# Synthesis and quantification of long chain monounsaturated fatty acid positional isomers occurred in fish and their bioactivities (魚中で発生した長鎖モノ不飽和脂肪酸の合成と定量そしてそれらの生物活性)

**Doctoral Dissertation**

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**March 2018**

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## ABSTRACT

Fish oil rich in long chain monounsaturated fatty acids (LC-MUFA) such as *cis*-eicosenoic acid (*c*-20:1) and *cis*-docosenoic acid (*c*-22:1) have shown beneficial health effects in modulating the risk factors associated with cardiovascular diseases, diabetes and obesity related metabolic dysfunctions. Thus, LC-MUFA may partially contribute to the well-known functional health benefits of fish oil. Some pelagic fishes and marine mammals contain a high quantity of LC-MUFA particularly *c*-20:1 and LC-MUFAs occur as a mixture of positional isomers (PIs) in fish oil. Limited data on health effects and occurrence of LC-MUFA PIs might be due to the unavailability of pure fatty acids (FA) standards. The objectives of the current study were to chemically synthesize the common LC-MUFA PIs and quantitatively analyze the distribution of LC-MUFA PIs in fishes from the Indian, Pacific and Atlantic Ocean. Further, functionality among bioavailable LC-MUFA PIs in fish oil was compared using chemically synthesized *c*-20:1 and *c*-22:1 isomers to examine their isomer specific effect on cellular lipid metabolism.

Positional isomers of *c*-20:1 (*c*5 (double bond locates at 5th carbon atom from carbonyl side, namely Δ5 position), *c*7, *c*9, *c*11, *c*13, and *c*15) and *c*-22:1 (*c*7, *c*9, *c*11, *c*13, and *c*15) were synthesized by a series of chemical reactions. The reaction pathway consisted of seven main reactions namely; bromination of diol, protection of hydroxyl group, coupling reaction, hydrolysis, bromination, and Grignard reaction. Most of reactions resulted a good yield (above 80% in average) with few exceptions. Synthesized PIs were analyzed by gas chromatography (GC)- flame ionization detector (FID) and GC-mass spectroscopy to determine the purity and confirmed the synthesis. GC analysis of synthesized isomers using high polar capillary column showed an elution order of FA PIs with an increasing Δ position. All the above-mentioned FA isomers except *c*5-20:1 were successfully synthesized with high purity (>97%) using the given protocol.

The occurrence and distribution of *c*-20:1 PIs in fishes from the Indian Ocean was investigated and compared to those from the Pacific and Atlantic Ocean. Lipids were extracted from the edible part of the fish and methyl esterified for GC analysis. The 20:1 methyl ester (ME) fraction was separated from total FAMEs by reversed-phase HPLC and quantitatively analyzed using a GC-FID equipped with a highly polar capillary column. The synthesized PIs in the previous study were used as standard to identify FA peaks in

fish oil samples and *c*14-20:1 was used as an internal standard. The results indicated that a high content of *c*-20:1 PIs were found in fishes from the Pacific Ocean (saury, 166.95±12.4 mg/g of oil), followed by the Atlantic Ocean (capelin, 162.7±3.5 mg/g of oil), and lastly from the Indian Ocean (goatfish, 34.39 mg/g of oil). With only a few exceptions, the most abundant 20:1 PI found in fishes of the Indian and Atlantic Ocean was the *c*11-20:1 isomer (>50%) followed by the *c*13-20:1 isomer (<25%). Unusually, the *c*7-20:1 isomer was predominantly found in some fishes such as the tooth ponyfish, longface emperor, and commerson's sole. The *c*9, *c*5, and *c*15-20:1 isomers were the least occurring in fishes from the Indian and Atlantic Ocean. In contrast, the *c*9-20:1 isomer was the principal isomer identified in fishes from the Pacific Ocean. The results revealed that the content and distribution of *c*-20:1 PIs varied among fishes in different oceans. The data presented in the current study are the first to report on the distribution of *c*-20:1 PIs in fishes from the Indian Ocean.

In the third study, six bioavailable *c*-20:1 PIs were examined for their effects on adipogenesis and lipogenesis using 3T3-L1 preadipocytes. Cells were cultured in the presence of experimental i.e. *c*-20:1 PIs-added (50 μM) or control (*c*9-18:1, 50 μM or no FA) growth medium. The treatment of *c*-20:1 PIs, decreased the cellular triglyceride (TG) content compared to that of *c*9-18:1-treated cells. Although, the levels of cellular phospholipids, total cholesterol, and non-esterified FA were not altered significantly ($p<0.05$) among treatments. FA composition of cellular lipids indicated that cells uptake *c*-20:1 PIs at significantly ($p<0.05$) different percentages and has altered the FA metabolism in cells. Among the tested *c*-20:1 PIs, *c*15 isomer down-regulated the transcriptional factors for adipogenesis (peroxisome proliferative activated receptor gamma (PPARγ**)** and CCCAT enhancer binding protein alpha (C/EBPα)) and lipogenesis (sterol regulatory element binding protein-1, SREBP-1) compared to that of *c*9-18:1 and other *c*-20:1PIs.

The effect of selected LC-MUFA PIs with different chain length was compared through an *in vitro* study using 3T3-L1 and HepG2 cells. Common LC-MUFA PIs, i.e. *c*-18:1 (13 isomers), *c*-20:1(6 isomers) and *c*-22:1(5 isomers), were screened based on their individual effect on the cellular lipid accumulation. Three LC-MUFA PIs (*c*5-18:1, *c*9-18:1, *c*11-18:1, *c*9-20:1, *c*11-20:1, *c*15-20:1, *c*7-22:1, *c*9-22:1, and *c*11-22:1) from each chain length were selected for further studies of their effect on the cellular lipid metabolism. The selected LC-MUFA PIs influenced differently on TG accumulation in 3T3-L1 cells. The

lowest and highest TG accumulation were showed in cells treated with *c*15-20:1 and *c*9-22:1, respectively. Further, cells treated with *c*9-20:1 followed by *c*15-20:1 indicated the lowest expression of transcription factors related to adipogenesis (PPARγ and C/EBPα). The cells treated with *c*15-20:1 indicated the lowest expression of transcription factors related to lipogenesis (SREBP-1). The results were significantly different ($p<0.05$) among treatments. The cells treated with *c*15-20:1 had a low level of mRNA expression of genes related to FA synthesis (stearoyl-Co-A desaturase-1 and FA synthase) compared to other PIs. In addition, experimental PIs showed different effects on the levels of mRNA expression of gene related to FA β oxidation (carnitine palmitoyltranferase-1a). Comparison of the functionality among *c*9 and *c*11 isomers of 18:1, 20:1, and 22:1 revealed that the bioactivities of *c*-LC-MUFA varied with the changes in the chain length. It was apparent that some experimental LC-MUFA PIs such as *c*15-20:1 and *c*9-20:1 improved the cellular lipid metabolism, by suppression of adipogenesis, lipogenesis, and enhancing the cellular FA β oxidation.

In conclusion, LC-MUFA PIs, i.e. *c*-20:1 and *c*-22:1, were chemically synthesized with a high purity (>97%) and used as standards for GC analysis and *in vitro* studies. The occurrence and distribution of *c*-20:1 PIs varied among fishes in different oceans, where *c*11-20:1 contributed to high proportion in the Indian and Atlantic Ocean fishes and *c*9-20:1 was predominant in the Pacific Ocean fishes. Results revealed that common *c*-20:1 PIs affected differently on adipogenesis and lipogenesis in 3T3-L1 cells and the position of the double bond influenced on the bioactivities. The selected LC-MUFA PIs from different chain lengths had different effect on the lipid metabolism at cellular level and *c*15-20:1 showed comparatively good anti-adipogenic, anti-lipogenic effect, and improvement of cellular lipid metabolism. Results revealed that the double bond position and number of carbon atoms in LC-MUFA influenced on their functionality at the cellular level.

## Table of content

## ABBREVIATIONS

| | |
|---|---|
| ACC | Acetyl-coA carboxylase |
| ANOVA | Analysis of variance |
| AOCS | American Oil Chemists' Society |
| ATP | Adenosine triphosphate |
| BCA | Bicinchoninic acid |
| BSA | Bovine serum albumin |
| *c* | *cis* |
| *c*9-18:1 | *cis* 9 - octadecenoic acid |
| *c*-20:1 | *cis*-eicosenoic acid |
| *c*-22:1 | *cis*-docosenoic acid |
| C/EBPα | CCCAT enhancer binding protein alpha |
| CLA | Conjugated linoleic acid |
| CoA | Coenzyme A |
| CPT-1a | Carnitine palmitoyltransferase 1a |
| Dex | Dexamethasone |
| DHA | Docosahexaenoic acid |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA | Deoxyribonucleic acid |
| EPA | Eicosapentaenoic acid |
| FA | Fatty acid |
| FAC | Fatty acid composition |
| FAME | Fatty acid methyl ester |
| FAS | Fatty acid synthase |
| FATP | Fatty acid transport protein |
| FBS | Fetal bovine serum |
| FID | Flame ionization detector |
| GC | Gas chromatography |
| HDL | High density lipoprotein |
| HMPA | Hexamethylphosphoramide |
| IBMX | 3-isobutyl-1-methyl-xanthine |
| IL | Ionic liquid |

| | |
|---|---|
| IS | Internal standard |
| IUPAC | International Union of Pure and Applied Chemistry |
| JOCS | Japan Oil Chemists' Society |
| LC-MUFA | Long chain monounsaturated fatty acids |
| LCFA | Long chain fatty acids |
| LDL | Low density lipoprotein |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| LPL | Lipoprotein lipase |
| mRNA | Messenger Ribonucleic acid |
| MS | Mass spectrometry |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NEFA | Non-esterified fatty acids |
| ODS | Octadecyl-silica |
| PIs | Positional isomers |
| PIPES | Piperazine-N, N`-bis-2-ethanesulfonic acid |
| PL | Phospholipids |
| PPARγ | Peroxisome proliferative activated receptor gamma |
| PUFA | Polyunsaturated fatty acid |
| $R_f$ | Relative response factor |
| RIPA | Radioimmunoprecipitation assay |
| RP-HPLC | Reverse phase-high performance liquid chromatography |
| $R_s$ | Resolution factor |
| RT | Room temperature |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SCD-1 | Stearoyl-Co-A desaturase-1 |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SFA | Saturated fatty acids |
| *s/n* | Signal-to-noise |
| SREBP-1 | Sterol regulatory element binding protein-1 |
| *t* | *trans* |
| TAG/ TG | Triacylglycerol/ Triglyceride |
| TBAI | Tetrabutylammonium iodide |

| | |
|---|---|
| TBS | Tris-buffered saline |
| TCh | Total cholesterol |
| THF | Tetrahydrofuran |
| THP | Tetrahydropyran |
| TLC | Thin layer chromatography |
| VLDL | Very low-density lipoprotein |
| 21:0 | Heneicosanoic acid |

# Chapter 01

# General introduction

## 1.1. Fish oil as functional lipids

Functional lipids have received a great attention by the lipid scientists as well as by the nutritionists. Consumption of the health food is highly considered among the society due to increase in the metabolic related syndromes such as obesity, hyperglycemia, hyperlipidemia etc. that leads to diseases such as diabetic, cardiovascular disease, and atherosclerosis. Fish oil is known as one of functional lipids that decrease blood triacylglycerol (TAG) and cholesterol levels, decrease liver fat accumulation, and exhibit anti-inflammatory effects[1, 2)].

Lipids in fish oil mainly consist of TAG in the neutral lipid fraction. The content of phospholipids (PL) and other lipid classes such as sterol esters, non-esterified fatty acids (NEFA), etc. are relatively low and vary depend on the species[3)]. Fatty acid serves as a ubiquitous component in each lipid class and primarily important structural component in TAG, as three fatty acids esterified with glycerol molecule to form TAG[4)]. Analysis of different marine fish oil have reported a unique fatty acid composition (FAC) due to the presence of high content n-3 polyunsaturated fatty acids (n-3 PUFA) [5-7)]. It was well documented that the beneficial effects of fish oil are mainly due to the presence of characteristic n-3 PUFA such as, eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) in fish oil and are recognized to contribute mainly to functionality of fish oil[8-10)]. Recent studies conducted using fish oil rich in long chain monounsaturated fatty acids (LC-MUFA) consisted of 20 or 22 carbon atoms (saury oil[11)], pollock oil[12)], and cod oil[13)] also reported to have beneficial health functions in modulating the risk factors associated with cardiovascular diseases and diabetics. Probably, both n-3 PUFAs and LC-MUFAs are important for the health function of fish oil; however, the health effect of LC-MUFAs in fish oil has not been confirmed using pure fatty acids. Thus, the current study mainly focuses on the occurrence and bioactivities of LC-MUFA characteristically contained in fish oil with a special consideration on their positional isomers.

## 1.2. Bioactive fatty acids

Bioactivity of a compound explains the effect of that compound on living organisms or cells or tissue; thus, showing a comprehensive meaning. Fatty acids have widely studied for their bioactivities since 1950s. Bioactive fatty acids play a vital role in immune regulation, inflammation, and maintenance of homeostasis[14].

Some saturated short chain fatty acids such as propionic acid, butyric acid were reported to inhibit the growth and proliferation of colon cancer cells[15]. Medium chain fatty acids such as caprylic (8:0) and capric (10:0) acids induce the thermogenesis without contributing to the weight gain[15]. Further, medium chain fatty acids reported to reduce blood levels of low density lipoprotein (LDL); thus, help in controlling or preventing the obesity and related diseases[15]. Health benefits of MUFA particularly food rich in oleic acid have been broadly studied and discussed in section 1.8. Studies on bioactivity of oleic acid and their derivatives such as oxygenated products have reported in the areas of anti-microbial[16], anti-inflammatory, anti-cancer[15], etc. Further, in a recent study, anti-inflammatory activity of extracts of spiny sea-stars which containing *cis*-11-eicosenoic acid (20:1) and *cis*-11, *cis*-14-eicosadienoic acid (20:2) as main fatty acids has reported in a cell model[17].

PUFAs exhibit the highest bioactivity compared to the saturated fatty acids (SFA) and MUFA. Among the PUFAs, n-3 PUFAs are the most important as bioactive fatty acids. PUFA-rich diet can decrease ApoB levels by a combination of reduced production and increased catabolism thereby reduced the risk of atherosclerosis and cardiovascular diseases[18]. The consumption of fish rich in n-3 PUFA have been related with improved immune response such as reduced risk of asthma-related symptoms. Further, n-3 PUFA rich diet helps to decrease the levels of proinflammatory compounds such as C-reactive proteins, interleukin-6, and prostaglandins[15]. In addition, lipids containing n-6 PUFA such as arachidonic acid are involved in controlling immunity and inflammation[19].

These dietary fatty acids have reported to provide health benefits through modification of tissue FAC and by induction of cell signaling pathways. Particularly, n-3 fatty acid-induced weight loss has been shown to involve decreased appetite, modulation of lipogenic gene expression, and tissue metabolism. The physiological functions and molecular actions of selected n-3 PUFA, conjugated fatty acids, medium chain fatty acids and other bioactive

lipids in the development of metabolic syndrome have reviewed by Nagao and Yanagita[18]. Accordingly, most of the bioactive fatty acids act through transcriptional regulation of lipid and glucose metabolism[18]. Thus, physiological studies are essential to demonstrate the therapeutic properties of any synthetic or natural product and thereby to use them as medicines/drugs or as food supplements.

### 1.3. LC-MUFA

Dietary fatty acids are categorized to SFA, MUFA, and PUFA based on the number of the double bonds in the hydrocarbon chain. MUFAs are chemically classified as fatty acids that contain one C-C double bond in their acyl structure. Fatty acids are further classified to short chain (4-8 C atoms), medium chain (8-12 C atoms), and long chain (>14 C atoms) based on the number of C atoms available in the hydrocarbon chain, for the convenience in studying their physical, chemical, and functional properties. Different authors have used different approaches in these classifications. The current study focuses on the LC-MUFA with 18, 20, and 22 C atoms in the hydrocarbon chain.

The physical, chemical, and functional properties of fatty acids vary based on many factors such as the number of C atoms in the acyl chain, availability of double bonds, position and geometric configuration of the double bond, etc.[20]. Accordingly, physico-chemical characteristics of LC-MUFA such as color, odor, melting point, boiling point, density, solubility, chemical reactivity, etc. vary among these fatty acids[4]. In general, LC-MUFAs are insoluble in water and soluble in organic solvent. Some LC-MUFAs are liquid at room temperature (25°C). But LC-MUFAs with C atoms > 20 show relatively high melting point and occur as semisolid or solid at room temperature. The pure form of the fatty acids is colorless and odorless, although impurities and environmental odors absorbed during industrial production could cause LC-MUFA to change the color pale to slightly yellowish color and may have some characteristic odor. Further, chemical reactivity of MUFA differ with the chain length and geometry and position of the double bond.

### 1.4. Dietary sources of LC-MUFA

In food, the predominant LC-MUFA is the oleic acid (*cis*-9-octadecenoic acids, *c*9-18: 1), and accounts more than 90% of the total *cis*-MUFA consumed[21]. Vegetable oil such as olive oil, sunflower oil, canola oil, etc. are the richest sources of oleic acid followed by nuts (macadamia, hazelnuts, pecans, almonds, etc.) and some fruits (avocado, olives,

etc)[21]. The second abundant LC-MUFA varies among plant and marine based foods. Some pelagic fishes such as herring, capelin, cod[22], saury[23], and some marine mammals such as seals and whales[24] are reported with a high content (>10-15% of lipid composition) of *cis*-eicosenoic acid (*c*-20:1) and *cis*-docosenoic acid (*c*-22:1). Therefore, LC-MUFAs consisted of 20 or 22 carbon atoms, are also known to be characteristic fatty acids in fish oil. It was reported that cold water or temperate water-fishes, particularly the North Atlantic and the North Pacific fishes have a high content of LC-MUFA (20:1 and 22:1) than that of the warm or tropical water-fishes[25-27]. Although, some of the plant foods such as rapeseed oil, mustard seed and vegetables belongs to *cruciferae* family are rich sources of *c*-22:1. Some fishes contain *cis*-tetracosenoic acid (*c*-24:1) in minor quantities. However, this study only focuses on the *cis*-LC-MUFA containing C chain length 18, 20 and 22, i.e. *c*-18:1, *c*-20:1, and *c*-22:1, due to their relative abundance in food particularly in fish. Comprehensive analysis of LC-MUFA in food reported the occurrence of different geometric and positional isomers of fatty acids.

### 1.5. Isomers of LC-MUFA

The presence of the double bond in MUFA forms the structural isomers, namely geometric isomers and positional isomers. Two types of geometric configurations are available in nature such as *cis* and *trans*. In the *cis* configuration, the hydrogen atoms attached to the double bond are in the same direction (Fig. 1.1 a). In contrast, the hydrogen atoms attached to the double bond are in the opposite direction in the *trans* configuration (Fig. 1.1 b).

a) *cis* b) *trans*

**Fig. 1.1** Geometric configurations of monounsaturated fatty acids a) *cis* and b) *trans*

#### 1.5.1. Geometric isomers

The *cis* configuration is abundant in raw or unprocessed foods. Thus, health studies of different fatty acids have mainly focused on the effect of *cis* configuration of unsaturated fatty acids. Although, *trans* fatty acids also have received great attention recently because of the adverse health effects shown by the some of the dietary *trans* fatty acids[28, 29]. A *cis*-double bond in a fatty acid introduces a permanent bend or kink in the

alkyl chain and result in looser packing in membranes or crystal structures. Physico-chemical characteristics of geometric isomers of MUFA differ significantly. For instance, *cis* isomer of a MUFA has considerably low melting point and oxidative stability than its *trans* isomer[4]. Further, functional properties of *cis* and *trans* isomers differ considerably.

### 1.5.2. Occurrence of positional isomers of LC-MUFA

Positional isomers (PIs) are the compounds sharing same molecular formula and the same functional groups but differ in the position of the double bonds/functional groups in the hydrocarbon chain. The structures of some common LC-MUFA PIs consisted of 18 C and 20 C atoms are shown in the Fig. 1.2.

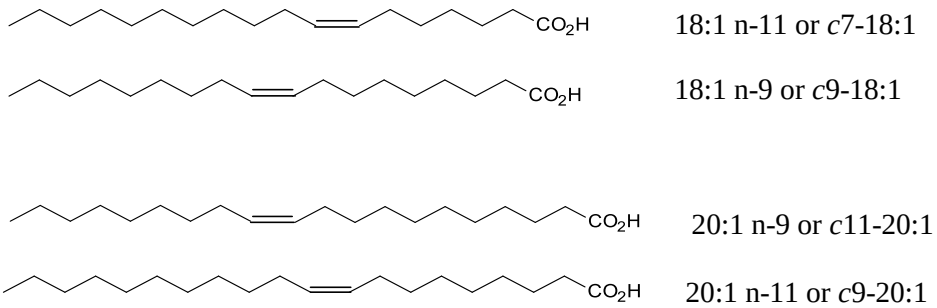


**Fig. 1.2** The structures of common LC-MUFA PIs consisted of 18 C and 20 C atoms

The double bond in MUFA can be denoted relative to the ω (methyl) end or the Δ (carboxylic) end of the chain. For instance, vaccenic acid could be written as 18:1 n-11 or *c*7-18:1. In IUPAC nomenclature of fatty acids carboxyl carbon is denoted by the number one, and positions in the chain and double are denoted with reference to it. Dietary lipids frequently contain families of MUFA with similar terminal structures, but different chain length and may have originated from a common precursor either by chain elongation or by β oxidation. Nomenclature using methyl end (n or ω notation) is useful for biochemists to identify such relationships.

A variety of LC-MUFA PIs exists in nature and their occurrence and distribution vary among food[30-32]. Total of 13 isomers of *c*-18:1 with double bond position shifting from Δ 4 to Δ 16 have reported in dietary fat and oil[33]. Further, 6 PIs of *c*-20:1 and 5 PIs of *c*-22:1 were reported in marine fish lipids[34]. The most common LC-MUFA PI found in nature is the *c*9-18:1 and the common double bond position for MUFA is between the 9th

and 10th carbon atom from the carboxyl end. Nevertheless, some exceptions are found in certain plants and animals. Bacterial lipids and sea buckthorn pulp contain *cis*-vaccenic acid (*c*7-18:1) in considerable amounts. Petroselinic acid (*c*6-18:1) contributes over 50% of seed oil fatty acids of *Umbelliferae* species such as carrot, parsley, and coriander[35]. Further, exceptions or unusual patterns of distribution of PIs were reported among C20 monoenes, for instance, meadowfoam (*Limnanthes alba*) seed oil contains *c*5-20:1 in large proportion (> 60%)[36], plants belong to brassica seed oil contain *c*11-20:1 and *c*13-20:1 isomers in high proportion and some fish oil contains a high level of *c*9-20:1 and *c*11-20:1 isomers[35]. In addition, erucic acid (*c*13-22:1) is found in high levels (40–50%) in *Cruciferae* family such as rapeseed and mustard seed. Dietary intake of LC-MUFA is mainly in the form of n-9 and n-11 positional isomers. The n-11 isomer is obtained mainly through diet where n-9 LC-MUFA can be formed by the *de novo* synthesis[37]. Biosynthesis of different LC-MUFA PIs is explained in section 1.7.1.

### 1.6. Digestion and absorption of dietary lipids

The digestion and absorption of fatty acids are well documented by many researchers. Dietary lipids mainly consist of TAG (>90%) and remainder contains cholesterol, cholesteryl esters, PLs, and NEFAs. Digestion and absorption of TAG are considered in this section. The digestion of dietary TAG mainly occurs by the activity of gastric and pancreatic lipase in the stomach and in the small intestine. This process is complicated that involve multi-step processes and highly controlled by hormonal action. Dietary lipids that enter to the small intestine are emulsified with bile salt and fat-soluble vitamins and undergo hydrolyses by the action of pancreatic lipase. The activity of lipase is specific and firstly hydrolyses the fatty acid in the 1st and 3rd position of the TAG molecule. The primary products of TAG hydrolysis are a mixture of free fatty acids and 2-monoacylglycerols[38].

After digestion, short chain and medium chain length fatty acids are directly entering into the blood stream via portal vein. The other products of dietary lipid digestion, such as long chain (both saturated and unsaturated) fatty acids, 2-monoacylglycerol, cholesterol, fat-soluble vitamins, etc. form a mixed micelle with bile salts. Later, lipid components in mixed micelle are absorbed into intestinal mucosal cells where they are assembled or packed in to chylomicrons (lipoprotein particles) and released to the lymphatic vessels.

Chylomicrons are passed through thoracic duct and left subclavian vein, and entered to the blood stream, and transported to target tissues or organs[38)].

### 1.7. Cellular metabolism of fatty acids

Chylomicrons circulating in the blood stream are picked up through apolipoprotein E and CII by the energy utilizing and energy storage tissues and organs such as muscles, adipose tissues, liver, etc.[38)]. Adipocytes and hepatocytes play a vital role in the fatty acid metabolism in the body. In adipocytes, TAG in chylomicrons are degraded to free fatty acids and 2-monoacylglycerol by lipoprotein lipase (LPL). Fat stored in the adipocytes is mainly derived from the dietary fatty acids (exogenous) and endogenous fatty acids synthesized in the liver. Cellular fatty acid metabolism in tissues significantly varied in fasting and absorptive state. For instance, in absorptive state, adipocytes uptake fatty acids, re-esterified to TAG, and stored. While in fasting state, adipose tissues increase the degradation of fat and release of fatty acids to circulation. Lipid metabolism referred both anabolism/ fatty acid synthesis and the catabolism/ fatty acid oxidation.

#### 1.7.1. Fatty acid synthesis

Fatty acids serve numerous physiological functions in the body, thus must be taken through diet or synthesized *de novo*. Liver is a main organ for the *de novo* synthesis of fatty acids. Fatty acid synthesis is a cytosolic process and favored by the availability of acetyl coenzyme A (CoA) (from glucose and amino acid metabolism) and NADPH (from glucose metabolism). In mammals, fatty acids are biosynthesized *de novo* from acetyl CoA by the action of fatty acid synthase (FAS) to yield palmitic acid[39)]. Synthesized SFAs can be further modified to long chain SFA, MUFA, or PUFA by the action of various elongases and desaturase enzymes. Acetyl-CoA carboxylase (ACC) catalyzes the rate limiting reaction for fatty acid synthesis which is the formation of malonyl CoA from acetyl CoA. The synthesized fatty acids can be esterified and converted into TAG molecules for storage. In the synthesis of oleic acid (18:1 n-9), palmitic acid is elongated by the action of elongase to yield stearic acid, which is then desaturated to oleic acid by stearoyl-coA desaturase (SCD-1)[40, 41)]. SCD-1 catalyzes the rate-limiting reaction of the synthesis of MUFAs, mainly palmitoleic acid (16:1 n-7) and oleic acid (18:1 n-9), which are the major endogenous MUFAs for membrane PLs, TAGs, wax esters, and cholesteryl

esters[42]. The action of Δ9 desaturase on SFA introduces a C-C double bond to the 9th C atom from the carboxyl end. LC-MUFA are synthesized by the elongation of 16:1 n-7 and 18:1 n-9 or else by the action of SCD-1 on LC-SFA.

Availability of different LC-MUFA-PIs in food is not due to presence of multiple desaturase enzymes. It has been reported that plants and animals have Δ9 desaturase enzymes mainly, thus the content of *c*9 MUFA is predominant in food. Mammals do not contain Δ12 and Δ15 desaturase[43] and unable to introduce a double bond near to the methyl end. Although, some reports indicate the presence of some other desaturase enzymes such as Δ5 desaturase and Δ6 desaturase in mammals[44] which could introduce a double bond near to the carboxylic end. During the biosynthesis of LC-MUFA in organisms, dietary MUFAs are modified by chain elongation or chain shortening[45] (Fig. 1.3)[46]. It could be assumed that high content of LC-MUFA PIs other than *c*9 isomers are mainly derived from the diet and have exogeneous origin.

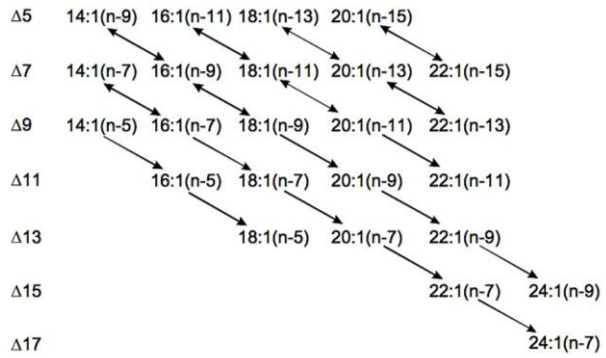


**Fig. 1.3** Probable pathways of monoenoic fatty acid synthesis[46]

Fatty acids themselves involve in the TAG synthesis and increase in the cellular lipid accumulation. Further, dietary fatty acids regulate the transcriptional factors such as peroxisome proliferator activated receptors (PPARs) and sterol regulatory element binding proteins (SREBPs) which are related to the lipid metabolism and ultimately influenced on the mRNA expression of the genes related to the lipid metabolism[47].

### 1.7.2. Fatty acid β oxidation

Fatty acid β oxidation or degradation occurs in many tissues such as muscles, heart, liver, and lesser extend in adipose tissue that require energy. Fatty acids provide main

source of energy for living beings. They are catabolized by both mitochondrial (C atoms <20) and peroxisomal β oxidation (C atoms >20) [48]. As shown in the reaction, fatty acids (R-COOH) must be activated to their CoA thioesters (RCO-SCoA) prior to any cellular reactions, i.e. synthesis or β oxidation. Fatty acids uptake into cells through plasma membrane carrier proteins and undergo activation by coenzyme A (CoASH) and forming their CoA thio esters. This reaction utilizes energy in the form of adenosine triphosphate (ATP)


(AMP- Adenosine monophosphate; PPi- Pyrophosphate)

The individual acyl CoA synthetases are grouped as short chain, medium chain, and long chain depending on their substrate specificity. Long chain acyl CoA synthetases in animals are membrane bound and associated with endoplasmic reticulum, mitochondrial outer membrane, and peroxisome[49]. LC-MUFAs (length atoms >20) undergo β oxidation mainly in peroxisome yielding shorter chain fatty acid, which enter to mitochondria for further oxidation. Peroxisomes cannot complete β oxidation of fatty acids thus involve only with chain reduction. After chain reduction, those fatty acids are catabolized in side the mitochondria. In cells, long chain acyl Co-A converted to long chain acyl carnitine through the action of carnitine palmitoyl transferase-1 (CPT-1) and enters to mitochondria and converts back to long chain acyl Co-A through CPT-2. Malonyl CoA inhibits CPT-1 enzyme related to fatty acid oxidation and serves as control point of fatty acid oxidation. Long chain acyl Co-A undergoes β oxidation where it breaks down to acetyl-CoA molecule per each cycle of oxidation. The number of acetyl-CoA molecules produced depends on the chain length of the fatty acid being oxidized. The fatty acid β-oxidation pathway is a complex series of reactions, which involves many enzymes. Acetyl-CoA molecules enter to the tricarboxylic acid cycle to produce FADH2 and NADH during the process of fatty acid β-oxidation. FADH2 and NADH are used by the electron transport chain to produce ATP[50]. Fatty acid β-oxidation regulates at several points throughout the process. For instances, regulation of the fatty acids uptake into the cells or regulating the level of malonyl CoA in cells which acts as an inhibitor of CPT-1, etc.

### 1.8. Health effects of MUFA

All the types of fatty acids provide energy[51)] and have specific biological functions in the body, thus abnormalities in the metabolism of fatty acids could lead to the development of diseases such as type 2 diabetes, cardiovascular disease, etc.[47)]. Numerous studies have focused on the health effects of MUFA with a special consideration to plant derived MUFA. Many studies have used oleic acid (*c*9-18:1) rich foods to study the health effect of *c*-MUFA. Oleic acid proposed to have a potential role in decreasing brain-related disorders such as dementia and Alzheimer's disease. Thus, consumption of oleic-acid rich diets could have beneficial effects on brain functions. Oleic acid has also been shown to have a potential role in the therapeutic management of colorectal cancer[15)]. The natural abundance of oleic acid in plant-based foods highlights the potential beneficial effects of this fatty acid in whole body functions. Replacement of SFA with MUFA and PUFA reported to decrease the plasma concentration of total and LDL cholesterol[52)].

Apart from oleic acid, some studies have used palmitoleic acid (16:1 n-7) to study the effect of *c*-MUFA on health particularly their effect on the insulin resistance[53)], obesity[54)], etc. Some systemic reviews and the meta-analyses[55)] show the health effect of MUFA enriched diet in relation to diseases such as cardiovascular, diabetes, atherosclerosis, etc. Further, consumption of high amount of *c*-MUFA showed no adverse effects on the health. Although, dietary recommendation for the MUFA has not been published under the dietary guidelines for Americans[56)]. Some other organizations have set guidelines for the intake of *c*-MUFA[57)].

#### 1.8.1. Past studies on health effects of fish oil rich in LC-MUFA

Comparatively, limited researches have done on the health effect of the LC-MUFA consisted of more than 18 C atoms. Recently, studies have conducted using fish oil rich in LC-MUFA particularly *c*-20:1 and *c*-22:1 using human, animals, and cell models. Human studies with LC-MUFA rich diet reported a low and different level of postprandial plasma level of LC-MUFA compared to that of n-3 PUFA[58)]. These data suggest a possible rapid metabolism of LC-MUFA and different metabolic pathways might be responsible for the mobilization rates of these different fatty acids. Studies conducted using seal and cod liver oil showed an increased level of high-density lipoprotein (HDL) cholesterol, decreased

level of TAG, and an improvement in serum lipid profile in human blood[59, 60]. In an animal study, it was reported that saury oil (10% w/w supplementation) which is a rich source of *c*-20:1 and *c*-22:1, suppressed gene expression related to adipogenesis and lipogenesis and induce gene expression related to fatty acid oxidation, thus resulted in improvements of glucose and lipid metabolism[11]. Another study reported reduced plasma TAG and very low-density lipoprotein (VLDL) cholesterol and increased the plasma HDL cholesterol in LDL receptor deficient mice fed with a high LC-MUFA diet[61]. Further, LC-MUFA rich fish oil reported to suppress the rise in proatherogenic LDL cholesterol without decreasing anti-atherogenic levels of HDL cholesterol[12] and helps to control atherogenesis[62]. These findings lead to further studies on LC-MUFA using concentrated or purified fatty acids for clear understanding on functionality of dietary LC-MUFA.

Recently, Yang *et al*. have published important findings indicating beneficial effects of LC-MUFA concentrates from fish oil on the lipid profile in mice[11, 63, 64] and cell models[65]. These findings include favorable alterations in the mRNA levels of genes related to the glucose/ lipid metabolism in diet induced obese mice fed with concentrated LC-MUFA from saury fish oil[63], reduced hyperinsulinemia, hyperleptinemia, adipocyte size and down regulate inflammatory genes and upregulate PPAR gamma in type 2 diabetic KK-Ay mice[64] and up regulation of PPAR family genes by LC-MUFA diet was also observed in Apo-E deficient mice[65]. Therefore, these findings show the importance of *c*-LC-MUFA consisted of 20 and 22 C atoms as bioactive lipids.

### 1.8.2. Isomer specific health effects of LC-MUFA

Among the studies relating to the metabolism of MUFA isomers, geometric isomers have received much attention and studied thoroughly on their effect to human health. There is a less published research on the health effects of MUFA-PIs on the health. As mentioned previously (section 1.5.), MUFA occurs as a mixture of PIs and their occurrence varies in food. Thus, the knowledge on specific effect of LC-MUFA PIs would be useful in further studies of their functionality/ bioactivity and for future practical applications. Studies have reported that PIs of some fatty acids (18:1[66], conjugated linoleic acid (CLA)[67]) showed differential effects on the lipid metabolism. Effect of dietary LC-MUFA on health may represent a synergistic effect or antagonistic effect of all the available LC-MUFA PIs in

that food. A recent study showed that the individual CLA-PIs and their mixtures had different effects on cellular lipid metabolism[68]. Although the beneficial effects of LC-MUFA containing more than 20 carbon atoms have reported, there are no published comparative data on the health effects of pure LC-MUFA or LC-MUFA PIs containing more than 20 carbon atoms. Thus, the effects of the double bond position and the number of C atoms in the chain length in LC-MUFA for health function have not been confirmed yet. Hence, further studies are needed to elucidate the potential bioactivity/ functionality of individual LC-MUFA PIs.

### 1.9. Overview of chapters

The background of the study and some of recent literature works carried out on the bioactivities and health effects of LC-MUFA with more attention to PIs have explained in the Chapter 01.

The Chapter 02 explains the chemical synthesis pathway of LC-MUFA PIs i.e. *c*-20:1 PIs and *c*-22:1 PIs. Seven positional isomers of *c*-20:1 and five positional isomers of *c*-22:1 targeted for the synthesis. Synthesized positional isomers of LC-MUFA was used as references or standards and to study functional properties or bioactivities of individual isomers.

The occurrence and the distribution of *c*-20:1 PIs in the Indian Ocean fishes was quantitatively analyzed and reported in the Chapter 03. The *c*-20:1 PIs synthesized in the first part of the experiment was used in this study. According to previous studies, some pelagic fishes from the Pacific and the Atlantic Ocean contain considerably high amount of *c*-20:1 and *c*-22:1. Although, no studies have done on the positional isomeric profile of *c*-20:1 in Indian Ocean fishes.

The Chapter 04 reports the effects of *c*-20:1 PIs on the adipogenesis and lipogenesis in 3T3-L1 cells. The main objective of this study was to investigate the effect of position of the double bond in LC-MUFA on the preadipocyte differentiation and lipogenesis in the 3T3-L1 cells.

*In vitro* studies were conducted using selected PIs of *c*-18:1, *c*-20:1, and *c*-22:1 on their individual effects on some aspects related to the lipid metabolism and described in the Chapter 05. Both adipocytes and hepatocytes play an important role in the lipid metabolism

in the body. Thus, LC-MUFA PIs having different carbon chain lengths and different double bond position were studied to investigate and to compare their individual effects on the lipid metabolism in 3T3-L1 and HepG2 cells.

The Chapter 06 summarizes the findings of all the experiments that explained in the Chapter 02-05 with perspectives for future studies.

## 1.10. Objective of the study

### 1.10.1. General objective:

The current study aims to compare the bioactivities or functionalities among individual LC-MUFA PIs consisted of 20 and 22 carbon atoms characteristically contained in fish oil using cell culture studies.

### 1.10.2. Specific objectives

1. To chemically synthesize the LC-MUFA PIs characteristically contained in fish oil
2. To quantitatively analyze the distribution of *cis*-20:1 PIs in marine fish from the Indian Ocean
3. To study and compare the bioactivity of LC-MUFA PIs in relation to cellular lipid metabolism

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# Chapter 02

## Synthesis of long chain monounsaturated fatty acid positional isomers

### 2.1. Introduction

Fatty acids are organic compounds comprised of C, H, and O in their basic structure and consisted of a hydrophobic hydrocarbon chain with a terminal carboxyl group thus also referred as carboxylic acids. The basic structure of a fatty acid could be written as $CH_3(CH_2)n$ COOH. The hydrocarbon chain is either saturated or unsaturated depend on the availability of double bonds[1)].

Over the past decades, fats and oils industry has grown to a top level. Thus, scientists in the field worked hard to understand the physical, chemical nature, and functionality of each fatty acid occurred in diverse sources of fats and oils, thereby to expand their utilization. The need for the organic synthesis of fatty acids arises due to the difficulty in isolation of the pure fatty acids from natural sources. Synthesis of fatty acids using enzymatic reactions[2,3)] and chemical splitting[4,5)] have been carried out in the past decades by many researchers and well documented. Although, the final products resulted from enzyme based fatty acid synthesis are a mixture of several fatty acids and other compounds leading to a relatively low purity of the target fatty acid. Thus, organic synthesis pathways have developed for the synthesis of carboxylic acid and their isomers of interest from a mixture of scratches and organic compounds to yield pure fatty acids. Synthetic or naturally occurring fatty acids have wide applications in different industries such as fortifying agents in food industry, dietary supplements or drugs in pharmaceutical industry, ingredients in cosmetic industry, etc. Pure forms of fatty acids are also important for the synthesis of isotope labelled fatty acid that are widely used in the physical, chemical and biological studies of fatty acids. The uses of isotope labelled fatty acids in cellular lipid metabolism have reviewed by many researchers[6-8)].

The synthesis of MUFA, particularly, *c*9-18:1 was firstly reported in 1934 by Nollar and Bannerot[9)]. Many synthesis routes of unsaturated fatty acids have developed and continuously improved over the past decades. The application of Wittig reaction is the popular and common strategy currently used for the synthesis of unsaturated fatty acids and their geometric isomers[10)]. Monoenoic acids could also be produced by olefinic acid by

partial synthesis via bromination followed by dehydrobromination[11,12]. The synthesis of all the PIs of *cis* and *trans* octadecenoic acid was reported by Gunstone and Ismail[13] and Barve and Gunstone[14]. Synthesis of medium chain MUFA reported by Gilman and Holland[15]. Further, synthesis of LC-MUFA containing chain length 24 to 28 have reported by Pomonis & Hakk in 1990[16].

LC-MUFA shows structural isomerism due to the presence of the olefinic bond. Types of structural isomers exist in LC-MUFA and their PIs are introduced in the Chapter 01. The physico-chemical characteristics of unsaturated fatty acids differ according to position and geometric configuration of the double bond[17]. Accordingly, the physical, chemical, and functional properties could differ among the PIs of LC-MUFA. Although, limited data available on the properties of LC-MUFA PIs containing chain length more than 20, particularly in relation to functional properties. Further, occurrence and distribution of LC-MUFA PIs in food have not studied thoroughly, might due to the unavailability of standards for quantitative analysis and expensive methods associated with the identification and quantification of LC-MUFA PIs. Thus, the current study focused on the organic synthesis of commonly occurring LC-MUFA PIs containing 20 and 22 C atoms. Figure 2.1 and 2.2 show the chemical structures of LC-MUFA PIs to be synthesized in the current study. The synthesized PIs will be used as standards for gas chromatography (GC) analysis in identification of LC-MUFA PIs in fish oil. Further, synthesized LC-MUFA PIs will be used to investigate their functional properties in cell cultures.

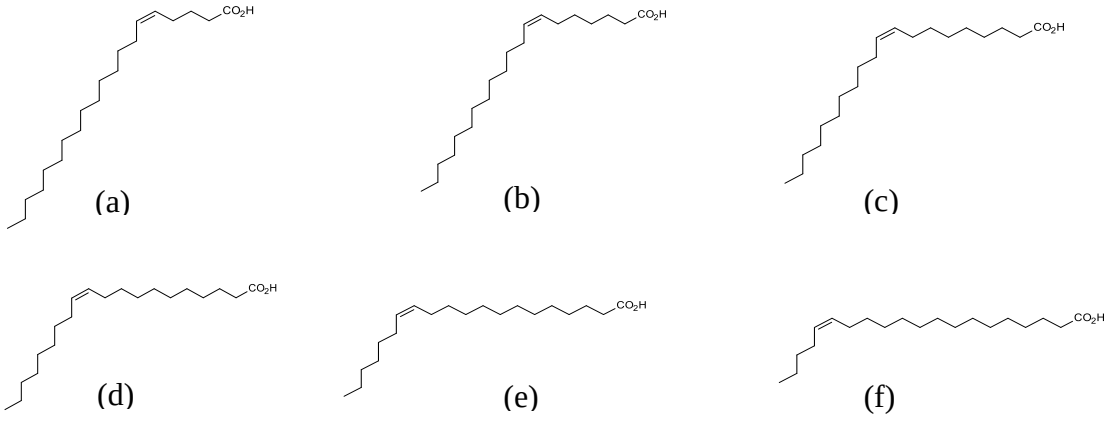



**Fig. 2.1** Chemical structures of common *c*-20:1 PIs (a) *c*5-20:1, (b) *c*7- 20:1, (c) *c*9-20:1, (d) *c*11-20:1, (e) *c*13-20:1, and (f) *c*15-20:1

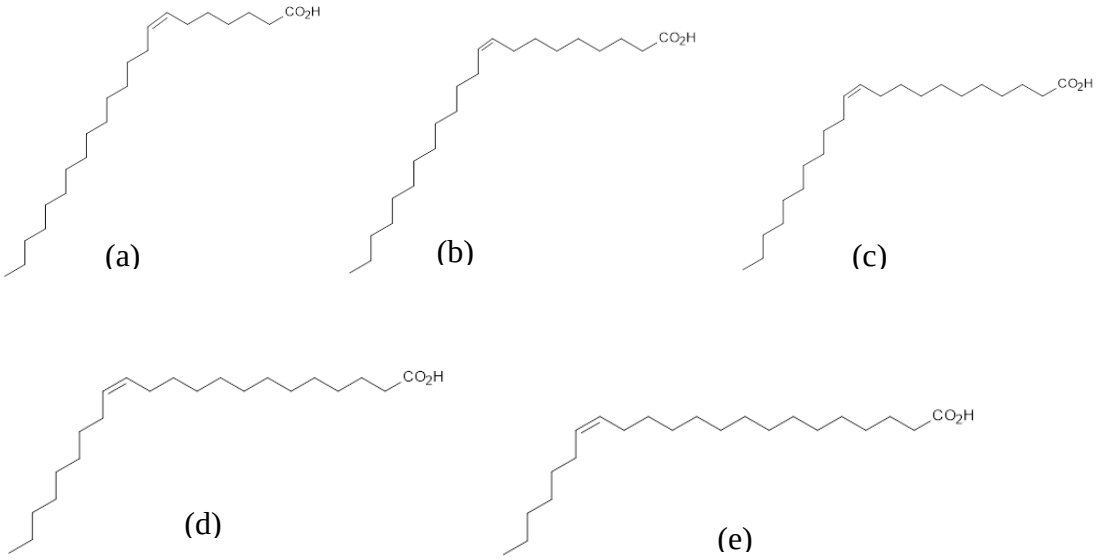



**Fig. 2.2** Chemical structures of common *c*-22:1 PIs (a) *c*7- 22:1, (b) *c*9-22:1, (c) *c*11-22:1, (d) *c*13-22:1, and (e) *c*15-22:1

## 2.2. Materials and Methods

### 2.2.1. Materials

Table 2.1 and 2.2 list the starting materials and alkyne used in the synthesis of *c*-LC-MUFA PIs. All the solvent and chemical reagents used in the synthesis were analytical grade and purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Sigma-Aldrich Japan K.K. (Tokyo, Japan) or Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). GC standards of *c*9-20:1 and *c*9-22:1 methyl esters were purchased from Sigma-Aldrich Japan K.K. The *c*5-20:1 fatty acid was obtained from Nu-Chek Prep, Inc. (Elysian, MN).

Table 2. 1 Initial reactant and alkyne used in synthesis of *c*-20:1 PIs

| No | Isomer | | Required diol | Required alkyne |
|---|---|---|---|---|
| 1 | n-5 | *c*15 | 1,13-Tridecanediol | 1-Hexyne |
| 2 | n-7 | *c*13 | 1,11-Undecanediol | 1-Octyne |
| 3 | n-9 | *c*11 | 1,9-Nonanediol | 1-Decyne |
| 4 | n-11 | *c*9 | 1,7-Heptanediol | 1-Dodecyne |
| 5 | n-13 | *c*7 | 1,5-Pentanediol | 1-Tetradecyne |
| 6 | n-15 | *c*5 | 1,3-Propanediol | 1- Hexadecyne |

Table 2. 2 Initial reactant and Alkyne used in synthesis of *c*-22:1 PIs

| No | Isomer | | Required diol | Required alkyne |
|---|---|---|---|---|
| 1 | n-7 | *c*15 | 1,13-Tridecanediol | 1-Octyne |
| 2 | n-9 | *c*13 | 1,11-Undecanediol | 1-Decyne |
| 3 | n-11 | *c*11 | 1,9-Nonanediol | 1-Dodecyne |
| 4 | n-13 | *c*9 | 1,7-Heptanediol | 1-Tetradecyne |
| 5 | n-15 | *c*7 | 1,5-Pentanediol | 1- Hexadecyne |

### 2.2.2. Common synthesis pathway of LC-MUFA-PIs

The following route of synthesis of LC-MUFA was adopted from a method developed for the synthesis of octadecenoic acid PIs by Tsukishima Foods Industry, Co. Ltd. (Tokyo, Japan). The synthesis route (Fig. 2.3) of LC-MUFA consists of seven or nine main reactions steps followed by purification after each step.

The synthesis pathway starts with bromination of relevant diol followed by lithiation. Then the resulted material will be coupled with alkyne under controlled condition. Next, protective group will be removed by hydrolysis and the material will be subjected to hydrogenation. The formed alkenyl alcohol will be brominated and finally Grignard reaction will be carried to obtain the respective fatty acid isomer. Each reaction step is given in briefly in the following text.

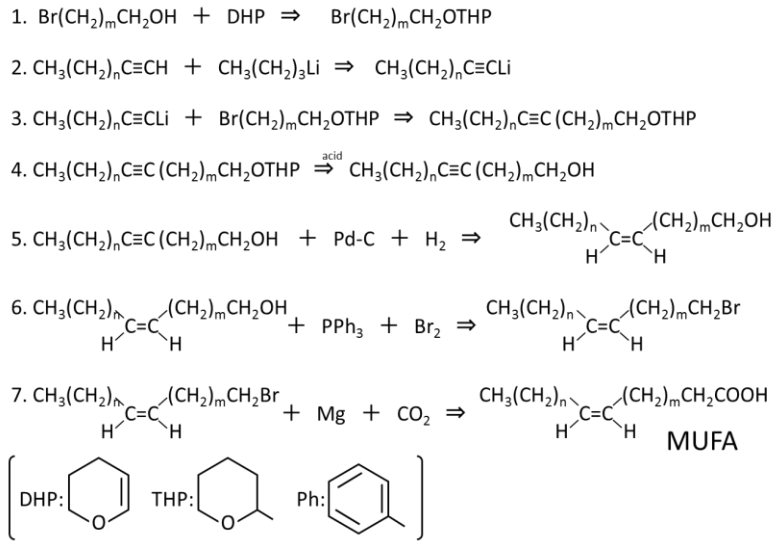



**Fig. 2.3** Synthesis route of LC-MUFA PIs

### 2.2.2.1. Bromination of diol

The respective diol (1.0 mol) (Table 1 or Table 2) and hydrobromic acid (approximately 8 mol) were mixed in a four-neck reaction flask using toluene as the solvent. Reaction mixture was refluxed at 100°C for 2-3 hrs using silicon oil bath with continuous stirring of the reaction mixture. The progress of the reaction was checked every 1 hr interval using thin layer chromatography (TLC, Silica gel 60 $F_{254}$, 5 X 10 cm, film thickness 0.25 mm, Merck kGaA, Darmstadt, Germany). At the completion of the reaction, mixture was neutralized and extracted using a suitable organic solvent. The product was purified by passing the reaction mixture through a silica gel column.

Chemical reaction:

$$HO\ (CH_2)_yOH\ + HBr \xrightarrow[\text{Reflux, 2-3 hrs}]{C_6H_5CH_3} Br\ (CH_2)_yOH\ + H_2O$$

### 2.2.2.2. Protection of hydroxyl group

The brominated material (1.0 mol) from previous step (2.2.2.1) was reacted with 3,4-dihydro-2H-Pyrane (1.2 mol) in dichloromethane for 2-3 hrs in the presence of a catalyst (*p*-toluenesulfonic acid, 0.01 mol) at room temperature (RT) to add tetrahydropyranyl (THP) grpup. Reaction was checked at the beginning and every 1 hr interval using TLC. At the completion of the reaction, mixture was neutralized, and the target material was extracted, washed, dewatered, and purified.

Chemical reaction:

$$Br\ (CH_2)_yOH\ + \text{  } \xrightarrow[\text{Stir, 2-3 hrs, 25°C}]{p\text{-TsOH, } CH_2Cl_2} Br\ (CH_2)_yOTHP$$

### 2.2.2.3. Coupling reaction with the alkyne

Initially, respective alkyne (2 mol) (Table 2.1 and 2.2) and *n*-butyllithium (2 mol) were reacted under Ar in super dehydrated tetrahydrofuran (THF) at below -5°C for 1.5 hrs. Then, hexamethylphosphoric triamide (HMPA) (approximately 100 mL), tetrabutylammonium iodide (TBAI) (0.01 mol), and the material obtained from previous step ($Br(CH_2)_nOTHP$, 1 mol) was added and reacted for overnight at 55–60°C. On the following day, reaction was checked for the completion using TLC and reaction mixture

was neutralized using 10% $H_2SO_4$ followed by the extraction, washing, dewatering, and finally purification.

Chemical reaction:

$$CH_3(CH_2)_xC{\equiv}CH + n\text{-BuLi} \xrightarrow[-5°C,\ 1.5\ hrs]{Ar,\ THF} CH_3(CH_2)_xC{\equiv}C^-\ Li^+$$

$$CH_3(CH_2)_xC{\equiv}\bar{C}\ Li^+ + Br\ (CH_2)_yOTHP \xrightarrow[60°C,\ 12\ hrs,\ H^+]{HMPA,\ TBAI} CH_3(CH_2)_xC{\equiv}C(CH_2)_yOTHP$$

### 2.2.2.4. THP de-protection

The THP de-protection (removal of the protecting group) was carried out by reacting the material prepared from previous step (2.2.2.3) with a mixture of acetic acid, water, and THF (4:2:1, v/v/v) at 45°C for 2-3 hrs with continuous stirring. The progress of the reaction was monitored using TLC. After completion of the reaction, the target compound was extracted with ethyl acetate, followed by washing dewatering and evaporation. Finally, products were purified by passing through a silica gel column.

Chemical reaction:

$$CH_3(CH_2)_xC{\equiv}C(CH_2)_yOTHP \xrightarrow[45°C\ for\ 2\text{-}3\ hrs]{AcOH\text{-}H_2O\text{-}THF} CH_3(CH_2)_xC{\equiv}C(CH_2)_yOH$$

### 2.2.2.5. Hydrogenation

The material ($CH_3(CH_2)_xC{\equiv}C(CH_2)_yOH$, 1 mol) obtained in the previous step (2.2.2.5) was mixed with quinoline (0.45 mol) and the catalyst (Lindlar's catalyst (5% Pd-$CaCO_3$ + $Pb(OCOCH_3)_2$ + quinoline), 0.02 mol) inside a glass tube using hexane as the solvent. Next, mixture was reacted with hydrogen inside a metal pressure reactor under pressure of 0.5 MPa for 18 - 20 hrs with continuous stirring. On the following day, the solvent with organic products was filtered and mixture was neutralized with 3N HCl, washed with NaCl(aq), followed by dehydration and purification. Reactants and products were analyzed for the completion of the reaction using Gas chromatography (GC) (TRACE GC ULTRA, Thermo Fisher Scientific, Waltham, MA.) fitted with an InertCap Pure WAX GC column (30 m x 0.25 mm, 0.25 μm, GL Sciences Inc., Tokyo, Japan) and a flame ionization detector (FID). Injector and detector temperatures were maintained at 250°C. Analysis was carried out using He (1.2 mL/min) as the carrier gas. The split ratio was 20:1

(v/v). The column temperature was maintained at 80°C for 3 min and gradually increased to 150°C with the rate of 10 °C/min afterward up to 250°C with the rate of 20 °C/min.

Chemical reaction:

$$CH_3(CH_2)_xC{\equiv}C(CH_2)_yOH \xrightarrow[H_2,\ 0.5\ MPa,\ 25°C]{\text{Quinoline, Lindlar's}} CH_3(CH_2)_xCH{=}CH(CH_2)_YOH$$

**2.2.2.6. Bromination**

Initially, triphenyl phosphine (1.5 mol) was reacted with bromine (l) (1.3 mol) in super dehydrated $CH_2Cl_2$ at below 10°C for 1 hr. Then, reaction mixture was neutralized using a mixture of same quantities of pyridine (1.6 mol) and acetonitrile (1.6 mol) followed by the addition of the reactant ($CH_3(CH_2)_xCH{=}CH(CH_2)_YOH$, 1 mol) into the mixture and reacted for 2-3 hrs at RT. The completion of reaction was checked using TLC, followed by evaporation and purification using silica gel column.

Chemical reaction:

$$CH_3(CH_2)_xCH{=}CH(CH_2)_YOH \xrightarrow[\text{Acetonitrile, 2-3 hrs, RT}]{Br_2,\ TPP,\ Pyridine,\ CH_2Cl_2,} CH_3(CH_2)_xCH{=}CH(CH_2)_YBr$$

**2.2.2.7. Grignard reaction**

The target carboxylic acid or the monounsaturated fatty acids was synthesized by preparing the respective Grignard reagent followed by carboxylation. At first, Grignard reagent was prepared by reacting with activated Mg pieces (1.05 mol) (using iodine and 1,2-dibromoethane) and in THF (super dehydrated). Synthesized alkene bromide (1 mol) was added to the activated Mg under controlled relative humidity and low temperature (below 15°C). Next, Grignard reagent was treated with carbon dioxide followed by acidification to synthesize carboxylic acid. The product was purified by passing the reaction mixture through a silica gel column.

Chemical reaction:

$$CH_3(CH_2)_xCH{=}CH(CH_2)_Y\,MgBr \xrightarrow{CO_2,\ H_3O^+} CH_3(CH_2)_xCH{=}CH(CH_2)_YCOOH$$

### 2.2.2.8. Additional chemical reactions

Two supplementary reactions, namely, methyl esterification and reduction of ester group, were used when the respective diol was not commercially available. Thus, the synthesis of *c*15-20:1 and *c*7-22:1 consisted of nine main reaction steps followed by purification after each reaction. The corresponding dienoic acid (i.e. 1,13 tridecanedioic acid) was methyl esterified by refluxing in a mixture of methanol and sulfuric acid (100:1, v/v) at 80–90°C for 2-3 hrs. The resulted dimethyl ester was purified and subjected to alcoholization. Briefly, the 1,13-dimethyltridecandiote (3.3 mol) was reacted with $LiAlH_4$ (2 mol) in THF (super dehydrated) under Ar at RT for 5 hrs. Resulted product was extracted using ethyl acetate and purified by passing through a silica gel column.

Chemical reactions:

$$HOOC(CH_2)_{11}COOH \xrightarrow{CH_3OH,\ H^+,\ 90°C} CH_3OOC(CH_2)_{11}COOCH_3$$

$$CH_3OOC(CH_2)_{11}COOCH_3 \xrightarrow[\text{Stir 5 hr}]{LiAlH_4,\ THF} HO(CH_2)_{13}OH$$

### 2.2.3. Thin layer chromatography (TLC)

The intermediate and final products in each synthesis step were checked using TLC with a suitable developing solution. The retention factor (Rf) values corresponding to each product were calculated using the formula given below.

$$\mathbf{R_f} = \frac{\text{Distance travelled by solute (from baseline)}}{\text{Distance travelled by solvent (from baseline)}}$$

### 2.2.4. The percentage yield of reactions

The yield of each reaction was calculated based on the percentage molar ratio of product to reactant.

### 2.2.5. Analysis of LC-MUFA PIs using GC-FID and GC-MS

Each synthesized positional isomer was analyzed using GC-FID to determine the purity and for confirmation of the synthesis. Fatty acid methyl esters (FAME) was prepared by reacting pure fatty acids (50 μg) with 14% $BF_3$– methanol solution. Prepared FAME was purified by passing through silica gel column and analyzed using GC-2014 (Shimadzu Corporation, Kyoto, Japan). FAME separation was carried out on a highly polar

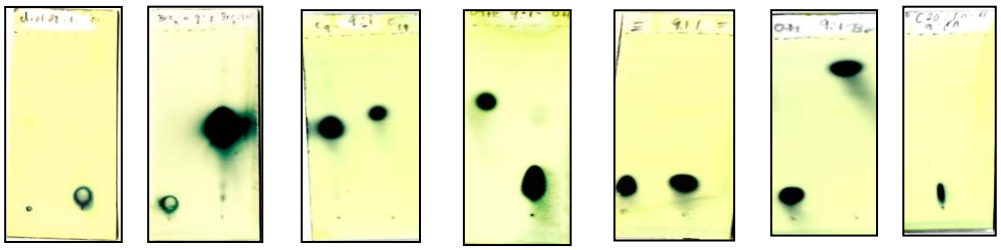
ionic liquid (IL) capillary column (SLB-IL111, 100 m x 0.25 mm, 0.2 μm, Sigma-Aldrich, Japan K.K.) and peak was identified by comparing with the retention time of standard *c*9-20:1. Injector and detector temperatures were maintained at 250°C. Analysis was carried out isothermally at 160°C using He (1.2 mL/min) as the carrier gas. The split ratio was 100:1. LC-MUFA PIs were analyzed out using GC (TRACE GC ULTRA, Thermo Fisher Scientific) – Mass spectrometry (MS) (ITQ1100, Thermo Fisher Scientific) fitted with a metal capillary GC column (Ultra ALLOY$^{+}$-65, 30m, 0.25 mm, 0.1μm, Frontier Laboratories Ltd., Koriyama, Japan) to confirm the molecular weight of respective fatty acids. Injector and detector temperatures were maintained at 300°C. The split ratio was 20:1 (v/v) and ionization mode was chemical ionization (CI).

### 2.3. Results

LC-MUFAs, i.e. *c*-20:1 and *c*-22:1 PIs, were synthesized as described in section 2.2.3. The intermediate and final products of synthesis were tested using TLC, GC-FID, and GC-MS as required.

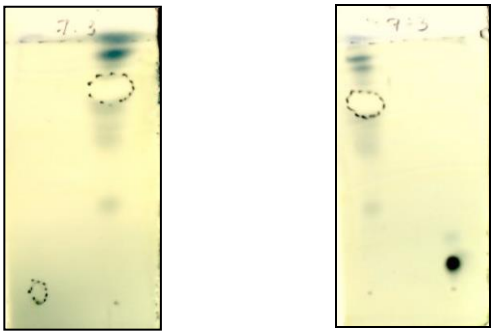
#### 2.3.1. TLC

The analysis of intermediate and the final product of synthesis was carried out using TLC. Figure 2.4 and 2.5 shows the TLC testing of reactants and products at the beginning (left side spot in each plate) and the completion (right side spot in each plate) of each reaction. The Rf values were calculated in each reaction of the synthesis of *c*11-20:1 (Table 2.3). Suitable solvent ratios of organic solvents were used to prepare the developing solution for the TLC based on the pre-tested Rf values.



(a) (b) (c) (d) (e) (f) (g)

**Fig. 2.4** TLC plates obtained in each reaction (before reaction–left side spot, after reaction-right side spot) (*n*-hexane: ethyl acetate =9:1, v/v); (a) Bromination of diol, (b) THP protection, (c) Coupling with alkyne, (d) Hydrolysis, (e) Hydrogenation, (f) Bromination, and (g) Grignard reaction



(a) (b)

**Fig. 2.5** TLC plates of obtained for additional reactions (a) Methyl esterification and (b) Reduction of ester group

Table 2.3 Rf values for the intermediate and final products of synthesis of *c*11-20:1 (20:1 n-9)

| | Rf values | | |
|---|---|---|---|
| Compound | Hex:Ethyl Ac = 9:1 | Hex:Ethyl Ac = 7:3 | Hex:Ethyl Ac= 6:4 |
| $HO(CH_2)_9OH$ | 0.02 | 0.06 | 0.1 |
| $HO(CH_2)_9Br$ | 0.08 | 0.29 | 0.43 |
| $Br(CH_2)_9OTHP$ | 0.49 | 0.65 | 0.71 |
| $CH_3(CH_2)_7\ C{\equiv}C(CH_2)_9OTHP$ | 0.55 | 0.73 | 0.82 |
| $CH_3(CH_2)_7\ C{\equiv}C(CH_2)_9\ OH$ | 0.16 | 0.43 | 0.66 |
| $CH_3(CH_2)_7\ CH{=}CH(CH_2)_9\ OH$ | 0.18 | 0.50 | 0.69 |
| $CH_3(CH_2)_7\ CH{=}CH(CH_2)_9\ Br$ | 0.83 | 0.87 | 0.92 |
| $CH_3(CH_2)_7\ CH{=}CH(CH_2)_9COOH$ | 0.08 | 0.45 | 0.69 |

Hex: *n*-hexane, Ethyl Ac: ethyl acetate

### 2.3.2. Percentage yield of reactions

Purification of the target products was carried out using silica gel column (flash column) after each reaction and the percentage yield was calculated based on the molar ratio of reactant to product. Table 2.4 and 2.5 show the percentage yield obtained for each reaction in synthesis of *c*-20:1 PIs and *c*-22:1 PIs, respectively. Accordingly, most of reactions gave a higher yield (> 75%) with few exceptions such as Grignard reaction.

Table 2.4 Yield of each reaction in synthesis of *c*-20:1 PIs

| Reaction | % yield | | | | | | |
|---|---|---|---|---|---|---|---|
| | n-5 (*c*15) | n-6 (*c*14) | n-7 (*c*13) | n-9 (*c*11) | n-11 (*c*9) | n-13 (*c*7) | n-15 (*c*5) |
| Methyl esterification | 94 | _ | _ | _ | _ | _ | _ |
| Reduction of ester group | 55 | _ | _ | _ | _ | _ | _ |
| Bromination | 79 | _ | _ | 87 | 75 | 85 | 82 |
| THP protection | 93 | 83 | 95 | 88 | 91 | 48 | 52 |
| Coupling reaction | 72 | 78 | 74 | 86 | 54 | 84 | <5 |
| Hydrolysis | 95 | 97 | 87 | 50 | 91 | 90 | |
| Hydrogenation | 98 | 98 | 93 | 95 | 96 | 93 | |
| Bromination | 91 | 94 | 91 | 97 | 95 | 85 | |
| Grignard reaction | 78 | 53 | 52 | 62 | 36 | 15 | |
| Overall yield | 19 | 29 | 27 | 18 | 22 | 10 | |

Table 2.5 Yield of each reaction in synthesis of *c*-22:1 PIs

| Reaction | % yield | | | | |
|---|---|---|---|---|---|
| | n-7 (*c*15) | n-9 (*c*13) | n-11 (*c*11) | n-13 (*c*9) | n-15 (*c*7) |
| Methyl esterification | 94 | - | - | - | - |
| Reduction of ester group | 60 | - | - | - | - |
| Bromination | 80 | - | 85 | 78 | 82 |
| THP protection | 91 | 89 | 84 | 92 | 48 |
| Coupling reaction | 77 | 80 | 51 | 75 | 79 |
| Hydrolysis | 86 | 84 | 80 | 89 | 91 |
| Hydrogenation | 96 | 98 | 72 | 90 | 99 |
| Bromination | 91 | 90 | 90 | 96 | 98 |
| Grignard reaction | 35 | 42 | 47 | 59 | 76 |

### 2.3.3. GC-FID and GC-MS analysis of synthesized LC-MUFA PIs

The GC analysis of synthesized LC-MUFA PIs was carried out to confirm the synthesis process and the results indicated the successful synthesis of most of the isomers with purity > 97% except for *c*15-22:1. Fatty acids eluted at different retention times in the SLB-IL 111 high polar capillary GC column with an increasing order of Δ position. Figure 2.6 and 2.8 show the gas chromatograms of the synthesized *c*-20:1 and *c*-22:1 PIs, respectively. Molecular weight of synthesized fatty acids was analyzed by GC-MS and confirmed the synthesis of particular LC-MUFAs by comparing the theoretical molecular weight of the major fragmentation ions with that of the respective mass spectrum. The GC-MS data obtained for the analysis of *c*9-20:1 (20:1 n-9) was given in the Fig. 2.7. The synthesis of *c*5-20:1 isomer was not completed due to the very low yield resulted after the coupling reaction.

## 2.4. Discussion

The chemical synthesis of LC-MUFA PIs was explained in this chapter and the synthesis of the most of targeted *c*-20:1 and *c*-22:1 PIs were successfully carried out using the method explained in the section 2.2. Thus, the given synthesis route could be adopted in the synthesis of medium chain, long chain, or very long chain MUFA PIs. Further, results confirmed the synthesis of *cis* configuration of LC-MUFA PIs with a higher purity (>97%). Although, synthesis of LC-MUFA PIs with the double bond closer to methyl end and carboxylic end requires more consideration due to unavailability of starting materials and due to incompatibility of order of reaction in the given synthesis route.

Analysis of final and intermediate products of synthesis were carried out using TLC and GC-FID. The Rf values of compounds differ due to differences in the polarity of the solvent or the analyte, variation in the chromatographic conditions such as stationary phase, mobile phase, temperature, etc. [18]. However, in TLC, identical Rf values will not always indicate identical compounds. Therefore, GC-FID or GC-MS analysis must perform in doubtful situations.

Some of the reactions resulted a low yield due to many inevitable experimental conditions. Yield of an organic synthesis reaction is highly dependent on many external and internal factors such as temperature, pH, concentration of reactants, chemical nature of

reactants and products, speed of mixing, rate of reaction, and stoichiometry of reactant to products, etc.[19]. Thus, optimum reaction conditions may vary significantly from compound to compound. In addition, the purification step also caused a low yield as material loss while transferring from one container to another or due to human errors. In the current study, optimization of reaction conditions was not given priority as prime objective was the successful synthesis of target PIs.

Reactions such as reduction of dimethyl brassylate, bromination of alkyl diol, THP protection of some diols (propanediol, pentanediol), and Grignard reaction resulted in a low yield. In reduction of dimethyl brassylate, initial reaction condition was not favorable in converting dimethyl brassylate in to 1,13-tridecanediol and resulted a very low yield. The reaction time was increased up to five hours to improve the yield of the reaction (55%). It was clear that reaction conditions had to be optimized to achieve a higher yield in this reaction. The order of reactions mentioned in section 2.2.2 was changed in the synthesis of *c*5-20:1, *c*7-20:1, and *c*7-22:1 because 1,3-propanediol and 1,5-pentanediol cannot be brominated directly using HBr due to formation of cyclic structure during the reaction. Thus, first THP protection of 1,3 propanediol and 1,5 pentanediol was carried out to synthesize $C_5H_8O(CH_2)_n$ OH, which resulted a low yield of the target compound.

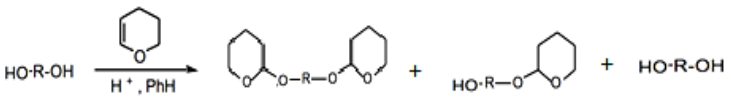



**Fig. 2.9** Possible products of THP protection of alkyl diol

This reaction leads to three possible products as shown in Fig. 2.9; consequently, the yield of targeted compound was low. Reaction conditions could be changed such as changing molar ratios of reactants to increase the forward reaction. Another option is use of the Appel reaction[20] using alkyl diol, triphenyl phosphine and bromine (as mentioned in section 2.2.2.6.), although it leads to form dibromoalkane if the reaction is not controlled properly.

Protecting groups are commonly used in organic synthesis to conduct a selective chemical reaction if compounds containing multifunctional groups. In this experiment, THP group was used (section 2.2.2.2.) to protect the hydroxyl group prior to the coupling

reaction. It is a widely used protective group due to low cost, ease of installation, general stability to most nonacidic reagents and ease of removal[21]. The coupling reaction serves as a major step in this synthesis pathway where a C-C triple bond is introduced through coupling of alkyl halides with terminal alkyne under Sonogashira type reaction[22]. Most of the reactions utilized specific catalysts that facilitate the reaction of interest and improves the yield of the target product. For instance, in coupling reaction, TBAI acts as a phase transfer catalyst that facilitates the interphase transfer of species allowing reactions between reagents in two immiscible phases[23]. Further, in hydrogenation reaction, partial hydrogenation was carried out using a selective catalyst. Partial hydrogenation of alkyne group could be achieved through Lindlar's catalyst[24]. Presence of little quinoline in the reaction medium reported to increase the production of *cis* isomer (yield> 98%). Another optional selective catalyst for the partial hydrogenation is borohydride–reduced Nickel developed by the Brown and Ahuja[25]. Nohair *et al.*[26] reported that the selectivity of the catalyst could be enhanced by the addition of Cu or Ni to the reaction medium. Impurities such as saturated fatty acids, traces of *trans* isomer could be present after the partial hydrogenation and best to be removed by silver ion silica chromatography. The hydrogenation reactions involved with unsaturated fatty alcohols are complicated by the fact that the double bond also tends to react. Thus, experimental conditions and the catalysts must be carefully selected to prevent saturation.

The Grignard reaction is used to create a variety of products in organic synthesis. This reaction was discovered by the Victor Grignard in 1900 and one of the widely used reaction in organic synthesis. The Grignard reagent is formed through the reaction of an alkyl or aryl halide with magnesium metal via a radical mechanism. Grignard reagent are stable in ethereal solution and in general quite reactive[27]. Thus, proper control of experimental conditions such as exclusion of atmospheric moisture and oxygen is required to get the target product. The synthesis of MUFA PIs also could be achieved using Wittig reaction with less number of reaction steps. The Wittig reaction is applicable to synthesize MUFA PIs with the double bond located closer to the methyl or carboxyl end of the acyl chain due to some mismatches of the reactions explained in this chapter. The position of the double bond in MUFA can be confirmed by GC-MS analysis of dimethyl disulfide (DMDS) adducts of MUFA fatty acid methyl esters as described by Nichols and Guckert[28]. In GC-MS analysis of DMDS derivatives of MUFA PIs, diagnostic and

characteristics ions are formed by the cleavage between the $SCH_3$ ions (Δ and ω fragments).

In the current study, LC-MUFA PIs were synthesized in the laboratory due to commercial unavailability of some PIs and difficulty in isolating pure PIs from natural sources. The given synthesis pathway provides a cost-effective way to obtain LC-MUFA PIs particularly in large scale synthesis.

## 2.5. Conclusion

Results revealed that the LC-MUFA PIs, i.e. *c*-20:1 PIs and *c*-22:1 PIs, could be synthesized successfully using the given synthesis route described in this chapter. GC analysis indicated that the synthesized *c*-LC-MUFA PIs had a high purity (>97%) and suitable to be used in the cell culture or animal studies. Further, synthesized *cis*-LC-MUFA PIs could also be used as standards for GC or high-performance liquid chromatography (HPLC).

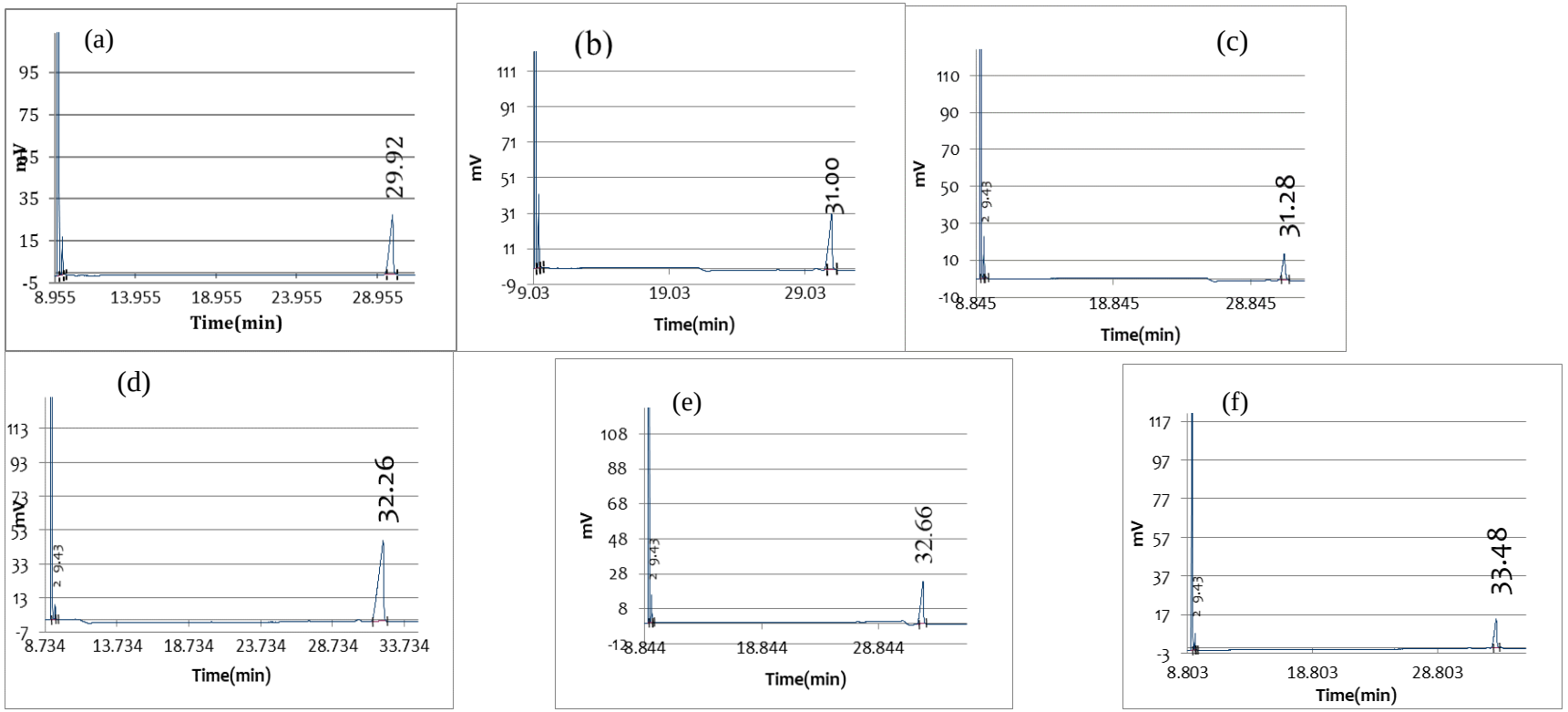



**Fig. 2.6** GC-FID chromatograms of *c*-20:1 PIs a) 20:1 n-15, b) 20:1 n-13, c) 20:1 n-11, d) 20:1 n-9, e) 20:1 n-7, and f) 20:1 n-5

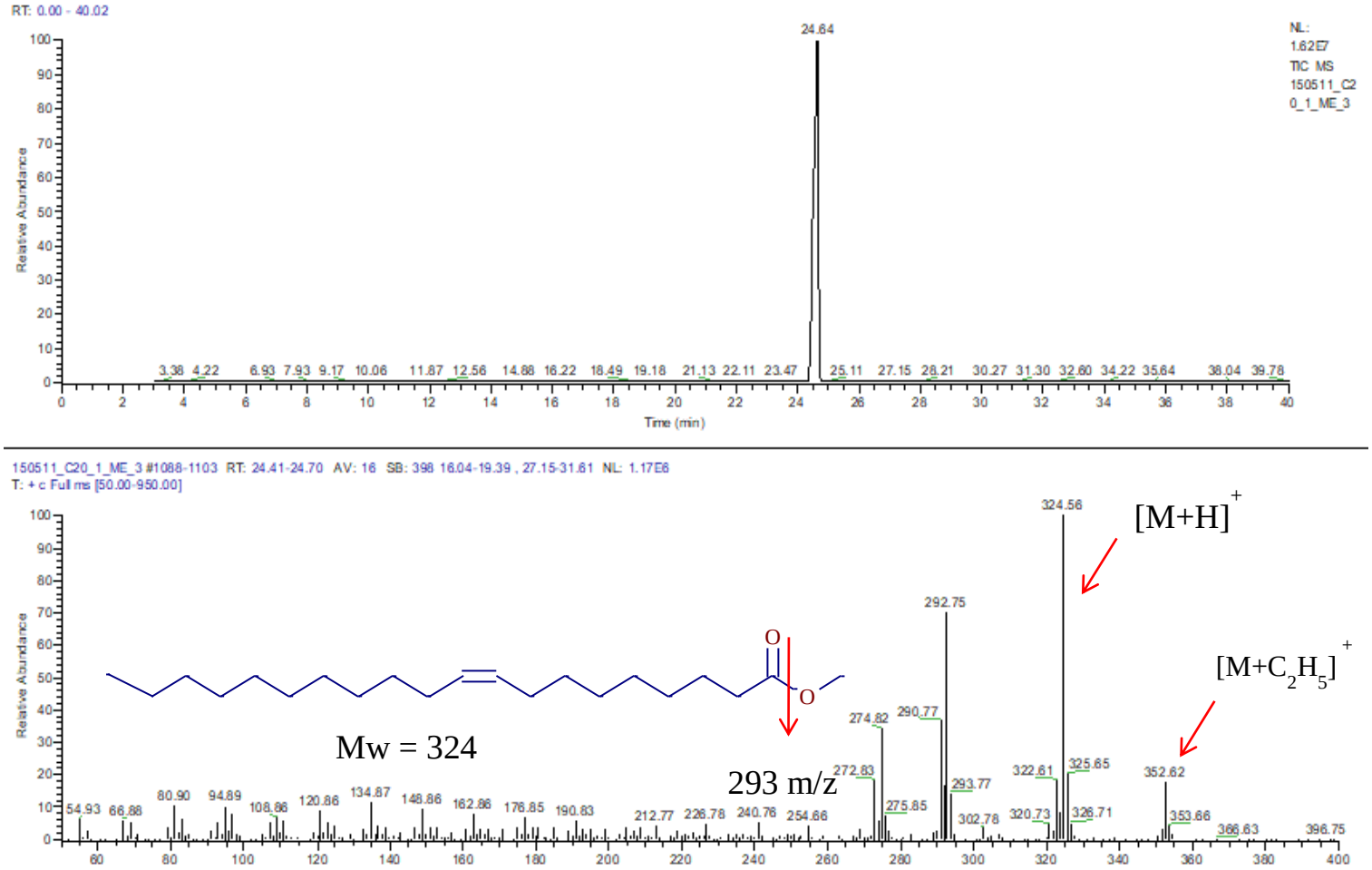



**Fig. 2.7** GC mass spectrum of *c*9-20:1 (20:1 n-11) isomer

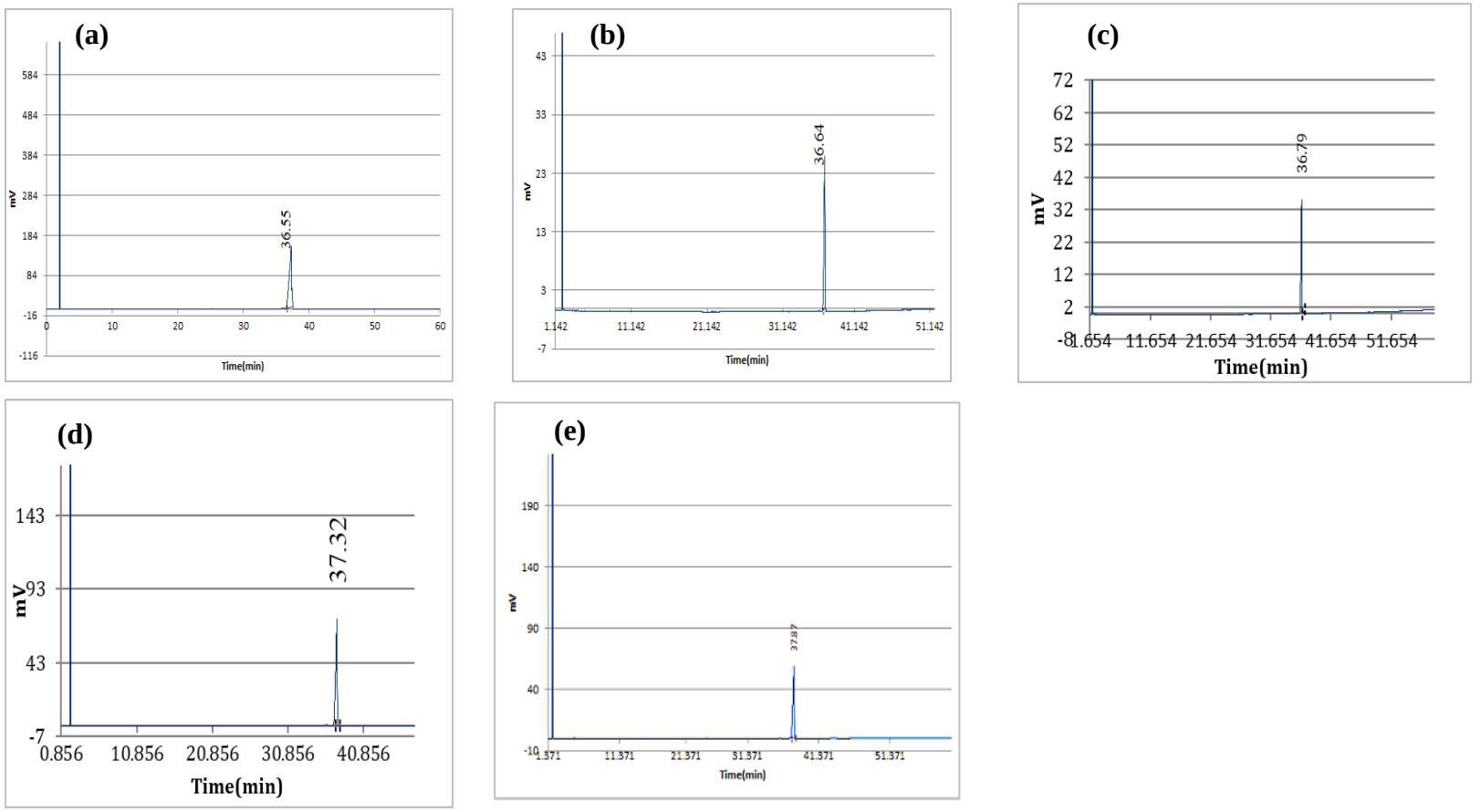



**Fig. 2.8** GC-FID chromatograms of *c*-22:1 PIs a) 22:1n-15, b) 22:1 n-13, c) 22:1 n-11, d) 22:1 n-9, and e) 22:1 n-7

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## Chapter 03

## Quantitative analysis of the distribution of *cis*-eicosenoic acid positional isomers in marine fishes

### 3.1. Introduction

*Cis*-eicosenoic acid (*c*-20:1) is a LC-MUFA consisting of a *cis*-olefinic bond and is commonly found in fish lipids[1)] and some plants belonging to the Sapindaceae, Brassicaceae, Limnathaceae, and Cruciferae families[2)]. Some pelagic and marine fishes contain high amounts of LC-MUFAs, especially *c*-20:1. In marine fishes such as herring, mackerel, capelin, pollock, flathead flounder, and cod, *c*-20:1 is one of the major fatty acids (FAs) accounting for 5–15% of total FA composition[3)]. LC-MUFAs may contribute to the well-known functional health benefits of fish oil. In the past decade, LC-MUFAs (>18 C atoms) in fish oil have received great attention due to their beneficial health effects, particularly in modulating risk factors associated with cardiovascular diseases and diabetes through their ability to decrease plasma levels of very low-density lipoproteins and triacylglycerols[4)], and their favorable effects on insulin sensitivity[5)].

Dietary FAs differ from each other due to differences in the constituent chain length, number of the double bonds, position of the double bond, and geometry (*cis* or *trans*) of the double bond. In most naturally occurring unsaturated FAs, the geometric configuration is mainly *cis* form, hence the analysis of FA composition in raw/unprocessed food mainly focuses on the *cis* form of FAs. Recently, the role of MUFA isomers in lipid metabolism has received renewed attention due to reported differences in health benefits of geometric and PIs[6)]. The present study focused on PIs of *c*-20:1 in fish oil. PIs are defined as compounds with the same molecular formula but different position/location of an atom or functional group[7)]. According to bioavailability, six PIs of *c*-20:1 have been reported in fishes, namely *c*5, *c*7, *c*9, *c*11, *c*13, and *c*15-20:1[8)]. Due to a greater understanding of the functional and physiological effects of different FA isomers, a more efficient method to analyze the isomeric profile of particular FAs has been needed.

Various methods have been developed by researchers to determine the profile of positional and geometric isomers of FAs in foods. The official method by the AOCS for the determination of the isomeric profile of food uses GC fitted with the 100 m SP-2560 or

CP-Sil 88 capillary column, operated at 180°C isothermally[9]. However, some PIs of *c*-20:1 found in fish oils are not clearly separated from each other, even on the 100 m long capillary column with a cyanopropyl siloxane phase[8,10]. Furthermore, *c*5, *c*7, *c*9, and *c*11 PIs of 20:1 elute as a single peak on polyethylene glycol-coated columns, such as Omegawax 320 and FAMEWAX GC columns, which are commonly used for FA analysis in fish oil[10]. Recent application of the ionic liquid (IL) stationary phase imparts more polar functionalities to the capillary GC columns[11], thus forming a useful tool in the identification of positional and geometric isomers of FAs in food. Nevertheless, the identification of LC-MUFA PIs from a complex mixture of fatty acid methyl esters (FAME) presents a major challenge due to overlap with other FAs. It has been previously reported that the geometric isomers of α-linolenic acid, γ-linolenic acid, and heneicosanoic acid (21:0) overlap with the isomers of *c*-20:1 on the SLB-IL111 GC column[12].

Pre-separation of *cis* and *trans* geometric isomers from total FAME prior to GC analysis allows the correct determination of the isomeric profile of the samples. Thus, pre-fractionation of geometric isomers using silver ion thin layer chromatography ($Ag^+$-TLC) or silver ion high performance liquid chromatography ($Ag^+$-HPLC) followed by GC analysis is widely practiced in the analysis of monoenoic isomers[9]. Recently, some researchers have used reversed-phase HPLC (RP-HPLC) as an alternative method in separating the MUFA fraction of samples prior to GC analysis[13].

The limited amount of data regarding the distribution of positional and geometric isomers of LC-MUFAs is mainly due to the unavailability of GC standards and appropriate GC columns. A few studies have investigated the distribution of PIs of *c*-20:1 and *c*-22:1 in fishes captured from the Pacific Ocean and Atlantic Ocean. In these studies, *c*-20:1 positional isomer was separated from flathead flounder and used as a reference[14]. Studies have reported the distribution of *c*-20:1 in the lipids of 38 species, including Japanese pelagic fish, benthic fish, shellfish, and crustaceans[10, 14, 15]. To date, no research has been conducted on the distribution of PIs of LC-MUFAs in fishes from the Indian Ocean. The current study aimed to quantify *c*-20:1 PIs in marine fish by RP-HPLC pre-separation followed by GC analysis using the chemically synthesized *c*-20:1 isomers (described in chapter 02) as a reference. The study also compared the isomeric profile of *c*-20:1 among fishes from the Indian, Pacific, and Atlantic Ocean.

## 3.2. Materials and methods

### 3.2.1. Chemicals and materials

Six PIs of *c*-20:1 (*c*7, *c*9, *c*11, *c*13, *c*14, and *c*15) were synthesized in the laboratory using the method described in chapter 02 and used as standards in the identification of *c*-20:1 PIs in fish oil. The chemical reagents were obtained from Wako Pure Chemical Industries, Ltd. and Sigma-Aldrich Japan K.K. The standard fatty acid methyl esters (FAMEs) (Supelco-37 component FAME mix) and 21:0 were purchased from Sigma-Aldrich Japan K.K. *c*5-20:1 methyl ester was obtained from Nu-Chek Prep, Inc., Elysian, MN.

### 3.2.2. Sample collection

Fishes caught from the Indian Ocean (n=45) were purchased from a fish-landing site in Negombo, Sri Lanka. The fishes were immediately chilled after purchase and brought to Japan under frozen and vacuum-sealed conditions. Fish from the Pacific Ocean (n=3) and the Atlantic Ocean (n=3) were purchased from a supermarket in Japan and stored under frozen conditions until use for oil extraction.

### 3.2.3. Sample preparation

Fish oil was extracted from the edible portion (flesh in large or medium sized fish, whole fish with or without the gut in small fish) of each fish using the Bligh and Dyer method[16], and the oil percentage of each fish sample was determined on a wet weight basis. All the crude oil samples were stored at -40°C until further analysis.

FAME was prepared from each crude oil sample (using the JOCS official method 2.4.1.2[17]) with 21:0 as an internal standard (IS). The fish lipids were saponified in 0.5 M NaOH–methanol solution at 90°C for 15 min and the resulting FAs were methylated using 14% $BF_3$–methanol solution. Each FAME sample was purified using silica gel column chromatography and used in HPLC fractionation.

### 3.2.4. HPLC separation of 20:1 FAME

Each FAME sample was added with a known quantity of *c*14–20:1 FAME (as an IS) prior to fractionation by HPLC. The eicosenoic methyl ester fraction from total FAME samples was obtained using RP-HPLC fitted with semi-preparative octadecyl (C18) silica gel column (Inertsil ODS, 250 mm, 10.0 mm i.d., 10 μm particle size, GL Sciences Inc., Tokyo, Japan) equipped with a solvent pump (1580-PU, JASCO Corporation, Hachioji, Japan) at a flow rate of 4 mL/min and a UV spectrometer (L-4000, Hitachi High-Technologies Corporation, Tokyo, Japan) at 210 nm wavelength and using a chromatopac integrator (C-R5A, Shimadzu Corporation, Kyoto, Japan). Separated *c*-20:1 FAME fractions were vacuum evaporated and subsequently used in the GC analysis.

### 3.2.5. GC-FID analysis

Each FAME sample was screened for the availability of *c*-20:1 by analyzing total FA composition using GC-2014 (Shimadzu Corporation) fitted with a FID. FAME separation was carried out on the Omegawax 320 capillary GC column (30 m x 0.32 mm x 0.25 μm, Sigma-Aldrich Japan K.K.) and peaks were identified using the Supelco 37 component FAME mix standard (data for total FA composition are not shown).

The *c*-20:1 FAME fractions obtained from HPLC were subjected to GC-FID analysis using GC-2014 (Shimadzu Corporation) fitted with a highly polar ionic liquid (IL) capillary column (SLB-IL111, 100 m x 0.25 mm, 0.2 μm, Sigma-Aldrich Japan K.K.). Injector and detector temperatures were maintained at 250°C. Separation was carried out isothermally at 160°C using helium (1.2 mL/min) as the carrier gas. The split ratio was 100:1 (v/v). Three replicate analyses (from step 3.2.3 to step 3.2.5) were performed per sample.

### 3.2.6. Quantification of *c*-20:1 PIs

The quantities of individual *c*-20:1 PIs (mg/g of oil) were measured using *c*14-20:1 as an IS. The total and individual FA results are presented as the mean ± SD for three replicates.

### 3.2.7. Gas chromatographic characteristics of *c*-20:1 PIs

#### 3.2.7.1. Preparation of calibration curves

Standard solution series were prepared for each isomer with a known concentration of *c*14-20:1 as an IS and used in GC analysis. A calibration curve for each isomer was obtained by plotting the ratio of test isomer concentration to IS vs the ratio of the peak area of the test isomer to IS. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using signal-to-noise (*s*/*n*) ratio and the values were defined as *s*/*n* = 3 and *s*/*n* = 10, respectively.

#### 3.2.7.2. Resolution and relative response factor

A standard solution containing all selected PIs of *c*-20:1 FAMEs was prepared, including *c*14-20:1 FAME as an IS. After GC analysis, the resolution factor of two *c*-20:1 methyl ester isomer peaks adjoining each other and the relative response factor of *c*-20:1 PIs were calculated using the equation[18] given below.

$$\text{Resolution factor (Rs)} = 2\,(tR2-tR1) / W1+ W2$$

where, $t_{R1}$ and $t_{R2}$ are retention times, $W_1$ and $W_2$ are the width of the first and second-eluted peaks, respectively.

$$\text{Relative response factor (Rf)} = PsCa / PaCs$$

where, $P_s$ and $P_a$ are peak areas, $C_s$ and $C_a$ are concentrations of IS and test positional isomer or analyte, respectively.

## 3.3. Results

### 3.3.1. Percentage of lipid content

Table 3.1 indicates the names (scientific and common) of analyzed fishes, the portion of the fish that was used to extract lipid and the percentage lipid content (wet weight basis). Fish from the Pacific Ocean demonstrated the highest percentage of lipid (true sardine: 27.71±1.53%, saury: 25.05±0.65%) followed by fish caught from the Atlantic Ocean (Atlantic mackerel: 24.69±1.02%) and the lowest percentage of lipids

recorded in fish from the Indian Ocean with levels ranging from 0.9% (longface emperor) to 9.2% (spotted sardinella).

### 3.3.2. HPLC separation of *c*-20:1

Figure 3. 1 (a) shows the HPLC chromatogram obtained for the mixture of *c*-20:1 PIs methyl esters prepared with commercial materials along with *c*14–20:1 as an IS. Figure 1 (b) shows the HPLC chromatogram obtained for total FAME in fish oil using the octadecyl silica gel column. The retention time corresponding to fractionation of *c*-20:1 PIs methyl esters was ranged from 14.8–16.5 min.

### 3.3.3. Gas chromatographic characteristics of 20:1 PIs

Relative response factor ($R_f$) and resolution factor ($R_s$) for *c*-20:1 PIs were calculated, and values are shown in Fig. 3.2 and Table 3.2, respectively. The relative response factor obtained for each isomer in comparison to the *c*14-20:1 isomer was close to 1 (ranged from 0.81–1.18). The $R_s$ values ranged from 0.7–1.7 between two adjoining *c*-20:1 PIs.

According to the partial gas chromatogram shown in Fig. 3. 3 (a), the elution order of PIs of *c*-20:1 was in the sequence of *c*5, *c*7, *c*9, *c*11, *c*13, and *c*15-20:1 at an isothermal temperature of 160°C on the highly polar SLB-IL111 GC column. The IS (*c*14-20:1) eluted (Fig. 3. 3 (b)) between the isomer peaks of *c*13 and *c*15. In most of the PIs, the baseline was resolved clearly except in the *c*13 and *c*14 isomer pair.

### 3.3.4. The occurrence and distribution of *c*-20:1 in fish

#### 3.3.4.1. The total content of *c*-20:1

The total content of *c*-20:1 isomer in fishes from each ocean, namely the Indian, Pacific, and Atlantic Ocean, along with the levels of individual PIs are shown in Table 3.3. The content of *c*-20:1 PIs in fishes varied widely among different oceans. Fish from the Pacific Ocean (saury, 166.95±12.44 mg/g of oil) had the highest levels of total *c*-20:1 followed by capelin from the Atlantic Ocean (162.69±3.51 mg/g of oil). Comparatively, fishes from the Indian Ocean exhibited a low content of *c*-20:1. Among the fishes from the Indian Ocean, goatfish contained the highest levels of *c*-20:1 FA (34.39±4.57 mg/g of oil)

followed by the long spine seabream (30.65±2.54 mg/g of oil) and the lowest levels were found in the long-jaw thryssa (1.33±0.02 mg/g of oil).

#### 3.3.4.2. Distribution of *c*-20:1 PIs

##### *c*5-20:1 isomer

The occurrence of the *c*5-20:1 isomer was very low or not detected in most of the fishes. The highest content of *c*5-20:1 isomer was found in two fishes from the Indian Ocean namely, long spine seabream (0.70±0.36 mg/g of oil) and goatfish (0.69±0.17 mg/g of oil).

##### *c*7-20:1 isomer

In the Indian Ocean fishes, all of the analyzed fishes contained *c*7-20:1 FA isomer contributing to the third highest occurring (≈ 18%) among the 20:1 PIs with the content ranging from 0.13±0.01 mg/g of oil to 19.5±6.75 mg/g of oil. The long spine seabream showed the highest content of *c*7-20:1 (19.5±6.75 mg/g of oil), which was the utmost value, reported among all the analyzed *c*-20:1 PIs among the Indian Ocean fishes. In some fishes, such as tooth pony, long face emperor, commerson's sole, Indian anchovy, and long spine-seabream, the *c*7-20:1 FA isomer contributed to the highest proportion among total *c*-20:1. In contrast, only horse mackerel (0.23±0.03 mg/g of oil) contained this isomer among the Pacific and Atlantic Ocean fishes.

##### *c*9-20:1 isomer

Most of the fishes from the Indian and Atlantic Ocean contained a low amount of *c*9-20:1 isomer, in contrast to the high proportion of *c*9-20:1 isomer in selected fishes from the Pacific Ocean. The saury from the Pacific Ocean contained the highest level (131.10±10.37 mg/g of oil) followed by the true sardine (58.99±10.70 mg/g of oil). Among the Indian Ocean fishes, the long face emperor contained the highest (1.17±0.01 mg/g of oil) amount of *c*9-20:1 isomer. This particular *c*-20:1 isomer was not detected in fishes analyzed from the Atlantic Ocean.

### *c*11-20:1 isomer

The predominant *c*-20:1 positional isomer in most of the fishes from the Indian and the Atlantic Ocean was *c*11-20:1. Among the Indian Ocean fishes, the highest amount of *c*11-20:1 was reported in the goatfish (15.97±2.28 mg/g of oil) followed by the crimson job fish (7.38±0.52 mg/g of oil). It is interesting to note that this isomer contributed >95% to the total *c*-20:1 content with an uppermost value of 156.60±3.35 mg/g of oil reported in capelin from the Atlantic Ocean. In case of the fishes from the Pacific Ocean, *c*11-20:1 was the second highest occurring (≈ 20 %) among total *c*-20:1 with the content ranging from 11.25±1.58 mg/g of oil (horse mackerel) to 34.06±1.70 mg/g of oil (saury).

### *c*13-20:1 isomer

The *c*13-20:1 isomer was the second largest occurring (≈ 25 %) in terms of total *c*-20:1 in fishes from the Indian Ocean with the highest content found in commerson's sole (12.2±3.6 mg/g of oil) and the lowest in squid (0.10±0.02 mg/g of oil). In some fishes such as mullet, Indian mackerel, and crimson jobfish, this particular isomer contributed to the highest proportion of the total *c*-20:1. The occurrence of *c*13-20:1 isomer in both the Pacific and Atlantic Ocean fishes was considerably low, with content <1 mg/g of oil.

### *c*15-20:1isomer

Almost all of the analyzed fishes from the three oceans contained a very low amount of *c*15-20:1 isomer, with <1.5% on average. Among the Indian Ocean fishes, rainbow sardine was reported as having the highest value (0.57±0.08 mg/g of oil) of *c*15-20:1, followed by the commerson's sole (0.52±0.29 mg/g of oil). The content of *c*15-20:1 isomer reported in the Pacific Ocean fishes was higher (ranging from 0.05–0.97 mg/g of oil) than that of fishes from the other two oceans, with the lowest content found in fishes from the Atlantic Ocean. Among the six types of tested *c*-20:1 PIs, *c*15 isomer contributed lowest proportion to the total *c*-20:1 content.

## 3.4. Discussion

The distribution of *c*-20:1 PIs in marine fishes mainly from the Indian Ocean were quantitatively analyzed and reported in the current study. The lipid content of fish from the Indian Ocean was comparatively low, which may be due to the removal of the head,

viscera, liver, etc. during sample preparation. Further, as reported by previous studies, the lipid content of the commercially important pelagic fish from the coastal sea along Sri Lanka varies between 0.69–14.6% and changes when compared with that of edible portion in some fish such as white sardinella[19]. In particular, fish from tropical regions have been previously reported to have lower amounts of lipids compared to those from Arctic and temperate regions[3]. This finding is logical as body lipids act as an insulator against temperature variation and also provide stored energy when required.

In this study, RP-HPLC separation of *c*-20:1 methyl esters was used as a pre-separation method prior to GC analysis. It was clearly indicated that all the *c*-20:1 PIs were well separated from other FAs, in particular the polyunsaturated FAs. Consequently, the ODS column demonstrated a good fractionation ability of the *c*-20:1 isomers in fish oil samples. The pre-separation of *c*-20:1 FAMEs by RP-HPLC eliminates polyunsaturated FAMEs that could be troublesome in the highly polar GC column and thereby increases the life span of the column. In a recent study, preparative RP-HPLC was used to separate the *cis* and *trans* isomers of unsaturated FAMEs contained in edible oils and reported a higher percentage of reproducibility of fractionation (98%)[20]. Some researchers have validated the RP-HPLC method of pre-fractionation by comparing data with $Ag^+$-TLC separation followed by GC analysis[21]. In the current study, the RP-HPLC method of separation provided a better alternative for $Ag^+$ chromatography for the pre-separation of FAMEs prior to GC analysis.

According to GC analysis, of 20:1 PIs were eluted in order of increasing **Δ** position, and this observation is in conformity with the findings of Delmonte *et al.*[12] and Nakamura *et al*.[14] for the same GC column and Ando and Sasaki[15] for the SLB-IL100 GC column. In the current study, SLB-IL111 GC column provided a good resolution between two adjacent isomer peaks. The clear separation of PIs on the GC column allowed for the correct identification of all the analyzed isomers. The GC column polarity, length, operating temperature, and flow rate of carrier gas play an important role in the resolution of isomer peaks. The SLB-IL111 GC capillary column has the stationary phase with the highest polarity that has been commercialized for use in capillary GC[12]. The column is coated with the polyionic liquid phase that exhibits a dual nature retention selectivity allowing it to separate both the polar and non-polar compounds[22]. The special functional properties of the IL used in the stationary phase of SLB-IL111[12] provides the highest

thermal stability to the column and allows operation at wider and higher temperatures with a low column bleeding[23].

The total content of *c*-20:1 vary among fishes from different oceans. Some researchers have reported levels of *c*-20:1 in pelagic fish along the coastal sea of Sri Lanka[19]. However, a comparison of data was not possible as these data were reported for whole fish, not for the edible portion. Several studies have reported a higher occurrence of *c*-20:1 in some Pacific Ocean fishes, namely saury, chum salmon, and Pacific mackerel[14], and Atlantic Ocean fishes, namely Atlantic herring, capelin, and mackerel[24]. The results of this study support these findings. Some researchers reported that LC-MUFAs (20:1, 22:1, and 24:1) have an exogenous origin and contribute to a higher proportion of total FA composition in fish oils from temperate and northern latitudes[2].

In previous studies, researchers have reported six PIs of *c*-20:1 in the lipids of some marine fishes[15,25]. This observation was true for some fishes analyzed in this study. The occurrence of a variety of double bond positions may not be due to the presence of multiple FA desaturase enzymes in fish since evidence of those desaturases is available mainly for Δ9 desaturase and few records of Δ5 desaturase[25]. Marine fishes such as turbot, gilthead, sea bream, and golden gray mullet have displayed limited Δ5 desaturase activity, which can introduce a double bond between carbon number 5 and 6 in an acyl chain of the carboxyl end[26]. All the fishes showed a very low content of *c*15-20:1 isomer. It has reported that with certain exceptions, animals in general lack Δ15 desaturase, which is required to produce the *c*15-20:1 isomer[27].

The results clearly indicated that some of the fishes from the Indian Ocean contained unusually high quantities of *c*7-20:1 instead of *c*11-20:1. The occurrence of the *c*7-20:1 isomer may be less documented in previous studies as this isomer overlaps with the *c*9-20:1 isomer in most of the GC columns that are used in the analysis of FA composition of fish lipids[10]. It was reported that *c*7-20:1 was a minor component in the total *c*-20:1 content of most pelagic fish, which is rich in either *c*11 or *c*9-20:1 isomers. In contrast, the *c*7-20:1 isomer was one of the major isomers contributing to the total *c*-20:1 content in benthic fishes and also their dietary animals[14]. In the current study, a high content of *c*7-20:1 was mainly recorded in benthic fish[28].

Some researchers have reported that in most of pelagic fishes, the main isomer is *c*11-20:1, except in the northwest pacific fishes in which the *c*9-20:1 isomer contributes the highest proportion of the total *c*-20:1[14]. Results from the current study are in accordance with the above observation. Ackman *et al*.[29] also reported that the *c*-20:1 PIs found in the marine environment show a predominance of n-9 (*c*11) and n-11 (*c*9) isomers, except for some marine invertebrates and some benthic organisms, such as brittle star and worms[25]. Apparently, LC-MUFAs biosynthesized by marine benthic organisms, and thereby marine fish, particularly those that have access to these sources, i.e. bottom feeders, accumulate higher levels of particular FAs in their body.

It was obvious that the content of each *c*-20:1 positional isomer in fish varied considerably according to the geographic location and/or habitat (whether pelagic or benthic). Subsequently, the feeding environment and pattern varies (surface or bottom feeder) among the fishes. In this study, among the Indian Ocean fishes, higher *c*-20:1 content was reported in bottom feeders (benthic), whereas in the fishes from the Pacific and Atlantic Ocean, pelagic fish contained a high content of *c*-20:1. FA composition of organisms in the same species and/or same genera are usually similar, but unique differences occur when some FAs are derived from their diet rather than synthesis *de nevo*[25]. According to Table 3.3, the distribution of *c*-20:1 isomers varied widely even within the same species such as *Nibea* sp., *Lutjanus* sp., *Thryssa* sp. etc. During the biosynthesis of LC-MUFAs in organisms, dietary MUFAs are modified by chain elongation or chain shortening of the FAs[25]. This fact also contributes to the occurrence of different PIs of MUFA and their wide variation in different organisms. A previous study[30] reported changes of FA content with the change of environmental temperature, among body parts, etc. Accordingly, *c*-20:1 occurs in high levels when environmental temperature fluctuates between 15–27°C, and beyond that range, levels begin to reduce. The biosynthesis and accumulation of *c*-20:1 in organisms is affected by environmental temperature, as enzymatic activity is governed by temperature. Eicosenoic acid may exhibit optimum biological function when environmental temperature falls between 15–27°C as it is in a semi-solid to liquid state in this temperature range (melting point of *c*-20:1 ≈ 22–24°C).

In previous studies, data regarding *c*-20:1 levels were derived from *c*11-20:1 levels as the reference since many commercial standard FAME mixes only contain the *c*11-20:1

positional isomer. As a result, the occurrence of *c*-20:1, reported as *c*11-20:1 may have been overestimated in many studies. This fact must be taken into consideration in future studies. The results clearly revealed that a wide range of *c*-20:1 PIs exist in fish oil and can be comprehensively analyzed using the SLB-IL111 highly polar GC capillary column, if reference materials are available.

To the best of our knowledge, the data presented in the current study are the first to report on the distribution of *c*-20:1 PIs in fishes from the Indian Ocean. These fishes are commonly consumed by the Asian population; thus, this study provides fundamental data about the dietary intake of *c*-20:1 PIs. The availability of FAs through diet depends on the percentage of these FAs in fish lipids as well as the total content of lipids in the edible portion of fish. Most of the species analyzed from the Indian Ocean had a comparatively low amount of *c*-20:1 than that of those from the Pacific and Atlantic Ocean, consequently their dietary availability is also low. The data presented in this study are a valuable resource for other researchers conducting comparative studies on fishes from different geographic locations.

### 3.5. Conclusion

The present study reported the occurrence and distribution of *c*-20:1 PIs in fishes mainly from the Indian Ocean and compared these to fishes from the Pacific and Atlantic Ocean. In fishes from the Indian and Atlantic Oceans, *c*11-20:1 isomer contributed to the highest proportion of total *c*-20:1, followed by the *c*13-20:1 isomer. In contrast, *c*9-20:1 was the predominant isomer in fishes from the Pacific Ocean. The *c*11-20:1 isomer was not always predominant in fishes from the Indian Ocean as some benthic living fishes contained a high content of *c*7-20:1 isomer. In the Indian Ocean-fish group, high content of *c*-20:1 was reported in bottom living fish, though the pelagic fish in the Pacific Ocean and Atlantic Ocean was reported to have a high content of *c*-20:1. The results demonstrated that the content and distribution of *c*-20:1 PIs varied with geographical location and habitat. Further studies are needed to study the effect of different PIs on health, as the distribution of these FAs was remarkably different among fishes from different oceans.

Table 3.1 The common name, scientific name, place of origin, % of lipid content, and part of fish used for lipid extraction in fishes from the Indian, Pacific, and Atlantic Ocean

| SR No | Common name | Scientific name | Place of origin/ Ocean | % Lipid content | Part used to extract oil |
|---|---|---|---|---|---|
| 1 | Anchovy | *Stolephorus commersoni* | Indian | 1.59 | Whole fish |
| 2 | Squid | *Loligo singhalensis* | Indian | 1.45 | Flesh |
| 3 | Rainbow sardine | *Dussumieria acuta* | Indian | 3.80 | Whole fish |
| 4 | Mullet | *Mugil cephalus* | Indian | 3.18 | Whole fish |
| 5 | White sardinella | *Sardinella albella* | Indian | 1.93 | Whole fish |
| 6 | Bagrid catfish | *Trachysurus sp* | Indian | 7.07 | Whole fish |
| 7 | Pickhandle barracuda | *Sphyraena jello* | Indian | 1.54 | Flesh |
| 8 | Yellowstriped goatfish | *Upeneus vittatus* | Indian | 4.11 | Flesh |
| 9 | Tooth pony | *Gazza minuta* | Indian | 2.13 | Whole fish |
| 10 | Blackspot snapper | *Lutjanus fulviflamma* | Indian | 1.32 | Whole fish |
| 11 | Malabar travelly | *Carangoide malabaricus* | Indian | 1.44 | Flesh |
| 12 | Yellow stripe scad | *Selaroides leptolepis* | Indian | 2.13 | Whole fish |
| 13 | Spotted sardinella | *Amblyguster sirm* | Indian | 9.23 | Whole fish |
| 14 | Indian mackerel | *Rastrelliger kanagurta* | Indian | 4.51 | Whole fish |
| 15 | Long face emperor | *Lethrinus olivaceus* | Indian | 0.92 | Flesh |
| 16 | Rainbow runner | *Elagatis bipinnulata* | Indian | 3.97 | Flesh |
| 17 | Big eye scad | *Selar crumenophtthalamus* | Indian | 2.48 | Flesh |
| 18 | Yellow fin tuna | *Thunnus albacares* | Indian | 2.34 | Flesh |
| 19 | Spanish mackerel | *Scomberomorus commersoni* | Indian | 2.75 | Flesh |
| 20 | Spotted croaker | *Protonibea diacanthus* | Indian | 1.59 | Whole fish |
| 21 | Cuttle fish | *Sepia sp* | Indian | 1.90 | Flesh |
| 22 | Commerson’s sole | *Synaptura commersoniana* | Indian | 1.93 | Whole fish |
| 23 | Rabbit fish | *Siganus canaliculatus* | Indian | 3.52 | Whole fish |
| 24 | Obilquebanded grouper | *Epinephalus radiates* | Indian | 1.74 | Flesh |
| 25 | Big eye barracuda | *Sphyraenea forsteri* | Indian | 2.38 | Flesh |
| 26 | Indian anchovy | *Stolephorus indicus* | Indian | 2.67 | Whole fish |
| 27 | Blacktip soldier fish | *Myripristis melanostictus* | Indian | 1.51 | Whole fish |
| 28 | Small scaled terapon | *Terapon puta* | Indian | 2.45 | Whole fish |

| | | | | | |
|---|---|---|---|---|---|
| 29 | Croaker | *Nibea coibor* | Indian | 2.50 | Whole fish |
| 30 | Blotched Croaker | *Nibea maculate* | Indian | 1.51 | Whole fish |
| 31 | Croaker | *Nibea sp* | Indian | 3.89 | Whole fish |
| 32 | Yellow tail scad | *Atule mate* | Indian | 2.98 | Flesh |
| 33 | Long jaw thryssa | *Thryssa setirostris* | Indian | 1.02 | Whole fish |
| 34 | Hamilton' s Thryssa | *Thryssa hamiltonii* | Indian | 3.33 | Whole fish |
| 35 | Indian Salmon | *Eleutheronema tetradactylum* | Indian | 3.94 | Whole fish |
| 36 | Brownstripe red snapper | *Lutjanus vitta* | Indian | 1.47 | Whole fish |
| 37 | Longspine-seabream | *Argyrops spinifer* | Indian | 6.90 | Whole fish |
| 38 | Orange striped emperor | *Lethrinus obsoletus* | Indian | 1.23 | Whole fish |
| 39 | Crimson jobfish | *Pristipomoides filamentosus* | Indian | NA | Whole fish |
| 40 | Bigeye snapper | *Lutjanus lutjanus* | Indian | 1.55 | Whole fish |
| 41 | Indian Snapper | *Lutjanus madras* | Indian | 1.73 | Whole fish |
| 42 | Longjaw thryssa | *Thryssa sterostis* | Indian | 4.41 | Whole fish |
| 43 | Sail fish | *Istiophorus platypterus* | Indian | 1.63 | Flesh |
| 44 | Malabar grouper | *Epinephalus malabariucs* | Indian | 1.08 | Flesh |
| 45 | Goatfishes | *Upeneus moluccensis* | Indian | 3.82 | Flesh |
| 46 | Pacific saury | *Cololabis saira* | Pacific | 25.1 | Flesh |
| 47 | True Sardine | *Sardina pilchardus* | Pacific | 27.71 | Flesh |
| 48 | Horse makerel | *Trachurus japonicus* | Pacific | 6.37 | Flesh |
| 49 | Atlantic mackerel | *Scomber scombrus* | Atlantic | 24.69 | Flesh |
| 50 | Atlantic Salmon | *Salmo salar* | Atlantic | 13.01 | Flesh |
| 51 | Atlantic Capelin | *Mallotus villosus* | Atlantic | 10.56 | Flesh |

NA-Not available

**Table 3.2** Resolution factors for *c*-20:1 PIs

| Double bond position | 5_7 | 7_9 | 9_11 | 11_13 | 13_14 | 14_15 | 13_15 |
|---|---|---|---|---|---|---|---|
| Resolution factor | 1.73±0.07 | 0.81±0.08 | 0.90±0.10 | 1.18±0.16 | 0.70±0.05 | 0.78±0.08 | 1.46±0.11 |

Data represents the mean ± SD of three replicates (n = 3).

Table 3.3 The total and individual content of *c*-20:1 PIs in lipids of edible portion of fish from the Indian, Pacific, and Atlantic Ocean

| SR. No. | Common name | Content of the individual isomers (mg/g of oil) | | | | | | | | | | | | Total *c*-20:1 (mg/g of oil) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *c*5 (n-15) | | *c*7 (n-13) | | *c*9 (n-11) | | *c*11 (n-9) | | *c*13 (n-7) | | *c*15 (n-5) | | | |
| | | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| 1 | Anchovy | ND | - | 0.19 | 0.03 | 0.10 | 0.06 | 0.66 | 0.08 | 0.52 | 0.08 | 0.02 | 0.03 | 1.48 | 0.14 |
| 2 | Squid | ND | - | 0.54 | 0.00 | 0.06 | 0.05 | 5.62 | 1.24 | 0.10 | 0.02 | ND | - | 6.32 | 2.22 |
| 3 | Rainbow sardine | 0.04 | 0.01 | 0.45 | 0.02 | 0.49 | 0.15 | 1.97 | 0.18 | 1.40 | 0.17 | 0.57 | 0.08 | 4.92 | 0.30 |
| 4 | Mullet | ND | - | 0.48 | 0.10 | 0.05 | 0.05 | 0.49 | 0.07 | 0.84 | 0.18 | 0.01 | 0.00 | 1.87 | 0.37 |
| 5 | White sardinella | 0.02 | 0.00 | 0.34 | 0.08 | 0.06 | 0.00 | 1.73 | 0.44 | 0.81 | 0.18 | 0.03 | 0.02 | 2.98 | 0.64 |
| 6 | Bagrid catfish | ND | - | 0.18 | 0.04 | 0.12 | 0.07 | 8.47 | 1.59 | 0.65 | 0.11 | 0.01 | 0.01 | 9.42 | 1.67 |
| 7 | Pickhandle barracuda | ND | - | 0.30 | 0.06 | 0.20 | 0.05 | 0.75 | 0.17 | 0.47 | 0.01 | 0.02 | 0.02 | 1.73 | 0.25 |
| 8 | Yellowstriped goatfish | 0.04 | 0.01 | 0.95 | 0.12 | 0.27 | 0.11 | 4.35 | 0.54 | 2.52 | 0.24 | 0.12 | 0.07 | 8.24 | 0.92 |
| 9 | Tooth pony | 0.24 | 0.04 | 6.60 | 0.94 | 0.03 | 0.05 | 3.97 | 0.62 | 2.30 | 0.50 | 0.14 | 0.00 | 13.28 | 0.66 |
| 10 | Blackspot snapper | 0.04 | 0.05 | 1.59 | 0.16 | 0.13 | 0.02 | 3.49 | 0.65 | 1.35 | 1.01 | 0.03 | 0.03 | 6.62 | 2.89 |
| 11 | Malabar travelly | ND | - | 0.24 | 0.00 | 0.17 | 0.18 | 1.11 | 0.14 | 0.56 | 0.11 | 0.03 | 0.03 | 2.11 | 0.19 |
| 12 | Yellow stripe scad | ND | - | 0.13 | 0.01 | 0.09 | 0.10 | 1.64 | 0.27 | 0.48 | 0.10 | 0.02 | 0.03 | 2.36 | 0.23 |
| 13 | Spotted sardinella | 0.04 | 0.04 | 0.64 | 0.07 | 0.21 | 0.06 | 5.10 | 0.54 | 1.92 | 0.53 | 0.09 | 0.05 | 8.00 | 0.69 |
| 14 | Indian mackerel | 0.06 | 0.02 | 0.78 | 0.11 | 0.15 | 0.08 | 1.88 | 0.35 | 2.88 | 0.53 | 0.20 | 0.00 | 5.95 | 1.04 |
| 15 | Long face emperor | 0.01 | 0.00 | 3.07 | 1.21 | 1.17 | 0.01 | 2.74 | 1.60 | 0.93 | 0.31 | ND | - | 7.92 | 3.94 |
| 16 | Rainbow runner | 0.01 | 0.01 | 0.46 | 0.03 | 0.10 | 0.06 | 1.94 | 0.32 | 0.59 | 0.09 | 0.03 | 0.01 | 3.12 | 0.33 |
| 17 | Big eye scad | ND | - | 0.36 | 0.07 | 0.11 | 0.07 | 1.59 | 0.22 | 0.60 | 0.12 | 0.03 | 0.02 | 2.69 | 0.35 |
| 18 | Yellow fin tuna | 0.01 | 0.00 | 0.58 | 0.13 | 0.22 | 0.09 | 4.04 | 0.57 | 1.15 | 0.19 | 0.10 | 0.04 | 6.09 | 0.58 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 19 | Spanish mackerel | ND | - | 0.35 | 0.03 | 0.37 | 0.13 | 4.57 | 0.56 | 0.66 | 0.09 | 0.10 | 0.03 | 6.05 | 0.49 |
| 20 | Spotted croaker | ND | - | 0.26 | 0.10 | 0.21 | 0.23 | 1.55 | 0.15 | 0.76 | 0.20 | 0.02 | 0.03 | 2.80 | 0.19 |
| 21 | Cuttle fish | 0.01 | 0.03 | 1.95 | 0.67 | 0.07 | 0.03 | 5.36 | 0.76 | 0.25 | 0.05 | 0.01 | 0.01 | 7.66 | 1.54 |
| 22 | Commerson’s sole | 0.24 | 0.12 | 13.06 | 4.31 | 0.03 | 0.00 | 2.48 | 0.83 | 12.19 | 3.60 | 0.52 | 0.29 | 28.52 | 8.67 |
| 23 | Rabbit fish | 0.04 | 0.04 | 2.98 | 0.48 | 0.12 | 0.05 | 3.62 | 0.65 | 3.33 | 0.98 | 0.09 | 0.02 | 10.18 | 2.05 |
| 24 | Obilquebanded grouper | ND | - | 0.67 | 0.13 | 0.17 | 0.04 | 5.60 | 0.85 | 1.35 | 0.23 | 0.06 | 0.05 | 7.85 | 1.16 |
| 25 | Big eye barracuda | ND | - | 0.48 | 0.03 | 0.20 | 0.09 | 1.86 | 0.32 | 0.76 | 0.15 | 0.03 | 0.04 | 3.34 | 0.31 |
| 26 | Indian anchovy | 0.06 | 0.04 | 1.69 | 0.20 | 0.03 | 0.05 | 1.02 | 0.13 | 1.16 | 0.15 | 0.05 | 0.05 | 4.02 | 0.05 |
| 27 | Blacktip soldier fish | ND | - | 0.67 | 0.07 | 0.24 | 0.07 | 6.92 | 0.52 | 1.10 | 0.29 | 0.04 | 0.05 | 8.98 | 0.67 |
| 28 | Small scaled terapon | ND | - | 0.25 | 0.07 | 0.14 | 0.02 | 1.39 | 0.43 | 0.73 | 0.16 | 0.01 | 0.02 | 2.53 | 0.63 |
| 29 | Croaker | 0.01 | 0.02 | 1.33 | 0.33 | 0.01 | 0.01 | 1.73 | 0.52 | 0.98 | 0.01 | 0.03 | 0.04 | 4.09 | 0.79 |
| 30 | Blotched Croaker | ND | - | 0.42 | 0.02 | 0.26 | 0.35 | 1.22 | 0.25 | 0.37 | 0.08 | 0.02 | 0.02 | 2.29 | 0.48 |
| 31 | Croaker | 0.06 | 0.07 | 2.01 | 0.29 | 0.06 | 0.05 | 4.35 | 0.36 | 2.80 | 0.65 | 0.11 | 0.05 | 9.39 | 1.39 |
| 32 | Yellow tail scad | 0.02 | 0.02 | 0.50 | 0.07 | 0.21 | 0.07 | 2.07 | 0.12 | 1.21 | 0.33 | 0.23 | 0.09 | 4.24 | 0.47 |
| 33 | Long jaw thryssa | ND | - | 0.21 | 0.15 | ND | - | 0.76 | 0.12 | 0.33 | 0.02 | 0.02 | 0.03 | 1.33 | 0.03 |
| 34 | Hamilton' s Thryssa | ND | - | 0.34 | 0.02 | 0.15 | 0.04 | 2.75 | 0.12 | 1.06 | 0.19 | 0.06 | 0.04 | 4.36 | 0.03 |
| 35 | Indian Salmon | 0.01 | 0.01 | 0.48 | 0.01 | 0.17 | 0.04 | 3.24 | 0.26 | 1.72 | 0.40 | 0.10 | 0.03 | 5.72 | 0.55 |
| 36 | Brownstripe red snapper | ND | - | 0.88 | 0.29 | 0.17 | 0.17 | 1.31 | 0.17 | 0.68 | 0.31 | 0.02 | 0.03 | 3.07 | 0.63 |
| 37 | Longspine-seabream | 0.70 | 0.36 | 19.48 | 6.75 | 0.04 | 0.05 | 4.01 | 1.99 | 6.12 | 1.80 | 0.29 | 0.09 | 30.65 | 2.54 |
| 38 | Orange striped emperor | 0.04 | 0.04 | 1.33 | 0.36 | 0.09 | 0.10 | 2.91 | 0.39 | 1.55 | 0.50 | 0.04 | 0.00 | 5.97 | 0.15 |
| 39 | Crimson jobfish | ND | - | 0.55 | 0.16 | 0.19 | 0.07 | 7.38 | 0.52 | 8.64 | 1.98 | 0.25 | 0.04 | 17.01 | 3.83 |
| 40 | Bigeye snapper | ND | - | 1.14 | 0.12 | 0.22 | 0.09 | 6.13 | 0.77 | 1.29 | 0.29 | 0.11 | 0.05 | 8.90 | 0.85 |
| 41 | Indian Snapper | ND | - | 0.48 | 0.02 | 0.22 | 0.26 | 1.89 | 0.54 | 0.68 | 0.30 | 0.03 | 0.03 | 3.30 | 0.45 |
| 42 | Longjaw thryssa | ND | - | 0.53 | 0.08 | 0.10 | 0.02 | 2.16 | 0.22 | 1.26 | 0.29 | 0.06 | 0.03 | 4.11 | 0.72 |
| 43 | Sail fish | ND | - | 0.33 | 0.07 | 0.27 | 0.11 | 1.16 | 0.03 | 0.22 | 0.11 | 0.02 | 0.03 | 2.01 | 0.08 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 44 | Malabar grouper | ND | - | 0.66 | 0.19 | 0.26 | 0.15 | 1.19 | 0.29 | 0.35 | 0.05 | 0.01 | 0.02 | 2.46 | 0.58 |
| 45 | Goatfishes | 0.69 | 0.17 | 11.64 | 1.63 | ND | - | 15.97 | 2.28 | 5.92 | 0.40 | 0.18 | 0.06 | 34.39 | 4.57 |
| 46 | Saury | ND | - | ND | - | 131.10 | 10.37 | 34.06 | 1.70 | 0.83 | 0.08 | 0.97 | 0.29 | 166.95 | 12.44 |
| 47 | True Sardine | ND | - | ND | - | 58.99 | 10.70 | 16.32 | 3.01 | 0.55 | 0.15 | 0.85 | 0.16 | 76.72 | 13.70 |
| 48 | Horse Mackerel | ND | - | 0.23 | 0.03 | 1.30 | 0.37 | 11.25 | 1.58 | 0.69 | 0.11 | 0.05 | 0.03 | 13.51 | 2.13 |
| 49 | Atlantic Mackerel | ND | - | ND | - | 1.93 | 1.15 | 85.95 | 13.75 | 0.67 | 0.13 | 0.13 | 0.00 | 88.67 | 15.03 |
| 50 | Salmon | ND | - | ND | - | 0.47 | 0.10 | 17.29 | 6.37 | 0.48 | 0.19 | ND | - | 18.24 | 6.66 |
| 51 | Capelin | ND | - | ND | - | 0.55 | 0.77 | 156.60 | 3.35 | 5.13 | 0.83 | 0.40 | 0.11 | 162.69 | 3.51 |

Data represents the mean ± SD of three replicates (n = 3); ND-Not detected

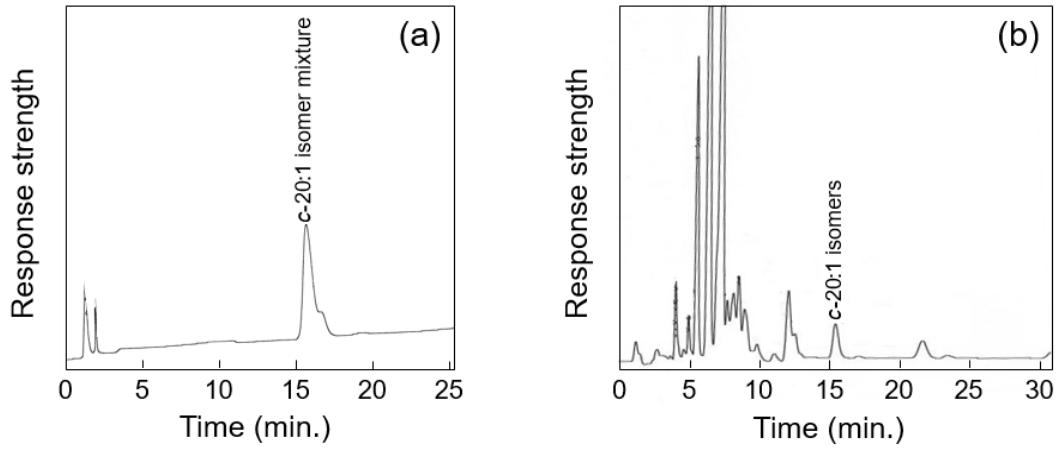


**Fig. 3.1** (a) HPLC chromatogram of the *c*-20:1 methyl ester mixture prepared with synthesize materials (b) HPLC chromatogram of fish oil (capelin) total FAME by ODS column.

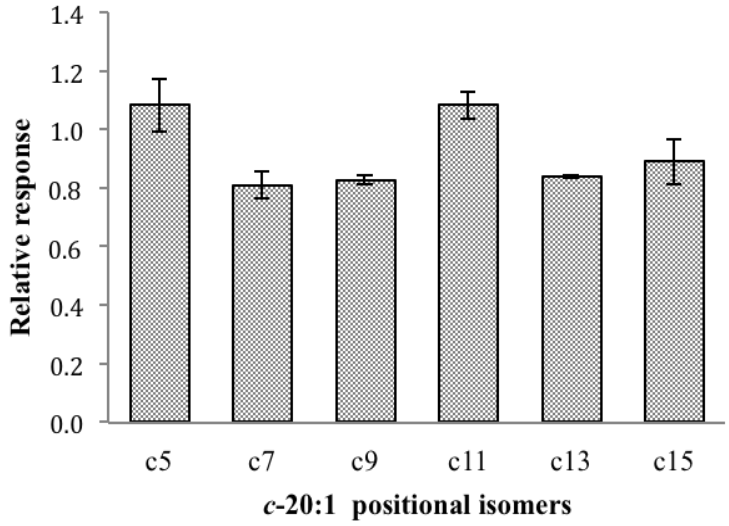


**Fig. 3.2** Relative response factor of *c*-20:1 PIs

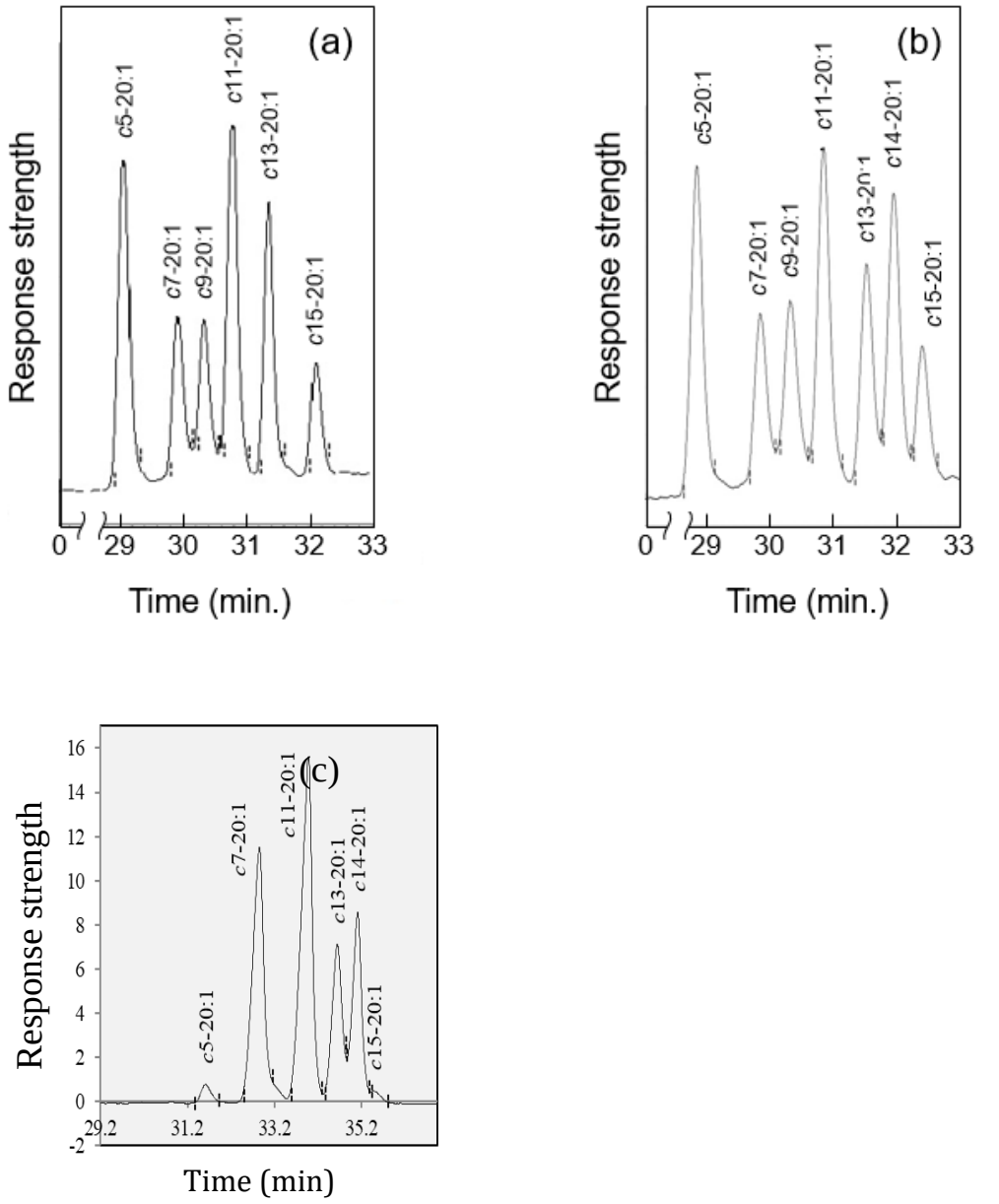


**Fig. 3.3** Partial gas chromatogram of *c*-20:1 methyl esters on SLB-IL 111 high polar GC column at 160°C isothermal temperature; (a) *c*-20:1 isomer mix without IS (*c*14-20:1), (b) *c*-20:1 isomer mix with IS (*c*14-20:1), (c) *c*-20:1 isomer from fish oil (goatfishes) with IS

IS: Internal Standard

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## Chapter 04

## Differential effect of *cis*-eicosenoic acid positional isomers on adipogenesis and lipogenesis in 3T3-L1 cells

### 4.1. Introduction

LC-MUFA including *c*-20:1 are characteristically found in some pelagic fishes such as herring, capelin, cod[1)], saury[2)], pollock[3)], etc. Fish lipids are well-known as functional lipids due to their health benefits arising from characteristic n-3 PUFA such as DHA and EPA. Recent studies reported that the ingestion of LC-MUFA-rich fish oils attenuates metabolic syndrome risk factors by reducing plasma glucose and lipid levels in diet induced obese mice[4,5)]. Undoubtedly, both n-3 PUFAs and LC-MUFAs in fish oil may contribute to the health functions of fish oil. However, the health effect of characteristic LC-MUFAs in fish oil has not been confirmed using pure FAs. MUFA has an olefinic bond in their acyl structure, thus exists in different positional (Fig. 4.1) and geometric configurations in nature. The common geometric configuration in unprocessed food is the *cis* form. According to the previous studies, six *c*-20:1 PIs have been reported in fish[6)] (described in section 3.1.). Further, their occurrence and distribution are notably varied among fishes in different Ocean[7,8)]. Thus, the current study focused on functionality or bioactivity individual *c*-20:1 PIs.

Adipogenesis or differentiation of preadipocytes into mature fat cells and lipid accumulation in adipocytes are related to the occurrence and the development of the obesity. Obesity is one of the main metabolic disorders prevailing in the world and leads to many diseases such as diabetes, cardiovascular diseases, osteoarthritis, cancer etc.[9)]. According to the stage of the obesity, preadipocytes stimulate and differentiate to adipocytes or just increase in fat accumulation in already differentiated adipocytes[10)]. Thus, researchers focus on the control of the adipogenesis and also the control of the lipid accumulation in adipocytes via improving the composition of dietary fat as a prevention measure for obesity and subsequent diseases.

Adipocytes are the main cells in adipose tissue and function as energy storage cells in the body. Adipose tissue also serves as an endocrine organ by secreting many cytokines

(known as adipocytokines) apart from its function as storage of body fat[11]. Thus, adipose tissue regulates many physiological functions including lipid metabolism. Peroxisome proliferator activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein alpha (C/EBPα) are two transcriptional factors occur in adipocytes and are known to be the master regulators of adipogenesis[12,13]. Control of these transcriptional factors is important to regulate the onset of obesity. Adipogenesis is accompanied by a dramatic increase in the expression of adipocyte specific genes and related protein including adipocyte fatty acid binding protein and lipid-metabolizing enzymes. Sterol regulatory element binding protein-1 (SREBP-1) is another transcription factor that controls and induces the entire process of the *de novo* lipogenesis[14] and are known to regulate the expression of lipogenic genes enzymes such as fatty acid synthase, stearoyl-Co-A desaturase -1 (SCD-1), Glycerol 3-phosphate acyl transferase[15].

The effect of dietary fats on human health depends not only on the quantity of the consumed fat but also the composition and the nature of the FAs. The metabolic functions of a particular FAs at cellular and molecular levels are ultimately determined by the number of carbon atoms in the acyl chain, availability of C-C double bonds, geometric configuration of the atoms, position, number of double bonds in the acyl structure, etc.[16, 17]. Many studies have proven the beneficial health effects of the MUFA rich diet over the SFA and carbohydrate rich diets[18, 19]. Furthermore, studies have carried out on the effects of the geometric and positional isomers of 18:1 using animal and cell model and shown to have different effects on health [16, 20]. Although, no studies have conducted so far on the health effects of individual *c*-20:1 PIs. Studies conducted by Yang *et al*.[21] demonstrate the positive health benefits of LC-MUFA, i.e. 20:1 and 22:1, using fish oil concentrates. Hence, *c*-20:1 is present in food as a mixture of PIs and due to their differences in the occurrence and distribution; the knowledge of isomer specific biological effects on health would be useful in understanding the effect of the position of double bond in *c*-20:1 on health and interpreting the results of previous studies done using animal models.

Thus, the objective of the current study to investigate the effect of six common *c*-20:1 PIs i.e. *c*5, *c*7, *c*9, *c*11, *c*13, and *c*15-20:1, (Fig. 4.1) with compared to *c*9-18:1 (oleic acid) and non-treated, on the adipogenesis and selected aspects of lipogenesis, in 3T3-L1 cells. Murine 3T3-L1 preadipocytes have frequently used in past decades in many studies related to adipogenesis and lipid accumulation, as they can differentiate into mature white

adipocyte under appropriate hormonal or experimentalmanipulation[22]. In the current study, we hypothesized that *c*-20:1 PIs may influence on the adipogenesis of 3T3-L1 preadipocytes and consequently effect on the lipogenesis at the cellular level. Thus, we studied the individual effect of selected six pure *c*-20:1 PIs (synthetic) on the changes of the main cellular lipid classes, fatty acid composition (FAC), Δ9 desaturase activity and the transcriptional factors that involve in adipogenesis (PPARγ and C/EBPα) and the lipogenesis (SREBP-1).

## 4.2. Materials and Methods

### 4.2.1. Materials

Murine 3T3-L1 preadipocytes were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Five common positional isomers of *c*-20:1 (purity > 98%) except *c*5-20:1 were synthesized in the laboratory using the method described in Chapter 02. The *c*5-20:1 was purchased from Nu-Chek Prep, Inc. (Elysian, MN.). Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS) Dominican Republic was bought from Biosera Europe (Nuaille, France). Triglyceride (TG), total cholesterol (TCh), phospholipids (PL), and non-esterified fatty acids (NEFA) enzymatic assay kits were purchased from the Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Penicillin – Streptomycin solution (x100) and FA free-bovine serum albumin (BSA) (30% w/v) solution were also purchased from Wako Pure Chemical Industries, Ltd. Dexamethasone (Dex), 3-isobutyl-1-methyl-xanthine (IBMX), and insulin were purchased from Sigma-Aldrich Co. LLC. Japan K.K. (Tokyo, Japan). All the reagents used in the study were analytical grade.

### 4.2.2. Cell culture

3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% FBS and 1% of penicillin-streptomycin (Basal medium) at 37°C in a humidified atmosphere under 5% $CO_2$. In experimental cultures, basal medium was added with experimental FAs (50 μM each *c*-20:1 PIs, i.e. *c*5, *c*7, *c*9, *c*11, *c*13, and *c*15) or control (50 μM, *c*9-18:1 or 0.5% FA free-BSA with 0.01% ethanol). FA-BSA complex was prepared by dissolving ethanolic solution of respective FAs in DMEM containing 5% FA free- BSA as previously described[23] with slight modifications. The concentrations of FA free-BSA and ethanol in

the final medium were adjusted to 0.5% and 0.01%, respectively. After two days of confluence, 3T3-L1 cells were induced for differentiation (Day 0; D0) using differentiation cocktail (500 μM IBMX, 1 μM Dex, and 10 μg/mL insulin) for 48 h. The treatment of cells with FAs was started in D0. Then, cells were incubated (D2 to D4) with insulin medium (Basal medium + 0.5% FA free BSA + 10 μg/mL insulin + 50 μM FA) followed by changing the medium (basal medium + 0.5% FA free-BSA + 50 μM FA) with 48 h interval till the D10 or a predetermined culture period.

### 4.2.3. Oil Red O staining

After culture period (D10), cells were washed with phosphate saline buffer (PBS) twice and fixed with 10% formalin solution. Following, cells were washed with a mixture of water and 2-propanol (40:60, v/v), subsequently stained with Oil Red O solution for 20 – 30 minutes. Then, stained cells were washed with the mixture of water and 2-propanol (40:60, v/v). After that the cells were further washed with PBS and observed under a microscope. The Oil Red O dyes absorbed into lipid droplets were extracted using 2-proponaol and the absorbance was read at 450 nm with a Bio-Rad iMark$^{TM}$ microplate absorbance reader (Bio-Rad Laboratories, Inc., Hercules, CA.).

### 4.2.4. Biochemical assays

3T3 L1 cells were cultured and treated as described in section 4.2.2. On the D10, cells were collected using lysis buffer (PIPES buffer and 0.1% Triton-X) and homogenized using an ultrasonic disruptor (TOMY Digital Biology Co., Ltd., Tokyo, Japan). Then, the mixture was centrifuged (Eppendorf centrifuge 5415 R, Eppendorf AG., Hamburg, Germany) to obtain the cell sap. Next, the cell sap was analyzed for cellular protein (Bicinchoninic Acid (BCA) method) (DC$^{TM}$ Protein Assay Reagent, Bio-Rad Laboratories, Inc., Hercules, CA) and for major lipid classes; TG, PL, TCh, and NEFA using enzymatic assay kits and data were normalized to the total protein content in the respective samples.

### 4.2.5. Gas chromatography analyses

#### 4.2.5.1. Analysis of fatty acid composition in cellular lipids

The cellular lipids were extracted by a modified method of Bligh and Dyer as previously described[24]. In brief, one part of cell sap was vortexed with 4 parts of a mixture

of dichloromethane and methanol (1:2, v/v) followed by the addition of 1.2 mL of dichloromethane and 1.6 mL of water. Then, the organic layer was separated and dried under a stream of nitrogen gas. A half portion of extracted lipids was methyl esterified using $BF_3$/MeOH and analyzed for the FAC in total lipids by gas chromatoghraphy (GC) (Shimadzu Corporation, Kyoto, Japan) fitted with Omegawax 320 capillary GC column (Sigma-Aldrich Japan K.K.) and peaks were identified using a standard fatty acid mix (Supelco 37 component fatty acid methyl ester mix, Sigma-Aldrich Japan K.K.) and a mixture of *c*-20:1 PIs used in this study.

#### 4.2.5.2. Δ 9 desaturase activity

The TG fraction was obtained from a portion of cellular lipids using a high-performance thin layer chromatography plate (10 cm x 10 cm, Merck KGaA, Darmstadt, Germany) followed by methylation and determined the FAC in the TG fraction using the GC–FID. The desaturase activity was calculated based on the ratio of monounsaturated to saturated FAs obtained using the TG fraction of the cellular lipids using the formula given.

Δ 9 desaturase activity = % Area 16:1/ % Area 16:0 or % Area 18:1/ % Area 18:0.

### 4.2.6. Western blotting

Three *c*-20:1 PIs (*c*15, *c*11, and *c*9-20:1) were selected based on the results of normalized TG values and relative abundance of *c*-20:1 PIs in food. 3T3-L1 cells were cultured using the same protocol mentioned in section 2.2 and collected on the day 5, using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl buffer (pH 8.0), 150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, and 1% (v/v) Nonidet P-40) added with 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich Japan K.K.) and protein content was determined by BCA method. Then, equal parts of cell lysate and sample buffer solution (2ME+) (x2) (Wako Pure Chemical Industries, Ltd.) were mixed and subjected to Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Approximately, 25 μg of cellular protein was loaded per lane along with a protein marker (Precision plus protein$^{TM}$ dual color standard, Bio-Rad Laboratories, Inc.). The separated proteins were transferred to a polyvinylidene difluoride membrane and blocked in 1% skim milk, incubated with the selected primary antibodies; PPARγ, C/EBPα, and SREBP-1 (Santa Cruz Biotechnology, Inc., Dallas, TX) for overnight. On the following day, the

membrane was washed with a mixture of Tris-buffered saline (TBS) and 0.1% polyoxyethylene sorbitan monolaurate (Tween 20) and incubated with an appropriate secondary antibody (Goat anti-mouse/anti-rabbit IgG-horseradish peroxidase-conjugated (Santa Cruz Biotechnology, Inc.)) for one hour. Next, bands were visualized using the ECL Prime western blotting detection kit (Bio-Rad laboratories, Inc.) and results were analyzed using IMAGE J software. β-actin was used as the internal standard.

#### 4.2.7. Statistical analysis

Results were expressed as the mean ± SD. The data groups were analyzed statistically to detect significant difference ($p<0.05$) between each group of the treatments and controls (*c*9-18:1, 0.5% FA-free BSA) using one-way ANOVA followed by the Tukey-Kramer *post-hoc* test (Kaleida Graph, Synergy software, Reading, PA).

### 4.3. Results

#### 4.3.1. Oil Red O staining

Lipid accumulation as a marker of adipogenesis was quantified by Oil Red O staining. Figure 4.2 illustrates the changes of the cellular lipids in 3T3-L1 cells treated with different *c*-20:1 PIs, *c*9-18:1 and 0.5% FA free-BSA. Among *c*-20:1 PIs treated cells, the highest and the lowest accumulation of lipids were indicated in *c*11-20:1 (1.25 ± 0.10) and *c*15-20:1 (1.02 ± 0.05), respectively. Results indicated that the experimental *c*-20:1 PIs affected significantly ($p<0.05$) different on the lipid accumulation in 3T3-L1 cells compared to *c*9-18:1. Further, all the treatment resulted in a lower level of cellular lipids with compared to that of *c*9-18:1 (1.60 ± 0.07).

#### 4.3.2. Effect of *c*-20:1 PIs on the main lipid classes in 3T3-L1 cells

#### 4. 3.2.1. TG content

The normalized values of TG in cells treated with different *c*-20:1 PIs, *c*9-18:1, and 0.5% FA free-BSA are shown in Fig. 4.3 (A). The treatment of 3T3-L1 cells with the *c*-20:1 PIs resulted a decrease in the cellular TG content with compared to that of *c*9-18:1 (0.65 ± 0.08 TG (mg/mg protein)). Among tested isomers, the lowest and the highest levels of cellular TG were shown by the cells treated with *c*15-20:1, 0.41 ± 0.08 TG mg/mg

protein and *c*13-20:1 FA, 0.63 ± 0.09 TG mg/mg protein, respectively. The intracellular TG contents in cells were significantly different ($p<0.05$) among experimental isomers and *c*9-18:1 with few exceptions.

#### 4.3.2.2. Cellular phospholipid, total cholesterol, and non-esterified fatty acid contents

The changes of normalized cellular PL, TCh, and NEFA contents in 3T3-L1 cells treated with *c*-20:1 PIs, *c*9-18:1, and 0.5% FA free-BSA are shown in Fig. 4.3 (B), (C), and (D), respectively. The tested concentration of the PIs didn't affect significantly ($p<0.05$) on any of the above-stated lipid classes among *c*-20:1 PIs and *c*9-18:1 treated 3T3-L1 cells. Although, values indicated by non-treated cells and *c*5-20:1 were relatively low with relevant to cellular PL and TCh when compared to that of *c*9-18:1. The NEFA was a minor component in 3T3-L1 cells and detected in small quantities in all cells.

### 4.3.3. Gas chromatography analyses of cellular lipids

#### 4.3.3.1. Fatty acid composition of the total lipids extracted from cells

Table 4.1 summarizes the FAC of total cellular lipids extracted from 3T3-L1 cells treated with common *c*-20:1 PIs, *c*9-18:1, and 0.5% FA free-BSA. In most of the samples except for *c*11-20:1 and *c*9-18:1treated cells, the abundant FA found in the cellular lipids was the palmitic acid (16:0) with values ranged from 18.24% ± 1.4 (*c*13-20:1) to 28.33% ± 5.0 (*c*5-20:1) among the treatments. With few exceptions, the second highest FA in cellular lipids was the *c*9-18:1 contributing approximately 5.75% ± 0.6 (*c*13-20:1) to 31.53% ± 10.5 (*c*9-18:1) to the total FAC. All the samples showed a relatively high content of stearic acid (18:0) irrespective of the treated *c*-20:1 PIs. The principal PUFA found in all samples was the eicosatrienoic acid (20:3) ranging from 4.63 ± 0.6 (*c*9-18:1) to 9.83% ± 1.7 (*c*11-20:1). Consequently, the level of total SFA, MUFA, and PUFA varied considerably among extracted cellular lipids depending on the treated *c*-20:1 PIs. Hence, the FAC in total cellular lipids were substantially altered by the FA isomer added to the growth medium.

According to FAC data, it was apparent that cells uptake *c*-20:1 PIs into cells at different percentages and value varied from 3.79% ± 0.8 (*c*15-20:1) to 21.29% ± 1.5 (*c*9-20:1), thus it exhibited unique variations among treatments. It was clear that *c*-20:1 PIs were not available in the basic cell culture medium as the FAC of 0.5% FA free-BSA cells

showed no *c*-20:1 PIs. The statistical analysis indicated that the *c*-20:1 PIs in cellular lipids were significantly different ($p<0.05$) among the treatments.

#### 4.3.3.2. The Δ 9 desaturase activity

The Δ 9 desaturase indices calculated from the FAC data (not shown in the paper) obtained from the TG fraction of cellular lipids are listed in Table 4. 2. The treatment of *c*-20:1 PIs resulted, different and low values of Δ9 desaturation indices (18:1/18:0) in cells with compared to that of *c*9-18:1 (4.4 ± 0.5) thus it affected differently on the SCD-1 enzyme activity. The Δ 9 desaturase activity indices were significantly different ($p<0.05$) among treatments and controls.

### 4.3.4. Expression of transcriptional factors related to adipogenesis and lipogenesis

#### 4.3.4.1. The expression of protein related to adipocyte differentiation

Figure 4.4 shows the normalized values of (A) PPARγ and (B) C/EBPα in cells treated with *c*15-20:1, *c*11-20:1, *c*9-20:1, *c*9-18:1, and 0.5% FA-free BSA. Accordingly, *c*15-20:1 significantly decreased ($p<0.05$) the expression of the key regulators of adipogenesis; PPARγ and C/EBPα compared to that of the *c*9-18:1. The values of PPARγ in cells treated with *c*15-20:1 was significantly different ($p<0.05$) from those of the *c*11-20:1. The levels of PPARγ and C/EBPα were lower in cells treated with *c*9-20:1 and *c*11-20:1 than that of *c*9-18:1, but not significantly ($p<0.05$) different.

#### 4.3.4.2. The expression of protein related to lipogenesis

The normalized values of SREBP-1 in 3T3-L1 cells treated with *c*15-20:1, *c*11-20:1, *c*9-20:1, *c*9-18:1, and 0.5% FA-free BSA are shown in Fig. 4.4 (C). Treatment of *c*15-20:1 and *c*11-20:1 has significantly ($p<0.05$) down-regulated the expression of SREBP-1 than that of *c*9-18:1 and *c*9-20:1. The highest and lowest expression of SREBP-1 was reported in cells treated with *c*9-18:1 and non-treated cells, respectively.

## 4.4. Discussion

Results of the present study clearly indicated that the individual *c*-20:1 PIs affected differently on the adipogenesis, lipid accumulation, and lipogenesis in 3T3-L1 cells. Results implied that the intracellular TG content was decreased by all kinds of tested *c*-

20:1 PIs compared to the *c*9-18:1, which is the abundant MUFA in the most of plant-based foods. Thus, *c*-20:1 PIs have beneficial effects in controlling the cellular TG content in adipocytes, subsequently may regulate the lipid accumulation in adipocytes. Intracellular lipid content is known to be controlled by adipocyte differentiation and lipid metabolism[15]. Thus, experimental *c*-20:1 PIs modulated the cellular TG content either by influencing on one or both above-mentioned processes. The *c*15-20:1 isomer showed significantly lower cellular TG content compared to the other *c*-20:1 PIs. The *c*15-20:1 isomer may act through suppressing adipogenesis or by inhibition of TG accumulation via decreased activity of the fatty acid synthase enzyme. The *c*15-20:1 isomer is present in small quantities in fish oil[7]. The presence of different positional isomers even in small quantities in a mixture may exhibit synergistic effects and contributes to the functional properties or health benefits of particular compounds.

3T3-L1 cells are primarily used in many studies related to the adipogenesis and researchers have used 3T3-L1 cells to investigate the effect of certain FAs including geometric and PIs, (i.e. conjugated linoleic acid and *trans* FAs), on the lipid metabolism[25, 26]. The main lipid class in mature adipocytes is the TG and may represent the composition of dietary FAs as adipocytes absorb FAs mainly from the plasma, but not exactly similar to the dietary FAC as adipocytes involve with *de-novo* lipogenesis[27]. The other lipid classes such as diacylglycerides, TCh, PL, and NEFA are present in adipocyte in minor quantities. Those represent a unique FAs profile and seldom resemble the composition of the dietary lipids[27]. Thus, in the calculation of the Δ9 desaturase indices, FAs derived from the TG fraction of cellular lipids should be obtained.

FAC data showed that the 3T3-L1 cells readily absorbed exogenous FA i.e. *c*-20:1 PIs but in different ratios. The differences in the uptake of these FA isomers into adipocytes could also account for the different effects exerted by these *c*-20:1 PIs. Comparision of the FAC of non-treated (0.5% FA-free BSA treated cells) and the *c*-20:1 PIs treated cells indicated that experimental FAs have considerably altered the FA metabolism in 3T3-L1 cells. Some studies have reported on the selective mobilisation of FA from adipocytes based on the chain length, the number of the double bonds etc.[28]. It may due to the preferential uptake of FA into adipocytes. The long chain FAs unable to diffuse directly to adipocytes thus entered into the cells via fatty acid transport protein (FATP)[29]. The fatty acid transporters; CD36 and FATP1 are induced by PPARγ ligands in

adipose tissue[30]. Changes in the activity of the FATP could lead to some pathological conditions such as diabetes, insulin resistance and obesity. The current study showed that the adipocytes showed selective uptake towards the positional isomers of *c*-20:1. This might be due to different levels of affinities of respective isomers to FATP. Results demonstrated that the position of the double bond plays an important role in the uptake of *c*-20:1 into adipocytes. According to the FAC data, several anonymous FAs were detected closer to the *c*9-18:1, *c*9-16:1 and *c*9-14:1 in cells treated with different *c*-20:1 PIs. These FAs may have originated by the β oxidation of the respective *c*-20:1 PIs. Intracellular catabolism and anabolism of respective *c*-20:1 PIs could be tracked using isotope labeled FAs. Or else, mass spectrometry analysis must be conducted to identify these unusual FAs. The *c*-20:1 PIs were not detected in cells treated with 0.5% FA-free BSA. In the study, FA free-BSA was used to avoid the extraneous FAs in the growth medium other than the experimental FA. Though, basal medium contained certain FAs from FBS added during the preparation of the medium[25], *c*-20:1 PIs were not available in the basal medium.

Results implied that SCD-1 enzyme activity decreased in cells treated with *c*-20:1 PIs compared to that of *c*9-18:1 and led to decrease FA esterification, thereby reduced synthesis and accumulation of TG. The values were calculated based on the ratio MUFA to SFA (ratio of 18:1 to 18:0 or ratio of 16:1 to 16:0 which are the main SFA and MUFA in cellular lipids) where SCD-1 enzyme acts on the respective SFA to biosynthesize MUFA. Δ 9 desaturase activity or SCD-1 enzyme activity is a predictor of metabolic disorders, including obesity, insulin resistance, diabetes, fatty liver, etc.[31]. Thus, reduction of the SCD-1 activity helps to control obesity and related metabolic diseases.

To examine the effect of selected *c*-20:1 PIs on the adipogenesis, we focused on the key transcriptional factors for pre-adipocyte differentiation; PPARγ and C/EBPα. The expression of PPARγ and C/EBPα mRNA is induced in 3T3-L1 cells after 48 h of incubation in a differentiation medium[32] and these express continuously in differentiated adipocytes. After PPARγ is activated, it induces various genes involved in lipid and glucose metabolism[33]. In this study, it was clearly shown that *c*15-20:1 comparatively down-regulated the expression PPARγ and C/EBPα, thus directly affected on the adipogenesis of 3T3-L1 preadipocytes. Subsequently, cellular lipid content was reduced in the cells treated with *c*15-20:1. It has reported that LCFA, i.e saturated and unsaturated LCFAs bind with all subclasses of PPARs, thereby regulate the energy metabolism by

acting as an agonistic ligand for the transcriptional factors PPARs[34]. Thus, it was apparent that treatment of LC-MUFA to the growth medium increases the intracellular TG than that of the cells treated only with FA free-BSA.

SREBP-1 is a transcriptional factor that regulates the expression of genes related to lipogenesis. The expression of SREBP-1 increases during the preadipocyte differentiation and maturation[35]. Thus, we investigated the effect of selected *c*-20:1 PIs on the protein expression of SREBP-1, to understand the mechanism underlying the reduced cellular TG after treatment of *c*-20:1 PIs. The treatment of *c*15-20:1 and *c*11-20:1 considerably down-regulated the expression of SREBP-1 with compared to *c*9-18:1 and *c*9-20:1. Reduction of the protein expression of the SREBP-1 is a good indication of consequent suppression of the TG synthesis resulting lower cellular TG. The detailed mechanism of suppression of SREBP-1 by *c*-20:1 PIs has to be investigated in future.

In foods such as fish oil[8], vegetable oil[36], nuts, and seeds, *c*-20:1 PIs available as a mixture and the distribution of these PIs are different among these foods. Yang and colleagues have indicated the beneficial effects of *c*-20:1 PIs mixture extracted from saury oil[5] and pollock oil[4] on attenuating obesity-related metabolic dysfunction and modulating hyperlipidemia and ameliorates hepatic steatosis in mice, respectively. In those studies, the predominant PI in the mixture was the *c*9-20:1 and the mixture have used up to the 4-7 % (w/w) in the preparation of diet for mice. Hence, the beneficial effects *c*-20:1 isomers has been proven previously using a mixture of these particular isomers. There were no studies have carried out up to present to study the individual effect of pure *c*-20:1 PIs, on the adipogenesis, lipid accumulation and lipogenesis at cellular levels or in animal models. To the best of our knowledge, the present study was the first study to investigate the effect of the pure *c*-20:1 PIs on the adipogenesis and the lipogenesis in a cell model. The results of the present study provided clear information on how the *c*-20:1 PIs acted on the adipogenesis and the lipogenesis at the cellular level.

### 4.5. Conclusion

Overall results revealed that individual *c*-20:1 PIs affected differentially on the lipid accumulation, adipogenesis, and lipogenesis in 3T3-L1 cells. Among the tested *c*-20:1 PIs, *c*15-20:1 showed an anti-adipogenic and anti-lipogenic effect compared to other PIs and *c*9-18:1. The reduction of TG content by *c*15-20:1 PI was due to down-regulation of

transcriptional factors related to adipogenesis and lipogenesis. The abundant *c*-20:1 PIs in fish oil, i.e. *c*9-20:1 and *c*11-20:1, have shown no considerable differences either in lipid accumulation or in the expression of transcriptional factors governing adipogenesis. This study showed that the position of the double bond in *c*-20:1 influenced on their functionality and indicated possible effects of individual *c*-20:1 PIs in relation to delaying the onset and the development of obesity. Thus, foods rich in 20:1 PIs, such as fish oil, plant seeds, and nuts are useful as functional foods for the prevention of obesity and related diseases. Further, studies are suggested to evaluate the individual effect of *c*-20:1 PIs on gene expression products related to lipid metabolism.

Table 4. 1 Effect of *c*-20:1 PIs on the fatty acid composition (%) of total cellular lipids in 3T3-L1 cells

| Fatty acid | Control | | *c*15-20:1 | | *c*13-20:1 | | *c*11-20:1 | | *c*9-20:1 | | *c*7-20:1 | | *c*5-20:1 | | *c*9-18:1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| 12:0 | 0.89 | ± 0.1 | ND | | ND | | ND | | ND | | ND | | ND | | 0.58 | ± 0.2 |
| 13:0 | 0.24 | ± 0.0 | ND | | ND | | ND | | ND | | ND | | ND | | ND | |
| 14:0 | 3.29 | ± 0.9 | 2.24 | ± 0.6 | 1.26 | ± 0.1 | 1.74 | ± 0.2 | 2.1 | ± 0.1 | 1.93 | ± 1.3 | 1.52 | ± 0.5 | 2.34 | ± 1.0 |
| 15:0 | 1.41 | ± 0.3 | 1.15 | ± 0.2 | 1.19 | ± 0.3 | 1.2 | ± 0.5 | 1.41 | ± 0.4 | 1.14 | ± 0.6 | 0.89 | ± 0.4 | 1.64 | ± 0.4 |
| 16:0 | 22.89 | ± 3.4 | 23.18 | ± 1.6 | 18.24 | ± 1.4 | 23.29 | ± 1.1 | 23.13 | ± 3.8 | 20.29 | ± 1.5 | 28.33 | ± 5.0 | 23.73 | ± 1.9 |
| 17:0 | ND | | ND | | ND | | ND | | ND | | ND | | ND | | 1.32 | ± 0.1 |
| 18:0 | 16.14 | ± 1.4 | 12.29 | ± 4.5 | 9.77 | ± 2.4 | 15.41 | ± 7.2 | 13.34 | ± 1.9 | 14.49 | ± 5.3 | 18 | ± 7.8 | 15.38 | ± 7.2 |
| 20:0 | 5.39 | ± 0.0 | 2.46 | ± 1.0 | 2.86 | ± 0.2 | 2.98 | ± 0.3 | ND | | ND | | 2.11 | ± 0.1 | 1.67 | ± 0.1 |
| 21:0 | 2.76 | ± 0.8 | ND | | 3.22 | ± 0.9 | 1.66 | ± 0.6 | ND | ± 0.5 | 4.02 | ± 0.9 | 2.69 | ± 0.1 | 1.89 | ± 0.5 |
| 22:0 | ND | | ND | | ND | | ND | | 0.34 | ± 0.1 | 3.94 | ± 0.7 | ND | | ND | |
| 24:0 | 0.88 | ± 0.0 | 1.96 | ± 0.1 | ND | | 3.61 | ± 0.9 | ND | | ND | | ND | | 0.9 | ± 0.0 |
| Total SFA | 48.5 | ±3.9 [a] | 42.0 | ±3.5 [ab] | 34.8 | ±2.0 [b] | 43.5 | ±8.1 [ab] | 40.9 | ±4.4 [ab] | 42.6 | ±3.4 [ab] | 51.9 | ±2.1 [a] | 46.1 | ±5.0 [a] |
| 14:1 n-5 (*c*9-14:1) | 1.41 | ± 0.3 | ND | | ND | | ND | | ND | | ND | | ND | | ND | |
| 15:1 | 3.98 | ± 0.1 | 2.04 | ± 0.3 | 1.13 | ± 0.2 | 1.69 | ± 0.3 | 1.78 | ± 0.8 | 1.74 | ± 0.2 | 3.3 | ± 0.5 | 0.99 | ± 0.0 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 16:1 n-7 (*c*9-16:1) | 3.13 | ± 2.1 | 4.02 | ± 1.2 | 5.93 | ± 1.7 | 2.18 | ± 0.6 | 3.03 | ± 0.1 | 3.8 | ± 0.6 | 6.1 | ± 0.6 | 3.38 | ± 1.3 |
| 17:1 | 0.43 | ± 0.0 | ND | | ND | | ND | | ND | | 2.48 | ± 2.3 | ND | | 0.6 | ± 0.0 |
| 18:1 n-9 (*c*9-18:1) | 18.3 | ± 1.8 | 18.79 | ± 1.4 | 5.75 | ± 0.6 | 28.83 | ± 6.9 | 20.61 | ± 5.0 | 9.75 | ± 6.0 | 10.51 | ± 4.1 | 31.53 | ± 10.5 |
| 20:1 | ND | | 3.79 | ± 0.9 [ab] | 9.74 | ± 3.0 [c] | 8.1 | ± 1.9 [c] | 21.3 | ± 1.5 [d] | 13.4 | ± 1.6 [e] | 7.77 | ± 0.4 [ac] | 0.85 | ± 0.1 [b] |
| 22:1 n-9 (*c*13-22:1) | ND | | ND | | 1.24 | ± 0.5 | ND | | ND | | ND | | ND | | ND | |
| 24:1 n-9 (*c*15-24:1) | 4.53 | ± 2.9 | ND | | 4.8 | ± 2.7 | 5.43 | ± 2.0 | 3.75 | ± 0.2 | 6.56 | ± 2.2 | 5.32 | ± 2.2 | 4.36 | ± 2.9 |
| Total MUFA | 29.2 | ±7.7 [a] | 25.6 | ±6.2 [a] | 27.97 | ±2.2 [a] | 43.7 | ±5.9 [bc] | 48.6 | ±6.0 [b] | 36.7 | ±2.6 [ab] | 31.9 | ±3.0 [ac] | 42.4 | ±7.2 [bc] |
| 18:2 n-6 | 1.37 | ± 0.4 | 13.07 | ± 1.5 | 3.54 | ± 0.7 | ND | | 0.84 | ± 0.1 | 1.27 | ± 1.3 | 1.53 | ± 0.4 | 1.08 | ± 0.3 |
| 20:2 n-6 | 1.35 | ± 0.71 | ND | | ND | | ND | | ND | | ND | | 0.65 | ± 0.1 | 0.63 | ± 0.0 |
| 20:3 n-3 | 7.75 | ± 6.1 | 7.55 | ± 8.1 | 6.39 | ± 1.7 | 9.83 | ± 1.7 | 6.93 | ± 1.1 | 9.42 | ± 3.2 | 7.11 | ± 4.1 | 4.63 | ± 0.6 |
| 20:4 n-6 | ND | | 10.78 | ± 0.8 | ND | | ND | | ND | | ND | | ND | | ND | |
| 20:5 n-3 | 1.58 | ± 0.0 | ND | | 1.54 | ± 0.2 | ND | | 1.07 | ± 0.0 | ND | | 1.69 | ± 0.1 | 0.76 | ± 0.0 |
| 22:6 n-3 | 0.7 | ± 0.0 | 1.68 | ± 0.1 | ND | | ND | | 3.88 | ± 0.1 | 6.18 | ± 0.9 | 6.88 | ± 0.7 | 3.73 | ± 2.0 |
| Total PUFA | 14.21 | ±2.1 [a] | 24.57 | ±9.5 [b] | 10.28 | ±1.0 [a] | 9.8 | ±1.4 [a] | 9.8 | ±2.7 [a] | 13.5 | ±2.3 [a] | 14.28 | ±3.4 [a] | 9.2 | ±1.4 [a] |
| Total other FA (NI) | 9.03 | ±1.34 | 16.94 | ±2.2 | 29.92 | ±1.6 | 8.58 | ±0.4 | 8.31 | ±3.6 | 11.54 | ±1.9 | 7.65 | ±2.4 | 7.20 | ±0.6 |

*Data represents the mean ± SD, n=4. ND: Not detected; NI: Not Identified, SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; The different letters in same row was significantly different ($p<0.05$).*

Table 4. 2 The effect of the treatment of *c*-20:1 PIs on the Δ 9 desaturation indices

| | | | *c*-20:1 isomer | | | | | | | | | | | | *c*9-18:1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Factor | Control | | *c*15 | | *c*13 | | *c*11 | | *c*9 | | *c*7 | | *c*5 | | | |
| 16:1/16:0 | 0.09 | ± 0.01[a] | 0.08 | ± 0.01[a] | 0.18 | ± 0.01[b] | 0.08 | ± 0.01[a] | 0.10 | ± 0.01[a] | 0.08 | ± 0.01[a] | 0.09 | ± 0.01[a] | 0.16 | ± 0.03[c] |
| 18:1/18:0 | 1.11 | ± 0.13[a] | 0.98 | ± 0.62[a] | 0.87 | ± 0.06[a] | 2.29 | ± 0.50[b] | 1.57 | ± 0.43[a] | 1.24 | ± 0.40[a] | 0.99 | ± 0.13[a] | 4.37 | ± 0.5[c] |

*Desaturation indices in the TG fraction of cellular lipids in 3T3-L1 cells treated with different c-20:1 PIs; Data represents the mean ± SD, n=4. The different letters in same row was significantly different (p<0.05).*

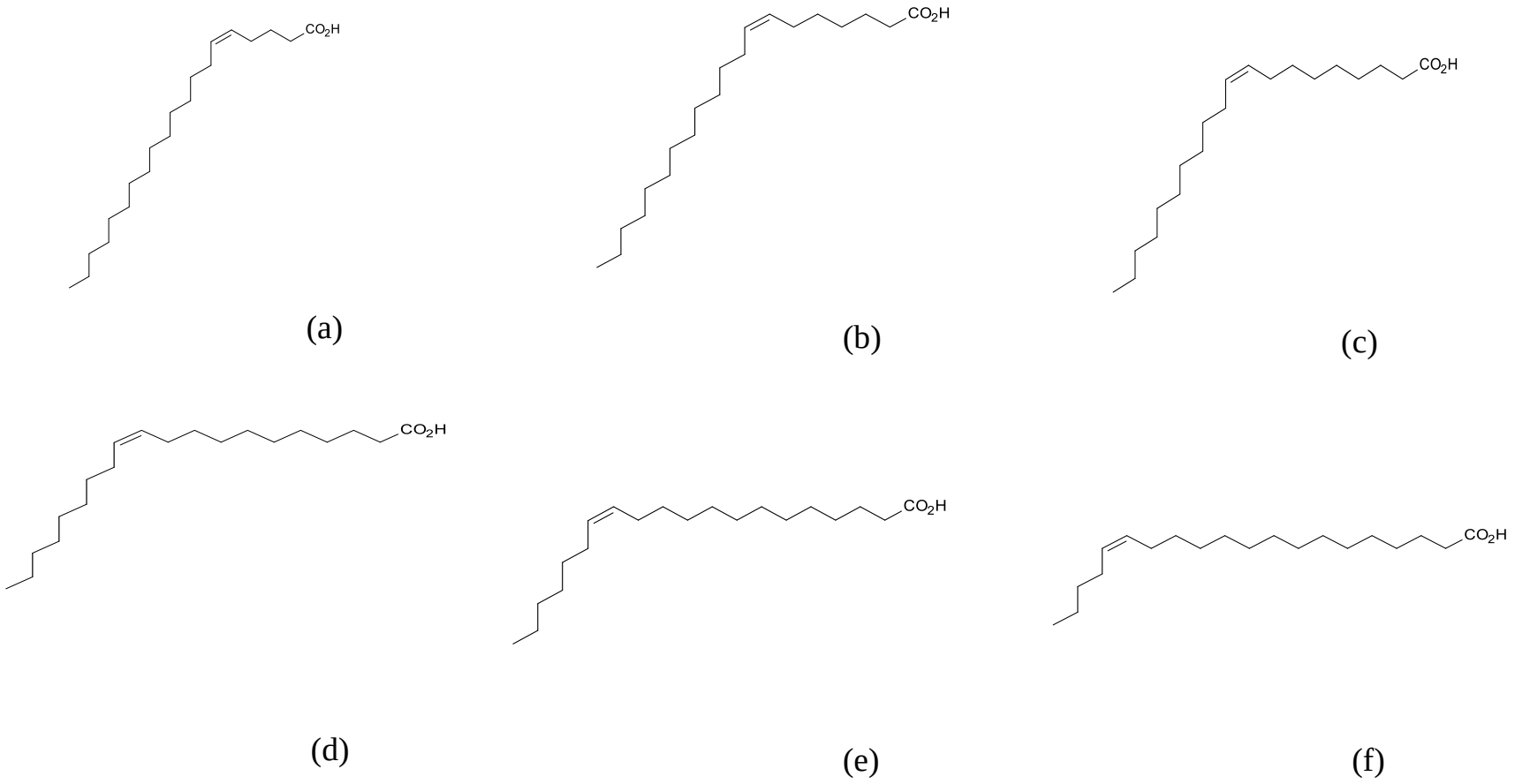



**Fig. 4.1** Chemical structures of selected *c*-20:1 PIs; (a) *c*5-20:1, (b) *c*7-20:1, (c) *c*9-20:1, (d) *c*11-20:1, (e) *c*13-20:1, and (f) *c*15-20:1

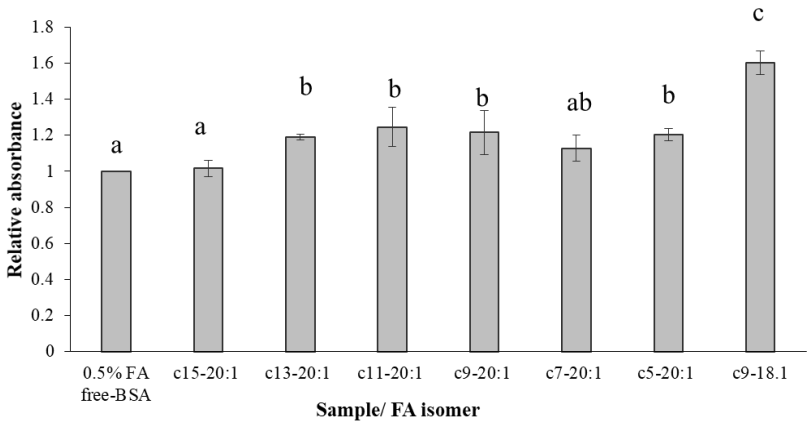
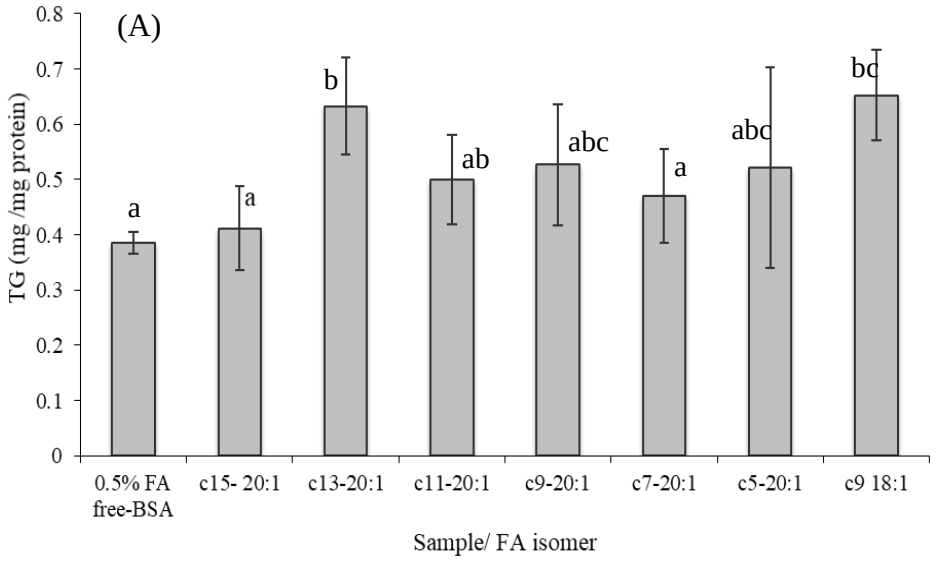
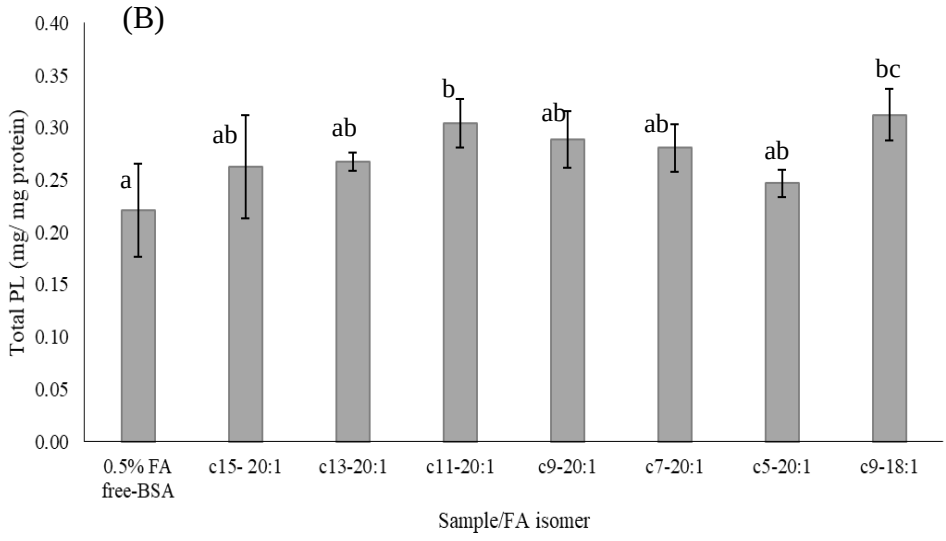



**Fig. 4.2** Relative intracellular lipid content in 3T3-L1 cells treated with different *c*-20:1 PIs, *c*9-18:1, and 0.5% FA free-BSA on day 10 of differentiation determined by Oil Red O Staining, Data are shown as mean ± SD, n=6, Means for a variable without a common letter differ at $p<0.05$



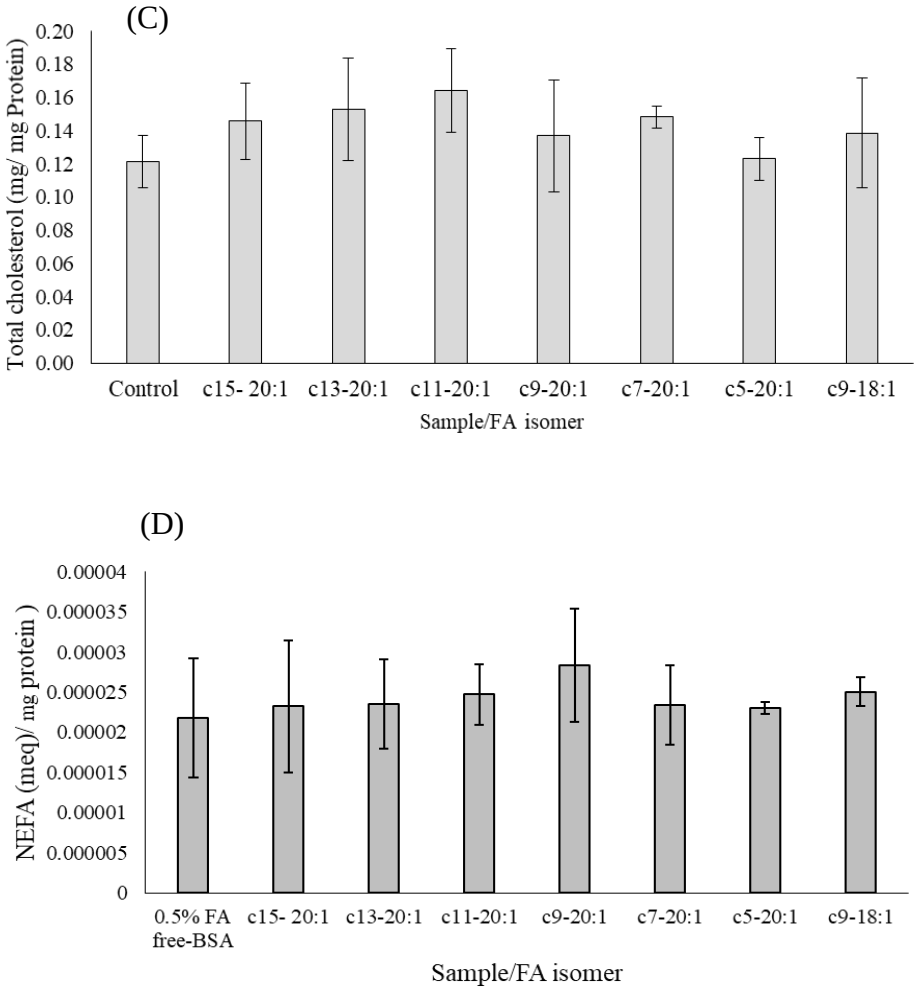



**Fig. 4.3** The effect of the different *c*-20:1 PIs, *c*9-18:1, and 0.5% FA free-BSA on (A) TG accumulation, (B) Phospholipids, (C) Total cholesterol, and (D) Non- esterified fatty acids in 3T3-L1 cells on the day 10 of differentiation, determined by enzymatic assay kits. Data are shown as mean ± SD, n=6, Means for a variable without a common letter differ at $p<0.05$

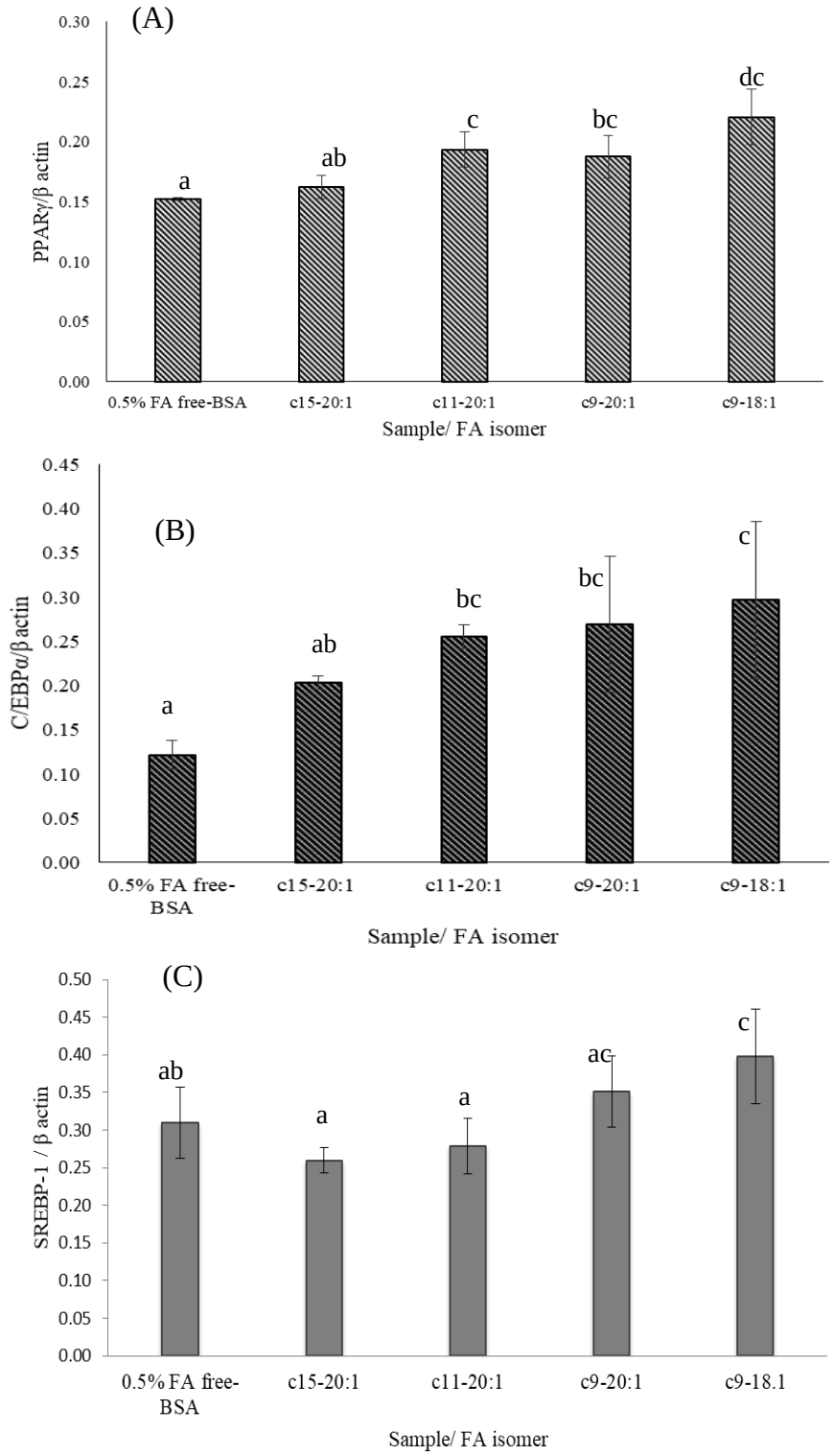



**Fig. 4.4** The effect of the different *c*-20:1 PIs, *c*9-18:1, and 0.5% FA free-BSA on protein expression of (A) PPARγ, (B) C/EBPα, and (C) SREBP-1 in 3T3-L1 cells on the day 5 of differentiation, determined by western blotting. Data are shown as mean ± SD, n=6, Means for a variable without a common letter differ at $p<0.05$.

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## Chapter 05

## Comparison of the effect of selected long chain monounsaturated fatty acids positional isomers on lipid metabolism - *in vitro* study

### 5.1. Introduction

LC-MUFAs are an important group of FAs in dietary lipids and the most of foods have a high content of LC-MUFA in their total lipids (approximately 40-60%), particularly as oleic acid (*c*9-18:1). In plant-based foods, *c*9-18:1 is a predominant LC-MUFA[1)]. Although, some of the marine fish lipids and plants belong to *cruciferae* family are reported to have a higher content of *c*-20:1 and *c*-22:1[2, 3)]. Thus, the total content of LC-MUFA changes among plant lipids and animal lipids specially fish lipids. A recent study conducted using different FAs belongs to n-3 PUFA showed that the both the number of carbon atoms and double bonds influence on their functionality[4)]. Although, comparison between the functionality of LC-MUFA with relevant to number of C atoms in the acyl chain or the position of the double bond have not investigated thoroughly.

As described in previous chapters, MUFA contains an olefinic bond in hydrocarbon structure and occurs naturally in *cis* configuration. The double bond in MUFA could be located at different positions in the hydrocarbon chain, but commonly found in between the 9th and 10th carbon atoms from the carboxyl carbon atom due to the action of the Δ9 desaturase[5)]. Various LC-MUFA PIs have reported in dietary lipids and their occurrence and distribution differ among food. Accordingly, 13 isomers of *c*-18:1[6, 7)], 6 PIs of *c*-20:1 and 5 PIs of *c*-22:1[8)] were reported in dietary lipids (explained in section 1.4.2.). Recent studies have shown that the functional properties of the FAs vary among their PIs[9)].

Health effects of n-3 PUFA and their PIs especially conjugated linoleic acid (CLA) and their PIs have broadly studied using different animal and cell models[9-12)] and reported to have different effects on the lipid metabolism. Although, n-3 PUFAs have a high biologically functionality, their usage is limited due to less oxidative stability that lead to production of oxidized FAs at higher processing temperatures and subsequent negative health effects. Oxidative stability of MUFA lies in between SFA and PUFA, where MUFAs have less oxidative stability than SFA but higher than that of PUFA[13)]. Many studies have proven the beneficial effects of MUFA rich diet such as cholesterol-lowering

effect and improving plasma lipoproteins balance, etc.[14] MUFA reported to exhibit more similar effects to that of n-3 PUFA when substitute MUFA instead of carbohydrate in the diet[15]. Epidemiological studies have carried out using LC-MUFA with plant origin (mainly *c*9-18:1 PIs)[16, 17] and fish origin (mainly *c*11-20:1, *c*9-20:1, and *c*11-22:1)[18, 19]. It was apparent that MUFA occurs as a mixture of their PIs in food[20]. Thus, the physiological effect exerted by these FAs might be a cumulative function (synergistic or antagonistic) of all naturally occurring PIs. Thus, knowledge on physiological effects of individual isomers on health would be useful in understanding the effect of the position of double bond on their functionality.

FAs are important as both structural components and fuel molecules[21] and play a vital role in lipid homeostasis. Disorders in the metabolism FAs lead to development of obesity and type 2 diabetes[22]. Different FAs act differently on the lipid metabolism at cellular level. A clear understanding of an effect of a particular fatty acid on the lipid metabolism at cellular level is important to review their metabolic functions. The effect of various FAs on cellular TG (equal to TAG) accumulation and lipid metabolism has studied broadly using different cell lines. Both adipocytes and hepatocytes have important roles in the lipid metabolism in our body and controls anabolism and catabolism of lipids. Hence, a substantial amount of studies has done using HepG2 cells (human hepatocyte carcinoma cells) [4, 23, 24] and 3T3-L1 cell[25-27] lines during past three decades to study the effect of FAs on the cellular lipid metabolism. The 3T3-L1 cell line is widely used for studying adipogenesis and the biochemistry of adipocytes. It has originated from swiss mouse embryonic cells, undergone commitment and could be differentiated to mature adipocytes through appropriate conditions. Adipocytes mainly involve in the storage of energy in the form of TAG and glycogenesis. The HepG2 cells and the 3T3-L1 cells are adherent cells and suitable for different experimental protocols.

Dysregulations of lipid metabolism in adipocytes directly related with the onset and development of the obesity and subsequent diseases. The PPARs and the SREBPs are major classes of transcriptional regulators of enzymes involved in the fatty acid metabolism and both exist in several isoforms. Generally, the PPAR γ and the SREBP-1 control processes involving lipogenesis[28] and play key role in cellular lipid metabolism. The PPAR γ stimulates fatty acid storage in adipose tissue by increasing both the storage

capacity and the fatty acid flux into adipocytes, thus predominantly expressed in adipose tissue[29]. It has found that unsaturated FAs such as oleic, linoleic, and arachidonic acids act as natural ligands of the PPAR γ[30]. Once activated, the PPAR γ increases the expression of numerous genes involving the lipid metabolism and uptake in adipocytes[31]. The family of SREBPs regulates the transcriptional activation of many genes involving regulation of lipid metabolism, particularly the lipogenesis[32] and high expression of SREBP-1c was reported in the liver and adipose tissue[33]. Further, it has reported that the dietary FAs and their metabolites are able to modulate the expressions of genes related to lipid metabolism such as fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD-1), and carnitine palmitoyltranferase 1a (CPT-1a) by several mechanisms. These FAs can affect the gene transcription in relation to fatty acid synthesis, messenger RNA processing and modulate the postprandial modulation of the protein[34, 35].

The physiological functions or bioactivities of LC-MUFA consist of more than 18 C atoms, i.e. 20:1, 22:1, 24:1, etc., have not studied and compared broadly based on their chain length and position of the double bond. Limited studies provided data on comparison of the effects of LC-MUFA isomers on cellular functions[36]. The double bond in LC-MUFA could be located near to methyl end or carboxylic end or at the middle of the acyl chain. Although, it was found that the functionalities of LC-MUFA are different, there are still many debatable questions about LC-MUFA-PIs and their functionalities. No studies provide comparative data on the functionality of LC-MUFA-PIs. Thus, the bioactivities of LC-MUFA PIs and the relevant molecular mechanisms are yet to be elucidated. In the current study, LC-MUFA PIs having different chain lengths (C18, C20, and C22) and double bond located at same or different positions were investigated to compare their individual effect on the lipid metabolism at cellular level using 3T3-L1 and Hep G2 cells.

## 5.2. Materials and Methods

### 5.2.1. Materials

Murine 3T3-L1 preadipocytes were obtained from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Human liver carcinoma (HepG2) cells were also purchased from the DS Pharma Biomedical Co., Ltd. *cis*-LC-MUFA PIs (purity > 97%) of *c*-20:1 and *c*-22:1 were synthesized in-house using the protocol described in Chapter 02. The *c*-18:1 PIs were

obtained from Tsukishima Foods Industry Co., Ltd. (Tokyo, Japan). Similar list of materials given in the section 4.2.1 were used in this experiment. All the reagents used in this study were analytical grade.

### 5.2.2. Screening of LC-PUFA-PIs

The common (naturally occurring) *cis*-LC-PUFA PIs (13 PIs of *c*-18:1, 6 PIs of *c*-20:1, and 5 PIs of *c*-22:1) were screened for their individual effect on cell viability using HepG2 cells (MTT assay) and effect on the cellular lipid accumulation using 3T3-L1 cells by Oil Red O staining. Results of the screening of LC-MUFA PIs were not given in the dissertation. Three representative LC-MUFA-PIs (*c*5-18:1, *c*9-18:1, *c*11-18:1, *c*9-20:1, *c*15-20:1, *c*11-20:1, *c*7-22:1, *c*9-22:1, and *c*11-22:1) from each chain length were selected for further studies based on their individual effect on the cellular lipid accumulation and their abundance in food in particularly fish lipids.

### 5.2.3. Cell culture

The 3T3-L1 preadipocytes or the HepG2 cells were maintained in DMEM supplemented with 10% FBS and 1% of penicillin-streptomycin (basal medium) at 37°C in a humidified atmosphere under 5% $CO_2$. Cells were transferred at three days interval with the change of fresh medium with two days interval and experiment was conducted using cells from passage 5 to passage 12 in the 3T3-L1 cells line and passage 6 to passage 15 in the HepG2 cells line.

In experimental cultures using the 3T3-L1 cells, basal medium was added with 50 μM of each selected LC-MUFA-PI (mentioned in 5.2.2) and 0.5% FA free-BSA with 0.01% ethanol as the control. FA-BSA complex was prepared as previously described[37] with slight modifications. The experimental conditions and protocols were similar to the method described in section 4.2.2. The cells were cultured till the D10 or predetermined culture period.

The cell viability or cytotoxicity of FAs was measured using the HepG2 cells by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The cells were maintained in a DMEM culture medium with above-mentioned experimental conditions. For the experimental cultures, the cells were cultured in 96 well plates using a

DMEM culture medium supplemented with 10% FBS and 1% of FA free-BSA. After the cells reached to 60-70% confluence, the cells were treated with 50 μM of LC-MUFA PIs (mentioned in 5.2.2) or 0.5% FA free-bovine serum with 0.01% ethanol as the control for 24 hrs. MTT assay was carried out according to manufacturer protocol to determine the cells viability and cytotoxicity (data are not shown).

### 5.2.4. Cellular triglyceride content

After culture period (on D10), the 3T3-L1 cells were collected and cell lysate was obtained (as described in section 4.2.4). Then cell lysate was analyzed for cellular TG content using enzymatic assay kit. The contents of TG in samples were normalized to the total protein content in the respective samples.

### 5.2.5. Analysis of cellular fatty acid composition of cellular lipids

The cellular lipids were extracted by a modified method of Bligh and Dyer as described by Lin *et al*[10]. Extracted cellular lipids were methyl esterified and analyzed for the fatty acid composition (FAC) in total lipids (explained in section 4.2.5.1).

### 5.2.6. Determination of effect of LC-MUFA PIs on the protein expression products related to the adipogenesis and lipogenesis in 3T3-L1 cells by western blotting

The 3T3-L1 cells were cultured using the same protocol mentioned above (section 5.2.3) and cells were collected on D5, using RIPA buffer added with 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich Japan K.K.). The cellular protein content was determined by BCA method. Then, cell lysate was mixed with equal part of sample buffer solution (2ME+) (x2) and subjected to SDS-PAGE followed by western blotting as described in chapter 04 (section 4.6). Results were analyzed using IMAGE J software and normalized to the level of β-actin in respective sample.

### 5.2.7. Determination of effect of LC-MUFA PIs on mRNA expression of genes related to lipid metabolism

Six LC-MUFA PIs (*c*5-18:1, *c*9-18:1, *c*15-20:1, *c*11-20:1, *c*7-22:1, and *c*11-22:1) were selected to study their effect on mRNA expression of genes related to lipid metabolism. After the culture period (D7), total mRNA was isolated from treated cells

using TRIzol reagent (Qiagen, Venlo, Netherlands) according to the manufacturer protocol. Next, complementary DNA was prepared and amplified using real-time RT PCR, which was performed using THUNDERBIRD Probe qPCR Mix (Toyobo Co., Ltd., Osaka, Japan) in a final volume of 20 μL. The expression was normalized to the internal standard β actin. Details of RT-PCR primers are listed in Table 5.1. Cycle parameters were 95°C for 2 min then 40 cycles of 95°C for 30 s, and 60°C for 30 s (FADS1, SCD-1, and CPT-1a). Expression was determined by the standard curve method.

### 5.2.8. Statistical analysis

All the analytical results were presented as the mean ± SD. Statistical analysis was carried out using one-way ANOVA followed by a Tukey-Kramer *post-hoc* test. Differences were considered significant when $p$ was less than 0.05 ($p<0.05$).

## 5.3. Results

### 5.3.1. Screening of LC-MUFA PIs

The effect of LC-MUFA PIs on the cytotoxicity or cell viability was measured by MTT assay using the HepG2 cells (data were not shown). Almost all the LC-MUFA PIs showed a higher cell viability (> 85%). The effect of the LC-MUFA PIs on the cellular lipid accumulation was determined by the Oil Red O staining and expressed as the relative cellular lipid content (data were not shown). The lipid accumulation in the 3T3-L1 cells treated with different LC-MUFA PIs was isomer specific and varied among same (among *c*-18:1 PIs, among *c*-20:1 PIs, or among *c*-22:1 PIs) and different (between groups of *c*-18:1 PIs, *c*-20:1 PIs, and *c*-22:1 PIs) groups of LC-MUFA PIs. Cells were changed their size and shape (Fig. 5.1) during the culture period. Micrographs of the 3T3-L1 cells showed that the most of cells were filled with lipid droplets by D10. Nine isomers (*c*5-18:1, *c*9-18:1, *c*11-18:1, *c*9-20:1, *c*15-20:1, *c*11-20:1, *c*7-22:1, *c*9-22:1, and *c*11-22:1) were selected after screening for further analysis considering the facts on their ability to reduce the cellular lipid accumulation and their relative abundance in dietary lipids.

### 5.3.2. Effect of LC-MUFA-PIs on cellular TG accumulation

Figure 5.2 illustrates the effect of selected LC-MUFA PIs on TG accumulation in the 3T3-L1 cells. Selected PIs influenced differently on TG accumulation in the 3T3-L1 cells. The lowest and highest cellular TG accumulation were shown by cells treated with *c*15-20:1 and *c*9-22:1, respectively. The level of TG in the treated cells increased in order of *c*15-20:1 < *c*5-18:1 < *c*11-20:1< *c*9-20:1 < *c*11-18:1 < *c*7-22:1 < *c*9-18:1 < *c*11-22:1< *c*9-22:1. The content of cellular TG was significantly different ($p<0.05$) among treatments. Further, it was apparent that *c*9-LC-MUFA in each tested chain length had a higher cellular TG content with compared to other PIs. It was apparent that the cells treated with *c*-22:1 PIs showed the highest accumulation of cellular TG.

### 5.3.3. Effect of LC-MUFA-PIs on the FAC of cellular lipids

The FAC data in cellular lipids treated with selected LC-MUFA PIs are summarized in the Table 5.2. The FAC of cellular lipids varied with the treated LC-MUFA PIs. Irrespective of the treated LC-MUFA PIs, the abundant FA in most cells were palmitic acid (16:0) followed by the oleic acid (18:1n-9, *c*9-18:1) with few exceptions. The prominent PUFA in most of cellular lipid was the 20:3. The content of total MUFA and PUFA were significantly ($p<0.05$) varied in the cells treated with LC-MUFA PIs (Fig. 5.3). Although, there was no significant ($p<0.05$) difference in the content of total SFA in cellular lipids among treatments. The FAC data revealed that cells uptake the treated LC-MUFA PIs into cells at different percentages. The cells treated with *c*9-18:1 showed the highest content of cellular lipids.

### 5.3.4. The expression of transcriptional factors related to adipogenesis and lipogenesis

#### 5.3.4.1. The expression of protein related to adipogenesis in 3T3-L1 cells

The effect of the selected LC-MUFA PIs on the protein expression products related to adipogenesis, i.e. PPAR γ and C/EBP α, was determined by western blotting. The changes in the expression of the PPAR γ in the 3T3-L1 cells treated with selected LC-MUFA PIs are shown in Fig. 5.4 (A). LC-MUFA PIs influenced significantly ($p<0.05$) different on the levels of the PPAR γ in the 3T3-L1 cells. The highest level of PPAR γ was

shown in cells treated with *c*9-22:1. The *c*9-20:1 followed by *c*15-20:1 reported the lowest levels of the PPAR γ.

Figure 5.4 (B) shows the changes in the level of the C/EBPα in the 3T3-L1 cells treated with the selected LC-MUFA PIs on the D5 of the differentiation. As indicated, the level of expression of the C/EBPα in the 3T3-L1 cells was isomer specific and significantly ($p<0.05$) varied among the treatments. The lowest and the highest expression of the C/EBPα in the cells treated with *c*9-20:1 followed by *c*15-20:1 and *c*9-22:1, respectively. It was apparent that the levels of expression of the PPAR γ and the C/EBPα were not significantly ($p<0.05$) different within the LC-MUFA PIs groups having 18 and 20 C atoms. For instance, the level of the PPAR γ or the C/EBPα were not significantly ($p<0.05$) different among cells treated with *c*5-18:1, *c*9-18:1, and *c*11-18:1. The cells treated with *c*-22:1 PIs showed the highest expression of the PPAR γ and the C/EBPα and the levels are significantly ($p<0.05$) different among the treated *c*-22:1 PIs.

#### 5.3.4.2. The effect of LC-MUFA PIs on transcriptional factors related to the lipogenesis in 3T3-L1 cells

The changes in the transcriptional factors related to lipogenesis in LC-MUFA PIs treated cells were determined in the 3T3-L1 and results are given in the Fig. 5.5. The protein expression of SREBP-1 was significantly ($p<0.05$) reduced by the treatment of *c*15-20:1 with compared to that of the other LC-MUFA PIs. The level of expression of SREBP-1 was increased in order of *c*-20:1 PIs < *c*-18:1 PIs < *c*-22:1. All the *c*-22:1 PIs treated cells showed considerably elevated levels of SREBP-1.

### 5.3.5. Effect of LC-MUFA PIs on mRNA expression related to glucose and lipid metabolism

To elucidate the effect of LC-MUFA PIs on the mRNA expression of genes related to glucose/ lipid metabolism, RT-PCR was performed. Figure 5.6 (A) and (B) show the changes in the mRNA expression of SCD-1 and FAS in 3T3-L1 cells treated with different LC-MUFA PIs, determined by RT-PCR on the 7th day of differentiation. The cells treated with *c*15-20:1 showed the lowest level of mRNA expression of SCD-1 and FAS than that

of other LC-MUFA PIs and control. The mRNA expressions of SCD-1 and FAS were significantly ($p$<0.05) different among the treatments.

### 5.3.6. The effect of LC-MUFA PIs on the mRNA expression of genes related FA β oxidation

The effect of the LC-MUFA PIs on the mRNA expression related to cellular lipid and glucose β oxidation, i.e. CPT-1a, was measured by RT-PCR on the D7 of the differentiation of cells. Figure 5.6 (C) shows the changes in the mRNA expression of the CPT-1a in cells treated with LC-MUFA PIs. The increase in the mRNA expression of the CPT-1a related to the increase in the energy metabolism in the cells. Among the tested isomers, *c*-20:1 PIs showed comparative increment in the mRNA expression of the CPT-1a than that of other LC-MUFA PIs. The effect exerted by individual LC-MUFA PIs was not significantly ($p$<0.05) different among treatments.

## 5.4. Discussion

The *c*-LC-MUFA PIs consisted of 18, 20 and 22 C atoms were tested for their individual effect on cellular lipid metabolism. The tested LC-MUFA PIs did not influence undesirably on the cell viability. Thus, the concentration of FAs (50 μM FA in the final medium) used in the experiment was not cytotoxic and did not influence on subsequent analysis. The MTT assay provides a quantitative and sensitive detection of cell proliferation since it measures the growth rate of cells by a linear relationship between cell activity and absorbance.

The lipid accumulation in the 3T3-L1 cells treated with different LC-MUFA PIs was isomer specific and varied among the treatments. The *c*9-LC-MUFAs showed a higher accumulation of TG compared to that of other PIs. The LC-MUFA with the double bond position at 9th carbon atom from the carboxylic acid synthesized mainly through the activity of Δ9 desaturase on respective SFA. The *c*9-MUFA are reported to the predominant monoenoic PIs due to abundance of Δ9 desaturase in plants and animals. The cellular content of TG was higher in all cells treated with FA PIs than that of control, indicating that FAs treatment enhances the lipid accumulation in 3T3-L1 cells. Similar observations reported by Kokta *et al.*[39]. Long chain FAs bind and activate all the

subclasses of the PPAR and emerged as strong endogenous ligand candidates of the PPARs, thus promotes the differentiation of pre-adipocytes in to mature adipocytes[40]. Consequently, they upregulate the activity of enzymes related to lipid synthesis, subsequently increasing the cellular lipid content.

In the current study, *c*-22:1 PIs showed a high lipid accumulation. Past studies conducted using erucic acid (22:1 n-9) rich food reported to have negative effects on the health causing myocardial lipidosis due to poor mitochondrial β oxidation. However, some studies reported 22:1 rich food showed only a transient lipidosis in some organs and it disappeared upon continued feeding, might due to increased activity of peroxisomal beta oxidation[41, 42]. Flatmark *et al.* showed a stimulatory effect of LC-MUFA rich fish oil on peroxisomal β oxidation in heart[43]. The predominant 22:1 PI in fish oil is the 22:1 n-11 (*c*11-22:1). Previous studies of health effects of 22:1 have carried out using food rich in 22:1 n-9 (*c*13-22:1) isomer such as rapeseed oil. In the current study, it was clearly shown that the 22:1 n-11 (*c*11-22:1) and 22:1 n-9 (*c*13-22:1) isomers showed a different effect on cellular lipid metabolism and *c*13-22:1 cause accumulation in lipids.

The FAC data of cellular lipids showed that the treatment of *c*-LC-MUFA PIs had changed the FA metabolism at cellular level. FAC data obtained in this experiment showed similar pattern as observed in the experiment conducted using *c*-20:1 PIs (section 4.3.3.1.). Recent studies showed LC-MUFA rich diet increased levels of LC-MUFA isomers in plasma, tissues, and other organs, particularly in adipose tissue[44]. The cells treated with LC-MUFA PIs showed an elevated level of n-3 PUFA, which may ultimately contribute to the beneficial effect on the factors related to the metabolic syndrome, thus showing positive health benefits. Similar observations were reported by some researchers who studied the cellular FAC of tissues after feeding LC-MUFA concentrated fish oil and further revealed that the treatment of LC-MUFA PIs resulted in decrease in the content of n-6 PUFA, which may contribute to the improvement in systemic inflammation and control of the metabolic syndrome[45, 46]. Studies have reported that the dietary FAs provide health benefits through changes in the cellular FA metabolism or induction of the cell signaling pathways[47].

It was apparent that the cells uptake the treated LC-MUFA PIs into cells at different percentages. Same observation was noted in the previous experiment described in chapter

04. The LC-MUFA cannot enter cells directly, hence enter via fatty acid transport binding protein (FATP). Based on the FAC data, adipocytes showed a selective uptake towards the different LC-MUFA PIs. LC-MUFA PIs with the double bond located at the middle position of the acyl chain, i.e. *c*9-18:1, *c*9-20:1, showed considerably higher level of uptake compared to that of LC-MUFA PIs (*c*15-20:1 and *c*7-22:1) with double bond located near to carboxyl or methyl end of the acyl chain. The structure of *cis*-MUFA is not linear and exhibits kink of the acyl chain at the C-C double bond. Hence, the structure of LC-MUFA PIs would be symmetric or asymmetric depend on the position of the double bond. Thereby, the position of the double bond in LC-MUFA may have affected on the binding affinity of the MUFA to the FATP and subsequently influence on the level of uptake of fatty acid into the cells. Another reason for the variation of LC-MUFA PIs in the FAC of cellular lipids could be due to the differences in the metabolic rates of particular PIs or isomerization to another form before incorporated into cellular lipids. Further studies are needed to understand the reason behind the differences in the levels of LC-MUFA PIs in cellular lipids.

The treatment of some LC-MUFA PIs such as *c*15-20:1 caused reduction in the expression of key regulatory transcriptional factors of adipogenesis, i.e. PPAR γ and C/EBPα, leading to reduction in the preadipocyte differentiation to mature adipocytes, subsequently reducing the cellular lipid content. Feeding fish oil rich in LC-MUFA was shown to decrease adipose tissue mass and suppress visceral fat accumulation in rats[46]. In the current study, it was shown that some LC-MUFA PIs, e.g. *c*15-20:1, had comparatively anti-adipogenic effects during differentiation in the 3T3-L1 cells. Adipocyte differentiation is accompanied by the occurrence of 2 critical events after initiation of differentiation, namely mitotic clonal expansion and irreversible commitment to differentiation[48]. Some FAs such as CLA[49], EPA[36], and DHA[50] are reported to exhibit strong anti-adipogenic activity in the 3T3-L1 cells in which levels of the PPAR γ and the C/EBP α were lower than those of control. Thus, these FAs may interfere with these processes resulting suppression of adipogenesis. In this experiment, none of the tested LC-MUFA PIs showed lower PPAR γ and C/EBP α than that of control. Although, the level of the PPAR γ in cells treated with *c*15-20:1 was nearly similar to that of the control.

Some of the tested LC-MUFA PIs such as *c*15-20:1 caused significant ($p<0.05$) reduction of the level of the SREBP-1 in cells and consequently the expression of genes related to lipogenesis would be reduced. Lipogenesis is upregulated during adipocyte differentiation leading to accumulation of cellular lipids particularly TG. Colin *et al*. reported that *de novo* lipogenesis in differentiating human adipocytes could provide all the FAs needed for the maturation[51]. It could be suggested that the position of the double bond and chain length in LC-MUFA plays a significant role in regulating the expression of the transcriptional factors related to adipogenesis and lipogenesis in the 3T3-L1 cells. In the current study, experimental FAs isomers were selected based on their individual function in reducing the TG content in the 3T3-L1 cells. Comparison of the individual effect among *c*9-LC-MUFA (or *c*11-LC-MUFA) isomers of different chain lengths showed the influence of number of C atoms in the in LC-MUFAs (same double bond position from the carboxylic end) on their functionality and showed differences among isomers.

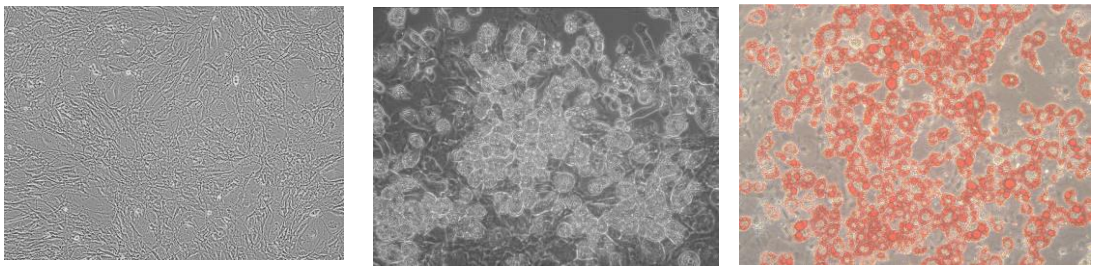
Lipid metabolism is a collective action of anabolism and catabolism of lipids. Cellular lipid metabolism governs by number of genes and proteins. In the current study, SCD-1 and FAS were selected to study the effect of *c*-LC-MUFA PIs on the FA synthesis. SCD-1 acts as an enhancer of metabolic syndrome[52]. Hence, reduction in the mRNA expression of the SCD-1 reduces the risk of metabolic syndrome. Changes in the mRNA expression related to lipogenesis directly influence the cellular lipid content. In mammals, fatty acid synthesis and metabolism are strictly controlled by nuclear transcription factor like SREBP-1c and regulates genes involving fatty acid synthesis and metabolism such as FAS and SCD-1[53, 54]. In the current study, tested *c*-20:1 PIs showed a favorable effect by comparatively reducing the mRNA expression of gene related to FA synthesis. The CPT-1a was used to measure the effect of LC-MUFA PIs on the mRNA expression of genes related to the cellular lipid and glucose oxidation. The increment of the level of CPT-1a is favorable for the reduction in cellular lipids. Hence, it could be suggested that the treatment of cells with *c*-20:1 PIs particularly, *c*15 isomer resulted in upregulation of FA oxidative gene, CPT-1a, and subsequent decrease in the cellular lipid content. It has reported that the dietary FAs and their metabolites are able to modulate the gene expressions by several mechanisms. FAs or their derivatives (acyl-CoA or eicosanoids) may interact with nuclear receptor proteins that bind to certain regulatory regions of DNA and thereby alter transcription of these genes. These FAs can affect the gene transcription

in relation to fatty acid synthesis, mRNA processing and modulate the postprandial modulation of the protein[55-57]. Several FAs such as EPA and CLA are reported to suppress the lipogenic gene transcription by downregulating the expression of specific genes such as SREBP. According to the results of the mRNA expression of genes, it was apparent that some experimental LC-MUFA PIs improved the glucose and lipid metabolism by suppression of lipogenesis and enhancement of the cellular lipid and glucose β oxidation. Previous studies have reported that PUFA PIs[58] and PIs of *c*-18:1[59] have different effect on the lipid metabolism in cell and animal models.

The current study provided the comparison between the activities of predominant LC-MUFA PIs characteristically contained in fish oil and plant oil and showed their differential effect on the lipid metabolism at cellular level. Further, this study demonstrated the influence of chain length in *c*-LC-MUFA on the functionality of individual PIs. Although, it was not possible to relate the increase in the chain length and the functionality of LC-MUFA due to limited data obtained on the health effect of PIs. However, results revealed that the functionality of LC-MUFA changes with the changes in the chain length. Thus, the effect of *c*-LC-MUFA on health may influence by the type of the available *c*-LC-MUFA, the predominant PIs and isomeric profile of LC-MUFAs in that food. Further studies need to be carried out to study the effect of LC-MUFA PIs on various aspects of metabolic syndrome related disorders.

### 5.5. Conclusion

Results revealed that the common LC-MUFA PIs affected differently on cellular TG accumulation, fatty acid composition of cellular lipids, expression of transcriptional factors related to adipogenesis and expression of genes related to lipid metabolism in the 3T3- L1 cells. Among the experimental LC-MUFA PIs, *c*-20:1 PIs showed improved cellular metabolism compared to that of *c*-18:1 PIs and *c*-22:1 PIs. Further, cells treated with *c*9-22:1 significantly increased the cellular lipid content by enhancing adipogenesis and lipogenesis. The selected LC-MUFA PIs from different chain lengths had different effect on the lipid metabolism at cellular level and *c*15-20:1 showed comparatively good anti-adipogenic, anti-lipogenic effect, and improvement of cellular lipid metabolism. The obtained results revealed that the position and the number of carbon atoms in *c*-LC-MUFA influenced on their functionality at the cellular level.



(a) (b) (c)

**Fig. 5.1** Micrographs of 3T3-L1 cells (a) D0 of differentiation, (b) D10 of differentiation, and (c) Oil red stained after D10 of differentiation

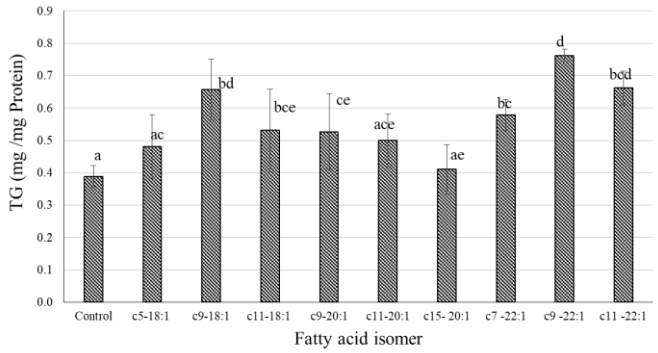



**Fig. 5.2** Changes in the cellular TG accumulation in 3T3-L1 cells treated with different LC-MUFA PIs and control on the D10 of differentiation determined by enzymatic assay kit. Data are shown as mean ± SD (n=6). Different letters indicate significant difference at $p < 0.05$.

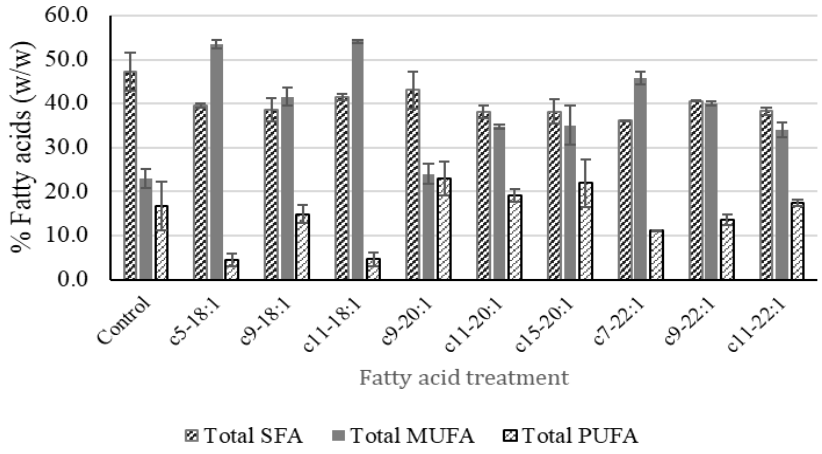



**Fig. 5.3** Effect of LC-MUFA PIs on the major fatty acid classes in cellular lipids in 3T3-L1 cells on the D10 of differentiation. Data are shown as mean ± SD (n=4)

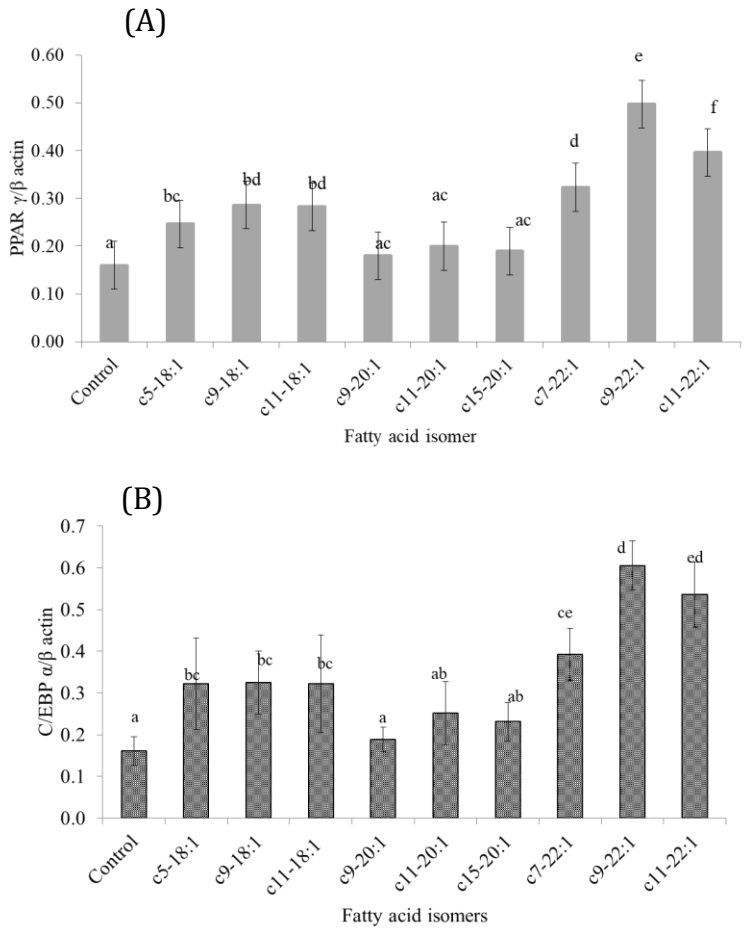



**Fig. 5.4** The effect of different LC-MUFA PIs and control (0.5% FA free-BSA) on expression of transcriptional factors related to adipogenesis (A) PPARγ and (B) C/EBPα in 3T3-L1 cells on the day 5 of differentiation, determined by western blotting. Data are shown as mean ± SD (n=6). Different letters indicate significant difference at $p < 0.05$.

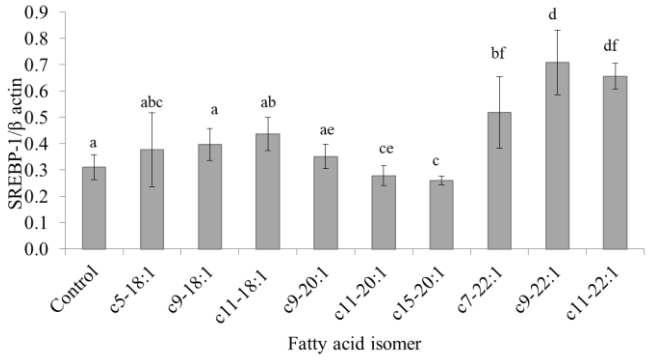



**Fig. 5.5** The effect of different LC-MUFA PIs and control (0.5% FA free-BSA) on expression of SREBP-1 in 3T3-L1 cells on D5 of differentiation determined by western blotting. Data are shown as mean ± SD (n=6). Different letters indicate significant difference at $p < 0.05$.

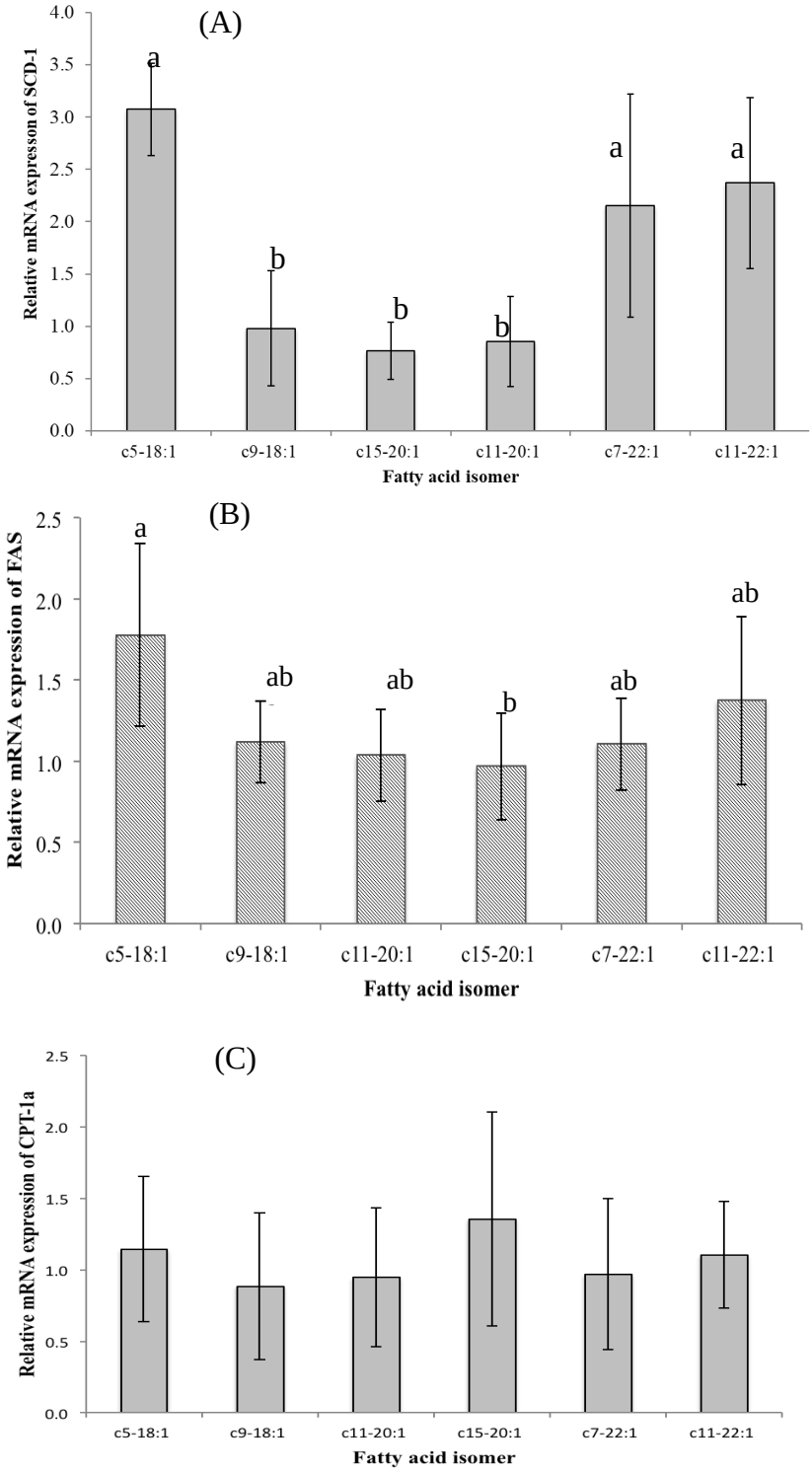



**Fig. 5.6** The effect of different LC-MUFA PIs and control (0.5% FA free-BSA) on the mRNA expression of (A) SCD-1, (B) FAS, and (C) CPT-1a relating to lipid metabolism in 3T3-L1 cells on D7 of differentiation determined by western blotting. Data are shown as mean ± SD (n=6). Different letters indicate significant difference at $p < 0.05$.

Table 5.1 GenBank Accession numbers and primer sequences used in Real-Time PCR experiments

| Gene | Primer sequence' | Accession number |
|---|---|---|
| SCD-1 | 5’ -TTCTTGCGATACACTCTGGTGC-3’ | NM_009127 |
| | 5’ -CGGGATTGAATGTTCTTGTCGT-3’ | |
| FAS | 5’ -GGAGGTGGTGATAGCCGGTAT-3’ | NM_007988 |
| | 5’ -TGGGTAATCCATAGAGCCCAG-3’ | |
| CPT1a | 5’ -CTCCGCCTGAGCCATGAAG-3’ | NM_013495 |
| | 5’ -CACCAGTGATGATGCCATTCT-3’ | |
| Actb | 5’-GACCCAGATCATGTTTGAGACC-3’ | NM_0311443 |
| | 5’-AGGCATACAGGGACAACACA-3’ | |

Table 5.2 Effect of LC-MUFA PIs on the fatty acid composition (%) of total cellular lipids in 3T3-L1 cells

| Fatty acid | Control | | *c*5-18:1 | | *c*9-18:1 | | *c*11-18:1 | | *c*9-20:1 | | *c*11-20:1 | | *c*15-20:1 | | *c*7-22:1 | | *c*9-22:1 | | *c*11-22:1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD | Mean | SD |
| 12:0 | 2.7 | 1.8 | 0.2 | 0.1 | 1.0 | 0.2 | 0.9 | 1.0 | 1.7 | | 0.6 | | 1.8 | 1.0 | 0.7 | | 1.2 | 0.6 | 1.2 | 1.0 |
| 14:0 | 2.7 | 0.9 | 4.1 | 0.1 | 2.4 | 0.8 | 2.7 | 0.3 | 1.7 | 0.5 | 1.7 | 0.0 | 2.3 | 0.5 | 1.5 | 0.5 | 3.0 | 0.3 | 2.0 | 0.8 |
| 15:0 | 1.3 | 0.0 | 6.3 | 0.2 | 1.6 | 0.3 | 7.0 | 1.2 | 1.0 | 0.1 | 1.7 | 0.3 | 1.7 | 0.1 | 0.5 | | 1.0 | 0.3 | 1.0 | 0.2 |
| 16:0 | 24.1 | 1.6 | 23.2 | 0.5 | 21.9 | 1.7 | 24.5 | 1.7 | 20.8 | 1.4 | 21.7 | 1.0 | 23.3 | 1.2 | 21.7 | 2.0 | 23.1 | 1.1 | 24.4 | 5.2 |
| 17:0 | 0.6 | 0.0 | 2.1 | 0.0 | 0.8 | 0.3 | 2.6 | 0.5 | 0.5 | | 1.0 | 0.2 | 1.0 | 0.4 | 0.7 | | 0.3 | | 0.4 | |
| 18:0 | 15.1 | 0.0 | 3.6 | 0.2 | 10.8 | 0.5 | 3.8 | 1.4 | 13.3 | 0.6 | 11.7 | 0.8 | 14.1 | 0.9 | 13.3 | 1.3 | 11.4 | 0.7 | 13.5 | 3.8 |
| 21:0 | 0.6 | 0.0 | nd | | nd | | nd | | nd | | nd | | nd | | 0.8 | 0.3 | 0.8 | 0.3 | 1.0 | |
| Total SFA | 47.2 | 4.3 | 39.5 | 0.5 | 38.6 | 2.6 | 41.5 | 0.7 | 43.1 | 4.2 | 38.1 | 1.4 | 38.2 | 2.7 | 36.2 | 0.0 | 40.6 | 0.1 | 38.3 | 0.9 |
| 14:1 n-5 | nd | | 0.6 | 0.0 | nd | | 0.3 | 0.1 | nd | | nd | | nd | | nd | | nd | | nd | |
| 15:1 | 3.2 | 1.2 | 0.6 | 0.0 | 1.9 | 0.1 | 0.9 | 0.2 | 3.2 | 0.3 | 2.7 | 0.1 | 2.3 | 0.2 | 5.3 | 1.1 | 4.3 | 0.2 | 4.1 | 0.8 |
| 16:1 n-7 | 5.8 | 0.5 | 21.1 | 0.8 | 5.4 | 3.2 | 19.4 | 2.5 | 4.0 | 0.3 | 2.9 | 1.0 | 5.4 | 0.2 | 4.9 | 1.1 | 6.8 | 1.9 | 5.4 | 0.1 |
| 17:1 | 0.9 | 0.2 | 4.5 | 0.1 | 0.6 | 0.0 | 4.5 | 0.9 | | | 0.6 | 0.1 | 0.6 | | 0.5 | | 0.8 | | 0.5 | 0.1 |
| 18:1 isomer | nd | | 16.5 | 0.5 | nd | | 19.6 | 2.0 | nd | | nd | | nd | | nd | | 3.0 | 0.4 | nd | |
| 18:1 n-9 | 9.9 | 0.7 | 9.0 | 0.2 | 29.9 | 1.7 | 8.5 | 0.8 | 14.7 | 1.6 | 21.3 | 0.6 | 7.5 | 1.2 | 9.2 | 2.6 | 12.1 | 3.0 | 19.5 | 1.7 |
| 20:1 isomer | nd | | 0.7 | 0.0 | 1.5 | 0.4 | 0.3 | 0.0 | 9.2 | 1.1 | 4.6 | 0.4 | 4.5 | 0.5 | 3.1 | | 0.9 | 0.4 | 4.2 | 0.2 |
| 22:1 isomer | nd | | nd | | | | | | | | | | | | 9.6 | 1.7 | 10.0 | 0.5 | 4.8 | 1.3 |
| 24:1 n-9 | 3.1 | 1.3 | 1.0 | | 2.2 | 0.2 | 0.8 | 0.2 | 3.9 | 2.0 | | | 4.1 | 0.6 | 3.2 | 0.6 | 2.5 | 0.4 | 6.7 | 0.9 |
| Total MUFA | 22.9 | 2.1 | 53.5 | 1.0 | 41.5 | 2.0 | 54.1 | 0.3 | 24.0 | 2.3 | 34.8 | 0.5 | 35.1 | 4.5 | 45.7 | 1.4 | 40.0 | 0.6 | 34.1 | 1.6 |
| 18:2 n-6 | 1.5 | 0.4 | 0.6 | | 1.1 | 0.0 | 0.8 | 0.5 | 1.6 | 0.5 | 1.1 | 0.0 | 1.3 | 0.2 | 1.1 | 0.4 | 1.2 | 0.2 | 1.4 | 0.3 |
| 20:2 n-6 | 0.5 | 0.1 | nd | | nd | | nd | | nd | | nd | | nd | | 0.9 | 0.4 | 0.8 | 0.1 | 0.5 | |

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 20:3 n-6 | 2.1 | 0.9 | 0.7 | | 1.4 | 0.4 | 0.6 | 0.0 | 2.2 | 0.3 | 1.7 | 0.3 | 2.9 | 0.4 | 1.9 | 0.5 | 2.1 | 0.3 | 1.7 | 0.3 |
| 20:3 n-3 | 9.7 | 3.6 | 2.0 | 0.2 | 7.1 | 2.2 | 1.7 | 0.1 | 11.8 | 1.0 | 9.1 | 0.4 | 11.2 | 0.6 | 8.1 | 1.6 | 6.7 | 0.4 | 8.2 | 1.4 |
| 20:5 n-3 | 1.4 | 0.6 | 0.2 | | 0.7 | | 0.2 | 0.1 | 1.2 | 0.3 | 1.2 | 0.0 | 1.5 | 0.1 | 1.5 | 0.1 | 1.4 | 0.3 | 1.0 | 0.4 |
| 22:6 n-3 | 1.6 | 0.0 | 1.7 | 0.2 | 4.8 | 0.7 | 1.2 | 0.8 | 5.5 | 3.3 | 6.0 | 1.3 | 6.3 | 2.9 | 3.9 | 2.3 | 1.4 | 0.2 | | |
| Total PUFA | 16.8 | 5.5 | 4.5 | 1.5 | 14.9 | 2.1 | 4.6 | 1.5 | 23.0 | 3.9 | 19.2 | 1.5 | 21.9 | 5.5 | 11.2 | 0.0 | 13.6 | 1.2 | 17.5 | 0.7 |
| Other FA -NI (total) | 14.2 | 3.2 | 2.5 | 0.1 | 5.1 | 1.6 | 0.3 | 0.2 | 5.5 | 1.5 | 8.0 | 0.5 | 9.2 | 1.5 | 10.2 | 0.0 | 5.5 | 1.5 | 6.2 | 1.5 |

*Data represents the mean ± SD, n=4. nd: Not detected; NI: Not Identified, SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids*

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## Chapter 06

## General conclusion

LC-MUFAs are an important group of FAs in our diet contributing more than 45% to the composition of total FAs in most of foods. LC-MUFA consisting of 20 and 22 C atoms are characteristically found in some marine fish oil. The olefinic bond in the LC-MUFA could be located at various positions creating different PIs. The current study investigated the occurrence of different *c*-LC-MUFA PIs in fish oil and their bioactivities; particularly, their individual effects on the factors related to lipid metabolism using *in vitro* studies.

The common *c*-LC-MUFA PIs consisting of 20 and 22 C atoms were chemically synthesized in the laboratory using the synthesis route described in the chapter 02. Accordingly, six *c*-20:1 PIs and five *c*-22:1 PIs were synthesized successfully using the given method and used in subsequent analysis. The method explained to synthesize LC-MUFA was cost effective and appropriate to be used in large scale production of monoenoic PIs. The synthesized *c*-LC-MUFA PIs had a high purity (>97%), thus, suitable to be used in the cell culture or animal studies. Further, synthesized *c*-LC-MUFA PIs could also be used as standards for GC, HPLC, or any other analytical techniques.

The Chapter 03 reported the occurrence and distribution of *c*-20:1 PIs in fishes mainly from the Indian Ocean and compared the values using fishes from the Pacific and the Atlantic Ocean. In previous studies, some pelagic fishes and marine mammals from the Pacific Ocean showed a higher content of *c*-20:1 in fish oil. The results of the current study, revealed that the content of *c*-20:1 in the Indian Ocean fishes was lower than that of the Pacific and Atlantic Ocean fishes. Further, distribution of *c*-20:1 PIs was varied considerably among fishes from three different Oceans. In fishes from the Indian and the Atlantic Ocean, *c*11-20:1 isomer contributed to the highest proportion of total *c*-20:1, followed by *c*13-20:1. In contrast, *c*9-20:1 was the predominant isomer in fishes from the Pacific Ocean. However, *c*11-20:1 was not always predominant isomer in fishes from the Indian Ocean as some benthic living fishes contain an unusually high content of *c*7-20:1 isomer. In the Indian Ocean fish group, a high content of *c*-20:1 was reported in bottom living fish, though the pelagic fish in the Pacific Ocean and the Atlantic Ocean was

reported to have a high content of *c*-20:1. The results demonstrated that the content and distribution of *c*-20:1 PIs varied with the geographical location and habitat. The differences in the occurrence and the distribution of *c*-20:1 PIs in fishes from different oceans provided a worthy cause for studying the bioactivities of individual *c*-20:1 PIs.

Chapter 04 explains the findings of the individual effects or the isomer specific effects of naturally occurring *c*-20:1 PIs at cellular level. *In vitro* studies were conducted to examine the effect of different *c*-20:1 PIs on the factors related to obesity such as adipogenesis and lipogenesis using 3T3-L1. Results revealed that individual *c*-20:1 PIs affected differentially on the lipid accumulation, major lipid classes, cellular fatty acid composition, adipogenesis, and lipogenesis in 3T3-L1 cells. Among the tested *c*-20:1 PIs, *c*15-20:1 and *c*7-20:1 isomers noticeably reduced the cellular TG accumulation. However, individual *c*-20:1 PIs did not influence significantly on other lipid classes such total Ch, PL, and NEFA. Among the tested isomers, the *c*15-20:1 isomer showed an anti-adipogenic and anti-lipogenic effect compared to other PIs and *c*9-18:1. The reduction of cellular TG content by *c*15-20:1 was mainly due to down-regulation of transcriptional factors related to adipogenesis and lipogenesis. The abundant *c*-20:1 PIs in fish oil, i.e. *c*9-20:1 and *c*11-20:1, have shown no considerable differences either in lipid accumulation or in the expression of transcriptional factors governing adipogenesis. Results of the this study revealed that the position of the double bond in *c*-20:1 PIs influenced on their functionalities and indicated possible effects of individual *c*-20:1 PIs in relation to delaying the onset and the development of obesity. Thus, foods rich in 20:1 PIs such as fish oil, plant seeds, and nuts might be useful as functional foods for the prevention of obesity and related diseases.

The occurrence and the distribution of *c*-LC-MUFA PIs vary among plant-based and animal-based foods. The Chapter 05 explains the bioactivities of selected LC-MUFA PIs having different chain lengths, i.e. 18, 20 and 22, at cellular level. Results revealed that the individual *c*-LC-MUFA PIs showed different effects on the cellular TG content, cellular FAC, and lipid metabolism in 3T3-L cells or pre-adipocytes. It was apparent that the treatment of *c*-LC-MUFA PIs resulted changes in the FA metabolism in 3T3-L1 cells. Further, selected *c*-LC-MUFA PIs influenced differently on the transcriptional factors related to adipogenesis and lipogenesis. Among the tested *c*-LC-MUFA PIs, *c*15-20:1

showed comparatively good anti-adipogenic, anti-lipogenic effect, and improved cellular lipid metabolism. The reduction of cellular TG content by *c*15-20:1 could be suggested due to the down-regulation of transcriptional factors related to adipogenesis, lipogenesis, suppression of mRNA expression related to FAs synthesis, and upregulation of mRNA expression related to FAs oxidation. The results revealed that the double bond position and the number of C atoms in *c*-LC-MUFA influenced on their functionalities at the cellular level.

Overall results revealed that the distribution and bioactivities of individual *c*-LC-MUFA PIs particularly *c*-20:1 PIs varied considerably. The availability of the highest bioactive isomer, *c*15-20:1 was low in all analyzed fishes from the Indian, Atlantic and Pacific Ocean-fishes. Although, the presence of bioactive *c*15-20:1 even in small quantities in a mixture of *c*-20:1 PIs may exhibit synergistic effect and contribute to the functional properties of fish oil. Predominant *c*-20:1 PIs in fishes, i.e. *c*9-20:1 and *c*11-20:1, had similar bioactivities in relation to the cellular lipid metabolism. It was noticeable that favorable effects exerted by all *c*-20:1 PIs on cellular lipid metabolism than that of *c*-18:1 PIs and *c*-22:1 PIs. Thus, the content and the composition of *c*-20:1 PIs in fish ultimately contribute to positive health benefits.

The current study mainly focused on *c*-LC-MUFA PIs that reduce the cellular lipid accumulation and improve the cellular lipid metabolism. Thus, bioactivities such as anti-lipogenic, anti-adipogenic, and anti-obesity activities of *c*-LC-MUFA PIs on adipocytes were studied and reported in this dissertation. Both adipocytes and hepatocytes play a key role in the lipid metabolism and govern by number of genes and proteins. Therefore, further studies could be carried out to investigate the effect of *c*-LC-MUFA PIs on lipid metabolism in different types of cells. The maintenance of homeostasis is a collective response of whole physiological conditions. Therefore, further studies such as the effect of individual PIs on inflammatory marker and/or the expression of adipocytokines are suggested to elaborate the bioactivities of LC-MUFA PIs. It is essential to confirm the finding of cell culture studies through animal studies prior to any practical applications related to human.

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