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Amino Acids in New Organic Fertilizer AnchoisFert

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The analysis via GC-MS of the amino acids present in AnchoisFert, a new organic fertilizer co-product of fish oil extraction from anchovy fillet leftovers using limonene, unveils the presence of 16 amino acids, essential, quasi-essential and non-essential. Leucine, glycine, glutamic acid and alanine are the most abundant AAs. Proline, aspartic acid, arginine, serine, lysine and phenylalanine are also relatively plentiful. Alongside the results of the techno-economic and life cycle assessment analyses, these outcomes suggest that the "LimoFish" circular economy process is highly effective in recovering valued AAs that otherwise would be lost in the environment. This greatly improves the sustainability of anchovy fishing, processing and consumption, further supporting the scale-up and industrialization of the process.

Introduction

AnchoisFert is the new organic fertilizer[1] derived in one pot as co-product of fish oil extraction with limonene from anchovy fillet leftovers.[2] Showing a remarkably high content of carbon (40%) and nitrogen (12%) as well as of flavonoids and valued minerals, the fertilizer was recently found to be largely superior to commonly used organic (manure) and chemical (NPK) fertilizers.[1] Such high fertilization activity is due to the large amount of mineral nutrients (calcium, sulfate, magnesium, potassium, phosphate), and to the concomitant presence of a large amount of bioactive compounds such as total phenols (8507 μg g^{-1} total phenol content expressed as tannic acid equivalent) and flavonoids (1868 μg g^{-1}, expressed as quercetin equivalent).[1]

Besides being rich in omega-3 lipids and vitamin D in bioavailable vitamin D_3 isomer form,[3] that make it suitable to replace highly refined fish oil used to produce omega-3 dietary supplements,[4] the "AnchoisOil" whole fish oil co-extracted with AnchoisFert microencapsulated in mesoporous silica has even shown powerful anticancer activity *in vitro*,[5] which has lately been ascribed to its ability to decrease IL-8 gene expression, miRNA-21 and transcription factor NF-κB nuclear expression.[6]

Taken individually, either the production of the new whole fish oil[7] and that of the new organic fertilizer[8] are technically feasible and economically convenient. In brief, thanks to the "LimoFish" circular economy process, biowaste available in huge amounts (anchovy is the world's most caught fish) is converted into valued bioproducts in high demand using a non-toxic solvent derived from orange peel (limonene) which is nearly entirely recovered after the oil and fertilizer co-production.[9]

The aim of this work is to identify the amino acids comprising the AnchoisFert proteins and assess their relative abundance. The analysis was carried out via a simple method based on their derivatization with ethyl chloroformate followed by gas chromatography-mass spectrometer (GC-MS) separation and identification of the resulting ethoxy carbonyl ethyl esters.[10]

Involved in key metabolic processes vital to the health, growth, development and reproduction of organisms, amino acids are the building blocks of proteins and play an essential role in energy metabolism, neurotransmission, reproduction and immunity.[11] Not being synthetized by the human body, essential amino acids (EAAs) are entirely assumed through the diet by eating animal or vegetal foods rich in proteins, even though the nutritive value of vegetal proteins is lower due to lower or unbalanced EAAs content.[12]

Anchovies, either fresh or processed (salted or canned), are a source of noble proteins widely consumed worldwide. For example, all the essential amino acids required in the human diet are present in fillets of Peruvian anchovy (*Engraulis ringens*).[13] Similarly, researchers in Turkey recently identified nine EAAs (except from tryptophan) and nine non-essential amino acids in European anchovy (*Engraulis encrasicolus*) fished in the Aegean, Black and Marmara Seas, with lysine found in the highest amount in all groups.[14] Reduction of protein loss through valorization of by-products has long been identified as a key solution to reduce food loss and improve the sustainability of anchovy consumption.[15]

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Results and discussion

The AnchoisFert biomaterial consists of a grey powder of relatively small particles that can be freely handled even after drying (no static charge accumulation). Figure S1 in the Supporting Information shows the GC chromatogram profile of AnchoisFert following derivatization with ethyl chloroformate to convert its amino acids in readily separated ethoxy carbonyl ethyl esters.

Table 1 shows the retention times and the ionic fragments for the 16 amino acids identified in AnchoisFert.

Table 2 shows the relative abundance of the amino acids found in AnchoisFert.

Leucine (1.63%), glycine (1.44%), glutamic acid (1.21%) and alanine (0.88%) are the most abundant AAs in AnchoisFert. Histidine (0.03%), isoleucine (0.07%), tyrosine (0.16%) and valine (0.14%) are the least plentiful. Lysine, that in fillets of fished in Turkey was the most abundant,[14] has an intermediate concentration (0.32%).

Table 1. Retention time and ionic fragments for amino acids identified in AnchoisFert.

Amino acid	Retention time (min)	Ionic fragments (*m/z*)
Alanine	8.77	116/44
Valine	9.44	98/72/144/116
Isoleucine	10.78	74/158/130/102
Leucine	11.20	158/102/72/43
Glycine	11.93	102/74
Proline	13.46	142/98/70
Aspartic acid	20.25	188/116/42/74/56
Threonine	20.60	129/101/74
Methionine	22.53	175/61/129
Glutamic acid	22.72	84/41/56
Serine	22.93	132/129/60
Phenylalanine	24.95	176/192/91/102
Arginine	32.91	149/167/113/71
Lysine	35	156/128/45/226
Histidine	36.48	238/254/154
Tyrosine	38.57	107/192/264

Table 2. Relative abundance of amino acids in AnchoisFert.

Amino acid	Abundance (wt%)
Alanine	0.88
Valine	0.14
Isoleucine	0.07
Leucine	1.63
Glycine	1.44
Proline	0.87
Aspartic acid	0.73
Threonine	0.23
Methionine	0.11
Glutamic acid	1.21
Serine	0.31
Phenylalanine	0.26
Arginine	0.59
Lysine	0.32
Histidine	0.03
Tyrosine	0.16
Total	8.97

Glycine is a powerful anti-inflammatory immunonutrient,[16] with a key structural (DNA, RNA and collagen synthesis), functional (heme, bile salts and creatin), and protective (glutathione and conjugation with drug molecules) role in human metabolism.[17]

Leucine, an essential AA that provides a signal that amino acids are available stimulating muscle protein synthesis,[18] is the second most abundant AA.

As put it by Jackson "only a small group of amino acids, alanine, aspartate and glutamic acid, are genuinely non-essential".[17] The three NEEAs are particularly abundant in AnchoisFert. A component of dipeptide carnosine concentrated in muscle and brain tissue in humans, alanine is widely used as a strength-enhancing supplement.[19] In plants, alanine is accumulated as a generic stress response molecule involved in protecting plants from temperature extremes, hypoxia, drought, as well as chemical and biotic stresses.[20]

Glutamic acid is the major neurotransmitter in humans, though it becomes toxic when present outside of protein in excess to dose healthy human can accommodate[21] (it is widely added as taste enhancer to many foods as sodium glutamate). Again, glutamate has a key signaling role in plants being involved in amino acid metabolism, and was recently found to reshape the plant microbial community protecting plants against pathogens.[22]

In addition, proline (0.87%), aspartic acid (0.73%), arginine (0.59%) and serine (0.31%) are also relatively abundant in AnchoisFert.

Aspartic acid plays a critical role in protecting fish from bacterial infections by enhancing the concentration of nitrogen oxides that induce phagocytosis of microbial pathogens boosting fish immunity.[23] In higher plants, aspartate is the common precursor of the essential amino acids lysine, threonine, methionine and isoleucine, the lack of which dramatically reduces the nutritive value of all cereal and legume crops.[24]

Dietary proline protects retinas from degeneration induced by the oxidative damage in the retinal pigment epithelium.[25] Accumulating in several plant species in response to environmental stress, proline plays a key rolein plant recovery from stress.[26]

Improving cardiovascular function and enhancing lean tissue mass, arginine is widely used as dietary supplement also to reduce obesity.[27] In plants, arginine, the AA with the highest N:C ratio amid the 21 proteinogenic amino acids, serves to store nitrogen as well as in defending plants against different stress agents.[28]

Called "a metabolic hub"[29] and formed only in glial cells, serine links glial metabolism with synaptic activity and plasticity to such an extent that its lack contributes to many brain disorders. In plants, furthermore, serine has an important function in plant metabolism and development via both the photorespiratory glycolate pathway and the non-photorespiratory phosphorylated pathway.[30]

Finally, lysine, the first limiting amino acid in nearly all developing countries where it is widely used today as dietary supplement to improve the nutritional status of populations,[31]

was found as mentioned above in intermediate levels (0.32%). In higher plants, lysine enhances also the abiotic and biotic stress responses.[32]

The aromatic amino acid phenylalanine (0.26 wt% in AnchoisFert) in plants exerts multiple biological functions and health-promoting properties, such as protection against abiotic and biotic stress as well as being required for protein biosynthesis and cell survival, acting as a precursor of numerous secondary metabolites.[33]

Conclusions

The analysis via GC-MS of amino acids present in AnchoisFert, a new organic fertilizer co-product of fish oil extraction from anchovy fillet leftovers using biosolvent limonene, unveils the presence of 16 amino acids, essential, quasi-essential and non-essential. Leucine, glycine, glutamic acid and alanine are the most abundant AAs. Proline, aspartic acid, arginine, serine, lysine and phenylalanine are also relatively plentiful.

Alongside the presence of abundant bioavailable organic carbon and valued minerals,[1] the broad and significant role of virtually all these AAs in plant growth and metabolism further explains the exceptional fertilization properties of this new organic fertilizer. However, the same AAs play multiple health-beneficial roles in humans. Accordingly, the activity of AnchoisFert as pharmaconutrient should be urgently investigated.

Along with the results of the techno-economic[8] and life cycle assessment[34] analyses, these outcomes suggest that the circular economy LimoFish process[9] is highly effective in recovering valued AAs that otherwise would be lost in the environment. Its widespread practical uptake will greatly improve the sustainability of anchovy fishing, processing and consumption,[15] thereby further supporting the scale-up and industrialization of the process.

Experimental Section

Reagents and materials

All chemicals (reagent grade) were purchased from Sigma Aldrich (Milan, Italy) and used with further treatment processes. Only chloroform was obtained from Fisher Chemical (Thermo Fisher Scientific, Rodano, MI, Italy). Ultrapure, de-ionized water was obtained with a Milli-Q water purification system (Merck-Millipore, Burlington, MA, USA).

Sample preparation

The AnchoisFert wet residue obtained after centrifugation of the AnchoisOil as described elsewhere,[2] was washed on a Bruckner filter using a 583 micron nylon filter mesh filter first with pure EtOH (to remove residual limonene) and then with ultrapure water. The clean AnchoisFert obtained was thus dried in an oven at 110 °C for 2 h. A 5 mg sample of dried AnchoisFert was added to a vial followed by a 200 μL aliquot of concentrated (9 M) aqueous HCl. Nitrogen gas was briefly insufflated with a glass pipette to remove oxygen from the mixture. The vial with the acidified sample was placed in a oven at 110 °C for 24 h, after which it was insufflated again with N_2, added with 300 μL ultrapure water and 300 μL of chloroform. The surnatant (50 μL) was transferred to another vial and added with 15 μL of aqueous $NaHCO_3$, 50 μL of ethanol-pyridine (4:1, v:v), 10 μL of ethyl chloroformate, 50 μL of ethyl chloroformate with 1% internal standard (ethyl lactate) and 15 μL of aqueous $NaHCO_3$. After formation of two layers, a sample retrieved from the bottom layer was injected in the GC-MS spectrometer for the analysis.

GC-MS analysis

The GC-MS analysis was carried out using a Trace 1310 gas chromatograph with ISQ LT single quadrupole mass spectrometer CG-MS spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).The GC was equipped with a Zebron ZB-WAX capillary column (bonded polyethylene glycol, 30 m × 0.25 μm film thickness × 0.25 mm i.d.) supplied by Phenomenex (Torrance, CA, USA). A 1 μL sample was injected in split mode (1/100) using the Triplus RSH autosampler and liquid handling system (Thermo Fisher Scientific). High purity helium gas (99.999%) was used as carrier gas with a flow rate of 1.0 mL min^{-1}. The oven temperature programming was as follows: the initial oven temperature held at 120 °C for 2 min, then increased to 240 °C at a rate of 4 °C min^{-1}, and then to 260 °C at a rate of 30 °C min^{-1} and hold for 10 min. The ion source and interface temperature were set at 280 °C and 265 °C, respectively. All samples were analyzed in selected ion monitoring (SIM) mode, in a mass range from 35 to 360 Da.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Amino acids · AnchoisFert · anchovy · amino acids · organic fertilizer

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