JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Soyasaponins Resist Extrusion Cooking and Are Not Degraded during Gut Passage in Atlantic Salmon (*Salmo salar* L.)

DAVID KNUDSEN,*,†,‡ ØYVIND RØN,‡ GUNVOR BAARDSEN,‡ JØRN SMEDSGAARD,§ WOLFGANG KOPPE,‡ AND HANNE FRØKIÆR†

Biochemistry and Nutrition Group, BioCentrum-DTU, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark, Skretting Aquaculture Research Centre, P.O. Box 48, N-4001 Stavanger, Norway, and Centre for Microbial Biotechnology, BioCentrum-DTU, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

The stability of soyasaponins in fish feed formulations was investigated. The level of soyasaponin Ab, Bb, Bc, Ba-2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (Ba-DDMP), Bb-DDMP, and Bc-DDMP was quantified in 15 samples of defatted soybean meal, two full fat soybean meals, and two soybean protein concentrates by reverse phase high-performance liquid chromatography. The total level of saponins in the 15 samples of commercial defatted soybean meal ranged from 4.8−6.8 μmol/g (5.1−7.0 g/kg). The two full fat meals contained 4.4 and 4.7 μmol/g whereas no saponins could be detected in the alcohol-extracted soybean protein concentrates. Fifteen batches of fish feed containing 20% defatted soybean meal were produced by twin-screw extrusion from the 15 different samples of defatted soybean meal. Extrusion did not reduce the total level of group B saponins, but the ratio between DDMP-conjugated group B saponins and non-DDMP-conjugated group B saponins was slightly reduced. A soybean-containing diet was fed to seawater adapted Atlantic salmon for 9 weeks. Yttrium oxide was included in the feed as an inert marker in order to estimate the disappearance of saponins during gut passage. High levels of intact non-DDMP-conjugated group B soyasaponins were found in feces whereas only low levels of DDMP-conjugated saponins could be detected. The overall disappearance of saponins was close to zero, and the concentration of intact saponins in dry feces reached levels several fold higher than dietary levels. The present work demonstrates that non-DDMP-conjugated group B soyasaponins resist extrusion cooking and remain intact during gut passage in Atlantic salmon. The latter is contrary to earlier findings in endothermic animals.

KEYWORDS: Soybeans; saponins; extrusion; stability; degradation; Atlantic salmon

INTRODUCTION

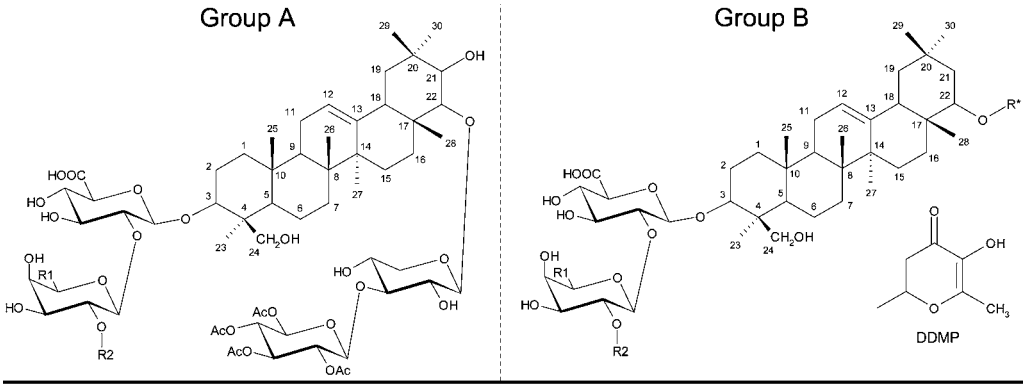
Saponins are naturally occurring amphiphilic molecules consisting of a sugar moiety linked to a steroid or triterpenoid aglycone. They are widely distributed in wild plants and are also present in cultivated crops like tea, ginseng, spinach, asparagus, egg plant, sugar beet, sunflower, oat, and legumes. Saponins are considered to be involved in the plants' defense system against pathogenic attack since many saponins have been found to display strong antibacterial and antifungal activities. Saponins have also been reported to have several biological activities in animals, both positive and negative, of which many can be ascribed to their action on cell membranes. The observed activities include cholesterol-lowering effects, immunostimulating effects, increase of intestinal permeability, reproduction effects, and glucocorticoid-like actions (*1*, *2*).

Soybeans are one of the major dietary sources of saponins for humans and domestic animals. Soyasaponins are triterpene glycosides and can be divided into two major groups: A and B (**Figure 1**) (*3*−*6*). Only few studies have investigated the bioavailability and absorption of soyasaponins. Gestetner et al. (*7*) reported that the majority of ingested soyasaponins was hydrolyzed to free aglycones by the intestinal microflora in chicks, rats, and mice. Recently, Hu et al. (*8*) obtained similar results in a human intervention study. No intact saponins could be detected in feces, and further research confirmed that the degradation was facilitated by the gut flora (*9*).

Limited supplies of fishmeal could be a bottleneck for future growth in the aquaculture industry, and much effort has therefore been made to find alternative protein sources for carnivorous fish like Atlantic salmon (*10*−*13*). Soybean meal is one of the most promising candidates due to high protein content and steady supply. Several studies have shown, however, that high levels of dietary soybeans reduce lipid digestibility and induce enteritis in the distal intestine of Atlantic salmon (*14*−*18*). Van de Ingh et al. (*14*, *15*) have demonstrated that the causative

* To whom correspondence should be addressed. Tel: +47 5182 5547. Fax: +47 5182 5501. E-mail: david.knudsen@skretting.com.
† Biochemistry and Nutrition Group, Technical University of Denmark.
‡ Skretting Aquaculture Research Centre.
§ Centre for Microbial Biotechnology, Technical University of Denmark.

10.1021/jf0604992 CCC: $33.50
Published on Web 07/26/2006



	Name	R1	R2[a]	R*	Formula	Mono isotopic mass
Group A	Ab	CH_2OH	Glc	-	$C_{67}H_{104}O_{33}$	1436.646 Da
	Ac	CH_2OH	Rha	-	$C_{67}H_{104}O_{32}$	1420.651 Da
	Af	CH_2OH	H	-	$C_{61}H_{64}O_{28}$	1274.593 Da
Group B	Ba	CH_2OH	Glc	H	$C_{48}H_{78}O_{19}$	958.514 Da
	Bb	CH_2OH	Rha	H	$C_{48}H_{78}O_{18}$	942.519 Da
	Bc	H	Rha	H	$C_{47}H_{76}O_{17}$	912.508 Da
	Bb'	CH_2OH	H	H	$C_{42}H_{68}O_{14}$	796.461 Da
	Bc'	H	H	H	$C_{41}H_{66}O_{13}$	766.450 Da
	Ba-DDMP (αg)	CH_2OH	Glc	DDMP	$C_{54}H_{84}O_{22}$	1084.545 Da
	Bb-DDMP (βg)	CH_2OH	Rha	DDMP	$C_{54}H_{84}O_{21}$	1068.551 Da
	Bc-DDMP (βa)	H	Rha	DDMP	$C_{53}H_{82}O_{20}$	1038.540 Da
	Bb'-DDMP (γg)	CH_2OH	H	DDMP	$C_{48}H_{74}O_{17}$	922.493 Da
	Bc'-DDMP (γa)	H	H	DDMP	$C_{47}H_{72}O_{16}$	892.482 Da

Figure 1. Structures of major saponins in soybeans. Glc, β-D-glucopyranosyl; Rha, α-L-rhamnopyranosyl; DDMP, 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one.

components for these adverse effects stick to the protein fraction during hexane extraction of the oil. Interestingly, alcohol-extracted soybean protein concentrate was found to be free from these components. Experiments with soybean molasses (the byproduct of alcohol extraction) revealed that the causative components were extracted from the defatted soybean meal rather than being deactivated (*15*).

Despite increased use of saponin-containing plant raw materials in commercial fish feed formulation, the impact of dietary saponins on fish has scarcely been investigated. Bureau et al. (*19*) found that Quillaja saponins reduced growth performance in Chinook salmon and Rainbow trout and caused extensive damage to the intestinal mucosa of the hindgut. It was also observed that soyasaponin-rich extracts had potent feeding deterrent properties for Chinook salmon. Krogdahl et al. (*20*) investigated the effect of soyasaponins on Atlantic salmon but did not find any effect on nutrient digestibility, growth performance, or intestinal morphology. The type of soyasaponins and the purity of the preparation used in this study were, however, not specified. Interestingly, Francis et al. (*21*, *22*) found positive effects of Quillaja saponins on growth performance in common carp and Nile tilapia when administered at low doses.

No studies have apparently been made to investigate the fate of soyasaponins in fish feed formulations. The objective of the present work was, therefore, to examine the effect of extrusion cooking on soyasaponins and to investigate whether soyasaponins are degraded in the digestive tract of Atlantic salmon.

A quantification method for soyasaponins was established using high-performance liquid chromatography (HPLC) with diode array detection (DAD). Identification of individual soyasaponins was verified by mass spectrometry. The method aimed to simultaneously quantify all major soyasaponins in their native forms as described by Decroos et al. (*23*) but by use of commercial soyasaponin standards as described by Hubert et al. (*24*).

MATERIALS AND METHODS

Soybean Samples. Nineteen different soybean samples were collected as follows: 15 batches of defatted soybean meal, two full fat soybean meals, and two soybean protein concentrates produced by alcohol extraction. The 15 batches of defatted soybean meal were obtained from different processing plants in Europe, the United States, and Brazil. Four of these batches were produced from non-GMO varieties grown in Brazil, and six other batches were known to contain varieties grown in the United States. The collected material was considered to reflect the compositional variation that can be expected within batches of commercial available defatted soybean meal.

Production of Experimental Feed. The 15 batches of defatted soybean meal were used as raw material for the production of 15 different batches of extruded fish feed. Feed production was done at Nutreco Technology Centre (Stavanger, Norway). The general recipe for the experimental feed was 45.3% fishmeal, 14.8% wheat starch, 20.0% defatted soybean meal, 19.8% fish oil, and 0.1% premixes. A preconditioner (DDC, Wenger Manufacturing, Inc., United States) coupled to a twin-screw extruder (TX57, Wenger) with a 6.5 mm × 3.4 mm diameter die was used for diet production. Steam and tap water were fed to the preconditioner to reach a moisture level of 25%. The feed rate of the extruder was 180 kg/h, and the screw speed was adjusted to get a wet bulk density after extrusion of 550 g/dm^3. The extruded pellets were dried by warm air (95 °C) in a single belt dryer (model 360, Wenger Manufacturing, Inc.) to reach a moisture level of approximately 7%. The overall residence time in the dryer was adjusted to 25 min. The dried pellets were finally coated with fish oil at 60 °C under reduced pressure (VCC coater, Dinnissen BV, Sevenum, The Nederlands).

Production of Feed for Fish Trial. The diet was produced as 4 mm pellets by twin-screw extrusion as described above. Yttrium oxide (Y_2O_3, Selectipur, Merck KgaA, Darmstadt, Germany) was included as an inert marker to facilitate evaluation of saponin disappearance during gut passage (*25*). The recipe is shown in **Table 1**.

Fish, Rearing Conditions, and Sampling of Feces. The feeding experiment was performed at Nutreco ARC Lerang Research Station (Jørpeland, Norway). Seawater-adapted Atlantic salmon (*Salmo salar* L.) with an initial average weight of 210 g were fed the soybean-containing diet for 62 days. At day 0 of the feeding period, 30 salmon

Table 1. Formulation of Diet for Fish Trials

fish meal (Scandinavian LT) (g/kg)	490.0
defatted soybean meal (Denofa, Norway) (g/kg)	200.0
wheat (g/kg)	109.0
fish oil (northern hemisphere) (g/kg)	198.5
minerals, vitamins, and pigment (g/kg)	2.4
yttrium oxide (Y_2O_3, Merck KgaA, Germany) (g/kg)	0.1
total (g/kg)	1000.0

were transferred to a circular 400 L fiberglass tank equipped with waste feed collection and continuously supplied with seawater (15 L/min). Water was pumped from 90 m depth and held at a constant temperature of 8 °C during the experiment. The fish were fed twice a day aiming at 20% overfeeding, and waste feed was collected. At the end of the feeding period, fish were stripped to collect feces according to Austreng (*26*). Fecal samples were frozen immediately after sampling and kept at −20 °C prior to analysis.

Calculation of Saponin Disappearance. Yttrium was quantified in feed and fecal samples by inductivity coupled plasma spectroscopy (Optima 3000 ICP, PerkinElmer Inc., Boston, MA) at Jordforsk Laboratory (Ås, Norway). The disappearance of saponins was calculated by the indirect method for determination of apparent digestibility (eq 1) according to Maynard and Loosli (*27*).

$$\text{disappearance}_{\text{saponins}} = 100 - \left(100 \times \frac{[\text{saponin}]_{\text{feces}}}{[\text{saponin}]_{\text{feed}}} \times \frac{[\text{yttrium}]_{\text{feed}}}{[\text{yttrium}]_{\text{feces}}}\right)$$

For the calculation of saponin disappearance, $\text{disappearance}_{\text{saponins}}$ is the disappearance coefficient of saponins in %, $[\text{saponins}]_{\text{feed}}$ is the concentration of saponins in feed (as is), $[\text{saponins}]_{\text{feces}}$ is the concentration of saponins in feces (as is), $[\text{yttrium}]_{\text{feed}}$ is the concentration of yttrium in feed (as is), and $[\text{yttrium}]_{\text{feces}}$ is the concentration of yttrium in faeces (as is).

Moisture, Dry Matter. Moisture levels in feed and feces were measured by drying to constant weight at 102−105 °C in two replicates.

Separation and Identification of Saponins. Separation of saponins was achieved on a Hewlett-Packard series 1050 HPLC-DAD system using a 250 mm × 4.6 mm i.d., 5 μm, Supelcosil ABZ+Plus, C_{18} reverse phase column (Supelco, Sigma-Aldrich). The mobile phases were 0.05% trifluroacetic acid in water (solvent A) and 0.05% trifluroacetic acid in acetonitrile (solvent B). The gradient elution was linear from 25 to 50% B, 0−65 min; linear from 50 to 60% B, 65−70 min; linear from 60 to 100% B, 70−75 min; isocratic at 100% B, 75−85 min; then linear from 100 to 25% B, 85−90 min; and finally isocratic at 25% B, 90−100 min. The flow rate was 0.5 mL/min, the injection volume was 50 μL, and the column temperature was 30 °C. Identification of soyesaponins was confirmed by HPLC retention time, UV absorption spectra recorded at 200−350 nm, and LC-MS (see below). Group A saponins and the non-DDMP-conjugated group B saponins have maximum UV absorptions at 200−205 nm and are difficult to identify by UV detection alone since they lack characteristic chromophores. DDMP-conjugated group B saponins, on the other hand, display a characteristic absorption maximum at 292 nm. The acetal linkage between the DDMP group and the C22 of the aglycone has been found to be more vulnerable to alkaline hydrolysis than the acetal bond at C3, which links the oligosaccharide to the aglycone (*6*). As a consequence, non-DDMP group B saponins can be obtained from gentle alkaline treatment of their DDMP-conjugated counterparts, a property that aids UV identification of non-DDMP-conjugated group B saponins. LC-MS was performed on a Hewlett-Packard series 1100 HPLC system connected to a Waters (Micromass) LCT time-of-flight mass spectrometer with an electrospray source including a reference spray (Lockspray). The separation was done using the same column and elution profile as above. The mass spectrometer was tuned for positive electrospray to a resolution better than 6000 fwhm and calibrated on a mixture of poly(ethylene glycol) (PEG 400−600−1000) dissolved in 50% acetonitrile with 0.2% formic acid. A solution of leucine−enkphaline (1 μg/mL in 50% acetonitrile with 0.2% formic acid) was used for the reference spray (lockmass) at approximately 5 μL/min. Spectra were collected in centroid mode from 100 to 1500 Da/e at a rate of 1 spectrum per second; every third spectrum was collected from the reference spray for internal calibration using the protonated mass of leucine−enkphaline of 556.2771 Da/e. The desolvation gas flow was at approximately 600 L/h at 400 °C, source block at 150 °C, capillary voltage at 3200 V, and cone at 25 V to minimize in-source fragmentation.

Quantification of Soyasaponins. The quantification was done using the HPLC-DAD system described above. An analytical standard of soybean saponin Bb (standard 1) was purchased from Chromadex, Inc. (Santa Ana, CA). The molar absorption at 205 nm was assumed to be approximately equal for all non-DDMP-conjugated group B saponins, and the change in solvent composition during elution was considered not to contribute significantly. A general calibration curve was therefore prepared by using standard 1 and applied to all non-DDMP-conjugated group B saponins. The error introduced by these approximations was assessed by use of another commercial saponin standard (standard 2) containing a mixture of non-DDMP-conjugated group B saponins (Soy Saponins Concentrate, 95%, Item #73891.95, Organic Technology, OH). Group A saponin concentrations were estimated from absorptions at 205 nm using the calibration curve for saponin Bb.

DDMP-conjugated group B saponins were quantified at 292 nm. In order to estimate how strongly DDMP-conjugated saponins absorb at 292 nm as compared to corresponding non-DDMP-conjugated forms at 205 nm, a mild selective alkaline hydrolysis of the DDMP group was conducted. Soybean molasses (kindly provided by Solae, Aarhus, Denmark) was used as a saponin-rich test sample for the alkaline hydrolysis. Briefly, 500 μL of diluted molasses was treated with either 500 μL of 100 mM NaOH in 70% EtOH ("alkaline treated") or 500 μL of 70% EtOH ("control") at room temperature. After 1 h of reaction, 500 μL of 100 mM HCl in 70% EtOH was added to the alkaline-treated sample and 500 μL of 70% EtOH was added to the control. The two samples were then centrifuged at 15000*g* and analyzed by HPLC. The increase of non-DDMP-conjugated saponins and the decrease of DDMP-conjugated saponins caused by alkaline hydrolysis were determined by comparison. The hydrolysis was repeated for several different concentrations of soybean molasses, and the results were plotted as peak area decrease of DDMP-conjugated forms at 292 nm vs peak area increase of non-DDMP-conjugated forms at 205 nm. The apparent absorption ratio between the DDMP-conjugated form at 292 nm and the non-conjugated form at 205 nm was found by linear regression.

Saponin Extraction. Saponins were extracted with 70% aqueous ethanol at room temperature. One hundred milligrams of finely ground soybean sample was extracted with 3 mL of solvent for 15 min on a vibrator (IKA-VIBRAX type VXR). After extraction, the suspension was centrifuged at 3000*g* for 5 min. The extraction procedure was repeated three times by resuspending the pellet. The four collected supernatants were pooled to get a total extraction volume of 12 mL. Soybean-containing fish feed samples were defatted prior to saponin extraction: 500 mg of finely ground fish feed sample was extracted twice with 5 mL of hexane for 15 min on the vibrator and subsequently centrifuged. The defatted pellet was then extracted with 4 × 3 mL 70% aqueous ethanol as described above. Saponins could be extracted more readily from fish feces: 1 g of wet feces was extracted with 3 × 2 mL 70% aqueous ethanol as described above to get a total extraction volume of 6 mL. All extracts were centrifuged at 15000*g* for 5 min prior to analysis.

Statistics. Standard curves were constructed from two commercial saponin standards using linear regression. The absorption ratios between DDMP-conjugated forms of soyasaponin Bb and Bc at 292 nm and the free forms of Bb and Bc at 205 nm were also found by linear regression. Five replicated measurements of the same soybean sample (grinded, extracted, and analyzed five times) were used to assess the precision of the quantification method. The effect of extrusion was evaluated by comparing the inclusion level of soybean meal (fixed at 20% for all diets) to the ratio between saponin levels found in feed and raw material. This comparison was done using a one-sample *t*-test at a confidence level of 95% according to Montgomery (*28*).

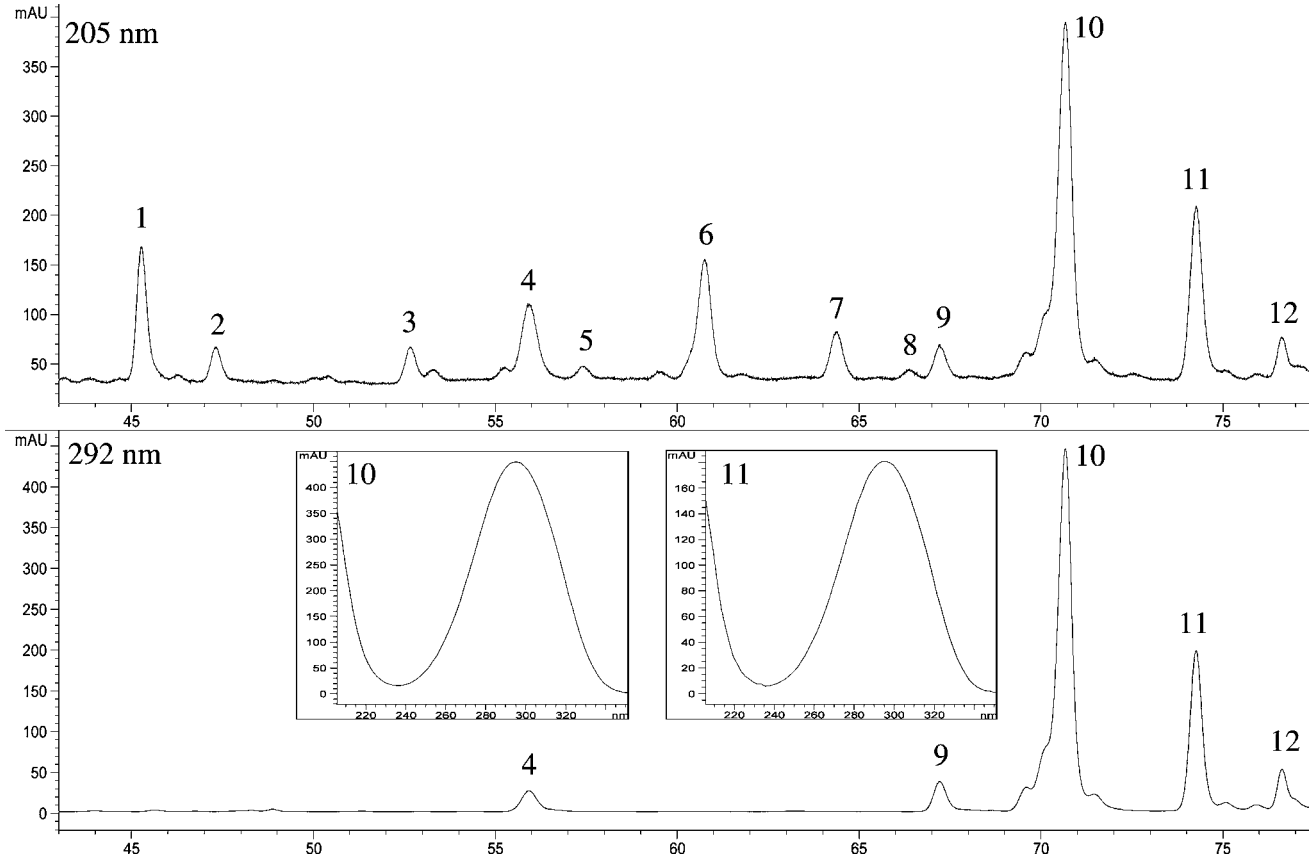


Figure 2. Chromatogram of soybean molasses recorded at 205 and 292 nm. Separation was achieved on a Supelcosil ABZ+plus, C18 reverse phase column (Supelco, Sigma-Aldrich). The identified soyasaponins eluted after 45–75 min. Peaks 1–3 are saponin Ab, Ac, and Af; peak 4 is genistein; and peaks 5–12 are saponin Ba, Bb, Bc, Bb′, Ba-DDMP, Bb-DDMP, Bc-DDMP, and Bb′-DDMP. The inserted spectra show the absorption pattern for Bb-DDMP and Bc-DDMP between 205 and 350 nm.

RESULTS

Identification of Soybean Saponins. LC-MS analyses using positive electrospray ionization were used to confirm the identity of the individual group B saponins and to identify group A saponins. A sample of soybean molasses (diluted 1:5 in 70% ethanol) and a stock solution of standard 2 (Soy Saponins Concentrate, 95%, Item #73891.95, Organic Technology) were used as test samples during the identification work. The following ions were used for tracing the saponins ($[M + H]^+$; $[M + Na]^+$): Saponin Ab (*m*/*z* 1437.6538; *m*/*z* 1459.6358), Ac (*m*/*z* 1421.6589; *m*/*z* 1443.6408), and Af (*m*/*z* 1275.6010; *m*/*z* 1297.5829); for saponin Ba (*m*/*z* 959.5216; *m*/*z* 981.5035), Bb (*m*/*z* 943.5266; *m*/*z* 965.5086), Bc (*m*/*z* 913.5161; *m*/*z* 935.4980), Bb′ (*m*/*z* 797.4687; *m*/*z* 819.4507), and Bc′ (*m*/*z* 767.4582; *m*/*z* 789.4401); and finally for saponin Ba-DDMP (*m*/*z* 1085.5532; *m*/*z* 1107.5352), Bb-DDMP (*m*/*z* 1069.5583; *m*/*z* 1091.5403), Bc-DDMP (*m*/*z* 1039.5479; *m*/*z* 1061.5297), Bb′-DDMP (*m*/*z* 923.0004; *m*/*z* 948.4824), and Bc′-DDMP (*m*/*z* 893.4899; *m*/*z* 915.4718). The sodium adduct $[M + Na]^+$ was seen in all spectra, and other characteristic fragmentation ions were seen consistent with literature (*3*−*6*). In all cases, the saponins were found with a high mass accuracy. Narrow ion traces (<60 ppm width) around the protonated and/or sodiated masses allowed a clear identification of all saponins. However, the ion trace chromatograms for Bb′ at *m*/*z* 797.4687 and Bc′ at *m*/*z* 767.4581 showed two chromatographic peaks, where the first eluting peaks corresponded to loss of Rha from Bb and Bc, respectively, confirmed by the retention times from the Bb and Bc traces. Similarly, the ion trace chromatograms for Bb′-DDMP at *m*/*z* 923.500 and Bc′-DDMP at *m*/*z* 893.490 show two chromatographic peaks, where the first eluting peak corresponded to loss of Rha from Bb-DDMP and Bc-DDMP, confirmed by the retention times from the Bb-DDMP and Bc-DDMP traces. Part of the UV chromatogram recorded at 205 and 292 nm for soybean molasses (diluted 1:5) can be seen in **Figure 2**. The identified saponins eluted after 45−75 min with adequate separation. Neither saponin Aa, Ad, Ae, Ag, nor Ah could be detected in any of the two test samples. **Figure 2** also contains the absorption spectra for Bb-DDMP and Bc-DDMP recorded from 205 to 350 nm. The absorbance at 292 nm was approximately 1.2 times higher than the absorbance at 205 nm for both Bb-DDMP and Bc-DDMP.

Quantification of Soybean Saponins. Standard 1 was used to construct a HPLC calibration curve for saponin Bb. Mass spectrometry of standard 2 revealed that this standard contained a mixture of saponin Ba, Bb, Bc, Bb′, and Bc′. Using the assumption of equal molar absorption, the molar ratios of the detected saponins were estimated to be 9, 48, 28, 10, and 5%, respectively, by comparing the relative peak areas recorded at 205 nm. These molar ratios were then used to determine the total molar concentration of saponins in stock solutions of standard 2, and an average calibration curve for all non-DDMP-conjugated group B saponins was constructed. **Figure 3** shows the absorption readings fitted with linear regression lines for both standard 1 and standard 2. The slopes of the two calibration curves were almost identical. The error introduced by quantifying all non-DDMP-conjugated group B saponins as saponin Bb equivalents was therefore regarded as negligible.

To estimate the relative extinction coefficient for DDMP-conjugated saponins at 292 nm as compared to non-DDMP-

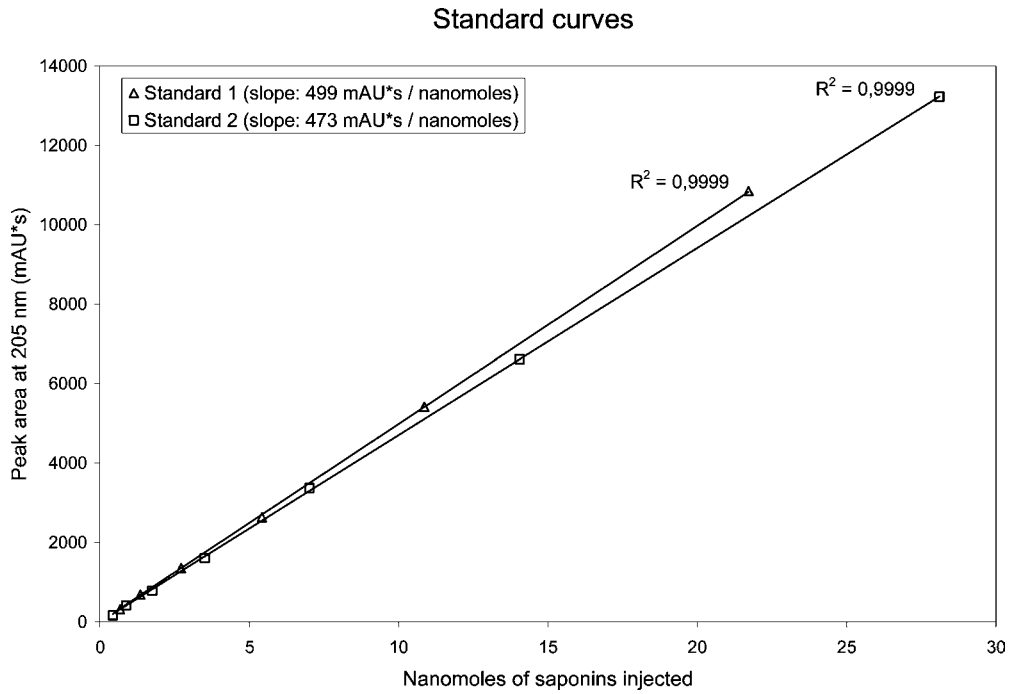


Figure 3. HPLC calibration curves. Serial dilutions in 70% ethanol were prepared from stock solutions of standard 1 (saponin Bb) and standard 2 (a mixture of non-DDMP-conjugated group B saponins). Calibration curves were obtained by linear regression.

conjugated saponins at 205 nm, a dilution series of soybean molasses were subjected to mild alkaline cleavage of the DDMP moiety. The peak area decrease of DDMP-conjugated forms at 292 nm after alkaline treatment and the corresponding peak area increase of non-DDMP-conjugated forms at 205 nm were found by comparing the alkaline-treated samples to corresponding control samples. The absorption ratios between the DDMP-conjugated forms at 292 nm and the free forms of Bb and Bc at 205 nm were found by linear regression to 1.23 ($R^2 = 0.9994$) and 1.27 ($R^2 = 0.9989$), respectively. A ratio of 1.25 was chosen as the general conversion factor for all DDMP-conjugated saponins. In summary, all non-DDMP-conjugated group B saponins were quantified using the standard curve for Bb, and all DDMP-conjugated group B saponins were quantified by reducing the recorded peak areas at 292 nm with a factor 1.25 before applying the standard curve for Bb. Saponin Ab, Ac, and Af were tentatively quantified as Bb equivalents. During the experiments with mild alkaline hydrolysis of the DDMP moiety, it was observed that saponin Ab was vulnerable to weak alkaline hydrolysis. This is consistent with the findings of Gu et al. (*6*), who reported that all acetylated group A saponins converted into the deacetylated forms after mild alkaline treatment with 50 mM NaOH.

The analytical precision of the method was assessed by five replicated analyses of the same soybean sample (ground, extracted, and analyzed five times). The coefficient of variation was found to be 10.8, 3.4, 6.9, 8.8, 1.7, 5.3, and 2.5% for Ab, Bb, Bc, Ba-DDMP, Bb-DDMP, Bc-DDMP, and total sum, respectively. The levels of Ac, Af, Ba, Bb′, Bc′, Bb′-DDMP, and Bc′-DDMP in soybean and feed samples were generally found to be too low for quantitative evaluation and were therefore omitted from the analysis.

Determination of Saponins in Various Soybean Products and Feed Samples. Table 2 shows the saponin level in various soybean and feed samples. The effect of extrusion was evaluated by comparing the observed ratio between saponin level in feed and raw material to the inclusion level (20%). A one-sample *t*-test was conducted at a confidence level of 95% according to Montgomery (*28*). The levels of saponin Ab, Ba-DDMP, Bb-DDMP, and Bc-DDMP were significantly reduced by extrusion while the levels of Bb and Bc were significantly increased. However, the overall level of saponins after extrusion was not affected significantly.

Disappearance of Soybean Saponins in Atlantic Salmon. Feed and feces were analyzed for saponins and yttrium in duplicate, and the disappearance coefficient was calculated according to eq 1 (**Table 3**). Dry matter content in feed and feces was found to be 96.6 and 8.0%, respectively.

DISCUSSION

In the present study, we have determined the concentration of soyasaponins in 15 samples of defatted soybean meal and the corresponding fish feed prepared from the soybean samples. By comparing the concentration of saponins found in soybean meal, fish feed, and fish feces, we have demonstrated that no or only minor degradation takes place during fish feed production and gut passage in Atlantic salmon, except for the conversion of DDMP-conjugated saponins to their nonconjugated forms during extrusion and, to a greater extent, during passage in the digestive tract of the fish.

An analytical method using reverse phase HPLC was established for simultaneous detection of group A, group B, and DDMP-conjugated group B soyasaponins. Identification of individual soyasaponins was confirmed by LC-MS. Commercial standards containing non-DDMP-conjugated group B soyasaponins were purchased from two different suppliers and gave rise to very consistent results (**Figure 3**). The quantification of non-DDMP-conjugated group B saponins in the present work is therefore considered to be of high precision. An indirect approach was used to quantify DDMP-conjugated group B saponins: The ratio between molar absorption of the DDMP-conjugated form at 292 nm and molar absorption of the non-DDMP-conjugated counter form at 205 nm was estimated to 1.25 by gentle alkaline hydrolysis of the DDMP moiety. Knowing this ratio, the DDMP-conjugated group B saponins

Table 2. Level of Soyasaponins in Various Soybean and Feed Samples[a]

	Ab	Bb	Bc	Ba-DDMP	Bb-DDMP	Bc-DDMP	sum
	Saponin Content (μmol/g)						
SBM 1[b]	0.49	2.12	0.94	0.13	1.78	0.88	6.33
SBM 2[b]	0.58	2.28	1.11	0.09	1.78	0.94	6.77
SBM 3[c]	0.49	2.13	0.93	0.12	2.05	1.01	6.72
SBM 4[c]	0.65	2.19	1.04	0.10	1.42	0.67	6.06
SBM 5[c]	0.44	2.38	1.27	0.07	1.18	0.54	5.87
SBM 6[d]	0.46	2.34	1.03	0.12	1.67	0.77	6.40
SBM 7[c]	0.49	2.20	0.97	0.13	2.02	0.97	6.78
SBM 8[b]	0.65	2.29	1.07	0.09	1.28	0.61	5.99
SBM 9[b]	0.55	2.70	1.34	0.07	1.06	0.51	6.23
SBM 10[b]	0.51	2.44	1.06	0.09	1.43	0.64	6.16
SBM 11[d]	0.69	1.54	0.62	0.09	1.34	0.55	4.84
SBM 12[d]	0.79	1.51	0.51	0.12	1.73	0.62	5.28
SBM 13[d]	0.95	1.67	0.64	0.16	1.94	0.77	6.13
SBM 14[d]	0.96	1.46	0.45	0.18	2.18	0.64	5.86
SBM 15[d]	0.86	1.42	0.52	0.12	1.99	0.77	5.69
FF-SBM 1	0.48	1.70	0.86	0.05	0.97	0.64	4.70
FF-SBM 2	0.35	1.95	1.00	0.06	0.69	0.35	4.40
SPC 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SPC 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00
feed 1	0.09	0.52	0.23	0.02	0.28	0.14	1.32
feed 2	0.11	0.50	0.26	0.02	0.30	0.15	1.33
feed 3	0.11	0.49	0.22	0.02	0.36	0.17	1.36
feed 4	0.09	0.57	0.25	0.02	0.24	0.12	1.32
feed 5	0.08	0.65	0.29	0.01	0.21	0.10	1.37
feed 6	0.09	0.53	0.25	0.02	0.28	0.13	1.30
feed 7	0.09	0.56	0.25	0.02	0.36	0.16	1.44
feed 8	0.10	0.54	0.25	0.01	0.20	0.09	1.23
feed 9	0.09	0.62	0.28	0.01	0.18	0.08	1.30
feed 10	0.10	0.57	0.24	0.02	0.22	0.10	1.27
feed 11	0.13	0.29	0.11	0.02	0.24	0.10	0.89
feed 12	0.14	0.40	0.14	0.02	0.27	0.10	1.09
feed 13	0.19	0.33	0.14	0.03	0.33	0.12	1.13
feed 14	0.19	0.36	0.14	0.03	0.38	0.14	1.23
feed 15	0.15	0.37	0.13	0.02	0.28	0.11	1.09
	Ratios between Saponin Level in Feed and Soybean Meal						
feed 1/SBM 1	0.19	0.25	0.24	0.14	0.16	0.16	0.21
feed 2/SBM 2	0.19	0.22	0.24	0.20	0.17	0.16	0.20
feed 3/SBM 3	0.23	0.23	0.23	0.19	0.17	0.17	0.20
feed 4/SBM 4	0.14	0.26	0.24	0.17	0.17	0.17	0.22
feed 5/SBM 5	0.17	0.27	0.23	0.20	0.18	0.18	0.23
feed 6/SBM 6	0.19	0.23	0.24	0.15	0.17	0.17	0.20
feed 7/SBM 7	0.19	0.25	0.26	0.17	0.18	0.17	0.21
feed 8/SBM 8	0.15	0.24	0.24	0.15	0.16	0.15	0.20
feed 9/SBM 9	0.16	0.23	0.21	0.16	0.16	0.16	0.21
feed 10/SBM 10	0.19	0.23	0.22	0.17	0.16	0.15	0.21
feed 11/SBM 11	0.19	0.19	0.18	0.16	0.18	0.18	0.18
feed 12/SBM 12	0.18	0.26	0.27	0.15	0.15	0.16	0.21
feed 13/SBM 13	0.20	0.20	0.21	0.16	0.17	0.16	0.18
feed 14/SBM 14	0.20	0.24	0.31	0.16	0.17	0.21	0.21
feed 15/SBM 15	0.18	0.26	0.25	0.17	0.14	0.14	0.19
average	0.18*	0.23*	0.23*	0.17*	0.16*	0.16*	0.20

[a] SBM, defatted soybean meal; FF-SBM, full fat soybean meals; and SPC, soybean protein concentrates. Presented data are single analyses. *, Significant difference from the inclusion level (20%) tested with a one-sample *t*-test at a 0.95 confidence level. [b] Contain soybeans of unknown origin. [c] Contain non-GMO varieties from Brazil. [d] Contain varieties grown in the United States.

could be quantified by applying the standard curve for non-DDMP-conjugated group B saponins. Hubert et al. (*24*) estimated the ratio between molar absorption of the DDMP-conjugated forms at 205 nm vs molar absorption of the free forms at 205 nm to be 1.05 and 1.20 for soyasaponin Bb and Bc, respectively. According to the recorded UV absorption spectrum of Bb-DDMP and Bc-DDMP shown in **Figure 2**, the absorbance at 292 nm was approximately 1.2 times higher than the absorbance at 205 nm for both Bb-DDMP and Bc-DDMP. The estimated value of 1.25 in the present work for the ratio between molar absorption of the DDMP-conjugated form at 292 nm and molar absorption of the non-DDMP-conjugated counter form at 205 nm is therefore in good agreement with Hubert et al. (*24*). Even though the quantification approach for DDMP-conjugated soyasaponins is an analytical compromise, the method is considered to quantify DDMP-conjugated group B saponins fairly accurately. Group A saponins, however, were only tentatively quantified by applying the standard curve for saponin Bb. In summary, an analytical method for simultaneously quantification of all major soyasaponins was developed, suitable for following the fate of soyasaponins in fish feed formulations. The repeatability of the method was assessed by five replicated analyses of the same soybean sample. The coefficient of variation was 10.8% for soyasaponin Ab and below 9% for all of the detected group B saponins. For the total sum of saponins, the coefficient of variation was 2.5%. This level of precision is acceptable considering that UV detection at 205 nm is associated with relatively high levels of noise.

The total level of saponins in the 15 analyzed samples of defatted soybean meal was found to vary between 4.84 and 6.78 μmol/g. These values are in good agreement with Hu et al. (*29*), who found the average level of group B saponins in whole seeds from 46 different soybean varieties to be 4.04 μmol/g. No significant difference ($\alpha = 0.05$) was found between the total saponins content of non-GMO varieties from Brazil and the varieties grown in the United States. The level of saponins in the two full fat meals was slightly lower than the levels found in defatted meals. This was expected since saponins follow the protein fraction during hexane extraction of the oil (*30*). No saponins could be detected in alcohol-extracted soybean protein concentrates, which is in agreement with Hu et al. (*29*), who only detected trace amounts of saponins in alcohol-extracted soybean concentrates. Gu et al. (*6*) found the total level of group A and group B saponins to be 11 g/kg in a sample of defatted soybean meal from a Chinese soybean variety. Our results suggest that the total saponin level in commercial batches of defatted soybean meal typically could be expected to be in the range of 5−7 g/kg. In general, impure standards lead to overestimation while insufficient extraction is a common cause of underestimation. The extraction procedures reported in the present work were based on several preliminary studies and especially designed to ensure complete extraction while minimizing degradation of the unstable saponins (using short extraction time and low temperature).

The variation in saponin content between the 15 batches of defatted soybean meal was surprisingly small. Hu et al. (*29*) found the level of group B saponins in whole soybeans to vary from 2.5 to 5.9 μmol/g in 46 tested soybean varieties, and Shiraiwa et al. (*31*) found the total saponin content in soybean hypocotyls from 457 different soybean varieties to vary between 0.6 and 6.2%. Possible explanations to the low variation observed in the present study could be that extensive mixing of soybean varieties takes place at the processing plants or that the variation in saponin content between the most commercial important soybean varieties is small.

Extrusion cooking did not affect the total level of saponins significantly even though the ratio between DDMP-conjugated saponins and nonconjugated forms was slightly decreased. The level of saponin Ab was slightly reduced after extrusion. This was probably due to partial degradation of the sugar chain attached to C22 (e.g., deacetylation) rather than degradation of the sugar chain attached to C3 since no degradation of group B saponins at C3 was observed.

The linkage between the DDMP moiety and the C22 of the aglycone has previously been found to be instable in aqueous

Table 3. Saponin and Yttrium Levels in Feed and Feces (Analyses Were Done in Duplicate)[a]

	yttrium (mg/kg)	saponin content (μmol/g)						
		Ab	Bb	Bc	Ba-DDMP	Bb-DDMP	Bc-DDMP	sum
feed (dry)	72.0 ± 2.1	0.125 ± 0.001	0.458 ± 0.026	0.168 ± 0.016	0.023 ± 0.003	0.340 ± 0.015	0.154 ± 0.005	1.270 ± 0.011
feces (dry)	195.1 ± 5.6	ND[b,c] < 0.015	2.081 ± 0.001	1.021 ± 0.018	ND[b,d] < 0.001	0.129 ± 0.004	0.063 ± 0.006	3.294 ± 0.026
saponin disappearance coefficient		100.0	−67.5	−123.8	100.0	86.0	84.9	4.3[e]

[a] Values are presented as means ± half the difference between duplicates. [b] Not detected. [c] Detection limit for Ab. [d] Detection limit for Ba-DDMP. [e] Calculated by inserting the sum of saponins measured in feed and feces, respectively, into eq 1.

and alcohol solution (*29*) and sensitive to pH changes (*6*). The reduced ratio between DDMP-conjugated saponins and non-conjugated forms caused by extrusion could therefore be due to the increased water activity during extrusion rather than a result of high temperature, pressure, and shear forces.

The level of soyasaponins and yttrium (the inert marker) was quantified in feed and feces in duplicates. The objective was to assess whether soyasaponins were degraded during gut passage in Atlantics salmon or not. High levels of saponin Bb and Bc were found in feces whereas only low concentrations of the DDMP-conjugated forms could be detected. The latter was expected considering the instability of the DDMP-conjugated forms in aqueous solution. The disappearance coefficients of the nonconjugated forms were strongly negative, which is very likely to be due to the conversion of DDMP forms to non-DDMP-conjugated forms, and the overall disappearance coefficient for saponins was close to zero. These findings demonstrate that group B saponins are neither absorbed nor degraded (except for the cleavage of the DDMP moiety) in the digestive tract of Atlantic salmon. No saponin Ab could be detected in feces; however, because non-DDMP-conjugated group B saponins remained intact during gut passage, the most likely explanation to the missing saponin Ab in feces is degradation of the sugar chain attached to C22 (e.g., deacetylation) rather than degradation of the sugar chain attached to C3. The lack of degradation caused the concentration of group B saponins to reach a level in dry feces that was 2−3 times higher than dietary level (**Table 3**). The fecal analyses were done on pooled feces from 30 fish. No conclusions can therefore be made, from the present data, regarding variability in soyasaponin degradation between individual fish or between different fish groups.

Previous studies in humans, chicks, mice, and rats (*7*−*9*) have demonstrated that the gut flora is able to cleave off the sugar chain attached to C3, which effectively disrupts the surface-active properties of saponins. The microbial community in the digestive tract of fish, however, is known to be substantially lower in number and less diverse than in endothermic animals (*32*). The bacterial density in the distal intestine of Atlantic salmon farmed in Norway and Scotland was recently found to be 1.5×10^7 and 6.8×10^7 per g of digesta, respectively, whereas the levels found in, e.g., the human colon has been reported to be in the range of 1×10^9 to 1×10^{11} (*33*, *34*). Limited bacterial fermentation could therefore explain the observed lack of saponin degradation during gut passage in Atlantic salmon. High levels of intact amphiphilic soyasaponins throughout the digestive tract could potentially affect micelle formation, interfere with endocytosis, or perhaps even disrupt the membranes of enterocytes. Further studies are currently being done to investigate if dietary soyasaponins are responsible for the intestinal inflammation and the reduced lipid digestibility that has been reported for Atlantic salmon fed high levels of low-processed soybean products. Further studies are also needed to examine if soyasaponins have any positive effect on Atlantic salmon and to investigate the fate of dietary soyasaponins in other fish species.

LITERATURE CITED

(1) Sparg, S. G.; Light, M. E.; van Staden, J. Biological activities and distribution of plant saponins. *J. Ethnopharmacol.* **2004**, *94*, 219−243.

(2) Francis, G.; Kerem, Z.; Makkar, H. P. S.; Becker, K. The biological action of saponins in animal systems: A review. *Br. J. Nutr.* **2002**, *88*, 587−605.

(3) Shiraiwa, M.; Kudo, S.; Shimoyamada, M.; Harada, K; Okubo, K. Composition and structure of "group A saponin" in soybean seed. *Agric. Biol. Chem.* **1991**, *55* (2), 315−322.

(4) Shiraiwa, M.; Harada, K.; Okubo, K. Composition and structure of "group B saponin" in soybean seed. *Agric. Biol. Chem.* **1991**, *55* (4), 911−917.

(5) Kudou, S.; Tonomura, M.; Tsukamoto, C.; Uchida, T.; Sakabe, T.; Tamura, N.; Okubo, K. Isolation and structural elucidation of DDMP-conjugated soyasaponins as genuine saponins from soybean seeds. *Biosci., Biotechnol., Biochem.* **1993**, *57* (4), 546−550.

(6) Gu, L. W.; Tao, G. J.; Gu, W. Y., Prior, R. L. Determination of soyasaponins in soy with LC-MS following structural unification by partial alkaline degradation. *J. Agric. Food Chem.* **2002**, *50*, 6951−6959.

(7) Gestetner, B.; Birk, Y.; Tencer, Y. Fate of ingested soybean saponins and the physiological aspect of their hemolytic activity. *J. Agric. Food Chem.* **1968**, *16*, 1031−1035.

(8) Hu, J.; Reddy, M. B.; Hendrich, S.; Murphy, P. A. Soyasaponin I and Sapongenol B have limited absorption by Caco-2 intestinal cells and limited bioavailability in women. *J. Nutr.* **2004**, *134*, 1867−1872.

(9) Hu, J.; Zheng, Y. L.; Hyde, W.; Hendrich S.; Murphy, P. A. Human fecal metabolism of soyasaponin I. *J. Agric. Food Chem.* **2004**, *52*, 2689−2696.

(10) Tacon, Albert. J. Aquaculture production trends analysis. *FAO Fisheries Circular No. 886, Rev. 2*; FAO: Rome, 2003; 95 pp.

(11) New, M. B.; Wijkström, U. N.; FAO. Use of fishmeal and fish oil in aquafeeds: further thoughts on the fishmeal trap. *FAO Fisheries Circular No. 975*; FAO: Rome, 2002; 61 pp.

(12) Naylor, R. L.; Goldburg, R. J.; Primavera, J. H.; Kautsky, N.; Beveridge, M. C. M.; Clay, J.; Folke, C.; Lubchenco, J.; Mooney, H.; Troell, M. Effect of aquaculture on world fish supplies. *Nature* **2000**, *405*, 1017−1024.

(13) Francis, G.; Makkar, H. P. S.; Becker, K. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture* **2001**, *199*, 197−227.

(14) van den Ingh, T. S. G. A.; Krogdahl, A.; Olli, J. J.; Hendriks, H. G. C. J.; Koninkx, J. G. J. F. Effects of soybean-containing diets on the mid and distal intestine in Atlantic salmon (*Salmo salar*)—A morphological study. *Aquaculture* **1991**, *94*, 297−305.

(15) van den Ingh, T. S. G. A.; Olli, J. J.; Krogdahl, A. Alcohol-soluble components in soybeans cause morphological changes in the distal intestine of Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* **1996**, *19*, 47−53.

(16) Baeverfjord, G.; Krogdahl, A. Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: A comparison with the intestines of fasted fish. *J. Fish Dis.* **1996**, *19*, 375−387.

(17) Bakke-McKellep, A. M.; Press, C. M.; Baeverfjord, G.; Krogdahl, A.; Landsverk, T. Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, *Salmo salar* L., with soybean meal-induced enteritis. *J. Fish Dis.* **2000**, *23*, 115−127.

(18) Krogdahl, Å.; Bakke-McKellep, A. M.; Baeverfjord, G. Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). *Aquacult. Nutr.* **2003**, *9*, 361−371.

(19) Bureau, D. P.; Harris, A. M.; Cho, C. Y. The effects of purified alcohol extracts from soy products on feed intake and growth of chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **1998**, *161*, 27−43.

(20) Krogdahl, A.; Roem, A.; Baeverfjord, G. Effects of soybean saponin, raffinose and soybean alcohol extract on nutrient digestibilities, growth and intestinal morphology in Atlantic salmon. In *Quality in Aquaculture. European Aquaculture Society Special Publication No. 23*; European Aquaculture Society: 1995; pp 118−119.

(21) Francis, G.; Makkar, H. P. S.; Becker, K. Dietary supplementation with a Quillaja saponin mixture improves growth performance and metabolic efficiency in common carp (*Cyprinus carpio* L.). *Aquaculture* **2002**, *203*, 311−320.

(22) Francis, G.; Makkar, H. P. S.; Becker, K. Quillaja saponins—A natural growth promoter for fish. *Anim. Feed Sci. Technol.* **2005**, *121*, 147−157.

(23) Decroos, K.; Vincken, J.-P.; Heng, L.; Bakker, R.; Gruppen, H.; Verstraete, W. Simultaneous quantification of differently glycosylated, acetylated, and 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one-conjugated soyasaponins using reversed-phase high-performance liquid chromatography with evaporative light scattering detection. *J. Chromatogr. A* **2005**, *1072*, 185−193.

(24) Hubert, J.; Berger, M.; Daydé, D. Use of simplified HPLC-UV analysis for soyasaponin B determination: Study of saponin and isoflavone variability in soybean cultivars and soy-based health food products. *J. Agric. Food Chem.* **2005**, *53*, 3923−3930.

(25) Austreng, E.; Storebakken, T.; Thomassen, M. S.; Refstie, S.; Thomassen, Y. Evaluation of selected trivalent metal oxides as inert markers used to estimate apparent digestibility in salmonids. *Aquaculture* **2000**, *188*, 65−78.

(26) Austreng, E. Digestibility determination in fish using chromix oxide marking and analysis of contents from different segments of the gastrointestinal tract. *Aquaculture* **1978**, *13*, 265−272.

(27) Maynard, L. A.; Loosli, J. K. Feeding experiments. The determination of digestibility. In *Animal Nutrition*, 6th ed.; McGraw-Hill: New York, 1969; 613 pp.

(28) Montgomery, D. C. Comparing a single mean to a specified value. in *Design and Analysis of Experiments*, 5th ed.; John Wiley & Sons: New York, 2001; Chapter 2-4.6, 684 pp.

(29) Hu, J.; Lee, S. O.; Hendrich, S.; Murphy, P. A. Quantification of the group B soyasaponins by high-performance liquid chromatography. *J. Agric. Food Chem.* **2002**, *50*, 2587−2594.

(30) Kitagawa, I.; Saito, M.; Taniyama, T.; Yoshikawa, M. Saponin and sapogenol. XXXVIII. Structure of saponin A from soybean. *Chem. Pharm. Bull.* **1985**, *33*, 598−608.

(31) Shiraiwa, M.; Harada, K.; Okubo, K. Composition and content of saponins in soybean seed according to variety, cultivation year and maturity. *Agric. Biol. Chem.* **1991**, *55* (2), 323−331.

(32) Ringø, E.; Strøm, E.; Tabachek, J. Intestinal microflora of salmonids: a review. *Aquacult. Res.* **1995**, *26*, 773−789.

(33) Holben, W. E.; Williams, P.; Saarinen, M.; Särkilahti, L. K.; Apajalahti, J. H. A. Phylogenetic analysis of intestinal microflora indicates a novel *Mycoplasma* phylotype in farmed and wild salmon. *Microb. Ecol.* **2002**, *44*, 175−185.

(34) Baron, S. Gastrointestinal tract flora, section 1.6. *Medical Microbiology*, 4th ed.; The University of Texas Medical Branch: Galveston, TX, 1996.

Received for review February 20, 2006. Revised manuscript received June 9, 2006. Accepted June 22, 2006.

JF0604992