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RESEARCH PAPER

COMPARISON OF METHODS OF EXTERNAL AND INTERNAL STANDARD FOR GC DETERMINATION OF DOCOSA-HEXAENOIC ACID

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Present study includes the comparison of methods of external and internal standard for the estimation of parameters like linearity and precision in gas chromatographic analysis of docosahexaenoic acid (DHA) with flame ionization detector at temperature 140°C - 280°C and using hydrogen as gas carrier. Linear dependence of the ratio of area of methylated DHA/area of methyl nonadecanoate (internal standard) to the DHA concentration was obtained. There was no observed difference between the correlation coefficients for two methods (R^2 = 0.9928). From the experimental results, it is obvious that related standard deviation (RSD) for internal standard method is more than RSD for external standard method, which demonstrates that nonadecanoic acid is not a suitable internal standard. The external standard method allows achievement of good linearity and precision and is appropriate for identification and determination of DHA and fatty acids in food supplements.

Key words: Docosahexaenoic acid, GC, External standard, Correlation coefficient, Linearity, Precision.

INTRODUCTION

Health benefits of omega-3 fatty acids include cardioprotection, control of arthritis and reduction of risk of Alzheimer's disease and dementia (Hamilton *et al* 2010). Fish oil has a wide variety of fatty acids, from 16-carbon chain length palmitic and palmitoleic acids to 20- and 22-carbon long fatty acids like docosahexaenoic acid (DHA; 22:6 n-3) and eicosapentaenoic acid (EPA; 20:5 n-3). Vegetable oils are mainly rich of 16-carbon and 18-carbon chain length fatty acids (Hernandez and Hosokawa, 2011). α-Linolenic acid (18:3 n-3) is found in walnuts, beans, oils of flaxseed and soybeans. It's conversion to the more functionally important long-chain omega-3 polyunsaturated fatty acids like DHA and EPA in humans is limited and varies among individuals (Djuricic *et al* 2014). DHA and EPA are associated with health benefits (Swanson *et al* 2012) as improvement of cognition (McCann and Ames, 2005) during fetal and infant development (Kleiner *et al* 2014) and of behavior in children with attention deficit/hyperactivity disorder (Milte *et al* 2012) (**Figure 1**). Higher DHA levels taken for six months improve memory and learning mental flexibility in older adults with mild memory deficiency (Yurko-Mauro *et al* 2010).

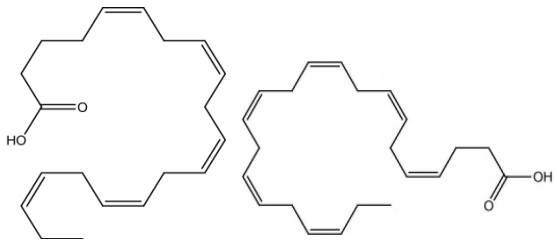

Fig. 1. Chemical structures of DHA and EPA

Epidemiological and clinical studies have established that DHA and EPA protect against coronary heart disease, are anti-arrhythmic agents which improve vascular endothelial function, lower blood pressure and serum triglyceride levels (Wijendran and Hayes, 2004). DHA and EPA supplementation during pregnancy not only benefits the fetus, but also leads to healthier pregnancies by increasing gestation duration and infant birthweights (Kleiner *et al* 2014).

DHA is an omega-3 fatty acid that is a primary structural component of the human brain, cerebral cortex, skin and retina and is important for function of central nervous system (Guesnet and Alessandri, 2011) and for cognition enhancement (Sharma and Yadav, 2012). DHA-derived neuroprotectin D1 has regulatory interactions with the molecular-genetic mechanisms, affecting beta-amyloid precursor protein and due to neurotrophic, anti-apoptotic and anti-inflammatory signaling, regulates brain cell survival.

Deficits of DHA are associated with inflammation, apoptosis and neuronal dysfunction in Alzheimer's disease (Lukiw and Bazan, 2008). DHA supplementation decreases serum C-reactive protein (Kelley *et al* 2009) and attenuates nuclear factor kappa B activity and resultant inflammatory mediators are interleukin 6, interleukin 8 and tumor necrosis factor α (Cotogni *et al* 2011). DHA produces a potent anti-inflammatory N-acyl ethanolamine that reduce expression of interleukin 6 (Balvers *et al* 2010).

An important role of DHA in the retina is suggested by its high levels in the tissue (Horrocks and Yeo, 1999). A neonatal dietary supply of DHA is required for the normal development of retinal function (Jeffrey *et al* 2001). For analysis of fatty acids in oils, fats, foods and biological samples, capillary electrophoresis (Oliveira *et al* 2001) is utilized with different detection possibilities like nonaqueous capillary electrophoresis with near-infrared fluorophore detection (Gallaher and Johnson, 2000) and indirect UV and conductivity detection (Surowiec *et al* 2004). Several methods have been reported for identification and quantification of DHA and EPA in dietary supplements and biological samples. The determination of DHA and EPA in fish oil has been reported by high-performance liquid chromatography (HPLC) (Dillon *et al* 2013) and HPLC with mass spectrometry (MS) detector (Lacaze *et al* 2017; Salm *et al* 2011).

For simultaneous determination of DHA, EPA and arachidonic acid in human plasma, HPLC with electrochemical detector using the reverse-phase C30 column and detection based on the voltammetric reduction of 3,5-di-*tert*-butyl-1,2-benzoquinone is used (Kotani *et al* 2016).

Fatty acids from different plants like *Macadamia integrifolia* (Carrillo *et al* 2017), *Caryodendron orinocense* Karst (Carrillo *et al* 2018), *Juglans neotropica* Diels (Vilcacundo *et al* 2018) are identified as methyl esters using the GC/MS method at the conditions such as column DB-WAX (60 m × 250 μm × 0.25 μm); oven temperature from 80 °C to 100 °C at 20 °C/min; injector and detector temperatures: 250 °C and helium as carrier gas at a linear flow velocity of 1.4 ml/min (Carrillo *et al* 2017; Carrillo *et al* 2018; Vilcacundo *et al* 2018).

Fatty acids from oil extracted from *Macadamia integrifolia* seeds are determined as 9.11 % palmitic acid, 3.93 % stearic acid, 83.36 % oleic acid, 3.79 % linoleic acid and 1.69 % linolenic acid (Carrillo *et al* 2017). Fatty acids from *Caryodendron orinocense* Karst seeds are determined as 7.0 % palmitic acid, t_R = 19.321 min; 3.47 % stearic acid, t_R = 25.955 min; 18.59 % oleic acid, t_R = 26.856 min; 68.04 % linoleic acid t_R = 28.628 min; 2.90 % linolenic acid, t_R = 31.092 min (Carrillo *et al* 2018). The fatty acids obtained from *Juglans neotropica* Diels are 5.05 % palmitic acid, t_R = 19.378 min; 2.26 % stearic acid, t_R = 26.057 min; 19.50 % oleic acid, t_R = 26.919 min; 65.81 % linoleic acid, t_R = 28.693 min and 2.79 % linolenic acid, t_R = 31.092 min) (Vilcacundo *et al* 2018).

GC/MS is reported with a highly polar capillary column (CP-Sil 88, 100 m) for simultaneously determination of DHA and EPA as active and fish oil nutritional capsules. For analysis of fish oil nutritional capsules, GC/MS with the conditions like DB-5 capillary column (0.25 μm × 30 m × 0.25 mm); oven temperature maintained at 80 °C for 1 min followed by increase at a rate of 10 °C/min to 250 °C, and then 8°C/min until 280 °C; helium as carrier gas at a flow rate of 0.8 ml/min; mass spectrometer in electron-impact mode with injection temperature 250 °C, is used (Yi *et al* 2014).

For alone analysis of DHA in infant preparations, GC method is reported at the following conditions like VB-wax capillary column (60 m × 0.32 mm × 0.25 μm); injector temperature 230 °C, flame-ionization detector temperature 250 °C, oven temperature to increase from

130 °C to 230 °C by 2 °C/min, helium as carrier gas at a flow rate of 2.0 ml/min (Harmita *et al* 2018).

The aim of current study was the comparison of methods of external and internal standard for the estimation of parameters such as linearity and precision in gas chromatograpic analysis of DHA with flame ionization detector on capillary column ZB-1701 (cyanopropyl methyl siloxane) (30 m × 0.25 mm × 0.25 μm); different temperature program like 5 min (140 °C), 140 °C – 240 °C at 4 °C/min, 20 min (240 °C), 240 °C – 280 °C at 6 °C/min, 20 min (280 °C) and hydrogen as gas carrier.

MATERIALS AND METHODS

Materials

Reference substances

All reagents used were of analytical grade quality like derivatizing reagent: boron trifluoride (99.5 %) (Sigma Aldrich, N:339963); *n*-hexane (Valerus, N:UN 1208); isooctane (99.7 %) (Sigma Aldrich, N:59030); methanol (99.9 %) (Sigma Aldrich, N:SZBD 063AV UN 1230); nitrogen (Messer Grisheim, N:00474); potassium hydroxide (Fluka, N:757 551) and toluene (99.8 %) (Sigma Aldrich, N:244511). Docosahexaenoic acid (≥ 98 % for GC) (Sigma Aldrich, N:D2534); Nonadecanoic acid (≥ 98 % for GC) (Sigma Aldrich, N:5252).

Methods

Gas chromatography equipment included gas chromatograph "Autosystem" (Perkin Elmer, USA), equipped with a split-splitter injector, flame ionization detector, capillary column ZB-1701 (cyanopropyl methyl siloxane) (30 m × 0.25 mm × 0.25 μm) (Phenomenex Inc.); hydrogen generator (HGH-300E, Beijing uiland, China); compressor for compressed air with a system of filters; analytical balance; air thermostat.

Chromatographic conditions

Inlet gas carrier (hydrogen) pressure 15 psi; temperature program: 5 min at 140 °C, increasing temperature to 240 °C at 4 °C/min, holding at 240 °C for 20 min, raising the temperature to 280 °C at a speed of 6 °C/min. and keeping temperature for 10 min at 280 °C.

Preparation of methylated standard solutions of DHA

200 μl of internal standard nonadecanoic acid solution in isooctane was introduced into reaction vessels and the solvent was removed by purging with nitrogen at room temperature. To the dry residue was added 2.0 ml of a 14 % boron trifluoride solution in methanol and respectively aliquots of 100 μl, 200 μl, 400 μl and 800 μl from 500 μg/ml DHA standard solution. The reaction vessels were immediately sealed with teflon laminated septum and purged with nitrogen. The reaction mixture slides were incubated for 12 min at 65 °C, then cooled and by a syringe, 1.0 ml of water and 1.0 ml of *n*-hexane were introduced into the vessels. The samples were shaken for 1-2 min for an extraction of the methylated acids, transferred rapidly to 10 ml tubes and after separation of the phases, 200 μl of *n*-hexane extract was introduced in an autosampler container. Aliquot parts of 1 μl of the methylated acid extract were introduced into the gas chromatograph for analysis.

RESULTS AND DISCUSSION

Due to the high polarity of the carboxyl group of the fatty acids and their ability to form hydrogen bonds, their direct gas chromatographic analysis requires particularly inert (special) stationary phases. The overcoming of the adverse effects associated with the carboxyl group polarity is achieved by converting the acids into esters, acyl or silyl derivatives. The methylation of the carboxyl group is most commonly used for this purpose.

According to the lnternational Conference on Harmonization (ICH), method validation was performed to ensure that an analytical methodology is appropriate. Analytical parameters like linearity, LOD (limit of detection), LOQ (limit of quantitation) and accuracy were estimated and compared by methods of internal and external standard in the specified concentration range of the analyte.

Selectivity

A "placebo" solution without the active substance DHA and internal standard nonadecanoic acid was prepared in the same manner like sample solutions. The selectivity of the applied GC method was proved by the fact, that on the chromatograms with "placebo" solution, there were no peak with retention time, corresponding to the methyl ester of DHA: t_R DHA methyl ester = 34.517 min or retention time of internal standard: t_R nonadecanoic acid methyl ester = 27.560.

Linearity

The dependence of the chromatographic peak area of DHA from the amount was examined by obtaining the chromatograms of a series of the methylated standard solutions of docosahexaenoic acid and an internal standard of the nonadecanoic acid. Chromatograms of methylated standard solutions of docosahexaenoic acid and internal standard are shown in **Figure 2**.

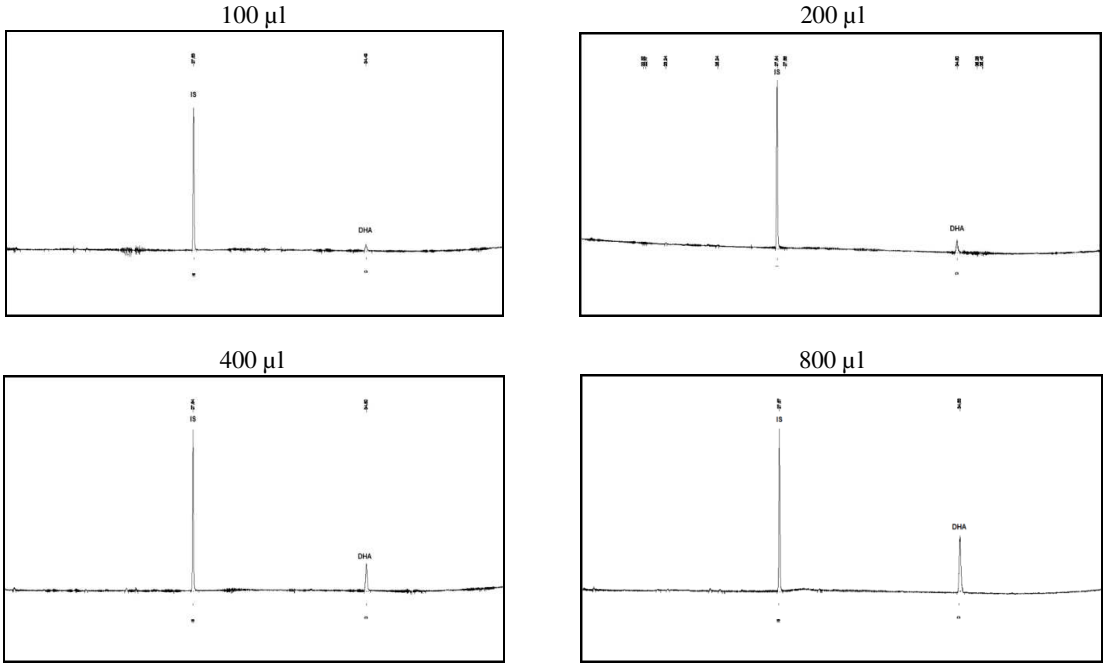

Fig. 2. Chromatograms of methylated DHA standard and methylated internal standard nonadecanoic acid

Linearity for methylated standard solutions of docosahexaenoic acid is illustrated in **Figure 3** by internal standard and in **Figure 4** by external standard.

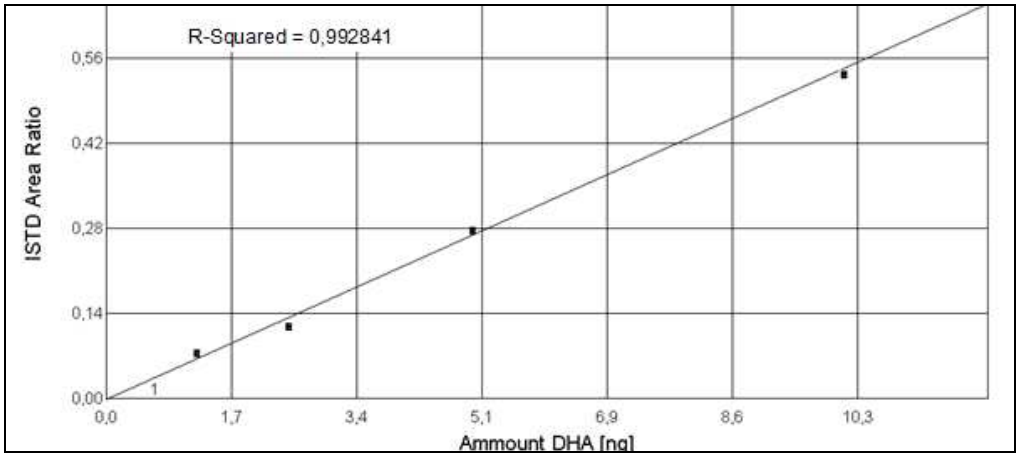

Fig. 3. Linearity for methylated DHA standard with methylated internal standard nonadecanoic acid

The standard graph showed that for chromatographic quantities up to 400 ng, the dependence of the ratio of area of the methylated DHA/area of methyl nonadecanoate (internal standard) to the DHA quantity is linear. There is not obtained difference between the correlation coefficients for methods of internal and external standard (R_2 = 0.9928).

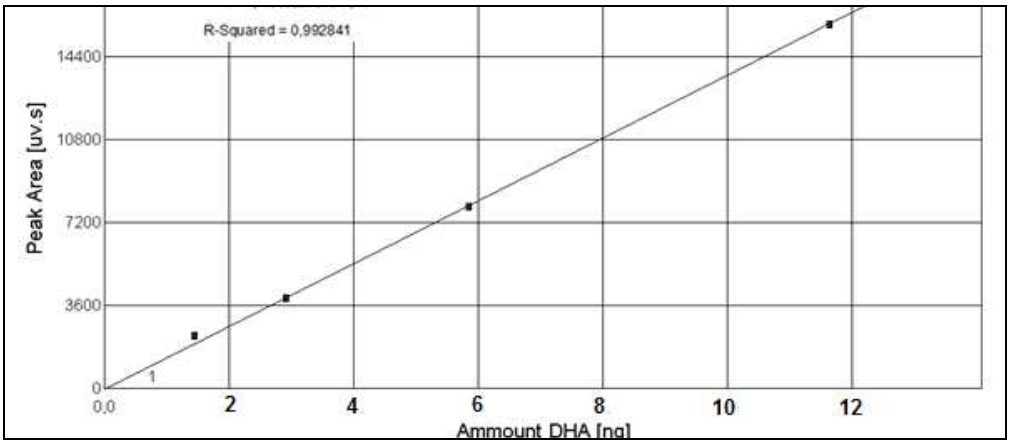

Fig. 4. Linearity for methylated DHA external standard

Estimation of analytical parameter sensitivity

The sensitivity of the analytical method is expressed by the ratio signal/noise (S/N). This method of expressing presents the sensitivity of the GC by LOD (concentration, at which the obtained ratio S/N is 3:1) and LOQ (concentration, at which the obtained ratio S/N is 10:1). For the determination of the detection limit, the standard graph was used from which the ratio S/N was determined at 100 μl solution of DHA at a concentration of 500 μg/ml, divided in 1 ml of hexane extract (which corresponds to concentration of 50 μg/ml), from which in the chromatograph was introduced 1 μl (50 ng) in a splitter ratio of 1 : 40 (LOQ = 1.25 ng, LOQ = 12.5 ng).

Study of repeatability in determination of DHA with external and internal standard

The precision (repeatability) of the measurement of peak area of MDHA (with an external standard), and the ratio of the areas of the chromatographic peaks of MDHA and the internal standard was determined by three reproduction of the analytical procedure for derivatization and extraction with *n*-hexane in the introduction of 400 μl standard solution of DHA at a concentration of 500 μg/ml (**Figure 5**).

REPORT FOR PRECISION OF DHA MEASUREMENT

Peak #	Time [min]	Area [uV*sec]	ISTD Resp Ratio	Component Name
28	27,565	28625,89	1,0000	IS
33	34,526	7887,46	0,2755	DHA

REPORT FOR PRECISION OF DHA MEASUREMENT

Peak #	Time [min]	Area [uV*sec]	ISTD Resp Ratio	Component Name
18	27,543	28669,08	1,0000	IS
22	34,496	7921,44	0,2763	DHA

REPORT FOR PRECISION OF DHA MEASUREMENT

Peak #	Time [min]	Area [uV*sec]	ISTD Resp Ratio	Component Name
27	27,572	28229,39	1,0000	IS
30	34,529	7872,12	0,2789	DHA

Fig. 5. Chromatographic peak areas of methylated DHA standard and methylated internal standard nonadecanoic acid

Precision (repeatability) was estimated by the uncertainty of the result, determined by standard deviation (SD) and relative standard deviation (RSD). In **Table 1**, for the estimation of precision, the results for the peak area (A): A_{MDHA}, A (nonadecanoic acid methyl ester); Chauvenet's criterion for area (U): U A_{MDHA}, U A (nonadecanoic acid methyl ester); arithmetical mean; standard deviation (SD) and relative standard deviation (RSD) [%] are summarized. RSD for method with the internal standard nonadecanoic acid (0.851) is more than RSD for method with external standard DHA (0.318).

Table 1. Area of peaks of methylated DHA standard and methylated internal standard nonadecanoic acid

N:	A_{MDHA}	U A_{MDHA}	$A_{Nonadecanoic\ acid\ methyl\ ester}$	$A_{MDHA}/A_{Nonadecanoic\ acid\ methyl\ ester}$
1	7872	0.84	28229	0.2789
2	7887	0.24	28626	0.2755
3	7921	1.12	28669	0.2763
$\overline{x}$	7893		28508	0.2769
SD	25.11		24.6	0.00178
RSD [%]	0.318		0.851	0.0064

Test for system suitability

The suitability of the system was confirmed by the lack of a statistically significant difference between the values of the chromatographic parameter retention time in the analysis of 3 samples of MDHA: SD = 0.018, RSD = 0.05 % and internal standard nonadecanoic acid methyl ester: SD = 0.015, RSD = 0.05 % (**Table 2**).

Table 2. Retention times for methylated DHA standard and methylated internal standard nonadecanoic acid

N:	$t_{R\ DHA\ (min)}$	U $t_{R\ DHA}$	$t_{R\ Nonadecanoic\ acid\ (min)}$	U $t_{R\ Nonadecanoic\ acid}$
1	34.529	0.67	27.572	0.80
2	34.526	0.50	27.565	0.33
3	34.496	1.17	27.543	1.33
$\overline{x}$	34.517		2 7.560	
SD	0.018		0.015	
RSD [%]	0.05		0.05	

CONCLUSION

The comparison of the correlation coefficients of the two standard graphs constructed respectively with the internal standard and the external standard method showed that the internal standard practically does not contribute to higher precision in the determination of DHA by the method described. RSD for the internal standard is more than RSD for DHA, which demonstrates that nonadecanoic acid is not a suitable internal standard for the determination of polyunsaturated fatty acids and their determination without internal standard allows achieving sufficiently good reproducibility.

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