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Monoethanolamine Treatment of Fish Wastes and Salmon Guts to Increase Its Palmitoylethanolamide and Anandamide Contents

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Abstract: This study was carried out to determine the palmitoylethanolamide (PEA) and arachidonoylethanolamide (AEA) or anandamide contents in selected fish wastes, treating the fish wastes with highest PEA and AEA with different concentration of monoethanolamine (MEA) solution, incubation temperature and time, as well as the ratio of MEA solution to fish waste to further increase its PEA and AEA contents.Based on the results of the preliminary experiment, a fractional factorial design experiments was done with 4 factors including MEA concentration, incubation time, incubation temperature and dosing ratio (MEA solution:salmon guts). The results showed that the MEA content ranged from 2.25 to 8.06 mg/g sample, the PEA content ranged from 17.4 to 300.2 µg/g sample while the AEA content ranged from 1.3 to 19.0 µg/g sample all on a wet weight basis of all the FD treated samples. The FD treated sample with the highest MEA, PEA and AEA using an MEA solution concentration of 250mM from pure MEA chemical, incubation time of 0.5 hour, incubation temperature of 6oC and a dose ratio of 6 mL MEA solution:100 g salmon guts. The MEA, PEA and AEA contents of the different samples were analysed using the Yates algorithm to determine which of the four factors were more important. The results showed that MEA, PEA and AEA contents were significantly affected by the concentration of MEA solution used in dosing the salmon guts, followed by the incubation time and then a slight effect of dosing ratio while the incubation temperature has no significant effect.

Keywords: Monoethanolamine, HPLC-UV, Palmitoylethanolamide, Anandamide, Fish Wastes, Salmon Guts

1. Introduction

Amide-linked fatty acids occur in nature in the form of ceramides as major components of sphingolipids and as N-acyl constituents of proteins. Among glycerophospholipids they are usually present in trace amounts as N-acylphosphatidylethanolamine (N-acyl PE) and N-acylphosphatidylserine (N-acyl PS) (Schmid et al., 1990). While both N-acyl PE and N-acyl PS may be functionally important components of biological membranes, the major interest in N-acyl PE has been its role as the precursor of N-acyethanolamines (NAEs), especially the endogenous cannabinoid, arachidonoylethanolamide (AEA) or anandamide (Di Marzo, 1998). Schmid (2000) hypothesized that different NAES, including anandamide, can mediate biological processes through targets other than cannabinoid receptors. The cellular levels of both N-acyethanolamines (NAEs) and N-acylphosphatidylethanolamine (N-acyl PE) appear to be tightly regulated under physiological conditions, these are increased in intact cells in response to stress and these are increased massively under conditions of cell and tissue degeneration and membrane degradation (Schmid and Berdyshev, 2002).

The identification and cloning of cannabinoid receptors (Pertwee, 1993; Howlett, 1995) in both brain (cannabinoid 1, CB1) (Matsuda et al., 1990) and peripheral tissues (cannabinoid 2, CB2) (Munro et al., 1993; Bayewitch et al., 1995) facilitated studies in the analgesic effects of cannabinoids. In addition, putative endogenous cannabinoid ligands have been described for both central CB1 (Devane et al., 1992; Fride and Mechoulam, 1993) and peripheral CB2 cannabinoid receptors (Facci et al., 1995). The brain constituent anandamide or AEA, has been shown to be produced by neuronal cells (Di Marzo et al., 1994) and have cannabimimetic effects (Smith et al., 1994; Mechoulam et al., 1996). Agonists at the CB1 receptor site have been shown to exhibit anti-nociceptive activity in models of acute (Smith et al., 1994; Stein et al., 1996) and neuropathic pain (Herzberg et al., 1997). As with endogenous opioid ligands, the duration of activity of AEA is thought to be short (Welch et al., 1995; Stein et al., 1996).

Activation of the CB2 receptor appear to be more involved in downregulation of the inflammatory response (Facci et al., 1995; Mazzari et al., 1996). It has recently become clear that CB2 receptors are expressed on cells of immune origin, including lymphocytes, mast cells and macrophages (Facci et al., 1995; Galiegue et al., 1995). Palmitoylethanolamide or PEA (a candidate for the endogenous ligand at the CB2 receptor) accumulates in inflamed tissue (Natarajan et al., 1982) and has been shown to reduce mast cell degranulation, plasma extravasation and hyperalgesia in a dose dependent manner (Mazzari et al., 1996). It has been proposed that the local production of PEA may lead to inhibition of both inflammation and sensitizing effects of inflammatory products on nociceptive processes (autocoid local inflammation, ALIA) (Levi-Montalcini et al., 1996); this may be a CB2 receptor mediated effect (Jaggar et al., 1998).

PEA is an endogenous fatty acid amide, an analog of the endocannabinoid anandamide (AEA), that belongs to the family of N-acylethanolamines NAE (Hansen, 2010). NAEs are released from cells in response to noxious stimuli. As all NAEs, also the PEA has a local effect, and its tissue levels are closely regulated through the balance of production and degradation activity (Passavanti et al., 2019).

The effects of the PEA are due to its interaction with several pathways: at first, it reduces, via the peroxisome proliferator-activated receptor alpha (PPARα), the recruitment and activation of mast cells at sites of nerve injury and the release of pro-inflammatory mediators from these cells (Costa et al., 2008; Cerrato et al., 2010); secondly, it inhibits the microglia activation and the recruitment of mast cells into spinal cord after peripheral nerve injury, as well as following spinal neuroinflammation or spinal cord injury (Genovese et al., 2008; Esposito et al., 2011). Sugiura et al. (2000) have demonstrated that PEA has just a very low affinity for cannabinoid receptor 2 (CB_2), clarifying why CB_2 antagonists do not inhibit some of its anti-inflammatory effects (Costa et al., 2002). PEA indirectly activates CB_2 and the cannabinoid receptor 1 (CB_1) (Petrosino and Di Marzo, 2017), down-modulating fatty acid amide hydrolase (FAAH), the enzyme responsible of the degradation of AEA, a CB_1 agonist (Di Marzo et al., 2001).

A proprietary method to highly enrich animal tissues with NAEs such as PEA and AEA using the process aid monoethanolamine (MEA) that increases their synthesis in situ was developed by Seperex Nutritionals (2008). In this process, NAEs are synthesized using the tissues own endogenous enzymes (acyltransferases and phospholipases) and phospholipid substrates (phosphatidylethanolamine, PE). Seperex Nutritionals Ltd. had already applied this process to green-lipped mussel meat and recommended its use on fish wastes. In New Zealand, there are several fish wastes from processing plants that this process can be applied like salmon guts, salmon mature and immature roes, hoki guts and hoki roe, as well as squid guts. Knowing the NAEs in these wastes is important since the waste with the highest NAEs would be the best sample to process with MEA to increase further its NAEs. This resulting product can be utilized as a pet food supplement particularly for older dogs and cats suffering from chronic pain and inflammation. Della Rocca and Gamba (2021) pointed out the use of micro-PEA for the chronic pain in dogs and cats.

The problem of fish wastes has increased over the years and becoming a global concern which is affected by several biological, technical, and operational factors as well as socio-economic drivers (Kim and Mendis, 2006; Arvanitoyannis and Kassaveti, 2008). It has been estimated that more than 50% of fish tissues including fins, heads, skin, and viscera are discarded as they are considered wastes. Every year discards from the world's fisheries exceed 20 million tons equivalent to 25% of the total production of marine fishery catch and include "non-target" species, fish processing wastes and by-products (Kim and Mendis, 2006; Mahro and Timm, 2007). Fish wastes and byproducts are increasingly gaining attention, as they offer a significant and sustainable source of high-value bio-compounds, due to their high content of collagen, peptides, chitin, polyunsaturated fatty acids (PUFA), enzymes and minerals, suitable for biotechnological or pharmaceutical applications with high market value (Shahidi et al., 2019; Shavandi et al., 2020). Hence, the process to produce treated fish wastes with high NAEs is an additional technology for fish wastes processing.

Determination of MEA content in samples was needed for the analysis. Several of methods based on the HPLC with refractive index detector (RID) system were published (Supap et al., 2006; Voice and Rochelle, 2013; Zhao et al., 2015) and HPLC with ultraviolet (UV) detector system with derivatization were also reported (Ngim et al., 2007; Larsen and Sansom, 2008; Liu et al., 2009).

Analysis of the PEA and AEA in food samples can be done using the LC-MS system as shown by Cawthron Institute (2009), Abramo et al. (2014) and Esposito et al. (2021).

This study was carried out to determine the PEA and AEA in selected fish wastes and treating the fish wastes with highest PEA and AEA with different concentration of MEA solution, incubation temperature and time, as well as the ratio of MEA to fish waste to further increase its PEA and AEA.

2. Materials and Methods

2.1. Materials

The Sanger reagent (2% 1-fluoro-2,4-dinitrobenzene in acetone), MEA (99.5% pure), sodium bicarbonate, methanol, and hydrochloric acid (HCl) were purchased from the (Sigma-Aldrich, Auckland, New Zealand). The HPLC grade chemicals (99.9%) like Acetonitrile and Formic Acid were procured from Fisher Chemical (Loughborough, UK). The salmon guts, salmon immature and immature roes were obtained from High Country Salmon, Glenbrook, Twizel, New Zealand Salmon while the hoki guts, hoki roe and squid guts from Sanford, Auckland, New Zealand.

2.2. MEA Measurement

The MEA measurement method developed by Larsen and Sansom (2008) was modified in order to use an isochratic pump mode instead of the low-pressure gradient pump mode. The mobile phase used was 50% Acetonitrile and 50% of 0.1% aqueous formic acid solution. After many

evaluations the final method used is summarised as follows. A high-performance liquid chromatography (HPLC) Shimadzu (LC-10AD VP liquid chromatograph) with system controller (SCL-10A VP) equipped with a pump and auto-injector (SIL-10AD VP) and UV-vis detector (SPD-10AV) was used in the analysis of MEA from the hydrolysed fish wastes and standard solutions. The chromatographic column was a Luna 5 µm C18 (2) 100 A, 250 mm x 4.6 mm with a security guard. The detector signal was analysed using the LC Solution software to obtain the integrated area of the peaks from the chromatogram. The mobile phase was a mixture of 50% acetonitrile and 50% of 0.1% aqueous formic acid solution in isochratic pump mode with a flowrate of 0.50 mL/min. HPLC-UVvis measurements were done at room temperature of about 20-25^{o}C. The UV-vis detector was set with a wavelength of 340 nm for Channel 1 and 254 nm for Channel 2. A 5 µL of the filtered sample was automatically injected into the system for measurement. The retention time and peak area of MEA can be obtained from the print-out of the software. The peak area of various MEA standard solutions was correlated using linear and polynomial regressions. The regression equation with high coefficient of determination (r^2) but at the same time have more realistic values (i.e. no negative values) will was used in converting the peak area of the samples into MEA concentrations (mg/mL). The MEA content of the sample (mg/g sample) was obtained by dividing the MEA concentration with 0.1 g sample which is the amount contained in a 1 mL liquid sample.

The derivatization of the MEA standard solutions and the fish wastes samples followed a modified procedure of Larsen and Samson (2008). The fish wastes samples were prepared by getting 30-40 g and then using the method as follows: a) get 1.0 g of the fish wastes sample and put into a 15-mL plastic container. Add purified water to the 10 mL mark and mix the contents by shaking using a mechanical shaker for 1 min; b) obtain 1 mL each of the mixture into two 1.5 mL Eppendorf tube and centrifuge at 10,000 rpm for 5 minutes; c) a sample of aqueous layer (400 µL) from the each Eppendorf tube was obtained and placed into a 6-mL plastic container, then 400 µL of 2% Sanger's reagent in acetone solution, 400 µL purified water and 160 µL of 1 M sodium bicarbonate solution were added; d) the mixture was mixed by manual shaking the container and then incubating in a water bath at 50^{o}C for 1 hour; e) at the end of incubation, the container was taken out of the water bath and cooled down at room temperature; f) the mixture was added with 100 µL of 2 M HCl solution and the contents were well mixed; g) obtain 800 µL of this solution and place into another 6-mL plastic container (green top) and dilute with 400 µL of Methanol, and 400 µL of 0.1% aqueous Formic Acid solution; and h) the mixture was mixed well by manual shaking and then filtered thru a 0.45 µm PTFE filter into amber vials for HPLC-UVvis measurement of MEA.

The MEA standard solutions were prepared as follows: a) a stock solution of MEA (10.17 mg/mL) was prepared in methanol. Subsamples of these MEA standard solutions were taken and derivatized to prepare a calibration curve from 0.013 to 1.017 mg/mL which would equate to approximately 0.13 to 10.17 mg/g sample; b) derivatization was done by mixing the MEA standard solution (400 µL), Sanger's reagent in 2% acetone (400 µL), purified water (400 µL) and 1 M sodium bicarbonate solution (160 µL) in a 6-mL plastic container. Then steps (d) to (h) for the sample preparation of fish wastes was followed.

2.3. MEA Treatment of Fish Wastes

A preliminary experiment was done on the treatment of selected fish wastes added with 130 mM concentration of MEA solution and incubated at 10^{o}C for one hour with a dosing ratio of 4 mL MEA solution: 100 g fish wastes and then was freeze dried. Based on the results of this experiment, 4 factors were identified to be important in the production of NAEs from the MEA-treated fish wastes that gave the highest PEA. The factors include MEA concentration, incubation time, incubation temperature and dosing ratio (MEA Solution:Fish Wastes). A fractional factorial design in 4 factors and 2 levels was carried out as shown in Table 1.

2.4. Moisture Content and Product Yield of the FD Treated Salmon Guts

The initial and final moisture contents of the freeze-dried (FD) treated fish wastes were determined at Cawthron Institute and the product yield was calculated as shown below:

Product Yield = 100 x (Amount of FD Product/Amount of Treated Fish Wastes) (1)

2.5. PEA and AEA Analysis

About 35 g of each freeze dried untreated and MEA-treated fish wastes samples were sent to Cawthron Institute for the determination of their PEA and AEA using the LC-MS method (Cawthron Institute, 2016). All the results were expressed in µg/g of sample.

3. Results and Discussion

3.1. Calibration Curve and Chromatogram of MEA Solution Concentration

Figure 1 shows the calibration curve of MEA with the MEA solution concentration on the y-axis and the HPLC-UV vis area on the x-axis. The separation times for MEA ranged from 7.66 to 8.09 minutes. A linear model can be fitted on the data as shown below:

MEA Concentration (mg/mL) = 0.5143 x (HPLC Area/1x10^{7}) + 0.0026 (r^2 = 0.9933) (2)

The MEA content of the sample was calculated further as shown below,

MEA Content (mg/g sample) = MEA Concentration (mg/mL)/0.1 g sample/mL (3)

The coefficient of determination (r^2) is high at 0.9933 indicating a good fit on the data as also shown in Figure 1.

Table 1. Fractional factorial design in 4 factors and 2 levels for the treatment of fish wastes (with highest PEA content) with different MEA solution concentration, incubation time, incubation temperature and dose ratio (MEA solution:salmon guts) and the resulting MEA, PEA and AEA of freeze-dried products.

Treatment	Factor 1 MEA Concentration	Factor 2 Incubation Time	Factor 3 Incubation Temperature	Factor 4 Dose Ratio
T1	50 mM (-)	0.5 hours (-)	6ºC (-)	2 ml: 100 g (-)
T2	250 mM (+)	0.5 hours (-)	6ºC (-)	6 ml: 100 g (+)
T3	50 mM (-)	5.5 hours (+)	6ºC (-)	6 ml: 100 g (+)
T4	250 mM (+)	5.5 hours (+)	6ºC (-)	2 ml: 100 g (-)
T5	50 mM (-)	0.5 hours (-)	14ºC (+)	6 ml: 100 g (+)
T6	250 mM (+)	0.5 hours (-)	14ºC (+)	2 ml: 100 g (-)
T7	50 mM (-)	5.5 hours (+)	14ºC (+)	2 ml: 100 g (-)
T8	250 mM (+)	5.5 hours (+)	14ºC (+)	6 ml: 100 g (+)

Note: Numbers in parentheses are coded factors where (+) – High Level, (-) – Low Level

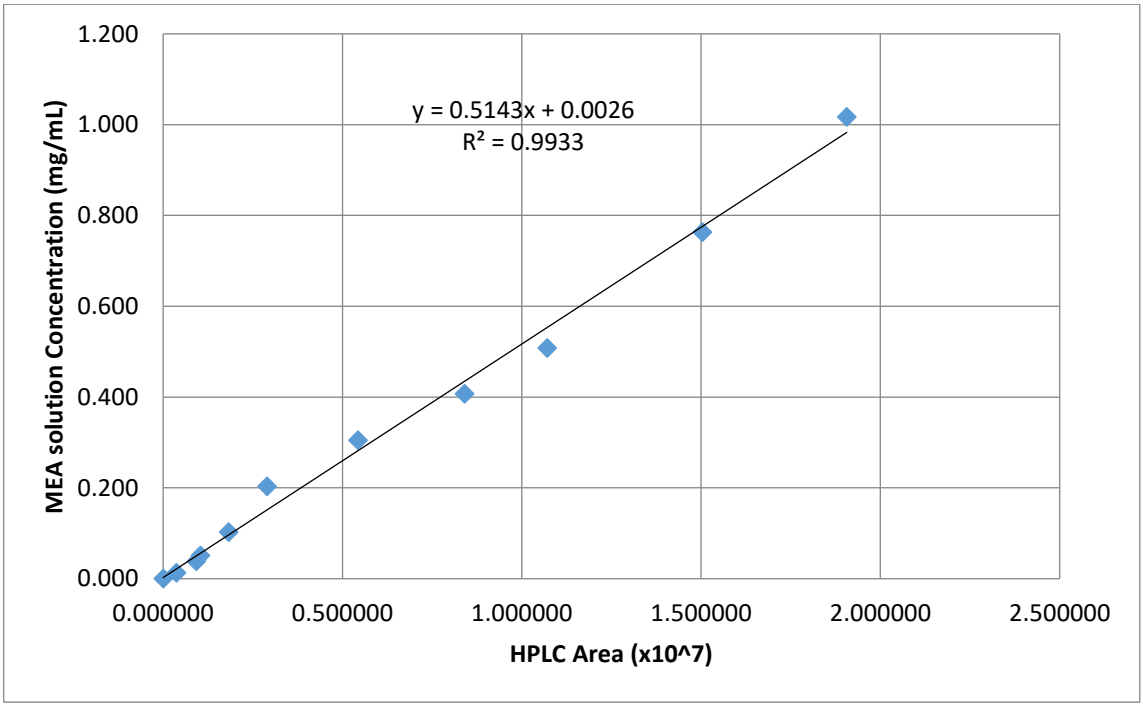

Figure 1. Calibration curve of MEA solution concentration.

The chromatogram of MEA solution with a concentration of 0.508 mg/mL is shown in Figure 2. The peak of the MEA came out at about 7.9 minutes. There were several peaks that also came out later at around 11.0, 13.6 and 14.3 minutes which were due to the mobile phase used. Figure 3 shows the chromatogram of the mobile phase only with 0 MEA concentration. As expected, no peak came out at around t=7.9 mins.

3.2. Preliminary Experiments on the Treatment of Selected Fish Wastes with MEA Solution

A preliminary experiment was done on the treatment of selected fish wastes added with 130 mM concentration of MEA solution and incubated at 10ºC for one hour with a dosing ratio of 4 mL MEA solution:100 g fish wastes and then freeze drying. Table 2 summarises the moisture, PEA and AEA contents of the different freeze-dried samples. The results show that the salmon guts gave the highest PEA (220 µg/g sample) and followed by the squid guts (130 µg/g sample) while the salmon immature roe the highest AEA (38 µg/g sample) and followed by the salmon mature roe (30 µg/g sample). The hoki roe and guts gave the lowest PEA. Based on the results, further experiments on the treatment of salmon guts with different concentration of MEA, incubation time, incubation temperature and dosing ratio (MEA solution:salmon guts).

De Luca et al. (2019) reported the PEA content of different fish meats ranged from 20 to 60 ng/g sample and even considering that fish guts might contain 10 times than the fish meat (~600 ng/g sample = 0.6 µg/g sample), would still be very low compared with the treated fish wastes obtained in this study. It must also be noted that the freeze-dried salmon mature roe gave the lowest moisture content (0.55% w.b.) while the squid guts had the highest moisture content (6.22% w.b.).

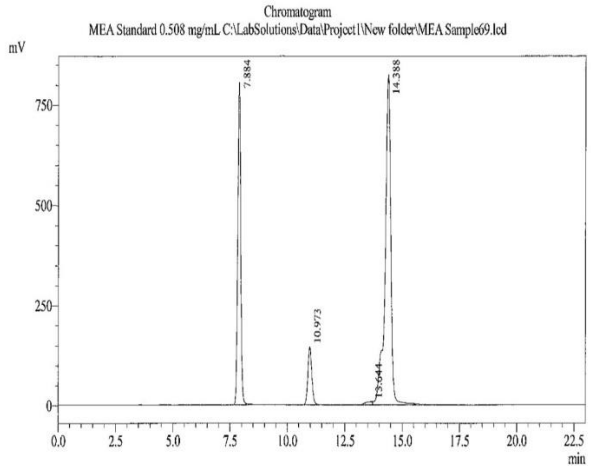

Figure 2. Chromatogram of MEA solution with a concentration of 0.508 mg/mL (MEA is shown at t=7.9 mins).

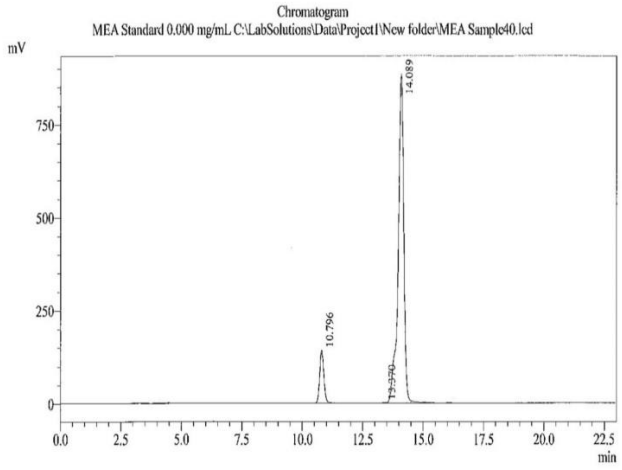

Figure 3. Chromatogram of the mobile phase only with 0 MEA solution concentration (no peak at t=7.9 mins).

3.3. Treatment of Salmon Guts with MEA Solution using a Fractional Factorial Design

Table 3 shows the moisture content, MEA content and percent yield of the different treated and freeze-dried samples using a fractional factorial design. The moisture contents ranged from 2.61 to 3.29 g/100 g sample, the MEA content ranged from 2.25 to 8.06 mg/g sample (wet weight basis) and 2.32 to 8.30 mg/g sample (dry weight basis) and the percent yield from 43.92 to 50.79%. While Table 4 shows the PEA and AEA contents of the different treated and freeze-dried samples. The PEA content ranged from 17.4 to 300.2 μg/g sample while the AEA content ranged from 1.3 to 19.0 μg/g sample.

It must be noted that the LD_{50} of MEA for oral dose in rabbits was at 1.0 to 2.9 g/kg body weight (Knaak et al., 1997). Since, the LD_{50} for dogs was not found, the lower dose of 1.0 g/kg or 1000 mg MEA/kg body weight was assumed to apply for dogs, then all the samples were within the allowable dose even ingesting as much as 100 g of the treated salmon guts for the sample with the highest MEA content. It is worth noting that the highest PEA content of the treated salmon guts increased by 1.5 times of the untreated salmon guts while the AEA increased by 1.7 times.

The MEA, moisture, PEA and AEA contents of the different samples (Tables 3 and 4) were analysed using the Yates algorithm to determine which of the four factors were more important (Myers et al., 2009). The results showed that MEA, PEA and AEA were significantly affected by the concentration of MEA solution used in dosing the salmon guts, followed by the incubation time and then a slight effect of dosing ratio. There is also a high interaction effect between the concentration of MEA solution and incubation time and a slight interaction effect between concentration of MEA solution and dosing ratio. The incubation temperature has no significant effect on all the properties.

Table 2. Moisture, PEA and AEA contents for the different treated fish wastes with 130 mM concentration of MEA solution, dosing ratio of 4 mL MEA solution:100 g fish guts and incubated at 10°C for one hour and then freeze dried at 60°C.

Fish Wastes	Moisture Content (% w.b.)	PEA (μg/g sample)	AEA (μg/g sample)
Salmon Mature Roe	0.55	89	30
Salmon Immature Roe	2.02	110	38
Salmon Guts	2.12	220	11
Hoki Roe	2.07	28	3
Hoki Guts	2.57	82	11
Squid Guts	6.22	130	7

Table 3. Moisture content, MEA content and percent yield of the different samples of freeze-dried treated salmon guts in the screening experiments.

Treatment	Moisture Content (g/100g) (wb)	MEA Content (mg/g) (w.w.b.)	MEA Content (mg/g) (d.w.b.)	Percent Yield
T1S	3.14	3.05	3.15	45.83
T2S	2.84	8.06	8.30	43.92
T3S	2.93	2.25	2.32	46.11
T4S	2.61	7.87	8.08	48.68
T5S	3.29	3.18	3.28	44.50
T6S	2.60	7.57	7.77	46.60
T7S	2.62	2.30	2.36	50.79
T8S	2.97	8.05	8.30	46.07

wwb – wet weight basis; dwb – dry weight basis; wb – wet basis

Table 4. PEA and AEA contents on a wet weight basis of the different samples of freeze-dried treated salmon guts in the screening experiments.

Treatment	PEA Content (µg/g) (wet basis)	AEA Content (µg/g) (wet basis)
T1S	17.4	1.3
T2S	300.2	19.0
T3S	34.6	2.4
T4S	120.6	7.0
T5S	28.9	2.4
T6S	252.1	17.5
T7S	44.1	2.8
T8S	138.4	9.1

4. Conclusion

The MEA treated salmon guts gave the highest PEA and followed by the squid guts while the salmon immature roe the highest AEA and followed by the salmon mature roe. The hoki roe and guts gave the lowest PEA.

The MEA, PEA and AEA were significantly affected by the concentration of MEA solution used in dosing the salmon guts, followed by the incubation time and then a slight effect of dosing ratio. There is also a high interaction effect between the concentration of MEA solution and incubation time and a slight interaction effect between concentration of MEA solution and dosing ratio. The incubation temperature has no significant effect on all the properties.

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Authors' contributions:

The article was written by LMD, as well as the data analysis.

Conflict of interest disclosure:

The author declares no conflict of interest on the written article.

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