Identification and quantification of bio-actives and metabolites in physiological matrices by automated HPLC-MS

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Identificatie en kwantificering van bio-aktieve stoffen en metabolieten in physiologische matrices met behulp van geautomatiseerde HPLC-MS.

(met een samenvatting in het Nederlands)

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Abbreviations

Instrumental

API	atmospheric pressure ionization
CID-MS^2	Collision-induced dissociation tandem mass spectrometry
CZE	capillary zone electrophoresis
GPC	gel permeation chromatography
HILIC	hydrophilic interaction chromatography
HPLC	high performance liquid chromatography
I.D.	internal diameter
IT-SPME	in-tube solid-phase micro extraction
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
LPME	hollow fibre membrane liquid-phase micro extraction
MRM	multiple reaction monitoring
MS	mass spectrometry
PCA	principle component analysis
PLS	partial least-squares
PPT	protein precipitation
RPLC	reversed-phase liquid chromatography
RPM	rotations per minute
SLM	supported liquid membrane
SPE	solid-phase extraction
SS-LLE	solid-supported liquid-liquid extraction
TIC	total ion current
UPLC	ultra performance liquid chromatography
UV	ultra violet

Compounds and chemicals

AT1	angiotensin I
AT2	angiotensin II
ACE	angiotensin I-converting enzyme
ACEI	ACE inhibitor
C	catechin
CG	catechin gallate
CXW-1-17	5-(3,4-dihydroxyphenyl)-γ-valerolactone
CXW-1-19	5-(3-methoxy-4-hydroxyphenyl)-γ-valerolactone
DMF	dimetyl formamide
EC	epicatechin
ECG	epicatechin gallate
EDTA	ethylenediaminetetraacetic acid
EGC	epigallocatechin

EGCG	epigallocatechin gallate
FAPGC	*N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine
GC	gallocatechin
GCG	gallocatechin gallate
H	hippuric acid
HL	histidyl-leucine
HHL	hippuryl-histidyl-leucine
ISOR	isorhamnetin
KAEM	kaempferol
LTP	lactotripeptide
MTBE	methyl tertiary butyl ether
MYR	myricetin
3/4-OMGA	3- or 4-*O*-methyl gallic acid
OPA	o-phthaldialdehyde
PEG	polyethylene glycol
PBS	phosphate buffer solution
RES	resveratrol
SMP	skimmed milk protein
TFA	trifluoroacetic acid
FA	formic acid
TNBS	trinitrobenzene sulfonic acid
WPI	whey protein isolate

Others

ANOVA	analysis of variance
AUC	area under curve
BMI	body mass index
EFSA	European food safety authority

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1 Introduction

1.1 General introduction

1.1.1 History

Food plays an important role in the wellbeing of mankind, not only as a source of energy (carbohydrates, fats, proteins) and essential micronutrients (vitamins, minerals), but also for health reasons. Hippocrates (460 – 377 BC), generally considered to be the father of modern medicine, claimed already that “good health implies the awareness of the powers of natural and processed foods”. In the eleventh to twelfth century the Salernitan School advised that doctors must observe and learn from the effect of food intake. In every civilization people were, and still are, aware of the importance of food for human health. In particular in the Chinese world traditional medicine has been used for the treatment of diseases for several thousands of years. Feng et al. provides an excellent overview of knowledge discovery by using databases concerning recent traditional Chinese medicine studies [1].

In the past years there has been a renewed interest in functional foods. Functional foods refers to foods that are consumed as part of a normal food pattern and that have beneficial effects on body functions that go beyond adequate nutritional effects and that are relevant to an improved state of health and well-being and/or a reduction of the risk (not prevention) of disease (cited from the web site of the International Life Science Institute).

Scientists in many academic and industrial research institutes have been involved in studying the effects of food on human health. Numerous specific foods or food ingredients have been studied for an equally wide range of positive health aspects. In Table 1 some examples are given of (classes of) natural compounds of which certain representatives can bring about such effects. It must be mentioned that many health claims so far are just proposed but often not yet confirmed by the European Food Safety Authority (EFSA) as it requires extensive, very well conducted epidemiological studies involving various consumer groups to provide hard evidence.

1.1.2 Functional foods

In line with the above mentioned statement functional foods contain “bioactive” compounds that have physiological benefits and/or reduce the risk of chronic disease. Functional foods can be similar in appearance to a conventional food product that is consumed as part of a usual diet [2]. As commercial alternatives fabricated functional food products have become very popular. A fabricated functional food is a processed food product

fortified with bioactive compounds which are not necessarily naturally occurring in the food raw materials. One example is margarine enriched with plant sterols claimed to have cholesterol lowering properties.

Table 1 Some examples of natural compound classes and their positive effect on human health.

Compound class *	Source	Proposed health effect	Applied in food product (e.g.)	References
small peptides	e.g. milk	blood pressure	yoghurt drinks, bars	14
flavonoids	plant	blood pressure cancer coronary heart disease anti-oxidative	coffee, tea, juices	15-21
sterols	plant	cholesterol level	margarines, yoghurt drinks	22-24
steroidal glycosides	e.g. succulents	obesity reduction	not yet in foods **	25-27
lipids	e.g. nuts	coronary heart disease	nutty bars	28
proteins	e.g. soy	cardiovascular health	soups, (milky) drinks	29
polyphenols	plant, fruits, legumes	coronary heart disease	tea, wine, beer, chocolate	28
catechins	e.g. tea	vascular condition	tea drinks	30
caffeine	coffee and tea	mood and alertness	coffee and tea drinks	31
DHA	fish	brain building/growth	fish-products	32

* The positive health effects are usually brought about by particular representatives of the compound class.

** Can be purchased in capsules and sprays; not yet applied in foods.

1.1.3 Analytical challence

The bioactives of interest very often are complex medium-sized biomolecules (molecular masses of few 100 – few 1000 Da). To understand the effectiveness of these bioactives in functional foods, knowledge of their composition and structure as well as of the structural modifications after consumption (metabolites) is essential. This information helps to understand the bio-availability, the rate and extent to which a substance is absorbed and circulated within the body, and their effect on the physiological process. Uptake, transport and fate of the compounds in the body can be followed by analyzing samples obtained from so-called Absorption, Distribution, Metabolism and Excretion (ADME) studies. The identification of bioactives often is hampered by the source of the compounds being usually rather

complex, e.g. plant extracts or fermented milk. Very often the activity of the functional ingredients changes after consumption of the product due to compound breakdown and/or metabolism.

The identification of the metabolic products in plasma, tissue, urine and faeces is difficult due to the complexity of the matrices and the very low levels at which the metabolites occur. This together with the often low stability of the bioactives makes the development of proper analytical approaches in ADME studies highly important.

1.1.4 Strategies for the analysis of functional food ingredients and their metabolites formed after consumption in the human body

Food ingredients

The actual bioactivity of a functional food product is often caused by one or a few specific ingredients present in the food product. For the identification of the bioactive compounds several analytical strategies can be followed. One option is to use an on-line bioassay hyphenated to a chromatographic separation method followed by structure identification using NMR and/or MS. Niederländer et al. reviewed for instance antioxidant activity assays coupled on-line with liquid chromatography [3]. In his review a highly detailed description and evaluation is given of the most common on-line assays. After the detection of the active compound, identification using MS and/or NMR is required often preceded by sample purification. This purification can involve fractionation, liquid-liquid extraction, solid-phase extraction and many other techniques. The method selected depends on the compound type and matrix to be analyzed. Purification needs to be carried out under mild conditions and the number of purification steps has to be as low as possible, because often compound degradation or adsorptive losses occur during the different steps.

Physiological effects

To get insight into the physiological fate and functioning of bioactive compounds analysis of body fluids, tissues and faeces is required. Especially body fluids have been analyzed extensively for this purpose; the main technique applied being LC-MS. In these analyses sample preparation plays an important role. Removal of interfering components is essential for the performance of the analytical system used. For example lipids can negatively affect the chromatographic separation of peptides while proteins may block the LC column. In principle everything that enters the MS source may also pollute the source resulting in a loss of sensitivity. To maintain optimum source performance it is therefore essential to purify the sample prior to analysis. Various sample preparation methods can be adopted for sample clean-up. The optimum method is often depending on the type of

compounds to be analyzed, the matrix and the instrument used for the analysis.

Many reviews can be found focussing on one or more of these items. For example Stalikas et al. [4] discusses methods for the extraction, separation, and detection for biologically and physiologically significant compounds such as phenolic acids, flavonoids, alkaloids, saponins and sesquiterpenoids in natural products. In this review a thorough summary is given of the sample preparation, derivatization, chromatographic separation method and type of detector used for individual compounds and classes of compounds. The article shows that the required sample preparation steps are strongly depending on the type of compound to be analyzed and the instrument used for the analysis. In case of NMR analysis, for instance, the sample preparation can be much more limited as compared to MS analysis. For the analysis in body fluids a different approach must often be followed compared to the analysis in natural products; as in bodyl fluids not only the matrix composition is completely different from that of natural products but also the amount of sample is limited and the concentration of the compounds of interest is generally much lower. Moreover, the stability of the bioactive compounds may be lower in body fluids compared to that in the original natural matrix. From the many reviews on sample preparation of biological samples three of them will be mentioned more specifically in this introduction. Xing et al. [5] describes the three most common sample extraction methods for serum/plasma clean-up. The authors discuss the influence of the extraction parameters such as pH, sample volume and composition on the effectiveness of the method. The article includes LC-MS, bio-assays and some applications for the analysis of alkaloids, saponins and sesquiterpenoids. Luque-Garcia et al. [6] reviewed the analysis of serum/plasma for biomarker identification and plasma profiling. Many parameters influencing the results of the sample preparation are discussed, varying from the sample collection, handling and storage via sample clean-up to the method of detection. The authors clearly demonstrate the large influence of the different preparation steps, starting with the effects of sample collection methods. The latter step is often excluded from literature concerning plasma measurements although it might even be the most important step of the entire process. One of the examples given by the authors is the huge effect of thawing on the peptide profile of plasma. After only a few repeated thawing steps the profile can change dramatically. Visser et al. [7] compares various sample preparation methods for the analysis of proteins and peptides in biological matrices. The recent developments regarding the coupling of sample preparation to liquid-chromatography (LC) and capillary zone electrophoresis (CZE) and the advantages and disadvantages of both methods are discussed.

Metabolite identification

For the identification of metabolites usually HPLC-MS is applied on samples collected from *in-vivo* or *in-vitro* experiments. Excellent reviews describing the analytical approach for screening and identification of metabolites are published by e.g. Tolonen et al. [8] and Holcapek et al. [9]. The authors discuss the bio-assays used, the sample preparation methods, chromatographic conditions and the advantages and disadvantages of the different MS instruments available for the identification of metabolites.

Data interpretation, metabolite prediction software

One of the problems in the evaluation of the results of for instance human or animal trials is the large amount of data produced. Therefore special software packages have been designed to analyze these data sets. A highly informative review comparing the different packages is published by Trygg et al. [10]. The authors clearly describe the different steps in the chemometric approach for metabonomic studies and give a detailed comparison of the software packages available for data analysis such as Principle Component Analysis (PCA) and Partial Least-Squares (PLS). A relatively new approach for the identification of metabolites is the use of metabolite prediction software. Kulkarni et al. reviewed four of such in-silico analysis packages [11]. The authors thoroughly investigated the parameters that can influence the results, such as the knowledge base used by the software, its construction, the chemical input, initial set-up and the reliability of simulation and prediction.

Quantification of bioactives and metabolites

Quantitative measurement of bioactive compounds and their metabolites in body fluids and tissues provides information essential for understanding the uptake, fate and functionality of the compounds in the human body. For the separation and identification of the compounds from the complex matrix in general GC or HPLC is applied, combined with different types of detectors amongst which mass spectrometry. Nowadays, the latter technique is the most popular detector due to its high selectivity and sensitivity, while also the reduced price and greatly improved ease of use of modern MS instruments play an important role. One must take into consideration, however, that other detectors such as the FID and UV detector in general show better linearities and larger dynamic ranges compared to MS. Nowadays HPLC is preferred over GC for the analysis of bioactives and their metabolites in plasma and urine since no complex sample preparation steps such as derivatisation are required and the compounds are analyzed in their natural aqueous environment.

Criteria required for a proper interpretation of the MS data are amongst others high chromatographic resolution, low detection limit, high

dynamic range and good reproducibility over a long time period. Although MS is a selective tool, chromatographic pre-separation is very often necessary to separate the analyte(s) of interest from the complex mixture prior to MS detection. The chromatographic method must first be optimized to prevent interaction of co-eluting compounds with the compound of interest unless (expensive) labelled standards are available. The still increasing sensitivity and robustness of the MS equipment, together with optimization of the sample preparation, enables extremely low detection limits. Due to the ongoing improvement of the HPLC column material a higher selectivity and longer column life-time can be obtained resulting in an increase in the numbers of samples that can be analyzed under identical conditions with stable retention times. As has been stated earlier, the latter is essential when the data are to be used for computerized interpretation. The latest development in the field of chromatography is the introduction of ultra performance liquid chromatography (UPLC). This system is capable of handling pressures up to approximately 1000 bar allowing chromatographic analyses with very small column particles (down to 1.5 μm). This results in either a very high chromatographic resolution or strongly reduced analysis times. At the time this thesis was written approximately 500 publications could be found describing the application of UPLC for the analysis of a large variety of compounds in different matrices. Examples are the articles of Musuamba et al. [12] and Avula et al. [13] demonstrating the power of UPLC for fast analysis and high resolution separation, respectively.

1.2 Scope of the thesis

The objective of this thesis is the optimization of the analytical process aiming at the identification and quantification of selected bioactive compounds and their metabolites in foods and (simulated) body fluids. The work is an integral part of the Unilever Research programmes and is focussed on three compound classes: small peptides, steroidal glycosides and small polyphenols. The matrices consist of natural source materials, blood, plasma, urine and microsomal cell extracts. Although many approaches for the identification and quantification of these types of analytes in biological samples have been published, most of them were designed for the analysis of only one specific compound in one type of matrix. The work described in this thesis is focussed on the development of improved, more widely applicable methods for sample preparation and strategies for identification of the mentioned types of bioactives and their metabolites, methods for the quantification of these compounds in complex matrices and finally automation of the sample preparation and quantification process.

The methods of choice applied in this work are based on thorough literature studies and preliminary testing and the results are described in

chapter 2 to 10. For the automation of the analytical process methods have been developed that use integrated sample preparation systems such as on-line SPE in combination with LC- or UPLC-MS.

1.2.1 Sample preparation

In **Chapter 2** of this thesis the applicability of different extraction methods for the analysis of peptides, steroidal glycosides and small polyphenols from source material is described. The aim of this work was to get an impression of the performance of the extraction methods for these compound classes covering a broad range of hydrophobicities and polarities. Secondly the robustness and ease of operation was evaluated. The potential for automation of each of the methods was investigated.

1.2.2 Identification of bioactive compounds

The development and application of a method for the identification of bioactive peptides in complex milk protein hydrolysates is described in **Chapters 3 and 4.** The method was designed for the analysis of small antihypertensive peptides present in hydrolyzed milk. The first step in the process concerned the separation of the bioactive peptides from the complex mixture of hydrolyzed milk by 2-dimensional liquid chromatography. In the second step the application of an at-line bio-assay combined with MS was investigated for the detection and identification of the bioactive peptides.

As the detection and identification of the active peptides is time consuming and laborious a fast method needed to be developed. In **Chapter 3** the development of an at-line LC-MS method for the detection and identification of angiotensin I-inhibiting peptides from casein hydrolysates is described. **Chapter 4** describes the application of at-line two dimensional LC-MS for the identification of the poorly eluting hydrophilic bioactive peptides in the milk hydrolysates described in chapter 3. The method was optimized and the effect of the method on the recovery of the total activity of the sample was determined.

1.2.3 Quantification of bioactives in plasma

Following the identification of the bioactive peptides the next step is to determine the physiological effect of the separate peptides. To be able to understand the process in the human body the determination of a bio-profile is necessary. This bio-profile displays the concentration of the specific peptides in the blood stream versus the time after consumption of the peptide and provides information on the extent and time-frame of the adsorption and metabolism of the peptide.

Chapter 5 describes the HPLC-MRM-MS method developed for the quantification of ACE-inhibiting peptides in plasma samples collected from human volunteers after the consumption of a peptide-enriched drink.

Different methods for the preparation of the plasma samples were compared. A simple and fast method for the precipitation of proteins from plasma was developed and validated. The new methods were applied to real life samples. In **Chapter 6** the quantification method described in the previous chapter was applied to human plasma samples collected from a placebo-controlled cross-over study. In this study the bio-availability of antihypertensive peptides was investigated. The bio-availability (*C-Max* value) and half-life time of peptides of interest could be calculated.

1.2.4 Identification of metabolites of bioactive compounds

In the milk peptide studies the amount of sample available for the identification and quantification of the bioactive peptides was sufficient to perform several analyses. In **Chapter 7** a method for the identification of metabolites of bioactive compounds is described that can be applied when only a very small amount of material is available. In this study bioactive, appetite-suppressing steroidal glycosides from a *Hoodia gordonii* plant extract and their metabolites formed in the liver needed to be identified. To optimally use the limited amount of material, metabolite prediction software in combination with an optimized HPLC-MRM-MS verification was selected for evaluation. The method was tested on plasma samples incubated with liver microsomes and on plasma samples collected from an animal trial.

1.2.5 Quantification of metabolites in plasma

In **Chapter 8** a method is described for the quantification of the *Hoodia* steroidal glycosides and their metabolites in plasma samples collected from an animal trial. The method is based on liquid-liquid extraction followed by HPLC-MRM-MS and is validated against the criteria set for linearity, detection limit and robustness.

1.3 General remarks

In **Chapter 9** some general conclusions are drawn with respect to the work described in the chapters 2 to 8. The methods for the identification and quantification of bioactive compounds and their metabolites are discussed. The instrumentation available for the quantification of complex medium-sized biomolecules is discussed and new instrumental developments and their applicability are looked at. A prognosis is given on the feasibility of setting-up one single, fully automated method for sample preparation, identification and quantification of medium-sized biomolecules with strongly varying structures and hydrophobicities in a broad range of matrices.

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2 Optimization of the sample preparation for quantification of biologically active compounds and metabolites in body fluids

Summary
Sample preparation plays an essential role in the analysis of bioactive compounds. In the past mainly liquid-liquid extraction (LLE) was applied for the preparation of samples for LC-MS analysis, but nowadays many alternative sample preparation techniques are available. Besides the more classical methods such as LLE and solid-phase extraction (SPE) many new techniques have been successfully developed in particular for application in automated systems. In this study three techniques were selected for comparison, SPE, solid-supported liquid-liquid extraction (SS-LLE) and Monotrap extraction. Monotrap extraction is new and has strong potential for fast screening analyses. The aim of the study was to make a quick reconnaissance of the different techniques and thus gain an impression of the applicability and limitations of each. We focussed on three classes of biologically active compounds: peptides, steroidal glycosides and small polyphenols. The results show that each technique has its advantages and disadvantages. The range of hydrophobicity of SS-LLE is comparable to that of LLE, but in contrast to the latter SS-LLE has potential for automation. High recoveries could be obtained for the three different compound classes. SPE is more generally accepted as compared to the other techniques but needs more fine-tuning of the parameters to obtain good recoveries. The method is, however, very easy to automate and dedicated instrumentation is already commercially available. Monotrap extraction shows a high potential for fast and easy sample preparation for qualitative analyses of hydrophobic or volatile compounds from liquids. This technique is an interesting option for further application studies.

The investigation confirms the generally accepted rule that the optimum sample preparation technique is strongly depending on the type of analytes and the composition of the matrix. Although there is an overlap in the applicability of the techniques there will always be specific cases which work best with only one of them. Therefore a good insight into the analytical problem is essential.

2.1 Introduction

In the past years there has been an increasing interest in functional foods and the effects of the active ingredients of food products on human health.

Many different natural products have been tested for their functionality in human and animal trials. Examples are the effect of peptides derived from milk and flavonoids from tea, grape and wine on antihypertensive [1-7], the effect of plant sterols on cholesterol lowering [8-10], the effect of steroidal glycosides on appetite suppression [11-13] and many other potential applications. To get insight into the absorption behaviour of the ingredients into the blood stream and metabolites formed, analysis of body fluids is required. Many reports have been published in the past years on the analysis of plasma after consumption of healthy food ingredients [14-19].

Sample preparation generally plays an important role in the analysis of body fluids. Removal of interfering compounds such as proteins and fats is essential for the chromatographic performance and lifetime of the HPLC column used. Proteins tend to block the column, while for the removal of fat column flushing is required. Also the sensitivity and condition of the mass spectrometry source can be influenced by the presence of e.g. salts and fat. A large number of techniques are available for sample preparation. A fast and simple sample preparation technique is the “dilute and shoot” method [20-22] also called protein precipitation (PPT) [23]. With this method proteins are removed from the sample by addition of an organic modifier followed by centrifugation. The method is often used in combination with fast chromatographic-(MS) analyses, but requires column flushing which increases the total analysis time. An alternative for protein removal from plasma is heating for several minutes followed by centrifugation [17]. More selective and commonly used techniques are LLE or solid-phase extraction (SPE) [24-29]. Later developments in the field are e.g. SS-LLE [30], hollow fibre membrane liquid-phase micro extraction (LPME) [31], in-tube solid-phase micro extraction (IT-SPME) [32], turbulent flow [33-35], or combinations of techniques such as supported liquid membrane (SLM) with SPE [36].

In this paper we describe the applicability of SS-LLE, SPE and Monotrap extraction for the isolation of potential bioactive compounds from plasma. SS-LLE and SPE were selected for their applicability on a broad range of compounds and their potential for automation. Monotrap extraction was tested as a recently introduced adsorption technique. The aim was not to fully optimize each method for the highest recovery but to get an impression which method has the highest general applicability within the range of test-compound classes. For high throughput analyses an automated system is preferred such as SPE and SS-LLE robotics. For fast screening or capturing hydrophobic compounds or volatile compounds from liquids the Monotrap system is preferred.

To investigate the techniques on a broad range of compounds with different hydrophobicity and polarity the following commercial food products were investigated: peptides from fermented milk, polyphenols from tea and

grape and steroidal glycosides from a plant extract. The peptides vary from the nonpolar hydrophobic dipeptide VP (214 Da) to the more hydrophilic hexapeptide DKEITP (701 Da). Most of the polyphenols are polar and hydrophilic except isorhamnetin which is nonpolar and hydrophobic. The molecular masses vary from 184 Da for 3- or 4-O-methyl gallic acid to 458 Da for epigallocatechin gallate. Due to the presence of two OH groups in the steroidal glycoside P57AS3 (878 Da) it is less hydrophobic than the homologue with the pentasacharide moiety (1006 Da) which contains only one OH group in the carbohydrate chain. The effect of various types of solvents, reaction times and other conditions were investigated. The aim of this study was to increase the sample throughput by using automation of the sample preparation combined with ultra performance liquid chromatography mass spectrometry (UPLC-MS).

2.2 Experimental

2.2.1 Materials and methods

Chemicals

The fermented milk powder was purchased from Calpis Co (Tokio, Japan). The plant extract containing the steroidal glycosides was donated by Cognis Iberia S.A.U. (Barcelona, Spain). All catechins, flavonols, stilbene and taxifolin were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 5-(3'-methoxy-4'-hydroxyphenyl)-γ-valerolactone were synthesized in house. 3- and 4-*O*-methylgallic acid were purchased from Extrasynthèse (Genay Sedex, France). The SPE cartridges tested were the Oasis® silica C18, the MAX and the HLB type of Waters (Etten-Leur, the Netherlands). All cartridges had a volume of 3 ml and contained 60 mg of column material. For the peptide analyses an extraction plate manifold was used consisting of a 96 well plate filled with MCX, WAX, MAX and WCX material (Waters). The SS-LLE cartridges were purchased from Varian (Middelburg, the Netherlands). The Monotrap materials were donated by ATAS GL (Veldhoven, the Netherlands). Human and porcine plasma were obtained from Innovative Research Inc. (Southfield USA). DMF, MTBE, acetonitrile, methanol, propanol-2 and ethyl acetate were of gradient grade (Merck, Amsterdam, the Netherlands). Formic acid was pro analysis grade also from Merck. The water was of Milli-Q quality. Milk was purchased at a local grocery.

Instrumentation

The peptides were analyzed on an HP1100 HPLC (Agilent, Amstelveen, the Netherlands) coupled to a Quattro-II triple quadrupole mass spectrometer

(Waters, Etten-Leur, the Netherlands). The polyphenols were analysed on a 1200 HPLC coupled to a 6410 triple quadrupole mass spectrometer (Agilent, Amstelveen, the Netherlands) and the steroidal glycosides on a 2795 HPLC coupled to a Quattro-Premier triple quadrupole mass spectrometer (Waters). Data interpretation was performed using either MassLynx vs 4.0 SP 4 software (Waters) or MassHunter vs B.01.04 software (Agilent).

2.3 Results and discussion

In the ideal situation one single robust automated method should be available for sample preparation applicable to a broad range of compounds. In the real situation this is practically impossible due to the large physico-chemical differences between molecules with varying structures. For instance for SPE a large number of different packing materials are available each designed for a specific group of compounds. Although there is an overlap in efficiency, no cartridge exists suitable for all types of compounds. To get an impression which of the three selected methods performed best for our selected group of compounds three criteria were evaluated: the percentage recovery, the sample clean-up efficiency and the potential for automation.

2.3.1 Solid phase extraction

SPE was performed on the three different groups of compounds. The cartridges used were silica C18, MAX and HLB generally advised for a wide range of applications. Various parameters were tested such as the concentration of the analytes, the composition of the eluting solvent, the volume of the eluting solvent and the sample matrix. The sample matrix was solvent, milk or plasma. The plasma used was human or porcine. The procedure used for conditioning and elution was as advised by the supplier of the cartridges. The regular procedure consisted of conditioning the cartridge with 2 ml of methanol followed by 2 ml of Milli-Q water. Then the sample solution (0.5 ml, diluted to 2 ml with water) was loaded followed by a washing step with 2 ml of a 5% aqueous methanol solution. Elution of the analytes was performed with 2 ml of methanol.

Polyphenols

C18, HLB and MAX SPE cartridges were tested on a mixture of polyphenols in a concentration for each individual compound of 100 ng ml^{-1}. Solvents tested were DMF, methanol, acetonitrile and water in various combinations and ratios, with or without addition of formic acid. Strongly different results were obtained. For example Figure 1 shows the effect of eluting the HLB cartridge with 70% DMF or using 100% acetonitrile. It is obvious that in this

experiment 70% DMF gives much better recoveries for most of the compounds while acetonitrile is very selective. The best results, however, were obtained using a combination of acetonitrile and aqueous 0.1% formic acid in combination with the C18 SPE cartridge.

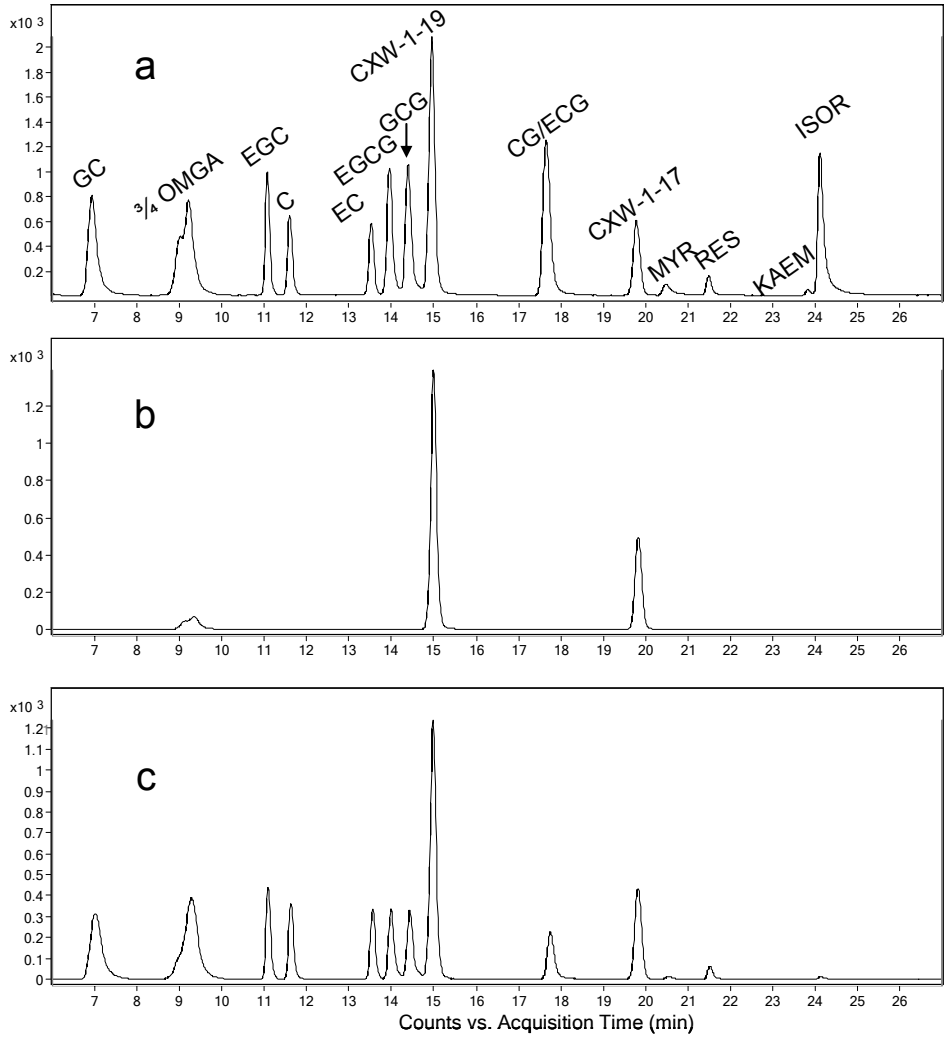

Figure 1 Mixture of polyphenol standards purified with HLB type SPE column, **a** without SPE treatment, **b** with 70% DMF, **c** with 100% acetonitrile.

In Table 1 the recovery of the polyphenols spiked in human plasma and eluted with various concentrations of acetonitrile are given. Myricetin and isorhamnetin were excluded from the table. These compounds showed a poor reproducibility probably due to their instability in solvents. The results clearly show that the percentage of acetonitrile is not very critical. Variation

of the concentration of the polyphenol mixture had no effect on the percentage recovery.

Table 1 Recovery percentages of human plasma spiked with polyphenols at a concentration for each individual compound of 100 ng ml^{-1} using a C18 SPE column at varying concentrations of acetonitrile in 0.1% aqueous formic acid. Myricetin and isorhamnetin are excluded due to their poor reproducibility.

Compound	Percentage acetonitrile (%)						
	40	**50**	**60**	**70**	**80**	**90**	**100**
3/4-OMGA	89	84	82	101	96	99	91
C	70	55	57	77	73	75	55
EGC	89	64	75	98	94	96	70
GC	68	53	54	71	65	68	50
CXW-1-19	94	95	88	104	101	103	100
EC	76	61	63	79	75	77	56
EGCG	95	78	88	106	103	104	77
GCG	90	74	92	109	106	108	77
CG/ECG	94	82	88	101	99	100	74
CXW-1-17	90	98	87	101	100	100	102
RES	104	96	91	115	97	106	93
KAEM	5	25	37	39	51	45	50

Steroidal glycosides

The steroidal glycoside mixture used was an extract from the *Hoodia gordonii* plant. As a typical example of these homologous compounds the structure of one of them is given in Figure 2 [37]. Full structural information on the homologues is given in chapter 7. The elution solvents tested were pure methanol and 80% to 100% of acetonitrile in water. No significant differences in recovery could be observed. For further experiments pure methanol was used as an eluent. In Table 2 the recovery of porcine plasma spiked with the *Hoodia* extract at a concentration for each individual compound of 100 ng ml^{-1} and purified on the three cartridges are displayed. The recoveries of the three cartridges are very similar, with the exception that the recovery of the very hydrophobic H.g.-17 is the highest on the HLB cartridge. The concentration level was varied between 5 and 1000 ng ml^{-1} and no effect on the percentage recovery was observed. Full scan analysis of the three extracts, however, showed that there were large differences between the clean-up efficiency of the C18 cartridge and the other two. In Figure 3 the Total Ion Chromatograms (TIC) of the C18 and the MAX extracts are displayed. Figure 4 displays the reconstructed ion traces of the

metabolites. Only the detected metabolites are listed. The other metabolites were either below the detection level of the system or were not formed. Later experiments on a broader range of steroidal glycosides showed that the HLB cartridge covers a broader hydrophobicity range than the MAX cartridge and is therefore preferred. The HLB and MAX cartridges remove proteins and fat from the plasma sample while the fat-soluble compounds are retained on the cartridge until elution.

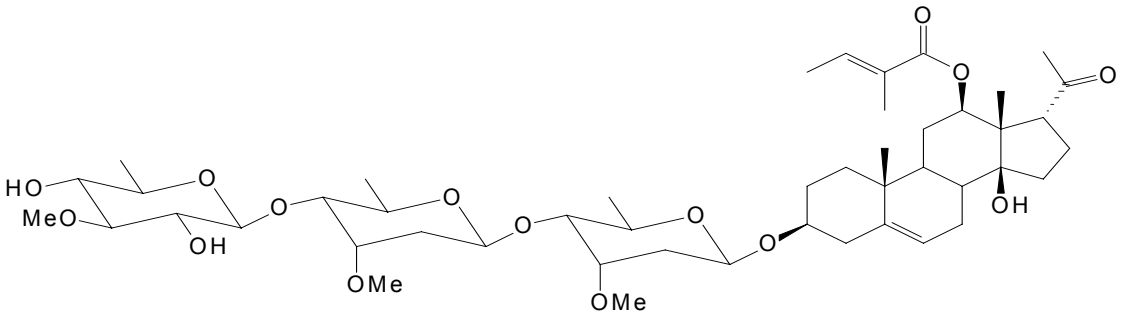

Figure 2 The structure of the steroidal glycoside H.g.-12 identified from a *Hoodia gordonii* extract.

Table 2 Recovery percentages of six *Hoodia gordonii* steroidal glycosides in porcine plasma at a concentration of 100 ng ml^{-1} using three different types of SPE cartridges**.** H.g.-12 consisted of two isomers coded I and II.

compound	Column type		
	C18	MAX	HLB
H.g.-12	83	82	95
Isomer H.g.-12	106	105	111
H.g.-22	99	99	100
H.g.-23	89	91	92
H.g.-24	90	87	97
H.g.-17	81	76	88

Peptides

Milk was spiked with the fermented milk powder in a concentration of 20 mg ml^{-1} and centrifuged for 30 min at 14000 RPM. The supernatant was used for the SPE clean-up. All experiments were carried out on 96 well plates containing different types of SPE material. The SPE materials were MCX, WAX, MAX and WCX. The pre-treatment of the plate was varied from regular to 0.1% phosphoric acid or 0.1% ammonium hydroxide in water. The eluent was varied from methanol to methanol/acetonitrile. The best results were obtained with the MCX material using methanol as eluent. In Table 3 the percentage recovery is given for a selected number of peptides with various length and hydrophobicity using different pre-treatment and eluent on the MCX cartridge. The hydrophilic peptides elute best with methanol, while the more hydrophobic peptides elute best with a combination of methanol and water. VAP is known to be instable in solution which explains

the relatively low recovery. A combination of acetonitrile and methanol, possibly combined with formic acid may further improve the results.

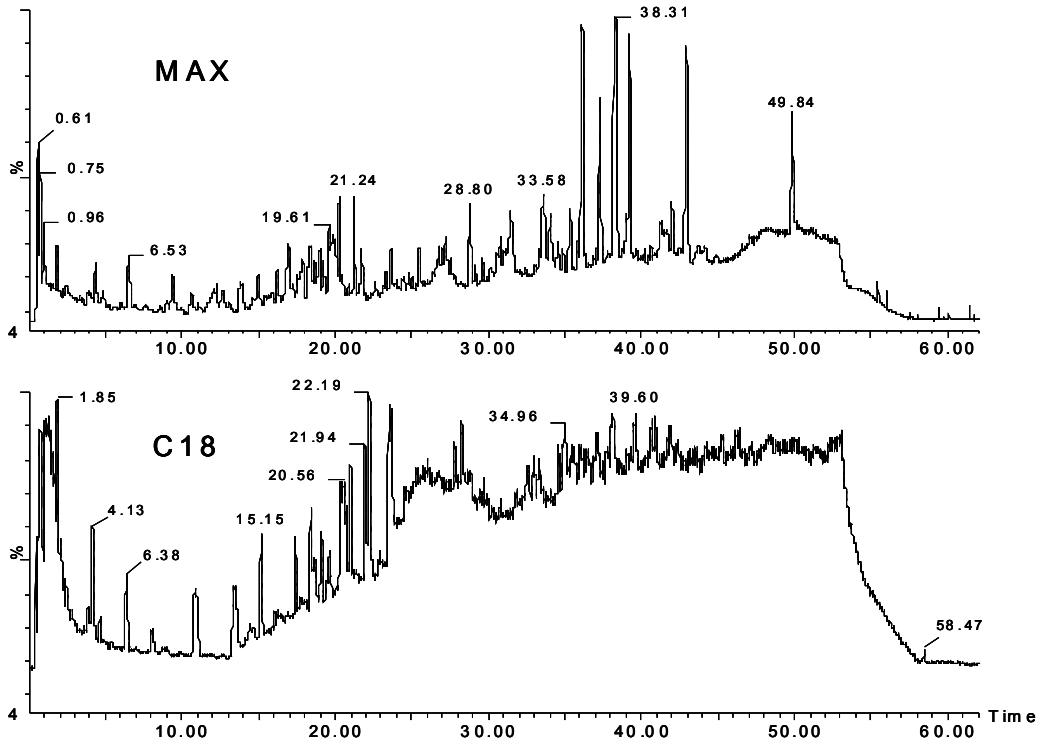

Figure 3 MS-TIC of the full scan LC-MS analysis of porcine plasma spiked with the *Hoodia gordonii* extract at a concentration of 100 ng ml^{-1} purified with the C18 and the MAX type SPE cartridges.

2.3.2 Solid-supported liquid-liquid extraction

In SS-LLE the liquid sample is taken-up by the column material (diatomaceous earth). After a few minutes an immiscible organic solvent (e.g. ethyl acetate or MTBE) is used to extract and elute the analytes from the column while proteins and other matrix materials remain adsorbed to the column material. The milk spiked with peptides was not analyzed with SS-LLE.

Polyphenols

Again the 100 ng ml^{-1} solution in human plasma was used to test the SS-LLE column. Duplicate analyses were performed on the same day and the measurements were performed on five different days covering a time period

of two weeks. The deviation between days varied up to a factor of 2 while the deviation of measurements carried out on the same day was only slightly less. In Table 4 the recovery in duplicate analyses carried out on the same day is given. Although there is a deviation between the duplicate measurements the results clearly show that this method covers a broad range of compounds. Probably the time used for the different steps of the procedure plays an important role in the recovery. It is hypothesized, for instance, that the time the eluent is in contact with the adsorbent (staionary) phase is sufficiently long to allow the transfer of the analytes from the aqueous to the organic phase.

Table 3 Percentage recovery of the MCX extraction of milk spiked with fermented milk powder. The SPE pre-treatment was 1: regular, elution with methanol, 2: 0.1% phosphoric acid, elution with methanol, 3: regular, elution with a mixture of methanol/acetonitrile 1/1 (v/v), 4: 0.1% ammonium hydroxide, elution with methanol.

	Percentage Recovery			
	Regular	H_3PO_4	regular	NH_4OH
Peptide	**MeOH**	**MeOH**	**MeOH/ACN**	**MeOH**
VP	65	40	20	0.5
VAP	54	26	15	0.3
VPP	60	35	28	0.5
LPP	90	43	58	0.9
IPP	84	46	80	0.8
FPP	65	48	83	1.6
LPVP	72	52	92	2.7
TPVVVPP	73	60	89	2.5
DKEITP	32	48	93	0

Table 4 Percentage recovery of a within-day duplicate analysis of SS-LLE of polyphenols spiked in human plasma at a concentration of 100 ng ml^{-1}.

	percentage recovery	
Compound	**exp 1**	**exp 2**
3/4-OMGA	93	99
C	54	35
EGC	53	33
GC	46	29
CXW-1-19	56	65
EC	50	30
EGCG	56	43
GCG	80	48
CG.ECG	71	46
CXW-1-17	88	96
MYR	95	111
RES	38	31
KAEM	58	66

Steroidal glycosides

For this experiment spiked porcine plasma was analyzed with concentrations of 25, 50, 100, 500 and 1000 ng ml^{-1} of the *Hoodia* extract. The extraction solvents tested were ethyl acetate, MTBE and a mixture of MTBE and 2-propanol in a ratio of 80/20 (v/v). The incubation time before elution with organic solvent varied between 5, 10 and 15 min. In Table 5 the recoveries of duplicate extractions of the *Hoodia* extract in porcine plasma in various concentrations are given. Pure MTBE was used for the extraction of the analytes. Although less pronounced than for the polypgenol analyses, still a relatively large deviation between the duplicate measurements is observed.

Table 5 Percentage recoveries of duplicate experiments of porcine plasma spiked with *Hoodia gordonii* extract at different concentration levels using solid-supported liquid/liquid extraction cartridges (SS-LLE).

Extract concen-tration in ng ml^{-1}	Percentage recovery											
	H.g.-12		H.g.-22		H.g.-21		H.g.-23		H.g.-24		H.g.-17	
	exp 1	exp 2	exp 1	exp 2	exp 1	exp 2	exp 1	exp 2	exp 1	exp 2	exp 1	exp 2
25	72	73	65	66	67	68	68	68	68	59	63	54
50	66	80	61	75	65	77	60	74	60	67	63	62
100	85	66	78	61	80	63	79	61	68	53	58	42
500	74	84	70	83	70	82	73	91	69	79	56	64
1000	83	72	91	79	91	79	94	84	78	75	66	64

2.3.3 Monotrap

The Monotrap is a new technique based on adsorption of analytes to carbon. The principle is the same as the octanol-water partitioning. Hydrophilic compounds have a preference for the aqueous phase whereas the hydrophobic compounds will be absorbed by the carbon disc. The reaction follows an equilibrium and therefore the contact time of the disc with the sample solution plays an important role. The major difference between Monotrap and the other two methods is that the Monotrap works according to an equilibrium and only part of the analyte will be adsorbed, while the other two methods are exhaustive extraction methods. The procedure given by the supplier is as follows: a small carbon disc is introduced into 3 ml of the sample solution and shaken for several minutes. After this the disc is washed with 2 ml of Milli-Q water and the analytes are then extracted from the disc with 500 µl of an organic modifier using 10 min of ultrasonic treatment. The experiments were limited to polyphenols and steroidal glycosides.

Polyphenols

The Monotrap extraction of polyphenols with a concentration of 100 ng ml^{-1} in water was not successful. This could be expected since the polyphenols selected for the experiments are all very hydrophilic. Only isorhamnetin could be extracted but only with a very low percentage of recovery. No further experiments were performed.

Steroidal glycosides

In the steroidal glycoside mixture (100 ng ml^{-1} in water) the distribution of the hydrophobicity is much wider than with the polyphenols which are mainly hydrophilic. In Figure 5 the adsorption time curve of 6 steroidal glycosides

with increasing hydrophobicity is given. The curves clearly show that the percentage adsorption increases with increasing hydrophobicity. The supplier advises an adsorption time of 20 min. The results further indicate that the percentage adsorption increases with increasing incubation times. After washing with Milli-Q water the disc was eluted with methanol. Figure 6 shows the percentage desorbed material extracted from the disc. The results show that even in the best case H.g.-17 87% of the material adsorbed on the disc remains on the disc even after 1 hour of extraction. Different types of solvents such as MTBE, toluene and methanol and combinations were tested without significant changes in desorption behaviour (data not shown). This combined with the limited adsorption efficiency on the disc means that this method is not very suited for quantification of steroidal glycosides in complex matrices. For qualitative analyses, however, the method is very fast and simple, providing that the concentration of the analytes is sufficiently high.

2.4 Conclusions

Three types of sample preparation methods were tested for their applicability on three different compound classes. The extraction methods were SPE, SS-LLE and Monotrap. The compound classes tested were polyphenols, steroidal glycosides and peptides. The aim of the experiments was to increase the sample throughput e.g. by automation preferably with one single method which could be applied to all groups of compounds.

The best results were obtained with SPE. This method covers the broadest range of compounds with varying hydrophobicity, has the highest percentage of recovery, the best reproducibility and can be fully automated. One drawback is that there is not a single cartridge which is applicable to all groups of compounds. For each class of compounds different cartridges must be tested to obtain optimal results. The SS-LLE method showed promising results for steroidal glycosides and for polyphenols. Peptides were not tested with this method. The major drawback was that a relatively low in-between day repeatability was observed. Automation can be performed by using the same instrumentation as used for SPE. The Monotrap showed promising results for the various groups of compounds. The adsorption and desorption time plays an important role with respect to the percentage recovery. This method seems in particular applicable for qualitative analyses of involatile and volatile compounds in complex mixtures e.g. for profiling or screening purposes.

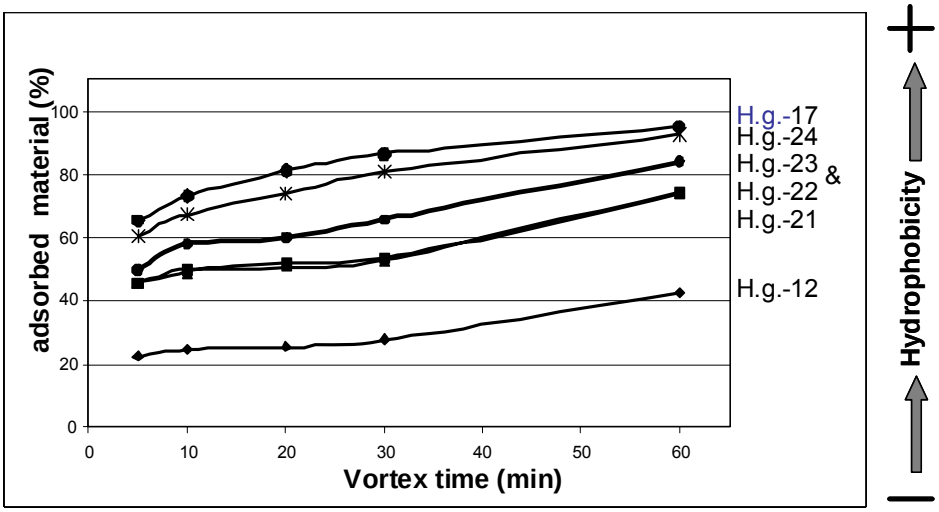

Figure 5 Time curve of the percentage adsorption of Hoodia compounds on the Monotrap disc.

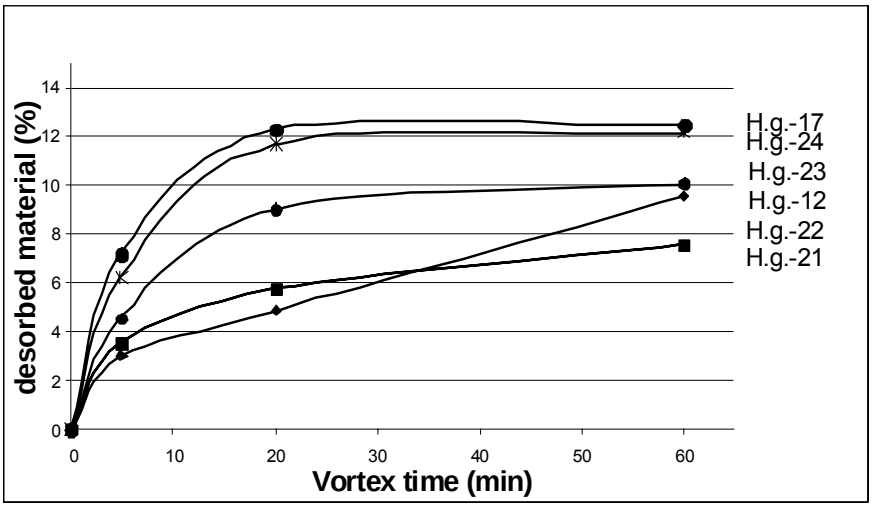

Figure 6 The percentage of steroidal glycosides extracted from the disc.

This study does not claim to get full insight into the applicability of the methods tested, or to get an overview of all possible sample preparation methods and their field of application. It shows merely the applicability of three types of methods on three types of compound classes and clearly demonstrates the advantages and disadvantages of the different methodes within this (limited) context. It should be obvious that the analytical question and type of compound still play an important role in the selection of the sample preparation method. To increase the sample throughput for our field of application automated SPE seems to be the best choice.

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3 Development of an at-line method for the identification of angiotensin I-inhibiting peptides in protein hydrolysates'

Summary
Knowledge of the structure of bioactive compounds is essential to understand the physiological mechanism of the compounds in the human and animal body. In this chapter a method for the identification of small, antihypertensive peptides is described. A fast at-line method was developed for the identification of ACE-inhibiting (ACEI) peptides in protein hydrolysates. The method consists of activity measurements of fractions collected from a two-dimensional HPLC fractionation of the peptide mixture followed by MS identification of the peptides in the inhibiting fractions. The assay is based on the inhibiting effect of ACEI peptides on the hydrolytic scission of the substrate Hippuryl-Histidyl-Leucine (HHL) during the ACE-catalyzed hydrolysis reaction. A fast LC method was developed for the quantification of Hippuric acid (H) and HHL, allowing a large number of fractions to be analyzed within a reasonable time period. The method is sensitive and requires only standard laboratory equipment. The limit of detection is 0.34 μM for the known ACEI peptide IPP. This is sufficiently sensitive for the identification of only moderately active peptides and/or ACEI peptides present at low concentrations. The relative standard deviation of the inhibition assay was 12% measured over a time period of 2 months. The IC50 value of IPP measured with the assay was 5.6 μM, which is comparable to the values of 5 μM and 5.15 μM reported in literature for the standard Matsui method. The assay was successfully applied in the identification of ACEI peptides in enzymatically hydrolysed caseinate samples. Two new, not earlier published ACEI peptides were identified; MAP (β-casein f102-104) and ITP (α-s2-casein f119-121) with IC50 values of 3.8 μM and 50 μM, respectively.

3.1 Introduction

Biologically active peptides play an important role in a large number of physiological processes in the human body such as immune stimulation, blood pressure regulation and cardiovascular functioning [1]. Hence peptides are attractive ingredients for so-called functional food products. In the past a variety of food-grade protein-containing sources, ranging from fish and meat to milk and maize have been investigated as potential sources for biologically active peptides [*e.g*. 2,3]. One of the fields that has been extensively studied is the use of small peptides to reduce hypertension [*e.g*. 4,5]. In the regulation of blood pressure certain small peptides are known to inhibit the formation of the potent vasoconstrictor angiotensin II (AT2) from angiotensin I (AT1) by inhibiting the angiotensin-converting enzyme (ACE). In order to get insight in the physiological behaviour of ACE inhibiting peptides, as well as to develop improved functional food products, knowledge on the identity and generation of these peptides is crucial.

The identification of active peptides from complex natural ACE-inhibiting food products or ingredients is a laborious task. Methods to do so generally rely on isolation of the active peptide(s) from the complex material using various sequential purification processes based on chromatography or electrophoresis, followed by identification of the peptides in the active fractions with *e.g.* Edman sequencing or mass spectrometry [*e.g.* 6]. Important aspects to consider when setting up a method aimed at the identification of active peptides in a complex sample include the selectivity and sensitivity of the activity assay used, as well as the cost of substrate and reagents and the complexity of the required instrumentation. Moreover, the assay should preferably mimic the processes in the human body as closely as possible to minimise *in-vitro* to *in-vivo* differences. In order to improve the sensitivity and selectivity of the ACE assay many different substrates and quantification methods have been described. Popular substrates for the ACE assay include HHL [7] and the internally quenched fluorescent substrate abz-FRK(dnp)P-OH [8] or N-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine (FAPGG) [9]. In our view the HHL substrate is attractive because it shows the closest resemblance to AT1, the physiological substrate. Techniques to monitor the conversion rate of HHL include chromatographic or electrophoretic quantification of HHL or its products H and HL [10,11], or colorimetric or fluorimetric detection after OPA [12] or TNBS derivatisation [13]. Chromatographic quantification minimises errors due to background peptides but clearly is much slower than colorimetric methods. The best performance in terms of sensitivity and selectivity is obtained with the substrate abz-FRK(dnp)P-OH and fluorescence detection [9]. A potential drawback here is that the substrate is rather dissimilar from the physiological

substrate AT1. All work aiming at the identification of ACE active ingredients published in literature so far is based on repeated off-line fractionation, activity measurement and identification. A notable exception is the work by van Elswijk et al. [8]. In their work these authors apply an elegant on-line system consisting of a highly efficient HPLC separation, a quenched fluorescent substrate conversion assay and mass spectrometric identification. The ACEI peptides are separated by HPLC. A small part of the column effluent is directed towards the mass spectrometer for identification of the detected peptides whereas the major fraction is sent to a continuous flow ACE assay providing a continuous read-out of the ACEI activity. The method is sensitive and does not require laborious fractionation steps. Losses of peptides due to adsorption are minimised as no collection vials are used. The major drawback of this method is, though, that sophisticated dedicated equipment is required and again the dissimilarity of the substrate from AT1.

In this chapter we present a sensitive and selective at-line method for the identification of ACE-inhibiting peptides in protein hydrolysates. HPLC followed by fraction collection is applied to fractionate the complex peptide mixtures. Activity measurements are based on the popular HHL-based Matsui assay as described by Cushman and Cheung [6]. Optimisation of this assay is discussed. The applicability of very fast LC-UV for quantification of the substrate conversion rate is demonstrated. Exact mass measurement is applied for the identification of the peptides in the fractions that show ACE-inhibiting activity. Statistical information is given on the assay and the limitations of the method are discussed. Finally, the applicability of the assay is demonstrated by the identification of the peptides responsible for the ACE inhibition of an enzymatically hydrolysed caseinate.

3.2 Experimental

3.2.1 Materials and methods

Chemicals

Model peptides were obtained from different suppliers. The peptide IPP (purity >98%) was purchased from Bachem (Bubendorf, Switzerland). The peptides MAP and ITP were synthesized at the University of Utrecht (Faculty of beta-sciences, departement of chemistry, Utrecht, the Netherlands). ACE and HHL were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). All other chemicals were of high purity and were purchased from Merck (Amsterdam, the Netherlands). Enzymatically hydrolysed caseinate was kindly provided by DSM Food Specialties (Delft, the Netherlands).

Instrumentation

All peptide fractionations and identifications were performed on a Waters Alliance 2795 HT HPLC coupled to a Micromass QTOF-Ultima hybrid Time-of-Flight mass spectrometer equipped with the lock spray option for accurate mass determination (Waters, Almere, the Netherlands).

Analytical separation

1 ml of water/acetonitrile 90/10 (v/v) was added to 10 mg of casein hydrolysate. The mixture was vortexed for 1 min, treated ultrasonically at room temperature for 30 min and was centrifuged at 14000 RPM. 50 µl of the supernatant was injected onto an 150 x 4.6 mm Inertsil 5 ODS3 analytical column with a particle size of 5 µm (Varian, Middelburg, the Netherlands), equipped with a reversed phase C8 guard column (Waters, Etten-Leur, the Netherlands). Mobile phase A consisted of a 0.1% TFA solution in Milli-Q water. Mobile phase B consisted of a 0.1% TFA solution in acetonitrile. The initial eluent composition was 100% A. The eluent was kept at 100% A for 5 min. After this a linear gradient was started to 5% B in 10 min, followed by a linear gradient to 70% B in 40 min. Then a linear gradient was used to 90% B in 5 min and the eluent composition was kept at 90% B for another 5 min. Finally the eluent was reduced to 100% A in 1 min and the system was allowed to re-equilibrate for 14 min. The total run time was 80 min. The eluent flow rate was 1.0 ml min^{-1} and the column temperature was set at 60 °C. UV detection was performed at 215 and 254 nm. 90% of the eluent flow was split to the fraction collector, while the remaining 10% was introduced into the MS-source. The home-made splitter consisted of a zero dead volume metal T-piece (Valco Benelux, Nieuw Vennep, the Netherlands) of which one line was coupled to a restriction consisting of a 10 cm piece of 75 µm I.D. PEEK tubing coupled to the tubing of the fraction collector. The other line, consisting of 40 cm of the same PEEK tubing, was connected to the MS-probe. Fractions of 900 µl each were collected from 0 to 65 min with interval times of 1 min in a polypropylene 1.4 ml 96-well plate (Micronic B.V., Lelystad, the Netherlands). The fractions were neutralized with 180 µl of a 0.15% aqueous ammonium hydroxide solution. The solvent was evaporated to dryness under nitrogen at 40 °C using an Ultravap 96-well evaporation device (Porvair, Shepperton, UK). The residues were reconstituted in 80 µl of Milli-Q water and vortexed for 1 min. Then the content of each well was equally divided over two standard polypropylene 300 µl 96-well plates (Nunc, Roskilde, Denmark). The plates were stored at –20 °C until further use. One plate was used to determine the ACE-inhibition of the fractions. For active fractions the corresponding well of the other plate was used for further fractionation on a 150 x 2.1 mm Biosuite™ column with a particle size of 3 µm *(*Waters, Etten-Leur, the Netherlands). The separation was performed at 40 °C and a flow rate of 0.2 ml min^{-1} using mobile phase C

consisting of a 0.1% formic acid (FA) solution in Milli-Q water and D consisting of 0.1% FA in methanol. The initial eluent composition was 100% C. A linear gradient was used to 70% D in 30 min, followed by a linear gradient to 90% B in 5 min. The eluent was reduced to 100% C in 1 min and the system was allowed to equilibrate for 14 min. The total run time was 50 min. From the Biosuite™ column fractions were collected with an interval time of 10 s in a polypropylene 300 µl 96-well plate. These fractions were than neutralized with 8 µl of aqueous 0.15% ammonium hydroxide solution. After neutralisation the fractions were dried and reconstituted in 50 µl of Milli-Q water using the equipment and conditions described above. 40 µl of each fraction was pipetted into another 300 µl polypropylene 96 well plate which was used for the at-line assay. The plate with the remaining 10 µl of each fraction was used for structural identification of the peptides in the fractions showing ACEI activity. Both plates were stored at –20 °C until further use.

At-line Matsui assay

ACEI activities of the collected fractions were measured in a 96 well plate with chromatographic read-out. The initial conditions used for the assay were as published by Cushman and Cheung [6]. The optimum ratio of HHL and ACE, the incubation time and temperature as well as the reaction time and temperature were determined experimentally (see below).

Fast-LC analysis

The activity of the ACE preparation was determined from the residual levels of the HHL substrate and the level of the product H formed. HHL and H were determined using fast-LC performed on a 25 x 4.6 mm Chromolith RP 18 Flash column (Merck, Darmstadt, Germany), equipped with a 10 x 4.6 mm guard column from the same supplier. The mobile phase composition, flow rate, and column temperature were optimized prior to starting the ACE-inhibition studies. In all experiments the injection volume was 30 µl. UV detection of HHL and H was performed at 280 nm.

Calculation of the percentage inhibition

The percentage inhibition of each fraction was calculated from the conversion of HHL to H relative to a blank measurement according to the following equation:

$I\ (\%) = (Cb - Cf)/Cb \times 100$

In this equation Cb represents the percentage conversion of HHL to H for a blank water measurement and Cf the percentage conversion observed for a fraction. The percentage conversion was calculated according to the equation:

C(%) = H/(H + HHL) x 100

Here H and HHL represent the measured peak areas of the two compounds.

Structural identification of ACEI peptides

For the identification of the peptides the remaining 10 µl fractions from the Biosuite™ column were diluted to 50 µl with an aqueous 0.1% TFA solution. 40 µl of each fraction was injected onto the LC-MS system. The separation was performed on the Biosuite™ column using the gradient described above. The source and desolvation temperatures were 100 °C and 300 °C, respectively. The cone and desolvation gas flows were 100 l h^{-1} and 800 l h^{-1}. The capillary voltage was 4 kV and the cone voltage 35 V. The collision energy was 10 eV. The collision gas was argon and the analyzer pressure was 4e-5 mbar. In the MS-MS mode the collision voltage was increased to 25 eV. In the lock spray probe a mixture of PEG 300, PEG 600 and PEG 1000 (Sigma Chemicals, St Louis, USA) was used in a 10 mM ammonium acetate solution in methanol/water 1/1 (v/v) at concentrations of 1, 2 and 4 µg ml^{-1}, respectively. The reference flow was 5 µl min^{-1}. Spectra were recorded at a resolution of 10,000.

3.3 Results and discussion

For the successful identification of ACEI peptides in complex mixtures the performance of the inhibition assay is crucial, i.e. it has to be very sensitive, fast and insensible to interferences. Evidently the actual performance of the assay strongly depends on the read-out method used to quantify the conversion of the substrate. Colorimetric or fluorimetric methods are fast, but have a low sensitivity and the presence of interfering substances often results in high blank values. Chromatographic quantification of the substrate HHL and/or the H and HL formed can overcome these problems, but is much slower. Deployment of a very fast chromatographic method can reduce this disadvantage significantly.

3.3.1 Compatibility and miniaturization of the method

The determination of the ACEI activity of the chromatographic fractions is a crucial step in the identification of the actives. Before this measurement can be performed, the conditions of the fractions, e.g. pH and organic solvent level, have to be adjusted. In the current assay even low concentrations of organic solvents negatively influence the sensitivity. In the on-line ACEI method described by van Elswijk [8], for example, the eluent is diluted using an inverse gradient to keep the methanol concentration at a constant level of 10%. The advantage of the at-line assay described in the present article is

that neutralization and solvent evaporation are carried out prior to the ACEI assay. Due to this all types of organic modifiers and high modifier concentrations are allowed in the chromatographic fractionation step. Moreover, the sensitivity of the assay is positively influenced by the concentration step. The only disadvantage of course is that the procedure becomes slightly more laborious. By performing the fractionations and reactions in microtiter plate format and using well plate evaporators, the analysis time per sample can be reduced strongly.

3.3.2 ACEI assay

Optimization of the Matsui ACEI assay was performed using a solution of the known inhibitor IPP in Milli-Q water at a concentration of 1 µg g^{-1}. Because the peptide concentrations in the HPLC fractions are relatively low, the ACEI assay should be as sensitive as possible. The performance of the assay in terms of sensitivity and speed strongly depends on the experimental conditions. The concentrations of the enzyme (ACE) and substrate (HHL), the incubation time and temperature as well as the reaction time and temperature were optimized starting from the values given by Cushman and Cheung [6]. The best results were obtained when 40 µl of the IPP solution was mixed with 25 µl of a 33.2 mU ml^{-1} ACE solution in phosphate buffered saline (PBS) pH 7.4 with a total chloride concentration of 400 mM. The optimum incubation time and temperature were 10 min and 37 °C, respectively. Variations in the ratio of ACE and HHL showed that the best results were obtained when 15 µl of a 0.35 mM HHL solution in PBS was added to each well. The highest percentage of conversion of HHL to H was reached when the mixture was allowed to react for 60 min at 50 °C. In order to stop the reaction the well plate was cooled in melting ice and stored at 4 °C prior to the fast-LC measurement. Using this ACE/HHL ratio, a conversion of HHL to H of 70% was obtained in the absence of inhibiting peptides.

3.3.3 Fast-LC quantification of HHL and its conversion products

To keep the overall run time of the ACEI determination of the chromatographic fractions at an acceptable level, the run time of the chromatographic separation should be as short as possible. For instance in the procedure described by Meng [9] the analysis time is 12 min which would result in a total run time of 12 hours when 60 fractions were to be analyzed. In order to reduce the total run time a faster chromatographic method was developed. A generic route towards faster LC is the use of columns packed with smaller particles. A drawback of the use of this type of columns, however, is the very high inlet pressure required. The recently introduced monolythic columns eliminate most of this drawback while still providing a very high separation speed. A Chromolith Flash RP18 column was tested for

its ability to give a fast separation of HHL, HL and H. Various mobile phase compositions, temperatures and flow rates were tested with the aim to reach adequate separation of HHL and H from each other and from interfering UV adsorbing amino acids or peptides in the shortest possible time. HL does not respond at the wavelength used and hence does not interfere with the measurement. Gradient separations were not investigated as these will necessitate the use of column re-equilibration at the end of the run. The best compromise between separation and speed was obtained for a mobile phase consisting of 0.1% TFA in water/acetonitrile 85/15 (v/v) at a flow rate of 2 ml min^{-1} and a column temperature of 40 °C. The analysis time of each fraction using these conditions is 1.5 min, resulting in a total run time of 90 min when 60 fractions are to be analyzed. The analysis time could even be reduced to 15 s when an eluent flow of 5 ml min^{-1} was used at a water/acetonitrile ratio of 90/10 (v/v). At these conditions, however, it is no longer possible to separate H from some UV-active amino acids and/or peptides present in some fractions.

A typical example of an ACEI measurement is given in Figure 1. This figure shows the analysis of part of the fractions collected from the analytical ODS3 column after an injection of hydrolysed caseinate. The peaks representing H and HHL are marked with a circle and a triangle, respectively. Clearly the HHL/H peak intensity ratios vary and provide a measure for ACEI activity in the hydrolysed caseinate. In Figure 2 the calculated inhibition versus the fraction number is graphically displayed. Obviously fractions 24 - 26 contain one or more ACEI compounds.

3.3.4 Assay sensitivity and reproducibility

In order to test the sensitivity of the at-line assay the inhibition of the known ACE inhibitor IPP was determined at concentrations varying from 0 to 2.24 µg ml^{-1}. Duplicate measurements were performed for each concentration. The results are graphically displayed in Figure 3. The limit of detection of the assay was found to be approximately 0.34 µM for IPP. At this concentration the inhibition is 5.8% and well above the background noise of the measurement of 2%. The IC50 value of IPP was calculated to be 5.6 µM. This value is in good agreement with the values of 5 µM to 5.15 µM reported in literature [5,17]. For the determination of the reproducibility the IC50 value of IPP was measured 8 times covering a time period of 2 months. The averaged IC50 value was 5.6 µM with a standard deviation of approximately 12%.

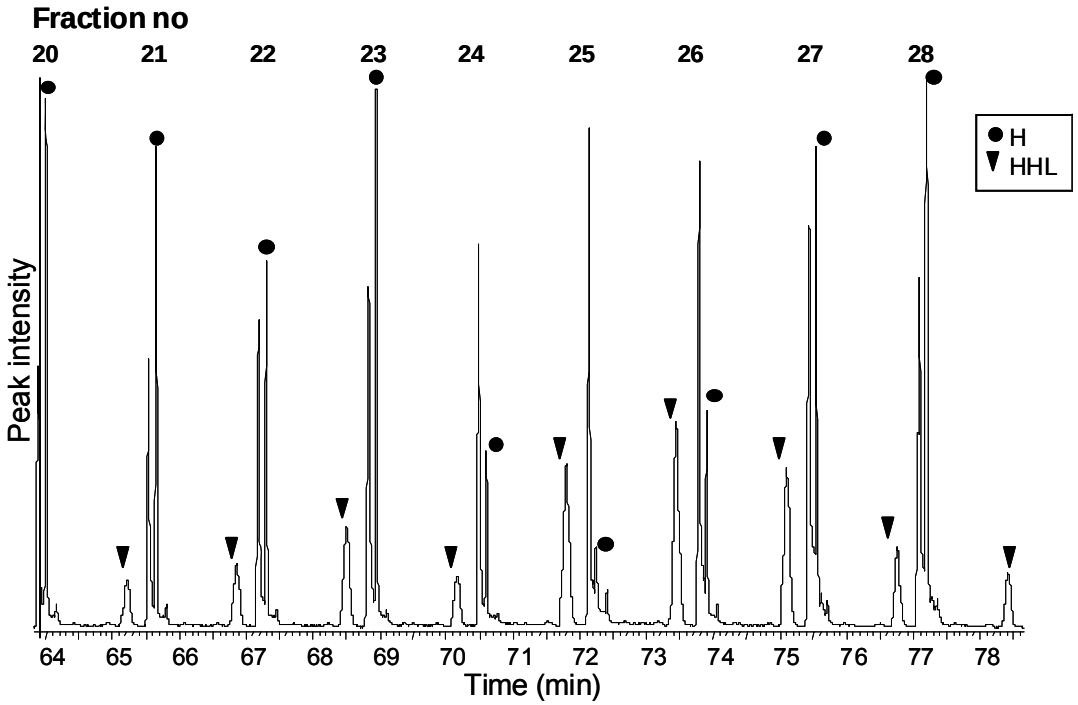


Figure 1 Fast-LC chromatogram of nine subsequent activity measurements of HPLC-fractions of a hydrolysed caseinate.

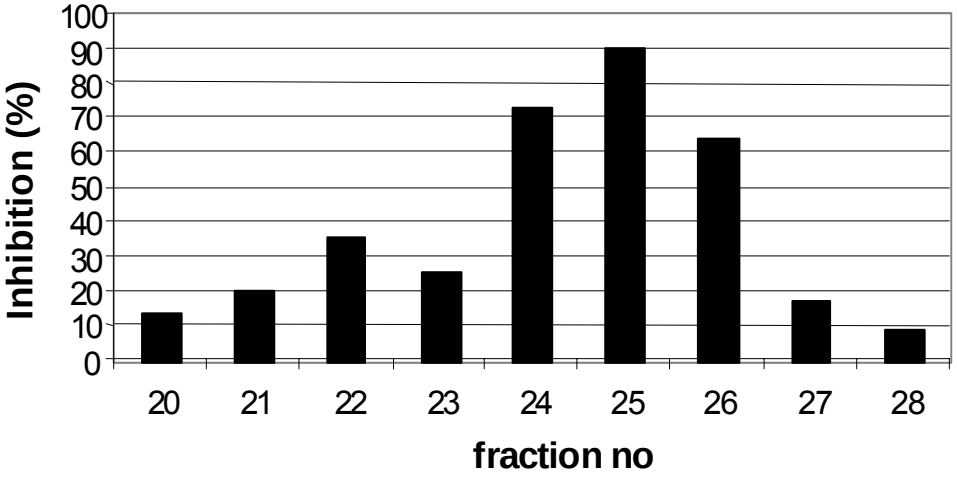
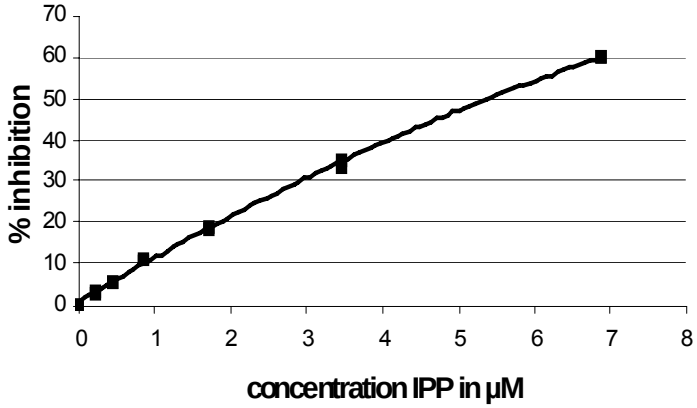


Figure 2 Activity profile derived from the data shown in Figure 1.

Figure 3 Graphical display of the inhibition of IPP at different concentrations.



3.3.5 Principle of the at-line assay

The principle of the full procedure for the identification of ACEI peptides is schematically displayed in Figure 4. In order to get an impression of the distribution of the activity over the various peptides in the sample, the sample is first injected onto the ODS3 octadecyl-modified column using the water/acetonitrile gradient.

Fractions are collected in a 96-well plate with interval times of 1 min. Half of the volume of each fraction is pipetted into a second 96-well plate for further analysis on the Biosuite™ column. The other half is than used to measure the ACEI activity for all fractions. Subsequently the fractions showing more than 5% inhibition are injected onto the Biosuite™ column using the water/methanol gradient. Fractions are collected in a third 96-well plate now with an interval time of 10 s. Part of the volume of each fraction is pipetted into a fourth well plate. The third and fourth 96-well plates are used to create a more detailed activity profile and to determine the molecular ions of the peptides in the chromatographic peaks with increased activity. Finally MS-MS is used for the identification of the peptides in the active sub-fractions.

3.3.6 Hydrolysed caseinate

In order to evaluate the applicability of the newly developed approach for real life samples, a hydrolysed caseinate was studied. 20 µl of a 10 mg ml^{-1} sample solution was injected onto the analytical ODS3 column and analysed using HPLC-UV-MS. In Figure 5A the UV trace at 215 nm and the MS-Total Ion Current (TIC) are given. The peptide distribution of the sample is obviously very complex. Many of the chromatographic peaks show more than one ion, each representing a different peptide. In Figure 5B the activity profile of the sample determined with the Matsui assay is given. The activity

of the product is clearly distributed over a large number of peptides. In the region between 13 and 18 min, however, a clear increase can be seen.

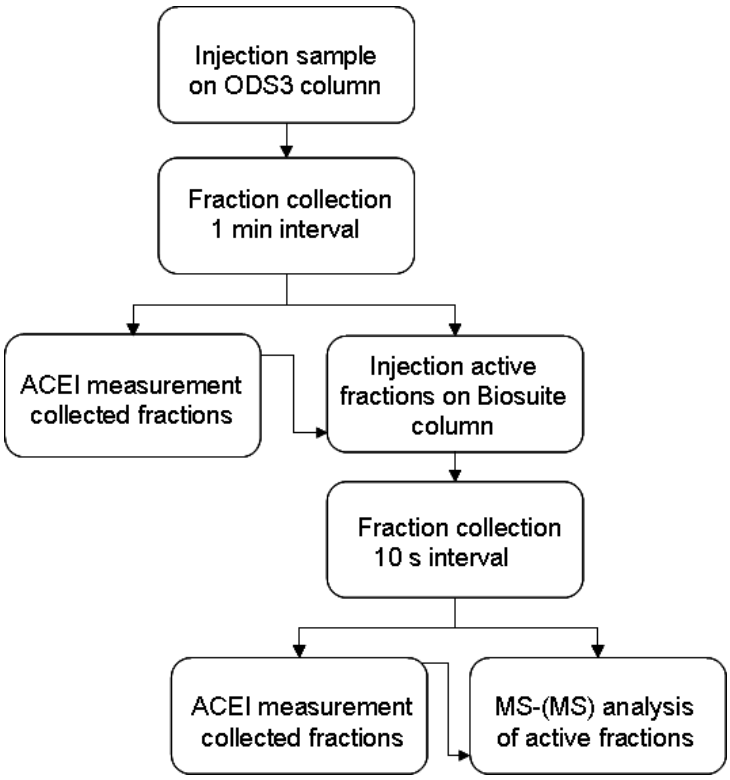


Figure 4 Schematic principle of the at-line method for the determination of ACE inhibiting peptides in complex mixtures.

The full scanning MS analysis indicates that the activity of the fractions 15 to 17 can be explained from the presence of the known ACEI peptides IPP and LPP in the sample [22]. These two peptides, with IC50 values of 5 μM and 9 μM, respectively, are known to be present in hydrolysed caseinate [5,18]. In order to confirm this, the hydrolysed caseinate was injected again, now collecting fractions between 13 and 20 min with interval times of 10 s. The ACEI activity of each fraction was determined and the activity profile was compared to the MS-traces of the detected molecular ions. The result of the analysis is given in Figure 6. For display purposes the peak height of the MS-traces was normalised to the measured activity of the peptide. This figure clearly shows that the profiles of the $[M+H]^+$ ion *m/z* 326.2 of IPP and LPP cover the activity of the peaks at 15.3 min and 16.7 min.

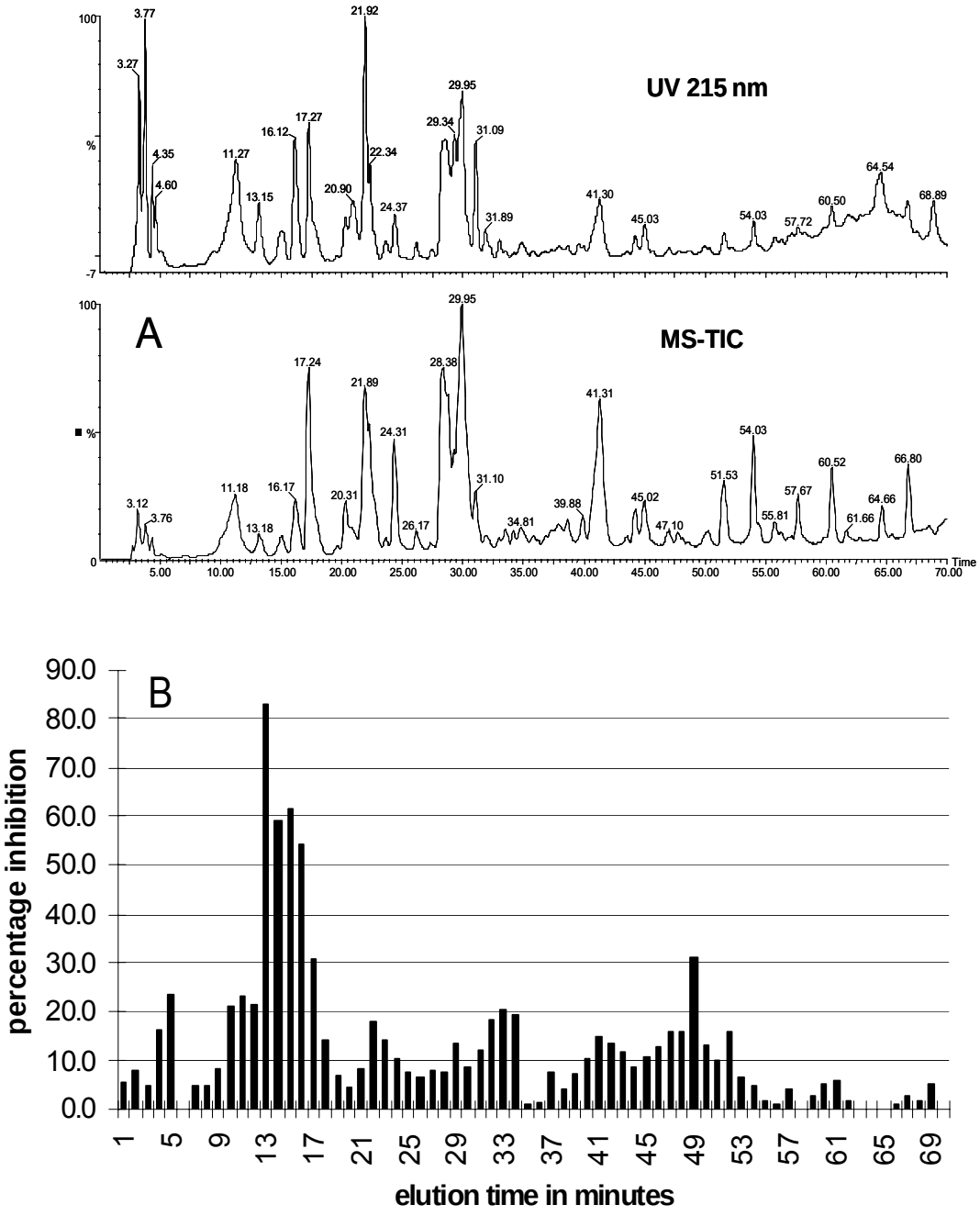


Figure 5 A) MS-TIC and UV (215 nm) chromatogram of a hydrolysed caseinate. **B)** Activity profile of hydrolysed caseinate analysed on the ODS3 column (Interval time = 1 min).

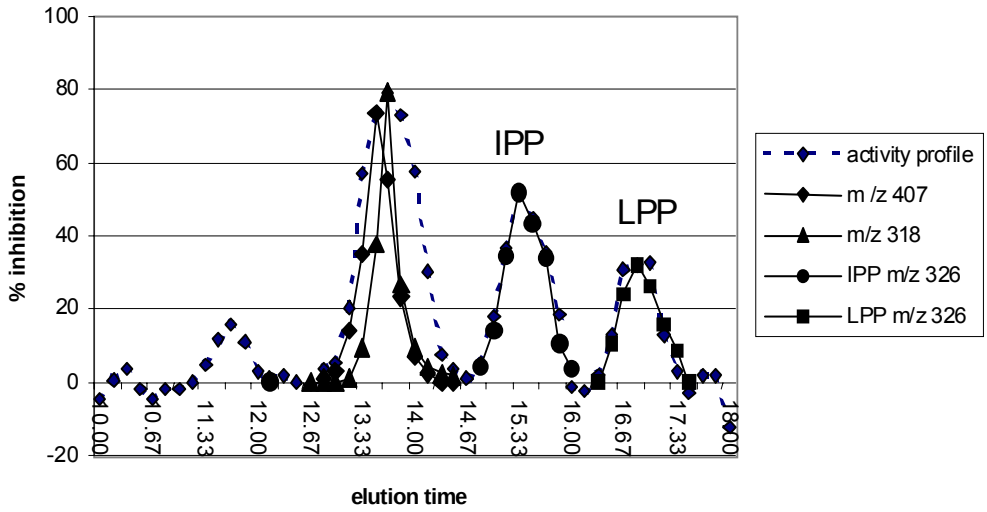


Figure 6 Detailed activity profile and identified molecular ions of hydrolysed caseinate.

The identity of the peptides that are responsible for the activity at 13 min to 14 min (fraction 13) is unknown. The spectra of the fractions that show a positive inhibition (from Figure 5 (B)) are very complex and represent a mixture of peptides. As an example the spectrum of the fraction collected at 13 min is given in Figure 7. The spectrum at 14 min contained largely the same ions, indicating that the peptides eluted partly in two fractions. A search of the most intense molecular ions present in the two fractions in a

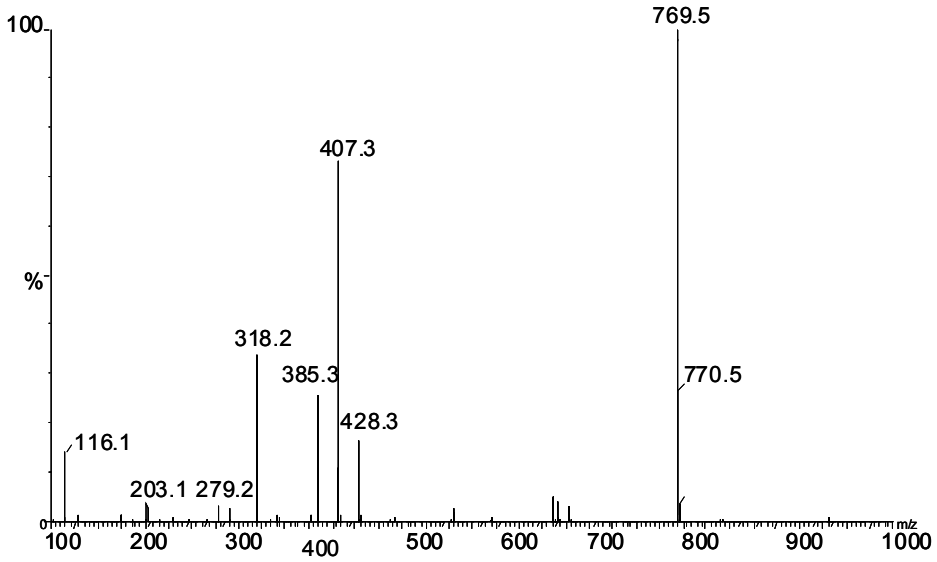


Figure 7 MS spectrum of fraction 13 on the ODS3 column.

home-created database of published ACEI peptides did not reveal the identity of the active peptides in these fractions. Only two peptides, with $[M+H]^+$ ions *m/z* 318 and *m/z* 407 from the spectrum of the fraction collected at 13 min match the activity profile of the peak eluting at 13.5 min (Figure 6). To determine which of the two peptides was responsible for the ACEI activity measured at 13.5 min, the fractions 13 and 14 were combined and injected on the analytical Biosuite™ column. Fractions were again collected with interval times of 10 s. Each fraction was split and one part was used to measure the ACEI activity, while the other part was used for later identification of the peptides. In Figure 8 the activity profile and the MS-TIC of fraction 13/14 are displayed. On the Biosuite™ column the compounds with the molecular ions *m/z* 407 and *m/z* 318 are now fully separated. The results clearly show that the peptide with the molecular ion *m/z* 318 is the source of the measured activity. For the identification of the ion at *m/z* 318 the exact mass was determined and the product-ion MS-MS spectrum was recorded. The exact mass of the ion was found to be 318.1467 Da. A library search of this mass using a search window of 0.07 Da in the spectra of the bovine proteins, α-s1-, α-s2-, β-, and κ-casein revealed only one hit, the tripeptide methionyl-alaninyl-proline (MAP) originating from β-casein f102-104 with a mass error of – 6.6 ppm. The MS-MS spectrum of the ion at *m/z* 318 was recorded and compared to the theoretical fragmentation pattern as described by Biemann and Roepstorff [19,20,21]. The MS-MS spectrum and the assigned fragments are given in Figure 9.

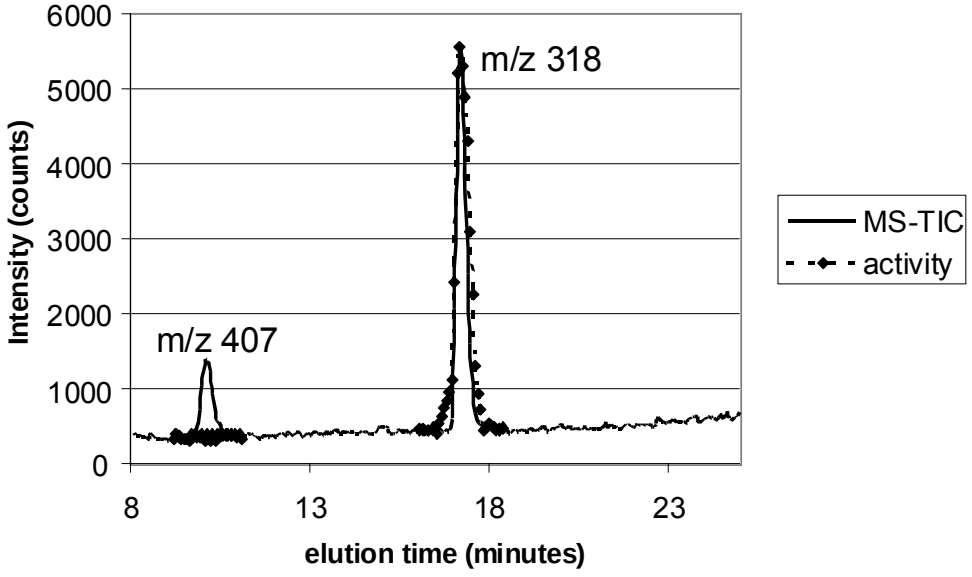


Figure 8 Activity profile and MS-trace of the combined fractions 13 and 14 analysed on the Biosuite™ column (interval time = 10 s).

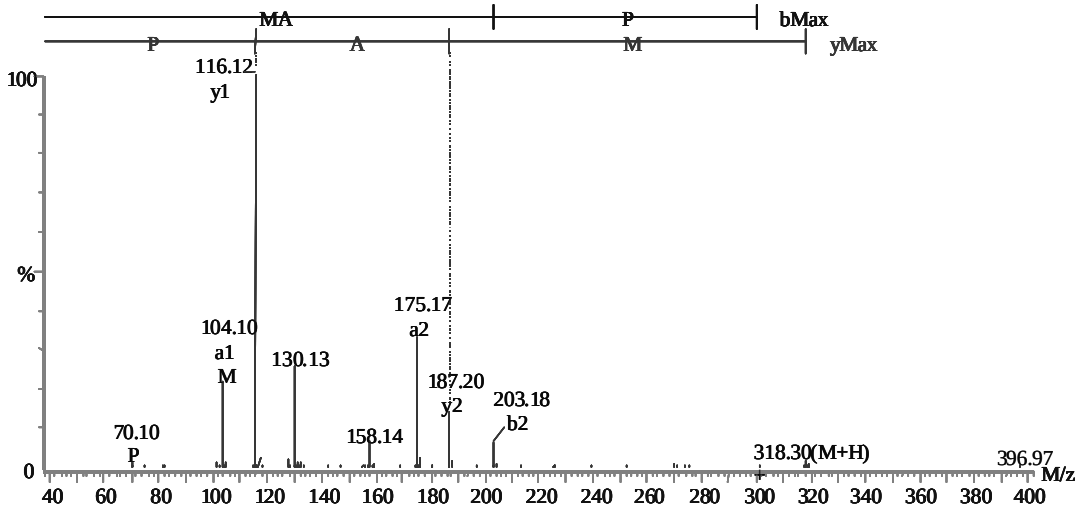


Figure 9 Product-ion spectrum of *m/z* 318.

The exact mass measurement and MS-MS fragmentation pattern confirmed the assignment of the sequence MAP to the peptide with the molecular ion at 318 Da. Another new ACEI peptide, responsible for part of the activity at 15 min in Figure 5 (B), is the peptide ITP originating from α-s2-casein f134-136. The measured $[M+H]^+$ ion *m/z* 330.2002 and the MS-MS spectrum confirmed the sequence of this peptide (not displayed). MAP and ITP were synthesised and MS and MS-MS measurements confirmed the sequence of the two peptides. The peptides with the highest contribution to the total ACEI capacity of the sample were IPP (IC50: 5.6 μM), LPP (IC50: 9.6), MAP (IC50: 3.8 μM) and ITP (IC50: 50 μM).

3.4 Conclusions

A rapid at-line method was developed for the identification of ACEI peptides in complex mixtures. The assay consists of a 2-dimensional approach using two different RPC18 HPLC columns operated at different gradients. In the first analysis a rough survey of the activity profile is obtained, while in the second analysis fractions collected from the first column are analysed in detail on a different column. The assay can be performed using standard laboratory equipment. The sensitivity of the assay is 0.34 μM for IPP. This is sufficient to identify even those peptides that have only a minor contribution to the total activity of an ACEI product. The sensitivity is comparable or even slightly better than the value of 2 μM for IPP reported for the on-line method

published by van Elswijk et al [8]. In comparison with this on-line method the newly developed at-line method offers the advantage that different types of solvents can be used for the HPLC separation. Additional advantages are that there is no limitation to the percentage of organic modifier in the eluent and there is no loss of sensitivity due to dilution. The only drawback of our at-line method is the more laborious sample handling procedure.

The ACEI activity of the hydrolysed caseinate is distributed over a large number of peptides. Approximately 50% of the activity, however, is caused by a small group of peptides including the tripeptides IPP and LPP. Two new, not earlier published ACEI peptides ITP (α-s2-casein f119-121) and MAP (β-casein f102-104) were identified using the at-line assay. The peptide ITP co-elutes with IPP and remained undetected in single dimensional chromatographic approaches. The IC50 values of ITP and MAP are 50 μM and 3.8 μM, respectively. The peptides MAP and ITP are not previously reported and especially MAP is extremely potent.

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4 Application of at-line two-dimensional liquid chromatography-mass spectrometry for identification of small hydrophilic angiotensin I-inhibiting peptides in milk hydrolysates

Summary

Very often it is not possible to explain the total bio-activity of a product from the bio-active compounds identified in the fractions collected from an HPLC-analysis of the product. This can be caused by poorly retained bio-actives which are not separated by the HPLC-column and co-elute with e.g. salts and fats. In this chapter a method is described that makes it possible to identify such compounds. A two-dimensional chromatographic method with mass spectrometric detection has been developed for the identification of small, hydrophilic Angiotensin I-inhibiting peptides in enzymatically produced milk protein hydrolyzates. The method involves the further separation of the poorly retained hydrophilic fraction from a standard C18 reversed-phase column on a HILIC column. The latter column is specifically designed for the separation of hydrophilic compounds. Narrow fractions collected from the HILIC column were analyzed for their Angiotensin I-converting enzyme (ACE) inhibiting potential in an at-line assay. Fractions showing a significant inhibition of ACE were analyzed with LC-MS for peptide identification. With this method the main peptides responsible for the ACE inhibition in the hydrophilic part of a milk hydrolysate could be determined. The ACE-inhibiting peptides RP, AP, VK, EK and EW represented more than 85% of the ACE inhibition potential of the hydrophilic fraction.

4.1 Introduction

Hypertension is one of the major health problems in the western world. A variety of food ingredients that can help to reduce blood pressure have been described. Particularly attractive are hydrolyzed proteins from fermented milk containing specific peptides that can inhibit the angiotensin I-converting enzyme (ACE), an enzyme involved in blood-pressure regulation [1-3]. For the development of optimized anti-hypertensive food products, it is crucial to know which specific peptide(s) in the complex protein hydrolysate is (are) responsible for the ACE inhibition.

Identification of active ingredients in complex mixtures is generally done using assay-guided repeated fractionation. For the specific case of ACE-inhibiting peptides in milk-protein hydrolysates often only part of the total activity of the product can be assigned to specific peptides [4]. A significant part of the remaining activity is present in fractions that are poorly retained by the usually applied reversed-phase columns in the fractionation procedure. Nakamura et al. [1] for example could only explain part of the activity of a casein hydrolysate by the tripeptides VPP and IPP eluting in one of the later fractions. The remaining activity was present in the unretained fraction. In our laboratory similar samples were seen to have over 30% of the total activity in the unretained fraction. Identification of the oligopeptides in this fraction was not further pursued but would have been extremely difficult because the fraction contains far too many compounds for an unambiguous activity assignment. Moreover, there are also too many interfering compounds present in the first-eluting fractions for reliable MS identification. Similar situations are also described by other authors [5-7]. In chapter 3 we presented a new at-line method for the identification of ACE-inhibiting (oligo)peptides in hydrolyzed caseinate [4]. Approximately 70% of the activity of these samples was caused by peptides nicely retained by the two reversed-phase columns used in the fractionation. Twenty percent of the activity was present in the hydrophilic fraction. The remaining 10% was caused by a large number of peptides with low ACE-inhibiting activity. Identification of the peptides in the hydrophilic fraction was impossible due to the large number of peptides present and the presence of co-eluting compounds, such as salts and sugars that resulted in severe suppression of the ionisation. Two reversed-phase columns were used in an at-line 2D set-up to provide improved resolution of the very complex mixture. Unfortunately, the highly hydrophilic peptides escaped identification since they were unretained on both columns. To identify the peptides responsible for the ACE inhibition of the unretained hydrophilic fraction improved chromatographic retention and separation is required. Hydrophilic interaction chromatography (HILIC) could be an attractive way of achieving this.

The use of HILIC for separation of hydrophilic substances such as proteins, peptides and nucleic acids was first described by Alpert in 1990 [8]. Since then HILIC has been used for the separation of many different substances, for example denaturants [9], polar oligomers [10], and pharmaceutical ingredients [11]. Many publications can be found describing the increased retention of hydrophilic peptides on HILIC columns in comparison with reversed-phase columns. A detailed review is published by Yoshida [12]. Literature describing the application of reversed-phase separation in combination with HILIC chromatography is scarce. A notable exception is the compositional analysis of an enzymatic protein hydrolysate obtained from acid-deaminated wheat gluten by Schlichtherie-Cerny [13]. The hydrolysate was first fractionated by GPC. The fraction corresponding to a molecular weight range of 100 – 750 Da was then further fractionated by RPLC. Finally, the peptides in the unretained peak from the RPLC column were separated and characterised by HILIC-API-MS. Using this method ten amino acids, eight dipeptides and one tripeptide were identified in this fraction.

In this chapter we describe the development of a method for the identification of ACE-inhibiting peptides in the poorly retained fractions of a reversed-phase fractionation of an enzymatically produced milk protein hydrolysate. The possibility of improving chromatographic resolution by using a HILIC separation as a second-dimension separation after the first reversed-phase isolation is studied. The aim of the new method is to extend the applicability of a previously developed system [1] for the identification of ACE-inhibiting peptides in complex peptide mixtures such as protein hydrolysates [4]. The applicability of the method is demonstrated by the identification of hydrophilic ACE-inhibiting peptides contained in such hydrolysates.

4.2 Experimental

4.2.1 Materials and methods

Chemicals

The model peptides VPP, IPP and LPP (purity >98%) were purchased from Bachem (Bübendorf, Switzerland). All other peptides were synthesised by JPT Peptide Technologies (Berlin, Germany). For the HPLC analyses acetonitrile, formic acid and TFA were purchased from Merck (Amsterdam, the Netherlands). Aqueous ammonia (Merck) was used for neutralization of the HPLC fractions. For instrument tuning PEG 300, PEG 600 and PEG 1000 were purchased from Sigma Chemicals (St. Louis, MO, USA). Ammonium acetate was obtained from Merck. For the at-line assay Angiotensin I-converting enzyme (ACE) and hippuryl-histidyl-leucine (HHL)

were purchased from Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands). PBS buffer was purchased from Gibco (Paisly, UK). The hydrolyzed milk powder was an enzymatically hydrolyzed milk protein obtained from Calpis (Tokyo, Japan).

Instrumentation

All peptide separations and identifications were performed with a Waters Alliance 2795 HT HPLC coupled to a Micromass QTOF-Ultima hybrid time-of-flight mass spectrometer equipped with a lock spray option for accurate mass determination (Waters, Almere, the Netherlands). Fractions were collected in polypropylene 300 µl 96-well plates (Nunc, Roskilde, Denmark) using a Mark IV fraction collector also from Waters. Solvent evaporation was performed using an Ultravap 96-well evaporation device (Porvair, Shepperton, UK). Quantification of HHL and HL in the at-line assay was performed on the QTOF instrument described above.

Two-dimensional analytical separation

The first-dimension reversed-phase separation was performed on two serially connected 150 x 2.1 mm Inertsil 5 ODS3 columns, particle size 5 µm, operating in the gradient mode (Varian, Middelburg, the Netherlands). The guard column was a 20 x 3.9 mm SymmetryShield RP8 column packed with 5 µm particles (Waters, Etten-Leur, the Netherlands). Mobile phase A consisted of 0.1% TFA in Milli-Q water. Mobile phase B consisted of 0.1% TFA in acetonitrile. The initial eluent composition was 100% A. After a 5 min hold a linear gradient was started to 5% B in 15 min, followed by a linear gradient to 70% B in 75 min. Finally, mobile phase composition was programmed to 99% B in 1 min and kept at 99% B for another 4 min. At the end of the gradient the eluent composition was reduced to 100% A in 6 min and the system was allowed to re-equilibrate for 14 min. The total run time was 120 min. The eluent flow rate was 0.2 ml min^{-1} and the column temperature was 60 °C. In-house experiments revealed that at this temperature isomeric peptides such as IPP and LPP are separated. UV detection was performed at 215 nm and 254 nm. Fractions of 200 µl were collected in 96-well plates. After neutralization and solvent evaporation the residues were reconstituted in 50 µl of a 80/20 mixture of solvents A and B for the second-dimension HILIC analysis.

The second-dimension HILIC analysis was performed on a 150 x 2.1 mm HILIC Atlantis column with a particle size of 3 µm (Waters, Etten-Leur, the Netherlands). Here solvent A consisted of 0.1% formic acid in acetonitrile and solvent B of a 10 mM aqueous ammonium acetate solution + 0.1% formic acid in Milli-Q water. The separation was performed at 40 °C, the maximum temperature specified by the manufacturer, and a flow rate of 0.2 ml min^{-1}. The initial eluent composition was 95% A. A linear gradient was

used to 70% B in 30 min, followed by a linear gradient to 90% B in 5 min where it was kept for another 5 min. At the end of the run the eluent was reduced to 95% C in 2 min. Then the eluent flow was increased to 0.3 ml min^{-1} in 1 min. This flow rate was maintained for another 7 min. Finally the flow was reduced to 0.2 ml min^{-1} in 1 min and the system was allowed to equilibrate for 3 min. The total run time was 54 min. 45 µl of each fraction collected from the ODS3 column was injected. At the outlet of the second dimension HILIC column fractions of 200 µl were collected in 96 well plates. The fractions were neutralized, evaporated and transferred to the activity assay (next section).

At-line Matsui assay

ACE inhibition by the collected fractions was measured in a 96-well plate assay according to the at-line method described in chapter 3 [1]. The method is based on the conversion of HHL into hipurate (H) and histidyl-leucine (HL) by the angiotensin I-converting enzyme (ACE). Briefly, after evaporation of the eluent the fractions were reconstituted in 50 µl PBS buffer and vortexed for 1 min. This solution (40 µl) was used for the at-line assay. Percentage inhibition was calculated from the peak areas of HHL and H at 280 nm. For the active fractions the remaining 10 µl was used for the identification of the peptides.

Structural identification of ACEI peptides

Peptide identification was performed on the QTOF MS instrument using full-scan HPLC-MS and product-ion MS-MS in the accurate mass mode. Fractions collected after the second dimension that showed activity were separated on the HILIC column now coupled to the MS using the gradient described above. The source and desolvation temperatures were 100 °C and 300 °C, respectively. The cone and desolvation gas flows were 100 l h^{-1} and 800 l h^{-1}, respectively. The capillary voltage was 4 kV and the cone voltage 35 V. The collision energy was 10 eV in full-scan mode and 25 eV in MS-MS mode. The collision gas was argon and the analyzer pressure was 4 x 10^{-5} mbar. In the lock spray probe a mixture of PEG 300, PEG 600 and PEG 1000 dissolved in 10 mM ammonium acetate in methanol/water 1/1 (v/v) at concentrations of 0.01, 0.02 and 0.04 µg ml^{-1}, respectively, was used. The reference flow was 5 µl min^{-1}. Spectra were recorded at a resolution of 10,000. MassLynx software (Waters) was used for data acquisition and evaluation.

4.3 Results and discussion

In a reversed-phase fractionation of a protein hydrolysate a significant percentage of the activity can be present in the unretained hydrophilic fraction. To be able to calculate the contribution of this fraction of the milk hydrolysate to the total activity, an ACE inhibition profile of the entire product was produced. To this end 20 µl of a 20 mg ml^{-1} solution of the milk hydrolysate in solvent A was injected on the 300 mm ODS3 column. Fractions of 200 µl each were collected from 0 to 65 min, with an interval time of 1 min in a 96-well plate. The ACE inhibition of each fraction was determined. Figure 1A shows the MS TIC profile and Figure 1B shows the activity profile of the fermented milk samples.

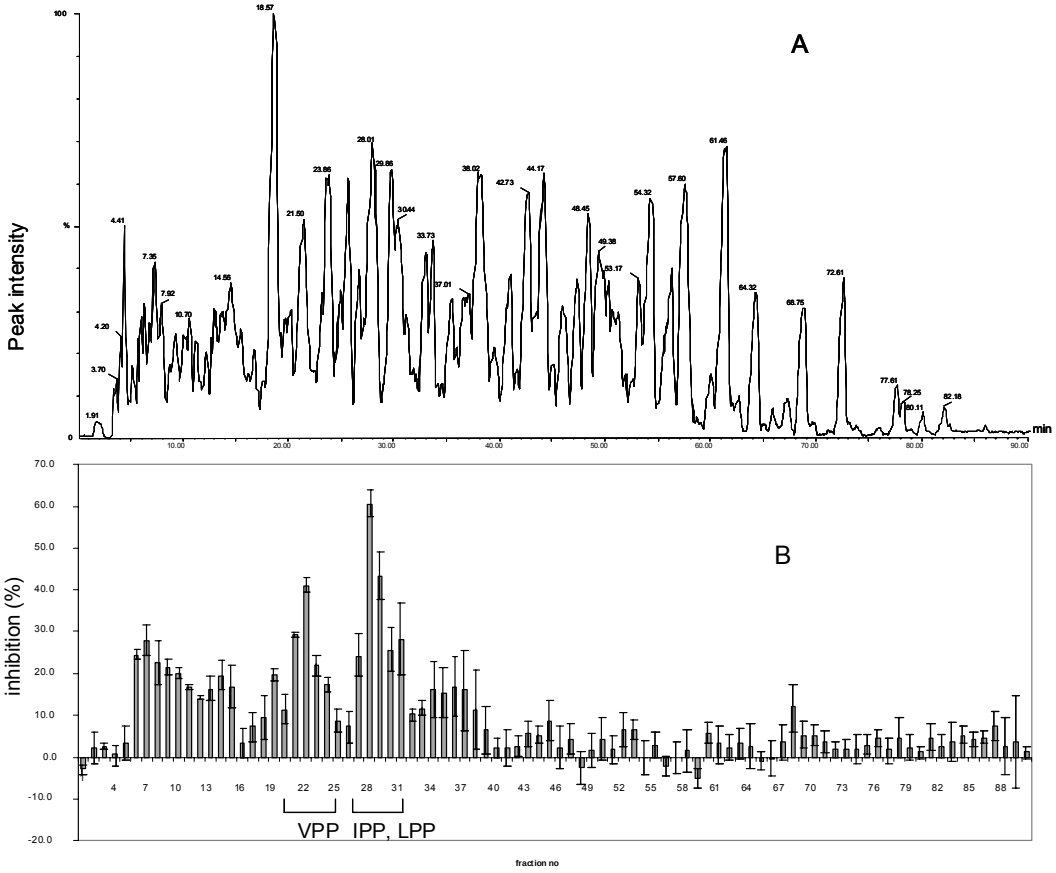

Figure 1 LC separation of the milk hydrolysate on the ODS-3 reversed-phase column **A)**: MS-TIC profile **B)**: ACE inhibition profile. Error bars indicate the standard deviation (n = 3). Fractions of 200 µl were collected. For other conditions see text.

From the data in Figure 1B it was calculated that the hydrophilic compounds in fractions 6 to 15 represent approximately 25% of the total ACE inhibition. MS-MS showed that the high activity in the fractions 27 to 31 resulted from the known ACE-inhibiting peptides IPP and LPP. The activity in the fractions 21 to 23 resulted from the peptide VPP. These peptides have been identified to be the main contributors to the ACE inhibition of a *Lactobacillus helveticus* fermented milk [2] and represent 40% of the total ACE inhibition. The remaining activity between the fractions 16 and 40 is most likely caused by a mixture of ACE-inhibiting peptides of different chain lengths. In the region of fractions 6 to 15 a large number of peptides co-elute and the spectra of the fractions are highly complex. Figure 2, for example, shows the mass spectrum of fraction 7 collected from the first dimension ODS 3 column. At least 16 ions can be observed with intensities above 10% of the base peak, all representing different peptides. In order to be able to assign the measured activity to one or more of these peptides a further separation in an additional chromatographic dimension is required. To achieve this, fractions collected from the first-dimension ODS column were injected on the second-

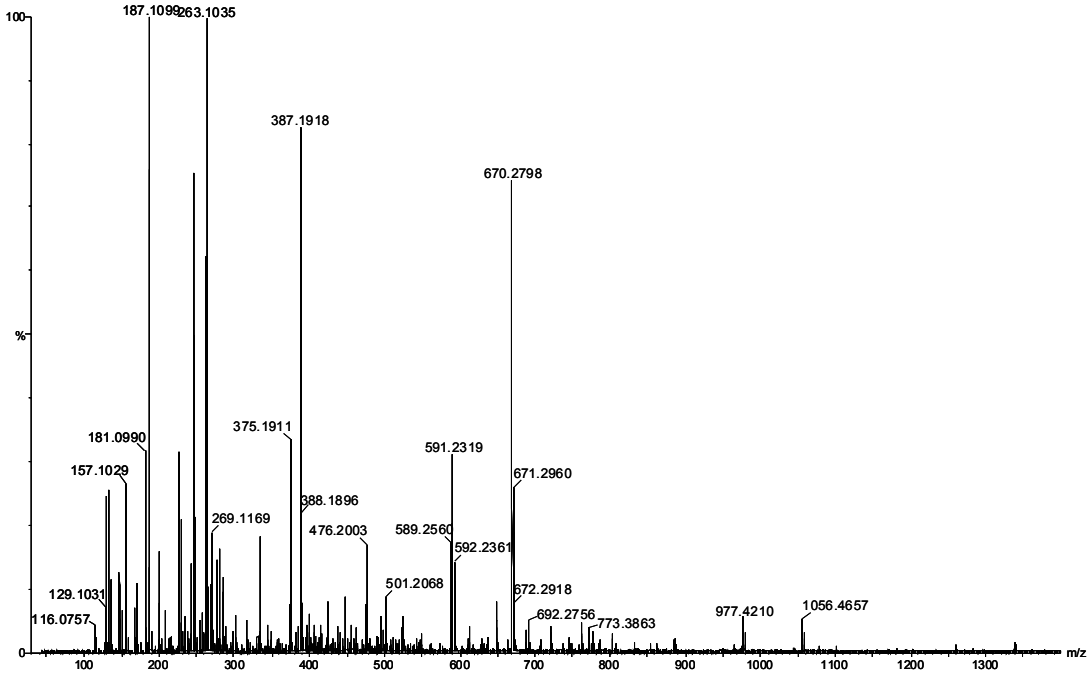

Figure 2 Mass spectrum of fraction 7 collected from the first dimension ODS3 column.

dimension HILIC column. Again 200 µl fractions were collected in 96-well plates. After neutralization and evaporation the residues were reconstituted in 50 µl Milli-Q water. 40 µl was used for ACE inhibition testing, the remaining 10 µl for MS identification. As an example of the results of these experiments, Figure 3 shows the TIC chromatogram obtained from the full-scanning analysis of fraction 7 of the ODS3 column, now separated on the second dimension HILIC column. The TIC chromatogram clearly shows that the composition of this fraction indeed is very complex.

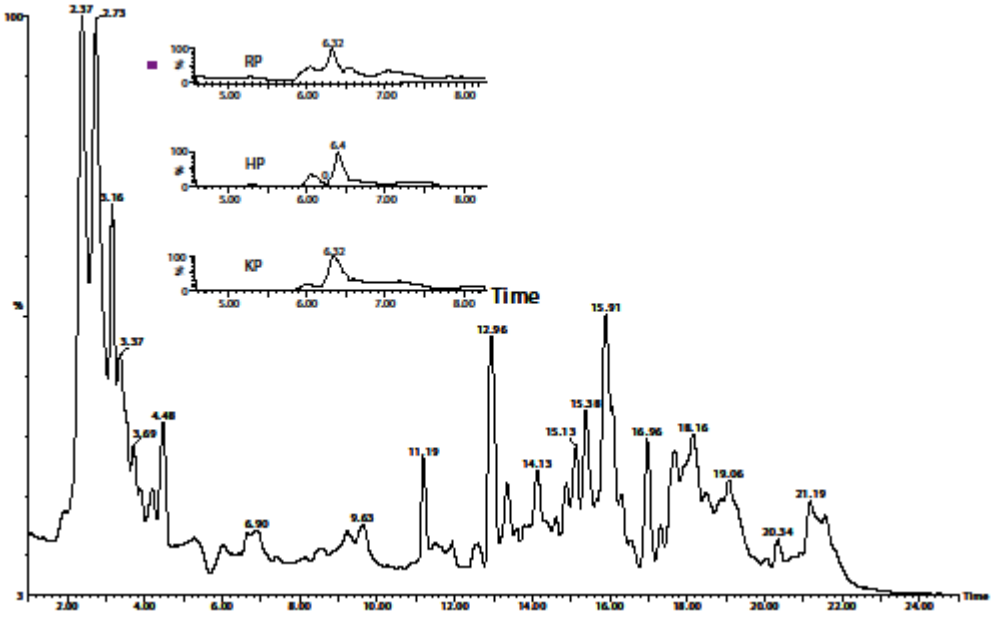

Figure 3 MS-TIC profile of fraction 7 from the ODS3 column analysed on the HILIC column. The insert shows the selected ion traces of three of the most active peptides

To identify the peptide(s) responsible for the ACE inhibition, the fractions collected on the 96-well plate were subjected to the ACE inhibition assay. As an example Figure 4 displays the activity distribution of ODS fraction 7. The data in Figure 4 are corrected for the background recorded using a blank gradient, i.e. a gradient without injection. A blank analysis of the HILIC column showed slightly negative values compared to a reference of pure PBS. The source of these negative responses is still unknown. The results in Figure 4 clearly show a strong activity in the HILIC fractions 17 to 19 and a somewhat lower activity in the fractions 12 to 16 of the HILIC column. LC-MS separations were performed on these fractions and the MS data were searched for peptides in the two active regions. In Figure 5 the mass spectrum of fraction 18 is given.

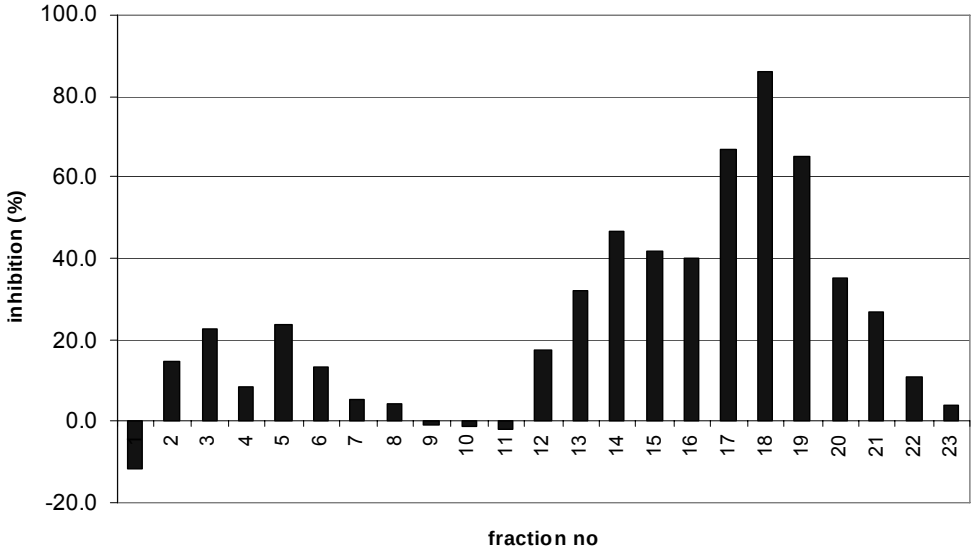

Figure 4 ACEI profile of fraction 7 from the ODS3 column analysed on the HILIC column.

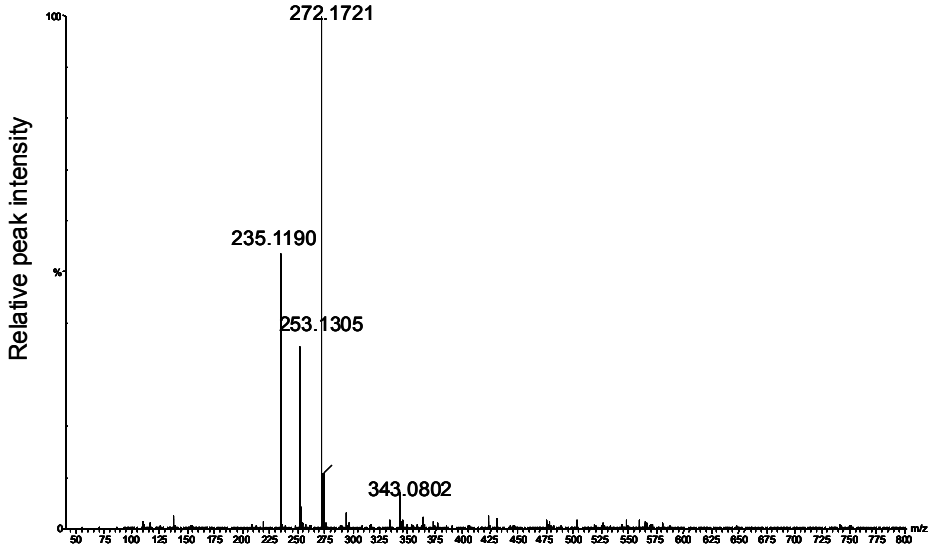

Figure 5 Mass spectrum of fraction 18 collected from the HILIC column.

In the spectrum three abundant ions can be observed. A library search and MS-MS measurements revealed that *m/z* 272.1721 represents the molecular ion of the dipeptide RP (- 0.7 ppm), while *m/z* 253.1305 represents the molecular ion of HP (- 2.1 ppm). The ion at *m/z* 235.1190 is formed by the loss of water from the ion at *m/z* 253.1305. Identification of the peptides in

the remaining fractions of the HILIC column showed that fraction 7 of the first-dimension ODS3 column consisted of 3 free amino acids, 19 dipeptides and 2 tripeptides while 6 compounds remained unidentified. The positively identified amino acids were E, Q and K and the dipeptides were QD, KY, ER, RE, KP, HP, RP, AP, VK, EK, EW and PH. The dipeptides ET, TP, TQ, PQ, KV, KE, HK and tripeptides APK and VRG were tentatively identified. Structure confirmation was based on the elution time, measured exact mass (error < 5 ppm) and MS-MS fragmentation pattern compared to those of the model compounds. Using this method the remaining hydrophilic fractions 5 to 15 collected from the ODS3 column were also analyzed in the 2D mode. In Figure 6 a three-dimensional plot of the activity distribution over the fractions of the two columns is given. In total 5 amino acids, 35 dipeptides, 13 tripeptides, 1 pentapeptide and 18 not yet identified compounds were found. The identity of all amino acids and the sequence of 27 dipeptides were again confirmed by model compounds. Table 1 lists the sequences of the identified peptides together with their reported IC50 values and ACE inhibition data at 20 µM, established in house [14]. For the peptides for which no model compounds were available, identification was based solely on their fragmentation pattern in MS-MS.

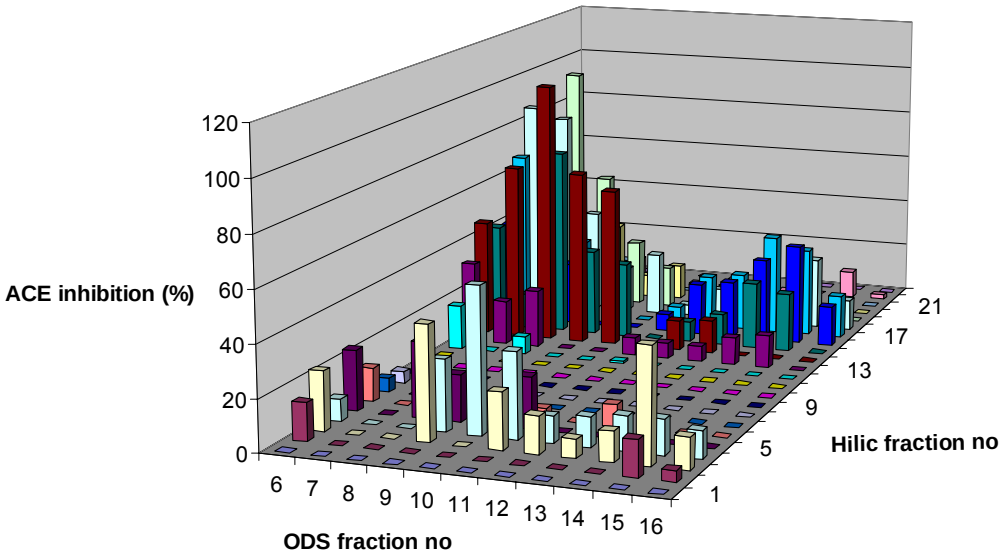

Figure 6 Three dimensional display of the ACEI activity distribution of the fractions collected from the ODS3 column and the HILIC column.

Table 1 Peptides identified in fractions 6 to 15 of the ODS3 column, analysed on the HILIC column. The sequences of the bold printed peptides were confirmed by MS-MS analyses of reference peptides. Annotation: n.r.: not reported in literature. n.m.: not measured.

Elution time (min)	Peptide sequence	IC50 value (µM) literature	Literature reference	Inhibition at 20 µM (%)	Peak area (counts)
11.64	**F**	n.r.			83
11.90	**Y**	n.r.			2274
12.02	**EI**	n.r.		38.2	2221
12.36	**IE**	n.r.		3.7	4591
12.40	YP	720	13	n.m.	58
12.59	**EV**	n.r.		25.4	622
12.59	**VE**	n.r.		41.0	22
12.59	**IP**	129	13	17.7	100
12.84	VP	575	13	45.9	801
13.21	**E**	n.r.		n.m.	288
13.26	TE	n.r.		26.0	344
13.44	**LQ**	n.r.		11.2	748
13.74	**KP**	16,22,30	13,15	33.7	144
13.81	VNE	n.r.			1378
14.08	LN	n.r.		13.7	271
14.10	**Q**	n.r.		n.m.	511
14.10	**QD**	n.r.		n.m.	791
14.28	TDVEN	n.r.			2019
14.50	**PT**	n.r.		3.1	1001
14.56	NVP	n.r.			2744
14.61	YQ	n.r.		n.m.	36
14.64	SPP	n.r.			335
14.72	**TQ**	n.r.		0.0	175
14.86	**AH**	n.r.		7.3	101
14.91	**PP**	2285	18	0.0	1759
15.01	**AP**	29,269	10,13	11.4	811
15.18	**PQ**	n.r.		0.0	1041
16.34	FP	1216	13	n.m.	631
16.41	HSM	n.r.			656
16.43	EPF	n.r.			186
16.43	DKI	n.r.			187
16.52	**KY**	2,7,8,13	13,16,17	n.m.	13
16.66	VYP	288	13		125
17.00	**EW**	n.r.		65.1	1578
17.22	**VK**	13	13	57.2	167
17.23	**ER**	n.r.		n.m.	236
17.30	SGY	n.r.			624
17.35	**ME**	n.r.		24.6	42
17.55	**K**	n.r.		n.m.	497
17.61	**EK**	n.r.		41.8	337
17.84	**RE**	n.r.		n.m.	166
17.86	**KE**	n.r.		13.4	83
17.89	**KV**	33		n.m.	235
18.04	VVR	n.r.			114
18.08	HP	n.r.		11.1	1427
18.18	KVP	n.r.			45
18.24	VPQ	n.r.			505
18.63	**RP**	21,91,182	13	36.4	1340
18.97	**PH**	n.r.		0.0	306
19.03	QP	n.r.		n.m.	402
19.11	**KP**	16,22,30	13,15	33.6	144
19.22	APK	n.r.		n.m.	266
20.18	VRG	n.r.		n.m.	313

To verify whether the identified peptides indeed fully explain the measured activity of the hydrophilic fraction the concentration of all peptides should be determined. Next a mixture should be prepared which is then analyzed using the two-dimensional method described in this chapter. The ACE inhibition profile of this mixture should be identical to that of the sample. Such an experiment will hardly be feasible because it requires the availability of all single components at high purity. To check the completeness of the inventory, the activity contribution of each identified peptide was estimated from the measured peak area and the IC50 value obtained from literature or from unpublished in-house data. To estimate the contribution of an individual peptide the following relationship was used: $CA \approx A_p / IC50_p$. In this equation CA represents the contribution of the peptide to the measured ACE inhibition, A_p the peak area and $IC50_p$ the IC50 value of the peptide. Clearly this proportionality equation is only approximate as it assumes that the MS response for all peptides is identical. When both the peak area and the IC50 value are taken into consideration it can be concluded that the dipeptides AP, RP, VK, and EK are the main peptides responsible for the measured ACE inhibition of the hydrophilic fractions 6 to 10 collected from the ODS3 column (Figure 7).

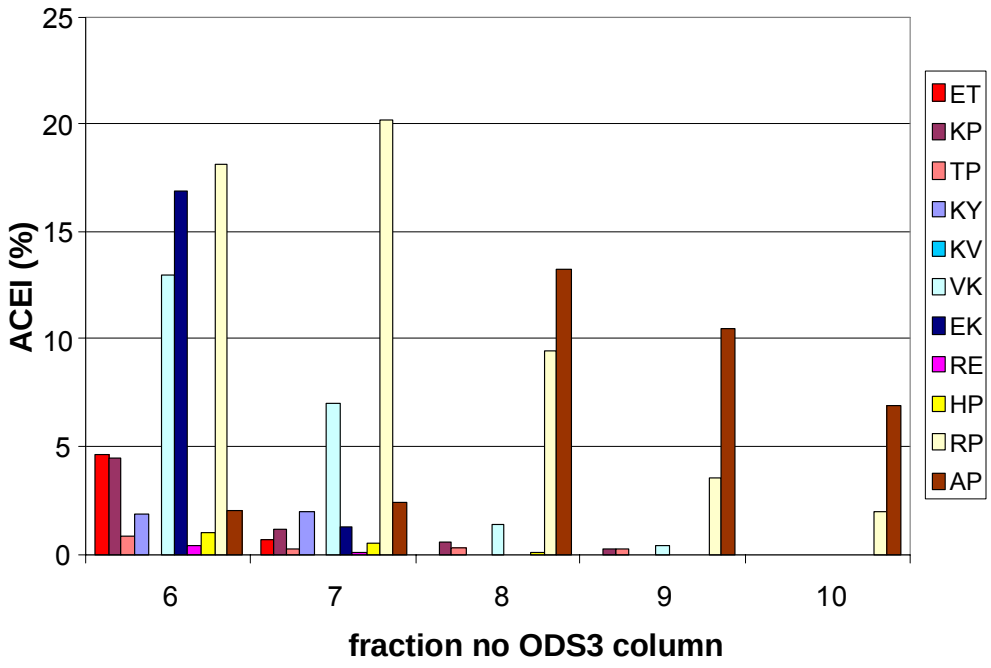

Figure 7 Distribution of the ACE-inhibiting activity calculated from Table 1 in the hydrophilic fractions 6 to 10 collected from the ODS3 column.

In Figure 8 the activities of all peptides in each fraction are summarized and the profile is compared to that of the measured profile. The two profiles are reasonably similar. The differences between the graphs can be explained from the fact that no correction was applied for differences in MS responses of the various peptides. The remaining fractions 11 to 15 of the ODS3 column were analyzed in the same way as the fractions 6 to 10. These analyses showed that the peptide EW is responsible for the major part of the ACE inhibition measured in these fractions. In total the five peptides identified in the fractions 6 to 15 are responsible for approximately 85% of the activity measured in the hydrophilic fractions. The most important contribution being the dipeptide RP which is responsible for 34%. Together with the peptides VPP, IPP and LPP now approximately 65% of the total activity of the product has been explained. The remaining 35% is distributed over a large number of peptides with different chain lengths and with relatively low individual contributions.

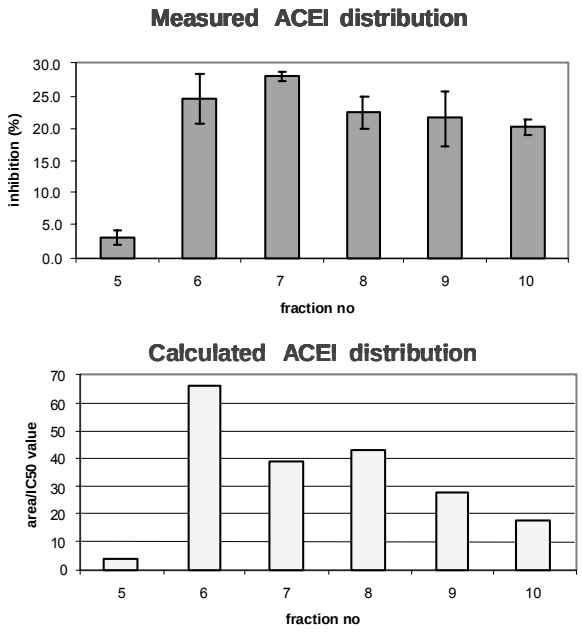

Figure 8 Calculated ACE inhibition profile (bottom) versus the measured profile (top). Error bars indicate the standard deviation (n = 3).

When compiling the list of published IC50 values for the peptides identified in the present study in some cases large differences were seen for the IC50 values of proline-containing peptides in different publications. The reported IC50 values for example for AP vary between 29 µM and 269 µM [12]. This large difference might be caused by variations in the *cis/trans* ratio of the proline present in synthetic model compounds as a result of differences in the synthesis routes (Fmoc or Boc) as was shown for the peptide DKIHP by Gómez-Ruiz [13]. In our calculations the *trans*-Pro value of 29 µM for AP was used since *trans*-Pro is known to be dominant in natural products [14]. Many of the identified dipeptides were found in milk hydrolysates for the first time. For most of the newly identified ACE-active peptides it is actually the first time they are reported at all. The long list of newly identified peptides found here clearly demonstrates the potential of the two dimensional separation approach involving reversed-phase HPLC and HILIC.

4.4 Conclusions

Two-dimensional liquid chromatography in combination with mass spectrometry was successfully applied for the identification of poorly retained peptides present in enzymatically hydrolyzed milk protein. A standard C18 reversed-phase column was used for the first separation followed by a second dimension separation on a HILIC column. This 2-dimensional procedure significantly improves the separation of hydrophilic peptides that elute almost unretained on a reversed-phase column and co-elute with numerous other compounds such as carbohydrates and salts. These compounds suppress the ionization and complicate the identification. The method enabled the identification of hydrophilic peptides in complex mixtures. In the hydrophilic fraction of the milk hydrolysate investigated 71 compounds were found of which 5 free amino acids, 35 dipeptides, 12 tripeptides, 1 pentapeptide and 18 not yet identified compounds. Five peptides, RP, AP, VK, EK and EW were responsible for approximately 85% of the total measured ACEI activity of the hydrophilic fraction, of which RP has the highest individual contribution (34%). None of the five peptides has, to our knowledge, been reported earlier in milk hydrolysates or related products such as yogurt or cheese. Most of the peptides involved remained undetected in one-dimensional chromatography.

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5 Quantification of ACE inhibiting peptides in human plasma using high-performance liquid chromatography-mass spectrometry

Summary

Once the bioactive compounds have been identified (Chapters 3 and 4) information on their behavior, e.g. adsorption and life-time in the human body is required. Therefore quantification of the bioactive compounds needs to be carried out in body fluids, tissues and faeces. In the present chapter a method is described for the quantification of the peptides identified in Chapters 3 and 4 in human plasma. An HPLC-MRM-MS method was developed for the quantification of seventeen small ACE-inhibiting (ACEI) peptides in plasma samples collected from human volunteers after the consumption of a peptide-enriched drink. The assay shows the high selectivity and sensitivity necessary to monitor small changes in the levels of the ACEI peptides after consumption of drinks developed to effect lowering of the blood pressure. Four different sample preparation methods were tested and evaluated. The final method selected is simple and effective and consists mainly of the removal of proteins by acidification and heating, followed by a large volume injection. Additional sample preparation steps such as solid-phase extraction and liquid-liquid partitioning were studied. Although they resulted in cleaner extracts, losses of specific peptides such as SAP were frequently seen. The isotope-labeled form of one of the peptides to be quantified, [U^{13}C]IPP, was used as an internal standard. The limit of detection of the assay is below 0.01 ng ml^{-1}. The limits of quantification were between 0.05 and 0.2 ng ml^{-1}, which corresponds to approximately 10% of the expected peptide concentration in plasma based on a normal diet. The intra- and inter-day relative standard deviations for all peptides were below 25% and the method has an accuracy of better than 75%. The long term stability is good. At least 200 samples could be analyzed before the system had to be cleaned. The assay has been successfully applied to blood samples collected from volunteers during a human trial.

5.1 Introduction

One of the key enzymes in the regulation of blood pressure is the angiotensin I-converting enzyme (ACE). ACE converts angiotensin I into the potent vasoconstrictor angiotensin II. Many protein-containing food products, ranging from fish and meat to milk and maize, are known to yield ACE-inhibitory peptides upon *in-vivo* proteolysis. So far, a wide range of different peptides with ACE-inhibitory effects has been identified using peptide purification methods and *in-vitro* ACE-inhibition assays [1-5], or more sophisticated on-line techniques [6]. Some of the identified peptides have been synthesised and proven to be active in both *in-vitro* ACE-inhibition assays as well as in relevant *in-vivo* animal models for hypertension.

Although many peptides from food proteins have been shown to inhibit ACE *in-vitro* and also lowered blood pressure in *in-vivo* studies, the proposed properties of these peptides in real life situations still need to be demonstrated. To exert their physiological effect in the body, the peptides need to be absorbed from the intestine and enter the blood circulation in an active form. So far, there is no direct evidence that any of the identified peptides are indeed absorbed and reach the blood circulation. Therefore, it is extremely important to demonstrate the presence of ACE-inhibiting peptides in blood after the consumption of a food product containing these peptides. In order to be able to measure these peptides in plasma, highly sensitive and accurate methods are required. The expected concentration levels are in the picogram per millilitre range.

Peptide identification, and to a lesser extent quantification, has received a great deal of attention in recent years due to the rapidly expanding interest in proteomics [7,8]. For the current work, however, that research is unfortunately only of limited or indirect relevance. This is because of the different matrices involved and the nature of the underlying question, i.e. identification of proteins versus the quantification of small peptides with known structures. A second area where peptide analysis is performed is in metabonomics research where peptide profiles of patients suffering from a specific disease are compared to that of healthy patients [9,10]. The peptides analysed are relatively large with a sequence of at least eight residues and molecular ions of 0.8 kDa to 5 kDa. Finally, an area where the quantification of known peptides in biological fluids is relevant is in the study of peptidic drugs. For example Yang et al. [11] developed a method for the quantitative analysis of an opiod pentapeptide and its cyclic prodrug in rat plasma. Chavez-Eng et al. [12] measured a cyclic hexapeptide with known antifungal properties in human plasma. In these studies the plasma samples are separated using HPLC with UV, electrochemical, fluorescence or mass spectrometric detection. Nowadays the very sensitive

mass spectrometric detector is preferred over fluorescence due to its much higher selectivity and the possibility to be applied without derivatisation. The sample preparation methods applied generally consist of removing proteins by precipitation or other techniques, followed by isolation and concentration of the peptide fraction. The typical detection limits of such procedures are in the low ng ml^{-1} range.

The limitations of the methods mentioned above are the relatively large volumes of blood that are needed and/or the extensive cleanup procedures that must be applied to isolate and concentrate the peptides of interest. Moreover, the target peptides in these peptidic drugs studies are relatively large, which is advantageous for the selection of ions for MRM-MS operation. Finally, measurements are also simpler if unique peptides, i.e. peptides with sequences that do not occur in the proteins normally consumed, have to be measured.

In our study healthy volunteers consumed fermented milk enriched in ACEI peptides in a human trial performed over a time period of several weeks. During the study the blood pressure was measured and blood samples were collected for quantification of previously identified ACEI peptides. Considering the peptide intake and assuming 0.01% to 1% effective absorption and transport to the systemic blood circulation, the expected concentration of the peptides in the blood should be in the high pg ml^{-1} range. In order to be able to quantify peptides at this very low level, the desired detection limit was set at 0.01 ng ml^{-1}.

The subject of this chapter is the development and validation of an analytical method for the quantification of small ACEI peptides in plasma in the pg ml^{-1} range. Liquid chromatography with atmospheric-pressure ionisation tandem mass spectrometry is selected as the quantification method. Various methods for sample clean-up are evaluated with emphasis on the suitability for the above-mentioned sensitivity range and long term stability. The full set of ACE-inhibiting peptides consists of 17 peptides with sequence lengths between 2 and 5 residues and different hydrophobicity. Statistical information on the performance of the HPLC-MS-MS method is reported and its inherent limitations are discussed. Finally the application of the method to the analysis of authentic blood samples is demonstrated.

5.2 Experimental

5.2.1 Materials and methods

Standards and chemicals

The peptides IPP and VPP (purity >98%) were purchased from Bachem (Bubendorf, Switzerland). The internal standard used was [U^{13}C]IPP (fully ^{13}C labelled in Isoleucine only) and was purchased from Bio Peptide (San

Diego, California, USA). All other peptides were synthesised at the University of Utrecht (Faculty of beta-sciences, department of chemistry, Utrecht, the Netherlands). The sequences of the peptides studied are given in Table 1. The purity of each peptide was determined in-house using HPLC-UV-MS. All purities were better than 90%. Acetonitrile, trifluoroacetic acid, propionic acid and 2-propanol were purchased from Merck (Amsterdam, the Netherlands). Porcine and bovine plasma were obtained from a local slaughter house.

Solid-phase extraction

Octadecyl modified silica solid-phase extraction cartridges (various sizes) were purchased from Waters (Etten-Leur, the Netherlands). The cartridges were preconditioned with 4 ml of methanol followed by 4 ml of Milli-Q water. After loading of the sample, the cartridge was washed with 1 ml of Milli-Q water and the peptides were eluted using 4 ml of methanol. The collected methanol fraction was evaporated to dryness under a stream of nitrogen at 40 °C and reconstituted in 500 µl of Milli-Q water.

Standard preparation

Peptide standard solutions were prepared either in Milli-Q water or in a five fold diluted SPE extract of bovine plasma (vide infra). The bovine plasma extract was prepared by addition of 20 µl of a 10% aqueous TFA solution to 1 ml of plasma. The mixture was vortexed for 2 min followed by heating at 95 °C for 2 min. After cooling and centrifugation at 13000 RPM the supernatant phase was purified by passing it through a C18 Sep-Pak SPE column (Waters Etten-Leur, the Netherlands). One ml of plasma was applied onto a 20 ml/5 g cartridge previously conditioned with 20 ml of methanol and 20 ml of Milli-Q water. The protein pellet in the centrifugation tube was discarded. Methanol (4 ml) was applied for elution of the cartridge. The methanol fraction was collected and evaporated under a stream of nitrogen at room temperature. The residue was reconstituted in 5 ml of Milli-Q water. Calibration standards were prepared starting from a mixture of the standard peptides with a concentration of 500 µg ml^{-1} for each peptide in Milli-Q water. Subsequent dilutions were made in the bovine plasma extract to yield the following concentrations: 0.01, 0.05, 0.1, 0.2, 0.5, 1 and 2 ng ml^{-1}. Each solution contained the internal standard [$U^{13}C$]IPP at a concentration of 1 ng ml^{-1}.

Instrumentation

All analyses were performed on a Waters 2790 HPLC coupled to a Micromass Quattro-Premier triple quadrupole mass spectrometer (Waters, Almere, the Netherlands).

Chromatographic conditions

All analyses were performed on a 150 x 2.1 mm Inertsil 5 ODS3 column with a particle size of 5 µm (Varian, Bergen op Zoom, the Netherlands), equipped with a 10 x 4.6 mm reversed-phase C8 guard column (Waters, Etten-Leur, the Netherlands). Mobile phase A consisted of 0.1% TFA in Milli-Q water. Mobile phase B consisted of 0.1% TFA in acetonitrile. The initial eluent composition was 100% A. The eluent was kept at 100% A for 5 min and increased to 5% B in 10 min, followed by a linear gradient to 30% B in 15 min. Then a linear gradient was used to 70% B in 5 min, and the eluent composition was kept at 70% B for another 5 min. The eluent was reduced to 100% A in 1 min and the system was allowed to equilibrate for 9 min. The total run time was 50 min. The eluent flow was 0.2 ml min^{-1} and the column temperature was set at 60 °C. The UV trace was recorded at 215 nm. The eluent of the first 5 min was directed to waste to avoid contamination of the mass spectrometer with salts and other highly polar compounds. The injection volume ranged from 10 to 150 µl depending on the purity of the sample after the sample preparation. If after sample pre-treatment the solution was viscous or unclear, a volume of 10 µl was injected. In all other cases the injection volume was 150 µl.

Post column additive

To overcome suppression of the ionisation due to the presence of TFA in the eluent, a mixture of propionic acid and 2-propanol (70/30 v/v) was added post-column using a separate pump. The solvent delivery system used was a P1000 isocratic pump (Spectra-Physics, Darmstadt, Germany). The flow rate was 0.05 ml min^{-1}. A piece of 30 cm of fused silica tubing (75 µm id) was used as a restrictor between the pump and the post-column T-piece in order to maintain a stable flow rate.

Mass spectrometric conditions

All measurements were carried out using high-performance liquid chromatography- atmospheric pressure ionisation-multiple reaction monitoring mass spectrometry (HPLC-API-MRM-MS) in positive ionisation mode. The capillary voltage was set to 4 kV. The source temperature was kept at 100 °C and the nebulizer temperature at 250 °C in order to prevent in-source condensation, especially at the starting eluent composition of 100% water. The desolvation gas flow and cone gas flow were 355 l h^{-1} and 188 l h^{-1}, respectively. Argon was used as collision gas at a gas pressure of 2.3×10^{-3} mbar. The dwell time was set at 0.5 s for each peptide, with an interscan time of 0.05 s. The optimum cone voltages and collision energies were determined for each peptide individually prior to the start of the experiments. Peptides were identified based on their retention time, their molecular ion and the ratio of the peak heights of two different characteristic

product ions for each peptide relative to those of the model compounds. A maximum variation of 0.2 min for the elution time and 10% for the product-ion ratios was accepted. Detailed information on the product-ions, cone voltages and collision energies is given in Table 1.

Table 1 Sequences, MRM conditions and product-ion ratios of the peptides measured in the assay.

Peptide	precursor ion *m/z*	V[a)]	Product ion 1 *m/z*	CeV-1[b)]	Product ion 2 *m/z*	CeV-2[c)]	Ratio Ion1/ion2
VPP	312.2	25	213.1	18	169.1	18	1.6/1
IPP	326.2	25	213.1	18	183.1	18	5.5/1
LPP	326.2	25	213.1	18	183.1	18	3.4/1
IPPL	439.2	25	326.2	16	183.1	16	2.5/1
HLP	366.2	25	251.2	14	86.0	30	15.3/1
HLPLP	576.3	60	251.2	24	183.1	24	4.7/1
VAP	286.2	25	116.2	12	70.0	15	13.2/1
SAP	274.1	25	187.1	11	70.0	17	11.6/1
IIAEK	573.3	60	347.2	20	86.0	30	12.7/1
FY	329.1	30	120.0	18	182.1	14	5.5/1
VF	265.1	30	72.0	16	166.1	11	1.3/1
VY	281.1	26	182.1	11	72.0	17	1.2/1
IY	295.1	23	86.0	18	182.1	11	3.4/1
AW	276.1	22	188.1	20	205.1	20	10.1/1
IW	318.2	24	205.1	14	86.0	18	1.8/1
KVLPVP	652.4	40	197.1	20	537.4	13	4.2/1
LW	318.2	24	205.1	14	86.0	18	1.2/1
[U^{13}C]IPP	332.2	25	213.1	18	189.1	18	2.2/1

a. Cone voltage.
b. Collision energy product-ion 1.
c. Collision energy product-ion 2.

5.3 Results and discussion

With the highly selective and sensitive state-of-the-art LC-MS-MS systems plasma samples can generally be analysed after only limited sample pre-treatment. In many cases protein removal by solvent or heat precipitation suffices [15]. In a first series of experiments we studied whether this simple “dilute and shoot” method [15] provided sufficient sensitivity for the current analysis. To minimize the usage of human plasma required for the method development porcine plasma, generally considered to be a reasonable alternative for human plasma, was used in this part of the work.

The initial sample preparation consisted of protein precipitation using organic solvents. Three different solvents were tested: methanol, ethanol and acetonitrile with plasma/solvent ratios of 1:1 or 2:1 followed by

centrifugation at 13000 RPM for 30 min. The volume of plasma pre-treated was 1 ml. The supernatant was concentrated five times under a nitrogen gas flow at ambient temperature. The recovery was determined using porcine plasma spiked at a high level (60 µg ml^{-1}) with IPP. An injection volume of 10 µl was used. The results are given in Table 2. The data show that the best recovery (~100%) was achieved with methanol.

Table 2 Recovery of IPP from spiked porcine plasma after solvent precipitation**.**

Plasma/solvent ratio	Recovery (%)
Plasma/methanol 1:2	105
Plasma/methanol 1:1	93
Plasma/acetonitrile 1:2	1
Plasma/acetonitrile 1:1	26
Plasma/ethanol 1:2	73
Plasma/ethanol 1:1	73

Subsequent analyses were performed using plasma/methanol in a ratio of 1:2. The limits of detection were determined using porcine plasma spiked with IPP and KVLPVP in the concentration range of 0.04 ng ml^{-1} to 4 ng ml^{-1} and using an injection volume of 150 µl. The LODs for KVLPVP and IPP were 0.4 ng ml^{-1} and 4 ng ml^{-1}, respectively. This was not low enough to quantify the peptides at the desired concentration level. Another drawback of this sample preparation protocol was that contamination of the MS source already occurred after approximately 10 injections causing a strong decrease in sensitivity. In order to improve the efficiency of the protein removal step, a solid-phase extraction purification step was incorporated in the method. After centrifugation at 13000 RPM for 30 min, 1 ml of the supernatant of the spiked porcine plasma samples was applied to a pre-conditioned SPE cartridge. The SPE eluent was evaporated under nitrogen at 40 °C and the residue was then reconstituted in 500 µl of Milli-Q water. After reconstitution the solution was cloudy, most likely as a result of proteins and lipids not being removed during the precipitation and SPE steps. Therefore it was decided to use a more rigorous method to remove the proteins from the plasma. In this method protein precipitation was obtained by acidification of the plasma and temperature treatment. 20 µl of a 10% aqueous TFA solution was added to 1 ml of plasma. This mixture was then heated at 100 °C for 2 min. After cooling to ambient temperature the SPE step described above was applied. After evaporation of the solvent and reconstitution in 500 µl of Milli-Q water the solution was clear, indicating that full removal of proteins and fat was obtained.

The recovery of the method outlined above was determined by spiking human plasma with the peptides VF, SAP, IPP, IIAEK and HLPLP at concentrations of 10, 50, 100, 500 and 1000 ng ml^{-1} plasma. The results of the experiments are summarised in Table 3. The results show that the acid/heat/SPE purification method can be used for hydrophobic peptides.

Hydrophilic peptides such as e.g. SAP, unfortunately, have poor recoveries because they are not, or only partly, retained on the SPE column. Experiments with model compounds in pure water confirmed this statement.

Table 3 Recovery of the peptides VF, SAP, IPP, IIAEK and HLPLP using SPE purification.

Concentration in ng l^{-1}	Recovery (%)				
	VF	**SAP**	**IPP**	**IIAEK**	**HLPLP**
10	96	35	96	103	73
50	100	54	76	95	75
100	97	33	85	71	77
500	80	35	72	70	70
1000	87	51	69	86	73

The long term reproducibility of the method appeared to be poor, with standard deviations of approximately 50%. To overcome this problem, the SPE step was omitted from the method. The final, strongly simplified method consisted of the addition of 20 µl of a 10% TFA solution to 1 ml of plasma. After vortexing for 30 s the mixture was heated to 100 °C for 2 min, rapidly cooled down using running cold water and centrifuged for 30 min at 13000 RPM. 150 µl of the approximately 200 µl of supernatant was then injected onto the HPLC-column. Such large injection volume did not result in significant peak broadening when compared to injections of smaller sample volumes.

During the experiments it appeared that the peptide levels decreased during storage in aqueous solutions, especially at concentration levels below 10 ng ml^{-1}. This even occurred when samples were stored at –20 °C and is probably due to binding of the peptides to active sites of the glass vial. Different vial types were tested, including polyethylene and silylated glass vials, however, without significant improvement. The best results were obtained when the peptides were dissolved in five times diluted SPE-purified bovine plasma. Most likely, the excess peptides present in the background efficiently prevent adsorption of the peptides of interest on the active sites.

The final method was validated according to the procedure described by Shah et al [13]. Calibration data were generated using either the SPE extract of bovine plasma or human pool plasma spiked with seventeen peptides that can be formed from bovine milk proteins upon digestion, at concentrations of 0, 0.01, 0.05, 0.1, 0.2, 0.5, 1 and 2 ng ml^{-1}. The concentration of the [$U^{13}C$]IPP internal standard in all samples was 1 ng ml^{-1}. The protein sources and the in-house determined Matsui IC50 ACEI values [14] of the peptides are given in Table 4.

Table 4 Protein sources and Matsui activities of spiked peptides.

Peptide	Protein source	Position	Matsui IC50 value in µM
VPP	β -casein	84-86	2.5
IPP	β -casein	74-76	2
LPP	β -casein	151-153	10
IPPL	β -casein	74-77	>50
HLP	β -casein	134-136	>100
HLPLP	β -casein	134-138	17
VAP	α-s1-casein lactotransferrin	25-27 610-612	1
FY	α -s1-casein α -s2-casein	145-146 and 153-154 103-104	10
VF	α -lactalbumin α -s1-casein β -lactoglobulin lactotransferrin	27-28 31-32 81-82 83-84 and 233-234	5 – 10
VY	α -s2-casein β -casein β -lactoglobulin	198-199 59-60 41-42	5
IY	Lactotransferrin	100-101 and 418-419	0.5
AW	α -s1-casein	163-164	5
IW	α -lactalbumin lactotransferrin	78-79 286-287	0.5
LW	α -s1-casein	198-199	5

The calibration curves were constructed from the peak area ratios of the analyte relative to the internal standard versus concentration. The concentrations of the peptides in the spiked pool plasma sample were calculated using a linear equation fitted through the different calibration curves. The limit of detection was defined as the lowest detectable chromatographic peak with a signal-to-noise ratio of 3:1. The limit of quantification was defined as the lowest concentration that could be measured with a maximum day-to-day relative standard deviation of 25% and a maximum deviation from the nominal value of 25%. The LOD and LOQ were determined by analysing the spiked plasma samples on five different days covering a time period of two weeks. The intra- and inter-day repeatability and reproducibility and the accuracy of the method were determined by analysing the spiked extract of SPE-purified bovine plasma in the concentration range of 0.01 to 2 ng ml^{-1} five times on five different days covering a time period of 11 days. The accuracy was expressed as the percentage of the measured concentration versus the spiked concentration. The stability of the samples was assessed by analysing 21 spiked plasma samples with concentration levels between 0.01 and 2 ng ml^{-1} stored for 24

or 72 hours at 5 °C and –20 °C. The percentage carry-over was determined by the injection of blank Milli-Q water following the analysis of plasma samples spiked at concentration levels of 0.5 and 5 ng ml^{-1}.

Linearity

The calibration curves were linear in the concentration range from 0.01 to 2 ng ml^{-1}. The linearity (R^2) for all peptides was better than 0.997. The LOD was below 0.01 ng ml^{-1} for all peptides. The LOQ values ranged between 0.01 and 0.03 ng ml^{-1}. The LOD and LOQ met the target values as described in the introduction. A summary of linearity data of the assay validation is given in Table 5. Representative chromatograms of seven of the peptides studied are given in Figure 1.

Table 5 Linearity data of peptide detection in human plasma using LC-MRM-MS.

Peptide	Limit of detection LOD (ng ml^{-1})	Limit of quantification (LOQ (ng ml^{-1})	Linear regression coefficient (R^2)
VPP	0.01	0.05	0.9987
IPP	0.005	0.01	0.9993
LPP	0.002	0.01	0.9991
IPPL	0.002	0.01	0.9989
HLP	0.001	0.01	0.9994
HLPLP	0.01	0.05	0.9974
VAP	0.01	0.05	0.9978
FY	0.005	0.01	0.9987
VF	0.001	0.01	0.9994
VY	0.001	0.01	0.9990
IY	0.003	0.01	0.9996
AW	0.003	0.01	0.9990
IW	0.005	0.01	0.9988
LW	0.002	0.01	0.9992

Repeatability, reproducibility and accuracy

Statistical evaluation of the results indicated an acceptable accuracy, repeatability and reproducibility of the method. The repeatability and reproducibility improved significantly at higher concentrations. The intra- and inter-day results were very similar. The results of the inter-day measurements are given in Table 6. The precision is expressed as the coefficient of variation (C.V.,%); n=5. The accuracy is expressed as [(mean calculated concentration)/(spiked concentration)] x 100.

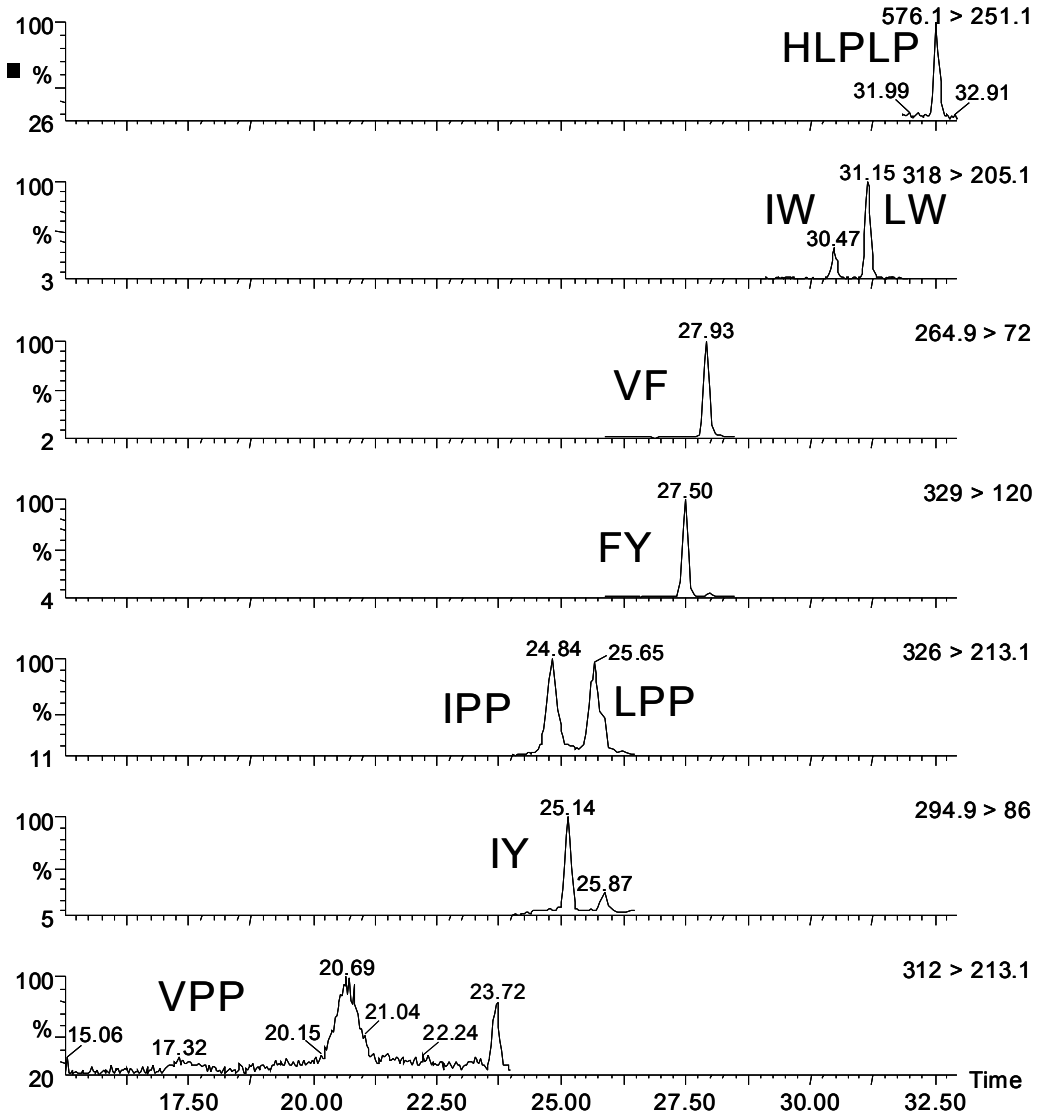

Figure 1 Typical chromatogram of a standard solution with a concentration of 0.05 ng ml^{-1} for each peptide. For display purposes only the chromatograms of seven randomly selected peptides are given, the chromatograms of the other peptides are very similar.

For some of the peptides the inter-day and intra-day precision values for the LOQ are above the generally accepted limit for quantitative bioanalyses. Despite that, the accuracy and precision of the method enable its application for monitoring changes in the concentration of the peptides upon the intake of a drink enriched in ACE-inhibiting peptides.

Table 6 Inter-day precision and accuracy of the assay.

Conc. in ng ml^{-1}	VPP		IPP		LPP		IPPL	
	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy
0.01	29.8	112.1	21.6	99.8	22.1	88.8	22.1	0.01
0.05	23.8	84.8	15.8	101.6	16.7	103.8	16.7	0.05
0.1	13.0	109.6	17.9	103.2	9.5	108.8	9.5	0.1
0.2	4.5	94.0	9.9	99.2	3.4	100.2	3.4	0.2
0.5	2.9	97.0	7.0	95.0	2.2	94.8	2.2	0.5
1	1.9	102.6	4.4	99.8	5.0	96.0	5.0	1
2	0.4	99.8	1.5	100.4	0.9	101.4	0.9	2

Conc. in ng ml^{-1}	HLP		HLPLP		VAP		FY	
	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy
0.01	23.0	85.8	26.7	85.8	28.5	119.4	23.6	112.0
0.05	19.9	84.6	19.7	87.2	24.5	101.4	14.7	85.4
0.1	13.8	101.6	21.2	110.8	11.3	118.8	8.3	113.6
0.2	11.3	98.0	4.2	100.6	6.4	95.2	1.4	100.0
0.5	3.5	96.4	6.3	95.2	8.6	94.6	2.0	97.2
1	3.1	104.6	5.1	104.8	5.6	99.8	2.5	104.8
2	0.8	99.2	1.3	98.8	1.5	100.2	0.9	99.4

Conc. in ng ml^{-1}	VF		VY		IY		AW	
	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy
0.01	21.5	112.1.	23.9	88.8	23.6	84.4	21.1	87.2
0.05	18.8	101.2	21.1	82.2	21.5	89.8	20.5	89.0
0.1	9.3	118.0	9.7	107.8	12.7	108.6	5.0	109.2
0.2	4.9	98.0	3.3	99.2	7.5	95.4	5.9	96.2
0.5	5.0	100.0	4.9	98.6	4.1	98.8	4.3	97.4
1	2.4	105.2	1.7	103.8	4.6	105.6	2.7	103.8
2	0.8	98.8	0.9	99.4	1.7	98.6	0.9	99.4

Conc. in ng ml^{-1}	IW		LW	
	Precision	Accuracy	Precision	Accuracy
0.01	24.8	89.8	22.7	111.7
0.05	17.9	88.0	15.8	108.2
0.1	4.0	112.6	6.2	99.1
0.2	5.5	96.6	5.1	98.6
0.5	3.5	98.8	3.8	99.4
1	2.3	104.4	2.0	99.0
2	1.1	99.2	0.9	98.8

Stability

No significant decrease could be detected in the concentration of peptide solutions prepared in the five fold diluted SPE extract of bovine plasma in concentrations of 0.05 ng ml^{-1} and at 2 ng ml^{-1} stored for 24 and for 72 hours at 5 °C or at –20 °C. The variations seen were within the reproducibility limits of the method.

Carry-over

The percentage carry-over was below 1% for VPP, IPP, LPP, IPPL, VAP, VY, AW, IW and LW measured at 0.5 ng ml^{-1} and at 5 ng ml^{-1}. For FY the carry-over was measured to be 5% at 0.5 ng ml^{-1} and 1% at 5 ng ml^{-1}. The carry-over for HLPLP, VF, and IY was 10% and 1% at concentration levels of 0.5 and 5 ng ml^{-1}, respectively. The relatively high carry-over for the latter peptides was probably caused by partial release of these peptides adsorbed to the column material during preceding runs. This adversely affects the accuracy of the method for these peptides.

Robustness

The method was used in a human trial where the influence of food peptides in relation to blood pressure was studied. The method proved to be robust. At least 200 plasma samples could be analysed before the MS source had to be cleaned. The HPLC-column did not show any sign of deterioration from this large number of samples. The only precaution that was taken was the replacement of the guard column after 250 samples. The method was applied to more than one thousand plasma samples collected during this human trial. Typical ion chromatograms obtained from a plasma sample collected during the trial are given in Figure 2. The levels found in this particular sample ranged from 0.05 ng ml^{-1} for VPP to 0.3 ng ml^{-1} for VF. Good peak shapes are seen for all peptides. The slight broadening seen for the early eluting peptides VY and VPP is the result of the large injection volume. The high selectivity of the chromatographic method is nicely reflected in the separation of the peak pairs IPP/LPP and IW/LW. Baseline separation is obtained for both peptide pairs.

The good sensitivity, the high selectivity and the excellent long term stability of the method clearly indicate that the process used for sample preparation, chromatographic separation and detection is in proper balance. This makes the developed method a useful tool in research aimed at the development of novel anthypertensive functional foods.

5.4 Conclusions

An HPLC-MRM-MS method has been developed and validated for measuring seventeen ACE inhibiting small peptides in human blood plasma in one single analysis. The sample preparation method was optimised. Various methods for protein removal were compared. The best results were obtained using acidification and heating. The application of SPE for protein removal resulted in poor reproducibilities. The limits of detection and quantification were between 0.001 and 0.05 ng ml^{-1} and are low enough to

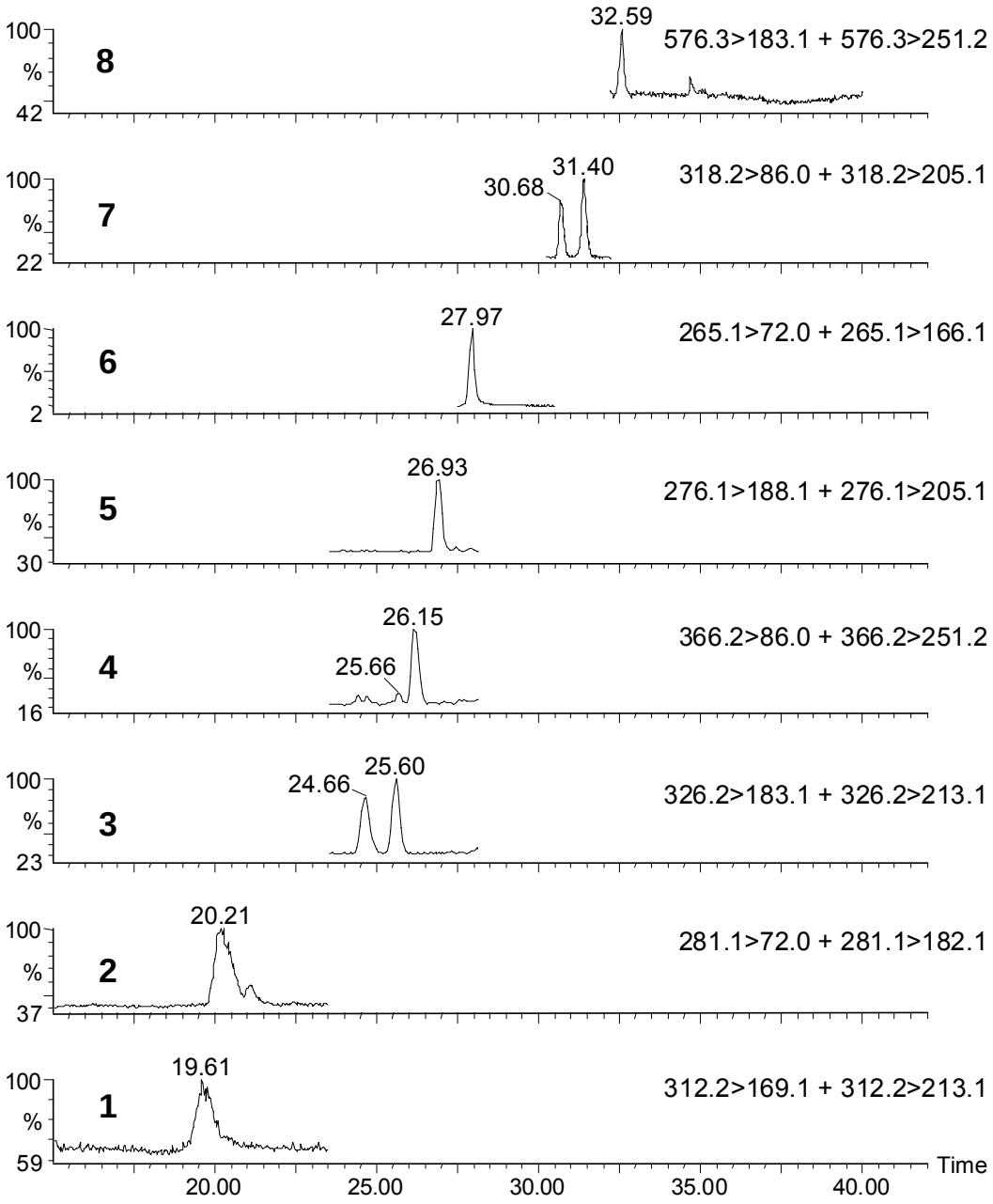

Figure 2 Typical chromatogram of a plasma sample collected during a human trial. The traces represent the summarized traces of the two product ions monitored for each peptide indicated in the chromatograms. Only the traces of the peptides detected in the sample are displayed. Trace 1: VPP, 2: VY, 3: IPP Rt 24.66 and LPP Rt 25.60, 4: HLP, 5: AW, 6: VF, 7: IW Rt 30.68 and LW Rt 31.40, 8: HLPLP.

monitor the peptides at the concentration levels occurring in case of a normal diet. The accuracy is better than 75% and intra- and inter-day

relative standard deviations are below 25%, enabling monitoring of even small changes in the concentration levels after consumption of a peptide-enriched drink. The method was successfully applied to large numbers of plasma samples collected during a human trial. At least 200 samples could be analysed before the MS source had to be cleaned making the method practically suitable for application in human trials where large numbers of samples have to be measured in time periods of weeks or months.

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6 Bioavailability of ACE inhibitory peptides

Summary
The method developed for the quantification of bio-active peptides in plasma as described in chapter 5, was applied to plasma samples collected from a cross-over study. The aim of this placebo-controlled crossover study was (a) to assess the bioavailability of IPP and seven other ACE-inhibiting peptides present in a lactotripeptide- (LTP) enriched yoghurt beverage and (b) to study possible effects of IPP on bioavailability. Six male and female subjects randomly consumed an LTP-enriched yoghurt beverage or a placebo in the fasted and an LTP-enriched yoghurt beverage in the fed or fasted state. The Area under Curve (AUC) of IPP after the LTP treatment in the fasted state was 2.1-fold of that after the placebo treatment ($P<0.001$). The maximum concentration after administration reached in the time/concentration curve (C_{max}) was greater after consumption of the LTP-enriched beverage (897 ± 157 pmol l^{-1}) than after the placebo treatment (555 ± 80 pmol l^{-1}, $P<0.001$) with a greater time at the C_{max} (T_{max}) in the placebo treatment ($P=0.06$). Plasma concentrations of the peptides LW, FY, IY, and LPP increased significantly compared to baseline ($P<0.05$) in the LTP-enriched and placebo treatment when consumed in the fasted state. However ΔC_{max}-values differed significantly between the placebo and LTP-enriched treatment only for LPP. IPP concentrations were affected by meal intake. When the beverage was consumed after a meal, the AUC of IPP was 1.3-fold ($P<0.05$) of the AUC derived from pre-meal intake. This was due to an increase in the plasma elimination half-life ($P<0.05$), C_{max} and T_{max} were not affected by meal intake. In summary, this is the first demonstration that the tripeptide IPP selectively escapes from intestinal degradation and reaches the circulation undegraded.

6.1 Introduction

Bioactive peptides are increasingly becoming of interest in the development of functional food products, as selected food proteins contain precursor sequences of peptides that may exert a physiological effect in the body when released from the parent protein [1-3]. One of the best-studied classes of bioactive peptides so far is the class of peptides with blood pressure-lowering activity. To date, several studies in spontaneously hypertensive rats and humans with elevated blood pressure demonstrated a antihypertensive effect of selected fermented or partly enzymatically hydrolyzed milk products [4-10]. In most cases, the antihypertensive effect of those bioactive peptides was explained by their angiotensin I-converting enzyme (ACE, EC 3.4.15.1) inhibitory activity. ACE is one of the key enzymes in blood pressure regulation, because it generates the vasoconstrictor angiotensin II and inactivates the vasodilator bradykinin [11]. Numerous ACE-inhibitory peptides, derived from milk protein fractions or from other food proteins, have been described *in-vitro* and predicted to be effective antihypertensives *in-vivo* [3,12-14]. However, it is difficult to establish a direct relation between ACE-inhibitory activity detected in these products *in-vitro* and antihypertensive activity *in-vivo*, because bioavailability of peptides is a major issue. On this account, it has been suggested that selected, very effective *in-vitro* ACE-inhibitory peptide hydrolysates from α-casein failed to lower blood pressure in models of hypertension because of intestinal breakdown of the peptides [15].

The underlying motivation for the research reported here is based on the assumption that crossing the peptidolytic barrier of the gastrointestinal tract is a prerequisite before any antihypertensive effect, whether via inhibition of ACE or other mechanisms, can be postulated, given that the bioactive peptides have to reach the cardiovascular system intact. However, whole peptide absorption was shown to be generally negligible [16,17], because the peptides have to resist the hydrolysis by gastric and pancreatic proteases as well as brush border amino- and carboxypeptidases, although a few exceptions have been reported [18]. Dipeptides and tripeptides are efficiently taken up by the enterocyte via Peptide Transporter 1 [19], but the effective degradation by cytosolic and plasma peptidases limits their bioavailability. Surprisingly, studies on bioactive peptides have generally failed to consider these aspects. Only a few simulated gastrointestinal digestion studies were performed and demonstrated that, depending on the amino acid sequence, some peptides, particularly C-terminal Pro- and Pro-Pro-containing peptides, were stable under simulated gastrointestinal conditions, making these peptides more likely to be effective antihypertensives [20]. However, *in-vitro* absorption studies with *in-vitro*

ACE-inhibitory peptides, stable under gastrointestinal conditions, showed very limited transport rates of peptides [21-23], implying only limited transport across the intestinal barrier of nonhydrolyzed peptides *in-vivo*.

To our knowledge, only two bioavailability studies with antihypertensive, food-derived peptides were performed *in-vivo* in human subjects [24,25]. A single oral administration of an aqueous solution containing the peptide VW led to a dose-dependent increase of VW up to nanomolar concentrations in the plasma of normotensive as well as mildly hypertensive subjects. [24,25]. Beside these two studies, bioavailability of food-derived blood pressure-lowering peptides has not been investigated in human subjects, to our knowledge. The aim of the present study was to characterize whether the blood pressure-lowering tripeptide IPP [8] derived from enzymatically hydrolyzed casein is bioavailable in human after oral administration. Furthermore, we wanted to investigate whether IPP and other ACE-inhibitory peptides encrypted in milk protein are liberated in the intestinal tract and absorbed partly in a nonhydrolyzed form. Therefore, we examined in healthy male and female subjects a detailed kinetic profiling of IPP after oral consumption of a lactotripeptide (LTP)-enriched yoghurt beverage and determined plasma concentrations of seven other peptides (LPP, AW, IW, LW, VY, IY, FW) with known high *in-vitro* ACE-inhibitory activities [26]. In addition, special attention was given to the fact that consumption of the LTP-enriched beverage together with a meal could affect IPP bioavailability. Thus, we investigated the effect of consuming the beverage either in a fasted state, i.e. in the morning 30 min before breakfast on an empty stomach, vs. a fed state i.e. 30 min after the start of a breakfast.

6.2 Experimental

6.2.1 Materials and Methods

Subjects

Participants in the age of 18 – 55 y were recruited among inhabitants of the town of Vlaardingen and surroundings. Subjects were screened to be generally healthy as assessed by means of a screening questionnaire, body mass index (BMI) assessment (19 – 27 $kg \cdot m^{-2}$), standard hematology, and urine. The subjects were all nonsmokers and had not donated blood at least 4 weeks (men) or 8 weeks (women) before the start of the study. A total of 17 subjects were screened and six subjects plus three reserve subjects were included in the study. Three persons withdrew from the study for personal and medical reasons; these subjects were replaced by the three reserve subjects. The six subjects (four female, two male) were randomly assigned to one of the six treatment sequences (see study design). Participants were informed about the study and all subjects signed an informed consent form before participation. The Medical Ethical Committee of Wageningen

University approved the study (approved November 2005). The baseline characteristics of the study participants are summarized in Table 1.

Study design

The study was a randomized, placebo-controlled, full crossover intervention study performed at the Unilever Food and Health Research Institute, Vlaardingen, The Netherlands. The study was conducted following the guidelines of Good Clinical Practice. The study consisted of three intervention days, which were separated by two 6 day washout periods. In the morning of each intervention day, the subjects who had fasted overnight came to the study facility and received one intervention. The subjects were randomly allocated to one of the six possible intervention sequences. In intervention 1, subjects consumed a 250 ml beverage containing 57 mg of LTP (20.4 mg IPP, 20.0 mg VPP and 16.5 mg LPP, in the following referred as "LTP-enriched" test beverage) after a 10 h over-night fast and 30 min prior to intake of a standardized breakfast. In intervention 2, subjects consumed an identical beverage 30 min after the start of a standardized breakfast. In intervention 3, the control intervention, a 250 ml beverage without added LTP equivalents was consumed after a 10 h over-night fast and 30 min prior to intake of a standardized breakfast. The LTP-enriched yoghurt beverage and the placebo beverage were similar with respect to their content in carbohydrate, fat, protein and calcium (Table 2). Blood was sampled directly before consumption of the beverage (t = 0) and at 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 75, 90, and 120 min after consumption of the beverage. In intervention 2, we took an additional sample before consumption of the breakfast (t = -30 min). During the study, the subjects were encouraged to minimize changes in composition of their habitual diet. In addition to the 10 h fasting period, the subjects had a two-day run-in period before each intervention day in which they were not allowed to consume fermented dairy products, such as cheese and yoghurt.

Table 1: General characteristics of study participants[1]

Parameter	**Male (n=2)**	**Female (n=4)**
Age, *y*	21.0 ± 2.0	41.8 ± 7.1
Weight, *kg*	73.3 ± 1.1	67.4 ± 3.7
Height, *cm*	186.5 ± 7.3	169.0 ± 3.0
BMI, $kg \cdot m^{-2}$	21.0 ± 1.0	23.5 ± 0.6

[1]Data are expressed as mean ± SD

Standardized breakfast

On the intervention days, we gave the subjects the LTP-enriched or the placebo yoghurt beverage and a standardized breakfast at the study facility. The standardized breakfast consisted of two slices of 70 g of whole wheat bread, 10 g of spread, 30 g of strawberry jam and 125 ml of semi-skimmed milk. During the intervention days, the subjects were allowed to drink a standardized amount of water. The breakfast provided 1.4 MJ energy, with 68 %, 14 % and 18 % of the energy derived from carbohydrate, protein and fat, respectively. Subjects consumed the same standardized breakfast on all study days.

Study product

All test beverages were produced from pasteurized, acidified semi-skimmed yoghurt. The LTP's in the LTP-enriched yoghurt beverages were derived from AmealPeptide™ powder (Calpis Co. Ltd., Tokyo, Japan). AmealPeptide™ powder was manufactured by enzymatic hydrolysis of milk casein followed by spray drying. AmealPeptide™ contains high concentrations of the ACE inhibiting tripeptides IPP, VPP and LPP. AmealPeptide™ powder was added to the semi-skimmed yoghurt at a concentration of 2.8%. An orange flavor concentrate was mixed with the yoghurt and the mixture was pasteurized and poured aseptically into 250 ml plastic bottles. The placebo beverage was similar to the LTP-enriched beverage, but did not contain AmealPeptide™ powder. The protein content was adjusted to the protein level in the LTP-enriched yoghurt beverage by adding 2.8% whey protein isolate. The nutritional composition of the LTP-enriched and the placebo yoghurt beverage is given in Table 2.

Collection of plasma samples

Venous blood samples were collected in chilled K_3-EDTA-treated tubes. Plasma was separated by centrifugation (3000 g, 15 min, 4°C) within 30 min of collection. TFA was added to a final concentration of 1% and samples were stored at –80°C until assayed.

Peptide analysis by on-line RPLC-MRM-MS

Plasma concentrations of IPP, LPP, AW, IW, LW, VY, IY and FY were determined using the LC-MRM-MS method described in chapter 5. Peptide separation was performed on a 150 x 2.1 mm ODS3 column (Varian, Bergen op Zoom, the Netherlands) with a particle size of 5 µm. The gradient started at 100% 0.1% TFA in Milli-Q water and ended at 70% 0.1% TFA in acetonitrile. To support the ionization a 70/30 mixture of propionic acid and propanol-2 was added post column at a flow rate of 50 µl min^{-1}. All analyses were carried out on an Alliance 2795 HPLC (Waters, Brussel, Belgium) combined with a Quattro Premier triple quadrupole mass spectrometer from

the same supplier. As internal standard [U^{13}C]Ile-Pro-Pro (BioPeptide, San Diego, USA) was used. Calibration standards varied between 3 nmol·l^{-1} and 6146 nmol·l^{-1} and the concentration of the internal standard was constant at 3073 nmol l^{-1}. The specificity and sensitivity of the described HPLC-MRM-MS method has been demonstrated in chapter 5 with limits of quantification between 31 pmol·l^{-1} (IPP, LPP, IW, LW and FY) and 36 pmol·l^{-1} (AW, IY and VY).

Table 2 Nutritional composition of the test beverages[1]

Nutritional Content	**LTP-enriched beverage**	**Placebo beverage**
Energy, kJ·l^{-1}	3349	3349
Carbohydrates, g·l^{-1} - Glucose - Fructose - Sucrose - Lactose - Maltose	125 13 42 45 23 <0.5	132 13 42 45 23 <0.5
Protein, g·l^{-1} - SMP[2] - LTP powder - WPI[2]	51 23 28 -	51 23 - 28
Lactotripeptide, mg·l^{-1} - IPP - LPP - VPP	 81.6 ± 1.6 66.0 ± 2.0 80.0 ± 1.2	 <0.5 <0.5 <0.5
Fat, g·l^{-1}	3.1	2.9
Ca^{2+}, mg·l^{-1}	1030	1000
Mg^{2+}, mg·l^{-1}	110	110
Na^{+}, mg·l^{-1}	760	330
Lactic Acid, g·l^{-1}	6.6	6.1
Citric Acid, g·l^{-1}	2.9	29
Acetic Acid, g·l^{-1}	0.1	<0.1
Ash, g·l^{-1}	7.3	6.4
Moisture, g·l^{-1}	810	802

[1]Values are single measures, lactotripeptide content expressed as means ± SD, n = 3.
[2]SMP, skimmed milk protein; WPI, whey protein isolate

Calculations and Statistics

The primary outcome in this study was the area under the plasma peptide concentration vs. time curve after the test treatment. The area under the curve (AUC) was calculated by the linear trapezoidal rule as follows:

$$AUC = \frac{1}{2}\sum_{i=1}^{n-1}(m_{i+1} - m_i)(H_{i+1} + H_i)$$

where m_i is the i^{th} min, H_i is the i^{th} available peptide value, and n is the number of min. Peptide bioavailability was determined over the period from 0 to 120 min; all values were considered in the $AUC_{0\text{-}120}$ calculation. The secondary outcomes were C_{max}, i.e. the maximum peptide plasma concentration, T_{max}, i.e. the time after ingestion when reaching C_{max}, $t_{1/2}$, i.e. the time in the linear elimination phase in which peptide plasma concentration is halved. C_{max} and T_{max} were directly derived from the experimental data, whereas $t_{1/2}$ was calculated from the data within the terminal elimination phase. The rate constant for elimination (λ) was estimated by log/linear interpolation by repeated regression using the last four non-zero concentrations, then the last five concentrations, etc. Data points prior to C_{max} were not used. For each regression an adjusted R^2 was computed and the λ derived from the regression with the largest adjusted R^2 was used. By definition, the terminal half-life $t_{1/2}$ was calculated as follows:

$$t_{1/2} = \ln(2)/\lambda$$

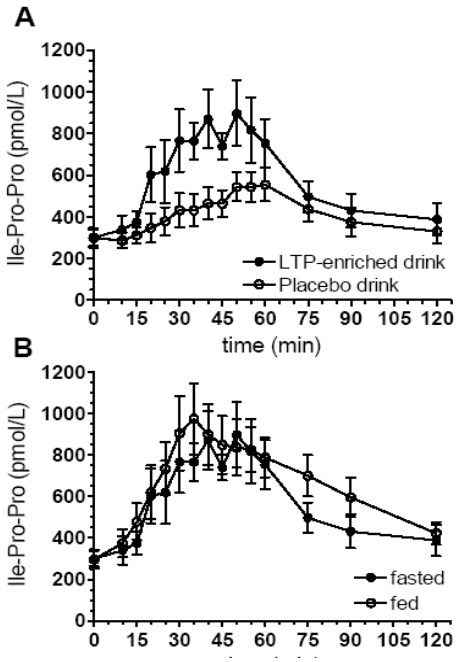

Figure 1 Plasma IPP concentrations in healthy subjects after consumption of LTP-enriched and placebo yoghurt beverages in the fasted state (A) and after consumption of the LTP-enriched yoghurt beverage in fasting and the fed states (B). Values are expressed as mean ± SD (n=6).

The statistical analysis was performed using the software package SAS 9.1 (SAS Institute, Cary, North Carolina, USA). Descriptive analyses consist of distribution statistics (number of available observations, mean, standard deviation and 95% confidence intervals) for continuous data. The

effects of treatments on both primary and secondary outcomes were evaluated by means of an analysis of variance (ANOVA) including treatment, period and subject in the model. The 3 treatments were compared to each other using the multiple comparison Tukey tests that assured an overall alpha error of 5%. The difference between each treatment was estimated on the basis of adjusted means. No adjustments were made for multiplicity due to testing multiple (secondary) variables. The secondary analyses were expected to support the primary results. All tests were 2-sided with a significance level of 5%.

6.3 Results and discussion

ACE inhibitory peptide composition of the test beverage

The amount of IPP, VPP and LPP present as free tripeptide was 20.4 mg, 20.0 mg and 16.5 mg in 20 ml of the LTP-enriched yoghurt beverage, respectively. The placebo product contained <0.13 mg of each peptide in 250 ml, demonstrating that the LTP-enriched beverage indeed contained a greater proportion of free IPP (Table 2).

Maximum IPP plasma concentration and time after bolus intake

The proportion of subjects that provided sufficient data to construct complete kinetic profiles using a one-compartment model was <50% of the data set. This was mainly due to a biphasic and scattered absorption phase (individual data not shown). Thus, kinetic parameters were calculated from all subject data by non-compartmental analysis without modeling the data. For each of the parameters, dot plots were constructed to evaluate the presence of extreme outliers. For each parameter all individual values were <3 SD from the mean and data from all subjects were included into the analysis. The mean plasma concentrations of IPP changed after an oral bolus of 250 ml of an LTP-enriched yoghurt (2.8% AmealPeptide™) or placebo (2.8% whey protein isolate) beverage (Figure 1).

In both cases, the beverage was consumed 30 min before intake of the breakfast. After a 10 h overnight fast before administration of the test beverages IPP plasma concentrations were about 300 pmol·l^{-1} and differed not between the LTP-enriched and the placebo treatment (Table 3). Consumption of the whey protein isolate containing placebo beverage led to a significant ($P \leq 0.05$) increase in IPP plasma concentrations to a peak plasma concentration (C_{max}) of 555 ± 80 pmol·l^{-1} plasma, as compared to baseline concentrations. However, after consumption of the LTP-enriched beverage the C_{max} value of IPP was 1.6-fold elevated of that after the placebo treatment. Although there was a significant ($P \leq 0.05$) difference between the C_{max} values of subjects consuming the LTP-enriched and placebo beverages their corresponding time to reach maximum plasma concentration (T_{max}) did not differ significantly (Table 3). However, we

observed a strong trend (P = 0.09) that T_{max} was reached faster after consumption of the LTP-enriched beverage compared to the placebo beverage consumption (Table 3).

Plasma bioavailability of IPP (net AUC) and curve progression

Since IPP was detectable at baseline in all subjects, and since these levels varied between subjects, net area under the curve (AUC) values for plasma IPP were calculated by correcting for baseline concentrations per subject. The *net* AUC for plasma IPP over a period of 120 min after subjects consumed the LTP-enriched yoghurt beverage in the fasted state was 2.2-fold higher ($P \leq 0.001$) than after consuming the placebo yoghurt beverage (Table 3). The kinetic curves showed that when subjects consumed the LTP-enriched yoghurt beverage, plasma IPP concentrations increased with almost no lag time and were significantly different ($P \leq 0.05$) from baseline values 15 min after ingestion, whereas in the placebo treatment those were significantly ($P \leq 0.05$) different from baseline after 25 min. In these concentrations the LTP-enriched and the placebo treatment, IPP concentrations returned almost to baseline by 120 min.

Plasma concentrations of other ACE inhibitory peptides

Beside IPP, we determined plasma concentrations of the ACE-inhibitory peptides LPP, AW, IW, VY, IY, FY, and LW in response to oral intake of an LTP-enriched and a placebo yoghurt beverage in the fasted state. Likewise, background levels of these peptides were present in varying amounts in plasma of fasting subjects. Especially the dipeptides LW, IW and IY showed much higher background levels in human plasma compared to the tripeptides. Plasma levels of all peptides increased in response to intake of the LTP-enriched and the placebo yoghurt beverage. The increase measured as ΔC_{max}, that is the difference between C_{max} and baseline concentrations, differed ($P \leq 0.05$) after the placebo (96 ± 34 pmol·l^{-1}) and LTP-enriched (152 ± 85 pmol·l^{-1}) treatments only for the tripeptide LPP.

Effect of meal intake on IPP bioavailability: comparison of fasted and fed state

To test whether concomitant food intake can affect IPP bioavailability, subjects consumed the LTP-enriched beverage in fasted and fed state, i.e. 30 min before and 30 min after starting to eat a breakfast. All subjects consumed breakfast in <15 min. Intake of the LTP-enriched yoghurt beverage shortly after a meal affected IPP bioavailability as judged from net AUC values. When the LTP-containing beverage was taken in the fasted state mean plasma AUC values were 1.2-fold of that after the placebo treatment (Table 3). Net AUC differed between the treatments ($P \leq 0.05$) (Table 3). The plasma half-life of Ile-Pro-Pro was prolonged ($P \leq 0.01$) when

the LTP-enriched yoghurt beverage was consumed after the breakfast (Figure 1; Table 3). The C_{max} and T_{max} values did not differ between the fed and fasted treatment ($P > 0.05$).

Table 3 IPP kinetic measurements in healthy subjects after consumption of an LTP-enriched and placebo yoghurt beverage in the fed and fasted state.

	Treatment		
Parameter	**Placebo** fasted	**LTP-enriched** fed	**LTP-enriched** fasted
dose[1] **Ile-Pro-Pro** *mg l*$^{-1}$ *µmol·l*$^{-1}$	<0.15[a] <1.50[a]	81.6 ± 1.6[b] 100 ± 11[b]	81.8 ± 1.5[b] 100 ± 12[b]
baseline, *pmol·l*$^{-1}$	299 ± 45 (157 − 436)	296 ± 65 (174 − 467)	300 ± 38 (175 − 428)
C_{max}, *pmol·l*$^{-1}$	555 ± 80[a] (344 − 845)	973 ± 180[b] (636 − 1514)	897 ± 157[b] (631 − 1663)
T_{max}, *min*	54.2 ± 7.9 (40 − 60)	41.7 ± 15.7 (20 − 60)	39.9 ± 9.9 (25 − 50)
AUC_{0-120}, *nmol·min·l*$^{-1}$	18.0 ± 5.7[a] (8.9 − 26.0)	47.5 ± 17.5[c] (27.7 − 77.4)	38.5 ± 19.9[b] (14.6 − 72.3)
HL[2]**,** *min*	n.d.	38.6 ± 13.5[b] (28.7 − 58.8)	26.4 ± 15.1[a] (5.7 − 44.1)
MRT, *min*	61 ± 4 (56 ± 66)	58 ± 5 (49 ± 66)	52 ± 5 (44 ± 60)

[1]Values are expressed as least square means ± SD (range), n = 6. Means in a row with superscripts without a common letter differ, $P \leq 0.05$.
[2]HL, plasma half-life; [3]n.d., not determined, [4]MRT, mean residence time.

6.4 Conclusions

Uptake of intact peptides from the gut lumen into the circulatory system is discussed controversial in literature. Here, using a highly sensitive and specific LC-MRM-MS method, we describe for the first time, to our knowledge, that the tripeptide IPP was present in plasma of human subjects and that its plasma levels increased after intake of an LTP-enriched yoghurt beverage. Furthermore, we show that IPP plasma concentrations are augmented by concomitant intake of the LTP-beverage and a meal.

Our double blind, placebo-controlled crossover study strongly suggests, although not directly demonstrates, that Ile-Pro-Pro is bioavailable after oral consumption of an LTP-enriched yoghurt beverage. Evidence for this was provided by the *net* AUC and C_{max} data for Ile-Pro-Pro that were significantly higher for the LTP-enriched yoghurt beverage compared with the placebo, in which the LTP powder was replaced with IPP-free whey protein isolate. In addition, ingesting the LTP-enriched beverage significantly increased plasma concentrations of the ACE-inhibitory peptide LPP compared with the placebo. The increased plasma concentrations of LPP detected in our study were similar to those of the dipeptide VY detected in plasma after oral dosing of 6 mg and 12 mg VY [24,25]. In contrast to the long half-life of 3.1 h of VY detected [24,25], IPP was cleared from plasma much faster with an elimination half-life of ~30 min. A third study, reporting a plasma half-life of 20 min for the dipeptide AQ after continuous intravenous infusion [27], supports our findings of rapid disappearance of peptides from plasma. In addition, the C_{max} of IPP was about 1 nmol·l^{-1}, which is far below its effective concentration on ACE inhibition determined *in-vitro* (50% inhibition constant = 5 µmol·l^{-1}) [28], although higher doses of IPP were used in our study than in human studies showing a antihypertensive effect in response to LTP consumption [29,30]. Thus, it remains questionable whether those peptides lower blood pressure solely via an ACE-inhibiting mechanism.

Surprisingly, plasma IPP concentrations were also significantly elevated above baseline concentrations after consumption of the whey protein isolate-containing placebo. This suggests that IPP was generated in the intestinal tract by luminal and brush border peptidases, as the placebo beverage contained no free IPP. Minor amounts of IPP-precursors were present in the yoghurt matrix, e.g. κ-casein, and in the milk protein consumed with the breakfast. The lagged increase in plasma concentration of IPP and the trend of higher T_{max} values after the placebo treatment (54.2 ± 7.4 min) than in the LTP-enriched treatment (39.9 ± 9.9 min) ($P = 0.09$) suggest that IPP after the placebo treatment was indeed generated during the digestion process in the intestinal tract. Evidence for the release of IPP from large peptide sequences in the intestinal tract was given in *in-vitro* digestion studies using simulated gastrointestinal fluids, which showed liberation of IPP from casein peptides [31,32]. The difference in *net* AUC of IPP between placebo and LTP-enriched treatment was small, although significant, compared with their large concentration difference in the test beverages. This raises the question whether hydrolysis of IPP-containing protein sources prior to consumption is necessary to increase the bioavailability of bioactive peptides. This question needs to be addressed in a separate study in which the bioavailability of IPP is directly compared between non-hydrolyzed casein sources and hydrolyzed casein.

The net AUC value of IPP (P ≤0.05) was larger after consumption of the LTP-enriched beverage in the fed compared with the fasted state. Furthermore, the elimination half-life of IPP was increased (P ≤0.01) after the fed treatment. A possible mechanism causing this effect could be that meal proteins act in an unspecific, competitive manner with the tripeptide for luminal, brush border and cytosolic peptidase activity and thus protect Ile-Pro-Pro from hydrolysis by saturation of peptidases. Furthermore, meal-derived peptidase inhibitors, e.g. antitrypsin and antichymotrypsin activity as described in wheat [33], could cause the increase in IPP plasma concentrations. We found that a variety of dipeptides, including the dipeptide Val-Tyr, were present after a 10 h fasting period in human plasma in pico- and nanomolar concentrations, as determined by means of LC-MRM-MS.

To our knowledge, this is the first demonstration of the existence of endogenous di- and tripeptides in human plasma. This is in contrast to the observation of Matsui et al. who showed the absence of the dipeptide VY in plasma of fasting subjects [25]. An explanation for the differences might be the analytical method used. However, it is difficult to predict from the results of our study what the origin of IPP, LPP and the other dipeptides in plasma at baseline is. Given the short half-life of IPP of ~30 min determined in this study, it is unlikely that the IPP detected before intake of the test beverages is derived from dietary origin. On the other hand, numerous human proteins contain the IPP sequence, e.g. heparanase and cadherin-14 [34,35], suggesting that the peptides detected at baseline could be derived from the constant turnover of proteins containing the respective sequences. The fast attainment of peak plasma concentrations in <1 h for IPP and the differences in *net* AUC between LTP-enriched and placebo treatment strongly suggests that those peptides are derived from the test beverages. Secretion of endogenous peptides in response to meal intake, however, should be considered. After both treatments, significantly increased plasma levels of LW, FY and IY were detected after intake of the test beverages. Because the LTP-containing beverage was not specifically enriched in dipeptides, no difference between treatments was detected. Interestingly, the levels of dipeptides in plasma tended to be generally higher than those of the tripeptides. This is in line with the observation that bioavailability of peptides increases with decreasing chain length [36].

The low plasma concentrations of IPP observed in this study confirms the low oral bioavailability of peptides and indicates that presystemic enzymatic degradation and poor penetration of the intestinal barrier determines this low bioavailability. In general, permeation of solutes across the gastrointestinal epithelium occurs via paracellular and transcellular absorption pathways, and can be influenced by active and polarized transport systems. Di- and tripeptides are taken up actively and quickly by enterocytes via the peptide transporter 1. The efflux of peptides into the

blood, however, is limited, because no active transport system at the basolateral side of the enterocyte supports their release [19,37]. The large variation in physicochemical properties (size, charge, lipophilicity) of peptides, which determines their permeability, makes it impossible to draw general conclusions about their potential to cross the intestinal barrier via paracellular transport or passive diffusion (38). IPP and LPP, however, are hydrophilic peptides with logP values < -2.2. Thus, those peptides are not to be expected to follow a transcellular pathway of absorption through passive diffusion [40]. The size of the paracellular space of 10 – 50 Å in the duodenum and jejunum would allow a limited paracellular flux of those peptides and may account for the low plasma concentrations detected in the present study. *In-vitro* transport studies across Caco-2 monolayers showing very low permeability constants for VV, VPP and GGYR support this [21-23]. Furthermore, peptides consisting of proteinogenic amino acids were rapidly hydrolyzed by apical and cytosolic peptidases in the Caco-2 system [23], indicating that the metabolic instability is a major factor in limiting peptide bioavailability.

In summary, in humans, the peptides IPP and LPP were detected in plasma in picomolar concentrations. Moreover, plasma concentrations increased significantly to nanomolar concentrations when ingested in the form of a LTP-enriched yoghurt beverage. In addition, concomitant intake of the LTP-enriched yoghurt and a meal further increased plasma concentrations of IPP.

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7 Liquid chromatography-mass spectrometric identification of metabolites formed upon incubation of *Hoodia gordonii* steroidal glycosides with human liver microsomes

Summary

In the chapters 3 and 4 the identification of ACE inhibitory small peptides present at μg ml^{-1} in fermented milk was described. In the present chapter the metabolites of *Hoodia gordonii* steroid glycosides formed in a liver microsomal incubation are identified. The structures of the parent steroidal glycosides are known but those of the metabolites are not. As the amount of incubation material is limited and metabolite concentrations are low, a new, sensitive mass spectrometric procedure has been developed for the metabolite identification. The method consists of screening for the molecular ion and some characteristic fragment ions of metabolites predicted by metabolism prediction software. The fragmentation mechanism of the metabolites in MS-MS was determined using model compounds. The MS ionization conditions were optimized to obtain maximum sensitivity, a high abundance of the molecular ion and efficient fragmentation in MS-MS. The best MS performance was obtained using mobile phases doped with lithium salt. With this method six not earlier published groups of metabolites were identified viz. the detiglated steroidal glycosides, the demethylated species, the demethyl-detiglated species and three groups of oxidation products. The method is sensitive and requires only a small amount of sample.

7.1 Introduction

The *Hoodia gordonii* (Masson) Sweet ex Decne is a perennial, succulent plant species from the *Apocycnacaea* family (previously named the *Asclepiadaceae* family) indigenous to the arid regions of South Africa, Botswana and Namibia and is originally found in the Kalahari Desert in Southern Africa. The potential of *Hoodia* extracts for weight management was first noted by the National Food Research Institute of the CSIR in South Africa which conducted a nationwide survey to determine the nutritional value of foods from the wild [1]. These studies showed a significant reduction in food intake upon consumption of *Hoodia* extracts. Patents on the plant extract were filed by the CSIR in 1998 [1]. Van Heerden et al. identified the oxypregnane-based trisaccharide component P57AS3 and a structurally similar molecule containing a tetrasaccharide unit to be responsible for the reduced food intake [2]. In this paper we will follow the nomenclature for *Hoodia* steroidal glycosides as proposed by Janssen et al. [3]. Following this nomenclature P57AS3 will be referred to as H.g.-12. The structure of H.g.-12 and its homologues discussed in this chapter is given in Figure 1.

Pure H.g.-12 was tested for its appetite suppression in rats after oral intake [2, 4]. In subsequent studies Avula et al. confirmed the structure of the two steroidal glycosides by using collision-induced dissociation tandem mass spectrometry (CD-MS^2) and identified six new homologues and isomers in extracts of the *Hoodia gordonii* plant [2, 5]. Pawar et al. identified eleven new oxypregnane-based homologues of H.g.-12 [6] and eleven calogenin-based glycosides [7]. Dall'Acqua finally reported nine oxypregnane-based glycosides representing isomers of the steroid structure with tetra- and pentasaccharide units. [8]. All authors noted that *Hoodia gordonii* extracts are very complex mixtures containing a wide range of structurally related compounds.

To get insight in the physiological behaviour of the different *Hoodia* steroidal glycosides knowledge of the metabolism and the structures of the metabolites formed in the body is required. To our knowledge no papers have so far been published describing the metabolism of *Hoodia* steroidal glycosides in the human physiology. In an excellent review Tolonen et al. summarized the analytical approaches for screening and identification of metabolites. [9]. One of the approaches is the verification of the presence of predicted or expected metabolites. For this approach preferably accurate-mass MS instruments should be used. The high sensitivity of these instruments, even if wide mass ranges are scanned, combined with the accurate mass feature and high resolution, strongly favour their use over other instruments such as triple quadrupole and ion-trap instruments. Nevertheless in some cases one of the other instruments may be preferred

over accurate mass instruments as will be demonstrated in this chapter. Although the verification of just one sample, preferably taken at the time point corresponding with C_{max}, should be sufficient to confirm the presence of the predicted metabolites, in general two samples are analyzed, one collected at C_{max} and a reference sample collected at the start of the experiment (t = 0).

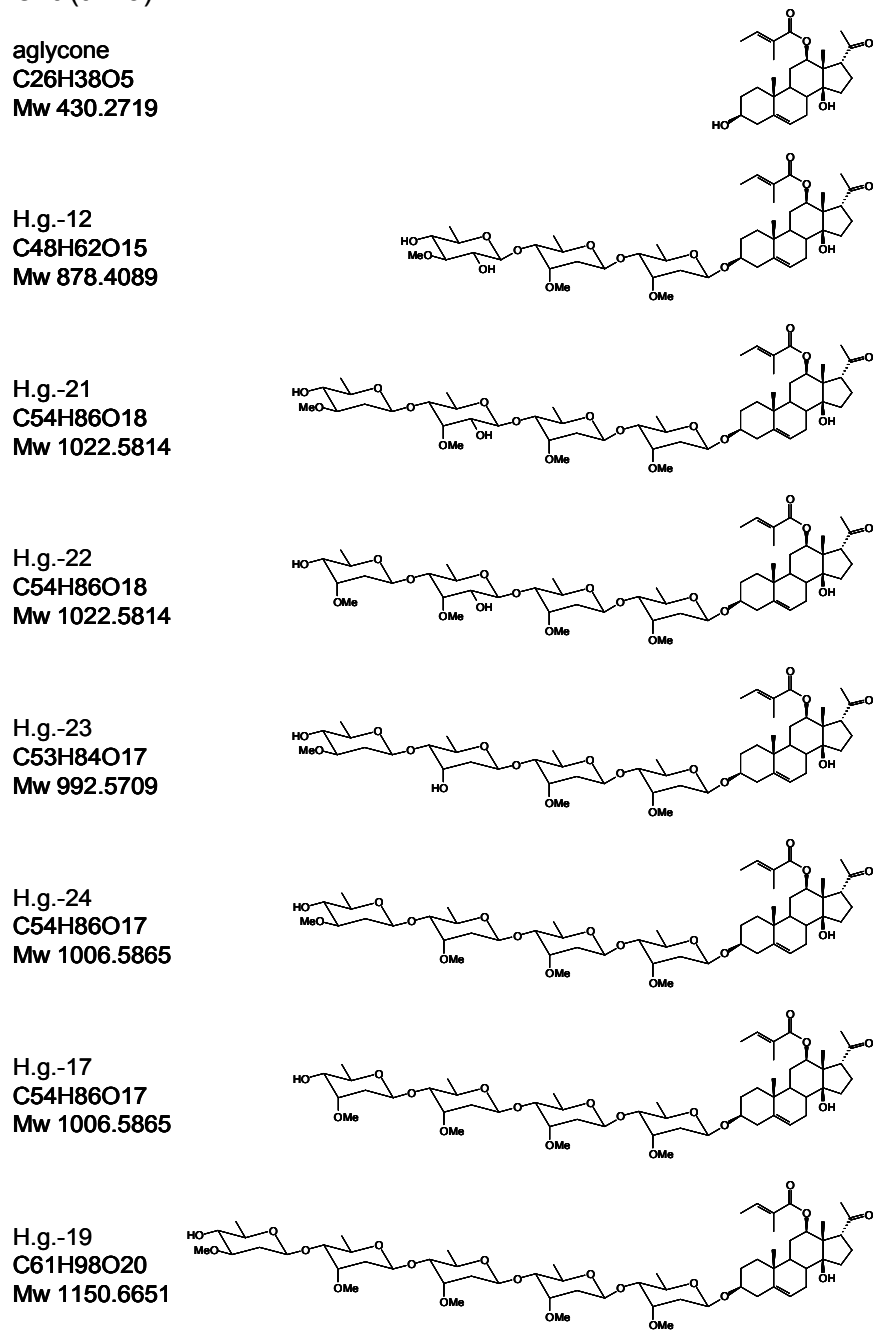

Figure 1 Structures of the main *Hoodia gordonii* glycosides.

The two chromatograms are compared at mass trace level looking for the pseudomolecular ions of the predicted metabolites or at TIC level to find both the predicted and unpredicted metabolites. The process is carried out either manually or using subtraction software and CID-MS^2 on the ions of interest in a second analysis for structure confirmation. The only drawback of this method is that relatively large volumes of sample are required, especially when the instrument available is not equipped with data-dependent scanning options. To limit animal testing, cell-based systems are more and more used in the initial stages of metabolism research. Such systems, however, usually generate only limited sample volumes. One alternative when only small sample volumes are available is nano-LC-MS. This method unfortunately is not very rugged. Another alternative is using HPLC in combination with a triple-quadrupole instrument in MRM-MS mode for monitoring characteristic precursor and fragment ions of metabolites. MRM-MS is very sensitive so that just minute sample volumes are needed. A drawback of MRM-MS, however, is that the precursor and product ions need to be selected a priori. Even when looking for the presence of specific compounds this can still be difficult because knowledge on the fragmentation mechanism of the metabolites is required. Since the predicted metabolites often will not be available as standards, the determination of the MS fragmentation pathway and ion selection has to be performed using model compounds with similar structures.

In this chapter we describe an LC-MS method for the highly sensitive detection of "software-predicted" *Hoodia* steroidal glycoside metabolites in *in-vitro* human liver microsomal incubation experiments of *Hoodia* extracts and of pure H.g.-12. The aim of the investigation was to study the applicability of semi-targeted MRM-MS for the identification of metabolites when only very small amounts of sample are available. The possibility to use sensitive MRM-MS on fragment ions predicted from the fragmentation pathways of model compounds was investigated. Ionization conditions were optimized to obtain maximum sensitivity.

7.2 Experimental

7.2.1 Materials and methods

Chemicals

The *Hoodia gordonii* extract was provided by Cognis Iberica SL (Barcelona, Spain). It contained approximately 85% of *Hoodia gordonii* steroidal glycosides. The microsome incubation studies were performed at TNO (Zeist, the Netherlands). Pig liver microsomes were purchased from BD Biosciences (USA). The enzyme activity of P450 and UDP-glycuronosyl transferase was tested to confirm the activity of key enzymes. 6β-

hydroxylation of testosterone served as positive control for cytochrome P450 activity and glucuronidation of 7-hydroxy-trifluoromethyl coumarin served as positive controls for UDP-glucuronosyl transferase activity.

Incubations were performed at 37°C in 200 µl incubation mixtures containing human liver microsomes at a concentration of 1.0 mg ml^{-1}, 0.1 M potassium phosphate buffer pH 7.4, 3 mM NADPH (cofactor for CYP3A4/5), 1 mM UDPGA (cofactor for UGP-GT) and 10 mM $MgCl_2$. Incubations were performed in quadruplicate for 0, 10, 20, 30 and 60 min. The reactions were started by the addition of the test compound (final concentration: 1 µg ml^{-1} H.g.-12). The amount of organic solvent in the incubation mixtures was 1.0% (v/v). After the incubation period, 100 µl ice-cold acetonitrile was added to terminate the enzymatic reaction. Subsequently, the samples were centrifuged and the supernatants stored at -70ºC until analysis. The total volume of sample available was 100 µl. The concentration of *Hoodia* extract used in the incubation assay was approximately 1 µg ml^{-1} resulting in a concentration for H.g.-12 of approximately 100 ng ml^{-1}. Pure H.g.-12 and H.g.-22 were obtained from Phytopharm (Godmanchester, U.K.). Acetonitrile, methanol and methyl-tertiary-butyl-ether (MTBE) were of gradient grade quality. Formic acid and ammonium acetate were of pro-analysis quality. Lithium chloride had a purity >99%. All chemicals were purchased from Merck (Amsterdam, the Netherlands). The deionised water was prepared using a Milli-Q water purification system (Millipore, Amsterdam, the Netherlands).

Instrumentation

All LC-MS analyses were performed on a Waters 2795 HPLC coupled to a Quattro-Premier triple quadrupole mass spectrometer (Waters, Etten-Leur, the Netherlands).

Sample preparation

The extracts of the microsome samples were prepared using the liquid/liquid extraction method developed for the quantification of steroidal glycosides in porcine plasma described in chapter 8 [10]. In short: 0.1 ml of the sample was diluted to 1 ml with Milli-Q water and two times extracted with 5 ml of MTBE. The MTBE fractions were combined, evaporated under nitrogen at 40 °C, and reconstituted in 240 µl of the initial HPLC eluent.

Chromatographic conditions

All analyses were performed on a 150 x 2.1 mm Inertsil 5 Zorbax Extend-C18 column with a particle size of 5 µm (Agilent, Amstelveen, the Netherlands). Mobile phase A consisted of aqueous 0.1% formic acid. Mobile phase B consisted of acetonitrile. Mobile phase C consisted of methanol. During the experiments two different additives were used; a 10

mM aqueous ammonium acetate solution and an aqueous lithium chloride solution. The concentration of the lithium chloride solution was optimized as will be discussed later. The initial eluent composition was A/B/C 80/14/6. The eluent was kept at the initial condition for 10 min and increased to A/B/C 10/75/15 in 35 min. It was kept at this composition for 5 min. At the end of a run the eluent composition was programmed back to the starting composition in 1 min and the system was allowed to equilibrate for 9 min. The total run time was 60 min. The eluent flow was 0.2 ml min^{-1} and the column temperature was set at 40 °C. The eluent of the first 10 minutes was directed to waste to avoid contamination of the mass spectrometer with salts and other highly polar compounds. The injection volume was 80 µl.

Mass spectrometric conditions

All measurements were carried out using high-performance liquid chromatography atmospheric-pressure ionization multiple-reaction monitoring mass spectrometry (HPLC-API-MRM-MS) in positive ionization mode. The capillary voltage was set at 4 kV. The source temperature was kept at 100 °C and the nebulizer temperature at 250 °C. The desolvation and cone gas flows were 800 l h^{-1} and 50 l h^{-1}, respectively. Argon was used as collision gas at a gas cell pressure of 4.2e-3 mbar. The dwell time was set at 0.3 s for each steroidal glycoside, with an interscan time of 0.03 s. These settings are typical for this instrument and were not further optimized. The cone voltage and collision energy were based on the optimum settings for H.g.-12 and were set at 80 V and 45 eV, respectively.

7.3 Results and discussion

The use of LC-MRM-MS is a logical choice for the detection of predicted metabolites when only small sample volumes are available. Due to the high selectivity and sensitivity of MRM-MS even metabolites present at only very low concentration levels can be detected.

7.3.1 Determination of the fragmentation mechanism of *Hoodia* steroidal glycosides

To be able to predict which fragments will be formed from the metabolites of the *Hoodia* steroidal glycosides in CID-MS^2, knowledge on the MS fragmentation mechanism of these compounds is required. Avula et al. [5] described the MS fragmentation pathway of the H.g.-12 molecule. After the loss of the tiglate group and/or a loss of water, monosaccharide units are lost one by one. In Figure 2 (A) the full scanning spectrum of the LC-MS analysis of H.g.-12 is given. Ammonium acetate was used to promote ionization. Next to the pseudomolecular ion at *m/z* 861.5, representing the loss of water from the protonated molecular ion, many other ions can be observed, such as e.g.

the sodium adduct at *m/z* 901.5, an intense ion at *m/z* 761.5 representing the loss of the tiglate group and ions at *m/z* 295.2 and *m/z* 313.2 representing the sterol core. When the $[M+H-H_2O]^+$ ion at *m/z* 861.5 is used as precursor ion in MRM, approximately 60% of the total number of fragment ions generated in the MS-source remains unused. Figure 2 (B) shows the CID-tandem mass spectrum of the precursor ion at *m/z* 861.5 together with part of the fragmentation pathway [3]. The two most characteristic fragment ions are at *m/z* 761.5 representing the loss of the tiglate group from the pseudomolecular ion and at *m/z* 295.2 which represents the steroidal core of the molecule. Each of these ions represents approximately 20% of the total ion current (TIC), meaning that 80% of the molecular ion sensitivity is lost when one of these ions is selected for further investigation. Because of the strong fragmentation, both in the MS source and the collision cell, only approximately 8% of the original ion current generated in the MS source is used. As a result of this it will be difficult to detect metabolites present at low concentrations. Additionally, strong in-source fragmentation makes it difficult to determine the molecular mass of the metabolites. Finally, when in later analyses quantification of the metabolites is required, the sub-optimal use of the ion current will negatively influence the detection limit of the method.

7.3.2 Effect of lithium salt addition

To overcome the problem of strong fragmentation in the MS source a stable molecular ion must be formed that does not fragment upon ionization. On the other hand, the ion should not be too stable because this would hinder fragmentation in the collision cell of the instrument in CID-MS^2 experiments. Lithium salts are frequently applied to enhance the formation of molecular ions from non-polar compounds. Unlike the highly stable sodium adducts formed when sodium salts are added to the sample, the addition of lithium salts results in the formation of stable lithium adducts from which structure informative fragments can be generated in the collision cell [11-13]. Lithium adducts are used in a broad field of analyses such as the characterization of underivatized *N*-linked glycans [13] and the analysis of low polarity compounds such as glycosphingolipids [11]. LC-MS analysis of H.g.-12 demonstrated that with the lithium chloride doped mobile phase mainly the $[M+Li]^+$ ion *m/z* 885.5 is formed while the abundance of the sodium adduct was low. Moreover, in-source fragmentation was near absent. The full scanning spectrum of H.g.-12 given in Figure 3 (A) clearly shows the $[M+Li]^+$ ion at *m/z* 885.5 representing 70% of the TIC with hardly any ions being generated by in-source fragmentation. The optimum concentration of lithium chloride for the *Hoodia* steroidal glycosides was determined by monitoring the pseudomolecular ion at *m/z* 885.5 during continuous introduction of H.g.-12 with the addition of increasing concentrations of lithium chloride by means of a three-way valve. In these experiments the lithium chloride

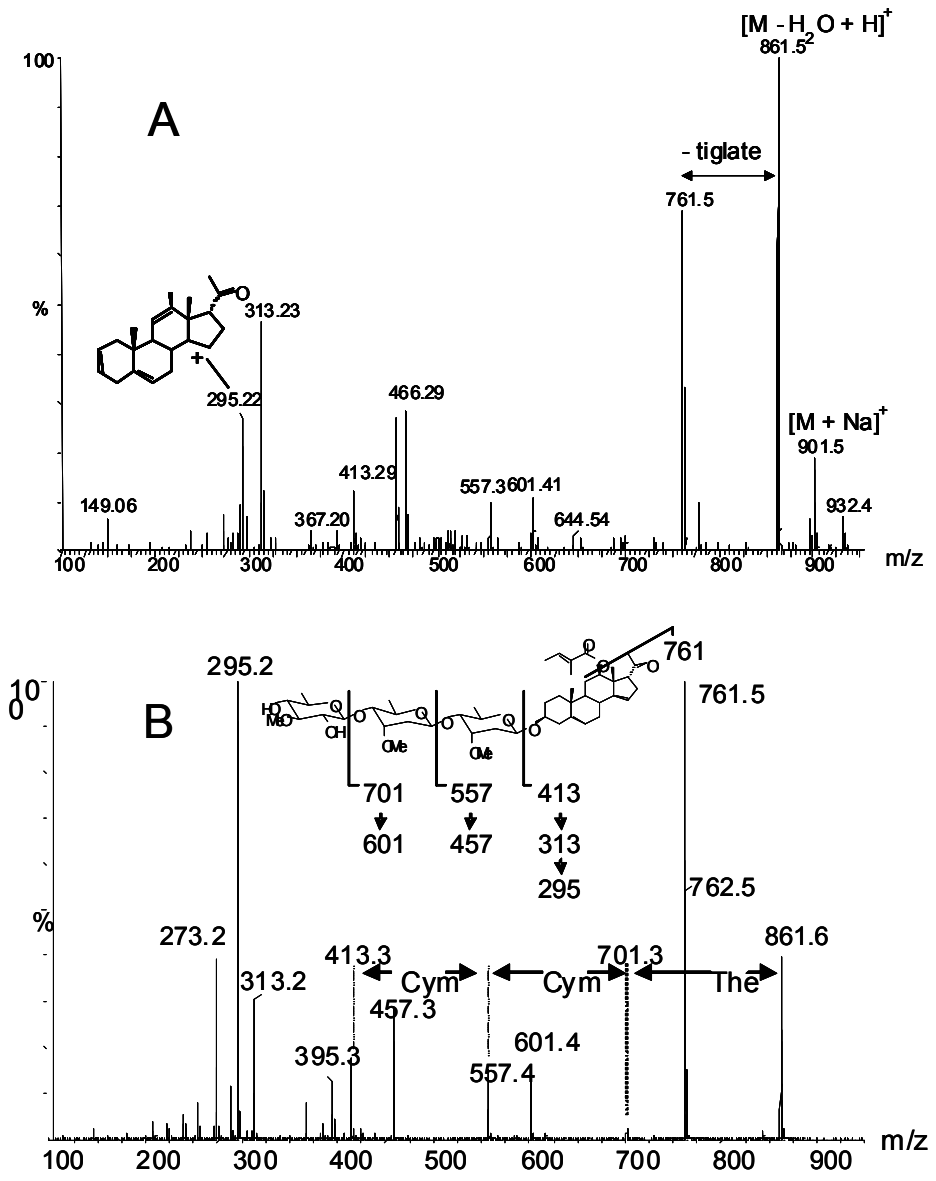

Figure 2 Mass spectra of H.g.-12 using ammonium acetate as eluent additive. A: full scanning mode. B: the product-ion MS spectrum of m/z 861.

concentration was varied between 0 and 0.1 mM in steps of 0.01 mM. The optimum concentration was determined to be 0.02 mM. In Figure 3 (B) the product-ion spectrum of the precursor ion at *m/z* 885.5 is given. Two intense and very characteristic fragment ions can be observed, at *m/z* 785.3, representing the loss of the tiglate group from the molecule, and at *m/z* 311.2 representing the terminal disaccharide part of the structure. These

ions account for approximately 50% of the total amount of ions generated, and thus 35% of the TIC is available for structure confirmation.

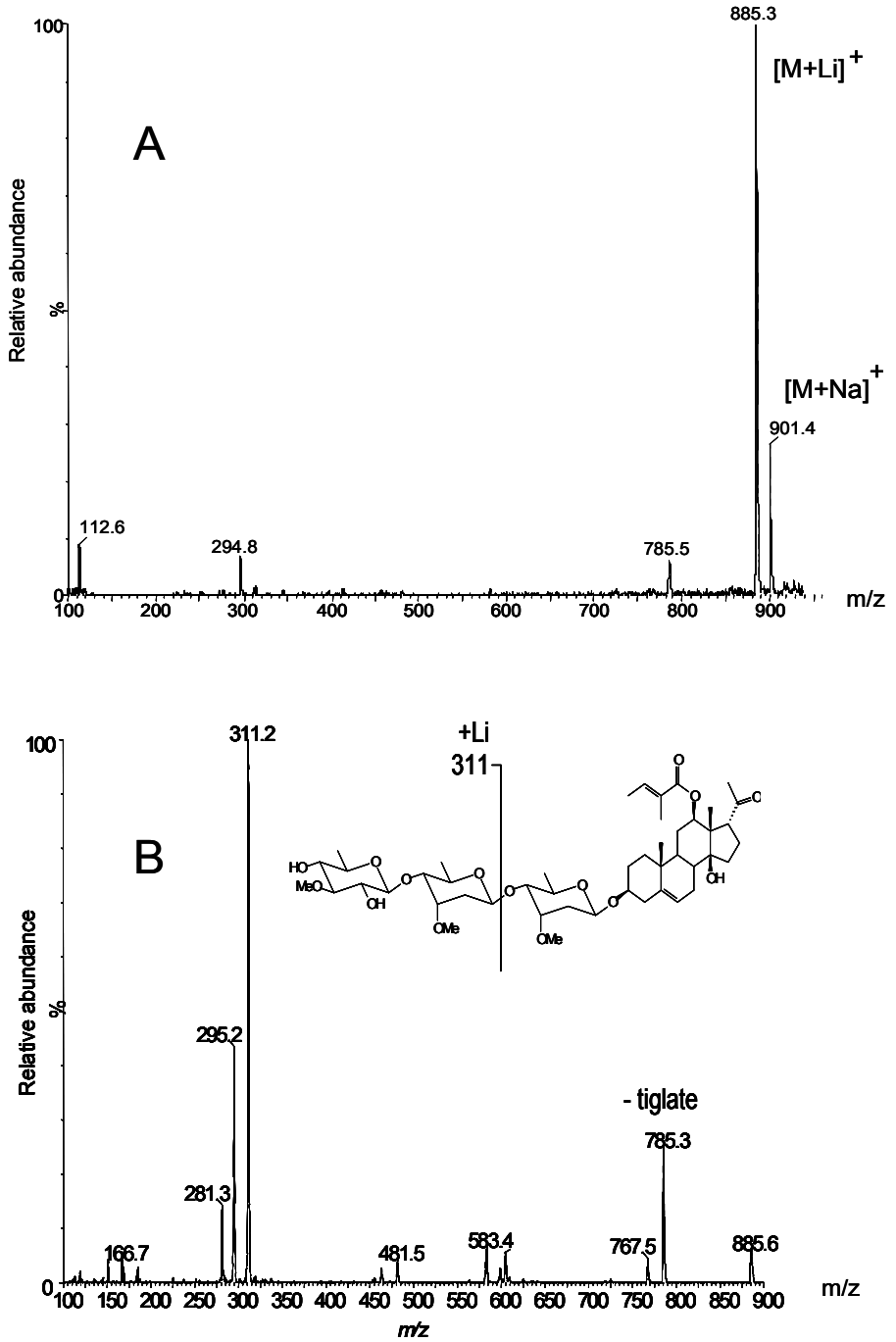

Figure 3 Spectra of H.g.-12 using lithium chloride as eluent additive in A: full scanning mode and B: the product-ion MS spectrum of *m/z* 885.

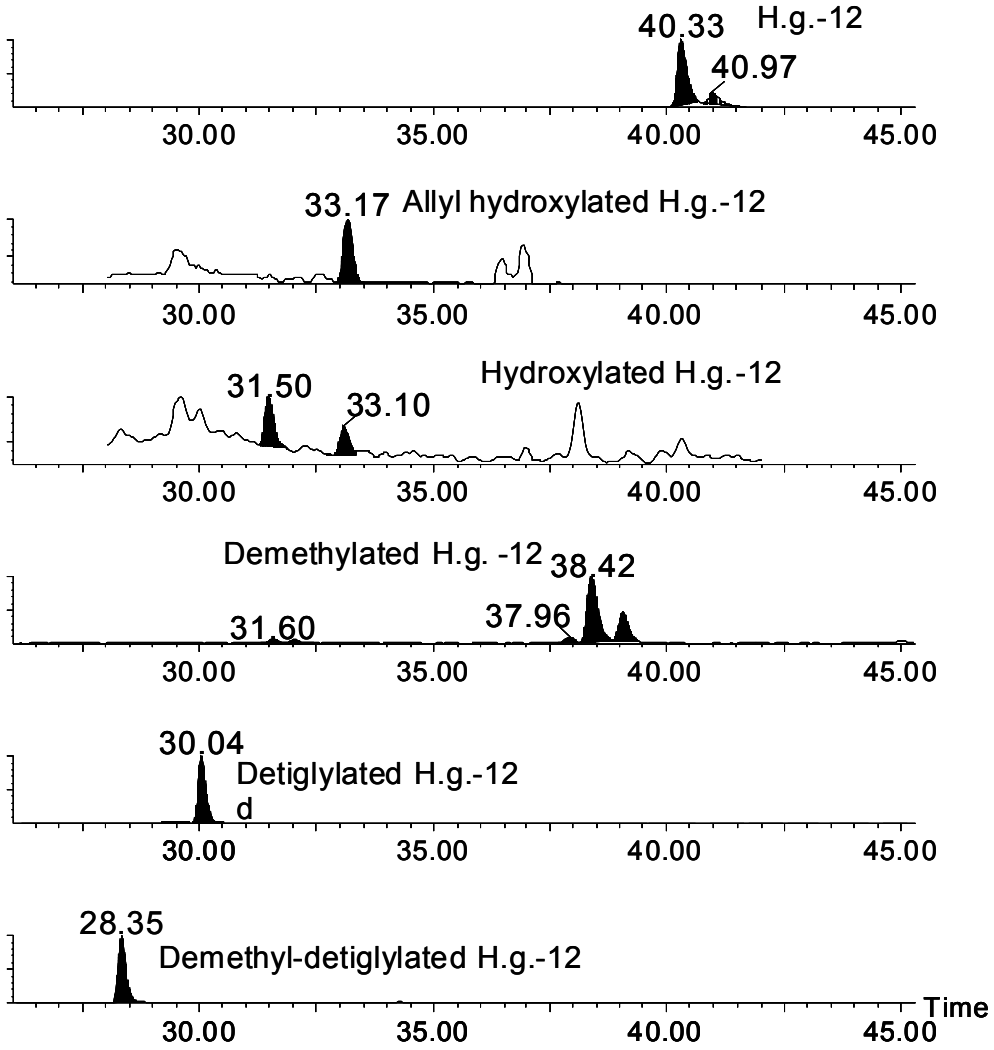

Figure 4 Reconstructed ion traces of the metabolites of H.g.-12 incubated with human liver microsomes. The metabolite peaks are shaded.

7.3.3 Metabolite identification

When LC-MRM is to be used as a very sensitive MS method for the detection of predicted metabolites at low concentration levels in complex (e.g. microsomal) preparations, knowledge on the fragmentation pathway of the metabolites is required. Ideally the predicted metabolites should be purchased as model compounds and their presence should be verified from retention times and fragmentation patterns. Unfortunately true *Hoodia gordonii* metabolites are not available. Therefore, CID-MS^2 studies were performed on *Hoodia gordonii* extracts containing H.g.-12 and a wide range

of homologues to build knowledge on the fragmentation pathway of structurally related *Hoodia* steroidal glycosides. CID-MS^2 was performed on the pseudomolecular ions of the steroidal glycosides and the fragmentation patterns were compared to elucidate the fragmentation mechanisms. The results are summarized in Table 1.

Table 1 Fragment ions of *Hoodia* steroidal glycosides. The most intense ion and the corresponding part of the terminal carbohydrate chain are printed in bold. Nomenclature and literature names as in reference [3].

Name	**Identified in literature**	**Precursor ion**	**Terminal carbohydrate fragments (*)**	
H.g.-12	Hoodigoside 12	*m/z* 885.5	*m/z* 151, **311**	**the-cym**-cym
H.g.-24	Formula 9	*m/z* 1013.5	*m/z* 151, **295**, 439	**ole-cym**-cym-cym
H.g.-17	Gordonoside 7	*m/z* 1013.5	*m/z* 151, **295**, 439	**cym-cym-**cym-cym
H.g.-22	Formula 7	*m/z* 1029.5	*m/z* **311**, 455	**cym-mda**-cym-cym
H.g.-21	Formula 12	*m/z* 1029.5	*m/z* **311**, 455	**ole-mda**-cym-cym
H.g.-19	Gordonoside 10	*m/z* 1157.5	*m/z* **295**, 439,583	**ole-cym**-cym-cym-cym
H.g.-23	Formula 8	*m/z* 999.5	*m/z* **281**, 425	**ole-dig**-cym-cym

* the = thevetose, cym = cymarose, ole = oleandrose, mda = 3-*O*-methyl-6-deoxyallose, dig = digitoxose.

The most intense ion in all spectra of the steroidal glycosides represents the terminal disaccharide unit. The ion representing [M – tiglate] was also present in all spectra at a moderate to high intensity (for practical reasons not included in the table). The table demonstrates that indeed *Hoodia* metabolites can be detected by performing CID-MS^2 on the pseudomolecular ion of the predicted metabolites while monitoring the ion representing the loss of the tiglate group and that representing the terminal disaccharide unit. For the *Hoodia gordonii* steroidal glycosides the types of monosaccharide moieties are limited. Therefore the most likely combination of monosaccharide moieties can be derived from the mass difference between the originating fragment ions. Of course MS cannot discriminate between isomeric monosaccharide units such as cymarose and oleandrose which both occur in the *Hoodia gordonii* preparations.

The previous paragraphs show that when only a very small volume of sample is available and the expected concentrations are low, HPLC-MRM-MS on lithium adduct ions can be used as a sensitive method for verifying the presence of predicted metabolites from *Hoodia gordonii* steroidal glycosides. To confirm this, pure H.g.-12 was incubated for 60 min with human liver microsomes and extracts of the reaction mixture were analyzed. In-house expertise combined with metabolite prediction software was used to predict which metabolites would be formed during this incubation. Based on the observed fragment ions of the *Hoodia* steroidal glycosides given in Table 1, the fragment ions of the expected metabolites are summarized in

Table 2. When compiling the table of expected ions we only looked for 1st level metabolites and combinations of two 1st level metabolism steps. When

Table 2 Predicted metabolites formed from H.g.-12 incubated with human liver microsomes, their molecular ions and expected fragment ions.

Metabolism	**Molecular ion [M+Li]+**	**Theoretical fragment ions**	
		- tiglate	**terminal disacch.**
Detiglation	*m/z* 803.5	no ion	*m/z* 311
Demethylation	*m/z* 871.5	*m/z* 771.5	*m/z* 297
Demethylation and detiglylation	*m/z* 789.5	no ion	*m/z* 297
Oxidation of secondary (alicyclic) alcohols	*m/z* 883.5	*m/z* 783.5	*m/z* 309
Hydroxylation of methyl carbon adjacent to an aliphatic ring or alpha to a carbonyl group	*m/z* 901.5	*m/z* 801.5	*m/z* 311 or *m/z* 327
Reduction of aliphatic ketones	*m/z* 887.5		
Allylic hydroxylation	*m/z* 901.5	*m/z* 801.5 or *m/z* 785.5	*m/z* 311
Glucuronidation of alicyclic alcohols	*m/z* 1061.5	*m/z* 961.5	*m/z* 487
Conjugation of alpha, beta-unsaturated compounds with glutathione	*m/z* 1048.5	*m/z* 785.5	*m/z* 311

2nd level and higher order metabolites would be included the number of ions to be scanned would be too large. This would cause the sensitivity of the method to be strongly reduced. Since the elution times of the metabolites are unknown, all MRM transitions must be scanned over a large time window. In Table 3 the results of the analysis are given. Figure 4 displays the reconstructed ion traces of the metabolites. Only the detected metabolites are listed. The other metabolites were either below the detection level of the system or were not formed. The results clearly show that HPLC-MRM-MS-MS can be used for the detection of (software) predicted metabolites of *Hoodia* steroidal glycosides. By monitoring the pseudomolecular $[M+Li]^+$ ions of the metabolites together with their characteristic terminal disaccharide unit ion and the $[M+Li\text{-tiglate}]^+$ ion, information is provided on the presence of the predicted metabolites in the mixture. From the peak areas, expressed as a percentage of the TIC corrected for response factors, it can be calculated that the detiglated species represent 86% of the total amount of metabolites formed, followed by the demethyl detiglated species which account for 5.5%. The oxidised species represent only a minor fraction of the metabolites. Glucuronidated species and glutathione conjugates were not found, possibly because these

Table 3 Metabolites found after a 60-min incubation of H.g.-12 with human liver microsomes. The carbohydrate fragments printed in bold have the highest intensity and represent the terminal disaccharide part of the molecule.

Metabolism	Elution time (min)	Carbohydrate fragments		Precursor ion *m/z*	Confirmation of tiglyl		Peak area (%)
		Fragment Ion *m/z*	Fragment structure		Fragment ion *m/z*	Fragment structure	
Detiglylation	30.04	151, **311**	the-cym	803.5	No ion		86.0
Demethylation	31.56	151, **297**	demethyl-the-cym	871.5	771.5	- tiglyl	0.1
	32.02	151, **297**	demethyl-the-cym	871.5	771.5	- tiglyl	0.1
	38.38	151, **297**	demethyl-the-cym	871.5	771.5	- tiglyl	1.4
	39.07	151, **297**	demethyl-the-cym	871.5	771.5	- tiglyl	1.4
Demethyl-detiglation	28.34	151, **297**	demethyl-the-cym	789.5	No ion		5.5
Hydroxykation of methyl carbon	31.50	**311**	the-cym	887.5	787.5	- tiglyl	0.8
Hydroxykation of methyl carbon	33.07	**311**	the-cym	887.5	787.5	- tiglyl	0.5
Demethyl allylic hydroxylation	31.44	**297**	demethyl-the-cym	887.5	771.5	- OH-tiglyl	1.0
Allyl hydroxylation	33.14	**311**	the-cym	901.5	785.5	- OH-tiglyl	3.2

compounds are not extracted in the sample preparation step. Moreover these compounds are negatively charged in solution and, consequently, are less efficiently ionized in the MS procedure. With the MS instrument used it was not possible to switch between positive and negative ionization mode in one single analysis and the small amount of material precluded separate analyses.

7.3.4 Determination of steroidal glycoside metabolites of the *Hoodia gordonii* extract incubated with human liver microsomes

Following the successful detection of metabolites formed from pure H.g.-12 in microsome studies also *Hoodia gordonii* extracts were incubated with human liver microsomes and samples were taken at different time intervals. The sample taken at 60 min was screened for the metabolites listed in Table 2. In Table 4 the metabolites detected in the sample are displayed. Clearly the table shows that only the detiglated, the demethylated and the demethyl-detiglated forms of the different steroidal glycosides could be found. This is

in good agreement with the results obtained after the incubation of the pure H.g.-12 in which the same metabolites were formed and where the oxidized species were present only at very low concentrations. The remaining sample material was analyzed for the steroidal glycosides listed in Table 4. The peak areas of the steroidal glycosides and identified metabolites were determined and displayed as a percentage of the peak area of H.g.-12 measured in the sample taken at t = 0. The results of these experiments are summarized in Table 5.

Table 4 Metabolites identified in *Hoodia gordonii* incubated with human liver microsomes.

Metabolite	Elution time (min)	Precursor ion	Product ion
Detiglated H.g.-12	30.04	803.5	311.2
Detiglated H.g.-21 or H.g.-22	32.69	947.5	311.2, 455.3
Detiglated H.g.-22 or H.g.-21	33.02	947.5	311.2, 455.3
Demethylated H.g.-12	31.56	871.5	297.2, 771.5
Demethylated H.g.-12	32.02	871.5	297.2, 771.5
Demethylated H.g.-12	38.38	871.5	297.2, 771.5
Demethylated H.g.-12	39.07	871.5	297.2, 771.5
Demethyl-detiglated H.g.-12	28.35	789.5	297.2
Demethyl-detiglated H.g.-21 or H.g.-22	30.84	933.5	297.2
Demethyl-detiglated H.g.-22 or H.g.-21	31.28	933.5	297.2

Note: The metabolites of H.g.-21 and H.g.-22 have identical molecular ions and can only be distinguished based on the retention times as obtained from model compounds.

Although the levels presented are relative, the data clearly show the strong increase in time of the detiglated species of H.g.-12 and, somewhat less pronounced, the increase of the demethylated form. Interestingly the concentration of H.g.-12 remains reasonably constant. Most likely during the incubation H.g.-12 is formed from larger homologues. The detiglated species of H.g.-21 and H.g.-22 also show an increase in concentration over time, but now, different from H.g.-12, the original species H.g.-21 and H.g.-22 do show a decrease in concentration over time. In case of H.g.-22 even a very strong decrease can be observed in the first 10 min. No increase could be observed for the detiglated species of the steroidal glycosides with more than four monosaccharide residues attached to the steroid backbone. Although no quantitative conclusions can be drawn from the results, the sum of the peak areas indicates that most of the metabolites formed have been accounted for. Hence the predictions and confirmation of the predicted structures must be close to complete with no important metabolites being missed. Clearly the approach applied here is feasible.

Table 5 Degradation of *Hoodia gordonii* steroidal glycosides and formation of metabolites in H.g.-12 incubated with pig liver microsomes quantified relative to the original H.g.-12 level.

Compound	incubation time (min)			
	0	10	20	30
Demethyl detiglyl H.g.-12	0.0	0.0	1.1	1.4
detiglyl H.g.-12	0.5	26.9	40.7	40.2
demethyl detiglyl H.g.-21 (or H.g.-22)	0.0	0.1	0.1	0.1
demethyl detiglyl H.g.-22 (or H.g.-21)	0.0	0.1	0.2	0.1
detiglyl H.g.-21 (or H.g.-22)	0.0	0.6	0.8	0.7
detiglyl H.g.-22 (or H.g.-21)	0.0	0.5	0.6	0.7
Aglycone of H.g.-12	27.7	0.1	0.3	0.1
demethyl H.g.-12	0.2	4.1	6.3	7.0
H.g.-12	100.0	114.9	104.4	84.2
H.g.-21	8.7	5.7	3.2	1.8
H.g.-22	24.4	1.2	0.9	0.5
H.g.-23	27.5	31.8	21.5	18.4
H.g.-24	21.7	28.1	22.7	16.9
H.g.-17	11.8	11.3	6.7	17.5
H.g.-19	0.6	0.5	0.3	0.2

7.4 Conclusions

A strategy for the identification of metabolites from *Hoodia* steroidal glycosides in liver microsomal incubation studies was developed. It involves MRM-MS to monitor fragment ions expected for the metabolites predicted by metabolism prediction software. The metabolites expected, and indeed found, shared two common features in their CID-MS^2 spectra: an indicator for the presence or absence of the tiglate group and one representing the last two sugar units of the carbohydrate chain. The method is sensitive and uses only small sample amounts. It was successfully applied for metabolite analysis in samples from *Hoodia* extract incubated with human liver microsomes. In these experiments six new groups of metabolites could be identified. The most intense metabolite formed was the detiglated species followed by the demethyl-detiglated species. Small amounts were found of the demethylated species and some oxidation products. The glucuronidated and glutathione conjugates were not detected, most likely due to their negative charge in solution. The only drawback of the method is that it is limited to the predicted species, all un-predicted species remain undetected. Mass balance considerations, however, indicate that in the present study a significant fraction of the metabolites must be covered by the set of metabolites monitored.

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8 Quantification of steroidal glycosides from *Hoodia gordonii* in porcine plasma using high performance liquid chromatography-mass spectrometry

Summary
In chapter 7 seven steroidal glycosides and their aglycone from the plant extract of the *Hoodia gordonii* plant were described. From plasma incubated with liver microsomes six metabolites of these steroidal glycosides could be identified. In the present chapter a method is described for the quantification of these compounds in plasma collected from a porcine trial. This method involves a liquid-liquid extraction in combination with HPLC-MRM-MS. The liquid-liquid extraction procedure covers the broad range of hydrophobicities of the target compounds. The LC separation method was designed in such a way that water/fat emulsions could be injected without adversely affecting the performance of the LC column. The deuterium-labelled analogue of one of the compounds was used as internal standard. The method was validated for the three most important glycosides P57AS3 (H.g.-12), its homologue (H.g.-22) and the detiglated form of H.g.-12 and was successfully applied also for the related glycosides and metabolites. The limits of quantification were 0.04 ng ml^{-1} for the two main steroidal glycosides and 0.1 ng ml^{-1} for the detiglated metabolite. The assay proved to be sensitive and selective and allows the monitoring of small changes in the concentrations of the compounds in plasma after consumption of the extract. The quantification limits are sufficiently low to allow the monitoring of the concentration-time profiles in plasma after consumption of *Hoodia gordonii* extract. The standard deviation of the intra-day measurements were better than 20% for concentrations below 5 ng ml^{-1} and better than 10% for concentrations above 5 ng ml^{-1}. Only one out of eight steroidal glycosides showed a poorer performance. The long term stability was good. The method was successfully applied to plasma samples collected from a porcine pharmacokinetic study.

8.1 Introduction

Obesity is a risk factor for diabetes, high blood pressure, cancer, high cholesterol, asthma, arthritis and generally poor health status [1, 2]. Recent studies suggest that approximately 300,000 deaths per year in the USA are attributed to obesity [3]. Economic and technological trends promoting a sedentary lifestyle with easy access to low-cost, high-calorific food make that the obesity epidemic is increasing its impact on society [4]. A healthy diet associated with exercise is clearly beneficial for reducing and controlling bodyweight and products that safely help consumers to control their calorie intake could contribute significantly to this. In the past, several species of *Hoodia* were included in a research program undertaken by the National Food Research Institute of the Council of Scientific and Industrial Research (CSIR) in South Africa. Their research showed that *Hoodia gordonii* contained a range of steroidal glycosides that decreased food intake and body weight in man and animals [5]. A number of these steroidal glycosides have been identified by several authors. For an overview of the structures and nomenclature see reference [6].

Recently, a fast method has been developed to quantify the *Hoodia gordonii* actives H.g.-12 (also referred to as P57AS3 or P57) and H.g.-22 [7] in plasma from different species [8]. This method enabled the analysis of a high number of samples for pharmacokinetic studies. However, *Hoodia* extracts contain a number of other steroidal glycosides that are structurally very similar to H.g.-12 and H.g.-22 (see Figure 1 Chapter 7) [9-11]. Moreover, recent *in-vitro* studies suggest that metabolites of H.g.-12 and its homologues can be formed *in-vivo* [12]. Important metabolic modifications observed were the loss of the tiglate group and demethylation of the methoxy groups of the carbohydrates. To get insight in the physiological behaviour and the mechanism of action of the steroidal glycosides and their metabolites it is important to be able to determine the concentration of all these compounds in blood samples collected after intake of *Hoodia gordonii* extract.

The subject of this paper was to develop and validate an analytical method for the quantification of steroidal glycosides originating from *Hoodia gordonii* and their metabolites in porcine plasma at ng g^{-1} concentration level and higher. This method enables studies aimed at understanding the metabolic pathway of *Hoodia* steroidal glycosides in the body. The set of compounds studied consists of the aglycone, seven steroidal glycosides originating from *Hoodia gordonii* and six metabolites. Isomers of several glycosides were also included in the quantification process, resulting in a total of 24 peaks to be quantified in one single analysis.

8.2 Experimental

8.2.1 Materials and methods

Standards and chemicals

The *Hoodia gordonii extract* was obtained from Cognis Iberia S.A.U. (Barcelona, Spain). Using the previously developed LC-UV method [7] the total steroidal glycoside content of the extract was determined to be 87% divided over at least 30 different compounds. H.g.-12, detiglated H.g.-12, H.g.-22 and the deuterium labelled H.g.-12 were purified or synthesized in-house. Porcine plasma was obtained from Innovative Research Inc. (Southfield, Michigan, USA). Methyl-tertiary butyl ether (MTBE), acetonitrile and methanol were of gradient grade (Merck, Amsterdam, the Netherlands). Formic acid was pro-analysis grade also from Merck. Demineralized water was prepared using a Milli-Q water purification device (Millipore, Bedford, New Hampshire, USA).

Standard preparation

Calibration standard solutions of the pure H.g.-12, detiglated H.g.-12 and H.g.-22 were prepared starting from a concentration of each steroidal glycoside of 1 mg ml^{-1} in Milli-Q water/acetonitrile 50/50 (v/v). Subsequent dilutions were prepared either in a solvent mixture consisting of aqueous 0.1% formic acid/acetonitrile/methanol 80/14/6 (v/v/v) or in porcine plasma to yield the following concentrations: 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000 ng ml^{-1}. Each solution contained the internal standard, deuterium labelled H.g.-12, at a concentration of 50 ng ml^{-1}.

Plasma sample preparation

The target analytes are rather non-polar species. For such analytes liquid-liquid extraction using non-polar solvents is typically employed. Here we opted for the use of a two-stage liquid/liquid extraction of plasma with MTBE carried out at -20 °C. At this temperature the analytes are stable and the solubility in the fat is low. For the broad range of *Hoodia* steroidal glycosides described in this paper parameters such as the extraction volume, number of subsequent extractions and temperature were varied. The best results were obtained when 500 µl of plasma was mixed with 50 µl of the 500 ng ml^{-1} deuterium labeled H.g.-12 solution in solvent. The sample and the internal standard stock solution were stored in melting ice. Next 5 ml of cold water was added and after vortexing for 10 s 5 ml of MTBE was added. From this point on all further steps were carried out at room temperature. After vortexing for 5 s the mixture was shaken for 15 min at 900 RPM using a mixing device. The layers were allowed to separate for 15 min and 4 ml of

the upper MTBE layer was pipetted into a second test tube. Another portion of 5 ml MTBE was added to the first tube and after vortexing it was mixed again at 900 RPM for 15 min. After 15 min of stabilization 5 ml of the top MTBE layer was pipetted into the test tube containing the first extract. The 9 ml of MTBE was evaporated to dryness under a stream of nitrogen at 37 °C. After addition of 60 µl of methanol the tube was shaken at 400 rpm for 10 min. Then 180 µl of 0.1% formic acid was added and the tube was shaken again at 400 RPM for another 10 min. Finally, the sample solution was pipetted into a 2 ml autosampler vial, treated ultrasonically for 5 min and stored at 15 °C until injection. All samples were injected on the HPLC system within 24 hours after preparation. After the addition of the 0.1% formic acid solution a finely dispersed emulsion is formed in which the steroidal glycosides are homogeneously distributed.

Instrumentation

All analyses were performed on a Waters 2795 HPLC coupled to a Quattro-Premier triple quadrupole mass spectrometer (Waters, Etten-Leur, the Netherlands).

Chromatographic conditions

All analyses were performed on a 150 x 2.1 mm Zorbax Extend C18 column with a particle size of 5 µm (Agilent, Amstelveen, the Netherlands). Mobile phase A consisted of 0.1% formic acid in Milli-Q water, mobile phase B was acetonitrile and mobile phase C was methanol. The initial eluent composition was A/B/C 80/14/6 v/v/v. All mobile phases contained 0.02 mM lithium chloride to improve the MS ionization properties (see below and chapter 7). After a 10 min hold a linear gradient was started to A/B/C 10/75/15 v/v/v in 35 min. The eluent was kept at this composition for 5 min and programmed back to the initial composition in 5 min. The system was allowed to re-equilibrate for 5 min. The total run time was 60 min. The eluent flow rate was 0.2 ml min^{-1} and the column temperature was set at 40 °C. The injection volume was 80 µl. The autosampler temperature was 15 °C; below this temperature crystallization of some of the steroidal glycosides could occur. In order to overcome the possible negative effects of fatty material from the sample on the column performance, after each set of 40 samples a column clean-up was carried out by flushing the column with the solvents A/B/C in a ratio of 5/5/90 (v/v/v) at an eluent flow of 0.2 ml min^{-1} for at least 4 hours. With this method the column performance was stable for at least 1000 injections.

Mass spectrometric conditions

All measurements were carried out using high performance liquid-chromatography atmospheric-pressure ionization-multiple-reaction

monitoring mass spectrometry (HPLC-API-MRM-MS) in positive ionization mode. The capillary voltage was set at 4 kV. The source temperature was kept at 100 °C and the nebulizer temperature at 250 °C. The desolvation and cone gas flows were 800 and 50 l h^{-1}, respectively. Argon was used as collision gas at a gas flow of 0.35 ml min^{-1}. The dwell time was set at 0.3 s for each steroidal glycoside, with an interscan time of 0.03 s. The optimum cone voltages and collision energies were determined for each steroidal glycoside individually prior to the start of the experiments. Steroidal glycosides were identified based on their retention time, their molecular ion and the ratio of two characteristic product ions for each steroidal glycoside, relative to those of the model compounds (see Table 1). A maximum variation of 0.1 min for the elution time and 10% for the product-ion ratio was accepted. The optimum settings of the cone voltages and collision energies for the *Hoodia gordonii* steroidal glycosides were determined by repeated injections of the *Hoodia* extract in the initial eluent at a concentration of 1000 ng ml^{-1}. The optimum MS settings for the metabolites were taken from a previous study in which *Hoodia gordonii* extract was incubated with human liver microsomes [13]. Successive analyses were performed in which the cone voltage was varied in the range of 10 to 120 V at steps of 5 V, with the collision energy being varied from 20 to 60 eV with intervals of 3 eV. Detailed information on the elution times, precursor ions, product ions, cone voltages, collision energies and product ion ratios is given in Table 1.

8.3 Results and discussion

At the start of this project, several methods for identifying and quantifying *Hoodia* steroidal glycosides in raw plant material and products had been described in literature [6, 8-10, 12]. Also, a rapid method for quantifying H.g-12 and H.g.-22 in plasma was available [7]. However, a method that would allow the quantification of a large set of structurally similar *Hoodia* steroidal glycosides and their most important metabolites was not available. Moreover, Madgula et al. provided evidence for *in-vitro* metabolism of H.g.-12 [14], which could be confirmed by Graf et al. [17]. Therefore, the aim of this study was to develop a bioanalysis method for the quantification of a range of steroidal glycosides from *Hoodia gordonii* and their metabolites in human plasma.

The determination of *Hoodia gordonii* compounds and their metabolites in plasma is a typical example of the analysis of low levels of complex compounds in a complex and interfering matrix. The levels that could be physiologically relevant are expected to be in the low ng ml^{-1} range [9]. At this level there are numerous compounds present in the plasma sample that could interfere with the analysis. The main interfering compounds evidently are lipids and proteins. From the physico-chemical

Table 1 Elution times, MRM conditions and product-ion ratios of *Hoodia gordonii* steroidal glycosides and metabolites.

Steroidal glycoside code [2])	Elution time (min)	Precursor Ion (*m/z*)	Cone Voltage (V) [1])	Product ion (*m/z*)	Collision energy (eV) [1])	Product ion ratio 1/2
detiglated aglycone	17.89	396.3	30	1: 319.3 2: 337.3	1: 35 2: 25	2.0/1
demethyl detiglated H.g.-12	28.57	789.5	80	1: 297.2	1: 50	n.a.
detiglated H.g.-12 I	29.58	803.5	80	1: 311.2	1: 50	n.a.
detiglated H.g.-12 II	30.29	803.5	80	1: 311.2	1: 50	n.a.
demethyl detiglated H.g. 22 I	30.84	933.5	80	1: 297.2	1: 50	n.a.
demethyl detiglated H.g. 22 II	31.14	933.5	80	1: 297.2	1: 50	n.a.
demethyl detiglated H.g. 22 III	31.57	933.5	80	1: 297.2	1: 50	n.a.
detiglated H.g. 22 I	33.02	947.5	85	1: 455.2 2: 311.2	1: 50 2: 50	4.3/1
detiglated H.g. 22 II	33.40	947.5	85	1: 455.2 2: 311.2	1: 50 2: 50	2.1/1
detiglated H.g. 22 III	34.03	947.5	85	1: 455.2 2: 311.2	1: 50 2: 50	3.1/1
Aglycone I	33.80	478.4	30	1: 319.3 2: 337.3	1: 35 2: 25	1.4/1
Aglycone II	34.46	478.4	30	1: 319.3 2: 337.3	1: 35 2: 25	1.2/1
Demethyl H.g.-12 I	38.91	871.5	80	1: 297.2 2: 771.5	1: 50 2: 40	1.6/1
Demethyl H.g.-12 II	39.50	871.5	80	1: 297.2 2: 771.5	1: 50 2: 40	1.4/1
H.g.-12 I	40.83	885.5	80	1: 311.3 2: 785.5	1: 52 2: 40	1.4/1
H.g.-12 II	41.48	885.5	80	1: 311.3 2: 785.5	1: 52 2: 40	1.3/1
H.g. 21	42.72	1029.6	95	1: 455.3 2: 931.5	1: 55 2: 45	8.9/1
H.g. 22	43.60	1029.6	95	1: 455.3 2: 931.5	1: 55 2: 45	17.5/1
H.g. 23 I	44.91	999.5	90	1: 425.2 2: 899.5	1: 54 2: 45	1.7/1
H.g. 23 II	45.55	999.5	90	1: 425.2 2: 899.5	1: 54 2: 45	1.5/1
H.g. 23 III	46.02	999.5	90	1: 425.2 2: 900.5	1: 54 2: 45	1.8/1
H.g. 24 I	47.15	1013.6	95	1: 439.2 2: 914.5	1: 55 2: 45	1.1/1
H.g. 24 II	47.75	1013.6	95	1: 439.2 2: 914.5	1: 55 2: 45	1.0/1
H.g. 17	48.18	1013.6	95	1: 439.2 2: 914.5	1: 55 2: 45	1.2/1
H.g. 19	49.41	1157.5	110	1: 584.4 2: 1059.5	1: 55 2: 45	2.3/1
Deuterium labelled H.g.-12	40.72	895.5	80	1: 311.3 2: 785.5	1: 52 2: 40	1.5/1

[1]) The cone voltage and collision energy were optimized for each individual compound.
[2]) For several compounds more than one chromatographic peak representing isomers could be observed, the separated isomers are indicated as I, II or III.

properties of the target analytes it is clear that simple protein removal methods such as acetonitrile precipitation or protein removal by heating can not be applied. The rather non-polar *Hoodia* steroidal glycosides will show a strong protein binding and are likely to be partly removed with the proteins when using standard protein precipitation methods. Liquid/liquid extraction or SPE are probably more suited for the sample preparation. A complication here is the presence of high levels of lipidic material in the samples. Another problem with relatively labile carbohydrate-like structures is that in the MS source strong fragmentation occurs. The fragment intensity is concentration dependent which results in highly non-linear correlation curves. To overcome this problem mobile phase additives are necessary that generate more stable molecular (-adduct) ions. Commonly used additives are ammonium, sodium, potassium and lithium salts [16]. Ammonium ions enhance the ionization but the adduct ions generated are unstable already in the MS source. Sodium and potassium ions result in very stabile molecular ions but require extremely high collision energies for fragmentation in the collision cell. Lithium has proven to be an excellent compromise. It gives stable molecular ions while still allowing easy fragmentation in the collision cell at relatively low collision energies. As a result of this the linearity of the calibration curves strongly improves. A minimum limit of quantification of 0.1 ng ml^{-1} was taken as acceptable as below this value no effective activity of the *Hoodia* compounds could be expected [9]. The final method was validated according to the procedure described by Shah. et al. [17].

Addition of lithium chloride

In chapter 7 we demonstrated that under standard HPLC-MS conditions the *Hoodia* steroidal glycosides in the MS-source not only give the protonated molecular ion, but also generate the sodiated ion and fragment ions [12]. The most intense ion observed represents the loss of the tiglate group from the molecule. In the CID-MS^2 mode many fragment ions are generated. This proofed to be extremely useful for the identification of unknown steroidal glycosides or for confirmation of molecular structures. For quantification purposes, however, the generation of multiple molecular ions and fragments in the MS source negatively influences the sensitivity of the method. Ideally only one molecular ion and preferably only a few fragment ions should be generated in the MRM-MS mode. Moreover, the calibration curves should be linear to allow accurate quantification. Unfortunately the two model compounds studied were found to have highly non-linear calibration lines when the protonated ion was used for the generation of fragment ions (data not shown). When lithium chloride was added to the eluent only one main molecular ion was generated in the MS source, the $[M+Li]^+$ ion. In the collision cell two fragment ions are generated, representing the loss of the tiglate group (if present) and the terminal disaccharide unit of the

carbohydrate chain. Calibration curves of the model compounds in the lithium adduct experiments showed good linearities in the relevant range. In Figure 1 the calibration curves of H.G.-12 are given generated with and without lithium chloride.

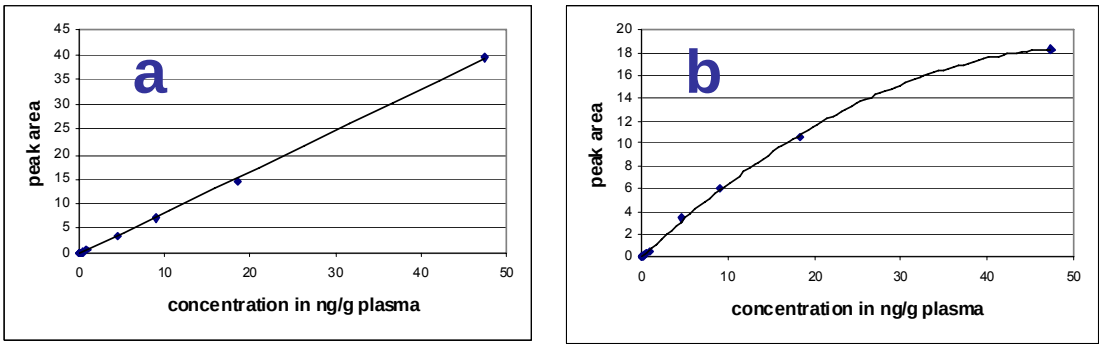

Figure 1 Calibration curves of H.g.-12 analyzed with (a) and without (b) lithium chloride in the HPLC eluent.

Ionization efficiency

For the accurate quantification of the individual steroidal glycosides model compounds would be preferred for each individual compound. Purified standards were, however, only available for H.g.-12, its detiglated form and H.g.-22. Since no synthesized standards were available for the other steroidal glycosides the *Hoodia* extract was used instead. This extract was analyzed using both the HPLC-UV method as described by Janssen et al [8] and the HPLC-MS method developed in here. In the LC-UV analyses it is reasonable to assume equal molar response factors for each of the analytes. Using one pure compound the levels of all steroidal glycosides can then be derived, and correction factors for the MS analyses can be calculated for each steroidal glycoside. This approach failed for the metabolites as the levels of these species were too low for LC-UV analysis. Ionization efficiency correction factors for the metabolites were set to 1.0 relative to H.g.-12. The response factor of H.g.-12, which was available as the pure material, was arbitrarily set to 1.00. The response factors of other steroidal glycosides were calculated relative to H.g.-12. The results are summarized in Table 2. As can be concluded from this table, most compounds have similar ionization efficiencies. A noticeable exception is H.g.-19, the steroidal glycoside with the longest oligosaccharide chain. The high correction factor of 9.45 negatively influences the sensitivity and accuracy of the method for this compound.

Table 2 MS correction factors for the *Hoodia gordonii* steroidal glycosides relative to H.g.-12 (set to 100%).

Compound	Correction factor	Relative standard deviation (n = 6)
H.g.-12	1.00	1.3%
Aglycone	1.00	7.3%
H.g.-21	1.60	2.7%
H.g.-23	1.19	2.3%
H.g.-24	1.34	1.9%
H.g.-17	1.58	2.4%
H.g.-19	9.45	11.3%

Linearity and detection limits

The linearity was calculated by the analysis of a set of calibration standards in porcine plasma. The concentration range studied ran from 0 to 1000 ng ml^{-1}. Deuterated H.g.-12 at a concentration level of 50 ng ml^{-1} was used as the internal standard. For the determination of the limit of detection samples spiked at 0.01 and 0.05 ng ml^{-1} were analyzed. All samples were prepared and analyzed on the same day. One set of standards was measured in ascending concentration order followed by the other set in descending concentration order. The two sets were separated by a blank sample.

The limits of detection (LOD) was calculated from the signal-to-noise ratio of the chromatographic peak using the peak to peak S/N option in the Masslynx software. The noise processing option was set to “no extra processing”. The LOD was defined as the concentration that gave a chromatographic peak with a signal to noise ratio of 3:1. The limit of quantification (LOQ) was defined as the lowest concentration that could be measured with a maximum relative standard deviation for the precision of 20% and a maximum deviation from the nominal value in the accuracy/recovery measurements of 20%. The results of the measurements are summarized in Table 3. The calibration curves for H.g.-12, detiglated H.g.-12 and H.g.-22 showed a good linearity in the concentration range from 0.1 to 1000 ng ml^{-1}. The detection limits were approximately 0.01 ng ml^{-1} for H.g.-12 and H.g.-22 and 0.05 ng ml^{-1} for the detiglated H.g.-12. These values are low enough to measure physiologically relevant levels in complex sample matrices.

Within-day variation

The within-day variation was determined by analyzing 8 samples of plasma spiked with the *Hoodia* extract at a concentration of 100 ng ml^{-1} within one day. The results of the analyses are summarized in Table 4.

Table 3 Linearities, LODs and LOQs of steroidal glycosides in porcine plasma using LC-MRM-MS (Concentration range 0.01 – 1000 ng ml-1).

Steroidal glycoside	Limit of detection LOD (ng ml^{-1})	Limit of quantification LOQ (ng ml^{-1})	Linear regression coefficient (R^2)
detiglated H.g.-12	0.05 ng ml^{-1}	0.1 ng ml^{-1}	0.9987
H.g.-12	0.01 ng ml^{-1}	0.04 ng ml^{-1}	0.9993
H.g. 22	0.01 ng ml^{-1}	0.04 ng ml^{-1}	0.9989

Table 4 Within-day variation of porcine plasma spiked with *Hoodia gordonii* extract (n = 8).

Meas. no	Concentration in ng g^{-1} plasma							
	H.g.-12	aglycon	H.g.-21	H.g.-22	H.g.-23	H.g.-24	H.g.-17	H.g.-19
1	21.60	0.16	4.99	19.73	8.45	10.27	3.70	0.33
2	21.12	0.17	4.80	17.15	8.06	10.10	3.62	0.34
3	21.15	0.17	4.83	17.60	8.54	11.23	3.79	0.44
4	21.15	0.16	4.78	17.70	8.36	11.21	3.44	0.39
5	21.04	0.15	4.97	17.37	8.69	10.23	3.43	0.37
6	20.62	0.15	5.10	17.02	8.73	9.82	3.32	0.35
7	20.82	0.15	5.23	17.70	8.70	10.20	3.22	0.36
8	21.39	0.39	5.18	18.50	8.34	11.53	3.30	0.42
Average	21.11	0.19	4.98	17.84	8.48	10.57	3.48	0.37
Rel STD	1.46	44.40	3.52	4.95	2.71	6.07	5.93	10.74

The standard deviations of all steroidal glycosides except the aglycone are within 20%. The poor result found for the aglycone is most likely caused by the low intensity of the peak of this compound which in turn is caused by both its low level and the poor ionization characteristics.

Precision and accuracy

The precision was determined by analyzing plasma samples spiked with the *Hoodia* extract at a concentration of 100 ng ml^{-1}. Duplicate samples were prepared and analyzed on five days in a time period of five weeks. The accuracy was determined by analyzing five replicate preparations of porcine plasma spiked with the three reference standards at concentrations of 0.1, 0.5, 1, 10, 100 and 500 ng ml^{-1}. The deuterium labelled H.g.-12 internal standard concentration was 50 ng ml^{-1} in all samples. The results of the measurements are given in Table 5. Statistical evaluation of the results indicated an acceptable precision and accuracy for all steroidal aglycones, except for the detiglated H.g.-12. For this compound, at a concentration of 0.1 ng ml^{-1}, the accuracy was 126.8% and thus outside the acceptance limit of 120% [16].

Table 5 Precision and accuracy of the assay within one day (n = 5).

Concentration (ng ml^{-1})	Detiglated H.g.-12		H.g.-12		H.g.-22	
	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy
0.1	16.2	126.8	10.7	112.6	12.1	89.4
0.5	11.6	106.3	8.3	97.3	9.2	92.5
1	9.3	94.6	6.0	91.7	6.4	88.5
10	7.2	98.2	4.3	101.5	5.2	91.6
100	4.1	97.3	1.7	98.4	2.5	89.1
500	2.5	99.1	1.3	95.6	1.6	84.5

Stability

No significant changes could be detected in the concentration of the steroidal glycosides in liquid/liquid extracts of porcine plasma spiked at concentration levels of 0.5, 5 and 50 ng ml^{-1} stored for 24 or 72 h at 15 °C or -20 °C. The variations were within the reproducibility of the method. The stability of the glycosides in plasma stored at -80 °C was not investigated. It is generally accepted that plasma samples stored at this low temperature will remain stable for at least one year.

Carry-over

The percentage carry-over was measured in a blank sample injected directly after the 1000 ng ml^{-1} measurement. It was found to be below 0.01% for all analytes.

Robustness

The method was used to analyse over 2000 porcine plasma samples from an intervention trial. The HPLC column was replaced once. Elution times remained stable, even on a contaminated column. The cone of the MS source was cleaned once a week, i.e. typically after ~100 samples (including calibration line samples, quality control samples and blanks). Cleaning of the source was carried out once every 3 months. Typical ion chromatograms obtained for a plasma sample collected during the porcine intervention study are given in Figure 2. Besides the mentioned steroidal glycosides and metabolites, a number of isomers could be observed. This resulted in a total of 24 compounds to be quantified in one single analysis. The concentration levels found in the plasma samples varied from 0.14 ng ml^{-1} for the detiglated aglycone to almost 700 ng ml^{-1} for the detiglated species of H.g.-12. Good peak shapes are seen for all steroidal glycosides, while most isomers were baseline separated.

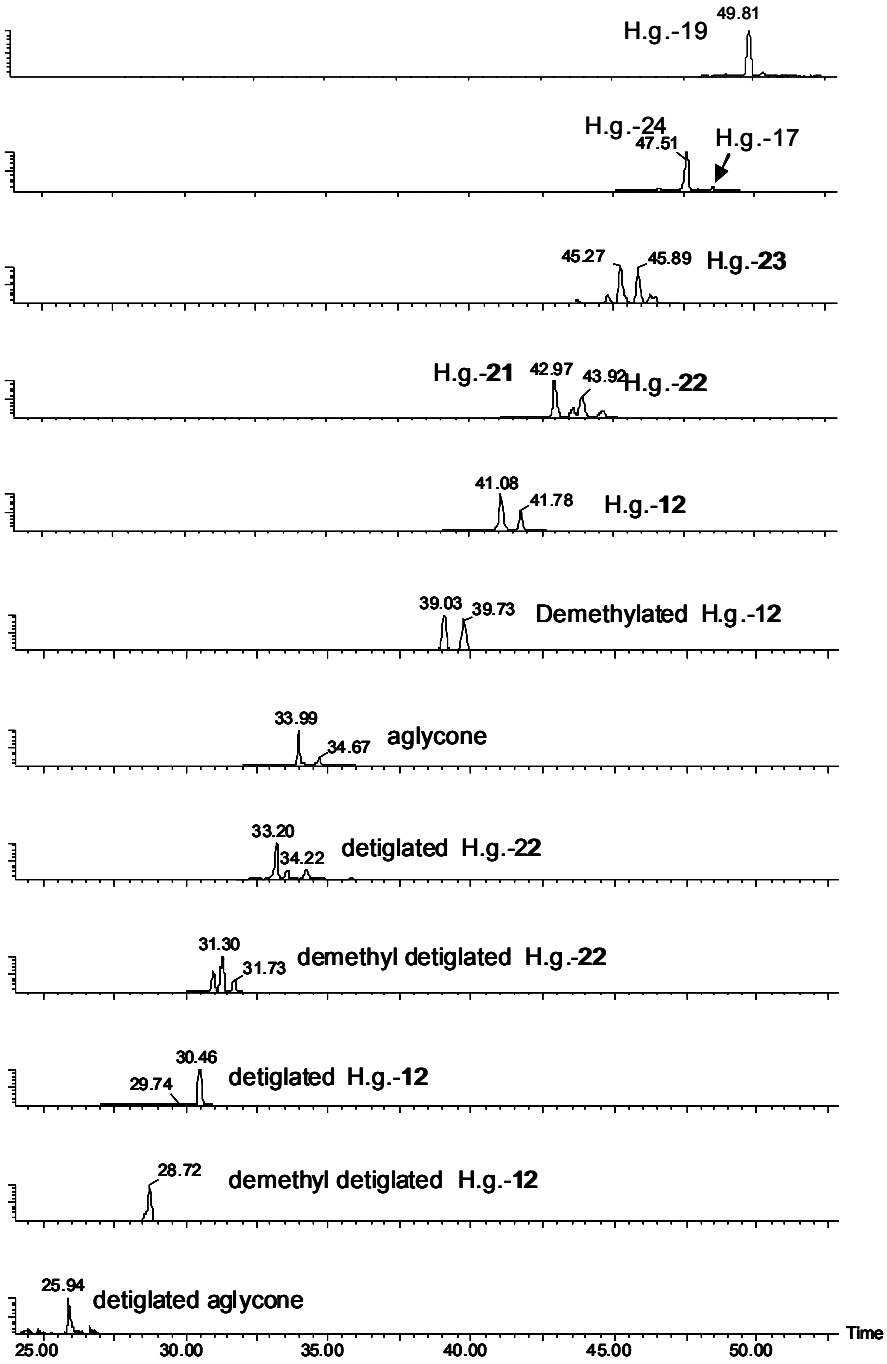

Figure 2 Typical ion chromatograms of a plasma sample. Summarized MRM traces of two product ions monitored for each steroid glycoside or metabolite are represented.

8.4 Conclusions

An HPLC-MRM-MS method has been developed for the quantification of eight steroidal glycosides, the detiglated metabolite of the main component (H.g.-12) and semi-quantitative analysis of five other metabolites in porcine plasma. Together with the isomers a total of 25 compounds were quantified in one single analysis. Careful optimization of the extraction conditions was needed to prevent the loss of the fat-soluble hydrophobic glycosides during sample clean-up. The limits of detection and quantification varied between 0.01 and 0.1 ng ml^{-1} and are low enough to monitor changes in plasma concentrations after intake of the *Hoodia* extract. The accuracy is better than 80% for H.g.-12 and H.g.-22 for concentrations between 0.1 and 500 ng ml^{-1}. A similar accuracy is also found for the detiglated H.g.-12. The precision was better than 20% enabling accurate monitoring of the time-concentration profiles of the steroidal glycosides in the porcine plasma samples. The good chromatographic separation and high stability combined with the excellent sensitivity and selectivity of the MRM-MS method makes the method developed especially suited for the quantification of steroidal glycosides and their metabolites in large numbers of plasma samples. The method is rugged, requires minimal maintenance or MS-source cleaning and was successfully applied to over 2000 samples collected from an intervention study in pigs [18].

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9 General Remarks

9.1 Conclusions drawn from the described work

The described work clearly shows that the preferred analytical approach used in foods research and development for the identification of bioactive compounds and their metabolites can vary strongly depending on the compound type and matrix to be analyzed. Often, a priori fractionation has to be applied for the isolation and purification of the active ingredient(s), whereas for identification mass spectrometry and/or NMR spectroscopy is combined with a bio-assay for the identification of the compounds. For quantification of the bioactives and their metabolites the choice of techniques and methods that can be used is much larger as in this case the matrix composition (natural product, e.g. plant extracts or physiological material, e.g. plasma, urine, tissue) is highly influential. In general it can be stated that the availability of analytical instrumentation and the experience with particular techniques and methods often play an important part in the choice of the analytical approach.

Liquid/liquid extraction (LLE) is often the preferred method for sample work-up. The main advantage of LLE is that it can be carefully tuned to the compounds to be analyzed by varying the pH and the types of extraction liquids. Due to this a high degree of purification can be achieved for the compounds to be analyzed. The main disadvantage is that parts of the extraction procedure must be carried out manually, which makes the method rather laborious and time consuming. From the results of the experiments described in chapters 2 - 8 it can be concluded that the selection of a general method for the isolation and purification of various groups of bioactives and their metabolites might be successful, in particular when the compounds to be analyzed show similarities in their chemistry, hydrophobicity and molecular size. This is often the case since the compounds must fit in the physiological system of the human body (pH, enzyme resistance; membrane permeability).

In the analytical MS process often accurate-mass measurement is used for identification of the bioactives while MRM is used for quantification. Although other MS techniques such as precursor-ion MS and neutral loss/gain MS are sometimes applied in specific applications, MRM is the preferred technique mainly because of its high selectivity and sensitivity. Of course full-scan MS would be preferred since bioactives identified in a later stage of the study can then still be quantified using the old data sets which is not the case when MRM was used to analyze the samples. A main problem is that the selectivity and sensitivity of the so far most frequently used

quadrupole instruments in full-scanning mode are insufficient. TOF instruments have higher sensitivity and selectivity but showed until recently a very limited dynamic range. Notably, in the latest generation of instruments this problem is solved and nowadays TOF instruments are becoming more and more applied in compound quantification.

9.2 Considerations on future developments

The most important requirements for the application of one, widely applicable general method for bio-sample analysis are:

1. Fast and simple optimization of the sample preparation for each new set of compounds and/or matrices.
2. Integrated sample work-up and analysis.
3. Easy to operate and (relatively) low cost equipment.
4. Short (chromatographic) analysis time.
5. Automated data interpretation.

The potential for automation is an important factor as it can strongly reduce the time needed to analyze series of samples, especially when the sample preparation is in-line with the analytical system preferably a combination of LC and (TOF or quadrupole) MS. Nowadays many types of sample preparation systems are available of which several can be combined with and/or controlled by other equipment. Currently two types of sample preparation systems are commonly used.

In the first type of system an in-line regenerable clean-up cartridge or column collects the bioactives and their metabolites and, after washing, applies them to the analytical system. Usually, such a system is very robust due to a minimum amount of handlings and moving parts. Disadvantages are that peak broadening might occur, in particular for highly hydrophilic compounds and the system handles only one sample at a time. By using a parallel system the sample throughput can be increased.

The second type of system consists of a robot that can be combined with various specialized units such as an SPE unit, cooling trays, injection valves, etc. Such systems can be used either off-line or on-line with LC-MS analytical instrumentation and usually have very high flexibility. Even bioassays at controlled temperatures and addition of internal standards can be performed prior to the sample clean-up. Also sample concentration can be performed by using a temperature-controlled flow of nitrogen. The system contains moving parts, the robotic arm, and might therefore be less robust as compared to the above described first type of system.

An interesting option, to our knowledge not yet described in literature, is to use such a robotic system in combination with so-called solid-supported

liquid/liquid cartridges (see Chapter 2). These cartridges have been recently introduced on the commercial market and can be used in combination with SPE sample clean-up systems. The advantage of this type of cartridge as compared to SPE, is that its principle is closer to that of manually performed LLE. The advantage of this system compared to e.g. manual LLE is that it can be used in automated analytical systems. Many other sample clean-up systems are available each with their own range of applicability. One example is turboflow combined with LC-MS. This system is, however, more suitable for the analysis of one (or only a few) compound(s) and is not very suited for large compound sets covering a broad range of hydrophobicity.

In general it can be stated that qualitative and quantitative analysis of complex, multi-component samples – as subject in this thesis – usually requires a complex system involving a suite of instrumental parts working in parallel or in sequence. The different parts have to be optimally coupled with each other. One must be aware that speeding-up one part of the analysis process can conflict with the other parts. Thus, the speed and analytical conditions of the sample clean-up process must be very well tuned and be compatible with the actual analytical process, the data handling and data interpretation. It is therefore always necessary to optimize the integral process. Shortening the LC-separation by using UPLC instead of HPLC can reduce the time of analysis by a factor of 3 to 5. As far as the data interpretation is concerned it is of utmost importance to select or develop the proper software to prevent unsurveyable data sets. A close corporation with the supplier(s) of the instrument(s) hardware and instrumental and data handling software is required to build an optimally integrated analytical system. For example the quantification software presently available on LC-MS instruments could be seriously improved by new algorithms to create a bio-curve from the measurements and to mark possible outliers for further examination.

Identification and quantification of bioactive compounds and their metabolites in complex matrices such as plant extracts, body fluids and tissues is a subject of ongoing interest in a wide range of applications such as medical sciences, pharmaceutical and foods R & D. In this context the further development of robust, fast and automated systems capable of analyzing large numbers of samples without the need of intensive instrument maintenance will remain to be an important subject.

Summary

In this thesis the feasibility of the development of a standard protocol for the identification and quantification of bio-active compounds and their metabolites in physiological matrices has been investigated. The aim of the investigation was to develop one fully automated and standardized method for physiological samples analyzed by LC-MS. The samples are generally obtained from *in-vivo* or *in-vitro* studies set-up to determine the effect of natural foodstuff on the human health. The method includes an automated procedure for sample preparation, optimized LC-MS separation and identification followed by automated data interpretation. The work was focussed on blood and urine.

The interest of (commercial) food companies for bio-active compounds with positive effects on human health and the application of those compounds in functional food products have a long history. In Chapter 1 an overview of compound classes, their proposed health effect and (natural) sources is given. Analytical strategies used up-to-now for the identification of functional food ingredients and quantification of those ingredients in physiological samples often obtained from human or animal trials are described. The analytical approach is often focussed on NMR on the whole sample or after a minimized sample preparation and/or identification and quantification of the bio-actives and their metabolites by using LC-MS. For the latter a more complex sample preparation is required. Special attention is given in this chapter to the three major steps of the analytical procedure: the sample preparation, the chromatographic separation and MS-analysis and the data interpretation.

Many different sample preparation techniques are available for the isolation of bio-active compounds and their metabolites from physiological fluids. From these, three techniques were selected for further investigation: solid-supported liquid/liquid extraction (SS-LLE), solid-phase extraction (SPE) and Monotrap extraction. The techniques were applied to polyphenols, steroidal glycosides and peptides. The compounds were spiked in aqueous solutions, plasma or milk. The results are described in chapter 2 and show that SPE has the highest potential for further investigation and application.

In Chapter 3 the development of a two-dimensional LC-MS method for the identification of small peptides with antihypertensive properties is described. A very fast method using a bio-assay for the bio-activity determination in combination with accurate mass LC-MS for the identification of the compounds was developed and applied to fermented milk resulting in the

identification of two, not earlier described, new tripeptides with a high bio-activity. In Chapter 4 the method was applied to fermented milk for the identification of small, strongly hydrophilic, peptides. New in this chapter is the application of hydrophilic interaction chromatography (HILIC) for the separation of peptides that are poorly retained on reversed phase columns.

Once the bio-active peptides have been identified quantification of these peptides in e.g. plasma collected from a human or animal trial is required. In Chapter 5 the development of a highly sensitive LC-MS method is described for the quantification of seventeen peptides in plasma in one single LC-MS analysis. The method was validated and successfully applied on several thousands of plasma samples collected from an animal trial.

Finally the method was applied to plasma samples collected from a human breakfast trial. In this placebo-controlled crossover study the effect of a breakfast on the bioavailability of the peptides in a peptide-enriched drink was investigated. The results are given in chapter 6.

Another class of compounds with potentially high bio-activity is the group of steroidal glycosides isolated from the *Hoodia gordonii* succulent. These compounds are claimed to have appetite-lowering properties. In Chapter 7 the development of a very sensitive LC-MS method is described which can be used when only very small amounts of sample are available, for instance in *in-vit*ro microsomal studies. The method combines the power of *in-silico* prediction of the metabolites to be formed with the high sensitivity of LC-MRM-MS. The advantages of this method are that only a very small amount of sample is required and the analyses can be performed using standard LC-MS instrumentation. The disadvantage is that only predicted metabolites will be identified and unpredicted species will be missed. The use of lithium chloride as eluent additive for the improvement of the sensitivity and accuracy of the LC-MS analysis is demonstrated. The method was successfully applied for the identification of metabolites of steroidal glycosides from a liver microsomal incubation of the *Hoodia gordonii* plant extract.

In Chapter 8 an LC-MS method is described for the quantification of the steroidal glycosides and metabolites described above. With this method 25 steroidal glycosides and their metabolites can be quantified in one single analysis. The method was validated and applied to several thousands of samples obtained from porcine trials.

In Chapter 9 conclusions are drawn from the described work. SPE shows the highest potential of the tested methods for the purification of plasma and

urine for a wide range of compounds with different polarities and hydrophobicities. The method is easy to automate, can be combined with an LC-MS and is highly robust. For future developments several considerations are given focussing on, amongst others, automation of the complete analysis process including the sample preparation and automation of the data interpretation.

Samenvatting

In dit proefschrift wordt de haalbaarheid onderzocht van de ontwikkeling van een standaard protocol voor de identificatie en kwantificatie van bio-actieve verbindingen en hun omzettingsproducten in humaan monstermateriaal. De monsters werden verkregen uit in-vitro studies of uit bloed en urine van proefpersonen die deelnamen aan voedingsproeven. Deze proeven werden uitgevoerd om de gezondheidseffecten te bestuderen van een aantal natuurlijke verbindingen die werden toegevoegd aan bepaalde voedings-middelen. De analysemethode omvat een geautomatiseerde procedure voor de monstervoorbereiding, geoptimaliseerde scheiding en identificatie met behulp van gekoppelde vloeistofchromatografie/massaspectrometrie (LC-MS), gevolgd door geautomatiseerde interpretatie van de gegevens.

De interesse in voedingsmiddelenbedrijven voor natuurlijke, bio-actieve verbindingen met positieve effecten op de menselijke gezondheid en de toepassing van deze verbindingen in voedingsmiddelen heeft een lange geschiedenis. In hoofdstuk 1 wordt een overzicht gegeven van een aantal natuurlijke verbindingen en hun veronderstelde effect op de gezondheid. Voorts worden analytische strategieën beschreven die tot nu toe zijn toegepast voor de identificatie en kwantificering van functionele voedingsingrediënten in fysiologische monsters. De analyse geschiedt vaak door middel van LC-MS analyse voorafgegaan door een bepaalde mate van voorscheiding. In dit hoofdstuk wordt speciale aandacht gegeven aan de vier belangrijkste onderdelen: de monstervoorbereiding, de chromatografische scheiding, de MS-analyse en de interpretatie van de gegevens.

Veel verschillende monstervoorbereidingstechnieken zijn beschikbaar voor de isolatie van bio-actieve stoffen en hun metabolieten uit fysiologische vloeistoffen. Uit deze technieken werden er drie geselecteerd voor nader onderzoek: solid-supported liquid/liquid extraction (LLE-SS), solid-phase extraction (SPE) en Monotrap extractie. De technieken werden toegepast op polyfenolen, steroïd-glycosiden en peptiden en geanalyseerd in waterige oplossingen, plasma en melk. De resultaten zijn beschreven in hoofdstuk 2 en laten zien dat SPE het hoogste potentieel heeft voor verdere toepassing.

In hoofdstuk 3 wordt de ontwikkeling van een twee-dimensionale LC-MS methode voor de identificatie van kleine peptiden met bloeddrukverlagende eigenschappen beschreven. Een zeer snelle methode waarbij gebruik gemaakt wordt van een bio-assay voor de bepaling van de bio-activiteit in combinatie met nauwkeurige massa LC-MS voor de identificatie van de stoffen werd ontwikkeld en toegepast op gefermenteerde melk. Dit heeft

geresulteerd in de identificatie van twee, niet eerder beschreven, tripeptiden met een hoge bio-activiteit. In hoofdstuk 4 werd de methode toegepast op gefermenteerde melk voor de identificatie van kleine, sterk hydrofiele, peptiden. Nieuw in dit hoofdstuk is de toepassing van hydrofiele interactie chromatografie (HILIC) voor het scheiden van peptiden die slecht gescheiden worden op reversed phase kolommen.

Zodra de bio-actieve peptiden zijn geïdentificeerd is hun kwantificering vereist in bijvoorbeeld plasma verzameld tijdens menselijke of dierlijke voedingsproeven. In hoofdstuk 5 wordt de ontwikkeling van een zeer gevoelige LC-MS methode voor de kwantificering van zeventien peptiden in plasma in één LC-MS analyse beschreven. De methode is gevalideerd en met succes toegepast op enkele duizenden plasma-monsters verzameld gedurende een dierproef.

Ten slotte werd de methode toegepast op plasmamonsters van een menselijke ontbijtproef. In deze placebo-gecontroleerde crossover studie werd het effect van een ontbijt op de biobeschikbaarheid van de peptiden in een peptide-verrijkte drank onderzocht. De resultaten zijn weergegeven in hoofdstuk 6.

Een andere klasse van verbindingen met potentieel hoge bio-activiteit is de groep van steroïd-glycosiden geïsoleerd uit de *Hoodia gordonii* plant. Deze verbindingen worden verondersteld eetlust-verlagende eigenschappen te hebben. In hoofdstuk 7 wordt de ontwikkeling van een zeer gevoelige LC-MS methode beschreven die gebruikt kan worden wanneer er slechts zeer kleine hoeveelheden monster beschikbaar zijn, bijvoorbeeld bij microsomale in-vitro studies. De methode combineert de kracht van in-silico voorspelling van de metabolieten welke gevormd kunnen worden met de hoge gevoeligheid van LC-MS-MRM analyse. De voordelen van deze methode zijn dat slechts zeer kleine hoeveelheid van het monster nodig zijn en dat de analyses uitgevoerd kunnen worden met behulp van standaard LC-MS apparatuur. Een nadeel is echter dat alleen voorspelde metabolieten zullen worden geïdentificeerd en dat niet voorspelde verbindingen worden gemist. De toevoeging van lithium chloride aan het eluens voor de verbetering van de gevoeligheid en de nauwkeurigheid van de LC-MS analyse is aangetoond. De methode werd met succes toegepast voor de identificatie van metabolieten van *Hoodia* steroïd-glycosiden gevormd bij incubatie van levermicrosomen.

In Hoofdstuk 8 wordt een LC-MS methode beschreven voor de kwantificering van de steroïd-glycosiden en metabolieten. Met deze methode kunnen 25 steroïd-glycosiden en hun metabolieten worden geanalyseerd in

één enkele analyse. De methode is gevalideerd en toegepast op enkele duizenden monsters verkregen uit voedingsonderzoeken met varkens.

In hoofdstuk 9 worden enkele conclusies getrokken uit het beschreven werk. SPE toont het hoogste potentieel van de geteste methoden voor de zuivering van een breed scala van verbindingen met verschillende polariteiten en hydrofobiciteit uit plasma en urine. De methode is gemakkelijk te automatiseren, kan worden gecombineerd met LC-MS en is zeer robuust. Voor toekomstig applicatieonderzoek worden verschillende overwegingen gegeven, gericht op onder andere de automatisering van het volledige analyseproces, met inbegrip van de voorbereiding van het monster en automatisering van de data-interpretatie.

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Chris