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A liquid chromatography–tandem mass spectrometry method to measure fatty acids in biological samples

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ABSTRACT

As pre-clinical and clinical research interest in ω-3 polyunsaturated fatty acids (PUFA) increases, so does the need for a fast, accurate and reproducible analytical method to measure fatty acids (FA) in biological samples in order to validate potential prognostic and predictive biomarkers, as well as establishing compliance in ω-3 PUFA intervention trials. We developed a LC–ESI-MS/MS method suitable for high throughput development to measure FAs and validated it in the context of treatment with the ω-3 PUFA eicosapentaenoic acid (EPA). Uniquely we directly compared the LC–ESI-MS/MS method to a GC–MS protocol. We demonstrated the LC–ESI-MS/MS method is accurate and reproducible, with coefficients of variation consistently below 15% for each PUFA analysed. The relative FA content values correlated well with those obtained by GC–MS ($r^2 = 0.94$, $p < 0.001$ for EPA) *in vitro*. The data obtained following analysis of FA content of liver tissues from mice fed an eicosapentaenoic acid enriched diet showed similar results to that of published studies in which GC–MS was used. The LC–ESI-MS/MS method allows concomitant analysis of unesterified (free, unbound) and esterified (bound) FAs in biological samples, allowing investigation of different PUFA pools in cells and tissues.

1. Introduction

Long-chain (>14 carbon atoms) polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, C20:5 ω-3) and arachidonic acid (AA, C20:4 ω-6) play crucial roles in normal human physiology and the pathophysiology of several major diseases, including cancer and neurodegenerative diseases [1,2]. Dietary intake, or administration of purified supplement formulations, of the ω-3 PUFAs EPA and docosahexaenoic acid (DHA, C22:6 ω-3) is increasingly recognised to have likely therapeutic benefit for a range of conditions such as cardiovascular disease [3,4], neurological disorders [5,6] and cancer [7,8].

Lipophilic PUFAs exist predominantly in the plasma membrane following dietary and supplement intake. This property means that measurements of relative and absolute PUFA content of cells and tissues can potentially be used as a disease risk biomarker in observational studies or as a predictive biomarker in intervention studies. In the context of intervention trials, specific PUFA levels in erythrocyte membranes or the plasma phospholipid fraction can be used as a measure of compliance, baseline dietary ω-3 PUFA intake and individual variability in PUFA content [9].

Therefore, there exists a requirement for a robust, reproducible method of PUFA measurement easily applicable to pre-clinical and clinical use. Traditionally, gas chromatography-mass spectrometry

Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl; DMAP, 4-(dimethylamino) pyridine; DAABD-AE, 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole; LNA, alpha-linolenic acid; AA, arachidonic acid; CRC, colorectal cancer; CV, coefficient of variation; LNA-d_{14}, deuterated alpha-linolenic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EMT, EPA for Metastasis Trial; FA, fatty acid; FFA, free fatty acid; GC–MS, gas chromatography–tandem mass spectrometry; LC–ESI-MS/MS, liquid chromatography electrospray ionisation triple quadrupole tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; LA, linoleic acid; OA, oleic acid; PA, palmitic acid; SA, stearic acid.

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(GC–MS) has been employed for relative quantification of FA content in the lipid phase of biological samples *in vitro* and *in vivo* [10]. However, there are limitations to GC–MS quantification of PUFAs. For example, electron ionisation typically involves high energies, causing structural rearrangement of lipids that produce overlapping spectra for different PUFAs, which can make data interpretation problematic [10], though using a suitable column, many of these can be separated chromatographically. Moreover, very high temperatures are required, which is not always suitable for analysis of PUFAs due to sensitivity to thermal degradation [11]. This can be problematic, however these high temperatures are not necessary for LC/MS/MS and therefore thermal degradation is unlikely to occur.

Liquid chromatography (LC) in combination with electrospray ionisation triple quadrupole tandem mass spectrometry (ESI-MS/MS) is now widely available in many laboratories. This has led to the development of several ESI-MS/MS methods with or without derivatisation steps [12]. A number of derivatisation agents, which are simple to incorporate during sample preparation and increase sensitivity of LC–ESI-MS/MS for PUFA analysis, are now available. Benzofuran and diazoalkane derivatisation has been reported to lower the limit of detection by enhancing fragmentation patterns during MS, thus increasing sensitivity [13–15]. However, no LC–ESI-MS/MS method has been compared directly to established GC–MS methodology for multiple FA measurement in biological samples. Herein, we describe a new LC–ESI-MS/MS method for measurement of fatty acids (FAs) using widely available equipment and amenable to high-throughput analysis. We compare the novel LC–ESI-MS/MS method to a well-established GC–MS protocol [16,17] for FA analysis in biological samples. We also confirmed applicability of the LC–ESI-MS/MS method to animal and human samples in the context of EPA supplementation experiments. Finally, we highlight the use of the LC–ESI-MS/MS method to compare relative changes in free (unesterified) and total (free + esterified) cellular PUFA content after exogenous EPA exposure.

2. Methods

2.1. Reagents

EPA free fatty acid (FFA) was provided by SLA Pharma AG (Watford, UK). Use of EPA-FFA in *in vitro* experiments has been described previously [17]. Acetonitrile, methanol, 2-propanol, water and chloroform used for fatty acid extraction were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were UHPLC–MS grade. Deionised purified water was generated using an Elga Maxima and Elga Purelab Option purifying system (18.2 MΩ-cm) (Veolia Water Technologies UK, High Wycombe). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide HCl (EDC), 4-(dimethylamino) pyridine (DMAP), 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE) and fatty acid standards; eicosapentaenoic acid (EPA C20:5 ω-3), docosapentaenoic acid (DPA, C22:5 ω-3), docosahexaenoic acid (DHA C22:6 ω-3), alpha-linolenic acid (LNA C18:3 ω-3), arachidonic acid (AA C20:4 ω-6), linoleic acid (LA, C18:2 ω-6), stearic acid (SA, C18:0), oleic acid (OA C18:1 ω-9) and palmitic acid (PA, C16:0), were also purchased from Sigma Aldrich. Internal standard, deuterated alpha-linolenic acid (LNA-d_{14}), was purchased from Cayman Chemical (Ann Arbor, MI, USA).

2.2. In vitro studies

MC38 mouse colorectal cancer (CRC) cells were a gift from Professor Daniel Beauchamp (Vanderbilt Institute, USA). HCA-7 human CRC cells were obtained from the American Type Culture Collection (UK). Cells were cultured in RPMI 1640 medium containing Glutamax® supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (all Thermofisher Scientific, UK), at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were routinely sub-cultured using 0.25% (w/v) trypsin (Thermofisher Scientific, UK). Viable cells were counted using a haemocytometer in the presence of 0.04% (v/v) trypan blue (Sigma–Aldrich, UK).

For each cell line, an equal number of viable cells were seeded in tissue culture flasks and left to reach 60–70% confluence. Cells were then treated with either ethanol carrier (0.6% v/v) or EPA-FFA and incubated for 24 h. Cells were then washed twice with sterile PBS (Thermofisher Scientific, UK) and harvested by trypsinisation. The cell suspensions obtained were counted and centrifuged at $500 \times g$ for 5 min at room temperature. The cell pellets were then stored at −80 °C until analysis.

2.3. In vivo studies

C57Bl/6 mice were housed in a specific pathogen-free environment. All experiments were undertaken with UK Home Office approval. Eight week-old female C57Bl/6 mice were administered one of two iso-caloric test diets (n = 16 per group) *ad libitum* for 28 days based on a modified AIN-93G diet base, with 7% corn oil. The diets contained either no EPA-FFA or 5% [w/w] EPA-FFA, replacing the equivalent amount of corn oil. Fresh diet was manufactured by IPS (London, UK) every 8 days and delivered within 48 h in vacuum-packed 100 g foil bags in order to minimise oxidation. The feed was replaced every day with fresh diet from a previously unopened bag. On day 15, 1×10^6 viable MC38 mouse CRC cells suspended in 100 μl sterile PBS were injected subcutaneously with a sterile 27G needle. Animals continued on the same diet and were weighed daily for a further 14 days until sacrifice. Immediately after sacrifice, liver samples were snap-frozen in liquid N_2 and stored at −80 °C. Tissues were prepared for extraction by homogenisation in 10 parts PBS using a mechanical homogeniser (Ultra Turrax, IKA, UK). A 50 μl aliquot was then used for extraction of FAs.

2.4. Human studies

We used data from 8 patients who took part in the EPA for Metastasis Trial (EMT, [16]), in which blood samples for plasma and erythrocyte extraction were obtained before and after intervention (EPA treatment median duration of 44 days). The full data set from erythrocyte membrane PUFA content analysis, of 88 patients (including the 8 samples included here) has previously been published [9].

2.5. GC–MS: extraction and analysis

The relative FA content of MC38 cells exposed to EPA-FFA was measured by GC–MS, as previously described [18,19]. In brief samples, into which internal standard was added (Heneicosanoic acid, 2.5 μg/sample), were homogenised in 0.5 ml saline, then purified by liquid/liquid extraction (Chloroform: Methanol 2:1 vol/vol, 3 + 2 ml, 0.5% di butylated hydroxytoluene [BHT]) twice. After centrifugation (2000 x G, 10 min, 25 °C), organic phases were combined and re-extracted with 2 ml Chloroform/H_2O (1:1 vol/vol), separated by centrifugation and the organic phase transferred to a new tube, then taken to dryness under a N_2 stream at 30 °C.

Analytes were derivatized into free fatty acids (FA) by a base-catalyzed reaction (2 ml of KOH 0.5 M in Methanol, 80 °C, 10 min) and then esterified to form fatty acid methyl esters (FAMEs) in an acid-catalyzed reaction using boron trifluoride in methanol, (1 ml BF_3, 80 °C, 10 min) [20] FAMES were then extracted twice with Hexane (3 + 2 ml) and then taken to dryness until used.

The FAMES mixture was then injected onto a gas chromatograph for separation (Agilent HP5890, equipped with PTV [Programmed Temperature Inlet] injector), then detected and identified by a mass spectrometric detector (MSD) (Agilent MS 5970) used in ESI mode (electron-impact ionisation, 60 eV). We used a SUPELCO SP™ 2330 column (30 mt × 0.25 mm × 0.2 μm film thickness). Helium was the carrier gas (initial flow 0.5 ml/min, constant pressure mode, 10.8 psi). Further detail is given in Courtney et al. [21].

2.6. LC/MS: Extraction of FAs from biological samples

FAs were extracted from samples using an isopropanol/chloroform method (Fig. 1) adapted from the protocol first published by Rose et al. [22]. Tissue homogenates or washed erythrocytes (50 μl) were mixed with 50 μl of distilled water, containing internal standard (LNA-d_{14}) at 2 μg/ml, and allowed to stand for 15 min. For blanks and controls, samples were substituted with PBS or a mix of FA standards (2 μg/ml). Isopropanol was added slowly to a volume of 650 μl with mixing, and following incubation for 1 h at room temperature, 350 μl of chloroform was added. After a further 1 h incubation, samples were centrifuged at 10,000 × *g* for 5 min. The supernatant was then evaporated to dryness in a rotary evaporator (EZ-2 plus rotary evaporator, Genevac Ltd, Suffolk, UK), and those extracts destined to undergo hydrolysis were reconstituted in 500 μl acetonitrile. Saponification by acid hydrolysis was performed with the addition of 50 μl 5 M HCl followed by incubation at 80 °C for 1 h. 50 μl of 5 M NaOH was then added to neutralise, before the addition of 350 μl chloroform. The samples were left to stand for 5 min, before isolating the top 800 μl for evaporation. Extracted FAs from hydrolysed and non-hydrolysed samples were reconstituted in 50 μl methanol prior to derivatisation.

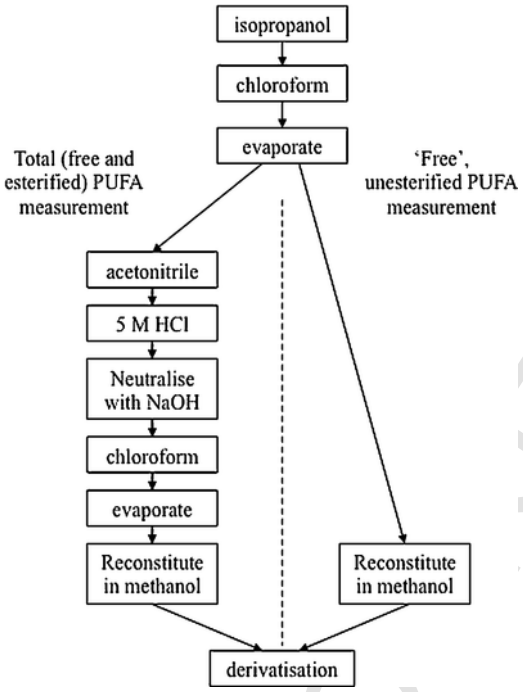

Fig. 1. FA extraction method.

2.7. LC/MS: Derivatisation of extracted FAs

50 μl of EDC, 50 μl of DMAP and 50 μl of the derivatising agent DAABD-AE (all 10 mg/ml in methanol), were mixed with each 50 μl sample of FA extract. The mixture was then incubated for 24 h at room temperature in amber glass HPLC vials. The resulting FA derivatives were measured by LC–ESI-MS/MS.

2.8. LC–ESI-MS/MS analysis

Samples were analysed using a Waters Alliance 2695 High Pressure LC separations module in combination with a Waters Micromass Quattro Ultima triple quadrupole mass spectrometer (Waters UK, Hertfordshire). 2 μl of derivatised sample was separated on a HiChrom RPB column (2.1 mm × 250 mm, 5 μm) (HiChrom Ltd, Berkshire UK). Mobile phase (MP) A consisted of 90% dH_2O, 10% MeOH, 0.1% formic acid and MPB consisted of 90% MeOH, 10% dH_2O and 0.1% formic acid. Gradient conditions were as follows: Starting at 80% MPB changing to 83% MPB over 8 min, then increasing to 95% MPB at 15 min, remaining at 95% MPB until 17 min before returning to starting conditions at 18 min. The overall run time was 25 min. The flow rate was set at 0.5 ml/min and split post-column with 0.3 ml/min delivered to the MS. Samples were analysed in MRM mode for the following fatty acids: EPA, DPA, DHA, LNA, AA, LA, SA, OA, PA and internal standard LNA-d_{14}. Instrument settings: Capillary voltage, 3 kV; Cone energy, 15 eV; collision energy, 25 eV; source temperature, 120 °C and desolvation temperature, 300 °C. MRM settings are detailed in Table 1.

Results are expressed for each FA as the percentage of the total analysed FA content for that sample, calculated as the sum of the ratio of each FA peak area to the internal standard peak area (%FA).

2.9. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6. Bland–Altman curves and linear regression analysis were used to compare the two MS methodologies. The statistical significance of differ-

Table 1
LC–ESI-MS/MS FA detection parameters and comparison with GC–MS.

Fatty acid	$(M+H)^+$	MRM transition[a]	Retention time, t_r (min)	Linear range (μg/ml)	Standard Curve linear Regression r^2 value	LOD (μg/ml)	LOQ (μg/ml)	GC–MS to LC–MS/MS linear regression
EPA	303.46	613.8 > 150.5	6.2	0.01–50	0.9953	0.01	0.01	$r^2 = 0.94, p < 0.0001$
DPA	331.5	641.9 > 150.5	9.4	0.01–50	0.9938	0.001	0.01	$r^2 = 0.97, p < 0.0001$
DHA	329.49	639.9 > 150.5	8.1	0.001–50	0.9984	0.001	0.01	$r^2 = 0.85, p < 0.0001$
LNA	279.43	589.8 > 150.5	6.1	0.01–50	0.9951	0.001	0.01	$r^2 = 0.38, p = 0.032$
AA	305.4	615.8 > 150.5	8.4	0.01–50	0.9944	0.001	0.01	$r^2 = 0.89, p < 0.0001$
LA	281.45	591.8 > 150.5	8.3	0.01–50	0.9981	0.001	0.01	$r^2 = 0.63, p = 0.0021$
SA	285.48	595.8 > 150.5	16.4	0.1–50	0.9908	[b]	0.1	$r^2 = 0.89, p < 0.0001$
OA	283.5	593.8 > 150.5	11.8	0.1–50	0.9942	0.001	0.1	$r^2 = 0.96, p < 0.0001$
PA	257.4	567.8 > 150.5	10.3	0.1–50	0.9921	[b]	0.1	$r^2 = 0.40, p = 0.026$

[a] Parent ion used for all FA MRM channels is FA *m/z* plus mass of DAABD-AE (310.4).
[b] Unable to determine LOD due to background levels.

ences in % FA were analysed using the multiple *T*-test with Holm–Sidak correction ($\alpha = 0.05$).

3. Results

3.1. LC–ESI-MS/MS performance for FA measurement

We developed a FA extraction and LC–ESI-MS/MS analysis method to quantify 9 FAs in a range of biological samples. Table 1 summarises the detection parameters for each FA. Typical MS traces are illustrated in the Supplementary Fig. S1. The limit of detection (LOD) for the FAs was routinely below 1 ng/ml equating to an on-column injection of 2 pg of FA, except for SA and PA, for which an accurate LOD could not be determined due to background contamination levels. The limit of quantification (LOQ) and linear range of quantification for each FA was also determined (Table 1). The linear range was established as 0.01–50 μg/ml for EPA, DPA, LNA, AA and LA. The DHA linear range was wider, from 0.001 to 50 μg/ml, whilst SA, OA and PA could only be quantified in concentrations ranging between 0.1 and 50 μg/ml because of high background levels (Table 1). To assess the reproducibility and accuracy of the method, we determined intra-day and inter-day coefficient of variations (CV) for absolute concentrations of each FA (Table 2). Calibration curves were prepared daily and injected in triplicate over 3 days. There was good reproducibility and accuracy with intra-day and inter-day %CVs consistently $<20\%$ and % accuracy within 10%. The intra- and inter-day %CV data were also calculated from the FA chromatographic peak area to internal standard peak area ratio to facilitate the comparison with published GC–MS data (Table 3). The LC–ESI-MS/MS method therefore allowed us to quantify each of the 9 FAs and express the amount of FA detected either as an absolute concentration (weight/volume) or as a percentage amount relative to the total FA weight or % amount relative to the total FA peak area. The latter is the most common way of expressing relative FA amounts in biological samples by GC–MS. We therefore expressed the LC–ESI-MS/MS data as the percentage of each FA relative to the total FA peak area (%FA) in order to facilitate the comparison with GC–MS data.

3.2. Comparison of LC–ESI-MS/MS and GC–MS for the measurement of FAs in vitro

We performed a direct comparison between GC–MS and LC–ESI-MS/MS FA measurements in biological samples. We used MC38 mouse CRC cells, which are established in our laboratory for studies of EPA anti-CRC activity, in order to compare the LC–ESI-MS/MS method on hydrolysed FA extract samples against GC–MS. MC38 mouse CRC cells were treated with three concentrations of EPA-FFA or an equal volume of ethanol carrier control for 24 h. The relative amount of each FA was determined independently by both techniques (Fig. 2). In untreated cells, only low levels of EPA could be measured with $1.98 \pm 0.18\%$ of FA determined by LC–ESI-MS/MS compared with $0.60 \pm 0.02\%$ of FA by GC–MS (Fig. 2A). Both methods showed a concentration-dependent increase in cellular EPA content following EPA treatment, accompanied by a concomitant decrease in relative AA content (Fig. 2B–D) but mainly at the expense of SA and OA content. There was a 14 ± 3 fold ($p = 0.0001$) and 27 ± 6 fold ($p = 0.001$) increase in EPA % of FA after treatment with 100 μM EPA compared with control cells, when

Table 2
Intra-day and Inter-day reproducibility and accuracy values calculated from QC standards ($n = 3$). Italic values are those obtained for concentrations below the LOQ.

FA	Conc. (ng/ml)	Intra-day			Inter-day		
		Measured	CV (%)	Accuracy (%)	Measured	CV (%)	Accuracy (%)
EPA	10	9.2 ± 1.5	16.0	92.0	7.2 ± 1.2	17.2	71.5
	100	84.8 ± 13.0	17.3	84.8	83.6 ± 3.4	4.1	83.6
	500	487.5 ± 74.6	15.3	97.5	484.3 ± 7.7	1.6	96.9
	1000	1026.6 ± 128.3	12.5	102.7	960.5 ± 133.9	13.9	96.0
DPA	10	8.4 ± 0.8	9.6	83.9	6.9 ± 0.6	8.1	68.6
	100	92.9 ± 9.5	11.5	92.9	85.9 ± 6.1	7.1	85.9
	500	487.7 ± 49.7	10.2	97.5	483.8 ± 6.1	1.3	96.8
	1000	1032.8 ± 80.6	7.8	103.3	970.8 ± 137.6	14.2	97.1
DHA	10	8.0 ± 0.8	9.9	79.7	6.8 ± 0.9	13.1	68.0
	100	95.2 ± 8.7	12.5	95.2	85.1 ± 9.8	11.5	85.0
	500	490.7 ± 44.7	9.1	98.1	475.3 ± 19.7	4.1	95.1
	1000	1031.3 ± 87.7	8.5	103.1	983.6 ± 152.2	15.5	98.4
LNA	10	8.9 ± 0.8	9.0	88.6	6.9 ± 1.2	17.5	68.8
	100	87.1 ± 11.8	13.3	87.1	86.9 ± 7.7	8.8	86.9
	500	494.7 ± 67.3	13.6	98.9	477.4 ± 18.8	3.9	95.5
	1000	1022.1 ± 125.7	12.3	102.2	976.8 ± 142.0	14.5	97.7
AA	10	9.1 ± 0.9	9.9	91.0	7.3 ± 0.2	3.0	73.1
	100	93.3 ± 8.6	14.5	93.3	85.8 ± 6.6	7.7	85.8
	500	492.0 ± 45.3	9.2	98.4	487.5 ± 5.6	1.1	97.5
	1000	1034.6 ± 78.6	7.6	103.5	965.2 ± 134.7	14.0	96.5
LA	10	12.8 ± 1.6	12.3	127.9	9.0 ± 0.4	3.9	89.8
	100	97.8 ± 8.2	10.2	97.8	117.5 ± 43.6	37.1	117.5
	500	494.2 ± 41.5	8.4	98.8	477.0 ± 23.0	4.8	95.4
	1000	1021.3 ± 94.0	9.2	102.1	958.0 ± 125.3	13.1	95.8
SA	*10*	*34.1 ± 7.4*	*21.8*	*340.7*	*−12.4 ± 5.6*	*45.1(–)*	*124.2(–)*
	100	93.7 ± 2.5	9.9	93.7	189.9 ± 163.4	86.0	189.9
	500	545.4 ± 14.7	2.7	109.1	490.9 ± 47.4	9.7	98.2
	1000	1002.0 ± 103.2	10.3	100.2	953.4 ± 136.0	14.3	95.3
OA	*10*	*50.2 ± 8.6*	*17.1*	*501.8*	*31.1 ± 4.2*	*13.5*	*310.8*
	100	115.2 ± 11.3	11.6	115.2	141.5 ± 56.3	39.8	141.5
	500	511.2 ± 50.1	9.8	102.2	492.5 ± 24.2	4.9	98.5
	1000	1017.0 ± 89.5	8.8	101.7	955.9 ± 133.3	13.9	95.6
PA	*10*	*60.1 ± 11.9*	*19.8*	*601.0*	*−38.5 ± 53.1*	*137.8(–)*	*385.2(–)*
	100	98.9 ± 3.7	7.9	98.9	117.6 ± 38.9	33.1	117.6
	500	530.5 ± 19.6	3.7	106.1	465.3 ± 60.2	12.9	93.1
	1000	1019.9 ± 66.3	6.5	102.0	934.3 ± 150.4	16.1	93.4

Table 3
Intra-day and Inter-day %CV values calculated from FA peak area to internal standard ratio, for QC standards at 4 concentrations (n = 3). Intra-sample %CVs were obtained by aliquoting one erythrocyte sample into 5 samples and proceeding to the extraction and analysis of each aliquot independently on the same day. Italic values are those obtained for concentrations below LOQ.

FA	Conc. (ng/ml)	CV (%)		
		Intra-sample	Intra-day	Inter-day
EPA	10		7.39	6.43
	100		3.15	5.87
	500		3.15	0.00
	1000		8.65	8.32
	Erythrocytes	2.25		
DPA	10		11.84	8.17
	100		11.15	10.31
	500		16.83	3.19
	1000		8.36	1.79
	Erythrocytes	3.87		
DHA	10		3.72	7.74
	100		15.13	12.02
	500		16.45	0.00
	1000		8.26	15.86
	Erythrocytes	5.53		
LNA	10		1.01	5.87
	100		17.85	6.14
	500		1.84	0.00
	1000		7.56	8.32
	Erythrocytes	3.52		
AA	10		3.94	1.70
	100		11.55	10.41
	500		17.70	0.94
	1000		12.95	6.06
	Erythrocytes	3.22		
LA	10		9.36	19.49
	100		17.95	9.59
	500		17.59	2.34
	1000		6.57	9.53
	Erythrocytes	3.96		
SA	*10*		*209.55*	*-67.01*
	100		122.14	38.60
	500		37.23	6.24
	1000		9.76	20.39
	Erythrocytes	55.06		
OA	*10*		*13.94*	*45.30*
	100		18.60	15.18
	500		21.75	1.26
	1000		11.27	16.08
	Erythrocytes	4.96		
PA	*10*		*-155.34*	*-97.71*
	100		55.37	36.36
	500		3.30	10.41
	1000		6.39	0.00
	Erythrocytes	20.55		

measured by LC–ESI-MS/MS and GC–MS respectively (Fig. 2C). The data expressed as Bland–Altman plots (Fig. 3) show excellent agreement between the two methods over a wide range of values, except for SA and OA, for which there was fixed bias dependent on the method used (Figs. 2 and 3). SA measurements between the two methods showed a mean bias of 9.6 ± 2.9 points with 95% limits of agreement from −3.9 to 15.3 (Fig. 3). OA measurements showed a mean bias of −17.5 ± 3.4 points (95% limits of agreement −24.2 to −10.8). Bias between the methods restricted to SA and OA might be explained by differences in glassware and plastic FA contamination between the laboratories [23], which performed the LC–ESI-MS/MS (Bradford) and the GC–MS (Bologna) analyses.

Linear regression analysis for each FA revealed a statistically significant correlation between the methods for all FAs, which was weakest for SA (Fig. 4 and Table 1). Taken together, these data reveal good correlation and agreement between the LC–ESI-MS/MS method and GC–MS for measurement of PUFAs in cultured cells in the context of ω-3 PUFA treatment.

3.3. Measurement of omega-3 PUFA incorporation in mouse liver

Next, we applied the LC–ESI-MS/MS method to the measurement of FA content in more complex biological samples. C57Bl/6 mice bearing MC38 mouse CRC cell tumours received EPA-containing or control diet for 4 weeks prior to sacrifice. LC–ESI-MS/MS analysis of hydrolysed lipid extracts from liver samples revealed a significant increase in the % amount of EPA, DPA and DHA, as well as a significant reduction in the relative amount of AA, LA and OA in the liver of mice fed the diet containing 5% (w/w) EPA (Fig. 5, $p < 0.05$). These FA values are similar to those obtained by GC–MS measurement in a previously published EPA treatment experiment [18]. Therefore, the LC–ESI-MS/MS method can detect omega-3 PUFA changes in mouse tissue following exogenous administration of EPA.

3.4. Measurement of omega-3 PUFAs in clinical samples

We then tested the applicability of the LC–ESI-MS/MS method for FA analysis in a clinical setting, using a subset of clinical samples from the EMT study [9,16]. We analysed the total FA content of erythrocytes from 8 participants before and after treatment with EPA-FFA 2 g daily using the hydrolysis method, *i.e.* total FA (Fig. 6). The % EPA content relative to the total FA pool was significantly increased after EPA treatment compared with baseline (2.26 ± 0.47% compared with 0.77 ± 0.06%). EPA was the only FA that demonstrated a statistically significant change in relative amount ($p < 0.05$; Fig. 6). Clinical samples were also split in 5 aliquots before extraction and injected the same day to assess reproducibility in clinical samples. The intra-sample variation data calculated after multiple extraction and analysis of one sample showed excellent reproducibility with %CV values between 2.2–5.5% for each FA, with the exception of SA and PA, which were much higher due to background (calculated from ratio to internal standard, Table 3).

3.5. Use of LC–ESI-MS/MS to distinguish changes in free FA and bound FA content in biological samples

Derivatisation and hydrolysis occur concomitantly during sample preparation for GC–MS analysis. Separation of the derivatisation step from the acid hydrolysis step during the sample preparation phase for LC–ESI-MS/MS allowed us to investigate differential effects of exogenous EPA exposure on 'free', unesterified FA content (determined from non-hydrolysed samples) compared with total (free, triglyceride- and phospholipid-bound) FAs released by acid hydrolysis. In the *in vitro* mouse MC38 cell experiment, C57Bl/6 mouse model and for the clinical erythrocyte samples, we compared lipid extracts from hydrolysed and non-hydrolysed samples. In MC38 mouse CRC cells treated with 100 μM EPA for 24 h, we observed that EPA constituted a smaller proportion of the pool of free FAs (18.27 ± 0.40%) than the total FA content (42.17 ± 2.37%) of these cells (Fig. 7A and B). By contrast, dietary EPA administration *in vivo* did not lead to preferential enrichment of free (17.6 ± 5.90%) or bound (18.27 ± 0.40%) pools of EPA in hepatocytes (Fig. 7C and D). The more modest changes in relative EPA content in erythrocytes following EPA treatment in the EMT study were also not associated with preferential EPA incorporation as either the free FA or within the bound FA pool (Fig. 7E and F). Differences in individual free and bound FA profiles in erythrocytes, both before and after EPA treatment (Fig. 7E and F), highlight that the FA compartment to which EPA and other PUFAs localise should be taken into account

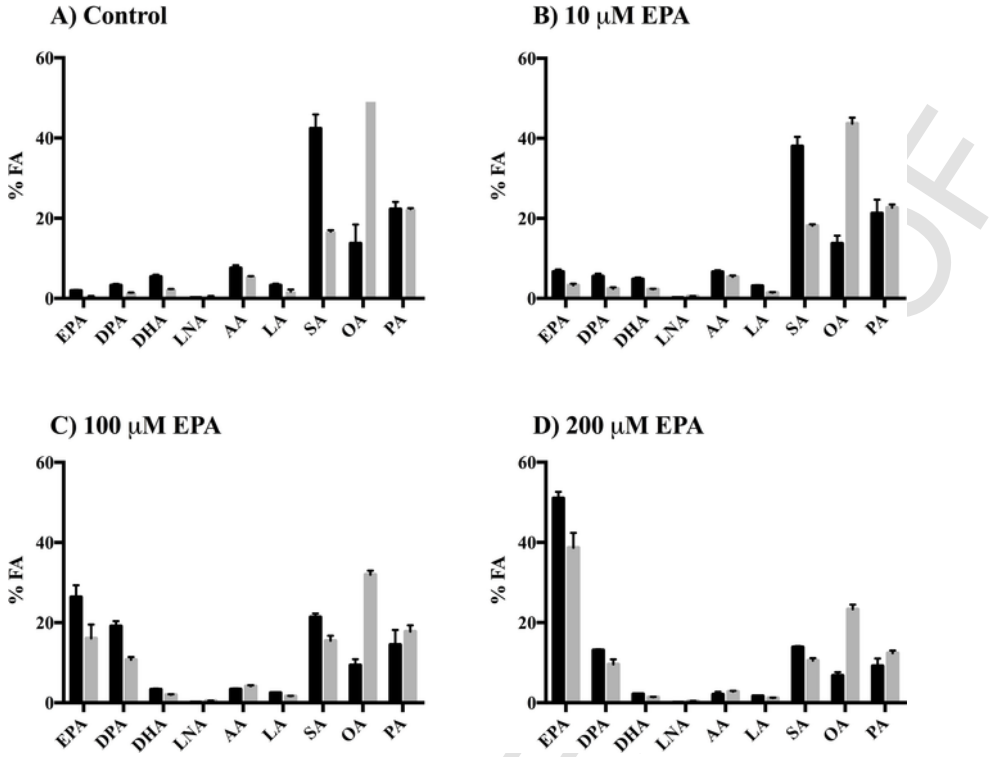

Fig. 2. Measurement of FA content in MC38 mouse CRC cells. Relative FA content of control and EPA-treated cells was determined by LC–ESI-MS/MS (■) or GC–MS (▒). MC38 mouse CRC cells were treated with ethanol carrier (0.1% v/v) (A), 10 μM EPA (B), 100 μM EPA (C) or 200 μM EPA (D). Data are expressed as the mean ± standard deviation for 3 independent replicates.

when inferences about biological activity of individual FAs are made based on relative cell and tissue levels.

4. Discussion

We describe an accurate and reproducible LC–ESI-MS/MS method for the analysis of FAs in cultured cells, and also animal and human samples. We found one prior study comparing LC–ESI-MS/MS method to GC-combustion-isotope ratio MS to measure [^{13}C16]-palmitate enrichment in volunteers [24]. However, this is the first direct comparison of LC–ESI-MS/MS to the 'gold-standard' GC–MS method for multiple non labelled FA relative content and it has confirmed excellent agreement for FA analysis between the methods.

Several LC–MS/MS protocols have previously been published for FA analysis [23,25–27], some of which use linear ion trap quadrupole tandem mass spectrometers, often reporting relatively high LOD (up to 1 μg/ml, [26]) while others have restricted methodology to EPA only [25] or EPA, DHA and AA [27]. Of note is the method of Yung et al. demonstrating an excellent LOD of 0.25 ng/ml, however this is for a urea-based analog of EPA [28].

Here we describe the comprehensive analysis of a range of FAs (DPA, DHA, AA, EPA, SA, OA, LA, LNA, PA) using triple quadrupole mass spectrometry, systems which are widely available in most analytical laboratories although the methodology is suitable for ion trap systems also.

The use of DAABD-AE as a derivatisation agent was first developed by Tsukamoto et al. [29], specifically for reactions with carboxylic acids and specifically for sensitive detection using ESI-MS. Use of DAABD-AE has been previously optimised [30], resulting in a convenient derivatisation process producing a highly sensitive analytical method when used in combination with LC/MS/MS. Fatty acids are known to produce a complex fragmentation when subject to ESI. However, DAABD-AE derivatisation stabilised all the FAs analysed, producing a characteristic product ion spectrum with a consistent major fragment ion at *m/z* 150.5. This characteristic fragment can be explained by the dimethylaminoethylaminosulfonyl fragment within DAABD-AE [30]. Indeed a precursor ion scan for *m/z* 150.5 would be a good opportunity to find more potential FAs in the chromatographic run, if needed.

Separation of the 9 FAs was satisfactory using our column of choice (2.1 mm × 250 mm, 5 μm particle size). However, the methodology would be easily adaptable to UPLC using 1.7 μm particles, if required. The assay is also suitable for the investigation of other disease states such as peroxisomal disorders, for example by the inclusion of other fatty acids including pristanic acid, phytanic acid. We appreciate that double bond positional isomers (*e.g.*, ω-3 *vs* ω-6 species with the same *m/z* value) may be present together in human and animal samples and have confirmed that we can, for example, separate and hence differentiate, between ω-6 DPA and ω-3 DPA (unpublished data).

The increased LOQ and LOD (up to 100 ng/ml) for PA, OA and SA was due to the small, but consistent background interference and was compensated by use of blank controls, which were subtracted from analysed samples. Background interference is a widely reported problem for FA analysis, in particular for SA and PA, resulting in a higher LOQ than for many other fatty acids [23]. This could be a substantial issue for analysis of free FA but is less so for analysis of membrane FA incorporation as the concentration of an individual FA being analysed is relatively high compared to background. It is important to take background FA presence into account using appropriate control samples.

Sensitivity is not usually a concern with the high concentrations of FA present in most biological samples. However, the low LODs reported here would enable the development of an assay relevant for micro-sampling techniques such as finger-prick blood sampling [31]. The straightforward methodology described here is also amenable to small sample analysis. Furthermore, the protocol has now been adapted to a

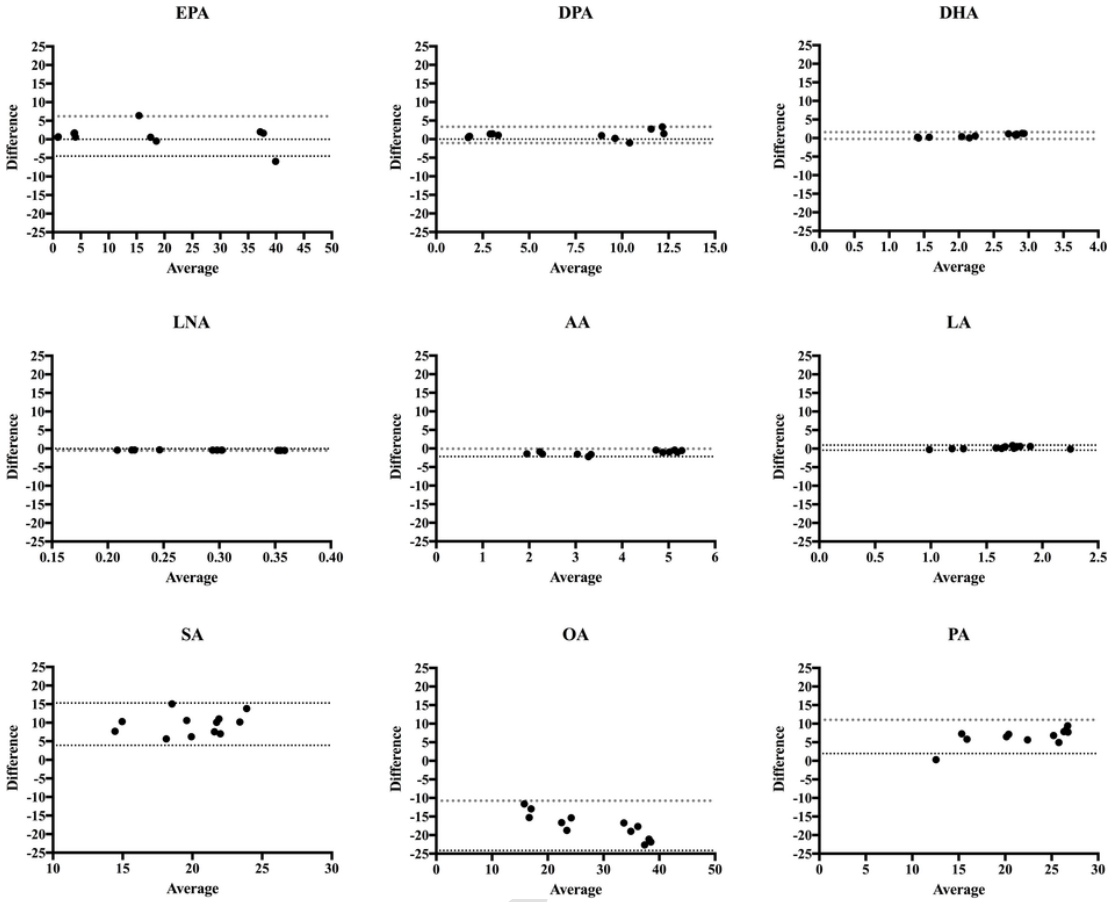

Fig. 3. Comparison of LC–ESI-MS/MS and GC–MS for the measurement of fatty acids in mouse MC38 CRC cells. Bland–Altman curves of GC–MS and LC–ESI-MS/MS data for each FA analysed (n = 12 per FA). Positive differences equate to higher measurement values by LC–ESI-MS/MS and negative differences denote a higher values by GC–MS.

high throughput format allowing FA extraction in 96-well plates using 2 ml Sirocco protein precipitation plates (Waters UK).

The suitability of the assay for analysis of complex tissue samples is demonstrated by the analysis of mouse liver FA content. The studies demonstrated significant changes in omega-3 PUFA levels following manipulation of the dietary intake of mice. Interestingly, a similar study was performed by Arnold et al. in 2010, where rats were fed an EPA and DHA rich diet and tissue FA levels were examined by GC–MS [32]. Their data, just like ours, showed an increase of EPA and DHA in several organs including the rat liver with a concomitant decrease in the relative amount of oleic acid whilst saturated acid levels remained unchanged [32]. The LC–ESI-MS/MS method is accurate and can detect tissue changes in FA profile in a comparable manner to GC–MS. Furthermore, the reproducible analysis of erythrocytes from patients receiving EPA-FFA with intra-day %CVs consistently $<$20% makes this methodology suitable for multicenter clinical studies.

The method is also versatile in that it is very easy to change between the analysis of hydrolysed and non-hydrolysed FA extracts from a range of sources. We are aware of one other report in which LC–MS/MS was used to distinguish between free (unesterified) and total FAs by avoidance of a hydrolysis step but the aim was to detect unesterified EPA in the plasma following oral administration of -EPA ethyl ester rather than investigating compartmentalisation in cells [25]. One other study looked at PUFA distribution in cells comparing free and esterified fluorescent PUFAs in cells overexpressing the liver–type fatty acid-binding protein [33]. This study showed that the proportion of free, unesterified EPA in cells is greater than for other PUFAs, which is consistent with our data [33]. These data, together with our study, indicate that FAs may compartmentalise differently depending on the tissue analysed, as well the absolute amount of each FA present within a biological sample. In turn, this could impact on the biological activity of each FA, as their availability for metabolism or interaction with proteins such as signalling receptors may be dependent on their presence as the FFA or as an esterified FA. Such information will be relevant when translating mechanistic *in vitro* studies to the clinical settings where the ω-3 PUFA tissue content and therefore their relative compartmentalisation may not be comparable.

In conclusion, we describe a validated methodology for the analysis of FAs using LC–ESI-MS/MS which is amenable to high throughput analysis techniques. This methodology compares favourably to GC–MS in the context of evaluation of EPA incorporation *in vitro* and *in vivo*. The extraction, separation and quantitation of FAs from biological tissues, demonstrates the flexibility of the assay for measurements of FAs in complex pre-clinical and clinical tissues such as liver and erythrocytes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2017.04.030.

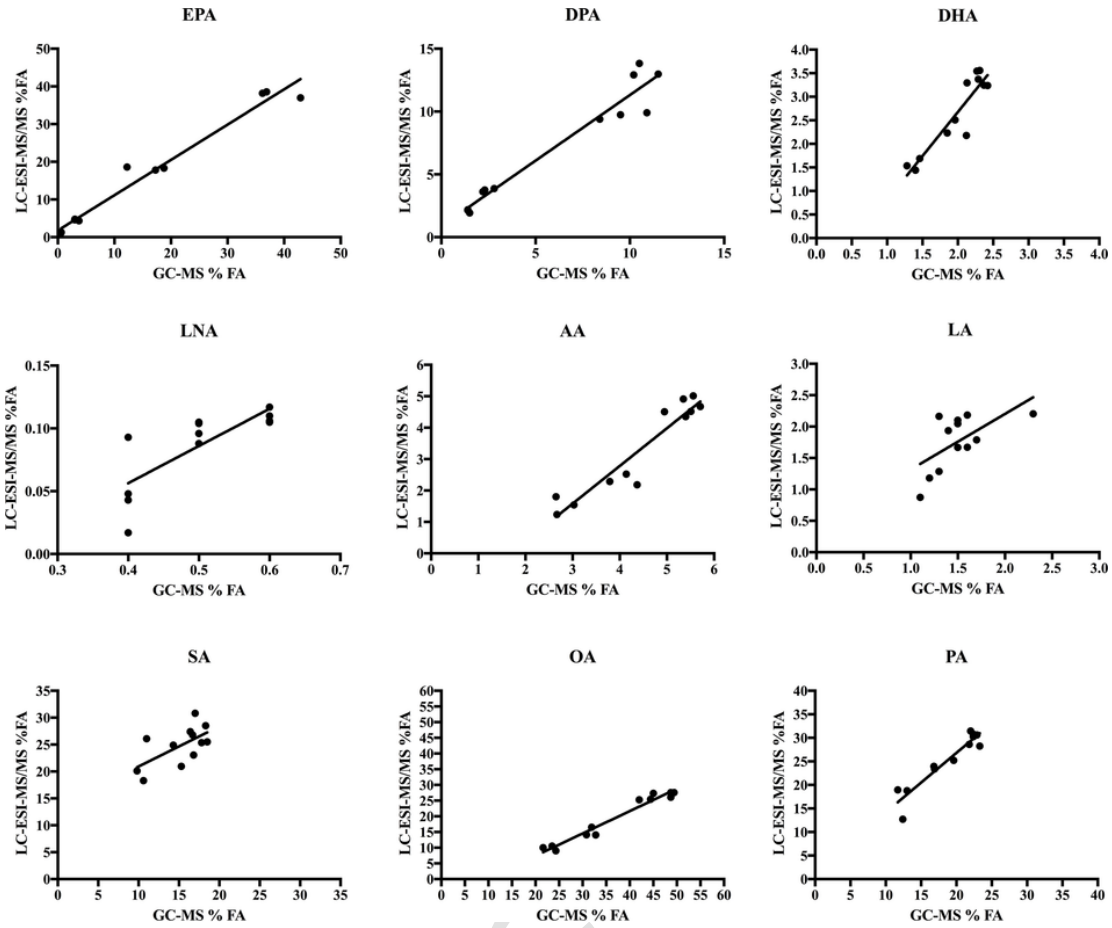

Fig. 4. Relationship between LC–ESI-MS/MS and GC–MS data. Linear regression analysis of GC–MS and LC–ESI-MS/MS for each of the 9 fatty acids analysed in MC38 mouse CRC cells (n = 12 per FA).

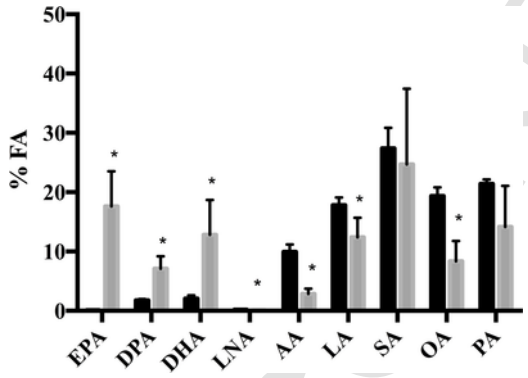

Fig. 5. Measurement of FA content in mouse liver samples. C57Bl/6 mice were fed control diet (■) or 5% EPA-containing diet (□). n = 5 per group. Data are expressed as the mean % FA ± standard deviation. *$p < 0.05$ for the difference between groups (multiple T-test with Holm–Sidak correction).

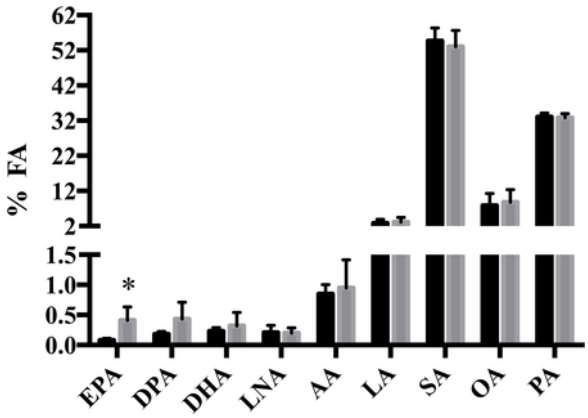

Fig. 6. Measurement of relative FA content in human erythrocytes. Fatty acids were measured in the hydrolysed lipid extract from erythrocytes obtained pre- (■) and post-treatment with EPA-FFA (□). Data are expressed as the mean %FA ± standard deviation, n = 8. *denotes a statistically significant difference in %FA between pre- and post-treatment samples ($p < 0.05$; multiple T-test with Holm–Sidak correction).

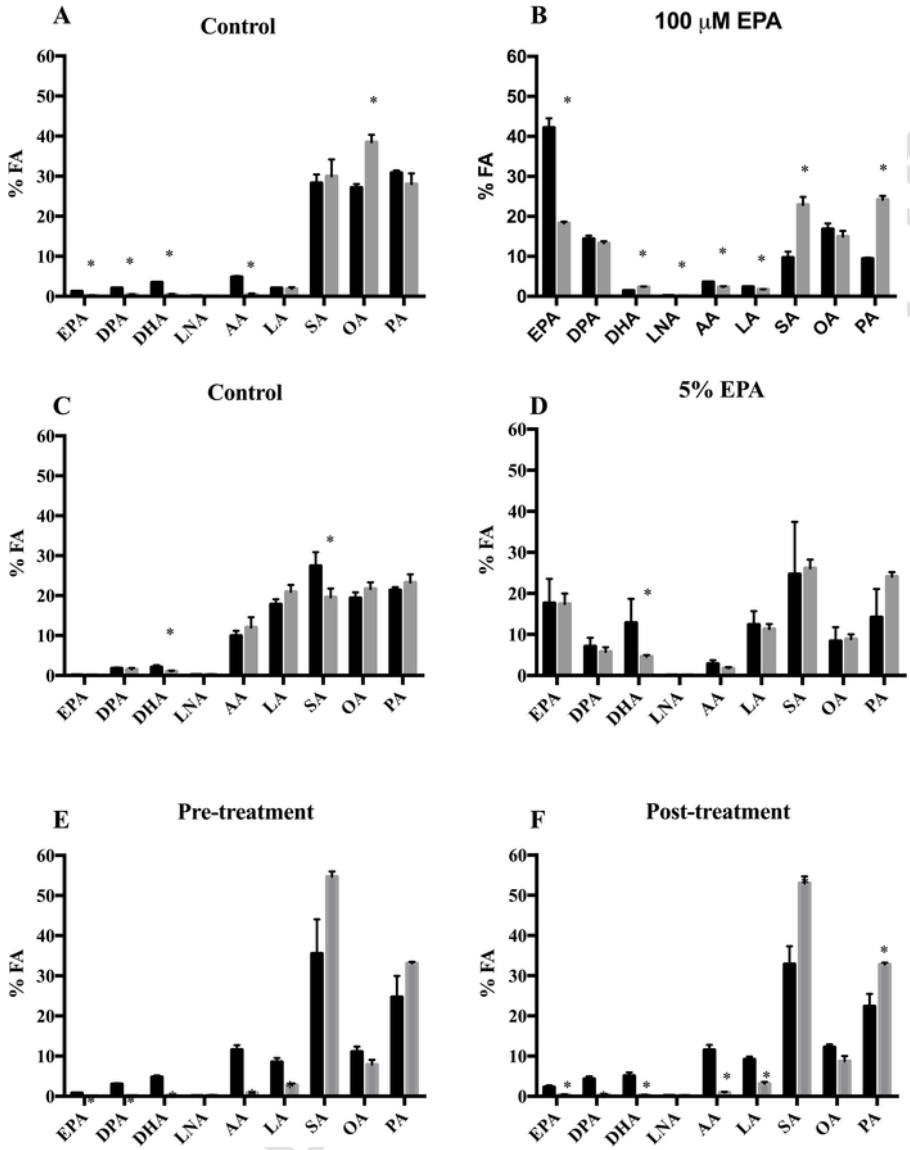

Fig. 7. Free (unesterified) and total (free + esterified) fatty acid distribution in biological samples. Relative FA content of mouse MC38 mouse CRC cells (A and B), mouse liver (C and D) and erythrocytes (E and F) in the absence or presence of EPA-FFA treatment. FA content was measured by LC–ESI-MS/MS either with (■) or without hydrolysis (■) during FA extraction in order to compare the relative amount of each FA total (■) or as the free FA only (■), respectively. In each case, data are the mean and standard deviation for $n = 12$ (A and B), $n = 5$ (C and D) and $n = 8$ (E and F). *denotes a statistically significant difference in %FA relative distribution between total (free + esterified) and free FAs.

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