A novel approach to the measurement of surfactant parameters in arthropod digestive juices

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ABSTRACT

In arthropods, the determination of two important parameters of digestive juices, i.e. the total surfactant concentration and the critical micelle concentration (CMC), is challenging due to small sample volumes and low surfactant concentrations. In this work, we report a successful implementation of potentiometric titrations using the surfactant ion-selective electrode (SISE) and the pyrene fluorescence method (PFM) for the determination of the total surfactant concentration and CMC in the digestive juice of terrestrial isopod crustaceans *Porcellio scaber*. Pooled digestive juice extracts of four (SISE) or two (PFM) animals were used per measurement run. In both cases, digestive juice extracts in 100 µL of deionized water were sufficient for one measurement run. The total surfactant concentration of *P. scaber* digestive juice was determined to be 9.2 ± 3.5 mM and the CMC was around 90 µM. Our work presents an important improvement towards easy CMC determination in small volume samples in comparison with the commonly used stalagmometric technique, where much larger sample volumes are usually needed. To date, the total surfactant concentration was not measured in the digestive juices of arthropods other than *Homarus vulgaris*, *Astacus leptodactylus* and *Cancer pagurus*, for which a complex separation and analytical techniques were required. Our results obtained by SISE and PFM therefore present the first successful quantification of surfactants and their CMC in small volumes of arthropod digestive juice without prior separation or purification techniques.

Keywords: terrestrial isopod, surfactant, critical micelle concentration, surfactant ion-selective electrode, pyrene fluorescence

INTRODUCTION

In invertebrates, digestive surfactants play various roles, many of which are analogous to those of bile salts in vertebrates (Vonk, 1962; Mayer et al., 1997). Digestive surfactants may solubilize food particles, activate or deactivate digestive enzymes, hold hydrophobic compounds in solution beyond their aqueous solubility by incorporating them into micelles, prevent the loss of digestive enzymes due to adsorption onto food, provide lubrication for the transport of food through the digestive system (Mayer et al., 1997), and perhaps play some other roles. In herbivore arthropods, specifically, digestive surfactants may also protect digestive enzymes and dietary proteins from being precipitated by tannin-rich food (Martin and Martin, 1984; Martin et al., 1987; Zimmer, 1997). In insects, digestive surfactants play a crucial role in plant-herbivore interactions, along with the facilitation of nitrogen assimilation from food (Mori and Yoshinaga, 2011). The knowledge of surfactant properties in invertebrate digestive juices is of interest in many fields, such as in crustacean aquaculture with the focus on improving feed digestibility (Sales, 2010; Yue et al., 2013; Perera and Simon, 2014), in plant physiology when studying the defensive reaction of plants to the injuries caused by herbivorous insects (Mori and Yoshinaga, 2011), in marine ecotoxicology with the aim of elucidating the susceptibility of animals to hydrophobic organic pollutants (Voparil et al., 2003), and in nanotoxicology with the goal of explaining the transformations of metal-containing nanoparticles after the consumption by test organisms (Diez-Ortiz et al., 2015; Romih et al., 2015).

The structure of digestive surfactants appears to be very similar throughout the arthropod phylum and, on the other hand, distinctly different from vertebrate surfactants. As opposed to vertebrates, arthropods are in general incapable of endogenous sterol synthesis (Zandee, 1967; Holwerda and Vonk, 1973), therefore the hydrophobic part of the surfactant molecule is

represented by a fatty acid chain instead of the cholic acid derivatives (van den Oord et al., 1965; Vonk, 1969; Holwerda and Vonk, 1973; Collatz and Mommsen, 1974; Tumlinson and Lait, 2005; Mori and Yoshinaga, 2011). The structure of the surfactants' polar head group in arthropods varies. It may consist of a sulphate group, such as in Chinese mitten crab, *Eriocheir sinensis* (Vonk, 1969), or of different combinations of amino acids or related compounds (such as sarcosine and taurine) in different crustacean, insect and spider species (van den Oord et al., 1965; Holwerda and Vonk, 1973; Collatz and Mommsen, 1974; Tumlinson and Lait, 2005; Mori and Yoshinaga, 2011). The arthropod surfactants may be either anionic or zwitterionic.

Besides structure and charge, two additional parameters of surfactants in the arthropod digestive juices are important: their total concentration and the concentration where micellization occurs, which is termed the critical micelle concentration (CMC), because for many surfactant functions it is important that sufficient amount of micelles is present. However, the information about these two parameters is scarce due to the challenging analysis of digestive juice composition, because only small volumes of juices are present in most arthropod species. To the best of our knowledge, the total concentration and the CMC of arthropod surfactants have so far been quantified only in decapod crustaceans *Cancer pagurus* (Vonk, 1969), *Homarus vulgaris* and *Astacus leptodactylus* (Holwerda and Vonk, 1973). The most likely reason for the lack of data on surfactants in arthropod digestive juices is that for this purpose methods including ion-exchange chromatography, gel filtration, acid hydrolysis, thin-layer chromatography and amino acid analysis were used which demand complex sample preparation and separations before analyses (Vonk, 1969; Holwerda and Vonk, 1973). For insects, such as lepidopteran larvae (*Manduca sexta*, *Lymantria dyspar*) and grasshoppers (*Schistocerca gregaria*), as well as for isopods (*Porcellio scaber*), the exact quantification of

the total surfactant concentration has not been attempted and their concentrations were expressed as multiples of (an unidentified value of) CMC (Martin and Martin, 1984; Martin et al., 1987; DeVeau and Schultz, 1992; Zimmer, 1997), which was measured by tensiometry according to the method of Ferguson (1933).

There is a need for methods that would enable the detection of low surfactant concentrations and a simple and rapid analysis, preferably with little or no sample preprocessing. One simple method for the determination of surfactant concentrations as well as of their CMC values, is titration with surfactant ion-selective electrodes (SISEs), which have been commercially available since approximately 1990. These electrodes enable the determination of a variety of anionic and cationic surfactants (Schmitt, 2001). The titration technique for concentration determination is based on the precipitation reaction between surfactants in the sample and an oppositely charged surfactant in the titrant solution. It is thus not selective for individual surfactants, but determines their total amount in the mixture. Special sample pretreatment is generally not necessary and the analysis of surfactants is possible in various complex matrices, such as wastewaters or commercial cosmetic products (Metrohm Application Bulletin No. 233/3 e). The titration conditions must be optimized for each type of the electrode, as well as for each surfactant, because SISEs from different manufacturers differ in their response time and in the potential difference registered during a titration (Schmitt, 2001).

An alternative method to tensiometry for the measurement of CMC in small-volume samples is the pyrene fluorescence method ([PFM] Kalyanasundaram and Thomas, 1977; Winnik and Regismond, 1996). In this method, pyrene is added to the surfactant solution as a fluorescent probe and the intensity ratio of the first and the third peak (I_1/I_3) in the characteristic pyrene fluorescence spectrum is calculated for various surfactant concentrations. The ratio of these

two peaks depends on the net hydrophobicity/polarity of the probe's immediate environment. Below the CMC, where no hydrophobic domains are present in aqueous surfactant solutions, the peak ratio value is high, corresponding to the polar environment of water. When surfactant micelles start to form, the ratio decreases and reaches a lower and roughly constant value above the CMC, because pyrene becomes incorporated in the hydrophobic regions of the micelles. The CMC is evaluated at the point where the pyrene polarity ratio as a function of the total surfactant concentration starts to decrease (Winnik and Regismond, 1996). The advantage of PFM over tensiometry for the determination of CMC is the considerably lower amount of solution needed for fluorescence measurements. This makes PFM appealing for the analysis of biological samples where volumes are often extremely limited, especially since fluorimeters have nowadays become common pieces of laboratory equipment.

The aim of the present study is to apply the commercial SISE and PFM to determine the total surfactant concentration and the CMC in the digestive juice of a model arthropod, the terrestrial isopod crustacean *P. scaber*. Terrestrial isopods *P. scaber* are among the most studied organisms in ecotoxicology, ecophysiology (Drobne, 1997), and recently also in nanotoxicology (Romih et al., 2015). We discuss the possibility of quantifying surfactant content and their CMC in small volumes of arthropod digestive juice without prior separation or purification techniques. This information is important for the understanding of processes occurring in the arthropod digestive systems.

MATERIALS AND METHODS

Reagents and solutions

All chemicals were of analytical grade purity. Silver nitrate ($AgNO_3$), *N*-dodecylpyridinium chloride (DPC), sodium dodecyl sulfate (SDS), sodium *N*-lauroylsarcosine (NLS), and pyrene

were purchased from Sigma Aldrich (Steinheim, Germany). Water used throughout the work was first deionized and then additionally purified using the Elix 10/Milli-Q Gradient unit (Millipore, Bedford, Massachusetts, USA).

Collection and cultivation of model arthropods

Terrestrial isopods were collected in April 2014 from a compost heap in a non-polluted area near Vrhnika, Slovenia. The animals were kept in a controlled chamber at a constant temperature (20 ± 2 °C) with a light regime (at 16 h light and 8 h darkness cycle) and fed dry common hazel leaves (*Corylus avellana*) for three weeks before the experiments were carried out.

The depuration and dissection of test animals

Immediately prior to the experiments, 50 adult *P. scaber* of both sexes, at the intermoult stage and with the average fresh body mass of 63 ± 19 mg, were collected from the culture and placed individually into plastic Petri dishes (9 cm in diameter) to depurate their guts. Pieces of filter paper of approximately 4 cm^2 in size were added to the Petri dishes to help maintain the moisture, but otherwise no food or soil was added. The lids were sprinkled with deionized water and the Petri dishes were placed in a large plastic container with moistened paper towels at the bottom. The container was sealed with plastic foil and kept for 4 days under the same conditions as the previous animal cultivation. The Petri dishes were checked daily to ensure appropriate moisture.

After the depuration stage, the animals were decapitated and their hindguts were isolated with tweezers. It was confirmed that none of the animals had consumed any filter paper; the hindguts that contained more than trace amounts of food were discarded. 43 out of 50

depurations were successful. The depurated hindguts were then divided into two groups: 25 of them were designated for the measurements of surfactant content in the digestive juice and 18 for the measurements of CMC values.

Analysis of hindgut volume

The hindguts designated for the measurements of CMC were placed on glass object slides and photographed under the Zeiss Stemi SV 11 stereomicroscope coupled to the Axiocam 105 color camera (Carl Zeiss AG, Jena, Germany) at 6-fold magnification. The hindgut length and the longitudinal cross-section area were measured with the AxioVision Release 4.8 software upon calibration of the stereomicroscope with an external scale (millimeter paper).

The shape of *P. scaber* hindgut can be mathematically considered as a solid of revolution. However, due to the limited number of parameters that could be reliably estimated from the photographs, we considered it sufficient for our purpose to approximate the hindgut shape by a cylinder. The volume (V) of an ideal cylinder is calculated as $V = \pi r^2 l$, where r is the cylinder's radius and l its length. However, since the hindgut radius is not constant along its longitudinal axis, its average value ($\bar{r}_h$) can be calculated from the hindgut longitudinal cross-section area (A_h) and its length (l_h) as $\bar{r}_h = \frac{A_h}{2l_h}$ and used in the evaluation of V. The A_h and l_h values were estimated from the photographs.

Digestive juice extraction

After the isolated hindguts had been photographed, they were transferred into 1 mL test tubes containing 100 µL of deionized water. For the measurements of surfactant concentrations, the samples were prepared from individual hindguts, whereas for the measurements of CMC, two hindguts were placed in the same test tube. The muscular wall of the hindguts was perforated

with the previously ethanol-washed and dried 1 mL (insulin) syringe and the hindgut content was blown out and into the aqueous solution. When the hindguts were considered empty, they were removed from the extracts and discarded. The water-suspended hindgut contents were centrifuged for 5 min at 14000 rpm to sediment the food remnants. The samples were frozen at –20 °C until use.

Measurements of total surfactant concentration with a surfactant ion-selective electrode

Cationic surfactant DPC was used as the titrant in all surfactant titrations. Concentrations of the DPC titrant solutions were standardized by precipitation potentiometric titration with a 0.1 M $AgNO_3$ solution, using a chloride ion-selective electrode (Metrohm AG, Herisau, Switzerland) as the indicator electrode coupled to a mercury/mercurous sulfate (Hg/Hg_2SO_4) electrode as the reference electrode. The standardizations were carried out in a 50 mL beaker at room temperature (23 ± 1 °C) during continuous stirring with a magnetic stirrer at 200 rpm.

The measurements of the total surfactant content were performed using a SISE from Metrohm AG (Herisau, Switzerland; order no. 6.0507.120) as the indicator electrode and a saturated calomel (Hg/Hg_2Cl_2) electrode as the reference electrode. The potential difference (E) between the electrodes was measured using the multimeter MA 5740 (Iskra, Ljubljana, Slovenia). The titrant was the previously standardized DPC solution. The measurements were carried out in the form of potentiometric precipitation titrations in a 20 mL glass cell connected to a circulation water bath in order to maintain the temperature at 25 °C. During the titration, the solution was continuously stirred with a magnetic stirrer at 600 rpm.

The proper functioning of the SISE was ensured by first checking its response to the concentration of commercial anionic surfactants recommended by the manufacturer, i.e. by

titrating 2.5 mM SDS and 5 mM NLS solutions with a 100 mM DPC solution. Second, the possibility of measuring low concentrations of anionic surfactants was explored by titrating a 0.25 mM SDS solution with a 4 mM DPC solution. SDS titrations were carried out at the solution's natural pH (= 6.6), while for NLS titrations the solution pH was adjusted to 10.8 following recommendations of the producer (Metrohm Application Bulletin No. 233/3 e). The DPC solution was added to the anionic surfactant solution in 50 or 100 μL volume increments by using a Gilmont micrometer burette (Cole-Palmer, Vernon Hills, Illinois, USA). The titration curves were constructed by plotting *E* versus the volume of the added titrant.

Finally, the putative anionic surfactants in *P. scaber* digestive juice (van den Oord et al., 1965; Holwerda and Vonk, 1973) were titrated with a 0.4 mM DPC solution. Samples of *P. scaber* digestive juice from four animals (i.e. four extracts of 100 μL each) were pooled and diluted to 10 mL with deionized water. The pH of the solution was adjusted to 9.9 before the titrations were started. DPC was added to the digestive juice solution in 5 or 10 μL increments. The rest of the procedure was the same as in the case of commercial surfactants.

The titration equivalent volumes were calculated from the second derivatives of the titration curves using standard procedures that are described in the **Supplementary Information** (**Eq. S1**–**S4**, **Table S1**).

Measurements of the critical micelle concentration with pyrene fluorescence spectroscopy

The CMC measurements of *P. scaber* digestive juice were carried out with pyrene-based fluorescence spectroscopy (Kalyanasundaram & Thomas, 1977; Winnik and Regismond, 1996). A saturated solution of pyrene in deionized water was prepared one day in advance by suspending pyrene in water and stirring overnight to ensure saturation. A 400 μL quartz

fluorescence cuvette with a 10 mm optical path was filled with 150 µL deionized water and 50 µL of the saturated aqueous solution of pyrene. Then, extracts of two *P. scaber* hindgut contents were added to the cuvette in 10 µL increments, usually up to the total added volume of 80 µL. The pyrene fluorescence spectra were recorded after each addition by using the luminescence spectrometer Perkin Elmer LS 50 (Perkin Elmer, Waltham, Massachusetts, USA). The excitation and emission slit widths were 2.5 mm, the excitation wavelength was 300 nm, and the scanning rate was 240 nm/min. All emission spectra were recorded in the wavelength region from 350 to 450 nm. Five scans were accumulated for each run and spectra were fully corrected before the ratio of the intensities of the first and third vibronic bands, I_1/I_3, was calculated. The I_1/I_3 ratio was plotted against the corresponding cumulative digestive juice volume in order to evaluate the volume signifying the CMC (i.e. V_{CMC}). The CMC was then evaluated from V_{CMC} and from the estimated concentration of surfactants in the digestive juice.

RESULTS AND DISCUSSION

P. scaber hindgut volume

Figure 1 represents an isolated hindgut from one of the test animals, containing traces of food, as it was inspected under a stereomicroscope. Both the length and the longitudinal cross-sectional area could be determined with satisfactory precision. The total hindgut volume was calculated to be 5.5 ± 1.8 (average ± standard deviation) mm^3 (µL) based on the data from 16 animals, which were intended for the measurements of CMC, with their fresh body mass of 54 ± 17 mg. Two hindguts were accidentally deformed during the section and were not included in the average volume calculations.

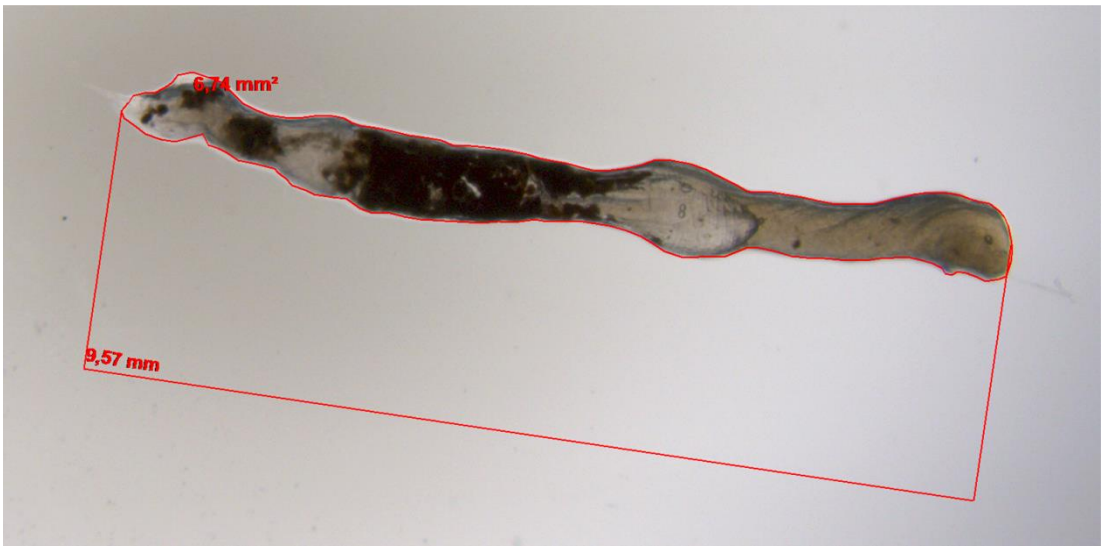


Figure 1. The isolated hindgut of *P. scaber* containing remnants of food, as seen under a stereomicroscope at 6-fold magnification. The hindgut length and the longitudinal cross-section area were measured upon calibration of the stereomicroscope with an external scale (millimeter paper). The markings of measured values in the photograph were produced automatically by the software.

The juice had an appearance of a light brown, opaque liquid and it is evident from **Figure 1** that it was unevenly distributed throughout the hindgut. Zimmer (1997) noted that 1.0 ± 0.3 µL of digestive juice was present in the gut lumens of adult *P. scaber* that he used for the measurements of the surface tension of the digestive juice. Animal body mass, hindgut dimensions or the procedure of digestive juice volume quantification were not reported in that study (Zimmer, 1997). However, since roughly 1–2 thirds of the hindgut lumens appeared to be filled with the digestive juice in our case, we estimate that the animals probably contained 2–3 µL of the digestive juice.

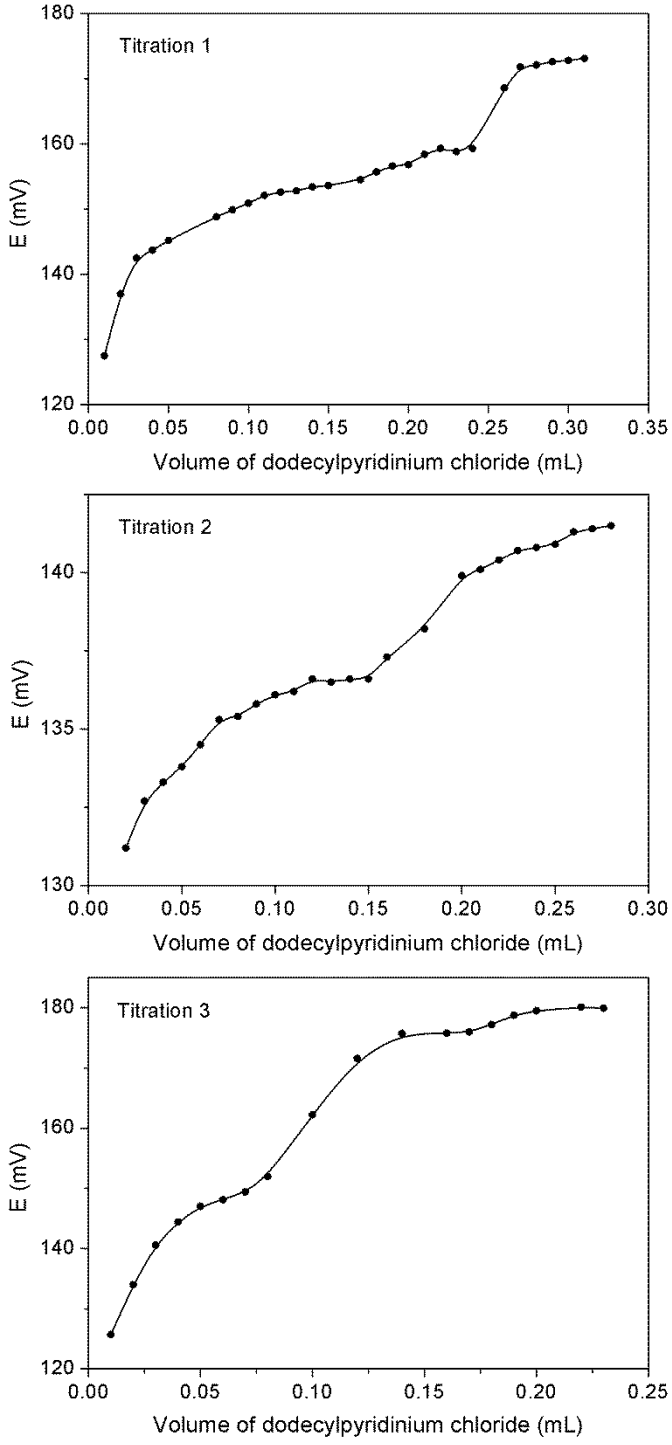
Total surfactant concentration in P. scaber digestive juice

The dependence of the measured *E* on the volume of the added DPC titrant solution into SDS and NLS solutions is presented in **Supplementary Information** (**Figures S1–S3**) for 2

titration runs for each surfactant. **Figures S2** and **S3** apply to two different concentrations of NLS. These experiments served as the quality control check.

Preliminary experiments for the titrations of *P. scaber* digestive juice (using 9 out of 25 animals) showed that the medium needs to be alkaline for the titrations to be successful, implying the carboxylate nature of the surfactants (Metrohm Application Bulletin No. 233/3 e). This reasoning is in line with reports in the literature noting that the vast majority of invertebrates, such as decapod crustaceans, insects, and spiders contain surfactants with carboxylic head groups (van den Oord et al., 1965; Vonk, 1969; Holwerda and Vonk, 1973; Collatz and Mommsen, 1974; Tumlinson and Lait, 2005; Mori and Yoshinaga, 2011). The only exception noted so far was Chinese mitten crab, *E. sinensis*, where the polar head consists of a sulphate group (Vonk, 1969). However, the determination of sulphated surfactants (with the precipitation reaction using an oppositely charged surfactant, as it is the case with SISE titrations) does not depend on the pH. We conclude that titration in alkaline conditions enables the determination of all negatively charged surfactants in the samples.

In subsequent experiments, the digestive juice of 4 isopods was pooled together in order to obtain a detectable inflection point in the titration curve; 3 out of 4 titrations (including the remaining 16 animals; see above) were successful. These titration curves are shown in **Figure 2**. Although the titration curves in **Figure 2** begin at different initial *E* values, it must be noted that such drift is common in measurements with ion-selective electrodes and may be accounted for by some suitable calibration using standard solutions (which is usually not applicable with real samples). However, because the position of the inflection point does not depend on the absolute value of *E*, the equivalent surfactant volume could be unambiguously evaluated for all three titration curves in **Figure 2**.



314

315 **Figure 2.** The dependence of *E* on the volume of the added SDS or NLS during the titration of *P. scaber*
316 digestive juice with a 0.4 mM dodecylpyridinium chloride (DPC) solution at the surfactant ion-selective
317 electrode; the initial solution volume was 10 mL. The DPC solution was added to the digestive juice solution in
318 10 μL increments, starting with 20 μL.

319

The calculations showed that the total concentration of detected surfactants in the pooled samples was 0.31, 0.24, and 0.14 mM, for titration 1, 2, and 3, respectively. Recalculated per one animal and by taking the estimate of 2.5 µL as the volume of digestive juice in the animal gut, this yields an average total surfactant concentration of 9.2 ± 3.5 mM. The value for the total surfactant concentration in *P. scaber* appears to be of the same order of magnitude, albeit slightly lower than the values for other decapod crustaceans, which were reported to be between 12 and 39 mM (more specifically 17.5 mM for *C. pagurus* [Vonk, 1969], 12–17 mM for *H. vulgaris*, and 39 mM for *A. leptodactylus* [Holwerda and Vonk, 1973]). A lower value in our case may be attributed either to biological differences between species or to the measurement errors due to sampling procedures, low sample volumes, etc. Nevertheless, our result for the total surfactant concentration is reasonably close to the values reported for other crustaceans.

Critical micelle concentration of P. scaber digestive juice

The intensity of pyrene fluorescence and the relative ratio of peak heights in the fine structure of its fluorescence spectrum depend on the immediate polarity of the pyrene's microenvironment. Plots of the pyrene fluorescence ratio (I_1/I_3) as a function of the volume of digestive juice are shown in **Figure 3.** Four measurements (out of 7 in total, i.e. for 8 animals) showed a characteristic break in this plot that signifies the CMC. The remaining 3 curves (corresponding to 6 animals) displayed a more or less linear decrease in I_1/I_3 and thus the evaluation of CMC was not possible (these data are not shown). A likely reason for this is that besides surfactants other common constituents of *P. scaber* gut contents, such as proteins, bacteria and digested food remnants (Zimmer, 2002), also affect the fluorescence spectrum of pyrene. If the contribution of these components to micro-polarity of the medium is dominant, they may mask that of the surfactants and prevent the determination of their CMC by PFM.

Incomplete gut depuration and biological variability may therefore be the main reasons that influence the detection of micelles in samples obtained from different animals.

The initial I_1/I_3 ratio (**Figure 3**) has a value around 1.6 and indicates polarity which is slightly lower than that of water (I_1/I_3 = 1.8–1.9). This is probably due to numerous components in the digestive juice and food remnants that may have partial hydrophobic character (e.g. surfactants, proteins, polyphenolic compounds from the digested food; Zimmer, 2002). The final I_1/I_3 value differs considerably from sample to sample. It is between 1.0 and 1.4 and indicates a lower polarity of the micellar interior (**Figure 3**). For example, in SDS and cetyltrimethylammonium chloride, CTAC, the value of I_1/I_3 in micelles is around 1.1 and 1.3 (Ringsdorf et al., 1991), respectively. The large span of values in our case is probably due to different compositions of digestive juice extracts, reflecting the variability of the depuration efficiency among individual animals during the sample preparation.

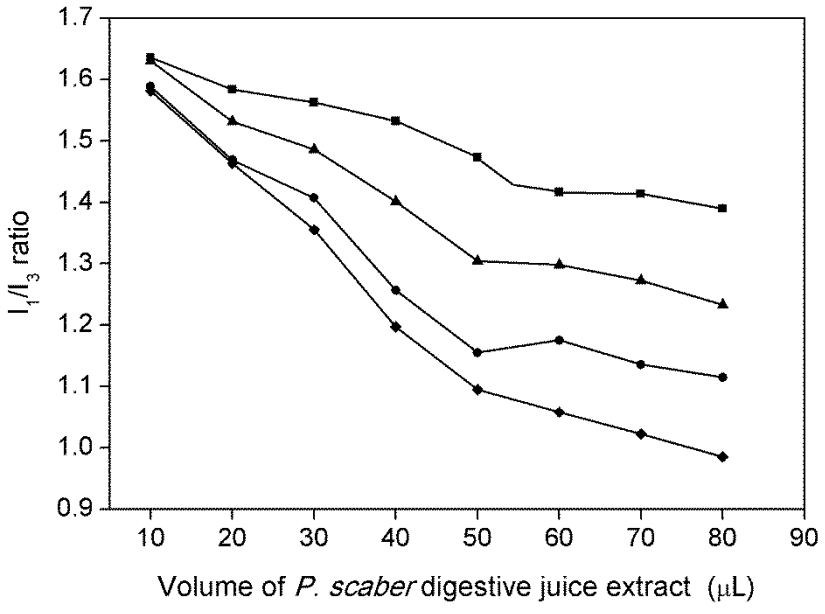


Figure 3. The variation of the I_1/I_3 intensity ratio obtained from the pyrene fluorescence emission spectra as a function of the cumulative volume of *P. scaber* digestive juice extract. Each curve applies to a sample obtained by extracting the digestive juice of two animals into 100 μL of the solvent.

The curves in **Figure 3** show a closely matching volume of *P. scaber* digestive juice at which the micellization occurs, evident from the points where the curves' slopes change: V = 50 µL (in 3 measurements) and around 55 µL (in 1 measurement). By taking into account the dilution of digestive juices by deionized water during extraction, their further dilution in the fluorimeter cuvette, the digestive juice volume per one animal (~2.5 µL), and the previously determined estimate of the total surfactant concentration in *P. scaber* digestive juice, the average CMC is around 90 µM. This value is around 100-times lower than the total surfactant concentration. A similar ratio (around 80) between the total surfactant concentration and the CMC was determined for *P. scaber* digestive juice by Zimmer (1997) using tensiometry.

This high ratio between the total surfactant concentration and the CMC in digestive juices is a physiological adaptation to food rich in tannins, which may cause a depletion of digestive surfactants by precipitation (Zimmer, 1997). In agreement with our results, the measurements of the CMC by tensiometry showed high ratios between the total surfactant concentrations and the CMC also for some phytophagous insect species. For example, the CMC was estimated to be around 10% of the total surfactant concentration in *S. gregaria* (Martin et al., 1987) and in the larvae of *M. sexta* (Martin and Martin, 1984; Martin et al., 1987), or around 12.5% of the total surfactant concentration in *L. dyspar* (DeVeau and Schultz, 1992).

The importance of the total surfactant concentrations and CMC for the solubilization properties of the arthropod digestive juices

The CMC depends predominantly on the surfactant structure (the size, charge and chemical nature of the polar head group, and the length and branching of the alkyl chain) and the solution characteristics (e.g. the concentration of simple salts like NaCl, which determines the ionic strength of the environment). In marine invertebrates, the presence of surfactant micelles

388 in digestive juices was found to correlate with the food source. In obligate carnivores and
389 suspension feeders, micelles are absent (Mayer et al., 1997) and lipids are transported through
390 the digestive system in the form of emulsions (Voparil et al., 2008); note that the surfactant
391 concentration for emulsion formation is different from the CMC, usually smaller. In
392 omnivores and deposit feeders, on the other hand, surfactant micelles are usually found in all
393 species, but not necessarily in every individual of these species (Mayer et al., 1997). The
394 correlation between the presence of micelles and food source, which exists in different
395 phyletic groups, could explain why micelles were not found in the digestive juices of *D.*
396 *marginalis* larvae (Vonk, 1969), *H. vulgaris* and *A. leptodactylus* (Holwerda and Vonk,
397 1973), all of which are carnivores. As an exception to the abovementioned rule, micelles were
398 found in the carnivore *C. pagurus* and the CMC of around 20% of the total surfactant
399 concentration was reported (Vonk, 1969). The synthesis of surfactants in living organisms at
400 concentrations above the CMC is costly in terms of the amount of material needed andenergy
401 spent; it can thus be expected that the total surfactant concentration will be much higher than
402 the CMC only in those animal species where emulsions would not suffice for successful
403 digestion and micelles are required for solubilization of ingested food particles (Voparil et al.,
404 2008). Examples of such animal species are deposit feeders, where micelles play a crucial role
405 in the solubilization of nutrients and also prevent the loss of digestive enzymes due to
406 adsorption onto sediments (Mayer et al., 1997).
407
408 The total surfactant concentration much higher than the CMC might also be adaptive for those
409 insect and isopod species which feed on tannin-rich food and in which surfactants prevent the
410 precipitation of digestive enzymes and dietary proteins (Martin and Martin, 1984; Martin et
411 al., 1987; Zimmer, 1997). Moreover, tannin-rich food may cause the depletion of surfactants
412 and consequently lead to lipid deficiency (DeVeau and Schultz, 1992; Zimmer, 1997). In

insects and isopods, the CMC appears to be lower than in some other invertebrates, such as crustaceans *C. pagurus* (3.5 mM; Vonk, 1969) or polychaetes *Arenicola marina* (2 mM; Smoot et al., 2003); it is approximately 90 μM in *P. scaber* according to our estimates and it was reported to be 25 μM for one of the most common surfactants in insects, *N*-linolenoyl-glutamine (Spiteller and Boland, 2003). High total surfactant concentration to the CMC ratio, such as in *P. scaber*, therefore indicates that the number of micelles in the digestive juice is very high. A high degree of micellization could enhance the solubilization potential of *P. scaber* digestive juice towards hydrophobic compounds and metals in food, analogously to the digestive juices of marine deposit feeders, which were found to solubilize metals and polyaromatic hydrocarbons from marine sediments in large quantities, i.e. orders of magnitude more than seawater alone (Chen and Mayer, 1998; Mayer et al., 1996, 2001). The solubilization potential of digestive juices needs to be taken into account when the contributions of arthropod digestive processes to energy and matter cycles are being considered in both the pristine and polluted environments.

CONCLUSIONS

In the present study, we have demonstrated a successful application of titrations with the commercial surfactant ion-selective electrode (SISE) and of the pyrene fluorescence intensity method (PFM) for the determination of the total surfactant concentration and/or the CMC in the digestive juice of *P. scaber* without previous purification or separation of surface active components from the juice. Our results demonstrate that titrations with the commercial SISE and the PFM are simple, rapid, and low-cost techniques that can be used for the analysis of surfactants in digestive juices of arthropod species where the sample volume is limited (a few μL only like in *P. scaber*) and the surfactant concentration is low (the order of magnitude of a few mM). We have also shown that the SISE is not sensitive to traces of food remaining in the

digestive juice after depuration, while for the PFM the presence of food traces in the digestive juices needs to be as limited as possible for a successful determination of the CMC. Overall, both SISE and PFM have been shown as a good alternative to the commonly employed complex and laborious procedures consisting of separations, various chromatographic techniques, and/or inductively-coupled plasma mass spectrometry needed to measure the total surfactant concentration, as well as to the stalagmometric technique used for the measurement of the CMC, where a major drawback is the requirement of relatively large sample volumes. To the best of our knowledge, this is the first attempt to use commercial SISE and PFM for the assessment of the surfactant properties in low-volume samples of arthropod digestive juices without previous purifications or separations. It is also the first report on the total concentration of surfactants in *P. scaber* digestive juice, which was determined to be 9.2 ± 3.5 mM. The CMC was determined to be around 90 μM, which is in line with the previous reports that the ratio between the total surfactant concentration and the CMC in the *P. scaber* digestive juice is at least 80:1.

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