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DETERMINING BLUBBER FATTY ACID COMPOSITION: A COMPARISON OF *IN SITU* DIRECT AND TRADITIONAL METHODS

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ABSTRACT

Fatty acids (FAs) are used to make inferences about the foraging behavior and diets of free-ranging marine mammals. However, several methods are currently available for determining the FA composition of blubber and these methods may produce different results. We compared *in situ* direct transesterification methods, where a small amount of tissue is sampled, with more traditional methods involving prior lipid extraction of the entire sample of interest. Using gray seal (*Halichoerus grypus*) and beluga whale (*Delphinapterus leucas*) blubber, we found that when the direct *in situ* method was used on a 2-mg sample of blubber, the resulting FA profile differed significantly from that produced when traditional full-extraction methods were employed. Regardless of where the small spot sample was taken within the blubber depth, it was not representative of the entire blubber FA composition, as blubber is non-homogeneous throughout its depth. We also modified the *in situ* direct method to allow analysis of the entire blubber layer. Results of this full-layer direct method compared quite favorably with traditional extraction methods and may provide a reasonable alternative for analyses. Although application of our full-layer direct method will require further verification in certain marine mammal blubber samples, we conclude that the large differences obtained when using the direct method are not a consequence of the chemical method itself. Rather, they arise from non-representative sampling of the blubber FA composition.

Key words: transesterification, methanolysis, blubber, fatty acid, direct, *in situ*, marine mammal.

Fatty acids (FAs) in blubber are used to make inferences about the diet and foraging ecology of marine mammals based on the knowledge that FA patterns in prey influence the lipid stores of their predators (*e.g.*, Klem 1935; Ackman and Eaton 1966; Hooper *et al.* 1973; Colby *et al.* 1993; Iverson 1993; Käkelä *et al.*

1993; Iverson *et al.* 1995; Smith *et al.* 1996; Iverson *et al.* 1997*a*, *b*; Kirsch *et al.* 2000). A blubber biopsy (100–500 mg) from a free-ranging animal can provide accurate and relatively non-invasive information about diet that is not dependent on the recovery of prey with hard parts, nor limited to only the last meal (Iverson *et al.*, in press). However, the manner in which FAs in blubber are analyzed can have a significant impact on the interpretation of FA composition. We compared two methods used in the analysis of marine mammal blubber in order to investigate potential sources of discrepancies and make recommendations for appropriate analyses.

Fatty acids must be converted to FA methyl esters (FAME) prior to analysis by the most common method, gas liquid chromatography (GLC). Traditional methods involve the initial extraction of all lipids from a tissue sample, followed by acidic (or basic) transesterification (*e.g.*, Christie 1982). Direct, or *in situ* transesterification, is an alternative method in which FAME are formed directly from tissue without prior isolation of lipids (Abel *et al.* 1963). This method has a number of advantages over traditional methods that require initial extraction of lipids. Most notably, the *in situ* method reduces costs by reducing the amount of time and reagents required for lipid extraction. Consequently, this method has received increasing attention (see Liu 1994, Carrapiso and García 2000 for reviews) and recently, direct transesterification has been used to determine the FA composition of marine mammal blubber using very small samples of tissue taken near the skin (Grahl-Nielsen and Mjaavatten 1991, Grahl-Nielsen *et al.* 2000).

Results using *in situ* and traditional methods on homogenous samples have compared favorably (Carrapiso and García 2000). Direct and conventional methods have produced nearly identical FA profiles from homogeneous samples of baby formula (Cantellops *et al.* 1999), fish tissues (Park and Goins 1994, Ulberth and Henninger 1995, Guillou *et al.* 1996), human blood, adipose tissue, milk, and feces (Lepage and Roy 1984, 1986), bovine muscle and liver (Park and Goins 1994), and various marine organisms (Whyte 1988). However, the suitability of the *in situ* method to determine the FA composition of blubber has not been adequately evaluated. Blubber is not homogeneous through its depth and stratification can be pronounced (Iverson 2002). In pinnipeds, while there is significant turnover and deposition of dietary FAs throughout the entire blubber layer, the inner layer responds more rapidly to recent diet (*e.g.*, Best *et al.* 2003; Iverson *et al.*, in press). Conversely, in many cetaceans, the inner layer appears to be the most metabolically active and where dietary FAs are readily stored (*e.g.*, Koopman *et al.* 1996, Koopman 2001). Therefore, our objective was to determine whether this efficient and cost-effective method is appropriate for analysis of marine mammal blubber. Furthermore, considering the small amounts of tissue used in some recent studies, we examined the feasibility of such "micro-sampling" as a means of determining the representative FA composition of the entire blubber layer or of the blubber layer most representative of diet.

METHODS

We compared four methods of determining the FA composition of blubber (Table 1). The first two methods ("BF_3" and "Hilditch") involve first isolating all lipids from the blubber prior to transesterification. We refer to these as the traditional extraction methods. The second two methods involve a single-step,

Table 1. Summary of four methods for determining fatty acid (FA) composition of marine mammal blubber[a].

Method	Traditional extraction or *in situ*[b]	Approx. tissue mass (g)	Description	Approx. solvent amounts[c]	References
BF_3	Extraction	0.2–0.5	Lipid extracted from full-depth or inner blubber biopsy (*i.e.*, muscle to skin or muscle to mid-depth, Fig. 1a, b, respectively); FAME subsequently prepared using BF_3 in methanol, heated at 100°C for 1 h.	14 ml (e) 6 ml (m)	*e.g.*, Morrison and Smith 1964; Iverson *et al.* 1997*a*, *b*; Ackman 1998.
Hilditch	Extraction	0.2–0.5	Lipid again first extracted from full-depth or inner blubber biopsy (Fig. 1a, b); FAME subsequently prepared using H_2SO_4 in methanol at room temperature for 3 d.	14 ml (e) 6 ml (m)	Hilditch and Williams 1964; Iverson 1988; Iverson *et al.* 1997*a*, *b*.
Micro-Direct	*In situ*	0.002	A 2-mg sample of blubber tissue is removed 0.5–1.0 cm beneath skin (Fig. 1c) and subjected to single-step methylation using 0.8 ml HCL in methanol, heated at 110°C for 2 h.	5 ml (m)	Viga and Grahl-Nielsen 1990, Grahl-Nielsen and Mjaavatten 1991, Grahl-Nielsen *et al.* 2000.
Full-Direct	*In situ*	0.2–0.5	Modification of Micro-Direct method (above): entire full-depth blubber biopsy (Fig. 1a) is subjected to single-step methylation using 8 ml HCL in methanol as above.	12 ml (m)	This study.

[a] The first three methods listed have been used in published studies; the fourth is a modified approach.
[b] Extraction: lipid is quantitatively extracted from the entire sample and an aliquot of the homogenate is subjected to methylation; *In situ*: fatty acids are directly methylated from the tissue sampled without prior extraction.
[c] (e): total solvents used in extraction and isolation of lipid; (m): total solvents used in methylation and isolation of FAME.

direct transesterification of blubber lipids *in situ.* The first three methods (BF_3, Hilditch and "Micro-Direct") have been used previously in marine mammal studies, while the fourth ("Full-Direct") is a modification of the Micro-Direct approach; it is identical to the Micro-Direct method except that the volume of HCl-methanol is increased ten-fold to accommodate the larger amount of tissue (approximately 100×) contained in a full-depth biopsy (Table 1). Attempting to increase the solvent volume to match the increase in tissue size (*i.e.*, using 80 ml of HCl in methanol) would make the Full-Direct method impractical. With our modification, amounts of reagents remain far in excess of that required to ensure the reaction goes to completion and our results demonstrate that this modification achieves a transesterification efficiency equal to or greater than Micro-Direct.

Gray Seal Biopsies

We collected full-depth blubber biopsies (Fig. 1a) from 31 wild-caught gray seals (*Halichoerus grypus*) on Sable Island, Nova Scotia, in September 2001, using a 6-mm biopsy punch (see Kirsch *et al.* 2000). We then compared the FA signatures obtained from these samples using traditional extraction (*i.e.*, BF_3 and Hilditch) and Micro-Direct methods. For Micro-Direct analysis, a 0.0023 ± 0.00040 g sample was taken from the biopsy sample 1 cm below the skin (Fig. 1c) following the methods of Grahl-Nielsen and Mjaavatten (1991) and Grahl-Nielsen *et al.* (2000). For BF_3 and Hilditch analyses, lipid was extracted from the entire biopsy (0.34 ± 0.070 g) after the skin and muscle had been removed and FAME were subsequently prepared. Although 31 gray seal samples were collected, only 18 samples yielded accurate analyses using the Micro-Direct method due to the small and highly variable amount of lipid obtained.

Beluga Whale

We collected three samples from each of two beluga whales (*Delphinapterus leucas*) killed by native hunters. Cetacean blubber is generally more highly stratified, with the inner layer reflecting diet and the outer layer serving more structural functions (*e.g.*, Koopman *et al.* 1996, Koopman 2001). Thus, our aim was to examine the conclusions that would be drawn about blubber FA composition by analyzing a representative sample of the inner layer using traditional methods *versus* using the Micro-Direct method on a sample near the skin. A full-depth blubber core was taken from skin to muscle at three sites on each animal. From each core, the inner-half of the layer (*i.e.*, from muscle to mid-depth, Fig. 1b; 0.57 ± 0.050 g) was analyzed using the two traditional extraction methods (*i.e.*, BF_3 and Hilditch) and these results were compared with those of the Micro-Direct method on a sample (0.0023 ± 0.00047 g) taken 1 cm below the skin, as described above (Fig. 1c).

Full-Direct vs. *Traditional Methods*

Comparisons between the traditional extraction methods and the Micro-Direct method are confounded by both tissue sample size and location, with the former methods analyzing all FAs extracted from an entire tissue depth or layer and the latter analyzing only those FAs contained in a small spot at a specific site. Thus, to remove these potential influences and compare the chemical methods themselves,

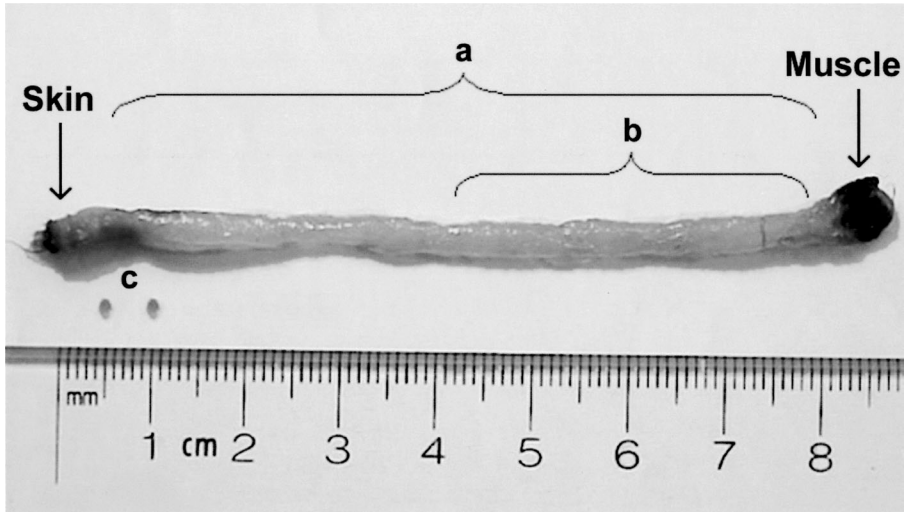

Figure 1. Illustration of a typical blubber biopsy from a gray seal and the samples used in FA analyses. (a) Full-depth blubber biopsy (~0.77 g) with skin and muscle still attached, all lipids extracted. (b) Inner layer of blubber (muscle to mid-depth), all lipids extracted. (c) Micro-Direct samples (0.0022 g each) shown relative to their location taken in previously published studies, *i.e.*, 0.5 cm or 1.0 cm beneath the skin (Grahl-Nielsen and Mjaavatten 1991, Grahl-Nielsen *et al.* 2000) and used in the present study.

we employed a modification of the Micro-Direct method, which would allow analysis of the full blubber layer. For this "Full-Direct" method (Table 1), we modified the Micro-Direct method to accommodate a larger tissue sample: *i.e.*, one that would represent the full blubber layer of interest in the case of pinnipeds. A large full-depth square of blubber (approximately 7 cm × 7 cm × 4.5 cm deep) was taken from a single adult female gray seal shortly after observed death on Sable Island in September 2001. We then used biopsy punches (6 mm in diameter) to take 36 full-depth cores from this block, mimicking normal field biopsies. We randomly assigned each of the 36 cores to one of three methods (*i.e.,* $n = 12$ each) for FA analysis: BF_3, Hilditch, and Full-Direct (see Table 1).

Effects of in situ *Sample Location*

Although samples taken near the skin and analyzed with the Micro-Direct method may not yield comparable results to traditional methods, it is possible that if samples were taken at greater blubber depths, the Micro-Direct method may be appropriate. Thus, we examined the effects of sample location within the blubber layer by comparing Micro-Direct samples taken at a point spot from the inner, middle, and outer portions of the blubber (see Fig. 1). We used the same piece of blubber from the gray seal described above and randomly assigned an additional 36 blubber cores to one of three location treatments (*i.e.*, $n = 12$ for each location). For each blubber core, a subsample (0.0024 ± 0.00055 g) was taken from 0.5 cm below the skin ("Outer"), 0.5 cm above the muscle ("Inner"), or precisely in the middle of the layer and analyzed using the Micro-Direct method. The remainder of the core was discarded.

Sample Analysis

Duplicate analyses of FAME from all methods were performed using temperature-programmed GLC according to Iverson *et al.* (1992; 1997*a*, *b*). Although the 17 overall most abundant FAs are illustrated, for all statistical analyses only the $n - 1$ most abundant FAs were used (*i.e.*, sample size, n, minus 1), and their values were normalized using a log transformation prior to comparison using MANOVA. The values for each FA were transformed by calculating the log of the ratio of each FA to 21:5n-3. In the beluga whale study, small sample size precluded statistical analysis.

Results

Gray Seal Biopsies

The FA analysis method had a significant effect on the FA profiles of gray seal biopsies (Fig. 2a; Wilks' $\Lambda = 0.041$, $P < 0.001$). BF_3 and Hilditch produced identical results, and both differed significantly from the Micro-Direct method ($P < 0.01$) for 14 of the 17 FAs. The Micro-Direct method underestimated the saturated FAs (*e.g.*, 14:0, 16:0, and 18:0) at less than half the levels found using traditional extraction methods. Micro-Direct also greatly underestimated long-chain monounsaturated FAs, such as dietary 20:1n-9 and 22:1n-11, and overestimated shorter- and medium-chain monounsaturates.

Beluga Whale

The differences between the Micro-Direct and traditional methods were pronounced. Although small sample size precluded rigorous statistical analysis of the beluga samples, Figure 2b illustrates that, again, BF_3 and Hilditch produced identical results across all FAs while the Micro-Direct method differed substantially. The Micro-Direct method yielded higher levels of shorter- and medium-chain monounsaturates than did the traditional extraction methods. It also underestimated the saturated FAs and the longer-chain unsaturated and dietary FAs (*e.g.*, 20:1n-9, 22:1n-11, 22:5n-3, 22:6n-3).

Full-Direct vs. *Traditional Methods*

When the Micro-Direct method was modified to allow analysis of the entire blubber layer, the results compared favorably with the traditional extraction methods (Fig. 3a). Although there were significant differences found among the three full-layer methods overall (11 most abundant FAs, Wilks' $\Lambda = 0.039$, $P < 0.001$), there were no significant differences among the three treatments for 5 of the 11 FAs. Furthermore, no method differed consistently from another in the remaining FAs, and the magnitude of differences was generally small. Thus, these differences are unlikely to be biologically relevant.

Effects of in situ *Sample Location*

Finally, we compared the effect of applying the Micro-Direct method to three locations in the blubber layer. The location of the sample taken within the blubber layer had a significant effect on the FA signature (Wilks' $\Lambda = 0.036$, $P < 0.001$) with

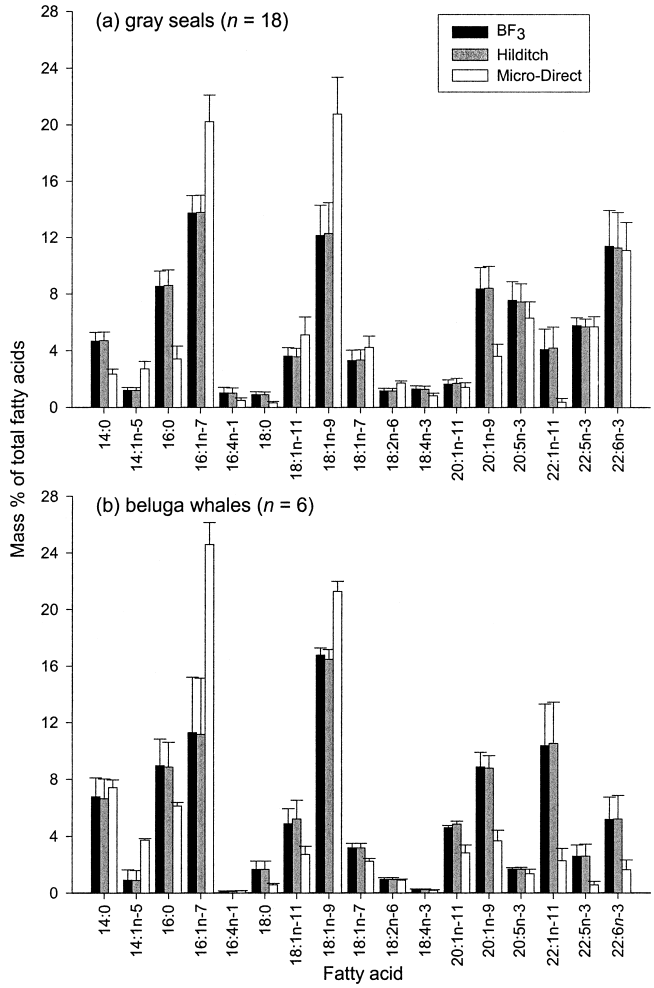

Figure 2. Comparison of traditional and Micro-Direct analyses of blubber samples collected from (a) wild-caught gray seals ($n = 18$) and (b) beluga whales ($n = 6$), using 17 most-abundant FAs found in the gray seal samples (mean mass % $\pm$ SD). Micro-Direct samples were taken 1 cm beneath the skin, while BF_3 and Hilditch methods were performed on lipid extracted from the entire depth of the blubber layer for gray seals and the inner half of the blubber layer for belugas (see Fig. 1 and text). For gray seals, Micro-Direct differed significantly from traditional extraction methods (MANOVA, Wilks' $\Lambda = 0.041$, $P < 0.001$).

all 11 FAs tested differing across location (Fig. 3b). Although the greatest differences were observed between the outer samples and the other two areas, there were also large significant differences between inner and middle samples. Additionally, none of the results from the individual spots were entirely representative of the FA composition of the full blubber layer (Fig. 3a). Compared to the BF_3 results for the full blubber layer (Fig. 3a), the outer-direct samples differed ($P < 0.02$) for all 11 FAs, the inner-direct samples differed for six FAs, and the middle-direct

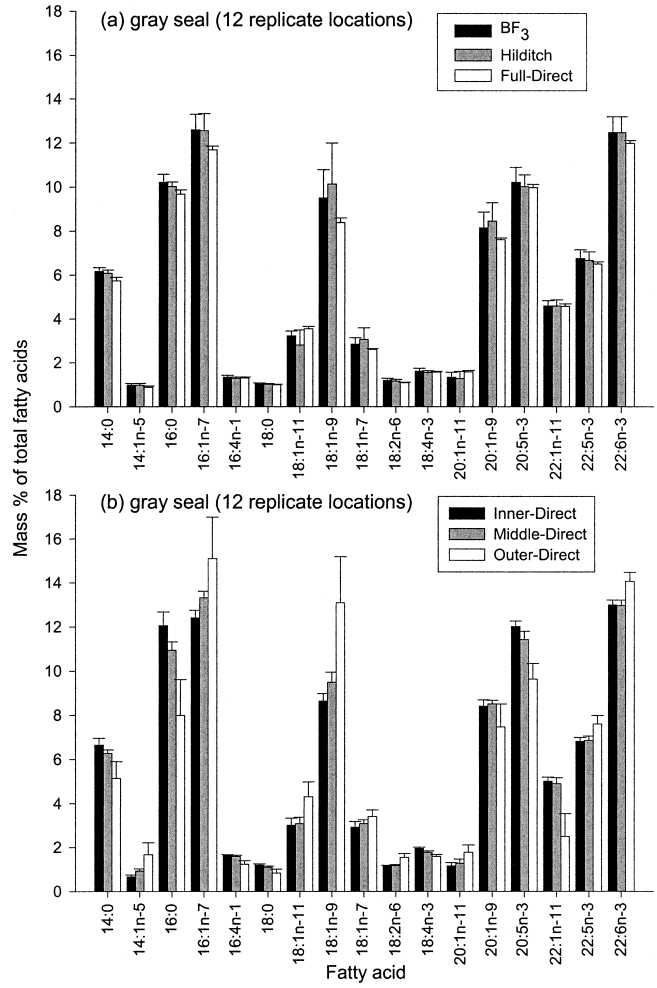

Figure 3. (a) Comparison of Full-Direct and traditional methods performed on samples collected from a single adult gray seal ($n = 12$ replicate samples each). While MANOVA on the 11 (*i.e.*, $n - 1$) most-abundant FAs indicates a significant method effect ($P < 0.001$), the three methods compare favorably for most of the FAs examined. (b) Comparison of Micro-Direct analyses of samples (2 mg) collected from the Inner, Middle, and Outer regions of gray seal blubber ($n = 12$ replicate samples each). Significant effects were found for all 11 most-abundant FAs (MANOVA, $P < 0.001$).

samples differed for two FAs. Compared to Full-Direct results (Fig. 3a), the outer samples differed for 10 FAs, the inner samples differed for four FAs, and the middle samples differed for three FAs.

Discussion

Results from this study demonstrate that a Micro-Direct sample taken near the skin does not reflect the true blubber FA store of either gray seals or beluga whales

(Fig. 2). Although pinniped blubber is generally less stratified than that of cetaceans, the differences found here were pronounced for both species. We conclude that the Micro-Direct method does not yield comparable results to traditional extraction methods in the analysis of marine mammal blubber, regardless of where the sample is taken within the blubber core. Given that blubber is not a homogeneous tissue, a single small subsample, even if taken deeper within the tissue, will not be representative of the larger blubber layer (Fig. 3b).

To use the FA composition of blubber to make inferences about diet, it is critical to sample and accurately characterize the part of the fat depot that is deposited from diet. In phocids, although the inner layer responds more rapidly to a shift in diet, even the FA composition of the outer layer will reflect dietary changes within 10 d (M. H. Cooper and S. J. Iverson, unpublished data). In fact, in captive gray seals, radioactively-labeled FAs are incorporated in the outer layer within 12 h of feeding (M. H. Cooper, S. M. Budge and S. J. Iverson, unpublished data). Therefore, either full-depth or inner-half blubber FA composition must be accurately analyzed, but with different aims. The diets of both gray and harbor seals (*Phoca vitulina*), integrated over several months, have been accurately determined by quantitative FA signature analysis (QFASA) using the FA composition of full-depth blubber samples (Iverson *et al.*, in press). However, recent evidence also indicates that the FA composition of the inner-half of the blubber layer provides a shorter-term estimate of diet (*i.e.*, weeks, M. H. Cooper, S. J. Iverson and W. D. Bowen, unpublished data). In contrast to pinnipeds, for many cetaceans only the inner half of the blubber layer may be appropriate for diet inferences (*e.g.*, Koopman *et al.* 1996, Koopman 2001).

Clearly, the location and size of a blubber sample must be carefully considered in any analysis of FA composition. Unfortunately, when taking a small subsample from a non-homogeneous tissue there is no way to resolve the effects of sample size and location. However, it is clear that the dramatically different results produced by the Micro-Direct methods are not a consequence of the chemical method itself, as the Full-Direct chemical method compared quite well with traditional methods. When a representative subsample of FA is available for analysis, both *in situ* and traditional approaches provide comparable results. In contrast to the Micro-Direct method, the Full-Direct method appears to offer a reliable means of reducing some of the analysis time and costs that are associated with traditional extraction methods. The Full-Direct method may also provide an effective alternative in the FA analysis of small, freeze-dried, individual prey (*e.g.*, copepods, amphipods) of some marine mammals. Nevertheless, investigators should be aware that the Full-Direct method does not allow the direct measurement of fat content that is necessary when using QFASA (Iverson *et al.*, in press). It is possible to estimate fat content with this method if the assumption is made that triacylglycerols are the only lipid class present (Carpenter *et al.* 1993). However, this is never true in marine mammal prey, and fat content estimated in this way will not be as accurate as direct measurement.

Although our tests of the Full-Direct method yielded encouraging results, we suggest that further evaluation is needed to determine whether the Full-Direct method will perform well on the blubber of other marine mammal species. For instance, water in the blubber may interfere with *in situ* transesterification. In the presence of water triacylglycerols precipitate and react more slowly, especially long-chain saturated FAs (see Carrapiso and García 2000 and references therein). Our Full-Direct *versus* traditional results showed no differences in the levels of saturated

FAs, and Lepage and Roy (1984) found that water levels below 10% had no effect on direct transesterification. Thus, water may not have been a factor in our samples. However, since the water content of pinniped blubber can increase from 5.5% to 17% during annual fasting cycles such as during molting and lactation (Bowen *et al.* 1992), direct transesterification may not be appropriate for blubber samples taken at certain times of year.

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