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ORIGINAL PAPER

Production of Aroma-Rich Extracts from Sardine Cooking Wastewaters: Exploring Their Potential for Modulating Feed Intake in European Seabass

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Abstract

Managing the canning industry's nutrient-rich and odorous liquid waste is a hurdle. Concurrently, the growing use of vegetable ingredients in aquafeeds diminishes palatability and feed consumption in carnivorous fish. Thus, we hypothesized that aromas could be extracted from cooking wastewaters at canning factories and added to plant-based diets to stimulate intake in European seabass. Sardine cooking wastewaters were collected and tested directly (CW-A) or after vacuum distillation (VD-A) or liquid/liquid extraction with soybean oil (LLE-A). Despite losses in aldehydes and short-chain alcohols, both processes were effective in removing off-flavours. VD-A displayed a higher concentration of most aromas compared to LLE-A. Extracts were included at 2 µg g^{-1} of 1-penten-3-ol, the most abundant compound in all extracts, in diets (CW, VD, LLE). A non-supplemented diet was used as control. Each diet was assigned to six groups of juvenile fish, fed a single meal until apparent satiation. Our emphasis was on this initial feeding to comprehend the hedonic control of feed intake, minimizing habituation effects and the impact of the long-term metabolic requirements. Feed intake was highest for the control group. No differences on plasma metabolites were observed, suggesting feed intake was primarily regulated by hedonic rather than homeostatic mechanisms. Moreover, the lower intake in the supplemented diets was partially associated with a lower expression of orexigenic (intake-promoting) neuropeptides and higher expression of anorexigenic (intake-reducing) neuropeptides in the brain, despite the lack of significant diet-related differences. Overall, this study presents a novel approach to valorise cooking wastewater from the canning industry, since cooking wastewaters extracts rich in aromas were successfully produced, however, in the tested concentration, had no positive impact on the short-term feed intake response of European seabass.

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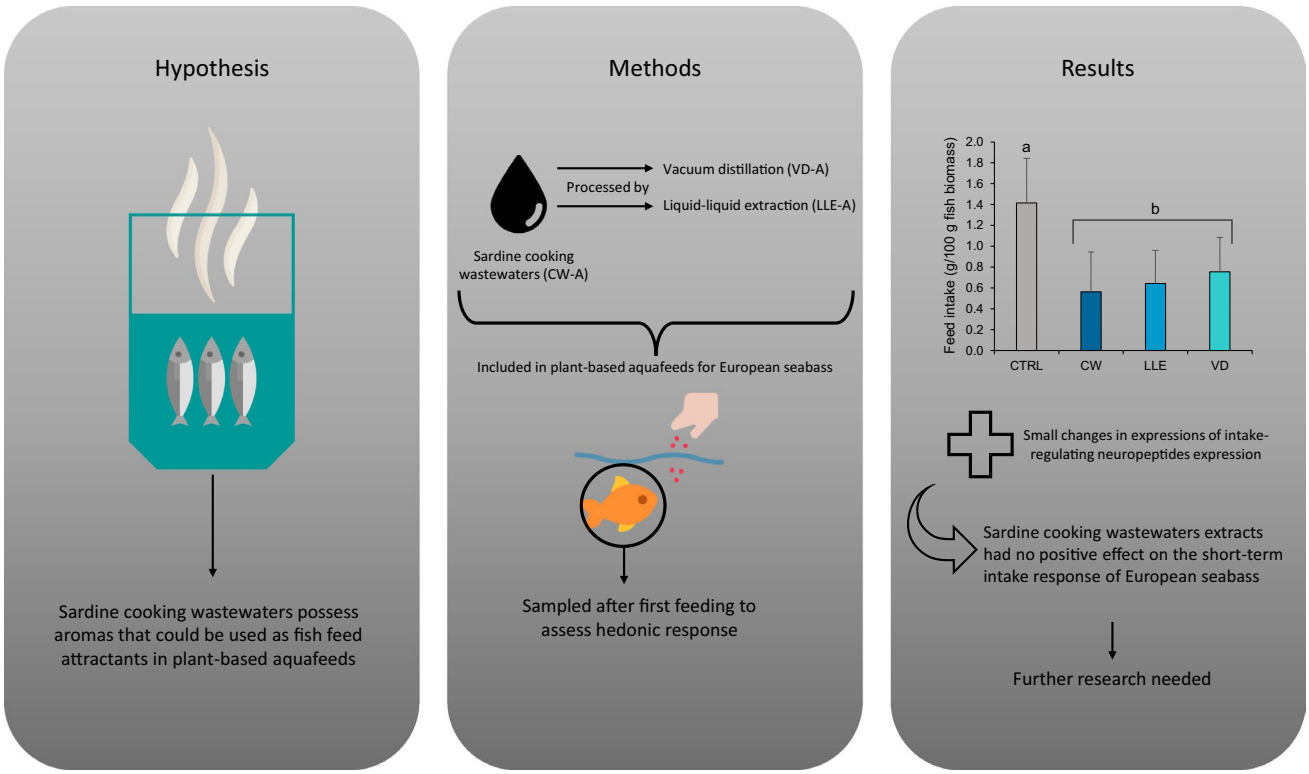
Graphical Abstract



Keywords Appetite stimulants · Palatability · Zero waste · Circular economy

Introduction

Currently, one of the key problems worldwide is waste management and this challenge is further aggravated by the growing complexity and quantities of industrial wastes produced all over the world. In this sense, reuse and valorisation of wastes and effluents is particularly significative.

The canning industry represents 11% of the worldwide fish market. In the particular case of Portugal, over 56 thousand tonnes of canned fish were produced in 2021, of which 20% corresponded to sardine products [1]. It is worth noting that for the production of each tonne of canned fish, up to 9 m^3 of liquid waste are generated [2]. This liquid waste is particularly complicated to process due to its high levels of salts, organic matter and odorous volatile compounds, including alcohols, aldehydes and ketones, which are released during the cooking process. Often, wastewaters are processed in conventional treatment plants with flotation devices, although other methods have been suggested, including evaporation processes [3], biotransformation [4, 5], and membrane filtration [4, 6]. Additionally, these novel methods may allow the recovery of concentrated fatty acids, proteins/peptides or even aromas, which could have further applications in other industries and contribute to the valorisation of this waste.

In parallel, the aquaculture sector faces several challenges regarding the sustainability of aquafeed production. Marine-based aquafeed ingredients, such as fishmeal (FM) and fish oil (FO), due to their scarceness, increasing prices and unsustainability, are being replaced by other sources, such as plant proteins and oils, which could minimize such issues [7]. Nevertheless, such replacement can be hazardous, due to the inclusion of antinutritional factors, inadequate amino acid or fatty acid profile and poorer nutrient digestibility [8, 9]. In addition, a major detrimental effect of these ingredients is the reduced palatability, and therefore reduced feed intake. Geurden et al. [10] noticed a reduction in feed intake when switching from a marine-based diet to a plant-based one, during the first week of feeding of rainbow trout (*Oncorhynchus mykiss*). Liang et al. [11] observed anorexia in *Lateolabrax japonicas*, when dietary FM was totally substituted by a plant protein blend, during the first 2 weeks of feeding. Similarly, Sabioni et al. [12] reported that the dietary inclusion of a soy protein concentrate above 50% diminished feed intake in dorado (*Salminus brasilensis*) by modulating certain appetite-regulating hormones.

Furthermore, Torstensen et al. [13] showed a reduced intake in Atlantic salmon (*Salmo salar*) smolts fed a diet with 80% of plant proteins and 70% vegetable oils, compared to a marine-based aquafeed, after three months of feeding. Even a mid-term reduction in feed intake followed by habituation to high-vegetable diets, where significant differences in feed intake patterns disappear, may affect overall growth performance of the fish, and cause large production losses [13], as this reduction may have impacted physiological and endocrine functions.

Moreover, feed can account for up to 40–60% of the production costs in fish farms [14], being a leading cause of waste production, because of leftover uneaten feed or dietary digestibility issues [15, 16]. Thus, the feed industry has been seeking novel strategies to stimulate feed intake, without compromising feed efficiency.

Feed intake modulation relies on the interaction of both homeostatic and hedonic signals, with the first relating to the animal's needs to fulfil energy requirements, and the latter resulting from pleasant sensations derived from feed [17]. While this is a complex regulating mechanism that involves several central and peripheral tissues and sensory organs, an integration can be found at the central nervous system level, mostly at the fish's hypothalamus and telencephalon [18, 19]. In this sense, feed attractants can directly interact with taste or olfactive receptors, promoting a hedonic response that results in increased feed intake. Interaction of food odours and olfactive receptors produces olfactory signals which are then transmitted to the brain, where they are integrated in the feed intake regulation network [20]. Although significant progress has been made, there remains ongoing exploration into the intricate mechanisms modulating feed intake regulation in fish. Ultimately, integration of homeostatic and hedonic signals affects the abundance of specific neuropeptides which may in turn affect feed intake, either suppressing it (anorexigenic) or stimulating it (orexigenic). These neuropeptides include the anorexigens cocaine and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC) and the orexigens agouti-related peptide (AgRP) and neuropeptide Y (NPY) [21, 22]. While neuropeptide expression in the hypothalamus has been closely linked with the homeostatic regulation, their role in the telencephalon might relate more to a hedonic regulation, although this is still unclear. Other mechanisms, such as the opioid system and endocannabinoid system, are also believed to play a role in the hedonic control of feed intake [23, 24], but their interaction with the expression of the aforementioned neuropeptides is still unclear. Over time fish can display habituation to diets, even if they are not particularly pleasing, due to nutrient necessities. Hence, evaluating feed intake and its regulation after short feeding trials can be an opportunity to screen for potential feed attractants and predict how they would affect fish feed intake [17, 22, 25], particularly regarding the impact of these attractants in the hedonic response, since the impact of metabolic needs in the first meal would be minimized.

Therefore, one way to overcome the issue of plant-based aquafeeds reduce palatability is the use of feed attractants [26]. Furthermore, fish display a very high sensibility to tastants and odorants, when compared to most mammals [20, 27]. In this regard, a number of feed attractants have been described in the literature, mostly including amino acids [28] and peptides/hydrolysates [29]. Nonetheless, compounds such as alcohols, aldehydes and organic acids, which have been reported to stimulate food intake in mammals [30, 31] are still widely unexplored in fish species. Moreover, feed attractants must be not only effective as such, but also economically and environmentally sustainable, and this could be achieved by valorising agri-food by-products, simultaneously contributing to circular economy policies [32]. As such, we have hypothesized that cooking wastewaters from the canning industry, characterized by a "fish smell", might be harnessed as a source of valuable aroma compounds for utilization as feed attractants. Thus, the goals of this work encompassed the extraction of aromas from sardine cooking wastewaters and the assessment of their potential as short-term feed intake stimulants in plant-based diets for European seabass.

Materials and Methods

Production of Aroma Concentrate Extracts

Effluent Collection and Reverse Osmosis

Sardine cooking wastewater from a local canning industry (A Poveira S.A., Laúndos, Portugal) was obtained after steaming sardines at 100 °C during 7 min. An antioxidant extract (acorn extract; 1% v/v) was added at the outlet of the cooking chambers to prevent lipid oxidation and aroma deterioration. This cooking wastewater aroma with the antioxidant, CW-A, was stored at −20 °C until further processed. A 100 L sample of CW-A was centrifuged (industrial West-Falia type ADB skimming centrifuge) for 30 min, resulting in one aqueous and one organic fraction. A reverse osmosis process with a TW30LE—2540 membrane (AMFOR, Inc, USA), with an area of 2.6 m^2, was then applied to the aqueous fraction to achieve a fraction richer in aromas.

Vacuum Distillation

This fraction (600 mL) was evaporated using a rotary evaporator (R-210, BÜCHI Labortechnik AG, Switzerland), under a feed temperature of 30 °C, and further distilled using a vacuum pressure of 25 mbar and a condensation temperature

of 0 °C. The final vacuum distillate extracted aroma, VD-A, was stored at −20 °C until further use.

Liquid–Liquid Extraction

The ratio *oil:feed* for liquid–liquid extraction with soy oil was set based on the ratio *final condensate:feed* achieved for the vacuum distillation process. Thus, extraction was performed at 30 °C, with agitation for 15 min at 240 rpm, using 10% soy oil, followed by centrifugation at 17,000 × *g* for 20 min. The liquid–liquid extracted aroma was named LLE-A and kept at −20 °C until use.

Diet Preparation

For the feeding trial, four isonitrogeneous (52% protein in dry matter, DM) and isolipidic (18% DM) diets were formulated, complying with European seabass nutritional requirements (National Research Council, 2011). The control (CTRL) was a practical plant-based diet, with 12.5% FM

Table 1 Identified aroma compounds in sardine cooking wastewaters and aromatic concentrates

Aroma compounds	CW-A		VD-A		LLE-A	
	Area	Conc.	Area	Conc.	Area	Conc.
Aldehydes						
Pentanal	342,274,014				242,047,008	
Hexanal	91,764,044				333,718,748	
Heptanal	55,695,101	0.008	3,057,639	0.050		
2-Hexenal, (E)-	224,708,703					
2-Heptenal, (Z)-					52,659,617	
Nonanal	62,709,909		91,162,319		11,237,254	
2-Octenal, (E)-	93,582,059		25,531,262		7,592,788	
2,4-Heptadienal	537,113,248	0.060	31,038,604	0.030	1,474,782	0.002
2,6-Nonadienal, (E,Z)-	128,500,271	0.060	122,048,941	0.059		
Alcohols						
1-Penten-3-ol	621,242,979	2.080	211,923,385	0.247	39,359,694	0.055
2-Penten-1-ol, (E)-	28,574,975		27,468,182		0	
2-Penten-1-ol, (Z)-	88,113,043.67	0.160	59,594,494	0.103	0	
1-Hexanol			212,159,530		71,875,385	
2-Hexen-1-ol, (E)-	38,769,652.33		23,485,942		9,079,827	
1-Octen-3-ol	155,667,784	0.005	252,085,522	0.010	16,925,971	0.002
1-Heptanol			143,858,319		5,919,625	
(5Z)-Octa-1,5-dien-3-ol	212,332,868		183,893,384		4,327,839	
1-Octanol			2,710,154,269		42,707,256	
1-Decanol					49,946,449	
Ketones						
2-Nonanone	72,661,377	0.003	104,099,006	0.005		
3,5-Octadien-2-one			81,267,303		16,549,597	
(3E,5E)-3,5-octadien-2-one	73,914,891		459,011,183		3,991,195	
2-Undecanone	31,415,463		191,942,580			
Acids						
Pentanoic acid					12,101,580	
Hexanoic acid					6,570,463	
Alkanes						
Pentadecane	66,233,128					
Heptadecane	152,304,028					
Sulphur compounds						
trans-2-(2-Pentenyl)furan	437,467,260					

Concentrations are shown in ppm

CW-A cooking wastewaters with antioxidant extract, *VD-A* extract from vacuum distillation, *LLE-A* extract from liquid–liquid extractions

and 4% FO. This was chosen due to the growing tendency of the use of plant-based ingredients and their known impact in feed intake [10–12]. Considering 1-penten-3-ol was the most abundant compound in the extracts (Table 1) and is also responsible for the flavour of fresh marine products [6, 33], aroma inclusion levels were set as to incorporate the same amount of this alcohol in all supplemented diets. The three experimental diets (CW, LLE, VD) were developed through the addition of each aromatic extract to the soybean oil fraction of the CTRL diet. All diets were extruded, as described in Resende et al. [34], and the oil fraction was further added by coating, in order to avoid extract degradation caused by extrusion temperatures during pellet manufacturing. Experimental diets were produced by SPAROS Lda. and stored at 4 °C until use. Table 2 describes diets ingredients and proximate composition.

Feeding Trial

Juvenile European seabass were acquired from a commercial fish farm (Atlantik Fish Lda., Portugal) and transported to the Fish Culture Experimental Unit of CIIMAR (Matosinhos, Portugal). Fish were kept in quarantine, in a 2000 L tank included in a recirculating saltwater system (RAS) for 2 weeks and, during this period, they were fed a commercial diet (Aquasoja, Sorgal S.A.; 50% crude protein, 20% crude fat, as DM basis) once daily. Nitrogenous compounds ($NH_4^+ \leq 0.05$ mg L^{-1}; $NO_2^- \leq 0.5$ mg L^{-1}; $NO_3^- \leq 5$ mg L^{-1}) salinity (35‰), temperature (21 ± 1 °C), dissolved oxygen (>90% saturation) and pH (7.5 ≤ pH ≤ 8.5) were regularly monitored and maintained at levels recommended for this species [35]. Photoperiod was a cycle of 12 h light/12 h dark.

After the quarantine period, fish were individually weighed (96.0 ± 13.8 g) and measured (total length, 20.7 ± 1.0 cm). Then, 24 homogeneous groups of 4 fish were randomly distributed by 50 L fiberglass tanks within a RAS (2.5 L min^{-1} flow rate). The remaining environmental conditions were the same as the quarantine.

After a 15-day acclimatisation period to the tanks, each diet was randomly assigned to six groups of fish that were fed until apparent satiation with the experimental diets. Sampling was performed 2 and 6 h after this single meal, where two fish were collected from three different tanks per diet (six fish/treatment) for each time point, to avoid handling stress at the second sampling point. Sampling times were selected based on previous similar studies [36, 37].

For the sampling, fish were collected and sacrificed by anaesthetic overdose (2-phenoxyethanol, Sigma-Aldrich, MO, USA). Fish were individually weighed (g) and measured (total length, cm). Blood was taken from the caudal vein using heparinized syringes and centrifuged (5000 × *g*, 10 min) for plasma collection, which was stored at −80 °C until analysis. Hypothalamus and telencephalon were collected, flash-frozen in dry ice and stored at −80 °C until mRNA extraction and quantification.

Table 2 Formulation and chemical composition of diets used in the feeding trial

	Diets			
	CTRL	CW	LLE	VD
Ingredients (%)				
Fishmeal[a]	12.5	12.5	12.5	12.5
Soy protein concentrate[b]	25.0	25.0	25.0	25.0
Wheat gluten[c]	15.0	15.0	15.0	15.0
Corn gluten meal[d]	10.0	10.0	10.0	10.0
Soybean meal[e]	11.0	11.0	11.0	11.0
Wheat meal[f]	9.1	9.1	9.1	9.1
Vitamin and mineral premix[g]	0.5	0.5	0.5	0.5
DCP[h]	1.5	1.5	1.5	1.5
Fish oil[i]	4.0	4.0	4.0	4.0
Soybean oil[j]	11.4	11.4	7.7	11.4
CW (mL)[k]		12.0		
LLE[l]			3.7	
VC (mL)[m]				100.0
Chemical composition (%DM)				
Dry matter	95.24	94.53	95.08	94.53
Ash	6.52	6.51	6.57	6.51
Crude protein	52.19	52.28	52.15	52.28
Crude fat	18.12	17.87	17.58	17.87
Energy (kJ/g)	22.65	22.84	22.68	22.84
Phosphorus	0.85	0.86	0.87	0.86
1-Penten-3-ol (µg/g)	0.98	2.10	2.16	2.10

[a]Fishmeal NORVIK LT, Sopropêche, France (72% crude protein, 7% crude fat)

[b]Soy protein concentrate Soycomil®-P, ADM, Animal Nutrition™, Netherlands (65% protein, 0.7% lipids)

[c]Wheat gluten composition: DM: 901 g kg^{-1}; protein: 838 g kg^{-1}; lipids: 16 g kg^{-1}

[d]Corn gluten feed from COPAM, Portugal (61% crude protein, 6% crude fat)

[e]Dehulled solvent extracted soybean meal, CARGILL, Spain (48% crude protein, 2.2% crude fat)

[f]Wheat meal from Casa Lanchinha Lda., Portugal (10.2% protein, 1.2% lipids)

[g]INVIVO 1%, Premix for marine fish, PREMIX Lda., Portugal

[h]Di-calcium phosphate

[i]Sardine oil, Sopropêche, France

[j]Henry Lamotte Oils, GmbH, Germany

[k]Extract from sardine cooking wastewaters

[l]Extract from sardine cooking wastewaters processed through liquid/liquid extraction

[m]Extract from sardine cooking wastewaters processed through vacuum distillation

Chemical Analysis

Diets were ground and analysed according to AOAC methods, as described in Resende et al. [34]. In short, dry matter (105 °C, 24 h); ash (combustion at 550 °C for 6 h, muffle furnace, Nabertherm L9/11/B170, Bremen, Germany); crude fat (petroleum ether extraction; Soxtec™ 2055, FOSS, Höganäs, Sweden); crude protein (N × 6.25; Leco nitrogen analyser FP-528, Leco Corporation, St. Joseph, USA); gross energy (adiabatic bomb calorimeter; Werke C2000, IKA, Staufen, Germany); and phosphorus (acid digestion of ashes, then phosphates quantification with ammonium molybdate via absorbance reading at 820 nm) were assessed in duplicate. Moreover, for identification of the present compounds and quantification of 1-penten-3-ol in aroma concentrate extracts and diets, SPME/GC–MS (solid phase microextraction followed by gas chromatography–mass spectrometry) was applied, according to the protocol reported in Pereira et al. [6]. A Shimadzu gas chromatograph (GCMS-QP2010) equipped with a WAX column (30 m × 0.25 mm i.d. × 0.25 µm) was used. The carrier gas was ultrapure helium, at 1 mL min^{-1}. The oven temperature program was as follows: 60 °C (4 min), followed by a gradual increase of 2 °C min^{-1} up to 180 °C. Injector temperature was 200 °C (limited by the desorption temperature expressed by the SPME fibre manufacturer). Detector temperature was fixed at 220 °C and ionization source at 200 °C. The ionization mode was electron impact with electron energy of 70 eV. A volume of 6 mL was then extracted by CAR/PDMS fibre during 15 min to 60 °C with and without stirring. The time of analyte desorption from the SPME fibre was set at 10 min. The injection was performed in the spitless mode for 2 min. After this period, the split ratio was set at 1:20 until the end of the chromatographic run. The internal standard was 2-nonanol. NaCl (2 g) was added to all analysed samples, in order to achieve a good extraction of the compounds.

Plasma Metabolites Quantification

Glucose, lactate, cholesterol and non-esterified fatty acids (NEFA) were examined enzymatically using commercial kits (Spinreact, Barcelona, Spain, for glucose, lactate and cholesterol; Wako Chemicals, Neuss, Germany, for fatty acids), adapted to microplate, as described in Velasco et al. [38]. All analysis were run in triplicate on a microplate spectrophotometer (BioTek Synergy HT, Vermont, USA).

mRNA Quantification of Neuropeptides Through RT-qPCR

Total RNA was extracted from whole hypothalamus or telencephalon using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations, with some modifications, followed by purification with a NZY Total RNA Isolation Kit (NZYTech, Lisbon, Portugal) as described by Ferreira et al. [39]. RNA quantity and purity was evaluated with a DeNovix DS-11FX spectrophotometer (Wilmington, DE, USA) and assessed based on the absorbance ratio 260:280 nm. Samples with a A_{260}/A_{280} ratio of 1.80–2.10 were considered for analysis. Then, 1.5 µg of RNA was reverse transcribed to cDNA with the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal), following the manufacturer's instructions.

Expression of neuropeptide y (*npy*), agouti-related peptide (*agrp2*), cocaine and amphetamine-related transcript (*cartpt2*) and pro-opio melanocortin (*pomca*) was assessed by real time quantitative PCR (RT-qPCR), using the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) as described in Basto et al. [40]. Neuropeptide forms were selected based on previous reports of their involvement in the intake modulation in European seabass [36, 40]. Reactions were performed with 40–400 nM of each primer (forward and reverse; Table 3), 5 µL of Green Supermix and 2 µL of cDNA, achieving a total reaction volume of 10 µL. Thermal cycling conditions were as follows: 95 °C for 2 min, followed by 40 cycles of two steps—first, 95 °C for 5 s, then primer annealing temperature (60–62 °C; Table 3) for 28 s. After the final PCR cycle, post-amplification dissociation curves were systematically monitored (60–95, 0.5 °C in each cycle) to guarantee reaction specificity. PCR efficiency was evaluated in serial twofold dilutions of cDNA through from a sample pool of all experiments, using the CFX Maestro 2.3 software (Bio-Rad, Hercules, CA, USA). Only values between 90% and 115% were accepted. All samples were analysed in duplicate and blanks without cDNA were run as negative control.

Ribosomal protein 40S (*rps40*), elongation factor 1 alpha (*ef1α*) and beta-actin (*βact*) were considered as housekeeping genes; the most stable genes or combination of genes were assessed using the qbase^{+} Software, considering the lowest M index and CV. The Pfaffl method was applied for relative quantification of target gene transcripts [41].

Table 3 Oligonucleotide sequences used to assess relative mRNA quantity through RT-qPCR

Gene	Primer sequence	Annealing temperature (°C)	PCR efficiency (%)	Accession number
agrp2	F: GGGCAGAGGACACAAAGAAA R: TGTGACTTTCCTGTGGTGGA	H: 62 T: 62	H: 114 T: 108	HE660087
npy	F: ACGGAGGGATACCCGGTGAA R: GCTGAGTAGTACTTGGCCAGCTC	H: 62 T: 60	H: 110 T: 113	AJ005378
cartpt2	F: CCGAACCTGACCAGCGAGAA R: GCTCCCCGACATCACACGTT	H: 62 T: 60	H: 107 T: 107	MZ441181
pomca	F: CCGGTCAAAGTCTTCACCTC R: ACCTCCTGTGCCTTCTCCTC	H: 62	H: 112	AY691808
β-act	F: CAAAGCCAACAGGGAGAAGATGA R: ACCGGAGTCCATGACGATAC	H: 60 T: 60	H: 113 T: 93	AJ537421
rps40	F: TGATTGTGACAGACCCTCGTG R: CACAGAGCAATGGTGGGGAT	H: 62 T: 62	H: 109 T: 106	HE978789
ef1α	F: AACTTCAACGCCCAGGTCAT R: CTTCTTGCCAGAACGACGGT	H: 60 T: 62	H: 94 T:112	AJ866727.1

Statistical Analysis

Data were examined for normality and homogeneity of variances according to Kolmogorov–Smirnov and Levene's tests, respectively, and, if needed, adequately transformed. Two-way ANOVA was applied to analyze data, using the Statistica software. If significant effects of treatments were found, means were compared through the pairwise Tukey multiple comparison test. When data did not comply with the ANOVA assumptions, a Kruskal–Wallis test was performed for each factor and the pairwise multiple comparison of mean ranks was carried out to identify significant differences between groups.

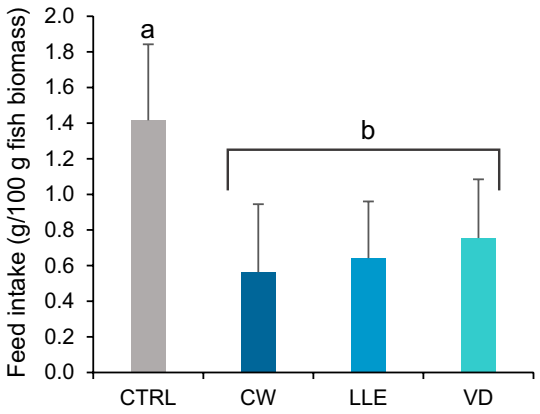


Fig. 1 Feed intake of fish fed the experimental diets after one meal. Values presented as mean + SD ($n = 6$). Different letters above the bars indicate significant differences among dietary treatments ($p < 0.05$)

Results

Extracts Characterization

LLE-A was the extract richest in aldehydes and organic acids, while VD-A displayed the highest amounts of alcohols and ketones (Table 1). No organic acids were observed in CW-A and VD-A extracts. Moreover, alkanes and sulphur compounds were only present in the CW-A and undetected in the processed aroma extracts. 1-Penten-3-ol was the most abundant compound in all fractions and, as expected, supplemented diets displayed similar levels of this compound and well above those found in the CTRL diet (0.9 vs. 2.1; Table 1).

Feed Intake

Figure 1 depicts the feed intake after the first single meal with the experimental diets. No significant differences could be found among supplemented diets and they all resulted in a significantly lower intake than that of the CTRL diet (0.5–0.8 vs. 1.4 g/100 g).

Plasma Metabolites

Plasma metabolites concentrations are reported on Table 4. No significant differences were observed for any of the analysed parameters, considering dietary treatments, sampling time and the interaction between these factors. However, the CW diet led to slightly higher plasma concentrations of NEFA, cholesterol and triglycerides, 2 h after the first feeding; these tendencies disappeared at the 6 h sampling point, where the CW diet actually led to slightly lower values of NEFA and triglycerides.

Table 4 Plasma metabolites of European seabass, 2 or 6 h after the first feeding with the experimental diets

	2 h				6 h				*p*-value		
	CTRL	CW	LLE	VD	CTRL	CW	LLE	VD	Diet	Sampling time	Interaction
Glucose	4.402 ± 0.582	6.281 ± 0.477	4.689 ± 0.395	5.014 ± 0.585	6.977 ± 0.616	4.116 ± 0.504	6.145 ± 0.765	5.246 ± 0.712	0.884	0.602	0.203
Lactate	3.969 ± 0.537	4.318 ± 0.810	3.582 ± 0.356	4.708 ± 0.457	4.085 ± 0.541	3.574 ± 0.819	4.642 ± 0.713	4.208 ± 0.578	0.827	0.968	0.456
NEFA	0.463 ± 0.186	1.474 ± 0.373	0.557 ± 0.183	0.467 ± 0.195	0.974 ± 0.296	0.411 ± 0.150	1.605 ± 0.375	0.881 ± 0.289	0.607	0.142	0.032*
Cholesterol	3.161 ± 0.541	4.995 ± 0.399	2.924 ± 0.282	3.427 ± 0.476	4.687 ± 0.592	3.250 ± 0.331	4.802 ± 0.725	5.151 ± 0.861	0.873	0.051	0.060
Triglycerides	8.246 ± 1.232	13.784 ± 1.641	7.430 ± 1.235	7.699 ± 1.522	8.708 ± 1.684	7.448 ± 1.654	12.355 ± 1.588	9.232 ± 1.595	0.429	0.893	0.052

Values presented as mean + SE ($n = 6$), in mmol L^{-1}

*Without significant differences after post-hoc test

Intake-Regulating Neuropeptides

No statistically significant differences were observed for the expression of neuropeptides involved in the regulation of feed intake, neither in the hypothalamus nor in the telencephalon (Fig. 2; Table 5). However, supplemented diets displayed a slight increase in the expression of orexigenic *agrp* on the telencephalon, 6 h after the feeding, compared to the 2 h sampling point. LLE also showed a similar tendency for the expression of *npy* in the hypothalamus. Regarding anorexigenic neuropeptides, the CTRL, CW and LLE diets displayed an increase in *cartpt* expression in the hypothalamus, 6 h after the feeding, a tendency which was not observed in the telencephalon. It was not possible to quantify *pomc* in the telencephalon, due to its low expression levels. In the hypothalamus, the expression of this neuropeptide was highest for the LLE diet.

Discussion

This work had the dual objectives of extracting aromas from sardine cooking wastewaters within the context of a circular economy and evaluating the influence of incorporating these aroma concentrate extracts into the diet on the short-term feed intake modulation of European seabass.

The developed aroma concentrate extracts were predominantly composed of alcohols and aldehydes, mostly derived from the breakdown of sardine oil during steaming [2]. The chemical aroma profile of the CW-A corresponds well to previous analysis of sardine cooking wastewaters supplemented with acorn extract [6]. Yet, some differences could be perceived as no quantifiable amounts of 1-octanol and hexanoic acid were observed in the present work, whilst pentanal was the third most abundant aldehyde in CW-A, contrarily to the previous study. The further processing of the cooking wastewaters extracts aimed at obtaining a fraction richer in aromas and without impurities. However, this caused losses in some compounds, including most aldehydes and all pentenols. In contrast, the vacuum distillation process led to an extract with higher concentration of long-chain alcohols (such as octanol and decanol) and ketones, while the LLE-A displayed higher amounts of organic acids. This suggests that aldehydes and short-chain alcohols (such as pentenols) are probably more prone to losses during processing. Most of these losses are thought to occur during the centrifugation and the liquid–liquid extraction or vacuum distillation phases. The reverse osmosis is precisely applied in order to have a more concentrated fraction prior to these latter procedures. This is confirmed by the higher concentrations of 1-penten-3-ol obtained by Pereira et al. [6], after reverse osmosis applied to the sardine cooking waters. Comparing the two processes, liquid–liquid extraction and

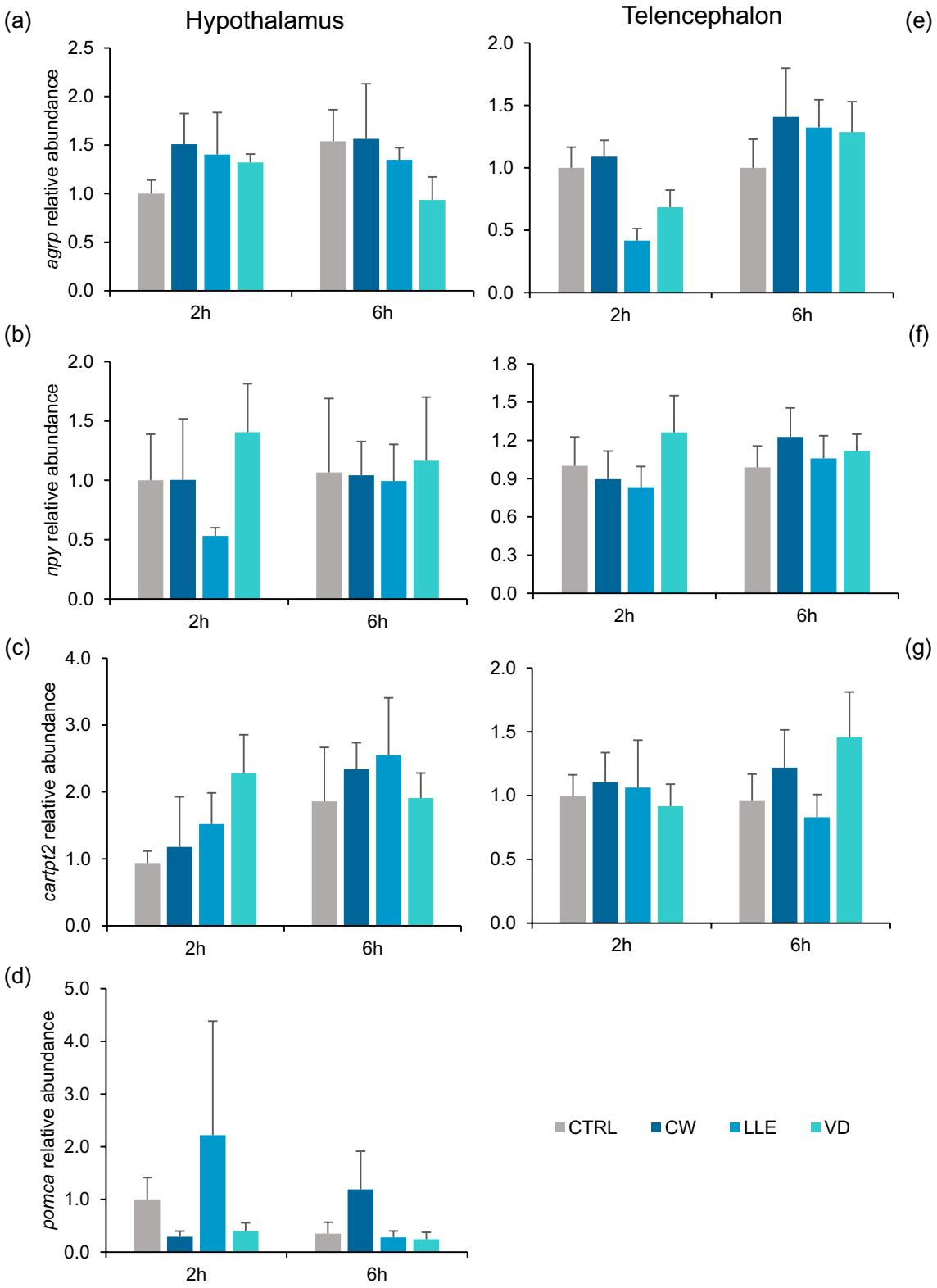


Fig. 2 Post-feeding mRNA abundance, in the hypothalamus (**a**–**d**) and telencephalon (**e**–**g**), of neuropeptides related to feed intake modulation of European seabass, after the first meal with the experimental diets. Results are presented as average + SEM ($n = 6$)

Table 5 *p*-Values of the two away ANOVA analysis of the neuropeptide expression in the hypothalamus and telencephalon of fish fed the experimental diets

	Neuropeptide	Diet	Sampling time	Diet × Sampling time
Hypothalamus	*agrp2*	0.602	0.857	0.604
	npy	0.811	0.550	0.273
	cartpt2	0.412	0.143	0.958
	pomca	0.560	0.336	0.489
Telencephalon	*agrp2*	0.439	0.008	0.062
	npy	0.791	0.482	0.921
	cartpt2	0.635	0.716	0.881

vacuum distillation, we observe that generally the VD-A displayed a higher concentration of several compounds, including the 1-penten-3-ol and 2,4-heptadienal, and thus seems more promising. This could be due to these compounds' lower hydrophobicity. However, it is important to note that the vacuum distillation process implies a cooling step, potentially increasing its price and energetic expenditure. Moreover, the aroma recovering processes are usually optimized considering the removal of off-flavours [42], generally defined based on literature regarding human sensory perception. Future studies in fish regarding the animals' response to individual aroma compounds, in order to better understand what is an off-flavour to the animals, would aid in the optimization of these processes towards application in aquafeeds. In any case, the developed processes allowed the successful removal of alkanes and sulphur-based compounds, generally classified as off-flavours and as having a "green beans" aroma [43, 44], from the CW-A, as they were not detected in the final extracts.

The European seabass is a carnivorous fish species, and thus the dietary inclusion of extracts rich in compounds such as hexanal, 2,4-heptadienal, 1-octen-3-ol, and 1-penten-3-ol, described as having a "fishy" or "meaty" odour [6, 45], could possibly be an innovative way to increase feed intake of plant-based diets. However, fish's response to odorants may differ from that of mammals, and thus not elicit this expected response [27, 46]. Senzui and Fukada [47] showed that supplementing a *Seriola quinqueradiata* diet with alanine, an amino acid with strong olfactory stimulant properties, promotes a "search" response in fish, without increasing feed intake. This suggests that olfactive properties of diet alone may not be enough to modulate the complex final response of feed intake. This could be one of the reasons that led to the worse response of fish to the aroma-supplemented diets, as reflected by the worse VFI displayed by these diets. In any case, these intake values are consistent with previous literature reports for this species fed traditional fishmeal diets [36]. The control diet led to VFI values which were significantly higher than all supplemented values, suggesting that the supplementation with these aromas was not effective at increasing feed intake. Studies using aroma enriched extracts as feed intake modulators are scarce, and thus understanding the reasons behind this poor efficacy is difficult. However, some literature reports have been performed, mostly based on electrophysiological studies. Aliphatic acids, particularly those with 3–5 carbons, have been reported as having stimulatory properties on Atlantic salmon (*S. salar*), common carp (*Cyprinus carpio*) and Japanese eel (*Anguilla japonica*) [28, 48], but long and medium chain fatty acids decrease feed intake [49, 50]. The LLE-A was the only extract in which fatty acids with lower carbon chains were found, but the hexanoic acid may already be deterrent, as observed by the lower VFI of this diet. Additionally, the CW-A had high levels of pentanal and 2,4-heptadienal, which may present a "rubber" and "rancid" odour [51], possibly being detrimental off-flavours in fish diets and hence resulting in decreased feed intake. Furthermore, most of the volatile compounds present in the tested extracts are a result of fish oil degradation during sardine cooking [2]. As such, and although they may have a "fish" odour to humans, they might be recognized by fish as being associated with rancidity rather than fresh fish, and thus act as deterrent. In this study, the response after a single meal was assessed. This approach avoids habituation responses, and also minimizes the effects of metabolic needs that are more influential in long-term trials, compared to the hedonic regulation of feed intake [40].

Analysis of neuropeptides in the brain regions most associated with feed intake regulation can provide information of the impact of dietary formulations on fish appetite. In this work, no statistically significant differences were observed among dietary treatments or sampling times, on both tissues assessed (telencephalon and hypothalamus). Likewise, in *Rachycentron canadum*, diets with different lysine to arginine rations that affected feed intake resulted in similar levels of *npy* expression [52]. In a short trial, variations in neuropeptide levels pre-feeding will be due to circadian response and would not provide additional information, as the fish have not been previously exposed to the experimental diets. However, in a longer trial, after daily exposed to the experimental diets, this approach may allow us to obtain information related to dietary-derived differences from a hedonic point of view. Additionally, it is worth noting that feed intake regulation depends on a complex integration of several neuropeptides and hormones, and thus the combination of non-significant changes in the individual expression of neuropeptides may result in significant alterations in the overall regulation of feed intake. In this sense, the LLE and VD diets displayed lower expression of the orexigenic *agrp* in the telencephalon, 2 h after the feeding, which may help explain the lower voluntary feed intake reported in fish fed such diets. Yet, this could not be observed 6 h after feeding,

which suggests that the expression of this neuropeptide might have already returned to its basal levels. In fact, at 6 h post-feeding, the expression of neuropeptides, in both brain tissues, seems rather similar among diets. This is in agreement with a previous report on Atlantic salmon (*S. salar*), in which expression of all neuropeptides in the brain was similar in unfed fish (representing basal levels) and 6 h after the feeding [53]. The higher expression of the anorexigenic *pomca* in the hypothalamus of fish fed LLE, 2 h after feeding, also partially explains the lower feed intake of this diet, when compared to the control. A similar rationale can be applied to the higher expression of the anorexigenic of *cartpt* in the hypothalamus of fish fed LLE, 2 h after feeding, compared to the control.

Stress has a clear impact on appetite regulation [54]. However, it should be noted that glucose and lactate levels, common stress indicators in fish [55], were statistically similar among diets and within expected values for this species [56]. Thus, it is not expected for the decrease of feed intake to be a result of stress of changing into a new diet. Feed intake is also modulated by nutrient metabolism (homeostatic regulation) [25]. Nevertheless, since diets were isolipidic, isoproteic and isoenergetic, and provide all essential amino acids and fatty acids for European seabass, it is not expected that alterations on feed intake are due to a consequence of homeostatic regulation, at least in the short-term. All fish had been previously fed the same diets for the whole quarantine and habituation period, and they should be at a similar metabolic state, which is unlikely to change drastically immediately after a single first meal. Moreover, in the literature, reports of short response times to changes in feed exist, probably due to an hedonic preference [57]. The results found for the plasma metabolites partially corroborate this hypothesis, as no significant differences on major metabolites (glucose, lactate, NEFA, cholesterol and triglycerides) were observed for any dietary treatments, either 2 or 6 h after feeding. This indicates a similar nutrient metabolism of all diets. Moreover, 6 h after feeding, the CW diet displays slightly lower levels of all plasma metabolites but displays a slight overexpression of anorexigenic neuropeptides. This suggests that the hedonic regulation is prevailing over the homeostatic regulation, since despite a decrease in circulating nutrients, fish were not responding with a potential increase on appetite. However, further analysis of putative hedonic signalling mechanisms, including markers from the endocannabinoid and opioid systems, along with dopamine or serotonin, are suggested in future work to verify this hypothesis. Something similar happened with the LLE diet, but 2 h after feeding—lower levels of plasmatic metabolites accompanied with an increase in the anorexigenic *pomca* and a decrease in orexigenic *npy*. It is also worth noting that these situations are similar, despite occurring at different post-prandial moments. In spite of the lack of significant changes in plasma metabolites between the two selected sampling points, 2 and 6 h post-prandial, in the long term these tendencies may lead to a significant effect on nutrient metabolism and possibly affect homeostatic intake modulation. Moreover, some aroma compounds, for example, heptanal, 2,4-heptadienal, 2,6-nonadienal, (E,Z) and 2-nonanone, often display antimicrobial activity [58–63] and could hence lead to microbiota alterations with a direct impact on nutrient metabolism. Therefore, a modulation of feed intake through homeostatic processes might occur, and at a longer term, this should be taken into account when assessing overall growth performance.

Further analysis of other molecules also involved in feed intake modulation, at central and peripheral level, such as ghrelin, cholecystokinin and orexins should also be performed in order to obtain a clearer picture of the impact of these diets on feed intake modulation, along with markers from the endocannabinoid and opioid systems, dopamine or serotonin, which are believed to be involved in the regulation of feed intake in fish. Analysis of fish feed behaviour could also shed light on whether these extracts promote search behaviours and if it is worth pursuing further optimization of these extracts.

Conclusions

Overall, we were able to extract several volatile compounds from sardine cooking wastewaters, highlighting the potential for waste valorisation. However, the supplementation of plant-based aquafeeds with such compounds, at the tested concentration, did not result in a positive short-term feed intake response for European seabass, neither considering the application of a direct cooking wastewaters, nor after further processing. To understand the full potential of these extracts to improve the feeding preferences of European seabass, further optimization of the extracts' production process, and/or of their inclusion levels in diets for European seabass should be considered. Additionally, it is worth noting that while the extracts did not yield the desired effects in European seabass, they may hold promise as attractants for other fish species or even for different animal models, and that hypothesis merits further evaluation.

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supervision, project administration, funding acquisition, writing—review and editing; CV: Conceptualization, methodology, validation, supervision, writing—original draft, writing—review and editing. All authors approved the final manuscript.

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Data Availability Data will be made available on request.

Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

Ethical Approval The animal experimental trial was approved by the CIIMAR ethical committee for Managing Animal Welfare (ORBEA) and conducted by accredited scientists in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGAV-Portugal), following FELASA category C recommendations. The trial was performed following the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals for scientific purposes.

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