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(54) **Title**: FEED ADDITIVES FOR AQUACULTURE AND AQUARIUM CULTURE

(57) **Abstract**: The present invention refers of feed additives for aquaculture and aquarium culture. These additives comprise the amino acid, 1-methyl-L-tryptophan, or its isomers with the objective of improving the attractiveness of feeds used in aquaculture and aquaria for fish, as well as other aquatic organisms, under culture conditions. Therefore, this invention has applications in the agriculture-food industry.

# DESCRIPTION

# "FEED ADDITIVES FOR AQUACULTURE AND AQUARIUM CULTURE"

## Technical area of the invention

The present invention is related to the agriculture-food industry in the production of feed additives for use in aquaculture and aquarium culture. As such, the objective of the present invention is to obtain new feed additives for aquatic organisms under culture conditions.

## Background to the invention

In the last few years the aquaculture and aquarium culture sectors have seen considerable growth. In 2003, aquaculture grew by 6.8%, surpassing the growth rates of other sectors such as aviculture (4.1%), fisheries (3.2%) and pig-farming (3.1%).

One of the species of great interest to the aquaculture industry, due to its high market value, is the Senegalese sole, *Solea senegalensis*.

However, one of the limitations that persist in the intensive production of the sole is its slow growth-rate due, in great part, to its reduced feed intake. This problem is also prevalent in other species generally considered with good aquaculture potential. Part of this problem results from the current use of generic feeds for Senegalese sole aquaculture which, not being specifically designed for the sole, are not attractive to it.

Analysis of the stomach contents of wild-caught Senegalese sole has shown that its principal natural prey organisms are

a polychaete (*Hediste diversicolor*) and a bivalve mollusc (*Scrobicularia plana*).

The Senegalese sole is nocturnal, spending most of its time buried in the substrate where chemical detection of predators and prey is carried out by the olfactory and gustatory systems. In fish, olfaction is used for initial detection of prey, although the gustatory system often has sensitivity to similar compounds as the olfactory system. Furthermore, the Senegalese sole, like other fish and aquatic invertebrates, has a highly developed olfactory system. The olfactory rosettes of the sole are relatively large and visible to the naked eye. The olfactory nerves and bulbs are also well-developed.

Following these observations, it was proposed that the identification of natural olfactory stimuli, being attractive and promoting feed ingestion, could be of great value to the aquaculture industry, given that they would not only promote food intake, with consequent increases in growth-rate, but also reduce the time taken for the detection and ingestion of feed, thus contributing to improvement of water quality.

Therefore, a laboratory study was developed to select and identify food-related stimuli in the sole, using a combination of electrophysiological and analytical chemical techniques.

**General Description of the Invention**

The present invention refers to the introduction of a specific amino acid as a feed additive for fish and/or other aquatic animals under culture conditions. As such, it

comprises of the addition of the amino acid 1-methyl-L-tryptophan, or its isomers, to feed with the aim of making these feeds more attractive at a gustatory and/or olfactory level.

The advantages of greater food ingestion are a smaller loss of uneaten food in culture tanks, resulting in greater feeding efficiency, and higher growth-rates and, consequently, greater efficiency for the producers. Moreover, there will be less need to clean the culture environment, contributing significantly to a better and more efficient maintenance of water quality in the culture of species of interest.

One of the principal requirements of aquaculture is rapid growth. However, some species show difficulty in ingesting feed in sufficient quantity to satisfy both their physiological needs and an adequate growth-rate. This may be due to the texture, flavour or smell of the feed or, indeed, other characteristics as yet not understood.

The addition of attractive substances that stimulate ingestion should, at least in part, solve this problem.

Previous studies have shown that some fish species, such as the Senegalese sole, have high sensitivity, specifically, olfactory, for an amino acid released by its preferred natural prey.
Therefore, the present invention is based on studies using this compound as additive to turn feed more attractive to economically important species such as fish, decapod crustaceans and cephalopod molluscs.

**Description of the Figures**

Figure 1. Olfactory response of the Senegalese sole to the amino acid 1-methyl-L-tryptophan. Semi-logarithmic plot of the normalized EOG amplitude (summary of the receptor potentials) against odorant concentration. Note the high olfactory sensitivity of the sole to 1-methyl-L-tryptophan with thresholds of detection less than $10^{-6}$ M. Data are shown as mean ± SEM (n=7).

Figure 2. Inhibitory effect of cross-adaptation to 1-methyl-L-tryptophan on the response top the C18 eluate of polychaete-conditioned water (x10 original concentration), recorded from the upper epithelium. Note that in the presence of excess 1-methyl-L-tryptophan, the response to the active compound in the eluate is reduced, suggesting that 1-methyl-L-tryptophan - or a similar compound capable of binding to the same olfactory receptor(s) is present in the eluate. Data are shown as mean ± SEM (n=6).

Figure 3. Chemical structure of 1-methyl-L-tryptophan.

**Detailed description of the invention**

The present invention refers to the use of 1-methyl-L-tryptophan in fish feed or feed for carnivorous invertebrates in culture, such as decapod crustaceans or cephalopod crustaceans. More specifically, the mentioned amino acid is preferably added to the feed in a quantity between 0.001 and 10 mg for each kilogram of feed.

1. Identification of Odorants

To identify food-related odorants, 'conditioned' seawater (35 ‰) was prepared. '"Conditioned water" in the scope of the present invention means water that contains 100 g of living polychaetes *H. diversicolor*, one of the main prey species of the sole, per litre such that this water contains substances produced by the polychaetes and released to the water.

The conditioned water was then lyophilized to reduce its volume and concentrate the substances released by the polychaetes. The olfactory potency of this concentrate was assessed by electro-olfactogram (EOG).

In brief, the EOG evaluates the olfactory sensitivity of a fish to a given compound. By EOG, it is possible to evaluate whether a fish can smell a given substance and, if so, it can give information on its relative olfactory potency by comparing with a standard.

The EOG measures changes in the electrical potential of olfactory receptor neurons when stimulated by specific substances. The lower the concentration necessary to evoke a change in electronic potential, the higher the sensitivity is to that substance.

To carry out EOG recording, fish were anaesthetized. For anesthesia purposes water containing 100 $mg.L^{-1}$ 3-aminobenzoic acid ethyl ester followed by an intra-peritoneal injection of 3 $mL.kg^{-1}$ Saffan™ (Schering-Plough Animal Health, Welwyn Garden City, UK) was used. Aerated water at 12 ‰ was pumped over the gills via a tube placed in the fish's mouth. The EOG was recorded using a Grass CP122

AC/DC amplifier (Grass Technologies, AStro-Med, West Warwick, RI, USA), and glass micropipettes filled with 3 M NaCl in 1% agar. The recording electrode was placed close to the olfactory rosette at a position which gave the maximal response to the standard stimulus ($10^{-3}$ M L-cysteine) and the reference electrode was placed lightly on the skin nearby. The signal was digitalized (Digidata 1322A, Molecular Devices, Inc., Foster City, CA, USA) and saved on a computer running Axoscope™ software (version 9.1, Molecular Devices, Inc.). All stimuli were dissolved directly into water at 12 ‰. One minute was allowed between successive stimuli. The different stimuli were given in a varied order, but always in order of increasing concentration.

Using the EOG, it was shown that the lyophilized water contained substances detected by the olfactory system of the sole.

The polychaete-conditioned water was then submitted to solid-phase extraction (SPE) by C18 cartridges, obtained commercially, thus separating relatively polar substances from non-polar substances. The cartridges were activated, following the manufacturer's instructions, with methanol followed by distilled water. Then a sample of 5 L (original volume) of lyophilized water was passed through the cartridge. The retained material was eluted with 50 mL, then concentrated by evaporation and stored at -20°C until analysis. The non-retained aqueous filtrate was also stored at -20°C.

The olfactory potency of both SPE fractions (retained fraction and non-retained aqueous filtrate) were tested by

EOG; the great majority of olfactory potency was found to be due to relatively non-polar compounds in the retained fraction.

Subsequently, the retained fraction containing relatively non-polar compounds was fractionated by high-performance liquid chromatography and the olfactory potency of the fractions obtained was assessed by EOG.

Liquid chromatography was done using an Agilent 1100 series system (Agilent Technologies, South Queensferry, West Lothian, UK), consisting of a quaternary pump, a de-gassing system, an auto-sampling injector, an automatic fraction collector, a column oven (maintaining the column at 28°C) and a diode array detector ranging between 200 and 300 nm. The column used was an Ascentis C18 (25 cm x 4.6 mm, 5 µm particles; Sigma-Aldrich, UK). The mobile phase consisted of distilled water for 5 minutes, followed by a linear water/methanol gradient over 30 minutes followed by 100 methanol for 5 minutes. The flow-rate was 1 mL per minute. Fractions were collected every two minutes. The absorption results were saved and analysed with the software Agilent ChemoStation.

Analysis by EOG showed that fractions 1 and 2 contained most of the olfactory activity. These two fractions were then subjected to liquid chromatography linked to mass spectrometry (LC-MS) for chemical identification.

The liquid chromatography conditions were similar to those outlined above, whilst mass spectrometry analysis was done with a Thermo-Finnigan LCQ Classic ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA) with a

binary LC pump (Series 200, Perkin Elmer, UK). The mass spectrometer was operated in positive ion electro-spray mode spray with a voltage of 4 kV. Nitrogen gas flows of 60 arbitrary units (sheath flow) and 20 arbitrary units (auxiliary flow) and a capillary temperature of 270°C were employed to produce stable spray conditions. Data were collected in the full-scan mode, over the range m/z 100 to 1200. Xcalibur software (Thermo Scientific, UK) was used to process the mass spectral data and produce total ion chromatograms for the separation.

Fractions 1 and 2 contained a principal peak with retention time of approximately 2.8 minutes and a molecular weight of m/z 219.4. Fractionation of this peak by MS-MS gave a fragment of m/z 205.3. This difference of 14 between the molecular weights of 219 and 205 corresponds with the loss of a methyl group. Furthermore, 205.3 is the molecular weight of tryptophan, suggesting that the compound present in fractions 1 and 2 is methylated tryptophan. Injection of commercially available 1-methyl-L-tryptophan (Sigma-Aldrich) showed identical retention time and mass spectra to fractions 1 and 2.

These results show that the compound with olfactory potency present in the water conditioned by *H. diversicolor* is methyl-L-tryptophan.

2. Preferred concentration of the amino acid in the feed

Given that the other existing isomers of methyl-L-tryptophan have the same mass spectra these were also considered in the scope of the invention.

In a preferred embodiment of the present invention the 1-methyl-L-tryptophan is used at concentrations between 0.001 and 10 $mg.kg^{-1}$ of feed. These relate to the concentration of such amino acid in the conditioned water and the comparison with the olfactory sensitivity of the sole which is lower than $10^{-6}M$, most likely $10^{-8}M$, based on electrophysiological studies and the fact that this is also the sensitivity of fish to amino acids fish. For fish or carnivorous aquatic invertebrates, which feed on the same or related natural diets as sole, and therefore are likely to smell 1-methyl-L-tryptophan, the most appropriate concentration will need to be determined. The concentration of 1-methyl-L-tryptophan to be used may also have to be adjusted according to the composition of the feed, e.g., if it has a higher percentage of vegetable components concentrations may need to be higher because carnivorous fish are less interested in plant material.

**Examples**

Example 1: Add 0.001 to 10 mg of 1-methyl-L-tryptophan to each kg dry weight of fish feed or carnivorous aquatic invertebrate feed.

## CLAIMS

1. Feed additives for aquiculture and aquarium culture **comprising** 1-methyl-L-tryptophan and/or its isomers.

2. Feed additives, according to the previous claim, 1-methyl-L-tryptophan and/or its isomers in concentrations between 0.001 and 10 mg per kilogram of feed.

3. Use of the amino acid 1-methyl-L-tryptophan and/or its isomers in the manufacture of feeds for aquaculture and aquarium culture.

4. Use of the amino acid 1-methyl-L-tryptophan and/or its isomers, according to the previous claim, as an attractive feed additive and inducer of ingestion.

5. Use of the amino acid 1-methyl-L-tryptophan and/or its isomers, according to any of the claims 3 or 4 at concentrations between 0.001 and 10 mg per kilogram of dry feed.

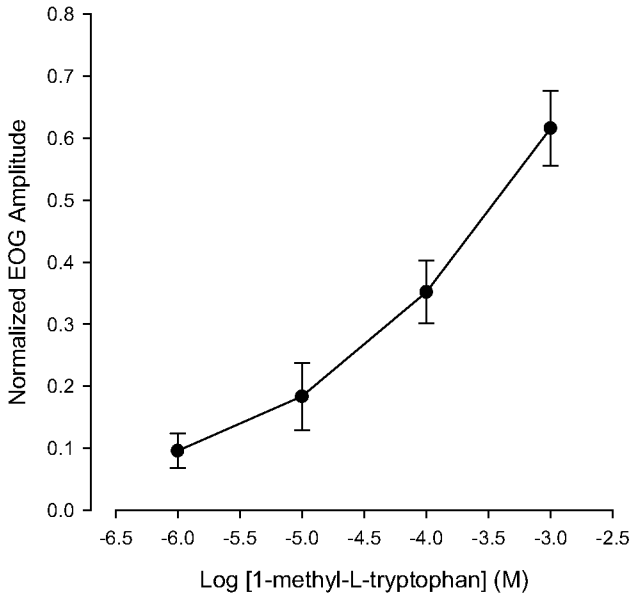
Normalized EOG Amplitude
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
-2.5
Log [1-methyl-L-tryptophan] (M)

Fig. 1

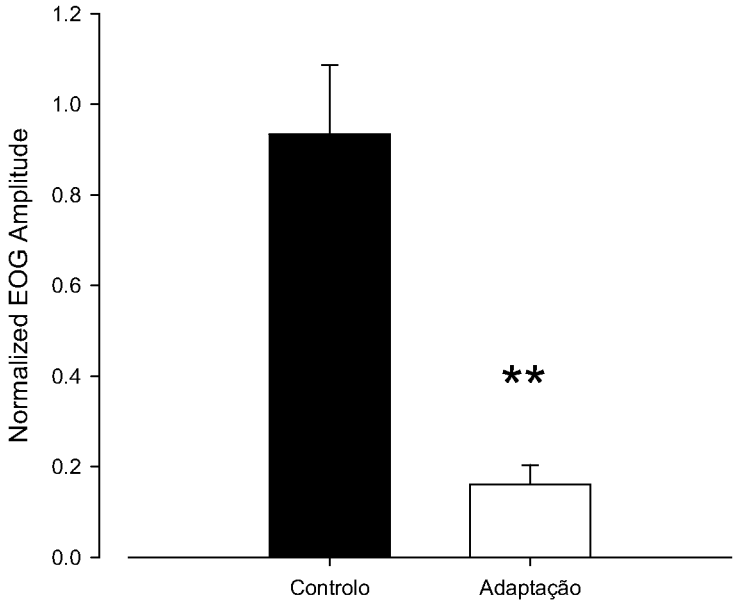
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Normalized EOG Amplitude
**
Controlo
Adaptação

Fig. 2

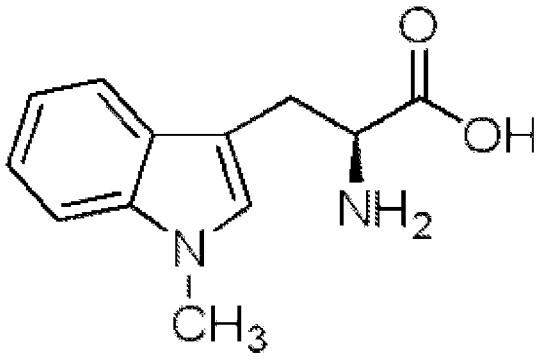


Fig. 3

# INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2010/053876

**A. CLASSIFICATION OF SUBJECT MATTER**
INV. A23K1/16 A23K1/18
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, EMBASE, FSTA, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | OH G S ET AL: "3-Hydroxyanthranilic acid, one of metabolites of tryptophan via indoleamine 2,3-dioxygenase pathway, suppresses inducible nitric oxide synthase expression by enhancing heme oxygenase-1 expression" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 320, no. 4, 6 August 2004 (2004-08-06), pages 1156-1162, XP027194349 ISSN: 0006-291X [retrieved on 2004-07-22] page 1157, left-hand column, last paragraph<br>-----<br>-/-- | 1,2 |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 12 November 2010 | 23/11/2010 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Smeets, Dieter |

# INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2010/053876

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 00/64274 A1 (WINBERG SVANTE [SE])<br>2 November 2000 (2000-11-02) | 1-3,5 |
| A | page 4, last paragraph - page 6, paragraph 3; claims | 4 |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2010/053876

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 0064274 A1 | 02-11-2000 | AU 4634200 A | 10-11-2000 |