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DISSERTATION

Online hyphenation of chromatography and mass spectrometry – elucidating the molecular composition of a biological adhesive from ixodid ticks

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Abstract

Amongst the omics studies, metabolomics aims the comprehensive and quantitative analysis of all metabolites in a biological system and plays a crucial role in understanding cellular reactions which yield very important information about biological systems and their metabolic status. In the first part of this thesis methodologies for the targeted analysis of metabolites, applying gas chromatography electron ionisation tandem mass spectrometry (GC-EI-MS/MS) and liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) were developed. Highly selective and sensitive analysis was achieved by the application of multiple reaction monitoring (MRM). Different metabolites from several metabolite classes (amino acids, carbohydrates and the respective phosphates and organic acids), yet all important in the primary carbon metabolism, were selected. Two derivatisation strategies for GC, trimethylsilylation (TMS) and methoximation in combination with TMS (MeOx/TMS) were investigated. It could be revealed that compared to exclusively perform TMS the combined method presents benefits regarding derivatisation products and kinetics of the reaction. The formation of up to seven derivatives for monosaccharides was obtained if mere TMS was applied. Furthermore, the amino acid lysine produced two derivatives: a single or double silylated ε-amino group. The combined derivatisation method prevents these drawbacks. However, an outstanding observation was the formation of two additional glucose derivatives in the presence of lysine after MeOx/TMS, possibly representing the α- and ß-anomer of the cyclic form of the hexose. Although several substances formed two derivatives after MeOx/TMS the chromatographic performance revealed benefits of the developed GC-EI-MS/MS method compared to LC-ESI-MS/MS: Separation of the sugar alcohols and monosaccharides, except ribose, could be achieved by GC, though only sum peaks for C5- and C6-sugars and sugar alcohols could be obtained by LC. In addition to that the GC peak widths had a maximum of 0.27 min compared to several minutes for LC. The obtained limits of detection (LODs) and quantification (LOQs) were in the low µM range and were predominantly lower for GC-EI-MS/MS than for LC-ESI-MS/MS.

With respect to potential applications in healthcare, pharmacy, the bonding industry, and cosmetics, biological adhesives produced from various animals are of high interest. Ticks, temporary ectoparasites, are feeding obligatorily on the blood of vertebrates and are known to secret such a bioadhesive called attachment cement prior and during their blood meal. This viscous and sticky substance is produced by the ticks' salivary glands and hardens after secretion. The main function of the cement seems to be the anchoring of the

tick by strengthening the attachment of the ticks' mouthparts to the host. However, all functions of the cement and the detailed composition are not fully understood or explored yet. Thus, the second part of this thesis focused on the characterisation of the tick attachment cement.

Tick attachment cement was collected from *in vitro* fed *Amblyomma hebraeum* and *Dermacentor marginatus* ticks using an artificial membrane feeding system and different feeding materials. Amino acid analysis of cement samples was performed by GC-EI-MS/MS and LC-ESI-MS/MS after acidic or alkaline hydrolysis using hydrochloric acid (HCl) or sodium hydroxide (NaOH). However, after studying the amino acid recovery of standard proteins, alkaline hydrolysis was found to yield very poor recoveries and acidic sample preparation was chosen for further analysis. Instrumental analyses on the basis of GC-EI-MS/MS and LC-ESI-MS/MS were validated and showed to be very suitable for cement analysis. High amounts of non-polar amino acids (Gly, Leu, Pro, Ala and Phe) were found to be present in the cements, more than 60%, with Gly being the major component (25% and 35% of the total cement in *Dermacentor marginatus* and *Amblyomma hebraeum*, respectively). The comparison of both species showed that both adhesives are rather similar with the largest difference in the amount of Gly.

The cement showed very poor solubility in different buffer systems, but using acidic conditions allowed for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation. Proteins of *Dermacentor marginatus* cement were identified after tryptic *in-gel* digestion using LC-ESI-MS/MS. The identification of glycine-rich proteins (GRPs), e.g. the cement protein RIM36, confirmed the findings from amino acid analysis. Additionally, proteins that inhibit tissue repair, others hampering the function of protein degrading enzymes or some with antimicrobial activity were found. These findings indicate that the cement is not only involved in attachment, but also in protecting the tick itself.

Zusammenfassung

Unter den Omics-Studien zielt Metabolomics auf die umfassende und quantitative Analyse aller Metaboliten in einem biologischen System ab und spielt eine entscheidende Rolle für das Verständnis von Zellreaktionen, welche sehr wichtige Informationen über biologische Systeme und ihren Stoffwechselstatus liefern. Im ersten Teil dieser Arbeit wurden Methoden für eine zielgerichtete Analyse von Metaboliten unter Einsatz von Gaschromatographie gekoppelt mit Elektronenionisations-Tandem-Massenspektrometrie (GC-EI-MS/MS) und Flüssigkeitschromatographie gekoppelt mit Elektrosprayionisations-Tandem-Massenspektrometrie (LC-ESI-MS/MS) entwickelt. Durch den Einsatz von Multiple Reaction Monitoring (MRM) konnten die Analysen mit hoher Selektivität und Sensitivität durchgeführt werden. Unterschiedliche Metaboliten verschiedenster Metabolitenklassen (Aminosäuren, Kohlenhydrate und die entsprechenden Phosphate und organische Säuren), alles wichtige Vertreter des primären Kohlenstoffkreislaufs, wurden ausgewählt. Hinsichtlich GC wurden zwei Derivatisierungsstrategien, Trimethylsilylierung (TMS) und Methoximierung in Kombination mit TMS (MeOx/TMS), näher untersucht. Es konnte gezeigt werden, dass die kombinierte Methode im Vergleich zur alleinigen Durchführung von TMS Vorteile in Bezug auf Derivatisierungsprodukte und Reaktionskinetik aufweist. Bei alleiniger Anwendung von TMS wurden bis zu sieben Derivate für Monosaccharide erhalten. Darüber hinaus bildete die Aminosäure Lysin zwei Derivate: eine einfach oder doppelt silylierte ε-Aminogruppe. Diese Nachteile konnten durch die kombinierte Derivatisierungsmethode vermieden werden. Eine besondere Beobachtung war jedoch die Bildung von zwei zusätzlichen Glukosederivaten in Gegenwart von Lysin nach erfolgter MeOx/TMS Derivatisierung, die möglicherweise das α- und β-Anomer der cyclischen Form der Hexose darstellen.
Obwohl MeOx/TMS bei mehreren Substanzen die Bildung von zwei Derivaten bewirkte, zeigte die entwickelte GC-EI-MS/MS Methode hinsichtlich der chromatographischen Leistung Vorteile gegenüber LC-ESI-MS/MS: die Zuckeralkohole und Monosaccharide, mit Ausnahme von Ribose, konnten durch GC getrennt, durch LC jedoch nur als C5- und C6-Zucker- und Zuckeralkohol-Summenpeaks erhalten werden. Die ermittelten Nachweis- (LODs) und Bestimmungsgrenzen (LOQs) lagen im niedrigen µM-Bereich und sind für GC-EI-MS/MS zumeist niedriger als für LC-ESI-MS/MS.

Der zweite Teil dieser Arbeit befasst sich mit der Charakterisierung eines von Zecken abgesonderten biologischen Klebstoffes. Im Hinblick auf mögliche Anwendungen im Gesundheitswesen, in der Pharmazie, in der Klebe- und in der Kosmetikindustrie sind

biologische Klebstoffe, hergestellt von verschiedensten Tieren, von großem Interesse. Zecken, temporäre Ektoparasiten, ernähren sich ausschließlich vom Blut von Wirbeltieren und sind bekannt vor und während ihrer Blutmahlzeit einen solchen biologischen Klebstoff, den sogenannten Zeckenzement, abzusondern. Diese viskose und klebrige Substanz wird von den Speicheldrüsen der Zecken produziert und härtet nach der Sekretion aus. Die Hauptfunktion des Zements scheint die Verankerung der Zecke durch die Verstärkung der Haftung der Mundwerkzeuge der Zecken am Wirt zu sein. Alle Funktionen des Zements und die detaillierte Zusammensetzung sind jedoch noch nicht vollständig bekannt und erforscht und werden daher hier behandelt.

Zeckenzement wurde von *in vitro* gefütterten *Amblyomma hebraeum* und *Dermacentor marginatus* Zecken unter Verwendung eines künstlichen Membranfütterungssystems und unterschiedlichen Fütterungsmedien gesammelt. Aminosäureanalyse von Zementproben wurde durch GC-EI-MS/MS und LC-ESI-MS/MS nach saurer oder basischer Hydrolyse unter Verwendung von Salzsäure (HCl) oder Natronlauge (NaOH) durchgeführt. Nach Ermittlung der Wiederfindungsraten für Aminosäuren unter Verwendung von Standards wurde jedoch festgestellt, dass basische Hydrolyse zu sehr schlechten Wiederfindungen führte, wodurch saure Probenvorbereitung für alle weiteren Analysen verwendet wurde. Instrumentelle Analysen auf Basis von GC-EI-MS/MS und LC-ESI-MS/MS Methoden wurden validiert und erwiesen sich für die Zementanalyse als sehr geeignet. Es wurde festgestellt, dass im Zement mehr als 60% an unpolaren Aminosäuren vorhanden sind (Gly, Leu, Pro, Ala, Phe), wobei Gly der Hauptbestandteil war (25% und 35% des gesamten Zements in *Dermacentor marginatus* bzw. *Amblyomma hebraeum*). Der Vergleich beider Spezies ergab, dass beide Klebstoffe sehr ähnlich zusammengesetzt sind und der größte Unterschied in der Menge an Gly besteht.

Der Zement zeigte sehr schlechte Löslichkeit in verschiedenen Puffersystemen, jedoch ermöglichten saure Probenvorbereitungsbedingungen eine Proteinauftrennungen mittels Natriumdodecylsulfat-Polyacrylamid-Gelelektrophorese (SDS-PAGE). Proteine von *Dermacentor marginatus* Zement wurden anschließend nach tryptischem In-Gel-Verdau mittels LC-ESI-MS/MS identifiziert. Die Identifizierung von Glycin-reichen Proteinen (GRPs), z.B. das Zementprotein RIM36, bestätigte die Ergebnisse der Aminosäureanalyse. Zusätzlich wurden Proteine, die die Gewebereparatur inhibieren, die die Funktion von Protein abbauenden Enzymen beeinträchtigen, oder einige mit antimikrobieller Aktivität, gefunden. Diese Resultate deuten darauf hin, dass der Zement nicht nur an der Anhaftung, sondern auch am Schutz der Zecke selbst beteiligt ist.

Aim of the Thesis

This thesis covers multiple important parts: most importantly multiple methods were developed for small molecule analysis from biological samples and secondly a comprehensive analysis of biological adhesives is presented where analytical approaches built on afore mentioned methods.

In the first part of this Ph.D. thesis methodologies for the analysis of 24 selected metabolites mainly belonging to the primary carbon cycle metabolism were developed. In particular amino acids, organic acids, sugars and sugar derivatives were studied.
The analytical performance of two techniques, gas chromatography electron ionisation tandem mass spectrometry (GC-EI-MS/MS) and liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS), was investigated and compared in terms of separation efficiency and detection sensitivity (**Manuscript I**, p. 33). For GC-EI-MS/MS two different derivatisation strategies, trimethylsilylation (TMS) and methoximation in combination with TMS (MeOx/TMS), were applied and results compared in terms of formed derivatives and kinetic of the derivatisation reaction (**Manuscript II**, p. 47). On both instruments limits of detection (LODs) and limits of quantification (LOQs) were determined and presented in this work.

The aim of the second part of the thesis was the characterisation of biological adhesives, in particular tick attachment cement from the ixodid ticks *Amblyomma hebraeum* and *Dermacentor marginatus*. Hence, the bioadhesive was collected from adult ticks that were fed in artificial feeding units on cattle blood and investigated after sample preparation by GC-EI-MS/MS and by liquid chromatography electrospray ionisation time-of-flight tandem mass spectrometry (LC-ESI-ToF-MS/MS) after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).
In order to get an overview of the current research status of the biological skin plug system of ticks, literature was screened (**Publication I**, p. 96). This allowed to get an insight into tick cement research, the biological significance, functions and structure of the cement as well as its histochemistry and biochemistry. Furthermore, cement was compared with other biological adhesives and the potential applications of tick attachment cement were outlined.
For biochemical analysis tick cement was grinded and homogenised and, since the sample amount was rather low, cement from several animals (female and male) was pooled. Amino acid analysis was established using GC-EI-MS/MS and the amino acid pattern of both

investigated tick species was determined (**Manuscript III**, p 118). In comparison, the amino acid composition of barnacle cement (*Lepas anatifera*) was investigated to better understand differences in adhesion processes.

It is known that tick attachment cement contains mainly proteins. To better understand its particular composition, the protein composition was investigated using a proteomics approach. To separate proteins by SDS-PAGE the sample has to be solubilised, but tick attachment cement has to have little solubility in buffer solutions, as it would otherwise easily dissolve during the feeding process of the tick. After developing an appropriate sample preparation for and separation by SDS-PAGE, protein bands were cut out of the gels and protein identification was performed after *in-gel* digestion and LC-ESI-ToF-MS/MS analysis (**Manuscript IV**, p. 152).

INTRODUCTION

1 Principle methodology for analyte separation and detection

1.1 Gas chromatography coupled to mass spectrometry

Gas chromatography coupled to mass spectrometry (GC-MS) is a very useful instrumentation with a high separation efficiency which can resolve very complex biological samples [1]. The typical peak widths are within the range of a few milliseconds and retention times are very reproducible [2]. Furthermore, GC-MS features versatile, selective and sensitive mass detection [3, 4]. The combination of GC with electron ionisation (EI) allows the use of spectral libraries for the identification of unknown compounds based on mass to charge (m/z) and intensity ratios of observed fragment ions. This was achieved through the standardisation of the electron energy (70 eV) of the emitted electrons with which the volatile analytes are bombarded in the high vacuum ion source (10^{-7}-10^{-5} mbar, 200-250 °C), producing highly reproducible fragmentation patterns for a particular molecule (**Figure 1**).

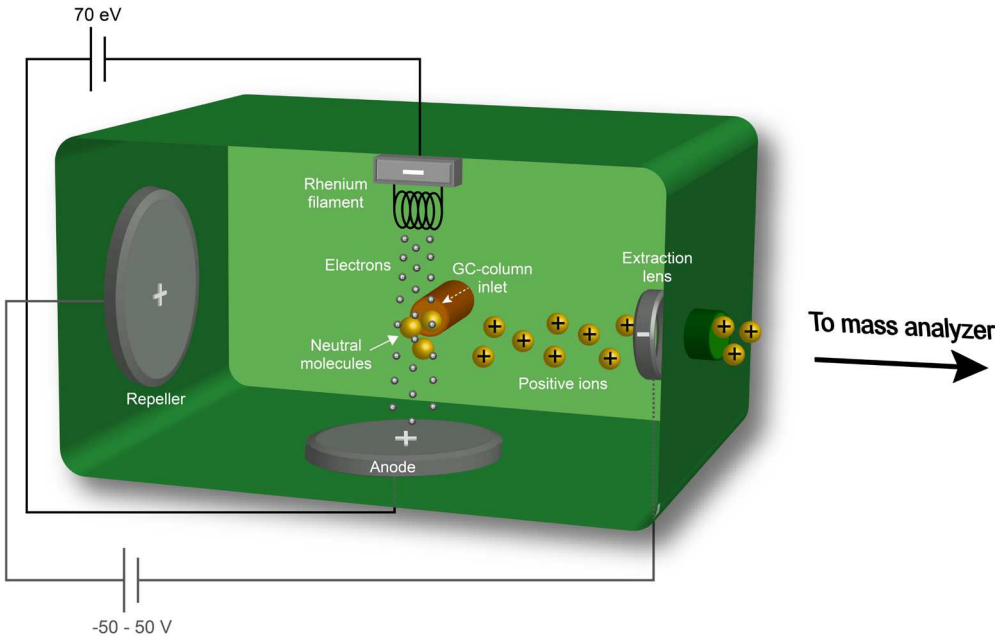

Figure 1: Scheme of an EI ion source for GC-MS analysis

Since EI mass spectra have been studied for several decades many libraries are available containing thousands of spectra [5]. The most comprehensive mass spectral libraries are available from the National Institute of Standards and Technology (containing more than

300,000 spectra) and Wiley (containing more than 750,000 spectra) [6, 7]. On the one hand EI mass spectra can be useful in determining the structure of an analyte since the high energy during ionisation results in extensive fragmentation, but on the other hand the molecular ion usually is not present in the spectra which makes structure elucidation and characterisation often complicated.

If there is the need for information about the molecular ion another ionisation method, chemical ionisation (CI), can be used which requires a lower amount of energy. The ionisation is based on the reaction of gas molecules ionised by EI with the analyte molecules.

In GC the separation of analytes takes place in the gas phase and thus the sample is heated to high temperatures (200-300 °C) during injection allowing the compounds to evaporate. However, many analytes contain polar functional groups that are thermally labile or the compound is not volatile at all. Thus, in order to reduce the polarity of functional groups and to increase the analytes volatilities, chemical derivatisation is usually performed prior to analysis. Details for that can be seen in **section 1.2, Chemical derivatisation of metabolites** (p. 21) and **section 2.2, Protein hydrolysis followed by amino acid analysis** (p. 27), in **Part I: Method Development**.

Limitations of GC:

The most obvious limitation of GC is sample preparation with its derivatisation reaction which usually contains several steps and can be time consuming. Furthermore, data complexity can be increased if multiple derivatives are formed from one analyte (e.g. silylation of saccharides). In addition to that several factors which influence the derivatisation yield have to be considered and often hinder analysis:

- The amount of derivatisation agent, usually in excess to allow a complete reaction, and the derivatisation time highly influence analysis outcomes. Ideally every analyte has entirely reacted, but typically the derivatisation is not totally completed. E.g. efficient derivatisation for amino acids is often only given after 30 h of silylation, which is not convenient especially in the context of high-throughput analysis. Additionally, during such long time derivatisation times degradation of derivatives can occur [8].
- Another obstacle for sample derivatisation is the investigation of complex mixtures since there is not one derivatisation agent working perfectly fine for all analytes,

not to forget optimal reaction times and temperatures for each analyte. Thus, usually a compromise has to be made to allow an acceptable analysis.

Besides derivatisation another limitation of GC is the fact that non-volatile or thermal labile compounds are not suited for analysis. For such compounds usually liquid chromatography (LC) is the method of choice and will be discussed in the following.

1.2 Liquid chromatography coupled to mass spectrometry

Liquid chromatography coupled to mass spectrometry (LC-MS) offers the ability of resolving a wider range of small molecules extending from low molecular weight compounds, also detectable by GC-MS, to non-volatile or thermally labile analytes. Typically, this can be achieved without the application of a derivatisation step. The latest development in LC is ultra high performance liquid chromatography (UHPLC). UHPLC uses particles with sub-2 µm diameter which increase the separation capacity significantly compared to conventional high-performance liquid chromatography (HPLC). Yet, such small particles cause higher back-pressures, up to 12,000 psi compared to about 6,000 psi in conventional chromatography using 3-5 µm particles [9]. This higher back-pressure can be reduced by performing separations at elevated temperature where the solvent viscosity is decreased. Such high temperatures allow higher flow rates resulting in reduced analysis times and it increases also the interaction of the analytes with the stationary phase due to enhanced kinetics and therefore can improve the resolution. Comparing particle sizes of 3 µm (HPLC) with 1.7 µm (UHPLC) shows that instead of 1,500 ions in 10 min, 5,000 ions in only 5 min can be detected [10]. This clearly demonstrates increased column efficiency and peak capacity. This results not only in a better peak resolution but also in reduced analysis times and lower detection limits [5, 11].

Reversed phase (RP) chromatography is the standard tool for the analysis of medium polar and non-polar analytes and represents a reliable, robust, repeatable and well-understood separation mechanism. It covers a wide range of molecular structures and therefore is well established and accepted [9]. Small molecule analysis is usually carried out using C_{18} stationary phases, whereas C_4 phases are used for proteins and larger or hydrophobic peptides [12, 13]. Many polar or charged analytes are not retained on standard RP columns and are eluted with the void volume, thus, in order to separate polar substances like ketones, aldehydes, alcohols, carboxylic acids or amines derivatisation can be used even though a derivatisation step makes sample preparation more complex like in GC. The formation of derivatives can enhance detection limits, separation efficiency, ionisation

efficiency and MS/MS detectability [14, 15]. Several MS/MS and LC-ESI-MS/MS suitable derivatisation agents for the mentioned substance classes can be found in literature [15, 16]. The alkylation of amino acids with propyl chloroformate (PCF) is one of these derivatisation strategies and is discussed in detail in **section 2.2**, **Protein hydrolysis followed by amino acid analysis** (p. 27) in **Part I: Method Development** and **Manuscript III** (p. 118) in **Part II: A First Investigation of Tick Attachment Cement**.

Besides RP, hydrophilic interaction liquid chromatography (HILIC) emerged as an interesting alternative. HILIC, which is already known since the early 1990s, allows the separation of highly polar substances and is orthogonal to RP and similar to normal phase (NP) chromatography [17]. However, the non-aqueous mobile phase in NP chromatography is replaced by an eluent with a high content of water miscible organic solvent, usually acetonitrile (ACN) or methanol (MeOH) in water or volatile buffer. The retention mechanism is not fully understood, but is based on hydrophilic interactions between the analyte and a water-enriched hydrophilic stationary phase [9, 18]. Since RP and HILIC are complementary methods it is also possible to combine both and further improve chromatographic separations and thus increase the obtained information. Details on the separation of selected metabolites using HILIC are discussed in detail in **Manuscript I** (p. 33) in **Part I: Method Development**.

The most commonly and widely used ionisation technique in combination with LC is electrospray ionisation (ESI). ESI allows sensitive analysis of polar and ionic compounds and offers high sensitivity and versatility [1, 11]. **Figure 2** shows the scheme of an ESI ion source. The ESI process is based on the generation of a Taylor cone resulting in an aerosol by the application of a high voltage on the mobile phase containing the analytes [19, 20]. The solvent evaporates and charged analyte ions are transferred into the mass analyser. The whole process is supported by the application of a nebuliser and drying gas flow (nitrogen, N_2). A special feature in the instrument used in this thesis is the application of a heating gas (dry air) next to the mentioned drying and nebulising gas which additionally facilitates solvent evaporation. The orthogonal geometry, as illustrated in **Figure 2**, is an improved and very robust ESI ion source configuration. This and the applied potential difference allows to transfer only charged ions and no solvent molecules or neutral ions into the mass analyser. There are two main theories which explain the formation of gas phase ions, i.e. the charged-residue model (CRM) and the ion evaporation model (IEM). The CRM, which most likely is considered for large molecules, suggests that the electrospray process generates droplets that contain only one analyte ion which is released when the solvent evaporates [21]. The IEM on the other hand is considered for small molecules and suggests that after the droplets reach a certain radius (through solvent

evaporation and Coulomb fission), where the field strength at their surface is sufficiently large, ions are directly emitted from the formed progeny droplets [22].

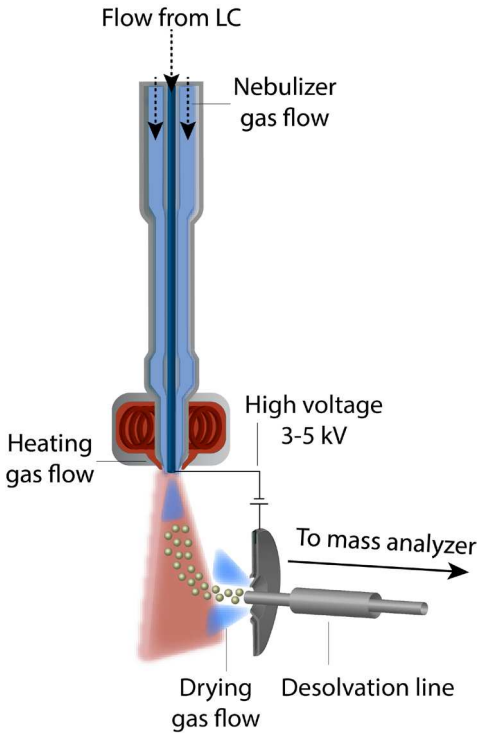

Figure 2: Scheme of an ESI ion source for LC with an orthogonal geometry.

Being a soft ionisation technique is a main advantage of ESI minimising by this analyte fragmentation. This assists structure elucidation and helps in the analytical interpretation of complex mixtures since molecular ions are present in the mass spectra [23]. Higher sensitivities (more than 100 fold) can be reached with nanoESI where the flow rates are reduced to a few nanoliters per minute [24]. If a more intense fragmentation is needed, e.g. for compound identification or a detailed structure analysis, ESI can be combined with collision induced dissociation (CID). Thus, more characteristic fragmentation patterns like in GC-EI-MS can be obtained.

A more comprehensive information on atmospheric pressure ionisation can be found in the review of Hayen and Karst where also methods like atmospheric pressure photoionisation (APPI), which is a more efficient alternative for the ionisation of less polar or neutral compounds, are discussed in detail [25].

Limitations of LC-MS:

Comparing GC-MS and LC-MS reveals that GC-MS has a higher reproducibility in terms of ionisation efficiency and chromatographic retention [11]. Compared to GC columns the peak performance of HPLC is far lower. Another limitation of LC-MS when analysing complex biological samples is the possibility of ion suppression caused by matrix effects [26, 27]. On the other hand, also ion enhancement can occur, which improves ionisation. Both, ion suppression and enhancement are a result of co-eluting compounds which change the degree of ionisation of a particular analyte and thus reduce or increase the signal [28-30]. In GC-EI-MS these effects are almost absent, but also can appear [31]. Another disadvantage of LC-MS is the lack of transferable libraries due to the high variability between different systems. This variability results from several sources like adduct formation in various systems altering relative ion abundances, differences in in-source fragmentation and tandem mass spectra fragments and the lack of LC reproducible retention indices [32].

1.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE is a well-accepted technique where charged particles like proteins migrate in gel-forming polymers upon application of an electric field. The migration or separation, respectively, is influenced by the present pore size of the gel which can be varied in dependence on the polymer to crosslinker ratio and their concentrations. Most often acrylamide and bisacrylamide are used as polymer and crosslinker, respectively. The implementation of an acrylamide concentration gradient obtained by mixing two solutions of different acrylamide concentration prior to polymerisation even allows the separation of complex mixtures in a single run [33]. SDS-PAGE separations are only dependent on the mass of the proteins but not the charge, by this enabling the determination of the molecular masses. This is achieved through the presence of the detergent SDS which acts as an anionic surfactant masking the intrinsic charge of the proteins resulting in a very similar charge to mass ratio [34]. For classical SDS-PAGE, denaturation of the proteins is required, thus additives like detergents or the chaotropic agent urea are added as well as reducing agents like dithiothreitol (DTT) or mercaptoethanol to break disulfide bonds.
After separation, the proteins are visualised using different staining methods like coomassie brilliant blue, silver or fluorescence staining. The choice depends on various factors like composition of the proteins of interest, availability of sample (concentration) or available equipment [35]. In the case of low concentrated samples usually silver staining is preferred having a far better sensitivity (below 1 ng per protein spot) than

coomassie brilliant blue [34, 36]. An even more sensitive method is fluorescence staining. It offers reliable quantification over a wide linear dynamic range, but it is more elaborate since a fluorescence scanner is needed for visualisation and the dyes are very expensive.

1.4 Available mass analyser

There are several mass analysers available and the selection strongly depends on the goal of the analytical approach and the analytical question itself.

Single quadrupole mass analysers are used in GC- but also in LC-MS instruments. Operated in full scan mode generates nominal mass fragmentation patterns for the measured compounds [32]. In the case of EI and CI (for GC-MS), measurements in full scan mode allow a comparison of the recorded mass spectrum with the database and thus identification of the analytes. However, higher sensitivity (factor 10 to 100) can be reached when the mass analyser is operated in selected ion monitoring (SIM) mode. Allowing only ions of interest to pass through the instrument decreases significantly the time needed for each ion, allowing therefore longer dwell times (time used to measure a certain m/z gathering therefor more data points) [37]. An even further improvement can be achieved using a triple quadrupole mass analyser (**Figure 3**) which is capable of four different acquisition modes: product ion scan, precursor ion scan, neutral loss scan or multiple reaction monitoring (MRM). When performing measurements in the product ion scan mode, information about the gained product ions after CID of precursor ions can be obtained facilitating the determination of the structure. The precursor ion scan on the other hand allows to scan for precursor ions giving a certain fragment after CID. In both modes the second quadrupole (Q2) acts as collision cell where the ions collide with gas molecules (typically argon). In the precursor ion scan the first quadrupole (Q1) scans through the mass range whereas the third quadrupole (Q3) is set to a constant m/z. In contrast to that Q1 is set to a constant m/z and Q3 scans through the mass range when the product ion scan is used. The concept of MRM is a combination of these two modes and features enhanced sensitivity to the low µM range (see **Manuscript I**, p. 33 and **Manuscript II**, p. 47) [38]. This can be achieved by the selection of parent ions in Q1, which are subjected to CID in Q2. Subsequently Q3 filters selected product ions allowing them to pass the mass filter towards the detector. Another interesting mode of measurement of triple quadrupole mass analysers, is the neutral loss scan, which allows to detect all precursors that generate a common neutral-loss fragment, further adding specificity to the mass spectral detection.

The benefit when combining EI with a triple quadrupole mass analyser is the increased specificity since the selected ions are very often already fragments of the substance of interest produced during the ionisation process. This can be also observed for ESI, but less often because of its soft ionisation character.

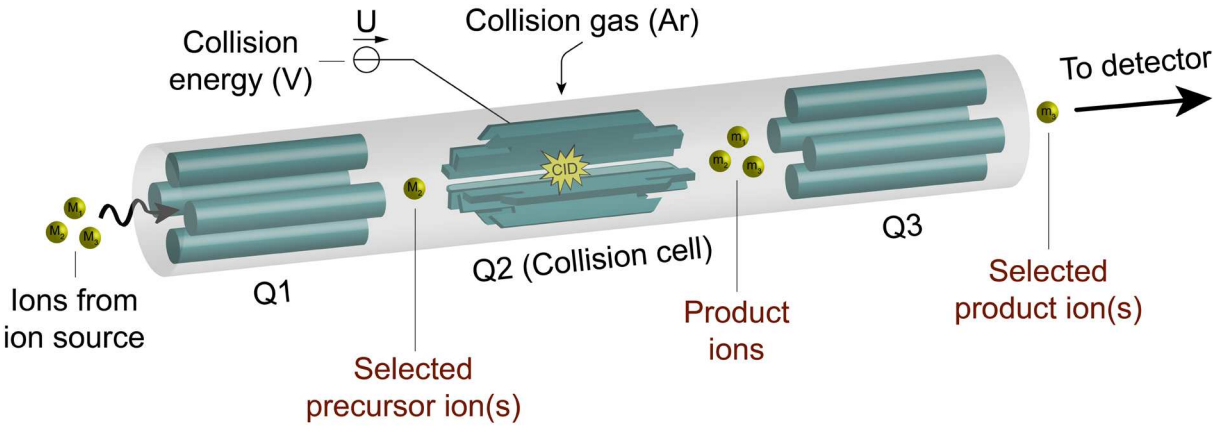

Figure 3: Scheme of a triple quadrupole (Q1, Q2 and Q3) mass analyser.

In addition to quadrupoles also ToF mass analysers can be used in combination with GC or LC. ToF mass analyser feature high sensitivity and fast acquisition rates (spectrum per sec) which allow the analysis of much more ions in the same time compared to a quadrupole mass analyser [39-41]. The very fast acquisition speed is especially important for GC or LC systems giving sharp eluting peaks, in order to provide an adequate number of data points per peak [42]. In contrast to a quadrupole mass analyser, which acts as a filter allowing only selected ions to pass through, no information is lost during analysis when using a ToF mass analyser since the full mass range is acquired at the same time. Moreover, the accessible mass range is increased a lot, i.e. > 1,000,000 Da in theory in the case of a linear ToF and ~ 15,000 Da in the case of a reflectron ToF. This is particularly important when analysing large molecules. Using ToF also allows the generation of accurate mass data, improving the ability of the identification of unknown substances. Furthermore, using MS^E allows the combined acquisition of precursor and product ions [43]. MS^E uses a collision cell before m/z separation in the ToF mass analyser. On the one hand low energy is applied to the collision cell resulting in no significant fragmentation and thus precursor ion information. On the other hand, when high energy is applied ions undergo fragmentation to provide fragment ion information. These two modes are alternating and thus providing MS and MS/MS spectra simultaneously. Since a collision energy ramp can be applied during high energy CID, MS^E eliminates the need to optimise collision energies for individual analytes. A further benefit of the combination of low- and

high-energy acquisition is the possibility of unambiguously identify analytes. However, the hyphenation of GC or LC with ToF is challenging since ToF usually requires pulsed ionisation techniques. Thus, ions cannot directly be transferred from the GC or LC ion source, but have to be introduced as pulsed packets. LC coupled to ToF-MS in combination with the acquisition mode MS^E was applied for protein identification in **Manuscript IV** (p. 152).

Mass resolution and accuracy can be increased a lot when using orbitrap or Fourier transform ion cyclotron resonance (FT-ICR) mass analyser, but it has to be considered that for these instrumentations the resolution is inversely proportional to the analysed mass [44, 45]. The increased mass accuracy is important for the analysis of proteins or peptides after separation by LC in order to correctly determine the amino acid sequences or at least limit the candidates to just a few sequences facilitating protein and peptide identification [46]. Beyond that, high mass accuracy allows the determination of the elemental composition of small molecules without performing MS/MS experiments, which is particularly important for untargeted metabolomic approaches. Another advantage of high resolution mass spectrometers is the ability of distinguishing between isobars (compounds with the same nominal mass) where a minimum resolving power of 20,000 to 30,000 can be necessary [5, 46]. Though the acquisition rates in general are too low to cover an extended mass range and therefore have to be adopted to be in sync with the time scale of GC or LC separations, but if the acquisition rates are decreased also the achievable resolution is decreased a lot [47].

Nevertheless, every mentioned mass analyser has its pros and cons and thus combinations, so called hybrid mass spectrometers are commonly used: e.g. ToF-ToF, quadrupole-ToF, ion trap-ToF, quadrupole-orbitrap, linear ion trap quadrupole-orbitrap or quadrupole-FT-ICR [41, 48].

Part I: Method Development

1 Method development for the analysis of metabolites

1.1 Overview

Metabolites are the end and by-products of enzyme-catalysed reactions that occur naturally in living cells [2]. Certain properties and characteristics must be met by a molecule to be called a metabolite [49]. First, metabolites realise a useful biological function in the cell. If a metabolite performs not directly a biological function, it will, after a structural modification, serve as a precursor for a further conversion into a biologically active compound. A major characteristic of metabolites is the finite half-life, which means that they are constantly taken up, produced, degraded or excreted by the cell and thus do not accumulate in cells. Furthermore, metabolites are recognised and acted upon by enzymes, which will change its properties by means of a chemical reaction.

The completeness of all reactions where metabolites play a role is called metabolism which is maintained and regulated to ensure constant supply of resources for the living cell and thus for the survival of the cell [23]. It can be divided into central or primary metabolism and secondary metabolism. The former is related to energy levels and the production of core structures in the cell, e.g. proteins and structural components. Primary metabolites, on which the focus is in this thesis, include ubiquitous compounds like amino acids, sugars, organic acids or nucleotides. Secondary metabolism is associated with the production of more specialised metabolites and these secondary metabolites do not participate directly in growth and development and therefore are non-essential for life.

The metabolites are characterised by its individual chemical structure which determines the physical and chemical properties of the compound and therefore each metabolite is unique and their features are specific. Some of the features determining the chemical properties of a metabolite are the molecular weight and size, the polarity, isomers and additional modifications, e.g. hydroxylation, phosphorylation, reduction, acetylation or amidation. Volatility, solubility, pK_a value and stability are further characteristics of the compounds and point out the huge variety of these molecules [1, 41]. In addition to that the abundance of each metabolite is very different and depends on the various reactions taking place inside the biological system resulting in huge concentration ranges [5].

As described above, metabolites are constantly transformed and changed in chemical reactions. The fast turnover and modification of metabolites requires specific and especially quick extraction methodologies in order to minimise degradation of the

compounds after sampling due to remaining enzymatic activity [5, 50]. The enormous chemical diversity and different concentration levels stress the need for a range of different separation and detection techniques.

The two main approaches for the analysis of metabolites are targeted and untargeted analysis, both with its inherent pros and cons [23]. In this thesis GC-EI-MS/MS and LC-ESI-MS/MS methodologies were investigated for performing targeted analysis.

Due to the superior separation efficiency and high sensitivity GC-MS is a versatile instrumentation for the analysis of metabolites allowing the separation of various analyte classes within a single measurement [8, 51]. However, GC-MS, in contrast to LC-MS, demands derivatisation prior to measurement in order to convert the usually polar metabolites into volatile, non-polar and stable derivatives [52].

1.2 Chemical derivatisation of metabolites

Besides the dominant reasons for derivatisation in GC, the increased volatility induced by decreased polarity of analytes, there are several other advantages and enhancements which can be realised when a derivatisation step is included in the sample preparation [53, 54]: i) increase of sample stability; ii) improvement of sensitivity (by several orders of magnitude), selectivity, chromatographic behaviour (retention time, peak symmetry) and separations; iii) facilitation of structural elucidation; However, the formation of by-products or the incomplete derivatisation of compounds with several functional groups has to be considered and potentially hinders analysis and often not all present analytes or functional groups can be derivatised by one reagent. Multiple derivatisation steps or even several independent analyses are the consequence.

The most versatile and universally applicable derivatisation agent for GC are alkylsilyl reagents (**Figure 4**), e.g. bis(trimethylsilyl)-acetamide (BSA), bis(trimethylsilyl)-trifluoro-acetamide (BSTFA) or N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) [31, 55-57]. MSTFA is the most volatile of the trimethylsilyl acetamides and therefore very suitable for GC-MS analysis [5]. Many functional groups that are problematic in GC analysis, such as hydroxyl, amine, amide, phosphate and thiol groups, can be converted to alkylsilyl derivatives.

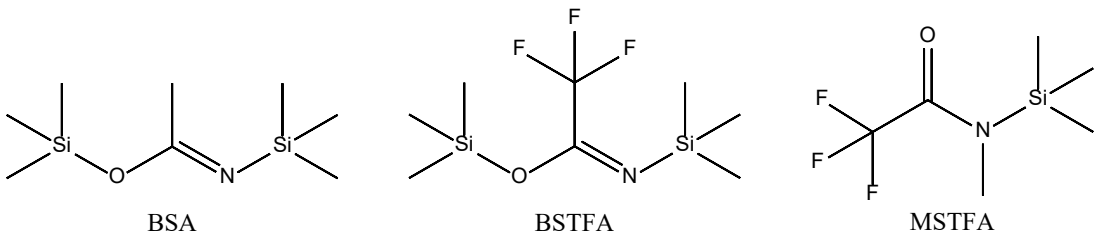

Figure 4: Most common alkylsilyl derivatisation reagents used for GC-MS analysis: bis(trimethylsilyl)-acetamide (BSA), bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA)

The TMS reaction introduces a trimethylsilyl group into the molecule by usually replacing an active hydrogen and it is believed that the reaction proceeds by an S_N2 mechanism (**Figure 5**) [55, 56]. S_N2 is a nucleophilic substitution reaction with a bi-molecular mechanism (two reacting species are involved) where the free electron pair of the nucleophile (here O) attacks at the positively charged atom (here Si). The resulting by-product N-methyltrifluoroacetamid (MTFA) represents the leaving group of the reaction and is the most volatile of all silylating reagents and elutes very early or even with the void volume [57]. TMS is typically performed at elevated temperatures (~30-40 °C) to improve the derivatisation yield. In addition to that basic solvents, like pyridine, are added to increase the TMS reaction rate by scavenging active protons in the reaction mixture [58].

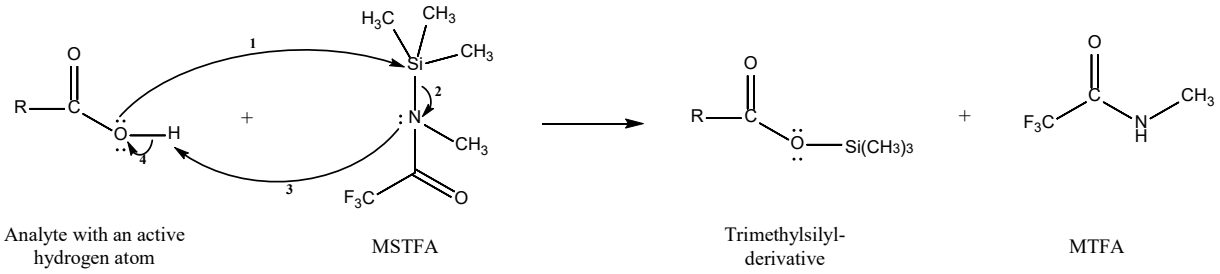

Figure 5: Reaction mechanism (S_N2) of an analyte with a replaceable hydrogen atom with MSTFA yielding the trimethylsilyl derivative and MTFA as by-product.

What has to be considered when performing TMS reactions is that analytes might get converted, e.g. Arg to Orn by the loss of urea as a trimethylsilyl derivative, and that the derivatives are susceptible towards hydrolysis and therefore sensitive to moisture, e.g. 1 µL of water will use about 20 µL of MSTFA [5, 31, 59]. Thus, samples have to be water free and thoroughly dried before derivatisation and contact with moisture has to be avoided. Another side effect of TMS is that reducing sugars can give multiple derivatives

resulting from the different tautomeric forms present in solution (**Figure 6**). The resulting derivatives include two pyranoses, two furanoses and one open ring form, and all of these derivatives can be separated by GC. This makes analysis and data evaluation tedious and error prone, especially when several reducing saccharides are present in the sample each giving raise to multiple peaks in the chromatograms.

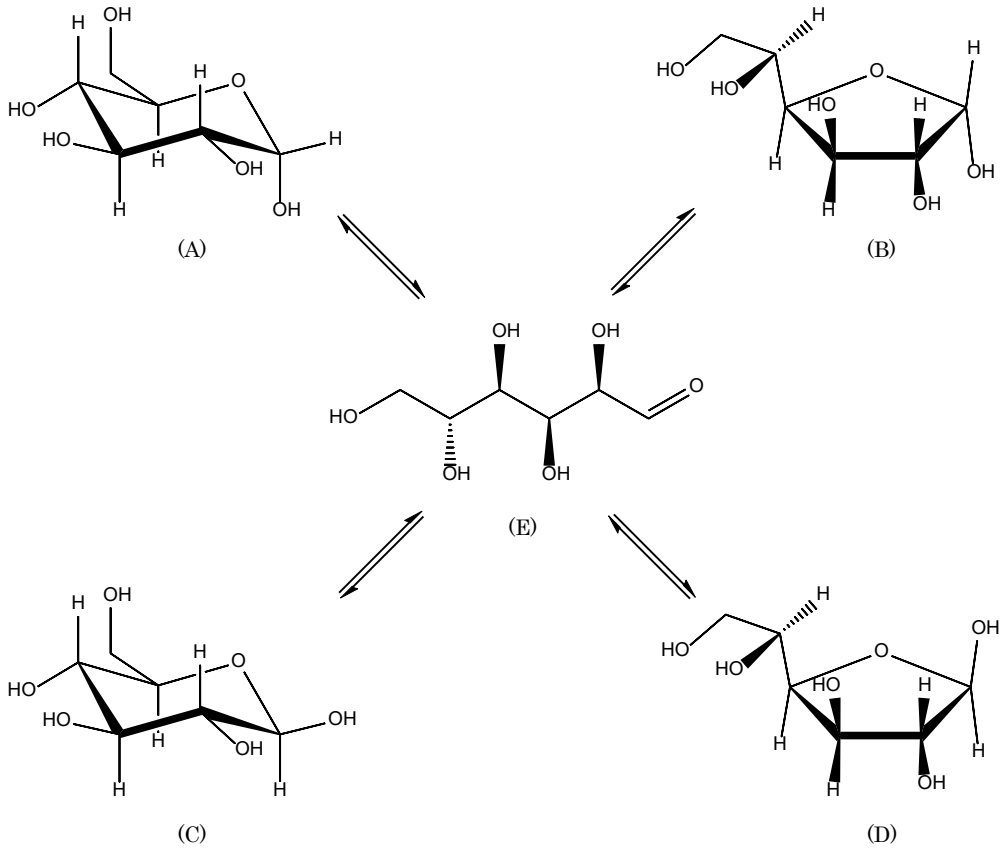

Figure 6: Tautomeric forms of glucose present in a dynamic equilibrium in solution. Illustrated are the alpha and beta pyranose (A, C) and furanose (B, D) forms of glucose as well as the open chain conformation (E).

In order to circumvent this problem, usually an oximation or a methoximation step prior to TMS is introduced, latter by using methoxyamine hydrochloride [3, 5]. This reaction locks sugars in the open-ring conformation, inhibiting cyclisation and only two isomers (syn and anti) with a constant proportion for each saccharide can be observed in the chromatograms (**Figure 7**) [8, 60, 61]. Compared to the sole TMS the combination of methoximation and TMS (MeOx/TMS) improves the analysis by decreasing the complexity of chromatograms. Today this method is predominantly used for GC-MS analysis, however it also has some limitations. As already mentioned the reaction and derivatives are moisture sensitive, multiple derivatives can be formed and furthermore unexpected signals can appear in the chromatogram that are usually difficult to interpret [62]. It has to be also considered that an increasing number of potential groups for derivatisation

increases the obtained molecular mass of the derivatives significantly, maybe even to an m/z which is no longer covered by the instrument in use (typically covered mass range for GC-MS analysis is m/z 50– 1100) or the compound can irreversible stick to the GC column. Details on MeOx/TMS derivatisation are discussed in **Manuscript I** (p. 33) and **Manuscript II** (p. 47).

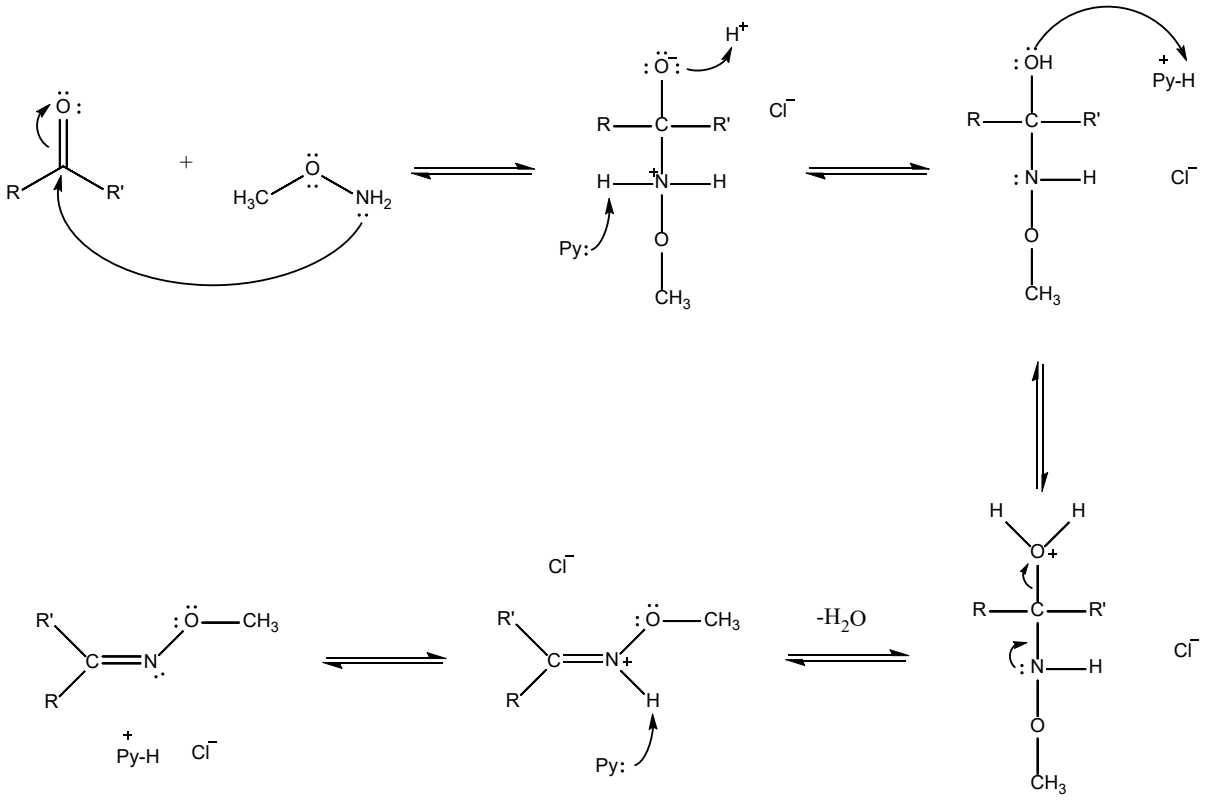

Figure 7: Methoximation of a carbonyl function with methoxyamine hydrochloride (CH_3ONH_2) in the presence of pyridine (Py) [63]. Depending on the orientation at the C=N bond the oxime derivatives can be formed as syn and anti-isomers regarding the methoxy group and R'.

As a good alternative, alkylation of acids or amines with alkyl chloroformates has to be mentioned and is discussed in detail in **section 2.2**, **Protein hydrolysis followed by amino acid analysis** (p. 27) [64].

2 Method development for protein analysis

2.1 Investigation of proteins

Protein solubilisation is the first step of protein analysis of biological samples and is achieved by the disruption of the macromolecular interactions, i.e. protein interactions, while keeping the proteins intact. The goal is the denaturation of the proteins in order to break non-covalent interactions, like electrostatic, Van der Waals, hydrophobic, charge-dipole, dipole-dipole or hydrogen bonds, and the reduction of disulfide bridges [65, 66].

The building blocks of proteins are amino acids which can be grouped by their side-chain chemical properties like hydrophobic/hydrophilic, charged/uncharged or polar/nonpolar. Hence, the specific protein properties and interactions with its surrounding environment are controlled by their amino acid composition and subsequent order in the sequence [67]. In addition to that, the amino acid composition plays an important role for protein solubility. Charged side chains for instance interact most likely with the solvent and are, therefore, expected to improve protein solubility in aqueous solutions [68]. Furthermore, amino acid hydration has an important effect on the solubility of proteins; e.g. Gln has less hydration compared to Glu and thus deamidation of proteins with a high content of Gln increases solubility [69]. Hydration is not only affected by side-chain properties but also by the primary protein sequence, since spatial proximity of amino acids influences each aqueous interactions [70]. Depending on the amino acid sequence local charge densities can be present promoting hydration in contrast to local increased hydrophobicity which reduces hydration. Moreover, the primary amino acid sequence of a protein influences the secondary, tertiary and quaternary structures that control surface exposure of amino acids to their surrounding environment and thus further influence protein solubility.

In addition to these intrinsic factors there are several extrinsic factors, which contribute to protein solubility, like pH, ionic strength and temperature. The net charge of a protein is zero at its isoelectric point (pI), where protein solubility reaches a minimum. Since the solubility of a protein increases with its net charge, acidic solvents are required for the solubilisation of proteins with high pIs, and alkaline solvents are used for those having low pIs [71]. The presence or absence of salts in the solubilisation solution can have a further impact since salts influence surface charges. High salt concentrations can create a competition for charged amino acid side chains between free water molecules and salt ions thereby reducing the solubility of the target protein [72]. The specific influence of various salts on the solubilisation of proteins is described by the Hofmeister series [73]. Next to that, the temperature of the surrounding medium can have an effect on protein solubility like the disruption of non-covalent intramolecular forces which stabilise the

higher-order protein structures. By thermal unfolding of the proteins enclosed residues may be exposed to the surrounding solvent thus adversely affecting solubility.

Protein solubilisation can be realised by three main types of reagents: chaotropes, surfactants and reducing agents [74]. Chaotropes are added at high concentrations to disrupt hydrogen bonds and inter- and intramolecular hydrophobic interactions and subsequently to maintain the proteins in solution and avoid aggregation and precipitation [75, 76]. Urea at concentrations of about 9.0 to 9.5 M is very often the chaotrope of choice, though, improvements in protein solubility can be achieved when combining 5-8 M urea with 1-2 M thiourea [75, 77-80].

Often detergents, or better said amphipathic molecules, which contain both a polar head and nonpolar tail, are added for solubilisation. These surfactants are important in preventing hydrophobic interactions, especially when using chaotropic reagents that expose hydrophobic protein domains [66]. The anionic detergent SDS is most widely used since it efficiently binds to proteins (approx. one SDS molecule per every two amino acids, which equals approx. 1.4 g SDS/g protein) and thus improves solubilisation significantly [76]. However, SDS interferes with several separation techniques e.g. isoelectric focusing, RP or anion exchange chromatography [75]. Further examples are non-ionic detergents like Tergitol NP-40 and Triton X-100 or zwitterionic detergents like 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and sulfobetaines. The latter ones have shown superior efficiency over non-ionic detergents and thus are the primary used surfactants [81, 82].

The breaking of disulfide bonds (disulfide reduction) can be achieved by an equilibrium displacement process using a large excess of free thiols like mercaptoethanol, thioglycerol or cysteamine [66]. However, these additives have to be used at high concentrations (≥0.2 M) to ensure maximum displacement yielding the free thiol form of the protein. DTT or dithioerythritol (DTE) are more often used as they shift the chemical equilibrium towards the free thiol group by forming an intramolecular, cyclic condensation product during oxidation. This happens already at much lower concentrations compared to e.g. mercaptoethanol. Another improvement of disulfide reduction and thus protein solubilisation is the use of phosphine derivatives, like tributylphosphine or tris-carboxyethylphosphine [75]. This is due to the fact that the reduction proceeds stoichiometric and that these reagents are not as sensitive as thiols to dissolved oxygen. Furthermore, phosphines allow reduction and alkylation to be performed during a single step since they do not contain a thiol group. Procedures such as agitation and ultra-sonication further can improve solubilisation.

After the proteins of interest are solubilised they can be investigated by a classical gel based proteomics approach by means of SDS-PAGE (see **section 1.3**, **Sodium dodecyl sulphate polyacrylamide gel electrophoresis**, p. 16) followed by proteolytic *in-gel* digestion and mass spectrometric analysis usually in combination with LC in order to allow separation of the generated peptides and by this reduce complexity of the peptide sample. For *in-gel* digestion protein bands of interest are excised from the SDS-PAGE gel and have to be destained in order to obtain peptide mass profiles [83]. For silver stained protein bands, as used in this thesis, a mixture of ferricyanide and thiocyanate is used for this. In order to retrieve sufficient enzymatic cleavage, proteins are denatured prior to digestion, since secondary and tertiary structures hamper enzymatic activity by steric hindrance of the cleavage sites [84]. This is usually accomplished by using DTT and a further alkylation step using iodoacetamide to prevent reformation. Several proteases such as AspN, chymotrypsin, GluC, LysC or trypsin can be used for enzymatic digestion and yield peptides from different specificities: AspN cuts the amino acid sequence N-terminal to Asp; chymotrypsin cuts at aromatic amino acids or Leu; GluC and LysC cleave peptide bonds C-terminal to Glu and Lys, respectively; trypsin cuts the amino acid sequence after Lys or Arg. To obtain a good digestion yield the destained gel slides are dehydrated using ACN allowing the penetration of the enzyme into the gel and maximising its concentration [75]. After addition of the enzyme the digestion itself is carried out in a few hours to overnight incubation at 37 °C.

In order to get an insight into the amino acid composition of protein samples amino acid analysis after protein hydrolysis can be performed and is discussed in the following section.

2.2 Protein hydrolysis followed by amino acid analysis

Amino acid analysis is a classical protein analysis method and is not only used to study the composition of proteins, it is also the most accurate method for protein quantification. For amino acid analysis all amino acids have to be released of the present substrate and afterwards recovered of the resulting hydrolysate. Ideally the whole process is carried out in a quantitative manner. The sample should be easily solubilised to allow an optimal interaction with the hydrolysis reagent. If that is not possible information can be lost or the obtained results of amino acid analysis can be incorrect. However, hydrolysis usually is carried out under very acidic or alkaline conditions supporting sample solubilisation. The method used the most for bioanalysis is hydrolysis under acidic conditions since it

allows the intact release of almost all amino acids, but it also leads to the conversion of amides into hydroxyl groups and various oxidations. Besides that, also alkaline hydrolysis or enzymatic digestion methods can be found in literature. In the review of Fountoulakis et al. various hydrolysis methods with their advantages and disadvantages are summarised, thus only a brief overview of the most important aspects is given here in the following paragraphs [85].

Today there is no hydrolysis method available which can keep all amino acids intact. Depending on the amino acids and thus on the present functional groups every method has its pros and cons. The completeness and duration of the process is affected by hydrolysis time, temperature, agent and additives. These parameters have to be carefully combined to obtain acceptable amino acid recoveries (close to 100%). According to the aim of the respective investigation the optimal method has to be chosen and usually compromises have to be accepted to get information on as many amino acids as possible within a single analysis.

Acidic hydrolysis usually is carried out using hydrochloric acid (HCl) or also methanesulfonic acid (MSA) at elevated temperatures for several minutes to hours (100 to 180 °C; up to 24 h). However, the use of MSA is less popular since it is not volatile, cannot be evaporated after hydrolysis and therefore introduces chemical background at rather high levels. Consequently, the hydrolysate can only be diluted to be used for further analysis and thus larger sample amounts are necessary to lower achievable LODs or LOQs [86]. The fact that MSA can be used for the determination of Trp and methionine sulfoxide (Mox), in contrast to HCl, also does not outbalance the mentioned disadvantages. Furthermore, both methods yield similar amino acid recoveries and thus hydrolysis with HCl is used routinely.

Since HCl can be evaporated it is possible to perform the hydrolysis with very little sample amounts (pmol to nmol range). Although hydrolysis by heating with HCl yields very good recoveries for most amino acids, there are some limitations which have to be considered and will be described in the following [85, 87-93].

As already mentioned above Trp cannot be determined after hydrolysis with HCl since it is almost completely destroyed (oxidation of the indole group side chain) [94]. However, various scavengers, like thioglycolic acid, mercaptoethanol, phenol, tryptamine or tryptamine[3-(2-aminoethyl)indole] can be added in order to protect Trp and increase the recovery. Nevertheless, reported recoveries vary between 40 and 100%, depending not only on the hydrolysis reagent but also on the presence of carbohydrates (glycosylated proteins) [90].

Cys is another amino acid affected by hydrolysis. It gets partially converted to cysteic acid and is therefore intentionally transformed to cysteic acid by performic acid oxidation or to S-carboxymethylcysteine by alkylation using iodoacetic acid or 4-vinylpyridine prior to hydrolysis. Both derivatives are stable towards acidic hydrolysis but these approaches are labour-intensive and time-demanding. In addition to that, various other methods can be found in literature: e.g. hydrolysis of the protein with sodium azide as additive resulting in the oxidation of Cys to cysteic acid, derivatisation of Cys by 3-bromopropylamine to S-3-aminopropylcysteine, determination as a mixed disulfide (S-((2-carboxyethyl)thio)-cysteine) in the presence of 3,3'-dithiodipropionic acid or thioglycolic acid and phenol in the hydrolysis solution (HCl).

Furthermore, the amides Gln and Asn are deaminated to the corresponding acids. Therefore, these two amino acids are measured as Glu and Asp and the obtained values for Glu and Asp represent the sum of the acid and amide derivative (Glx and Asx).

The recoveries for Met can be decreased if not all oxygen is removed from the system since it can be oxidised to Mox and methionine sulfone. Thus, it is important to flush the used hydrolysis agent and the vessel with inert gases, e.g. N_2. Higher recoveries for Met can be also obtained when thioglycolic acid or 2-mercaptoethanol is added to the hydrolysis solution preventing oxidation of Met [90, 95]. Another possibility is the quantitative oxidation to methionine sulfone by performic acid prior to acidic hydrolysis.

Next to that, hydroxyproline (Hyp), Ser and Thr can be partially destroyed during acid hydrolysis and losses of 10% to 15% for Ser and 5% to 10% for Thr are reported in literature. The use of reduced hydrolysis times again can increase the recoveries.

As already mentioned, phenol is often added to the hydrolysis as a scavenger for halogen radicals increasing by this the recovery of Tyr, which can undergo halogenation. It is also reported that Leu, Lys, Phe and Pro show higher yields when phenol was added.

For the very hydrophobic amino acids Ile, Leu and Val it is mentioned in literature that additional hydrolysis time, e.g. up to 72 h instead of 24 h, may be necessary since their peptide bonds are very strong and difficult to break.

Hydrolysis with HCl provides usually very suitable results and although there are some limitations the recoveries can be improved a lot by the use of additives (details can be seen in **Manuscript III**, p. 118 in **Part II: A First Investigation of Tick Attachment Cement**).

Besides acidic hydrolysis alkaline hydrolysis with sodium hydroxide (NaOH), lithium hydroxide (LiOH) or barium hydroxide (BaOH) is used [88]. However, alkaline solutions are chemically aggressive towards glass ware and alkali and heavy metal ions can be leached from vessels. These ions can destroy amino acids as in the case of metals or

increase unwanted alkali ions in the sample solution. The use of Teflon or polypropylene containers is recommended. Generally alkaline hydrolysis is exclusively used for the determination of Trp, since it is stable under basic conditions and gets destroyed during acidic hydrolysis, but a recovery of 100% is not reported in literature when using any of the alkaline hydrolysis procedures [93]. The amino acids Arg, Cys, Ser and Thr are destroyed and the other amino acids get racemised. It has to be considered that the success of using the mentioned hydroxides depends not on the used alkali hydroxide but more on degassing of the samples, the laboratory where the analysis is carried out and the samples themselves. Another problem is the fact that the used hydroxide solutions cannot be evaporated after hydrolysis but have to be diluted for further analysis. Consequently, the concentration of Trp, which is already low because of its low occurrence in proteins, is decreased even more. Hence, Trp analysis is often omitted or carried out after acidic hydrolysis with varying low recoveries. Thus, quantitative statements usually are difficult to be made.

Another option is enzymatic hydrolysis of proteins allowing the quantification of Asn, Gln and Trp and other sensitive residues, which are otherwise destroyed, or amino acids carrying side chain modifications [85, 89]. The major drawback of this approach is the relative specificity of the proteases for certain amino acids which can result in skewing quantitative results. Furthermore, a complete digestion requires the consecutive use of various enzymes prolonging the digestion process and using several enzymes can lead to a total reaction time of up to 40 hours. Moreover, the instability of some enzymes is problematic. Consequently, this method is hampered to be used for serial analyses.

Subsequently to protein hydrolysis the released amino acids can be analysed using GC-MS. As already mentioned in **section 1**, **Method development for the analysis of metabolites** (p. 25) GC-MS demands derivatisation prior analysis. The mentioned two step derivatisation, MeOx/TMS, can be also applied here. However, after protein hydrolysis only amino acids are the analytes of interest and thus an optimal derivatisation strategy, i.e. derivatisation using PCF, without compromises can be used.
Using PCF under the presence of pyridine as catalyst allows the derivatisation of amino acids to the corresponding propyl ester and carbamate derivatives in propanol (PrOH) giving only one derivative per amino acid [96]. The fact that only one derivative is formed is a big advantage compared to the mentioned MeOx/TMS reaction. The type of ester formed during the reaction is directly dependent on the used alcohol since the alkoxy group in the derivative corresponds to the alcohol in the reaction medium, but not to the alkyl

group of the chloroformate [97]. Usually both, the chloroformate and the alcohol, feature the same alkyl group (like the use of PCF and PrOH), but also treatment with reagents and alcohols of different alkyls can be used for special applications [98]. The reaction proceeds via a mixed carboxylic carbonic anhydride (intermediate) which undergoes an exchange reaction with the present alcohol (**Figure 8**) [64, 97, 99]. The alkyl chloroformate derivatisation has several advantages [1, 62, 96]: i) it is fast; ii) no heating is required; iii) the amino acids can be derivatised directly in aqueous solution, followed by extraction with an organic solvent; iv) it is a robust reaction; v) one single stable derivative is obtained in the majority of cases;

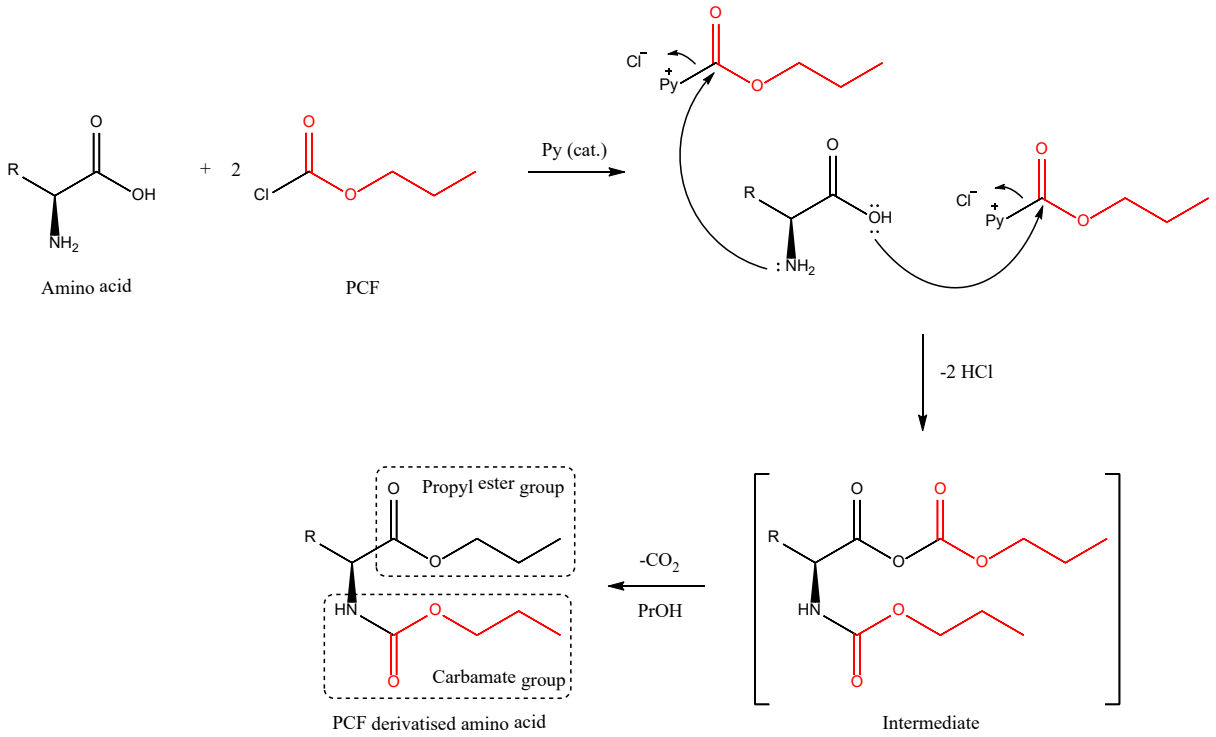

Figure 8: Reaction scheme of PCF derivatisation of an amino acid in the presence of pyridine (Py) as catalyst. The reaction proceeds via a mixed carboxylic carbonic anhydride intermediate followed by an exchange with PrOH. Reproduced from ref. [64]

Details on PCF derivatisation of amino acids are discussed in **Manuscript III** (p. 118) in **Part II: A First Investigation of Tick Attachment Cement**.

3 Manuscripts

In this section the manuscripts related to method development for the analysis of metabolites are presented.

Manuscript I – Method development for the analysis of selected metabolites by GC-EI-MS/MS and LC-ESI-MS/MS

Benedikt Engel, Peter Suralik, Gregor Mikl, Martina Marchetti-Deschmann

Institute of Chemical Technologies and Analytics, TU Wien, Vienna, Austria

Highlights of this manuscript

- Method development for the analysis of metabolites by GC-MS/MS and LC-MS/MS
- Optimisation of a two-step derivatisation strategy for GC-MS/MS: Methoximation in combination with trimethylsilylation
- Comparison of different pH for separation of metabolites by LC-MS/MS
- Comparison of results of GC-MS/MS and LC-MS/MS methods

Method development for the analysis of selected metabolites by GC-EI-MS/MS and LC-ESI-MS/MS

1. Introduction

Biological systems are composed of many different compounds and molecules building a highly complex network of reactions and functions. In order to investigate and understand such complex systems several fields of study have emerged, which can be summarised as omics technologies (**Figure 1**). Metabolomics, which represents the comprehensive analysis of all metabolites in a biological system, is one very important field within these studies [1]. It complements the information received through genomics, transcriptomics and proteomics by providing quantitative statements of low molecular weight analytes (<1800 Da). These analytes express the metabolic status of the system and in addition to that allow to investigate phenotype and phenotype changes when studying organisms [2].

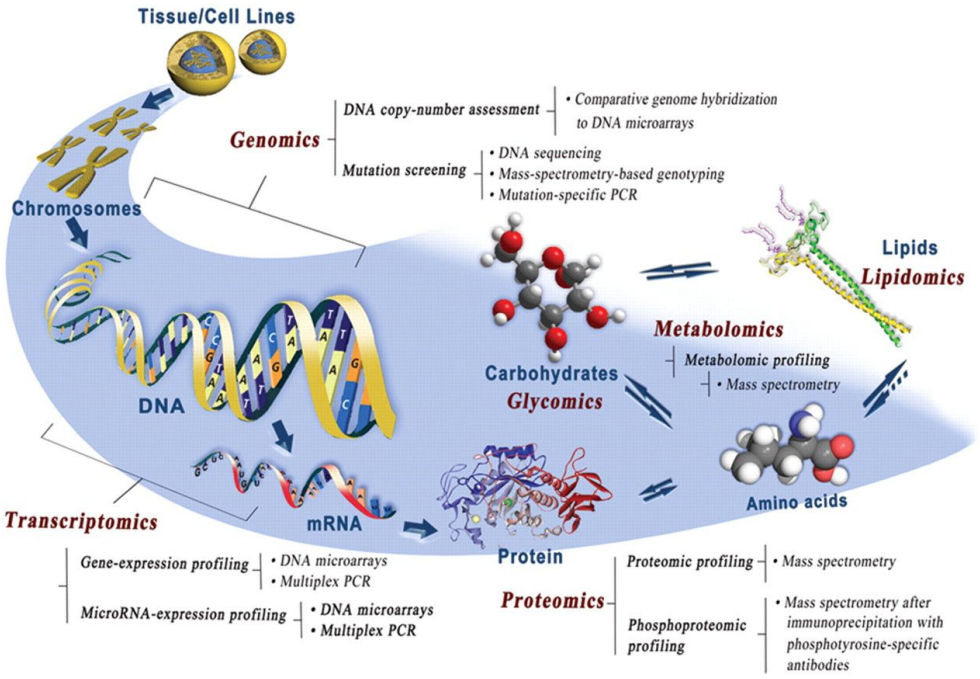

Figure 1: Schematic representation of the omics technologies, their corresponding analysis targets and assessment methods [3].

The fact that metabolic analytes are present in a huge variety and in different analyte classes results in the application of many different techniques, methods and analytical strategies. The two main approaches are targeted and untargeted analysis, both with its inherent pros and cons [4]. Here, two high performance chromatographic methods, gas

chromatography (GC) and liquid chromatography (LC), both in combination with triple quadrupole mass spectrometry applying low energy collision induced dissociation (GC-EI-MS/MS and LC-ESI-MS/MS), were investigated and compared for their respective performance parameters in targeted analyses.

The complete work was done in the course of two bachelor theses carried out by Peter Suralik and Gregor Mikl [5, 6].

2. Materials and methods

2.1. Chemicals and reagents

Double distilled water (ddH_2O, 18.2 MΩ.cm) was prepared using a Simplicity system Millipore, Billerica, MA, USA. Acetic acid (≥ 99.8%), acetonitrile (ACN, ≥ 99.9%), ammonium acetate (≥ 98.0%), ascorbic acid (≥ 99.7%), citric acid (≥ 99.5%), D-arabinose (≥ 99.8%), D-(-)-fructose (≥ 99.0%), D-(+)-glucose (≥ 99.5%), D-glucose-6-phosphate sodium salt (Sigma grade), D-lactose monohydrate (≥ 99.5%), D-mannitol (≥ 99.5%), D-(+)-mannose (≥ 99.0%), D-sorbitol (≥ 99.5%), D-2-phenylglycine (99.0%), D-(-)-ribose (≥ 99.0%), D-(+)-xylose (≥ 99.0%), glycine (≥ 99.0.%), α-ketoglutaric acid (≥ 99.0%), L-arginine (≥ 98.0%), L-cysteine (97.0%), L-glutamic acid (≥ 99.5%), L-lysine (≥ 98.0%), L-norvaline (≥ 99.0%), myo-inositol (≥ 99.0%), N-acetyl-L-glutamine, β-nicotinamide adenine dinucleotide-phosphate hydrate (NADP, ≥ 95.0%), pyridine (≥ 99.8%), sucrose (≥ 99.5%) and sodium pyruvate (≥ 99.0%) were obtained from Sigma-Aldrich, St. Louis, MO, USA. n-Hexane (≥ 99.0%) was obtained from VWR International, Rednor, PA, USA. D-(-)-α-(4-hydroxyphenyl) glycine (≥ 99.0%) was obtained from Tokyo Chemical Industry, Tokyo, Japan. N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, p. a.) was obtained from Macherey-Nagel, Düren, Germany. O-methoxyamine hydrochloride (≥ 98.0%) was obtained from Supelco, Bellefonte, PA, USA. Ammonia solution 25% (p. a.) was obtained from Merck, Darmstadt, Germany. Argon (≥ 99.999 Vol-%), helium (≥ 99.999 Vol-%) and nitrogen (≥ 99.999 Vol-%) were purchased from Messer Austria, Gumpoldskirchen, Austria.

2.2. GC-EI-MS/MS:

Sample derivatisation was performed in two steps: methoximation (MeOx) using 50 µL methoxyamine hydrochloride in pyridine (20 mg/mL) followed by trimethylsilylation (TMS) using 50 µL MSTFA. Prior to MeOx 5 µL of the internal standard norvaline

(5 mg/mL) were added and the samples were dried in a vacuum centrifuge (Univapo 100 H) from UniEquip, Planegg, Germany. Both reactions were performed under a nitrogen atmosphere. The reaction temperature and duration of MeOx and TMS were optimised to yield maximum signal intensities for all compounds. The following reaction conditions were the result of the optimisation: MeOx at 37 °C for 90 min followed by TMS at 37 °C for 30 min using a constant temperature oven.

The derivatised samples could be directly analysed using a Shimadzu GC2010 (Kyoto, Japan) gas chromatograph, coupled to a TQ8040 triple quadrupole mass spectrometer. The gas chromatographic system consisted of a Zebron ZB50 fused silica column (30 m × 0.25 mm × 0.25 µm, Phenomenex, Torrance, CA, USA) with a diphenyl dimethyl polysiloxane (50:50) phase. Linear velocity (36.7 cm/sec) was used as flow control mode, which resulted in a helium carrier gas flow rate on the column of 1.0 mL/min. The initial column oven temperature was 70.0 °C and was held for 5 min after injection followed by a 5 °C/min ramp to reach 245 °C. After an additional minute the temperature was quickly increased to 310 °C (heat rate of 250 °C/min) and held for 5 min. The total GC run time was 46.26 min. 1 µL of the samples was injected with a split ratio of 1:20 and the temperature of the injector was kept at 230 °C. The interface temperature was 250 °C. The mass spectrometer was equipped with an EI ion source (70 eV, rhenium filament, 200 °C) operated in positive ionisation mode. Masses were recorded after a solvent cut time of 5 min, using the Q3 scan or the multiple reaction monitoring (MRM) mode. The Q3 scan mainly was used for method development while the MRM mode (loop time 0.3 sec, minimum dwell time 11 msec), with a maximum of 6 ions measured simultaneously, was used for all further measurements. MRM optimisation was carried out using the Smart MRM tool provided within the GCMSsolution software. Argon was used as collision gas. All the data were acquired using the GCMSsolution (v. 4.20) from Shimadzu and structures were identified based on similarity search against the NIST11 and Wiley9 mass spectral library, respectively.

2.3. LC-ESI-MS/MS:

LC-ESI-MS/MS analysis was performed using a Shimadzu LCMS 8030+ Liquid Chromatograph Mass Spectrometer from Shimadzu, Kyōto, Japan. The stationary phase used was a Luna® NH_2 column (fully porous silica modified with amino groups, 150 × 2.0 mm, particle size 3.0 µm, pore size 100 Å, Phenomenex, Torrance, USA). The following gradients of the mobile phase were applied: gradient 1: starting at 80% B; decreased to 0% B in 25 min; holding 0% B for 20 min; increasing to 80% B in 1 min; equilibration at 80%

B for 14 min resulting in 60 min total run time; gradient 2: starting at 80% B; decreased to 0% B in 40 min; holding 0% B for 5 min; increasing to 80% B in 1 min; equilibration at 80% B for 14 min resulting in 60 min total run time; The mobile phase consisted of ddH_2O (10 mM ammonium acetate pH 3.0 or 9.9, A) and ACN (B) and the total flow was 0.2 mL/min. The column oven temperature was held at 25 °C. The desolvation line temperature was set to 250 °C and the heat block to 400 °C. Argon was used as collision gas. Nitrogen was used as nebulising (3 L/min) and drying gas (15 L/min) and generated from a Mistral LCMS Evolution N_2-Generator from DBS, Vigonza, Italy.

Masses were recorded using the Q3 scan or the MRM mode. The Q3 scan mainly was used for method development while the MRM mode was used for all further measurements. MRM optimisation was carried out using the optimisation tool provided within the Lab Solutions software. All data was acquired using LabSolutions (v. 5.89) from Shimadzu.

3. Results and discussion

Two methods, GC-EI-MS/MS and LC-ESI-MS/MS, for qualitative and quantitative analysis of selected metabolites of the primary carbon cycle (**Table 1**) were developed and compared. This selection does not include nucleotides, like adenosine mono-, di or triphosphate since these compounds were not included in our investigations. The MRM acquisition mode was used for both instruments and the parameters for the mass analyser (lens voltages and collision energies, CEs) were optimised beforehand. In the following sections the findings and developed methods of these two works are summarised. It has to be mentioned that in this work only pure standard substances and mixtures of them were used for analysis, thus all findings and statements are based on that. The influence of any sample matrices cannot be estimated and therefore not discussed and evaluated here.

Table 1: List of the investigated metabolites from the primary carbon cycle, chosen from different analyte classes (carbohydrates and the respective phosphates and alcohols, amino acids, organic acids, coenzymes) in alphabetical order.

Arabinose	Glycine	NADP
Arginine	α-(4-Hydroxyphenyl) glycine	2-Phenylglycine
Ascorbic acid	Lactose	Ribose
Citric acid	Lysine	Sodium pyruvate
Cysteine	α-Ketoglutaric acid	Sorbitol
Fructose	Mannose	Sucrose
Glucose	Mannitol	Xylose
Glucose-6-phosphate	Myo-inositol	
Glutamic acid	N-acetyl-glutamine	

3.1. GC-EI-MS/MS [38]:

The applied derivatisation method (methoximation in combination with trimethyl-silylation, MeOx/TMS) resulted in the formation of two isomeric forms in the case of monosaccharides and lactose. The oxygen of the carbonyl group is replaced by a N-O-CH_3-group and depending on the orientation at the C=N bond a syn- and anti-isomer, based on the methoxy group and the alkyl chain, is formed.

The derivatisation reaction was optimised to yield maximum peak areas for the selected metabolites. Glucose, fructose, α-ketoglutaric acid, glucose-6-phosphate and cysteine, representing four different metabolite classes (sugars, organic acids, sugar phosphates and amino acids) were chosen for optimisation of the two-step derivatisation reaction. Three reaction temperatures (room temperature (22.7 °C), 45 °C and 65 °C) and times (30, 90 and 150 min) were selected and varied systematically.

The following reaction conditions were the result of the optimisation: MeOx at 37 °C for 90 min followed by TMS at 37 °C for 30 min (for details see **section 3.3**, **Silylation in combination with preceding methoximation (MeOx/TMS)** in **Manuscript II**, p. 47). These parameters were used for the investigation of the chromatographic performance of the method and the determination of retention times, limits of detection (LODs) and limits of quantification (LOQs) of the metabolite mixture. The results for MRM optimisation are summarised in **Table 2**. In the case of arginine data evaluation was not possible, as it gets converted to ornithine and citrulline [7]. For the derivative of NADP no peak could be obtained, possibly due to the fact that the m/z value of the derivative was above the measurement range of the instrument (m/z 1090). The sensitivity of N-acetyl-glutamine and ascorbic acid was too low and therefore quantitative data evaluation could not be achieved. Nevertheless, retention times could be obtained, showing the suitability of the derivatisation for these substances.

Ribose could not be separated from arabinose and xylose, respectively. Hence, ribose had to be removed from the metabolite mixture and would require better chromatographic resolution. Regarding the two formed isomers for monosaccharides the more intense peak was used for data evaluation. In the case of mannose and glucose only one peak was observed. The reason for this is analyte co-elution: mannose overlaps with sorbitol and glucose with citric acid. Since mannose and sorbitol yield the same fragment ions the signal of sorbitol is falsified when mannose is present. The fragment ions of glucose and citric acid are different and therefore citric acid can be quantified without limitations. The resulting GC-EI-MS/MS chromatogram can be seen in **Figure 2**. The peak widths ranged between 0.06 and 0.27 min.

Table 2: Results of MRM optimisation. Transition 1 was used for quantification and the other two as qualifier transitions. [a,b]Compounds were not used for quantitative data evaluation ([a]lower intensity of the formed isomers; [b]too low sensitivity)

Compound	Retention time (min)	MRM transition 1	CE (V)	MRM transition 2	CE (V)	MRM transition 3	CE (V)
Sodium pyruvate	9.615	174.0>74.1	19	174.0>89.1	9	174.0>59.1	19
Norvaline	12.144	144.0>73.1	15	218.0>147.1	11	218.0>73.1	25
Glycine	13.695	174.0>73.1	15	174.0>86.1	11	248.0>147.1	15
Cysteine	21.448	218.0>73.1	15	220.0>73.1	15	218.0>100.1	9
2-Phenylglycine	21.603	178.0>73.1	15	73.0>58.0	15	178.0>91.1	15
Xylose 1[a]	22.277	217.0>73.1	15	307.0>73.2	27	217.0>129.1	9
Arabinose 1[a]	22.320	217.0>73.1	15	217.0>129.1	9	307.0>73.1	23
Arabinose 2	22.486	217.0>73.1	15	307.0>73.1	25	217.0>129.1	9
Xylose 2	22.602	217.0>73.1	15	307.0>73.1	25	217.0>129.1	9
Glutamic acid	22.832	246.0>128.1	15	246.0>73.1	25	230.0>147.1	15
α-Ketoglutaric acid	24.177	147.0>73.1	15	198.0>73.1	15	198.0>170.1	5
Mannitol	26.019	319.0>73.1	23	217.0>73.1	19	319.0>129.1	11
Fructose 1	26.175	217.0>73.1	19	307.0>73.1	29	307.0>103.1	11
Mannose 1	26.243	319.0>73.1	23	319.0>129.1	11	205.0>73.1	21
Sorbitol	26.314	319.0>73.1	25	319.0>129.1	11	217.0>73.1	21
Fructose 2[a]	26.510	217.0>73.1	15	307.0>73.1	25	307.0>103.1	15
Glucose 1	26.711	319.0>73.1	25	319.0>129.1	11	205.0>73.1	19
Citric acid	26.951	273.0>73.1	19	273.0>183.1	11	375.0>147.1	19
Lysine	27.347	156.0>73.1	15	317.0>156.2	5	317.0>73.2	27
α-(4-Hydroxyphenyl) glycine	27.541	266.0>73.1	23	73.0>58.0	15	266.0>194.1	15
myo-Inositol	29.312	217.0>73.1	19	318.0>73.2	27	318.0>147.2	25
Ascorbic acid[b]	29.667	147.0>73.1	15	332.0>147.1	25	332.0>73.2	27
N-Acetyl-glutamine[b]	33.280	156.0>73.1	11	215.0>156.1	7	215.0>73.1	23
Glucose-6-phosphate 1	35.693	299.0>73.2	25	387.0>73.2	29	299.0>147.1	21
Glucose-6-phosphate 2[a]	35.877	387.0>73.1	27	299.0>73.1	23	299.0>147.1	21
Sucrose	38.646	217.0>73.1	19	437.0>73.2	27	437.0>257.1	13
Lactose monohydrate 1	39.168	217.0>73.1	21	361.0>73.1	27	361.0>169.1	11
Lactose monohydrate 2[a]	39.393	217.0>73.1	19	361.0>73.2	27	361.0>169.2	9

The determined LODs and LOQs are in the low µM range (**Table 3**) and were calculated by means of the residual standard deviation of the regression lines ($S_{y/x}$) in the range of the LOD [8]. Furthermore, the investigation of linearity, by studying calibration curves, yielded acceptable coefficients of determination (R^2, **Table 3**): $R^2 \geq 0.98$ except for arabinose (0.972), glucose (0.976), sorbitol (0.979) and xylose (0.977). It has to be mentioned that the results of sorbitol are negatively influenced by the presence of mannose since the second isomer of mannose coelutes with sorbitol.

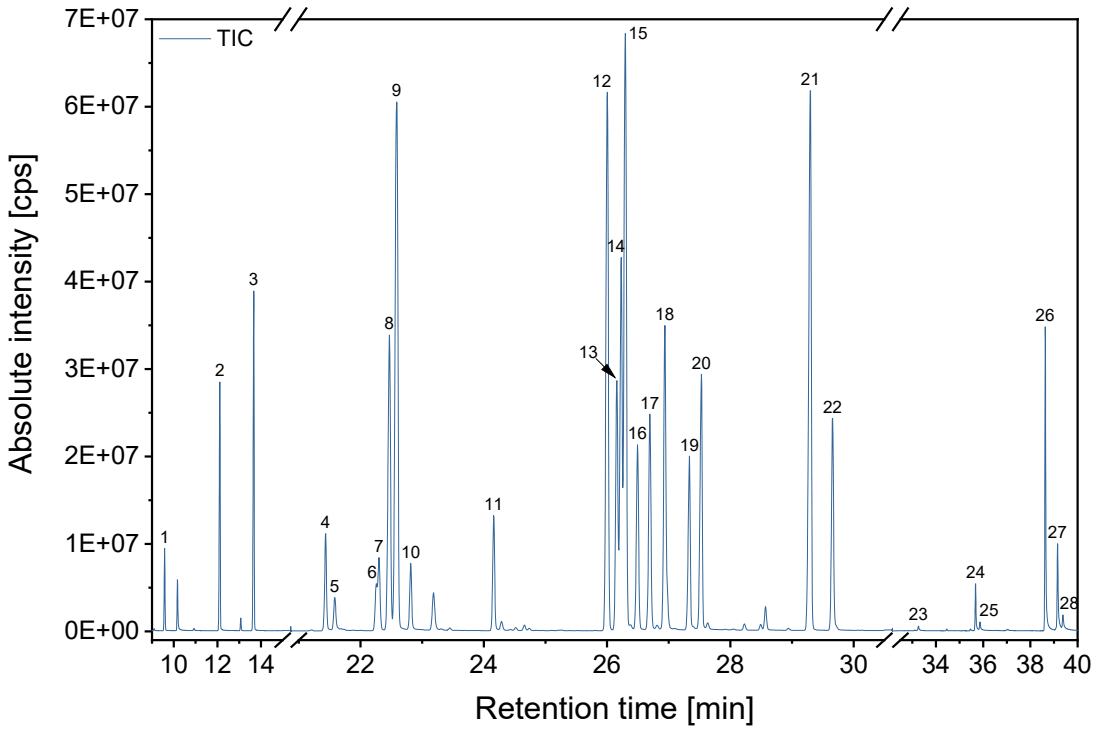

Figure 2: Chromatogram of the metabolite mixture acquired in Q3 scan mode. Peak identification: 1, pyruvate; 2, norvaline; 3, glycine; 4, cysteine; 5, 2-phenylglycine; 6, xylose 1; 7, arabinose 1; 8, arabinose 2; 9, xylose 2; 10, glutamic acid; 11, α-ketoglutaric acid; 12, sorbitol; 13, fructose 1; 14, mannose; 15, mannitol; 16, fructose 2; 17, glucose; 18, citric acid; 19, lysine; 20, α-(4-hydroxyphenyl) glycine; 21, myo-inositol; 22, ascorbic acid; 23, N-acetyl-glutamine; 24, glucose-6-phosphate 1; 25, glucose-6-phosphate 2; 26, sucrose; 27, lactose monohydrate 1; 28, lactose monohydrate 2;

Table 3: Resulting R^2, LOD and LOQ values for the GC-EI-MS/MS method.

Compound	R^2	LOD (µM)	LOQ (µM)
2-Phenylglycine	0.985	1.45	4.40
Arabinose	0.972	5.10	15.45
Citric acid	0.980	11.89	36.04
Cysteine	0.991	23.15	70.16
Fructose	0.980	7.25	21.96
Glucose	0.976	25.07	75.97
Glucose-6-phosphate	0.991	15.07	45.67
Glutamic acid	1.000	0.51	1.56
Glycine	0.997	4.05	12.28
Lactose monohydrate	1.000	0.98	2.97
Lysine	0.983	21.29	64.51
Mannitol	0.982	6.80	20.60
Mannose	0.982	21.15	64.10
myo-Inositol	0.992	3.47	10.51
Sodium pyruvate	0.996	13.40	40.62
Sorbitol	0.979	9.31	28.20
Sucrose	0.990	8.16	24.72
Xylose	0.977	10.76	32.60
α-(4-Hydroxyphenyl) glycine	0.996	9.09	27.54
α-Ketoglutaric acid	0.984	51.63	156.44

3.2. LC-ESI-MS/MS [100]

LC-MS has the big advantage that no derivatisation is needed for analysis. Thus, the metabolites can be measured directly after dilution. In this work hydrophilic interaction liquid chromatography using a Luna® NH_2 column was applied for all analyses. The silica surface of this stationary phase is modified with amino groups which serve as a weak anion exchanger and offer polar selectivity. For the separation of the analytes two mobile phases with different pH (3.0 and 9.9) were tested. It could be shown that a pH of 9.9 yielded better results regarding peak intensity, peak area and peak width for almost all substances (**Figure 3**). On the contrary, at pH 3.0, α-ketoglutaric acid, citric acid, glucose-6-phosphate, NADP and sodium pyruvate showed no peak at all and glucose a double peak. These results clearly demonstrate that the different pH highly affects retention times and ionisation efficiencies. This is obvious when considering the different pKa values, dissociation states at the two tested conditions and the distribution of the analytes between the ACN-rich mobile phase and the water-enriched layer adsorbed onto the hydrophilic stationary phase.

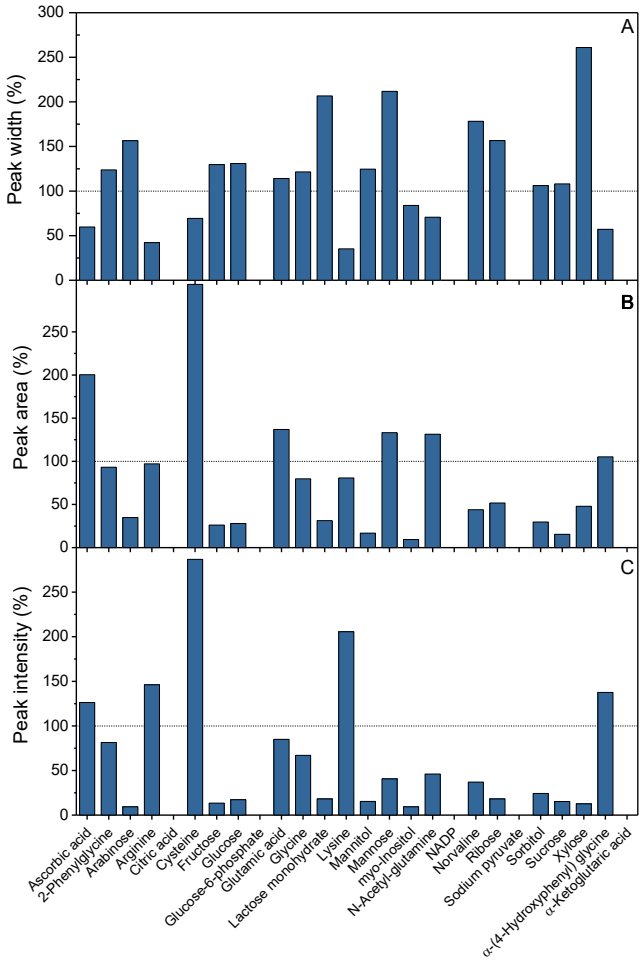

Figure 3: Peak width (A), peak area (B) and peak intensity (C) of all measured compounds at pH 3.0 in relation to pH 9.9. The dashed line (at 100%) corresponds to the values at pH 9.9.

Based on these findings all further measurements were performed with the mobile phase at pH 9.9, thus, the following results refer to alkaline conditions. In the following table the results of the MRM optimisation are shown.

Table 4: Results of MRM optimisation (pH 9.9). Transition 1 was used for quantification and the other two as qualifier transitions.

Compound	Retention time (min)	Ion mode	MRM transition 1	CE (V)	MRM transition 2	CE (V)	MRM transition 3	CE (V)
C5-sugars	4.943	neg.	149.15>89.00	7	149.15>71.05	11	-	-
C6-sugars	5.426	neg.	179.15>89.00	8	179.15>71.05	16	179.15>119.10	10
Mannitol/Sorbitol	5.955	neg.	181.15>89.05	14	181.15>71.05	21	181.15>101.00	14
Norvaline	6.571	pos.	118.15>72.15	-13	118.15>30.15	-21	-	-
Sucrose	7.317	neg.	341.10>89.05	20	341.10>179.20	13	341.10>119.05	19
myo-Inositol	7.545	neg.	179.15>87.05	17	179.15>161.15	14	179.15>70.90	23
Lactose monohydrate	7.934	neg.	341.20>160.95	7	341.20>101.00	16	341.20>179.15	8
2-Phenylglycine	8.058	pos.	152.05>77.10	-35	152.05>135.05	-14	-	-
Glycine	9.054	pos.	76.15>30.00	-15	-		-	-
α-(4-Hydroxy-phenyl) glycine	9.880	pos.	168.15>151.05	-12	168.15>77.00	-34	168.15>95.05	-23
Arginine	10.816	pos.	175.20>70.15	-23	175.20>60.15	-14	175.20>116.10	-17
N-Acetyl-glutamine	10.992	pos.	189.00>84.00	-26	189.00>130.00	-16	189.00>172.00	-11
Lysine	12.946	pos.	147.20>84.05	-18	147.20>130.10	-14	-	-
Glutamic acid	13.667	pos.	148.15>84.10	-16	148.15>56.10	-27	148.15>130.10	-14
Sodium pyruvate	13.777	neg.	87.25>42.95	12	-		-	-
Cysteine	16.187	pos.	121.95>76.15	-15	121.95>87.05	-16	-	-
α-Ketoglutaric acid	16.970	neg.	145.10>101.05	11	145.10>57.10	12	-	-
Glucose-6-phosphate	17.073	neg.	259.10>97.00	18	259.10>78.85	41	259.10>169.00	10
NADP	20.095	pos.	744.10>136.05	-54	744.10>603.90	-21	744.10>508.00	-29
Citric acid	20.159	neg.	191.10>111.00	11	191.10>87.05	17	-	-

The sensitivity of the method to measure ascorbic acid was too low and in addition to the peak exhibited an extreme width (15 min). Therefore, ascorbic acid was not included in the final method and quantitative data evaluation could not be achieved. It was not possible to separate the monosaccharides arabinose, ribose and xylose (C5-sugars), as well as fructose, glucose and mannose (C6-sugars). Hence, the C5- and C6-sugars could only be evaluated as sum peaks. The same was true for the sugar alcohols mannitol and sorbitol. A less steep elution profile (gradient 2, see **section 2.3**) could also not solve these problems. In these cases, a differentiation of the compounds by mass spectrometry was also not possible since the compounds of these three groups each have the same structure and molecular weight. The obtained peak widths of all substances ranged between 1.0 and 3.8 min (**Figure 4**) except for arginine (7.8 min), cysteine (8.0 min), lysine (7.3 min), α-ketoglutaric acid (5.9 min) and sodium pyruvate (4.9 min). In the case of the amino acid

cysteine it has to be mentioned that, when diluted with the mobile phase, a white precipitate could be seen after six hours, indicating a poor stability of cysteine in this solution. This has to be considered if samples are stored for a longer period before measurement.

The resulting LODs and LOQs are in the µM range (**Table 5**) and the limits were calculated by means of the residual standard deviation of the regression lines ($S_{y/x}$) in the range of the LOD [7]. The investigation of linearity, by studying the calibration curves, resulted very acceptable R^2 values (**Table 5**) which were ≥ 0.99, except for cysteine (0.9887).

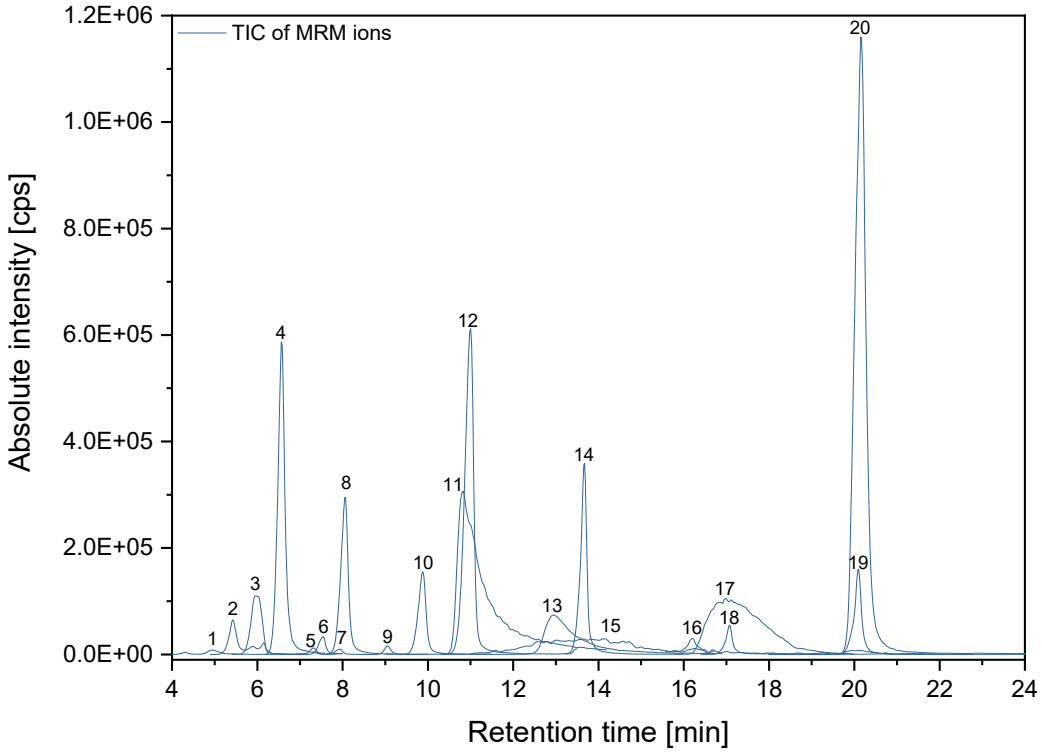

Figure 4: Chromatogram of the metabolite mixture acquired in MRM mode at pH 9.9. Shown is the TIC of the measured MRM transitions. Peak identification: 1,C5-sugars (arabinose, ribose, xylose); 2, C6-sugars (fructose, glucose, mannose); 3, mannitol/sorbitol; 4, norvaline; 5, sucrose; 6, myo-inositol; 7, lactose monohydrate; 8, 2-phenylglycine; 9, glycine; 10, α-(4-hydroxyphenyl) glycine; 11, arginine; 12, N-acetyl-glutamine; 13, lysine; 14, glutamic acid; 15, sodium pyruvate, 16, cysteine, 17, α-ketoglutaric acid; 18, glucose-6-phosphate; 19, NADP; 20, citric acid; Peaks 6, 15 and 16 are magnified by a factor of 10 (peak 6) or 50 (peaks 15 and 16), respectively.

Table 5: Resulting R^2, LOD and LOQ values for the LC-ESI-MS/MS method.

Compound	R^2	LOD (µM)	LOQ (µM)
2-Phenylglycine	1.0000	4.12	12.48
Arginine	0.9983	27.11	82.14
C5-sugar	0.9990	76.47	231.72
C6-sugar	0.9979	86.34	261.64
Citric acid	0.9959	4.39	13.29
Cysteine	0.9887	156.97	475.68
Glucose-6-phosphate	0.9917	2.30	6.97
Glutamic acid	0.9999	8.40	25.46
Glycine	0.9997	27.77	84.14
Lactose monohydrate	0.9990	2.79	8.45
Lysine	0.9997	13.89	42.10
Mannitol/Sorbitol	0.9997	21.90	66.35
myo-Inositol	0.9944	52.02	157.63
N-Acetyl-glutamine	0.9997	10.62	32.18
NADP	1.0000	1.11	3.37
Norvaline	1.0000	3.56	10.80
Sodium pyruvate	0.9987	57.92	175.50
Sucrose	0.9999	3.81	11.54
α-(4-Hydroxyphenyl) glycine	0.9982	29.83	90.39
α-Ketoglutaric acid	0.9968	2.75	8.32

3.3. Comparison of both methods

The big advantage of the LC method is, that no laborious sample preparation is necessary. There is no need for derivatisation and samples can be measured directly if present in an aqueous solution. On the other hand, samples intended to be measured by GC, have to be free from water, thus evaporation of the water has to be performed prior the derivatisation step. Furthermore, with respect to the stability of the derivatives, derivatisation and sample preparation was carried out under nitrogen atmosphere, additionally making GC analysis more complex.

Comparing the chromatographic performance reveals that the GC method has to be favoured when compared to the LC method at pH 9.9, although several substances formed two derivatives (**Figure 2** and **Figure 4**). The sugar alcohols and monosaccharides, except ribose, could be separated by GC, though only sum peaks for C5- and C6-sugars and sugar alcohols could be obtained by LC. In addition to that the GC peak widths had a maximum of 0.27 min compared to several minutes for LC, while the time for the total chromatographic run is comparable: 46 min (GC-EI-MS/MS method) and 60 min (LC-ESI-MS/MS method), respectively. Looking at the effective separation time shows that the analytes are separated within 40 min by GC-MS and 22 min by LC-MS. However, column equilibration for the latter increases the total run time a lot.

The obtained LODs and LOQs which are in the low µM range predominantly are lower for GC-EI-MS/MS than for LC-ESI-MS/MS: factor of 3 to 4 (2-phenylglycine, lactose monohydrate, α-(4-hydroxyphenyl) glycine, sodium pyruvate); factor of 7 (cysteine, glycine); factor of 15 to 16 (myo-inositol, glutamic acid). On the other hand, the following compounds have lower limits when measured by LC-MS: citric acid (factor of 3), glucose-6-phosphate (factor of 7), lysine (factor of 2), sucrose (factor of 2) and α-ketoglutaric acid (factor 19; **Table 3** and **Table 5**). Monosaccharides and sugar alcohols cannot be compared directly, as only sum peaks could be obtained in the case of LC-MS. Ribose, arginine, NADP and N-acetyl-glutamine could not be measured by GC-MS. Furthermore, sorbitol is falsified since it coelutes with one isomer of mannose. Ascorbic acid on the other hand could not be measured with both methods.

Concluding the comparison reveals that, depending on the analytical question both methods are suitable for analysis: the benefits of the GC-MS method on the one hand are the better chromatographic resolution and the lower LODs and LOQs, the advantages of LC-MS on the other hand are the higher linearity of the calibration curves as well as a less laborious sample preparation.

Further investigation of the MeOx/TMS derivatisation strategy can be found in **Manuscript II** (p. 47). In addition to that, a more detailed study on the quantitative analysis of amino acids was carried out during the investigations of tick attachment cement. Details can be seen in **Manuscript III** (p. 118) in **Part II: A First Investigation of Tick Attachment Cement**.

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Abbreviations

ACN, acetonitrile; **CE**, collision energy; **ddH_2O**, double distilled water; **GC**, gas chromatography; **GC-MS**, gas chromatography coupled to mass spectrometry; **GC-EI-MS/MS**, gas chromatography electron ionisation tandem mass spectrometry; **LC**, liquid chromatography; **LC-MS**, liquid chromatography coupled to mass spectrometry; **LC-ESI-MS/MS**, liquid chromatography electrospray ionisation tandem mass spectrometry; **LOD**, limit of detection; **LOQ**, limit of quantification; **MeOx**, methoximation; **MeOx/TMS**, methoximation in combination with trimethyl-silylation; **MSTFA**, N-methyl-N-(trimethylsilyl) trifluoroacetamide; **MRM**, multiple reaction monitoring; **NADP**, ß-nicotinamide adenine dinucleotide-phosphate hydrate; **R^2**, coefficient of determination; **TMS**, trimethylsilylation;

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Manuscript II – Critical considerations for trimethylsilyl derivatives of 24 primary metabolites measured by GC-MS/MS

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Highlights of this manuscript

- Method development for the analysis of metabolites
- Investigation of two derivatisation strategies:

  Trimethylsilylation and methoximation in combination with trimethylsilylation
- Comparison of results of both derivatisation techniques

Critical considerations for trimethylsilyl derivatives of 24 primary metabolites measured by GC-MS/MS

Running Title:

Targeted metabolomics using GC-EI-MRM and derivatization techniques

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Keywords: GC-MRM-MS, metabolites, methoximation, silylation, targeted metabolomics

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Non-standard abbreviations:

collision energy (CE), collision induced dissociation (CID), electron ionization (EI), glucose-6-phosphate (G6P), methoximation (MeOx), multiple reaction monitoring (MRM), N-methyl-trimethylsilyltrifluoroacetamide (MSTFA), trimethylsilylation (TMS)

Abstract:

Cellular reactions are very important for biological systems and understanding metabolic changes is therefore crucial. Here a GC-MS/MS method based on multiple reaction monitoring is presented increasing specificity and sensitivity of the method. 24 metabolites of the primary carbon metabolism were selected for method development as representatives of different analyte classes (carbohydrates and their phosphates, amino acids, organic acids). The derivatization strategy necessary for analysis includes trimethylsilyl derivatization or its combination with methoximation. Derivatization products and reaction kinetics were carefully studied and compared. It is shown in detail that mere silylation results in up to seven monosaccharide derivatives and that irregular derivatization products were observed for particular amino acids showing either one or two silylation products for the ε-amino groups. Additionally it was found that there is not a defined endpoint for this reaction. Methoximation/silylation was also optimized and studied in detail (time, temperature, kinetics) and showed reduced complexity for the derivatization products. However, some metabolites exhibited significantly lower signal responses. Most interestingly it was observed that in the presence of amino acids the derivatisation products for monosaccharides are altered when compared to monosaccharide standards, as demonstrated by the detailed discussion of glucose derivatisation in the presence of lysine.

1. Introduction:

Amongst the omics studies, metabolomics is the universal analysis of all metabolites in a biological system [1]. It completes the information received through genomics, transcriptomics and proteomics by providing quantitative statements of low molecular weight analytes (< 1800 Da), which express the metabolic status of a biological system [2]. The increasing number of publications in the field shows the importance of this research area and indicates that it is an important tool to study phenotypes and their changes [3].

There are two main approaches in metabolomics, targeted and untargeted analysis [4]. Each method has its inherent pros and cons which have to be considered for the whole analytical process. An untargeted approach is the analysis of the global metabolite profile of a sample, it produces an extensive amount of raw data, which makes identification and characterization of analytes a grand challenge. Due to different physicochemical properties and different abundance levels of these metabolites a complete coverage is very difficult. In addition to that, sample preparation techniques are often biased towards certain analytes (e.g. polar versus non-polar) and the sensitivity and/or selectivity of a chosen analytical technology limit the possibilities for untargeted approaches. In contrast, a targeted approach is the analysis of known, well-defined analytes, which makes method development less challenging, nevertheless still demanding because of the different nature of the analytes.

Chromatographic methods allow the simultaneous measurement of a large number of analytes and in this context, GC has the big advantage of a far better chromatographic resolution when compared to LC [5-7]. However, GC analysis very often demands derivatization of analytes prior to analysis to increase volatility by decreasing the polarity of analytes. Today, there are many different derivatization methods available to modify polar functional groups, *i.e.* alkylation, silylation or

acylation. In this work a thorough investigation of trimethylsilylation (TMS) with N-methyl-trimethylsilyltrifluoroacetamide (MSTFA) is presented [8-11] as it gives the most volatile of the TMS acetamides [3]. MSTFA introduces trimethylsilyl groups into molecules with hydroxyl (-OH), carboxyl (-COOH), thiol (-SH) and/or amine groups (-NH_2) by replacing one or two active hydrogen atoms (-H) forming O-, S-, N^1- (one H replaced) or N^2- (two H replaced) TMS derivatives. Additionally, a systematic investigation of the combination of TMS with a prior methoximation (MeOx/TMS) using methoxyamine hydrochloride is presented. MeOx converts aldehyde and keto groups into oximes reducing by this the number of tautomeric forms due to limited rotation along the C=N bond (mainly syn- and anti-isomers) [12].

Furthermore, a method based on triple-quadrupole technology in combination with GC and electron ionization (GC-EI-MS/MS) is discussed. The separation of a biological sample through GC reduces the complexity and MS in general is more sensitive and selective than other detectors allowing high throughput analysis at relatively low costs [13, 14]. Above this, the implementation of triple quadrupole technology as the latest technological advancement, has the benefit of producing highly characteristic fragment ions for each analyte by collision induced dissociation (CID) experiments of selected ions, often already characteristic fragments generated in the EI source. This can in the end be considered as a highly specific MS^3 experiment. However, when running experiments in the so-called multiple reaction monitoring (MRM) mode care has to be taken about the selection of appropriate transitions (precursor / product ion pair) to achieve maximum specificity [15].

Here we present a GC-EI-MRM method to detect 24 metabolites of the primary carbon cycle metabolism after MeOx/TMS derivatization. We give details on pitfalls occurring for data analysis resulting from derivatization and present LODs and LOQs

for representative analytes, i.e. 8 amino acids, 4 organic acids, 3 hexoses, 3 pentoses, 2 disaccharides, 1 sugar phosphate and 3 sugar alcohols.

2. Material and methods

2.1. Reagents and solvents:

Glycine (≥99%), L-arginine (≥98%), L-cysteine (97%), L-glutamic acid (≥99.5%), L-lysine (≥98%), N-acetyl-L-glutamine (≥98%), L-norvaline (≥99%, used as internal standard), citric acid (≥99.5%), L-ascorbic acid (≥99.7%), α-ketoglutaric acid (≥99%), sodium pyruvate (≥99%), D-(-)-arabinose (≥99.8%), D-(-)-fructose (≥99%), D-(+)-glucose (≥99.5%), D-(+)-mannose (≥99%), D-(-)-ribose (≥99%), D-(+)-xylose (≥99%), myo-inositol (≥99%), D-lactose monohydrate (≥99.5%), D-glucose-6-phosphate sodium salt (≥98%, G6P), D-mannitol (≥99.5%) and D-sorbitol (≥99.5%) were obtained from Sigma Aldrich (St. Louis, MO, USA). D-(-)-α-(4-hydroxyphenyl)-glycine (≥99%), D-2-phenylglycine (≥99%) and sucrose (≥99.5%) were obtained from SERVA (Heidelberg, Germany). Pyridine (anhydrous, 99.8%) and methoxyamine hydrochloride (≤100%) were purchased from Sigma Aldrich. MSTFA (≥99%, 1 mL vials) was obtained from Macherey-Nagel (Düren, Germany). Water used for this work was purified (18.2 MΩcm resistivity at 25 °C) with a Milli-Q water purification system (Millipore, Billerica, MA, USA).

2.2. Metabolite mixture

Aqueous stock solutions of every standard were prepared at 10 mg/mL with the exception of D-2-phenylglycine and L-glutamic acid for which 2 mg/mL stock solutions were used due to poor solubility in water. A mixture of the 24 metabolites was prepared

using these stock solutions resulting in a concentration of 416.7 µg/mL for each analyte except D-2-phenylglycine and glutamic acid where a concentration of 83.3 µg/mL was obtained.

2.3. Sample derivatizations

Prior to derivatization the samples had to be dried because of moisture sensitivity of the reagents and the derivatives. Therefore, 5 µL of each standard or 50 µL of the metabolite mixture were pipetted into 200 µL glass inserts (Macherey-Nagel, Düren, Germany). 5 µL of L-norvaline (5mg/mL in water) were added with a micro syringe as internal standard. Subsequently, samples were dried using a vacuum centrifuge (UniEquip, Planegg, Germany) and placed into 1.5 mL glass vials (Macherey-Nagel, Düren, Germany).

2.3.1. Trimethylsilylation (TMS)

Dried samples were dissolved in 50 µL pyridine and 50 µL MSTFA were added under a slight nitrogen stream to remove oxygen from the system. The reaction conditions were optimized during this work; in the end, optimized conditions were 37 °C for 30 min for which the samples were placed in a constant temperature oven after sealing the glass vials with crimp caps. The derivatized samples could be measured by GC-MS/MS directly after cooling of the reaction vial.

2.3.2. Methoximation in combination with trimethylsilylation (MeOx/TMS)

The second derivatization method was carried out with freshly prepared methoxyamine hydrochloride in pyridine (20 mg/mL). For this, 50 µL of the reagent

were added to dried samples. Reaction conditions were optimized during this work; in the end, 37 °C over 90 min in a temperature oven were the optimum. After this, TMS was carried out by adding 50 µL of MSTFA to the samples; reaction at 37 °C for 30 min after sealing the glass vials with crimp caps. Samples were measured by GC-MS/MS directly after cooling of the reaction vial.

2.4. GC-MS/MS analysis

Derivatized samples were analysed using a Shimadzu GC2010 (Kyoto, Japan) gas chromatograph, coupled to a TQ8040 triple quadrupole mass spectrometer. A Zebron ZB50 fused silica column (30 m × 0.25 mm × 0.25 µm, Phenomenex, CA, USA) with a diphenyl dimethyl polysiloxane (50:50) phase was used. Linear velocity (36.7 cm/sec) was used as flow control mode, which resulted in a carrier gas (helium 5.0) flow rate of 1.0 mL/min in the column. The initial column temperature in the oven was 70.0 °C and was held for 5 min after injection followed by a 5 °C/min ramp to reach 245 °C, held again for 1 min, then a quick increase to 310 °C followed (heat rate 250 °C/min) which was held for 5 min (total run time 46.26 min). 1 µL of the samples were injected via a deactivated inlet liner (Shimadzu, Kyoto, Japan) at a split ratio of 1:10 and 230 °C. The interface temperature was set to 250 °C. The mass spectrometer was equipped with an EI ion source (70 eV, rhenium filament, 200 °C) operated in positive ionization mode. Ions were recorded after a solvent cut time of 5 min, using the Q3 scan for method development and molecular identifications or by using the MRM mode for quantitative analyses. MRM was run with a loop time of 0.3 sec, a minimum dwell time of 11 msec and a maximum of 6 ions measured simultaneously (details below). Argon 5.0 was used as collision gas and collision energies (CEs) were optimized for each analyte using the vendor provided software, GCMSsolution v. 4.20

(Shimadzu). All data were acquired using the same software and structures were identified based on similarity search against the NIST11 and Wiley9 mass spectral libraries, respectively. In case of missing database entries all mass spectra were interpreted manually for structure confirmation.

2.5. Multiple Reaction Monitoring (MRM)

MRM optimization was carried out using the Smart MRM tool provided with the vendor's software. In brief, for each sample the signals obtained in a Q3 scan was integrated after confirmation of substance identification. Subsequently, product ion scans from characteristic fragment ions of each analyte were recorded with different CEs ranging from 3 to 45 V (3 V steps). CEs producing most abundant and highly specific transitions were chosen for each analyte.

2.6. Analytical parameters

To determine calibration functions 14 dilutions (1:10 to 1:7500) were prepared from the stock solution containing all 24 compounds giving analyte concentrations from 15.43 nM to 0.56 mM. D-2-phenylglycine and L-glutamic acid were limited by their solubility and were included in the calibration between 7.35 nM and 0.22 mM. Limit-of-detection and limit-of-quantificatioin (LOD/LOQ) calculations are based on the calibration function using only signals with a S/N above 3. LODs are reported as 3.3-times and LOQs as 10-times the absolute standard error of the regression function ($S_{y/x}$).

3. Results and discussion

The aim of this work was the investigation of 24 selected metabolites of the primary carbon metabolism by means of GC and MRM after optimized derivatization conditions. The selection of analytes represents four biologically important metabolite classes: sugars, organic acids, sugar phosphates and amino acids. Derivatization methods were optimized first using one metabolite per class. After that, calibration functions, LODs and LOQs were determined for the mixture of the 24 analytes.

3.1. MRM optimization

Measuring analytes in MRM mode after EI can be considered as a highly specific measurement of analytes as fragment ions generated in the EI source are selected and further fragmented by CID corroborating analyte identity. The crucial benefit of this technique is significantly reduced signal-to-noise ratios (S/Ns). Moreover, it allows to differentiate substances in chromatographically overlapping peaks using either the respective parent ions (different molecular weights) or fragment masses (isobars).

As shown in Supplementary Table 1 for most of the investigated substances, parent and fragment ions were highly characteristic, except for the investigated sugars where all isomers have the same parent and in most of the cases also identical fragment ions differing only in their respective intensities. For monosaccharides it was essential to achieve optimal separation by GC. Excellent separation and MS identification based on MRM transitions could be achieved for most of these analytes, except furanoses, in particular arabinose, ribose and xylose, could not be separated in an optimal manner. Every optimization step for these pyranoses however resulted in disadvantageous separations of all the other analytes and so for further method

development the decision was made to remove Ribose from the mixture (co-elution with Arabinose and Xylose, further details see chapter 3.4).

All metabolites were separated by GC within 48 min and a maximum of three transitions were identified for each analyte. The most intensive transition was used for quantification while two others were considered as qualification transitions. Figure 1 shows 12 MRM events found to be optimal for the 48 min GC separation to monitor all 24 metabolites with a maximum of 6 analytes measured per MRM event.

3.2. TMS of metabolites by the use of MSTFA

TMS is a common derivatization strategy and many different protocols in terms of sample volumes, temperature and derivatization time are available. To establish a methodology valid for future biological samples an internal standard, norvaline which gave a constant signal over time, was added already at this stage of method development to correct for sample losses or enrichments eventually occurring during sample preparation. Areas normalized against norvaline are reported throughout the rest of this publication.

Although most of the analytes give one distinct signal in chromatography and a mass spectrum matching to the respective spectrum of the database, it was noticed that glycine and lysine were each converted into two derivatives. Both amino acids showed TMS at the COOH-group (O-TMS), but differences for the NH_2-groups as identified by database search and manual spectral interpretation. Figure 2A and B show the mass spectra with the proposed structures for the two potential derivatives of glycine (9.00 and 13.58 min), one having a single (N^1-TMS) and the other having a double (N^2-TMS) silylation at the α-NH_2-group. Due to the fact that lysine exhibits also two NH_2-groups for the side chain, four derivatives were expected. Interestingly, lysine

yielded only two derivatives (23.64 and 27.25 min; Figure 2C and D), both with a single silylation at the α-NH_2-group but again differing in the silylation grade of the ε-NH_2-group. The formation of two derivatization products can be explained by (a) sequential and slow reactions of the H-atoms of the α-NH_2-group of glycine and the ε-NH_2-group of lysine and (b) steric hinderance inhibiting consecutive derivatization of the α-NH_2-group of lysine. To ensure completeness of reaction, kinetic studies were carried out. 12 identical samples, containing 10 mg/mL glycine and lysine each, were prepared. The development of peak areas of the derivatives over 32 hours of silylation was monitored (Figure 3). It was observed that the TMS reaction did not stop and that the end point was not reached even after 30 hours. The peak areas for the N^2-TMS derivative increased constantly over time, whereas the peak area of the N^1-TMS derivative (α-N^1 in the case of glycine and ε-N^1 in the case of lysine) decreased. Furthermore, it has to be stated, that the sum for all derivatives was not constant, but increased by 64% and 62% for glycine and lysine, respectively (Figure 3B).

It was also observed that all analysed monosaccharides were converted into several products, all separable by GC-MS. In particular, pentoses yielded five derivatives except for arabinose showing only four. All hexoses yielded seven derivatives giving by this the highest number of derivatives. An explanation for this is the free aldehyde group allowing the formation of furanose and pyranose hemiacetals besides open-chain derivatives. Details for glucose are discussed exemplarily: After TMS, glucose showed seven peaks which were separated by GC within a rather narrow time window from 25.92 to 28.46 min (Supplementary Figure 1). An exact structure/retention time correlation could not always be achieved. For the α- and β-1,2,3,5,6-pentakis-O-(trimethylsilyl)glucofuranose (structure I), the mass spectra observed for peaks 1 and 2 are similar and the signals clearly baseline separated (25.92 and 26.34 min). The same is true for α/β- 1,2,3,4,6-pentakis-O-

(trimethylsilyl)glucopyranose (structure II), which had the highest probability for identifications when searching the databases (3, 4, 5, 6 and 7 at 26.76, 26.99, 27.41, 27.94 and 28.46 min). Additionally, 2,3,4,5,6-pentakis-O-(trimethylsilyl)glucose (structure III) could be obtained as the most probable structure for the peaks eluting at 26.76 and 28.46 min (peaks 3 and 7). Purified derivatives were not available during this study making unambiguous peak assignment/confirmation of mass spectra impossible. Structures were denoted solely based on database searches giving mass spectral similarities (≥ 93%, except 82% for the peak at 25.92 and 26.34 min, and 83% for the peak at 27.41 min). The kinetics of derivatization was studied in detail also for monosaccharides (Figure 4) and a rapid decrease of five peak areas was observed, while the portion of two other derivatives were increasing over the first 10 hours. Nevertheless, this reaction was almost complete after 20 hours showing a further increase of only 6% (peak at 26.76 min) and 7% (peak at 28.46 min). It can be stated that TMS continues over the first hours and is reaching its endpoint at about 10 hours. The final products which could be obtained were pyranose hemiacetals of glucose or the open-ring chain form, respectively.

In summary, TMS is difficult to keep constant for monosaccharides and some amino acids. It produces many derivatives and has no clear endpoint making quantification challenging. Although the obtained derivatives for each monosaccharide were baseline separated, the resulting high number of derivatives for all studied saccharides is highly unfavourable as such derivates can co-elute or have same MRM transitions making identification and quantification impracticable.

3.3. Silylation in combination with preceding methoximation (MeOx/TMS)

To overcome isomerization and other reaction by-products, MeOx was introduced as published previously by Roessner et al. [10]. This step has the big advantage that aldehyde- and ketone-groups of reducing sugars are protected and uncontrolled ring formation during TMS is prevented by stabilizing carbonyl moieties in the β-position [10, 16]. The only fact which has to be considered is, that MeOx gives two different stereoisomers in the case of monosaccharides: syn- (Z) and anti- (E) isomers [11, 17], regardless of whether a D- or L-monosaccharide is the original analyte. By this, the number of signals per monosaccharide was significantly reduced, but retention time shifts had to be considered for the new products.

Derivatization conditions were optimized for five selected metabolites being candidates for each compound class (sugars, organic acids, sugar phosphates and amino acids), i.e. glucose, fructose, ketoglutaric acid, G6P and cysteine. The optimization process started with a MeOx reaction at 30 °C for 90 min which was combined with TMS at 37 °C for 30 min [10]. Optimization was evaluated according to characteristic mass spectra and GC performance (peak shape, area). Fixed TMS conditions (30 min, 37 °C) were first combined with varying MeOx conditions. Three different reaction times (30, 90 and 150 min) and temperatures (room temperature RT, 45 °C and 65 °C) were systematically changed. First, the three different reaction temperatures were investigated using a fixed reaction time of 90 min and subsequently the three reaction times were examined, while the optimized temperature was used. The optimization of the TMS reaction was carried out the same way using the optimized conditions for the MeOx step, a fixed reaction time of 30 min during the temperature optimization and subsequently varied reaction times at a fixed temperature (Figure 5).

For MeOx a derivatization temperature of 45 °C yielded the best results (Figure 5A). Especially fructose, α-ketoglutaric acid and cysteine guided the decision making

process as significantly higher reaction yields were achieved at this temperature. Regarding TMS, the optimum temperature was RT, except in the case of G6P where 45 °C produced the most abundant signal (+ 27% compared to RT; Figure 5C). The aim of the optimization was to get settings that allow the use of one constant temperature oven to have a quick and easy sample preparation. Therefore, a compromise was made by performing all further derivatization reactions at 37°C.

The optimization of the MeOx reaction times were found to show an optimum at 90 min (Figure 5B). In the case of TMS, maximum peak areas were obtained already after 30 min for glucose, fructose and α-ketoglutaric acid (Figure 5D). G6P showed similar peak areas after 30 and 90 min of derivatization. Only TMS of cysteine yielded the significantly higher signals after 90 min. Neverthelss peak areas obtained after 30 min were well acceptable showing the lowest absolute standard deviation. Thus, reaction times of 90 min for MeOx and 30 min for TMS were selected as final parameters, representing the optimal values for all selected substances with reasonable compromises. In summary the final parameters for the MeOx/TMS derivatization were as follows (literature values in parentheses): MeOx at 37 °C for 90 min (30 °C, 90 min) followed by TMS at 37 °C for 30 min (37 °C, 30 min) [10, 18, 19].

Kinetics for the optimized MeOx/TMS derivatization were again investigated. Although still two signals were observed for glycine, namely a α-N^1- and α-N^2-TMS derivative (9.00 and 13.58 min), more stable signals over time were gained, showing that the derivatization was complete after the first four hours (Supplementary Figure 2A). The observed peak areas in combination with standard deviations of only 11.90% and 2.90% for the α-N^1-TMS and the α-N^2-TMS signal gave only one product for lysine after 10 hours (RSD of 3.30%), namely the α-N^1- / ε-N^2-TMS derivative (Supplementary Figure 2A). Increasing the derivatization time to 10 hours seems to be beneficial for

this derivative, but shows less favourable results for the remaining substances (two exceptions: glucose and glycine). Moreover, such long reaction times increase the total time of analysis. Thus, it can be concluded that the presence of methoxyamine hydrochloride stabilizes reaction products giving better reproducibility for metabolite quantification.

In respect to the multiple hexose TMS derivatives it was found that beside the positive effect of significantly reducing the chromatographic complexity the observed peak areas were also much more constant over time after MeOx (Supplementary Figure 2B). This was confirmed by the relative standard deviation for the two glucose derivatives observed after MeOx/TMS (instead of 7 after mere TMS), 4.25% (26.68 min) and 3.95% (26.95 min).

However, an outstanding observation has to be mentioned. Two additional glucose derivatives were formed in the presence of lysine, but not glycine. Derivatization of mere glucose formed two expected isomeric products eluting at 26.68 and 26.95 min. In the presence of lysine two additional peaks at 26.80 and 28.49 min were present. Database search indicated that these peaks can be the α- and β-form of 1,2,3,4,6-pentakis-O-(trimethylsilyl)-D-glucopyranose which is the cyclic form of the derivatized hexose. It can be assumed that a competitive oxidation reaction in the presence of lysine is allowing the open ring form to reform its respective hemiacetal and thus preventing MeOx of the carbonyl group of glucose. However these signals are only minor (1.35% of main form) and can be neglected for more complex biological samples.

3.4. Analysis of 24 metabolites of the primary carbon cycle applying the optimized MeOx/TMS method

Optimized MeOx/TMS conditions were applied in the investigation of a mixture containing all 24 metabolites. First, retention times and chromatographic performance were studied (Supplementary Table 1) and the obtained structures of the derivatized analytes were identified based on similarity search (≥ 80%) against the NIST11 and Wiley9 spectral mass libraries (Supplementary Table 2). Databases did not contain spectra for the glycine and glutamine derivatives, α-(4-hydroxyphenyl)-glycine and N-acetyl-L-glutamine, but manual spectra interpretation confirmed the proposed structures. Monosaccharides were expected to exhibit two separated peaks for respective isomers and GC separation was achieved accordingly for all investigated pentoses (arabinose, xylose) and hexoses (fructose, glucose, mannose), for which mass spectra were identical. However, the intensity ratio of the two isomers was different for the individual monosaccharides (first peak : second peak): arabinose (~ 1:5), fructose (~ 1:1), xylose (~ 1:6), glucose (~ 6:1) and mannose (~ 5:1). Exemplary structures and mass spectra are shown in Figure 6 for a pentose (arabinose) and two hexoses (glucose and fructose). The peak ratios can be explained by the varying structures, i.e. number of carbon atoms and especially the position of the carbonyl function. In addition to the investigated monosaccharides also lactose and sucrose, both disaccharides, and G6P, a phosphorylated hexose, were studied. The formation of Z and E isomers (Supplementary Figure 3) resulted in two peaks with an intensity ratio of ~ 4:1 for G6P eluting at 35.66 and 35.84 min and lactose eluting at 39.14 and 39.36 min. In the case of lactose, it was hypothesized that one of the two monosaccharide building blocks gets methoximated and is remaining in its open form, while the other one remains cyclic while getting trimethylsilylated. This hypothesis could be confirmed by the obtained mass spectra. Sucrose on the other hand only

resulted in the formation of one derivative (38.62 min) showing TMS at eight OH-groups and no MeOx (Supplementary Figure 3).

As already mentioned above two peaks for glycine were obtained: N^1-TMS and N^2-TMS. These two derivatives were also present after TMS derivatization (Figure 2A and B). Lysine formed one derivative having a single silylation at the α- and a double silylation at the ε-NH_2 group (α-N^1,ε-N^2-TMS), and all other investigated amino acids formed one derivative having a single silylation at the NH_2-group. Cysteine additional was silylated at the SH-group yielding a N^1,O,S-TMS derivative.

The investigated organic acids were each converted to one derivative having all OH-groups silylated: ascorbic acid (O,O,O,O-TMS at 29.64 min), citric acid (O,O,O,O-TMS at 26.91 min), α-ketoglutaric acid (O,O-TMS at 23.20 min) and pyruvate (O-TMS at 9.62 min). The latter two additionally showed MeOx. Exemplary the structure and mass spectrum of the α-ketoglutaric acid derivative is shown in Supplementary Figure 4.

The total ion chromatogram of the GC-MRM-MS separation of all 24 metabolites is shown in Figure 7. As it can be seen the chromatographic resolution for all compounds was very good, providing baseline separation for many peaks, except for xylose/arabinose (peaks 8 and 9) and fructose/mannose/sorbitol (peaks 15, 16 and 17). Due to co-elution mannose/glucose exhibited only one signal, i.e. mannose overlaps with sorbitol (26.30 min) and glucose overlaps with citric acid (26.91 min). Although the molecular weights (MWs) of mannose and sorbitol derivatives are different, 570.10 and 615.26 g/mol respectively, the obtained precursor and fragment ions, thus monitored MRM transitions, are identical, so a separation by GC-MRM-MS was not possible. In contrast to that the co-eluting glucose (MW of the derivative: 570.11 g/mol) and citric acid (MW of the derivative of 408.67 g/mol) showed different transitions and thus differentiation by MS was possible. Nevertheless quantification

was possible for all compounds by using the second isomer of glucose (peak 19 at 26.68 min) and mannose (peak 16 at 26.22 min).

At this point it has to be considered that the presence of mannose influences the signal of sorbitol. In the case of C5 sugars, ribose could not be separated from arabinose and xylose. The precursor and fragment ions are the same and the GC-MRM-MS method did not allow separation and quantification of single compounds.

3.5. Determination of LOD and LOQ

Most of the analytes showed excellent linear responses with $R^2 > 0.99$ (17 substances), a few only between 0.98 and 0.99 (6 substances) and the resulting LODs, LOQs and R^2 values are shown in Table 1. Analytical parameters for the organic acids could be determined without issues since all compounds were baseline separated. The co-elution of citric acid with one of the glucose isomers made analyses more tedious, yet possible. As reported above, two signals were observed for the sugar derivatives, but, most importantly, it was validated, that the ratio of the obtained isomers remained constant for all concentration levels and therefore either the sum of the peak areas or only one peak could be considered for LODs and LOQs. In this study ribose was excluded from the metabolite mixture since both of its derivatives co-eluted either with one derivative of arabinose or one of xylose. Both fructose peaks (26.15 and 26.49 min) could be obtained in the final chromatogram and yielded almost identical regression parameters and limits. Therefore, the final reported values represent averages resulting in an RSD of 0.03% for R^2 and 1.8% for LOD and LOQ. Arabinose and xylose were quantified by using the more intense peaks at 22.45 and 22.57 min for high sensitivity measurements. As described above two signals were also observed for glycine and the disaccharide lactose and again the more intensive was used for

quantification (glycine 13.65 min, lactose 39.14 min). In the case of glucose (26.68 min) and mannose (26.22 min) the only peak observed was of course used for data evaluation. Both isomers observed for G6P at 35.65 and 35.84 min showed same performance characteristics, thus both peaks were used for evaluation.

In conclusion the found LODs/LOQs can be considered as a very satisfactory, since biologically relevant concentrations of metabolites in biological systems are very often in the low µM to a few hundred mM range [20], which is easily covered by the developed methodology.

4. Concluding remarks

GC coupled to EI-MS/MS showed to be a reliable platform for targeted metabolomics. The MRM technique allows highly sensitive and selective measurements by reducing background signals and thus increasing S/N. Although derivatization makes analysis more vulnerable to errors due to an increased number of preparation steps, the MeOx/TMS derivatization strategy allowed the analysis of a broad range of substance classes at biologically relevant LOD levels, i.e. 10^{-1} to 10^{-6} M.

Detailed studies of derivatization mechanisms and kinetics showed that derivativatization is highly reproducibile allowing comparison between biological samples exhibiting very low expression levels changes. This will in the end provide a method fit for metabolomics applications targeting key analytes of the primary carbon metabolism and therefore energy levels of biological systems. However, it has to be noted that in complex samples co-elution can alter the determined absolute concentration levels, as observed for the measurement of arabinose/xylose in the

presence of ribose or the altered derivatization behaviour of glucose in the presence of lysine but not glycine.

Acknowledgements

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Figure legends

Figure 1: Time segments of the final GC-MRM-MS method for 24 metabolites after MeOx/TMS derivatization.

Figure 2: Mass spectra and chemical structures of glycine and lysine derivatives after TMS derivatization. The corresponding retention times in GC analysis using the parameters outlined in the method section are: (A) 9.00 min, (B) 13.58 min, (C) 23.64 min and (D) 27.25 min. Structure elements in red indicate the NH_2-groups giving two derivatization products.

Figure 3: Kinetic study of glycine and lysine derivatization over 32 hours using TMS. (A) Peak areas of the four obtained derivatives and (B) sum of peak areas for both glycine and lysine derivatives. N^1 and N^2 represent the TMS derivatization at the corresponding α- or ε-NH_2-groups. All areas are corrected against norvaline (internal standard).

Figure 4: Kinetic study of glucose derivatization over 32 hours using TMS. All areas are corrected against norvaline (internal standard).

Figure 5: Comparison of derivatization conditions for five selected metabolites, glucose, fructose, ketoglutaric acid, G6P and cysteine. Peak areas for (A) different temperatures and (B) derivatization durations for MeOx (TMS reaction conditions constant at 37 °C for 30 min). TMS reaction outcomes are compared for (C) different temperatures and (D) reaction durations while MeOx conditions were constant (37 °C, 90 min). Error bars represent absolute errors of three

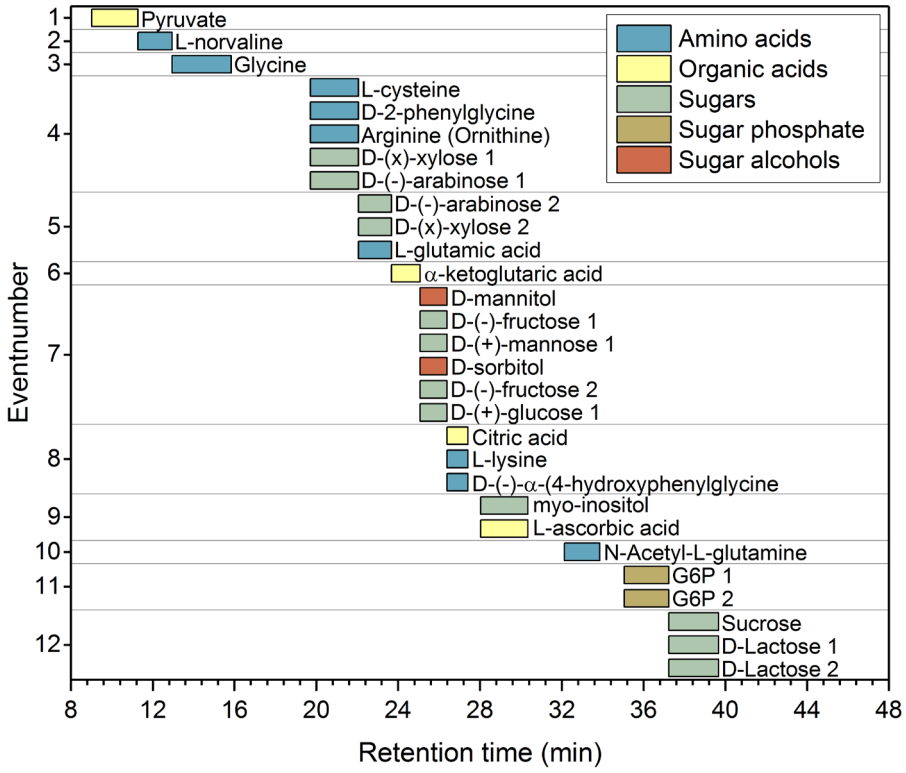
technical replicates. All areas are normalized against norvaline, G6P and cysteine results are magnified.

Figure 6: (A) Mass spectra and (B) structures of E- and Z-MeOx/TMS isomers of arabinose, fructose and glucose. An accurate alignment of structures and mass spectra is not possible at this point due to the lack of purified standards.

Figure 7: GC-MS chromatogram of a mixture of 24 metabolites belonging to the primary carbon metabolism. (A) Complete chromatogram, 5.00-45.00 min. (B) Zoom into the important time range between 21 and 30 min.

Peak assignment: 1, glycine (N^1,O-TMS); 2, pyruvate (MeOx O-TMS); 3, norvaline (N^1,O-TMS); 4, glycine N^2,O-TMS; 5, cysteine (N^1,O,S-TMS); 6, 2-phenylglycine (N^1,O-TMS); 7, ornithine (α-N^1,δ-N^1,O-TMS); 8, xylose (MeOx tetrakis-O-TMS, 1Z/1E); 9, arabinose (MeOx tetrakis-O-TMS, 1Z/1E); 10, arabinose (MeOx tetrakis-O-TMS, 1Z/1E); 11, xylose (MeOx tetrakis-O-TMS, 1Z/1E); 12, glutamic acid (N^1,bis-O-TMS); 13, α-ketoglutaric acid (MeOx bis-O-TMS; 14, mannitol (hexakis-TMS); 15, fructose (MeOx pentakis-O-TMS, 1Z/1E); 16, mannose (MeOx pentakis-O-TMS, 1Z/1E); 17, sorbitol (hexakis-TMS); 18, fructose (MeOx pentakis-O-TMS, 1Z/1E); 19, glucose (MeOx pentakis-O-TMS, 1Z/1E); 20, citric acid (tetrakis-TMS); 21, lysine (α-N^1,ε-N^2,O-TMS); 22, α-(4-hydroxyphenyl)-glycine (N^1,O-TMS); 23, myo-inositol (hexakis-O-TMS); 24, ascorbic acid (tetrakis-TMS); 25, N-acetyl-glutamine (N^2,O-TMS); 26, G6P (MeOx hexakis-TMS, 1Z/1E); 27, G6P (MeOx hexakis-TMS, 1Z/1E); 28, sucrose (octakis-TMS); 29, lactose (MeOx octakis-TMS 1Z/1E); 30, lactose (MeOx octakis-TMS 1Z/1E);

Figure 1:



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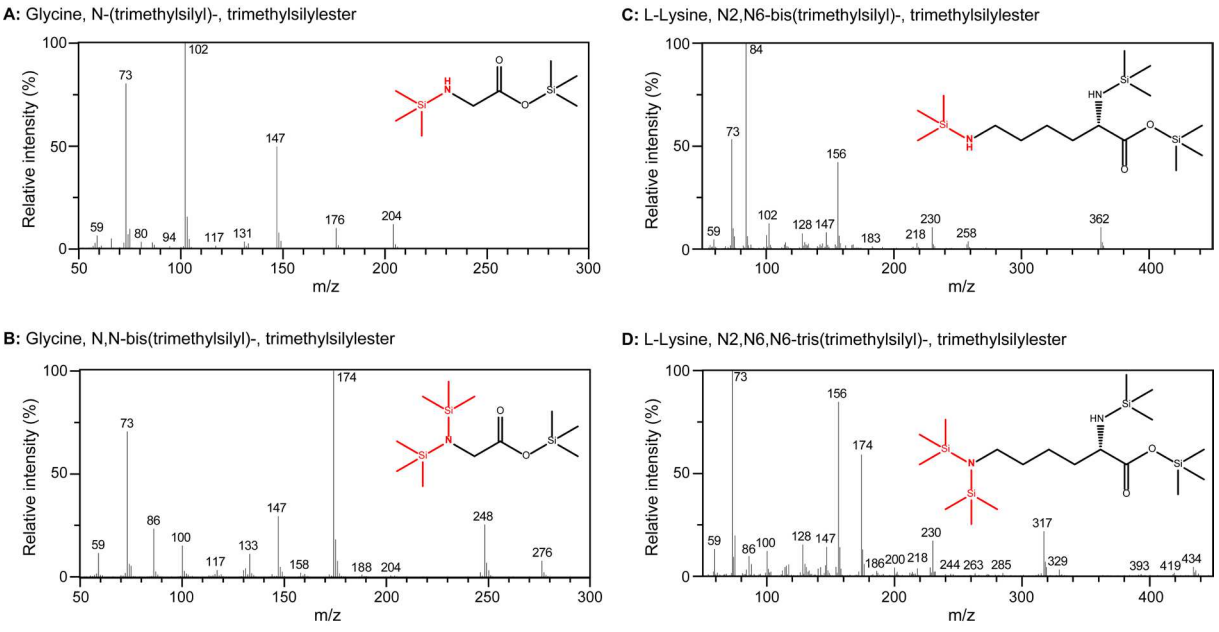

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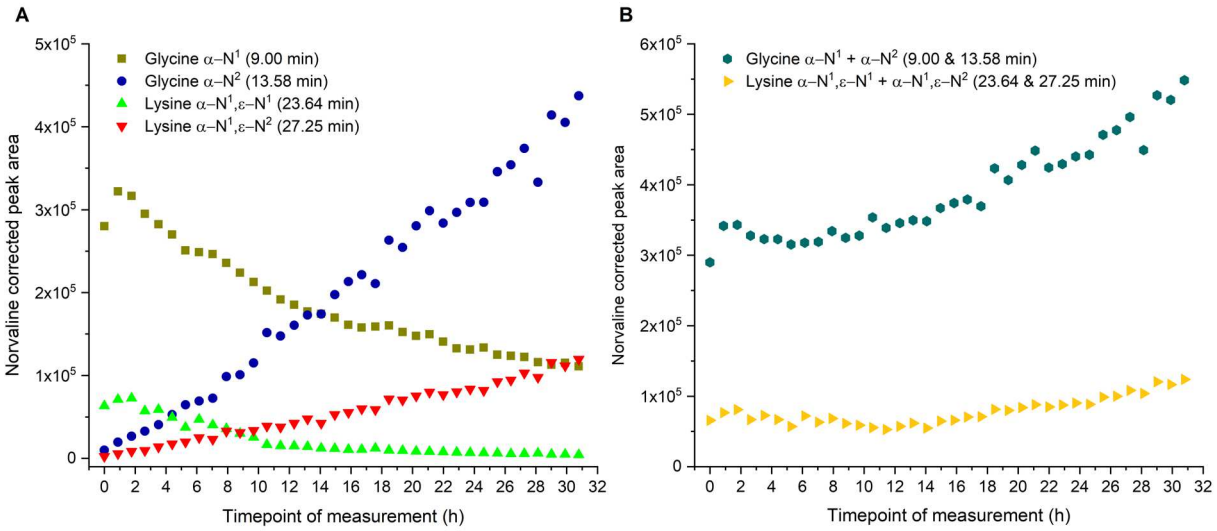

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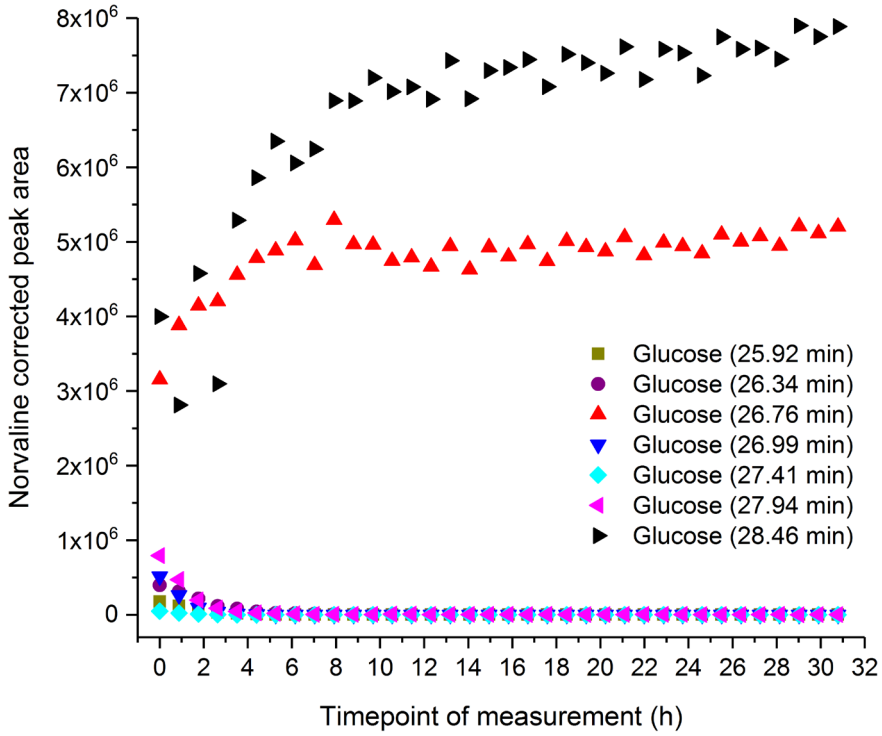

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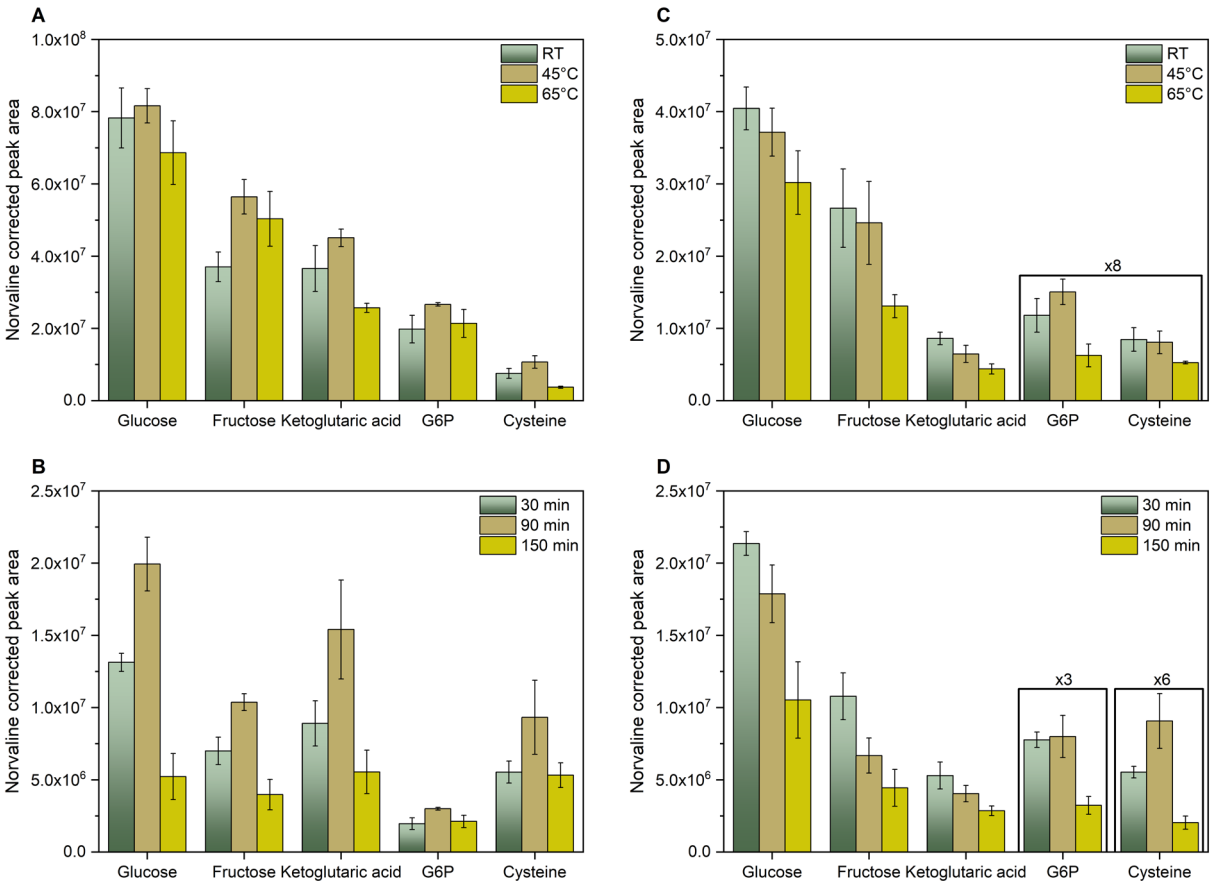
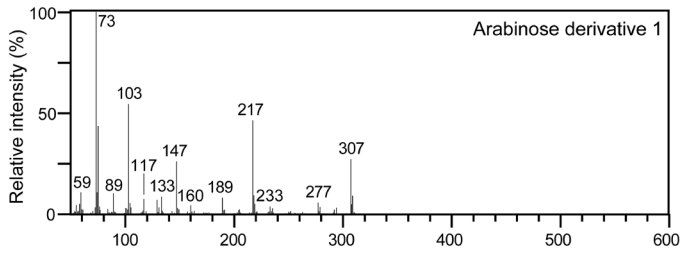
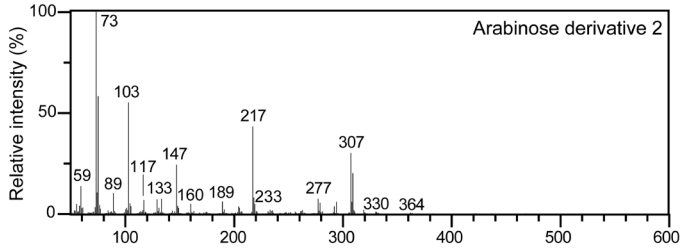
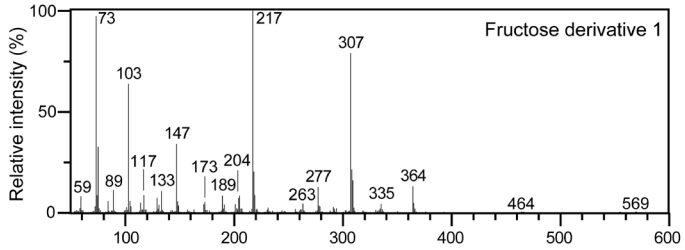


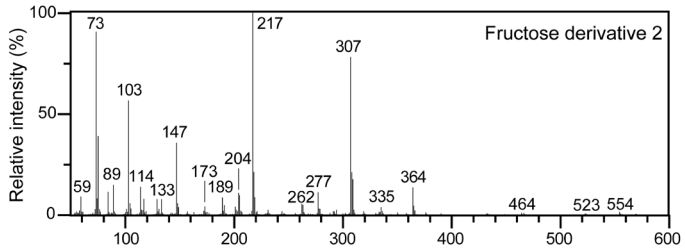
Figure 6:

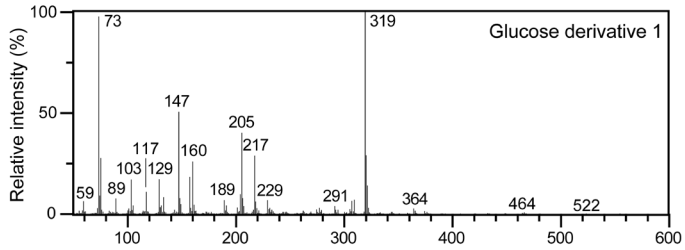
A: Mass spectra

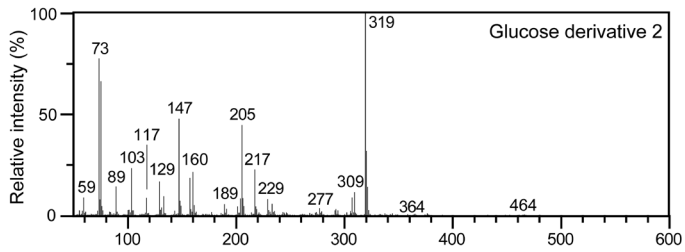




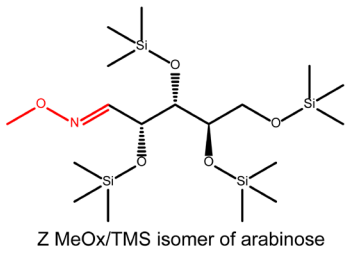




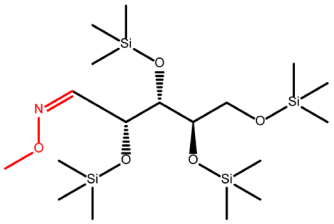




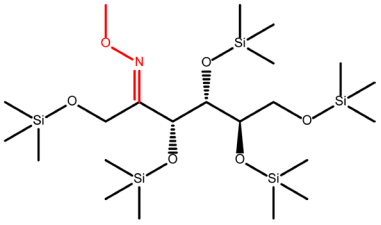
B: Proposed structures



Z MeOx/TMS isomer of arabinose



E MeOx/TMS isomer of arabinose



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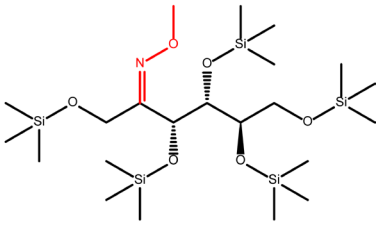

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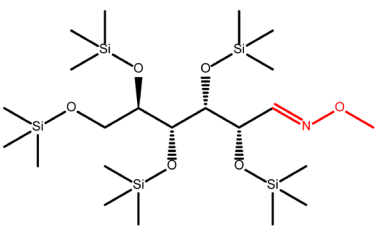
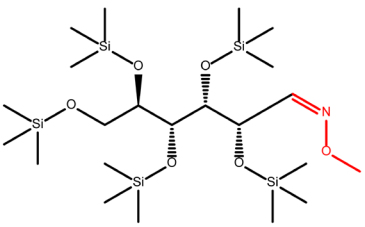


Z MeOx/TMS isomer of glucose



E MeOX/TMS isomer of glucose

Figure 7:

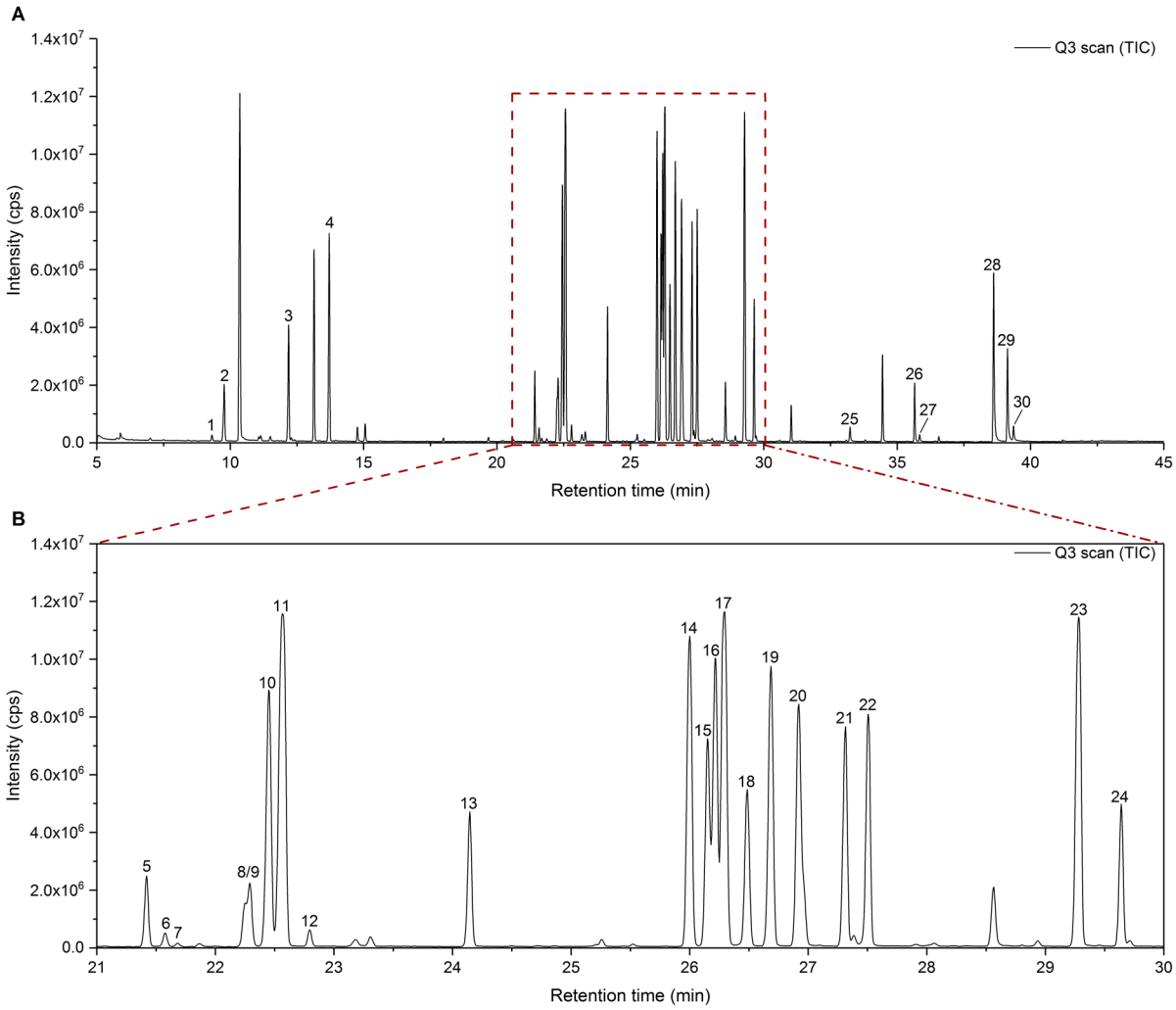


Table 1: LODs, LOQs and R^2 values for 24 representative metabolites of the primary carbon metabolism determined by GC-MRM

Compound	LOD (µM)	LOQ (µM)	R^2
Glycine	30.05	91.06	0.9974
L-arginine	128.06	426.87	0.9875
L-cysteine	4.64	14.05	0.9911
L-glutamic acid	4.22	12.78	0.9921
L-lysine	37.82	114.61	0.9875
N-acetyl-L-glutamine	13.77	41.71	0.9966
D-(-)-α-(4-hydroxyphenyl)-glycine	18.10	54.83	0.9925
D-2-phenylglycine	0.77	2.32	0.9905
Citric acid	1.25	3.77	0.9883
L-ascorbic acid	3.52	10.68	0.9939
α-ketoglutaric acid	5.21	15.80	0.9837
Pyruvate	0.42	1.27	0.9959
D-(-)-arabinose	1.00	3.02	0.9949
D-(-)-fructose	3.98	12.05	0.9921
D-(+)-glucose	0.26	0.80	0.9971
D-(+)-mannose	0.80	2.42	0.9847
D-(-)-ribose	-	-	-
D-(+)-xylose	1.22	3.69	0.9928
myo-inositol	2.50	7.57	0.9962
D-lactose	2.35	7.13	0.9887
Sucrose	0.57	1.73	0.9917
D-glucose-6-phosphate 1	6.88	20.86	0.9927
D-glucose-6-phosphate 2	4.91	14.88	0.9990
D-mannitol	3.02	9.16	0.9948
D-sorbitol	3.73	11.30	0.9921

Supplementary Material

Critical considerations for trimethylsilyl derivatives of 24 primary metabolites measured by GC-MS/MS

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Institute of Chemical Technologies and Analytics, TU Wien, Vienna, Austria

Contents:

Supplementary Table 1 summarizes the retention times, precursor and fragment ions obtained for all of the metabolites after MeOx/TMS derivatization.

Supplementary Figure 1 shows the GC-MS chromatogram and acquired mass spectra in addition to the suggested structures for the glucose derivatives after TMS derivatization.

Supplementary Figure 2 shows the results of the investigation of the reaction kinetics of MeOx/TMS derivatization of glycine and lysine (A) and glucose (B). In the case of glycine (9.00 and 13.58 min) and glucose (26.68 and 26.95 min) two signals could be obtained. This is caused by the single and double silylation of the NH_2 group of glycine and the formation of an E and Z MeOx/TMS isomer of glucose. Lysine only showed one very stable derivative (27.25 min), namely the single TMS at the α-NH_2-group and double TMS at the ε-NH_2-group.

Supplementary Table 2 summarizes the obtained derivatives for all analysed substances after MeOx/TMS derivatization at 37 °C for 90 min (MeOx) and 30 min (TMS). The obtained structures were identified based on similarity search (≥ 80%) against the NIST11 and Wiley9 spectral mass library.

Supplementary Figures 3 and 4 show the mass spectra and proposed structures of the MeOx/TMS derivatives of G6P, lactose, sucrose and α-ketoglutaric acid. In the case of G6P and lactose E and Z isomers were obtained due to the orientation at the C=N bond which is introduced by MeOx. Due to the lack of purified standards an accurate alignment of structures/mass spectra/retention times is not possible in the case of G6P and lactose.

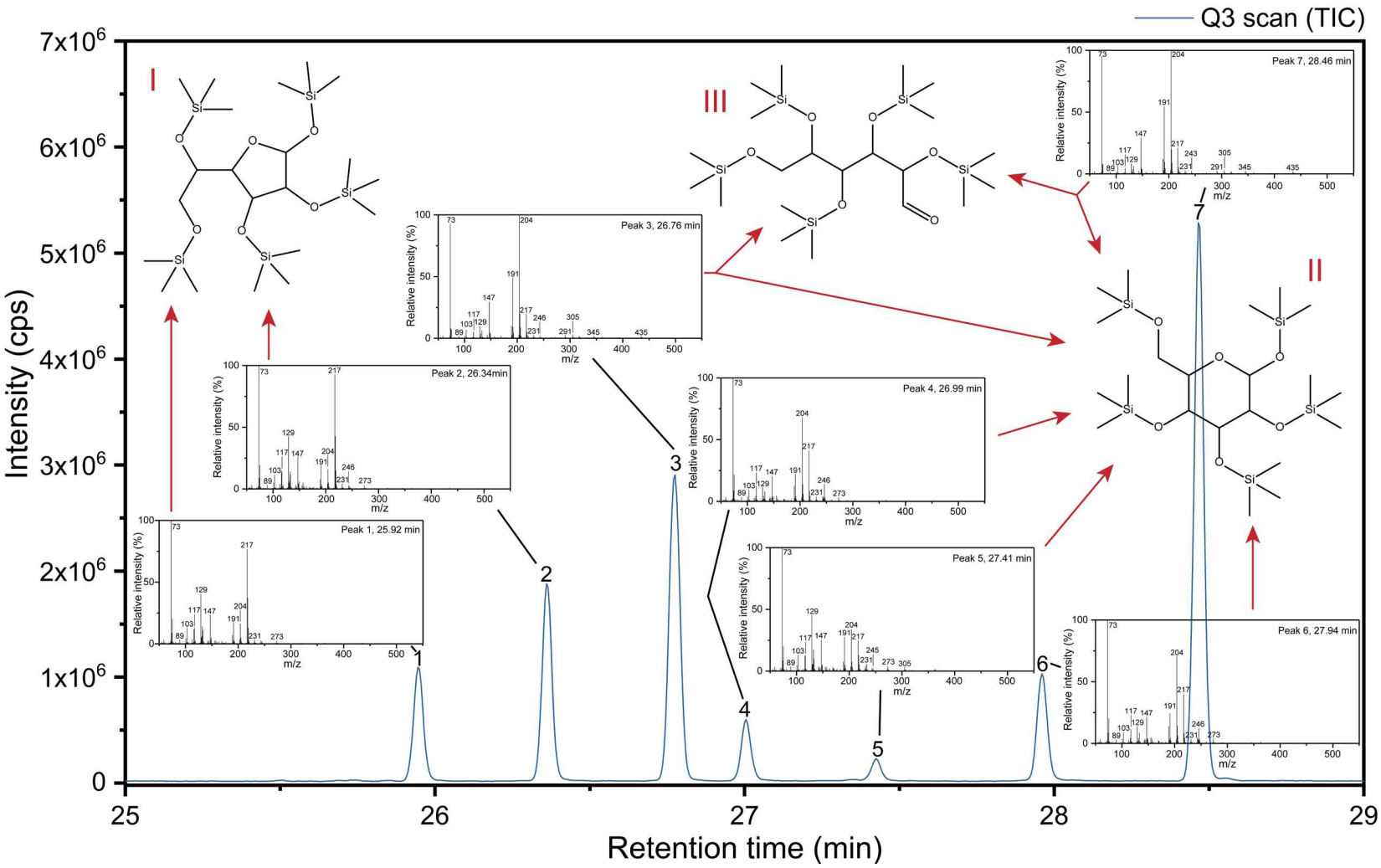
Supplementary Table 1: Retention times and MRM transitions of all analysed metabolites. The most intensive transition was used as quantification transition (transition 1). Transition 2 and 3 were considered as qualification transitions. Here the order is according to intensity (intensity of transition 2 > transition 3). Table is sorted by retention time.

Substance	Retention time (min)	Transition 1	CE (V)	Transition 2	CE (V)	Transition 3	CE (V)
Glycine 1	9.161	102.00>73.10	5	73.00>58.00	17	102.00>58.00	5
Pyruvate	9.625	174.00>89.10	5	174.00>99.10	5	174.00>74.10	17
L-norvaline (IS)	10.201	144.00>73.10	9	144.00>102.10	7	73.00>58.10	15
Glycine 2	13.655	174.00>73.10	11	174.00>86.10	9	174.00>100.10	7
L-cysteine	21.425	220.00>73.10	17	220.00>132.10	7	220.00>146.10	7
D-2-phenylglycine	21.582	178.00>73.10	11	73.00>58.10	17	178.00>58.10	25
L-arginine	21.685	142.00>73.10	7	73.00>58.00	15	142.00>105.00	19
D-(+)-xylose 1	22.257	103.00>73.10	7	217.00>73.10	17	217.00>129.10	9
D-(-)-arabinose 1	22.295	307.00>73.10	23	147.00>73.10	21	307.00>217.20	7
D-(-)-arabinose 2	22.454	217.00>73.10	17	307.00>73.10	25	217.00>129.10	7
D-(+)-xylose 2	22.567	103.00>73.10	7	307.00>73.10	25	307.00>103.10	11
L-glutamic acid	22.797	246.00>128.10	11	128.00>73.10	11	246.00>73.10	17
a-ketoglutaric acid	23.204	147.00>73.10	17	73.00>58.10	17	147.00>131.00	11
D-mannitol	26.004	319.00>73.10	23	217.00>73.10	19	319.00>129.10	11
D-(-)-fructose 1	26.154	217.00>73.10	19	307.00>73.10	29	217.00>129.10	9
D-(+)-mannose 1	26.215	319.00>73.10	23	319.00>129.10	11	205.00>73.10	17
D-sorbitol	26.297	319.00>73.10	23	217.00>73.10	17	319.00>129.10	11
D-(-)-fructose 2	26.486	217.00>73.10	17	307.00>73.10	23	217.00>129.10	9
D-(+)-glucose 1	26.681	319.00>73.10	25	319.00>129.10	11	205.00>73.10	19
Citric acid	26.913	273.00>73.10	19	273.00>183.10	9	347.00>147.10	23
L-lysine	27.298	156.00>73.10	15	174.00>73.10	17	174.00>86.10	9
D-(-)-a-(4-hydroxyphenyl)-glycine	27.492	266.00>73.10	19	266.00>194.10	17	73.00>58.10	15
myo-inositol	29.271	217.00>73.10	19	305.00>73.10	23	305.00>217.10	15
L-ascorbic acid	29.645	147.00>73.10	17	332.00>147.10	23	332.00>73.20	27
N-Acetyl-L-glutamine	33.238	156.00>73.10	11	215.00>156.10	7	215.00>73.10	23
D-glucose-6-phosphate 1	35.655	299.00>73.20	25	387.00>73.20	29	299.00>147.10	25
D-glucose-6-phosphate 2	35.843	299.00>73.20	25	387.00>73.10	29	299.00>147.10	21
Sucrose	38.620	361.00>73.10	25	361.00>169.10	11	217.00>73.10	19
D-Lactose 1	39.144	204.00>73.10	17	361.00>73.20	25	204.00>189.10	9
D-Lactose 2	39.363	204.00>73.10	17	361.00>73.20	25	204.00>189.10	9

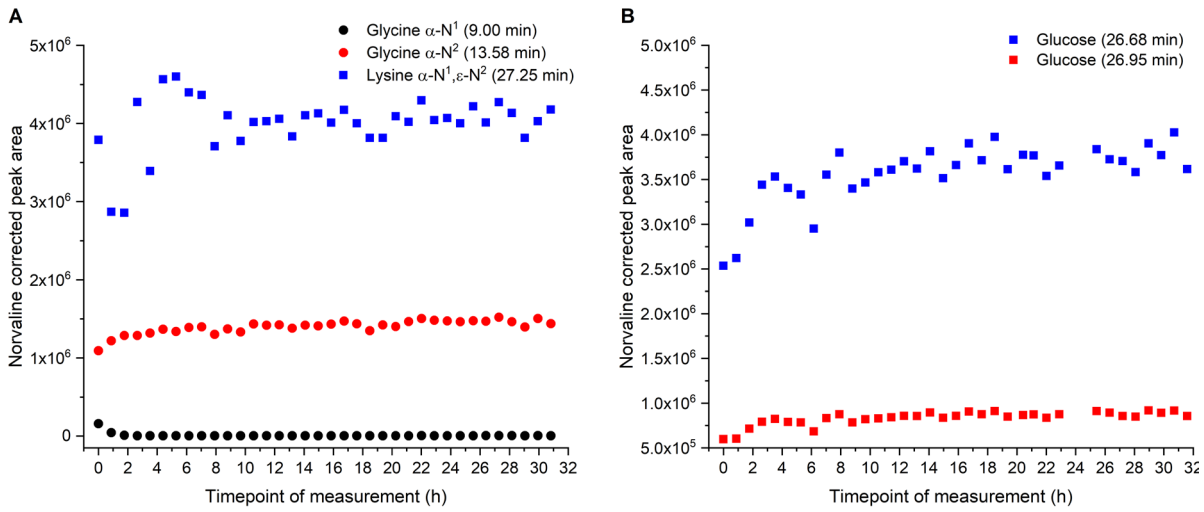
Supplementary Table 2: Obtained derivatives for all the analysed substances after MeOx/TMS derivatization at 37 °C for 90 min (MeOx) and 30 min (TMS).

Substance	MW[a]	Derivatives (NIST11 database entries)	MW[b]
Glycine	75.07	Glycine, N,N-bis(trimethylsilyl)-, trimethylsilyl ester	291.61
L-arginine	174.20	Ornithine, tri-TMS	348.71
L-cysteine	121.16	L-Cysteine, N,S-bis(trimethylsilyl)-, trimethylsilyl ester	337.70
L-glutamic acid	147.13	L-Glutamic acid, N-(trimethylsilyl)-, bis(trimethylsilyl) ester	363.68
L-lysine	146.19	L-Lysine, N2,N6,N6-tris(trimethylsilyl)-, trimethylsilyl ester	434.92
N-acetyl-L-glutamine	188.18	Trimethylsilyl N2-acetyl-N5-(trimethylsilyl)-L-glutaminate[c]	332.55
D-(-)-α-(4-hydroxyphenyl)-glycine	167.16	Benzeneacetic acid, alpha-[(trimethylsilyl)amino]-4-[(trimethylsilyl)oxy]-, trimethylsilyl ester[d]	383.71
D-2-phenylglycine	151.16	Glycine, N-phenyl-N-(trimethylsilyl)-, trimethylsilyl ester	295.53
Citric acid	192.12	1,2,3-Propanetricarboxylic acid, 2-[(trimethylsilyl)oxy]-, tris(trimethylsilyl) ester	480.85
L-ascorbic acid	176.12	L-Ascorbic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl)-	464.85
α-ketoglutaric acid	146.10	Pentanedioic acid, 2-(methoxyimino)-, bis(trimethylsilyl) ester	319.50
Pyruvate	110.04	Propanoic acid, 2-(methoxyimino)-, trimethylsilyl ester	189.29
D-(-)-arabinose	150.13	D-Arabinose, tetrakis(trimethylsilyl) ether, trimethylsilyloxime (1Z/1E)	467.90
D-(-)-fructose	180.16	D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime (1Z/1E)[e]	570.11
D-(+)-glucose	180.16	D-Glucose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z/1E)	570.11
D-(+)-mannose	180.16	D-Mannose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme (1Z/1E)	570.11
D-(-)-ribose	150.13	D-(-)-Ribose, tetrakis(trimethylsilyl) ether, trimethylsilyloxime (1Z/1E)	467.90
D-(+)-xylose	150.13	D-(+)-Xylose, tetrakis(trimethylsilyl) ether, trimethylsilyloxime (1Z/1E)	467.90
D-lactose	360.13	D-Lactose, octakis(trimethylsilyl) ether, methyloxime (1Z/1E)	948.80
Sucrose	342.30	Sucrose, octakis(trimethylsilyl) ether	919.75
D-glucose-6-phosphate	282.12	D-Glucose, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, o-methyloxime, 6-[bis(trimethylsilyl) phosphate] (1Z/1E)	722.27
D-mannitol	182.17	D-Mannitol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	615.26
D-sorbitol	182.17	D-Sorbitol, hexakis(trimethylsilyl) ether	615.26
Myo-inositol	180.16	Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	613.25

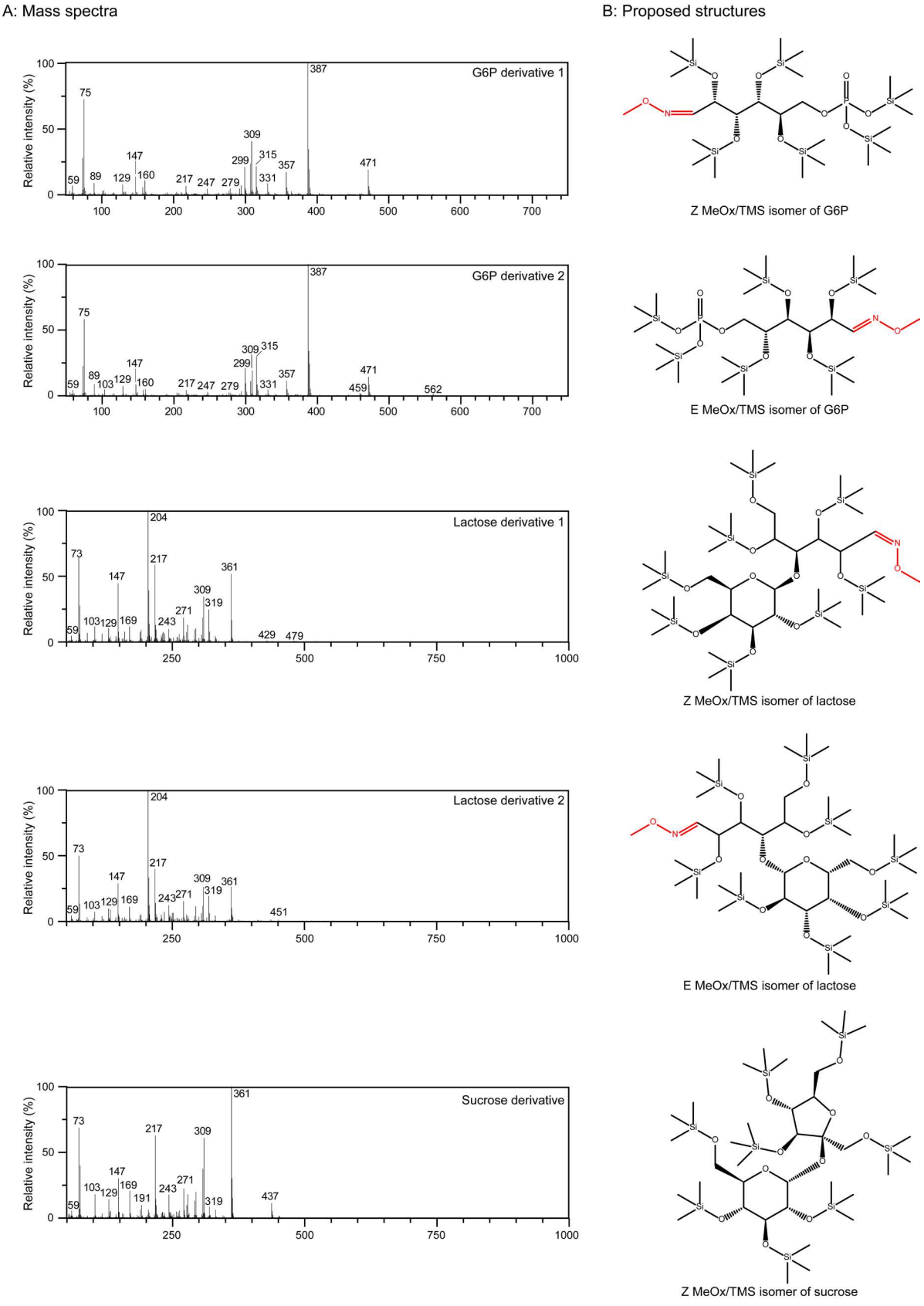
[a]…molecular weight of the substances in g/mol
[b]…molecular weight of the derivatives in g/mol
[c]…not found in database; manual identification using the mass spectrum; compound name from ChemDraw 15.1;
[d]…not found in database; manual identification using the mass spectrum; compound name from SciFinder;
[e]…Wiley9 database entry



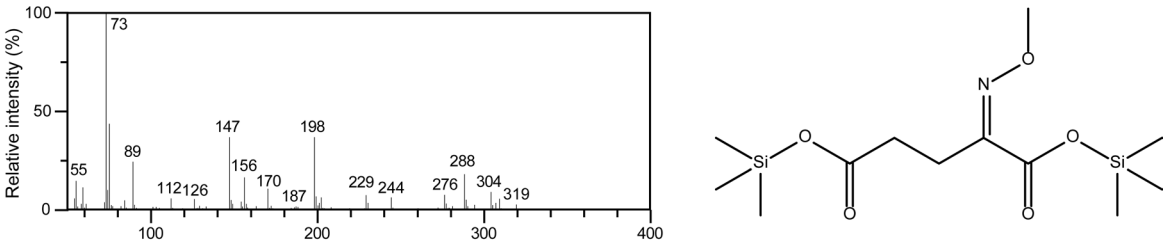
Supplementary Figure 1: GC-MS chromatogram of glucose after TMS including the corresponding mass spectra and proposed structures. Structure I represents α/β-1,2,3,5,6-pentakis-O-(trimethylsilyl)glucofuranose at 25.92 and 26.34 min, structure II 2,3,4,5,6-pentakis-O-(trimethylsilyl)glucose at 26.76 and 28.46 min and III α/β-1,2,3,4,6-pentakis-O-(trimethylsilyl)glucopyranose at 26.76, 26.99, 27.41, 27.94 and 28.46 min.



Supplementary Figure 2: Norvaline corrected peak areas of the MeOx/TMS derivatives of (A) glycine and lysine and (B) glucose over 32 hours after derivatization.



Supplementary Figure 3: Mass spectra (A) and structures (B) of E- and Z-MeOx/TMS isomers of G6P and lactose and of the MeOx/TMS derivative of sucrose. An accurate alignment of structures and mass spectra in the case of G6P and lactose is not possible at this point due to the lack of purified standards.



Supplementary Figure 4: Mass spectrum and structure of the MeOx/TMS derivative of α-ketoglutaric acid

Part II: A First Investigation of Tick Attachment Cement

1 Biological adhesives

1.1 Overview

Biological adhesives, produced from various animals, are a promising field of research due to their potential use in medicine. The problems of currently established adhesives in medicine is, that they either contain toxic substances, like cyanoacrylates or glutaraldehyde, or have weak bonding forces, like fibrin glues [101-103]. Thus, there is the need for new gluing materials, ideally based on biomimetic principles and biological degradable.

Biological attachment systems can be subdivided into several groups according the following principles: fundamental physical mechanism (to which the system operates), biological function of the attachment device and duration of the contact (permanent, temporary or transitory) [104]. The eight fundamental biological attachment mechanisms found are summarised in **Figure 5** [105]. However, biological systems may also apply various combinations of these mechanisms.

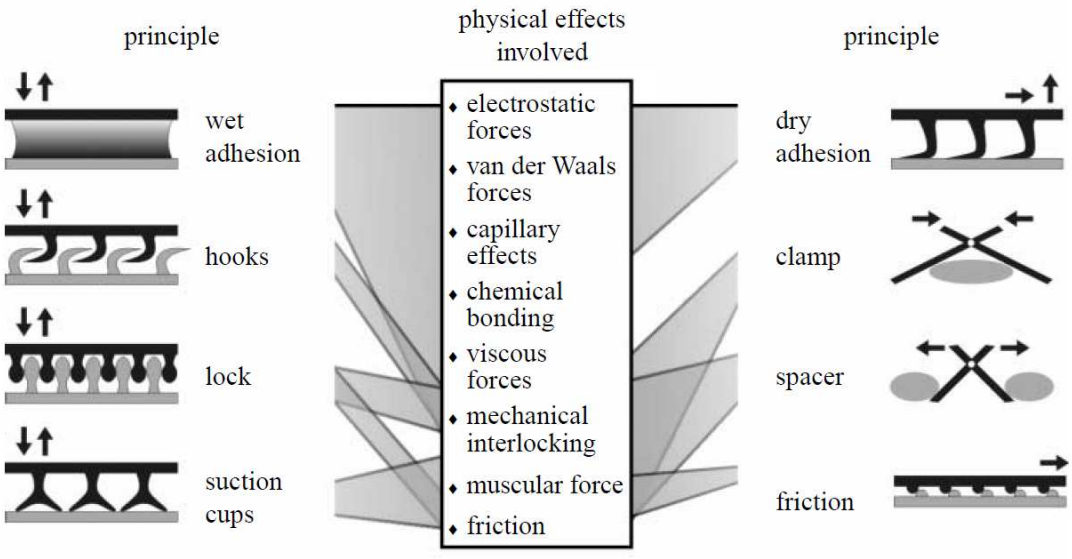


Figure 5: Fundamental principles of biological attachment systems and involved physical effects [104].

The attachment itself is required to fulfil a number of biological functions:

- position maintenance
- locomotion
- attachment to animal or plant host (e.g. for feeding)
- prey capture
- temporary attachment between body parts
- attachment to mating partner during copulation
- particle manipulation

The attachment often occurs in wet environments and withstands strong forces, e.g. underwater attachment of barnacles, mussels or sea urchins, or has the ability of rapidly developing strong contact forces, e.g. prey capturing of spiders. Furthermore, these biological adhesives are usually biodegradable, since they mainly consist of proteins, lipids or carbohydrates. Thus, the investigation of these glues is a very promising and interesting field of research.

In the following the biological adhesives of two animals, ticks and barnacles, will be discussed in detail. This selection is based on the fact that the dried adhesive of both is very similar in terms of hardness and solubility and furthermore both act in a humid environment. This suggests similarities in the adhesive's properties and thus makes a comparison very interesting.

1.2 Tick attachment cement

Ticks are small arachnids and belong to the subclass Acari (mites), the superorder Parasitiformes, the order Ixodida and the superfamily Ixodoidea. They live on their vertebrate host and feed on blood by penetrating into the skin with their teeth located on the hypostome of their mouthparts. Apart from feeding on their hosts, ticks do not need any nutrients from plants and can endure longer periods without any meal. This is accomplished by preventing desiccation and by relying on resources from a previous feeding [106]. In addition to that they have a notably long life cycle and can live for two or even three years [107].

The whole procedure of feeding refers to a complex of behavioural processes and can be resolved into nine main events:

1. Appetence (hunting or seeking a host)
2. Engagement (adherence to the skin of fur of the host)
3. Exploration (searching a suitable attachment site on the skin)
4. Penetration (insertion of the mouthparts)
5. Attachment (establishing feeding site)
6. Ingestion (taking up of blood and other fluids)
7. Engorgement (partial or complete meals of blood is taken)
8. Detachment (withdrawal of the mouthparts)
9. Disengagement (tick dropping of the host)

The focus of this work is on the event of attachment which is based on the secretion of a sticky substance called "tick cement" forming a cement cone. These adhesives, originating in the salivary glands, allow a firm adhesion and in addition to that protect the sensible mouthparts. Besides the attachment of the tick's mouthparts also other functions of the rapid hardening cement are suggested in literature, like the sealing of the feeding lesion or antimicrobial activity. A detailed summary of the suggested functions can be looked up in **Publication I** (p. 96).

In literature three tick families are reported: Nuttalliellidae (one species), Argasidae (193 species) and Ixodidae (702 species) [108]. Ticks of the family Argasidae lack of producing cement, possibly due to the fact that their feeding period is very short compared to ixodid ticks, whereas it is not known for the family Nuttalliellidae [109, 110]. Usually ixodid ticks feed for several days or even more than a week and require larger blood meals than argasids [111]. Depending on the species there are differences in the type of attachment to the host skin. **Figure 6** shows the formation of superficial cones of varying sizes with distinct flanges, the support of the inserted mouthparts or the infiltration of the host epidermis (*Dermacentor variabilis*). It was also found that there are differences between specific portions of the cement, like the core cement, which is deposited at the beginning, and the cortical cement, which is placed around the core cement [109, 112, 113]. Over the years different terminologies for the cement and cement portions evolved (details see **Publication I**, p. 96).

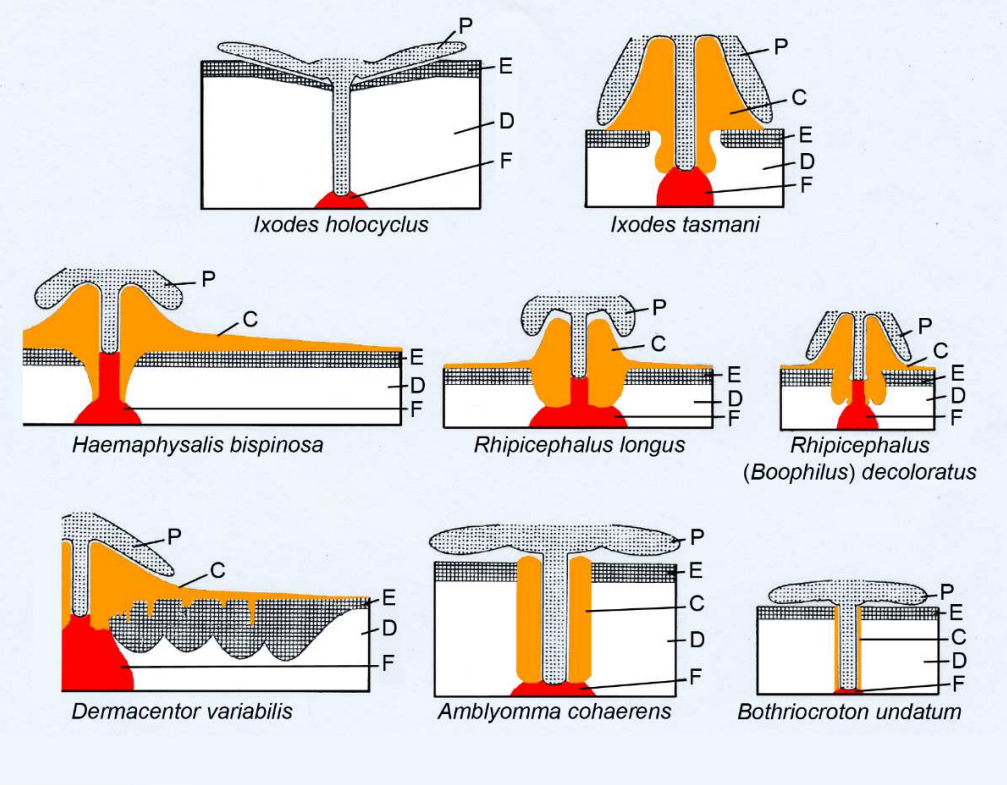


Figure 6: Examples of types of attachment of female ixodid ticks. *Ixodes holocyclus* is given as a comparison for a species not producing cement. Symbols: C, cement; D, dermis; E, epidermis; F, feeding pool; P, palps. [114]

Histochemistry revealed that the main components of tick cement are proteins, but it also contains carbohydrates, lipids, glyco- and lipoproteins [113, 115, 116]. Yet the process of hardening is unknown and proteins involved are poorly characterised. However, histological stains showed the presence of phenols or phenolic groups and phenol oxidase in salivary glands which could play a role in the hardening process [113, 117]. Biochemical analyses indicated the presence of high amounts of Leu, Ser, Tyr and especially Gly, but only low concentrations of His and Met [112].

These findings from literature could be also confirmed through amino acid analysis (see also **section 2.2**, **Protein hydrolysis followed by amino acid analysis**) carried out in this thesis, where for the first time cement collected from *Amblyomma hebraeum* and *Dermacentor marginatus* ticks was studied (**Manuscript III**, p.118).

The identification of proteins is more challenging than amino acid analysis, due to the poor solubility of the cement. The hydrolysis conditions used for amino acid analysis, which are usually concentrated acids or bases able to dissolve the cement, are far too harsh to bring the proteins into solution without destroying them. Additionally, the presence of various binding mechanisms may support the poor solubility [112, 113]. Therefore, moderate

solubilisation techniques have to be used, at the risk of incomplete solubilisation. But even the identification of solubilised proteins is not easy, since the current protein databases do not contain a lot of reviewed and thus confirmed protein entries of the tick species. Yet several proteins are known to be present in tick attachment cement, e.g. a 94 kDa protein detected from various species, a 20 kDa protein of *Amblyomma americanum* or a 15 kDa protein of *Rhipicephalus appendiculatus* named 64P [118-121]. Due to the fact that large amounts of Gly are present in tick cement several glycine rich proteins (GRP), containing more than 20% of Gly, could be found. It is hypothesised that GRPs stabilise and strengthen the cement cone causing also the water insolubility of the cement [122].

The presence of GRPs could be also confirmed by protein analysis of *Dermacentor marginatus* cement (see **section 2.1**, **Investigation of proteins** in **Part I: Method Development**). Details can be seen in **Manuscript IV** (p. 152).

1.3 Barnacle cement

Barnacles, marine sessile crustaceans, also produce a cement-like substance in order to attach to various underwater substrata (natural or manmade) like rocks, wood or ship hulls or even other living organisms [123]. The huge range of materials barnacles can attach to makes this secretion very special, but also problematic since barnacles attach to marine installations or cargo ships (bio fouling) [124]. Therefore, knowledge about the adhesive is needed for preventing this attachment or removing attached animals. Nevertheless, the secreted cement is promising for applications in medicine like tick cement. The biological effectiveness is highlighted by tensile strength measurements, which revealed that the tenacity of the barnacle adhesive is stronger than of limpets on slate or byssus pads of mussels on slate, both animals also producing a sticky substance, yet weaker than commercial dental adhesives [125-127].

Barnacles belong to the group of Cirripedia, which have a unique larval form: the cyprid. These cyprids use the secreted glue during the exploration phase to temporarily adhere to the substratum [128]. If a spot has been chosen, permanent glue is produced which hardens over the course of one to three hours forming “cement” [123]. Once attached the cyprid develops into the juvenile and later into an adult barnacle, which remains attached a lifelong [129]. The following figure shows an aggregation of *Lepas anatifera* barnacles and also attachment to a nylon rope.

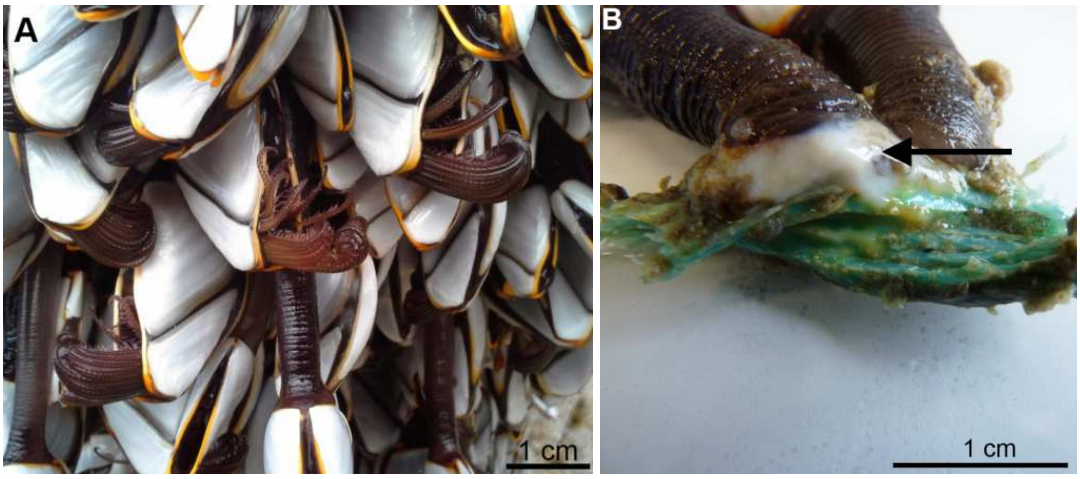


Figure 7: Aggregation of *Lepas anatifera* barnacles (A) and adhesive plaque of *Lepas anatifera* (black arrow) adhered to a nylon rope (B). [130]

In literature the cement layer between the calcareous base and the foreign substratum has been reported to be approx. 5 µm in thickness and the cement originates in the rounded or oval cement gland cells with a diameter of up to 200 µm [131, 132]. Furthermore, the adhesive has already been carefully characterised and two types can be differentiated: primary and secondary cement, based on the mode of formation. Primary cement is the material between the calcareous base and the substratum when the animal is naturally attached, whereas secondary cement is secreted after the barnacle has been removed from the substratum in order to reattach. Both types of cement have been found to mainly consist of proteins [133]. Moreover, the protein composition of both cement types seems to be highly similar, confirmed by amino acid analysis, peptide pattern comparison by cyanogen bromide protein fragmentation and SDS-PAGE and immunoblot analysis [133, 134].

The underwater attachment per se is proposed to be a multi-step process which only can be achieved if the requirements are fulfilled in the correct order and with proper timing [129, 135]. The multi-functionality of the cement, ranging from displacing seawater until curing to protection of the cement layer from water erosion and microorganisms, is shown in **Figure 8**. The variety of involved functions and processes indicate the presence of a multi-protein complex in which each protein has unique characteristics allowing the firm attachment of the animal.

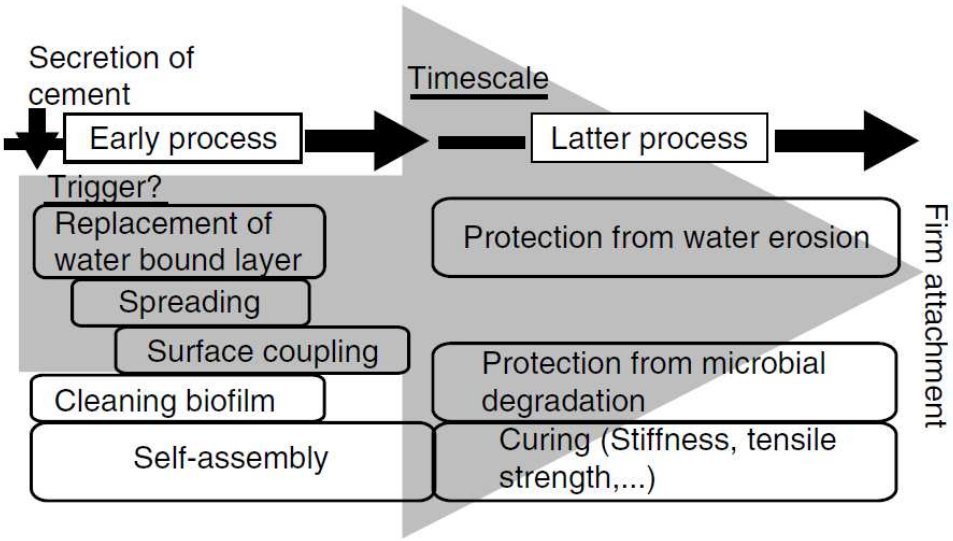


Figure 8: The functions of barnacle cement and involved processes during the attachment mechanism [129].

In addition to proteins, the primary component of barnacle cement, also small amounts of carbohydrates and lipids could be found to be present [136, 137]. In literature it can be found that the main amino acids present are Ser, Glu, Leu, Asp, Gly and Ala [133, 136, 138, 139]. However, interspecific differences were found.

Within this work it was possible to partly confirm literature findings by amino acid analysis (see also **section 2.2**, **Protein hydrolysis followed by amino acid analysis**) of *Lepas anatifera* cement (**Manuscript III**, p. 118). High amounts of Leu, Glu/Gln, Asp/Asn, Ala and Gly could be found, but also higher concentrations of Val and Ile were present. Compared to tick cement, in which Gly predominantly occurs, the concentrations are homogenously distributed. The differences in amino acid composition between ticks and barnacles as found in this work already suggests that the mechanism of attachment and gluing is different. This can be considered as a logical consequence of the fact that barnacles attach under water, whereas tick attachment takes place at the skin of their hosts, a less humid environment.

Barnacle cement contains more than 10 cement proteins (CPs), but not all are identified yet. Furthermore, the detailed functions are still not clear. The following table summarises proteins and their possible functions as identified so far. These proteins were exclusively identified by their apparent molecular weight, as estimated by SDS-PAGE but not by MS. The molecular weight information can be found in the corresponding protein name.

Table 6: Characteristics and functions assigned to proteins identified and studied in barnacle cement.

Proteins	Characteristics/possible functions	Ref.
CP-100k, CP-52k	Highly hydrophobic, low number of Cys residues (<2%) Providing an insoluble framework for other proteins	[140, 141]
CP-100k	Leu, Val, Ile and Ser (39% of the total), low number of Cys residues (<2%)	[142]
CP-68k	Glycosylated, dominated hydroxyl groups Ser, Thr, Ala and Gly (60% of the total) Priming and surface-coupling molecule	[129] [133] [129]
CP-20k	Abundance of hydrophilic and charged amino acids and Cys Abundance of Cys Interaction with metal ions Coupling agent to barnacle's own calcareous base	[143, 144] [145] [143] [144]
CP-19k	Ser, Thr, Gly, Ala and Val (67% of the total) No posttranslational modifications Coupling to foreign material surface	[146, 147] [147] [129, 147]
CP-16k	Lytic activity - protection from microorganisms	[148]

The major proteins found in barnacle cement are CP-100k, CP-52k and CP-68k. However, the different functions and properties of the proteins reveal the fact that this unique protein composition is necessary for barnacle attachment and settlement. Furthermore, the CPs are unique among underwater adhesive proteins (mussels, tubeworms) and no homologous proteins have been found in other species so far [149]. Clearly, more protein analysis and identification is essential to further understand the detailed mechanism of attachment.

2 Sample collection and preparation for bioadhesives

Until the early 1990s, information on tick attachment cement was primarily based on histological descriptions of host skin biopsies (details of historical perspectives on cement research can be found in **Publication I**, p. 96). The focus of cement research shifted in the last 40 years, as molecular biology-based techniques advanced from histological investigations to the identification of cement constituents, e.g. proteins. The first step, sample collection, is the most challenging one due to the size of the animals and the consequently little available sample amount. In the case of tick attachment cement two main methods can be found for cement collection: *in vivo* or *in vitro*. Since ticks only secrete their bioadhesive when feeding, cement can only be collected after their feeding process. This can be achieved by placing ticks on different animal hosts like cattle, chickens, rabbits or sheep for *in vivo* samples [122, 150, 151]. The problem is that the cement can be easily contaminated with proteins or other substances from the host hair or skin making further analysis very demanding. In order to diminish this problem special feeding systems, using artificial membranes, were developed as an alternative [122, 152-154]. These so called feeding units (**Figure 9**) allow to feed the ticks in a highly controlled environment and also the feeding material, typically bovine blood, can be easily maintained. However, contaminations with microorganisms still are possible, because of the lack of a host immune system, therefore, antifungal medication and antibiotics are added to protect the feeding system against infections. Furthermore, hair or pheromones can be added to the feeding units to mimic *in vivo* conditions in order to encourage the ticks to feed via the membrane. Finally, after several days of feeding the ticks can be removed and the cement cone can be collected and used for further analysis.

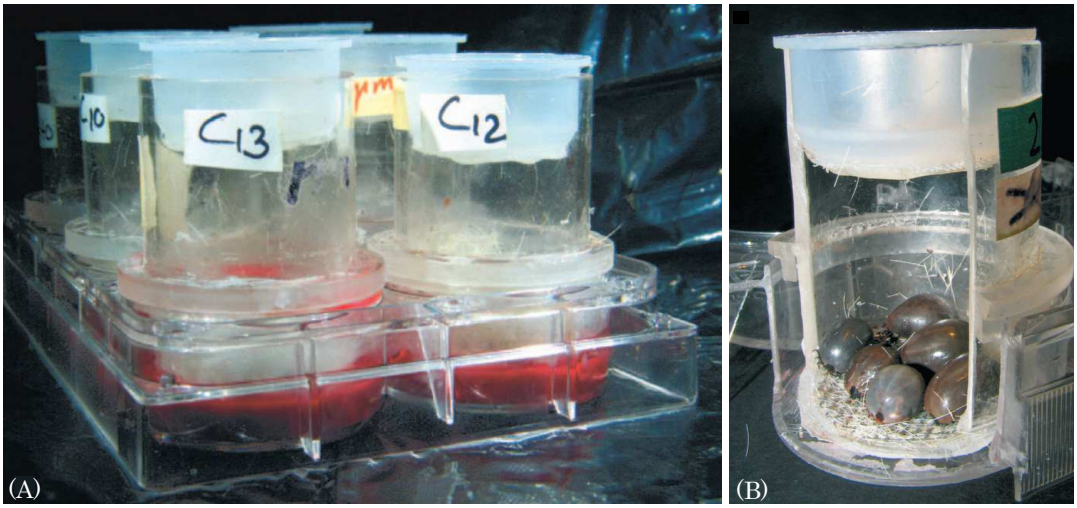


Figure 9: Artificial membrane feeding system. Six-well plate with feeding units over blood (A) and schema of a section through one feeding unit illustrating ticks feeding on blood through the membrane (B). [154]

The approach of using an artificial membrane feeding system was also used in this work for cement collection (see **Manuscript III**, p. 118 and **Manuscript IV**, p. 152).

The second bioadhesive, barnacle cement, which is discussed in this work, usually is collected from the wild where animals adhered to various substrata: glass, painted metal (data-buoys), rope or wood [140, 155]. After removing the attached animals from the substratum the cement sample can be scraped off from the calcareous base. Since the animals are difficult to find due to almost hidden localisation and the fact that there is only a very thin layer of cement, samples are not easy to access. However, it is also possible to raise cypris larvae from collected barnacles in the laboratory or to let adult animals settle on polymer films or glass plates [136, 156]. After days or a few weeks cement can be collected. Special attention has to be paid to avoid any possible organic/inorganic or environmental contamination during sampling, e.g. inorganic contaminations caused by small scrapings of the barnacle base during cement collection [133, 136]. Contaminations from the membranous base or the organic matrix of the base shell can be problematic and have to be considered since they can falsify the results of the analysis [157].

Collected cement samples in general are stored in a freezer at very low temperatures, e.g. -70 °C until further sample treatment. This inhibits protease activity which would cause sample destruction or information loss. Depending on the type of information one is interested in, different sample preparation protocols have to be considered. However, the insoluble characteristics of these biological adhesives makes sample preparation challenging. If not much is known about the binding mechanism of the secreted adhesives, general solubilisation methods and protocols can be used for solubilisation of proteins. The requirement of strong denaturing and/or reducing conditions for protein solubilisation of several biological adhesive systems was already shown and can be found in literature [158]. Details on protein solubilisation can be also found in **section 2.1**, **Investigation of proteins** (p. 25) in **Part I: Method Development**. In contrast to that, harsh solubilisation conditions like hot acids (HCl or MSA) or bases (NaOH, BaOH or LiOH), which are known to solubilise the adhesives very well, only can be used for amino acid analysis due to the destruction of the proteins' primary structure.
As already mentioned, biological adhesives are very complex samples, thus there is the need for high performance separation techniques like chromatography in order to reduce sample complexity. In addition to that the coupling of chromatographic methods to MS allows a highly sensitive and selective analysis as well as a high throughput and depth of coverage [159]. Thus, the hyphenation of chromatography and MS is an important key for

the investigation of complex biological samples like biological adhesives and offers tremendous opportunities (details on the methodologies can be found at the beginning of this thesis in **section 1**, **Principle methodology for analyte separation and detection**, p. 11).

After a successful method development (**Part I: Method Development**, p. 20) the techniques mentioned in this work were applied for the first investigation of tick and barnacle cement (see **Manuscripts III** and **IV**, p. 118 and p. 152, respectively). The focus of these manuscripts was on amino acid and protein analysis of the adhesives.

3 Manuscripts and Publications

In this section the manuscripts and publications related to the first investigation into tick attachment cement are presented.

Biological Reviews (2018), 93, pp. 1056-1076

Publication I – Tick attachment cement – reviewing the mysteries of a biological skin plug system

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Highlights of this publication

- Historical perspectives on tick cement research
- Biological significance, functions and deposition of the cement
- Comparison of the cement with other biological adhesives
- Potential applications of tick attachment cement

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Tick attachment cement – reviewing the mysteries of a biological skin plug system

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ABSTRACT

The majority of ticks in the family Ixodidae secrete a substance anchoring their mouthparts to the host skin. This substance is termed cement. It has adhesive properties and seals the lesion during feeding. The particular chemical composition and the curing process of the cement are unclear. This review summarizes the literature, starting with a historical overview, briefly introducing the different hypotheses on the origin of the adhesive and how the tick salivary glands have been identified as its source. Details on the sequence of cement deposition, the curing process and detachment are provided. Other possible functions of the cement, such as protection from the host immune system and antimicrobial properties, are presented. Histochemical and ultrastructural data of the intracellular granules in the salivary gland cells, as well as the secreted cement, suggest that proteins constitute the main material, with biochemical data revealing glycine to be the dominant amino acid. Applied methods and their restrictions are discussed. Tick cement is compared with adhesives of other animals such as barnacles, mussels and sea urchins. Finally, we address the potential of tick cement for the field of biomaterial research and in particular for medical applications in future.

Key words: ticks, Ixodidae, blood feeding, cement plug, artificial feeding, bioadhesives, salivary glands.

CONTENTS

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I. INTRODUCTION

Numerous animals produce chemical adhesives. Not surprisingly, the different lifestyles of these animals have led to the evolution of adhesives with different chemical compositions and properties. Remarkably, these gluing substances can solidify rapidly and bond to diverse surfaces, even under water (Flammang & Santos, 2015). Additionally, they are biodegradable after different periods of time and, due to their biological origin, in most cases they are also biocompatible.

Due to these properties, biological glues have significant potential for special applications, particularly in medicine but also for industry (Stewart, Ransom & Hlady, 2011). Nevertheless, only a few adhesives have been studied in detail, such as the byssus threads of mussels (Qin *et al.*, 2016), barnacle cement (Kamino *et al.*, 2000), and the glue used by Sandcastle worms (Zhao *et al.*, 2005). Most of these aquatic organisms use glues based on modified amino acid side chains, especially phosphorylated serines and hydroxylated tyrosines [e.g. 3,4-dihydroxyphenylalanine (DOPA)] (Stewart *et al.*, 2011). However, for the majority of other biological adhesives the composition and bonding mechanisms are largely unknown.

One example is a substance called 'cement', which is produced by ticks to anchor their mouthparts firmly into the skin tissue of their hosts during a blood meal. The natural function of this cement suggests that this material might have adhesive properties that could potentially be useful in the development of medical tissue glues or sealants. Here, we summarize current knowledge about the morphology, composition and mechanisms of attachment cement in ticks.

Ticks (Ixodida) are subdivided into three families: Nuttalliellidae (one species), Argasidae (193 sp.) and Ixodidae (702 sp.) (Guglielmone *et al.*, 2010). During their life cycle, they develop over successive egg, larva and nymph (up to eight in Argasidae) stages to adults (Oliver, 1989; Apanaskevich & Oliver, 2014). All species are temporary ectoparasites feeding obligatorily on the blood of vertebrates (Coons & Alberti, 1999). During feeding, the blood is ingested from a cavity in the host tissue (feeding pool or lesion) and the animals alternate between blood uptake and the injection of saliva into it (Sonenshine, 2005).

The production of attachment cement is known in the family Ixodidae, whereas it seems to be absent or at least uncommon in Argasidae (Binnington & Kemp, 1980; Kemp, Stone & Binnington, 1982). There are no reports of cement production in *Nuttalliella namaqua* Bedford, 1931, the single species in the family Nuttalliellidae (Mans *et al.*, 2012). The presence of cement seems to be related to different tick feeding habits; nymphs and females of *Nuttalliella namaqua* (Mans *et al.*, 2011) and most life stages in the Argasidae complete their blood meal within minutes to hours (Oliver, 1989; Apanaskevich & Oliver, 2014) and therefore may not need additional anchorage. Furthermore, the Argasidae enter the host skin deeply (Sauer *et al.*, 1995) with well-developed mouthparts (Binnington & Kemp, 1980) and usually become active when their host animals are resting or sleeping in their nests or burrows (Oliver, 1989), perhaps making cement production unnecessary. Exceptions might be found in larval stages of some genera in Argasidae and the larvae of *Nuttalliella namaqua* (Mans *et al.*, 2012), which take blood meals over several days (Oliver, 1989; Mans *et al.*, 2012; Apanaskevich & Oliver, 2014). However, to date, cement has only been reported for feeding larvae of *Argas pusillus* Kohls, 1950 (Stiller & Ranchitham, 1975), although this observation is not undisputed (Kemp *et al.*, 1982). It is likely that larval stages of the Argasidae do not need cement because the majority of this family is nidicolous (i.e. live in nests or burrows of their hosts) and therefore easily relocate a host after incidential detachment.

In contrast to Argasidae and Nuttalliellidae, long feeding periods are typical for all blood-feeding life stages in Ixodidae, usually taking several days or even more than a week (Oliver, 1989). Accordingly, cement is thought to be produced in all genera of this family with the only known exceptions being some species in the genus *Ixodes* (Kemp *et al.*, 1982).

II. HISTORICAL PERSPECTIVE ON CEMENT RESEARCH

The attachment cement of ticks was first described in the early 20th century, when it was termed 'cement-like substance' (Cowdry & Danks, 1933) or 'homogeneous eosinophilic mass' (i.e. with a strong affinity to acid dyes like eosin) (Hoeppli & Feng, 1931). Such structures around the mouthparts were histologically reported in skin biopsies from attached ixodid ticks on a variety of host animals (e.g. mice, hamsters, guinea pigs, sheep, dogs) (Hoeppli & Feng, 1931; Foggie, 1959; Gregson, 1960; Saito & Ohara, 1961; Theis & Budwiser, 1974).

Studies comparing the attachment of different tick species revealed differences in the position and shape of cement formations, as well as the insertion depth of the mouthparts (Saito & Ohara, 1961; Moorhouse, 1969). In agreement with these findings, genus-specific patterns of attachment were distinguished (e.g. '*Ixodes* type' and '*Haemaphysalis* type') (Moorhouse, 1969). Among species of the genus *Ixodes* attachment types were found to be so variable that they were further subdivided (e.g. '*persulcatus* type', '*japonensis* type' '*Ixodes* group 1', '*Ixodes* group 2'). In species of this genus cement could be totally absent, restricted to the area around the mouthparts within the host tissue, or additionally deposited on the skin surface (Saito & Ohara, 1961; Moorhouse, 1969).

For descriptive purposes, new terminologies (Table 1) were introduced for specific portions of cement (e.g. 'perirostral cement'; Saito & Ohara, 1961). Some of these terms are related to the position of a cement portion only, while others also describe a particular composition (e.g. lipoprotein for the 'internum'; Moorhouse, 1969).

Despite the frequent observation of cement, opinions on its nature and origin differed widely with some suggesting that cement was a host product. For instance, 'conical

Table 1. Terminology for cement portions

Terminology	Cement portion	References
Core cement	First portion of cement deposited at the attachment site, 'internum' (see below)	Kemp *et al.* (1982)
Cortical cement	Portion of cement laid down around the core cement and invading between the layers of the stratum corneum, 'cortex' (see below)	Binnington & Kemp (1980)
Internum	One of the two main portions of cement which consists of lipoprotein	Moorhouse & Tatchell (1966)
Cortex	One of the two main portions of cement which consists of carbohydrate-containing protein (in *R. microplus*)	Moorhouse & Tatchell (1966)
External cement	Cement portion deposited on the skin surface, comprising a conical part (or cone) surrounding the mouthparts of the tick and lateral wing-like extensions	Chinery (1973)
Internal cement	Cement portion below the skin surface, further separated into an inner and outer zone The compact inner zone consists of a tapering tube and the outer zone forms strands which are intermeshed with the fibrils of the surrounding dermal tissue	Chinery (1973)
Primary cement	Cement portion laid down on the first day of attachment, the cortex and internum can be clearly distinguished; the cement around the mouthparts	Binnington & Kemp (1980) and Moorhouse & Tatchell (1966)
Secondary cement	Portion of cement laid down approximately 24 h before the final engorgement process; cortical material is added to the base of the original cone extending it more deeply into the feeding cavity; the cement which is secreted into the feeding lesion	Binnington & Kemp (1980) and Moorhouse & Tatchell (1966)
Perirostral cement	Portion of cement laid down around the hypostome and cheliceral shafts, linked with the connective fibres in the corium of the host skin.	Saito & Ohara (1961)
Cover cement	Portion of cement which surrounds the greater part of the perirostral cement and covers the skin surface at the site of infestation	Saito & Ohara (1961)

or sleeve-like papillae' were described macroscopically on preserved tick-infested skin samples (*Dermacentor* sp., *Amblyomma* sp., *Rhipicephalus* sp.). These papillae were interpreted as outgrowths of the skin, probably resulting from attempts by the host to engulf the parasite (Snodgrass, 1948). Based on histological observations from other tick species [*Ixodes ricinus* (Linnaeus, 1758), *I. trianguliceps* Birula, 1895], it was also suggested that the collagen in the host skin aggregates around the tick's mouthparts to form a tight-fitting sheath, allowing firm attachment of the tick (Arthur, 1970, 1973; Whitwell, 1978).

Among those in favour of a tick-derived origin, several sources were discussed: regurgitated (back flowing) material from the gut, the coxal glands and the salivary glands. The idea that cement was regurgitated fluid from the gut was rejected due to the absence of material with similar histological staining properties in the digestive tract (Cowdry & Danks, 1933). Coxal glands also were ruled out (Hoeppli & Feng, 1931; Cowdry & Danks, 1933), because secretory activity could not be observed, macroscopically nor in histological sections (Hoeppli & Feng, 1931). Indeed, coxal glands are characteristic of non-cement-producing Argasidae but are absent in cement-producing Ixodidae (Chinery, 1973). Salivary glands were suggested to be the most likely source of the cement (Hoeppli & Feng, 1931; Gregson, 1960; Moorhouse & Tatchell, 1966; Moorhouse, 1969; Chinery, 1973): they become highly active during feeding and contain large amounts of secretory products (Cowdry & Danks, 1933), portions of the salivary glands react to histological staining in a similar way to the cement (Moorhouse & Tatchell, 1966; Moorhouse, 1969; Chinery, 1973), and a rapidly hardening fluid is secreted between the mouthparts of *Dermacentor andersoni* Stiles, 1908 following attachment to mouse ears (Gregson, 1960). Some evidence did challenge a salivary gland origin, for example intradermal injections of whole gland extracts did not produce cement-like structures in laboratory animals (Foggie, 1959). However, an increasing number of reports on similar reactions of cement and intracellular granules of certain salivary gland cells to histochemical dyes (Coons & Roshdy, 1973; Gill & Walker, 1988), the absence of such cells and their products in ticks that do not secrete cement (Roshdy & Coons, 1975; Binnington & Kemp, 1980) and findings that the gland cells and the cement contain similar immunoreactive polypeptides (Jaworski *et al.*, 1992) consolidated the salivary glands as the production site of cement.

Until the early 1990s, information on cement was primarily based on histological descriptions of host skin biopsies. Improved technologies paved the way for the modern molecular biological techniques that now dominate research on salivary glands and their products. Cement proteins with antigenic properties (e.g. 64P) were identified, steering research towards anti-tick vaccines (Mulenga *et al.*, 1999; Bishop *et al.*, 2002; Trimnell, Hails, & Nuttall, 2002; Trimnell *et al.*, 2005; Zhou *et al.*, 2006). The establishment of complementary DNA (cDNA) libraries allowed the comparison of putative

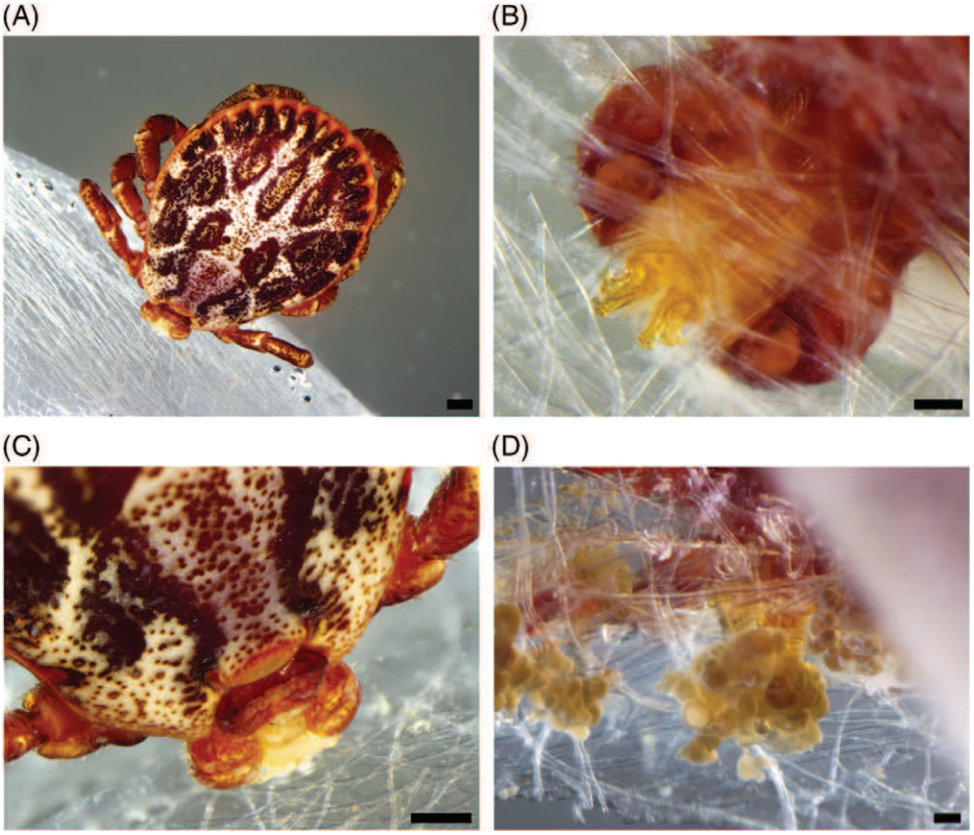

Fig. 1. Artificial feeding of ticks. (A) *Dermacentor marginatus* (Sulzer, 1776), male attached to a silicone membrane. (B) Chelicerae visible after cutting through the membrane. (C) Cement deposition between the palps at the upper side, and (D) at the underside of the membrane. Scale bars: A, C = 300 μm; B, D = 100 μm.

cement proteins in different tick species showing the variety in salivary gland composition (Maruyama *et al.*, 2010). Nevertheless it became clear that the sheer quantity of proteins and other products of the salivary glands (Alarcon-Chaidez, 2014) make assignment to particular functions such as cement formation difficult. To address this problem, temporary expression patterns of the salivary glands were analysed (Radulovic *et al.*, 2014; Kim *et al.*, 2016; Bullard, Williams & Karim, 2016), showing which salivary products were produced at certain points during feeding. These subsets of proteins were then tested for their functions by specific inactivation in living ticks and verification in feeding trials (Kim, Curran & Mulenga, 2014). However, some proteins of salivary glands have very similar nucleotide sequences, hampering efforts at specific silencing (Bullard *et al.*, 2016).

Another approach in cement analysis was to obtain material free of host skin tissue (Bullard *et al.*, 2016) by *in vitro* feeding of ticks on artificial membranes (Fig. 1). Feeding on animal-derived and artificial membranes is well established for the non-cement-producing Argasidae but attempts to transfer these systems to Ixodidae initially failed because of more sophisticated requirements for mimicking specific host cues for acceptance and attachment to such membranes. The long feeding periods of Ixodidae present an additional challenge, demanding several blood changes per day and careful monitoring and chemical additives to prevent microbial growth (Kuhnert, 1996). Silicone membranes have been used successfully to feed adults of *Dermacentor nuttalli* Olenev, 1928 and *Hyalomma excavatum* Koch, 1844 (formerly *H. anatolicum excavatum*) (Habedank, Hiepe & Montag, 1994) and all life stages of *Amblyomma hebraeum* Koch, 1844 (Kuhnert, Diehl & Guerin, 1995) and *Ixodes ricinus* (Kroeber & Guerin, 2007). Even though the technique must be adapted and modified for use with each tick species according to the length of the mouthparts (Kroeber & Guerin, 2007), examples of successful feeding of other species such as *Rhipicephalus sanguineus* Latreille, 1806, *Hyalomma dromedarii* Koch, 1844 and *H. anatolicum* Koch, 1844 (formerly *H. anatolicum anatolicum*) (Tajeri & Razmi, 2011; Fourie *et al.*, 2013) suggest this is a useful approach for cement research (Fig. 1). Compared to *in vivo* feeding, this method allows monitoring the progress of cement deposition and contamination-free collection of samples (Bullard *et al.*, 2016).

III. BIOLOGICAL SIGNIFICANCE AND FUNCTIONS OF THE CEMENT

Attachment cement is a rapidly hardening substance produced by the salivary glands of Ixodidae in preparation

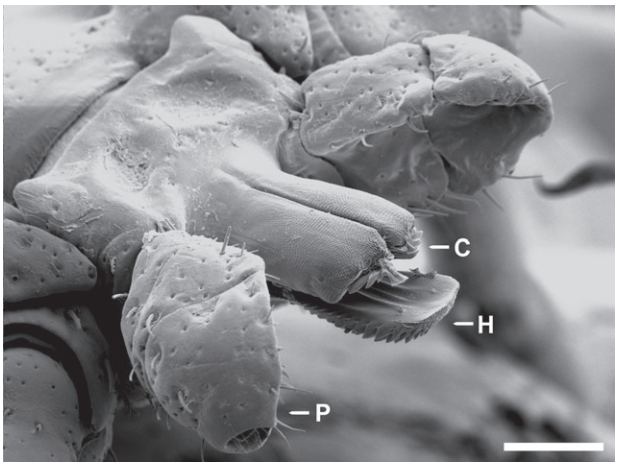

Fig. 2. Mouthparts of a female *Dermacentor marginatus*. Between two palps (P) there are paired chelicerae (C) and the hypostome (H). The chelicerae are extendable and cut into the host tissue during attachment. The hypostome acts dorsally as channel for saliva and blood, ventrally there are rows of teeth anchoring the tick to the cement and host tissue. The palps remain at the skin surface and fulfil sensory functions. Scale bar = 200 μm.

for and during feeding. Before cement secretion is finished, attached ticks can easily be pulled out of the skin (Sonenshine, 2005), but in manual tests considerable force is required to detach a tick after cement formation is completed (Gregson, 1960; Moorhouse & Tatchell, 1966). This demonstrates the anchoring function of the cement by strengthening the attachment of the ticks' mouthparts (Fig. 2) to the host. An effective form of attachment is essential for Ixodidae due to long feeding periods, during which the tick is at risk from host movement and scratching.

Besides its anchorage function, the deposited cement also fills any gaps between the inserted mouthparts and the host skin (Fig. 1). This sealing prevents loss of fluids and increases the effectiveness of the muscular pharynx, which is responsible for blood uptake (Saito & Ohara, 1961; Coons & Alberti, 1999). Intense bleeding at the feeding sites of *Amblyomma americanum* (Linnaeus, 1758) after silencing presumed cement compounds (Kim *et al.*, 2014) corroborates this assumption.

In addition, it was reported that tick cement might have antimicrobial properties (Alekseev *et al.*, 1995), but it remains unknown if these effects are derived from cement compounds themselves or from other salivary compounds in the cone (Francischetti *et al.*, 2009). A related function might be the exclusion of bacteria on the host skin surface from the feeding pool (P.M. Guerin, personal communication). The suggested functions of cement are summarized in Table 2.

Cement production probably provides other advantages. The adhesive substance allows them to attach to a host with minimal invasion of the mouthparts into the host tissue, which are kept in place by the superficial cement cone. It has been noted for *Haemaphysalis* sp., which have this type of attachment, that the host skin is less damaged and that the healing process progresses more rapidly than for *Ixodes* species (Saito, Ohara & Unagami, 1960). Species of the latter genus in this study produced no, or only small cement deposits at the host skin surface (Saito & Ohara, 1961). It might be the case that host animals are more likely to develop defence mechanisms against tick species causing more damage. This might represent a selective advantage for tick species which invest into deposits of cement on the host skin surface instead of long mouthparts for secure attachment.

It is well known that ticks are able to reattach following involuntary detachment from hosts (Gregson, 1960; Jaworski *et al.*, 1992; Wang, Henbest & Nuttall, 1999; Weiss & Kaufman, 2004). Cement production allows superficial attachment of ticks to the host and it might be speculated that this is advantageous during grooming by the host to remove the parasite. Gregson (1960) reported that the cement cone is usually pulled away with the mouthparts, as observed for *Dermacentor andersoni*. The cement might therefore function to protect the tick mouthparts during forceful detachment.

IV. THE SEQUENCE OF CEMENT DEPOSITION

Throughout this review, we will use the terms 'core' and 'cortical cement' introduced by Kemp *et al.* (1982) (see Table 1).

Cement secretion by ixodid ticks starts within 5–30 min of the insertion of the mouthparts into the host skin (Kemp *et al.*, 1982). The first portion of secretion is the core cement, which hardens rapidly. About 24 h later a second portion, the cortical cement is secreted, which solidifies more slowly (Kemp *et al.*, 1982).

In *Rhipicephalus* (formerly *Boophilus*) *microplus* (Canestrini, 1888) a distinct core and cortical cement can be observed about 24 h after attachment. With continued feeding and growth of the cement the two portions become difficult to distinguish by histochemistry (Moorhouse & Tatchell, 1966). One possible explanation might be that the cortical cement is able to enter the core cement through small perforations (Kemp *et al.*, 1982). Meanwhile, the cortical cement also spreads over and penetrates into the host skin close to the feeding site (Kemp *et al.*, 1982) where it fills gaps, resulting in firm adhesion of the feeding tick. In general, cement deposition ceases after a few days of attachment (Kemp *et al.*, 1982). However, some reports indicate that further cement can be deposited as secondary cement during the final stages of feeding (Moorhouse & Tatchell, 1966; Moorhouse, 1969).

In the three-host tick (i.e. the host is left after the blood meal and each life stage has to seek a new host) *Haemaphysalis spinigera* Neumann, 1897, a species lacking secondary cement production, it was reported in females that cement production is almost complete after 36 h (Chinery, 1973). At feeding sites of female *Rhipicephalus sanguineus* no substantial increase in the amount of cement was observed after 24 h of attachment (Theis & Budwiser, 1974); however, secondary cement production was observed in another report on this species (Moorhouse, 1969).

Table 2. Suggested functions of tick cement cones during feeding

Function	References
Firm attachment to the host	Coons & Alberti (1999), Sauer *et al.* (1995) and Theis & Budwiser (1974)
Sealing of the feeding lesion, to prevent the loss of fluids, enhance blood uptake and prevent the entry of air	Coons & Alberti (1999) and Saito & Ohara (1961)
Completing the channel formed by the mouthparts and directing the saliva more effectively into the host tissue	Arthur (1970); Tatchell (1969)
Obtaining blood (and other host fluids) from skin layers located much deeper than the distal ends of the mouthparts	Balashov (1984)
Blocking the expansion of the feeding pool into the tissues lateral to the mouthparts to maintain effective attachment	Arthur (1970); Tatchell (1969)
Preventing host immune molecules from coming into contact with the tick mouthparts	Binnington & Kemp (1980), Bishop *et al.* (2002) and Mulenga *et al.* (1999)
Confining the feeding pool within the host to prevent bacterial contamination from the host skin surface	P. M. Guerin (personal communication)
Antimicrobial activity	Alekseev *et al.* (1995) and Francischetti *et al.* (2009)

Secondary cement is similar to cortical cement in its composition (Moorhouse & Tatchell, 1966) and adds material to the base of the original cone. As a result, the cement cone extends deeper into the feeding cavity and forms a flange (Binnington & Kemp, 1980). Some ticks (e.g. *Haemaphysalis* and *Dermacentor* sp.) have strong lateral cement flanges on the skin surface with little secondary cement production, while in others (e.g. *Rhipicephalus* spp.) there is an internal flange of secondary cement (Moorhouse, 1969).

For *Rhipicephalus microplus*, a one-host tick (i.e. all parasitic stages feed on the same host individual) secondary cement production was observed on the third, 11th and 17th day for larvae, nymphs and females, respectively (Moorhouse & Tatchell, 1966), following initial attachment at the larval stage. It was suggested that these additional depositions of cement help the tick to remain securely attached following extension of the feeding cavity below the initial cone (Moorhouse & Tatchell, 1966), but they probably also protect the tick from detachment during the moulting process which also occurs on the host. Whereas moulting progresses uniformly all over the body of three-host ticks, in nymphs of *R. microplus* this process begins earlier in the legs than in the mouthparts, which are still involved in feeding. Ticks of this life stage begin to become immobile on the 11th day following attachment (Jorgensen & Kemp, 1986), i.e. corresponding to the period of secondary cement production in nymphs (Moorhouse & Tatchell, 1966). However, no secondary cement deposition was found in histological studies of the three-host ticks *Haemaphysalis spinigera*, *Hyalomma anatolicum* and *R. appendiculatus* Neumann, 1901 (Chinery, 1973; Walker & Fletcher, 1986; Gill & Walker, 1988).

V. THREE SALIVARY GLAND CELLS AS ORIGIN OF THE CEMENT

There is now general agreement that cement compounds originate from the tick paired salivary glands (Alarcon-Chaidez, 2014; Sonenshine & Roe, 2014) which undergo remarkable structural changes to act both as a secretory organ and fluid-transport system during feeding (Bowman, Ball & Sauer, 2008). These complex glands resemble clusters of grapes (Nicholson *et al.*, 2009) with a single structural unit termed an acinus. The salivary glands consist of a large number of acini with three to four different types of secretory regions and a supporting duct system. In female *Rhipicephalus appendiculatus* each branch of the paired glands consists of about 1400 acini and there are about 1350 in males (Walker, Fletcher & Gill, 1985). In general, agranular (type I) and granular (types II, III and IV) acini can be distinguished. With the exception of type IV, each acinus type contains different groups of cells (Fig. 3). Cement seems to originate from the cell class a- of type II acini, and from d- and e-cell classes in type III acini (Sauer *et al.*, 1995; Coons & Alberti, 1999). Early observations of type IV acini were all from cement-producing tick species (Fawcett, Binnington & Voigt, 1986), but there are no histochemical indications that they contribute material to the cement cone (Furquim, Bechara & Camargo Mathias, 2010). Indeed, type IV acini are also found in males of *Ixodes holocyclus* Neumann, 1899, a species which does not produce cement (Moorhouse, 1969) and in which males do not feed (Stone & Binnington, 1986).

Within their respective acini, a-, d- and e-cells are located close to the junction with the salivary duct system (Fig. 3) (Kemp *et al.*, 1982). These cells contain secretory granules before attachment and the period of their secretion corresponds with that of cement deposition (Kemp *et al.*, 1982; Gill & Walker, 1988). Similar staining of the secretory granules and the deposited cement in detailed histochemical studies of *Hyalomma anatolicum* (Gill & Walker, 1988) supports the hypothesis that a-, d- and e-cells are the origin of cement. Jaworski *et al.* (1992) localized a polypeptide in the d- and e-cells in type III acini of the salivary glands of female *Dermacentor variabilis* (Say, 1821) by immuno-staining which also appeared to be part of the cement (type II acini were not examined). The labelling in d-cells was

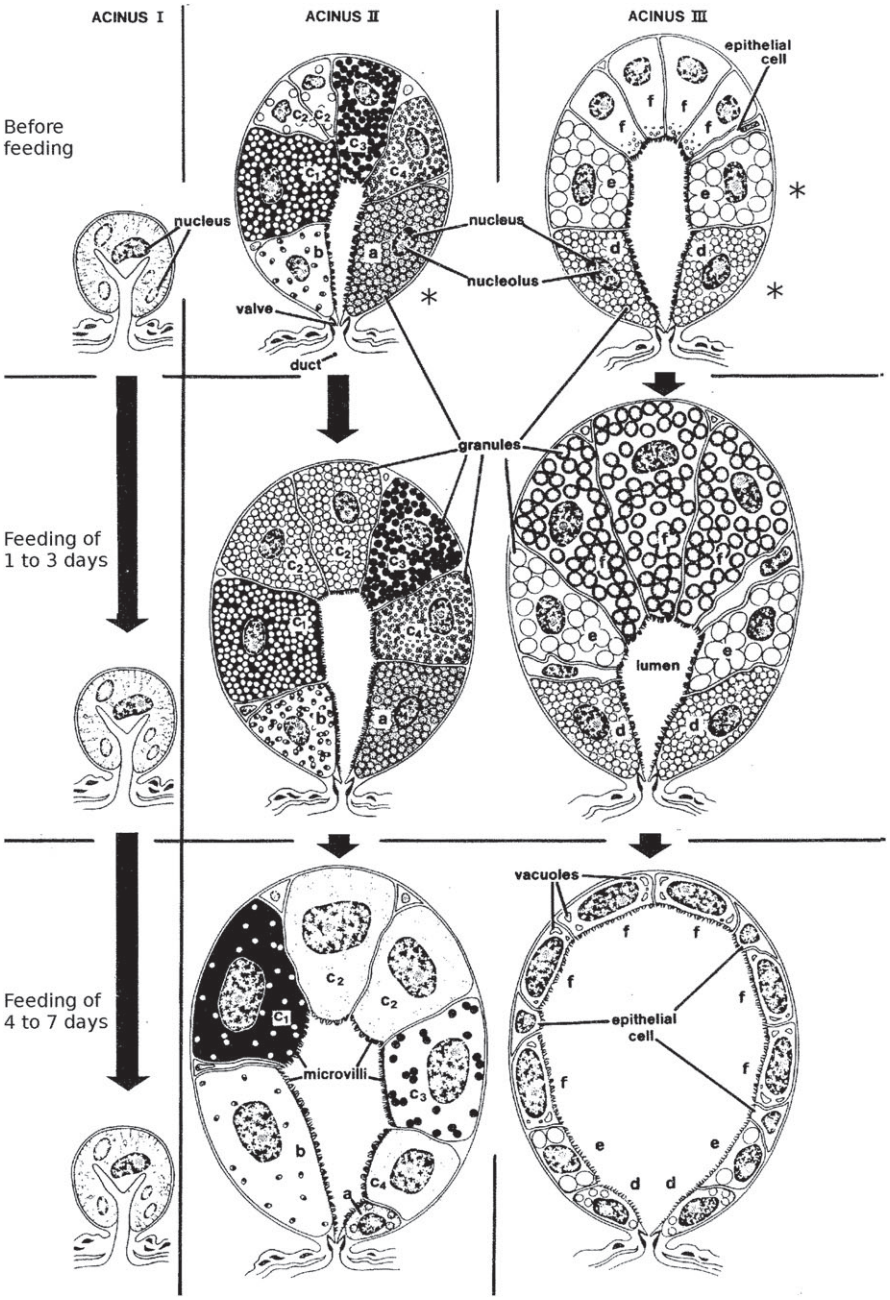

Fig. 3. Acini and cell types of the salivary glands of the tick *Rhipicephalus microplus* (female) and changes that occur during feeding. Type I acini remain unchanged during feeding, whereas there are pronounced alterations in acini II and III. The position of a-, d- and e-cells involved in cement production are shown by asterisks. Image adapted from Kemp *et al.* (1982). (Reprinted with kind permission of Elsevier).

particularly evident and appeared stronger in cells from the unfed stage than in fed ticks. Interestingly, this particular polypeptide also seems to be present in the salivary glands of *Amblyomma americanum* and *Rhipicephalus sanguineus* (Jaworski *et al.*, 1992).

Additionally, *Rhipicephalus* immuno-dominant molecule 36 (RIM 36 protein) cloned from a cDNA was found to be present in the salivary glands as well as the cement cones of *Rhipicephalus appendiculatus*. It was highly abundant in e-cells, present in d-cells and absent in type II acini (Bishop *et al.*, 2002).

It has been observed in *Rhipicephalus appendiculatus* that d- and e-cells of females are depleted of granules early in the feeding process and diminish in size afterwards, indicating that granule secretion contributes to cement deposition. By contrast, these cells remain synthetically active in males and lose fewer granules. This could be explained by the longer on-host periods in males and repeated cement production

Table 3. Differences in nomenclature for granular salivary gland cells. Cement precursor cells are highlighted in bold

Acinus	Most common cell type classification (Binnington, 1978)	Less-common cell type classification (Chinery, 1973; Yanagawa *et al.*, 1987)
II	**a**	**a**
	b	b
	c (c1–c4)	—
III	**d**	**c**
	e	**d**
	f	e
IV	g	—

(Fawcett *et al.*, 1986). During copulation, male ixodid ticks change position to feed together with the female as a couple (Feldman-Musham, 1986). Accordingly, cement might be needed more than once by the males (Binnington, 1978).

In a less-common cell-class classification, some studies (Chinery, 1973; Yanagawa *et al.*, 1988) report a- (acinus II), c- and d- (both in acinus III in this classification) cells as involved in cement production. Differences in the cell types observed and in the nomenclature of salivary gland cells by different authors (Table 3) complicate the picture. Nevertheless, the similarities of these cells (Yanagawa *et al.*, 1987) suggest that the same cell types were described.

In comparison, salivary glands of non-cement-producing Argasidae are less complex with only one granular acinus type (Roshdy & Coons, 1975; Binnington & Kemp, 1980; Coons & Alberti, 1999). *Argas persicus* (Oken, 1818) (Argasidae) contains two cell types, with the described i-cells resembling b-cells of type II acini in *Haemaphysalis spinigera* (Ixodidae) (Chinery, 1974). Three cell types (a-, b-, and c-) were described in the granular acinus of *Argas arboreus* Kaiser, Hoogstraal and Kohls, 1964 (Roshdy & Coons, 1975) and *Ornithodoros moubata* (Murray, 1877) (El Shoura, 1985). However, their granules all lack the microstructure described for Ixodidae (see Section VI), supporting a role of those structures in Ixodidae in the production of cement (Roshdy & Coons, 1975; El Shoura, 1985). No information could be found regarding the morphology and number of acini in *Nuttalliella namaqua* salivary glands, the non-cement-producing single species of the family Nuttalliellidae (Mans *et al.*, 2011).

VI. HISTOCHEMISTRY AND ELECTRON MICROSCOPY OF THE INTRACELLULAR GRANULES

Granules produced in the salivary gland cells have been subjected to histochemical testing in several species. Correlative histochemical data are available on gland cell granules (Table 4) and the cement material (Table 5) for four different tick species (*Haemaphysalis spinigera*, *Hyalomma anatolicum*, *Rhipicephalus appendiculatus* and *R. microplus*). These are the only species in which histochemical data been published for both gland cell granules and cement material.

All gland cells and cements stain for protein as well as the amino acids tyrosine and tryptophan. Lipids or lipoprotein were found in the granules of all cement-producing salivary gland cells, except for *Haemaphysalis spinigera*, which only gave faint positive reactions in the cement and a negative reaction in the gland cells (Chinery, 1973).

Based on the Periodic acid Schiff reaction (PAS), carbohydrates are present in the cement of some, but not all species (Moorhouse, 1969). However, the PAS does not allow further differentiation so it is unknown whether these carbohydrates are free or bound to proteins, or if they are mono- or polysaccharides. For *Dermacentor variabilis*, which also contains carbohydrate in the cement (Moorhouse, 1969), faint or moderate reactions in granules of unfed males were reported (Coons & Roshdy, 1973). Surprisingly, there was no positive reaction for carbohydrate in the granules of *R. microplus* (Binnington, 1978), even though the (cortical) cement of this species stains intensively with the PAS (Moorhouse & Tatchell, 1966).

In the three species tested for phenolic groups, the granules in the gland cells tested positive, in accordance with the cement staining for two of these species (Binnington, 1978; Walker *et al.*, 1985; Gill & Walker, 1988). Phenols can be converted into quinones, therefore their presence might indicate that a tanning process that crosslinks proteins is involved in cement formation (see Section IX).

Enzymatic activities were tested in the granules for two of the four species (*H. anatolicum* and *R. appendiculatus*). Activity of acid phosphatase, catechol oxidase, sulphatase and aminopeptidase observed in one or both species might be associated with modifications of secretory products in the granules. It is also possible that these enzymes are involved in other functions of tick saliva, such as the formation of the feeding pool. Granules in both species reacted positively for acid phosphatase. In *H. anatolicum*, granules of a- and d-cells reacted strongly; in *R. appendiculatus*, a strong reaction was only found in a-cell granules (Walker *et al.*, 1985; Gill & Walker, 1988).

R. appendiculatus granules in all three cell types tested positive for catechol oxidases. In addition, d-cell granules show moderate positive reactions for aminopeptidase and peroxidase (Walker *et al.*, 1985). Sulphatases were tested for in *H. anatolicum* only and detected in e-cell granules which gave a weak reaction (Gill & Walker, 1988). Catechol oxidase activity might be involved in quinone formation, again suggesting a possible role for tanning in cement formation. However, this enzymatic activity might also be associated with blood feeding; a salivary peroxidase of the mosquito *Anopheles albimanus* Wiedemann, 1820 also shows catechol oxidase activity and is known to cause vasodilation (Ribeiro & Valenzuela, 1999; Ribeiro, Mans & Arca, 2010). Similarly, the observed weak peroxidase activity might arise from a glutathione peroxidase, which is known from tick saliva and probably acts as an anti-inflammatory substance at the

Table 4. Summary of histochemical results on granules in salivary gland cells of different tick species. Histochemical methods applied in different studies may differ slightly, meaning that comparability is restricted to some extent (for details, see original publications). Symbols: +, positive reaction; −, negative reaction; 0, not tested; x, tested, but no data. Note, a-, c- and d-cells in *Haemaphysalis* are the same cell types as the a-, d- and e-cells in the other listed species. DOPA, 3,4-dihydrophenylalanine

	Tick species											
	Haemaphysalis spinigera			*Hyalomma anatolicum*			*Rhipicephalus appendiculatus*			*Rhipicephalus microplus*		
	(Chinery, 1973)			(Gill & Walker, 1988)			(Walker *et al.*, 1985)			(Binnington, 1978)		
	Cell types											
Components and enzymatic activities tested in the salivary granules	a	c	d	a	d	e	a	d	e	a	d	e
Carbohydrate	−	−	−	−	−	−	−	−	−	−	−	−
Acid mucopolysaccharide	−	−	−	0	0	0	0	0	0	0	0	0
Non-specific protein	+	+	+	+	+	+	+	+	+	+	+	+
Lipoprotein	0	0	0	0	0	0	0	0	0	+	+	+
Tryptophan	+	+	+	x	+	+	−	+	+	+	+	+
Tyrosine	+	+	+	+	+	+	+	+	+	−	+	+
Arginine	−	−	−	0	0	0	0	0	0	0	0	0
Amino group	−	−	−	0	0	0	0	0	0	0	0	0
Sulphydryl group	+	+	−	+	+	+	+	+	+	+	+	+
Disulphide group	+	+	−	+	+	+	0	0	0	−	−	−
Phenolic group	0	0	0	+	+	+	+	+	+	+	+	+
Lipid	−	−	−	+	+	+	+	+	+	+	+	+
Nucleic acids	−	−	−	−	−	−	0	0	0	0	0	0
Esterase	0	0	0	−	−	−	−	−	−	0	0	0
Aminopeptidase	0	0	0	−	−	−	x	+	x	0	0	0
Sulphatase	0	0	0	−	−	+	0	0	0	0	0	0
β-glucuronidase	0	0	0	−	−	−	0	0	0	0	0	0
Acid phosphatase	0	0	0	+	+	−	+	−	−	0	0	0
Alkaline phosphatase	0	0	0	−	−	−	−	−	−	0	0	0
Adenosine triphosphatase	0	0	0	−	−	−	−	−	−	0	0	0
Catechol oxidase/DOPA oxidase	0	0	0	−	−	−	+	+	+	0	0	0
Cytochrome oxidase	0	0	0	−	−	−	0	0	0	0	0	0
Monoamine oxidase	0	0	0	−	−	−	−	−	−	0	0	0
Peroxidase	0	0	0	0	0	0	−	+	−	0	0	0
NADPH diaphorase	0	0	0	−	−	−	0	0	0	0	0	0

feeding site (Tirloni *et al.*, 2015). Observations of moderate aminopeptidase activity may correspond with the presence of dipeptidases in the saliva. Dipeptidases are thought to inhibit pain responses of the host following tick attachment by destruction of bradykinin (Tirloni *et al.*, 2015).

Transmission electron microscopy (TEM) observations on the granules in salivary gland cells have revealed typical microstructural details, best described in *Rhipicephalus appendiculatus*. The ultrastructure of the granules in this species is very similar to that in comparable cell types in other tick species (Yanagawa *et al.*, 1987).

The a-cells of acinus II contain membrane-bound secretory granules of up to 3 μm with round or oval subunits of 0.5 μm, surrounded by electron-dense rod-like structures. The granules are embedded in a substance which is usually more electron-dense than the granules themselves; however, in some observations the granules appeared more dense than the surrounding substance (Walker *et al.*, 1985; Fawcett *et al.*, 1986). In females, the a-cells remain conspicuous until the end of feeding, although they do become smaller. In males, they remain relatively unchanged during feeding (Walker *et al.*, 1985).

The d-cells of acinus III have granules very similar in appearance to those of a-cells. The granules are 3–4 μm in diameter, often with very dense subunits of 0.4–0.6 μm in an electron-lucent substance, often also containing irregular strands. These might represent a different granule component or another form of the contents from the dense subunits (Fawcett, Buscher & Doxsey, 1982). Similar to a-cells, the density of the granule components may vary. It is not clear whether this is due to maturation processes or the result of differing fixation and dehydration steps in the various studies. High numbers of granules are found in unfed ticks, which are greatly reduced at the onset of

Table 5. Summary of histochemical results from cement cones of *Haemaphysalis spingera*, *Hyalomma anatolicum*, *Rhipicephalus appendiculatus* and *Rhipicephalus microplus*. Histochemical methods in the different studies may differ slightly (for details, see original publications). Symbols: +, positive reaction; −, negative reaction; +/−, faint reaction; 0, not tested; (+), indirectly proven. DOPA, 3,4-dihydrophenylalanine

Components and enzymatic activities tested in cement	Tick species			
	Haemaphysalis spinigera (Chinery, 1973)	*Hyalomma anatolicum* (Gill & Walker, 1988)	*Rhipicephalus appendiculatus* (Walker & Fletcher, 1986)	*Rhipicephalus microplus* (Moorhouse & Tatchell, 1966)
Carbohydrate	−	−	−	+
Acid mucopolysaccharide	−	0	0	0
Non-specific protein	+	+	+	0
Carbohydrate-containing protein	0	0	0	+
Basic protein	0	0	0	+
Tryptophan	+	+	+	+
Tyrosine	+	+	+	0
Arginine	−	0	0	−
Amino group	−	0	0	0
Sulphydryl group	+	+/−	+	+
Disulphide group	+/−	+/−	0	(+)
Phenolic group	0	0	+	+
Lipid	+/−	+	+	+
Nucleic acid	−	0	0	0
Esterase	0	+/−	0	0
Aminopeptidase	0	+	+	0
Sulphatase	0	−	0	0
β-glucuronidase	0	−	0	0
Acid phosphatase	0	+/−	0	0
Alkaline phosphatase	0	−	0	0
Adenosine triphosphatase	0	−	0	0
Catechol oxidase/DOPA oxidase	0	−	−	0
Cytochrome oxidase	0	+/−	0	0
Monoamine oxidase	0	−	0	0
NADPH diaphorase	0	−	0	0

feeding, when cement deposition starts. The observation of early granule stages within the Golgi apparatus suggests that d-cells continue to produce their secretory product for some days, before the cells diminish in size in females. In males, a reduction in granules is less obvious, with TEM revealing that the cells continue to be synthetically active, which seems to be related to the capacity for repeated cycles of cement production and feeding in order to mate with several females on their host (Fawcett *et al.*, 1986).

The cytoplasm of e-cells in acinus III contains the largest granules seen in the glands of unfed ticks (4–6 μm). They are less electron-dense than the granules in a- and d-cells and spherical or ovoid in shape (Fawcett, Doxsey & Buscher, 1981). At lower magnifications, the granules appear amorphous but higher resolution reveals close packaging of subunits (50–100 nm) within a denser substance, giving the granules a fine reticular pattern. Additionally, small numbers of electron-dense inclusions (100–500 nm) are randomly distributed throughout these granules. Similar to d-cells, the e-cells in males are active for longer than in females; in the latter the cells regress after exocytosis of their secretory products during attachment (Fawcett *et al.*, 1981, 1986).

Electron microscopy is very limited regarding the identification of substances, unless special staining methods are applied. However, the granular ultrastructure of a- and d-cells indicates that similar secretory products could be stored within these cells, whereas the content of e-cell granules is obviously different in both structure and density. These electron microscopic observations correspond with histochemical data on unfed males of *D. variabilis* (Coons & Roshdy, 1973) where a- and d-cell granules showed a moderate PAS reaction (for carbohydrate), stained blue with aniline blue black (ABB, for proteins) and violet with Toluidine Blue (for metachromasia). Granules of e-cells, by contrast, showed only a faint reaction with PAS, blue-to-green staining with ABB and orthochromasia with Toluidine Blue. It would be beneficial to perform further studies comparing the histology and ultrastructure of the granules in a larger number of tick species at different points during feeding.

VII. THE STRUCTURE OF CEMENT

Macroscopically, tick cement is usually white or pale brown in colour (Kemp *et al.*, 1982). The material has been described as cell free (Foggie, 1959; Moorhouse & Tatchell, 1966)

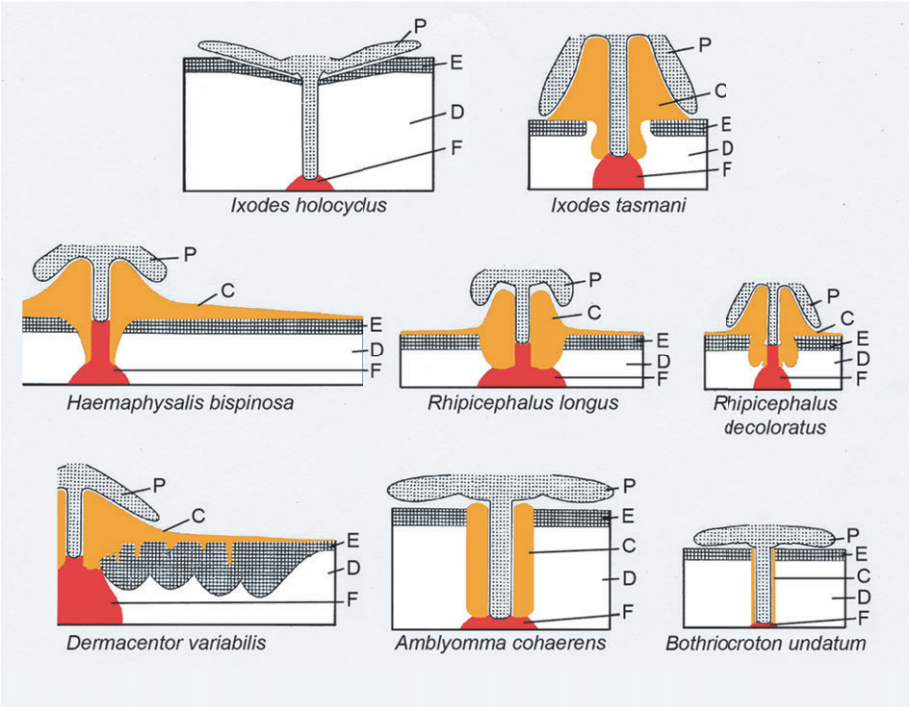

Fig. 4. Examples of types of attachment by female ixodid ticks. The cement can form superficial cones of different sizes, with pronounced lateral flanges, or support the inserted mouthparts. Additional cement can infiltrate the host epidermis around the feeding site in *Dermacentor* females. *Ixodes holocyclus* is given for comparison, as this species does not produce cement. Symbols: C, cement; D, dermis; E, epidermis; F, feeding pool; P, palps. Image adapted from Moorhouse (1969). (Reprinted with kind permission of Akadémiai Kiadó).

and homogeneous except for some striae, giving the cement a lamellate appearance which presumably results from its discontinuous deposition (Cowdry & Danks, 1933; Arthur, 1953; Moorhouse & Tatchell, 1966; Theis & Budwiser, 1974). In the cement of *Rhipicephalus appendiculatus*, Cowdry & Danks (1933) observed fine granular layers alternating with homogeneous layers. At the margin of the cement, strand-like extensions are seen to intermesh with the surrounding dermis (Chinery, 1973; Walker & Fletcher, 1986) and imprints of the tick mouthparts are frequently described from sectioned material (Saito & Ohara, 1961; Chinery, 1973; Theis & Budwiser, 1974).

Amongst ixodid ticks, cement cones differ in size and shape (Moorhouse, 1969). In general, two types of cement deposition can be distinguished (Fig. 4): (*i*) some species restrict the area of cement deposition close to the introduced mouthparts in the skin (e.g. ticks with long mouthparts found in the genera *Amblyomma* and *Bothriocroton*); (*ii*) many species secrete additional deposits of various dimensions onto the host surface. These surface deposits can form high cones at the feeding site (e.g. *Ixodes tasmani* Neumann, 1899) or form lateral flat flanges of various dimensions (e.g. *Haemaphysalis bispinosa* Neumann, 1897, *Dermacentor variabilis*, *Rhipicephalus longus* Neumann, 1907). High cones are known from species with long mouthparts, and lower cones from those with short mouthparts. In some species (e.g. *Rhipicephalus* spp.) which produce less-extensive lateral flanges on the skin surface, internal flanges of secondary cement can be found instead (Moorhouse, 1969). Cement aggregations on the skin allow ticks with small mouthparts a secure attachment, but some tick species with longer mouthparts also use superficial cement cones, meaning that only a fraction of the mouthparts is inserted into the host tissue and their proximal parts are fixed by the cement cone above the skin surface. An example of this is *Ixodes tasmani* (Fig. 4), whose females insert their long mouthparts only 130 μm into the host, whereas the cement cone supporting the rest of the mouthparts at the skin surface measures 340 μm (Moorhouse, 1969).

Although there is some uniformity in cement shapes within tick genera (Fig. 4), the association of a particular cement configuration with a specific genus is not always possible (Mueller-Doblies & Wikel, 2005). Chinery (1973) found differences between the cement cones of *Haemaphysalis spinigera* and the *Haemaphysalis* species described by Moorhouse (1969). Additionally, particular cement cone shapes may be influenced by the insertion angle of the mouthparts (Chinery, 1973).

VIII. HISTOCHEMISTRY OF THE CEMENT

In different species, the cement substance can be either strongly eosinophilic (Saito & Ohara, 1961; Theis & Budwiser, 1974) or not eosinophilic (Cowdry & Danks, 1933).

Moorhouse & Tatchell (1966) stated that the eosinophilic behaviour of the cement depends on the fixation and stain used, which may explain the reported variations.

Based on histochemical staining, the cement of *Rhipicephalus microplus* consists of protein (Bromophenol Blue) with additional lipid (Sudan Black B) in the core cement and carbohydrate-containing (PAS) proteins in the cortical cement (Moorhouse & Tatchell, 1966). In addition to proteins, free lipid structures and carbohydrates are also found in cement cones from some, but not all, tick species examined to date (Moorhouse, 1969). Cement cones from the genera *Amblyomma* and *Bothriocroton* (formerly *Aponomma*) (Moorhouse, 1969) as well as *Haemaphysalis spinigera* (Chinery, 1973), *Hyalomma anatolicum* (Gill & Walker, 1988) and *Rhipicephalus appendiculatus* (Walker & Fletcher, 1986) seem to lack carbohydrates. As with the salivary granules mentioned above, PAS tests did not allow further differentiation of carbohydrates.

Lipids and/or carbohydrates are either found in distinct regions of a core and cortical cement or form a series of folded lamellae, each containing lipids and carbohydrates together with proteins, as described for *Dermacentor* species (Moorhouse, 1969). These patterns presumably develop from the sequence, dynamics and material properties of deposition.

Despite the longer activity of the cement-producing salivary gland cells in males, at the histochemical level there are no differences between the cement produced by males and females of *Haemaphysalis spinigera* (Chinery, 1973).

Within cement, enzymes may be expected to be present as residuals from intracellular cement formation, the curing process or as stored catalytic material required for later cement degradation. A strong and homogeneous histochemical reaction for amino peptidase (leucyl naphthylamide method) was reported in the cement cones of *Hyalomma anatolicum* (Gill & Walker, 1988), *Rhipicephalus appendiculatus* (Walker & Fletcher, 1986) and in cement produced by larval *Rhipicephalus microplus* ticks (Schleger & Lincoln, 1976). Amino peptidases catalyse the cleavage of amino acids from the amino terminus of proteins or peptide substrates (Taylor, 1993). The function of amino peptidase in the largely proteinaceous cement material is unclear (Gill & Walker, 1988). Due to its presence throughout the cement cone, it is unlikely that this enzyme is involved only in modifications of fresh cement material. It may be a remnant of saliva portions used in the generation of the feeding pool. This might explain positive reactions for amino peptidase in areas of the host dermis at the attachment sites of *R. microplus* larvae (Schleger & Lincoln, 1976). Alternatively, this enzyme may assist in the detachment of the mouthparts from the cement after feeding, although there is no supporting evidence for this to date.

A weak reaction for acid phosphatase was identified with the Azo dye/lead method in cement cones of *Hyalomma anatolicum* (Gill & Walker, 1988), leading to suggestions that this enzyme has a gluing function in the cone (Walker *et al.*, 1985; Gill & Walker, 1988), but this was not further investigated. Activity of acid phosphatase during the curing process would contribute to protein aggregation or polymerization through an increase in negative charge.

Histochemical staining (fast red salt B, diazotization) was also applied to test cement cones of *R. microplus* (Moorhouse & Tatchell, 1966) and *R. appendiculatus* (Walker & Fletcher, 1986) for phenolic groups. Phenol oxidase was identified in the salivary glands of the same tick species (Binnington, 1978; Walker *et al.*, 1985), leading to suggestions that it was involved in a hardening process similar to insect cuticular tanning. However, positive phenol reactions were restricted to the cortical cement of *R. microplus* (Moorhouse & Tatchell, 1966), meaning that either tanning is only one of several curing mechanisms, or that phenols have other functions in tick cement.

As discussed above for intracellular granules, it is possible that the observed enzymatic reactions are not involved in cement formation, but relate to other functions of the saliva. Particularly surprising is the strong positive reaction for aminopeptidase; high concentrations of this enzyme would appear counterproductive in the protein-rich cement, raising the question of how the cement cone is protected from such activities.

IX. THE CURING PROCESS OF CEMENT

The curing of adhesives can take place by different mechanisms: hardening by loss of water or another solvent, cooling, or chemical reactions (Petrie, 2007). The latter include: (*i*) formation of helices from polysaccharides or proteins after cooling, supported by formation of hydrogen bonds and hydrophobic interactions (e.g. agar, gelatin); (*ii*) formation of ionic bonds between polysaccharides further stabilized by cations like Ca^{2+} (e.g. pectin); (*iii*) crosslinking of proteins by disulfide bonds, hydrophobic bonds (e.g. barnacle cement) or DOPA-rich regions (e.g. mussel byssal plaques) (Smith, 2002).

It remains unclear which chemical process is responsible for the solidification of tick cement. It was suggested to involve a quinone tanning process (Moorhouse & Tatchell, 1966) or a reaction similar to the coagulation of haemolymph (Kemp *et al.*, 1982). Quinone tanning is the chemical crosslinking of proteins by quinones of different structure. It is known to be involved in the sclerotization of insect cuticles and the formation of water-resistant adhesives in marine mussels (Hong, Lee & Lee, 2014). Insect sclerotization is caused by crosslinking of phenols or primary and secondary amines of cuticular proteins with N-acetyl-catecholamines. In mussel adhesives, the oxidation of DOPA induces DOPA–quinone linking which rapidly forms covalent bonds with basic amino acids such as lysine and histidine (Hong *et al.*, 2014).

The presence of phenolic groups both in cement cones and salivary glands of *R. microplus* and *R. appendiculatus* could suggest that a hardening process, similar to insect

cuticular tanning (Binnington, 1978) or quinone tanning of neighbouring sulphydryl groups (Moorhouse & Tatchell, 1966) takes place, as in the cuticular tanning of the mite *Acarus siro* Linnaeus, 1758 (Hughes, 1959). However, reported low levels of sulphur-containing proteins in the cement of *R. microplus* makes the latter mechanism unlikely (Binnington & Kemp, 1980). Further, phenolic groups were localized to cortical cement of *R. microplus* (Moorhouse & Tatchell, 1966) so a tanning process cannot explain the solidification of the core cement. In *Hyalomma anatolicum*, tanning similar to *A. siro* is unlikely to occur because sulphydryl and disulfide groups were detected at only very low levels and DOPA oxidase activity was not present (Gill & Walker, 1988).

Coagulation of the haemolymph was suggested to explain rapid solidification of core cement (Kemp *et al.*, 1982). However, preliminary studies in *R. microplus* revealed differences in the amino acid composition of coagulated haemolymph and cement, and it was questioned whether solidified haemolymph has the necessary insolubility required for cement to persist over days in host tissue (Kemp *et al.*, 1982).

Two chitinase-like proteins [glycoside hydrolase 18 (GH-18) family] were identified in the salivary glands of *Amblyomma americanum*. Their inactivation (silencing by RNA interference) impaired cement deposition leading to a significant decrease in attachment; treated ticks could be detached with a light touch. Furthermore, their feeding sites often showed extensive bleeding (Kim *et al.*, 2014). Both proteins lack putative chitin-binding domains and are also expressed in other parts of the tick body (Kim *et al.*, 2014). Recently, two chitinases have been identified from the saliva of *Ixodes scapularis* Say, 1821, one of them inactive and with 64–65% similarity to the chitinase-like proteins from *Amblyomma americanum*. However, the role of these proteins was not investigated (Kim *et al.*, 2016).

Differences in curing and grade of solubility between the core and cortical cement may mean that several different processes contribute to the solidification of cement.

X. TICK DETACHMENT

The mechanism behind tick detachment from the cement when leaving the host is not known. *Dermacentor andersoni* are able to separate within 1–2 min from the cement. Forced removal of tick and cement from ears of mice (Gregson, 1960) showed that the mechanical retraction of the chelicerae might be sufficient to achieve rapid detachment, but the secretion of a saliva component that dissolves the cement before detaching was also discussed (Kemp *et al.*, 1982; Sauer *et al.*, 1995). Bullard *et al.* (2016) reported that microlitre amounts of saliva can dissolve tick cement rapidly. Due to the high protein content of the cement, it might be expected that a protease is involved.

XI. BIOCHEMISTRY OF CEMENT

Based on biochemical analyses of cement, the main amino acids present are leucine, serine, tyrosine and glycine, but only low concentrations of histidine and methionine are present (Kemp *et al.*, 1982). Due to the fact that the cement cannot be easily dissolved, protein identification is very difficult. The presence of various binding mechanisms may support its poor solubility (Moorhouse & Tatchell, 1966; Kemp *et al.*, 1982). The best solubilization can be achieved by using hot bases or acids (Kemp *et al.*, 1982), which, however, makes proteomic analysis almost impossible because of hydrolysis. Several proteins are known to be present in tick cement: examples are a 94 kDa protein detected from various tick species, a 20 kDa protein of *Amblyomma americanum* and a 15 kDa protein of *Rhipicephalus appendiculatus*, named 64P (Brown, Shapiro & Askenase, 1984; Shapiro, Voigt & Fujisaki, 1986; Shapiro, Voigt & Ellis, 1989; Havlikova *et al.*, 2009).

The introduction of artificial feeding systems on silicone membranes allowed the isolation of cement cones of *Amblyomma americanum* free from host skin tissue for comparison with cement from *in vivo*-fed ticks of this species (Bullard *et al.*, 2016). Cement originating from *in vitro* feeding allowed the identification of more proteins than cones harvested from *in vivo* systems. In cement from membrane-fed ticks, 26 proteins were identified with liquid chromatography tandem mass spectrometry (LC-MS/MS), including some intracellular proteins, glycine-rich proteins (GRPs; containing more than 20% glycine), serine protease inhibitors, metalloproteases and unclassified proteins. Bullard *et al.* (2016) hypothesised that GRPs may stabilize and strengthen the cement cone and contribute to its insolubility.

The metalloproteases identified in cement may prevent blood clotting during feeding. Blood clotting may also be reduced by the inhibition of thrombin by protease inhibitors. The function of many of the identified proteins remains unknown, especially as these proteins are not yet characterized. Moreover, the low levels of arginine and lysine residues present in larger peptides after a typical enzymatic approach using trypsin for protein identification makes detection and sequence determination by MS and MS/MS difficult (Tan *et al.*, 2015).

Among amino acids, glycine is the best understood to date. Glycine was also reported to be the most abundant amino acid in the cement of *Rhipicephalus microplus* (Kemp *et al.*, 1982), and high concentrations of this amino acid are also known from other biological adhesives. High glycine contents were found in isolated saliva proteins of *Haemaphysalis longicornis* Neumann, 1901 and *Rhipicephalus appendiculatus* with a proven or putative role in cement formation (Mulenga *et al.*, 1999; Bishop *et al.*, 2002; Trimnell *et al.*, 2005; Zhou *et al.*, 2006). To date, only one GRP has been isolated from *in vitro* cement cones of *Amblyomma americanum* (Bullard *et al.*, 2016). This might be because only a certain cement fraction was investigated (Bullard *et al.*, 2016). Current opinions on the

Table 6. Suggested functions for glycine-rich proteins (GRPs) in tick cement cones and similarities to other proteins

Functions/properties of GRPs	References
Mimic components of the vertebrate host • to use host-derived enzymes for the cement hardening process • to inhibit rejection by the host • to facilitate the binding between cement and host tissue	Bishop *et al.* (2002), Havlikova *et al.* (2009) and Trimnell *et al.* (2002)
Similarity to extracellular matrix proteins (e.g. keratin, collagen, loricrin)	Bishop *et al.* (2002), Francischetti *et al.* (2009), Havlikova *et al.* (2009) and Trimnell *et al.* (2002)
Similarity to spider silk proteins	Francischetti *et al.* (2009) and Maruyama *et al.* (2010)
Similarity to peptides with antimicrobial activity from *Caenorhabditis elegans* Maupas, 1900	Francischetti *et al.* (2009)

function of GRPs in the cement of ticks are summarized in Table 6.

If GRPs are involved in cement production, then it might be the case that species with short mouthparts produce more GRPs than species with long mouthparts, because they depend on cement as part of their anchoring mechanism. Similarly, one-host ticks, which spend most of their life attached to the host, might produce larger quantities of these proteins. The identification of nucleotide sequences which encode potential GRPs from cDNA libraries of three different tick species [*Amblyomma cajennense* (Fabricius, 1787), *Rhipicephalus microplus*, *R. sanguineus*] seems to confirm these suggestions (Maruyama *et al.*, 2010). However, GRPs may have other functions: they are also found in antimicrobial substances and are related to immune evasion (Francischetti *et al.*, 2009; Havlikova *et al.*, 2009). Proteins of this class are also found in the family Argasidae (Maruyama *et al.*, 2010), which do not produce cement. Additionally, some GRPs in tick saliva are shorter than proteins usually involved in animal adhesives such as spider silk (Bullard *et al.*, 2016).

In the saliva of *Ixodes scapularis*, 17 GRPs have been identified that were present in the early phase of attachment and also at the end of feeding and after detachment. It was suggested that the latter might be the result of degenerating processes of the salivary glands at that point (Kim *et al.*, 2016). Further analysis of tick cement composition used Fourier transform infrared spectroscopy attenuated total reflectance (FTIR-ATR) to obtain information about the secondary structures of proteins at the surface of the cement cone (Bullard *et al.*, 2016). They observed significant differences between *in vivo* and *in vitro* collected cement cones. Both contained β-sheet structures and usually β-turns. Cement cones from *in vivo*-fed ticks additionally contained helical protein conformations (perhaps due to proteins synthesized by the host skin) but most of the structures were β-sheets. In comparison, the cement cone of *in vitro*-fed ticks exhibited proteins with mainly random coil structures, seen in cement after 72 h of feeding, with β-sheets and β-turns appearing later, after 5–7 days. These findings indicate that a conformational shift is involved in the curing/hardening process.

XII. COMPARISON OF CEMENT WITH OTHER BIOLOGICAL ADHESIVES

Other animals also produce cement-like adhesives for attachment. The barnacle *Semibalanus balanoides* (Linnaeus, 1767) (formerly *Balanus balanoides*), uses a special glue to adhere to surfaces under water. The cypris larvae (the final larval stage of barnacles) uses its attachment organ to anchor to the substratum while exploring it for permanent attachment at a later stage (Nott, 1969). Its glue production is located in round or oval unicellular gland cells, with a diameter of up to 200 μm (Fyhn & Costlow, 1976). During exploration of new habitats, proteinaceous 'footprints' of antennular secretions from previously arrived larvae stimulate settlement and may enhance the attractiveness of a surface, resulting in gregarious settlement even if no conspecific adult is present (Yule & Walker, 1984; Clare, Freet & McClary, 1994; Matsumura *et al.*, 1998). When the final site has been selected, permanent glue, a clear non-viscous fluid, is produced, which increases in viscosity and cures over the course of minutes to hours forming an approximately 5 μm layer of a multiprotein complex called 'cement' (Saroyan, Lindner & Dooley, 1970; Cheung, Ruggieri & Nigrelli, 1977; Kamino, 2008). The tensile strength of the cement of adult barnacles was shown to be stronger than mussel or limpet adhesives, but weaker than commercial dental adhesives (Yule & Walker, 1984; Nakajima *et al.*, 1995). The cement contains more than 90% protein (Walker, 1972; Kamino, Odo & Maruyama, 1996). However, DOPA – a common component in the foot proteins of the mussel – is not known from barnacle cement (Kamino *et al.*, 1996). Total protein hydrolysis showed that serine, threonine, alanine, glycine and proline are the most abundant amino acids in barnacle cement, but interspecific differences were found (Walker, 1972; Kamino *et al.*, 1996; Kamino, 2008). Lipids and carbohydrates are present in trace amounts, and silicon, calcium, aluminium and iron are the major inorganic residues (Walker, 1972). Several soluble proteins (hydrophobic, six amino acid biased, charged amino acid rich) were identified, but their functions are not fully understood. Hypotheses about underwater attachment

functions including priming, spreading and condensation and possible protection against bacterial degradation by lytic activity have been suggested (Kamino, 2006). Accurate protein analysis of the barnacle cement is again prevented by its solubilization. High solubilization (>90% of the cement of *Megabalanus rosa* Pilsbry, 1916) was enabled using guanidine hydrochloride (GdnHCl), a strong chaotrop and one of the strongest denaturants, under reducing conditions using dithiothreitol (DTT) (Kamino *et al.*, 2000). This non-proteolytic solubilization method revealed the importance of disulfide bonds for the stability of the cement and that certain bulk proteins are responsible for cement firmness (Kamino, 2008).

Like barnacles, the *Mytilus* genus of mussels also attaches to various substrata (e.g. rocks, wood, seaweed, other animals or ship hulls). This is mediated through a sectorial organ called the byssus, which can be divided into three main parts: the proximal part (root) and the distal part (stem) of the collagenous threads, and the attachment plaque (Brown, 1952). The adhesive proteins are produced within the foot organ, accumulated and then secreted into the sea water, where they produce byssal threads and the adhesive plaque through a curing process within minutes (Silverman & Roberto, 2007; Bandara, Zeng & Wu, 2013). Qin *et al.* (2016) identified 48 byssal proteins including collagens, protease inhibitors, enzymes, and other unknown proteins. Of these 48 proteins, 11 were exclusively from the plaque and therefore directly involved in the adhesion process. Particularly interesting was the identification of plaque enzymes including tyrosinase, superoxide dismutase, amino oxidase, glycosyl-hydrolase and peroxidase. DOPA is a common post-translationally modified tyrosine in mussels, and thus the presence of tyrosinase enzymes is expected: eight such enzymes were found, oxidizing tyrosine to DOPA and further DOPA to o-quinone leading to tanning, which has been proposed to play a focal role in mussel adhesion and cohesion (Waite *et al.*, 2005; Anderson *et al.*, 2010; Niklisch & Waite, 2012). The identified peroxidase enzymes may also be part of this redox balance system. Other abundant amino acids are proline, glycine, tyrosine, lysine and asparagine (Gantayet, Rees & Sone, 2014). A thin cuticular layer (5–10 μm) coating the byssal threads protects the mechanical integrity of this fibrous structure. This coating has an unusual combination of high hardness and extensibility, attributed to the formation of protein–metal complexes between three DOPA molecules from the mussel foot protein 1 (mfp-1) and one metal ion (Fe, V, Al) (Schmitt *et al.*, 2015).

Another animal that secretes a proteinaceous glue is the sandcastle worm *Phragmatopoma californica* (Fewkes, 1889), a marine polychaete, which uses the adhesive to build a surrounding protecting tube. The cementing material, which adheres sand and shell fragments together, is produced in bilateral glands and secreted from a special organ (Jensen & Morse, 1988). The cement bonds and the whole tubular construction must be strong and stable to withstand the turbulent, high-energy environment of the intertidal zone (Stewart *et al.*, 2004). After secretion, the cement appears creamy white or light tan and turns reddish to dark brown as it ages (Jensen & Morse, 1988; Stewart *et al.*, 2004). It sets in only 30 s, but takes several hours to cure into a solid foam (Stevens *et al.*, 2007). The adhesive material is largely proteinaceous containing 59.5% of total amino acid residues as short-chain amino acids (alanine, glycine, serine) and 2.6% DOPA which is thought to act as a cross-linker, stabilizing (tanning) the cement (Jensen & Morse, 1988; Stewart *et al.*, 2004; Stevens *et al.*, 2007). In addition, the cement also contains considerable amounts of phosphorus, calcium, magnesium and sulfur (Stewart *et al.*, 2004; Sun *et al.*, 2007; Wang & Stewart, 2013). Several charged polyelectrolytic proteins are present mainly composed of glycine, lysine, tyrosine (mostly as DOPA) and (phospho)-serine (Waite, Jensen & Morse, 1992; Zhao *et al.*, 2005; Endrizzi & Stewart, 2009). Zhao *et al.* (2005) proposed a process of cement formation where some or all of the precursors are accumulated with Ca and Mg as multiphase coacervates. These coacervates release the glue, which gelates in the sea water because of the lower solubility of Ca/Mg phosphate, and irreversible cysteine–DOPA cross-links are formed (dopaquinones). This cross-linking is catalysed through monophenoloxidase and catechol oxidase activity of a tyrosinase, which is present in the secretory cells (Solomon, Sundaram & Machonkin, 1996; Klabunde *et al.*, 1998; Wang & Stewart, 2013).

The sea star *Asterias rubens* Linnaeus, 1758 secretes an adhesive material used for dynamic attachment to withstand wave movement or to grip and pry open a mussel during feeding. Several studies (Hennebert, Wattiez & Flammang, 2011; Hennebert *et al.*, 2012, 2015) investigated the proteome of the sea star and the composition of the temporary adhesive, which mainly consists of proteins and carbohydrates. Several proteins of the glue secreted from the tube feet could be identified, despite its low solubility. Again, electrostatic interactions (polar and hydrogen-bonding of functional groups of glycan chains) and cross-links between the proteins may be responsible for its cohesive and adhesive strength. In addition, mucin-like proteins were identified which could be involved in the formation of a structural network through cross-linking or oligomerization to other molecules. Furthermore, proteins similar to metalloendopeptidases were identified in the glue and may be involved in the detachment process by degrading adhesive proteins. Several lectins were used to recognize oligosaccharide motifs on tube foot sections and on whole footprints. For the latter concanavalin A (Con A), wheatgerm agglutinin (WGA), ricin (RCA) and *Dolichos biflorus* agglutinin (DBA) showed positive reactions. No detailed information regarding the molecular mechanisms underlying sea star adhesion is yet available.

Another echinoderm, the sea urchin, also secretes a proteinaceous and carbohydrate-rich, reversible adhesive *via* its tube feet. Many proteins in the adhesive and non-adhesive part of this secretion have been identified, but only some have been characterized further (Lebesgue *et al.*, 2016). Lebesgue *et al.* (2016) proposed a molecular model for sea urchin reversible adhesion with putative functions of the identified

proteins. Nectin is one of the main adhesive proteins in addition to cohesive proteins, proteoglycans, curing and antimicrobial proteins. Again proteases and glycosylases may be the main de-adhesive secretion components.

The biochemical analysis of tick cement is still at an early stage. There are no detailed data regarding the metabolites, proteins, lipids and carbohydrates present in tick cement. Most proteins identified to date are from the salivary glands and their presence in the cement is speculative. Moreover, the adhesive mechanisms on which the secreted cement material is based remain unknown. Detachment mechanisms after the blood meal also remain theoretical (Gregson, 1960; Kemp *et al.*, 1982; Sauer *et al.*, 1995). However, there are several similarities between tick cement and bioadhesives from other animals. In all cases, the poor solubility makes analysis challenging. Tick and barnacle cements are mainly proteinaceous (above 80 and 90%, respectively), but protein identification is difficult, requiring a mild solubilization strategy. However, it is known that the amino acid composition of tick cement is similar to other adhesives. Glycine, serine, proline, alanine and tyrosine are abundant in the cement in all cases. A high content of short-chain amino acids may be correlated with cross-linking or hardening processes of the cement giving it high stability. Furthermore, the presence of DOPA is essential for the gluing function of the adhesives of mussels and sandcastle worms, but it has not yet been proven in tick or barnacle cement. The cement of both the sandcastle worm and the tick contains phenolic groups and a phenol oxidase. Tyrosinase activity is important in the formation of cross-links and phenolic groups could be involved in the hardening process of the adhesive.

XIII. TICK SALIVA AS A SOURCE OF TOXINS, TICK-BORNE PATHOGENS AND NEW THERAPEUTICS

The saliva produced by ticks is extremely complex and contains several hundred proteins (Brossard & Wikel, 2008) and other non-proteinaceous biomolecules (Oliveira *et al.*, 2011) that are secreted into the host. Some of these are irritating and skin-sensitizing substances or toxins. For instance, proteinases in tick saliva may act as digestive enzymes (Tirloni *et al.*, 2015). Proteins of the lipocalin family are found in Argasidae and Ixodidae and are thought to modulate inflammation processes; some lipocalins are responsible for 'sand tampan toxicoses' resulting from bites of *Ornithodoros savignyi* (Audouin, 1826). In addition, a number of proteins (e.g. cystatin, kunitz, metalloprotease) in tick saliva are known as components of toxic secretions from venomous animals (Estrada-Peña & Mans, 2014).

Tick saliva also constitutes the main route for the transmission of tick-borne pathogens which include viruses, bacteria and parasites (e.g. Tick-borne encephalitis virus, Crimean-Congo haemorrhagic fever virus, *Borrelia burgdorferi sensu lato*, *Rickettsia rickettsii*, *Francisella tularensis*, *Ehrlichia chaffeensis*, *Coxiella burnetii* and *Babesia microti*). Once acquired from a tick vector, pathogens can be transmitted from larvae to nymphs or nymphs to adults by transstadial transmission and/or to the next generation by transovarial transmission (Macaluso & Paddock, 2014; Nuttall, 2014). The immunomodulatory activity of saliva which allows the tick to feed successfully also leads to a phenomenon called saliva-assisted transmission (Labuda & Nuttall, 2008). The local conditions at the tick feeding sites, comparable to protected niches within the host body (Nuttall & Labuda, 2008), facilitate the survival of pathogens and transmission to previously uninfected ticks (Randolph, 2011).

Compounds of tick saliva have been considered as medical therapeutics in recent years. Investigations have included studies on anti-tumoral effects (Chudzinski-Tavassi *et al.*, 2010; Abreu *et al.*, 2014; Sousa *et al.*, 2015), applications against inflammatory diseases like myasthenia gravis (Hepburn *et al.*, 2007), new anticoagulants (Koh *et al.*, 2011) and the treatment of asthma (Horka *et al.*, 2012).

XIV. POTENTIAL APPLICATIONS OF TICK CEMENT

The natural adhesive and sealing functions of tick cement in vertebrate skin tissue suggest potential applications in medicine, in particular as a tissue glue or sealant. However, such tick cement-based glues will depend on as yet unknown properties of the adhesive molecules, which need to be identified and carefully studied.

There is a high demand for adhesives in medicine, as established tissue glues contain either toxic substances (cyanoacrylates, glutaraldehyde) or have weak bonding forces (fibrin glues) (Fuerst & Banerjee, 2005; Schneider, 2009; Zhang *et al.*, 2014*b*). Furthermore, existing tissue glues do not cover all possible fields of application: sutures, staples, screws and plates could be replaced by glues in some applications, if they can provide particular properties (Duarte *et al.*, 2012). Additionally, the trend towards minimally invasive surgical methods (laparoscopic, endoscopic, and robotic techniques) further increases the requirement for adhesives, as suturing and similar procedures are difficult through small incisions (Spotnitz & Burks, 2008; Schneider, 2009).

Tick cement may be an exciting answer that opens up new fields of application. Despite the fact that the properties of the adhesive molecules in tick cement remain unknown to date, observations suggest that tick cement contains two types of adhesives that differ functionally in curing time: a rapidly curing (core cement) and a slower hardening (cortical cement) fraction (see Section IV). Fast-curing adhesives might be used to close wounds or to stop bleeding, for example in liver lacerations. The cortical cement might have applications where longer time frames are required for tissue manipulation such as during reconstruction of complex fractures.

Tick cement has a viscous consistency. If this is also true for its adhesive component, it could allow fixing of small bone fragments too small for screw fixation and

currently stabilized by sutures or pins. Since autologous bone material is still considered to be the best material for bone regeneration, the preservation of small bone granules is of considerable importance. Cement might also be used for larger unloaded bone fragments such as the skullcap to avoid screwing and metal plate fixation. Gluing of tendons and ligaments without suturing or screwing would improve refixation, but is especially challenging as exceptional tensile strength is required. Fluent formulations could be applied to large surfaces in skin regeneration or hernia net fixation and in a similar method to fibrin spray applications (Sawamura *et al.*, 1999).

The likely presence of both a rapid- and a slow-hardening fraction in cement makes possible the sequential use of two components: prefixation with a faster curing glue and final stabilization with the slow-curing type. A mixture could result in a glue with hybrid properties, giving more application flexibility. In Section X we considered how ticks appear able to detach from their hosts by dissolving the cement with their saliva. A putative cement-dissolving portion of saliva enzymes could thus be considered as a component for a reversible tissue glue. Today, no enzyme-based, reversible glue is available to our knowledge, but this novel concept would facilitate repositioning of tissue fragments in complicated surgery or their removal at a later time point.

Recently it has been shown that it is advantageous to glue intravenous catheters to the patient's skin to avoid dislodgement, micromotion leading to vein irritation or bacterial invasion from the skin (Bugden *et al.*, 2016). This could be another application of cement-based adhesives. Similarly, the fixation of cannulas which must remain in place for longer time periods, e.g. in intracerebral brain infusion in which the curved skull shape makes it difficult to fix cannulas properly (Sike *et al.*, 2017) might be possible.

Besides medical applications, the cosmetic industry is always exploring new concepts and components for their products. Among the proteins applied in cosmetics are collagen, elastin and the silk protein fibroin, which all have a high content of the amino acid glycine (Secchi, 2008). Tick cement contains high levels of GRPs (see Table 6) making it a potential new constituent for cosmetics.

Application of biological compounds to industrial products requires a long process of isolation, identification and manipulation of ingredients to achieve optimal results in durability, applicability and biocompatibility. As such, biotechnological processing and chemical modification very often improve the final products. Biochemical techniques may allow the introduction of chemical modifications to tick-cement constituents, to insert beneficial protein sequences into sequences of tick-cement proteins or to combine other components to form new adhesives. Such engineering has already been successfully used for DOPA, an important component in mussel adhesion (see Section XII). Today, DOPA is copolymerized with vinyl monomers to form dopamine-co-acrylate, a hyperbranched polymer with a fourfold increase in wet adhesion strength compared to commercial fibrin sealants (Zhang *et al.*, 2014*b*). In another study, a new DOPA-based adhesive for rapid emergency re-entry after sternotomies was designed by using initially stable, but gradually hardening substances (Zhang *et al.*, 2014*a*). DOPA was also combined with polyethylene glycol to result in a photopolymerizable gel with sufficient strength for applications in skin and heart in *ex vivo* animal models (Zhang *et al.*, 2015).

We conclude that tick cement has much potential as a template for a biomimetic tissue adhesive and is an exciting resource for future developments in biomedical engineering.

XV. CONCLUSIONS

(1) The production of cement appears to be an exclusive feature of ixodid ticks and is related to the high complexity of the salivary glands in this tick family. Relatively few species of this tick family feed without the production of the adhesive, emphasising the importance of this substance to the enormous success of the Ixodidae as long-term blood feeders. Besides its more obvious functions as an adhesive and sealant, cement production might also allow the tick to avoid detection by the host and its defence systems. The reduction of tissue damage, the exclusion of bacteria from the host skin surface and the nearly complete isolation of the mouthparts from surrounding tissues are all facilitated by superficial cement deposition.

(2) As the largest family of ticks, the Ixodidae offer a number of opportunities for comparative research on cement. If differences in cement exist among species they will most likely be detected by studies on ticks with a narrow host spectrum or extreme host specificity. Such species could act as model organisms for adaptations of the adhesive to particular vertebrate groups or species. Comparative research on host-specific ticks may also help to identify components that are evolutionarily conserved and might therefore be critical for adhesiveness. Such studies will also contribute to our understanding of rare host specialisations, like feeding on amphibians. Subsequent identification of genes coding for cement components could also result in suitable alternative markers for molecular-based phylogenetic studies and tick systematics and consequently improve our knowledge on the phylogenetic relationships among Ixodidae.

(3) The artificial feeding of ticks on silicone membranes appears to be a suitable tool for studies on cement material, offering the possibility of uncompromised samples (free of skin tissue). Artificial feeding allows access to the adhesive material at virtually any time during feeding, something which is not possible *in vivo*. In this way the progress of cement deposition can be monitored. The development and standardization of mechanized feeding systems, allowing automatic and simultaneous artificial feeding of large numbers of ticks in order to yield quantities of cement material for analyses would be desirable. To obtain samples of high purity, a further improvement of the method might be the introduction of artificial feeding media to exclude host compounds derived from blood.

(4) The medical importance of ticks is generally associated with their vector role in disease transmission, but there are increasing examples that some tick products might be beneficial to man. Some salivary gland products are promising starting compounds for new therapeutics and, similarly, tick attachment cement might become a rich source of innovative products in medicine. In contrast to many other adhesives considered for future medical applications, tick cement is used in nature in a similar way, suggesting that this adhesive might be especially suitable for the development of new medical adhesives. Due to the natural function of the attachment cement in adhesion to and sealing of vertebrate skin, it might be especially promising as a tissue adhesive or sealant. Apart from the gluing function, there are two other characteristics of interest for medical applications: (*i*) the observed similarities of cement components to proteins found in vertebrate tissue might suggest good integration of its components and tissue compatibility; (*ii*) its possible antimicrobial activity.

(5) Research on tick cement is still in its infancy and currently little is known about the compounds involved in the formation and solidification of cement. Most information available comes from only a small number of tick species and much remains speculative. By contrast, more molecular biological information is available for potential compounds in the saliva of ticks. These components are probably also present in the cement, either as components or residuals. Besides the identification of the chemical components involved in the gluing function and the processes responsible for solidification, studies on the mechanical properties (e.g. elasticity, hardness, tensile strength) and surface interactions of cement are also lacking. Such studies will be of major importance for possible applications of cement-based adhesives. Concerted interdisciplinary efforts and highly sophisticated methodologies are needed to reach a better understanding of cement and its underlying adhesion mechanisms.

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Manuscript III – Revisiting amino acid analyses for bioadhesives including a direct comparison of tick attachment cement (*Dermacentor marginatus*) and barnacle cement (*Lepas anatifera*)

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Highlights of this manuscript

- Method development and validation for the analysis of amino acids by GC-MS/MS and LC-MS/MS after propyl chloroformate derivatisation
- Comparison of acidic and alkaline protein hydrolysis
- Improvement of amino acid recovery after acidic hydrolysis by the addition of phenol
- Comparison of amino acid composition of two bioadhesives

Revisiting amino acid analyses for bioadhesives including a direct comparison of tick attachment cement (*Dermacentor marginatus*) and barnacle cement (*Lepas anatifera*)

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Abstract

With respect to potential applications in healthcare, pharmacy, the bonding industry, and cosmetics, biological adhesives produced from various animals are of high interest. A large number of species in the family Ixodidae produce an adhesive substance called attachment cement. This study focuses on the detailed investigation of the amino acid composition of the attachment cement from ticks (*Dermacentor marginatus*) and comparison with cement samples from barnacles (*Lepas anatifera*).

Strong emphasis was laid on sample preparation of both types of cement and included the complete hydrolysis of proteins under either basic or acidic conditions. Stability of propyl chloroformate derivatives of the amino acids were measured by liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) and gas chromatography electron ionisation tandem mass spectrometry (GC-EI-MS/MS) and were validated in terms of reproducibility and precision of analyses using NIST certified amino acid as well as protein standards. For the majority of the amino acids analytically valid recovery rates between 86 and 111% were achieved using GC-EI-MS/MS analysis after acidic hydrolysis.

The method was applied for direct comparison of two biological glue systems, tick and barnacle cement. An outstandingly high content of glycine was present in the tick attachment cement; as were significant concentrations of leucine, serine and proline. In contrast, high levels of leucine, serine, aspartic acid/asparagine, glycine, glutamic acid/glutamine, alanine and valine were determined for barnacle cement. None of the samples showed the presence of 3,4-dihydroxy-phenylalanine (DOPA), yet tick attachment cement exhibited significantly higher concentration levels of tyrosine, the precursor of DOPA. It can clearly be stated that published results for amino acid analysis of barnacle cement show significantly under-/overestimation for some amino acids.

Distinct differences in amino acid presence and concentration were observed for the two bio-adhesives. Although both have cement-like properties the data implied that different attachment mechanisms are involved for each of the organisms.

Keywords

biodegradable adhesive (A), antimicrobial adhesion (A), biological adhesion (D), water resistance (D), amino acid analysis, tick attachment cement

1. Introduction

Ticks (Ixodida) are small arachnids from the superorder Parasitiformes and live temporarily on vertebrate hosts feeding on blood. Extendable chelicerae and backward curved teeth on the hypostome of the mouthparts allow the penetration and firm attachment to skin tissue [1]. Prior to, and during feeding, several ixodid species also produce a sticky secretion from the salivary glands that aids anchoring of the mouthparts to the host. This substance is called attachment cement. Other possible functions of the cement include aspects such as sealing of the feeding lesion and antimicrobial activity. A summary is given in a recent review [2].

The cement and adhesive properties thereof are still largely unexplored. Thus, this is an interesting and promising field of research to obtain an understanding of the cement composition and to potentially utilise the bonding properties of this substance. From the literature it is known that this cement is primarily comprised of proteins with additional lipid and carbohydrate constituents [3-5]. These findings, however, are based on histochemical staining and hence the nature and identity of the proteins is unknown. Thus, there is an obvious need to apply biochemical methods, *e.g.*, amino acid (AA) analysis, to obtain a deeper insight into the composition of attachment cement.

In this study, the adhesive of the tick cement from *Dermacentor marginatus* was compared to a bio-adhesive that has already been investigated in more detail [6-8]; namely, the barnacle cement from *Lepas anatifera*. These were selected because both adhere to surfaces in a moist/damp environment, and both have cement-like properties. A significant lifestyle difference between these systems is that barnacles adhesives must function under water and in high ionic (salt) conditions while tick adhesives have to maintain their function under physiological conditions at the skin-air biointerface. Thus, the comparison should prove to be highly interesting and reveal similarities and/or differences.

Sample preparation for water insoluble bio-adhesives is very hard and complete dissolution for biochemical analysis is difficult to guarantee. It was anticipated that either acidic or basic total hydrolysis of both cement types followed by AA analysis would provide the most complete solubilization and therefore the most comprehensive information. Method development included not only the most common amino acids but

also a post-translationally modified version of Tyr called 3,4-dihydroxy-phenylalanine (DOPA). DOPA is known to play a crucial role in the adhesive process of some marine organisms (*e.g.* mussels, sandcastle worms) that are also adherent in damp environments [9-11]. Due to the fact that proteins consist of several AAs and each AA has distinct physico-chemical properties (*i.e.* basic/acidic, hydrophilic/hydrophobic, charge/no charge, length of side chain), it is from the chemical point of view obvious that each type of hydrolysis, *e.g.*, acidic or alkaline hydrolysis, will have advantages and will result in different recovery rates.

The standard procedure for protein hydrolysis is to heat with 6 N hydrochloric acid (HCl) for about 24 h; and for most AAs very good recoveries are achieved [12, 13]. There are, however, limitations [13-16]: the amides glutamine (Gln) and asparagine (Asn) are deaminated to the corresponding acids and therefore the obtained values for glutamic acid (Glu) and aspartic acid (Asp) represent the sum of the acid and amide derivative and are often annotated as Glx and Asx. Even traces of oxygen convert methionine (Met) to methionine sulfoxide and methionine sulfone, which ultimately lowers recovery rates if sample preparation is not optimally performed. Tryptophan (Trp) and cysteine (Cys) are completely destroyed under acidic conditions, and hydroxyproline (Hyp), serine (Ser) and threonine (Thr) are partially destroyed, therefore their occurrence is usually underestimated. Tyrosine (Tyr) can undergo halogenation, however, this is counteracted by adding phenol to the reaction. Slower reaction kinetics for the hydrolysis of the hydrophobic AAs isoleucine (Ile), leucine (Leu) and valine (Val) are usually overcome by longer hydrolysis times. To determine the level of Trp in proteins, alkaline hydrolysis with sodium hydroxide (NaOH) is usually used [13]. Only Trp, however, is reported to be suitable for basic conditions because many of the other AAs are destroyed or racemise, *i.e.* change their conformation from D- to L-configuration.

In this study, alkaline and acidic hydrolysis methods performed on tick and barnacle attachment cement were compared by determining AA recovery. Gas chromatography (GC) and liquid chromatography (LC) were innovatively combined with highly-sensitive and specific multiple reaction monitoring (MRM) experiments. This enabled achievement of low limits of detection (LOD) and quantitation (LOQ) to be achieved for 21 AAs.

2. Materials and Methods

2.1 Chemicals and reagents:

Double-distilled water (ddH_2O, 18.2 MΩ.cm) was prepared using a Simplicity system Millipore, Billerica, MA, USA. HCl (37%), isopropanol (iPrOH, LC-MS grade), methanol (MeOH, LC-MS grade), phenol (100%) and NaOH (≥99%) were obtained from Merck (Darmstadt, Germany). The protein standards bovine serum albumin (BSA, ≥96%) and albumin from chicken egg white (ovalbumin, ≥98%) and ammonium formate (≥99.9%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). A 200 nmol/mL protein hydrolysate AA standard mixture and a norvaline (Nor) solution (200 nmol/mL in 10% n-PrOH and 20 mM HCl) were provided in the EZ:faast™ kit from Phenomenex (Torrance, CA, USA). The Nor solution was used as an internal standard (IS). AAs included were: alanine (Ala), arginine (Arg), aspartic acid (Asp), cystine (Cys), glutamic acid (Glu), glycine (Gly), histidine (His), hydroxylysine (Hyl), hydroxyproline (Hyp), leucine (Leu), isoleucine (Ile), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val). In addition, 3,4-dihydroxy-L-phenylalanine (DOPA) (both ≥98%) was obtained from Sigma-Aldrich. All reagents used for AA purification, derivatisation and extraction were also provided in the kit: eluting medium A (0.33 N NaOH), eluting medium B (n-PrOH:3-picoline 80:20, v/v), derivatisation reagent (chloroform:propyl chloroformate:iso-octane 60:20:20, v/v/v), extraction solution (iso-octane:chloroform 90:10, v/v) and re-dissolution solvent (iso-octane:chloroform 80:20, v/v).

For method validation, certified NIST (National Institute of Standards and Technology) reference standards were obtained from LGC-Standards (Teddington, United Kingdom): AAs at different mmol/L concentrations were available as a mixture in 0.1 mol/L HCl, and BSA was prepared as a 7% solution in sodium chloride (0.02 M).

2.2 Blood samples, tick and barnacle cement

Bovine blood was received from a Viennese slaughterhouse and stored at -20 °C before use. Female and male adult ticks of the species *Dermacentor marginatus* were obtained from Insect Services (Berlin, Germany). Tick attachment cement was collected and pooled from ticks after *in vitro* feeding for several days on bovine blood using an artificial membrane feeding system as previously described [17]. Barnacle cement from the gooseneck barnacle *Lepas anatifera* was collected from beach-

stranded animals that had washed up on glass bottles after fouling them. The hardened cement was carefully cut from the attachment interface with a scalpel, washed in ddH_2O and stored at -70 °C before analysis.

Both adhesives were ground and homogenised using an agate mortar and pestle (see section 3.5). Aliquots of 100-400 µg were weighed into Eppendorf tubes or 1.5 mL crimp neck GC vials (11.6 × 32 mm), capped with aluminium crimp caps (all from VWR, Vienna, Austria) and stored at -70 °C until further use.

2.3 Protein hydrolysis

Acidic hydrolysis:

6 N HCl was used with and without the addition of phenol (5%, w/v). To remove oxygen from the reaction solution, the HCl was flushed by bubbling nitrogen (N_2) through the solution prior to transfer into a GC vial [14]. 200 µL 6 N HCl were transferred into GC vials containing solid tick or barnacle cement. For liquid samples, *i.e.*, bovine blood and standard protein samples, the concentration of the HCl was increased and the volume decreased to obtain a final volume of 200 µL 6 N HCl. The vials were flushed with N_2 and capped with an aluminium crimp cap. Hydrolysis was performed at 105 °C for 24 h in a constantly heated oven. After hydrolysis, the remaining solvent was evaporated in a water bath (60 °C) under a gentle stream of N_2. The total hydrolysis product was then used for sample preparation. Highly-concentrated products were dissolved in 110 µL iPrOH (10%) and further diluted to appropriate concentrations.

Alkaline hydrolysis:

5.6 N NaOH was used without any additives and added to GC vials containing standard protein material. The vials were flushed with N_2 and capped with an aluminium crimp cap. Hydrolysis was performed at 105 °C for 24 h in a constantly heated oven. Alkaline hydrolysed products were directly used for sample preparation.

Acidic hydrolysis of certified protein standards:

The certified concentration value and the AA sequence are provided with the reference protein. After dilution of the original NIST standard, 50 µL (33.7 µg BSA) were transferred to a GC vial and hydrolysed as described above. Experiments were performed in quintuplicate. Assuming a 100% recovery, the concentration of the standard was chosen such that all AAs would fall within the limits of the calibration

curves. The occurrence of the AAs in BSA is variable and as such, one concentration level would not be appropriate to estimate the recovery rates for both low- and highly-abundant AAs. Thus, the residue of the hydrolysate was dissolved in 110 µL iPrOH (10%) and two aliquots (30 and 70 µL for high- and low-abundance AAs, respectively) were used for sample preparation.

2.4 AA derivatisation

AAs were derivatised before GC and LC analyses according to literature [18] and the vendor-provided product information [16]. Briefly, 100 µL and 25 µL of the IS (200 nmol/mL Nor) for GC and LC, respectively, were added to all AA standards and protein hydrolysates. The mixture was passed through a solid-phase extraction tip and AAs were eluted with 200 µL freshly-prepared elution solution (medium A:medium B, *i.e.*, 0.33 NaOH : (n-propanol:3-picoline 8:2), 3:2, v/v). By adding 50 µL of the derivatisation reagent, the AAs were converted into propyl ester and carbamate derivatives that were then extracted with 100 µL extraction solution [19]. Under a gentle stream of N_2, 50 µL of the obtained chloroform layer was evaporated to dryness.

2.5 MRM optimization

Multiple reaction monitoring (MRM) is the monitoring of multiple product ions from a selected precursor ion after inducing fragmentation [20]. An MRM event can be generated for different precursor ions to produce characteristic precursor/fragment pairs, or so-called, transitions. Energy levels for different transitions, however, are dependent on the type of chemical bond and are therefore not identical *per se*. To achieve the best ion intensity/characteristic analyte fragmentation ratio for sensitive and selective measurements, optimisation is of fundamental importance.

Collision energy (CE) levels for MRM transitions were optimised on a mixture of all AA (200 nmol/mL). CEs were ramped from 3 to 45 V and from 10 to 50 V in 3 V steps for GC and LC experiments, respectively. The three most intense MRM transitions were automatically selected, and the intensities were plotted against the CEs. The CE that resulted in the highest intensity for each transition was then chosen for the final MRM method. The transition that produced the most intensive signal was chosen for quantitation; and two additional transitions were selected for confirmation of AA identity. Final MRM methods contained time windows for each AA whereby particular transitions were recorded to enable highly-sensitive and selective measurements.

Suppl. Table 1 and 2 provide details on CEs, transitions and chromatographic retention times for GC- and LC-MS/MS, respectively. In the final GC-MS/MS method, only one confirmatory transition was obtained for Ala; and no confirmation transition was available for Gly. This is because both AAs have rather small molecular weights. As such, little to no characteristic fragments are generated during collision-induced dissociation. To minimise the reporting of false positives, attention was focused on avoiding non-specific transitions for all the other AAs.

2.6 GC-EI-MS/MS analysis

For these analyses, the sample residue was dissolved in 200 µL of the re-dissolution solvent. For gas chromatography electron ionisation tandem mass spectrometry (GC-EI-MS/MS), a Shimadzu GC2010 (Kyoto, Japan) gas chromatograph coupled to a TQ8040 triple quadrupole mass spectrometer (TQMS) equipped with a Zebron ZB-AAA column (10 m × 0.25 mm) was used. Samples (2 µL) were injected with a split ratio of 1:15 at 300 °C. The initial column oven temperature was 110 °C and was firstly raised to 150 °C at 30 °C/min; then to 220 °C at 50 °C/min; and finally, to 320 °C at 30 °C/min. The helium (≥99.999 Vol-%) carrier gas flow was maintained at 1.1 mL/min and the total analysis time was 6.07 min. The interface temperature was 320 °C and the MS was equipped with an electron ionisation (EI) ion source (70 eV, rhenium filament, 240 °C). In the Q3 scan or MRM mode, masses were recorded after a solvent cut-time of 0.71 min. The Q3 scan was primarily used during method development; whilst the MRM mode (event times between 30 and 75 msec), with a maximum of 9 ions measured simultaneously, was used for all further measurements. Fragment-ion information was provided from the product data in the EZ:faast™ kit (Phenomenex). Argon (Ar; ≥99.999 Vol-%) was used as the collision gas. All data were acquired with GCMSsolution (v. 4.20) from Shimadzu.

2.7 LC-ESI-MS/MS analysis

For the LC-MS/MS analyses, the dry samples were dissolved in 200 µL mobile phase A. A Nexera X2 LC-30 AD LC system (equipped with a SIL-30AC auto-sampler and a CTO-20AC column oven) connected to a LCMS-8060 TQMS (Shimadzu, Kyoto, Japan) with an electrospray ionisation (ESI) ion source was used for liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS). Mobile phase A and B were comprised of 10 mM ammonium formate in ddH_2O and 10

mM ammonium formate in MeOH, respectively. The derivatised AAs were separated on an endcapped LiChrospher RP18 column (125 × 2.0 mm) with a particle size of 5 µm (Machery-Nagel, Germany) that was operated at a flow rate of 0.6 mL/min. The gradient of the mobile phase was as follows: 0-13.8 min: 40% B; 13.8-17.0 min: ramped from 40% to 83% B and maintained for 3 min; 20.0-22.0 min: ramped from 83% to 40% B and maintained for 3 min. The temperatures of the column oven, mass spectrometer interface, desolvation line and heat block were 35 °C, 300 °C, 250 °C and 400 °C, respectively. *m/z* values were recorded in Q3 scans or by MRM in positive-ion mode. Q3 scans were used for method development; whilst the MRM mode was used for all further measurements. Fragment-ion information was provided from the product data in the EZ:faast™ kit (Phenomenex). Instrument specific settings were the dwell times for MRM experiments (between 15 and 80 msec). Ar (≥99.999 Vol-%) was used as the collision gas, N_2 as the nebulising (3 L/min) and drying gas (10 L/min). In addition, a heated gas flow (10 L/min) was used to heat the ESI source. Nebulising, drying and heating gas were generated from a ZEFIRO 8050 N_2 generator (CINEL S.r.l., Italy). All data were acquired with LabSolutions (v. 5.89) from Shimadzu.

2.8 Method validation

As sample preparation (purification, derivatisation and extraction) is prone to volume changes and/or sample losses, all measurements were normalised to the peak area of the IS, Nor, to compensate for possible errors. Nor is a non-proteinogenic AA that is very suitable as an IS for the methods used in this study. Nor was chosen because of the absence from most biological systems and the fact that the molecule has similar physicochemical properties to the analytes under investigation. Sample preparation included sample purification via solid-phase extraction (SPE), AA derivatisation using PCF (conversion of AAs into propyl esters and carbamate derivatives) and extraction of the derivatives. The first step of the sample preparation, SPE, is based on cation exchange. As all AAs were present in an acidic medium, Nor was also dissolved in an acidic solvent. The acidic environment is important for SPE to ensure that the AAs are in an anionic form [18]. Correction factors were determined by calculating area ratios (area of AA divided by area of IS). Further details on quantification and the IS is provided later in the manuscript (see section 3.2).

Concentration levels for the linearity studies were prepared and measured on three consecutive days (one series per day) to give triplicates and to assess day-to-day

variation. LODs and LOQs were calculated from calibrations at low concentrations with at least five concentration levels. Precision, defined as repeatability in terms of RSD (coefficient of variation, CV), was obtained by preparing a derivatised AA standard solution in triplicate on three different days (9 samples). This enabled the calculation of intra- and inter-day precision plus injection and retention time precision.

Chromatographic peaks were automatically integrated using LabSolutions or, if necessary, manually. For data evaluation, peak ratios (peak areas normalised to the IS) were plotted against concentration. Different concentration levels were analysed, and linearity was considered satisfactory when the square correlation coefficient (R^2) was > 0.99. Limits of detection (LOD) and quantitation (LOQ) were calculated from the calibration functions at low concentrations [21, 22]. According to the International Conference on Harmonisation guidelines, residual standard deviation of the linear regression was multiplied by a factor of 3.3 for LOD and 10 for LOQ, respectively, and divided by the slope of the regression line.

Intra- and inter-day accuracy (percentaged difference between nominal and determined concentration) and precision (repeatability in terms of relative standard deviation, RSD) of the method were evaluated using a certified NIST AA standard. DOPA and tryptophan are not included in the NIST standard, therefore an appropriate aliquot of each was added to the mixture. The two purchased AAs were weighed on a Sartorius SE2-F filter microbalance (Vienna, Austria) and diluted with ddH_2O.

For GC-EI-MS/MS, 10 AA concentrations (0.1 to 200 nmol/mL) were prepared for method validation. The concentration of the NIST AA standard was 50 nmol/mL (25 nmol/mL for C-C). DOPA and tryptophan were added to the NIST standard to obtain a final concentration of 50 nmol/mL.

For LC-ESI-MS/MS, 13 calibration levels (in the range 0.01 to 200 nmol/mL) were analysed. The concentration of the certified NIST AA standard for precision and accuracy analysis was 20 nmol/mL (10 nmol/mL for C-C). DOPA and tryptophan were added to the NIST AA standard in the first sample preparation step to obtain a concentration of 20 nmol/mL.

3. Results and Discussion

3.1 MRM optimisation and chromatographic performance

For both, the GC- and LC-MS/MS method, similar or even the same transitions were obtained for isomeric AAs (Ile and Leu, Nor and Val). As all these AAs could be chromatographically separated by GC, this was not a disadvantage. Unfortunately, Nor and Val were not baseline separated during LC. Although not optimal, normalisation against Nor was still feasible and could be implemented in further downstream analyses of the data. Asp also co-eluted with Nor and Val, however, this AA had characteristic MRM transitions that enabled straightforward analysis. For Ile and Leu, no chromatographic and mass spectrometric separation could be achieved by LC-ESI-MS/MS. Consequently, quantification of these two AAs was only possible using the summed peak area for both AAs. Trp and Tyr had the same product ion for the quantification transition in GC-MS/MS but could be chromatographically resolved. When analysed by LC-MS/MS, several other AAs overlapped or co-eluted: Arg and Ser; Gly, Hyp and Thr; Pro, Hly and Met; His, Glu and Lys; Trp, Leu/Ile and Phe; and C-C and Tyr. Excluding Leu and Ile, different MRM transitions were obtained for these AAs which is of high importance to differentiate the AAs.

Comparison of the GC and LC methods led to the conclusion that chromatographic separation and peak shape improved with GC (Fig. 1). The separation of 21 AA was achieved in 5.5 min (GC) compared to 18.0 min (LC), on average the peak widths (full-width-at-half-maximum) were only 9.57 ms, and only 2 AAs co-eluted compared to 20 in the LC analyses. Due to the fact that the chloroformate derivative of Arg is involatile [18], this AA cannot be analysed by GC-MS/MS. Silylation using *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) is also not suitable because Arg decomposes to Orn during the reaction [23-25]. Therefore, throughout the remainder of this work, Arg was measured by LC-MS/MS after propyl chloroformate (PCF) derivatisation; whereas all other AAs were measured by GC-MS/MS after PCF derivatisation.

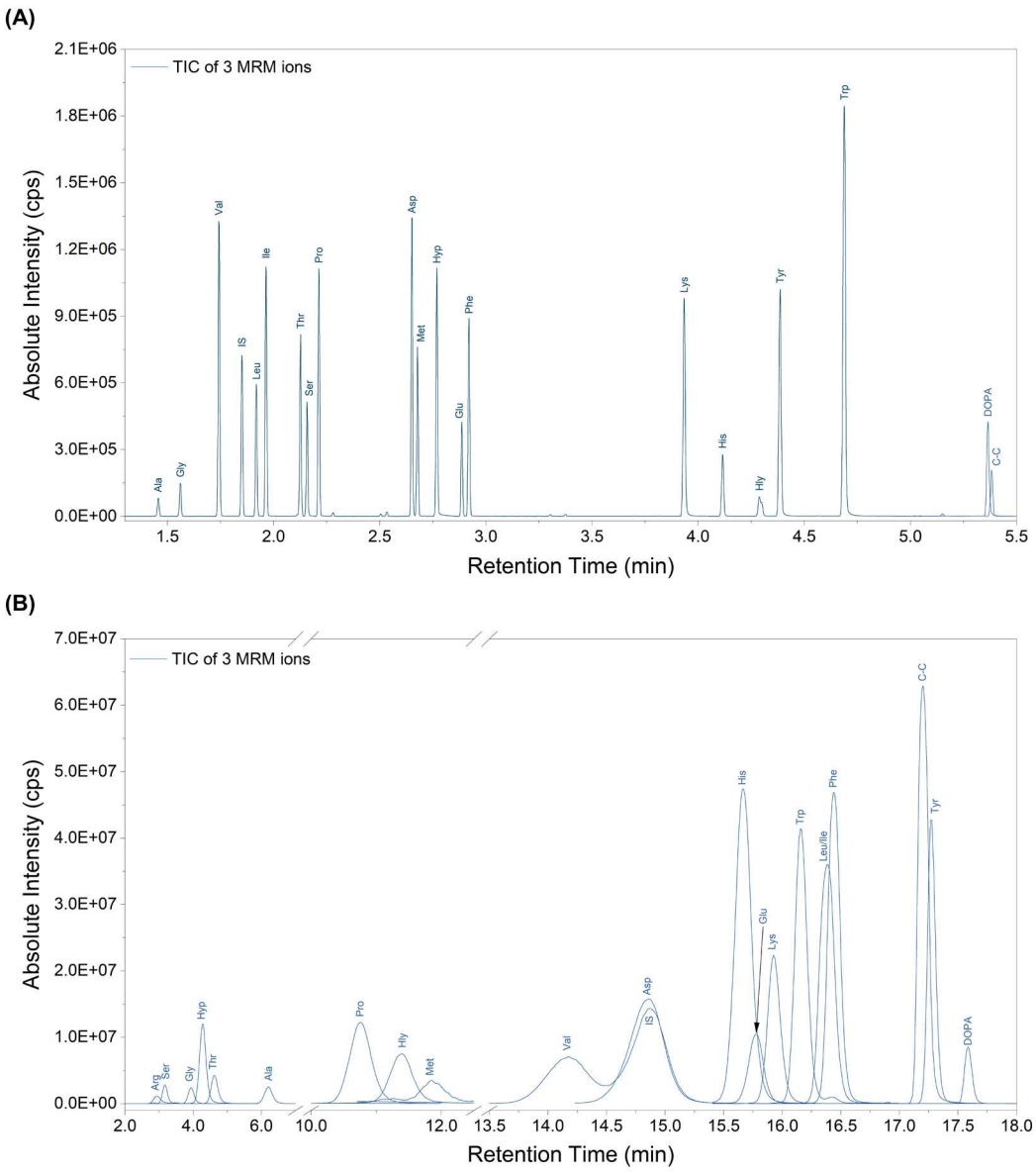

Fig. 1. Comparison of AA separation by GC-MS/MS and LC-MS/MS using a standard mixture: (A) GC-EI-MRM and (B) LC-ESI-MRM chromatogram of the AA standard mixture respectively containing 21 (200 nmol/mL) and 22 (25 nmol/mL) AAs, respectively. Norvaline (Nor) is included as an internal standard (IS). Ala, alanine; Arg, arginine; Asp, aspartic acid; C-C, cystine; DOPA, 3,4-dihydroxyphenylalanine; Glu, glutamic acid; Gly, glycine; His, histidine; Hly, hydroxylysine; Hyp, hydroxyproline; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Pro, proline; Trp, tryptophan; Phe, phenylalanine; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine; cps, count per seconds.

3.2 Method validation

By analysing a certified NIST AA standard (in triplicate on three days to produce 9 samples), the accuracy of the methods was investigated. Intra- and inter-day accuracy is expressed as the percentaged difference between the nominal and the determined concentration of the NIST standard. DOPA and Trp are not included in the NIST standard and were therefore supplemented. All results are summarised in Table 1 and discussed below. Results from LC-ESI-MS/MS method validation are provided in Suppl. Table 3 and are not discussed here. The exception is Arg because only LC-MS/MS was used for the analysis of this AA. At this point Asn and Gln were not included since the aim of this study was the analysis of samples after acidic hydrolysis, in which Asn and Gln are deaminated to the corresponding acids and thus not present in hydrolysed samples.

Linear calibration functions were achieved for up to 2 orders of magnitude except for C-C, DOPA, Hly, Met and Ser where only 1 order of magnitude was attained. Excluding Hly (0.9839), the adjusted R^2 values showed excellent linearity for all AAs (>0.99). To determine LODs and LOQs, five concentration levels at low concentrations were used for the majority of the AAs. Due to the reduced linear range, however, three and four concentrations, respectively, were used for C-C and Hly. Limits < 5 µM were obtained for all AAs; except for the LOD of Met (7.81 µM), and the LOQ of C-C (13.82 µM), DOPA (5.92 µM), Hly (11.09 µM) and Met (23.66 µM). For most of the AAs, the investigation of intra- and inter day precision yielded values with CVs < 10%. Arg (12.44 and 11.50% CV) and Glu (11.34 and 11.66% CV) showed slightly higher values for intra- and inter-day precision. DOPA (24.73% CV) and Met (13.17% CV) resulted in higher values for inter-day precision. The methodology enabled very precise retention times for all AAs (< 0.3% CV). Excluding C-C (3.3% CV), DOPA (7.6% CV) and Met (5.4% CV), the injection precision was also very high (< 3% CV). Furthermore, the accuracy values obtained (range 91 to 111%) showed that the method employed enabled highly accurate measurement of the AAs.

Table 1. Validation data of AA analysis acquired with GC-EI-MS/MS and (for Arg only) LC-ESI-MS/MS in MRM mode using a standard mixture of amino acids. All data were normalised to the IS (Nor). Precision and accuracy values were measured at a concentration of 50 nmol/mL (except C-C and Arg that were 25 and 20 nmol/mL, respectively). LOD and LOQ were calculated from calibration curves at low concentrations (also see section 2.7). Hly and Hyp were not included in the determination of accuracy.

CV: coefficient of variation;

RT: retention time

AA	RT (% CV)	Adj. R^2	LOD (µM)	LOQ (µM)	Linear range (nmol/mL)		Precision (% CV)			Accuracy (%)	
							Injection	Intra-day	Inter-day	Intra-day	Inter-day
Ala	0.15	0.9989	0.08	0.24	5.0	200.0	0.47	2.21	3.45	97.64	95.94
Arg	0.29	0.9996	1.01	3.06	0.5	200.0	1.03	12.44	11.50	107.84	107.58
Asp	0.06	0.9985	0.11	0.33	2.0	200.0	0.63	1.47	2.07	98.90	95.08
C-C	0.04	0.9972	4.56	13.82	41.6	166.4	3.33	3.37	8.00	104.52	108.55
DOPA	0.05	0.9968	1.95	5.92	25.0	200.1	7.65	7.31	24.73	104.95	104.03
Glu	0.06	0.9968	0.26	0.78	1.0	200.0	1.49	11.34	11.66	92.01	91.99
Gly	0.14	0.9996	0.16	0.50	2.0	200.0	0.45	1.02	6.54	105.43	97.80
His	0.04	0.9979	0.66	2.01	5.0	200.0	1.80	3.05	6.77	101.41	93.02
Hly	0.05	0.9839	3.66	11.09	10.0	200.0	2.47	6.24	6.34	-	-
Hyp	0.06	0.9988	0.25	0.77	2.0	200.0	1.54	3.51	3.49	-	-
Ile	0.10	0.9987	0.07	0.22	5.0	200.0	0.20	1.00	2.17	101.39	103.95
Leu	0.11	0.9985	0.03	0.10	5.0	200.0	0.15	0.65	0.94	106.21	107.84
Lys	0.05	0.9962	0.13	0.41	2.0	200.0	1.46	6.43	7.35	96.72	91.27
Met	0.06	0.9986	7.81	23.66	10.0	200.0	5.38	5.74	13.17	105.86	96.37
Phe	0.06	0.9995	0.07	0.22	2.0	200.0	1.63	2.20	2.48	102.48	103.34
Pro	0.09	0.9998	0.05	0.14	1.0	200.0	0.13	1.47	2.21	101.32	102.90
Ser	0.08	0.9977	0.27	0.81	10.0	200.0	1.98	2.72	4.18	97.77	96.74
Thr	0.09	0.9988	0.71	2.16	5.0	200.0	2.08	1.80	1.81	98.36	97.90
Trp	0.05	0.9972	1.56	4.72	2.0	100.0	1.94	3.14	5.37	102.82	110.83
Tyr	0.05	0.9995	0.21	0.64	1.0	100.0	1.95	1.79	3.04	97.51	98.44
Val	0.12	0.9999	0.03	0.10	2.0	200.0	0.08	0.52	2.44	101.19	98.89

3.3 AA recovery after acidic and alkaline hydrolysis

Using BSA and ovalbumin protein standards, acidic and basic hydrolysis were compared. Both proteins were hydrolysed with 6 N HCl or 5.6 N NaOH at 105 °C for 24 h. Due to unavailable experimental and vendor data, the AA sequences are not

precisely known for these proteins. Thus, sequences from the UniProt database (entries P02769 and P01012) were used to calculate AA recovery. As the database entries may not truly reflect the exact sequences of the proteins used, the obtained recovery values may not be completely correct. Nevertheless, this deviation does not influence the two methods and the final comparative outcome.

Fig. 2 shows the obtained recovery values. It is clear that alkaline hydrolysis resulted in very low recovery rates; < 40% for the majority of AAs. Following hydrolysis of BSA, only Lys, Tyr, Leu, Met and Phe showed recovery rates between 42 and 103%. Although high recovery rates were expected, Trp was not detected. The reason for this is that the SPE during sample preparation is compromised by the high concentration of NaOH in the hydrolysis solution. Na^+ ions bind to the cation exchange material of the SPE cartridges and hinder the binding and elution of the AAs. A dilution to 2 mL (dilution factor of ten) did not increase binding efficiency. In an attempt to improve recovery, several SPE steps with multiple cartridges of the same type were performed on the same sample solution. After three SPE steps, however, recovery had still not increased and AAs were still present in the solution. A dilution factor of, *e.g.*, ≥100 should enable the analysis, however, the concentration of AAs is too low for such a high dilution.

In contrast, acidic hydrolysis showed very good recoveries for many AAs. Previously reported oxidation of Met was observed; as was the partial destruction of Ser and Thr and the complete destruction of Trp. As His analysis did not show irregularities during derivatisation and GC-MS/MS analysis, it was assumed that the extraordinarily high recovery rate for His, 158 and 222%, is most probably due to imprecise sequence information. This can also explain recovery rates > 100% for the other AAs. Additionally, it was observed that, in general, ovalbumin hydrolysis yielded lower recoveries than BSA. This can be explained by the fact that ovalbumin is a glycoprotein and, to some extent, the glycan structures can hinder effective hydrolysis. No further attempts were made to reach complete hydrolysis.

To determine Trp, alkaline hydrolysis is necessary. However, the performance of alkaline hydrolysis across all AAs was not satisfactory; therefore, the decision was made to exclusively perform acidic hydrolysis. Due to the certified concentration and sequence information provided, hydrolysis of the BSA NIST standard (see section 3.4) enabled investigation of the accuracy of the protein hydrolysis method.

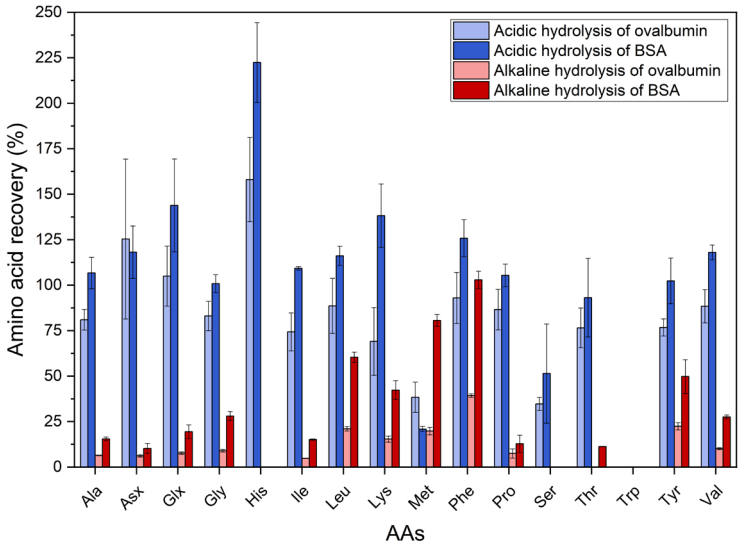

Fig. 2. AA recovery after acidic and alkaline hydrolysis of ovalbumin and BSA. Hydrolysis was performed with 6 N HCl or 5.6 N NaOH for 24 h at 105 °C.

3.4 Addition of phenol improved AA recovery following acidic hydrolysis

To improve AA recovery after acidic hydrolysis, phenol was added to HCl. This has been previously reported to enhance the recovery of some AAs [26-28]. Phenol acts as a halogen scavenger, thereby preventing halogenation of Tyr and also stabilising labile AAs. Acidic hydrolyses with 6 N HCl with, and without, the addition of 5% w/v phenol were compared. The experiment was performed over four weeks (two triplicates per week on different days, Fig. 3). The AA standard solution (19 AAs) plus DOPA was used at a concentration of 50 nmol/mL. Asn and Gln are not present in the AA standard, thus recoveries for Asp and Glu, instead of Asx and Glx, are visualised in Fig. 3.

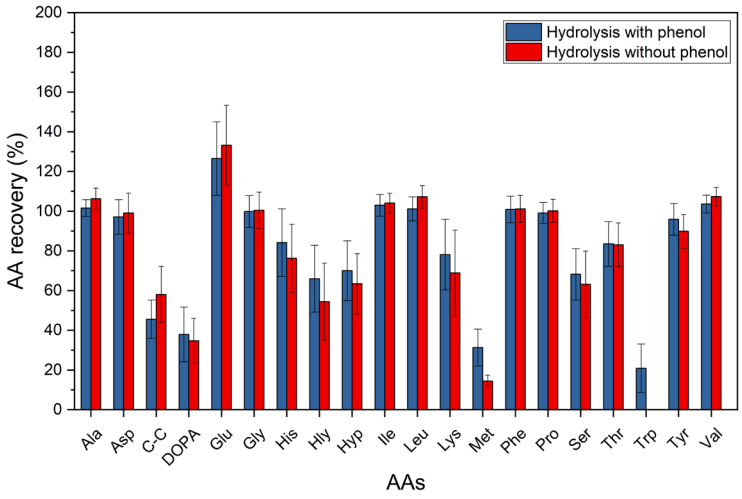

Fig. 3. AA recovery after acidic hydrolysis (24 h at 105 °C) of an AA standard mixture using 6 N HCl (with and without 5% phenol, w/v). Hydrolysis was performed in triplicate on eight different days within four weeks (two per week). The concentration for each AA was 50 nmol/mL.

As demonstrated in Fig. 3, recovery rates are similar for the compared methods (see also Suppl. Table 4). For method comparison, a 5% difference was defined as a significant improvement or deterioration. Thus, the addition of phenol increased the recovery of His (76.3% to 83.1%), Hly (54.4% to 65.6%), Hyp (63.4% to 69.2%), Lys (68.9% to 77.4%), Met (14.4% to 30.9%), Tyr (89.9% to 95.2%) and Trp (0.0% to 20.4%). Decreased recovery was observed for Ala (106.3% to 101.0%), C-C (58.0% to 45.1%), Glu (133.2% to 124.8%) and Leu (107.3% to 100.4%). According to this criterion (± 5%), recovery for all other AAs was considered identical for both methods. Of particular note is that there was a significant improvement for Met (+16.5%) and Trp (+20.4%). The latter was undetectable during hydrolysis without phenol. RSDs for both AAs are rather high however (Met: 30.3% CV, Trp: 60.2% CV) and increased considerably for Met with phenolic hydrolysis. Very good RSD values (3 to 11% CV) were obtained for Ala, Asp, Gly, Leu, Ile, Phe, Pro, Tyr and Val. For Glu and Thr, RSD values were between 12 and 16% CV; and for C-C, DOPA, His, Hly, Hyp, Lys, Met and Ser these values were between 18 and 37% CV. Comparison of the variation in the values indicated that the RSD decreased for all AAs except DOPA and Met. Thus, in general, the addition of 5% phenol improved sample preparation. The reason for the high recovery of Glu (>120%) is not yet clear and was not further investigated in this study.

The accuracy of the optimised hydrolysis was verified by acidic hydrolysis of the NIST BSA reference standard. To obtain information on the precision of Arg determination following protein hydrolysis, this investigation also included analysis of Arg by LC-MS/MS. The data revealed that AA recovery ranged from 94 to 111% (Fig. 4). Similar to the aforementioned experiments, values > 120% were obtained for Glx and His. The sequence information is provided with the certificate of the standard; thus, the reason for these high recoveries cannot be explained by an incorrect AA sequence. As such, an explanation for these effects has not yet been determined. Conversely, Ile showed a lower recovery of 86.4% and Trp was undetected. This latter observation is due to the fact that the majority of Trp was destroyed (only 20% Trp recovery in the presence of phenol) and only two Trp residues are present in BSA. The low recovery combined with the low abundance of this AA resulted in the concentration of Trp falling below the LOD. Thus, detection and quantitation of Trp was not possible. The information given in the provided certificate that there are 17 disulphide bonds (corresponding to 17 C-C molecules) in the NIST standard enabled calculation of a C-C recovery of 47.2%. In addition, Arg had a low recovery of 32.9%. The investigation of the accuracy of AA hydrolysis demonstrated that the recovery for the assessed AAs is very good; and only Arg, C-C and Trp showed high losses. Furthermore, the method enables adjustment of AA concentrations obtained for real samples by calculating correction factors.

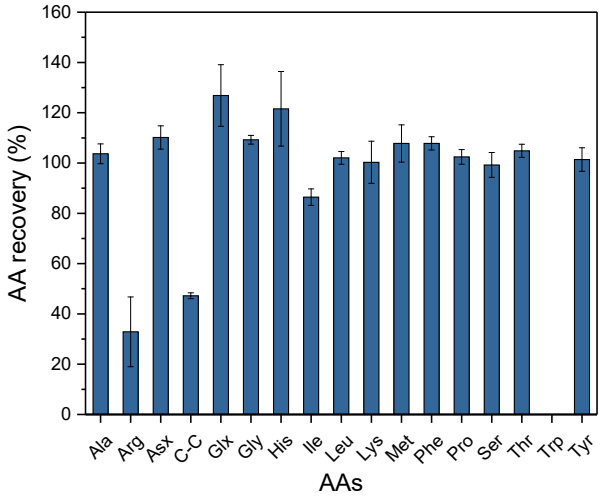

Fig. 4. AA recovery after acidic hydrolysis (24 h at 105 °C) of the certified NIST BSA protein standard using 6 N HCl (5% phenol, w/v).

3.5 Analysis of biological samples

Compared to ticks, barnacles are relatively large (0.5 cm to several cm in diameter) and thus, large sample amounts can be collected. In contrast, only very low quantities of biological adhesive is deposited when ticks adhere to the host organism via the mouthparts; albeit, the secreted amount is also species-dependent. The tick species, *Dermacentor marginatus*, secreted too little sample per individual tick to have sufficient material to measure all AAs including those of also low-abundance. Thus, samples from several ticks (up to 60 male and female animals) were pooled to obtain sufficient cement material for sample preparation. The higher quantity of the *Lepas anatifera* barnacle cement enabled further evaluation of the method before analysing the lower amounts of tick attachment cement.

Both adhesives were weighed on a microbalance and ground with an agate mortar and pestle (Fig. 5). The amounts used for a single analysis ranged between 100 and 200 µg and 200 and 400 µg for tick and barnacle cement, respectively. The low quantity of tick cement and the fact that the cement is highly electrostatically-charged made weighing difficult. Acidic hydrolysis was performed in triplicate, as described above. The AA concentrations from tick and barnacle cement are compared in Fig. 6 and Suppl. Table 5.

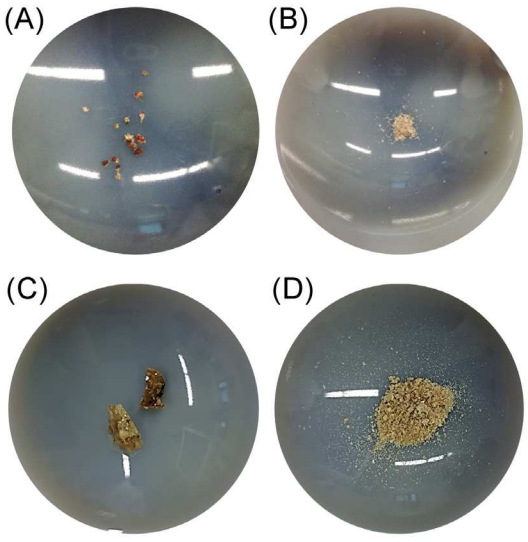

Fig. 5. Grinding and homogenisation of tick (*Dermacentor marginatus*) and barnacle (*Lepas anatifera*) cement samples using an agate mortar and pestle. Tick cement (A) before, and (B) after grinding. Barnacle cement (C) before, and (D) after grinding. Portions of the ground cement were used for acidic hydrolysis.

For the barnacle cement, many of the AAs had RSD values < 10% CV. Exceptions were: C-C (12.5% CV), Hly (14.3% CV), Hyp (23.5% CV), Lys (11.9% CV), Met (17.1% CV), Ser (12.5% CV), Thr (16.3% CV) and Tyr (12.7% CV). The main AAs in barnacle cement were mostly hydrophobic and non-polar, and included Leu, Ser, Gly, Ala, Val and Ile (Fig. 6). High amounts of Glx and Asx were obtained, representing the sum of the corresponding acid and amide derivative. In total, these AAs represented 4.9 nmol/µg or 68.7% of all observed AAs. As mentioned in the introduction, DOPA plays an important role in the adhesive apparatus of mussels; however, this AA was not observed in barnacle cement. This finding led to the conclusion that the adhesive mechanism is different in mussels and barnacles.

Nevertheless, in this study findings of AA levels for barnacle cement are moderately in agreement with previously published results where similar concentrations for Asx, Glx, Pro, Ser, Tyr and Val (all ± 10%) and Ile, Leu, Met and Thr (all ± 20%) were reported [8]. Compared to the findings of the present work, however, some differences were apparent. In particular, for Ala (+73%), Arg (+67%), C-C (-67%), Gly (+115%), His (+27%), Lys (+31%) and Phe (-26%). These differences are most likely due to the different methodology used in 1975 for cement analysis (LC-UV analysis after hydrolysis on an Amino Acid Analyser from Jeol) and/or biological variations of the cement sample showing the importance of the careful analysis presented here and importance to reconsider previously published data.

Analysis of tick attachment cement revealed that reproducibility is very good and not affected by the low sample quantity. For the majority of the AAs, RSD values were < 10% CV. Exceptions were: Ser (10.4% CV), Gly (12.6% CV), Trp (13.3% CV), Lys (14.4% CV) and Arg (29.4% CV). Tick cement is mainly composed of non-polar AAs and the major component was Gly with a concentration of 1.8 nmol/µg. Representing 27.2% of all observed AAs, this high amount of Gly is in accordance with the literature where glycine rich proteins (GRPs) are discussed in the context of salivary glands and cement extracts [5, 29, 30]. These proteins play a key role in the entire attachment and feeding process. The potential functions and properties of these GRPs are summarised in a recent review [2]. In addition to Gly, Leu (13.0%), Ser (12.4%) and Pro (9.7%) are highly-represented in the cement and together, all four comprised 4.21 nmol/µg or 62.4% of all observed AAs. The AAs C-C, DOPA, Hly, Hyp and Met were not present in *Dermacentor marginatus* cement.

An important point to keep in mind is that during artificial tick feeding bovine blood is in direct contact with the attachment cement produced by the animal. Some cement samples were slightly red in colour which was undoubtedly a consequence of this blood contact. As it is impossible to differentiate between AAs from the cement or blood, acidic hydrolysis of the blood was also performed to compare the AA composition with the cement (Suppl. Fig. 1). By comparing the very low concentrations of the individual AAs in the blood (0.1 to 2.0 nmol/mL, corresponding to fmol/µg concentrations) to the cement (0.02 to 1.84 nmol/µg), it was concluded that the most likely source of AA contamination appeared to have a negligible effect on the results. Hence, the high Gly content in tick cement in particular, does not appear to be related to the feeding material.

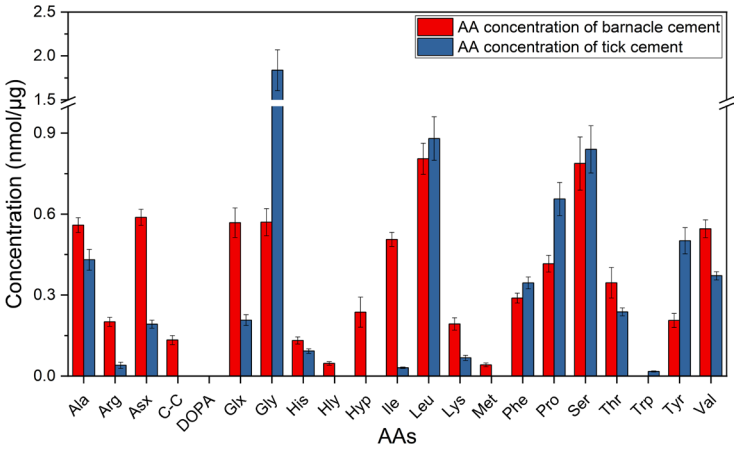

Fig. 6. AA concentration of barnacle cement (*Lepas anatifera*) and tick attachment cement (*Dermacentor marginatus*) after acidic hydrolysis. Hydrolysis was performed with 6 N HCl for 24 h at 105 °C. Concentration is given in nmol per µg cement.

Comparison of the AA composition of both adhesives showed that tick attachment cement consists of a large amount of Gly and also Leu, Ser and Pro (total 62.4%). In contrast, the AAs Leu, Ser, Asx, Gly, Glx, Ala, Val and Ile (total 68.7%) are the main components of barnacle cement. In tick cement, however, Gly appears to be the most dominant component, probably related with the adhesion mechanism. Hyp and C-C were not identified in the tick cement but were present in barnacle cement. The significant differences, however, led to the conclusion that ticks, and barnacles probably have different adhesion mechanisms. Nevertheless, in both of these biological adhesives, the content of His, Leu, Phe and Ser was very similar (difference < 0.1 nmol/µg). Due to the absence of DOPA, the adhesive mechanisms of both ticks

and barnacles are also different from mussels. Nevertheless, it has to be stated at this point that Tyr, the precursor of DOPA, is present in both samples and of significant higher concentration in tick than barnacle cement.

4. Conclusions

A highly-sensitive and selective MRM method for the analysis of AAs was developed. Furthermore, for both GC-EI-MS/MS and LC-ESI-MS/MS, detailed method validation was performed. Due to the thermal instability of the obtained Arg derivative, the latter approach was successfully applied to the analysis of this AA. It was demonstrated that the chromatographic performance and analysis time plus precision and accuracy are superior for most of the studied amino acids when using GC-MS/MS. LC-MS/MS, however, exhibits increased sensitivity. Moreover, the comparison of acidic and alkaline hydrolysis revealed reduced efficiency for glycoproteins, which always has to be kept in mind if AA analysis results are studied in the context of biological adhesives, long with the disadvantages of hydrolysis when using NaOH. In addition, it was proven that phenol as an additive during acidic hydrolysis improved the recovery of many AAs. The developed method was successfully applied to the analysis of tick and barnacle cement. Distinct differences in the composition were observed. The adhesive of *Dermacentor marginatus* ticks consists of high amounts of Gly and also Leu, Ser and Pro and together, these represented 62.4% of all observed AAs. Conversely, high amounts of Leu, Ser, Asx, Gly, Glx, Ala, Val and Ile (total 68.7%) are present in *Lepas anatifera* barnacle cement. Secreted tick cement is in direct contact with the feeding material (bovine blood). Nevertheless, it was shown that this most likely source of contamination did not appear to influence the results. To confirm this, a non-blood-based feeding material could be assessed.

Although both adhesives have cement-like properties and adhere in damp environments, the differences presented in this study indicated that ticks and barnacles have very different composition and therefore probably also different adhesion mechanisms. In addition, the absence of DOPA (an AA that plays a crucial role in the adhesive mechanism of mussels) in both cements showed that there are huge differences between these biological glues and well-studied systems such as e.g. mussels or sandcastleworms (all DOPA containing systems). Lastly, while analysis of bulk cements gives a hint about general similarities and differences from well-studied

systems (like mussels), only information on individual proteins (*e.g.* identified by proteomics approaches) which comprise the bulk cement, along with associated sugar or lipid components, can bring deeper understanding of adhesive mechanisms.

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CRediT author statement

Benedikt Engel: conceptualization, investigation, validation, writing – original draft, revision**, Johannes Suppan:** methodology, writing – revision**, Sylvia Nürnberger:** conceptualization, methodology, resources, funding acquisition, writing – editing&revision**, Anne Marie Power:** data curation, writing – review&editing**, Martina Marchetti-Deschmann:** conceptualization, methodology, supervision, resources, funding acquisition, writing – original draft&revision

Conflict of Interest

All authors declare no competing financial interests.

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Revisiting amino acid analyses for bioadhesives including a direct comparison of tick attachment cement (*Dermacentor marginatus*) and barnacle cement (*Lepas anatifera*)

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Suppl. Table 1: Optimised GC-MS/MS parameters listing quantitation and confirmation transitions for all MRM events relevant for AA analyses. The table is sorted according to retention times. For Gly, no confirmation transition was observed and for Ala, only one transition was obtained.

AAs	Retention time (min)	Quantitation transition		Confirmation transitions			
		Transition 1	CE (V)	Transition 2	CE (V)	Transition 3	CE (V)
Ala	1.46	130.00>88.10	5	130.00>86.20	5	-	-
Gly	1.56	102.00>74.10	5	-	-	-	-
Val	1.75	158.00>116.10	6	116.00>98.10	7	116.00>55.10	15
Nor	1.85	158.00>72.10	7	158.00>116.10	5	158.00>74.10	10
Leu	1.92	172.00>86.20	7	172.00>116.20	5	172.00>74.10	16
Ile	1.97	172.00>130.20	6	130.00>74.10	7	172.00>74.10	12
Thr	2.13	143.00>101.10	5	101.00>83.00	7	101.00>73.00	8
Ser	2.16	146.00>60.10	8	104.00>60.10	5	146.00>104.10	4
Pro	2.22	156.00>70.10	9	156.00>114.10	6	114.00>70.10	9
Asp	2.65	130.00>88.10	5	216.00>130.20	6	216.00>88.10	9
Met	2.68	203.00>101.10	9	143.00>101.10	5	203.00>143.10	5
Hyp	2.77	172.00>86.10	8	86.00>68.10	8	172.00>68.10	15
Glu	2.89	170.00>142.20	4	230.00>142.20	8	230.00>170.20	5
Phe	2.92	190.15>148.10	5	148.00>103.10	20	148.00>91.10	23
Lys	3.94	170.00>128.20	8	170.00>84.20	16	128.00>84.10	8
His	4.12	180.00>94.10	7	282.00>110.20	16	282.00>196.20	7
Hly	4.29	129.00>87.10	5	169.00>154.20	5	169.00>127.10	5
Tyr	4.39	107.10>77.10	18	164.00>147.10	15	164.00>118.10	15
Trp	4.69	130.00>77.10	26	130.00>103.10	19	130.00>128.10	16
DOPA	5.39	123.00>77.10	18	123.00>51.00	24	165.00>123.10	9
C-C	5.37	248.00>146.10	9	174.00>60.10	9	174.00>132.10	6

Suppl. Table 2: Optimised LC-MS/MS parameters listing quantitation and confirmation transitions for all MRM events relevant for AA analyses. The table is sorted according to retention time.

AAs	Retention time (min)	Quantitation transition		Confirmation transitions			
		Transition 1	CE (V)	Transition 2	CE (V)	Transition 3	CE (V)
Ala	2.95	303.20>70.20	-37	303.20>156.20	-21	303.20>286.20	-16
Gly	3.18	234.20>174.15	-9	234.20>146.20	-11	234.20>216.10	-7
Val	3.95	204.10>102.15	-11	204.10>118.25	-10	204.10>144.20	-8
Nor	4.29	260.20>172.20	-11	260.20>200.20	-8	260.20>130.25	-16
Leu	4.63	248.30>160.25	-10	248.20>188.25	-9	248.30>230.20	-7
Ile	6.22	218.10>130.20	-10	218.10>158.30	-8	218.10>88.15	-16
Thr	10.79	244.30>156.25	-11	244.30>114.20	-20	244.30>184.20	-8
Ser	11.41	377.20>125.25	-25	377.20>317.20	-9	377.20>359.15	-8
Pro	11.89	278.10>190.15	-10	278.10>142.20	-15	278.20>218.10	-9
Asp	14.21	246.30>158.20	-11	246.30>116.20	-17	246.30>186.20	-8
Met	14.88	304.20>216.20	-12	304.20>130.20	-18	304.20>244.20	-8
Hyp	14.89	246.40>158.30	-10	246.40>116.25	-16	246.10>72.15	-21
Glu	15.68	370.40>196.20	-21	370.40>110.20	-32	370.40>284.25	-16
Phe	15.79	318.20>172.20	-14	318.20>258.15	-9	318.20>230.20	-12
Lys	15.93	361.20>301.25	-10	361.20>170.25	-18	361.20>128.25	-24
His	16.15	333.30>245.20	-16	333.30>159.20	-27	333.30>273.15	-11
Hly	16.38	260.10>172.20	-11	260.10>130.15	-16	260.10>116.25	-17
Tyr	16.45	294.10>206.20	-11	294.10>120.25	-23	294.10>164.25	-16
Trp	17.21	497.30>248.15	-17	497.30>206.15	-24	497.30>306.15	-15
DOPA	17.27	396.30>222.20	-21	396.30>136.20	-29	396.30>308.20	-14
C-C	17.58	498.10>352.20	-14	498.10>178.15	-33	498.10>238.25	-27

Suppl. Table 3: LC-MS/MS validation results for all investigated AAs. All data were normalised to the IS. Precision and accuracy values were measured at a concentration of 20 nmol/mL (except C-C at 10 nmol/mL). LOD and LOQ were calculated from calibration curves at low concentrations (see section 2.7). Hly and Hyp were not included in determination of accuracy. CV: coefficient of variation; RT: retention time

AA	RT (% CV)	Adj. R2	LOD (µM)	LOQ (µM)	Linear range (nmol/mL)		Precision (% CV)			Accuracy (%)	
							Injection	Intra-day	Inter-day	Intra-day	Inter-day
Ala	0.16	0.9999	0.59	1.80	1.00	200.00	2.00	12.31	14.09	92.24	90.14
Arg	0.29	0.9996	1.01	3.06	0.50	200.04	1.03	12.44	11.50	107.84	107.58
Asp	0.10	0.9997	0.04	0.11	0.05	25.00	1.41	6.81	7.48	99.08	103.54
C-C	0.01	0.9983	0.04	0.14	0.50	10.00	0.89	8.54	10.55	106.44	107.39
DOPA	0.02	0.9988	0.06	0.20	0.10	25.52	2.33	6.89	16.01	85.76	86.16
Glu	0.05	0.9985	0.01	0.03	0.05	25.00	0.51	10.56	10.47	91.90	87.68
Gly	0.14	0.9997	0.06	0.18	1.00	100.00	2.11	1.68	4.48	103.85	102.76
His	0.05	0.9999	0.02	0.08	0.50	25.00	0.24	8.53	9.74	105.94	105.85
Hly	0.24	0.9994	0.73	2.21	2.00	200.00	2.32	2.61	3.95	-	-
Hyp	0.17	0.9999	0.01	0.04	0.03	25.00	1.93	3.89	4.03	-	-
Leu/Ile	0.03	0.9905	0.06	0.18	0.10	25.00	0.59	1.71	4.55	89.99	85.32
Lys	0.04	0.9994	0.02	0.07	0.10	25.00	0.91	15.87	14.50	113.14	110.32
Met	1.62	0.9999	0.13	0.39	50.00	200.00	3.87	120.11	119.92	284.30	274.21
Phe	0.03	0.9982	0.03	0.08	0.50	25.00	1.03	8.11	9.57	110.86	113.75
Pro	0.17	1.0000	0.01	0.02	0.10	50.00	1.77	1.63	3.22	101.20	97.38
Ser	0.17	0.9999	0.05	0.16	0.05	50.00	0.83	8.29	11.32	112.36	115.89
Thr	0.19	0.9999	0.03	0.10	0.10	25.00	2.05	5.49	6.00	102.57	108.75
Trp	0.04	1.0000	0.04	0.13	0.50	25.00	0.16	5.07	5.83	119.46	117.85
Tyr	0.02	0.9997	0.09	0.28	0.50	25.00	1.80	7.13	7.88	109.97	108.49
Val	0.14	0.9996	0.70	2.11	2.00	200.00	6.71	6.18	6.77	105.64	102.64

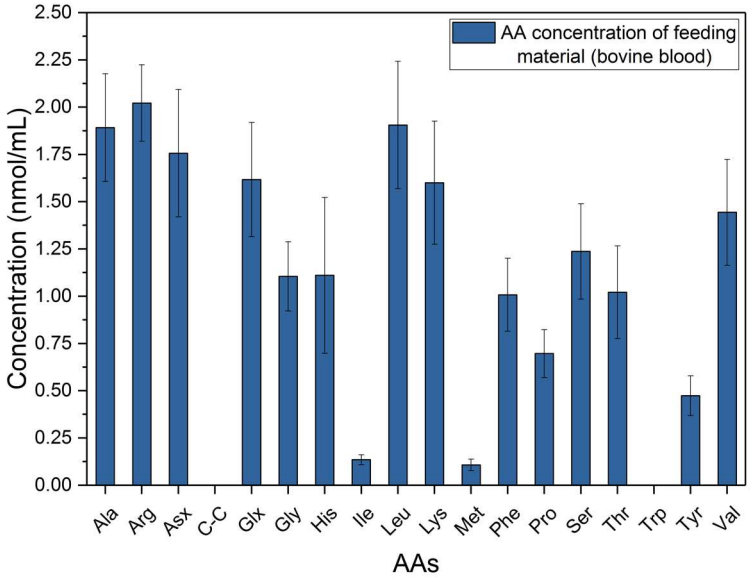
Suppl. Table 4: AA recovery values after acidic hydrolysis for 24 h at 105 °C of an AA standard mixture using 6 N HCl (with and without 5% phenol, w/v). Hydrolysis was performed in triplicate on eight different days within four weeks (two per week). The AA concentration was 50 nmol/mL for each AA. CV: coefficient of variation;

AA	AA recovery with phenol (%)	RSD (% CV)	AA recovery without phenol (%)	RSD (% CV)
Ala	100.97	3.08	106.28	5.04
Asx	96.17	8.15	99.08	10.14
C-C	45.06	21.32	58.05	24.40
DOPA	37.96	36.42	34.75	32.41
Glx	124.79	13.57	133.22	15.17
Gly	99.21	7.74	100.47	9.05
His	83.08	19.94	76.26	22.49
Hly	65.57	25.99	54.43	35.58
Hyp	69.23	21.65	63.44	23.94
Ile	102.31	4.39	104.17	4.71
Leu	100.36	4.76	107.30	5.25
Lys	77.44	23.07	68.95	31.34
Met	30.92	30.25	14.43	20.77
Phe	100.05	5.55	101.15	6.71
Pro	98.33	3.98	100.18	5.83
Ser	67.33	18.61	63.20	26.53
Thr	82.51	12.67	83.11	13.26
Trp	20.42	60.19	-	-
Tyr	95.22	7.83	89.90	9.47
Val	102.95	3.27	107.43	4.29

Suppl. Table 5: AA concentration of barnacle (*Lepas anatifera*) and tick (*Dermacentor marginatus*) cement after acidic hydrolysis using 6 N HCl (5% phenol, w/v) for 24 h at 105 °C. CV: coefficient of variation;

AA	Barnacle cement (nmol/µg)	RSD (% CV)	Tick cement (nmol/µg)	RSD (% CV)
Ala	0.56	4.91	0.43	8.89
Arg	0.20	8.32	0.04	29.42
Asx	0.59	5.09	0.19	7.75
C-C	0.13	12.55	-	-
DOPA	-	-	-	-
Glx	0.57	9.64	0.21	9.60
Gly	0.57	8.79	1.84	12.64
His	0.13	9.95	0.09	8.93
Hly	0.05	14.27	-	-
Hyp	0.24	23.49	-	-
Ile	0.51	5.10	0.03	9.05
Leu	0.81	7.14	0.88	9.15
Lys	0.19	11.92	0.07	14.36
Met	0.04	17.14	-	-
Phe	0.29	6.27	0.35	6.34
Pro	0.42	7.43	0.66	9.32
Ser	0.79	12.52	0.84	10.43
Thr	0.35	16.34	0.24	6.21
Trp	-	-	0.02	13.33
Tyr	0.21	12.66	0.50	9.65
Val	0.55	6.01	0.37	4.12

Suppl. Fig. 1: AA concentration of bovine blood (tick feeding material) in an artificial membrane feeding system.



Manuscript IV – Protein identification of the attachment cement of the ixodid tick *Dermacentor marginatus*

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Highlights of this manuscript

- Tick cement protein solubilisation using different buffer systems
- Separation of proteins by means of SDS-PAGE
- Protein identification using LC-ESI-ToF-MS/MS

Protein identification of the attachment cement of the ixodid tick *Dermacentor marginatus*

1. Introduction

It is known from literature that the cement of ticks mainly contains proteins and small amounts of lipids and carbohydrates probably in the form of lipoproteins and glycosylated proteins [1-3]. These facts display the high importance of the present proteins for the adhesion mechanism. However, not much is known about the involved proteins and adhesion machinery. Here, tick cement specific proteins were identified using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography electrospray ionisation time-of-flight tandem mass spectrometry (LC-ESI-ToF-MS/MS) to get a deeper insight into the composition of the attachment cement and a possible understanding of the adhesion process.

2. Materials and methods

2.1. Chemicals and reagents

Ultra-pure water (18.2 MΩ.cm) was prepared using a Simplicity system from Millipore, Billerica, MA, USA (for sample preparation for SDS-PAGE) and a Mili-Q water purification system (Mili-Q model Pacific TII 12) from Thermo Scientific, MA, USA (for *in-gel* digestion and LC-ESI-ToF-MS/MS analysis). Acetic acid (≥ 99.8%), ammonium bicarbonate (NH_4HCO_3, ≥ 99.5%), 3-[(3-cholamiopropyl)dimethylammonio]-1-propanesulfonate (CHAPS, ≥ 98%, TLC), DL-dithiothreitol (DTT, ≥ 99.5%), iodoacetamide (IAA, ≥ 99%), 2-mercaptoethanol (> 99%), potassiumhexacyanoferrat ($K_3Fe(CN)_6$, ≥ 99%), sodium thiosulphate ($Na_2S_2O_3$, ≥ 98%) and thiourea (> 99.0%) were obtained from Sigma-Aldrich, St. Louis, MO, USA. Acetonitrile (ACN, LiChrosolv) and urea (p. a.) was obtained from Merck, Darmstadt, Germany. Formic acid (≥ 97.5%) was obtained from Honeywell Fluka, Morristown, NJ, USA. Sequencing grade trypsin from bovine was from Roche, Mannheim, Germany. The albumin standard (2.0 mg/mL bovine serum albumin in 0.9% saline and 0.05% sodium azide) was obtained from Thermo Scientific, Rockford, IL, USA. NuPAGE 4-12% Bis-Tris gels, 4x lithium dodecyl sulphate (LDS) sample buffer (106 mM Tris HCl, 141 mM Tris Base, 2% LDS, 10% glycerol, 0.51 mM EDTA (ethylenediaminetetraacetic acid), 0.22 mM Serva Blue G250, 0.175 mM Phenol Red, pH 8.5), 20x 3-(N-morpholino)propanesulfonic acid (MOPS) SDS running buffer (50 mM

MOPS, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7), and BenchMark Protein Ladder were acquired from Life Technologies from Carlsbad, CA, USA.

2.2. Bioadhesive preparation

Tick attachment cement was collected from *in vitro* fed *Amblyomma hebraeum* and *Dermacentor marginatus* ticks and due to low sample amounts, cement of female and male animals was pooled. Barnacle cement samples (*Lepas anatifera*) were obtained from Anne Marie Power (National University of Ireland). Homogenisation was performed by grinding the adhesive in an agate mortar with pestle and portions of that were used for sample preparation. Proteins were solubilised using three different solubilisation buffers:

- Buffer 1: LDS sample buffer, ultra-pure water and DTT (50 mM) (2.5:6.5:1, v/v/v)
- Buffer 2: acetic acid (5%), rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS) and 2-mercaptoethanol (15:1:11, v/v/v)
- Buffer 3: ultra-pure water, rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS) and 2-mercaptoethanol (15:1:11, v/v/v)

Protein concentration was determined using the Bradford or Pierce™ 660 nm protein assay from Thermo Scientific, Rockford, IL, USA and a Nano Photometer by Implen, Munich, Germany and bovine serum albumin was used for calibration.

2.3. SDS-PAGE

Samples (1.00 µg) were applied in LDS sample buffer and 50 mM DTT. SDS-PAGE analyses were carried out on NuPAGE 4-12% Bis-Tris gels using MOPS SDS running buffer at 120 V (const.) and 60 mA (max.) in a XcellSurelock Mini Cell electrophoresis system from Life Technologies, Carlsbad, CA, USA. BenchMark Protein Ladder was used for molecular weight determination. Protein bands were visualised by silver staining suited for further mass spectrometric analyses and gels were stored in 1% acetic acid at 4 °C for further investigations [4].

2.4. LC-ESI-ToF-MS/MS

After SDS-PAGE protein bands were excised and proteins were identified by LC-ESI-ToF-MS/MS analysis after gel destaining and tryptic *in-gel* digestion [5]. Briefly, excised gel bands were destained in 100 mM $Na_2S_2O_3$ / 30 mM $K_4Fe(CN)_6 \cdot 3H_2O$ (1:1, v/v). Gel pieces were treated with ACN, rehydrated (100 mM NH_4HCO_3), reduced (10 mM DTT in 100 mM

NH_4HCO_3, 56 °C, 45 min), alkylated (50 mM IAA in 100 mM NH_4HCO_3, 22 °C, 30 min), and finally dried in a vacuum centrifuge. After rehydration in approx. 10 µL 50 mM NH_4HCO_3 (pH 8.5) containing 5% ACN and 125 ng trypsin and overnight incubation at 37 °C, peptides were extracted with 50 mM NH_4HCO_3/ACN (1:1, v/v) and 0.1% formic acid / ACN (1:1, v/v). All extracts of selected lanes were dried in a vacuum centrifuge. After reconstitution in 0.1% acetic acid samples could be directly used for analysis on the Synapt G2-Si using a C18 Acquity UPLC trap and analytical column (all from Waters, Manchester, United Kingdom). The mobile phase consisted of ultra-pure water (A) and ACN (B), both containing 0.1% acetic acid, and a gradient was used for elution (**Figure 1**).

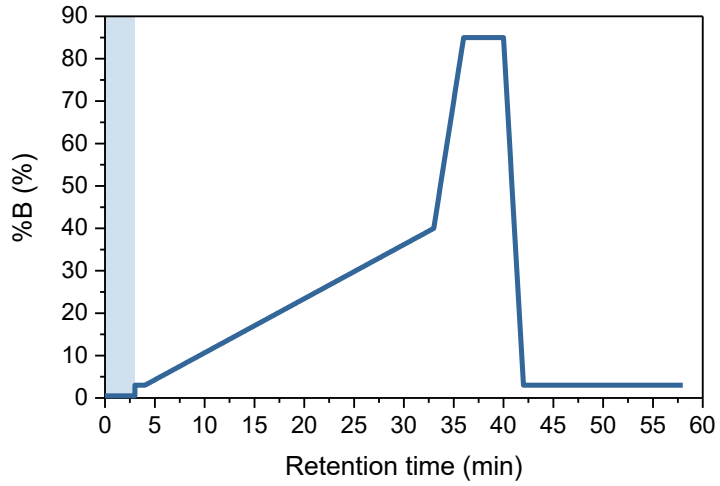

Figure 1: Mobile phase gradient used for peptide separation on the Synapt G2-Si. Mobile phase consisted of ultra-pure water (A) and ACN (B), both containing 0.1% acetic acid. The first three minutes were used for trapping the samples (blue marked area).

Mass spectrometric analysis was performed in the MS^E mode from m/z 50 to 2000. Every 60 sec a lock mass ([Glu1]-Fibronopeptide B, 50 fmol/µL and Leucine Enkephalin, 100 pg/µL; both from Waters, Manchester, United Kingdom) was measured for internal mass calibration. Protein identification was performed using PLGS (ProteinLynx Gobal SERVER). The processing included ion detection, lock mass correction, charge-state deconvolution and finally the database search. The search parameter are summarised in **Table 1**. The database (version 20180103) contained in total 191,736 UniProt sequences from several organisms which all use biological adhesion in different forms (**Table 2**).

Table 1: PLGS search parameter

Search parameter	Value
Intensity threshold	750 counts
Low energy threshold	150 counts
High energy threshold	25 counts
Min. fragment ion matches per peptide	3
Min. fragment ion matches per protein	7
Min. peptide matches per protein	1
Primary digest reagent	Trypsin
Missed cleavages	1
Fixed modifications	Carbamidomethyl C
Variable modifications	Oxidation M

Table 2: UniProt entries used for protein identification (version 20180103). All selected organisms produce bioadhesives and use them in different forms.

Organism	Database entries	
	Reviewed	Unreviewed
Barnacles (Thoracica)	8	4,609
Mussels (Mytilus)	83	3,901
Sandcastle worm (*Phragmatopoma californica*)	-	68
Sea star (*Asterias rubens*)	6	107
Sea urchins	257	33,572
Ticks (Ixodidae)	94	149,031

3. Results and discussion

3.1. Protein solubilisation

The protein solubilisation method of choice should neither destroy nor modify the proteins and thus, gentle conditions have to be used. It is known from literature that tick attachment cement is easily soluble in hot bases and acids [1]. However, this would destroy the proteins and therefore in this work three solubilisation buffers were investigated and compared by the amount of solubilised proteins using different photometric protein assays. Buffer 1 contains a conventional sample buffer (LDS) for protein solubilisation and DTT as reductant. Buffer 2 is mainly based on solubilisation facilitating acidic conditions (acetic acid) and a rehydration solution containing the chaotropes urea and thiourea which are quite efficient in disrupting hydrogen bonds. Buffer 3 is similar to buffer 2 but ultra-pure water was used instead of acetic acid. 2-mercaptoethanol was used as reductant for the latter buffer systems.

The comparison of the efficiency of the three buffer systems for solubilisation was studied for *Amblyomma hebraeum* ticks and *Lepas anatifera* barnacles. It could be revealed that buffer 2 has the highest ability of protein solubilisation (**Figure 2**). This suggests the presence of proteins with a high pI which have an increased solubility at acidic conditions [6]. The determination of the protein concentration also showed that both applied protein assays yielded very similar results.

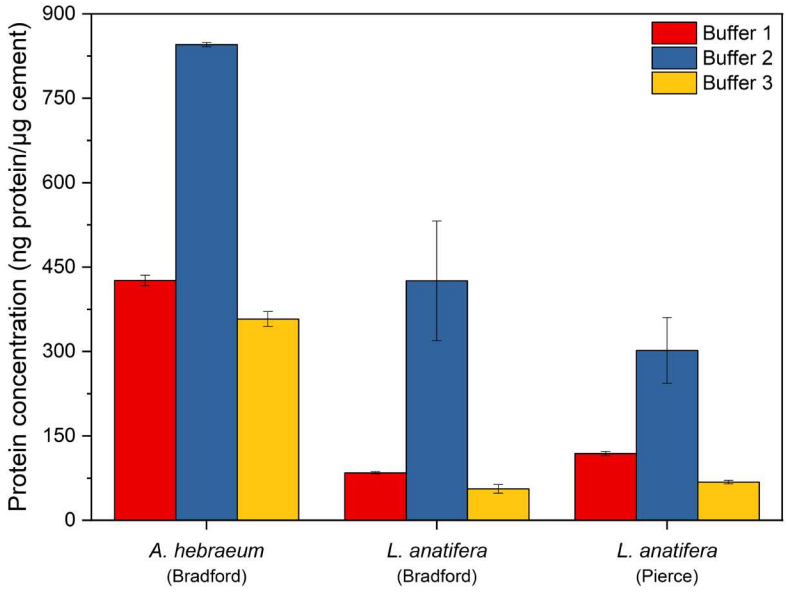

Figure 2: Results of protein solubilisation using three different buffer systems. Cement of *Amblyomma hebraeum* ticks and *Lepas anatifera* barnacles was used for this investigation. Protein concentration was determined either by a Bradford or Pierce assay.

3.2. Separation of tick attachment cement proteins

About 1.00 µg of the solubilised proteins were further treated with LDS buffer and DTT and separated on NuPAGE 4-12% Bis-Tris gels using a MOPS SDS running buffer. Ticks are often fed on blood-based feeding materials like bovine blood in order to mimic *in vivo* conditions. By this the secreted cement comes into direct contact with the blood and thus contamination of the cement with blood proteins has to be considered. **Figure 3** shows obvious differences for blood-fed ticks (Lane 2) when compared to cement harvested from ticks fed on blood-free medium (Lane 3). Lane 1 represents the blood based feeding material. The more intensely stained background of Lane 2 results from proteins present in the feeding material. In lane 3 a significant change of the protein pattern can be observed: the single band at 15 kDa in lane 2 is not present in lane 3 but two protein bands can be obtained. Furthermore, the intensity of the protein band at 70 kDa is decreased a lot in lane 3. In the range of 20 to 70 kDa a noticeable background intensity reduction can be seen allowing the visualisation of several tick cement protein bands. In addition to that in contrast to lane 2 four protein bands between 120 and 160 kDa can be obtained in lane 3.

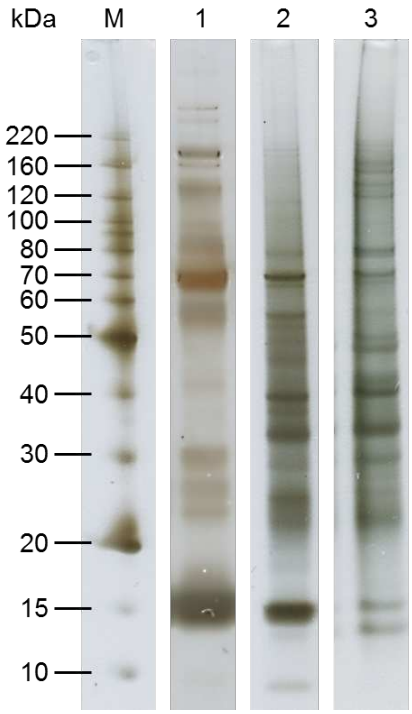

Figure 3: SDS-PAGE of a blood-based feeding material (Lane 1) and tick cement proteins from ticks fed with blood-based (Lane 2) and blood-free (Lane 3) feeding materials. Lane M represents the molecular weight marker

The two very intense bands (15 and 70 kDa, lane 2) are highly comparable to the bands in lane 1 and show that the attachment cement is contaminated by the blood-based feeding

material. This contamination makes further interpretation and especially protein identification difficult since proteins from the feeding material co-migrate with proteins originating from the tick cement, like the ones at 15 and 70 kDa. The absence of these bands in lane 3 and the noticeably reduction of background staining, yields improved SDS-PAGE results.

3.3. Protein identification

After SDS-PAGE all visible protein bands were excised and sample preparation for LC-ESI-ToF-MS/MS analysis was performed. Mass spectrometric analysis was performed in MS^E mode which allowed the generation of low energy and high energy collision induced dissociation spectra and thus precursor and product ion information of each peptide could be obtained by a single measurement.

The prominent bands at 15 and 70 kDa in Lane 1 in **Figure 3** were confirmed as haemoglobin and bovine serum albumin, respectively. These two proteins could be also identified in tick cement fed with bovine blood. This reveals the contamination of the cement and indicates the importance of a feeding material free from blood in order to allow optimal protein identification conditions. Therefore, the findings in the following refer to cement samples from ticks fed with non-blood-based feeding material.

The used protein database contained in total 191,288 unreviewed protein entries, from which 149,031 are specific for ticks (Ixodidae) and only 341 for *Dermacentor*. All other entries, i.e. 42,257, are non-tick related. Missing tick entries and homology to proteins found in other species are therefore the reason for many of the following protein identifications related to other species but *Dermacentor* using bioadhesion in all different forms. The drawback of an unreviewed protein database is especially the fact that the presence and function of the proteins are not clear or confirmed, making identification and interpretation of results challenging.

Several proteins in the range of 25 to 35, 60 to 160 kDa and at 15 kDa could be identified (**Figure 4**, Lane 1', bands A to L; **Table 3** and **Table 4**). Detailed information to the identified proteins can be found in **Supplementary Table 1** (p. 165). In the mass range of 35 to 60 kDa no proteins could be identified. The reason for that is the poor quality of the obtained mass spectra showing only noisy mass spectra with little to no signals.

Most striking is the fact, that more than one SDS-PAGE band contained so called Glycine-rich proteins (GRPs) (**Table 3**). The high abundance of glycine was already mentioned in literature and also our own amino acid analysis confirmed these findings (**Manuscript I**, p. 33 in **Part I: Method Development**). This suggests that glycine plays an essential role

in the attachment of ticks. The identified GRPs, having isoelectric points (pI) above 9, can be described as basic proteins. The identification of basic proteins nicely fits with the solubilisation behaviour (see **section 3.1**) as solubility increases with a protein's net charge [6]. Thus, acidic solvents are required for the solubilisation of proteins with high pIs, and alkaline solvents would be well suited for proteins with low pIs. One of the identified GRPs is RIM36, a protein described to be predominantly located in the e cell granules of the type III salivary gland acini inducing strong antibody response in cattle exposed to feeding ticks [7].

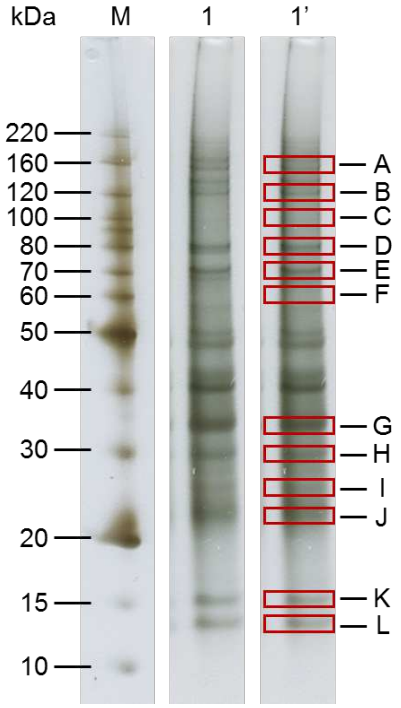

Figure 4: SDS-PAGE of tick cement proteins from ticks fed with blood-free feeding material (Lane 1). The boxes indicate protein bands which could be identified (Lane 1', A-L). Lane M represents the molecular weight marker.

GRPs were also characterised in plants and it was found that these proteins play a crucial role for cell wall structures, cell elongations, signal transductions, defences, RNA binding and some of them show antimicrobial activity [8, 9]. In insects these proteins are described to be involved in cuticle construction and show increased expression in response to stress. However, not much is known about the functions of these GRPs in ticks. The fact that a high glycine content is a feature of vertebrate extracellular matrix proteins (e.g. keratin or collagen) suggests that tick cement components may mimic components of vertebrate skin in order to use host-derived enzymes during the cement hardening process [7]. The identified putative collagen triple helix repeat protein is also an indicator that ticks secret

proteins closely related to the surrounding skin environment to get bonding due to tissue similarity and therefore some kind of resorption of the tick cement in the environmental collagen structure of the skin. Furthermore, GRPs seem to give the cement cone its strength and insoluble characteristics [10].

Other further interesting proteins identified in tick cement are the putative ixostatin which is described to be involved in the inhibition of tissue repair, Lipocalin which is a protein secreted by ticks in their salivary glands as an important strategy to interfere with the immune response of hosts and the putative Kunitz protein, which inhibits the function of protein degrading enzymes [11-13]. Both proteins can be considered as support during the feeding on the host. Identified proteins associated with saliva can be explained by the fact that the cement is produced by the salivary glands of Ixodidae in preparation for and during feeding. High enzymatic activity of the tick cement can be expected due to the fact that many of the identified proteins were found to be enzymes or enzyme inhibitors, like the salivary cystatin, a protein small peptide of approx. 15 kDa belonging to a group of functionally specific protease inhibitors [14].

Interestingly, a putative bitil peptide was also identified. Such peptides contain the TIL domain and generally consist of 56–84 amino acid residues having a total molecular weight of approx. 23 kDa, which nicely fits to the SDS-PAGE analysis. It was shown that a typical peptide of the TIL family is able to inhibit proteinase, and thus play roles in biological processes, such as inhibition of anticoagulation and participation in immune response [15-18].

In addition to that also proteins with antimicrobial activity, two tick specific and two non-tick specific ones, could be found which indicates the possible function of the cement to protect the tick and the feeding lesion (**Table 4**).

Table 3: Identified tick (cement) specific proteins with the corresponding position on the SDS-PAGE gel. *A., Amblyomma*; *D., Dermacentor*; *H., Hyalomma*; *I., Ixodes*; *R., Rhipicephalus*

Protein no.	Gel position	Protein name (UniProt database)	Species
1	D	Alpha-N-acetylgalactosaminidase	*R. appendiculatus*
2	K	Amblyomma 40-33 family member	*R. appendiculatus*
3	J	Glycine rich superfamily member	*R. appendiculatus*
4	K	Hypothetical glycine rich secreted cement protein	*H. rufipes*
5	I	Lipocalin	*R. zambeziensis*
6	G	Pre mRNA splicing factor 38a	*R. zambeziensis*
7	I	Putative bitil peptide	*R. pulchellus*
8	G	Putative cement protein RIM36	*D. variabilis*
9	H	Putative collagen triple helix repeat protein	*R. pulchellus*
10	B	Putative conserved plasma membrane protein	*A. cajennense*
11	A	Putative erythrocyte membrane-associated antigen	*A. cajennense*
12	L	Putative eukaryotic translation initiation factor 4 gamma	*I. ricinus*
13	H	Putative glycine rich protein	*R. pulchellus*
14	I	Putative glycine rich protein	*R. pulchellus*
15	F	Putative ixostatin	*I. ricinus*
16	J	Putative Kunitz-BPTI protein	*D. variabilis*
17	B	Putative PHD and ring finger domain-containing protein 1	*I. ricinus*
18	F	Putative ribonuclease t2 family	*I. ricinus*
19	K	Putative secreted salivary protein	*A. americanum*
20	I	Putative serine protease	*I. ricinus*
21	K	Putative tick salivary cystatin	*R. pulchellus*
22	E	Putative transcription termination factor rho	*I. ricinus*
23	B	Putative vitellogenin receptor *I. scapularis* vitellogenin receptor	*A. cajennense*
24	J	Sulfotransferase	*R. appendiculatus*
25	K	Tick serine protease	*R. appendiculatus*
26	B	Uncharacterised protein	*A. americanum*
27	C	Uncharacterised protein	*I. ricinus*
28	K	Uncharacterised protein	*I. ricinus*
29	G	Uncharacterised protein	*A. maculatum*
30	I	Uncharacterised protein	*I. scapularis*
31	J	Very acidic secreted protein putative	*I. scapularis*

Table 4: Identified proteins with antimicrobial activity with the corresponding position on the SDS-PAGE gel. *E., Echinus*; *H., Haemaphysalis*; *M., Mytilus*; *R., Rhipicephalus*

Protein no.	Gel position	Protein name (UniProt database)	Organism	Species
32	A	Female-specific histamine-binding protein 2	Tick	*R. appendiculatus*
33	C	Longicornsin	Tick	*H. longicornis*
34	B	Myticin C	Mussle	*M. galloprovincialis*
35	L	Strongylocin 2	Sea urchin	*E. esculentus*

3.4. Conclusion

This work allowed the identification of 35 tick cement specific proteins and two non-tick specific ones. Additionally, the presence and importance of GRPs for tick attachment was confirmed and corroborated hypotheses available in literature stating that GRPs stabilise and strengthen the cement cone causing the insolubility of the cement. Furthermore, the identification of proteins with antimicrobial activity reveals that the cement acts not only as a glue, but also has other functions like protecting the tick and the feeding lesion. However, the lack of database entries and especially of reviewed sequences makes the process of identification very challenging. Moreover, a complete solubilisation of the cement could not be achieved since insoluble debris remained in the investigated buffers. Therefore, future work should concentrate on the improvement of solubilisation and of available protein databases.

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Abbreviations

ACN, acetonitrile; **CHAPS**, 3-[(3-cholamiopropyl)dimethylammonio]-1-propanesulfonate; **DTT**, dithiothreitol; **GRP**, glycine-rich protein; **IAA**, iodoacetamide; **LC-ESI-ToF-MS/MS**, liquid chromatography electrospray ionisation time-of-flight tandem mass spectrometry; **LDS**, lithium dodecyl sulphate; **MOPS**, 3-(N-morpholino)propanesulfonic acid; **MS**, mass spectrometry; **MS/MS**, tandem mass spectrometry; **RNA**, ribonucleic acid; **SDS-PAGE**, sodium dodecyl sulphate polyacrylamide gel electrophoresis;

Supplementary Material

Protein identification of the attachment cement of the ixodid tick *Dermacentor marginatus*

Supplementary Table 1: Detailed information of identified proteins. *A.*, *Amblyomma*; *D.*, *Dermacentor*; *E.*, *Echinus*; *H.*, *Haemaphysalis*; *Hy.*, *Hyalomma*; *I.*, *Ixodes*; *M.*, *Mytilus*; *R.*, *Rhipicephalus*; rev., reviewed; unrev., unreviewed;

Protein no.	UniProt accession no.	Protein name (UniProt database)	Database entry	Organism	Species	Probability (%)	Protein false positive rate	Average mass (Da)	Sequence coverage (%)	Matched Peptides	Digested Peptides (theoretical)	Precursor RMS mass error (ppm)
1	A0A131Z8E1	Alpha-N-acetylgalactosaminidase	unrev.	Tick	*R. appendiculatus*	95	5.00	52889.77	43.8	9	28	9.9
2	A0A131Z253	Amblyomma 40-33 family member	unrev.	Tick	*R. appendiculatus*	95	0.00	22647.98	24.2	3	18	7.7
3	A0A131YF72	Glycine rich superfamily member	unrev.	Tick	*R. appendiculatus*	95	0.00	45770.30	30.7	5	20	6.5
4	E2J6U6	Hypothetical glycine rich secreted cement protein	unrev.	Tick	*Hy. rufipes*	95	0.00	17448.10	24.7	3	10	3.1
5	A0A224YCQ3	Lipocalin	unrev.	Tick	*R. zambeziensis*	95	4.55	27873.32	22.1	3	14	1.7
6	A0A224Z5B8	Pre mrna splicing factor 38a	unrev.	Tick	*R. zambeziensis*	95	0.00	46374.70	55.3	21	711	11.8
7	L7LTJ2	Putative bitil peptide	unrev.	Tick	*R. pulchellus*	95	0.00	25041.09	13.8	3	19	4.9
8	B7SP63	Putative cement protein RIM36	unrev.	Tick	*D. variabilis*	95	0.00	19733.73	78.8	4	4	5.6
9	L7M4K5	Putative collagen triple helix repeat protein	unrev.	Tick	*R. pulchellus*	95	0.00	33065.99	29.8	2	1	3.5
10	A0A023FE85	Putative conserved plasma membrane protein	unrev.	Tick	*A. cajennense*	95	0.00	31979.63	34.7	5	14	17.4
11	A0A023FF22	Putative erythrocyte membrane-associated antigen	unrev.	Tick	*A. cajennense*	95	0.00	29906.70	74.5	21	170	11.5
12	A0A147BGN0	Putative eukaryotic translation initiation factor 4 gamma	unrev.	Tick	*I. ricinus*	95	0.00	17308.89	57.5	14	203	17.8
13	L7MCS4	Putative glycine rich protein	unrev.	Tick	*R. pulchellus*	95	0.00	19575.40	25.8	3	10	10.4

14	L7MCP6	Putative glycine rich protein	unrev.	Tick	*R. pulchellus*	95	5.56	15970.52	16.1	2	7	3.0
15	A0A0K8R4W4	Putative ixostatin	unrev.	Tick	*I. ricinus*	95	4.35	12831.50	57.0	3	6	3.9
16	B7SP29	Putative Kunitz-BPTI protein	unrev.	Tick	*D. variabilis*	95	0.00	25800.71	13.6	3	15	1.0
17	A0A131Y8Z6	Putative phd and ring finger domain-containing protein 1	unrev.	Tick	*I. ricinus*	95	2.86	199950.68	28.5	36	527	19.8
18	V5H8M5	Putative ribonuclease t2 family	unrev.	Tick	*I. ricinus*	95	4.17	31885.46	34.7	4	21	24.1
19	A0A0C9QZ61	Putative secreted salivary protein	unrev.	Tick	*A. americanum*	95	2.86	12911.46	39.1	4	11	3.4
20	A0A0K8RR00	Putative serine protease	unrev.	Tick	*I. ricinus*	95	5.26	9271.40	54.3	3	6	15.8
21	L7LPR5	Putative tick salivary cystatin	unrev.	Tick	*R. pulchellus*	95	0.00	15369.61	9.3	1	8	1.8
22	A0A131Y172	Putative transcription termination factor rho	unrev.	Tick	*I. ricinus*	95	0.00	19719.21	62.7	14	183	29.0
23	A0A023FRH2	Putative vitellogenin receptor ixodes scapularis vitellogenin receptor	unrev.	Tick	*A. cajennense*	95	4.35	45872.62	56.6	14	26	15.3
24	A0A131Z5Z3	Sulfotransferase	unrev.	Tick	*R. appendiculatus*	95	0.00	23575.20	22.8	5	19	1.7
25	A0A131YWJ2	Tick serine protease	unrev.	Tick	*R. appendiculatus*	95	3.23	56147.30	18.5	7	37	9.0
26	A0A0C9RSV8	Uncharacterised protein	unrev.	Tick	*A. americanum*	95	0.00	17747.60	47.8	2	12	0.1
27	V5H732	Uncharacterised protein	unrev.	Tick	*I. ricinus*	95	0.00	23684.67	7.1	2	20	3.7
28	G3MNT5	Uncharacterised protein	unrev.	Tick	*A. maculatum*	95	0.00	38319.70	18.2	5	22	15.5
29	V5I1U8	Uncharacterised protein	unrev.	Tick	*I. ricinus*	95	0.00	31096.42	25.4	3	24	8.3
30	B7Q3C3	Uncharacterised protein	unrev.	Tick	*I. scapularis*	95	7.14	6787.84	86.9	3	5	3.6
31	B7PAL5	Very acidic secreted protein_ putative	unrev.	Tick	*I. scapularis*	95	0.00	14747.17	64.8	11	48	16.3
32	O77421	Female-specific histamine-binding protein 2	rev.	Tick	*R. appendiculatus*	50	0.00	21691.87	86.8	7	12	13.2
33	B2MW54	Longicornsin	rev.	Tick	*H. longicornis*	50	0.00	9236.71	75.6	9	7	18.3
34	A7DWU6	Myticin C	unrev.	Mussle	*M. galloprovincialis*	50	3.57	11497.15	28.0	2	9	12.4
35	A0A144LVM3	Strongylocin 2	rev.	Sea urchin	*E. esculentus*	50	0.00	10640.15	86.5	9	9	13.4

Determination of cortisol in hair

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Highlights of this manuscript

- Method development for the analysis of glucocorticoids by LC-ESI-MS/MS
- Optimisation of triple quadrupole mass analyser settings
- Investigation of horse hair extraction procedures
- Determination of LODs and LOQs

Determination of cortisol in hair

1. Introduction

Cortisol and cortisone are important endogenous glucocorticoids and belong to the corticosteroids, a class of steroid hormones. Both are produced in the adrenal cortex which is regulated by the hypothalamic pituitary adrenal axis (HPA axis) [1]. The activation of the HPA axis is known as a primary response to a stressful stimulus mediating the negative effects of stress by secretion of cortisol [2-4]. Consequently, increased cortisol production can be seen as a biomarker for stress [5, 6]. Furthermore, the alteration of glucocorticoid levels can be related to several diseases, like adrenal hyper function (Cushing's syndrome) or adrenal insufficiency, thus the measurement and concentration determination of steroids is crucial for the diagnosis of these disorders and chronic stress [7, 8]. In **Figure 1** the relationship between stress and HPA axis activation and the consequences are demonstrated [9]: Stress affects the brain function directly (indicated by the green arrow). This stress is balanced by cortisol, which also blocks the activation of inflammatory immune cells (healthy individual, left).

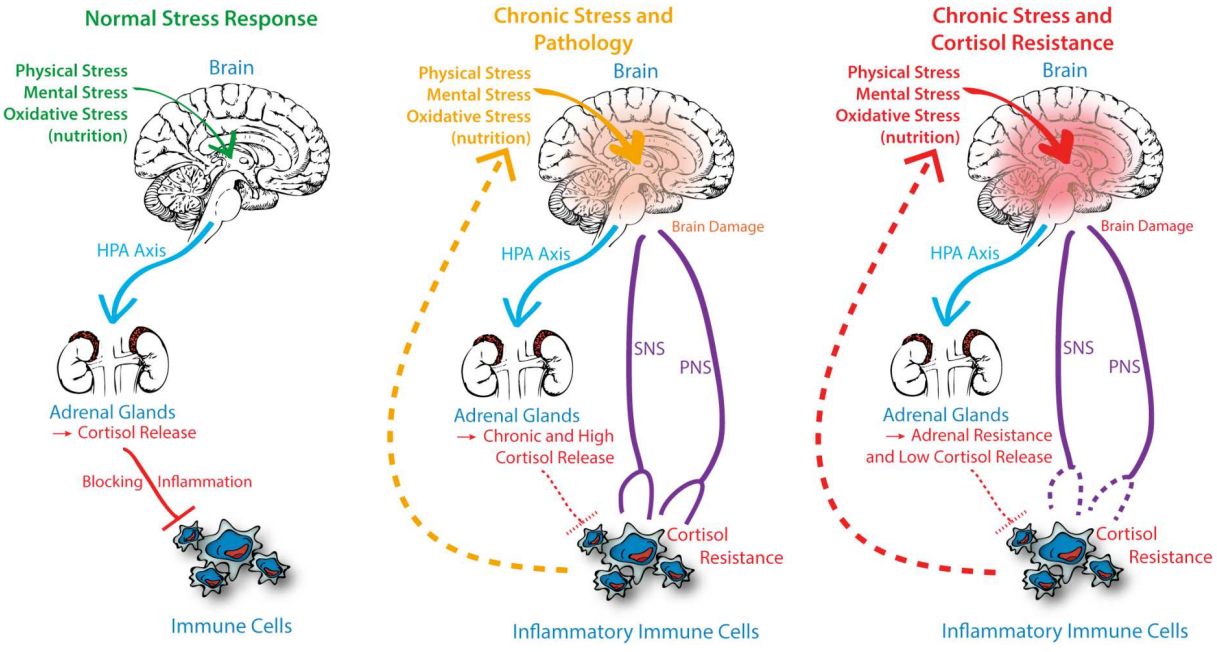

Figure 1: The role of stress induced activation of the HPA axis, cortisol and sympathetic nervous system (SNS) and parasympathetic nervous system (PNS) neurotransmitter release to combat immune cell activation. Figure taken from [9].

Chronic stress leads to a continuously activation of cortisol, however, the inflammatory immune cells downregulate their receptors and contribute to stressing brain regions by

inflammatory cytokines (yellow dashed line). In that state of stress, the so-called cortisol resistance, cortisol fails to down modulate the inflammatory cells (middle panel). In the case of even more stress the adrenals try to help the body to resist prolonged stress by producing persistently increased levels of cortisol. Subsequently, the adrenal glands are not able to make even normal amounts of cortisol, known as adrenal exhaustion or adrenal fatigue. In this situation, the communication and regulatory function of the sympathetic nervous system (SNS) and parasympathetic nervous system (PNS) is also affected, which may account for various aspects of immune dysfunction (right).

It has been also found that athletes show an increased release of cortisol or have an elevated corticosteroid profile when recently competed a marathon [10, 11]. The reason for this may be an increased stress level when such efforts are demanded from the organism. However, glucocorticoids are also known to be used for doping, since they raise the performance by a certain euphoria and an increase in motor activity [12-14]. Thus, a fast and simple determination of cortisol and other steroid hormones is fundamental to recognise health problems or also drug abuse.

Endogenous glucocorticoids can be found in various biological fluids like blood, salvia or urine [8]. However, the concentration of cortisol in these fluids is subjected to circadian changes and daily fluctuations, thus giving only point assessments, which are highly variable [15, 16]. In addition to that food intake and stress that may have occurred shortly before or during sampling, can lead to variations. Therefore, blood, saliva or urine provide only a reflection of cortisol secretion over a relatively short period of time [17]. Anyway, these biological fluids are well suited for capturing dynamic aspects of endocrine activity, but less useful as markers for the long-term secretory patterns [2].

In order to get a longer detection window of cortisol levels, the determination of cortisol in hair seems to be a promising field of research and it was already shown that hair cortisol can be used as a biomarker for stress during pregnancy [18]. Hair originates from hair follicles, which are surrounded by a capillary system that provides the growing hair with necessary metabolic material [19]. Yet it is not fully clear how drugs or other metabolites (for instance cortisol) are incorporated into hair. However, some incorporation models are mentioned in literature (**Figure 2**) [3, 19]: i) passive diffusion from blood capillaries into the hair follicle or from deep skin compartments during the hair shaft formation; ii) delayed incorporation from surrounding tissue; iii) deposition by diffusion from sweat or sebum secretions into the completed hair shaft as the most important alternative mechanism; iv) or from external sources; Next to that hair follicles itself produce small amounts of cortisol and the decomposition of present cortisol through external influences like bleaching or UV radiation has to be considered [18, 20].

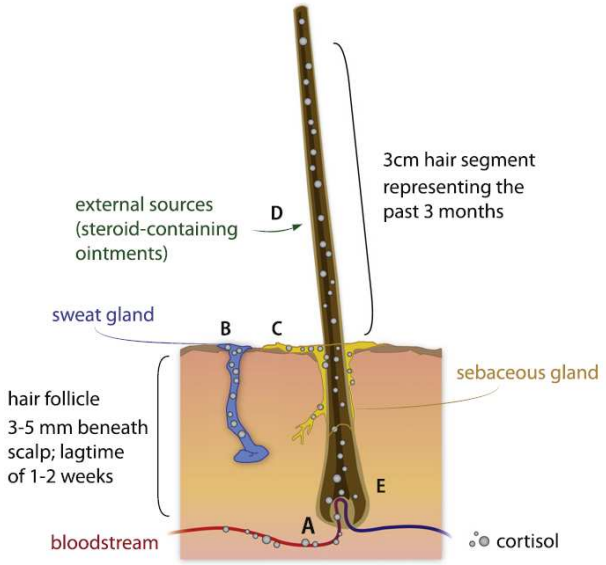

Figure 2: Incorporation of cortisol in hair via diffusion from blood (A), sweat (B) and/or sebum (C) as well from external sources (D). Additionally cortisol produced from the hair follicle itself can contribute to hair cortisol concentrations (E). Figure taken from [3].

It is assumed that hair has an average growth rate of 1 cm/month and thus a 3 cm segment, for example, represents a cumulative cortisol level over 3 months [21]. Therefore, compared to biological fluids, the analysis of hair allows to go back further in time. Anyhow, it has to be considered that there is a decrease of cortisol levels from the distal to the proximal parts of the hair due to a wash out effect [3]. Consequently, there is a natural limit to the period that can be retrospectively examined, yet still improved in comparison to urine analysis giving information up to 24 h [6].
In addition to the retrospective analysis there are more benefits of using hair for cortisol determination compared to blood, saliva and urine [5, 12, 15, 19, 22]:

- hair samples are easy to ship and store
- little sample amount is required
- sampling is non-invasive and easy (can be carried out by non-professionals)
- sample can be stored at room temperature (RT)

Usually, prior to mass spectrometry (MS), the steroid hormones were measured by immunoassays due to speed, simplicity and sensitivity [7, 23]. However, there are several limitations of immunoassays used for steroid hormone analysis: limited dynamic range, matrix effects and lack of specificity due to cross-reactivity of other steroid hormones,

causing an overestimation and the possibility of false positives [7, 24-26]. Thus, the use of chromatographic methods coupled to MS were getting more and more important replacing immunoassays in steroid analysis. In this respect, liquid chromatography coupled to mass spectrometry (LC-MS) prevailed over gas chromatography coupled to mass spectrometry (GC-MS), since GC often requires derivatisation, causing labour intensive sample preparation and low throughput [7]. On the other hand, LC-MS has sufficient analytical sensitivity and specificity. It has high speed and robustness for single steroid analysis and is far less laborious than GC-MS [27]. Consequently, a highly sensitive and selective liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) method for the quantification of cortisol and cortisone of human and horse hair was developed in this work.

All work on cortisol hair analysis was carried out in the course of student internships and a diploma thesis in cooperation with the Medical University of Vienna. The results are summarised in the following section.

2. Materials and methods

2.1. Chemicals and reagents

Double distilled water (ddH_2O, 18.2 MΩ.cm) was prepared using a Simplicity system Millipore, Billerica, MA, USA. Formic acid (98.0-100.0%), isopropanol (iPrOH, LC-MS grade) and methanol (MeOH, LC-MS grade) was obtained from Merck, Darmstadt, Germany. Cortisol, cortisone (TraceCERT®), 6-α- and 6-β-hydroxycortisol (both ≥ 98.0%), tetrahydrocortisone-2,2,3,4,4-d_5 (≥ 98.0%, d5-THE) and tetrahydrocortisol-2,2,3,4,4-d_5 (≥ 98.0%, d5-THF) were obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Sample preparation

In order to remove dirt, the complete hair strands from several, randomly selected horse tails were washed several times with tap water until the washing water showed no further staining. This was followed by a washing step with ddH_2O. Afterwards the hair was dried for at least 12 hours at RT before it was cut into 0.5 cm segments. Only the first 7 cm of the strands (distal to the scalp) were used for further sample preparation since in the following sections the concentration of cortisol should be rather low (wash out effect).

After cutting, the hair fragments were washed with ddH_2O and iPrOH: two times with ddH_2O in an ultrasonic bath and two times with iPrOH by shortly shaking followed by drying in a desiccator under vacuum. Finally, the washed and cut hair sections were pulverised in a mortar with pistil with the help of liquid nitrogen and dried in the hood at RT for about 2 h.

For the extraction 30 to 70 µg of the hair powder was weighted into a 1.5 mL glass vial and 1 mL of MeOH/formic acid (0.1%) was added. The extraction time was performed on a shaker for two to three days. After extraction the solution was transferred into a 2 mL Eppendorf tube and centrifuged (15 min, 15,000 g). The supernatant was transferred into a new Eppendorf tube and the solvent was evaporated using a vacuum centrifuge. Afterwards the sample was dissolved with 100 µL MeOH/formic acid (0.1%) and used for LC-ESI-MS/MS analysis.

2.3. LC-ESI-MS/MS

LC analysis was performed using a Shimadzu Nexera X2 LC-30 AD LC system (SIL-30AC auto sampler; CTO-20 AC column oven), connected to a Shimadzu LCMS-8060 TQMS. The stationary phase used was an ACQUITY UPLC C18 column (100 × 2.1 mm, particle size 1.7 µm, Waters, Manchester, United Kingdom). The gradient of the mobile phase was as

follows: starting at 20% B; raised to 90% B in 7 min; decreased to 20% B in 2 min and finally 3 min of equilibration. The mobile phase consisted of ddH_2O (0.1% formic acid, A) and MeOH (0.1% formic acid, B) and the total flow was 0.4 mL/min. The column oven temperature was held at 40 °C. The ESI interface temperature was set to 300 °C, the desolvation line to 250 °C and the heat block to 400 °C. Argon was used as collision gas, nitrogen as nebulising (3 L/min) and drying gas (10 L/min). In addition a heating gas flow (10 L/min) was used for heating the ESI source. Nebulising, drying and heating gas were generated from a ZEFIRO 8050 N_2 generator (CINEL S.r.l., Italy). All data was acquired using LabSolutions (v. 5.89) from Shimadzu.

3. Results and discussion

3.1. Method development

For the analytes cortisol, cortisone, 6-α- and 6-β-hydroxycortisol, d5-THE and d5-THF a highly sensitive and selective LC-ESI-MS/MS method was developed using MRM as MS acquisition mode. The mentioned compounds could be baseline separated within 5.5 min and the total run time of the method was 12 min (**Figure 3** top chromatogram). The aim of the method development was the determination of the optimal MRM transitions and collision energies (CEs) for the investigated analytes. The final method settings and retention times can be seen in **Table 1**.

Table 1: Final measurement parameter for the investigated analytes. MRM transitions of each compound are sorted by decreasing intensity of the product ion.

Compound	Retention time (min)	Measurement window (min)	MRM transitions	CE (V)
6-β-hydroxy-cortisol	1.73	1.5-2.0	423.20>347.05 423.20>313.05 423.20>377.05	20.0 35.0 14.0
6-α-hydroxy-cortisol	1.95	1.7-2.2	423.00>347.15 423.00>313.10 423.00>377.05	20.0 33.0 14.0
Cortisone	4.17	4.0-4.5	405.00>329.05 405.00>301.05 405.00>359.05	16.0 22.0 10.0
Cortisol	4.39	4.2-4.7	407.00>331.15 407.00>297.10 407.00>282.10	19.0 33.0 39.0
d5-THF	5.05	4.8-5.3	416.40>340.15 416.40>306.10 416.40>370.15	20.0 39.0 15.0
d5-THE	5.21	5.0-5.5	414.00>338.15 414.00>310.20 414.00>368.15	20.0 27.0 15.0

Table 2: LODs and LOQs of the investigated compounds calculated by means of the residual standard deviation of the regression lines in the range of the LOD.

Compound	LOD		LOQ	
	nM	ng/mL	nM	ng/mL
6-β-hydroxy-cortisol	0.25	0.09	0.74	0.28
6-α-hydroxy-cortisol	0.23	0.09	0.70	0.27
Cortisone	0.46	0.17	1.40	0.50
Cortisol	0.41	0.15	1.24	0.45
d5-THF	0.35	0.13	1.05	0.39
d5-THE	0.53	0.19	1.60	0.59

Compared to GC-MS, with limits in the range of 5 to 11 ng/mL, or to enzymatic techniques, with LODs in the range of 1 ng/mL, the obtained LODs are highly acceptable [8, 12]. This can be also confirmed when looking at LODs in literature for various LC-MS methods [28].

3.2. Results of horse hair analysis

Very low amounts of cortisol and cortisone could be found in the extracted horse hair. An example can be seen in **Figure 3** (bottom chromatogram). Since the extraction process also leaches out a lot of matrix components, visible through various peaks and an increased baseline at higher retention times (indicating the presence of very non-polar substances) the LC method had to be adapted to clean the column in order avoid a carryover effect and column clogging. This would be circumvented by a solid phase extraction prior LC analysis. However, this additional sample preparation step possibly causes a loss of the extracted cortisol or cortisone and was not investigated here.

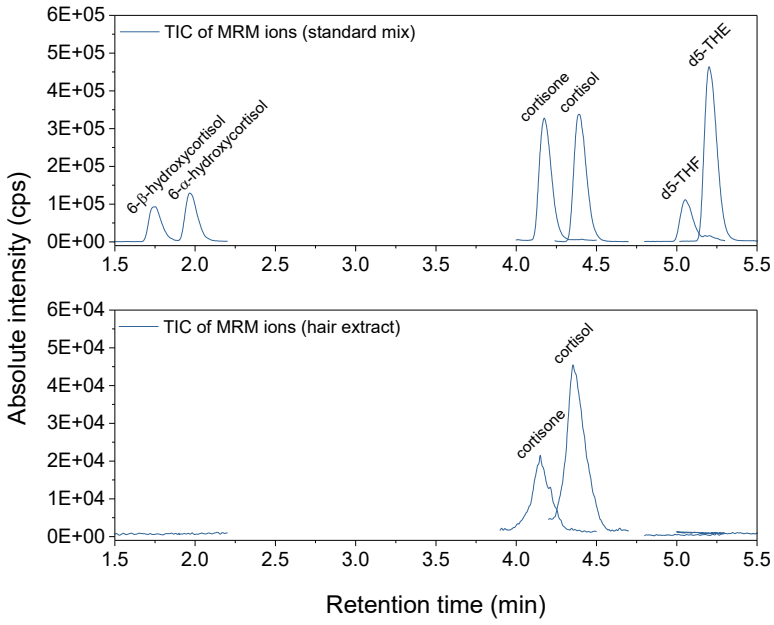

Figure 3: LC-ESI-MS/MS chromatogram of a standard mixture containing the six investigated compounds (top chromatogram) and a pulverised hair sample (bottom chromatogram).

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Abbreviations

CE, collision energies; **ddH_2O**, double distilled water; **ESI**, electrospray ionisation; **GC-MS**, gas chromatography coupled to mass spectrometry; **HPA** axis, hypothalamic pituitary adrenal axis; **iPrOH**, isopropanol; **LC**, liquid chromatography; **LC-MS**, liquid chromatography coupled to mass spectrometry; **LC-ESI-MS/MS**, liquid chromatography electrospray ionisation tandem mass spectrometry; **LOD**, limit of detection; **LOQ**, limit of quantification; **MeOH**, methanol; **MS**, mass spectrometry; **PNS**, parasympathetic nervous system; **RT**, room temperature; **SNS**, sympathetic nervous system; **THE**, tetrahydrocortisone; **THF**, tetrahydrocortisol; **UV**, ultraviolet;

Conclusions

The huge variety of metabolites caused by their individual structures, the physical and chemical properties and the fast turnover rates and modifications in biological systems demands the utilisation of high performance separation and detection techniques like GC-EI-MS/MS and LC-ESI-MS/MS as presented in this work. Furthermore, the different abundance levels and complex biological samples stress the need for a highly sensitive and also selective methodology such as MRM.

In **Manuscript I** (p. 33) it could be shown that both investigated methodologies, GC-EI-MS/MS and LC-ESI-MS/MS, are suited for the analysis of several metabolites from different substance classes and quantification is possible in the low µM range. However, comparing the chromatographic performance reveals the benefits of GC-EI-MS/MS, even though various compounds formed two derivatives after MeOx/TMS derivatisation. Several substances (sugar alcohols and monosaccharides, except ribose) could be separated by GC, but only sum peaks for pentoses, hexoses and sugar alcohols could be obtained by LC. The structural similarity of these substances also makes differentiation by MS using MRM impossible and thus the information obtained of a biological sample is quite limited. In addition to that the GC peak widths had a maximum of 0.27 min compared to several minutes for LC, which clearly points out the separation efficiency of the developed GC-EI-MS/MS method. What has to be considered is the fact that samples used for analysis by GC have to be free from water, demanding evaporation of the solvent prior to derivatisation. In addition to that sample preparation has to be carried out under a nitrogen atmosphere to facilitate stability of derivatives. This makes analysis by GC more complex and challenging, but still can be favourable when compared to the LC-ESI-MS/MS method.

The comparison of the two investigated derivatisation strategies for GC clearly showed the advantages of the combined MeOx/TMS reaction in contrast to mere TMS by reducing the complexity of the resulting chromatograms a lot due to less formed derivatives (**Manuscript II**, p. 47). This is especially important in the case of monosaccharides where up to seven peaks could be obtained after TMS. But also for amino acids, where Lys formed two derivatives. Furthermore, methoximation and the presence of methoxyamine hydrochloride stabilises compounds like monosaccharides or amino acids as could be shown by study of reaction kinetics. Latter also demonstrated that derivatives could be formed with high reproducibility which allows the comparison between technical replicates. Thus, any variation observed between biological replicates and experiments can only be of biological origin and will not be influenced by the developed methodology.

The research field of tick attachment cement, or in general of biological adhesives, is very interesting and emerging. This is caused by the search of new, sustainable and maybe better performing adhesives or glues applicable in medicine since currently available tissue glues like cyanoacrylates, glutaraldehyde or fibrin glues have toxic properties and/or low binding forces. Tick attachment cement and bioadhesives from other organisms like barnacles or mussels work in a wet environment facilitating the removal of water or moisture during the attachment process. Therefore, these bioadhesives have a high potential for medical applications as biomimetic glues.

Publication I (p. 96) gives an overview about the current research status of the biological skin plug system. This allowed to get an insight into tick cement research, the biological significance, functions and structure of the cement as well as its histochemistry and biochemistry. Furthermore, cement was compared with other biological adhesives and the potential applications of tick attachment cement were outlined. Cement research already has a long history and was first described in the early 20th century. Starting with histological investigations of the secretion in skin biopsies from attached ixodid ticks on a variety of host animals. Over the years improved technologies paved the way for the modern molecular biological techniques that now dominate research. Furthermore, artificial feeding of ticks on silicone membranes, as performed in this work, offered the possibility of the generation of uncompromised samples and allowed further improvement of tick cement analysis. In that way the adhesive material is accessible at virtually any time during feeding and the feeding can be easily monitored. Moreover, the development and standardisation of mechanised feeding systems allows automatic and simultaneous artificial feeding of large numbers of ticks in order to yield quantities of cement material required for analyses.

In **Manuscript III** (p. 118) it could be shown that cement of *Dermacentor marginatus* mainly contains non-polar amino acids (Gly, Leu, Pro, Ala and Phe; > 60%) with Gly as the major component (> 25%). Additional analyses revealed that cement from *Amblyomma hebraeum* has a very similar composition with a higher Gly content (> 35%). These findings prove the importance of Gly in the attachment process. Furthermore, the amino acid composition of *Dermacentor marginatus* was compared with the bioadhesive of *Lepas anatifera* barnacles. It could be shown that there are clear differences between tick and barnacle cement which suggests a different adhesion mechanism. The fact that barnacles adhere completely under water and ticks "only" in a wet environment supports this statement.

In addition to amino acid analysis protein analysis of *Dermacentor marginatus* attachment cement was carried out (**Manuscript IV**, p. 152). Acidic conditions of the

solubilisation buffer showed to be ideally suited for solubilising proteins which could subsequently be separated by SDS-PAGE. However, cement originated from ticks fed with blood-based feeding materials showed a high background in silver stained SDS-PAGE gels caused by the contamination of the adhesive with blood components from the feed. This could be confirmed by the identification of bovine serum albumin or haemoglobin in such cement samples. Thus, in order to obtain cement samples of high purity, artificial media, which are not based on blood, were introduced enabling the exclusion of host compounds derived from blood. In total 35 tick specific (cement) proteins and two non-tick specific ones could be identified. The identification of GRPs confirms the findings from amino acid analysis and the significance of these proteins and Gly for tick attachment. The high content of Gly and the identification of the putative collagen triple helix protein suggests that ticks secrete proteins closely related to the surrounding skin environment supporting the attachment process. The identification of proteins with antimicrobial activity, which inhibit tissue repair or hamper the function of protein degrading enzymes reveals that the cement not only acts as a glue, but also has other functions like protecting the tick. This is corroborated by the identification proteins, which interfere with the immune response of hosts or inhibit anticoagulation. The latter allowing the tick to feed on the blood.

Finally, it can be stated that the developed analytical methodologies are highly suitable for the investigation of complex biological samples. Due to the high complexity of the investigated biological adhesives the use of several diverse analytical tools, which are highly sensitive and selective, is essential. Moreover, the presence of many different analyte classes demands the application of separation techniques. Here, chromatographic methods were used and, in combination with MS and in particular MRM, these methods are highly eligible for the investigation of bioadhesives.

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Abbreviations

ACN	acetonitrile
Ala	alanine
APPI	atmospheric pressure photo ionisation
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Asx	asparagine and aspartic acid
BaOH	barium hydroxide
BSA	bis(trimethylsilyl)–acetamides
BSTFA	bis(trimethylsilyl)trifluoroacetamide
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CI	chemical ionisation
CID	collision induced dissociation
CP	cement protein
CRM	charged-residue model
Cys	cysteine
DTE	dithioerythritol
DTT	dithiothreitol
EI	electron ionisation
EI-MS	electron ionisation mass spectrometry
ESI	electrospray ionisation
FT-ICR	Fourier transform ion cyclotron resonance
GC	gas chromatography
GC-EI-MS	gas chromatography electron ionisation mass spectrometry
GC-EI-MS/MS	gas chromatography electron ionisation tandem mass spectrometry
GC-MS	gas chromatography coupled to mass spectrometry
Gln	glutamine
Glu	glutamic acid
Glx	glutamine and glutamic acid
Gly	glycine
GRP	glycine rich protein
HCl	hydrochloric acid
HILIC	hydrophilic interaction liquid chromatography

His	histidine
HPLC	high-performance liquid chromatography
Hyp	hydroxyproline
IEM	ion evaporation model
Ile	isoleucine
LC	liquid chromatography
LC-ESI-ToF-MS/MS	liquid chromatography electrospray ionisation time-of-flight tandem mass spectrometry
LC-MS	liquid chromatography coupled to mass spectrometry
Leu	leucine
LiOH	lithium hydroxide
LOD	limit of detection
LOQ	limit of quantification
Lys	lysine
m/z	mass to charge ratio
MeOH	methanol
MeOx/TMS	methoximation in combination with trimethylsilylation
Met	methionine
Mox	methionine sulfoxide
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSA	methane sulfonic acid
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
MTFA	N-methyltrifluoroacetamid
N2	nitrogen
NaOH	sodium hydroxide
NP	normal phase chromatography
Orn	ornithine
PCF	propyl chloroformate
Phe	phenylalanine
pI	isoelectric point
Pro	proline
PrOH	propanol
Q1	first quadrupole
Q2	second quadrupole/collision cell

Q3	third quadrupole
RP	reversed phase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	serine
SIM	selected ion monitoring
Thr	threonine
ToF	time-of-flight
Trp	tryptophan
Tyr	tyrosine
UHPLC	ultra-high-performance liquid chromatography
Val	valine

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Curriculum Vitae

Name:	Benedikt Engel
Date of Birth:	01.06.1988
Place of Birth:	Wr. Neustadt
Nationality:	Austria

Education

12/2014 - present **Doctoral programme in natural sciences, TU Wien**

Institute of Chemical Technologies and Analytics, working group Mass spectrometric Bio and Polymer Analytics, Assoc. Prof. Martina Marchetti-Deschmann, PhD

PhD thesis: *„Online hyphenation of chromatography and mass spectrometry – elucidating the molecular composition of a biological adhesive from ixodid ticks“*

- Analysis of small molecules using GC-MS/MS und LC-MS/MS
- Separation and characterisation of biomolecules using SDS-PAGE and LC-MS/MS
- Sample preparation of bio adhesives
- Project planning in agreement with the project partner (Medical University of Vienna)
- Supervision of students

11/2012-11/2014 **Master studies, TU Wien**

Field of study: Biotechnology und Bioanalytics

Master thesis: *„Characterization of recombinant Peroxidases in respect to efficiency and reaction characteristics for selected substrates“* (Assoc. Prof. Martina Marchetti-Deschmann, PhD, working group Omics Technologies)

- Implementation of enzyme reactions
- Identification and characterisation of reaction products using TLC, GC-MS/MS and LC-MS/MS
- Project planning in agreement with the project partner (EUCODIS Bioscience)

09/2007-11/2012 **Bachelor studies, TU Wien**

Field of study: Technical Chemistry

Bachelor thesis: *„Comparison of identification efficiency of unlabelled biomarkers by MALDI-TOF imaging mass spectrometry on rat kidney tissue untreated vs FFPE embedded“* (Assoc. Prof. Martina Marchetti-Deschmann, PhD)

- Method development for mass spectrometry imaging
- Identification of biomarkers on kidney tissue sections

09/1998-06/2006 **Grammar school, Kirchberg am Wechsel**

Grammar school with focus on natural sciences

Work Experience

01/2020 – present **Marinomed Biotech AG**

- Scientist at the department Marinosolv
- Tasks: Analytical and scientific supervision of development projects, supervision of chemical-technical assistants, development and implementation of analytical methods with a focus on chromatography for the characterisation and control of development products, cooperation with external laboratories for the application of further analysis methods and GLP compliant analysis, collaboration in project teams, preparing statistical evaluation of data, documentation and writing of reports

10/2019-12/2019 **Parental leave**

12/2014-07/2019 **TU Wien**

- University-/Project assistant at the Institute of Chemical Technologies and Analytics (working group Omics Technologies as well as Mass spectrometric Bio and Polymer Analytics, Assoc. Prof. Martina Marchetti-Deschmann, PhD)
- Student tutor for the lab course “Bioprocess technology and bioanalytics“ (SS 2016, SS 2017, SS 2018, for students of the master studies Biotechnology and Bioanalytics as well as Technical Chemistry)
- Student tutor for the lab course “Analytical methods and separation techniques“ (SS 2015, SS2016, SS 2017, SS 2018, for students of the master studies technical chemistry)
- Assistant in the lab course “Qualitative Inorganic Analysis“ (SS 2015, for students of the bachelor studies Technical Chemistry)
- Assistant in the lab course “Quantitative Analytical Chemistry” (WS 2018, for students of the bachelor studies Technical Chemistry)
- Assistant in the lab course “Instrumental and Bioanalytical Laboratory” (SS 2019, for students of the bachelor studies Technical Chemistry)

04/2013-05/2015 **Billa AG**

- Minor employment as cashier
- Tasks: Advice and support of customers, responsibility for product availability and presentation in the checkout area

10/2014-11/2014 **TU Wien**

- Student tutor for the lab course “Quantitative Analytical Chemistry” (for students of the bachelor studies Technical Chemistry)

11/2013-07/2014 **SYNDT Consulting GmbH/ESW Consulting WRUSS**

- Free service contract
- Tasks: Characterisation of waste or recyclable materials to be dumped, including the preparation of assessments, temporary job in the laboratory of ESW Consulting WRUSS

10/2013-02/2014 **TU Wien**

- Student tutor for the lab course “Quantitative Analytical Chemistry” (for students of the bachelor studies Technical Chemistry)

11/2012-09/2013 **Educational leave**

01/2007-11/2012 **Tank Roth GmbH**

- Cashier and waiter
- Tasks: Advice and support of customers, responsibility for product availability and presentation, kitchen activities

04/2010-07/2012 **ENSOWA GmbH (Termination due to bankruptcy)**

- Free service contract
- Tasks: Characterisation of poured excavated soil material in the port of Vienna, hydrocarbon analysis of soil samples, sampling of sewage water

09/2010 **OMV Refining & Marketing GmbH**

- Internship
- Tasks: Analysis of kerosene

08/2009-09/2009 **STAMAG GmbH**

- Internship
- Tasks: Quality control of grain supplies

07/2006-01/2007 **Martinek casern Baden**

- Completion of the military service

Benedikt Engel, MSc ◆ Strohberggasse 31/8 ◆ 1120 Wien ◆ +43 664 1242938 ◆ benedikt.x.engel@gmail.com

Skills

Methods:

- Chromatographic separation techniques (GC, LC)
- Triple quadrupole mass spectrometry in combination with GC and LC
- MALDI-ToF mass spectrometry
- Gel electrophoretic based separation techniques (SDS-PAGE)
- Protein identification after SDS-PAGE and LC-ESI-MS/MS using ProteinLynx Global SERVER

Languages:

- German (first language), English (business fluent in spoken and written)

Computer literacy:

- Microsoft Windows and Office applications, EndNote, Origin, ChemDraw

Other:

- Driving licence class B (16.8.2005)

Publications

2012 S. Fröhlich, **B. Putz**, H. Schachner, D. Kerjaschki, G. Allmaier, M. Marchetti-Deschmann: “Renopathological microstructure visualization from formalin fixed kidney tissue by MALDI-TOF mass spectrometry imaging”; Balkan Journal of Medical Genetics, 15 (2012), S. 13 – 16.

2017 J. Suppan, B. Engel, M. Marchetti-Deschmann, S. Nürnberger: “Tick attachment cement – reviewing the mysteries of a biological skin plug system”; Biological reviews

Publications (submitted)

2020 **B. Engel**, P. Suralik, M. Marchetti-Deschmann: “Critical considerations for trimethylsilyl derivatives of 24 primary metabolites measured by GC-MS/MS”; submitted to Journal of Separation Science.

2020 **B. Engel**, J. Suppan, S. Nürnberger, A. M. Power, M. Marchetti-Deschmann: “Revisiting amino acid analyses for bioadhesives including a direct comparison of tick attachment cement (*Dermacentor marginatus*) and barnacle cement (*Lepas anatifera*)”; submitted to International Journal of Adhesion and Adhesives

Conference Contributions (presenting author underlined)

03/2012 S. Fröhlich, **B. Putz**, H. Schachner, D. Kerjaschki, G. Allmaier, M. Marchetti-Deschmann: "Renopathological microstructure visualization from formalin fixed tissue by MALDI-RTOF-MSI"; MACPROGEN, Skopje, North Macedonia (Oral presentation)

08/2013 **B. Putz**, M. Bonta, C. Gierl-Mayer, H. Danninger, A. Limbeck: "LA-ICP-MS imaging of the distribution of non-metallic additives in powder metallurgic steel samples"; 17th European Conference on Analytical Chemistry, Waschau, Poland (Poster presentation)

02/2016 **B. Putz**, P. Suralik, G. Allmaier, M. Marchetti-Deschmann: "Challenges in targeted metabolomics using GC-EI-MS/MS and derivatization techniques"; 27th MassSpec Forum, TU Wien, Vienna, Austria (Oral presentation)

06/2016 **B. Putz**, G. Mikl, P. Suralik, G. Allmaier, M. Marchetti-Deschmann: "Challenges in targeted metabolomics – a comparison of capillary GC and LC triple quadrupole MS"; 12. ASAC-JunganalytikerInnenforum, Karl-Franzens-Universität Graz, Graz, Austria (Oral presentation)

04/2017 **B. Putz**, J. Suppan, S. Nürnberger, M. Marchetti-Deschmann: "Tick attachment cement – a still largely unexplored biological adhesive"; ANAKON 2017, Tübingen, Germany (Poster presentation)

06/2017 J. Suppan, **B. Putz**, M. Marchetti-Deschmann, S. Nürnberger: "Structural insights into tick attachment cement"; YSA PhD Symposium, Medical University of Vienna, Vienna, Austria (Poster presentation)

09/2017 S. Nürnberger, J. Suppan, **B. Engel**, M. Marchetti-Deschmann: "The sticky aspect of ticks - the tick plug cement for bionic bioadhesive research"; International Symposium on Tick-Borne Pathogens and Disease, Vienna, Austria (Oral presentation)

09-11/2017 **B. Engel**, J. Suppan, S. Nürnberger, M. Marchetti-Deschmann: "*Amblyomma hebraeum*: Analysis of tick attachment cement":

- ISSS 2017, TU Wien, Vienna, Austria (Poster presentation)
- ITPD 2017, Medical University of Vienna, Vienna, Austria (Poster presentation)
- ViCEM Inaugural Meeting 2017, Medical University of Vienna & TU Wien, Vienna, Austria (Poster presentation)

02/2018 **B. Engel**, J. Suppan, S. Nürnberger, M. Marchetti-Deschmann: „Tick attachment cement of *Amblyomma hebraeum* – An insight into its biochemical composition"; Tissue Cluster Meeting, FH Technikum Wien, Vienna, Austria (Oral presentation)

11/2018 J. Suppan, M Fürsatz, **B. Engel**, M. Marchetti-Deschmann, L. Moreno Ostertag, M. Valtiner, S. Nürnberger: "Tick attachment cement - first characterization of a potential tissue glue"; 3rd International Conference on Biological and Biomimetic Adhesives (ICBBA), Haifa, Israel (Oral presentation)

05/2019 **B. Engel**, J. Suppan, S. Nürnberger, M. Marchetti-Deschmann: "An insight into the bioadhesives of the ixodid ticks *Amblyomma hebraeum* and *Dermacentor marginatus*"; TERMIS EU 2019, Rhodes, Greece (Poster presentation)

J. Suppan, M. Fürsatz, **B. Engel**, M. Marchetti-Deschmann, L. Mears, M. Valtiner, O. Andriotis, P. Thurner, S. Nürnberger: "Morphological and biochemical properties of the attachment cement from Dermacentor marginatus"; TERMIS EU 2019, Rhodes, Greece (Poster presentation)

11/2019 L. L. E. Mears, M. Valtiner, L. Moreno Ostertag, C. Cupak, C., J. Suppan, M. Fürsatz, **B. Engel**, M. Marchetti-Deschmann, O. Andriotis, P. J. Thurner, S. Nürnberger: "Bio-Adhesion Properties of the Attachment Cement from the Tick Species *Dermacentor Marginatus*"; AIChE Annual Meeting 2019, Orlando, Florida, USA (Oral presentation)

Internationale Experience

10/2017 **Study visit:**
University of Rijeka, Department of Biotechnology, Rijeka, Croatia: Identification of tick cement proteins after one dimensional SDS-PAGE using LC-ESI-ToF-MS/MS

01/2018 **Study visit:**
University of Rijeka, Department of Biotechnology, Rijeka, Croatia: Identification of tick cement proteins after one dimensional SDS-PAGE using LC-ESI-ToF-MS/MS