Standard Operating Protocol: Determination of trimethylamine oxyde + trimethylamine in cod samples as an instrument to discriminate light salted vs. desalted cod products.

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1. INTRODUCTION / EXECUTIVE SUMMARY.

The present Standard Operating Procedure is part from the deliverables of the SALDICOD project, whose main objective was to provide analytical tools for the correct identification of cod products. The method detailed below has been developed to be readily incorporated by control laboratories, and in order to prevent fraud by discriminating light salted vs. desalted cod products.

Cod heavy salting and desalting leads involves intense variation in the muscle structure as well as the release of some muscle compounds to brine. Cod trimethylamine oxide (TMAO) levels in muscle are significant, even within fish groups, and this compound has been studied as a potential indicator of the heavy salting process. Results of the analysis performed in the SALDICOD project indicate that there is a significant difference in the concentration of this compound in cod muscle between products submitted to heavy salting and desalting (desalted cod) and materials that have not undergone intensive salting (unprocessed cod or light salted). Trimethylamine oxide undergoes degradation to trimethylamine (TMA) and/or dimethylamine (DMA) and formaldehyde (FA) during shelf-life depending on the storage conditions. This is the reason why the levels of TMA are considered as a freshness index for several species. The method selected is independent of the degree of spoilage of the product since it includes the contribution of both TMAO and TMA and expressed as the sum of the nitrogen present from TMAO and TMA. Even though the levels of DMA are not considered, the low values detected even in severely spoiled cod products within the range used for discrimination, shall not affect the final result.

Statistical modelling of about 110 results, including trials at pilot plant and laboratory and real market samples, indicate that a practical threshold for discrimination of the samples can be set at 12 mg N /100g Therefore, with a confidence level of a 95%, samples with contents below threshold shall be considered as desalted products (submitted to heavy salting) meanwhile samples with results above threshold shall be considered as not submitted to heavy salting (light salted / unprocessed).

2. SCOPE AND REFERENCES.

The method is based on the studies about the degradation of trimethylamine oxide compounds in fish from Parkin & Hultin (1982). The determination of TMAO is carried out by means of a preliminary reduction of this compound to TMA, using titanium trichloryde, and then measure total TMA with the traditional Dyer´s method (1945), including the Amano, Enokihara, Tozawa (1971) modifications. Some additional modifications have been included by ANFACO-CECOPESCA.

– *Parkin K. L. and Hultin H. O. (1982) Fish muscle microsomes catalyze the conversion of trimethylamine oxide to dimethylamine and formaldehyde. FEBS Lett. 139,61-64.1982.*

– *W. J. Dyer. (1945). Colorimetric Determination of Trimethylamine as the Picrate Salt. Amines in Fish Muscle: I. Journal of the Fisheries Research Board of Canada, Vol. 6d, No. 5 : pp. 351-358; 1945.*

– *Amano K; Enokihara K; Tozawa H. (1971) Proposed modification of Dyer's method for trimethylamine determination in cod fish. Fish inspection and quality control. p. 187-190. 1971.*

3. MATERIAL AND EQUIPMENTS.

- Laboratory blender (sample homogenization).
- Measuring cylinders, 500 ml, 250 ml, 100 ml, 50 ml.
- Ultraturrax homogenizer. .
- Volumetric flasks 100ml, 50 ml, 25 ml.
- Beakers 100 ml, 250 ml.
- Funnels.
- Laboratory Centrifuge.
- Filter paper quantitative analysis.
- Analytical weighing scale.
- Glass Pipettes 25 ml, 10 ml, 5 ml.
- Micropipettes 10-100 µL, 100-1000 µL, 500-5000 µL.
- Glass test tubes with screw caps (20x150 mm - 30 ml, and 13x100mm-13ml, or similar)
- PerkinElmer's LAMBDA 25/35/45 UV/Vis spectrophotometer system, or equivalent.
- Water purifying system, bi-distilled or Milli-Q grade.
- Polypropylene tubes with screw cap (50 ml).
- Vortex mixer.

4. REAGENTS AND STANDARDS.

4.1 Reagents and reagent preparation.

- Hydrocloric acid (HCl) 37% . reagent grade.
- Trichloroacetic acid, reagent grade,
- Formaldehyde, solution 37%, reagent grade.
- Picric acid (in 35% water) .
- Toluene, analytical grade.
- Potassium nitrate, analytical grade.
- Titanium(III) chloride solution about 15%.
- Sodium sulphate anhydrous.

- <u>25% hydrochloric acid</u>: Pour approximately 67 ml of commercial HCl (37%) in a measuring cylinder, and make up to 100 ml with distilled water.
- <u>5% (w/v) trichloroacetic acid (TCA)</u>: Weigh 50 g of TCA in a beaker and make up to 1 L with distilled water in a volumetric flask.
- <u>Formaldehyde solution 10%</u>: Pour 25ml of 37% formaldehyde in a measuring cylinder, and make up to 100 ml with distilled water.
- <u>Potassium hydroxide 25% (w / w)</u>: Weight 25 g of potassium hydroxide in a beaker and dissolve it with 75g of distilled water in a beaker.
- <u>Stock solution of 2% picric acid:</u> Weight 2,00 ± 0.01 g of dry [1] picric acid in a beaker . Dilute with aprox. 50 ml toluene, transfer to a 100 ml (Class A) volumetric flask and make up to 100 ml with toluene. ***(Carefully read the Safety data Sheet of picric acid before preparing the solution).***

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[1] *Picric acid with moisture contents below 10% can be highly explosive if not properly handled. Avoid the use of heat and contact to metal materials. Proper drying of small quantities of material can be achieved using*

- Standard solution of picric acid (PA) 0.02% (w / v): Pipette 1 ml of the stock solution of picric acid to a 100 ml (Class A) volumetric flask with toluene.
- Sodium hydroxide solution 35%: 350 g of NaOH dissolved in 1 liter of distilled water.
- 10% potassium nitrate: 5 g of KNO_3 are weighed and brought to 50 ml with Milli-Q water.
- Hydrochloric acid (10% approx): 25 ml commercial HCl (37%) are taken and brought to 100 ml with Milli-Q H_2O.
- HCl (10%): 27 ml commercial HCl (37%) are taken and brought to 100 ml with Milli-Q H_2O.
- Titanium trichloryde (6%): 10 ml of titanium trichloryde (15%) are taken and brought to 25 ml with HCl (10%).

4.2 Standards and Standard preparation.

– Trimethylamine N-oxide 98% . (Aldrich)

- Stock solution of trimethylamine oxide (1mg N-TMAO / ml): Weight 136.9 mg of TMAO (Aldrich-98%) in a weighing funnel and take to a 25 ml volumetric flask (Class A) with distilled water and 250 µL of HCl (37%).

- Standard solution of trimethylamine oxide (0.05 mg N-TMAO / ml): Perform a 1:20 dilution of the Stock solution of TMAO. Pipette 2.5 ml of the stock solution and make up to 50 ml in a volumetric flask (Class A) with distilled water with previous addition of 500 µL of HCl (37%).

5. *OPERATIONAL PROCEDURE.*

5.1 Calibration solutions:

The yellow coloration obtained follows the Lambert-Beer law in the proposed range of 0.004 and 0.04 N-TMAO/TMA. Different calibration standards are prepared as follows:

In consecutive 25 ml flasks add 0 (STD Blank: 0 mg N-TMAO): 400 uL (STD 1: 0.004 mg N-TMAO), 1 ml (STD 2: 0.01 mg N-TMAO), 2 ml (STD 3: 0.02 mg N-TMAO), 3 ml (STD 4: 0,03 mg N-TMAO), and 4 ml (STD 5: 0, 04 mg N-TMAO). Dilute standards with the respective amount of TCA (5%) to get a final volume of 15 ml before adding 3 ml of $TiCl_3$ (6%). Swirl carefully and place the flasks inside a bath at 60 ° C for 10 min. After that and once cooled down, add 500 uL of KNO_3 (10%) and made up to 25 ml with TCA (5%).

5.2 Extraction of the compounds from cod sample and reduction of TMAO to TMA:

- Select muscle pieces from cod excluding any skin, visceral or bone parts. Introduce them in a laboratory blender and mix to create a soft paste.
- Weight 10 g of the sample and homogenize with 25 ml. approximately 5% TCA in a 50 ml Falcon Tube using an ultraturrax instrument for at least 20 seconds.

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petri dishes, and a desiccator / vacuum desiccator. Follow safety conditions strictly when manipulating this compound.

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- Stored in a refrigerator for 15 minutes to precipitate protein. Centrifuge at 3500 rpm for 10 min at 4°c. Filter the material through a funnel coated with a quantitative filter paper to a 100 ml volumetric flask (Class A). Repeat the process with additional volume of TCA (5%) (approx.15 ml). Use additional 5% TCA to transfer sample to funnels, filtrate and take to the final 100 ml.
- Depending on the expected contents, pipette from 1 to 5 ml of this extract, to a 25 ml volumetric flask. Then; as the case of standards, 10-14 ml of TCA (5%) (up to 15 ml sample), and 3 ml of $TiCl_3$ (6%) are added. Similarly the flasks are taken to a bath at 60° C for 10 min. After cooling down, 500 uL of KNO_3 (10%) is added. Wait until bubbling slows down and made up to 25 ml with TCA (5%). Transfer the sample to 50 ml tubes (PP).

5.3 Transfer of TMA to organic phase and color development.

- Take 5 ml of the standard and sample solutions to 30 ml glass test tubes. Add 1 ml of 10% formaldehyde, then 10 ml of toluene, and finally 3 ml of 25% potassium hydroxide. Cap the tubes, vortex for 10 seconds, and heat them in a bath at 30° C for 5 minutes. Vortex again for 10 seconds and let stand until the two phases are separated.
- Pipette 8 ml of the organic phase (top) to clean and dry glass test tubes (approx.13 ml) containing approximately 100 mg of anhydrous sodium sulfate. Vortex and let precipitate sodium sulphate.
- Pipette 5 ml of the dried organic phase to another tube already containing 5 ml con of standard solution of picric acid. Cap the tube and vortex for 5 seconds.

5.4 UV-VIS spectroscopy measurements.

- Turn on the instrument and wait 15 min. to warm up lamps.
- Open software and set instrument to single wavelength measurements mode at 410 nm.
- Type in the sequence chart sample names standard names and concentration.
- Set instrument to 0 absorbance using the STD blank solution.
- Consecutively fill the spectroscopy cell with STD and samples and read absorbance.
- Print absorbance and calibration results.

6. RESULTS.

6.1 Calculation of final results.

Final results are expressed as mg of nitrogen from TMAO and TMA present in 100 g of sample, and calculated as follows:

The Standard Blank is not included in calibration and the intercept is not set to 0. By linear regression, the calibration curve is calculated: y = a + bx, where:

x = mg of N-TMA in each STANDARD.
y = absorbance values for the samples.
a = intercept
b = slope of the curve.

Extrapolating the value of the absorbance of the sample on the calibration curve is calculated. The final result is calculated according to the expression:

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$$\frac{mg\ N-TMA\ (x)}{Final\ aliquot\ (5\ ml)} * \frac{Flask\ volume\ (25ml)}{Extract\ aliquot\ (1-5\ ml)} * \frac{Extract\ volume\ (50\ ml)}{g\ sample\ (10g)} * 100$$

$$= \frac{mg\ N-TMA}{100\ g}$$

g sample = weight of sample used to extract (20 g).
Extract volume = total volume extract acid sample extract (100 ml).
Final aliquot = Volume of the aliquot taken from the 25 ml flask to tubings (5 ml).
Extract aliquot = aliquot of extract taken to 25 ml flask (1-5 ml).

6.2 Interpretation of results.

A practical threshold for discrimination of the samples has been set at 12,8 mg N /100g at a confidence level of a 99%. Samples with values over threshold are considered as light salted samples, meanwhile samples with values equal to or below 12,8 mg N /100g are identified as desalted samples.

7. *INTERNAL CONTROL FOR QUALITY EVALUATION.*

7.1 Blank test:

A blank sample (aliquot of 5 ml of TCA 5%) will be prepared along with the samples. Interpolation of its absorbance in the calibration shall not lead to a result higher than 0.001mg N-TMA.

7.2 Linearity:

The R^2 of the curve shall be higher than 0,99. The ratio between the observed value and the expected value for each one of the standards shall be between 90 -110%.

7.3 Sensitivity:

The slope of the calibration curve shall be within the range (12,0 -14,5).

7.4 Trueness:

A spiked sample of 12 mg N-TMA will be also prepared along with the samples. Results will be accepted if the verification sample leads to a final result between 9.6 and 13.2 mg N / 100g.

Standard Operating Protocol: Determination of creatine in cod samples as an instrument to discriminate light salted vs. desalted cod products.

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1. INTRODUCTION / EXECUTIVE SUMMARY.

The present Standard Operating Procedure is part from the deliverables of the SALDICOD project, whose main objective was to provide analytical tools for the correct identification of cod products. The method detailed below has been developed to be readily incorporated by control laboratories, and in order to prevent fraud by discriminating light salted vs. desalted cod products.

Cod heavy salting and desalting leads involves intense variation in the muscle structure as well as the release of some muscle compounds to brine. Cod creatine levels in muscle are significant, and this compound has been studied as a potential indicator of the heavy salting process. Results of the analysis performed in the SALDICOD project indicate that there is a significant difference in the concentration of this compound in cod muscle between products submitted to heavy salting and desalting (desalted cod) and materials that have not undergone intensive salting (unprocessed cod or light salted).

Statistical modelling of 47 results, including trials at pilot plant and laboratory and real market samples, indicate that a preliminary practical threshold for discrimination of the samples can be set at 40 mg/100 g although this value shall be further revised with a larger set of data.

Therefore, with a confidence level of a 99%, samples with contents below threshold shall be considered as desalted products (submitted to heavy salting) meanwhile samples with results above threshold shall be considered as not submitted to heavy salting (light salted / unprocessed).

2. SCOPE AND REFERENCES.

The identification and quantification of the dipeptides (creatine, carnosine, anserine) was carried out according to HPLC method described by Mora et al. (2007). The method had to be adapted to fish samples since the original method developed by Mora et al. (2007) was intended to be applied in meat (pork). Successive trials lead to the introduction some modifications by ANFACO-CECOPESCA. The working range has been adapted to the levels present in light salted and desalted cod. The calibration range goes from 2,5 µg/L to 100 µg/L, which corresponds to 5 mg/100g to 200 mg/100g in the samples, although dilution of the sample extracts could be included to lengthen the working range.

– Mora, L.; Sentandreu, M.A. and Toldrá, F. (2007) Hydrophilic chromatografic determination of carnosine, anserine, balenine, creatine and creatinine. J. Agric. Food Chem., 55:4664-4669.

3. MATERIAL AND EQUIPMENTS.

- 20 ml, 25 ml,50 ml, 100 ml and 1000 ml(Class A) flasks.
- 6 cm diameter funnels.
- Micropipettes 100-1000 ul, 0.5-5 ml and tips.
- 10 ml pipette.
- Falcon Tubes (PE) 50 ml and 10 ml.

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- – Tubes (PE) with cap 10 ml.
- – Glass wool.
- – Volumetric cilinder 10 ml.
- – Plastic Pasteur pippetes
- – Ultraturrax homogenizer.
- – Centrifuge.
- – Eppendorf vials (1.5 ml).
- – HPLC vials with PTFE /silicone septum cap.
- – GHP syringe filter and membrane filter 0.22 μm
- – HPLC, coupled to an Atlantis HILIC (4.6 x 150 mm, 3 μm particle size) column and UV-detection.
- – Vortex mixer.

4. REAGENTS AND STANDARDS.

4.1 Reagents and reagent preparation.

- – Hydrocloric acid (HCl) 37% reagent grade.
- – Ammonium acetate. For analysis grade. .
- – Acetonitrile, HPLC grade.
- – Methanol, HPLC grade.
- – Water (double distilled and deionized or Milli-Q grade).

- – **HCl 1 N**: From commercial HCl al 37% (= 1,19 g/ml), pipette 8,3 ml to a 100 ml flask. Add Milli-q water to volume.

- – **HCl 0,01 N:** From HCl (1N) solution pipette 10 ml to a 1000 ml flask. Take to final volume with Millli-Q water.

- – **Mobile Phase A (** *Ammonium acetate 0.65mM (pH 5.5) in H_2O Milli-Q: Acetonitrile (25:75, v:v)).* Dissolve 0.052 g of Ammonium acetate in 250 ml of Milli-q water and equilibrate pH at 5,5 using HCl (0,01N). Afterwards mixture both solvents in a volumetric cylinder in 25:75 ratio.

- – **Mobile Phase B** *(Ammonium acetate 4.55mM (pH 5.5) in H_2O Milli-Q: Acetonitrile (70:30,v:v)).* Dissolve 0.364 g of Ammonium acetate in 700 ml of Milli-q water and equilibrate pH at 5,5 using HCl (0,01N). Afterwards mixture both solvents in a volumetric cylinder in 70:30 ratio.

- – **H_2O Milli-Q with 5% Methanol.**

4.2 Standards and Standard preparation.

- – <u>Stock solution of creatine (5mg/ml)</u>, Weigh 260,5 mg de creatine monohydrate (*C-3630 Sigma*) and take to 50 ml in a Class A volumetric flask with with HCl (0,01 N).

– Intermediate solution of Creatine (0,5 mg/ml). Pipette 5 ml of the Stock solution and take to 50 ml in a Class A volumetric flask with HCl (0,01N).

Calibration Standards.

Make the following dilutions of the Intermediate Solution of Creatine as follows:

– **STD Level 5 (100 µg/ml):** Make a 1:5 dilution using Millli-q water :acetonitrile (25:75, v:v) in Class A volumetric flasks.

– **STD Level 4 (50 µg/ml):** Make a 1:10 dilution using Millli-q water :acetonitrile (25:75, v:v) in Class A volumetric flasks.

– **STD Level 3 (25 µg/ml):** Make a 1:20 dilution using Millli-q water :acetonitrile (25:75, v:v) in Class A volumetric flasks.

– **STD Level 2 (10 µg/ml):** Make a 1:50 dilution using Millli-q water :acetonitrile (25:75, v:v) in Class A volumetric flasks.

– **STD Level 1 (2,5 µg/ml):** Make a 1:200 dilution using Millli-q water :acetonitrile (25:75, v:v) in Class A volumetric flasks.

5. *OPERATIONAL PROCEDURE.*

5.1 Calibration:

A calibration curve from 0.01 mg/ml to 0.30 mg/ml was prepared as specified in section 4.2, by injecting (triplicate) 20 µl of each of the prepared standards.

5.2 Extraction of the target compounds:

The extraction of dipeptides is carried out as follows:

– 10.0 g of cod muscle was homogenized with 20 ml of 0.01 N HCl in water in a 50 ml Falcon tube, using an Ultraturrax at 5000 rpm 30 seconds. Clean the ultraturrax with additional 5 ml inside the Falcon tube. Keep the tubes at 4°c for at least 15 min.

– The extract was centrifuged at 4.500 rpm for 15 minutes at 4°C. The supernatant was filtered in a funnel containing glass wool or a quantitative paper filter to a volumetric flask 25 ml (Class A).

– The precipitate was re-suspended with 10 ml of 0.01 N HCl, shake vigorously for at least 5 minutes, centrifuge and the supernatant filtered to the same volumetric flask. Repeat the process with additional 6 ml of 0.01 N HCl. Afterwards the flask is taken to a final volume of 25 ml with 0.01 N HCl. *(This extract can be stored at -20ºC up to 3 months).*

– Transfer 10 ml of the extract to a PE tube and centrifuge again in order to clean the extract.

– 300 µl of the centrifuged extract becomes de-proteinized with 900 µl of acetonitrile in a 1.5 ml Eppendorf vial. Keep in the refrigerator at 4ºc for approximately 20 min. Afterwards, the sample was centrifuged at 10.000 rpm for 10 min at 4ºC.

– An aliquot of the supernatant was filtered through GHP syringe filter (WATERS) 13 mm diameter and 0.20 µm of pore size, and directly analyzed by HPLC system.

- **Chromatographic conditions.**

The peptides creatine, carnosine and anserine were analyzed by HPLC, through a silica column Atlantis HILIC (4.6 x 150 mm, 3 µm particle size) from WATERS. The instrument is a HPLC Alliance 2695, Waters coupled with UV detection (214 nm).

The mobile phases filtered through membrane filter GHP 0.22 µm prior analysis consisted in:
– Solvent A, 0.65 mM ammonium acetate pH 5.5 in water/acetonitrile (25:75, v/v).
– Solvent B, 4.55 mM ammonium acetate pH 5.5 in water/acetonitrile (70:30, v/v).

Flow rate: 1.4 ml/min.
Gradient conditions:

Minutes	Solvent A	Solvent B
0	100%	0%
6.5	50%	50%
12	100%	0%

The average time of retention of the creatine peak is 4,43 min ± 0.08min.

6. *RESULTS.*

6.1 Calculation of final results.

By linear regression, the calibration curve is calculated for each dipeptide as y = a + bx, where, "y" is the area, "x" is the mg of dipeptide, "a" the point of intersection with Y axis, and "b" de slope of the curve. A specific curve was obtained for each one of the target compounds.

To calculate the rate of creatine in muscle (**mg compound / 100 g):**

$$\frac{mg\ creatine\ (x)}{ml} * \frac{final\ vial\ volume\ (1,2\ ml)}{Final\ aliquot\ (0.3\ ml)} * \frac{flask\ volume\ (50\ ml)}{g\ sample\ (10\ g)} * 100$$
$$= \frac{mg\ creatine}{100\ g}$$

6.2 Interpretation of results.

A practical threshold for discrimination of the samples has been set at 40 mg creatine / 100 g at a confidence level of a 99%. Samples with values over threshold are considered as light salted samples meanwhile samples with values equal to or below 40 mg creatine / 100 g are identified as desalted samples.

7. *INTERNAL CONTROL FOR QUALITY EVALUATION.*

7.1 Precisión:

The RSD (%) of the peak areas among triplicate standards in each level of the calibration will not exceed 10%.

7.2 Linearity:

The r^2 of the curve shall be higher than 0,99. The response factor (RF: calculated as the slope of the curve between the origin and the average result of the triplicates, divided by the slope of the curve) for each one of the standards shall be between 90-110%. The calibration curve will be revised every three months

7.3 Verification Standard.

A verification standard of 20 µg/ ml (corresponding to 40 mg/100g) will be included in each set of analysis in order to detect any deviation of the calibration and to assure the accuracy of the results. The time of retention (tR) of the creatine peak will not deviate more than 0.5 min from the standards. The result of the verification standard will be within the range (38 mg/100g - 42 mg/100g).

Only in the case of a satisfactory result of the calibration standard, the samples will be analyzed. In the case the value is not within the permitted range, a new verification standard will be prepared. If the result continues to be unsatisfactory, the instrument and the method will be out of order until revision.

7.4 Blank:

In order to check any interference in the chromatographic system, a standard blank can be injected. Blank chromatogram shall not include any detectable peak within the time of retention of creatine.

7.5 Confirmation Criteria.

The time of retention of the peak of creatine in the verification standard and the unknown sample shall not deviate more than a 0,3 min.

Standard Operating Procedure: FT-IR Analysis of Cod Samples to discriminate Light Salted vs. Desalted Cod Products

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Contents

1 Purpose

This SOP describes the procedure for the analytical discrimination of desalted heavy-salted (HS) and light-salted (LS) cod samples by Fourier transform infrared spectrometry (FT-IR) and partial least squares discriminant analysis (PLS-DA).

FTIR analysis is used as sample fingerprinting technique. The fingerprint is then processed with a discriminant algorithm (PLS-DA) to discriminate HS from LS samples.

2 Safety requirements

- o Laboratory coats and gloves must be worn at all times in the work area.
- o Wear closed, flat shoes.
- o Long hair should be tied back.
- o The work area should be clean and tidy.
- o No eating, drinking or applying of cosmetics in the laboratory.

3 Equipment

- o Fourier Transform Infra-red (FTIR) spectrometer with Horizontal Attenuated Total Reflection (HATR) accessory (geometry: 45° parallelogram with mirrored angled faces, with nominal fifteen internal reflections).
- o UltraTurrax Homogeniser with support.
- o Electronic balance weighing to 0.1 g
- o Consumer laptop or desktop PC with proper statistical software (allowing to build PLS-DA models)

4 Consumables

- o Supply of local drinking water
- o Wash bottle for cleaning
- o 50 mL FALCON tubes
- o Deionised water
- o Acetone

5 Procedures

5.1 Sample preparation

Cut about 40 grams of cod loin.

Mince the muscle with a normal kitchen knife, removing bone, skin, fins, black membrane and possible foreign bodies (see Figure 1)
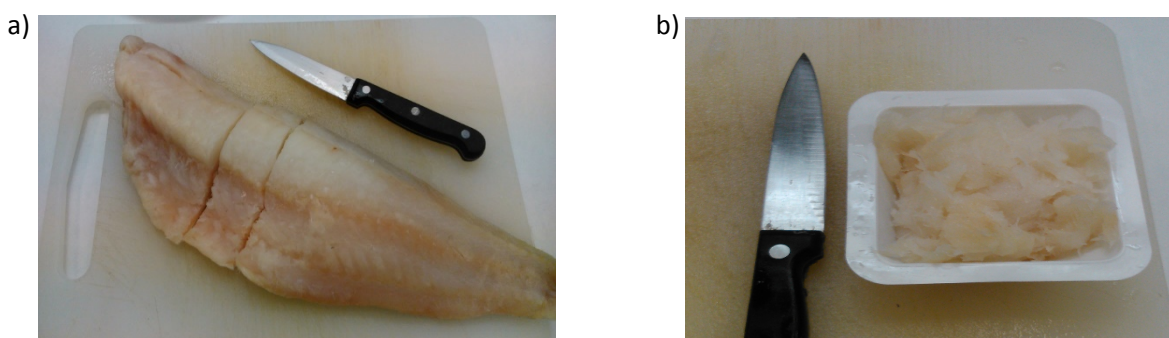
a)

b)

Figure 1. Cod loin before (a) and after (b)

Place the minced muscle into a 50 mL FALCON tube.

Homogenize the sample by an UltraTurrax equipped with dispersing element S 25 N - 8 G.

Homogenisation protocol is:

- 1 min at 6500 rpm;
- 1 min at 13500 rpm;
- 1 min a 24000 rpm.

During homogenization, keep the FALCON tubes on ice.

Homogenised sample must be kept covered until further sample handling.

For the analysis, each homogenised sample is split into three parts (aliquots) to be analysed sequentially, immediately upon preparation.

5.2   FTIR analysis

For each analysis, the sample must be overlaid on the HATR crystal to give total crystal coverage.

Spectra are collected at room temperature in a FT-IR Spectrometer equipped with an Horizontal Attenuated Total Reflection accessory (HATR), including transfer optics within the chamber through which infrared radiation is directed to a detachable ZnSe crystal plate. The crystal geometry is a 45° parallelogram with mirrored angled faces, with nominal fifteen internal reflections.

Collect the background spectrum before each sample measurement.

Thirty-two scans (at least) are performed to record each spectrum (nominal resolution of 4 cm^{-1} or better; spectral region of 4000–650 cm^{-1}).

After recording the spectrum, the sample is removed by the crystal with a scraper/spatula. The crystal is then cleaned with drinking water, deionised water, rinsed with acetone, and dried by using a lens cleaning tissue and a gentle cold air stream (see Figure 2).
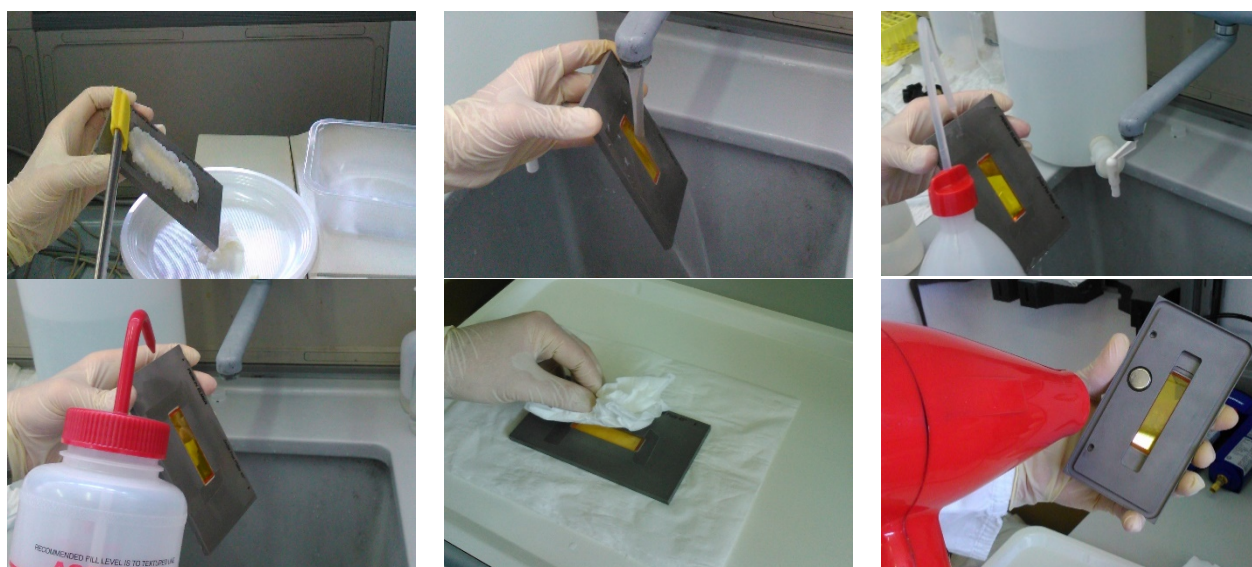

Figure 2. HATR crystal cleaning procedure

The cleaned crystal is carefully examined visually to ensure that no residue from the previous sampling is retained on the crystal surface, and it is checked with the background spectrum. Spectra are collected and used for multivariate analysis.

5.3 PLS-DA

5.3.1 Building the model

Import the spectra into a statistical software (MatLab or equivalent statistical software), also remarking the class they belong to (HS or LS).

Calculate the average spectrum for each sample (the mean of the spectra of the three aliquot of the same sample).

A sample library must be built, representing all the major variability sources that are expected in the future analyses (e.g. if samples from different countries are expected, the model samples must be from different countries). Samples are required to belong to both classes and to be from well-known origins. They have to be collected from different producers and treated in different ways (e.g. chilled, frozen, vacuum-packed, MAP-packed). A minimum of 10 samples for each category (category meaning a packaging kind per single producer, see Table 1), which derive from a minimum of 10 different fish per each producer, are required. Analysis of sample from each category must span from 0 to (at least) 7 days after packaging.

Higher number of samples increase the method performance.

Table 1. Example of sample library with minimum number of samples required. VP= Vacuum packed. HPP= High Pressure Processed

Class	Producer	Final format	Time after packaging (days)	Minimum number of samples
HS	Producer 1	Desalted-VP-Chilled	1, 2, 4, 7	10
		Desalted-HPP-VP-Chilled	1, 2, 4, 7	10
		Desalted-VP-Frozen	1, 2, 4, 7	10
	Producer 2	Desalted-VP-Chilled	1, 2, 4, 7	10
		Desalted-HPP-VP-Chilled	1, 2, 4, 7	10
		Desalted-VP-Frozen	1, 2, 4, 7	10
	Producer 3	Desalted-VP-Chilled	1, 2, 4, 7	10
		Desalted-HPP-VP-Chilled	1, 2, 4, 7	10
		Desalted-VP-Frozen	1, 2, 4, 7	10
LS	Producer 1	Light salted-VP-Chilled	1, 2, 4, 7	10
		Light salted-HPP-VP-Chilled	1, 2, 4, 7	10
		Light salted-VP-Frozen	1, 2, 4, 7	10
	Producer 2	Light salted-VP-Chilled	1, 2, 4, 7	10
		Light salted-HPP-VP-Chilled	1, 2, 4, 7	10
		Light salted-VP-Frozen	1, 2, 4, 7	10
	Producer 3	Light salted-VP-Chilled	1, 2, 4, 7	10
		Light salted-HPP-VP-Chilled	1, 2, 4, 7	10
		Light salted-VP-Frozen	1, 2, 4, 7	10

Calculate the 2nd derivative spectrum for each sample by using the Savitzky-Golay algorithm with a 15 points window and third-degree interpolating polynomial.

Build a PLS-DA model by using the auto-scaled 2nd derivative spectra also performing a "venetian blind" cross validation.

Look the minimum classification error in Cross Validation (CV), take the lower number of LVs (Latent Variables) providing the smallest CV classification error (in the case of the smallest CV error (achieved by both 1 and 2 LVs, then select 2).

The training data set can be improved through time by adding new samples from known origins. Each time the data set is increased, remember to check the CV classification error plot to verify the correct number of LVs to use.

5.3.2   Analysing unknown samples

Once the model is built, record three spectra (as reported in the section "FTIR Analysis") for each unclassified sample (a spectrum for each sample aliquot).

Calculate the average spectrum of the three aliquots.

Calculate 2nd derivative by using the Savitzky-Golay algorithm as reported in the previous section.

Insert the sample into the built model as validation sample and assign it to the class with the highest probability (calculated Y closer to 1).

6   Good laboratory practice and notes

- o   Avoid mixing melted ice with sample during homogenisation.
- o   The FTIR should be switched on 30 minutes before using, to allow the electronics to stabilise.
- o   Be sure that the HATR crystal is totally covered by the sample and that homogenised cod is well pressed onto the crystal surface (see Figure 3).
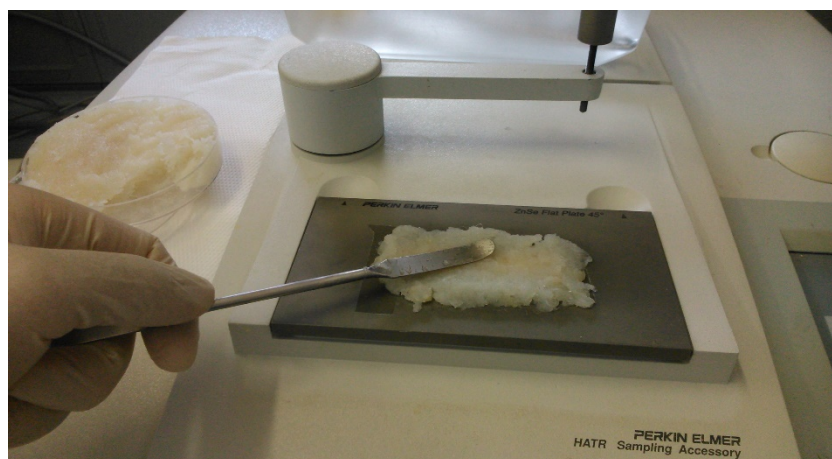- o   Use a non-metal scraper/spatula to remove homogenised cod from the HATR crystal (see Figure 2).

Figure 3. Sample overlaid on the HATR crystal before analysis.