Electronic Supplementary Information (ESI)

Monitoring metabolites change in ice stored ghol fish (*Protonibea diacanthus*) by ^{1}H NMR technique: Identification of pyruvate as spoilage marker

Ratish R. Nair,[a+] Nidhi Joshi,[b,+] Vinod P. Boricha,[a] Soumya Haldar,[*b,c] and Pabitra B. Chatterjee[*a,c]

[a]Analytical Division and Centralized Instrument Facility (AD&CIF), CSIR-CSMCRI, G. B. Marg, Bhavnagar-364002, Gujarat, India.

[b]Marine Biotechnology and Ecology Discipline, CSIR-CSMCRI, G. B. Marg, Bhavnagar-364002, Gujarat, India.

[c]Academy of Scientific and Innovative Research (AcSIR), CSIR-CSMCRI, G. B. Marg, Bhavnagar-364002, Gujarat, India.

Contents **Page No.**

Experimental section

Sample collection

Total 20 numbers of *Protonibea diacanthus* (a member belonging to the sciaenidae family), commonly known as ghol fish, with average body weight 300 - 400 g were collected from the fisherman immediately after catch from the western part of Gulf of Khambat (GoK), Gujarat, India. Subsequently, the fishes were stored in cool pack with sufficient ice and brought to the laboratory within 2 h. Whole fishes were stored in ice pack throughout the study. ^{1}H NMR based investigations were carried out from capture day (D-1) through D-12. For experimental purpose, fish tissue samples were collected from the underneath of dorsal fins, adjacent to the skeleton, which were commonly used for the preparation of fish fillets and considered to be the best part for processing and export purposes. To improve the representativeness, samples were prepared by randomly selecting and mixing 5 representative tissue samples from five different fishes which were taken from a batch of 20 fishes. All the sampling processes were carried out in aseptic condition with sterile spatula and forceps and were used for both ^{1}H NMR and microbiological investigations. Briefly, ~4 g was used for ^{1}H NMR studies and NMR spectroscopy were performed on real-time scale.

Sample preparation for ^{1}H NMR spectroscopy

Samples were prepared as perchloric acid extracts following literature procedure.[1] In brief, ~4 g of fish muscle tissue was freezed in a mortar using liquid nitrogen and subsequently crushed into fine powder by manual grinding using pestle. Thereafter, 8 mL of 7 % perchloric acid was added slowly to this powdered sample over a period of 10 minutes. The mixture was then mixed thoroughly in a homogenizer over a period of 10 minutes. The liquid acid mixture, so-obtained after homogenization, was transferred into a 15 mL centrifuge tube and the solution was neutralised using 5 M KOH at room temperature (RT). The pH was adjusted to ~7.8 and the solution mixture was then centrifuged at 3500 rpm for

25 minutes in order to remove potassium perchlorate as precipitates. Resulting supernatant was filtered using 0.2 μm membrane and was further used for ^{1}H NMR analysis.

^{1}H NMR data acquisition and processing

All ^{1}H NMR experiments with $HClO_4$ extracts of muscle tissues were carried out at 22 °C on a Bruker, Avance II 500 MHz NMR spectrometer (500.1324 MHz) equipped with a 5 mm BBO BB-1H probe-head. A total of 600 μL of sample was vortexed gently before acquisition. Sodium salt of 3-(trimethylsilyl) propionic acid-2,2,3,3-d_4 ($C_6H_9D_4O_2SiNa$, TMSP-d_4), dissolved in D_2O, was taken in an internal capillary for lock and referencing purposes. ^{1}H NMR spectra were acquired using a standard Bruker pulse program to which a presaturation pulse was added in order to reduce the water signal. The duration of presaturation is 5 s and the amplitude is 50 Hz. The free induction decays (FIDs) were acquired with 65536 data points during a 2.62 s acquisition time with a relaxation delay of 5 s. 90^o pulse was used to acquire the spectra and the length of the 90^o pulse is 11.25 μs. The acquired data were processed using TOPSPIN 2.1 software (Bruker Biospin). Resulting data were Fourier transformed after multiplied by an exponential window function using a line broadening function of 0.3 Hz. All NMR spectra were referenced to the CH_3 signal of TMSP-d_4 at δ 0 ppm. Due to our limitation, fish muscle tissues were analysed in duplicates on D-1, D-4, D-6, D-8, and D-12. Baseline was corrected by fitting to the polynomial function and no zero filling was performed.

Comparative quantification of metabolites

In each spectrum, relative proportions of all quantifiable metabolites were calculated by measuring the area of TMSP-d_4 which was taken in a sealed internal capillary. Throughout the experiment, same internal capillary was used and therefore, the intensity of TMSP-d_4 signal remained consistent in both fresh and spoiled samples as noted by the visual inspection of the ^{1}H spectra of all samples. For individual metabolite signal, the area was calculated with

respect to the area of the CH_3 protons of TMSP-d_4 at δ 0 ppm. Inclusion of an internal standard allowed direct quantification of different metabolites. After measuring the relative intensities of few selected metabolites in each of the fresh and spoiled spectra, the highest ratio of a particular metabolite with respect to TMSP-d_4 was considered as 100 %. Relative percentage variations among these metabolites were then calculated.

Fig. S1. 2D bar plot showing percentage variations (mean ± standard error of mean) of selected metabolites based on their concentrations change with ice storing time.

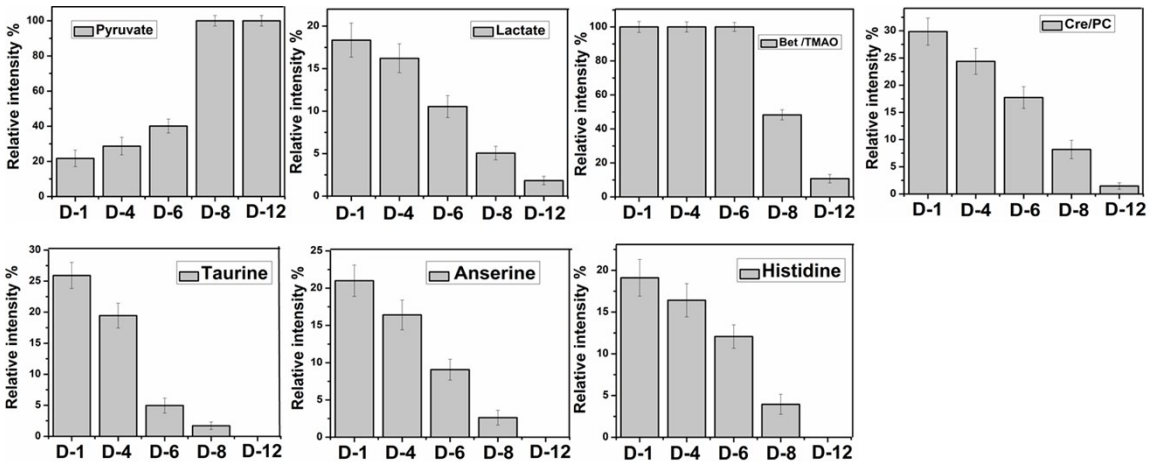

Table S1

Peak No.	^{1}H Chemical Shift (ppm)	Compound (multiplicity)	Proton type
1	0.99	Leucine (Leu) (d)	δ/δ′-CH3
2	1.02	Isoleucine (ILeu) (d)	γ-CH3
3	1.04	Valine (Val) (d)	γ′-CH3
4	1.16	Ethanol (t)	-CH_3
5	1.19	β-Hydroxybutarate (BHT) (d)	-CH_3
6	1.33	Lactate (Lac) (d)	-CH_3
7	1.48	Alanine (Ala) (d)	-CH_3
8	1.72	Lysine (Lys) & Leucine	-CH_2
9	1.91	Lysine	-CH_2
10	2.09	Acetate (Ac) (s)	-CH_3
11	2.13	Glutamine (Glm)	-CH_3
12	2.23	Pyruvate (Pyv) (s)	-CH_3
13	2.35	Glutamate (Glu) (s)	-CH_2
14	2.40	Glutamine (s)	-CH_2
15	2.73	Citrate (Cit) (s)	-CH_2
16	2.89	Aspartate (Asp) (s)	-CH_2
17	2.92	Trimethylamine (TMA) (s)	-CH_2
18	3.04	Creatine (Cre)/Phosphocreatine (PC) (s)	-N-CH_3
19	3.19	Tyrosine (Tyr)	β-CH_2
20	3.22	Choline (Cho) (s)	-$N(CH_3)_3$
21	3.26	Betaine (Bet) /	-N-CH_3

		Trimethylamine N-oxide (TMAO) (s)	
22	3.36	Phosphorylcholine (PCho)/Glycerophosphorylcholine (GPCho) (m)	$-N(CH_3)_3$
23	3.43	Taurine (Tau) (t)	$-N-CH_2$
24	3.51	β Glucose (β Glu)	-CH
25	3.56	Anserine (Anser) (s)	$-CH_2$
26	3.90	Anserine (s)	$-N-CH_3$
27	3.92	Creatine /phosphocreatine (s)	$-CH_2$
28	4.02	Sucrose (Suc)	-CH
29	4.12	Lactate (q)	-CH
30	4.36	Sucrose	-CH
31	4.43	Glycerophosphorylcholine	$\alpha-CH_2$
32	4.51	Anserine (m)	α-CH(His)
33	6.15	Inosine 5’ monophosphate (IMP) (d)	-CH
34	6.18	Inosine (Ino) (d)	-CH (Rib)
35	6.73	Histidine (His) (s)	-CH
36	8.19	Hypoxanthine (Hx) (s)	-CH
37	8.21	ADP (s)	-CH
38	8.23	Inosine 5’ monophosphate (s)	-CH
39	8.34	Hypoxanthine (s)	-CH
40	8.57	Formate (For) (s)	-CH
41	8.70	Inosine 5′ monophosphate	-CH
42	8.94	Nicotinamide (s)	-CH

References

1. S. Francesco, P. Gianfranco, B. Anna, F. Paolo, C. Francesco and S. Engelsen, 2010, *Food Chem.*, 2010, **120**, 907.