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Gas Chromatography Results Interpretation: Absolute Amounts Versus Relative Percentages

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1. Introduction

1.1 Fatty acid functions

Impaired fatty acid metabolism has been implied in a number of diseased states such as multiple sclerosis, sudden cardiac death, insulin resistance, atherosclerosis and hypertension (Blaak, 2003; Oram & Bornfeldt, 2004; Pilz et al., 2007; Sarafidis & Bakris, 2007; Hon et al., 2009a, 2009b). The fatty acid status may be assessed in plasma, platelets, red blood cells, white blood cells and brain tissue (Zamaria, 2004, Hon et al., 2009a). However, the plasma fatty acid profile may vary considerably, whereas red blood cell membrane fatty acids are said to reflect dietary fat intake in relation to the biological half life of cells (Romon et al., 1995; Zamaria, 2004). Furthermore, red blood cell membranes, in contrast to other cells, lack the desaturase enzymes and must get their fatty acids preformed from plasma (Allen et al., 2006). Fatty acids may be saturated, monounsaturated (the n-7 and n-9 fatty acid series) or polyunsaturated (the n-3 and n-6 fatty acid series) and membrane fatty acids may further be non-esterified or esterified into glycerophospholipids (Koay & Walmsley, 1999).

1.1.1 Cell membrane fluidity – Phospholipids

Cell membrane phospholipids differ in their head-groups and hydrocarbon chains (fatty acids) (Hazel & Williams, 1990; Williams, 1998; Barenholz, 2002). Their polar head-groups can be choline, ethanolamine, serine, inositol, inositol phosphates or glycerol (Koay & Walmsley, 1999). Cell membrane phospholipids as well the type of fatty acids contained within the different phospholipid fractions are responsible for a variety of cellular functions (Manzoli, 1970; Caret et al. 1997; Williams, 1998). Cell membrane phospholipids determine membrane structure and fluidity. Phosphatidylethanolamine phospholipids are ordered-crystalline-phase lipids and can pack closely in membranes, while phosphatidylcholine phospholipids are liquid-crystalline-phase lipids, and do not pack close in the membrane

(Harlos & Eibl, 1981; Williams, 1998; Hamai et al. 2006). A combination of ordered-crystalline-phase lipids and liquid-crystalline-phase lipids are needed in cell membranes to regulate membrane fluidity.

1.1.2 Cell membrane fluidity – Fatty acids

Polyunsaturated fatty acids influence membrane fluidity, are involved with neurotransmission and prostaglandin formation, and are involved in enzyme and receptor expression (Horrobin & Manku, 1990; Horrobin, 1999; De Pablo & De Cienfuegos, 2000; Nakamura & Nara, 2004; Zamaria, 2004). Similar to the role of cell membrane phospholipids, a balance in the saturated and unsaturated fatty acids composition is needed for optimal membrane fluidity (Allen et al. 2006). Saturated fatty acids contain single carbon-carbon bonds, while mono- and polyunsaturated fatty acids contain double carbon-carbon bonds which allow for greater flexibility of these chains (Horrobin, 1999; Allen et al. 2006). Membrane fluidity is an important function of cell membranes and organisms adjust the fluidity of their cellular membranes in response to changes in their physiochemical environment (Hazel & Williams, 1990; Williams, 1998; Barenholz, 2002; Allen et al. 2006). In this regard, phagocytosis is an important mechanism in many cells for the elimination of micro-organisms or foreign particles, and membrane fluidity plays an important role in this process (De Pablo & De Cienfuegos, 2000). Although the underlying cause of multiple sclerosis remains unknown, both an auto-immune and viral aetiology has been hypothesized as contributing factors to the disease (Stinissen et al. 1997; Brown, 2001; Hunter & Hafler, 2000).

1.1.3 Fatty acids in inflammation and infection

Polyunsaturated fatty acids are also precursors for eicosanoid production. Eiosanoids are inflammatory mediators of inflammation and their functions depend on each precursor fatty acid (Horrobin & Manku, 1990; De Pablo & De Cienfuegos, 2000; Zamaria, 2004). The effects of monounsaturated fatty acids on eicosanoid metabolism are less clear than that of the polyunsaturated fatty acids (Harwood & Yaqoob, 2002). However, studies suggest that diets rich in monounsaturated fatty acids may decrease the expression of adhesion molecules on peripheral blood mononuclear cell membranes, and may therefore have specific anti-inflammatory effects (Yaqoob et al. 1998; Harwood & Yaqoob, 2002). Saturated fatty acids have been shown to display anti-viral and -bacterial properties (Sands, 1977; Cordo et al. 1999; Narasimhan et al. 2006).

1.1.4 Fatty acid supplementation

It is important to be able to separate and quantify the individual fatty acids because they differ in structure and function. Altered lipid metabolism in the cell membrane is believed to contribute to central nervous system injury (Adibhatla & Hatcher, 2007) and influences the function of immune cells (Calder, 2007). Fatty acids have been implicated in the pathogenesis and treatment of multiple sclerosis (Zamaria, 2004; Harbige & Sharief, 2007). However, although some studies reported an improvement in symptoms during fatty acid supplementation (Bates et al. 1978; Nordvik et al. 2000; Weinstock-Guttman et al. 2005; Harbige & Sharief, 2007) others found no improvement in the disease progression of patients (Paty et al. 1978; Farinotti et al. 2007). Therefore, it is important to investigate fatty

acid metabolic abnormalities and possible treatment with fatty acid supplements in a disease such as multiple sclerosis carefully and comprehensively.

1.2 Gas chromatography applications of fatty acids

The ability of gas chromatography to separate and quantify fatty acids makes it possible to measure fatty acids in human cells. Generally, the results obtained from gas chromatography analysis are expressed either as absolute amounts or relative percentages. The preferred method for the quantification of fatty acids is in absolute amounts, using calibration methods and internal standards. However, quantification in relative percentages may in some cases supply additional information and this method should therefore not be rejected without evaluating its potential. This paper discusses the discrepancies arising when reporting results quantified in either absolute amounts or relative percentages when investigating the fatty acid profile in a diseased state.

1.3 Fatty acids quantified in relative percentages and in absolute amounts

It is important to know whether fatty acids are present in optimum amounts in relation to one another in a diseased state. For example, fatty acids from the n-6 and n-3 fatty acid series fulfil different functions as precursors for pro- and anti-inflammatory mediators respectively. Prostaglandin E2, which is derived from the metabolism of C20:4n-6 (see Table 1 for common names) is highly pro-inflammatory and prostaglandin E1 derived from the metabolism of C20:3n-6 has intermediate properties (Horrobin & Manku, 1990; Simopoulos, 2002; Bagga et al., 2003; Haag, 2003). In contrast, prostaglandin E3 derived from the metabolism of C20:5n-3 and docosanoids derived from the metabolism of C22:6n-3 have anti-inflammatory properties (Bagga et al., 2003; Haag, 2003; Zamaria, 2004; Farooqui et al., 2007; Chen et al., 2008). The n-3 fatty acids, C20:5n-3 and C22:6n-3 compete with the n-6 fatty acid, C20:4n-6 for enzymatic metabolism (Simopoulos, 2002; Bagga et al., 2003; Culp et al., 1979; Calder, 2007) and in a disease such as multiple sclerosis which is inflammatory in nature, it could be useful to know whether the n-6 and n-3 fatty acids are present in relative percentages comparable to that from control subjects.

The long-chain n-6 and n-3 polyunsaturated fatty acids are also required in large amounts in the brain, which requires four times the amount of C20:4n-6 than C22:6n-3 4 times the amount of C20:4n-6 than C22:6n-3 on a daily basis (Harbige & Sharief, 2007; Rapoport et al., 2007). Furthermore, the composition of membrane phospholipids and their fatty acids determines the degree of membrane fluidity and the degree of fatty acid unsaturation plays an important role in maintaining required physiological levels of fluidity (Mouritsen & Jorgensen, 1998; Zamaria, 2004). In a diseased state it may be useful to know their relative percentages as measured against that of total fatty acids present as compared to that of control subjects. However, using relative percentages as a measure could bias results when comparisons are made between subjects. Comparing similar percentages of different absolute values could become misleading when these absolute values differ substantially and it has been suggested that relative percentages do not represent a true reflection of the different fatty acid compounds present in biological fluids/tissue. Absolute values are also an indication of the movement of fatty acids between phospholipid classes within the cell membrane and could therefore reflect their possible availability for metabolite synthesis, particularly inflammatory signalling.

1.4 Fatty acids in multiple sclerosis

Reports on fatty acid composition in blood and brain tissue from patients with multiple sclerosis may vary substantially (Harbige & Sharief, 2007; Hon et al., 2009a, 2009b). Cultural and ethnic differences as well as dietary variability have been implicated in these differences (Harbige & Sharief, 2007). However, a further cause for these differences could possibly be related to the quantification method used. Measurement of the fatty acid composition in absolute amounts and relative percentages could both be informative, but could well differ in the information given. The absolute amounts of fatty acids are identified in relation to the phospholipids and membrane content whereas the relative percentage composition is a measure of each fatty acid present in relation to the total fatty acids identified, that is changes in one fatty acid will affect the percentage of all other fatty acids. Therefore, ideally, fatty acid analysis and interpretation should be evaluated according to outcome required. In this chapter we evaluate whether the two methods of measurement of fatty acids in control subjects would show sufficient agreement and to evaluate possible deviations from these relationships in patients with multiple sclerosis. Furthermore, to evaluate the two methods of measurement when correlated/associated with inflammation as measured by C-reactive protein as well as disease outcome as measured by the Kurtzke Expanded Disability Status Scale (Kurtzke, 1983). C-reactive protein is a recognised marker of inflammation and has been shown to correlate with infectious episodes and clinical relapses in patients with multiple sclerosis (Giovannoni et al., 1996; Giovannoni et al., 2001; Sellner et al., 2008). Thin-layer chromatography and gas chromatography were used for the extraction and separation of lipid classes and quantification of fatty acids in plasma, red blood cell and peripheral blood mononuclear cell membranes from control subjects and patients with multiple sclerosis (Folch et al., 1957; Gilfillan et al., 1983; Van Jaarsveld et al., 2000; Hon et al., 2009b; Hon et al., 2011a).

2. Methodology

2.1 Ethics statement

Ethics approval for the study was obtained from the Health and Applied Sciences Research Ethics Committee of the Cape Peninsula University of Technology. Patients with multiple sclerosis were contacted and recruited through the Multiple Sclerosis Society, Western Cape Branch, South Africa. The study population consisted of 31 female patients with multiple sclerosis and 30 age- race- and gender-matched control subjects. Informed written consent was obtained from all study participants. All results were treated confidentially.

2.2 Study population

The diagnoses of the recruited patients were verified by a neurologist based on clinical, laboratory and magnetic resonance imaging findings. Six of the patients had active disease, 11 had a relapse 5-12 months prior to recruitment and 14 did not relapse for more than a year. The number of years since the patients were diagnosed was 7 (11) years (median and quartile range). Ten patients were using non-steroidal anti-inflammatory drugs and five patients were using immunosuppressive medication. Exclusion criteria included the use of interferon, steroids, and fatty acid supplements.

2.3 Blood sampling and processing

Venous blood was collected after an overnight fast into anti-coagulant ethylenediaminetetraacetic acid tubes (Beckman Coulter, Cape Town, South Africa). Blood was separated into its different components using histopaque-1077 separation medium as per manufacturer's instructions (Sigma-Aldrich, Cape Town, South Africa). Blood was layered onto histopague in a ratio of 15 mL blood per 12 mL histopague and centrifuged at 400 x g for 20 min at room temperature. The plasma layer was kept, spun twice at 1500 x g for 5 min to remove platelet contaminants, and frozen in 1 mL aliquots. The peripheral blood mononuclear cell interface was recovered, washed twice with 0.85 % saline solution, resuspended in 1 mL of a 0.85 % saline solution and frozen. Three mL red blood cells were washed twice with 0.85 % saline solution and frozen as packed cells without added saline. The samples were frozen at -80°C immediately after separation of blood fractions. A 0.85 % saline solution was used in this study instead of the prescribed balanced phosphate buffered saline solution as additional tests such as membrane phosphate determination were to be carried out at a later stage.

Solvent mixtures and thin-layer chromatography was used for the extraction and separation of fatty acids respectively, with further conversion to fatty acid methyl esters for quantification by gas chromatography as described by Folch et al. (1957), Gilfillan et al. (1983), Van Jaarsveld et al. (2000), Hon et al. (2009b), Hon et al. (2011a). The following sections summarises the methodology used for quantification of the fatty acids in human tissue, plasma, red blood cell and peripheral blood mononuclear cell membranes.

2.4 Thin – Layer chromatography

The plasma, red blood cell and peripheral blood mononuclear cell membrane lipids were extracted according to a modified method of Folch et al. (1957), Van Jaarsveld et al. (2000), and described in Hon et al. (2009b), Hon et al. (2011a). All the extraction solvents were of high-performance liquid chromatography-grade and contained 0.01 % butylated hydroxytoluene (Sigma-Aldrich, Cape Town, South Africa) as an antioxidant. The red blood cell samples were extracted and the dried lipid residue resuspended in 80 µl chloroform/methanol (ratio 2:1 v/v) for thin-layer chromatography. Of this 20 µl was used for esterified fatty acid analysis and 40 µl for non-esterified fatty acid separation on thin-layer chromatography. The peripheral blood mononuclear cell samples were resuspended in 70 µl chloroform/methanol; 20 µl was used for esterified fatty acid analysis and 30 µl for non-esterified fatty acid analysis. Plasma samples were resuspended in 80 µl chloroform/methanol of which 20 µl was used for esterified fatty acid analysis and 30 µl for non-esterified fatty acid analysis.

Neutral lipids (non-esterified fatty acids, mono- di- and triacylglyceryl and cholesteryl fatty acid esters) were separated from the total phospholipid fraction by thin-layer chromatography on pre-coated silica gel plates (10 x 10 cm), using the solvent system petroleum benzene (boiling point 40-60°C)/diethyl ether (peroxide free)/acetic acid (90:30:1; v/v/v) (Sigma-Aldrich, Cape Town, South Africa) as previously described (Van Jaarsveld et al., 2000). Individual phospholipid classes were separated by thin-layer chromatography on pre-coated silica gel plates (10 x 10 cm) using chloroform/petroleum benzene/methanol/acetic acid/boric acid (40:30:20:10:1.8; v/v/v/v/w) as solvent (Gilfillan et al., 1983).

The lipid bands containing phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM) were identified by comparison to a known mixed phospholipid standard run in parallel to the samples and visualized with long wave ultraviolet light after spraying the plates with chloroform/methanol (ratio 1:1; v/v) containing 10 mg per 100 mL 2,5-bis-(5´-tert-butylbenzoxazolyl-[2´])thiophene (Sigma Chemical Company, Cape Town, South Africa). These bands were scraped off the thin-layer chromatography plates and used for further fatty acid analysis.

Fatty acids	
Abbreviated chemical formulae	**Common name**
C18:3n-3	Alpha-linolenic acid
C18:4n-3	Stearidonic acid
C20:4n-3	Eicosatetraenoic
C20:5n-3	Eicosapentaenoic acid
C22:5n-3	Docosapentaenoic acid
C22:6n-3	Docosahexaenoic acid
C18:2n-6	Linoleic acid
C18:3n-6	Gamma-linolenic acid
C20:2n-6	Eicosadienoic acid
C20:3n-6	Dihomo-gamma-linolenic acid
C20:4n-6	Arachidonic acid
C22:4n-6	Adrenic acid
C22:5n-6	Docosapentaenoic acid
C16:1n-7	Palmitoleic acid
C18:1n-7	Vaccenic acid
C18:1n-9	Oleic acid
C20:1n-9	Gadoleic acid
C14:0	Myristic acid
C16:0	Palmitic acid
C18:0	Stearic acid
C20:0	Arachidic acid
C22:0	Behenic acid
C24:0	Lignoceric acid

Table 1. Abbreviated chemical formulae and common names of major plasma and blood cell membrane fatty acids. References: Nightingale et al., 1990; Horrobin, 1999; Pereira et al., 2003.

2.5 Gas chromatography

The lipid fractions scraped off the thin-layer chromatography plates were transmethylated, using 5 % sulphuric acid/methanol at 70°C for 2 hours (sphingomyelin fraction: 18 hours) (Van Jaarsveld et al., 2000; Hon et al., 2009b; Hon et al., 2011a). After cooling, the resulting fatty acid methyl esters were extracted with 1 mL of distilled water and 2 mL of n-hexane. The top hexane layer was removed and evaporated to dryness in a waterbath at 37°C under nitrogen gas, re-dissolved in carbon disulphide and analyzed by gas chromatography

(Finnigan Focus Gas Chromatography, Thermo Electron Corporation, USA, equipped with flame ionization detection), using a 30 meter BPX 70 capillary columns of 0.32 mm internal diameter (SGE International Pty Ltd, Australia). Gas flow rates were as follows: nitrogen, 25 mL per min; air, 250 mL per min; and hydrogen (carrier gas), 25 mL per min and a split ratio of 20:1. Temperature programming was linear at 5°C per min, with initial temperature of 140°C, final temperature 220°C, injector temperature 240°C, and detector temperature 250°C. The fatty acid methyl esters were identified by comparison of the retention times to those of a standard fatty acid methyl ester mixture (Nu-Chek-Prep Inc., Elysian, Minnesota). The individual fatty acid methyl esters were quantified against an internal standard with known concentration (C17:0, Sigma-Aldrich, South Africa). Plasma fatty acids were quantified in absolute values in µg fatty acids per mL plasma analyzed. Red blood cell membrane fatty acids were quantified in absolute values in µg fatty acid per mL packed red blood cells analyzed. Peripheral blood mononuclear cell membrane protein was measured and fatty acids were quantified against membrane protein present in µg fatty acids per mg protein (see protein assay below). All individual fatty acids were also reported as relative percentage of the total fatty acids identified.

2.6 Protein analysis

The bicinchoninic acid protein determination assay was used to determine the protein content of peripheral blood mononuclear cell membranes (Kaushal & Barnes, 1986) for quantification of membrane fatty acids. Peripheral blood mononuclear cell membrane lipids were quantified against membrane protein present because of the high variation normally found in white blood cell counts. Of the starting material prepared in saline, a 200 µl sample aliquot was diluted with 2 % sodium dodecyl sulphate solution (Fluka, Sigma-Aldrich, Cape Town, South Africa) to denaturate protein prior to protein determination and assayed for protein content in triplicate. Bovine serum albumin (Sigma-Aldrich, Cape Town, South Africa) was used to prepare a standard curve. Sample and standard optical density was read on a spectrophotometer at a wavelength of 562 nm. Sample values were obtained from the bovine serum albumin linear standard curve. Peripheral blood mononuclear cell membrane fatty acids were quantified against membrane proteins and expressed in µg fatty acids per mg protein.

2.7 Phospholipid determination

Red blood cell and peripheral blood mononuclear cell membrane phospholipids were determined using a colorimetric assay with malachite green dye, as previously described (Itaya & Ui, 1966; Smuts et al., 1994). Phospholipid classes, identified and recovered as described above, were reduced to inorganic phosphates and quantified according to their phosphorous (Pi) content.

The different factors used for conversion of phosphorous to phospholipids were: for phosphatidylcholine: 25.4; phosphatidylethanolamine: 23.22; phosphatidylserine: 25.4; and sphingomyelin: 24.21. Red blood cell membrane phospholipid quantification was expressed in µg phospholipid per mL packed cells and peripheral blood mononuclear cell membrane phospholipid in µg phospholipid per mg protein (see protein assay).

2.8 C-reactive protein determination

Plasma C-reactive protein concentrations were determined in a routine Chemical Pathology laboratory using a Beckman nephelometer auto-analyser using reagents from Beckman, Cape Town, South Africa. A positive diagnostic value is considered as a C-reactive protein value equal to or greater than 5 μg per mL plasma.

2.9 Statistical analysis

A statistics programme, STATISTICA 9 {StatSoft, Inc. (2009). STATISTICA} was used to perform all statistical analyses. Correlation studies using Spearman's Rank correlation coefficient were used to evaluate the measure of agreement between the two methods (absolute amounts and relative percentages) in the plasma, red blood cell and peripheral blood mononuclear cell membranes from control subjects and patients with multiple sclerosis (Table 2). Linear regression analyses were used to measure strength of association between fatty acids quantified in absolute amounts and relative percentages, and C-reactive protein as well as the Kurtzke Expanded Disability Status Scale (Tables 3 and 4). The results were considered significant if P-values were less than 0.05.

3. Results

3.1 Method of measurement comparison

correlation studies between fatty acids quantified in absolute amounts and relative percentages are summarized in Table 2, Figure 1 and Results: sections 3.1.1, 3.1.2 and 3.1.3: Overall, the absolute amounts and relative percentage fatty acid composition results were moderately and mostly positively associated with each other in both study groups, although a number of fatty acids correlated not significantly in both groups (data not shown). However, it should be noted that though these correlations were significant, they were not strong as most of the R values were less than 0.5. Differences in the strength of association between the two study groups are listed below.

3.1.1 Plasma fatty acids

SM C20:0 showed significant correlations in controls only, and non-esterified fatty acid C18:2n-6 and PC C16:0 showed significance in patients only. Further, SM C18:0 showed weaker correlations in plasma from patients than in controls. Non-esterified fatty acids C20:4n-6, C16:0 and C18:0 showed no correlation in either of the two study groups.

3.1.2 Red blood cell membrane fatty acids

PS C22:4n-6, SM C16:0 and PS C18:0 showed significant correlations in controls only and PS C20:4n-6, PS C22:6n-3, PC C18:1n-9 and non-esterified fatty acid C22:0 were significantly associated in patients only. PE C18:0 showed a stronger association between absolute amounts and relative percentage fatty acids in red blood cells from patients with multiple sclerosis than in control subjects. Furthermore, PE C20:4n-6, PC C16:0, PE C16:0, PC C18:0, PE C18:0 and non-esterified fatty acid C16:0 showed no correlation in either of the two study groups.

	Total group		Controls		Patients	
	R	**P-value**	**R**	**P-value**	**R**	**P-value**
Plasma fatty acids						
Polyunsaturated fatty acids						
NEFA C18:2n-6	**0.42**	**0.0008**	0.31	0.0961	**0.48**	**0.0060**
Saturated fatty acids						
PC C16:0	**0.33**	**0.0095**	0.35	0.0601	**0.36**	**0.0469**
SM C18:0	**0.64**	**< 0.0001**	**0.80**	**< 0.0001**	**0.50**	**0.0039**
SM C20:0	**0.49**	**0.0001**	0.65	**0.0001**	0.34	0.0644
Red blood cell membrane fatty acids						
Polyunsaturated fatty acids						
PE C20:4n-6	**0.36**	**0.0041**	0.35	0.0583	0.28	0.1245
PS C20:4n-6	**0.40**	**0.0012**	0.17	0.3699	**0.50**	**0.0043**
PS C22:4n-6	**0.53**	**< 0.0001**	**0.74**	**< 0.0001**	0.18	0.3413
PS C22:6n-3	**0.45**	**0.0002**	0.31	0.0951	**0.57**	**0.0008**
Monounsaturated fatty acids						
PC C18:1n-9	**0.40**	**0.0012**	0.31	0.0992	**0.47**	**0.0072**
Saturated fatty acids						
NEFA C22:0	**0.41**	**0.0012**	0.27	0.1528	**0.45**	**0.0113**
PC C18:0	**0.23**	0.0737	0.33	0.0780	0.18	0.3363
PE C16:0	**0.32**	**0.0118**	0.34	0.0622	0.27	0.1373
PE C18:0	**0.34**	**0.0075**	0.30	0.1099	0.35	0.0518
PS C18:0	**-0.34**	**0.0079**	**-0.37**	**0.0461**	-0.26	0.1557
SM C16:0	**0.41**	**0.0011**	**0.58**	**0.0009**	0.36	0.0509
Peripheral blood mononuclear cell membrane fatty acids						
Polyunsaturated fatty acids						
PC C18:2n-6	**0.65**	**< 0.0001**	0.32	0.1156	**0.81**	**< 0.0001**
PE C20:4n-6	**0.56**	**< 0.0001**	0.38	0.0599	**0.69**	**0.0001**
Monounsaturated fatty acids						
NEFA C18:1n-9	**0.69**	**< 0.0001**	**0.89**	**< 0.0000**	**0.39**	**0.0463**
PC C18:1n-9	**0.62**	**< 0.0001**	**0.42**	**0.0351**	**0.78**	**< 0.0001**
Saturated fatty acids						
NEFA C18:0	-0.10	0.4700	**-0.43**	**0.0337**	0.08	0.6903
PE C18:0	**0.39**	**0.0051**	**0.49**	**0.0121**	0.31	0.13153
PS C18:0	**0.38**	**0.0064**	**0.40**	**0.0448**	0.34	0.0924
SM C16:0	**0.51**	**0.0001**	0.24	0.2432	**0.69**	**0.0001**
SM C18:0	**0.51**	**0.0002**	0.22	0.3168	**0.74**	**< 0.0001**

Table 2. Method of measurement comparison: correlation studies between fatty acids quantified in absolute amounts and relative percentages. Key: NEFAs: Non-esterified fatty acids, PC: Phosphatidylcholine, SM: Sphingomyelin, PE: Phosphatidylethanolamine, PS: Phosphatidylserine.

3.1.3 Peripheral blood mononuclear cell membrane fatty acids

PE C18:0, PS C18:0 and non-esterified fatty acid C18:0 showed significant correlations in controls only and PC C18:2n-6, PE C20:4n-6, SM C16:0 and SM C18:0 were significant in patients only. PC C18:1n-9 showed stronger correlations between absolute amounts and relative percentage fatty acids in peripheral blood mononuclear cells from patients with multiple sclerosis, while non-esterified fatty acid C18:1n-9 showed stronger correlations in controls.

3.2 Differences between plasma, red blood cell and peripheral blood mononuclear cell membrane fatty acids from control subjects and patients with multiple sclerosis

Measures of central tendencies have been published previously. Polyunsaturated fatty acids measured in plasma, red blood cell and peripheral blood mononuclear cell membranes from control subjects and patients with multiple sclerosis showed similar results when measured in absolute values or relative percentages. They include the following: PC C20:4n-6 quantified in absolute values (Hon et al., 2011a) and relative percentages (data not shown) was decreased in plasma from patients. PC C20:4n-6 quantified in absolute values (Hon et al., 2009a) and relative percentages (data not shown) was decreased in red blood cell membranes from patients. PE C22:4n-6 and PS C22:4n-6 quantified in absolute values as well as relative percentages were decreased in peripheral blood mononuclear cell membranes from patients with multiple sclerosis (Hon et al., 2009b).

3.3 Association studies between C-reactive protein and PC fatty acids are summarized in Table 3

3.3.1 Plasma fatty acids

C-reactive protein showed no association with any of the fatty acids quantified in absolute amounts in the PC phospholipid fraction in plasma from controls, but a positive association with PC C20:3n-6 in plasma from patients. It showed a weak association with PC C20:3n-6 quantified in relative percentages in plasma from controls and a strong association in plasma from patients similar to that found with the fatty acid quantified in absolute amounts. It further showed a positive association with PC C16:1n-7 quantified in relative percentages in plasma from controls and an inverse association with PC C18:1n-9 in plasma from patients.

3.3.2 Red blood cell membrane fatty acids

The C-reactive protein showed a positive association with PC C16:1n-7 (Hon et al., 2010) quantified in absolute amounts in the red blood cells from controls and a positive association with PC C20:3n-6 in red blood cells from patients. Similar to results for that of plasma PC fatty acids, it showed a weak association with PC C20:3n-6 quantified in relative percentages in red blood cells from controls and a strong association in red blood cells from patients. Also, similar to results for that of plasma PC fatty acids, the C-reactive protein showed a positive association with PC C16:1n-7 quantified in relative percentages in red blood cells from controls and an inverse association with PC C18:1n-9 in red blood cells from patients, but this inverse correlation was also found in red blood cells from controls (Hon et al., 2010).

	Controls				Patients			
	Absolute amounts		Percentages		Absolute amounts		Percentages	
	b*	P-value	b*	P-value	b*	P-value	b*	P-value
Plasma fatty acids								
Polyunsaturated fatty acids								
PC C20:3n-6	0.30	0.1242	0.33	0.0973	**0.39**	**0.0378**	**0.49**	**0.0071**
Monounsaturated fatty acids								
PC C16:1n-7	0.32	0.1152	**0.40**	**0.0444**	0.15	0.4485	0.12	0.5416
PC C18:1n-9	-0.01	0.9653	-0.12	0.5585	-0.16	0.4044	**-0.46**	**0.0126**
Saturated fatty acids								
PC C18:0	-0.01	0.9570	-0.19	0.3390	0.33	0.0840	0.21	0.2694
Red blood cell membrane fatty acids								
Polyunsaturated fatty acids								
PC C20:3n-6	0.20	0.3059	0.36	0.0625	0.34	0.0679	**0.52**	**0.0041**
PC C22:4n-6	0.18	0.3826	0.37	0.0601	-0.26	0.1759	-0.24	0.2113
Monounsaturated fatty acids								
PC C16:1n-7	**+ Association***		**0.39**	**0.0468**	No association*		0.14	0.4788
PC C18:1n-9	No association*		-0.34	0.0817	No association*		**-0.41**	**0.0264**
Peripheral blood mononuclear cell membrane fatty acids								
Polyunsaturated fatty acids								
PC C20:3n-6	0.22	0.3255	0.24	0.2818	0.16	0.4482	0.37	0.0714
PC C22:4n-6	0.27	0.2262	0.38	0.0773	-0.14	0.5093	0.07	0.7534
PC C22:5n-6	**0.45**	**0.0358**	**0.46**	**0.0326**	0.07	0.7629	0.23	0.2806
PC C20:5n-3	-0.05	0.8133	-0.06	0.7823	-0.36	0.0842	-0.36	0.0944
Monounsaturated fatty acids								
PC C16:1n-7	**+ Association***		**0.54**	**0.0095**	No association*		0.12	0.5970
PC C18:1n-7	**+ Association***		0.42	0.0537	No association*		**0.46**	**0.0230**
PC C18:1n-9	No association*		**-0.45**	**0.0353**	No association*		-0.02	0.9398
Saturated fatty acids								
PC C14:0	0.30	0.1772	0.29	0.1953	-0.34	0.0992	-0.25	0.2426
PC C18:0	0.04	0.8744	-0.03	0.8887	**-0.47**	**0.0204**	-0.24	0.2694
PC C20:0	-0.07	0.7563	-0.14	0.5429	-0.36	0.0866	-0.14	0.5003
PC C22:0	-0.39	0.0762	-0.40	0.0680	-0.16	0.4487	0.04	0.8700

Table 3. Association studies between C-reactive protein and PC fatty acids in plasma, red blood and peripheral blood mononuclear cell membranes. * Results: Hon et al. (2010)

3.3.3 Peripheral blood mononuclear cell membrane fatty acids

The C-reactive protein showed a weak association with PC C20:3n-6 quantified in relative percentages in peripheral blood mononuclear cell membranes from patients. It showed positive associations with PC C22:5n-6, PC C16:1n-7 (Hon et al., 2010) and PC C18:1n-7 (Hon et al., 2010) quantified in both absolute amounts and relative percentages in peripheral blood mononuclear cell membranes from controls, while only PC C18:1n-7 quantified in

relative percentages showed a positive association in peripheral blood mononuclear cell membranes from patients. It also showed an inverse association with PC C18:1n-9 quantified in relative percentages in peripheral blood mononuclear cell membranes from controls, in contrast to results in plasma and red blood cells where this association was found in patients.

3.4 Association studies between the Kurtzke Expanded Disability Status Scale and fatty acids from patients with multiple sclerosis are summarized in Table 4

3.4.1 Plasma fatty acids

The Kurtzke Expanded Disability Status Scale showed near-significant inverse associations with non-esterified fatty acid C20:4n-6 and SM C20:0 quantified in relative percentages, but not with absolute amounts in plasma from patients. It also showed a significant inverse correlation with non-esterified fatty acid C14:0 quantified in relative percentages, but a near-significant positive association with non-esterified fatty acid C14:0 quantified in absolute amounts.

	Patients with multiple sclerosis				
	Absolute amounts		**Relative percentages**		
	b*	**P-value**	**b***	**P-value**	**R^2**
Plasma fatty acids					
Polyunsaturated fatty acids					
NEFA C20:4n-6	-0.04	0.8188	-0.34	0.0716	0.05
Saturated fatty acids					
NEFA C14:0	0.42	0.0550	**-0.48**	**0.0307**	0.11
NEFA C16:0	0.26	0.1532	-0.31	0.0869	0.08
SM C20:0	-0.01	0.9683	-0.34	0.0828	0.05
Red blood cell membrane fatty acids					
Polyunsaturated fatty acids					
PC C20:2n-6	-0.42	0.2154	**0.72**	**0.0397**	0.12
PE C20:3n-6	-0.30	0.2806	**0.61**	**0.0322**	0.12
PS C22:5n-3	-0.06	0.7511	**-0.41**	**0.0320**	0.14
Saturated fatty acids					
PC C16:0	-0.31	0.0771	-0.31	0.077	0.13
PC C18:0	-0.13	0.4928	**0.41**	**0.0372**	0.09
PE C16:0	-0.06	0.7650	0.34	0.0835	0.04
Peripheral blood mononuclear cell membrane fatty acids					
Polyunsaturated fatty acids					
PE C20:5n-3	0.95	0.1296	-1.10	0.0791	0.06
Monounsaturated fatty acids					
PC C24:1n-9	2.14	0.0708	-1.99	0.0927	0.07

Table 4. Association studies between the Kurtzke Expanded Disability Status Scale and absolute amounts and relative percentage fatty acids in plasma, red blood cell and peripheral blood mononuclear cell membranes from patients with multiple sclerosis

3.4.2 Red blood cell membrane fatty acids

The Kurtzke Expanded Disability Status Scale showed significant positive associations with PC C20:2n-6, PE C20:3n-6 and PC C18:0 as well as a near-significant positive association with PE C16:0 quantified in relative percentages, but not with absolute amounts in red blood cell membranes from patients. In contrast, it showed a significant inverse correlation with PS C22:5n-3 quantified in relative percentages in red blood cell membranes from patients.

3.4.3 Peripheral blood mononuclear cell membrane fatty acids

The Kurtzke Expanded Disability Status Scale showed no significant associations with the fatty acids in peripheral blood mononuclear cell membranes from patients, but a near-significant inverse correlation with PE C20:5n-3 quantified in relative percentages.

4. Discussion

The objective was to establish whether using absolute amounts in quantifying fatty acids in biological tissue would provide sufficient information to investigate abnormalities in a diseased state, in this case investigating the fatty acid profile in patients with multiple sclerosis. Results showed that correlation strengths between fatty acids quantified in absolute amounts and relative percentages varied in some instances in plasma, red blood cell as well as peripheral blood mononuclear cell membranes between study groups (Table 2). Furthermore, even when a correlation was observed between absolute amounts and relative percentages the R value was not always close to the value one as would have been expected if these theoretically represent each other. Of note is that some of these correlations were lost or gained in diseased subjects. For example, PC C18:1n-9 showed stronger correlations in peripheral blood mononuclear cells from patients, than in control subjects. In contrast, PS C22:4n-6 showed significance in red blood cell membranes from controls, but not in patients with multiple sclerosis (Figure 1).

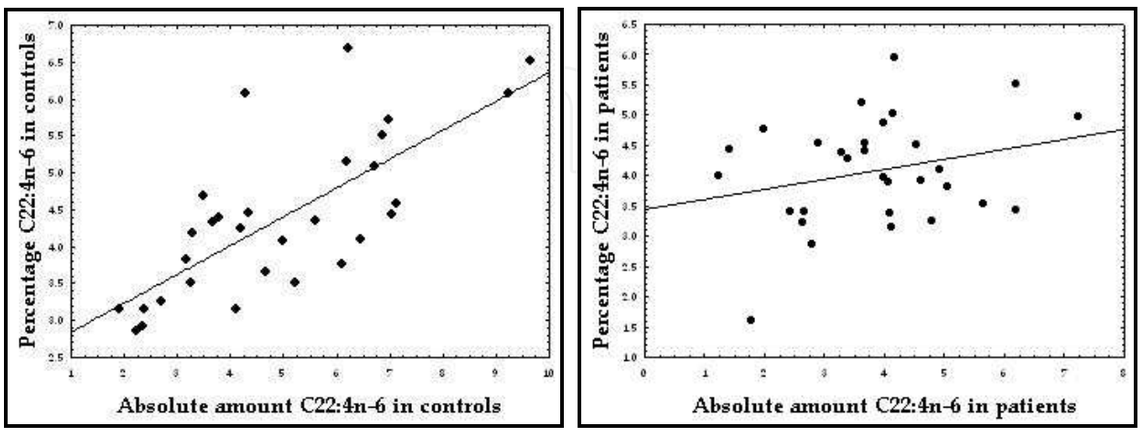

Fig. 1. Measurement comparison of PS C22:4n-6 quantified in absolute amounts and relative percentages in red blood cell membranes. Controls, R = 0.74; P = < 0.0000. In patients with multiple sclerosis the correlation was not significant, R = 0.18; P = 0.3413.

Multiple sclerosis is an inflammatory disease of the central nervous system in which an abnormal fatty acid profile has been reported, but with inconclusive findings (Cherayil, 1984; Navarro & Segura, 1989; Harbige & Sharief, 2007; Hon et al., 2009a, 2009b). The n-6 and n-3 polyunsaturated fatty acids, C18:2n-6, C20:4n-6 and C20:5n-3 have been reported to be decreased in plasma and blood cell membranes from these patients (Baker et al., 1964; Cherayil, 1984; Holman et al., 1989; Nightingale et al., 1990; Hon et al., 2009a, 2009b), but other research groups have found no differences compared to control subjects (Cumings et al., 1965; Fisher et al., 1987; Evans & Dodd, 1989; Koch et al., 2006). Disturbed metabolic relationships between C18:2n-6 and C20:3n-6 (Harbige & Sharief, 2007), as well as between C20:3n-6 and C20:4n-6 (Harbige & Sharief, 2007; Hon et al., 2009b) in the peripheral blood mononuclear cell membranes from patients with multiple sclerosis have also been reported. Homa et al. (1980) showed the relationship between C18:2n-6 and C20:4n-6 in red blood cells from patients to be disturbed, while Hon et al. (2009a) reported disturbances between PC C18:2n-6 and PC C20:3n-6, PC C18:2n-6 and PC C20:4n-6, PC C20:3n-6 and PC C20:4n-6 in red blood cells from patients. The lack of consensus on the role of fatty acids in the pathogenesis of multiple sclerosis, may be due to the variable results observed in previous studies which may be attributed to the different forms of assessments used (absolute amounts and relative percentages).

4.1 Quantification of polyunsaturated fatty acid C20: 4n-6

The n-6 polyunsaturated fatty acid PC C20:4n-6 (data not shown) showed significant correlations between absolute amounts and relative percentage composition in plasma from both the control group and patients with multiple sclerosis, but showed a wide variation in red blood cell membranes. PE C20:4n-6 showed a weak correlation in controls only, while PS C20:4n-6 in contrast showed a strong correlation in red blood cells from patients. PE C20:4n-6 showed a strong correlation in peripheral blood mononuclear cell membranes from patients and a weak correlation in controls. In contrast to the differences in correlations between absolute amounts and relative percentage composition of C20:4n-6 in red blood cell and peripheral blood mononuclear cell membranes from patients with that of the control group, the n-3 polyunsaturated fatty acids in general showed similar strong associations between absolute amounts and relative percentage in plasma, red blood cell and peripheral blood mononuclear cell membranes from controls and patients (Table 2). These results have demonstrated that in control subjects there is a wide range of association strengths between the n-6 fatty acids quantified in absolute amounts and relative percentages with these associations being further disturbed in patients with multiple sclerosis. On the other hand, the n-3 polyunsaturated fatty acids showed close associations between absolute amounts and relative percentages and that this was maintained in patients with multiple sclerosis.

It is unclear why the n-6 polyunsaturated fatty acid C20:4n-6 should show such a wide variation in association between absolute amounts and relative percentages (from very weak to strong) in plasma, red blood cell and peripheral blood mononuclear cell membranes from controls. Furthermore, it is unclear why C20:4n-6 should have stronger associations between absolute amounts and relative percentages specifically in peripheral blood mononuclear cell membranes from patients, unless the reversal of association strength in these immune cells are part of the pathogenesis of multiple sclerosis. C20:4n-6 is released from cell

membranes during inflammation as precursor for pro-inflammatory prostaglandin E2 production (Horrobin & Manku, 1990; Simopoulos, 2002; Bagga et al., 2003; Haag, 2003), while its precursor fatty acid in the n-6 fatty acid series, C20:3n-6 is the precursor fatty acid for the synthesis of prostaglandin E1, a less inflammatory mediator of inflammation, than prostaglandin E2. In contrast, prostaglandin E3 derived from the metabolism of C20:5n-3 and docosanoids derived from the metabolism of C22:6n-3 has anti-inflammatory properties (Bagga et al., 2003; Haag, 2003; Zamaria, 2004; Farooqui et al., 2007; Chen et al., 2008). In a disease such as multiple sclerosis, which is inflammatory in nature, it is useful to know whether the n-6 and n-3 polyunsaturated fatty acids show any metabolic abnormalities and these results suggested abnormalities within the n-6 polyunsaturated fatty acid series rather than in the n-3 polyunsaturated fatty acids.

Results from previous studies (Hon et al., 2009a, 2009b; Hon et al., 2011a) and this study showed similar decreases in polyunsaturated fatty acid C20:4n-6 and its elongation product C22:4n-6 when quantified in absolute amounts or relative percentages in plasma, red blood cell and/or peripheral blood mononuclear cell membranes from patients with multiple sclerosis as compared to controls. Therefore, investigating measures of central tendencies in fatty acids from control subjects and patients with multiple sclerosis using both quantifications methods does not explain the differences in reports of C20:4n-6 composition in patients with multiple sclerosis as compared to control subjects. However, the variations in association strengths between C20:4n-6 quantified in absolute amounts and relative percentages in patients compared to control subjects (Table 2) showed that other factors must contribute to the change in association. In this regard, the C-reactive protein has been shown to correlate inversely with PE C20:4n-6 and PE C22:4n-6 quantified in absolute values in red blood cell membranes from patients with multiple sclerosis (Hon et al., 2009a) and also inversely with PC C20:4n-6, PS C20:4n-6, PS C22:4n-6 and PI C22:4n-6 quantified in absolute values in peripheral blood mononuclear cell membranes from patients with multiple sclerosis (Hon et al., 2009b). It is possible therefore to expect C-reactive protein concentrations to show associations with blood fatty acids depending on the inflammatory status of the patients.

4.2 Association between C-reactive protein and polyunsaturated fatty acids

In this study, results (Table 3) showed that the C-reactive protein showed very few associations with fatty acids quantified in absolute amounts in plasma, red blood cell or peripheral blood mononuclear cell membranes from either control subjects or patients with multiple sclerosis. In contrast, it showed a number of associations with fatty acids quantified in relative percentages in both controls and patients. Furthermore, these results indicated that the association between the C-reactive protein and certain fatty acids quantified in relative percentages are valid, because the same associations were found in plasma and red blood cell membrane fatty acids. For example, the C-reactive protein showed positive associations with PC C20:3n-6 quantified in absolute amounts as well as in relative percentages in plasma from patients but an association in plasma from controls only with this fatty acid quantified in relative percentages. The same association was found between the C-reactive protein and PC C20:3n-6 quantified in relative percentages in red blood cells from controls and patients but not in peripheral blood mononuclear cell membranes from

patients or controls. These and previous results showed that association and correlation studies between the C-reactive protein and fatty acids as well as using absolute amounts and relative percentages of fatty acids in evaluating their role in inflammation in patients with multiple sclerosis gave different outcomes, and that relative percentages gave additional information. Specifically, these results showed that correlation studies highlighted the inverse correlations between the C-reactive protein and C20:4n-6 as well as C22:4n-6 quantified in absolute amounts in patients, while association studies showed a positive association between C-reactive protein and C20:3n-6 and that this association was found primarily with the relative percentages.

These results are important because C20:4n-6 and C20:3n-6 are precursors for prostaglandins E2 and E1 respectively and of these E1 is the less inflammatory mediator, and clearly, the C-reactive protein showed opposite correlations/associations with these two fatty acids. It is not clear from the results of this study whether the positive association between the C-reactive protein and C20:3n-6 was due to the membrane content of the fatty acid or whether it was due to the amount made available for prostaglandin E2 production, that is whether a higher membrane content suggested lower levels of the less inflammatory prostaglandin. However, fatty acid metabolic abnormalities have been reported between C20:3n-6 and C20:4n-6 in the peripheral blood mononuclear cell (Harbige & Sharief, 2007; Hon et al., 2009b) and red blood cell (Hon et al., (2009a) membranes from patients.

4.3 Association between C-reactive protein and monounsaturated fatty acids

Furthermore, the C-reactive protein showed positive associations with PC C16:1n-7 quantified in relative percentages in plasma from controls and with PC C16:1n-7 quantified in both absolute amounts (Hon et al., 2010) and relative percentages in both red blood cell and peripheral blood mononuclear cell membranes from controls (Table 3). In contrast, it showed no association with PC C16:1n-7 in any of these blood compartments from patients. The associations between the C-reactive protein and monounsaturated fatty acid C16:1n-7 in control subjects suggested a specific role for this fatty acid in the immune cells in the inflammatory process. The absence of this association in patients suggested an irregularity in the immune process in patients with multiple sclerosis. The C-reactive protein did show a positive association with PC C18:1n-7 in peripheral blood mononuclear cell membranes from patients but only with the percentage composition.

In contrast to the positive associations with the n-7 fatty acids, the C-reactive protein showed an inverse association with PC C18:1n-9 quantified in relative percentages in plasma and red blood cells from patients, but in peripheral blood mononuclear cell membranes from controls only. These results again showed the importance of including relative percentages when evaluating the relationship between inflammation and fatty acids in patients with multiple sclerosis. Monounsaturated fatty acids may have anti-inflammatory effects, although their role in inflammation is not as well defined as that of the polyunsaturated fatty acids (Yaqoob et al., 1998; Harwood & Yaqoob, 2002). The results from this and previous studies showed that the altered associations between the C-reactive protein and the n-7 and n-9 monounsaturated fatty acids may have had an effect on their anti-inflammatory functions and may therefore have contributed to the pathogenesis of the disease.

4.4 The relationship between polyunsaturated fatty acids and the Kurtzke Expanded Disability Status Scale

Previous studies have shown an inverse correlation between the Kurtzke Expanded Disability Status Scale and PC C20:4n-6 quantified in absolute values in red blood cell membranes from patients (Hon et al., 2009a), but a positive correlation with PC C20:3n-6 in peripheral blood mononuclear cell membranes from patients with multiple sclerosis (Hon et al., 2009b). Results from this study showed no association between the Kurtzke Expanded Disability Status Scale and C20:4n-6, but it did show positive associations with PC C20:2n-6 and PE C20:3n-6 quantified in relative percentages in red blood cell from patients (Table 4). These findings are important because the C-reactive protein has been shown in previous studies to show inverse correlations with C20:4n-6 quantified in absolute amounts in patients, while a positive association was shown in this study with PC C20:3n-6 quantified in relative percentages in peripheral blood mononuclear cell membranes from patients. Furthermore, previous studies have shown that the C-reactive protein also showed a positive correlation with the Kurtzke Expanded Disability Status Scale in patients with multiple sclerosis (Hon et al., 2009b).

Although these results would need further investigation, their associations/correlations would suggest that with an increase in the C-reactive protein, there is a decrease in blood cell membrane C20:4n-6 and an increase in C20:3n-6, with an accompanying increase in the Kurtzke Expanded Disability Status Scale in patients with multiple sclerosis. It is possible to hypothesize that the decrease in membrane C20:4n-6 could have been due to the release of this fatty acid for eicosanoid production because this fatty acid is the major fatty acid to be released for eicosanoid production during the inflammatory processes, as measured by the C-reactive protein (Calder, 2007). In addition, these results further suggested that an imbalance in C20:4n-6 and C20:3n-6 release for prostaglandin E2 and E1 production respectively could be associated with the inflammatory condition experienced by patients with multiple sclerosis and that this effect could possibly be a constant condition, hence the significant correlation/association with the Kurtzke Expanded Disability Status Scale respectively.

4.5 Membrane phospholipids

Membrane lipids consist of phospholipids, into which saturated and unsaturated fatty acids are incorporated, as well as cholesterol. Choline containing phospholipids, phosphatidylcholine and sphingomyelin are mainly on the outer leaflet of plasma membranes, while phosphatidylethanolamine and phosphatidylserine are located on the inner leaflet. The properties of the different phospholipid molecules depend on their different head-groups as well as on the type of fatty acids they contain (Williams, 1998; Horrobin, 1999) and their composition in cell membranes determines the degree of fluidity of the membrane structures. Phospholipids from both red blood cell (Hon et al., 2009c) and peripheral blood mononuclear cell (Hon et al., 2011b) membranes have been reported in detail and specifically their function in determining cell membrane fluidity and their role in disease outcome in patients with multiple sclerosis.

5. Conclusions

The validity of using fatty acid composition in relative percentages and/or absolute amounts has been discussed with regards to measurements of these in the blood

components of patients with multiple sclerosis, an inflammatory disease of the central nervous system, in which various fatty acid imbalances have been described, but without any firm conclusions as to cause or consequences. The results of this study showed that using a combination of fatty acids quantified in both absolute amounts and relative percentages could be relevant, especially in a disease state such as multiple sclerosis. This was further confirmed by the association between C-reactive protein, a marker of inflammation and the fatty acids. Therefore, it is recommended that fatty acids should be analysed and interpreted using both absolute amounts and relative percentages.

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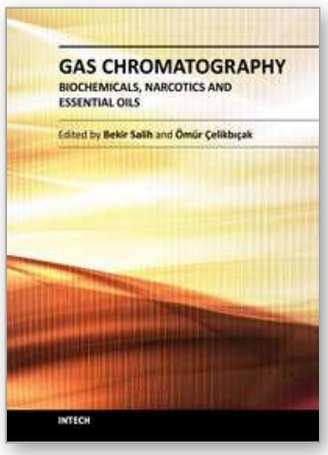

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Gas Chromatography involves the study of various vaporizable molecules in chemistry and the other related research fields. This analytical method has a number of features and advantages that make it an extremely valuable tool for the identification, quantification and structural elucidation of organic molecules. This book provides detailed gas chromatography information to applications of biochemicals, narcotics and essential oils. The details of the applications were briefly handled by the authors to increase their comprehensibility and feasibility. This guide should be certainly valuable to the novice, as well as to the experienced gas chromatography user who may not have the enough experience about the specific applications covered in this book. We believe this book will prove useful in most laboratories where modern gas chromatography is practiced.

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