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Analiza vsebnosti omega-3 maščobnih kislin v prehranskih dopolnilih

Analysis of omega-3 fatty acids contents in dietary supplements

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POVZETEK

Dandanes z navadno prehrano vnesemo premalo nujno potrebnih snovi za normalno delovanje organizma. Z intenzivnim kmetovanjem siromašimo prst in posledično se to kaže v hrani, ki ni dovolj bogata z vitamini in minerali. Nadomeščanje vitaminov, mineralov in drugih hranil se je razvilo v obliki prehranskih dopolnili, ki so postala velika tržna niša. Zaradi ohlapnega nadzora nad njimi se postavlja vprašanje o njihovi kakovosti, zato smo se odločili, da preverimo vsebnosti omega-3 maščobnih kislin v prehranskih dopolnilih. Naša prehrana vsebuje kompleksne zmesi različnih lipidov, ki vključujejo trdne, poltrdne in tekoče maščobe (olja), katerih osnovno strukturo sestavljajo maščobne kisline zaestrene z glicerolom v trigliceride. Pomembno vlogo pri nemotenem delovanju organizma imajo omega-3 maščobne kisline, ki jih naše telo ne zmore samo sintetizirati, zlasti EPK in DHK, ki ju sintetizirajo morski organizmi, plankton. Ker z navadno prehrano vnesemo dovolj omega-6 maščobnih kislin, je pomembno, da zaužijemo tudi dovolj omega-3 maščobnih kislin, če ne gre drugače, občasno tudi v obliki prehranskih dopolnil.

Namen magistrskega dela je analizirati naključno izbrane izdelke prehranskih dopolnil, ki vsebujejo omega-3 maščobne kisline in primerjati deklarirane vsebnosti na ovojnini izdelka s podatki, pridobljenimi z eksperimentalnim delom. Analize maščobnih kislin smo se lotili s pretvorbo v pripadajoče metilne estre, ki smo jih naredili po farmakopejski metodi B in paralelno z metodo *in situ* preestrenja. Slednja se je izkazala za bolj učinkovito. Vsebnost nastalih metilnih estrov maščobnih kislin smo ugotovili z uporabo plinskega kromatografa, sklopljenega z masnim spektrometrom. Koncentracije metilnih estrov maščobnih kislin v vzorcih smo kvantificirali s pomočjo njihovih standardov in jih nato preračunali v miligrame maščobnih kislin na gram olja (vzorca). Rezultati opravljenih analiz so zbrani v obliki tabele, kjer lahko iz vsakega vzorca razberemo, koliko je prisotne določene omega-3 maščobne kisline in odstotek odstopanja od deklarirane vrednosti pri klasični farmakopejski metodi B in *in situ* metodi. Glede na deklarirane vrednosti in priporočila (USP DSC) večina analiziranih prehranskih dopolnil vsebuje navedene koncentracije maščobnih kislin.

Vsebnosti maščobnih kislin, ugotovljenih s klasično farmakopejsko metodo B, so bistveno nižje od *in situ* metode priprave vzorcev. Razlog je v nepopolni derivatizaciji, zato je farmakopejska metoda primerna samo za ugotavljanje razmerja posameznih maščobnih kislin, ne pa za absolutno kvantifikacijo.

ABSTRACT

Today, consumption of ordinary food does not provide enough essential substances for the normal function of the human body. With intensive farming the soil became depleted of and essential components, which has a strong impact on plants we cultivate for food. Food is not rich enough in minerals, vitamins and other nutrients. Therefore the use of dietary supplements became popular with consequent development of a big market niche. Regulation and supervision of dietary supplements is very loose, which brings in question the quality of such products. Therefore, we decided to examine the contents of omega-3 fatty acids in dietary supplements.

Our food contains a complex mixture of different lipids, which they are in solid, semisolid and liquid states. The main part is composed of fatty acids esters with glycerol (triglicerides). Omega-3 fatty acids have an important role in human physiology and they can not be synthesized in a human body, especially EPA and DHA, which are synthesised by marine microorganisms and plankton. With ordinary food we get enough omega-6 fatty acids, but we must also provide enough omega-3 fatty acids, occasionally also with dietary supplements if there is no other option.

The purpose of this thesis was to analyse randomly selected dietary supplementary products containing omega-3 fatty acids and to compare declared content with experimental results. We started the analysis of fatty acids with conversion of fatty acids into corresponding methyl esters. The method described in Europeian Pharmacopoeia, which was modified was used parallel with *in situ* method. Latter subsequently proved to be more efficient. The content of the generated fatty acid methyl esters was then determined using a gas chromatograph connected to mass spectrometer. Concentrations of fatty acids in the samples were quantified by reference compounds (standards) and then converted into mg of fatty acids per gram of sample(s)/oil. All results are collected in a table, where the content of omega-3 fatty acids and deviation from declared content are presented according to Pharmacopoeia method B and *in situ* method.

The contents of fatty acids defined with Pharmacopoeia method B, are lower then contents of fatty acids obtained with *in situ* method. The main reason is incomplete derivatisation, which makes Pharmacopoeia method B appropriate for the determination of fatty acids ratios only and not for the absolute quantification.

SLOVARČEK IN SEZNAM OKRAJŠAV

α- LA ali ALA - (angl. alpha-linolenic acid) alfa linolenska kislina

BF_3 – borov trifluorid

DHA ali DHK - (angl. docosahexaenoic acid) dokozaheksaenojska kislina

DPA ali DPK - (angl. docosapentaenoic acid) dokozapentaenojska kislina

EPA ali EPK - (angl. eicozapentaenoic acid) eikozapentaenojska kislina

F.A.M.E. Mix C20:1-C20:5 – (angl. Fatty Acid Methly Esters) metilni estri maščobnih kislin Mix C20:1-C20:5

FID – (angl. Flame Ionisation Detector) plamenski ionizacijski detektor

GC – (angl. Gas Chromatography) plinska kromatografija

GC-MS – (angl. Gas Chromatography - Mass Spectrometry) plinska kromatografija sklopljena z masno spektrometrijo

HDL – (angl. High Density Lipoproteins) lipoproteini visoke gostote

IUPAC – (angl. International Union of Pure and Applied Chemistry) Mednarodna zveza za čisto in uporabno kemijo

LDL – (angl. Low Density Lipoproteins) lipoproteini nizke gostote

LK ali LA – (angl. linoleic acid) linolna kislina

MEMK – metilni estri maščobnih kislin

MK – maščobna kislina

m/z – (angl. mass-to-charge value) razmerje mase z nabojem

nevrogeneza – delitev, razvojna usmeritev in celotni razvoj nezrelih celic do zrelega nevrona

nevrotransmiter – živčni prenašalec (endogena kemikalija), ki prenaša signale po nevronih preko sinapse

RDA – (angl. Recommended Dietary Allowance) priporočen dnevni odmerek

RPM – (angl. Rotations Per Minute) obrati na minuto

S/N – (angl. Signal-to-Noise ratio) razmerje med signalom in šumom

TMK – *trans* maščobna kislina

USP DSC – (angl. United States Pharmacopeial Convention Dietary Supplement Standards) Konvencija ZDA za standardizacijo prehranskih dopolnil

VNMK – večkrat nenasičene maščobne kisline

1 UVOD

Z večanjem števila prebivalstva na Zemlji se povečuje tudi potreba po hrani. Ta je na žalost še vedno neenakomerno porazdeljena po zemeljski obli. Kmetijstvo ne dohaja vseh potreb po kakovostno pridelani hrani. Z intenzivnim kmetovanjem in obdelovanjem s ciljem hitrejše rasti pridelka prst osiromašimo mineralov. Današnja težnja modernega človeka je zdrav način življenja, ki vključuje zdravo prehrano, dovolj gibanja in obvladovanje stresa. Kot pravi rimski pesnik Juvenalis, avtor latinskega pregovora: "*Mens sana in corpore sano*" (zdrav duh v zdravem telesu).

Znanstveniki ugotavljajo, da v razvitem svetu kar okrog 95 % ljudi ne uživa tistega, kar telo potrebuje za ohranitev dobrega zdravja, in prek 70 % ljudi uživa hrano, ki zdravju celo škoduje. Vse bolj se uveljavlja mnenje, da je prav pomanjkanje vitaminov in mineralov v živilih eden najpomembnejših vzrokov za naraščanje kroničnih degenerativnih bolezni v sodobnem svetu. Zato se je povečalo povpraševanje po izdelkih, ki naj bi pripomogli k našemu zdravju tudi v povezavi z rastjo športnih in zdraviliških centrov, uspešne dejavnosti glavnih ponudnikov (proizvajalcev) na trgu ter dejavnejšega osveščanja potrošnikov. Vedno več ljudi se odloča za preventivo, s katero lahko preprečijo razvoj bolezni, trendi pa kažejo tudi rastoče povpraševanje po naravnih proizvodih. Želja po zdravem načinu življenja, višanje stroškov zdravljenja in staranje prebivalstva so ustvarili trg funkcionalnih živil, naravnih proizvodov in prehranskih dopolnil (1).

1.1 Prehranska dopolnila

Pojem ali termin prehranska dopolnila opredeljuje Pravilnik o prehranskih dopolnilih, ki je bil sprejet 30. 07. 2003 (datum prihoda v veljavo je 5. 9. 2003) in povzema Direktivo evropskega parlamenta in Sveta o prehranskih dopolnilih, št. 2002/46/EC.

Pravilnik o prehranskih dopolnilih je objavljen v Uradnem listu Republike Slovenije in opredeljuje prehranska dopolnila kot »živila, katerih namen je dopolnjevati običajno prehrano. So koncentrirani viri posameznih ali kombiniranih hranil ali drugih snovi s hranilnim ali fiziološkim učinkom, ki se dajejo v promet v obliki kapsul, pastil, tablet in drugih podobnih oblikah, v vrečkah s praškom, v ampulah s tekočino, v kapalnih stekleničkah in v drugih podobnih oblikah s tekočino in praškom, ki so oblikovane tako, da se jih lahko uživa v odmerjenih majhnih količinskih enotah« (1, 2).

Pravilnik podaja razlago, da naj pod »hranili« razumemo samo vitamine in minerale, a na drugi strani pa najdemo v zakonodaji razlago, da hranilo pomeni tudi beljakovine, ogljikohidrate, maščobe, prehranske vlaknine in natrij. V skladu s tem lahko prehranska dopolnila poleg vitaminov in mineralov vsebujejo tudi aminokisline, alge, maščobne kisline in lecitin, prehranske vlaknine, rastline in rastlinske izvlečke, mikoorganizme, méd in čebelje pridelke, encime, olja ter druge snovi s hranilnim ali fiziološkim učinkom, pod pogojem, da je njihova varnost v prehrani ljudi znanstveno utemeljena (1, 2, 3).

Prehranska dopolnila so živila, hrana. Čeprav po svoji obliki in vsebini ter načinu uporabe lahko spominjajo na zdravila, to niso (1). Na začetku smo definirali, kaj je prehransko dopolnilo. Na tem mestu še je treba definirati, kaj je zdravilo. Po Zakonu o zdravilih, je zdravilo vsaka snov ali kombinacija snovi, ki so predstavljene z lastnostmi za zdravljenje, preprečevanje ali ugotavljanje bolezni pri ljudeh ali živalih. Za zdravilo velja tudi vsaka snov ali kombinacija snovi, ki se lahko uporabljajo pri ljudeh ali živalih ali se daje ljudem ali živalim z namenom, da bi se ponovno vzpostavile, izboljšale ali spremenile fiziološke funkcije prek farmakološkega, imunološkega ali presnovnega delovanja, ali da bi se določila diagnoza (4).

Kljub nekim skupnim točkam med zdravilom in prehranskim dopolnilom, obstajajo bistvene razlike glede namena uporabe ter dokazne učinkovitosti za zdravljenje bolezni in bolezenskih stanj. Zdravilo lahko vsebuje prehransko dopolnilo, hranilo, obratno pa to ne gre. Če je izdelek s prehranskim dopolnilom predstavljen potencialnemu potrošniku kot zdravilo ali da dobi kupec vtis (ovojnina, specifikacija, oglaševanje, navodila za uporabo…), da gre za zdravilo, se izdelek avtomatično razvrsti med zdravila. V takem primeru preide v pristojnost JAZMP (Javna agencija za zdravila Republike Slovenije). Izdelek ne sme biti na trgu, dokler ne pridobi dovoljenja za promet z zdravilom. Dovoljenje za promet z zdravilom je zagotovilo za kakovost, varnost in učinkovitost zdravil. Le pri zdravilih z dovoljenjem za promet pristojni državni organi preverijo njihovo kakovost, varnost, učinkovitost (preden gre izdelek v prodajo). Zdravila so izdelana po principu in v skladu dobre proizvodne prakse, ki je pod ustreznim inšpekcijskim nadzorom. Zdravila imajo v navodilu za uporabo in na ovojnini verodostojne informacije o področju uporabe, indikacijah, opozorilih, učinkovitosti, kontraindikacijah, previdnostnih ukrepih, interakcijah, neželenih učinkih. Enako velja za registrirana zdravila rastlinskega/naravnega izvora, vključno s tradicionalnimi zdravili (2).

Kadar pride do dvoma o tem ali gre za zdravilo ali prehransko dopolnilo, se uporabljajo določbe 7. člena Zakona o zdravilih, kjer se s posebnim ugotovitvenim postopkom ugotavlja, ali se izdelek uvršča med zdravila ali prehranska dopolnila. Mejni izdelki med zdravili in prehranskimi dopolnili se pojavijo predvsem v povezavi z učinkom na fiziološke funkcije. Prehranska dopolnila fiziološke funkcije ohranjajo in jih podpirajo, medtem ko jih zdravila lahko tudi ponovno vzpostavijo, spreminjajo ali celo izboljšajo (2).
Kadar se prehransko dopolnilo prvič daje v promet v Republiki Sloveniji, mora proizvajalec, uvoznik izdelka, nosilec živilske dejavnosti, ki daje prvič v promet izdelek, o tem obvestiti pristojno ministrstvo (Ministrstvo za zdravje RS) z izpolnjeno vlogo v skladu s Pravilnikom o prehranskih dopolnilih. Nadzor nad prehranskimi dopolnili izvaja v okviru načrtovanega nadzora Zdravstveni inšpektorat Republike Slovenije (2).

1.1.1 Namen in uporaba prehranskih dopolnil

Zanimanje širše javnosti za uporabo prehranski dopolnil se iz leta v leto veča in s tem tudi njihovo uživanje. Raziskava »Nacionalna prehrana in prehranjevanje« (angl. *National Diet and Nutrition*) je pokazala, da kar 40 odstotkov Britancev starih od 19 do 64 let uporablja prehranska dopolnila. V Veliki Britaniji je prodaja prehranskih dopolnil v letu 2005 štela okrog £ 326 milijonov (3).
Prehranska dopolnila niso ekvivalent za uravnoteženo prehrano, saj le-ta vsebujejo vitamine in minerale v takih količinah, kot jih naše telo potrebuje. Obstajajo pa posamezniki in tudi določene skupine ljudi, z visokim tveganjem za pomanjkanje dnevno zaužitih hranil, ki upravičeno konzumirajo multivitaminska in mineralna dopolnila in jim le-ta prinašajo korist. Te segmentne skupine so:

- ženske, otroci, najstnice in starejši
- strogi vegetarijanci ali vegani
- kronični dietiki, ki dnevno zaužijejo manj kot 1200 kalorij
- ljudje, ki ne morejo uživati vseh mlečnih izdelkov ali imajo omejitve glede izpostavljenosti soncu
- kronični bolniki (rak, diabetes, AIDS…), ki jemljejo zdravila, sledijo posebnim dietam ali okrevajo po operaciji ali travmi
- alkoholiki
- kadilci (3).

1.1.2 Varnost, interakcije in neželeni učinki prehranskih dopolnil

Prehransko dopolnilo mora biti varno za uporabo z vidika sestave in primerno označeno ter predstavljeno potrošniku. Varno uporabo prehranskih dopolnil med drugim omogoča tudi ustrezno označevanje. Neustrezna označba izdelka lahko predstavlja tveganje za uporabnika, še posebej v primerih, ko niso navedene vse sestavine izdelka (vključno z navedbo vseh možno prisotnih alergenih snovi), rok uporabnosti, kot to določajo predpisi, in predpisana opozorila. Poleg z zakonom predpisanih opozoril in navedb mora označba prehranskega dopolnila navajati morebitna druga opozorila za uporabnika (npr. če obstajajo posebne skupine, ki se naj proizvodu izogibajo). Izdelek mora ustrezati mikrobiološkim merilom za živila, ne sme presegati mejnih vrednosti nečistot, ne sme vsebovati nedovoljenih novih živil, nedovoljenih aditivov oziroma aditivov v koncentracijah, višjih od predpisanih (dovoljenih). Onesnaževalo ali nečistota je snov, ki ni namerno dodana hrani. Prisotnost le-te je posledica proizvodnje, izdelave, predelave, priprave, obdelave, pakiranja, transporta ali hrambe take hrane ali kot posledica onesnaženosti okolja. Nekaj možnih oziroma prisotnih nečistot v prehranskih dopolnilih: ostanki težkih kovin (svinec, kadmij, živo srebro), ostanki pesticidov, spojine, ki vsebujejo benzenov obroč… Zato se pri preverjanju ustreznosti prehranskih dopolnil glede ustreznosti nečistot uporablja Uredba Sveta (EC) št. 315/93 in kasneje sprejeta Uredba Komisije (ES) št. 1881/2006 o določitvi mejnih vrednosti nekaterih nečistot v živilih (7).

Prehransko dopolnilo mora ustrezati splošni živilski zakonodaji. Živil, ki niso varna, ni dovoljeno dajati v promet. Šteje se, da živilo ni varno, če je izkazana škodljivost za zdravje ali neustreznost za prehrano ljudi. Pri tem se upoštevajo navodila za izvajanje Uredbe (ES) št. 178/2002, kjer je natančno popisan in obrazložen 14. člen uredbe, ki govori o varnosti živil (2, 7).

Za neželen učinek zdravila ali prehranskega dopolnila se šteje kakršen koli škodljiv in nenameren odziv telesa na zdravilo, kadar se pojavi pri odmerkih, ki se navadno uporabljajo za profilakso, diagnozo, za zdravljenje bolezni ali za modifikacijo fiziološke funkcije, medtem ko se toksični učinki pojavijo pri odmerkih, ki so večji od priporočenih. Za varno se šteje še uživanje mineralov in vitaminov v priporočenih odmerkih do 3 RDA (Recommended Dietary Allowance- priporočeni dnevni odmerek), razen za vitamin A in D, ki se smeta dalj časa jemati samo v odmerku 1 RDA. Med številnimi izdelki se zaradi neurejene zakonodaje na trgu pojavljajo tudi manj kakovostni primerki prehranskih dopolnil (proizvodnja zdravil mora delati po načelu dobre proizvodne prakse, medtem, ko

za prehranska dopolnila to ni zakonsko obvezujoče). Ko želimo izvedeti, kdo sme uporabljati prehranska dopolnila, kateri so možni neželeni učinki, interakcije, kontraindikacije, najpogosteje naletimo na naslednje odgovore: ni omejitev, ni interakcij, ni neželenih učinkov, ni kontraindikacij, kar pa ne drži popolnoma. Dolgo so zanemarjali neželene učinke in interakcije, ki jih lahko povzročijo prehranska dopolnila rastlinskega izvora. Preskok v razmišljanju se je spremenil, ko so odkrili, da lahko šentjanževka (lat. *Hypericum perforatum*) in sok grenivke pomembno vplivata na metabolizem številnih zdravil (9).

Podatkov kliničnih študij, ki bi kaj več povedale o samih interakcijah med zdravili in prehranskimi dopolnili ni dovolj. Veliko je poročil o posameznih primerih, a malo uradnih kliničnih raziskav. Vedno pogosteje se izvajajo *in vitro* raziskave o možnih farmakokinetičnih interakcijah zdravilo-prehransko dopolnilo, predvsem take, ki preučujejo vpliv na citokrom P_{450}. Tovrstne raziskave so pomembne, saj se veliko zdravil in prehranskih dopolnil presnavlja v jetrih prav preko tega citokroma (posledično so lahko prehranski dodatki induktorji ali inhibitorji citokroma P_{450} in posledično vplivajo na farmakokinetiko zdravil) (9).

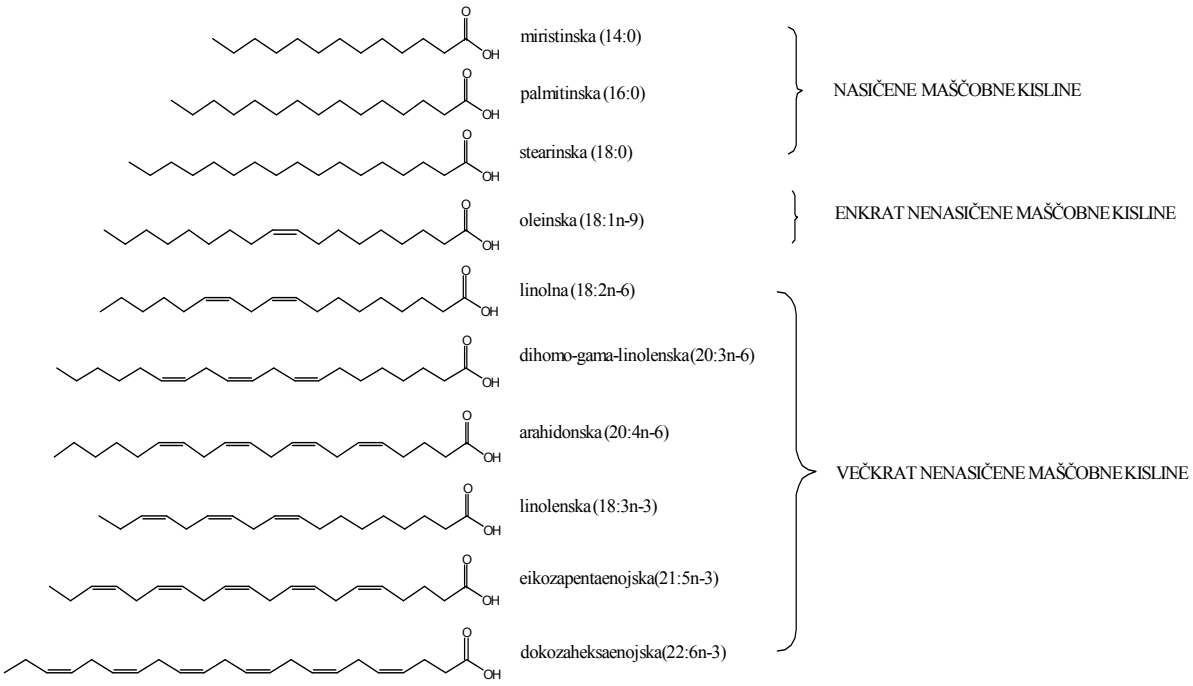
Samozdravljenje ali uživanje prehranskih dopolnil je treba prekiniti, če domnevamo, da je prišlo do neželenih učinkov in interakcij z zdravili. Nedvoumnega odgovora na vprašanje ali je določena prehranska dopolnila varno jemati skupaj z zdravilom, ni mogoče zatrdno odgovoriti (9). Zelo pomembno je, da zdravniki in farmacevti poznajo možne neželene učinke in interakcije med zdravili ter prehranskimi dopolnili, ter da se o njih pogovorijo z bolniki. Pomembnega dejstva ne smemo zanemariti: prehranska dopolnila niso zdravila in zato nimajo nobenih dokazov, da so učinkovita za zdravljenje bolezni (9).

1.2 Maščobne kisline

Naša prehrana vsebuje kompleksne zmesi različnih lipidov, ki vključujejo trdne, poltrdne in tekoče maščobe (olja), katerih osnovno strukturo sestavljajo maščobne kisline zaestrene z glicerolom v trigliceride (10). Maščobne kisline so sestavljene iz elementov vodika, kisika in ogljika, ki so združeni v ravno ogljikovo verigo različnih dolžin s končno karboksilno skupino. Ali drugače povedano: maščobne kisline so biološke molekule, ki vsebujejo polarno karboksilno skupino (-COOH) vezano na nerazvejano alifatsko verigo.

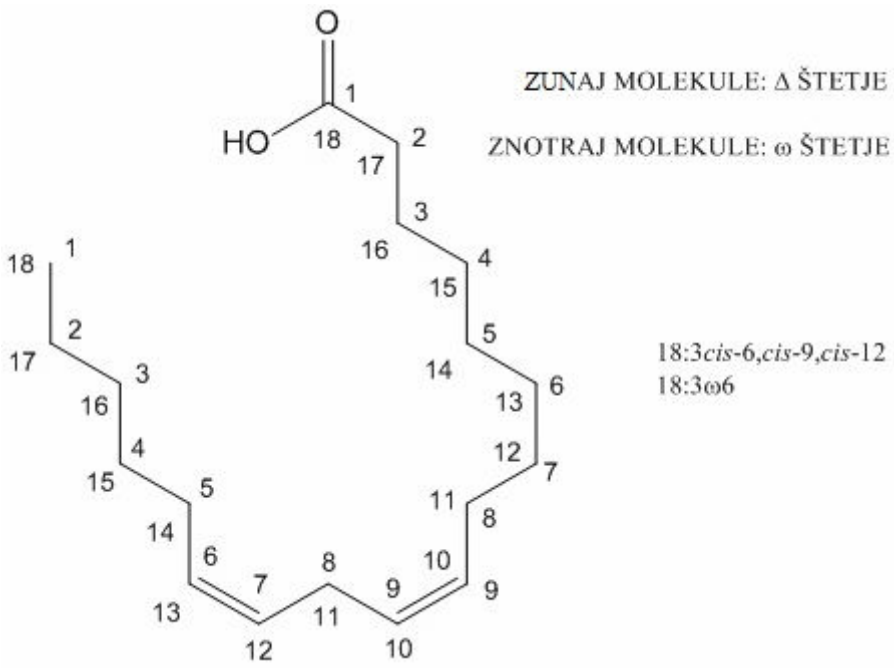
Polarne lastnosti, včasih ionski naboj izkazuje karboksilna kislina, ogljikova veriga pa kaže nepolarne lastnosti (11).

Maščobne kisline lahko na podlagi kemijske strukture razdelimo na: nasičene MK, enkrat nenasičene (mononenasičene- ena dvojna vez) MK in večkrat nenasičene (polinenasičene- dve ali več dvojnih vezi) MK (slika 3). V celicah se maščobne kisline vežejo na glicerol v obliki estrov. V prosti obliki jih navadno ne najdemo (12). Ogljikova atoma v alifatski verigi, ki sta vezana na eno ali drugo stran ogljika z dvojno vezjo, se lahko pojavita v *cis* ali *trans* konfiguraciji (14). Večina maščobnih kislin se v naravi pojavlja v *cis* obliki (11).



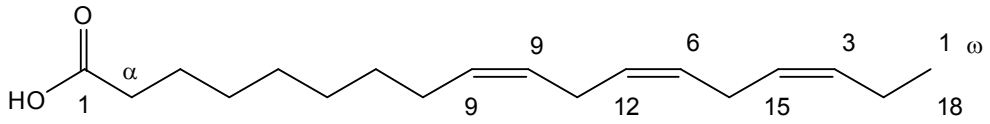
Slika 1: ***Strukturne formule nekaterih nasičenih, enkrat in večkrat nenasičenih maščobnih kislin (12).***

Poimenovanje maščobnih kislin se v literaturi različno navaja. Zato je IUPAC predlagal sistemsko nomenklaturo. Je najbolj striktna, saj izključuje katerokoli možnost napačne identifikacije. Sistematska metoda poimenuje maščobne kisline le na podlagi števila C-atomov ter števila in položaja nenasičenih vezi glede na karboksilno skupino. Določi se položaj in identiteta (slika 4) substituiranih skupin ter optična aktivnost in konfiguracija dvojnih vezi (11). Glede na lego prve dvojne vezi, gledano z metilnega konca molekule, delimo nenasičene maščobne kisline na omega-3 in omega-6 maščobne kisline (10).

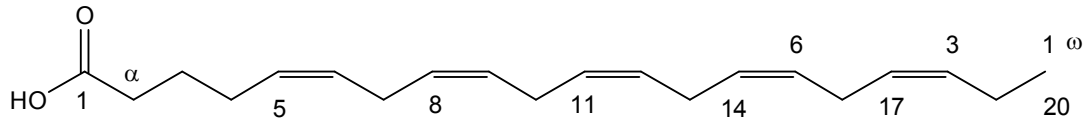


Slika 2: ***IUPAC štetje (Δ in ω) ogljikovih atomov maščobnih kislin (13).***

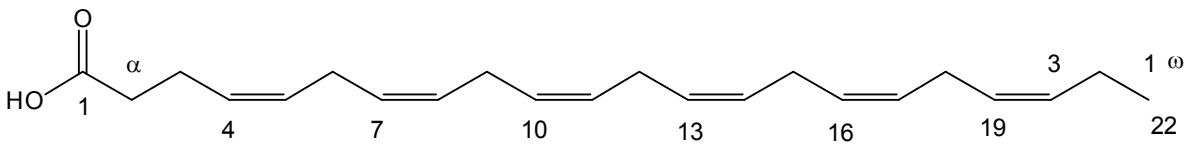
Omega-3 maščobne kisline so polinenasičene maščobne kisline dolgih verig (najmanj 18 C atomov), od katerih se prva od mnogih dvojnih vezi začne s tretjim atomom ogljika glede na metilni konec molekule (10). Pod omega-3 MK štejemo α-linolensko kislino (ALA; 18:3n-3), eikozapentaenojsko kislino (EPA; 20:5n-3) in dokozaheksaenojsko kislino (DHA; 22:6n-3). α-Linolenska kislina in derivati te kisline so esencialne MK. Človeško telo jih ni sposobno sintetizirati, zato jih moramo dobiti s hrano.



α-linolenska kislina



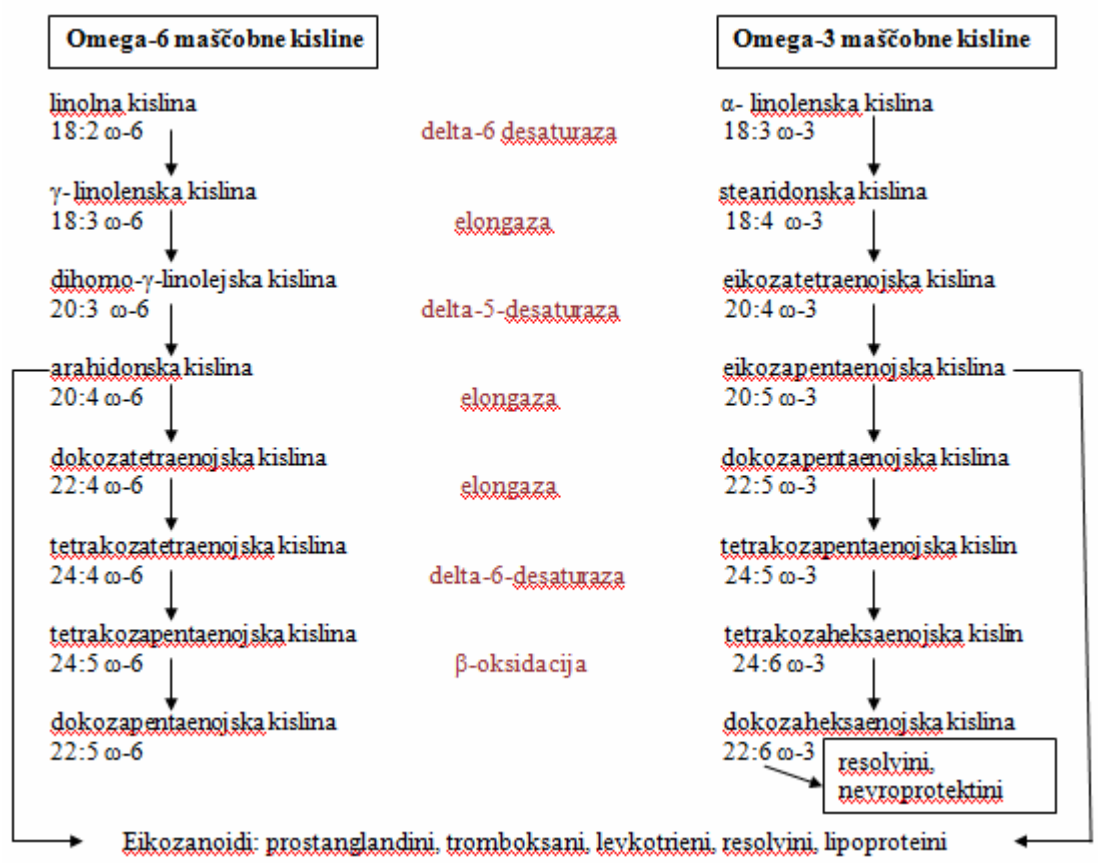
eikozapentaenojska kislina (EPA)



dokozaheksaenojska kislina (DHA)

Slika 3: ***Strukturne formule omega-3 maščobnih kislin (10).***

Pod omega-6 MK štejemo linolno kislino (LA; 18:2n-6), kjer se ta molekula v telesu pretvori v γ-linolensko (18:3n-6) in arahidonsko kislino (20:4n-6) (10, 12).



Slika 4: ***Presnova linolne (omega-6) in α-linolenske (omega-3) kisline (10).***

1.2.1 Pomen in vloga maščobnih kislin za organizem

Od izvora oziroma od sestave lipidov je odvisna naša kakovost zdravja in bivanja. Nepravilen izbor lipidov in količina le-teh povzroča sodobne zdravstvene tegobe (prekomerna telesna teža, sladkorna bolezen tipa II, srčno-žilni zapleti, visok krvni tlak). Na drugi stani pa, če se izogibamo lipidom v prehrani, pa nam kaj hitro zmanjka energije, volje, oslabi se imunski sistem, poveča se razdražljivost (12). Ker so maščobe pomemben element v prehranski piramidi, se dandanes govori z negativnim prizvokom. Poudarjajo se predvsem negativne lastnosti, premalo pa pozitivni učinki.

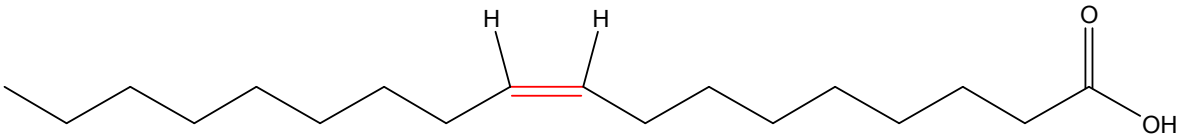
Ko so leta 1923 odkrili VNMK (večkrat nenasičene maščobne kisline), so jih uvrstili med vitamine, natančneje vitamin F. Leta 1930 je sledila ponovna razvrstitev VNMK, ko sta Burr in Miller dokazala, da VNMK bolj sodijo med lipide kakor med vitamine(14).

Nenasičene MK skrbijo in vzdržujejo celovitost celičnih membran. Za nasičene MK je značilna ravna veriga C-atomov, medtem ko za nenasičene MK, ki imajo v svoji strukturi eno ali več dvojnih vezi, to omogoča, da celična membrana ni tako toga (postane fluidna). Ustrezno razmerje nasičenih in nenasičenih MK v lipidnem dvosloju določa fizikalne značilnosti celice, na površini katere potekajo encimske pretvorbe. To je pomembno predvsem v živčnem tkivu, nevronih. Nenasičene MK so udeležene v imunskih procesih (pomanjkanje vodi v dojemljivost za okužbe), so prekurzorji eikozanoidov (tromboksani, prostaglandini, levkotrieni) (12,14). Arahidonska kislina in EPK sta prekurzorja številnih eikozanaidov. Prostaglandini in tromboksani nastanejo s pomočjo encima ciklooksigenaze, levkotrieni pa z encimom 5-lipooksigenazo. Omenjene spojine uravnavajo številne procese v telesu, na primer aktivacijo levkocitov in trombocitov, bronhokonstrikcijo, bolečino, sekrecijo v želodcu in vnetne procese (10). Koncentracije različnih eikozanoidov morajo biti v ravnotežju. Kakšno pa bo ali je to v ravnotežju, pa je odvisno od prisotnosti omega-3 in omega-6 MK, saj eikozanoidi nastajajo po delovanju istih encimov, vendar imajo različno biološko funkcijo. Posledično to pomeni, da je mogoče s količino in razmerjem ω-3 in ω-6 vplivati na razmerje eikozanoidov oziroma na fiziološke procese, ki jih regulirajo (12). VNMK sodelujejo pri metabolizmu in uravnavanju koncentracije holesterola. ω-6 VNMK niža vrednost celokupnega holesterola, a minimalno vpliva na trigliceride. Medtem, ko ω-3 VNMK vplivajo na koncentracijo trigliceridov v krvi. ω-3 VNMK ščitijo pred razvojem srčnih aritmij in zmanjšajo možnost tromboembolijskih zapletov. MK skrbijo tudi za epidermalno bariero. Nepogrešljivo vlogo imajo VNMK pri rasti in razvoju otroškega centralnega živčnega sistema, možganskega tkiva in vida (zadnje trimesečje nosečnosti in prva leta otrokovega razvoja). Zato je takrat povečana potreba po VNMK, predvsem DHK (14).

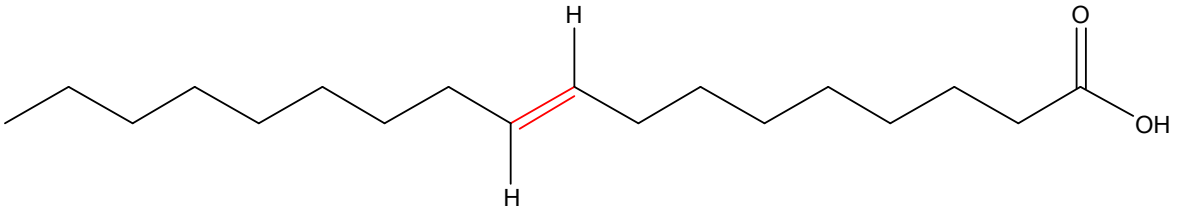
1.2.2 Vloga *trans* maščobnih kislin

V naravi je večina MK v *cis* obliki, MK s *trans* konfiguracijo se pojavljajo v predelanih živilih (slika 7). *Trans* oblike nastanejo pri procesu hidrogeniranja, cvrtju in toplotni obdelavi živalskih maščob, rastlinskega in ribjega olja, pri čemer se spremenijo plastičnost in tališče. Leta 1984 je bilo ocenjeno, da *trans* MK predstavljajo 6-8 % skupno zaužitih lipidov, vendar se je ta številka do danes spremenila (zmanjšala). Vnos *trans* MK je postavljen pod 1 % celotnega vnosa energije. Predpostavlja se, da imajo *trans* MK enak neugoden učinek na pojav srčno žilnih zapletov, kakor nasičene maščobne kisline. *Trans*

MK vplivajo na koncentracijo LDL holesterola in zmanjšujejo nivo HDL holesterola v krvi. Poleg tega pa tudi *trans* izomere zavirajo delovanje Δ6-desaturaze, ki ima funkcijo podaljševanja ali krajšanja verige esencialnih MK, zmanjšujejo rojstno maso, povečujejo telesno maso, zmanjšujejo vključevanje drugih MK v celično membrano. Viri *trans* MK vključuje vsa pečena živila, ki vsebujejo delno hidrogenirane rastlinske maščobe. Nekaj primerov: čips, piškoti, hitro pripravljena ocvrta hrana … (11, 12, 14, 15).



oleinska kislina



elaidinska kislina

Slika 5: ***Oleinska kislina in njen trans izomer elaidinska kislina (11).***

1.2.3 Vnos VNMK v telo

V uravnoteženi prehrani je vnos energije iz lipidov omejen na 20 do 30 oziroma 35 %. Odstotek je večji pri majhnih otrocih (30-35 %) in pri adolescentih (25-35 %). Delež nasičenih maščobnih kislin naj ne bi presegel 10 % energijskih potreb. Delež enkrat nenasičenih MK naj bi se gibal okoli 10 % in delež večkrat nenasičenih MK okoli 7 % energijskih potreb (tabela I). Pomembno dejstvo pri tem je, da moramo paziti na pravilno razmerje med ω-6 in ω-3 VNMK. V navadni prehrani je dovolj linolne kisline, ki je prekurzor za sintezo ω-6. Zato, do pomanjkanja le-te ne pride. Problem je α-linolenska, ki je premalo zaužijemo, poleg tega pa z linolno kislino tekmujeta za iste encimske procese v telesu. Ker je (praviloma) linolne kisline več, je pri izrabi procesov uspešnejša, kakor njena tekmica α-linolenska kislina, ki je nujno potrebna. Ustrezno razmerje med ω-3 in ω-6 VNMK je 1:5 ali manj. Na žalost pa je to razmerje v običajni prehrani 1:10 ali celo 1: 20-25 v prid ω-6. To razmerje je pomembno pri vegetarijancih, pri katerih je edini vnos ω-3 MK, prav α-linolenska kislina (12,14).

Oskrba organizma s pravilnim razmerjem ω-3 in ω-6 VNMK in ustrezno količino, je pomembna skozi celotno življenje posameznika. Najbolj pa pride do izraza, ko je otrok še v materinem telesu. Potrebe po VNMK so takrat največje, saj se takrat formirajo in oblikujejo možgansko tkivo, živčni sistem in vid. Tudi kasneje, ko otrok ni več del matere, je treba vnašati zadostno količino VNMK, da se poleg maloprej naštetih stvari še dokončno izoblikujejo psihomotorične sposobnosti otroka. Tako je med nosečnostjo (zadnje tri mesece) in dojenjem priporočljiv odmerek 100 do 200 mg DHK dnevno. Otroci stari od 6 mesecev in do 24 mesecev bi naj dnevno zaužili 100 mg DHK. Priporočljiva dnevna količina za otroke od 2 do 18 let je enaka kakor za odraslo populacijo (1 do 2 obroka mastnih rib na teden ali 250 mg EPK in DHK na dan) (12).
Ker ni nekih poenotenih smernic za jemanje VNMK, je tudi temu primerno več podatkov in priporočil kaj in koliko jemati.

Tabela I: Priporočila za uživanje esencialni maščobnih kislin pri odraslih (15).

MAŠČOBNA KISLINA	g/DAN (PRI 2000 KCAL/DAN)	% ENERGIJE
LINOLNA	4,44	2,0
LINOLNA – GORNJA MEJA	6,67	3,0
α-LINOLENSKA	2,22	1,0
DHA + EPA	0,65	0,3
DHA NAJMANJ	0,22	0,1
EPA NAJMANJ	0,22	0,1

Klinične in epidemiološke študije priporočajo dnevni vnos od 200 mg do 650 mg dolgoverižnih ω-3 MK in 18 g VNMK (15).

1.2.4 Viri esencialnih maščobnih kislin

Ker z navadno prehrano vnesemo dovolj ω-6 MK, je zato treba nameniti več pozornosti temu, kaj zaužiti, da bo se razmerje med ω-3 in ω-6 VNMK zmanjšalo. Viri ω-6 MK so: sončnično, sezamovo, bučno, koruzno olje, hrana živalskega izvora (meso in mlečni izdelki). Ker je ω-6 MK na dnevnem jedilniku dovolj, je treba povečati vnos hrane bogate z ω-9 in ω-3 MK. Živalski vir ω-3 MK so mastne ribe globokih severnih morij (divji losos, polenovka, skuša, slanik, sardela, tuna, sled …). Od rastlinskih virov pa najbolj izstopa

laneno seme oz. iz njega iztisnjeno olje. Izvrsten je zaradi razmerja med ω-3 in ω-6 VNMK (3,5:1 v korist ω-3 MK). Laneno seme vsebuje le α-linolensko kislino. Tudi olje črne koprive (*Perilla frutescens*) je bogat rastlinski vir ω-3 MK, saj vsebuje 60 % α-linolenske MK. Na trgu so dostopna tudi jajca, obogatena z ω-3 MK (če je vonj po ribah nezaželen). Vir DHK in EPK pa so morski organizmi, ki se hranijo s planktonom, saj sinteze DHK in EPK ne zmorejo. Vir ω-3 MK so tudi stročnice, žitarice, alge, orehi, vendar pa so le morski organizmi vir DHK in EPK (sinteza poteka v algah in fitoplanktonu, ki predstavlja njihovo hrano). Ni dovolj, da poskrbimo za pravilno prehranjenost z maščobami, potrebno je tudi varovanje pred oksidacijo maščob, zadosten vnos vlaknin, ki v črevesu vežejo nasičene maščobe in mehčajo blato. Ker se pri metabolizmu maščob tvorijo reaktivne kisikove spojine, je pomembno ob vnosu maščob v telo tudi vnesti antioksidante (10, 12, 14).

1.2.5 Uporaba ω-3 maščobnih kislin

- srčno-žilne bolezni

Za nastanek srčno-žilnih bolezni je vzrok genske narave in vpliv okolja. Slednji je tisti, na katerega lahko vplivamo z načinom življenja in prehrane. Omenjene maščobne kisline tako zmanjšajo možnost nastanka ateroskleroze, nižajo vrednost krvnega tlaka, znižujejo vrednost trigliceridov, zmanjšajo možnost za nastanek strdkov, zmanjšajo možnost ponovnega infarkta. Priporočen dnevni odmerek je 1 g EPK in DHK.

- vnetne in avtoimune bolezni

Številne študije potrjujejo, da ω-3 maščobne kisline lahko uravnavajo vnetje in imunske reakcije (metabolizem eikozanoidov). Rezultati so privedli do spoznanja, da je možno lajšati tovrstne tegobe.

- rak

Opravljene študije na živalih in tudi na ljudeh potrjujejo, da redno uživanje rib, ribjega olja, v glavnem produktov, bogatih z ω-3 maščobnimi kislinami, zmanjšajo tveganje za nastanek raka širokega črevesa, prostate in prsi. Učinek naj bi bil posledica številnih mehanizmov v spremembi rakastih celic kakor v spremenjenih lastnostih gostiteljevih celic. Povsem natančno mehanizmi niso znani, saj so še predmet raziskav. Kljub temu pa številni strokovnjaki zagovarjajo uporabo ω-3 maščobnih kislin pri rakavih bolnikih, da se okrepi njihov imunski sistem in da preprečijo prevelike izgube telesne mase.

- depresija

Študije opravljene na bolnikih z diagnozo depresije, so pokazale pomanjkanje ω-3 maščobnih kislin. To kaže, da imajo molekule ω-3 maščobnih kislin določeno vlogo v patogenezi depresije. S študijami, opravljenimi na podganah, so znanstveniki dognali, da je pomanjkanje ω-3 maščobnih kislin povezano z moteno nevrotransmisijo v serotoninergičnem in dopaminergičnem sistemu, kar vodi do zmanjšanega števila D2 in povečanega 5-HT2 receptorjev v frontalnem korteksu. Slednje bi naj imelo pomembno vlogo pri patofiziologiji depresije. Zato obstaja verjetnost, da se bodo ω-3 maščobne kisline, v prihodnosti uveljavile kot alternativno zdravljenje depresije in drugih motenj razpoloženja.

- nosečnost in dojenje

Za rast, razvoj in delovanje živčnih celic so pomembne ω-3 maščobne kisline, predvsem DHK. Zmanjšana količina DHK v obdobju razvoja možganov lahko privede do napak v nevrogenezi, metabolizmu nevrotransmiterjev, oteženem učenju, motenj v vidu. Novorojenčki in dojenčki, ki uživajo materino mleko, so odvisni od količine DHK v mleku. Zato mora mati s prehrano zagotoviti zadostno količino ω-3 maščobnih kislin, saj maščobne kisline prehajajo skozi materino mleko do otroka. Posledice zmanjšanega vnosa se pri otroku kažejo v slabšem vidu in okrnjenem razvoju možganov. Zaradi možnosti vnosa težkih kovin z uživanjem morske hrane se je priporočljivo izogibati prevelikemu uživanju rib (morski pes, tuna, skuša, mečarica) in na raje posežejo po prehranskih dopolnilih z ω-3 maščobnimi kislinami, kjer ni prisotnih težkih kovin. Nikakor pa to ne pomeni, da se odpovedo ribjim jedem.

Priporočila

ω-3 maščobne kisline so del prehrane in jih vnašamo v telo. Primerne so za vse ljudi, ker imajo kar nekaj pozitivnih učinkov in ne predstavljajo nevarnosti za zdravje. Dobro jih uživati že iz vidika, saj jih v telo ne vnesemo v zadostnih količinah. Kot prehransko dopolnilo se priporoča:

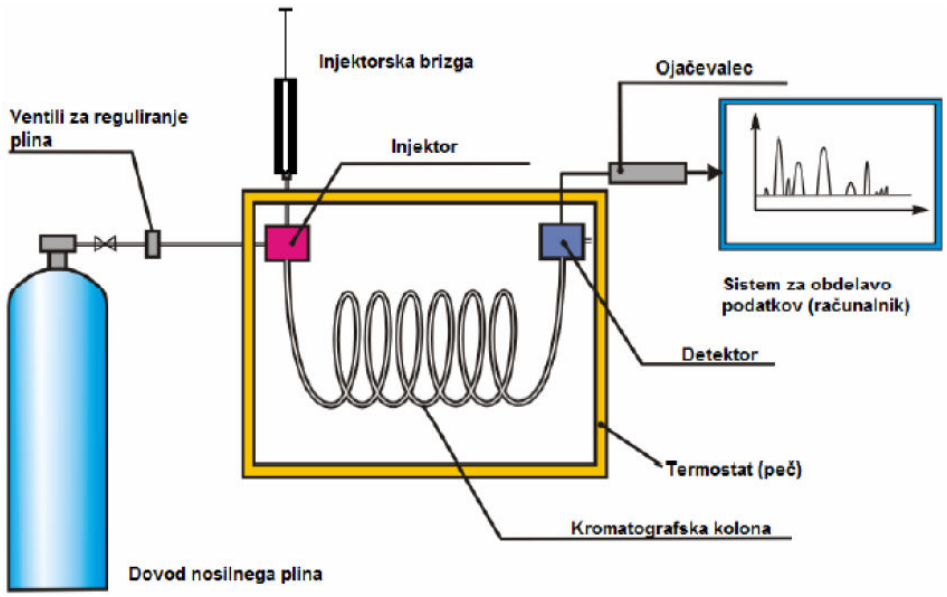
- bolnikom z večjim tveganjem za srčno-žilne bolezni
- bolnikom, ki so že preboleli miokardni infarkt
- bolnikom s hipertenzijo
- bolnikom s hipertrigliceridemijo
- pri težavah z luskavico
- bolnikom z revmatiodnim artritisom
- pri vseh vnetnih procesih

- nosečnicam in doječim materam
- in vsem, ki z nezadostnim vnosom maščob dobijo iz raznolike prehrane premalo ω-3 maščobnih kislin (10, 12, 15).

1.3 Plinska kromatografija z masno spektrometrijo (GC-MS)

Kromatografija, separacijska tehnika, je bila "rojena" leta 1903, ko je ruski botanik M.S. Tsvet na varšavski univerzi pripravil javno predavanje, kjer je predstavil novo separacijsko tehniko. Naslov predavanja, ki ga je botanik M.S. Tsvet imel, je bil: "*On a New Category of Adsorption Phenomena and Their Application to Biochemical Analysis*". Javno je razkril oziroma predstavil širokopotezne rezultate raziskav, ki jih je opravil na rastlinskih listih (listni pigmenti). Raziskave, ki jih je do tedaj opravil, so vodile v izpopolnjevanje metode (tehnike), ki je dovoljevala ali omogočala separacijo barvil (pigmentov) z rastlinskih listov. V nadaljnjih letih je novo tehniko izpopolnil- kasneje je postala znana pod imenom kromatografija (16). Kromatografija je separacijska tehnika, pri kateri posamezne komponente v zmesi ločimo med seboj. V praksi je pomembno, da to dosežem tudi v realnem času (17).

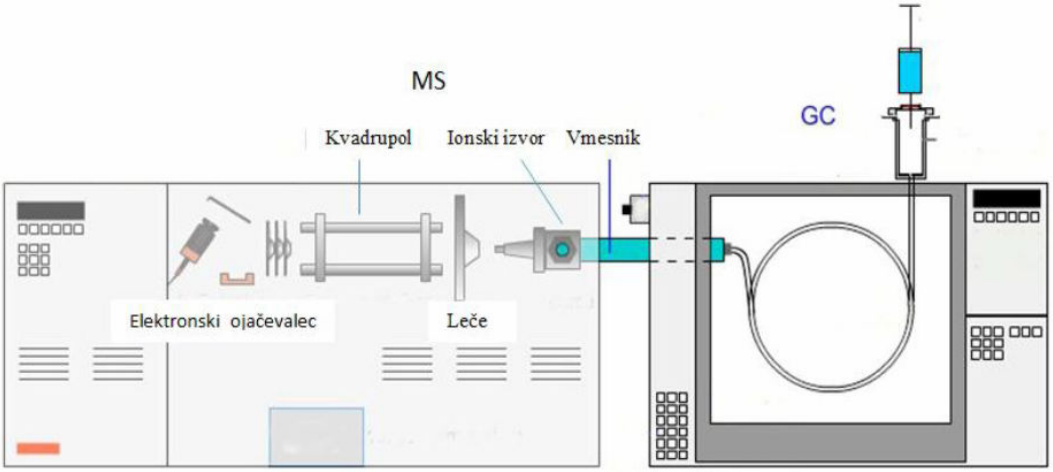
Plinska kromatografija (angl. Gas Chromatography), je analizna separacijska metoda, katere začetki uporabe segajo več kot 60 let nazaj in je zelo razširjena v današnjem času. Z njo ločimo hlapne komponente. Prednosti metode so v njeni ponovljivosti, natančnosti in dobri ločljivosti komponent ter vsestranskosti (17,18). Pri plinski kromatografiji (slika 8) se vzorec po injiciranju pri temperaturi nad vreliščem uplini in loči zmes na sestavne delce. Nosilni plin prenese zmes spojin skozi kolono, ki je napolnjena z adsorbentom ali nosilcem stacionarne faze v detektor. Najpomembnejša naloga pri plinski kromatografiji je izbor stacionarne faze. Velja pravilo, da za nepolarne spojine uporabimo nepolarne stacionarne faze in za polarne spojine polarne stacionarne faze (19). Nosilni plin (mobilna faza) ima nalogo, da potuje skupaj z vzorcem do detektorja. Kot nosilni plini v mobilni fazi se uporabljajo zelo čisti, inertni plini, ki ne vsebujejo vlage, kisika in ogljikovodikov. Pogoj je, da se ne vežejo na adsorbent oziroma stacionarno fazo. Pogosto uporabljamo helij in vodik, manj pa argon in dušik (20).



Slika 6: ***Shema osnovnih delov plinskega kromatografa (GC) (18).***

Injektor, ki je sestavni del GC aparata ima vlogo aplikatorja, ki da preiskovano spojino ali snov v / na kolono. Izbor injektorja je odvisen od vrste kolone. Injiciranje je ključen postopek pri analizi, zato ga je treba izvesti v čim krajšem času (17). Na vzorec delujejo različne kemijske interakcije s stacionarno fazo. Spojine imajo različne lastnosti in porazdelitvene koeficiente, glede na te parametre pride do ločbe (18). Posamezni koeficienti so odvisni od topnosti spojine v stacionarni fazi in od parnega tlaka spojine v mobilni fazi. Kolona je jedro plinske kromatografije, saj poteka ločba uparjene zmesi. Gradient za ločbo zagotovimo s spreminjanjem (višanjem) temperature kolone v termostatu (pečici), ki je od zunanje okolice dobro izolirano. Kolone so izdelane iz jekla, stekla, medenine in najlona. Večinoma se uporabljajo kapilarne kolone s premerom od 0,2 mm do 0,4 mm in dolžine nad 50 m. Tovrstna kolona je brez polnila, stacionarna faza je nanesena v tankem filmu po notranji strani kapilare. Prednost tovrstne kapilarne plinske kromatografije je v izredno veliki ločljivosti, čas analize pa je kratek. Kapaciteta kapilarne kolone je zelo majhna, zato se uporablja indirektno injiciranje vzorca. Injiciran vzorec se pomeša z inertnim, nosilnim plinom. Homogena zmes plina in vzorca se razdeli na dva dela; en zapušča sistem, drug gre na kolono (20). Na koncu kromatografske kolone je nameščen detektor, ki zazna komponente, ki se izločijo iz kolone po vrstnem redu in glede na retencijske čase komponent v koloni (18). Masna spektrometrija (MS) je nepogrešljiva analizna tehnika v naravoslovnih vodah. Osnovni princip MS temelji na generiranju ionov iz anorganskih in/ali organskih spojin s primerno metodo, ločitev teh ionov z njihovim razmerjem masa-naboj (m/z) in kvalitativno ter kvantitativna detekcija teh ionov glede na

posamezne m/z. Masni spektrometer je sestavljen iz izvora ionov, masnega analizatorja in detektorja, ki delujejo pod pogoji visokega vakuuma. MS proces je destruktivna metoda. Cilji dela z MS so ugotoviti molekulsko maso, molekulsko formulo in molekulsko strukturo iz fragmentov (19).



Slika 7: ***Shema instrumenta GC-MS (19).***

Plinska kromatografija in masna spektrometrija sta ločena analizna postopka, ki pa med sabo zelo dobro sodelujeta (slika 7 in 8). Plinska kromatografija loči komponente mešanice, medtem ko masna spektrometrija vsako komponento posebej identificira. Analitik lahko s kombinacijo teh dveh analiznih tehnik kvalitativno, z uporabo referenčnih spojin ali standardov še kvantitativno, ovrednoti posamezne spojine v analizirani zmesi (12).

Slika 8: ***GC-MS analizator na Katedri za farmacevtsko biologijo (Fakulteta za farmacijo).***

2 NAMEN DELA

Uporaba prehranskih dopolnil je zelo priljubljena in stalno raste, vendar ti izdelki niso tako strokovno nadzorovani, kot je to v veljavi pri zdravilih. Cilj naloge je z ustrezno metodologijo ugotoviti vsebnost ω-3 maščobnih kislin v naključno izbranih prehranskih dopolnilih, ki jih je možno kupiti na slovenskem trgu (lekarne, specializirane prodajalne, trgovine z živilskim blagom …). Preveriti oziroma primerjati želimo vsebnosti ω-3 maščobnih kislin, ki so navedene na ovojnini in vrednosti, ki jih bomo dobili z eksperimentalnim delom ustrezne metodologije in ugotoviti ali ustrezajo navedeni specifikaciji, oziroma za koliko odstopajo.

Najprej bomo poskušali ugotoviti vsebnost in sestavo maščobnih kislin z modificirano farmakopejsko metodo (Ph. Eur.). Pri tej metodi bo ekstrakciji lipidov sledila pretvorba maščobnih kislin v pripadajoče metilne estre z brezvodnim metanolu v alkalnem mediju in analiza s plinsko kromatografijo. Zaradi večje zanesljivosti bomo paralelno izvedli še *in situ* metodo derivatizacije maščobnih kislin z BF_3 v metanolu v alkalnem mediju brez predhodne ekstrakcije lipidov.

3 MATERIALI IN METODE

3.1 Materiali

3.1.1 Vzorci

Vzorci ali prehranska dopolnila z omega-3 maščobnimi kislinami, smo kupili v živilski prodajalni Spar®, specializirani prodajalni-drogeriji DM® in v Lekarni Nove Poljane. Pri tem moramo izpostaviti dejstvo, da je bil izbor prehranskih dopolnil naključen in z opravljenimi analizami želimo izvedeti, koliko določeno prehransko dopolnilo ne glede na proizvajalca vsebuje omega-3 nenasičenih maščobnih kislin. Večina preiskovanih vzorcev je v obliki želatinastih kapsul, razen farmakopejskega ribjega olja (*Iecoris Oleum*), ki je ustekleničeno.

Tabela II: Seznam analiziranih prehranskih dopolnil.

Ime izdelka, prehranskega dopolnila	**Proizvajalec, dobavitelj**	**Ime izdelka, prehranskega dopolnila**	**Proizvajalec, dobavitelj**
MOR EPA®	Minami Nutrition	**Cod Liver Oil**	Health Spark
Cod Liver Oil Omega 3	Natural Wealth	**Krill olje**	Biopharma Norway
Iecoris Oleum – ribje olje	Lekarna Nove Poljane Ljubljana	**Lososovo olje omega 3**	Hafesan
Neptune Krill Oil	Now® Foods	**Lyprinol**	Medicinalis+
Omega 3 forte	Dr. Böhm	**Neptune Krill Oil**	Natura Medica
Omega 3 ribje olje v kapsulah	Pharma Nutrilab®	**Omega 3 Cardio**	Natural Wealth
Fit Omega 3	Vital Slim by Maxximum	**Omega 3**	Drogerie Markt
Omega 3 Junior	Natural Wealth	**RibaMed® 1000**	Fidimed
Omega 3 Natal	Natural Wealth	**Seefischoel Omega 3**	Doppel Herz®, Quiesser Pharma
Omega 3 Neuro	Natural Wealth	**SensOmega**	Sensilab®
Omegavit	Marifit	**Super Krill Omega 3® - krilovo olje**	Medicinalis+
Super Omega 3	Now® Foods		

3.1.2 Reagenti

Topila

- prečiščena voda (Fakulteta za farmacijo, Univerza v Ljubljani)
- heptan, p.a. (Carlo Erba, Milano, Italija)

Reagenti za pripravo metilnih estrov

- metanol, p.a. (Panreac, Barcelona, Španija)
- dimetilkarbonat (Sigma-Aldrich, Steinheim, Nemčija)
- natrijev hidroksid, p.a. (Merck, Darmstadt, Nemčija)
- 14 % borov trifluorid v metanolu, pro synthesis (Merck, Darmstadt, Nemčija)
- natrij (Ridel de Haën, Seelze bei Hannover, Nemčija)

Standard

- F.A.M.E. Mix C20:1 – C20:5 (SUPELCO, Sigma Aldrich, Steinheim, Nemčija)

Ta standard smo uporabili, da smo preverili primernost metode. Ker smo želeli kvantificirati točno določene omega 3 maščobne kisline v prehranskih dopolnilih smo zato raje izbrali posamezne standarde izbranih omega 3 maščobnih kislin.

- DHA (dokozaheksaenojska kislina) 10,0 mg/mL v heptanu (SUPELCO, Sigma Aldrich, Steinheim, Nemčija)
- EPA (eikozapentaenojska kislina) 10,0 mg/mL v heptanu (SUPELCO, Sigma Aldrich, Steinheim, Nemčija)
- DPA (dokozapentaenojska kislina) 10,0 mg/mL v heptanu (SUPELCO, Sigma Aldrich, Steinheim, Nemčija)
- α-LA (alfa linolenska kislina) 10,0 mg/mL v heptanu (SUPELCO, Sigma Aldrich, Steinheim, Nemčija).

3.1.3 Aparature in laboratorijska oprema

Tehtanje

- analizna tehtnica KERN ALS 120-4 (Kern & Sohn GmbH, Balinger, Nemčija)
- analizna tehtnica PC 2000 (Mettler Toledo, Zürich, Švica)

Raztapljanje

- ultrazvočna kadička Sonorex Digitec (Bandelin, Berlin, Nemčija)

Ekstrakcija

- avtomatske pipete Proline 0,5-10 µL, 10-100 µL, 100-1000 µL (Biohit, Helsinki, Finska)
- plastične epruvete z zamaškom na navoj, 14 mL (TPP, Trasadingen, Švica)
- plastične epruvete z zamaškom Standard Micro Test Tube 3810X (Eppendorf, Hamburg, Nemčija)

Centrifugiranje

- centrifuga Centric 400 R (Tehtnica, Železniki, Slovenija)

Plinska kromatografija z masnim detektorjem (GC-MS)

Sistem: GCMS – QP2010 Ultra (Shimadzu Corporation, Kyoto, Japonska)

Kolona: kapilarna kolona Rxi-5 Sil MS, 30 m x 0,25 mm, 0,25 µm 5 % difenil / 95 % dimetilpolisiloksan (Restek, Bellefonte, PA, ZDA)

Računalniški program GCMS Solution 2.3 (Shimadzu Corporation, Kyoto, Japonska)

3.2 METODE

3.2.1 Meja detekcije in kvantifikacije standardov

Mejo detekcije in kvantifikacije smo izračunali iz koncentracije in razmerja S/N (signal/šum) za α-linolensko kislino v raztopini standarda:

c(A) koncentracija α-linolenske kisline v standardu (0,100 mg/mL)

S/N razmerje signal s šumom (33,97)

Mejna koncentracija za detekcijo: c(A) × 3 ÷ (S/N) =

= 0,100 mg/mL × 3 ÷ 33,97 =

= 0,00883 mg/mL = 8,83 ×10^{-3} mg/mL

Mejna koncentracija za kvantifikacijo: c(A) × 10 ÷ (S/N) =

= 0,100 mg/mL × 10 ÷ 33,97 =

= 0,00294 mg/mL = 2,94 ×10^{-3} mg/mL

Mejo detekcije in kvantifikacije smo izračunali iz koncentracije in razmerja S/N za EPK v raztopini standarda:

c(A) koncentracija EPK v standardu (0,100 mg/mL)

S/N razmerje signal s šumom (5,50)

Mejna koncentracija za detekcijo: c(A) × 3 ÷ (S/N) =

= 0,100 mg/mL × 3 ÷ 5,50 =

= 0,0546 mg/mL = 5,46 ×10^{-2} mg/mL

Mejna koncentracija za kvantifikacijo: c(A) × 10 ÷ (S/N) =

= 0,100 mg/mL × 10 ÷ 5,50 =

= 0,182 mg/mL = 1,82 ×10^{-1} mg/mL

Mejo detekcije in kvantifikacije smo izračunali iz koncentracije in razmerja S/N za DHK v raztopini standarda:

c(A) koncentracija DHK v standardu (0,100 mg/mL)

S/N razmerje signal s šumom (5,26)

Mejna koncentracija za detekcijo: c(A) × 3 ÷ (S/N) =

= 0,100 mg/mL × 3 ÷ 5,26 =

$= 0{,}0570 \text{ mg/mL} = 5{,}7 \times 10^{-2} \text{ mg/mL}$

Mejna koncentracija za kvantifikacijo: $c(A) \times 10 \div (S/N) =$

$= 0{,}100 \text{ mg/mL} \times 10 \div 5{,}26 =$

$= 0{,}190 \text{ mg/mL} = 1{,}90 \times 10^{-1} \text{ mg/mL}$

Mejo detekcije in kvantifikacije smo izračunali iz koncentracije in razmerja S/N za DPK v raztopini standarda:

c(A) koncentracija DPK v standardu (0,100 mg/mL)

S/N razmerje signal s šumom (5,81)

Mejna koncentracija za detekcijo: $c(A) \times 3 \div (S/N) =$

$= 0{,}100 \text{ mg/mL} \times 3 \div 5{,}81 =$

$= 0{,}0516 \text{ mg/mL} = 5{,}16 \times 10^{-2} \text{ mg/mL}$

Mejna koncentracija za kvantifikacijo: $c(A) \times 10 \div (S/N) =$

$= 0{,}100 \text{ mg/mL} \times 10 \div 5{,}81 =$

$= 0{,}172 \text{ mg/mL} = 1{,}72 \times 10^{-1} \text{ mg/mL}$

Linearnost umeritvene premice je ovrednotena z r^2. Vrednost $r^2 = 0{,}9999$ (za standardne raztopine). Koncentracije pripravljenih standardov ali referenčnih raztopin so bile: 1,0 mg/mL, 0,50 mg/mL in 0,10 mg/mL, kar predstavlja tudi območje umeritvenih premic. Referenčnih spojin nismo združevali v skupni raztopini, ampak smo jih analizirali posamezno. Iz teh treh koncentracijskih območij smo dobili (izmerili) umeritveno premico.

α- linolenska kislina $r^2 = 0{,}9999$

EPK $r^2 = 0{,}9999$

DHK $r^2 = 0{,}9998$

DPK $r^2 = 0{,}9999$

4 EKSPERIMENTALNI DEL

4.1 Ugotavljanje sestave omega-3 maščobnih kislin z modificirano farmakopejsko metodo B

Za analizo sestave omega-3 maščobnih kislin v naključno izbranih prehranskih dopolnilih smo uporabili modificirano farmakopejsko metodo B. Najprej smo natehtali 0,1 g (100 mg) vzorca. Ker je večina vzorcev v obliki olj v želatinastih kapsulah, smo morali pridobiti vsebino olja vzorca tako, da smo z iglo zbodli v kapsulo in vsebino iz nje iztisnili v ependorfko. Natehtani vzorci smo prenesli v 14 mL plastične epruvete z zamaškom. Vzorcem smo dodali 1 mL heptana in 1 mL dimetilkarbonata. Epruvete smo zamašili in krepko mešali ter greli vzorce v ultrazvočni kadički pri temperaturi 50 – 60 °C. Ko je bila zmes še topla, smo ji dodali 1 mL raztopine natrijevega metilata, ki smo ga pripravili z raztapljanjem 1,2 g natrija v 100 mL brezvodnega metanola. Temu je sledilo 5 minutno mešanje. Po približno petih minutah smo dodali 3 mL prečiščene vode in 30 sekund intenzivno mešali. Sledilo je centrifugiranje za 15 minut pri 1500 g. S tem smo dosegli, da sta nastali dve fazi. Zgornjo fazo smo ločili iz epruvete in jo odpipetirali v vialo. Redčili smo jo v razmerju 1:20 (50 μL vzorca + 950 μL heptana). Redčen vzorec smo odpipetirali v novo vialo in aplicirali v GC-MS. Postopek smo ponovili za vse vzorce, ki smo jim želeli ugotoviti vsebnost omega-3 maščobnih kislin v prehranskem dopolnilu.

Plinska kromatografija z masno spektroskopijo (farmakopejska metoda B)

Kromatografske razmere:

- kapilarna polidimetilsiloksanska kolona, 30 m x 0,25 mm, 0.25 μm debela stacionarna faza (v farmakopeji je stacionarna faza makrogolna)
- nosilni plin: helij s pretokom 1 mL/ min (v famakopeji je pretok 0,9 mL/ min)
- način injiciranja: split (1:100)
- temperaturni program: začetno temperaturo kolone smo nastavili na 160°C, potem je temperatura naraščala za 3°C/min do 250°C
- temperatura injektorja: 250 °C
- temperatura ionskega izvora: 200 °C
- temperatura vmesnika: 280 °C

- volumen injiciranja: 1 µL
- napetost na detektorju: 1 kV
- celokupen čas analize: 30 min

4.2 Ugotavljanje sestave omega-3 maščobnih kislin z metodo *in situ* preestrenja

Vsebnost in sestavo omega-3 maščobnih kislin v preiskovanih vzorcih smo poleg klasične farmakopejske metode ugotavljali z modificirano metodo po Parku in Goinsu (12). Najprej smo natehtali 0,1 g preiskovanega vzorca v plastično epruveto z zamaškom na navoj (volumen epruvete 14 mL). Točno vrednost zatehte smo si zapisali in k vzorcu dodali 1 mL 0.5 M NaOH v metanolu. Epruveto smo zamašili z zamaškom in segrevali na vodni kopeli 10 minut pri 90°C. Po desetih minutah smo epruveto vzeli iz kopeli in jo ohladili na sobno temperaturo. Nato smo dodali 2 mL 14 % BF_3 v metanolu, zamašili epruveto z zamaškom in jo dali v vodno kopel za 10 minut pri 90°C. Po iztečenem času smo epruveto vzeli iz vodne kopeli in jo ohladili na sobno temperaturo. Nato je sledil dodatek 1 mL heptana v epruveto ter enominutno močno stresanje vsebine v zamašeni epruveti. Sledilo je desetminutno centrifugiranje pri 3000 RPM (22°C). Po končanem centrifugiranju, ko sta v epruveti nastali dve plasti, smo gornjo odpipetirali v označeno vialo ter vzorec aplicirali na GC-MS aparaturo. Ostanek v plastični epruveti smo zavrgli.

Zaradi visoke koncentracije nekaterih estrov maščobnih kislin smo morali za kvantifikacijo teh estrov vse vzorce 20-krat redčiti, ki smo jo izvedli tako, da smo 50 µL vzorca odpipetirali v novo vialo in dodali 950 µL heptana ter dali v GC-MS na analizo.

Plinska kromatografija z masno spektroskopijo (*in situ* metoda)

Kromatografske razmere:

- kapilarna polidimetilsiloksanska kolona, 30 m x 0,25 mm, 0.25 µm debela stacionarna faza
- nosilni plin: helij s pretokom 1 mL/ min
- način injiciranja: split (1:100)
- temperaturni program: začetno temperaturo kolone smo nastavili na 160 °C, potem je temperatura naraščala za 3°C/min do 250°C

- temperatura injektorja: 250°C
- temperatura ionskega izvora: 200°C
- temperatura vmesnika: 280°C
- volumen injiciranja: 1 µL
- napetost na detektorju: 1 kV
- celokupen čas analize: 30 min

Kromatografske razmere so enake, kakor pri modificirani farmakopejski metodi B.

4.3 Izračun vsebnosti omega-3 maščobnih kislin na gram vzorca (vsebine olja)

Koncentracije, ki nam jih je podal računalnik po končani analizi, so bile v enotah mg/mL. To velja za vzorce analizirane z *in situ* metodo in za vzorce analizirane s farmakopejsko metodo. Ker pa smo vse vzorce predhodno redčili 20-krat in je bila zatehta vzorca 0,1 g, smo vrednosti analiziranih vzorcev, ki nam jih je podal računalnik pomnožili s faktorjem 200, da je preračunana koncentracija omega-3 maščobne kisline izražena na 1000 mg olja.

Račun: m (preračunana) = [mg ω-3 m.k. ÷ 1000 mg olja]

mg ω-3 m.k. = masa pridobljena iz rezultata analize in pomnožena s faktorjem redčitve (200 krat)

1000 mg olja = je enota, na katero smo izračunali maso prisotne določene ω-3 maščobne kisline.

4.4 Priprava standardov

Raztopine standardov smo pripravljali dvakrat. Prva priprava standardnih raztopin je vsebovala mešanico (F.A.M.E. mix $C_{20:1}$ – $C_{20:5}$). Naredili smo jo v treh različnih koncentracijah. Prva raztopina je vsebovala 400 µL (EPA+DHA+DPA+α-LA) F.A.M.E. mix-a h kateri smo dodali 600 µL heptana. Tako smo dobili t.i. MIX 1. Druga raztopina standarda je vsebovala 150 µL MIX 1 raztopine kateri smo dodali 150 µL heptana (MIX 2). Tretja raztopina standarda je vsebovala 100 µL MIX 1 raztopine kateri smo dodali 900 µL heptana (MIX 3). Ker se ta vrsta standarda ni obnesla na kromatografski ločbi (ne dovolj razločni vrhovi posameznih ω-3 maščobnih kislin in kopice drugih prisotnih

maščobnih kislin, ki niso del našega eksperimentalnega dela), smo se odločili, da naredimo nove raztopine standardov.

Druga priprava standardov je bila narejena brez F.A.M.E. mešanice. Odločili smo se, da bomo naredili standardne raztopine iz vsake certificirane viale z določeno ω-3 maščobno kislino. Prva raztopina standarda je vsebovala (30 µL EPA + 30 µL DHA + 30 µL DPA + 30 µL α-LA) skupno 120 µL posameznih certificiranih omega-3 maščobnih kislin, katerim smo dodali 180 µL heptana. Druga raztopina standarda je vsebovala (15 µL EPA + 15 µL DHA + 15 µL DPA + 15 µL α-LA) skupno 60 µL posameznih certificiranih omega-3 maščobnih kislin, katerim smo dodali 240 µL heptana. Tretja raztopina standarda je vsebovala 3 µL EPA + 3 µL DHA + 3 µL DPA + 3 µL α-LA) skupno 12 µL posameznih certificiranih omega-3 maščobnih kislin, katerim smo dodali 288 µL heptana.

Slednja priprava standardov, se je izkazala za veliko boljšo in iz umeritvene krivulje je lažje bilo definirati, katere in koliko določene omega-3 maščobne kisline je prisotne.

Koncentracije pripravljenih raztopin standardov so naslednje:

RS1: 1,0 mg/mL

RS2: 0,5 mg/mL

RS3: 0,1 mg/mL

Pripravljene raztopine standardov smo aplicirali na GC-MS sistem, pri enakih kromatografskih razmerah kot smo aplicirali raztopine vzorcev, pripravljenih s farmakopejsko in z *in situ* metodo (glej poglavje PRILOGE).

5 REZULTATI IN RAZPRAVA

V eksperimentalni del naloge smo vključili naključno izbrana prehranska dopolnila, ki vsebujejo ω-3 nenasičene maščobne kisline. Število vzorcev, ki smo jih z *in situ* metodo in s klasično, modificirano farmakopejsko metodo B, je bilo 23. Rezultati eksperimentalnega dela so predstavljeni v nadaljevanju v tabeli III. Pri analizi ω-3 nenasičenih maščobnih kislin smo se osredotočili na rezultate ali na t.i. prisotnost izbranih ω-3 nenasičenih maščobnih kislin. Kvantitativno smo želeli z obema analiznima metodama ovrednotiti α-linolensko kislino, EPK (eikozapentaenojsko kislino), DHK (dokozaheksaenojsko kislino) in DPK (dokozapentaenojsko kislino), katerih vsebnosti so navadno podane na ovojnini prehranskega dopolnila.

Evropska farmakopeja poleg ugotavljanja omega-3 maščobnih kislin s postopkom plinske kromatografije, predpisuje tudi ugotavljanje le-teh s tekočinsko kromatografijo visoke ločljivosti (22). Razlogov, za kaj se nismo odločili za izbiro te metode, je kar nekaj: nerazpoložljivost HPLC aparature, uporaba diferencialnega refraktometra, kompleksnejša priprava vzorcev, daljši čas analize in ne nazadnje farmakopeja preferira s to metodologijo le detekcijo EPK in DHK. Pri farmakopejski metodi B in pri *in situ* metodi vrednotimo α-linolensko kislino, EPK (eikozapentaenojsko kislino), DHK (dokozaheksaenojsko kislino) in DPK (dokozapentaenojsko kislino).

Tabela III: Analizirana prehranska dopolnila z *in situ* metodo in farmakopejsko metodo B vsebnosti omega-3 maščobnih kislin.

		IN SITU METODA				FARMAKOPEJSKA METODA			
Prehransko dopolnilo	Ester omega 3 maščobne kisline	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA
MOR EPA Minami	α-linolenska kislina (α-LK)	0,0860	17,2	5	244	0	0	5	-100
	eikozapentaenojska kislina (EPK)	4,51	903	580	55,6	2,53	506	580	-12,8
	dokozaheksaenojska kislina (DHK)	1,39	278	130	114	0,403	80,6	130	-38,0
	dokozapentaenojska kislina (DPK)	0,293	58,6		/	0,393	78,6		/
COD LIVER Oil NW	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,423	84,6	82	3,2	0,316	63,2	82	-23
	dokozaheksaenojska kislina (DHK)	0,487	97,4	77	27	0,229	45,8	77	-41
	dokozapentaenojska kislina (DPK)	0,126	25,2		/	0,230	46,0		/

		IN SITU METODA				FARMAKOPEJSKA METODA			
Prehransko dopolnilo	Ester omega 3 maščobne kisline	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA
COD LIVER OIL Sparks	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,280	56,0	81	-31	0,222	44,4	81	-45
	dokozaheksaenojska kislina (DHK)	0,442	88,4	107	-17,4	0,228	45,6	107	-57,4
	dokozapentaenojska kislina (DPK)	0,112	22,4		/	0,229	45,8		/
Iecoris Oleum (ribje olje)	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,606	121		/	0,259	51,8		/
	dokozaheksaenojska kislina (DHK)	1,00	201		/	0,253	50,6		/
	dokozapentaenojska kislina (DPK)	0,139	27,8		/	0,252	50,4		/
Krill olje Biopharma	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,471	94,2	49	92	0,236	47,2	49	-3,7
	dokozaheksaenojska kislina (DHK)	0,215	43,0	17	153	0,113	22,6	17	33
	dokozapentaenojska kislina (DPK)	0	0		/	0,121	24,2		/

		IN SITU METODA				FARMAKOPEJSKA METODA			
Prehransko dopolnilo	Ester omega 3 maščobne kisline	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA
Losos. olje Hafesan	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,989	198	180	10,0	0,588	118	180	-34,4
	dokozaheksaenojska kislina (DHK)	0,723	145	120	20,8	0,265	53	120	-55,8
	dokozapentaenojska kislina (DPK)	0,164	32,8		/	0,264	52,8		/
Lyprinol medicinalis +	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,370	74,0		/	0,139	27,8		/
	dokozaheksaenojska kislina (DHK)	0,230	46,0		/	0,107	21,4		/
	dokozapentaenojska kislina (DPK)	0,101	20,2		/	0,116	23,2		/
Neptun Krill Oil	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,908	182	150	21,3	0,281	56,2	150	-62,5
	dokozaheksaenojska kislina (DHK)	0,601	120	90	33	0,149	29,8	90	-67
	dokozapentaenojska kislina (DPK)	0	0		/	0,155	31		/

		IN SITU METODA				FARMAKOPEJSKA METODA			
Prehransko dopolnilo	Ester omega 3 maščobne kisline	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA
Neptun Krill NOW	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	1,12	223	150	48,7	0,486	97,2	150	-35,2
	dokozaheksaenojska kislina (DHK)	0,797	159	85	87	0,204	40,8	85	-52
	dokozapentaenojska kislina (DPK)	0,101	20,2		/	0,207	41,4		/
Dr.Böhm Omega3	α-linolenska kislina (α-LK)	0,683	137	156	-12,2	0,593	119		/
	eikozapentaenojska kislina (EPK)	0,763	153	220	-30,5	0,909	182		/
	dokozaheksaenojska kislina (DHK)	0,481	96,2	147	-34,6	0,405	81,0		/
	dokozapentaenojska kislina (DPK)	0,132	26,4		/	0,395	79,0		/
NutriLab Omega3	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,992	198	180	10,0	0,481	96,2	180	-46,6
	dokozaheksaenojska kislina (DHK)	0,678	136	120	13,3	0,223	44,6	120	-62,8
	dokozapentaenojska kislina (DPK)	0,159	31,8		/	0,225	45,0		/

		IN SITU METODA				FARMAKOPEJSKA METODA			
Prehransko dopolnilo	Ester omega 3 maščobne kisline	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA
Omega3 NW Cardio	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	1,59	318	300	6,00	1,01	202	300	-32,7
	dokozaheksaenojska kislina (DHK)	0,865	173	200	-13,5	0,502	100	200	-50,0
	dokozapentaenojska kislina (DPK)	0,165	33		/	0,485	97,0		/
Omega 3 DM	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,821	164	180	-8,89	0,565	113	180	-37,2
	dokozaheksaenojska kislina (DHK)	0,620	124	120	3,33	0,248	49,6	120	-58,7
	dokozapentaenojska kislina (DPK)	0,146	29,2		/	0,247	49,4		/
Omega 3 FI	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	1,34	267	180	48,3	0,564	113	180	-37,2
	dokozaheksaenojska kislina (DHK)	1,28	257	120	114	0,333	66,6	120	-44,5
	dokozapentaenojska kislina (DPK)	0,187	37,4		/	0,327	65,4		/

		IN SITU METODA				FARMAKOPEJSKA METODA			
Prehransko dopolnilo	Ester omega 3 maščobne kisline	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA
Omega 3 Junior NW	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,343	68,6		/	0,370	74,0		/
	dokozaheksaenojska kislina (DHK)	1,31	262	250	4,80	0,933	187	250	-25,2
	dokozapentaenojska kislina (DPK)	0,133	26,6		/	0,891	178		/
Omega 3 Natal NW	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,269	53,8		/	0,251	50,2		/
	dokozaheksaenojska kislina (DHK)	0,975	195	250	-22,0	0,629	126	250	-49,6
	dokozapentaenojska kislina (DPK)	0,122	24,4		/	0,605	121		/
Omega 3 Neuro NW	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,374	74,8	180	-58,4	0,270	54,0	180	-70,0
	dokozaheksaenojska kislina (DHK)	0,513	103	220	-53,2	0,214	42,8	220	-80,5
	dokozapentaenojska kislina (DPK)	0,159	31,8		/	0,216	43,2		/

		IN SITU METODA				FARMAKOPEJSKA METODA			
Prehransko dopolnilo	Ester omega 3 maščobne kisline	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA
Omegavit MARIFIT	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	1,90	380	300	26,7	0,984	197	300	-34,3
	dokozaheksaenojska kislina (DHK)	1,70	340	200	70,0	0,489	97,8	200	-51,1
	dokozapentaenojska kislina (DPK)	0,274	54,8		/	0,474	94,8		/
Ribamed	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,922	184	190	-3,16	0,681	136	190	-28,4
	dokozaheksaenojska kislina (DHK)	0,923	185	190	-2,63	0,442	88,4	190	-53,5
	dokozapentaenojska kislina (DPK)	0,18	36		/	0,429	85,8		/
Seefischöl Doppel Herz	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	1,15	230	180	27,8	0,547	109	180	-39,4
	dokozaheksaenojska kislina (DHK)	0,845	169	120	40,8	0,25	50	120	-58,3
	dokozapentaenojska kislina (DPK)	0,171	34,2		/	0,249	49,8		/

		IN SITU METODA				FARMAKOPEJSKA METODA			
Prehransko dopolnilo	Ester omega 3 maščobne kisline	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA	c(ANAL.)	c(PRER.) [mg kisline / 1000 mg olja]	DEKLARIRANA VSEBNOST	% ODSTOPANJA
SensOmega Sensilab	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	1,26	252	330	-23,6	1,09	217	330	-34,2
	dokozaheksaenojska kislina (DHK)	1,07	215	220	-2,27	0,64	128	220	-41,8
	dokozapentaenojska kislina (DPK)	0,182	36,4		/	0,615	123		/
SuperKrill Omega 3	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	0,669	134	150	-10,7	0,303	60,6	150	-59,6
	dokozaheksaenojska kislina (DHK)	0,418	83,6	90	-7,1	0,161	32,2	90	-64,2
	dokozapentaenojska kislina (DPK)	0	0		/	0,166	33,2		/
Super Omega 3 NOW	α-linolenska kislina (α-LK)	0	0		/	0	0		/
	eikozapentaenojska kislina (EPK)	2,41	481	360	33,6	0,877	175	360	-51,4
	dokozaheksaenojska kislina (DHK)	2,10	419	240	74,6	0,442	88,4	240	-63,2
	dokozapentaenojska kislina (DPK)	0,334	66,8		/	0,430	86,0		

Opomba: / - podatek ni razpoložljiv.

5.1 Razvoj klasične metode ugotavljanja maščobnih kislin

Prva metoda priprave in ugotavljanja koncentracij metilnih estrov maščobnih kislin v vzorcih je bila farmakopejska metoda. In sicer metoda B, ki pa smo jo morali modificirati (21). Upoštevali volumne oziroma količine potrebne za pripravo vzorcev za analizo. Ker smo kromatografske razmere spremenili, smo morali organske faze vzorcev pred analizo redčiti. Farmakopeja navaja za detekcijo molekul FID (flame ionisation detector = plamensko ionizacijski detektor), mi smo pa za detekcijo molekul estrov maščobnih kislin uporabili bolj občutljiv MS (masni detektor). V farmakopeji je predvidena končna temperatura kolone 225 °C, vendar smo jo v svojem primeru povečali na 250 °C, z namenom, da bi detektirali sterole in da bi kolono tudi očistili težko hlapnih spojin. Farmakopejska metoda B navaja časovno graduirano stopnjevanje temperature. Poleg tega pa čas analize po farmakopeji traja 61 minut. Heptan je hlapno organsko topilo, ki je prvi zapustil kolono. Snemanje spektra je potekalo 30 minut, da smo na spektru dobili želene, iskane spojine metilnih estrov maščobnih kislin. Ugotovili smo, da ne pride do popolne derivatizacije maščobnih kislin. Razlog za to tiči v podatkih iz farmakopeje, da je treba vzorec greti med 50 in 60 °C, mešati približno 5 minut. Verjetno je temperatura gretja prenizka, saj ne pride do popolne reakcije in posledično do (popolne) derivatizacije. Ocenjujemo, da gre bolj za semikvantitativno metodo, kot pa za čisto, absolutno kvantitativno metodo, saj je namen ugotavljanja farmakopejske metode razmerje maščobnih kislin, ne pa absolutna kvantifikacija, za kar zadostuje tudi delna derivatizacija.

5.2 Razvoj *in situ* metode preestrenja maščobnih kislin

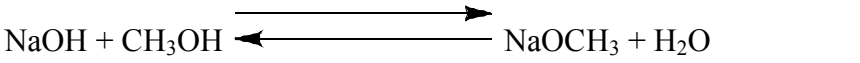
Običajna ali klasična metoda priprave metilnih estrov maščobnih kislin (MEMK) vključuje ekstrakcijo lipidov iz biološkega materiala in nadaljnjo preestrenje izoliranih lipidov. Sam proces je dolgotrajen in kompleksen. V izogib tem težavam, so avtorji opisali alternativni postopek, ki združuje ekstrakcijo in preestenje v eni stopnji (12).

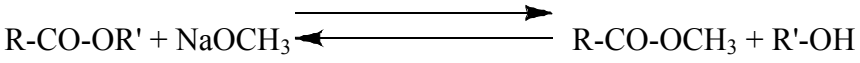
Ker smo želeli ugotoviti ustreznost in primernost klasične metode, smo se lotili razvoja t.i. *in situ* metode preestrenja maščobnih kislin.

Maščobne kisline navadno pretvorimo v pripadajoče metilne estre predvsem zato, da povečamo hlapnost in zmanjšamo tvorbo repov pri vrhovih v plinskem kromatogramu (12).

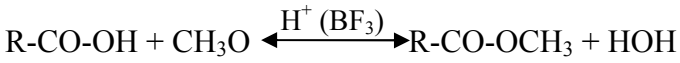
Metoda direktne priprave MEMK z raztopino BF_3 v metanolu je najbolj priljubljena metoda. Kot derivatizacijski reagent lahko uporabimo tudi raztopino natrijevega hidroksida v metanolu. Po pregledu literature smo izbrali metodo, ki sta jo uporabila Park in Goins. Raziskala sta *in situ* preestrenje z alkalno hidrolizo z natrijevim hidroksidom v metanolu in z nadaljnjo metilacijo z BF_3 v metanolu (23).

S hitrimi reakcijami smo v **bazičnem mediju** preestrili maščobne kisline:





Proste maščobne kisline smo zaestrili z reakcijo v **kislem mediju**:



V prvi stopnji smo izvedli preestrenje z NaOH v metanolu, naknadno kislo katalizo pa smo izvedli z BF_3 v metanolu (12). Reakcije potečejo hitro na polarnih lipidih, kot so proste maščobne kisline in fosfolipidi, saj se le-ti dobro raztapljajo v metanolu. Po drugi strani pa nepolarni lipidi, predvsem trigliceridi, so slabo topni v metanolu, zato je bilo potrebno v reakcijsko mešanico dodati diklorometan (12).

Metodo smo optimizirali ali modificirali tako, da smo uporabili dvakratno količino reagentov, kot je to navedeno v literaturi (23), da smo zagotovili popolno derivatizacijo v metilne estre in s tem pravilno kvantifikacijo. Poleg tega pa smo vse vzorce s to metodo še redčili v razmerju 1:20, da smo ohranili podobne razmere za analizo kot pri vzorcih s klasično metodo. Potek poskusa je ostal enak kakor pri klasični metodi ugotavljanja maščobnih kislin. Prav tako smo ohranili enake kromatografske razmere, kakršni so bili pri farmakopejski metodi ugotavljanja maščobnih kislin.

Pri vzorcih, analiziranih z modificirano farmakopejsko metodo B opazimo, da je pri vseh vzorcih vrednost α-linolenske kisline enaka 0. Razen pri vzorcu Dr. Böhm, kjer je preračunana vrednost 137 mg α-linolenske kisline na 1000 mg olja. Menimo, da zagotovo vrednost 0 ni realni odraz prehranskih dopolnil in zagotovo je α-linolenska kislina prisotna. Ker se vrh te kisline v kromatogramu zlije z oleinsko kislino (glej poglavje PRILOGE), je ne moremo kvantificirati. Prisotnost α-linolenske kisline smo ugotovili z analizo masnega

spektra fragmenta kromatografskega vrha. Videli smo, da je prisotna molekula α-linolenske kisline, vendar v tako nizkih koncentracijah, da so pod mejo kvantifikacije.

Naslednja velika opazka pri vseh analiziranih vzorcih z modificirano farmakopejsko metodo B je, da noben analiziran vzorec ni v deklariranih, predpisanih mejah. Pravzaprav le pri enem vzorcu (Krill olje, Biopharma) in še to pri DHK je vrednost odstopanja od deklarirane, predpisane vrednosti v pozitivno smer (33 %). Za vse ostale vzorce pa tabela kaže odstopanja od deklariranih vrednosti v negativno smer, manj od predpisanih na ovojnini. Tudi, če na hitro primerjamo stolpec koncentracije posameznih maščobnih kislin, izmerjenih z eno in drugo metodo, je razlika očitna. In veliko vzorcev ima bistveno nižjo izmerjeno koncentracijo maščobnih kislin z modificirano farmakopejsko metodo. Razlog za tak rezultat tiči v izboru metode in njenih pogojih. Farmakopejska metoda očitno ni dovolj specifična glede pogojev derivatizacije, saj navaja segrevanje vzorca med 50 in 60 °C (glej prilogo ali poglavje 4.1). Ni dovolj striktnih navodil, pogojev priprave vzorca. Posledično ne zreagira celoten vzorec, ali pride le do delne, nepopolne esterifikacije. Povečali oziroma podvojili smo tudi količino reagentov za nastanek metilnih estrov s farmakopejsko metodo. Žal rezultati tega ne kažejo. Zato smo paralelno (vzporedno) delali analize vzorcev z *in situ* metodo (glej poglavje PRILOGE). Izidi primerjalne metode, kot je že iz tabele z rezultati razvidno, so bistveno boljši in ni toliko negativnih odmikov od priporočenih, navedenih vrednosti na ovojnini izdelkov. Se pravi, da smo z *in situ* metodo dosegli derivatizacijo - nastanek metilnih estrov maščobnih kislin v večji meri. Glede na to, da v vzorcih nismo zaznali prostih maščobnih kislin, lahko domnevamo, da je bila pretvorba v metilne estre skoraj popolna. Uspeh lahko pripišemo sami metodi, saj ima višje temperaturne pogoje (90°C) za nastanek metilnih estrov. Prav tako je izbor drugih reagentov pri tej metodi boljši. In temu lahko pripišemo, da smo z *in situ* metodo dobili boljše ali primerljivejše rezultate s tistimi, ki so navedeni na izdelku prehranskega dopolnila.

Za kvantifikacijo rezultatov zato farmakopejska metoda ni primerna, je bolj primerna *in situ* metoda. Za večjo zanesljivost pa bi bila smiselna tudi validacija metode. Farmakopejska metoda je primerna za izračun razmerja nastalih estrov maščobnih kislin med eno in drugo metodologijo, saj za kvantifikacijo potrebujemo metodo, kjer pride do popolne derivatizacije in nastanka metilnih estrov maščobnih kislin. Zato farmakopejska metoda žal, ni metoda izbora, za kvantifikacijo maščobnih kislin.

Sama interpretacija rezultatov pridobljenih z eksperimentalnim delom je povezana s priporočili, standardi, ki jih dajejo farmakopeje. Lahko pa so to priporočila različnih strokovnih organizacij. Ena takih je USP DSC (United States Pharmacopeial Convention Dietary Supplement Standards), ki ima oddelek, ki se ukvarja z normativi v prehranskih dopolnilih (7). V našem primeru, kjer smo analizirali prehranska dopolnila z vsebnostjo, prisotnostjo omega 3 maščobnih kislin, USP DSC priporoča ali navaja, da mora biti vsebnost minimalno 95 % v prehranskem dopolnilu z omega 3 maščobnimi kislinami od deklarirane (navedene) na embalaži, ovojnini izdelka (7).

Iz tabele z rezultati je razvidno, da večina ali skoraj vsa prehranska dopolnila (razen izdelka MOR EPA® in Dr. Böhm® Omega 3 forte) nimajo deklarirane ali navedene vrednost za α-linolensko kislino. V prvem primeru gre za precejšnje odstopanje (244 %) v pozitivno smer od deklarirane vrednost na izdelku. Pri slednjem pa gre za negativni odklon od deklarirane vrednosti (-12,2 %), ki pa je po merilu USP DSC organizacije prevelik. V oči zbode tudi podatek, viden iz tabele, da nobeno analizirano prehransko dopolnilo z vsebnostjo omega 3 maščobnih kislin, nima na ovojnini navedene vrednosti (prisotnosti) dokozapentaenojske kisline (DPK). Zato kljub analiznim vsebnostim DPK molekule v prehranskih dopolnilih, ki so vidne, iz tabele žal nismo mogli ugotoviti odstotka odstopanja od deklarirane vsebnosti (saj jih ne navajajo). Kljub temu, da smo jo eksperimentalno ugotovili njeno prisotnost v prehranskih dopolnili. Tako, da največ podatkov o odstopanjih v eno ali drugo smer imamo pri EPK in DHK. Očitno sta to dve najpomembnejši omega 3 maščobni kislini s strani proizvajalcev, ki sta v očeh potrošnika najpomembnejši za njihovo in našo korist. Upoštevali smo tudi priporočilo USP DSC organizacije, da mora biti minimalno 95 % prisotne vsake maščobne kislin, ki je navedena ali deklarirana na izdelku.

Izdelki, ki imajo najvišje pozitivno odstopanje EPK in DHK so: Krill olje Biopharma, MOR EPA® Minami Nutrition, fit omega 3 Vital Slim® by Maxximum in Neptun Krill Oil, NOW®. Izdelki, ki imajo najvišje negativno odstopanje EPK in DHK so: Omega 3 Neuro by Natural Wealth®, Dr. Böhm® Omega 3 forte, Cod Liver Oil by Health Spark®. Večina prehranskih dopolnil pa se drži predpisanih vrednosti za DHK in EPK v svojih izdelkih. Nekateri produkti imajo negativen odklon od deklarirane vrednosti za EPK maščobno kislino in hkrati pozitiven odklon za DHK maščobno kislino. In obratno. Pozitiven odklon za EPK maščobno kislino in negativen odklon za DHK maščobno

kislino. Dva izdelka še posebej izstopata. To sta ribje olje in Lyprinol® by Medicinalis+. Ribje olje (Iecoris Oleum) nima navedenih nobenih vrednosti, koliko omega 3 maščobnih kislin bi naj vsebovalo. Kljub tej pomanjkljivosti, smo mnenja, da ga je kljub temu varno in smiselno uživati. Že preteklost in izkušnje nas učijo, da so z ribjim oljem pomagali človeškim zdravstvenim tegobam in jih uspešno premagovali. Negativno presenečenje je produkt Lyprinol. Na ovojnini izdelka je navedena celotna ali celokupna vsebnost omega 3 maščobnih kislin. Na žalost iz tega podatka ni moč sklepati kakšen je delež posameznih omega 3 maščobnih kislin.

Če povzamemo, priporočamo jemanje vseh prehranskih dopolnil z vsebnostjo omega 3 maščobnih kislin, ki imajo najvišje pozitivno odstopanje od deklarirane vrednosti. In vsa prehranska dopolnila, ki imajo manj kot pet odstotkov odstopanja od negativnega odklona vrednosti po priporočilih USP DSC organizacije. Prehranskih dopolnil, ki imajo prevelik negativni odklon od navedenih (deklariranih) vsebnosti ni primerno uživati. Saj z njimi ne bomo dosegli priporočene dnevne količine omega 3 maščobnih kislin (s tem mislimo predvsem na EPK in DHK, ki ju človeško telo ne zmore samo sintetizirati). Priporočen dnevno vnos EPK in DHK je 250 mg.

6 ZAKLJUČEK

V okviru magistrske naloge smo z naborom naključno izbranih prehranskih dopolnil z vsebnostjo omega-3 maščobnih kislin želeli z ustrezno metodologijo kvantitativno vrednotiti vsebnost teh kislin v naključno izbranih prehranskih dopolnilih. Metodi izbora sta bili modificirana farmakopejska metoda B in paralelna *in situ* metoda. S primerjavama metod smo želeli dokazati katera je boljša za kvantifikacijo omega-3 maščobnih kislin. Analizni sistem za kvantifikacijo je bil GC-MS. Poleg kvantifikacije omega-3 maščobnih kislin smo želeli hkrati tudi preveriti ustreznost z deklariranimi vrednostmi in vrednostmi, ki jih priporočajo farmakopeje ali za to pristojne inštitucije (USP DSC).

Ugotovili smo, da je *in situ* metoda boljša in primernejša za kvantifikacijo omega-3 maščobnih kislin, vendar bi bila za zagotavljanje večje zanesljivosti smiselna validacija metode. Modificirana farmakopejska metoda B, ni primer dobrega analiznega postopka za kvantifikacijo omega-3 maščobnih kislin, ker derivatizacija do metilnih estrov ni popolna. Rezultati analiz so pokazali, da je večina (14 od 23) prehranskih dopolnil v predpisanih deklariranih mejah USP DSC konvencije. Na področju deklariranih vrednosti omega-3 maščobnih kislin vlada zmeda. Različne države priporočajo različne vrednosti omega-3 maščobnih kislin (24, 25). Glede zgornje meje količine zaužitih omega-3 maščobnih kislin je Ameriška agencija za zdravila in hrano (FDA ali U.S. Food and Drug Administration) postavila zgornjo mejo pri 3 g/dan. Večje količine niso priporočljive zaradi neželenih učinkov. Če pa so opravičene ali zaželene glede na posameznikovo zdravstveno stanje, mora biti pod zdravniškim nadzorom. Za dve (2) prehranski dopolnil ni na voljo podatkov, zato ne vemo, ali so ustrezna ali ne. Dve prehranski dopolnili sta delno ustrezni- to pomeni, da le ena omega-3 maščobna kislina ne doseže priporočil USP DSC konvencije. Pet prehranskih dopolnil je neustreznih oziroma ne dosega želenega odstotka odstopanja od priporočil USP DSC. Ne pomeni pa to, da bo tako tudi v prihodnje, saj je zaradi ohlapne zakonodaje in minimalnega nadzora kakovost tovrstnih izdelkov vedno pod vprašajem, zato je nakup žal bolj stvar zaupanja.

Ker sama prehranska dopolnila niso tako strogo nadzorovana, kot je to značilno za zdravila, ne bi bilo slabo, da bi ob prvi prijavi proizvajalec (tisti, ki zaprosi za dovoljenje na trg s prehranskimi dopolnili na Ministrstvu za zdravje), podal tudi deklarirane vsebnosti za vse omega 3 maščobne kisline (α-linolenska kislina, EPK, DHK, DPK). Zaradi lažjega nadzora s strani Zdravstvenega inšpektorata in hkrati v poduk potencialnim kupcem, ki

bodo vedeli, kaj kupujejo in koliko oz. katere omega-3 maščobne kisline so prisotne v prehranskem dopolnilu. Smiselno bi bilo tudi poenotiti deklarirane vrednosti omega-3 maščobnih kislin med državami (znotraj EU, če že ne v svetovnem merilu), saj prihaja do razlik v priporočilih, koliko je potrebno na dan zaužiti omega-3 maščobnih kislin, da bomo zagotovili dnevne potrebe organizma. Ker dandanes z raznoliko hrano in načinom življenja, ki ga živimo ter z različnimi zdravstvenimi ali fiziološkimi stanji organizma, ne vnesemo dovolj ustreznih hranil, ki bi "nasitila" naš organizem, je smiselna občasna uporaba prehranskih dopolnil. Vedno pa moramo imeti v mislih, da so to dopolnila in ne nadomestilo za raznovrstno prehrano. Previdnost in dodatno izobraževanje na tem področju ni odveč, saj je trg tovrstnih izdelkov zelo velik. Ker pa si ne želimo škodovati, se je treba o smiselnosti jemanja prehranskih dopolni prej ustrezno podučiti in posvetovati z zdravnikom ali farmacevtom.

7 VIRI IN LITERATURA

1. Vodopivec Miha: Odnos porabnikov do prehranskih dopolnil. Diplomsko delo, Univerza v Ljubljani, Ekonomska fakulteta, maj 2011.

2. Kos Mitja: Vloga in pomen prehranskih dopolnil, Prehranska dopolnila I, urednika Aleš Obreza in Tomaž Vovk: strokovno izobraževanje, Ljubljana: Fakulteta za farmacijo, 2009: 7-14.

3. Bukovec Anja: Analiza panoge prehranskih dopolnil v Sloveniji. Diplomsko delo, Univerza v Ljubljani, Ekonomska fakulteta, september 2010.

4. Zakon o zdravilih, Ur.l. RS, št. 31/2006. Spremembe: Ur.l. RS, št. 45/2008 http://www.uradni-list.si/1/content?id=72463 (dostop: 20.5.2013).

5. Tuš M., Prehransko dopolnilo ali zdravilo? Na recept ali brez?, Lekarne Maribor http://www.mb-lekarne.si/slo/svetovalec/pogosta_vprasanja/-1/ni_teme/-1/ni_razvrscanja/65/prehransko_dopolnilo_ali_zdravilo__na_recept_ali_brez__ (dostop: 25.04.2013).

6. http://www.mz.gov.si/si/delovna_podrocja/javno_zdravje/sektor_za_varnost_in_zdravstveno_ustreznost_hrane/postopek_prve_prijave_notifikacije_prehranskih_dopolnil/ (dostop: 21.04.2013).

7. Pravst I., Blaznik U., Krbavčič A., Kreft S., Kušar A., Mičović E., Razinger B., Recek M., Repe J., Rogelj I., Stibilj V.: Priporočila za zagotavljanje kakovosti prehranskih dopolnil, pregleden znanstveni članek, Farmacevtski vestnik 2012; 63 http://www.dlib.si/details/URN:NBN:SI:DOC-VNDLKB9V (dostop: 25.04.2013).

8. Recek M., Triler M.: Prehranska dopolnila; novinarska konferenca, 1.2.2012 http://www.mz.gov.si/fileadmin/mz.gov.si/pageuploads/mz_dokumenti/delovna_podrocja/javno_zdravje/prehranska_dopolnila/predstavitev_prehranska_dopolnila_010212.pdf (dostop: 25.4.2013).

9. Sonc M.: Interakcije in neželeni učinki prehranskih dopolnil. ONKOLOGIJA/za prakso, leto XI, številka 1, junij 2007 http://www.onko-i.si/fileadmin/onko/datoteke/dokumenti/Onkologija_1_2007_22.pdf (dostop: 21.04.2013).

10. Rihtarič M.: Omega-3 nenasičene maščobne kisline, Ljubljanske lekarne http://www.lekarnaljubljana.si/public/datoteke/clanki_omega---zadnaj-verzija.pdf (dostop: 03.05.2013).

11. Gerbič M.: Vsebnost maščobnih kislin v povrhnjici in semenih različnih kultivarjev sliv. Diplomsko delo, Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za živilstvo, Ljubljana 2010.

12. Tomsič J.: Ugotavljanje maščobnokislinske sestave svežih in staranih kokošjih jajc iz različnih virov z uporabo plinske kromatografije. Diplomsko delo, Univerza v Ljubljani, Fakulteta za farmacijo, Ljubljana 2013.

13. Akoh C.C., Min B.D.: Food Lipids- Chemistry, Nutrition and Biotechnology, 3rd edition, CRC Press, 2008.

14. Zvonar Alenka: Funkcionalni lipidi kot nutricevtiki in prehranska dopolnila, Prehranska dopolnila II, urednika Aleš Obreza in Tomaž Vovk: strokovno izobraževanje, Ljubljana: Fakulteta za farmacijo, 2010: 32-41.

15. Kure V.: Vsebnost maščobnih kislin v svežih in konzerviranih morskih ribah. Diplomsko delo, Univerza v Ljubljani, Biotehniška fakulteta, Oddelek za živilstvo, Ljubljana 2006.

16. Ettre S. L.: M. S. Tswett and the invention of Chromatography, LC-GC Europe, september2005 http://www.chromatographyonline.com/lcgc/data/articlestandard//lcgceurope/382003/69718/article.pdf (dostop: 15.5.2013).

17. Pušaver S.: Določanje izbranih spojin v hidrolizatu biomase s plinsko kromatografijo in masno spektrometrijo. Diplomsko delo, Univerza v Mariboru, Fakulteta za kemijo in kemijsko tehnologijo, september 2012.

18. Lekše S.: Izolacija α- in β-pinena iz rastlinskih materialov. Diplomsko delo, Univerza v Mariboru, Fakulteta za kemijo in kemijsko tehnologijo, september 2011.

19. Miuc A.: Priprava vzorcev za določevanje spojin obstojnih onesnaževal z metodo GC/MS. Diplomsko delo, Univerza v Mariboru, Fakulteta za kemijo in kemijsko tehnologijo, marec 2012.

20. Kolar M.: Uporaba plinske kromatografije z masno spektrometrijo za določanje atrazina v vodi. Diplomsko delo, Univerza v Mariboru, Fakulteta za kemijo in kemijsko tehnologijo, december 2008.

21. European Pharmacopoeia (Ph. Eur. 7.0), elektronska verzija: 2.4.22. Composition of fatty acids by GC. European Directorate for the Quality of Medicines and HealthCare, Strasbourg, Francija.

22. European Pharmacopoeia (Ph. Eur. 7.0), elektronska verzija: Fish Oil, Rich in Omega-3 acids. European Directorate for the Quality of Medicines and HealthCare, Strasbourg, Francija; 2019-2020.

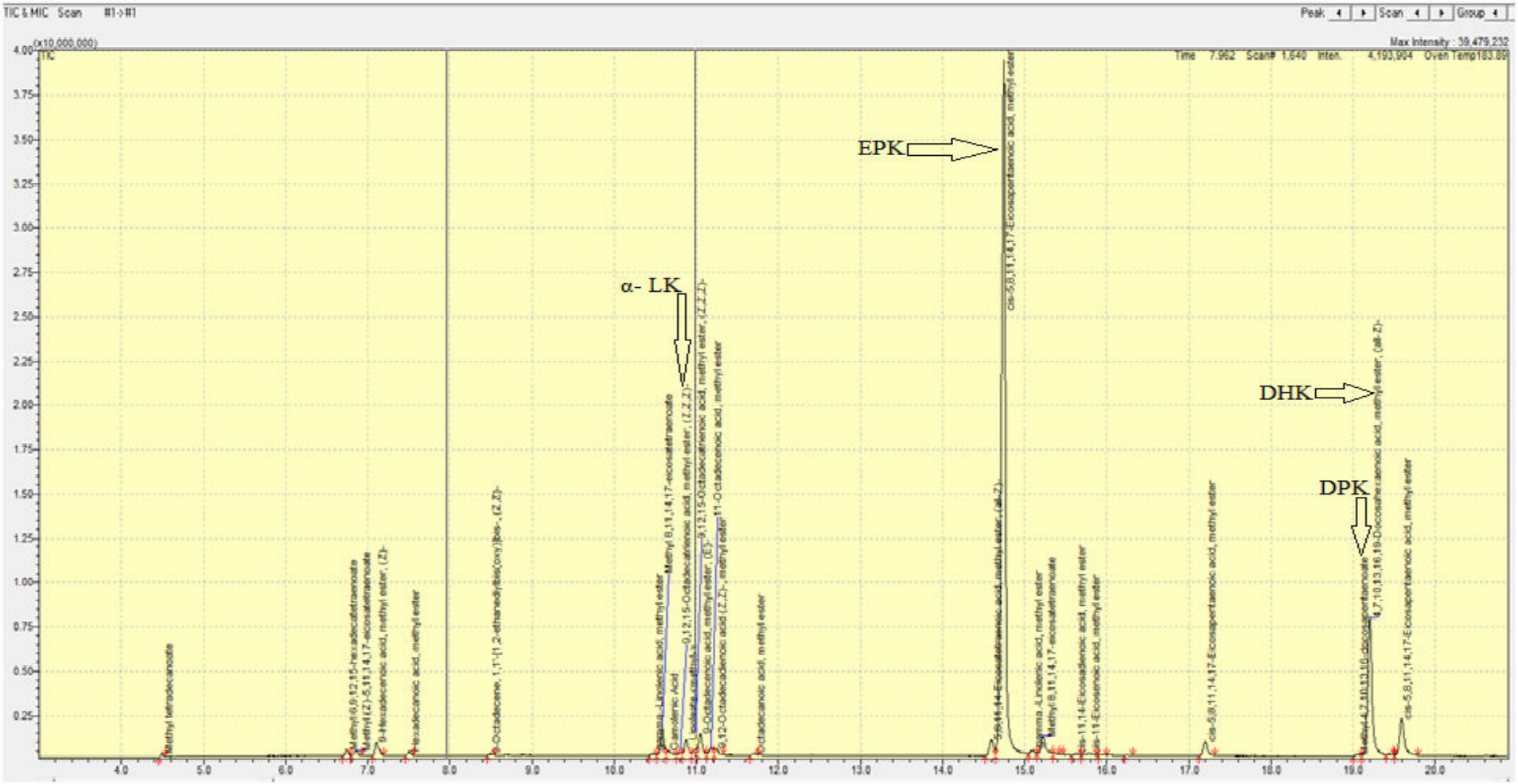
23. Park P.W., Goins R.E.: *In Situ* Preparation of Fatty Acid Methly Esters for Analysis of Fatty Acid Composition in Foods. Journal of Food Science Vol. 59, no.6, 1994; 1262-1266

24. White Paper: Long Chain Omega-3 Fatty Acids in Human Health, Council for Responsible Nutrition, Washington DC, ZDA, julij 2005 http://www.crnusa.org/pdfs/CRNo3wg_whitepaper.pdf (dostop: 31.08.2013)

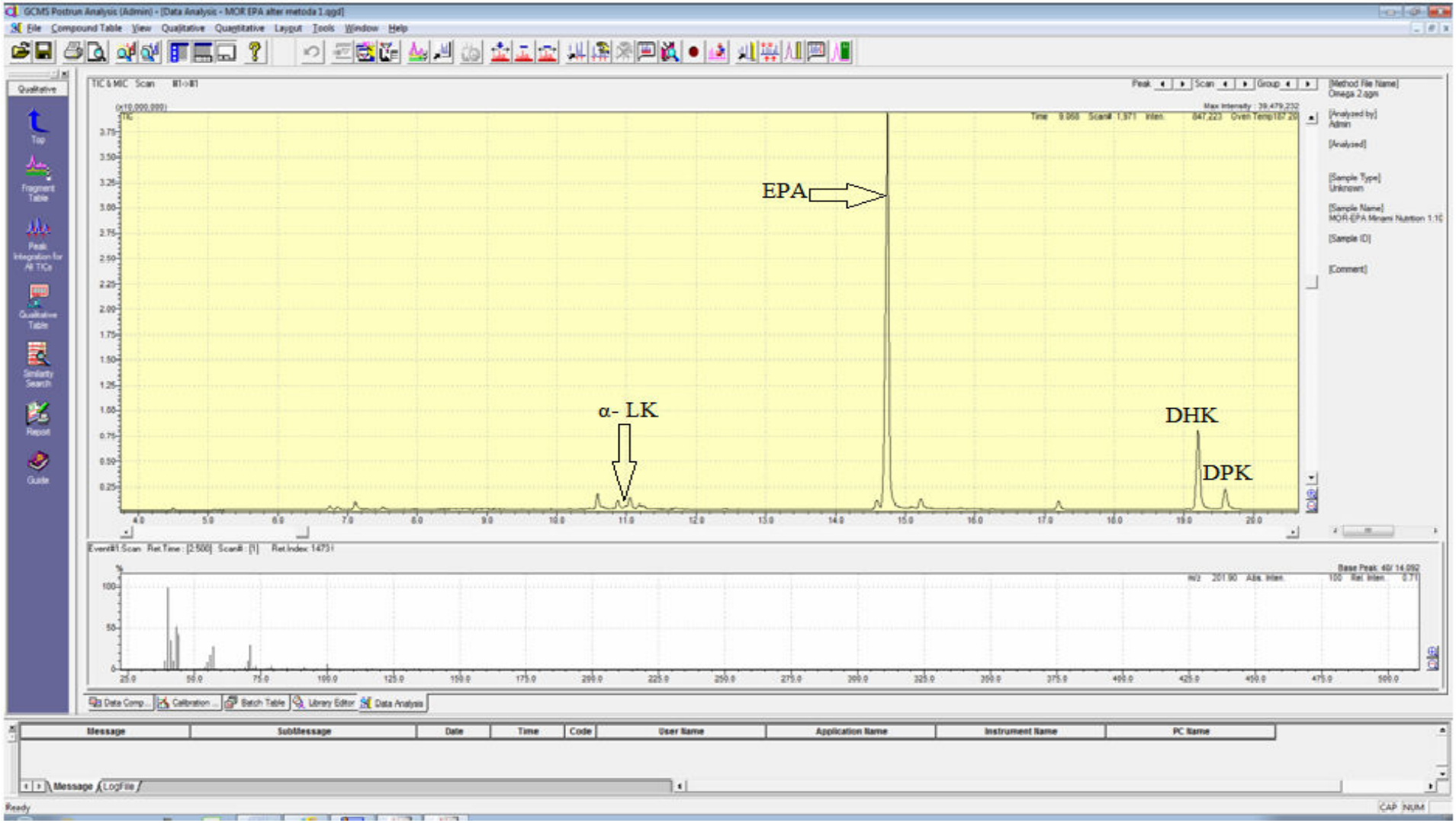
25. Nelson G.: American Heart Association Calls for Eating Fish Twice Per Week - What's a Vegetarian To Do?, Vegetarian Journal, September/Oktober 2001 http://www.vrg.org/journal/vj2001sep/2001sepomega3.htm (dostop: 31.08.2013)

8 PRILOGE

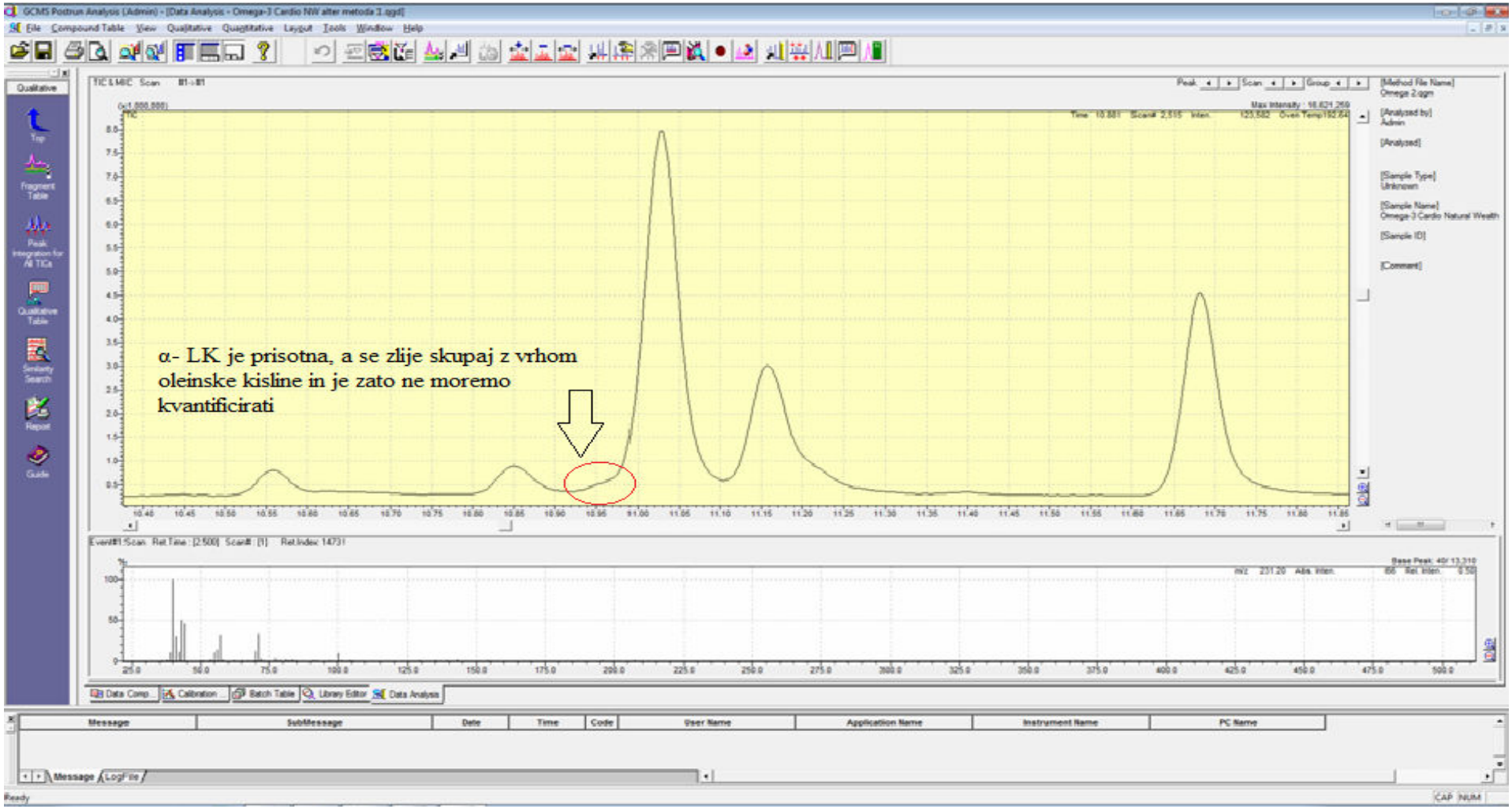
Prehransko dopolnilo MOR EPA Minami Nutrition – primer kromatografa vzorca



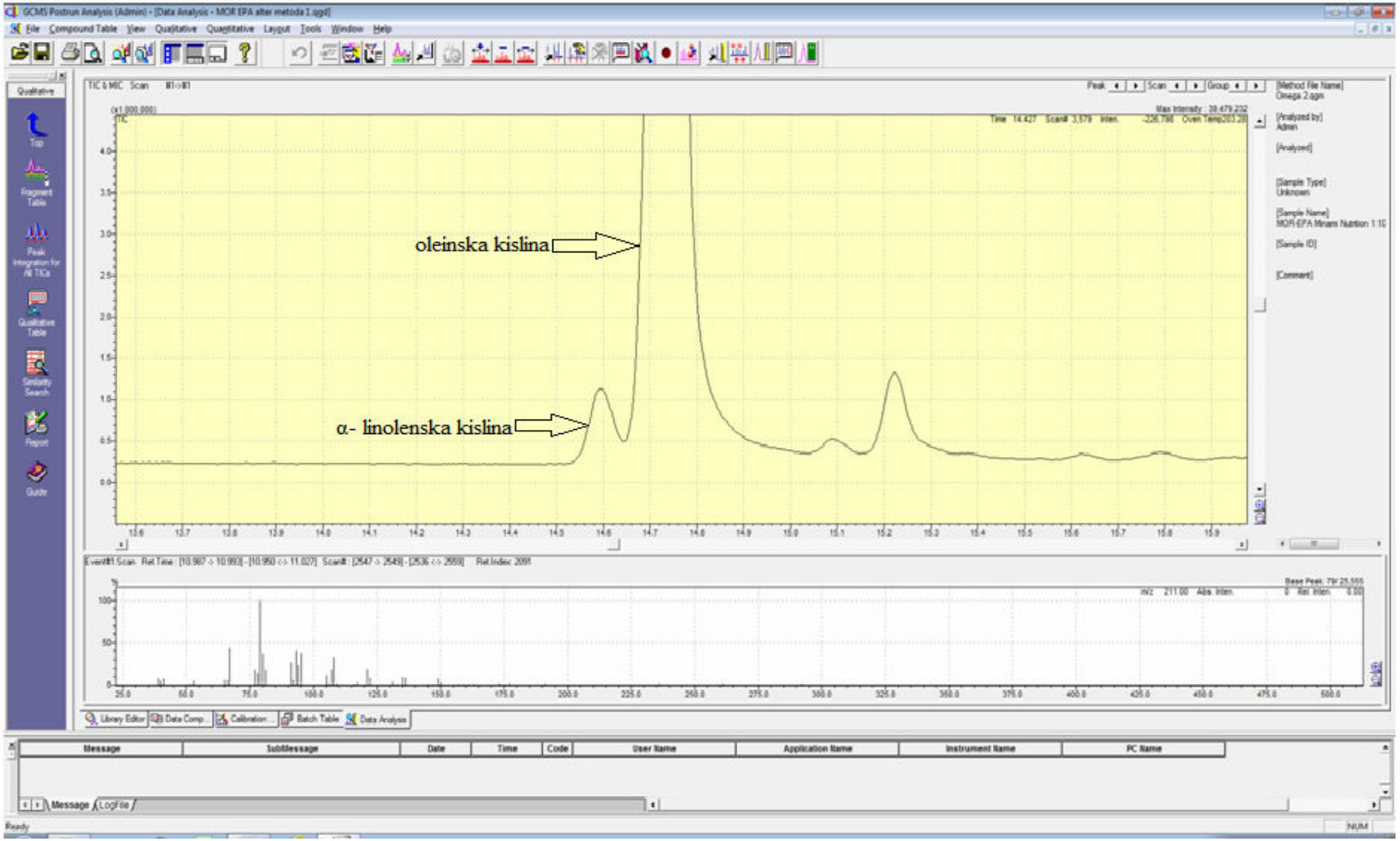
Prehransko dopolnilo MOR EPA Minami Nutrition – vrhovi omega-3 maščobnih kislin



Natural Wealth® Omega 3 Cardio



MOR EPA – povečan izsek kromatograma (kjer vidimo prisotno α-linolensko kislino in oleinsko kislino; na navadnem kromatogramu tega ne vidimo, vidimo le vrh oleinske kisline).



Certifikat analize DHK

Certificate of Analysis

DESCRIPTION: All cis-4,7,10,13,16,19-Docosa-

CATALOG NO.: 47570-U MFG DATE: May-2012

LOT NO.: LB92354 EXPIRATION DATE: May-2015

SOLVENT: HEPTANE

ANALYTE	CAS NUMBER	PERCENT PURITY(1)	WEIGHT(2) CONCENTRATION	ANALYTICAL(3) CONCENTRATION	STD DEV	SUPELCO LOT NO
ALL CIS-4,7,10,13,16,19-DOCOSA	2566-90-7	99.9	10000	10006	+/- 69.0	LB85950

(1) Determined by capillary GC-FID, unless otherwise noted.
(2) NIST traceable weights are used to verify balance calibration with the preparation of each lot. Concentration of analyte in solution is ug/ml +/- 0.5%, uncertainty based upon balance and Class A volumetric glassware. Weights are corrected for analytes less than 98% pure.
(3) Determined by chromatographic analysis against an independently prepared reference lot. Mean of replicate injections.

Elwood Doughty
QA Manager

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SUPELCO Analytical
595 North Harrison Road
Bellefonte, PA 16823-0048 USA
Phone(814)359-3441

Certifikat analize DPK

Certificate of Analysis

DESCRIPTION: Methyl cis-7,10,13,16,19-Docosapentaenoate

CATALOG NO.: 47563-U MFG DATE: Dec-2011

LOT NO.: LB89311 EXPIRATION DATE: Dec-2014

SOLVENT: HEPTANE

ANALYTE	CAS NUMBER	PERCENT PURITY(1)	WEIGHT(2) CONCENTRATION	ANALYTICAL(3)	STD DEV	SUPELCO LOT NO
METHYL CIS-7,10,13,16,19-DOCOS	108698-02-8	99.4	10000	10090	+/- 290.5	LB77632

(1) Determined by capillary GC-FID, unless otherwise noted.
(2) NIST traceable weights are used to verify balance calibration with the preparation of each lot. Concentration of analyte in solution is ug/ml +/- 0.5%, uncertainty based upon balance and Class A volumetric glassware. Weights are corrected for analytes less than 98% pure.
(3) Determined by chromatographic analysis against an independantly prepared reference lot. Mean of replicate injections.

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Certifikat analize EPK

Certificate of Analysis

DESCRIPTION: cis-5,8,11,14,17 Eicosapentaenoic, ME

CATALOG NO.: 47571-U MFG DATE: Apr-2011

LOT NO.: LB83970 EXPIRATION DATE: Apr-2014

SOLVENT: HEPTANE

ANALYTE	CAS NUMBER	PERCENT PURITY(1)	WEIGHT(2) CONCENTRATION	ANALYTICAL(3)	STD DEV	SUPELCO LOT NO
METHYL CIS-5,8,11,14,17-EICOSA	2734-47-6	99.9	10000	9825	+/- 446.4	LB83268

(1) Determined by capillary GC-FID, unless otherwise noted.
(2) NIST traceable weights are used to verify balance calibration with the preparation of each lot. Concentration of analyte in solution is ug/ml +/- 0.5%, uncertainty based upon balance and Class A volumetric glassware. Weights are corrected for analytes less than 98% pure.
(3) Determined by chromatographic analysis against an independently prepared reference lot. Mean of replicate injections.

Elwood Doughty
QA Manager

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SUPELCO Analytical
595 North Harrison Road
Bellefonte, PA 16823-0048 USA
Phone (814) 359-3441

Certifikat analize F.A.M.E. Mix C20:1-C20:5

Certificate of Analysis

DESCRIPTION: F.A.M.E. Mix C20:1-C20:5

CATALOG NO.: 18912-1AMP MFG DATE: Apr-2011

LOT NO.: LB83870 EXPIRATION DATE: Apr-2014

ANALYTE (1)	CAS NUMBER	PERCENT PURITY (2)	WEIGHT% (3) CONCENTRATION			ANALYTICAL (4) STD DEV	SUPELCO LOT NO
CIS-11,14-EICOSADIENOIC ACID M	2463-02-7	99.9	25.1	25.5	+/-	1.03	LB81408
METHYL CIS-5,8,11,14-EICOSATET	2566-89-4	99.6	25.0	25.6	+/-	1.19	LB79764
METHYL CIS-5,8,11,14,17-EICOSA	2734-47-6	99.9	25.1	25.8	+/-	1.11	LB83057
METHYL EICOSENOATE	2390-09-2	98.1	24.9	24.2	+/-	1.02	LB76597

(1) Listed in alphabetical order.
(2) Determined by capillary GC-FID, unless otherwise noted.
(3) Weight percent of analyte, calculated by using analyte weights. The Total may not equal 100% due to rounding. Weight concentrations may not remain stable after opening, even if resealed. NIST-Traceable weights are used to verify balance calibration with the preparation of each lot.
(4) Determined by chromatographic analysis against an independently prepared reference lot. Mean of replicate injections.

Elwood Doughty
QA Manager

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Certifikat alfa linolenske kisline

SIGMA-ALDRICH®

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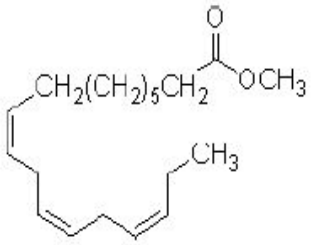
3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Methyl linolenate – ≥99% (GC)

Product Number:	**L2626**
Lot Number:	**SLBB3252V**
Brand:	SIGMA
CAS Number:	301-00-8
MDL Number:	MFCD00135851
Formula:	C19H32O2
Formula Weight:	292.46 g/mol
Storage Temperature:	Store at -20 °C
Quality Release Date:	28 NOV 2011
Recommended Retest Date:	NOV 2018



Test	Specification	Result
Appearance (Color)	Colorless to Very Faint Yellow	Colorless
Appearance (Turbidity)	Clear	Clear
Appearance (Form)	Liquid	Liquid
Proton NMR spectrum	Conforms to Structure	Conforms
13C NMR Identity	Conforms to Structure	Conforms
Purity (GC)	≥ 99 %	99 %
Recommended Retest Period 7 years	----------	----------

Rodney Burbach, Manager
Analytical Services
St. Louis, Missouri US

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Ph Eur - Composition of fatty acids by GC

To 0.5 mL of the test solution and of each of the reference solutions in test-tubes, add 5.0 mL of a freshly prepared 0.5 g/L solution of *methylbenzothiazolone hydrazone hydrochloride R*. Close the tubes, shake and allow to stand for 60 min. Add 1 mL of *ferric chloride-sulfamic acid reagent R* and allow to stand for 15 min. Measure the absorbance (*2.2.25*) of the solutions at 628 nm. Calculate the content of formaldehyde in the vaccine to be examined from the calibration curve established using the reference solutions. The test is invalid if the correlation coefficient (*r*) of the calibration curve is less than 0.97.

Emulsions. If the vaccine to be examined is an emulsion, the aqueous phase is separated using a suitable procedure and used for preparation of the test solution. The following procedures have been found suitable.

(a) Add 1.0 mL of the vaccine to be examined to 1.0 mL of *isopropyl myristate R* and mix. Add 1.3 mL of *1 M hydrochloric acid*, 2.0 mL of *chloroform R* and 2.7 mL of a 9 g/L solution of *sodium chloride R*. Mix thoroughly. Centrifuge at 15 000 *g* for 60 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*. If this procedure fails to separate the aqueous phase, add 100 g/L of *polysorbate 20 R* to the sodium chloride solution and repeat the procedure but centrifuge at 22 500 *g*.

(b) Add 1.0 mL of the vaccine to be examined to 1.0 mL of a 100 g/L solution of *sodium chloride R* and mix. Centrifuge at 1000 *g* for 15 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*.

(c) Add 1.0 mL of the vaccine to be examined to 2.0 mL of a 100 g/L solution of *sodium chloride R* and 3.0 mL of *chloroform R* and mix. Centrifuge at 1000 *g* for 5 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*.

01/2008:20419

2.4.19. ALKALINE IMPURITIES IN FATTY OILS

In a test-tube mix 10 mL of recently distilled *acetone R* and 0.3 mL of *water R* and add 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *alcohol R*. Neutralise the solution if necessary with *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide*. Add 10 mL of the oil to be examined, shake and allow to stand. Not more than 0.1 mL of *0.01 M hydrochloric acid* is required to change the colour of the upper layer to yellow.

01/2008:20421

2.4.21. FOREIGN OILS IN FATTY OILS BY THIN-LAYER CHROMATOGRAPHY

Examine by thin-layer chromatography (*2.2.27*) using *kieselguhr G R* as the coating substance. Impregnate a plate by placing it in a chromatographic tank containing the necessary quantity of a mixture of 10 volumes of *liquid paraffin R* and 90 volumes of *light petroleum R* so that the plate dips about 5 mm beneath the surface of the liquid. When the impregnation mixture has risen by at least 12 cm from the lower edge of the plate, remove the plate and allow the solvent to evaporate for 5 min. Carry out the chromatography in the same direction as the impregnation.

Preparation of the mixture of fatty acids. Heat 2 g of the oil with 30 mL of *0.5 M alcoholic potassium hydroxide* under a reflux condenser for 45 min. Add 50 mL of *water R*, allow to cool, transfer to a separating funnel and extract with three quantities, each of 50 mL, of *ether R*. Discard the ether extracts, acidify the aqueous layer with *hydrochloric acid R* and extract with three quantities, each of 50 mL, of *ether R*. Combine the ether extracts and wash with three quantities, each of 10 mL, of *water R*; discard the washings, dry the ether over *anhydrous sodium sulfate R* and filter. Evaporate the ether on a water-bath. Use the residue to prepare the test solution. The fatty acids may also be obtained from the soap solution prepared during the determination of the unsaponifiable matter.

Test solution. Dissolve 40 mg of the mixture of fatty acids obtained from the substance to be examined in 4 mL of *chloroform R*.

Reference solution. Dissolve 40 mg of the mixture of fatty acids obtained from a mixture of 19 volumes of *maize oil R* and 1 volume of *rapeseed oil R* in 4 mL of *chloroform R*.

Apply to the plate 3 µL of each solution. Develop over a path of 8 cm using a mixture of 10 volumes of *water R* and 90 volumes of *glacial acetic acid R*. Dry the plate at 110 °C for 10 min. Allow to cool and, unless otherwise prescribed, place the plate in a chromatographic chamber, with a tightly fitting lid, that has previously been saturated with iodine vapour by placing *iodine R* in an evaporating dish at the bottom of the chamber. After some time brown or yellowish-brown spots become visible. Remove the plate and allow to stand for a few minutes. When the brown background colour has disappeared, spray with *starch solution R*. Blue spots appear which may become brown on drying and again become blue after spraying with *water R*. The chromatogram obtained with the test solution always shows a spot with an R_F of about 0.5 (oleic acid) and a spot with an R_F of about 0.65 (linoleic acid) corresponding to the spots in the chromatogram obtained with the reference solution. With some oils a spot with an R_F of about 0.75 may be present (linolenic acid). By comparison with the spot in the chromatogram obtained with the reference solution, verify the absence in the chromatogram obtained with the test solution of a spot with an R_F of about 0.25 (erucic acid).

01/2008:20422
corrected 6.8

2.4.22. COMPOSITION OF FATTY ACIDS BY GAS CHROMATOGRAPHY

The test for foreign oils is carried out on the methyl esters of the fatty acids contained in the oil to be examined by gas chromatography (*2.2.28*).

METHOD A

This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, hydroperoxy-, cyclopropyl or cyclopropenyl group, or those that contain a large proportion of fatty acids of chain length less than 8 carbon atoms or to oils with an acid value greater than 2.0.

Test solution. When prescribed in the monograph, dry the oil to be examined before the methylation step. Weigh 1.0 g of the oil into a 25 mL round-bottomed flask with a ground-glass neck fitted with a reflux condenser and a gas port into the flask. Add 10 mL of *anhydrous methanol R* and 0.2 mL of a 60 g/L solution of *potassium hydroxide R* in *methanol R*. Attach the reflux condenser, pass *nitrogen R* through the mixture at a rate of about 50 mL/min, shake and heat to boiling. When the solution is clear (usually after about 10 min), continue heating for a further 5 min. Cool the flask under running water and transfer the contents to a separating funnel. Rinse the flask with 5 mL of *heptane R* and transfer the rinsings to the separating funnel and shake. Add 10 mL of a 200 g/L solution of *sodium chloride R* and shake vigorously. Allow to separate and transfer the organic layer to a vial containing *anhydrous sodium sulfate R*. Allow to stand, then filter.

Reference solution (a). Prepare 0.50 g of the mixture of calibrating substances with the composition described in one of the 2.4.22 tables, as prescribed in the individual monograph (if the monograph does not mention a specific solution, use the composition described in Table 2.4.22.-1). Dissolve in *heptane R* and dilute to 50.0 mL with the same solvent.

Reference solution (b). Dilute 1.0 mL of reference solution (a) to 10.0 mL with *heptane R.*

Reference solution (c). Prepare 0.50 g of a mixture of fatty acid methyl esters that corresponds in composition to the mixture of fatty acids indicated in the monograph of the substance to be examined. Dissolve in *heptane R* and dilute to 50.0 mL with the same solvent. Commercially available mixtures of fatty acid methyl esters may also be used.

Column:

- *material*: fused silica, glass or quartz;
- *size*: *l* = 10-30 m, Ø = 0.2-0.8 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.1-0.5 µm) or another suitable stationary phase.

Carrier gas: *helium for chromatography R* or *hydrogen for chromatography R.*

Flow rate: 1.3 mL/min (for a column Ø = 0.32 mm).

Split ratio: 1:100 or less, according to the internal diameter of the column used (1:50 when Ø = 0.32 mm).

Temperature:

- *column*: in isothermal conditions, 160-200 °C, according to the length and type of column used (200 °C for a column 30 m long and coated with a layer of *macrogol 20 000 R*); if a linear temperature programming is necessary, raise the temperature of the column at a rate of 3 °C/min from 170 °C to 230 °C, for example;
- *injection port*: 250 °C;
- *detector*: 250 °C.

Detection: flame ionisation.

Injection: 1 µL.

System suitability when using the mixture of calibrating substances in Table 2.4.22.-1 or Table 2.4.22.-3:

- *resolution*: minimum 1.8 between the peaks due to methyl oleate and methyl stearate in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 5 for the peak due to methyl myristate in the chromatogram obtained with reference solution (b);
- *number of theoretical plates*: minimum 30 000, calculated for the peak due to methyl stearate in the chromatogram obtained with reference solution (a).

System suitability when using the mixture of calibrating substances in Table 2.4.22.-2:

- *resolution*: minimum 4.0 between the peaks due to methyl caprylate and methyl caprate in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 5 for the peak due to methyl caproate in the chromatogram obtained with reference solution (b);
- *number of theoretical plates*: minimum 15 000, calculated for the peak due to methyl caprate in the chromatogram obtained with reference solution (a).

ASSESSMENT OF CHROMATOGRAMS

Avoid working conditions tending to give masked peaks (presence of constituents with small differences between retention times, for example linolenic acid and arachidic acid).

Qualitative analysis. Identify the peaks in the chromatogram obtained with reference solution (c) (isothermal operating conditions or linear temperature programming).

When using isothermal operating conditions, the peaks may also be identified by drawing calibration curves using the chromatogram obtained with reference solution (a) and the information given in Tables 2.4.22.-1, 2.4.22.-2 or 2.4.22.-3.

Table 2.4.22.-1. – *Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture to be examined be added to the calibration mixture, when the qualitative analysis is done using calibration curves)*

Mixture of the following substances	Composition (per cent *m/m*)
Methyl laurate R	5
Methyl myristate R	5
Methyl palmitate R	10
Methyl stearate R	20
Methyl arachidate R	40
Methyl oleate R	20

Table 2.4.22.-2. – *Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture to be examined be added to the calibration mixture, when the qualitative analysis is done using calibration curves)*

Mixture of the following substances	Composition (per cent *m/m*)
Methyl caproate R	10
Methyl caprylate R	10
Methyl caprate R	20
Methyl laurate R	20
Methyl myristate R	40

Table 2.4.22.-3. – *Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture to be examined be added to the calibration mixture, when the qualitative analysis is done using calibration curves)*

Mixture of the following substances	Composition (per cent *m/m*)
Methyl myristate R	5
Methyl palmitate R	10
Methyl stearate R	15
Methyl arachidate R	20
Methyl oleate R	20
Methyl eicosenoate R	10
Methyl behenate R	10
Methyl lignocerate R	10

Measure the reduced retention time (t'_R) of each peak in the chromatogram obtained with reference solution (a). t'_R is the retention time measured from the solvent peak and not from the time of injection. Plot the straight line:

$$log\ (t'_R) = f\ (equivalent\ chain\ length)$$

The logarithms of t'_R of unsaturated acids are situated on this line at points corresponding to non-integer values of carbon atoms known as 'equivalent chain lengths'; the equivalent chain length is the length of the theoretical saturated chain that would have the same t'_R as the fatty acid to be identified. For example, linoleic acid has the same t'_R as the theoretical saturated fatty acid having 18.8 carbon atoms.

Identify the peaks in the chromatogram obtained with the test solution by means of the straight line and the reduced retention times. Equivalent chain lengths are given in Table 2.4.22.-4.

Quantitative analysis. In general, the normalisation procedure is used in which the sum of the areas of the peaks in the chromatogram, except that of the solvent, is set at 100 per cent.

The content of a constituent is calculated by determining the area of the corresponding peak as a percentage of the sum of the areas of all the peaks. Disregard any peak with an area less than 0.05 per cent of the total area.

In certain cases, for example in the presence of fatty acids with 12 or less carbon atoms, correction factors can be prescribed in the individual monograph to convert peak areas in per cent *m/m*.

Table 2.4.22.-4. – *Equivalent chain lengths (this value, which is to be calculated using calibration curves, is given as an example for a column of macrogol 20 000 R)*

Fatty acid	Equivalent chain length
Caproic acid	6.0
Caprylic acid	8.0
Capric acid	10.0
Lauric acid	12.0
Myristic acid	14.0
Palmitic acid	16.0
Palmitoleic acid	16.3
Margaric acid	17.0
Stearic acid	18.0
Oleic acid	18.3
Linoleic acid	18.8
Gamma-linolenic acid	19.0
Alpha-linolenic acid	19.2
Arachidic acid	20.0
Eicosenoic acid (gondoic acid)	20.2
Arachidonic acid	21.2
Behenic acid	22.0
Erucic acid	22.2
12-Oxostearic acid	22.7
Ricinoleic acid	23.9
12-Hydroxystearic acid	23.9
Lignoceric acid	24.0
Nervonic acid	24.2

METHOD B

This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, hydroperoxy-, cyclopropyl or cyclopropenyl group or to oils with an acid value greater than 2.0.

Test solution. Introduce 0.100 g of the substance to be examined into a 10 mL centrifuge tube with a screw cap. Dissolve with 1 mL of *heptane R* and 1 mL of *dimethyl carbonate R* and mix vigorously under gentle heating (50-60 °C). Add, while still warm, 1 mL of a 12 g/L solution of *sodium R* in *anhydrous methanol R*, prepared with the necessary precautions, and mix vigorously for about 5 min. Add 3 mL of *distilled water R* and mix vigorously for about 30 s. Centrifuge for 15 min at 1500 *g*. Inject 1 µL of the organic phase.

Reference solutions and assessment of chromatograms. Where there is no specific prescription in the individual monograph, proceed as described under Method A.

Column:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

Carrier gas: *helium for chromatography R.*

Flow rate: 0.9 mL/min.

Split ratio: 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 15	100
	15 - 36	100 → 225
	36 - 61	225
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: 1 µL.

METHOD C

This method is not applicable to oils that contain glycerides of fatty acids with epoxy-, hydroepoxy-, hydroperoxy-, aldehyde, ketone, cyclopropyl and cyclopropenyl groups, and conjugated polyunsaturated and acetylenic compounds because of partial or complete destruction of these groups.

Test solution. Dissolve 0.10 g of the substance to be examined in 2 mL of a 20 g/L solution of *sodium hydroxide R* in *methanol R* in a 25 mL conical flask and boil under a reflux condenser for 30 min. Add 2.0 mL of *boron trifluoride-methanol solution R* through the condenser and boil for 30 min. Add 4 mL of *heptane R* through the condenser and boil for 5 min. Cool and add 10.0 mL of *saturated sodium chloride solution R*, shake for about 15 s and add a quantity of *saturated sodium chloride solution R* such that the upper phase is brought into the neck of the flask. Collect 2 mL of the upper phase, wash with 3 quantities, each of 2 mL, of *water R* and dry over *anhydrous sodium sulfate R*.

Reference solutions, chromatographic procedure and assessment of chromatograms. Where there is no specific prescription in the individual monograph, proceed as described under Method A.

01/2008:20423

2.4.23. STEROLS IN FATTY OILS

SEPARATION OF THE STEROL FRACTION

Prepare the unsaponifiable matter and then isolate the sterol fraction of the fatty oil by thin-layer chromatography (*2.2.27*), using a *TLC silica gel plate R* with a 0.2 mm to 0.5 mm layer.

Test solution (a). In a 150 mL flask fitted with a reflux condenser, place a volume of a 2 g/L solution of *betulin R* in *methylene chloride R* containing betulin corresponding to about 10 per cent of the sterol content of the sample used for the determination (e.g. in the case of olive oil add 500 µL, in the case of other vegetable oils add 1500 µL of the betulin solution). If the monograph requires the percentage content of the individual sterols in the sterol fraction, the addition of betulin may be omitted. Evaporate to dryness under a current of *nitrogen R*. Add 5.00 g (*m*) of the substance to be examined. Add 50 mL of *2 M alcoholic potassium hydroxide R* and heat on a water-bath for 1 h, swirling frequently. Cool to a temperature below 25 °C and transfer the contents of the flask to a separating funnel with 100 mL of *water R*. Shake the liquid carefully with 3 quantities, each of 100 mL, of *peroxide-free ether R*. Combine the ether layers in another separating funnel containing 40 mL of *water R*, shake gently for a few minutes, allow to separate and reject the aqueous phase. Wash the ether phase with several quantities, each of 40 mL, of *water R*, until the aqueous phase is no longer alkaline to phenolphthalein. Transfer the ether phase to a tared flask, washing the separating funnel with *peroxide-free ether R*. Distil off the ether with suitable precautions and add 6 mL of *acetone R* to the residue.

Ph Eur - Vegetable fatty oils

For avian vaccines, the safety test is generally carried out using 10 SPF chickens (*5.2.2*), except that for vaccines not recommended for use in chickens it is carried out using 10 birds of one of the species for which the vaccine is recommended, the birds being free from antibodies against the disease agent for which the vaccine is intended to provide protection.

3-8. **Potency**. The vaccine complies with the requirements of the test mentioned under Immunogenicity (section 2-3-1) when administered by a recommended route and method.

4. STORAGE

Store protected from light at a temperature of 5 ± 3 °C, unless otherwise indicated. Liquid preparations are not to be allowed to freeze, unless otherwise indicated.

5. LABELLING

The label states :

- that the preparation is for veterinary use,
- the volume of the preparation and the number of doses in the container,
- the route of administration,
- the type or types of bacteria or viruses used and for live vaccines the minimum and the maximum number of live bacteria or the minimum and the maximum virus titre,
- where applicable, for inactivated vaccines, the minimum potency in International Units,
- where applicable, the name and amount of antimicrobial preservative or other excipient,
- the name of any substance that may cause an adverse reaction,
- for freeze-dried vaccines :
  - the name or composition and the volume of the reconstituting liquid to be added,
  - the period within which the vaccine is to be used after reconstitution,
- for vaccines with an oily adjuvant, that if the vaccine is accidentally injected into man, urgent medical attention is necessary,
- the animal species for which the vaccine is intended,
- the indications for the vaccine,
- the instructions for use,
- any contra-indications to the use of the product including any required warning on the dangers of administration of an overdose,
- the doses recommended for different species.

01/2008:1579
corrected 6.4

VEGETABLE FATTY OILS

Olea herbaria

DEFINITION

Vegetable fatty oils are mainly solid or liquid triglycerides of fatty acids. They may contain small amounts of other lipids such as waxes, free fatty acids, partial glycerides or unsaponifiable matters. Vegetable fatty oils are obtained from the seeds, the fruit or the pit/stone/kernel of various plants by expression and/or solvent extraction, then possibly refined and hydrogenated. A suitable antioxidant may be added if necessary.

Virgin oil: an oil obtained from raw materials of special quality by mechanical procedures (e.g. by cold expression or centrifugation).

Refined oil: an oil obtained by expression and/or solvent extraction, and subsequently either alkali refining (followed by bleaching and any deodorisation) or physical refining.

Hydrogenated oil: an oil obtained by expression and/or solvent extraction, and subsequently either alkali refining or physical refining, then possible bleaching, followed by drying, hydrogenation and subsequent bleaching and deodorisation.

Only alkali-refined oils are used in the manufacture of parenteral preparations.

PRODUCTION

Measures are taken to ensure that the oil complies with the limit for benzo[a]pyrene decided by the competent authority. A limit of 2.0 ppb is set in Commission Regulation (EC) No. 208/2005.

OBTENTION OF A CRUDE OIL

Where the plant has a high oil content, the oil is generally obtained by expression under heating followed by an extraction; where the plant has a low oil content, the oil is generally obtained by direct extraction.

Mechanical procedures

A. Expression

High-pressure screw-pressing. It consists of some or all of the following steps: cleaning, drying, dehulling or decorticating, grinding, cooking and flaking.

During *cleaning* the foreign matter is eliminated. *Drying* may be necessary if the seed moisture content is higher than desirable for downstream processing. *Decorticating* is useful to obtain a high-protein meal by reduction of fibre and to reduce impurities in the oil. *Cooking* serves various purposes: completion of the breakdown of oil cells, lowering of the viscosity of the oil, coagulation of the protein in the meal, adjustment of the moisture level, sterilisation of the seed, detoxifying undesirable seed constituents (gossypol for cottonseed) and fixing certain phosphatides in the cake thus lowering subsequent refining losses. The efficacy of the expression process is such that only 3 per cent to 6 per cent of the oil is left in the cake.

Wet screw-pressing. The bunches are loaded into cages (for palm fruit) and moved into a horizontal steriliser with application of live steam and heating. The purposes of this steriliser are inactivation of enzymes, loosening of the fruit on the bunch, coagulation of proteins, etc. After heating in a digester, the pulp is fed to a screw-press. The oil is centrifugally clarified and vacuum-dried.

Pre-pressing followed by solvent extraction. The same sequence of steps is performed as above. The main function of pre-pressing is to obtain a cake of excellent permeability for the following solvent extraction stage. The extraction is performed either in a percolation-type or in an immersion-type apparatus. The efficacy of the solvent extraction process is such that residual oil levels in meal are generally below 1 per cent.

B. Centrifugation

Centrifugation separates the oily phase from the aqueous phase, which contains water-soluble components and residual solid particles. This operation can be carried out using:

- self-cleaning bowl or disc centrifuges;
- super-decanters, which are horizontal turbines equipped with a cylindrical bowl that tapers slightly at one end and which contains a continuously turning screw that scrapes the sides of the bowl; the screw and the bowl rotate at different speeds; the solid particles are discarded from the tapered end of the bowl and the oil flows out from the other end.

Solvent extraction. Prior to extraction, the following steps are carried out: the seeds are tempered for about a week at a temperature below 24 °C in order to loosen the hull from the seed and allow the seed moisture to attain equilibrium, then the seeds are cleaned, ground, dehulled and flaked. The most widely used solvent is a mixture of mainly *n*-hexane and methylpentanes (bp: 65-70 °C) commonly referred to as 'hexane'. Due to the major fire and explosive risks of this mixture, liquified gases and supercritical gases may also be used.

REFINING

The objective of refining is to remove impurities and contaminants of the oil with the least possible damage to the triglycerides and with minimal loss of oil. The contents of the following substances are reduced:

- free fatty acids, which may cause deterioration of the oil by oxidation, a smoked taste when heated and a sharp flavour (by alkali refining);
- water, which favours the enzymatic hydrolysis reactions (by alkali refining, drying);
- partial glycerides, which may cause foaming and a bitter taste (by neutralisation, washing);
- phosphatides and phosphorous compounds, which have emulsifying properties and may cause deposits, a darkening of the oil when heated, a cloudy appearance and bad organoleptic stability (by alkali refining);
- colouring matters such as chlorophyll (by alkali refining) and carotenoids (by bleaching);
- glycolipids, which may form colloidal solutions with water;
- free hydrocarbons, paraffin, waxes and resinous materials;
- metals (Fe, Cu, Pb, Sn, Pt, Pd, etc.), which are strong oxidation catalysts;
- pigments such as gossypol (in cottonseed oil) or mycotoxins such as aflatoxin (mainly in arachis seeds);
- pesticides;
- oxidation products (aldehydes, peroxides);
- proteins having possible allergic reactions;
- unsaponifiable matters (sterols, tocopherols and other vitamins);
- polycyclic aromatic hydrocarbons.

Alkali refining. It involves the following steps: degumming if necessary, neutralisation using alkali, washing and drying.

Degumming. During this step of the refining, i.e. treatment with water and/or phosphoric acid and/or sodium chloride, the phosphatides, phosphorous compounds and metals are eliminated. The use of this step depends on the nature of the oil.

Neutralisation with alkali. This step reduces the free-fatty-acid content below 0.1 per cent; the fatty acids are converted into oil-insoluble soaps, also called 'soapstocks'. Other substances may be removed by adsorption on these soaps: mucilaginous substances, phosphatides, oxidation products, colouring matters, etc. All substances that become insoluble in the oil on hydration are removed. Neutralisation with alkali has the disadvantage of saponifying a portion of neutral oil if the neutralisation is not well conducted.

Washing. This operation consists in removing the excess of soaps and alkali as well as the remaining traces of metals, phosphatides and other impurities, using hot water.

Drying. The remaining water is eliminated under vacuum before any further steps, such as bleaching.

Physical refining. It involves a steam treatment of the oil under high vacuum at a temperature greater than 235 °C. This technique can only be applied to oils naturally low in phosphatides and metals (palm and coconut) or from which phosphatides and metals have been removed by an acid treatment using concentrated phosphoric acid followed by an adsorptive treatment with activated bleaching earth (for sunflower, rapeseed, soya-bean). Moreover, it cannot be used for heat-sensitive oils (cottonseed oil), which darken.

Bleaching. The common method of bleaching is by adsorption treatment of the oil, which is generally heated at 90 °C for 30 min under vacuum, with bleaching earth (natural or activated) or carbon (activated or not); synthetic silica adsorbents may also be added. Substances that have not been totally removed during refining are eliminated, for example carotenoids and chlorophyll.

Deodorisation. Deodorisation eliminates odours, volatile substances and any residual extraction solvents; it involves injecting dry vapour into the oil, which is kept under vacuum at a high temperature. Different temperatures are used according to the oil: 200-235 °C for 1.5-3 h or greater than 240 °C for 30 min.

One of the main side reactions is thermic decolourisation due to the destruction of carotenoids when the temperature is greater than 150 °C. This technique provokes a loss of substances that may be distilled (free fatty acids, sterols, tocopherols, part of the refined oil), and may cause *cis-trans* isomerisation of the unsaturated fatty-acid double bonds.

WINTERISATION

Elimination of solids and waxes by filtration at low temperature (also called dewaxing). These solids and waxes could affect the appearance of the oil and cause deposits.

HYDROGENATION

The hydrogenation of the dried and/or bleached oil is performed using a catalyst (e.g. Ni, Pt, Pd), at a temperature of about 100-200 °C under hydrogen pressure. The catalyst is then removed by filtration at 90 °C. The hydrogen must be pure: free of poisons for the catalyst, water-free, and low in carbon dioxide, methane and nitrogen contents. Small amounts of polymers may be obtained. *Trans*-fatty acids are formed during partial hydrogenation.

CHROMATOGRAPHIC PURIFICATION

In high-purity applications, mainly for parenteral uses, the oil may be further purified by passing the oil through a column containing an activated earth. A solvent may sometimes be used to improve the efficiency. High-polarity molecules, such as oxidised materials, acids, alcohols, partial glycerides and free sterols, are preferentially removed.

When the oil is used in the manufacture of parenteral preparations, the limits set in the monograph for the acid value, the peroxide value and the water content may be different.

LABELLING

The label states:

- where applicable, that the oil was obtained by expression or extraction;
- where applicable, that the oil is suitable for use in the manufacture of parenteral preparations.

Članek *In situ* preparation of fatty acid methyl esters for analysis of fatty acid composition in foods

In Situ Preparation of Fatty Acid Methyl Esters for Analysis of Fatty Acid Composition in Foods

P.W PARK and R.E. GOINS

ABSTRACT

Lipid extraction preceding fatty acid methyl esters (FAME) preparation for gas chromatography is time-consuming and cumbersome. We omitted the lipid extraction and performed *in situ* transesterification (ISTE) by heating lipid-containing foods at 90°C for 10 min after adding 0.5N NaOH in methanol for methanolysis and continued heating another 10 min for further methylation after adding 14% BF_3 in methanol. FAME prepared by ISTE showed fatty acid composition virtually identical to FAME prepared after lipid extraction from powder, liquid, phospholipid-rich, and tissue products. Due to its simplicity, speed, and reduced organic solvent usage, ISTE should be useful to determine overall fatty acid composition of foods.

Key Words: fatty acid, methyl esters, *in situ* transesterification, gas chromatography

INTRODUCTION

FATTY ACIDS are usually converted into corresponding methyl esters (FAME) to improve volatility and to reduce peak tailing for gas chromatography (GC). Usually, FAME can be conveniently prepared by heating lipids with a large excess of either acid- or base-catalyzed reagents. Whereas acid-catalyzed reagents form FAME from both free fatty acids and O-acyl lipids (Morrison and Smith, 1964; Christie, 1992), base-catalyzed reagents cause only transesterification, i.e. conversion of O-acyl lipids to FAME and do not usually form FAME from free fatty acids (Luddy et al., 1968; Glass, 1971). A base-catalyzed reaction is a useful alternative to acid-catalyzed reactions when a lipid sample contains acid-labile fatty acids such as cyclopropenoids, present in seed oils from the Malvales order, such as cottonseed oil (Park and Rhee, 1988; Gaydou et al., 1993). To effect solubilization of nonpolar lipids such as triacylglycerols in methanol, the predominant solvent in derivatization reagents, benzene is frequently added to the derivatization reaction mixture (Sukhija and Palmquist, 1988; Sattler et al., 1991; O'Keefe et al., 1994). However, due to health concerns, use of benzene is discouraged. To help solubilize nonpolar lipids, methylene chloride (Einig and Ackman, 1987; Iversen et al., 1992), toluene (Ulberth and Henninger, 1992), and tetrahydrofuran (Christie, 1992) have been used in place of benzene.

Conventionally, a lipid extract has been used for FAME preparation regardless of transesterification procedure and solubilizing reagents employed (Koletzko and Bremer, 1989; Wang et al., 1990; Hoffman and Uauy, 1992; Morehouse and Ku, 1992; Park and Washington, 1993; Sigurgisladottir and Palmadottir, 1993; O'Keefe et al., 1994). Lipid extraction from products in which lipids are associated with proteins such as emulsified formulated products or muscle foods is cumbersome and time-consuming. Also, inefficient lipid extraction could lead to erroneous analysis of fatty acid composition. If the extraction step could be omitted and FAME could be prepared *in situ* from lipid-containing foods, the simplified procedure would save labor, increase sample throughput and reduce organic solvent waste.

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Direct FAME preparation without prior lipid extraction was used on microorganisms (Abel et al., 1963) and cereal grains (Welsh, 1975) without comparison to other methods. By heating feedstuffs and feces in benzene and methanolic HCl, Outen et al. (1976) prepared FAME which had a fatty acid composition comparable to FAME prepared through saponification followed by methylation or transesterification of lipid extract (Sukhija and Palmquist, 1988). That method, however, required pretreatment of samples for moisture removal in addition to prolonged heating of a reaction mixture (2 hr at 70°C). Moisture at > 2.5% of the reaction mixture hindered the transesterification reaction (Ulberth and Henninger, 1992). To minimize the interference from water, Browse et al. (1986) included 2,2'-dimethoxypropane in the methanolic HCl and improved the transesterification of leaf lipids. However, unreacted reagent was ascribed to spurious peaks on the chromatograms (Shimasaki et al., 1977). Dahmer et al. (1989) prepared FAME by heating soybean seed meal in hexane and methanolic sulfuric acid which resulted in < 70% conversion of the seed oil to methyl esters. This might have been due to a relatively short heating time (20 min at 90°C) since a 98% recovery was obtained with tripalmitin using similar reagents and a longer heating of 3 hr at 95°C (Welsh, 1975). Nonetheless, that inference was not persuasive considering the complete methylation of lipids in biological materials exposed to similar reagents at −65°C (Dugan et al., 1966). By heating lyophilized lipoprotein with methanolic BF_3 (90 min at 110°C), Sattler et al. (1991) obtained higher FAME yields than from the transesterification of lipid extract. Lepage and Roy (1986) reported FAME preparation by heating at 100°C for 60 min various biological specimens in methanol-benzene (4:1, v/v) following addition of acetyl chloride. This method, after modification to a milder reaction condition, was used to selectively methylate plasma free fatty acids (Lepage and Roy, 1988; Welz et al., 1990). Compared with acid catalysis, only a few methods have utilized base-catalyzed reagents for direct FAME preparation (Hougen and Bodo, 1973; Long et al., 1988). This may be partly due to the greater interference of water in base-catalyzed transferification, because free fatty acids resulting fom lipid hydrolysis are not methylated.

Methanolic BF_3 alone or in combination with methanolic sodium hydroxide is a popular method to prepare FAME from lipid extracts. We explored *in situ* transesterification through alcoholysis with methanolic sodium hydroxide followed by further methylation with methanolic BF_3, which had been successful in preparing FAME directly from ground peanuts (Barnes and Holaday, 1972). We found that FAME could be obtained directly from dried and also liquid samples with < 1 hr total sample preparation time. Such FAME had fatty acid compositions virtually identical to those obtained by transesterification of lipid extracts. Our objective was to study the *in situ* transesterification procedure for FAME preparation and its broad application potential as demonstrated with various samples.

MATERIALS & METHODS

Materials

Infant formulas, Ready-to-use Enfamil® and ProSobee® (Mead Johnson Nutritionals, Evansville, IN), and Ready-to-feed Similac® and Isomil® (Ross Laboratories, Columbus, OH) were purchased from a local

supermarket. Powdered formula (containing about 30% fat on a dry basis) was produced in a pilot plant; it was similar in composition to the Enfamil® Premature Formula (Mead Johnson Nutritionals) except that it contained different amounts of corn oil, soybean oil, coconut oil, and medium-chain triacylglycerols. A sample of human milk was obtained from a breast feeding mother at about 38 wk lactation. Fresh whole egg (Grade AA), calf liver, rainbow trout, and beef (USDA choice) were also purchased from a local supermarket. All standard lipids and quantitative reference mixtures were purchased from Nu Chek Prep, Inc. (Elysian, MN). Methanolic BF_3 (14%, w/v) was purchased from Pierce Co. (Rockford, IL). Reagent grade chemicals were hexane, anhydrous sodium sulfate, sodium hydroxide, sodium chloride, chloroform, and methanol.

In situ transesterification (ISTE)

The following amounts of lipid-containing foods were transferred into glass test tubes (13 × 100 mm): 50 mg powdered infant formula, 100 µL liquid infant formulas, and 150 µL human milk. Egg yolk (1g), after separation from the egg white, was diluted with 4 mL distilled water (D.W.), and a 150 µL aliquot was transferred into the test tube. Calf liver (1g) was homogenized with 2 mL D.W. using a Tissumizer (model SD18-10, Tekmar, Cincinnati, OH), and 300 µL homogenates were used. After removal of visible fat, 2g each of rainbow trout and beef muscle was homogenized similarly with 4 mL D.W., respectively. Aliquots of homogenates (100 µL) were transferred into the test tube. The ISTE procedure was a modification of the AOCS Offical Methods (1980) for FAME preparation from fats and oils. Methylene chloride (100 µL) and 1 mL 0.5N NaOH in methanol were added to each aliquot of lipid-containing foods. After being flushed with nitrogen and capped with Teflon-lined screw caps, the test tubes were heated in a water bath at 90°C for 10 min. The test tubes were removed from the water bath and allowed to cool briefly before addition of 1 mL 14% BF_3 in methanol. After nitrogen flushing and screw-capping, the heating continued in a water bath at 90°C for 10 min. Test tubes were removed and cooled to room temperature (≈23°C). One mL D.W. and 200–500 µL hexane were added to the test tubes and then FAME were extracted by vigorous shaking for about 1 min. Following centrifugation, the top layer was transferred into a vial for GC analysis.

Lipid extraction and transesterification (LETE)

Transesterification was carried out as described except it was done with the lipid extract. Lipids were extracted from all samples except powdered infant formula according to the method of Folch et al. (1957). Briefly, 60 mL $CHCl_3$-CH_3OH (2:1, v/v) was used to extract 3 mL liquid infant formula and human milk. Egg yolk (1g) was extracted with 20 mL chloroform-methanol. Calf liver, rainbow trout, and beef muscle (1g each) were homogenized with 20 mL chloroform-methanol, respectively. Tissue slurries were chilled in ice water during homogenization. Homogenates were filtered through Whatman filter paper (no. 1) and filtrates were collected. The filter paper and residue were re-extracted with 10 mL chloroform-methanol and filtered again. The combined filtrates were washed with 0.2 volume NaCl solution (0.73%, w/v) to remove nonlipid contaminants. The lower layer was collected after centrifugation. Moisture in the lipid extract was removed by drying over anhydrous sodium sulfate. Solvents were removed by rotary vacuum evaporation (Buchi Rotavapor R110, Brinkman, Westbury, NY) followed by nitrogen flash evaporation (N-evap, Organomation Associates, South Berlin, MA). The powdered infant formula (2g) was reconstituted into 12 mL D.W. and then extracted with 28 mL cyclohexane-ethyl acetate (9:1, v/v). The top layer after centrifugation was similarly freed of moisture and solvents. The lipid extracts were redissolved into methylene chloride so that 100 µL aliquots contained 2–6 mg extracts.

Thin layer chromatography (TLC)

The progress of the ISTE reaction was qualitatively examined by TLC of lipids extracted after alcoholysis and further methylation, respectively. Prior to extraction, the reaction mixture resulting from alcoholysis was acidified with 10N HCl (< pH 2) and then immediately extracted with 1 mL hexane. The extracts were freed of solvents and redissolved into 200 µL hexane. The extracts (2 µL) were spotted onto a silica gel G chromatoplate (Analtech, Inc., Newark, DE), developed in a solvent system of hexane:diethyl ether:acetic acid (85:15:1, v:v:v), and then charred with a spray of 50% sulfuric acid-dichromate followed by heating.

GC analysis

The fatty acid composition of FAME was analyzed by GC using a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a 7673 autosampler (Palo Alto, CA). Chromatographic data were recorded and stored in an IBM computer (PS/2 model 80) using Turbochrom software (PE Nelson, Cupertino, CA). The analytical column was a fused silica capillary Supelcowax 10 (0.32 mm × 30 m, 0.25 µm film thickness) from Supelco, Inc (Bellefonte, PA). The oven temperature was programmed from 100°C to 190°C at 15°C/min and then to 245°C at 5°C/min. Split injection was made at an 80 to 1 ratio. The carrier gas was H_2 and the make-up gas, N_2. The injector was maintained at 250°C and detector at 300°C. FAME were identified by comparison of retention times with standards and quantified by directly taking area percent as weight percent. This was based on the observation that area percents of FAME in quantitative reference mixtures (Nu Chek Prep, Inc., catalog no. 17A, 3B, and 3C) were in the range of 98.1 to 104.3% of the weight compositions. For weight composition of FAME of infant formulas, peak areas were corrected using theoretical FID response factors calculated according to the method of Ackman and Sipos (1964). This was due to considerable deviation in area percent of methyl caprylate and methyl caproate from stated weight compositions in the reference mixture (19A from Nu Chek Prep, Inc.).

Statistical analysis

All data are mean values ± standard deviation (M ± S.D.) from six preparations with a single injection each, unless otherwise stated.

RESULTS & DISCUSSION

Powder sample

For conventional FAME preparation, the lipid extracts are reacted in organic solvents which are mainly methanol. Since derivatization reagents are moisture-sensitive, moisture contamination in the lipid extracts must be avoided to assure complete methylation reactions. We carried out ISTE for a powdered infant formula first because it contained only a small amount of moisture, usually < 2.5% (w/w). Our hypothesis for ISTE was that lipids, among the other ingredients, would be the primary constituent affected by ISTE reactions, i.e., base-catalyzed methanolysis followed by further methylation. Thus the resulting FAME would be the major reaction products preferentially extracted with hexane. Throughout the reaction process, no clumping of powdered samples in the organic solvent medium was observed. However, following addition of methanolic sodium hydroxide and shortly after heating began, the reaction mixture turned brown, indicating Maillard reactions. Powdered infant formulas contain high amounts of proteins and carbohydrates, (≈12% and 54% respectively) and glycosylamine formation for Maillard reaction is facilitated in basic solutions (Whistler and Daniel, 1985). Thus we expected that brown coloration would increase under the basic conditions of ISTE. Nevertheless, when FAME were extracted with hexane, no apparent indication of brown color contamination occurred in the extract. This result seemed reasonable because Maillard browning reaction products would hardly be extracted into the nonpoar solvent due to the many remaining alcoholic groups on the sugar moiety.

Qualitative examination of the ISTE reaction was carried out by TLC (Fig. 1). Triacylglycerols in the reaction mixture were no longer observed after heating the powder sample in the methanolic sodium hydroxide for 10 min at 90°C; instead, FAME and free fatty acids appeared (lane 2). When the reaction mixture was heated for 5 min, the similar result was observed (not shown) indicating both transesterification and saponification occurred. This suggested that direct transesterification by use of only base-catalyzed reagent, as reported by Long et al. (1988) who reacted oilseed meals with 0.8 N methanolic sodium methoxide:hexane (3:2, v/v) at 80°C for 10 min, may not be satisfactory. The production of both FAME and unintended free fatty acids could be misleading although the disintegration of O-acyl lipids would be satisfactory. Glass (1971) reported that methanolysis preceded saponification. Thus FAME were produced predominantly with trace amounts of free fatty acids when corn

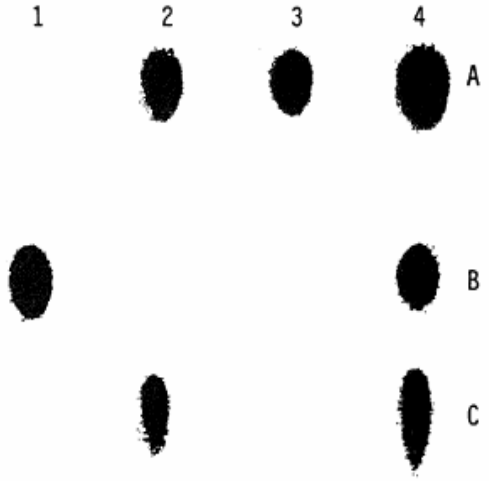

Fig. 1—Thin layer chromatogram of lipid extracts from *in situ* transesterification of powdered infant formulas. Lane 1, before reaction; lane 2, after heating in methanolic sodium hydroxide; lane 3, after further reaction with methanolic BF_3; and lane 4, standards containing methyl oleate (A), triolein (B) and oleic acid (C).

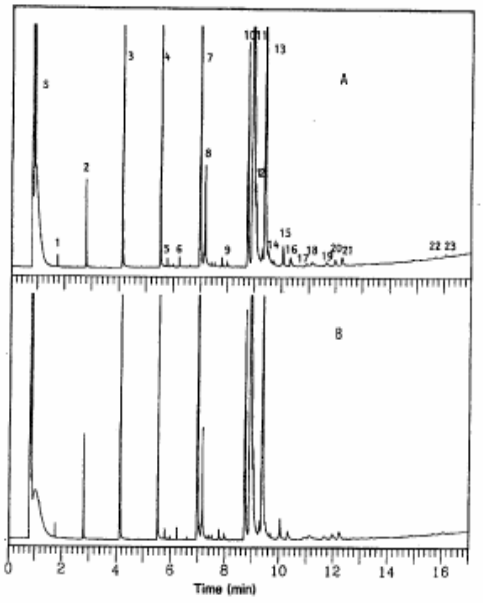

Fig. 2—Typical gas chromatogram of fatty acid methyl esters prepared from human milk by: A, *in situ* transesterification and B, lipid extraction and transesterification. Peaks: S, solvent; 1, 8:0, 2, 10:0, 3, 12:0, 4, 14:0, 5, 14:1ω5; 6, 15:0, 7, 16:0, 8, 16:1ω7; 9, 17:0; 10, 18:0; 11, 18:1ω9; 12, 18:1ω7(?); 13, 18:2ω6; 14, 18:3ω6; 15, 18:3ω3; 16, 18:4ω3; 17, 20:0; 18, 20:1ω9; 19, 20:2ω6; 20, 20:3ω6; 21, 20:4ω6; 22, 22:5ω3; and 23, 22:6ω3.

oil was heated in 0.5N methanolic sodium hydroxide for 3 min. However, as heating time was prolonged, saponification became more prevalent than transesterification leading to more free fatty acids than FAME (Glass, 1971). After further reaction with methanolic BF_3, free fatty acids disappeared and only FAME were noted (lane 3) which confirmed previous reports (Glass, 1971). These results indicated that our ISTE procedure led to FAME preparation directly from samples without prior lipid extraction.

GC analysis of FAME prepared by the ISTE method produced reproducible fatty acid profiles and peaks ranging from methyl caprylate to arachidate which we anticipated based upon the known composition of oils in the powdered formula. Gas chromatograms showed no extraneous peaks which might have originated from reactions other than the intended transesterification reaction. A very high precision of quantitation was observed, coefficient of variation < 1.5% (Table 1). Fatty acid composition of FAME prepared by ISTE was virtually identical to that of FAME prepared by conventional lipid extraction and transesterification (LETE). Although linolenic acid was present at low levels as the most unsaturated acid, good agreement was observed for its content between FAME by ISTE and FAME by LETE. This suggested that powdered infant formula could be subjected directly to transesterification and that prior lipid extraction was unnecessary. No difference was observed without use of methylene chloride and nitrogen flushing of headspace, but they were included due to ease and potential benefits (solubilization and oxidation suppression).

Liquid samples

Water would interrupt the transesterification reaction and also may hydrolyze the ester bond in FAME. The ISTE applicability to aqueous samples was tested with liquid infant formulas as an example of emulsified, formulated samples with high moisture content. During base-catalyzed methanolysis, the reaction mixture remained a solution, and again intense browning developed as heating continued. GC analysis of FAME by ISTE of Enfamil® liquid formulas revealed fatty acid compositions similar to FAME by LETE (Table 1). Fatty acid composition of FAME prepared by the two methods displayed practically no difference for the other liquid formulas tested, Similac® (Table 1), ProSobee®, and Isomil®. Fatty acid composition of ProSobee® and Isomil® (from 4 replicates), was virtually identical to that of Enfamil® and Similac® (data not shown). These liquid formulas contained about 87% water. Thus, the sample aliquot used (100 μL) provided about 4.3% (v/v) water in the derivatization mixture. Lepage and Roy (1986) reported that the presence of water up to about 5% of reaction volume did not adversely affect ISTE for which lipid-containing samples were heated in methanol-benzene (4:1, v/v) with acetyl chloride. Liquid formulas had different protein compositions, i.e. predominantly bovine whey proteins (Enfamil®), predominantly bovine caseins (Similac®), and soy protein isolates (ProSobee® and Isomil®). They had many other nutrients to serve as a sole food for infants, thus the effectiveness of ISTE with liquid infant formulas indicate its broad applicability.

This was further confirmed by the virtually identical fatty acid composition of human milk for which FAME were prepared by ISTE and LETE, respectively (Table 1). Unlike commercial infant formulas, human milk contains small amounts of arachidonic (ARA) and docosahexaenoic acids (DHA), which are long-chain ω-6 and ω-3 fatty acids, respectively (Harzer et al., 1983; Martin et al., 1993). Our human milk specimen displayed DHA at trace levels (below 0.1%) by both methods. Good agreement was observed for ARA; the difference in ARA between the ISTE and LETE methods was about 10% at a minor level (.36% vs .4%). Gas chromatograms of FAME prepared by the two procedures (Fig. 2) show despite severe browning reactions during ISTE, no extraneous peaks occurred other than those of FAME, and the fatty acid profiles matched. The highest moisture level tolerable for ISTE was not specifically determined. However, successful results were obtained with a 200 μL aliquot of human milk, suggesting that about 8.5% (v/v) water in the total reaction mixture did not impair the ISTE.

Phospholipid-rich product

Lipids of infant formulas and human milk consist of about 98% triacylglycerols (Jensen and Jensen, 1992). Egg yolks are rich in phospholipids, comprising about one-third of the lipid fraction (Powrie and Nakai, 1985). To test whether ISTE could

Table 1—Fatty acid composition (weight %) of FAME prepared by two different procedures for powdered and liquid foods

Fatty acid[a]	Powdered formula		Ready-to-use Enfamil		Ready-to-feed Similac		Human milk[e]	
	ISTE[b]	LETE[c]	ISTE	LETE	ISTE	LETE	ISTE	LETE
8:0	24.62±0.36[d]	25.21±0.15	1.28±0.03	1.31±0.02	2.66±0.07	2.56±0.10	tr	tr
10:0	11.94±0.03	12.39±0.04	1.05±0.02	1.08±0.01	2.27±0.03	2.21±0.08	1.71±0.02	1.66±0.13
12:0	9.38±0.03	9.55±0.03	8.57±0.09	8.58±0.01	18.32±0.09	18.50±0.49	6.54±0.20	6.50±0.23
14:0	3.95±0.02	3.97±0.04	3.98±0.06	3.99±0.01	7.28±0.03	7.31±0.16	5.97±0.12	5.88±0.12
16:0	7.25±0.06	6.98±0.03	23.60±0.36	23.25±0.16	9.99±0.07	9.98±0.25	16.58±0.27	16.59±0.07
16:1ω7	tr	tr	tr	tr	tr	tr	2.57±0.08	2.65±0.03
18:0	2.21±0.02	2.07±0.02	4.86±0.05	4.86±0.06	4.15±0.01	4.03±0.02	7.23±0.08	7.18±0.03
18:1ω9	13.08±0.12	13.41±0.09	37.04±0.43	36.66±0.14	17.72±0.14	17.85±0.08	37.61±0.51	37.65±0.62
18:2ω6	25.45±0.13	24.31±0.17	17.45±0.78	17.72±0.43	32.53±0.10	33.25±0.67	16.48±0.50	16.50±0.14
18:3ω3	1.81±0.01	1.79±0.01	1.65±0.04	1.64±0.01	4.91±0.19	4.96±0.04	0.66±0.04	0.73±0.02

[a] First figure refers to number of carbons; second figure to number of double bonds.
[b] *In situ* transesterification.
[c] Lipid extraction and transesterification.
[d] Mean ± standard deviation.
[e] For fatty acids not presented (< .4%), see Fig. 2.
tr: trace.

Table 2—Fatty acid composition (weight %) of FAME prepared by two different procedures for egg yolks and tissue products[a,b]

Fatty acid	Egg yolk		Calf liver		Rainbow trout		Beef muscle	
	ISTE	LETE	ISTE	LETE	ISTE	LETE	ISTE	LETE
14:0	0.37±0.01	0.36±0.01	1.21±0.03	1.23±0.02	4.20±0.09	4.16±0.10	1.29±0.07	1.36±0.01
Unk	nd	nd	1.02±0.05	1.02±0.03	nd	nd	3.14±0.13	3.21±0.07
16:0	26.50±0.07	26.61±0.22	13.49±0.17	14.24±0.23	20.00±0.12	19.82±0.07	18.59±0.82	17.74±0.26
16:1ω7	3.32±0.01	3.37±0.08	1.55±0.04	1.64±0.04	7.55±0.41	7.53±0.32	5.59±0.47	4.80±0.09
17:0	nd	nd	nd	nd	0.54±0.02	0.57±0.01	1.33±0.11	1.35±0.08
16:3(?)	nd	nd	0.34±0.02	0.32±0.03	0.23±0.01	0.25±0.00	1.57±0.17	1.82±0.06
18:0	8.90±0.01	8.72±0.06	23.24±0.14	23.13±0.24	4.83±0.06	4.77±0.04	8.49±0.21	8.76±0.08
18:1ω9	44.66±0.08	44.94±0.38	18.82±0.23	18.49±0.19	20.52±0.16	20.36±0.15	36.23±0.98	37.47±0.47
18:2ω6	11.45±0.15	11.73±0.08	17.16±0.23	17.43±0.18	10.40±0.07	10.26±0.06	11.62±0.39	10.33±0.11
18:3ω3	0.17±0.00	0.16±0.00	0.46±0.03	0.43±0.02	1.10±0.03	1.11±0.03	tr	tr
20:1ω9	0.20±0.00	0.18±0.01	0.38±0.02	0.32±0.01	1.15±0.03	1.20±0.01	tr	tr
20:3ω6	0.27±0.00	0.24±0.01	4.84±0.07	4.83±0.07	1.19±0.02	1.18±0.03	1.01±0.07	1.23±0.06
20:4ω6	1.63±0.02	1.58±0.02	9.26±0.14	9.38±0.17	0.94±0.01	0.97±0.02	6.59±0.09	6.19±0.06
20:5ω3	nd	nd	0.26±0.01	0.24±0.01	5.47±0.02	5.37±0.05	0.91±0.06	0.86±0.02
22:5ω3	nd	nd	1.65±0.02	1.65±0.06	1.96±0.02	2.01±0.01	tr	tr
22:6ω3	0.27±0.00	0.28±0.01	2.43±0.05	2.35±0.09	14.87±0.19	14.55±0.07	nd	nd

[a] Legends same as Table 1.
[b] Fatty acids (< 0.6%) not presented include: 16:1t(?) and 21:5ω3 in egg yolk; 16:1t(?), 16:2(?), and 20:2 in calf liver; 15:0, 16:2(?), 20:4ω3, and 21:5ω3 in rainbow trout; and 14:1, 16:1t(?), 16:2(?), and 16:4(?) in beef muscle.
nd: not detected, tr: trace.

be applied to foods containing phospholipids, we subjected egg yolks to ISTE. Prior to reaction, egg yolks were diluted with water so they could remain in contact with reactants. Unlike infant formulas and human milk, the egg yolk reaction mixture did not develop brown color, which could be attributed to the low carbohydrate level in egg yolks (Powrie and Nakai, 1985). The fatty acid composition of FAME prepared by ISTE agreed well with that of FAME obtained after LETE (Table 2).

Tissue products

The applicability of the ISTE method to preparing FAME from membrane lipids was tested with calf liver, rainbow trout, and beef muscle. Cell membranes were disintegrated through homogenization to expose lipids to ISTE reagents. The soft liver tissues facilitated homogenization with D.W. for ISTE, and with chloroform-methanol for LETE. GC analysis revealed virtually identical fatty acid compositions between FAME prepared by the two procedures (Table 2). Fatty acid composition reportedly varied with the part of the fish body (Kinsella et al., 1977). To improve sample homogeneity, the fish meat was homogenized with water and divided into two groups. One group was used for ISTE, and the other was further homogenized with chloroform-methanol for lipid extraction. The two different FAME preparations showed excellent agreement in fatty acid composition. Both preparations were rich in ω-3 fatty acid showing almost identical amounts of eicosapentaenoic acid (EPA) and DHA (Table 2). Because only fish flesh was used for fatty acid analysis, most polyunsaturated fatty acids (PUFA) likely came from muscle cell membranes. These results verified the use of the ISTE method to prepare FAME from structural lipids of tissue samples rich in PUFA.

Beef muscle was the only sample which did not initially yield reproducible fatty acid composition data. Although good agreement was observed for most fatty acids, a notable difference in ARA between the two methods was frequently found. ARA was higher to varying extents in FAME by the ISTE method than by the conventional LETE method. The overall fatty acid composition showed relatively small amounts of ARA, compared to its high content in phospholipid fraction (Allen and Foegeding, 1981). Thus we suspected that insufficient homogenization prior to lipid extraction may block leaching by the extracting solvents of membrane lipids associated with proteins. The more harsh conditions of ISTE (base-heating) would help disintegrate membrane lipids leading to higher ARA. Unlike the soft tissues of calf liver and fish muscle samples, prolonged homogenization of beef muscle samples with chloroform-methanol eventually led to fatty acid composition similar to that by the ISTE method (Table 2). Although a few unidentified peaks were noted, only one reached a notable level (about 3.2%). That peak was present in both FAME preparations indicating that it was not caused by the method difference.

CONCLUSIONS

THE ISTE METHOD omitted lengthy lipid extractions. Instead, FAME were prepared directly from base-catalyzed methanolysis followed by further methylation reactions. GC analysis of FAME prepared by the ISTE revealed fatty acid composition virtually identical to that of FAME prepared from lipid extracts of various samples including powder, liquid, phospholipid-rich, and tissue products. Although brown coloration developed during ISTE of some samples, it did not interfere with GC analysis of fatty acid composition. The successful performance of the

ISTE method with complex samples demonstrated its broad applicability for FAME preparation. Due to its simplicity, speed, and precision, the method should be useful in fatty acid analysis to determine overall fatty acid composition of foods rather than individual lipid classes, e.g. for nutrition labelling purposes.

REFERENCES

Abel, K., Deschmertzing, H., and Peterson, J.I. 1963. Classification of microorganisms by analysis of chemical composition. Feasibility of utilizing gas chromatography. J. Bacteriol. 85: 1039.

Ackman, R.G. and Sipos, J.C. 1964. Application of specific response factors in the gas chromatographic analysis of methyl esters of fatty acids with flame ionization detectors. J. Am. Oil Chem. Soc. 41: 377.

Allen, E.C. and Foegeding, E.A. 1981. Some lipid characteristics and interactions in muscle foods-a review. Food Technol. (5): 253.

AOCS. 1980. Method Ce 2-66. *Official and Tentative Methods of the American Oil Chemists' Society*, AOCS, Champaign, IL.

Barnes, P.C. Jr. and Holaday, C.E. 1972. Rapid preparation of fatty acid esters directly from ground peanuts. J. Chromatogr. Sci. 10: 181.

Browse, J., McCourt, P.J., and Somerville, C. 1986. Fatty acid composition of leaf lipids determined after combined digestion and fatty acid methyl ester formation from fresh tissue. Anal. Biochem. 152: 141.

Christie, W.W. 1992. Preparation of fatty acid methyl esters. INFORM 3: 1031.

Dahmer, M.L., Fleming, P.D., Collins, G.B., and Hildebrand, D.F. 1989. A rapid screening technique for determining the lipid composition of soybean seeds. J. Am. Oil Chem. Soc. 66: 543.

Dugan, Jr. L.R., McGinnis, G.W., and Vadehra, D.V. 1966. Low temperature direct methylation of lipids in biological materials. Lipids 1: 305.

Einig, R.G. and Ackman, R.G. 1987. Omega-3 PUFA in marine oil products. J. Am. Oil Chem. Soc. 64: 499.

Folch, J., Lees, M., and Stanley, G.H.S. 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226: 477.

Gaydou, E.M., Ramanoelina, A.R.P., Rasoarahona, J.R.E., and Combres, A. 1993. Fatty acid composition of Sterculia Seeds and oils from Madagascar. J. Agric. Food Chem. 41: 64.

Glass, R.G. 1971. Alcoholysis, saponification and the separation of fatty acid methyl esters. Lipids 6: 919.

Harzer, G., Haug, M., Dieterich, I., and Gentner, P.R. 1983. Changing patterns of human milk lipids in the course of the lactation and during the day. Am. J. Clin. Nutr. 37: 612.

Hoffman, D.R. and Uauy, R. 1992. Essentiality of dietary ω-3 fatty acids for premature infants: Plasma and red blood cell fatty acid composition. Lipids 27: 886.

Hougen, F.W. and Bodo, V. 1973. Extraction and methanolysis of oil from whole or crushed rapeseed for fatty acid analysis. J. Am. Oil Chem. Soc. 50: 230.

Iverson, S.J., Sampugna, J., and Oftedal, O.T. 1992. Positional specificity of gastric hydrolysis of long-chain n-3 polyunsaturated fatty acids of seal milk triglycerides. Lipids 27: 870.

Jensen, R.G. and Jensen, G.L. 1992. Specialty lipids for infant nutrition. I. Milks and formulas. J. Pediatric Gastroenterol. Nutr. 15: 232.

Kinsella, J.E., Shimp, J.L., Mai, J., and Weihrauch, J. 1977. Fatty acid content and composition of freshwater finfish. J. Am. Oil Chem. Soc. 54: 424.

Koletzko, B. and Bremer, H.J. 1989. Fat content and fatty acid composition of infant formulas. Acta Paediatr. Scand. 78: 513.

Lepage, G. and Roy, C.C. 1986. Direct transesterification of all classes of lipids in a one-step reaction. J. Lipid Res. 27: 114.

Lepage, G. and Roy, C.C. 1988. Specific methylation of plasma nonesterified fatty acids in a one-step reaction. J. Lipid Res. 29: 227.

Long, A.R., Massie, S.J., and Tyznik, W.J. 1988. Rapid direct extraction derivatization method for the determination of acylglycerol lipids in selected sample matrices. J. Food Sci. 53: 940.

Luddy, F.E., Barford, R.A., Herb, S.F., and Magidman, P. 1968. A rapid and quantitative procedure for the preparation of methyl esters of butteroil and other fats. J. Am. Oil Chem. Soc. 45: 549.

Martin, J-C., Bougnoux, P., Antoine, J-M., Lanson, M., and Couet, C. 1993. Triacylglycerol structure of human colostrum and mature milk. Lipids 28: 637.

Morrison, W.E. and Smith, L.M. 1964. Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol. J. Lipid Res. 5: 600.

Morehouse, K.M. and Ku, Y. 1992. Gas chromatographic and electron spin resonance investigations of γ-irradiated shrimp. J. Agric. Food Chem. 40: 1963.

O'Keefe, S.F., Wiley, V., and Gaskins, S. 1994. Geometrical isomers of essential fatty acids in liquid infant formulas. Food Res. Internat. 27: 7.

Outen, G.E., Beever, D.E., and Fenlon, J.S. 1976. Direct methylation of long-chain fatty acids in feeds, digesta and feces without prior extraction. J. Sci. Fd. Agric. 27: 419.

Park, Y.W. and Washington, A.C. 1993. Fatty acid composition of goat organ and muscle meat of Alpine and Nubian breeds. J. Food Sci. 58: 245.

Park, S.W. and Rhee, K.C. 1988. A capillary GC determination of cyclopropenoid fatty acids in cottonseed oils. J. Food Sci. 53: 1497.

Powrie, W.D. and Nakai, S. 1985. Characteristics of edible fluids of animal origin: eggs. Ch. 14. In *Food Chemistry*, (Ed.) O.R. Fennema, p. 833. Marcel Dekker, Inc., New York.

Sattler, W., Puhl, H., Hayn, M., Kostner, G., and Esterbauer, H. 1991. Determination of fatty acids in the main lipoprotein classes by capillary gas chromatography: BF_3/methanol transesterification of lyophilized samples instead of Folch extraction gives higher yields. Anal. Biochem. 198: 184.

Shimasaki, H., Phillips, F.C., and Privett, O.S. 1977. Direct transesterification of lipids in mammalian tissue for fatty acid analysis via dehydration with 2,2'-dimethoxypropane. J. Lipid Res. 18: 540.

Sigurgisladottir, S. and Palmadottir, H. 1993. Fatty acid composition of thirty-five Icelandic fish species. J. Am. Oil Chem. Soc. 70: 1081.

Sukhija, P.S. and Palmquist, D.L. 1988. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. J. Agric. Food Chem. 36: 1202.

Ulberth, F. and Henninger, M. 1992. One-step extraction/methylation method for determining the fatty acid composition of precessed foods. J. Am. Oil Chem. Soc. 69: 174.

Wang, Y.J., Miller, L.A., Perren, M., and Addis, P.B. 1990. Omega-3 fatty acids in Lake Superior fish. J. Food Sci. 55: 71.

Welsh, R.W. 1975. Fatty acid composition of grain from winter and spring sown oats, barley and wheat. J. Sci. Fd. Agric. 26: 429.

Welz, W., Sattler, W., Leis, H., and Malle, E. 1990. Rapid analysis of non-esterified fatty acids as methyl esters from different biological specimens by gas chromatography after one-step esterification. J. Chromatogr. 526: 319.

Whistler, R.L. and Daniel, J.R. 1985. Carbohydrates. Ch. 3. In *Food Chemistry*, (Ed.) O.R. Fennema, p. 103. Marcel Dekker, Inc., New York.

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