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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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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.

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

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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. A subsample of the hardened cement was carefully cut from the attachment interface with the glass bottle (i.e. not from the interface with the animal's basal membrane) with a scalpel, the edges of the plaque were removed on all sides, the subsample was 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

2.3.1. 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.

2.3.2. 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.

2.3.3. 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 optimisation

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. Tables 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 (0.1–200 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 0.01–200 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.

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 91–111%) showed that the method employed enabled highly accurate measurement of the AAs.

The analytical performance of the method presented here is in good agreement with literature. Validation data obtained from analysis of PCF derivatised AAs by GC in combination with a FID (flame ionisation detector) revealed similar results regarding the linear range (1–200 μM) and precision (<10%), but the LODs were significantly higher on average (0.5–1 μM) [18]. The same is true when comparing our data with GC-MS data from Kaspar et al. where again PCF derivatised AAs were analysed and an accuracy of 94–105% and LODs of 0.03–12 μM were obtained [23]. Coefficients of determination, on the contrary, are lower when Nor was used as IS. Higher values were obtained with stable-isotope labelled IS's. Linearity ranged from 0.3 to 2000 nmol/mL and thus is extended in both directions compared to our data. However,

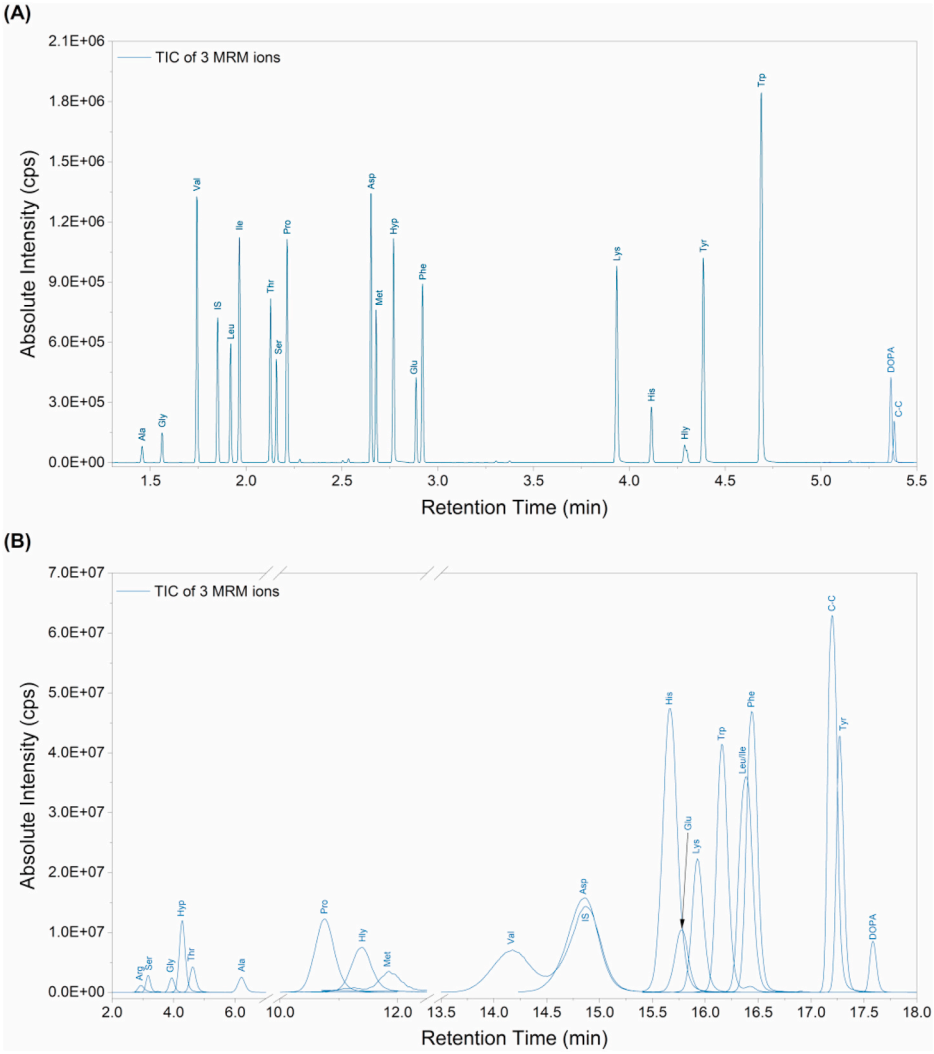

Fig. 1. Comparison of AA separation by GC-MS/MS and LC-MS/MS using a standard mixture: (A) GC-EI-MRM and (B) LC-ESI-MRM chromatogram of the AA standard mixture respectively containing 21 (200 nmol/mL) and 22 (25 nmol/mL) AAs, respectively. Norvaline (Nor) is included as an internal standard (IS). Ala, 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.

concentrations beyond 200 nmol/mL were not included in our study. Krumpochova et al. performed AA analysis by comparing GC-MS, RPLC-MS (both after PCF derivatisation) and HILIC-MS (without derivatisation) regarding linearity, LODs, precision and accuracy [26]. Precision and accuracy are very similar to the data presented here: precision is below 10% (intra-day) and below 14% (inter-day); accuracy is between 96 and 106% (intra-day) and 93 and 111% (inter-day). Linear ranges are a little bit extended to lower values (GC-MS: 0.05–200 nmol/mL; HILIC-MS: 0.03–100 nmol/mL) compared to our findings, but R^2 values are again comparable. Yet, LODs are lower (<100 nM). In that respect it has to be mentioned that the absolute sample amount applied for the RPLC- and HILIC-MS as presented by Krumpochova et al. is 20-times higher than we were using in our LC-EIS-MS/MS method. A fact which is contributed to the very low amount of attachment cements collected from *Dermacentor maginatus*.

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

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

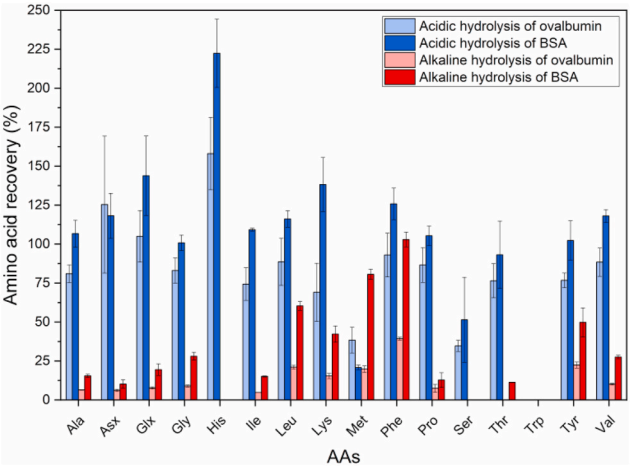

Fig. 2. AA recovery after acidic and alkaline hydrolysis of ovalbumin and BSA. Hydrolysis was performed with 6 N HCl or 5.6 N NaOH for 24 h at 105 °C.

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.

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 [27–29]. 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.

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%–83.1%), Hly (54.4%–65.6%), Hyp (63.4%–69.2%), Lys (68.9%–77.4%), Met (14.4%–30.9%), Tyr (89.9%–95.2%) and Trp (0.0%–20.4%). Decreased recovery was observed for Ala (106.3%–101.0%), C–C (58.0%–45.1%), Glu (133.2%–124.8%) and Leu (107.3%–100.4%). According to this criterion (±5%), recovery for all other AAs was

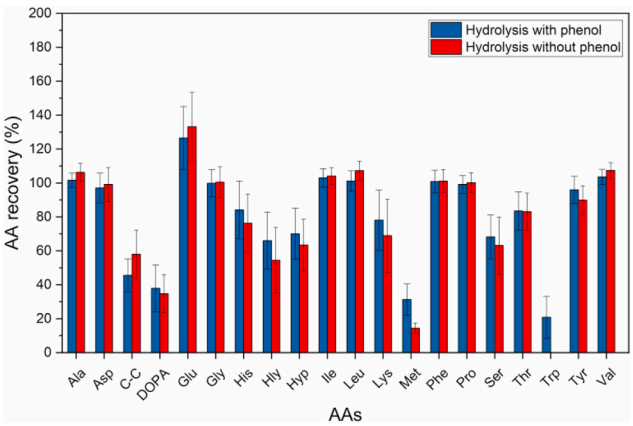

Fig. 3. AA recovery after acidic hydrolysis (24 h at 105 °C) of an AA standard mixture using 6 N HCl (with and without 5% phenol, w/v). Hydrolysis was performed in triplicate on eight different days within four weeks (two per week). The concentration for each AA was 50 nmol/mL.

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 (3–11% 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.

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.

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.

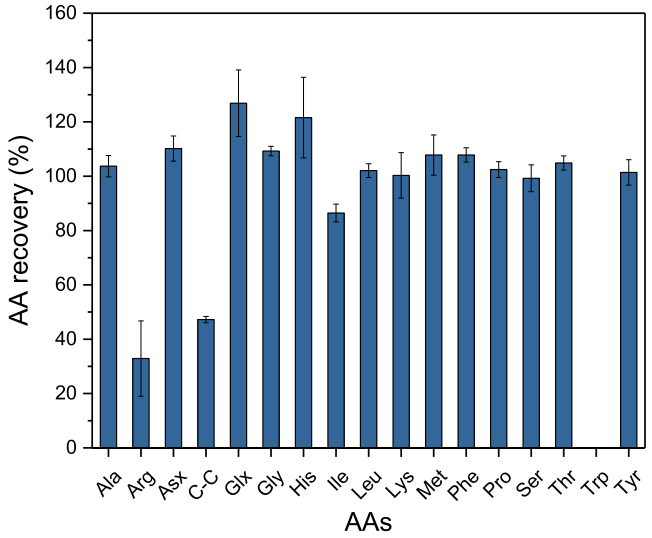

Fig. 4. AA recovery after acidic hydrolysis (24 h at 105 °C) of the certified NIST BSA protein standard using 6 N HCl (5% phenol, w/v).

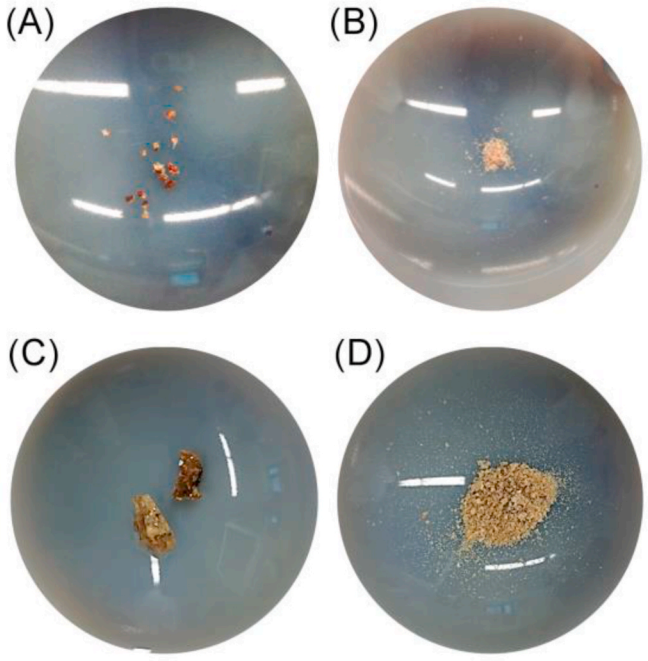

Fig. 5. Grinding and homogenisation of tick (*Dermacentor marginatus*) and barnacle (*Lepas anatifera*) cement samples using an agate mortar and pestle. Tick cement (A) before, and (B) after grinding. Barnacle cement (C) before, and (D) after grinding. Portions of the ground cement were used for acidic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

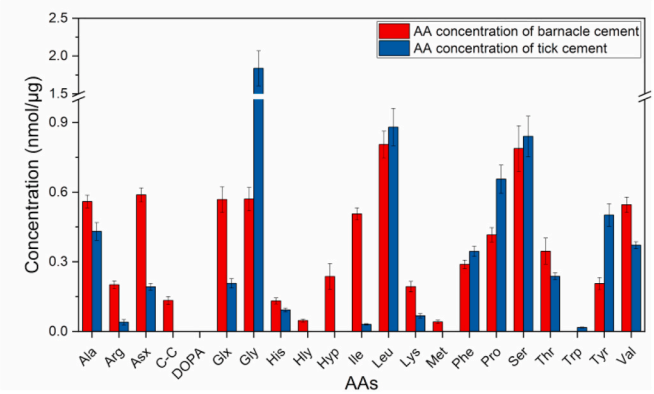

Fig. 6. AA concentration of barnacle cement (*Lepas anatifera*) and tick attachment cement (*Dermacentor marginatus*) after acidic hydrolysis. Hydrolysis was performed with 6 N HCl for 24 h at 105 °C. Concentration is given in nmol per µg cement.

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.

Additionally, the revealed AA composition was compared with the AA content of adhesive proteins of cement glands of *Lepas anatifera* (Suppl. Table 6). Therefore, peptides of adhesive proteins identified by mass spectrometry followed by *de novo* sequencing [30] were searched against the predicted protein database of the recently generated transcriptome [31]. Peptides identified from the cement float of *Dosima fascicularis* were also included in this peptide search since this species is closely related and belongs to the same family as *Lepas anatifera* barnacles [32]. In total 36 proteins could be extracted from the predicted protein database. When adding together all AAs of these proteins, the most abundant AAs are Leu, Glx (both 9.4%), Ala (9.1%), Ser (8.8%), Pro (8.0%), Arg, Asx (both 7.9%), Gly (7.2%) and Val (6.1%) representing 74% of all AAs. This fits very well to the AA composition presented here: Leu, Ser, Asx, Gly, Glx, Ala, Val and Ile represent 68.7% of all AAs. It has to be mentioned that this comparison contrasts AAs found from several proteins (studies [1–3]) with the AA composition of the complete cement complex (this study); with both sets of AAs coming from the same species (or closely related in the case of *Dosima fascicularis*). Thus, any differences are not species related but are a result of methodological artefacts like incomplete protein lists, partial protein sequences or errors during the annotation process.

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 [5,33,34]. 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 (0.1–2.0 nmol/mL, corresponding to fmol/µg concentrations) to the cement (0.02–1.84 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.

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.

Declaration of competing interest

All authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijadhadh.2020.102798.

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 – review & editing. Anne Marie Power: Data curation, Writing – review & editing. Martina Marchetti-Deschmann: Conceptualization, Methodology, Supervision, Resources, Funding acquisition, Writing – original draft, revision

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