# PRODUCTION OF OMEGA- 3 FATTY ACID CONCENTRATES FROM FISH AND ALGAL OILS AND THEIR STRUCTURAL ASSESSMENT BY NMR

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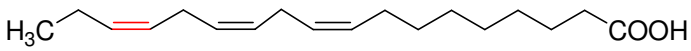
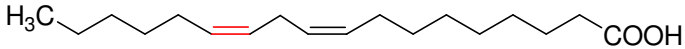
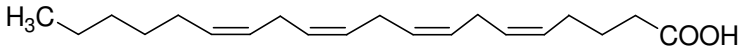
# LC-PUFA, OMEGA-3 vs. OMEGA-6



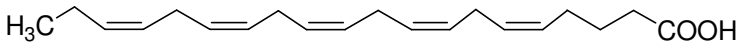

**α-linolenic acid (ALA, C18:3, n-3)**



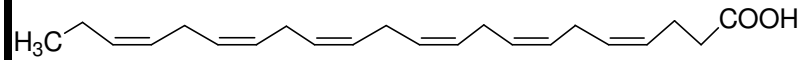

**linoleic acid (C18:2, n-6)**




**arachindonic acid (ARA, C18:4, n-6)**




**eicosapentaenoic acid (EPA, C20:5, n-3)**




**docosahexaenoicoic acid (DHA, C22:6, n-3)**

- LC-PUFAs are categorized by the position of the 1st double bond counting from the $CH_3$-terminal
- Location of the 1st double bond dictates biological activity
- All double bonds are $CH_2$-interruped
- Omega-3 series starts with α-linolenic acid (ALA,18:3n-3)
- Omega-6 series starts with linoleic acid (LA,18:2n-6)
- The most studied omega-3 FA are EPA and DHA

Table: **Summary of the physiological roles and potential clinical benefits of very long chain ω-3 fatty acids**

| Physiological role of very long chain ω-3 fatty acids | Potential clinical benefit | Target |
|---|---|---|
| Regulation of blood pressure<br>Regulation of platelet function | Decreased blood pressure<br>Decreased likelihood of thrombosis | Hypertension; CVD<br>CVD |
| Regulation of blood coagulation | Decreased likelihood of thrombosis | CVD |
| Regulation of plasma triglyceride concentrations | Decreased plasma triglyceride concentrations | Hypertriglyceridemia; CVD |
| Regulation of vascular function | Improved vascular reactivity | CVD |
| Regulation of cardiac rhythm | Decreased arrhythmias | CVD |
| Regulation of inflammation | Decreased inflammation | Inflammatory diseases (arthritis, inflammatory bowel diseases, psoriasis, lupus, asthma, cystic fibrosis, dermatitis, neurodegeneration …); CVD |
| Regulation of immune function | Improved immune function | Compromised immunity |
| Regulation of bone turnover | Maintained bone mass | Osteoporosis |
| Regulation of insulin sensitivity | Improved insulin sensitivity | Type-2 diabetes |
| Regulation of tumour cell growth | Decreased tumour cell growth and survival | Some cancers |
| Regulation of visual signaling (rhodopsin) | Optimised visual signaling Signalbildung | Poor infant visual development (especially pre-term) |
| Structural component of brain and central nervous system | Optimised brain development – cognitive and learning processes | Poor infant and childhood cognitive processes and learning |

Abbreviation used: CVD, cardiovascular disease

Calder. Schweiz. Zeit. Ernährungmedizin **2009**, *5*, 14-19

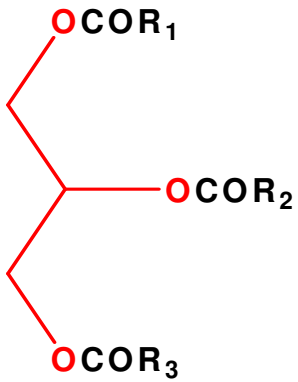
# PRODUCTION OF EPA/DHA CONCENTRATES

## STARTING OMEGA-3 OIL SELECTION

- The richest and the cheapest source of EPA/DHA is fish oil
- Starting oils with right FA profiles and EPA/DHA content ~30%!
  - anchovy/sardine (18/12TG, for high level EPA concentrates)
  - tuna (05/25TG, for high level DHA concentrates)

## CORE OMEGA-3 CONCENTRATION TECHNOLOGIES

- Fish oils are in the form of triglycerides →

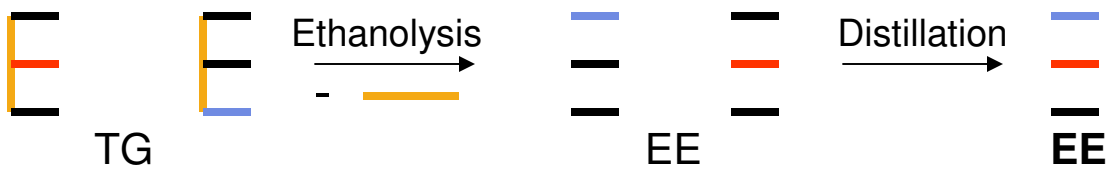



  - Winterization
  - Cold Solvent Precipitation
  - Urea Clathration
  - Ethanolysis + Fractional Distillation ⇒ Ethyl Ester (EE) Concentrates
  - **Enzyme-assisted Concentration ⇒ Triglyceride (TG) Concentrates**

# EE CONCENTRATES

## ETHANOLYSIS AND DISTILLATION

TG 30% —Ethanolysis→ EE 30% —Distillation→ **EE** 60%




Splitting TG molecules lowers the boiling points !

DHA-TG bp ~ 910 °C, vs. DHA-EE bp ~ 450 °C)

- Medium chain or medium chain EE
- DHA residues or DHA-EE
- EPA residues or EPA-EE
- Glycerol backbone or glycerol

Popular technology towering over the other options

$$O = CI^{1.67}$$

O, output

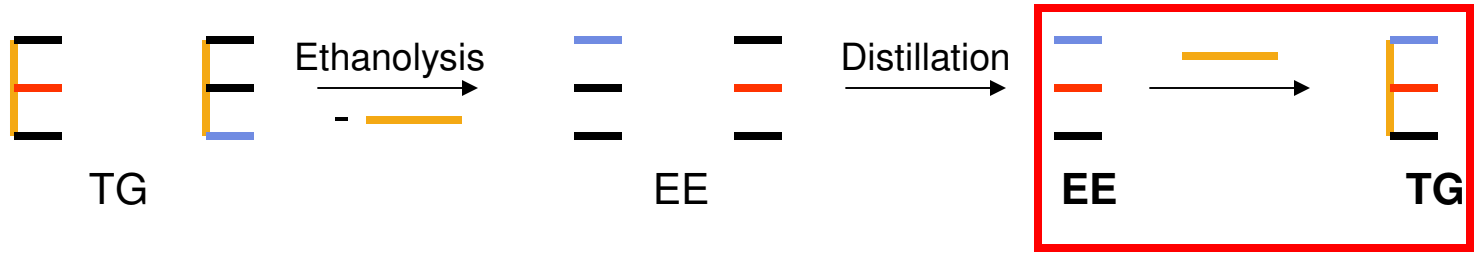
CI, capital investment

⇓

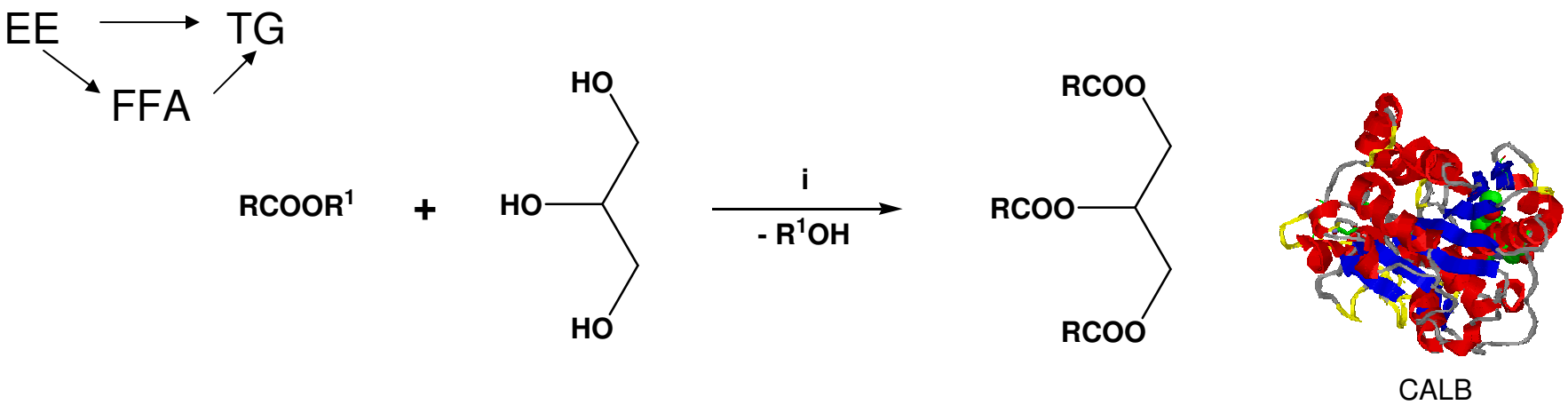
Economic advantage of large throughput!

# CONCENTRATION STRATEGY A

## ETHANOLYSIS / DISTILLATION / GLYCEROLYSIS



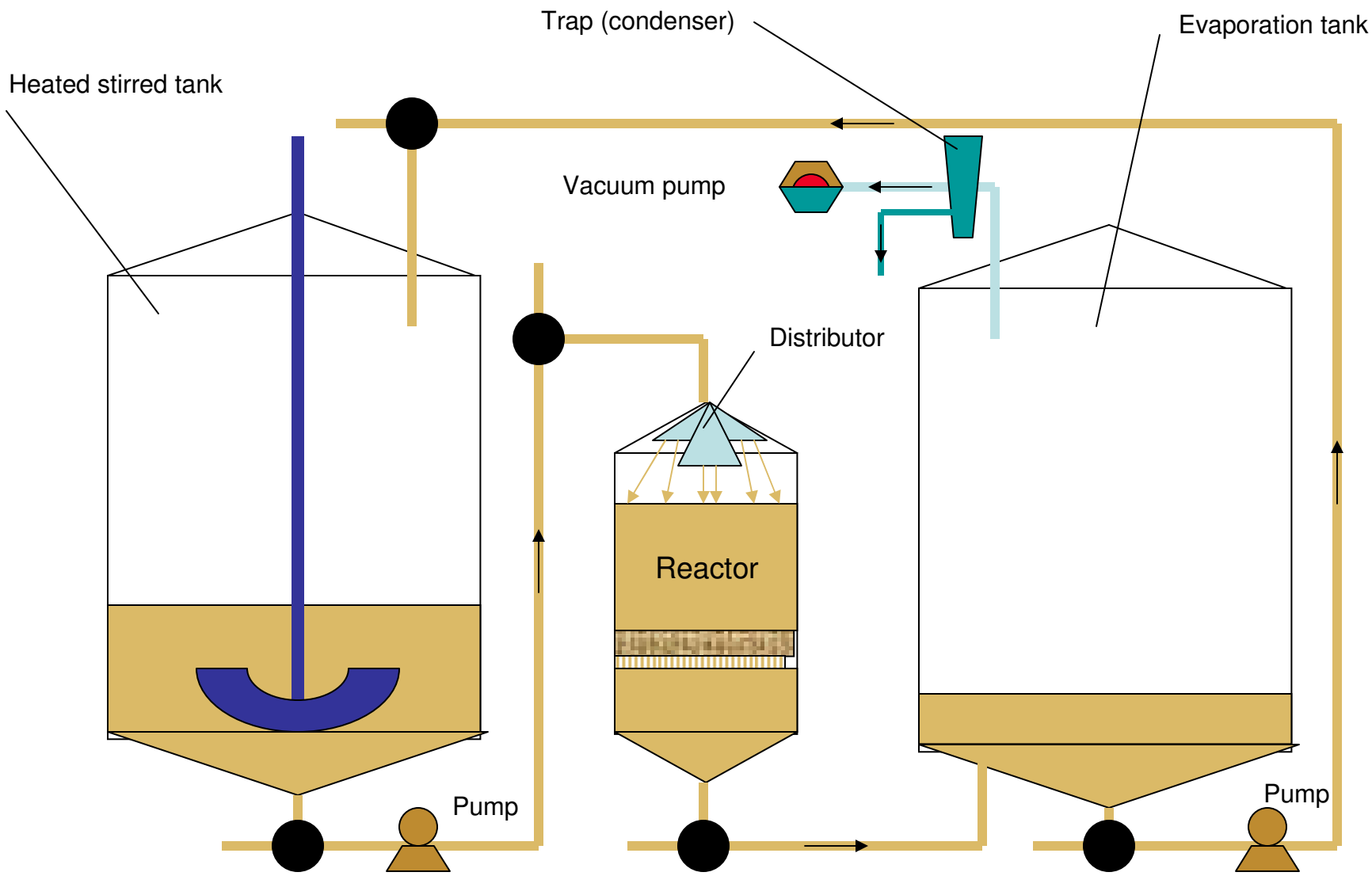

## GLYCEROLYSIS




R, a mixture of C14-C24 hydrocarbon residues,, primarily those of EPA and/or DHA
R1, H or Et; i, CALB, 60-85 C, vacuum

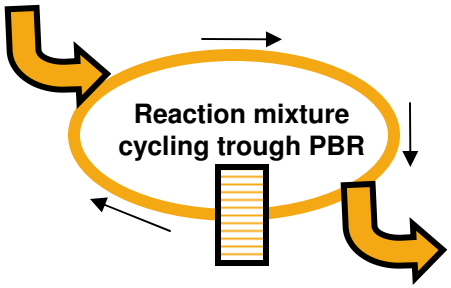
- Immobilized ***Candida antarctica*** lipase B (CALB) in **Packed Bed Reactor**
- The reaction is driven forward by EtOH or water removal under vacuum

# PACKED BED REACTOR



# MANUFACTURING OF TG CONCENTRATES

- Green technologies (free or immobilized lipases)
- Packed bed reactors





- First to introduce food grade immobilized lipase for EE –TG conversion !

$$\text{EE} \xrightarrow{\text{i}} \text{FFA} \xrightarrow{\text{ii}} \text{TG}$$

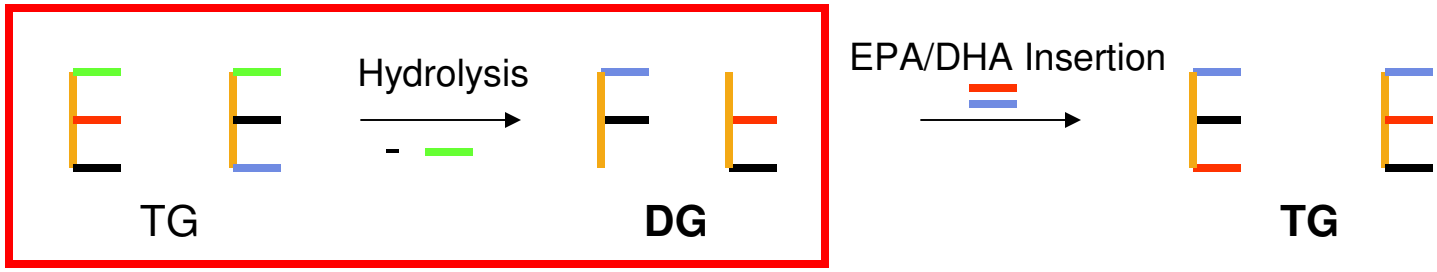
**i: CALB-L, EtOH/$H_2O$, 50 °C, vacuum**

**ii: CALB-FPX66, glycerol, 70 °C, vacuum**

**~ 80 reactions on 1 batch of immobilized. enzyme!**

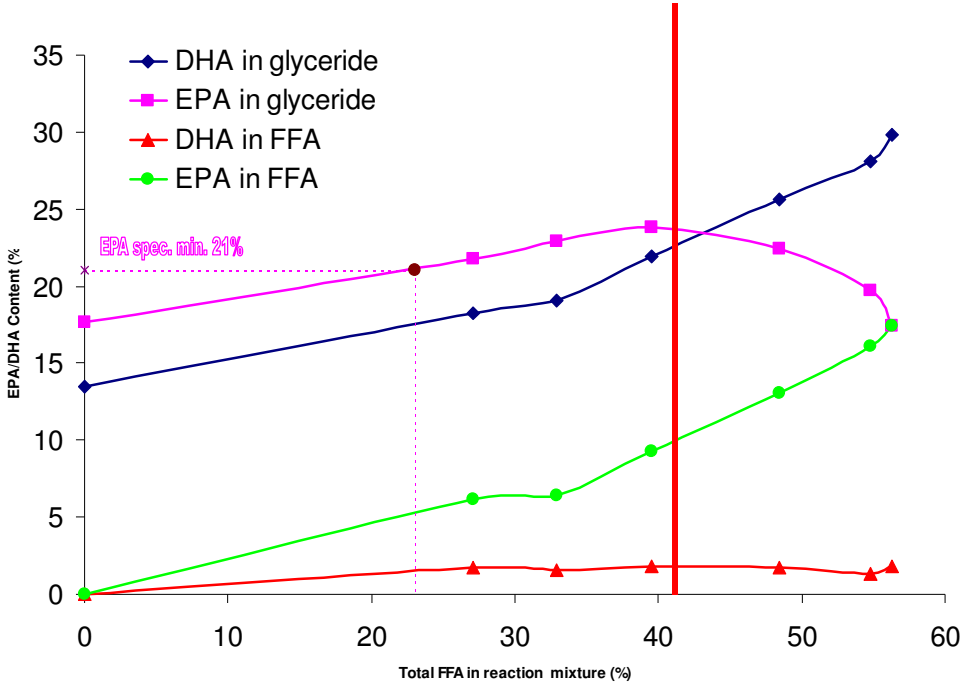
# CONCENTRATION STRATEGY B

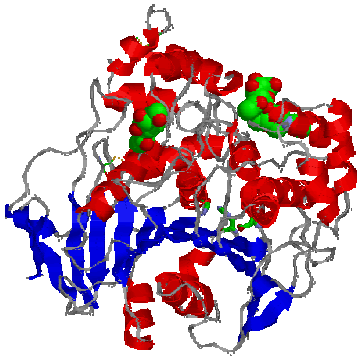
## HYDROLYSIS AND EPA (DHA) INSERTION




Hydrolysis: Lipo-JD, P buffer pH7.5, 40 °C, 42 h; EPA/DHA insertion: CALB, EPA/DHA source, 70°C, 24h

## TIME COURSE OF 18/12TG HYDROLYSIS



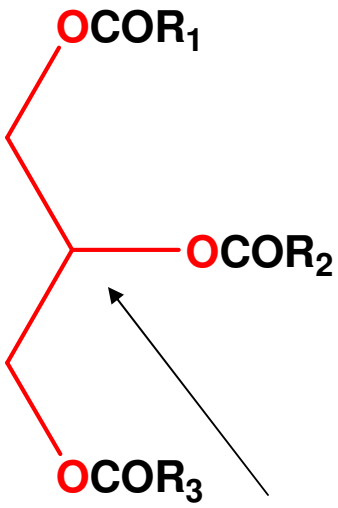



*Candida rugosa* lipase

# POSITIONAL DISTRIBUTION (PD) OF EPA/DHA IN TG OILS

It is very likely that the oxidative stability and thus also sensory qualities of EPA/DHA rich TG oils and their TG concentrates correlate with the positional distribution of fatty acid residues, primarily EPA and DHA in TG molecules.

## FACTS AND STRATEGY




If $R_1 \neq R_2$, $C_2$ is chiral

### Complexity

- Fish oil contains 30 to 60 different fatty acids
- Assuming 30 and taking into account positional distribution, theoretically 27000 different TGs

### Chromatography

- Complex mixtures, need for non-aqueous conditions

### Mass spectrometry

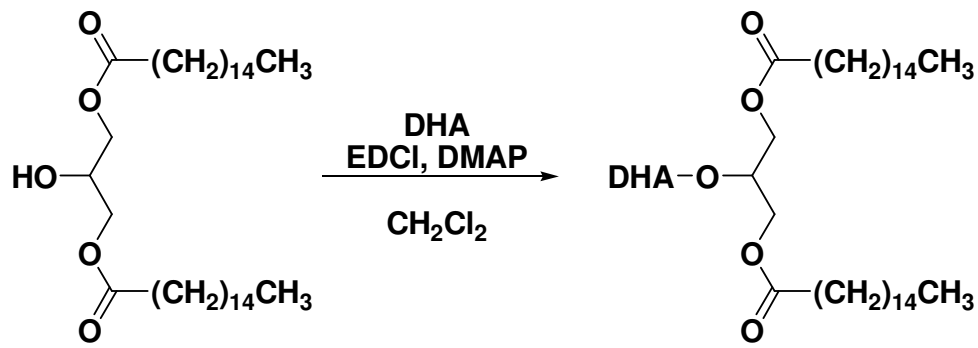
- Neutral compounds - poor ionization, difficulty adding reagents to mobile phase for adduct formation

### NMR

- Complex mixtures, limited data in the literature

# SYNTHESIS OF STANDARDS

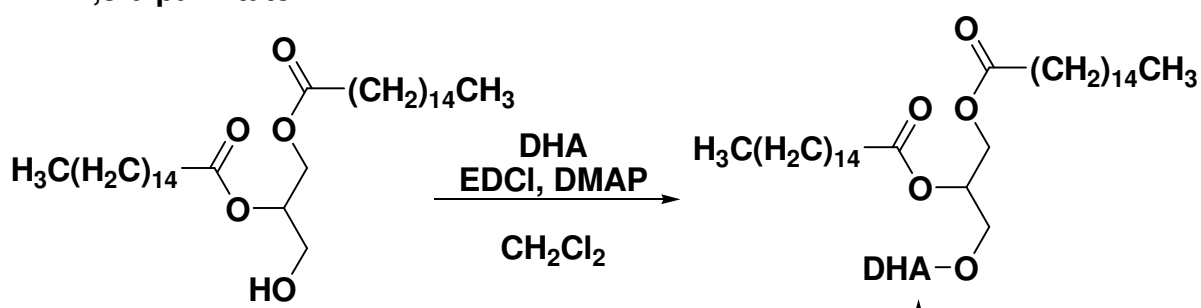
## SYMMETRIC



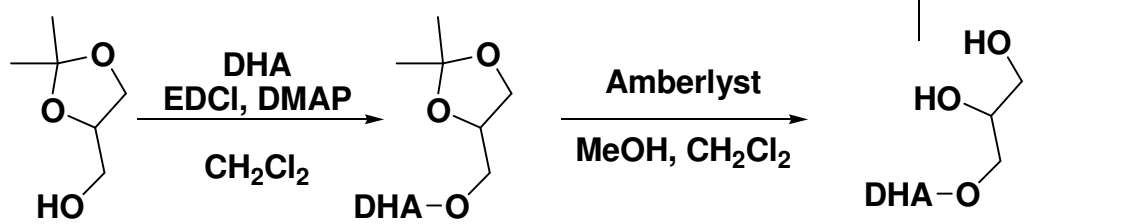

1,3-dipalmitate

## ASYMMETRIC

### Method A




1,2-dipalmitate




Wijesundera et. al. *J. Amer. Oil Chem. Soc.* **2007**, *84*, 11-21

# POSITIONAL DISTRIBUTION OF EPA AND DHA BY NMR

## $^{13}C$ NMR (preferred)

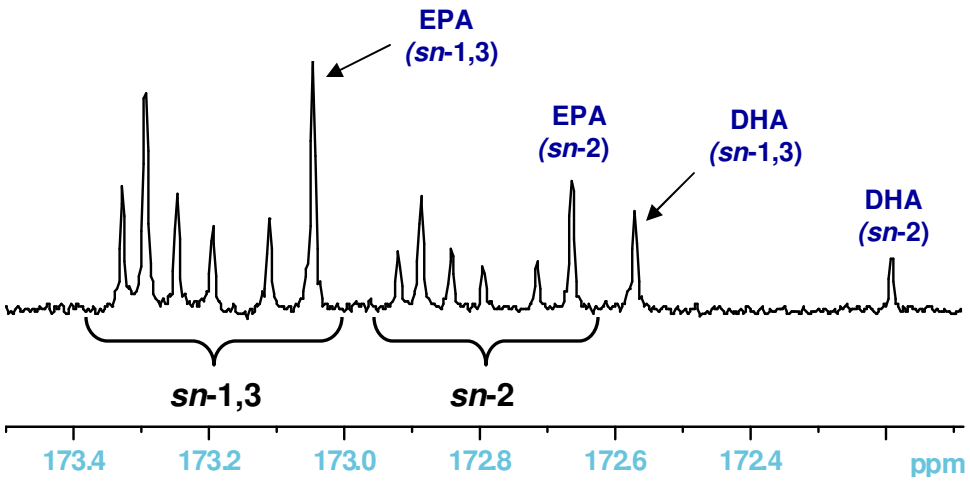
- Better resolution (larger spectral range) than $^{1}H$ NMR, although lower sensitivity

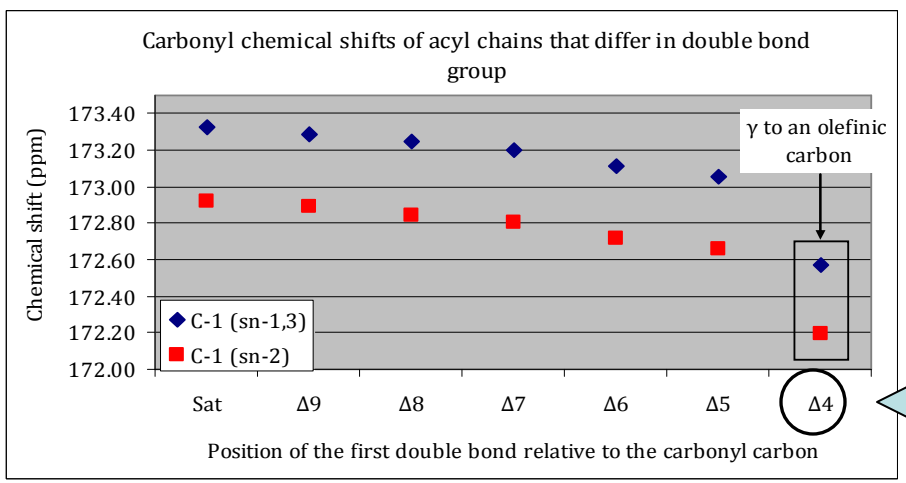
## CARBONYL (C-1) CARBONS (preferred)

- Carbonyls from sn-1,3- and sn-2 chains form clusters of peaks
- Within each cluster, chemical shifts differences between acyl chains occur as a result of the proximity to a neighboring double bond
- Unable to discriminate between *sn*-1 and *sn*-3 positions

# POSITIONAL DISTRIBUTION OF EPA AND DHA BY NMR

Carbonyl region of the 125 MHz $^{13}C$ NMR spectrum of a mixture of TG standards of seven fatty acids




DHA

## BASIC FACTS

- ❑ Carbonyls from *sn*-1,3 chains resonate at higher chemical shifts than *sn*-2 chains
- ❑ Within each cluster carbonyls from saturated chains resonate at a higher chemical shift
- ❑ Chemical shifts are in a direct correlation with the distance of the carbonyl carbon to the first double bond
- ❑ Unsaturation on the γ-position (Δ4, DHA) induces a greater shift on carbonyl chemical shifts; DHA carbonyls resonate in a unique position, which allows a straightforward assignment

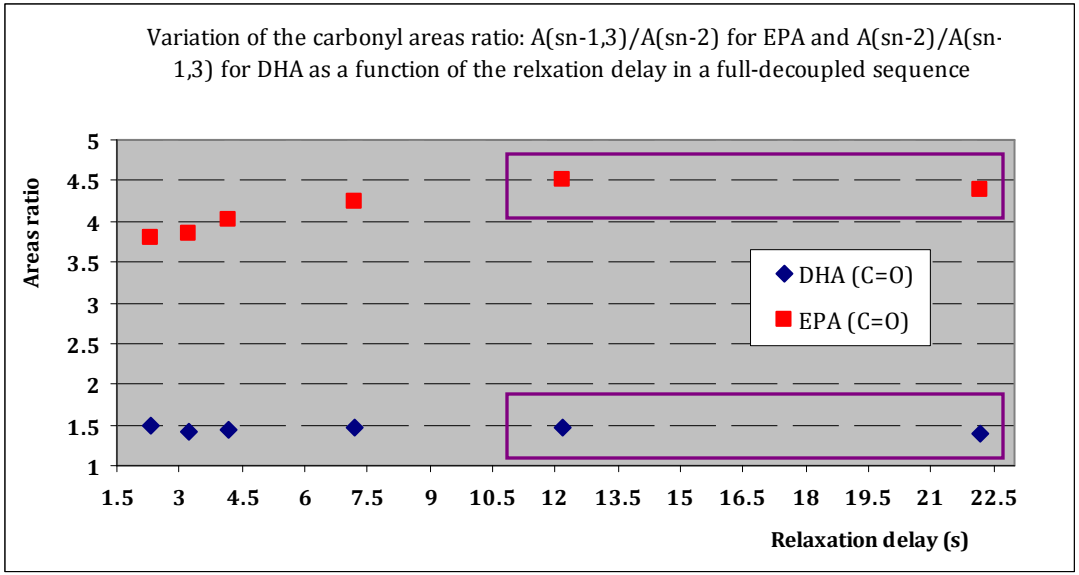
# POSITIONAL DISTRIBUTION OF EPA AND DHA BY NMR

## INVESTIGATED FISH OILS

- 18/12TG (sardine oil)
- Re-esterified 18/12TG
- 05/25TG (tuna oil)

## QUANTITATIVE $^{13}C$ NMR

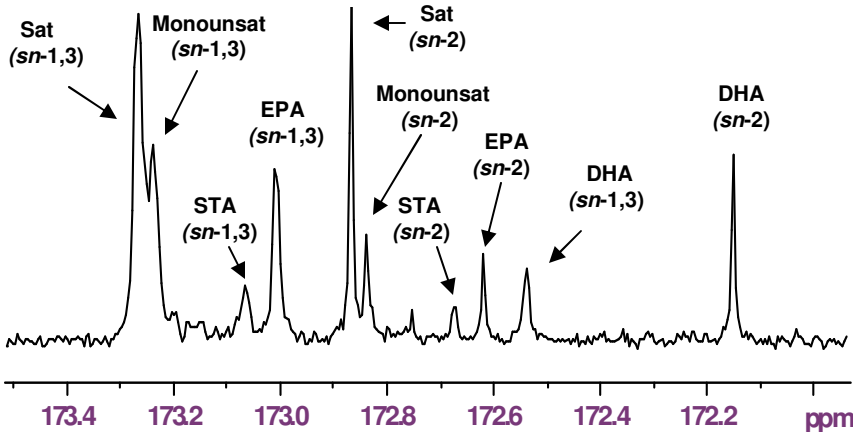
- Under full-decoupling conditions nOe build up may differ for carbonyls of *sn*-1,3 and *sn*-2 chains
- *Sn*-1,3/*sn*-2 area ratios were measured for EPA and DHA carbonyls as a function of the relaxation delay for 18/12TG (100mg/0.7mL $CDCl_3$)



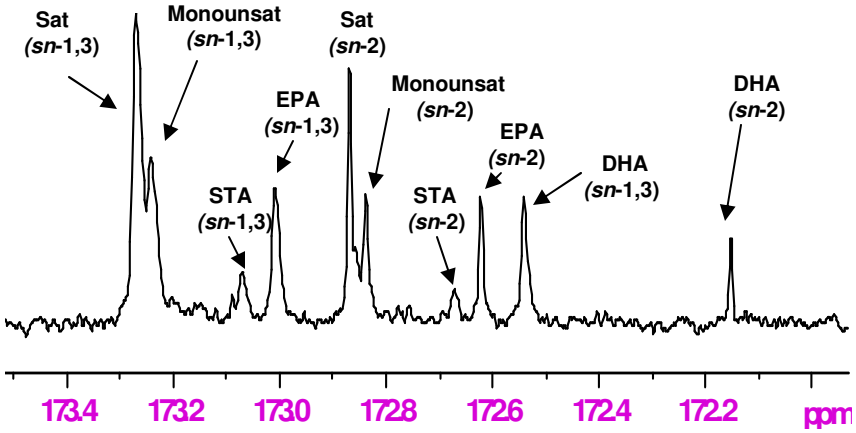

Area ratios for both EPA and DHA carbonyls remain constant after 12s delay (working range)

# POSITIONAL DISTRIBUTION OF EPA AND DHA BY NMR

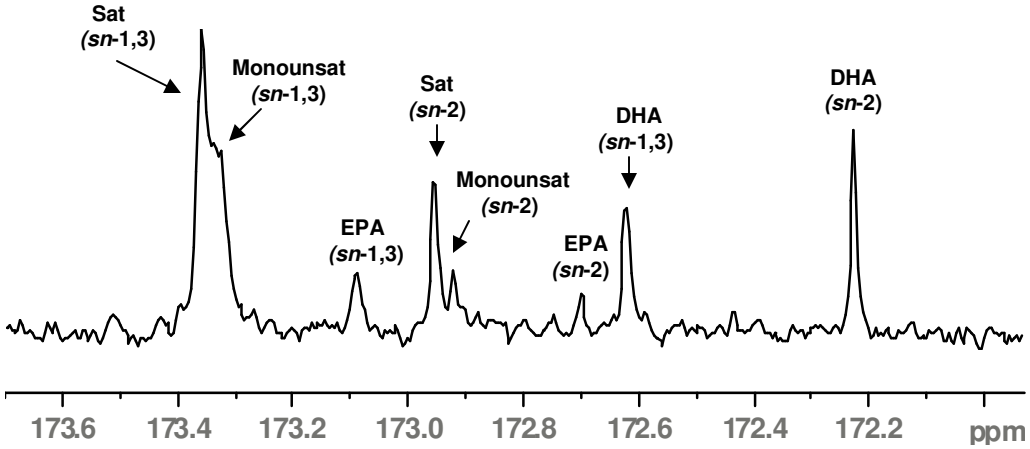
Carbonyl region of the 125 MHz $^{13}C$ NMR spectrum of **18/12TG oil** (100 mg/ 0.7 mL $CDCl_3$)



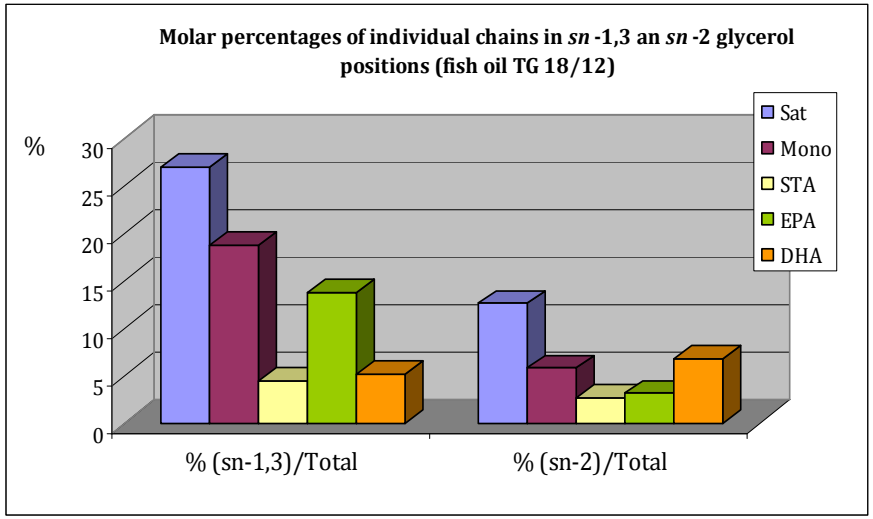

Carbonyl region of the 125 MHz $^{13}C$ NMR spectrum of **Re-esterified 18/12TG oil** (100 mg/ 0.7 mL $CDCl_3$)

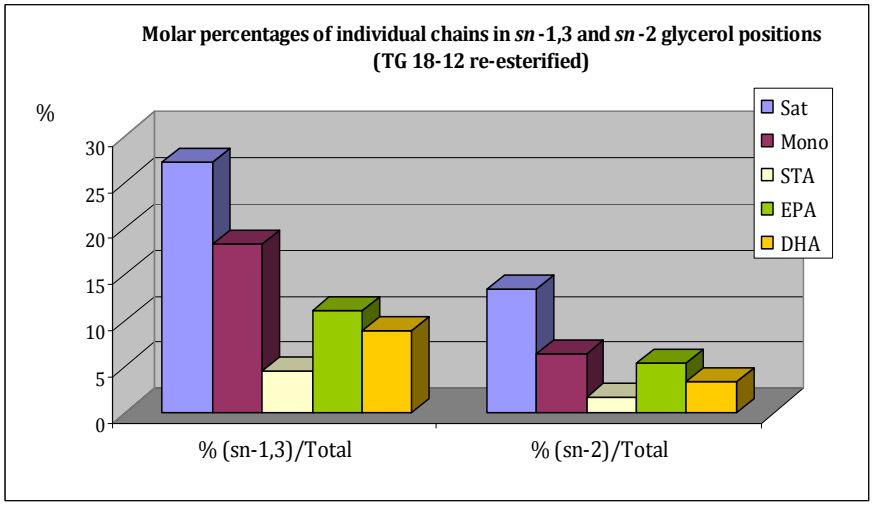



Carbonyl region of the 125 MHz $^{13}C$ NMR spectrum of 0525TG **tuna oil** (100 mg/ 0.7 mL $CDCl_3$)

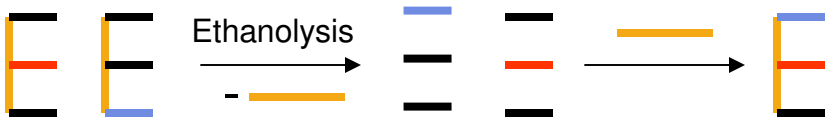


# POSITIONAL DISTRIBUTION OF EPA AND DHA BY NMR



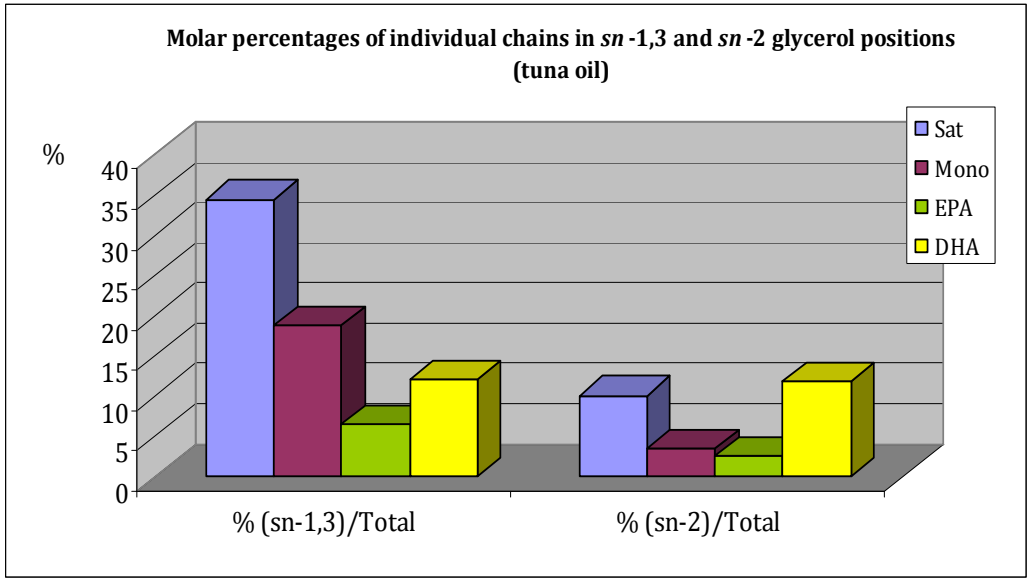

## RE-ESTERIFICATION OF 18/12TG




**(No concentration of EPA or DHA)**

- The distribution of saturated and mono-unsaturated chains as well stearidonic acid (STA) remains unaffected
- The distribution of EPA and DHA chains is affected
  - EPA that was mostly in *sn*-1,3 is randomized on re-esterification
  - DHA that was mostly in *sn*-2 is located mostly in *sn*-1,3 on re-esterification

# POSITIONAL DISTRIBUTION OF EPA AND DHA BY NMR




## 05/25TG TUNA OIL

- Saturated and monounsaturated chains are preferably located in *sn*-1,3 positions
- EPA is present in equal amounts in both *sn*-1,3 and *sn*-2 positions
- DHA shows a slight preference for the *sn*-2 position

# SUMMARY

- The evidence for the beneficial effects of omega-3 fatty acids FA, particularly EPA and DHA is undisputable.

- Science and innovation are the major drivers of dietary supplement and pharmaconutrient industry.

  - To deliver high quality omega-3 products the replacement of traditional chemistry by enzymatic technologies for manufacturing omega-3 based products is becoming more and more important.

  - The understanding of the positional distribution (PD) of the FA residues in the oils is of crucial importance since PD might be important for the stability, sensory and biological effects.

- ONC is leading the way in implementation of sophisticated manufacturing technologies and thorough understanding of physiochemical properties of omega-3 fatty acids.

# THANK YOU !