GC-MS fingerprinting of Fatty acids of somatic hybrid mushrooms derived from theprotoplast fusion between Pleurotus sajor- caju and Calocybe indica

Piu Das[1], Sohini Chatterjee[1], Amalesh Samanta[1*]

[1]*Division of Microbiology and Biotechnology, Department of Pharmaceutical Technology, Jadavpur University,*

Abstract: **GC-MS is an instrument that is used for lipid profiling and biomarker identification. The study aims to identify and quantified fatty acids (FAs) from six hybrid mushrooms (APS) with their parents *P.sajor caju* and *C.indica* by using GC-MS fingerprinting. By using TR-WaxMS Colum methyl ester form of fatty acids could be analysed. In the present study, we have identified twelve fatty acids. It was also found unsaturated fatty acids (UFA) are more predominant than saturated fatty acids (SFA). Saturated fatty acids, Palmitic acid, and Stearic acids are abundantly present in the studied samples and ranged from 0.345% to 30.339%. It was also noticed that monounsaturated fatty acids (MUFA), Oleic acid was profusely present in some hybrid mushroom strains (APS-3, APS-5, and APS-6) ranging between 0.170% to 12.327%, whereas polyunsaturated fatty acids (PUFA) are only abundantly present in hybrid APS-6 (22.908%). Overall, the unsaturated fatty acids were at higher concentrations than saturated fatty acids in hybrids than in parent strains.**

Key words: Hybrid edible mushrooms, fatty acid composition, GC-MS

I. INTRODUCTION

Lipids are one of the essential components of the cell membrane, with a structure typically composed of a glycerol backbone, 2 fatty acid tails (hydrophobic), and a phosphate group (hydrophilic). Lipids are generally hydrophobic or amphiphilic, and this diversity of lipids allows them to perform several bodily functions like energy storage for long-term use (triglycerides), several hormonal roles (steroids such as estrogen and testosterone), Insulation – both thermal (triglycerides) and electrical (sphingolipids), protection of internal organs (triglycerides and waxes) and as structural components of cells (phospholipids and cholesterol)(1,2). Among the different groups of lipid molecules, fatty acids (FAs) are an important category of biomolecules that regulate the key metabolic pathways in the body and take part in maintaining the proper health of an individual. Along with this, the composition of fatty acids also alters with age, time, environmental conditions, etc. which in turn makes FA a potential indicatorof clinical conditions (3,4).

Gas Chromatography-Mass Spectrometry (GC-MS) is one of the popular sensitive analytical techniques to allow the identification and quantification of individual components in fats and oils(5), it shows high sensitivity in the detection of volatile compounds similar to Liquid Chromatography-Mass Spectrometry (LC-MS)(6). Analysis ofa varied range of compounds such as essential oils, wax, eicosanoids, esters, perfumes, and terpenes can be done in GC-MS by selecting proper columns for tests (6,7). The principle of the GC-MS instrument includes the separation of chemical mixtures (the GC component) and eventually identifying the components at a molecular level (the MS component) (8). It is one the most precise tools for analysing environmental samples like volatile organic compounds (8). The GC working basis states that a mixture will be separated into individual components once heated and the heated gases will be carried through specified columns with an inert gas (such as helium). Asthe separated substances elute out from the columns, they flow into the MS where the Mass spectrometry works by identifying compounds by the mass of the analyte molecule. GC-MS thus combines the separation capability of GC with the fragments identification capability of MS making it more confirmatory concerning GC. Fatty acids(FAs) as explained earlier are the basic building block of most lipids, hence, the fatty acid levels in the blood are closely related to many diseases including cardiovascular diseases, blood pressure, and arthritis(9). The human body is unable to synthesize two dietary essential fatty acids: linoleic acid and linolenic acid, where the former isthe precursor of ω-6 arachidonic acid, the substrate for prostaglandin synthesis while the latter is the precursor ofother ω-3 fatty acids serving an important role in the growth and development(10). Edible mushrooms serve as one of the natural sources of essential fatty acids(10). With the expansion in knowledge concerning the human nutritional benefits and pharmacological actions, consumption of wild mushrooms has increased considerably (11). Mushrooms have been shown to have lower levels of calories and fats, they contain essential fatty acids, vegetable proteins, and valuable vitamins and minerals (12–14). Many researchers have examined the fatty acid composition of various mushrooms and have clarified the importance of a diet containing mushrooms (15,16). In the previous study, two edible mushrooms species, *Calocybe indica* and *Pleurotus sajor-caju* were chosen as parent strains to develop new somatic hybrid lines by hybridization through the protoplast fusion technique to enhance the shelf life, shorten the cropping period and make the new hybrids tolerant to high temperature(17), while in this present article we analyse and compare the fatty acid content in the previously cultivated hybrid mushrooms by GC-MS fingerprinting with TR-WaxMS column showing precise results.

Table no. 1: Saturated (S) & Unsaturated (U)Fatty acids are detected by GC-MS TR-Wax MS column; peaks were identified by NIST library.

Sl.no.	Compounds identified: Saturated fatty acids & Un saturated fatty acids.	Sample -1 (C.I.)	Sample -2 (APS-1)	Sample -3 (APS-2)	Sample -4 (APS-3)	Sample -5 (APS-4)	Sample -6 (APS-5)	Sample -7 (APS-6)	Sample -8 (P.S.)	GC-MS peck code no.
Saturated Fatty acids:										
1.	Tri Decanoic Acid	0.236±0.094	nd	nd	nd	nd	1.753±0.765	1.085±0.185	nd	S1
2.	Palmitic Acid	30.339±1.62	2.263±0.359	nd	7.498±0.548	0.345±0.136	nd	nd	nd	S2
3.	Stearic Acid	21.767±1.60	nd	2.066±0.358	0.332±0.061	nd	nd	nd	nd	S3
4.	Hepta decanoic acid, methyl ester	nd	nd	nd	0.212±0.117	nd	nd	nd	nd	S4
5.	Methyl tetra decanoic acid	nd	nd	nd	nd	nd	nd	nd	0.255±0.097	S5
Unsaturated Fatty acids:										
1.	Hexa decanoic acid, 14-methyl ester	0.327±0.062	nd	1.761±0.648	0.127±0.81	nd	nd	nd	nd	U1
2.	Dodecanoic acid, 2-methyl dodecanoic acid	2.568±0.529	nd	0.489±0.143	nd	nd	nd	nd	0.646±0.131	U2
3.	Oleic acid	nd	nd	0.170±0.048	nd	12.347±0.86	0.378±0.206	nd	nd	U3
4.	Tetra decanoic acid, 12-methyl ester	nd	nd	nd	2.410±0.581	nd	nd	nd	nd	U4
5.	Dodecanoic acid, 10- methyl ester	nd	nd	nd	nd	14.293+1.08	nd	nd	nd	U5
6.	Linoleic acid	nd	nd	nd	nd	nd	nd	22.908 ± 0.83	nd	U6
7.	Tridecanoic acid, 12-methyl ester	nd	nd	nd	nd	nd	nd	0.311+0.051	19.103+0.861	U7

Results were shown as mean+- SD(n=3)
Nd=Not detected

II. MATERIALS AND METHODS

Mushroom species

All hybridized mushroom samples (APS) including their parent samples Pleurotous sajor-caju and Calocybe indica were cultivated and collected from Madhyamgram Experimental Farm (MEF) of Bose Institute West Bengal. After collection, the fruit body of mushroom samples was immediately lyophilized by a lyophilizer (IIC Instrumentation Corporation, Kolkata).

Sample preparation

Lipid Extraction and preparation of Fatty Acid Methyl Esters: Crude fatty acids were extracted by the Soxhlet apparatus. The fatty acids obtained from the extraction were trans-esterified with methanol: sulphuric acid (95%): toluene 2:1:1 (v/v) and kept for 12 hours at a magnetic stirrer at 50°C and 160 rpm. After esterification,3 ml of deionized water was added and vortex to get phase separation. Now 3 ml of diethyl ether was added to the sample by vigorously shaking and vortex to recover the fatty acid methyl esterase and the upper phase was collected. Furthermore, to eliminate water from the sample was passed through microcolumn sodium sulphate anhydrous. Finally, the sample was recovered in a Teflon vial and before GC-MS, filtered through 0.22 µm Syringe-Driven Filters(HIMEDIA)(18)

Fatty Acid Analysis by GC-MS using TR-WaxMS Column

The Gas Chromatography-Mass Spectrometry (GC-MS) analyses of the FAs were carried out using a GC (Trace GC Ultra, Thermo Fisher Scientific India Pvt. Ltd.) equipped with a capillary column (TR-WaxMS, 30 m × 0.25mm [ID] × 0.25 µm film thickness) and an MS (POLARISQ, Thermo Fisher Scientific India Pvt. Ltd.) attached to it. The MS conditions were as follows; ionization voltage 70 eV, Mass range of 40-500, and the scan time equalto the GC run time. The individual constituents shown by GC were identified and quantified by comparing the retention times and by using the NIST library (version 2.0,2008) (18).

III. RESULT &DISCUSSION

Fatty Acid Analysis by GC-MS using TR-Wax MS Column

Analysis of fatty acids by GC-MS:

Gas chromatography-mass spectrometry (GC/MS) is an instrumental technique, comprising a gas chromatograph(GC) coupled to a mass spectrometer (MS), by which complex mixtures of chemicals may be separated, identified, and quantified. For a compound to be analysed by GC/MS it must be sufficiently volatile and thermally stable. Gas chromatography-mass spectrometry (GC-MS) is used to detect compounds using the relative gas chromatographic retention times and elution patterns of components of a mixture in combination with the mass spectral fragmentation patterns, which is the characteristic of a compound's chemical structure.

In the present study, saturated fatty acids (SFA) and unsaturated fatty acids (UFA) of mushroom extracts were analysed by GC-MS and identified by the NIST library. GC-MS was used for the analysis of a wide range of biological markers including fatty acids. By using the TR- WaxMS column methyl ester form of fatty acids couldbe analysed. A total of twelve fatty acids were identified, where we observed that unsaturated fatty acids (UFAs) were more predominant than saturated fatty acids (SFAs). Most of the abundant saturated fatty acids present in the studied samples were tri-decanoic acids (C13:0), palmitic acids (C16:0), and stearic acids (C18:0). Two other saturated fatty acids were also found in the studied samples in less quantity. On the other hand, seven unsaturatedfatty acids were found in the ethanol extracted mushroom samples and were shown in **Table no. 1**. In general, palmitic acid was present in the order CI>APS-3>APS-2>APS-4 and ranged between 0.345% to 30.339%. In caseof stearic acid, we noticed that *C. indica* exhibited the highest value i.e. 21.767% whereas APS-3 showed the lowest value (0.332%). In this study it was observed that parent *P. sajor-caju* contained only one saturated fatty acid, the methyl tetra decanoic acid in a very less amount i.e. 0.255%, it also possessed two unsaturated fatty acidswhich include tridecanoic acid 12-methyl ester and dodecanoic acid, 2-methyl dodecanoic acid; among these two the former was found in higher amount (19.10%). Sample-7 i.e. APS-6 comprised of two unsaturated fatty acids,where Linoleic acid was found majorly with a value of 22.90%. For hybrid strains, dodecanoic acid, 10-methyl ester was present insignificantly (14.29%) only in APS-4. APS-2 was composed of three different unsaturated fatty acids in a very trace amount. In this experiment, we found that parent *Calocybe indica* contained two unsaturated fatty acids. Hexadecanoic acid, 14- methyl ester, and Dodecanoic acid, 2- methyl dodecanoic acid(shown in **table no.1**).

The fat content of mushroom samples studied from GC-MS analysis was very low, but consisted of saturated and unsaturated fatty acids. In this experiment, we found that the unsaturated fatty acids were dominant over the saturated fatty acids. It was observed that palmitic acids were the most common 16 carbon saturated fatty acid found in plants and animals, which were present in the diet or could be synthesized endogenously via de novo. It acts as an emulsifier and surfactant. The excess palmitic acid, which induces lipotoxicity in hepatocytes, had beenimplicated in the development of non-alcoholic fatty liver disease also associated with insulin resistance. In this experiment, it was observed that the parent *C. indica* consisted of the highest level of palmitic acid i.e., 30.33%, and APS-3 exhibited a relatively higher value among the hybrid. Stearic acid was possibly thrombogenic. Stearic acid was present in two of the hybrid mushrooms (APS-3 and APS-4) samples in a limited amount. Stearic acids do not exhibit detrimental effects on human health, it always shows a neutral effect on serum cholesterol level (19). Methyl tetra decanoate was another saturated fatty acid with fourteen carbon backbone, methyl ester resulting from the formal condensation of the carboxy group of tetra decanoic acid (Myristic acid) with methanol. It has a role as a plant metabolite, a flavouring agent, and in perfumery. Heptadecanoic acid or margaric acid is a rare fatty acid and is present in very trace amounts. In this experiment, *P. sajor-caju* showed only 0.25% of saturatedfatty acid.

Unsaturated fats are known to be healthy dietary fats. It was observed from the fatty acid profile that, unsaturatedfatty acids are predominant. One of the important polyunsaturated fatty acids (PUFA) detected in studied samplewas linolenic acid, which are also known as "essential fatty acids". Linolenic acids are not synthesized in the human body, they are obtained from the diet and sufficient amount of linoleic acid may protect the body against heart diseases and diabetes. It is also established that linoleic acid is the precursor of 1-Octen-3-ol, known as the alcohol of fungi, which is the principal aromatic compound in most fungi and might contribute to mushroom flavour (20). Another major unsaturated fatty acid is oleic acid, it is a monounsaturated fatty acid(MUFA) and isfound in the ω-9 family. Oleic acid is not regarded as essential fatty acid because the human body has enzymes, which can synthesize oleic acid. Under the severe conditions of essential fatty acid deficiency, mammals elongateand desaturate oleic acid to produce eicosatrienoic acid (C20:3 n-9). It was reported in previous studies that oleicacid has the least negative effect on the relative carcinogenicity of fatty acids (19).

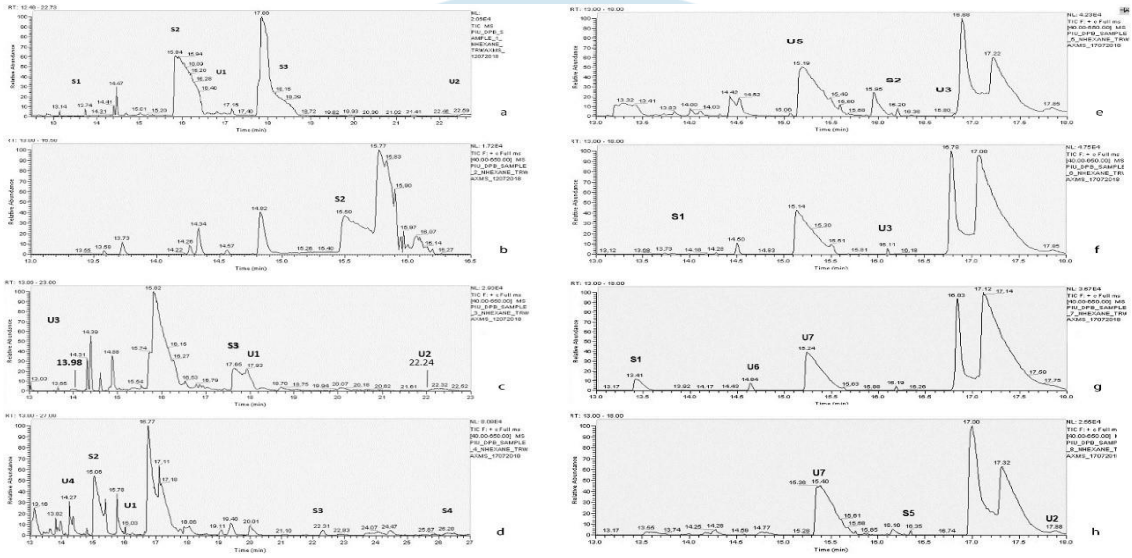

Figure 1: The GC-MS finger printing produced by the hybrid mushroom samples (APS-1, APS- 2, APS-3, APS-4, APS-5 & APS-6), along with their parents *C. indica* (a. upper) & *P.sajor-caju* (h. lower). In these chromatograms, saturated fatty acids were denoted by (S1- S5) and unsaturated fatty acids were denoted by (U1-U7). The codes representing different fatty acids.

IV. CONCLUSION

In this experiment, we analysed and compared a total of twelve fatty acids, which were obtained from hybrid mushroom samples. Previously, two edible varieties of mushrooms namely: *P.sajor caju* and *C.indica* were takenand allowed to undergo fusion using protoplast fusion technique, resulting in somatic hybrids of parent mushrooms. The newly cultivated hybrid mushroom species showed prominent levels of unsaturated fatty acids compared to saturated fatty acids, thereby making the new varieties more acceptable for consumption as unsaturated fatty acids has shown greater health benefits.

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