obtained from a 0.01 mg/mL solution of succinic acid (corresponding to 0.1%, referred to an Asp test solution of 10 mg/mL) was calculated.

Forced degradation experiments:

Unless otherwise specified, a 10 mg/mL aqueous test solution of Asp (containing 0.066% of malic acid and 0.008% of fumaric acid) was used. The test solution was exposed to the following conditions. Thereafter, the amount of impurities was determined using the validated method:

- Exposure to UV light at 254 nm and 366 nm for 18 h.

- Heating to 100 °C in a water bath for 4h.

- 500 mg of Asp were mixed with 40 mL of water after addition of hydrogen peroxide 30% v/v the solution was stirred and heated to 60 °C for 4h. To obtain complete dissolution, the solution was further heated to 80 °C for 10 min and injected.

For the analysis of the unknown impurities occurring after heating, the LC was coupled to TOF-MS detection using a post-column split of the mobile phase of 1/10. The TOF-MS detector was operated in the ESI negative mode.

7.2.3.4 Alanine

For the purity testing of Ala the above method was slightly modified: the concentration of PFHA was increased to 1.5 mmol/L. This was found to be necessary to obtain resolution of Ala and Glu at high concentrations of Ala. For peak identification and quantification the same solution as described under 7.2.3.3 was employed.

7.2.3.5 LC-MS/MS quantification of Asn

Quantification of Asn in 5 batches of pharmaceutical grade Asp was performed using the standard addition method spiking a sample containing 1 mg/mL of Asp with 100 – 200 – 300 ppm of Asn.

LC was performed injecting in triplicate 5 µL of the above solutions on a Waters Acquity UPLC BEH C8 column (100 mm x 2.1 mm, 1.7 µm). The column temperature was set to 50 °C. A mobile phase containing 1.0 mmol/L PFHA in water was used at a flow rate of 0.3 mL/min. The quantification was performed using the QqQ-MS detector with electrospray positive ionization in the multi-reaction-mode (MRM). As a precursor ion for Asn a m/z of 133.1 was selected. The quantification was performed using the m/z = 87.0 product ion. The m/z = 44.1 ion was used as qualifier. The source parameters were as follows: gas temperature: 300 °C, nitrogen gas flow: 12 L/min, nebulizer gas pressure: 45 psi, capillary voltage: 4500 V, fragmentor voltage: 70 V, collision energy: 16 V for m/z = 44.1 and 4 V for m/z = 87.0.

7.3 <u>Comparison of different detection systems for the purity control of L-Alanine</u>

7.3.1 Reagents and Chemicals

Water was delivered by an ELGA PureLab Ultra system (Elga Antony, France). Methanol puriss. p.a. and perfluoroheptanoic acid (PFHA) 99% were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The organic acids, Asp and Glu were of 99% purity. The reagents were either supplied by Sigma-Aldrich (St-Quentin Fallavier, France), Fluka (St-Quentin Fallavier, France), or Acros (Noisy le Grand, France). Alanine used for the preparation of the spiked test sample was supplied by Degussa Rexim (Nanning, China). Nitrogen + 99% was delivered by a Peak Systems NM18LA or NM30LA nitrogen generator (Lab Gaz Systems, Massy, France). Nitrogen N 50 used as MS/MS-collision gas came from Air Liquide (Illkirch, France). For the water determination Riedel-de Haen Hydranal Coulomat AG supplied by Sigma-Aldrich (Seelze, Germany) was used as a sample solvent.

The Inertsil ODS 3 column (Varian, CA, USA) used for HPLC-CAD/NQAD/ELSD method was purchased from Interchim (Montlucon, France). For HPLC-MSD an Agilent Zorbax Eclipse XDB C18 column (Waldbronn, Germany) was used.

Deuterated water (D_2O, 99.9 atom % D) was purchased from Euriso-top (Saarbrücken, Germany).

7.3.2 Equipment

A Waters Alliance Separation Module 2695 including thermostated autosampler, quarternary pump and column oven (St. Quentin-en-Yvelines, France) equipped with Waters Empower Pro data processing software was used for liquid chromatography coupled with ELSD, CAD and NQAD. Detection by CAD was performed by means of a Corona CAD (ESA Bioscience INC. - Vendor: Eurosep Instruments, Cergy Pontoise, France).

Evaporative light scattering detection was performed using a Polymer Laboratories PL-ELS 2100 Evaporative Light Scattering Detector (Marseille, France). The Quant NQAD Analytical Detector was kindly provided by Grace Davison Discovery Sciences (Templemars, France). For LC/MSD analysis an Agilent 1200 Series Rapid Resolution HPLC system coupled with an Agilent 6400 Series Triple Quadrupole LC/MSD (Waldbronn, Germany) was employed. ^{1}H NMR spectra were recorded on a

Bruker Avance 400 MHz and Bruker Avance 600 MHz spectrometer operating at 400.13 MHz and 600.57 MHz, respectively. All data were processed using Bruker's Topspin-NMR software version 2.1 (Bruker Analytik, Rheinstetten, Germany). All NMR samples were measured in Wilmad 5 mm od 527-PP-7 NMR tubes (Biebesheim, Germany).

The spiked test sample of Ala was produced using a Büchi Mini Spray Dryer B-290 with Inert-Loop B295 (Flawil, Switzerland). Coulometric water determination was performed using a Methrom 756 KF coulometer (Courtaboeuf, France). The loss on drying was determined using a Memmert drying oven UNE 400 (Schwabach, Germany).

7.3.3 Methods

7.3.3.1 Production of spiked Ala sample by spray drying

19.94 g of Ala, 91.3 mg (0.46%) malic acid, 81.9 mg (0.41%) fumaric acid, 76.8 mg (0.39%) Asp, and 195.6 mg (0.98%) Glu were dissolved in 200 mL of water and spray-dried using a Büchi Mini Spray Dryer B-290 with Inert-Loop B295. The spray-dryer parameters were as follows: peristaltic pump: 60%, aspiration 100%, inlet temperature 175 °C, gas-flow: 35 L nitrogen/min, outlet temperature 60-70 °C. After homogenization, a triplicate determination of water by coulometry was performed using 25 mg of the substance to be examined, directly introduced into the titration vessel. Moreover, the loss on drying was determined in a 6-fold determination drying 100 mg for the substance at 105 °C for 4 h. The combined average value of both determinations of 0.6% was considered negligible for the determination of the impurity content.

7.3.3.2 Purity determination of Ala by LC-CAD / NQAD / ELSD

LC-method: The separation was performed on an Inertsil ODS 3 column (150 mm x 4.6 mm; particle size 5 µm) at a column temperature of 30 °C. A mixture containing 1.5 mmol/L of PFHA in water / methanol (96/4 v/v) was used as mobile phase. The method run-time was set to 35 min applying a mobile phase flow rate of 1.0 mL/min. The injection volume was 40 µL. For the purity testing a 10 mg/mL solution of Ala in water was used.

Detector settings: 1) CAD-detection was performed using a gas pressure of 35 psi in the 100 pA detection range. 2) The NQAD detector was used with a nitrogen gas-

pressure of 40 psi, evaporation of the HPLC mobile phase at ambient temperature and a detector gain of 20. In order to increase the repeatability of injection, the linearization function was set to "off". 3) For detection by ELSD a nebulizer and drift tube temperature of 50 °C and a detector gas-flow of 1.0 L/min were found to be most suitable.

The quantification by NQAD was performed using aqueous dilutions of malic acid, fumaric acid, Asp, Glu and Ala corresponding to 0.1% of the concentration of the alanine test solution as external standards. For Glu, which was present in the spiked sample at about 1%, the result was calculated taking into account a linear calibration curve from 0.1% to 2.0%. For the CAD, an external standard solution containing 0.38% of malic acid and Asp, 0.48% of fumaric acid and 1.2% of Glu was employed. For ELSD, quantification was performed using double-logarithmic calibration curves.

7.3.3.3 Purity determination of Ala by LC-MSD

The LC separation was performed at 30 °C on a Zorbax Eclipse XDB C18 column (50 mm x 4.6 mm; particle size: 1.8 µm) using the mobile phase described under 6.3.3.2 at a flow rate of 0.5 mL/min for 7 min. The injection volume was 3 µL. Under these conditions, the peak due to alanine eluted at about 6.2 min.

MS detection was performed using electrospray ionization (ESI) in the negative mode. The source parameters were as follows: gas temperature: 290 °C, nitrogen gas flow: 12 L/min, nebulizer gas pressure: 45 psi, capillary voltage: 4500 V. From 0 to 1.8 min the dwell time was 200 msec, with a fragmentor voltage of 50 V to detect fumaric acid and malic acid at m/z = 115 in MS2-SIM mode. From 1.8 to 7.0 min the dwell time was set to 100 msec with a fragmentor voltage of 70 V to detect aspartic acid and glutamic acid at m/z = 132 and m/z = 146, respectively, in MS2-SIM mode.

7.3.3.4 Purity determination of Ala by ^{1}H NMR spectroscopy

Sample Preparation: For quantification of the potential impurities an amount of 50 mg of the Ala batch analyzed was dissolved in 750 µL D_2O and constantly shaken for several minutes. Thereof a standardized sample volume of 700 µL was transferred to a 5 mm NMR tube.

NMR-method. For all ^{1}H NMR spectra (400 MHz), 128 scans were collected into 64K data points over a spectral width of 5592.84 Hz (14 ppm) with the transmitter offset at 4.00 ppm, yielding a digital resolution of 0.17 Hz per point. The acquisition time was

5.86 s, followed by a relaxation delay of 39 s, resulting in a total pulse recycle time of 44.86 s to ensure full T_1 relaxation of the protons of Ala and its impurities. The spectra were recorded at 300 K using a flip angle of 90° and the chemical shifts were reported to trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP-d_4) as external standard at 0.00 ppm using a special tube containing 1% TSP-d_4 in D_2O (Bruker Analytik, Rheinstetten, Germany). All experiments were run in a non-spinning mode to avoid rotational sidebands. An exponential line broadening window function of 0.3 Hz was used in the data processing. After Fourier transformation of the free induction decays, the NMR spectra were phased, baseline corrected, and manually integrated in the appropriate regions of interest by excluding ^{13}C-satellites.

The ^{13}C decoupling experiments for the elimination of the ^{13}C-satellites were performed on a 600 MHz spectrometer (Bruker Avance III NMR, Rheinstetten, Germany) equipped with a 5 mm QNP cryoprobe. 16 scans were collected into 64K data points over a spectral width of 12335.53 Hz (20.54 ppm) with a transmitter offset at 6.175 ppm. A flip angle of 30° was used. The acquisition time was 2.66 s, followed by a relaxation delay of 20 s. All other parameters were set as mentioned above.

T_1 relaxation time measurement. The T_1 relaxation times were obtained using the inversion-recovery pulse sequence (400 MHz NMR spectrometer). For quantitative NMR analysis the relaxation delay was generally set to five times (flip angle: 90°) and three times (flip angle: 30°), respectively, the value of the longest spin-lattice relaxation time T_1 among resonances of interest, in order to avoid distortion of integrated signal intensity due to relaxation effects.

7.4 Development of a LC-CAD method for the control of impurities in streptomycin sulfate

7.4.1 Reagents and Chemicals

Water was delivered by an ELGA PureLab Ultra system (Elga Antony, France). Methanol puriss. p.a., perfluoroheptanoic acid (PFHA) 99 %, perfluorononanoic acid (PFNA) 99%, perfluoropropionic acid (PFPA) 97%, trifluoroacetic acid 99% (TFA), heptafluorobutyric acid (HFBA) 98%, 1-octanesulfonic acid sodium salt 99%, D(+)-glucose ACS reagent, sodium sulfate 99%, potassium dihydrogen phosphate 99%, ammonium acetate 99%, D(+)mannose 99%, phosphoric acid 85% (m/m), sulfuric acid 98%, hydrochloric acid 25%, 1,3-dihydroxynaphthalene 99%,

sodium(meta)perjodate 99%, glacial acetic acid, sodium hydrogensulfite ACS reagent, N-acetylglucosamine, potassium permanganate +99%, acetone p.a., acetonitrile for chromatography, tetrahydrofurane (THF) 99% and toluene 99% were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Streptidine sulfate as well as samples of streptomycin sulfate originating from VMD, Ludeco (Brussels, Belgium), Continental Pharma (Brussels, Belgium), Dopharma (Raamsdonksveer, The Netherlands), Office Chimique (Waterloo, Belgium) and Kela (Hoogstraten, Belgium) were kindly provided by Prof. Hoogmartens (Faculty of Pharmacy, University of Leuven, Belgium). Additionally, a sample of streptomycin sulfate Ph.Eur. CRS 3 was made available by the EDQM. All samples and reagents were used without further purification. Nitrogen + 99% was delivered by a Peak Systems NM18LA or NM30LA nitrogen generator (Lab Gaz Systems, Massy, France). Nitrogen N 50 used as MS/MS-collision gas came from Air Liquide (Illkirch, France).

7.4.2 Equipment

A Waters Alliance Separation Module 2695 including thermostated autosampler, quarternary pump and column oven (St. Quentin-en-Yvelines, France) equipped with Waters Empower Pro data processing software was used for liquid chromatography coupled UV and CAD. UV detection was carried out using a Waters UV Dual Wavelength Detector Module 2489 (St. Quentin-en-Yvelines, France). Detection by CAD was performed by means of a Corona CAD (ESA Bioscience INC. - Vendor: Eurosep Instruments, Cergy Pontoise, France). For LC/MSD analysis an Agilent 1200 Series Rapid Resolution LC system coupled with an Agilent 6400 Series Triple Quadrupole LC/MSD (Waldbronn, Germany) was employed. Exact mass measurement was conducted with a Bruker µ-TOF-MS (Wissembourg, France). TLC was performed using a rectangular TLC developing tank (L × H × W 12.1 cm × 10.8 cm × 8.3 cm) from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The silica gel G TLC plate came from Macherey Nagel (Hoerdt, France). The Inertsil ODS 3, YMC Pack ODS-AQ, YMC Pack Pro and Obelisc N columns were purchased from Interchim (Montlucon, France), the Supelcosil ABZ column from Supelco (Bellfonte, USA), the Zorbax Eclipse XDB C18 and Zorbax SB C18 columns from Agilent (Waldbronn, Germany), the Symmetry C18 column from Waters (St. Quentin-en-Yvelines, France), the Aquasil C18 from Fisher Bioblock Scientific (Illkirch, France),

the RepoSil Fluosil C8 column from Dr. Maisch GmbH (Ammerbuch, Germany), the ZIC-HILIC column from Merck (Darmstadt, Germany).

7.4.3 Methods

7.4.3.1 TLC for streptomycin B

For the test solutions, 200 mg of streptomycin sulfate was dissolved in a freshly prepared mixture of methanol / sulfuric acid 98% (97/3 v/v) and diluted to 5 mL with the same mixture of solvents. The solutions were heated under a reflux condenser for 1h and diluted to 20 mL with methanol. For the preparation of the reference solution, 36 mg of mannose were treated in the same way with the exception that a dilution with methanol to 50 mL was performed after heating. 5 mL of the mannose solution was further diluted to 50 mL using methanol. According to the Ph.Eur. 1 mg of mannose is equivalent to 4.13 mg of streptomycin B.

For the TLC, 10 µL of the test solutions and reference solution were applied to a silica gel coated TLC plate and developed using a mixture of 25 v of glacial acetic acid, 25 v of methanol and 50 v of toluene. After drying, the plate was sprayed with a freshly prepared mixture of equal volumes of 1,3-dihydroxynaphthalene in ethanol and a 20% (v/v) solution of sulfuric acid. Subsequently the plate was heated to 110 °C for 5 min.

7.4.3.2 Analysis of streptomycin sulfate by RP-IPC with UV detection

For the preparation of the mobile phase consisting of 14 g sodium sulfate and 1.5 g disodium 1-octanesulfonate were dissolved in 400 mL of water. After addition of 50 mL of 0.2 molar phosphate buffer (pH 3.0) and 50 mL of acetonitrile, the solution was diluted to 1 L with water. The phosphate buffer solution was prepared by dissolving 2.72 g of potassium dihydrogen phosphate in 80 mL of water. After adjustment to pH 3.0 with phosphoric acid 85%, the buffer solution was further diluted to 100.0 mL with water. A Supelcosil ABZ alkylamide column (250 mm x 4:6 mm, 5 µm) was employed for the LC separation at a temperature of 45 °C. 20 µL of the aforementioned test and reference solution was injected at a flow-rate of 1.0 mL/min. The method run-time was 50 min. Detection was performed by UV at 205 nm.

The streptomycin sulfate test solutions were prepared dissolving 50 mg of substance in 20.0 mL of water (2.5 mg/mL). The limit of quantification (based on a signal-to-

noise ratio of 10:1) of streptomycin sulfate was extrapolated from the detector response of a solution containing 12.5 µg/mL of streptomycin sulfate. The same solution was used as external standard for the quantification of the impurities.

For the calculation of the streptidine content a correction factor of 0.51 was applied. This factor was determined by HPLC measuring the detector response of solution containing 250 µg/mL of streptidine sulfate and 250 µg/mL of streptomycin sulfate in water.

7.4.3.3 Streptomycin sulfate HILIC-CAD method development

The test solution was prepared by dissolving 50 mg of streptomycin sulfate sample 10 in 10.0 mL of water. The solution was injected on two different HILIC columns using different mobile phases.

The different LC conditions tested are described in Table 7-4. Detection was performed by CAD at a gas pressure of 35 psi in the 100 pA range.

Table 7-4. LC conditions tested for the development of a HILIC method for streptomycin sulfate.

Columns	
ZIC HILIC (150 x 4.6 mm, 3.5 µm)	Obelisc N (150 x 4.6 mm, 5 µm)
Mobile phases	
a) 20 mmol/L ammonium acetate (pH 6.8) / acetonitrile at: 15/85; 20/80; 25/75; 30/70 (v/v) b) 20 mmo/L ammonium acetate (pH adjusted to 4.9 with acetic acid) / acetonitrile 25/75 (v/v)	a) 20 mmol/L ammonium acetate (pH 6.8) / acetonitrile 20/80 (v/v); b) Acetonitrile / water 80/20 (v/v) c) 20 mmo/L ammonium acetate (pH adjusted to 4.9 with acetic acid) / acetonitrile at: 15/85; 20/80; 30/70 (v/v)
LC Conditions	
Injection volume: 20 µL Flow rate: 0.5 mL/min Column temp: 25 °C Run-time: 60 min.	Injection volume: 20 µL Flow rate: a) and c) 0.5 mL/min b) 1:0 mL/min Column temp.: 25 °C Run-time: 60 min.

7.4.3.4 Development of an IPC-CAD method for the identification and quantification of impurities in streptomycin sulfate.

Column selection

The test solution was prepared dissolving 50 mg of streptomycin sulfate sample 10 in 10.0 mL a mixture of water / methanol (80/20 v/v) containing 1 mmol/L of HFBA. Additionally a 0.5% dilution of this solution was prepared using the same solvent mixture. 20 µL of the two solutions was injected in duplicate on a Symmetry C 18 column (150 mm x 3.9 mm, 5 µm), a Zorbax Eclipse XDB C18 column (150 mm x 4.6 mm, 5 µm) and an Inertsil ODS 3 column (150 mm x 4.6 mm, 5 µm) at a LC flow-rate of 1.0 mL/min. The column was kept at 30 °C and the run-time was 30 min. The mobile phases used were a) water / methanol (80/20 v/v) containing 1 mmol/L of HFBA and b) water / methanol (95/5 v/v) containing 1 mmol/L of HFBA. Detection was done by CAD at a gas pressure of 35 psi in the 100 pA detection range.

Selection of the ion pair reagent

The test solution was prepared dissolving 25 mg of streptomycin sulfate sample 10 in 10.0 mL a mixture of water (2.5 mg/mL). 20 µL were injected using an Inertsil ODS 3 column (150 mm x 4.6 mm, 5 µm) at 30 °C and a LC flow-rate of 1.0 mL/min. Detection was performed by CAD as given above. A 30 min linear gradient from 100% to 40% (v/v) of eluent A was performed. Several experiments were conducted adding different ion pair reagents at increasing concentrations to both elutent A (water) and eluent B (methanol). The following ion pair reagents and concentration were tested: TFA, HFBA, PFHA at 0.5 mmol/L, 2 mmol/L and 5 mmol/L. PFNA at 0.4 mmol/L and 1.6 mmol/L. In between, the column was flushed with methanol / water for 2 h and equilibrated with the new mobile phase at the initial gradient conditions for 4h.

Development of isocratic method conditions using HFBA and PFPA

Conditions as described above with the difference that isocratic elution over 90 min was applied. For the experiments performed with a purely aqueous mobile phase, the concentration of the test solution was increased to 5 mg of streptomycin sulfate per mL. The different mobile phase compositions tested are listed in Table 7-5.

Table 7-5. Overview of the different mobile phase compositions tested for HFBA and PFPA.

Ion pair reagent	Concentration (mmol/L)	Amount of methanol in the mobile phase (% v/v)
HFBA	7	60; 40; 30
	5	50; 40; 20
	2	40; 20
	0.5	10
PFPA	10	25; 10; 5
	5	25; 10; 5; 2; 0
	3; 6; 7	0

In addition to the above experiments, an ion pair gradient was tested with a 100% aqueous phase starting with 10 mmol/L PFPA and going to 2 mmol/L PFPA in 40 min. For the method optimization, a mobile phase containing 6 mmol/L of PFPA in water was used employing eluent flow rates of 0.8 mL/min, 1.0 mL/min and 1.2 mL/min. At each flow rate a column temperature of 25 °C, 30 °C, 35 °C, 40 °C, and 50 °C was tested.

Using the optimized method conditions, the LC was coupled with a triple-quadrupole MS. In order to reduce the eluent flow to the MS from 0.8 mL/min to 0.4 mL/min, a post column T-split of the eluent was installed. An ESI source (positive mode) was used for all experiments. The triple-quadrupole MS was operated in MS2-scan from 50 to 1300 m/z, nebulizer gas-pressure 25 psi; dry gas, 10 L/min; dry heater 330°C; capillary, 4000V; fragmentor, 135V; collision energy 0-60 eV.m/z 50 to 2000, scan time 500 msec.

To achieve resolution between streptomycin B and streptomycin a set of experiments was performed using the above LC-MS conditions and the mobile phases given in Table 7-6 at a flow rate of 0.8 mL/min with a 1:1 post column split of the mobile phase. The experiments were performed employing a 250 mm x 4.6 mm YMC-Pack Pro C18 column with a particle size of 3 µm.

Table 7-6. Overview of the different mobile phase compositions tested using LC-MS in order to optimise the method selectivity and to attain resolution between streptomycin B and streptomycin – For acetone it is noted that not all possible combinations of different concentrations of PFPA and organic modifier were tested.

Organic modifier	Concentration of PFPA (mmol/L)	Amount of organic modifier in the mobile phase (% v/v)
Acetonitrile	5, 9, 12, 17	3.75; 5; 10
Acetone	6; 12; 13, 15, 16; 17, 18; 20; 24; 25, 27, 28 32	0.5; 1; 2.5; 3.75; 5
THF	6; 16	3,75; 5

For the mobile phase containing 20 mmol/L of PFPA and 1% (v/v) of acetone as organic modifier, further experiments were performed varying the flow rate between 0.4 mL/min and 1.0 mL/min and changing the column temperature at a flow rate of 0.8 mL/min between 20 °C, 30 °C and 40 °C.

7.4.3.5 Identification and quantification of impurities in streptomycin sulfate

The HPLC-CAD and MS analysis was performed using a YMC-Pack Pro column (250 mm x 4.6 mm; particle size 3 µm) at a temperature of 40 °C. A mobile phase containing 20 mmol/L of PFPA in water / acetone (99/1 v/v) was used. The method run-time was 70 min at a flow-rate of 0.8 mL/min. 10 µL of a 5 mg/mL solution of streptomycin sulfate in water was injected as a test solution.

Calibration and CAD-response factor

The calibration curve used for the quantification was recorded using 0.1% (5 µg/mL), 0.2% (10 µg/mL), 0.5% (25 µg/mL), 1.0% (50 µg/mL), 2% (100 µg/mL) and 3% (150 µg/mL) dilutions of a 5 mg/mL solution of streptomycin sulfate sample 11. All dilutions were prepared from a 5 mg/mL stock solution using water as a solvent. For the verification of the linearity of the range, a 5% (250 µg/mL) dilution of the streptomycin sulfate test solution was injected instead of a 3% dilution.

The determination of the CAD correction factor of streptidine sulfate was carried out measuring the detector response of streptidine sulfate and streptomycin sulfate in solutions containing 25 µg/mL and 50 µg/mL of both substances, respectively.

The CAD limits of quantification (based on a signal-to-noise ratio of 10) were extrapolated from the detector response of a solution containing 5 µg/mL of streptomycin sulfate and 5 µg/mL of streptidine sulfate.

In-situ formation of impurities

For the in-situ formation of the hydrogensulfite adducts, 20 µL of a 100 mg/mL solution of sodium hydrogensulfite was added to a solution of 5 mg of streptomycin sulfate in 1 mL of water. The solution was subsequently analysed by LC.

For the generation of streptomycin acid, 20 µL of a 0.1 N potassium permanganate solution was added to a 5 mg/mL aqueous solution of streptomycin sulfate. 20 µL of each solution was analyzed by LC using the aforementioned method with MS detection.

For the verification of the identity of the impurity 1 with an m/z-ratio $[M+H]^+$ of 222, a 5 mg/mL test solution of streptomycin sulfate sample 10 spiked with about 0.5% of N-acetylglucosamine was analysed using the LC method described above and detection by means of CAD.

Detector settings

(i) CAD-detection was performed using a gas pressure of 35 psi in the 100 pA detection range.

(ii) TOF-MS

A 1/10 T-split of the mobile phase eluting from the LC column was applied for the LC-TOF-MS coupling. The TOF-MS parameters were as follows: source type and polarity, ESI-positive; nebulizer gas pressure, 2.0 bar; drying gas, 8.0 L/min; dry heater, 250 °C; capillary, 4500 V; end plate, 500 V; scan range, 50 – 3000 m/z; capillary exit, 100 V; hexapole RF, 100 V; skimmer 1, 50 V; hexapole 1, 25 V; pulsar pull, 0 V, pulsar push, 825 V; reflector, 1709 V, flight tube, 8589 V, detector, 1880 V.

(iii) MS/MS

The MS/MS parameters were as follows: source type and polarity, ESI-positive; nebulizer gas-pressure 35 psi; dry gas, 12 L/min; dry heater 350°C; capillary, 4000V; scan range, 50-1300 m/z; fragmentor, 135V; collision energy 0-60 eV.

7.5 Application of LC-CAD for the characterization of horse-chestnut standardised dry extract

7.5.1 Reagents and Chemicals

Water was delivered by an ELGA PureLab Ultra system (Elga Antony, France). Trifluoroacetic acid 99% (TFA), formic acid 98%, sulfuric acid 99%, phenolphthalein pH indicator, sodium hydroxide 1 M volumetric solution, methanol puriss. p.a and acetonitrile for chromatography were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The samples of purified ß-Aescin were provided by Kneipp (Würzburg, Germany), Euromed (Marseille, France), Schwabe (Karlsruhe, Germany), Le-Si (Lubljana, Slovenia), Fluka (Saint-Quentin Fallavier, France) and Extrasynthese (Genay, France). Protoaescigenin came from Schwabe (Karlsruhe, Germany). Methylsalicylate CRS and ibuprofen CRS were donated by the EDQM. All samples and reagents were used without further purification. Nitrogen + 99% was delivered by a Peak Systems NM18LA or NM30LA nitrogen generator (Lab Gaz Systems, Massy, France). Nitrogen N 50 used as MS/MS-collision gas came from Air Liquide (Illkirch, France).

7.5.2 Equipment

A Waters Alliance Separation Module 2695 including thermostated autosampler, quarternary pump and column oven (St. Quentin-en-Yvelines, France) equipped with Waters Empower Pro data processing software was used for liquid chromatography coupled UV and CAD. UV detection was carried out using a Waters UV Dual Wavelength Detector Module 2489 (St. Quentin-en-Yvelines, France). For the determination of the absorbance of protoaescigenin at different wavelength, a Waters Photodiode Array Detector Module 2996 (St. Quentin-en-Yvelines, France) was used. Detection by CAD was performed by means of a Corona CAD (ESA Bioscience INC. - Vendor: Eurosep Instruments, Cergy Pontoise, France). The loss on drying was determined using a Memmert drying oven UNE 400 (Schwabach, Germany). The 10 mL standard glass burette used for the titration, the silica crucibles (51 x 51 mm) for the sulfated ash test and the glass crucibles with lids (2 cm x 2 cm) for the loss on drying test were delivered by Schott (Clichy, France). For LC/MSD analysis an Agilent 1200 Series Rapid Resolution LC system coupled with an Agilent 6400 Series Triple Quadrupole HPLC/MSD (Waldbronn, Germany) was employed. The Nova Pak C18 column was purchased from Waters (St. Quentin-en-Yvelines,

France), the Grace Vydac C18 column came from Grace Davison Discovery Sciences (Templemars, France). Off-line UV measurements were carried out using a Varian Carry 1E UV-VIS spectrophotometer (Les Ulis, France) with 1 cm quartz cuvettes from Hellma (Müllheim, Germany).

7.5.3 Methods

7.5.3.1 LC-MS analysis of purified ß-aescin

The LC separation was performed at 25 °C on a Novapak C18 column (150 mm x 3.9 mm; particle size: 4 µm). The following step gradient was applied at a flow rate of 0.7 mL/min: 65% mobile phase A from 0 to 15 min, 50 % mobile phase A from 15.1 to 25 min and 10% mobile phase A from 25.1 to 30 min. Mobile phase A was composed of 0.1% (v/v) of formic acid in water, mobile phase B was acetonitrile for chromatography. The test solution was prepared as follows: 20 mg of aescin sample 2 was suspended in 2 mL of acetonitrile and sonicated for 5 min. After dilution to 5.0 mL with water, complete dissolution was obtained. 20 µL of this solution was injected. MS detection was performed using electrospray ionization (ESI) in the negative mode. The source parameters were as follows: gas temperature: 350 °C, nitrogen gas flow: 12 L/min, nebulizer gas pressure: 40 psi, capillary voltage: 4500 V, mass scan from m/z 100 to 1400 with a scan time of 500 ms, fragmentor voltage 135 V. Detection Mode: TIC.

7.5.3.2 Elaboration of a reference standard strategy for the determination of aescin in horse-chestnut standardized dry extract

Characterization of the aescin samples

The loss on drying and the determination of the sulfated ash was performed as described in the Ph.Eur. Chapters 2.2.32 d and 2.4.14, respectively. For the loss on drying a triplicate determination using 100 mg of the substance to be examined dried at a temperature of 105 °C to constant weight was performed. The sulfated ash test was carried out as a single determination using 1 g of substance. The assay titration for aescin was conducted dissolving 800 mg of substance in a mixture of 10 mL of water and 30 mL of methanol. The methanol was previously neutralized with 0.1 molar sodium hydroxide using 1 mL of Ph.Eur. phenolphthalein solution R as a color indicator. The titration of the samples was performed immediately after dissolution:

For the calculation of the aescin content, 1.0 mL of 0.1 molar sodium hydroxide was considered to be equivalent to 113.1 mg of aescin ($C_{55}H_{86}O_{24}$).

Comparison of the aescin samples by LC-UV according to the draft monograph

LC-method: The separation was performed injecting 20 µL of the different test solutions on an Grace Vydac C18 column (250 mm x 4.6 mm; particle size 5 µm) at a temperature of 25 °C using the following linear binary gradient at a flow-rate of 1.0 mL/min.: 0-15 min. mobile phase A 70%; 15-25 min. mobile phase A 70% to 65%; 25-35 min. mobile phase A 65%; 35-65 min. mobile phase A 65% to 50%; 65-70 min. mobile phase A 50% to 10%. Detection was performed by UV spectrophotometry at 210 nm using a variable wavelength detector. For the comparison of the response at 206 nm, 210 nm and 214 nm, a diodearray detector was used.

The test solutions and reference solution of the aescin candidate CRS were prepared dissolving 50 mg of the different aescin samples in an internal marker solution (containing 0.1 mg/mL of methyl salicylate and 0.3 mg/mL of ibuprofen in a mixture of acetonitrile / 0.1% aqueous formic acid (40/60; v/v)) and dilution to 10.0 mL with the same solution.

For the preparation of the protoaescigenin reference solution, 5 mg of protoaescigenin was suspended in 4 mL of acetonitrile. After 10 min of sonication and further dilution to 10.0 mL with acetonitrile, a clear solution was obtained. 2.0 mL of this solution was mixed with 2.0 mL of acetonitrile and diluted to 10.0 mL with a 0.05% aqueous solution of TFA.

The comparison of the detector response of protoaescigenin at different detector wavelength was carried out injection the above sample of protoaescigenin whilst simultaneously measuring the detector response at 206 nm, 210 nm, and 214 nm.

For the off-line measurement of the absorbance curve of protoaescigenin between 190 nm and 220 nm, a 0.04 mg/mL solution of protoescigenin in acetonitrile / 0.05% aqueous TFA (40/60; v/v) was used.

7.5.3.3 Use of the CAD for method improvement

LC-method: The separation was performed injecting 20 µL of the different test solutions on an Grace Vydac C18 column (250 mm x 4.6 mm; particle size 5 µm) at a temperature of 25 °C and a flow-rate of 1.0 mL/min using isocratic elution with a mobile phase containing acetonitrile / 0.05 % aqueous TFA (35/65; v/v). After 21

minutes a 10 min. "washing step" using acetonitrile / 0.05% TFA (90/10; v/v) was applied. Thereafter, the column was re-equilibrated with the mobile phase for 10 min before the next sample was injected.

Detection was performed either by UV spectrophotometry at 210 nm using a variable wavelength detector or by CAD at detector gas pressure of 35 psi in the 100 pA detection range. For the quantification by CAD, a calibration curve was recorded using 11 dilutions of a 0.8 mg/mL protoaescigenin stock solution at the following concentration levels: 0.002 mg/mL, 0.004 mg/mL, 0.008 mg/mL, 0.016 mg/mL, 0.032 mg/mL, 0.048 mg/mL, 0.064 mg/mL, 0.16 mg/mL, 0.24 mg/mL, 0.32 mg/mL, 0.40 mg/mL. For the quantification by UV, a calibration curve was recorded using 4 dilutions of a 0.8 mg/mL protoaescigenin stock solution at the following concentration levels: 0.008 mg/mL, 0.032 mg/mL, 0.16 mg/mL, 0.80 mg/mL. The stock solution was prepared by dissolution of 40 mg of protoaescigenin in 50.0 mL of methanol. For the preparation of the test solutions analysed by CAD, the subsequently specified amounts of sample were suspended in 2 mL of acetonitrile. After 10 min of sonication the solution was further dilution to 5.0 mL with 0.05% TFA: sample 3 and 7: 13 mg (2.6 mg/mL); sample 4 and 6: 10 mg (2 mg/mL); sample 5: 7 mg (1.4 mg/mL); sample 1: 5 mg (1 mg/mL). The test solutions for UV 210 nm detection were prepared in the same was but using 25 mg (5 mg/mL) of the substance to be examined.

7.6 Use of collision induced dissociation MS for the identity verification of peptides

7.6.1 Reagents and Chemicals

For gonadorelin, goserelin, buserelin, leuprorelin human insulin, insulin lispro and insulin aspart the corresponding reference standards of the Ph.Eur. (EDQM, Strasbourg, France) were used. Acetonitrile of LC grade was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany), water for chromatography was delivered by an ELGA PureLab Ultra system (Elga Antony, France), HCl solutions were prepared from dilutions of 0.1 M HCl TitriPUR from Merck (Darmstadt, Germany), ammonium hydrogen carbonate p.a., formic acid 98-100% p.a. and ammonium acetate puriss. for HPLC were either delivered by Sigma-Aldrich (St. Quentin Fallavier, France), Fluka (St. Quentin Fallavier, France) or Acros (Noisy le Grand, France). Endoproteinase Glu-C sequencing grade (V8 protease) was

supplied by Roche (Mannheim, Germany). Nitrogen +99% used as nebulizer and drying gas was delivered by a Peak Systems NM30LA nitrogen generator (Lab Gaz Systems, Massy, France). Nitrogen N 50 used as MS/MS collision-gas came from Air Liquide (Illkirch, France).

7.6.2 Equipment

CID-MS experiments were performed using an Agilent Triple Quad LC/MS 6410 (m/z range: 20-2000) equipped with an ESI-source and coupled to an Agilent 1200 Binary SL liquid chromatography system (Waldbronn, Germany).

7.6.3 Methods

1 mg/mL solutions of gonadorelin acetate, goserelin, buserelin and leuprorelin in acetonitrile / water (80/20, v/v) were prepared. For human insulin, insulin lispro and insulin aspart 1 mg/mL solutions in 1 mmol/L hydrochoric acid were used. Digested human insulin, insulin lispro and insulin aspart was prepared by addition of 20 µL of a 12 mg/mL solution of insulin dissolved in 10 mmol/L hydrochloric acid to 0.5 mL of 0.1M ammonium hydrogen carbonate. After addition of about 50 µg of V8 protease the solutions were heated in a water bath at 37 °C for four hours, and subsequently quenched with 20 µL of formic acid.

Sample infusion was performed by flow injection analysis (FIA) using an injection volume of 10 µL (MS2-scan) and 20 µL (CID-MS), respectively, at a flow rate of 0.2 mL/min of a mixture of acetonitrile / water (80/20, v/v) for the synthetic peptides and a mixture of acetonitrile / 0.07 g/L ammonium acetate solution (50/50, v/v) for the insulins. An ESI source (positive mode) was used for all experiments. The triple-quadrupole MS was operated in MS2-scan mode from a mass-to-charge-ratio (m/z-ratio) of 200 to 2000, scan time 500 msec, fragmentor voltage at 135 V. For the CID experiments a collision energy of 75 eV for the singly charged ions and of 20 eV for the doubly charged ions was applied. The ESI product ion scan was performed for an m/z-ratio of 50 to 2000. MS-source parameters: capillary -4000 V, nebulizer pressure 25 psi, dry gas-flow 10 L/min, gas temperature 330 °C.

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