



Quantification of sulphur amino acids by ultra-high performance liquid chromatography in aquatic invertebrates



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ARTICLE INFO

Keywords:

Ultra-high performance liquid chromatography
Invertebrate
Cysteic acid
Methionine sulfone

ABSTRACT

We examined the performance of an ultra-high performance liquid chromatography method to quantify protein-bound sulphur amino acids in zooplankton. Both cysteic acid and methionine sulfone were linear from 5 to 250 pmol ($r^2 = 0.99$), with a method detection limit of 13 pmol and 9 pmol, respectively. Although there was no matrix effect on linearity, adjacent peaks and co-eluting noise from the invertebrate proteins increased the detection limits when compared to common standards. Overall, performance characteristics were reproducible and accurate, and provide a means for quantifying sulphur amino acids in aquatic invertebrates, an understudied group.

Introduction

Sulphur amino acids, methionine and cysteine, play a fundamental role in protein synthesis, structure, and function [1] and in the storage of contaminants such as the neurotoxin methylmercury (MeHg) [2]. MeHg binds to sulphur amino acids during uptake, storage, and elimination in aquatic organisms including phytoplankton, bacteria, and fish [3–5] and is almost exclusively present as MeHg-cysteine in fish muscle proteins [6,7].

Accurate quantification of protein-bound sulphur amino acids is therefore desirable, but current methods present several challenges. Under classic hydrolysis conditions (6 N HCl, 110 °C, 24 h [8]), methionine and cysteine are destroyed to various degrees; consequently, they are usually oxidized to methionine sulfone (MSO) and cysteic acid (CYA), respectively, using performic acid (PFA) prior to acid hydrolysis [9]. Most studies reporting amino acid compositions of tissues are often lacking data for cyst(e)ine and methionine because of the extra time for this oxidation process, loss of other amino acids during PFA oxidation, and low levels of sulphur amino acids present [8,10,11]. Such data are particularly lacking in invertebrate samples, which have a different hydrolysate matrix than mammalian or plant tissues.

The newer ultra-high performance liquid chromatography (UPLC) systems increase speed, performance, and sensitivity for amino acid analyses when compared with previous technologies [12], thereby requiring less sample mass which is a particular advantage for some environmental samples. Although PFA oxidation with HPLC is a well-

established method, to our knowledge the current work represents the first time a UPLC was used to validate the detection of sulphur amino acids.

Samples

Zooplankton are microcrustaceans living in the water column of diverse aquatic habitats and form a key food resource for larval fish. Zooplankton (> 153 µm, bulk - Branchiopoda & Maxillopoda classes) from Kejimikujik National Park, Nova Scotia, Canada was selected as the invertebrate taxa for this study because it was possible to collect a large mass relatively easily. The bulk sample was collected in 2013 by towing a Wisconsin net in the pelagic zone of a lake and kept on ice until frozen. Prior to analysis, the sample was lyophilized, homogenized, and stored at -20 °C, consistent with standard procedures [8].

Chromatography

UPLC analysis was performed using a Waters Acquity system with a binary solvent manager, autosampler, column heater, and fluorescence detector (Milford, MA, USA) set up according to the Waters UPLC system guide [13]. Briefly, all chromatographic separations were performed with an in-line filter and a Waters AccQ-Tag_{ultra} reverse-phase column (2.1 × 100 mm, 1.7 µm) and fluorescence detector with excitation and emission wavelengths of 266 and 473 nm, respectively. The column heater was set at 55 °C and the mobile phase flow rate was

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<http://dx.doi.org/10.1016/j.ab.2017.10.022>

Received 6 May 2017; Received in revised form 13 October 2017; Accepted 25 October 2017

Available online 26 October 2017

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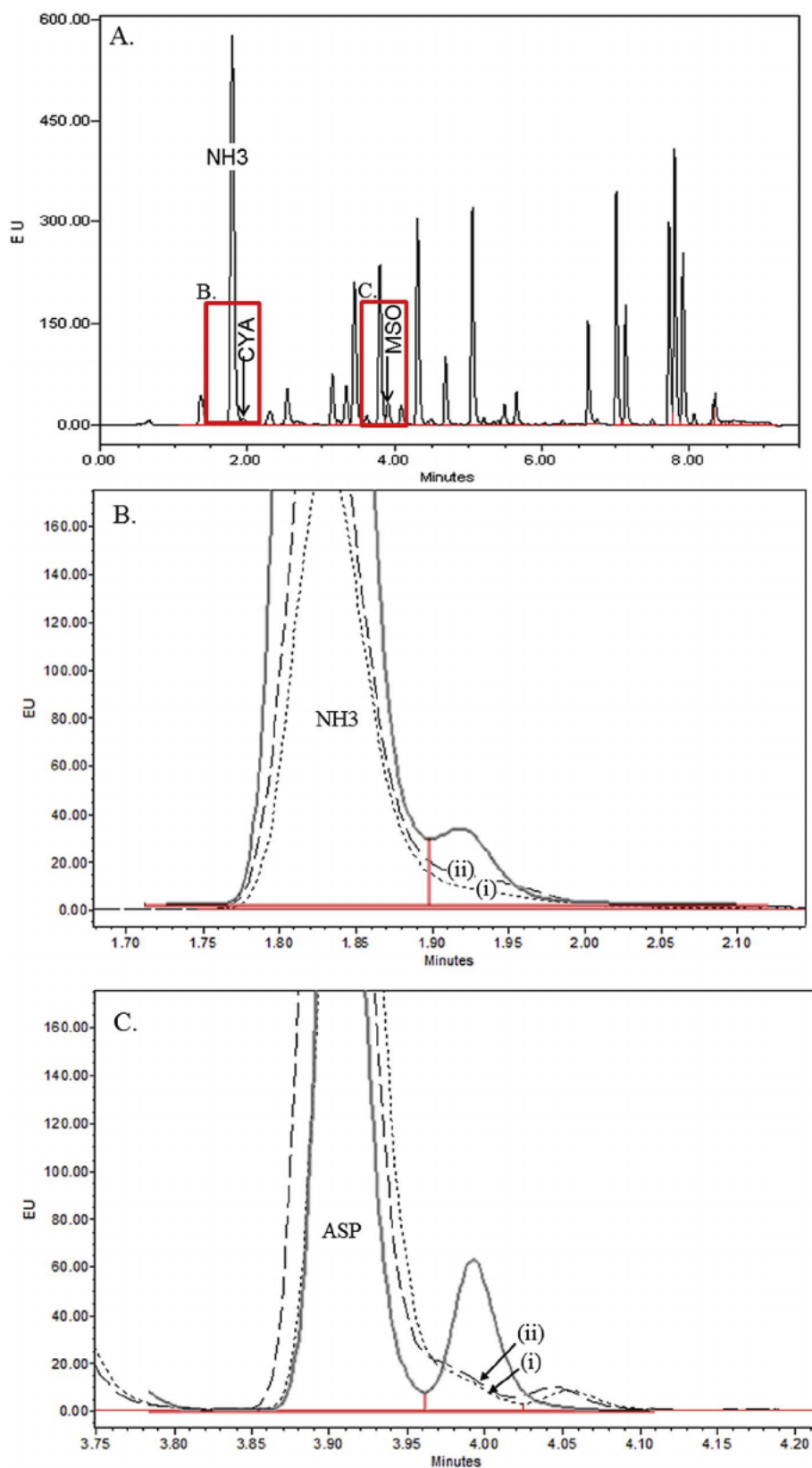


Fig. 1. Ultra-high performance liquid chromatography with fluorescence detection chromatogram of amino acids from (A) a zooplankton hydrolysate with performic acid (PFA) oxidation showing the location of ammonia (NH₃), cysteine acid (CYA), and methionine sulfone (MSO) peaks. Chromatograms of unoxidized zooplankton hydrolysates spiked with (B) CYA and (C) MSO standards of (i) 0 and (ii) 12.5 pmol/μL.

maintained at 0.7 mL/min. Eluent A was 5% (v/v) AccQ-Tag_{ultra} eluent A concentrate: 95% (v/v) Milli-Q ultrapure water and eluent B was 98% (v/v) acetonitrile: 2% (v/v) formic acid. The gradient profile was 0–0.54 min (99.9% A), 5.74 min (90.0% A), 7.74 min (78.8% A), 8.04 min (40.4% A), 8.05 min (10.0% A) 8.73–10.00 min (99.9% A).

Injection volume was 1 μL. Empower 3 (Waters) chromatography software was used for data acquisition.

Peak identification

Accurate determination of CYA at low concentrations in biological samples was tested since it elutes adjacent to a large ammonia (NH₃) peak (Fig. 1A and B). To determine the influence of this NH₃ peak on the CYA peak, unoxidized biological samples spiked with CYA and MSO standards were used. This would produce a typical NH₃ peak area for the sample matrix with known CYA and MSO peak areas. A 200 mg sample of zooplankton was hydrolyzed with 6 N HCl at 110 °C for 24 h [8]. The hydrolysate was filtered (0.45 µm Whatman filter) and divided into 1 mL samples. Each sample was spiked with different concentrations of CYA and MSO standards; norvaline (NVAL; 20 µL of 25 mmol/L) was added as an internal standard. Samples were then dried under vacuum. The pellet was reconstituted in 1 mL 0.1 N HCl and vortexed. As background controls, a hydrolysis tube with only HCl (hydrolysis blank) and blank derivatizations were included; in all controls, NH₃ (and amino acid) peaks were negligible compared to peaks from samples (data not shown), indicating that NH₃ was not from contamination from reagents or glassware.

Although a distinct CYA peak was not detectable at < 25 pmol spike, both the 5 and 12.5 pmol spiked samples had acceptable RSD. This was in contrast to standard “H” - and bovine serum albumin (BSA) (Pierce Chemical, USA) - spiked hydrolysates where there were detectable peaks, good recovery, and linearity from 2.5 pmol (data not shown), suggesting that the larger NH₃ peak in zooplankton hydrolysates does influence the readability of CYA. Changing the buffer profile did not improve the separation of CYA from NH₃. Similar to CYA, a distinct MSO peak in the zooplankton hydrolysate was not observed at < 25 pmol spike. Co-eluting “noise” was observed at the tail of the aspartic acid (ASP) peak in the non-spiked zooplankton hydrolysate (Fig. 1C). This “noise” after the ASP peak was also observed in the pig liver hydrolysate, but not in the BSA and the standard “H” hydrolysates spiked with a MSO standard. The linear range of 1.25–250 pmol observed in the latter matrices (data not shown) was therefore not possible with the invertebrate hydrolysate.

Linearity

Linearity was assessed using unoxidized zooplankton hydrolysate spiked with 8 concentrations of CYA and MSO standards in the range 0–250 pmol/µL, in triplicate, varying in the order of injection. The results were analyzed by linear regression. There was no difference among the peak areas for 0, 1.25, and 2.50 pmol/µL, with peak areas only increasing with concentrations > 5 pmol/µL. The linear ranges were 5–250 pmol for both CYA and MSO, with regression coefficients (r^2) of 0.9957 and 0.9903, respectively (Table 1). There was no significant difference among regression slopes (ANCOVA: CYA, $p = 0.0814$; MSO, $p = 0.4663$) from standard curves using zooplankton, pig liver, BSA, or standard “H” hydrolysates, indicating there were no matrix effects on the linearity of response.

Relative standard deviation (RSD)

In zooplankton hydrolysate, the RSD for CYA between 5 and 250 pmol was $5.6 \pm 3.1\%$ (mean \pm SD), ranging from 1.7% (250 pmol) to 10.6% (5 pmol). The RSD for MSO averaged $7.4 \pm 3.3\%$, ranging from 11.9% (12.5 pmol) to 2.3% (250 pmol).

Precision

The precision of detection was determined using a single zooplankton sample spiked with 25 pmol/µL of CYA and MSO standard and injected five times in a row on the same day (intra-day) or four times over seven days (inter-day). The peak area and retention time RSDs ranged from 2.0%–8.8% and from 0.2%–1.2%, respectively (Table 1), indicating sample stability over time. The precision of derivatization

Table 1
Analytical data from a zooplankton hydrolysate.

Measurement	Cysteic acid	Methionine sulphone	Sample size, frequency
Linear range, pmol	5–250	5–250	n = 6, triplicate
Linearity, r^2	0.9957	0.9903	
Precision, RSD %			
Peak area: intra-day	4.58	2.00	n = 5
inter-day-7 d	8.74	8.82	n = 4
Derivatization	5.55	1.19	n = 5
Retention time: intra-day	0.34	0.21	n = 5
inter-day-7 d	1.18	0.21	n = 4
Limit of detection ^a , pmol	10	8	n = 5
Limit of quantification ^b , pmol	34	27	n = 5
Method detection limit ^c , pmol	13	9	n = 5
Recovery, % (RSD %)			n = 3
25 pmol	96.2 (6.82)	110.9 (9.32)	
50 pmol	87.4 (3.77)	118.7 (8.07)	
125 pmol	101.7 (3.18)	94.8 (5.60)	
Mean \pm SD ^d , nmol mg ⁻¹ tissue			
Zooplankton, > 153 µm	8.11 \pm 2.98	18.32 \pm 5.67	n = 78
Dragonfly (Aeshnidae)	4.23 \pm 0.96	9.34 \pm 3.90	n = 114
Mayfly (Heptageniidae)	6.56 \pm 0.87	14.47 \pm 2.26	n = 56
Caddisfly (Limnephilidae)	4.80 \pm 0.72	18.05 \pm 4.12	n = 69
Yellow perch (<i>Perca avescens</i>)	28.83 \pm 7.76	51.05 \pm 11.91	n = 108

^a Signal/noise ratio = 3.

^b Signal/noise ratio = 10.

^c Standard deviation (SD) of a 12.5 pmol standard multiplied by the Student's T value.

^d Mean \pm SD of aquatic invertebrates (composite sample) and fish muscle collected in 2013 and 2014 from lakes in Kejimikujik National Park, Nova Scotia, Canada.

was found by derivatizing the same sample five times; the peak area RSDs were 5.6% and 1.2% for CYA and MSO, respectively (Table 1). These values were considered acceptable, but slightly higher than for other amino acids, potentially due to the small peak sizes and more noise from the sample matrix.

Limit of detection (LOD)

LOD was 10 pmol for CYA and 8 pmol for MSO, estimated using a 12.5 pmol spiked zooplankton hydrolysate as the concentration required to give a signal-to-noise ratio of 3 (n = 5) (Fig. 1B and C). This corresponded to limits of quantification (LOQ; signal-to-noise ratio of 10) of 34 pmol and 27 pmol, respectively (Table 1). These values were similar to the method detection limits (MDL) of 13 pmol for CYA and 9 pmol for MSO. The MDL was calculated as the standard deviation of a 12.5 pmol standard multiplied by the student's T value (2.776, n = 5). The LODs and LOQs were higher than those previously reported [14] with Waters AccQ-Tag on HPLC, although those LODs used amino acid standards and blanks.

The mean concentration in zooplankton samples for CYA and MSO was 8.11 ± 2.98 and 18.32 ± 5.67 nmol mg⁻¹ tissue, respectively (Table 1). These values were similar to other aquatic invertebrates collected from this system, although below a more typical protein, fish muscle (Table 1). The results were also comparable to the literature for freshwater zooplankton [15] and terrestrial invertebrates [16]. The LODs reported were well below all the matrices examined and therefore the sensitivity of the instrument and method was acceptable.

Recovery

The accuracy of the method was established by spiking three concentrations (25, 50, and 125 pmol) of CYA and MSO standard in zooplankton samples (200 mg) followed by PFA oxidation [9] and

hydrolysis in HCl, in triplicate. The recovery values were 96.2, 87.4, and 101.7% for CYA, respectively, and 110.9, 118.7, and 94.8% for MSO, respectively (Table 1). These recoveries are comparable to previous sulphur amino acid studies [11,14,17] with values between 91 and 106% for CYA and 98–106% for MSO.

Conclusions

This study aimed to validate the fast and sensitive AccQ-Tag^{ultra} UPLC method for protein-bound sulphur amino acid quantification in a zooplankton hydrolysate. The performance characteristics were reproducible, accurate, and comparable to previous technologies using standards or mammalian tissues. The sensitivity of the method was adequate for zooplankton. However, isolation of CYA was influenced by the adjacent NH₃ peak reducing sensitivity; moreover, MSO identification in zooplankton was influenced by co-eluting matrix noise. Therefore, for the determination of protein-bound sulphur amino acids, it is recommended that all new matrices develop specific detection limits using spiked samples, as LODs found in the literature may not be achievable in alternative matrices such as environmental invertebrate samples. In conclusion, this method was successful in determining accurate results in zooplankton hydrolysates and can be used to assess levels of sulphur amino acids in similar samples from other systems and regions.

Acknowledgments

The authors thank M. Nosworthy and A. Mercer for their suggestions and T. Kankayaliyan for technical assistance. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Program [429389-2012 and 312237-2012].

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