

# Lipid extractions from marine deep-sea invertebrates: modifications to the Bligh & Dyer method to increase lipid yields

SILVIA LINO, B. SOLÉ, V. DE MATOS, R. PEREIRA, J.R. XAVIER, R.S. SANTOS & A. COLAÇO



Lino, S., B. Solé, V. de Matos, R. Pereira, J.R. Xavier, R.S. Santos & A. Colaço 2013. Lipid extractions from marine deep-sea invertebrates: modifications to the Bligh & Dyer method to increase lipid yields. *Arquipelago. Life and Marine Sciences* 31: 9-14.

The deep-sea environment is difficult to sample, and often only small quantities of samples can be obtained when using less destructive methods than dredging. When working with marine animals that are difficult to sample and with limited quantities of tissue to extract lipids, it is essential to ensure that the used method extracts the maximum possible quantity of lipids. This study evaluates the efficiency of introducing modifications to the method originally described by Bligh & Dyer (1959). This lipid extraction method is broadly used with modifications, although these usually lack proper description and evaluation of increment in lipids. In this study we consider the improvement in terms of amount of lipids extracted by changing the method. Lipid content was determined by gravimetric measurements in eight invertebrates from the deep-sea, including deep-sea hydrothermal vents animals, using three different approaches. Results show increases of 14% to 30% in lipid contents obtained from hydrothermal vent invertebrate tissues and whole animals by placing the samples in methanol for 24 hours before applying the Bligh & Dyer mixture. Efficiency of the extractions using frozen and freeze-dried samples was also compared. For large sponges, the use of lyophilized materials resulted in increases of 3 to 7 times more lipids extracted when compared with extractions using frozen samples.

Key words: Bligh & Dyer extraction, deep-sea hydrothermal vent animals, large deep-sea sponges, marine lipids

*Silvia Lino (email: silvialino@uac.pt), Berta Solé, Valentina de Matos, Ricardo S. Santos, Ana Colaço, IMAR – University of Azores, LARSys – Associated Laboratory & Department of Oceanography and Fisheries (DOP) of the University of the Azores, PT-9901-862 Horta, Portugal; Raquel Pereira, DOP, University of the Azores, PT-9901-862 Horta, Portugal; Joana R. Xavier, CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Pólo dos Açores – Departamento de Biologia da Universidade dos Açores, PT-9501-801 Ponta Delgada, Portugal & CEAB, Centre d'Estudis Avançats de Blanes (CSIC), Camí d'accés a la Cala S. Francesc, 14, ES-17300 Blanes (Girona), Spain.*

## INTRODUCTION

Worldwide, only a few scientific institutions have the capacity to access the deep ocean and to sample the deep-sea hydrothermal vents in which extreme temperatures further complicate

sampling procedures (Allen & Jaspars 2009). Additionally, the available time for the use of submersibles and/or Remotely Operated Vehicle (ROVs) may be limited and collection of biological materials for studies at a given site often result in scarce sampling. When working with hydro-

thermal vent and deep-sea invertebrate samples that are hard to access and only result in small sizes and/or low quantities of tissues to work with, it is essential to obtain the maximum quantity of material without losing the quality of the compounds.

Marine lipids are objects of study all over the world, first for ecology since the variety of lipids is highly related with the characteristic living environments of these animals, and more recently with an increasing interest for biotechnology by exploring these molecules as source of biologically active products (eg. Ravichandran et al. 2001; Bergé & Barnathan 2005; Dembitsky 2006). Marine invertebrates have already proved to be potent sources of bioactive metabolites with some specific lipids occurring naturally in these animals. One example is the marine prostaglandins that have been isolated particularly from soft corals and gorgonians (Bhakuni & Rawat 2005). Here, we present improvements to previously described lipid extraction techniques in order to increase total lipid yields, in deep-sea invertebrates for further studies. The method described by Bligh and Dyer in 1959 is most commonly used in the extraction of lipids from several types of tissues from both plants and animals. It is a simple method, without major requirements in terms of equipment. Xiao et al. 2012 recently compared the efficiencies of 3 extraction methods, two Soxhlet and the Bligh & Dyer (B&D), in different matrices of fish and krill. It was shown that the Soxhlet method had lower efficiencies with particularly large differences found in samples with highest relative proportions of phospholipids and the B&D method had slightly higher extraction efficiency. Another study, by Iverson et al. (2001), showed that in samples containing more than 2% lipid, the B&D method produced significantly lower estimates of lipid content compared to other extraction techniques (such as Folch). This underestimation was seen to increase by up to 50% with lipid content of the sample. In animals like invertebrates, with high lipid content, these underestimations can be of special concern. Improvement of the method for invertebrates is, for that reason necessary in order to achieve better lipid yields. Since differences in the lipid yields are observed, even when slight modifications are made in the original method, describing

all modifications to the procedures and solvent/sample ratios is of highest importance (Iverson et al. 2001). White et al. (1979) made some modifications to the original method for the extraction of Sedimentary Microbial Biomass by introducing phosphate buffer to the methanol and chloroform mixture for the first step of the extraction and chloroform: water (50:50) for the second step. They also stated that volumes could be changed as long as the proportions of water: methanol: chloroform of 0.8:2:1 (V/V) for the single-phase extraction and of 0.9:1:1 (V/V) after separation into the second phase, were maintained. In the present study, we evaluated the lipid yields quantified by gravimetric measurements, with the introduction of an extra initial step using only methanol for the extraction of marine invertebrates, maintaining the ratios described by White et al. (1979). Smedes & Thomasen (1996) evaluated the B&D method and concluded that lipids have to dissolve in the aqueous phase first, before extraction to the organic phase. The kinetics of the extraction is then proportional to the solubility of the lipids in the aqueous phase. They also consider that higher methanol content will result in a consequently higher solubility in the aqueous phase. This is expected to result in a faster extraction and therefore a better yield. The introduction of an extra step-using methanol in our protocol is expected to prove this hypothesis. Application of a larger amount of methanol will result in an increase in methanol content in both phases (organic and aqueous). Therefore, the ratio between the solubility of co-extracts in both these phases will not change dramatically (Smedes & Thomasen 1996). Does the time embedded in methanol during the extraction, influence the yields? The purpose of our experimental study presented here, was to answer this question. Other modifications to the original method included the replacement of chloroform by the comparably less harmful dichloromethane for all samples. For sponge samples where we had larger quantity of tissue available, the same samples were extracted using both frozen material and freeze-dried material for comparison.

Comparisons in the quantity of total lipids extracted were made between:

1) Frozen and freeze-dried material of hard mega sponges: (using the same extraction modified B&D method based in White et al., 1979)

2) Dried samples of several deep-sea marine invertebrates:

- A) Extracted with B&D method (White et al. 1979)
- B) Pre treated with methanol for 24 hours followed by B&D method (White et al. 1979)
- C) Pre treated with methanol for 48 hours followed by B&D method (White et al. 1979).

## MATERIAL AND METHODS

### 1. SAMPLING AND PRESERVATION

A total of 136 specimens of marine invertebrates belonging to 8 species (3 hydrothermal and 5 from deep sea habitats), were collected between the summer of 2010 and the end of 2011 in the Azores archipelago (Northeast Atlantic Ocean). The hydrothermal vent animals (mussel, shrimp and polychaetes) were collected using the ROV Quest onboard R/V *Meteor* at Menez Gwen hydrothermal vent (ca. 836 m depth) and the ROV *Victor* 6000 onboard R/V *Porquoi Pas?* at Lucky Strike (ca. 1700 m). These specimens were frozen onboard using liquid nitrogen, stored at  $-80^{\circ}\text{C}$  during the missions, transported on dry ice from the ship and kept inland at  $-80^{\circ}\text{C}$  until being extracted. The mussels were subsampled according to type of tissue – gills, digestive glands and rest of muscle content, in a cold chamber onboard at  $10^{\circ}\text{C}$  to prevent chemical and biological alterations. Samples were frozen in pools of 10 individuals. The shrimps and polychaetes were also separated in the cold chamber and frozen in pools. The sponges and cold-water corals came from depths of 200 to 400 m and were collected accidentally (fisheries by-catch) during the annual longline fisheries monitoring campaigns conducted off the Azores islands onboard R/V *Arquipélago*. The samples were preserved immediately at  $-20^{\circ}\text{C}$  and, after being transported in ice to the laboratory on land, the associated macro fauna was removed by hand and the samples were cut into smaller portions (all procedures were performed in a cold chamber at  $10^{\circ}\text{C}$ ). The sponges were also washed in filtered seawater to remove the debris and sub-samples were taken for

the extractions from frozen material (to be compared with extractions done using frozen-lyophilized material). The samples were lyophilized frozen using a refrigerated vaportrap (*RVT 400*) with a *Valupump (VL1120)*, both from *Savan*, which took from 48 to 72 hours to dry completely (depending on the amount of material). Afterwards, the samples were reduced to powder using either a mullite mortar (for small quantities of tissue) or a mill *Masterchef* from *Fagor*. All materials used were cleaned with acetone to avoid cross contaminations.

### 2. TOTAL LIPID EXTRACTIONS & QUANTIFICATION

The B&D method used was based on the described by White et al. (1979). For extractions of frozen material we used frozen sponges previously cut in small pieces: for each 100 g of frozen sponge (shown to have at least 55% of water content), we added 150 ml of methanol and 75 ml of dichloromethane, to respect the proportion methanol: dichloromethane: water of 2:1:0.8 (v/v/v). The mixture was left to extract during two hours with a magnet in an *IKA R10 Power* shaker. After this time the mixture was transferred to a decantation ampoule, 60 ml of dichloromethane and 60 ml of distilled water were added to have a biphasic mixture with a final proportion of the three compounds of 2:1:0.9 (v/v). The mixture was then left to decant during 18 hours, after which, the organic phase was recovered and filtered onto a *Whatman 2V* filter from *Merck*, to a glass cylinder (pre-weighed). The organic volume was reduced by evaporation in a bath at  $37^{\circ}\text{C}$  (*Buchi B-490*) using a *Buchi* rotavapor *R-200* with a *Vac V500* pump and the extracted lipids quantified by weight in a precision balance *Mettler Toledo AB204 -S/FACT*.

For lyophilized sample extractions we added (for each 10 g of dried powder): 80 ml of methanol, 40 ml of dichloromethane and 32 ml of phosphate buffer ( $\text{K}_2\text{HPO}_4$ ,  $\text{H}_2\text{O}$  50mM neutralized with HCl 1N until  $\text{pH}=7,4$ ) to a final mixture of 2:1:0,8 (v/v/v). The remaining protocol was as described previously, with the exception of the volume of dichloromethane and distilled water added: 40 ml each to maintain the final proportion in the mixture of 2:1:0.9.

### 3. INTRODUCED MODIFICATIONS

For each 10 g of powder-lyophilized material, 80 ml of methanol was added and left to extract in an orbital shaker (*Heidolph Promax 2020*) at 120 rpm (position 4) for *i*) 24 hours and *ii*) 48 hours. After that time, 40 ml of dichloromethane and 32 ml of phosphate buffer was added and the above described extraction protocol was followed. In order to test if there were significant differences in the lipid yields between samples pre-treated with methanol or not, the amount of extracted lipids was grouped together per method and pairwise Mann-Whitney comparisons were conducted.

## RESULTS AND DISCUSSION

Total lipids extractions were made for 136 specimens of 8 species of marine invertebrates: the mussel *Bathymodiolus azoricus*, the commensal

polychaete *Branchiopolynoe seepensis*, the shrimp *Mirocaris fortunata*, the sponges *Neophrissospongia nolitangere*, *Petrosia* sp., *Leiodermatium* cf. *pfeifferae* and *Axinella vasonuda* and the coral *Callogorgia verticillata*. Of these specimens, the mussel *B. azoricus*, the scale worm *B. seepensis* and the shrimp *M. fortunata* are restricted to hydrothermal vent communities. The sponges *N. nolitangere*, *Petrosia* sp., *L. cf. pfeifferae*, *A. vasonuda* and the gorgonian coral *C. verticillata* are common components of deep-sea cold water communities in the Azores region. To calculate the % of lipids for the sponges and corals we used the values for mg lipid/g of dry mass for the method that presented the higher value as we considering it is extracting in total the lipids present in the sample. For the smaller samples, for which we cannot disregard the error associated with weighing such small amounts, we considered the minimum and the maximum values obtained in all methods. These percentages are presented in Table 1.

Table 1. Type of sample and species of marine deep-sea invertebrate (136 specimens), water and lipid content and quantity of lipids extracted using the three methods tested. A) Modified Bligh & Dyer; B) 24 hours in Methanol plus Bligh & Dyer; C) 48 hours in Methanol plus Bligh & Dyer. \*Considering the higher lipid yield for calculations.

Type of sample <i>Specie</i>	Dry weight (g)	Percentages (w/w)		Lipids as mg g <sup>-1</sup> of dry mass			Increment in Lipids
		Lipids*	Water	A	B	C	
Hard sponge <i>Neophrissospongia nolitangere</i>	2634	0.43*	55.66	1.59 ±0.43	4.19 ±0.14	3.88 ±0.11	60%
Hard sponge <i>Petrosia</i> sp. ind.1	1401	2.02*	56.90	13.03	14.06 ±0.90	15.29 ±6.9	15%
Hard sponge <i>Petrosia</i> sp. ind.2	375	0.87*	56.86	6.57	8.09 ±0.84	7.81 ±0.91	19%
Hard sponge <i>Leiodermatium</i> cf. <i>pfeifferae</i>	668	0.59*	55.90	3.17 ±0.40	5.85 ±0.099	3.65 ±0.56	46%
Soft sponge <i>Axinella vasonuda</i>	13	2.03*	88.30	20.30	-	16.06	-
Hard coral <i>C. verticillata</i>	8	1.86*	24.85	18.63	10.12	11.03 ±4.9	-
Hydrothermal inv. tissue: Gills of <i>B. azoricus</i>	14.3	3.9-9.9	-	76.34 ±1.50	91.31 ±13.41	61.19 ±21.61	16%
Hydrothermal inv. tissue: dig. glands of <i>B. azoricus</i>	5.4	7.8-17.2	-	97.02 ±17.16	126.69 ±33.53	79.60 ±7.04	23%
Hydrothermal inv. tissue: muscle of <i>B. azoricus</i>	26.4	4.7-9.7	-	72.20 ±8.00	83.63 ±1.56	69.07 ±25.51	14%
Hydrothermal inv.: Polychaete <i>B. seepensis</i>	2.0	12.8-15.4	-	128.85	153.85	47.12	16%
Hydrothermal inv.: Shrimp <i>M. fortunata</i>	3.1	6.3-9.6	-	67.27	96.23	83.05	30%

Although lipids from hydrothermal vent animals are object of many studies from all over the world due to their great importance for both ecology and biotechnology, the small size of these animals and the difficulty in obtaining large amounts of samples has become a challenge. In a total of 61 extractions using the three versions of the Bligh & Dyer modifications (Table 1) we showed that it is possible to get higher yields of lipids from both small and big size invertebrates by leaving these samples 24 hours in methanol. In the shrimp *Mirocaris fortunata*, which had on average 31 mg of dry weight, it was possible to extract 30% more lipids and in the several tissues of the mussel *Bathymodiolus azoricus* we extracted between 14% to 23% more lipids in the muscle and digestive gland respectively. Studying lipids from tissues of small animals like the vent mussel (gills are on average about 30% of the animal's weight and digestive glands about 11% of the animal's weight) is even a great challenge due to the reduced amount sample, hence to increase the lipids extracted 10 to 20% can be analytical important for the following studies.

Results also show that we were able to increase substantially the lipid yields for all the large sponges tested, by 15% for *Petrosia* sp. and 60% for the *Neophrissospongia nolitangere* by soaking them in methanol previously to the B&D extraction. The only sample for which the yields were higher using 48 hours in methanol instead of 24 hours was the *Neophrissospongia nolitangere*, the largest animal extracted in this study (2.6 kg) and with lower percentage of lipids (0.43%). Of *Petrosia* sp. we had two specimens of the same genus that were analyzed as individual 1 and individual 2 since the size varied significantly. The quantities of lipids were relatively different: 1.66% in specimen 1 and 0.69% in specimen 2. This is most probably related to the differences in size, since individual 1 was almost 4 times larger than individual 2.

Considering the 8 species of invertebrates tested, the only species where there was not an increase of lipids extracted by soaking them previously in methanol, were *Axinella vasonuda* and *Callogorgia verticillata*. Both species showed a decrease in extracted lipid quantities of about 20% using the modified protocols. The fact that

these were the only soft sponge and gorgonian coral tested seems relevant.

When comparing all extracted samples, the B & D extractions after storage of 48 hours in methanol ( $p=0.95$ ) yielded highest lipid concentrations, whilst only pre-treatment with methanol for 24 hours, gave a significantly lower amount of lipids ( $p<0.05$ ). Taking into account the overall results, we recommend adding the initial step of soaking the samples in methanol for 24 hours for lipid extraction of small invertebrates as molluscs, crustaceans and tissues of these animals and especially for hard sponges. In case of soft sponges and corals we advise to try other possible modifications to the B&D protocol to get better yields.

To compare extraction efficiencies of dry powders and frozen samples, the results for three large marine deep-sea sponges were analyzed (sample type and characteristics are described previously in Table 1). All large deep-sea sponge species used in this study had about the same percentage of water content (ca. 55%) and low lipid content (0.4 to 2.02%). However, the increase in the quantity of lipids extracted using lyophilized material was 3 times higher for *Neophrissospongia nolitangere* and 7 times higher for *Petrosia* sp. (Table 2). This shows that although the process of freeze-drying takes more time in the process of extraction, it has a greater ability to concentrate lipid and should be an option to consider in case of working with small quantities of starting material.

#### ACKNOWLEDGEMENTS

The authors would like to thank the chief scientists and the crew members at the scientific cruises ARQDAÇO-32-P10, MENEZMAR, ARQDAÇO-35-P11, CONDOR(1)2011 and MOMARSAT2011 which permitted the sample collection for this work and specially the ROV crew from *Quest 4000* and *Victor 6000* for the sampling at the hydrothermal vent sites. We are grateful to colleagues from Department of Oceanography and Fisheries (DOP), University of the Azores, for the taxonomic identifications and other valuable information for all deep-sea

specimens used in this study. We also thank the reviewer for helpful comments and improvement of the paper. The research was funded by CHE-MECO (EURODEEP/0001/2007) and DEEPFUN (FCT/ PTDC/MAR/111749/2009) projects and it was only possible with the cooperation of projects: CORALFISH, CONDOR and ARQDAÇO. This research was also supported by the funds provided by FCT to LARSyS Associated Laboratory & IMAR-University of the Azores / the Thematic Area E of the Strategic Project (OE & Compete) and by the DRCTC – Government of the Azores pluriannual funding. Silvia Lino and Joana R. Xavier were supported by PhD and postdoctoral grants from Fundação para a Ciência e Tecnologia, (FCT - Ministry of Science and Technology of Portugal), with references SFRH/BD/72154/2010 and SFRH/BPD/62946/2009 respectively.

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Received 22 Jul 2013. Accepted 21 Nov 2013,  
Published online 31 December 2013.