Flatbed Scanning as a Novel Approach for Examining Lipid Distribution in Fish: Comparison with MRI and Traditional Chemical Methods

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ABSTRACT

Depending upon species, the concentration and distribution of lipid in fish flesh impacts both processing requirements and eating quality. Dispersal of lipid within fish muscle may be manipulated by diet, feeding strategy, and through selective breeding. Several methods are currently used to examine lipid deposition in fish, but these are either arduous, costly, or reliant upon noxious chemicals. The need exists for a rapid, inexpensive, and safe method for examining lipid distribution in fish flesh. A technique that satisfies the preceding criteria was developed. Fish cutlets were stained with oil red O, scanned, and the images saved in 600 dpi *.tiff format. Oil red O was employed to differentiate muscle tissue from lipid. Cutlets were examined using computer-assisted image analysis and lipid presence in each cutlet recorded in percent terms. The results were compared to data generated from the same cutlets using Magnetic Resonance Imaging to separate muscle from lipid. No differences were detected between methods with regards to lipid distribution, which followed an anterior to posterior decline in the body. Lipid dispersal did not differ with gender. Estimates of total lipid in scanned images were identical to those recorded using chemical analysis.

INTRODUCTION

In fish, the spatial distribution of body fat, and in particular its dispersal through the muscle, is important in determining flesh texture, juiciness, taste, and odor (Johansson 2001). The ability to monitor and control lipid accumulation in aquacultured animals has become increasingly important due to the emergence of a progressively sophisticated and health-conscious consumer. Traditionally, techniques for determining the impact of genetic selection and husbandry manipulations upon quality traits, such as muscle lipid accumulation, include dissection and quantitative chemical analyses in combination with multiple samplings at designated spatial locations. These methods are frequently arduous and costly (Rye 1991), and also require the use of hazardous chemicals. Several alternative techniques have been examined in an effort to reduce dependence upon traditional methods, and to speed the prediction of carcass composition. Near Infrared Reflectance Analysis (NIRA) has been used in the inspection of fish carcass composition (Valdes *et al.* 1989) but is limited in application because of the need to grind samples. Gjerde (1987) examined the utility of Computerized Tomography (CT) with some

success. CT has since been employed in various studies to examine cutlet lipid and protein deposition in salmon (Rye 1991; Bjerkeng *et al.* 1997; Einen *et al.* 1998).

Other biomedical imaging techniques are also available that permit the non-invasive examination of the internal structures of animals. In this regard, Magnetic Resonance Imaging (MRI), in particular, has proven exceptional due to its precision, resolving power, and general availability. Although costly, MRI has been employed to examine various aspects of seafood end-product quality. These include studies upon trimethylamine oxide, trimethylamine and dimethylamine presence (Howell *et al.* 1996), and the impact of freeze-thawing on product deterioration (Nott *et al.* 1999a,b). MRI has also been exploited to examine liver lipid content in burbot (*Lota lota*) (Alanen *et al.* 1991; Komu and Alanen 1994). An obvious extension of such investigations would be to evaluate the usefulness of MRI to map lipid deposition processes in fish flesh.

Alternatives to costly medical imaging technologies and time-consuming and hazardous chemical analyses are nevertheless still needed to enable rapid on-site evaluation of the impact of breeding programs and/or dietary treatments upon carcass characteristics. Rønsholdt *et al.* (2000) employed flatbed scanning techniques and computer-assisted image analysis to examine carcass composition of farmed trout. These studies, however, involved manual image processing of cutlets that may have induced bias during analysis. Removal of partiality can be achieved using automated image processing. A requirement for automatic image evaluation of predominantly white-fleshed fish is a high contrast between parameters of interest, for example, lipid and muscle tissues. One method of differentiating between these tissue types is to use biological stains. In general, most histochemical techniques have been developed for microscopic analyses or for the study of macroparasites. Staining methods suitable for gross examination of whole body samples however, are sparse.

A number of lipid-soluble dyes are used for histochemical detection of fat with oil red O (ORO) being particularly suitable, as it reveals lipids with an orange-red tint (Lillie 1944a,b). An ability to highlight lipid-based fractions of, for example, a fish cutlet or steak permits automated image processing to the pixel level and hence eliminates analytical bias. The objective of the present study was two-fold. Firstly, to develop an economical, safe, and easy-to-use technique, with a capacity for on-site use, that would enable examination of gross distribution of lipid in fish;

and, secondly, to compare the method developed against traditional chemical analysis and MRI.

MATERIALS AND METHODS

Animals

Eight tilapia, *Oreochromis aureus,* (4 males, 4 females; 103.6±4.31 mm and 18.5±2.8 g), that had been fed an experimental diet (Table 1) for a period of eight weeks, were sacrificed by anaesthetic overdose (MS 222; Sigma Chemical Co., St. Louis, MO, USA) and subsequently frozen (-18°C) until examination. Each fish was used for MRI and flatbed scanning evaluations. A duplicate number of animals (n = 8; 4 male and 4 female; 104.3±2.50 mm and 20.1±1.5 g) were employed for chemical analysis.

Image preparation

MRI image acquisition

MRI images were obtained using a Siemens Magnetom Symphony (Siemens AG, Munich, Germany) imaging console connected to a 1.5 Tesla superconducting magnet (Siemens AG, Munich, Germany). Prior to MRI analysis, animals were thawed and gutted. Subsequently, individual animals were embedded in gelatin to eliminate air-based artefacts during imaging. T1 weighted and water saturated T1 weighted images (see Hornack (2002) for details; Fig. 1; slice thickness 5 mm) were achieved at a recovery delay (TR) of 400 and echo time (TE) of 15. Derived images were transferred from MRI film (enlarged 400%) by scanning (hp ScanJet 4C; Hewlett-Packard, Palo Alto, CA, USA) and saving each image in 600 dpi *.tiff format. For each animal, nine images (Figures. 1 and 2) were collected. Immediately following MRI, all fish were refrozen (-18°C).

Flatbed image acquisition

Refrozen fish from the MRI analysis were sliced into cutlets using an industrial meat-slicer at the same points at which MRI images were acquired (Figs. 1 and 2). Thawed cutlets were rapidly rinsed in 70% isopropanol and then stained using oil red O (Sigma Chemical Co., St. Louis, MO, USA) dissolved in isopropanol (99.9%) for 20 min. Subsequently, the cutlets were rinsed of excess ORO in 70% isopropanol (45°C) for 15 min and finally re-hydrated in tap water (45°C) for 5 min.

Table 1. Composition and proximate analysis of experimental diet employed during the present study.

Ingredient (g kg-1)	
Fish meal[a]	250
Soybean oil[b]	200
Wheat bran	145
Wheat flour	100
Corn starch	30
Corn oil	20
Cod liver oil	250
Vitamins and minerals[c]	5
Proximate composition (g kg-1)	
Total lipid	83
Crude protein	274
Ash	80
Moisture	144

[a]*Anchovy meal, 66.3% crude protein*
[b]*Solvent extracted, 44.2% crude protein*
[c]*From Goddard and McLean (2001)*

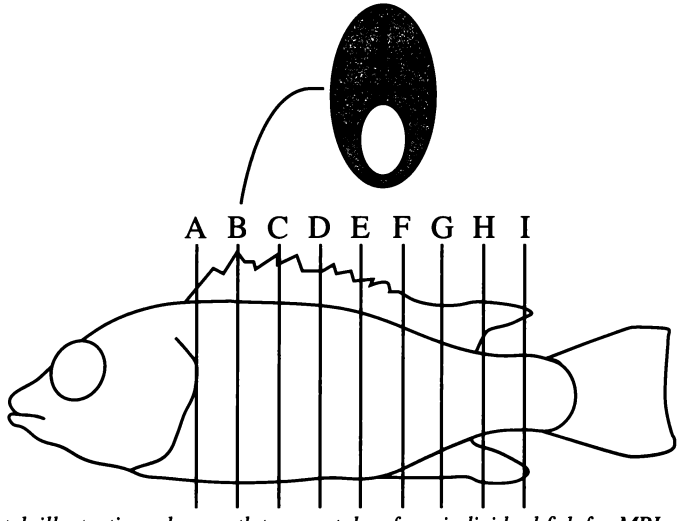

Figure 1. Sketch illustrating where cutlets were taken from individual fish for MRI and flatbed scanning and image analysis.

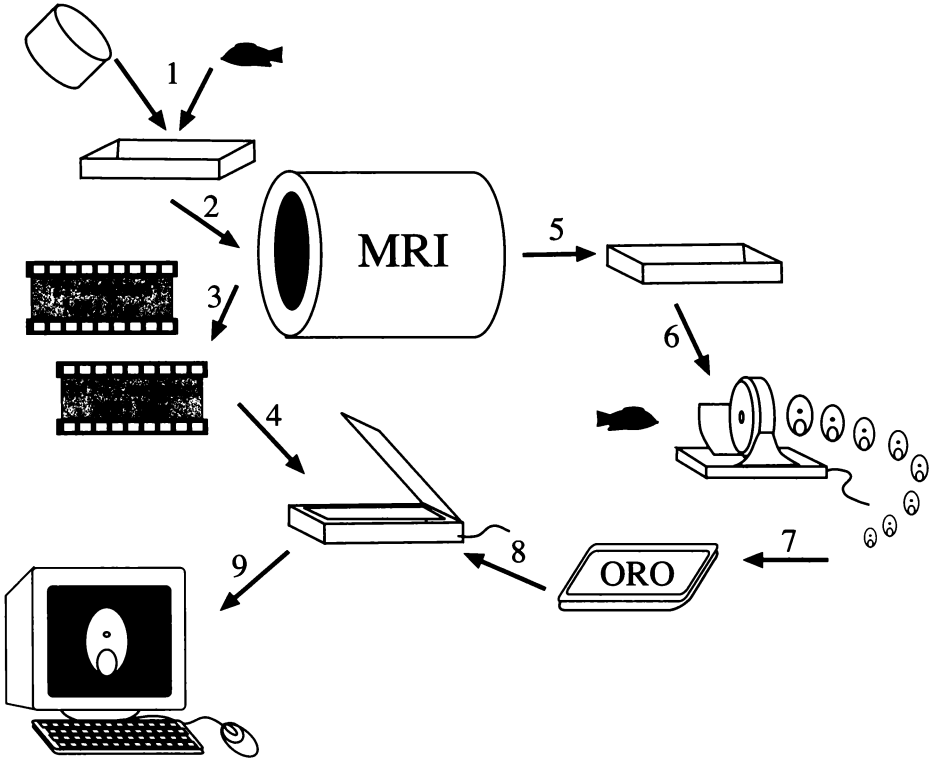

Figure 2. Illustration of the preparation of MRI and flatbed scanned images. 1) Animal embedding in gelatin, 2) MRI analysis, 3) MRI image acquisition, 4) Scanning of MRI film and saving at 600 dpi resolution, 5) Animals were refrozen, 6) Slicing of frozen animals, 7) Staining of cutlet segments using oil red O, 8) Scanning of stained cutlet segments (600 dpi), 9) Computerized analysis of acquired images.

Rinsing and re-hydration of cutlets from individual fish was accomplished by placing the sample in a 250 ml lidded container under continuous shaking. Finally, the cutlets were gently wiped to remove excess fluid and placed on an overhead film (Pelikan Transparent film TF 80, Pelikan Holding AG, Baar, Switzerland) anterior-most side down, scanned (hp ScanJet 4C) with an open lid under dark conditions (enlarged 400%) and saved in 600 dpi *.tiff format.

Image analysis

Built in functions in MatLab® v. 5.2 (MathWorks, Natick, MA, USA) were used to enable lipid analysis of stained images. The program employed the red, green, blue (RGB) spectrum and was able to separate lipid from muscle using reference values (mean +/- SD; see Fig. 3) down to the pixel level. Resolved data was also evaluated in percentage and illustrative terms. A separate program was developed in MatLab® to enable lipid

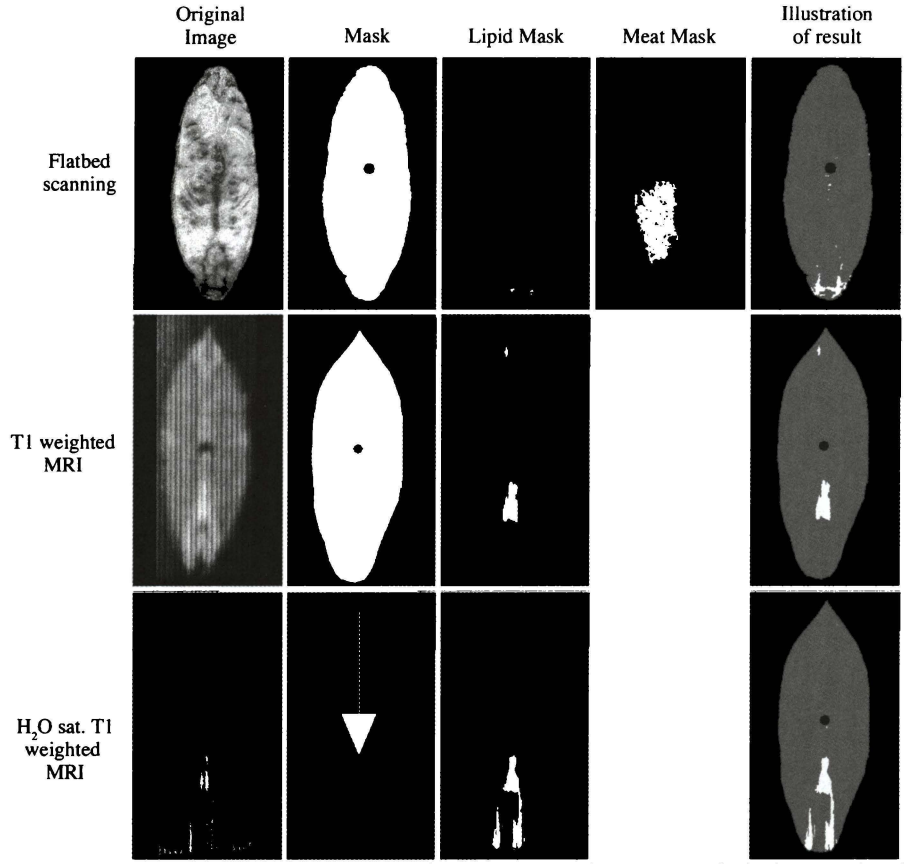

Figure 3. Illustration of the methods employed to examine lipid presence in flatbed scanned and MRI derived images of tilapia (see Methods text for more details).

analysis of the two types of MRI images (Fig. 3). It also operated in the RGB spectrum, but employed manually-separated reference images, because both T1 weighted and water saturated T1 weighted MRI films were developed in gray scale. Additionally, since the resolving power of the MRI signal was affected by lipid concentration, fat deposits in areas farther from the ventral region (belly) were less discrete and hence their analysis was more complex.

The RGB spectrum

Digital images consist of numerous dots, termed *pixels*. The number of pixels in a digital image is dependent upon image resolution, which is commonly defined in dots per inch (dpi). Each pixel is assigned a binary value (0-1) according to tone, e.g. black = 0 and white = 1. Digital images may be bitonal (black and white), grayscale, or in color. These image

types differ in the number of bits used to define each pixel. Whereas a bitonal pixel is defined by one bit, grayscale pixels range between 2-8 bits and color pixels from 8-24 bits. In a 24-bit color image, bits are often separated into three 8-bit groups - *viz.* red, green, or blue hues. A combination of the bits defines additional colors. MatLab® works separately in all three hues, with three bit values for each color/bit being defined. In a color image therefore, three values are employed: one for each hue. In contrast, in grayscale, different hues are discarded, leaving only the intensity of the single color.

Image appraisal

MRI and flatbed (stained) images were examined for percent lipid presence in each cutlet. An estimate of percent lipid for each fish was obtained using the following method: cutlet surface area (A and a, Fig. 4) expressed in pixels and height (h; set to 5 mm) was used for the determination of cutlet radius. Cutlet volume was calculated using the formula for assessing the volume of a cone. Estimated cutlet volume and total body lipid was calculated according to the following equations:

$$\text{Estimated cutlet volume (pixels)} = 1/3 \bullet \pi \times h \bullet (R^2 + R \times r + r^2) \bullet 100$$

$$\text{Estimated body lipid (\%)} = \%A \bullet (V_A / V_T) + \%B \bullet (V_B / V_T) + \ldots + \%_I \bullet (V_I / V_T)$$

Where percent lipid was $\%_{A, B, \ldots I}$; surface area was A, B, ... I, V_T was total cutlet volume in pixels, and V A, B, ... I the estimated cutlet volume in pixels (A, B, ... I).

Flatbed images were appraised as follows (Fig. 3): three masks were obtained using tools in Paint Shop Pro 6.0 (Jasc Software, Eden Prairie, MN, USA). Firstly, the spinal column was identified and assigned the color black. The remaining cutlet area was assigned the color white to provide a mask for analysis in MatLab®, which was then saved. From

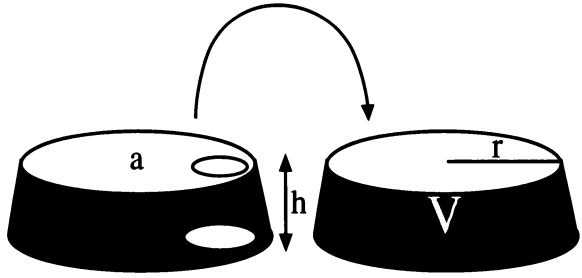

Figure 4. Illustration of the cone conversion method used to quantify cutlet lipid presence.

the original image, areas that stained intensively red were assigned to white, while the remainder were assigned to black, thereby providing a lipid mask. A meat mask was generated in a similar manner (Fig. 3). The original image, mask, lipid mask, and meat mask were identical in terms of size and location of the spinal column, such that pixels referring to, for example, meat on the meat mask were similarly identified on the original image. The four images were then analyzed in MatLab®. The lipid and meat masks provided mean and standard deviation of positively assigned pixels. This enabled MatLab® to analyze each pixel of the original image corresponding to the mask, relative to the distance to mean and standard deviation in the RGB spectrum. The following formula was applied within MatLab®:

d_meat=sqrt(((r(rr,cc)-meat_red_m)/meat_red_s)^2+((g(rr,cc)-meat_green_m)/meat_green_s)^2 +((b(rr,cc)-meat_blue_m)/meat_blue_s)^2);

where d_meat was the overall distance calculated, r(rr,cc) the pixel value in the red spectra, meat_red_m the mean value in the red spectra for pixels positively assigned to meat by the meat mask, meat_red_s the standard deviation in the red spectra of the pixels positively assigned to meat by the meat mask, etc. A similar formula was used for calculating the distance according to the lipid mean and standard deviation for the same pixel. Subsequently, the pixel analyzed was assigned to either meat or lipid depending on which distance was the shortest. MatLab® provided details relating to lipid/meat % and an illustration of the result.

MRI images were analyzed as follows: the T1 weighted MRI image was used to prepare a white mask covering the surface area of the cutlet but excluding the spinal column in Paint Shop Pro 6.0. This mask was used for analysis of both MRI image types. Secondly, for both MRI types lipid areas were assigned using tools in Paint Shop. The lipid areas were assigned to white and the remainder to black and saved as a lipid mask. Lipid or meat pixels were assigned manually. Subsequently, the original image, mask, and lipid mask were analyzed in MatLab®, which provided lipid/meat % and an illustration of the result.

Chemical analysis

An additional eight animals were employed for the chemical quantification of lipid. Individual fish were homogenized prior to lipid analysis using the method described by Bligh and Dyer (1957). All samples were analyzed in duplicate.

Statistical analyses

Statistical analyses were performed using SPSS ver. 10.0 (Data Description Inc., Ithaca, NY, USA). The model applied for the 3 x 9 factorial design was as follows:

$$y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{ijk}$$

where m was the true mean, A_i the analysis method, B_j the cutlet, ABij the interaction of the fixed factors, and finally e_{ijk} the residual effect. Differences between treatment means were analyzed by three-way ANOVA and differences between treatments ($P<0.05$) isolated using Tukey's test. Estimated lipid percent of cutlet A-I of each animal was analyzed by one-way ANOVA (flatbed scanning, T1 weighted and water saturated T1 weighted MRI).

RESULTS

Table 2 summarizes percent lipid levels in individual cutlets as determined by image analysis of ORO stained cutlets and MRI (T1 weighted and water-saturated T1) samples. Animal gender had no effect upon lipid distribution in the sections examined, regardless of method of analysis. No differences ($P > 0.05$) were discerned in cutlet lipid presence or in interactions between analytical technique and cutlet for any of the methods examined. Fish exhibited a general anterior to posterior decrease in lipid presence, with differences ($P=0.001$) in the amount of lipid detected being distinguishable between cutlets (Table 2). The first three cutlets (A-C) of all fish expressed a higher ($P < 0.05$) percentage of lipid than more posterior cuts (H and I), with cutlet I containing a lower ($P < 0.01$) percent lipid than cutlets D-G. Examination of sections D-H revealed equivalency in terms of percent lipid deposition (Table 2). A comparison of percent whole body lipid presence using imaging techniques and the Bligh and Dyer method did not reveal differences (>0.05) between the methods (Table 3).

DISCUSSION

Lipid distribution in fish varies with species, muscle type, and sampling point (Katikou *et al.* 2002) and may be influenced by season, feed type, and feeding strategy (Jobling *et al.* 1998; Johansen and Jobling 1998; Johansson *et al.* 2000). Lipid presence in fish flesh is arguably the most important trait that influences total quality of the final product. Lipids

Table 2. Comparison of flatbed scanning and MRI techniques in determining lipid percentage per cutlet examined (see Fig. 1). Statistical analysis for the 3 x 9 factorial design is indicated in the category columns. No interaction between method and cutlet segment was demonstrated. Separate one-way ANOVA for differences in cutlets is noted in the respective column. n = 8 per analysis. $P < 0.05$.

	Method		
Cutlet no.	Flatbed analysis[a]	T1 weighted[a]	H_2O saturated T1[a]
A[a]	2.54±1.20[a]	3.12±1.38[a]	2.87±1.66[a]
B[a]	2.51±1.41[ab]	3.14±2.06[a]	2.06±1.83[a]
C[a]	1.90±0.88[abc]	2.53±1.81[ab]	2.58±2.24[a]
D[ab]	1.44±0.70[abc]	2.33±1.00[ab]	2.78±1.97[a]
E[ab]	1.51±0.77[abc]	1.88±1.04[ab]	2.13±0.56[a]
F[ab]	1.43±0.59[abc]	2.42±2.07[ab]	2.60±1.55[a]
G[ab]	1.39±1.05[abc]	1.90±1.26[ab]	2.11±1.84[a]
H[bc]	1.03±0.75[bc]	0.98±1.17[ab]	1.00±0.98[a]
I[c]	0.84±0.48[c]	0.18±0.25[b]	0.19±0.46[a]

Table 3. Chemical analysis (n=6; Bligh and Dyer 1959) and estimates of lipid presence using flatbed, T1 weighted, and water saturated T1 MRI methods (n=8).

Method	Bligh and Dyer	Flatbed analysis	T1 weighted	Water saturated T1
Lipid content / Estimated lipid content.	1.71±0.46[a]	1.77±0.32[a]	2.29±1.19[a]	2.12±0.74[a]

impact the appearance, flavor, tenderness, and juiciness of fresh and processed products (Rasmussen *et al.* 2000; Johansson *et al.* 2000; Lie 2001). Lipids are also the main component responsible for the accumulation of smoked and flavoring/seasoning compounds that impart a final product's flavor (Beltran *et al.* 1991). The nutritional quality and quantity of lipid in fish flesh is of interest from a human health perspective, since it has long been recognized that n-3 PUFA reduces the incidence of heart disease, decreases risk of some cancers, and may act to stimulate the immune system.

Several studies have demonstrated the changes in fatty acid elasticity of fish flesh following dietary modifications (Viegas and Guzman 1998; Samuelsen *et al.* 2001; Bell *et al.* 2001). It is well established that lipid presence affects the technological quality of fish flesh from the processing perspective. Lipids impact sliceability (McLean and Devlin 2000), fillet yield, and trimming loss (Rasmussen *et al.* 2000), as well as the salting and drying process during smoking (Cardinal *et al.* 2001), and may play a role in fillet gaping (Lie 2001). Whole body lipid levels are also important in the bioaccumulation process (Stow *et al.* 1997; Zhou *et al.* 1999) and hence impact the safety of final products.

A trend in the aquaculture feed industry has been to formulate diets that are high in lipid (> 40%) as a method to spare protein and reduce environmental impact (Cho and Bureau 2001). Potential negative aspects of such diets however, are increased lipid accumulation and an overall decline in end product quality (Shearer 2001). A growing need exists to develop methods that enable safe and rapid assessment of lipid accumulation in the edible component of farmed fish. Such methods could support research and development in the formulation of novel diets, provide insights into lipid dynamics following application of various feeding strategies, assist during selective breeding programs, and provide a basis for on-farm product quality management.

In pigmented species of fish, such as the salmonids, it is often possible to visually detect fat deposits within muscle tissues. In contrast, visual recognition of lipid deposits in white-fleshed animals remains problematic. MRI provides the means to examine internal structures of animals non-destructively and permits separation of lipid from muscle tissues (Hornack 2002; Fig. 3). In mammals, including humans, the kinetics and distribution of body fat (Kamel *et al.* 2000; Fusch *et al.*

1999) has been examined by MRI. Clearly, similar studies with teleosts would provide great rewards with regards to understanding lipid dynamics over the life cycle and establishing the effects of different manipulations on depositional processes. In the present study with tilapia, MRI was able to distinguish lipid from muscle tissue in cutlets, demonstrating a cranial-caudal decline in cutlet lipid presence, which is in accord with chemical (Katikou *et al.* 2001) and CT (Gjerde 1987; Rye 1991) analyses of salmonids. Noteworthy in the present study was the lack of a difference in lipid presence or spatial distribution between genders.

Irrespective of the resolving power of MRI and its companion medical imaging technologies, however, such equipment is sophisticated, demanding, and expensive. In the practical sense, medical imaging technologies are of limited value to aquaculture. In contrast, the simplicity of highlighting lipids using fat-soluble stains such as oil red O, as used herein, or alternatives such as Sudan red B, Sudan black B and others, however, provides a rapid and inexpensive means of distinguishing and quantifying lipid deposits in edible flesh and viscera. The described method can be adapted and applied to all species wherein muscle and lipid tissues are difficult to separate visually (e.g., in unpigmented fleshed animals). Preliminary studies with summer (*Paralichthys dentatus*) and southern (*P. lethostigma*) flounders confirm the utility and general applicability of the method. Furthermore, use of stains such as Sudan black B would enable greater differentiation of lipid deposits even in pigmented species. Differences nevertheless exist when employing lipid-soluble histochemical dyes for macro samples such as fish cutlets/steaks, fillets, etc. Specifically, distinctions are met in incubation conditions, which likely occur due to sample thickness. When compared to MRI, the flatbed scanning method provided identical results with respect to lipid distribution and percent cutlet lipid. Moreover, quantitative estimates of lipid presence using flatbed- and MRI-derived data duplicated lipid values calculated by traditional chemical methods. Accordingly, the histochemical staining-flatbed scanning technique provides a safe, reproducible, and economical method of isolating and monitoring whole body, cutlet, or fillet lipid presence. The resolving power of this technique may be further enhanced using different or additional biological stains that are more specific. The major drawback of the flatbed method when compared to MRI was the time required to prepare samples prior to computer-assisted analysis, with individual fish requiring approximately 45 minutes. In contrast, with MRI, multiple images of multiple specimens

were acquired in a matter of minutes. However, the need to embed samples in gelatin to avoid artifacts in the MRI scan required overnight preparation, demanding two person hours total. Overall, the time required for the preparation of the flatbed-scanned images was similar to that of traditional chemical analysis. The analysis of both MRI and flatbed-scanned images (9 slices per fish) required approximately 4 hours per method. However the results generated provided more detail than traditional chemical analysis. The flatbed technique developed may prove useful in a wide range of basic and applied research.

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