

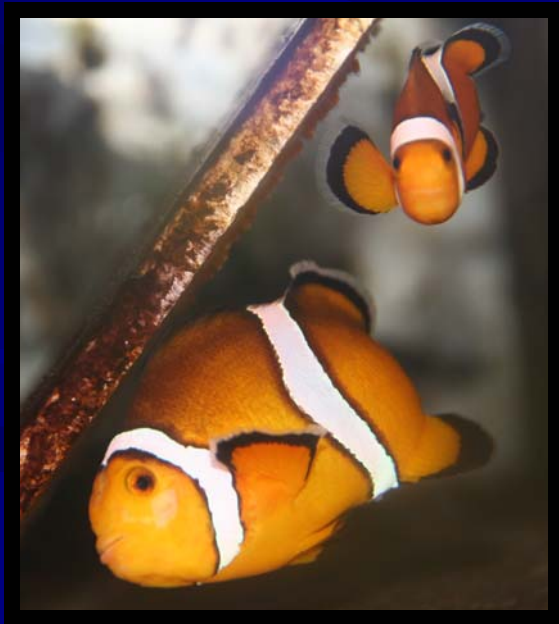
**A MORE EFFICIENT METHOD OF FATTY ACID
ANALYSIS AND THE DETERMINATION OF
CAROTENOID CONTENT IN THE STUDY OF BROOD
STOCK AND JUVENILE NUTRITION IN MARINE
ORNAMENTAL FISHES**

CoSMoS 2006



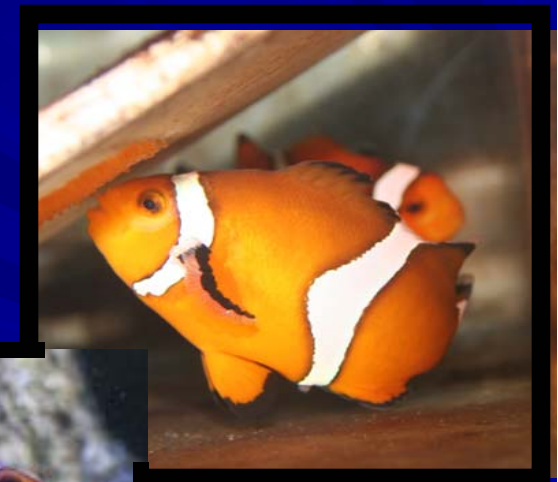
**Stephen O'Shea, Nancy Breen,
Brad Bourque and Skip Pomeroy**

Clownfish are one of the most common residents in private saltwater aquariums kept by hobbyists around the world, creating a high demand for high quality fish.



Current Status of Clownfish Market

- **Effects on environment**
 - dynamite**
 - cyanide**
 - wild populations**
- **Prefer aquacultured fish to wild caught**
 - better suited to aquarium environment**
- **Free from disease**



Production

Balancing market demands with cost and time of production

- **Successful breeding pairs**
2 year old fish followed by 6 month courtship
- **Fecundity and egg quality**
- **High survival rate through to juveniles**
- **Size**
Fast growth rate for quick sale (fish > 1")
Good proportions
- **Coloration**
Vibrant deep orange/red
Bright white stripes
Bold black accents
- **Health**
Strong immune system
Well formed with no abnormal deformities



Broodstock Nutrition

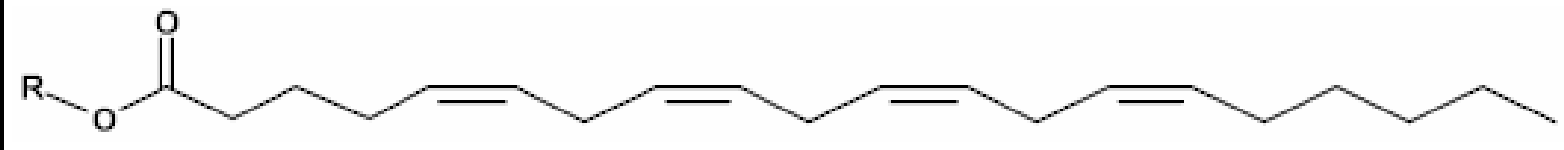
- **Broodstock nutrition plays an important role in egg quality and fecundity**
- **Fish have essential fatty acid requirements.**
 - PUFAs - polyunsaturated fatty acids**
 - HUFAs - highly unsaturated fatty acids**
- **This study focuses on HUFAs, the omega-3 and omega-6 fatty acids**
- **The importance of essential fatty acids in fish nutrition has been extensively investigated during the past 20 years**

Fatty Acids are Essential

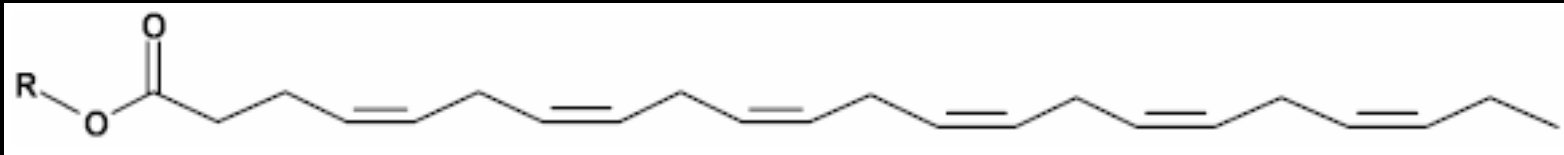
- Gonadal development
- Egg and sperm quality
- Sperm motility
- Egg fertilization
- Embryo development
- Fecundity
- Offspring survival

Essential Fatty Acids

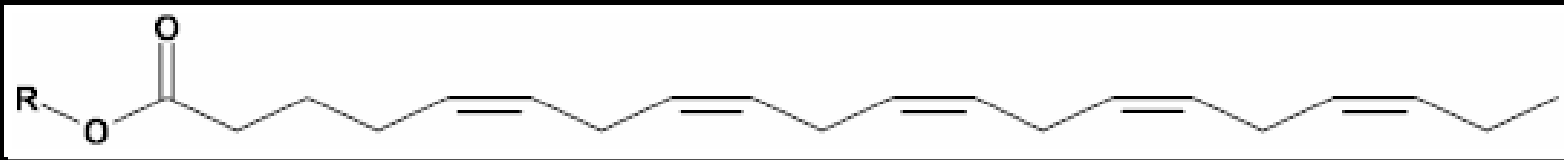
Arachidonic Acid (AA) 20:4n-6



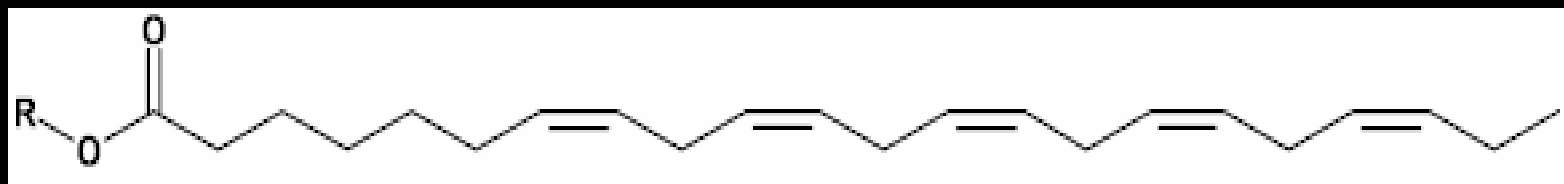
Docosahexaenoic Acid (DHA) 22:6n-3



Eicosapentaenoic Acid (EPA) 20:5n-3



Docosapentaenoic Acid (DPA) 22:5n-6



Essential Fatty Acids

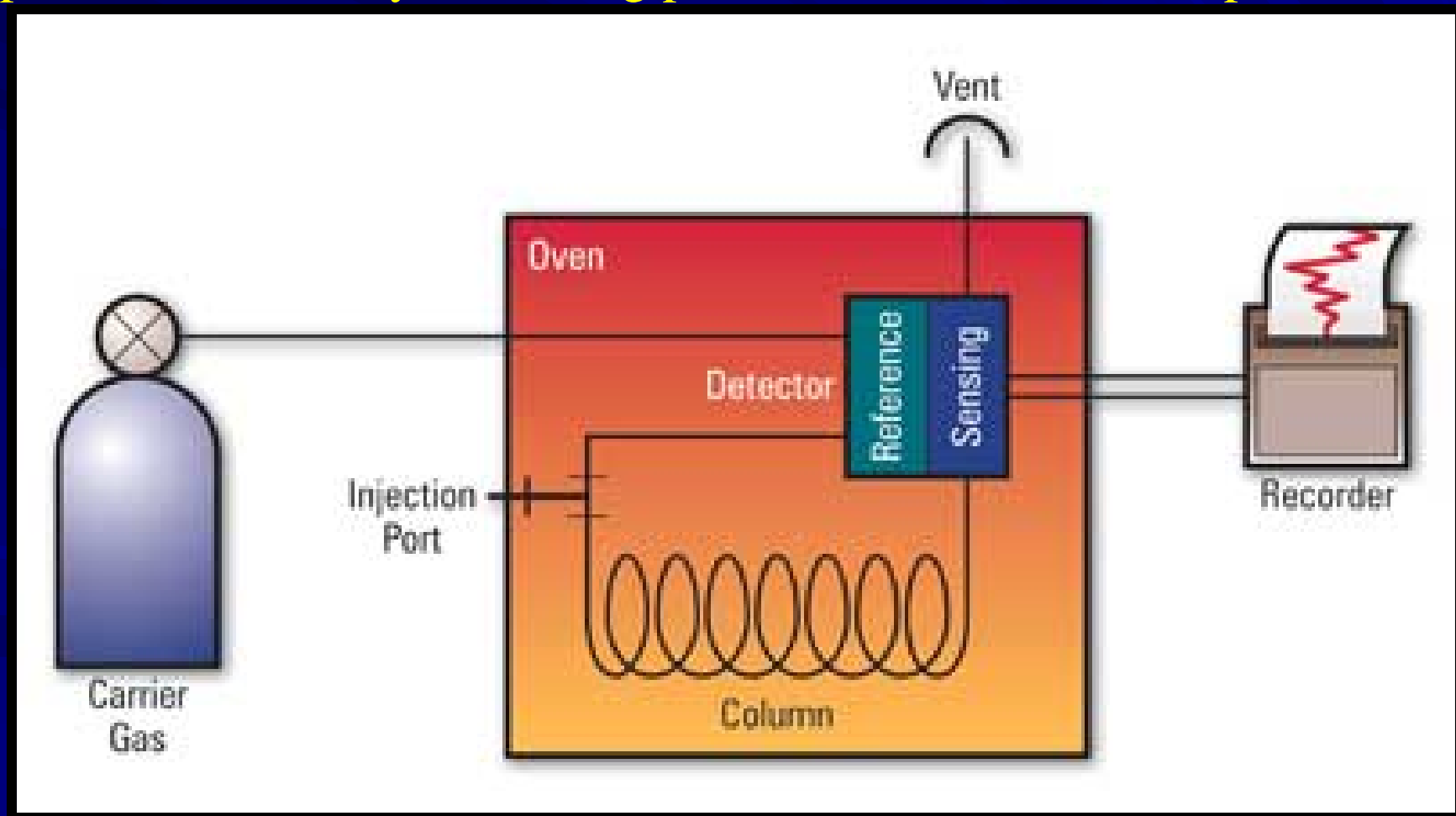
- **DHA** – high concentrations are found the in brain and retinal polar lipids; neural membrane structure and function
- **EPA and AA** – important structural components, precursors to biologically active compounds that regulate growth
- **DHA and AA** - increase larval resistance to stress and pathogen infections
- **The n-3 HUFAs** are also regarded as major energy sources during early embryonic development

Current Study

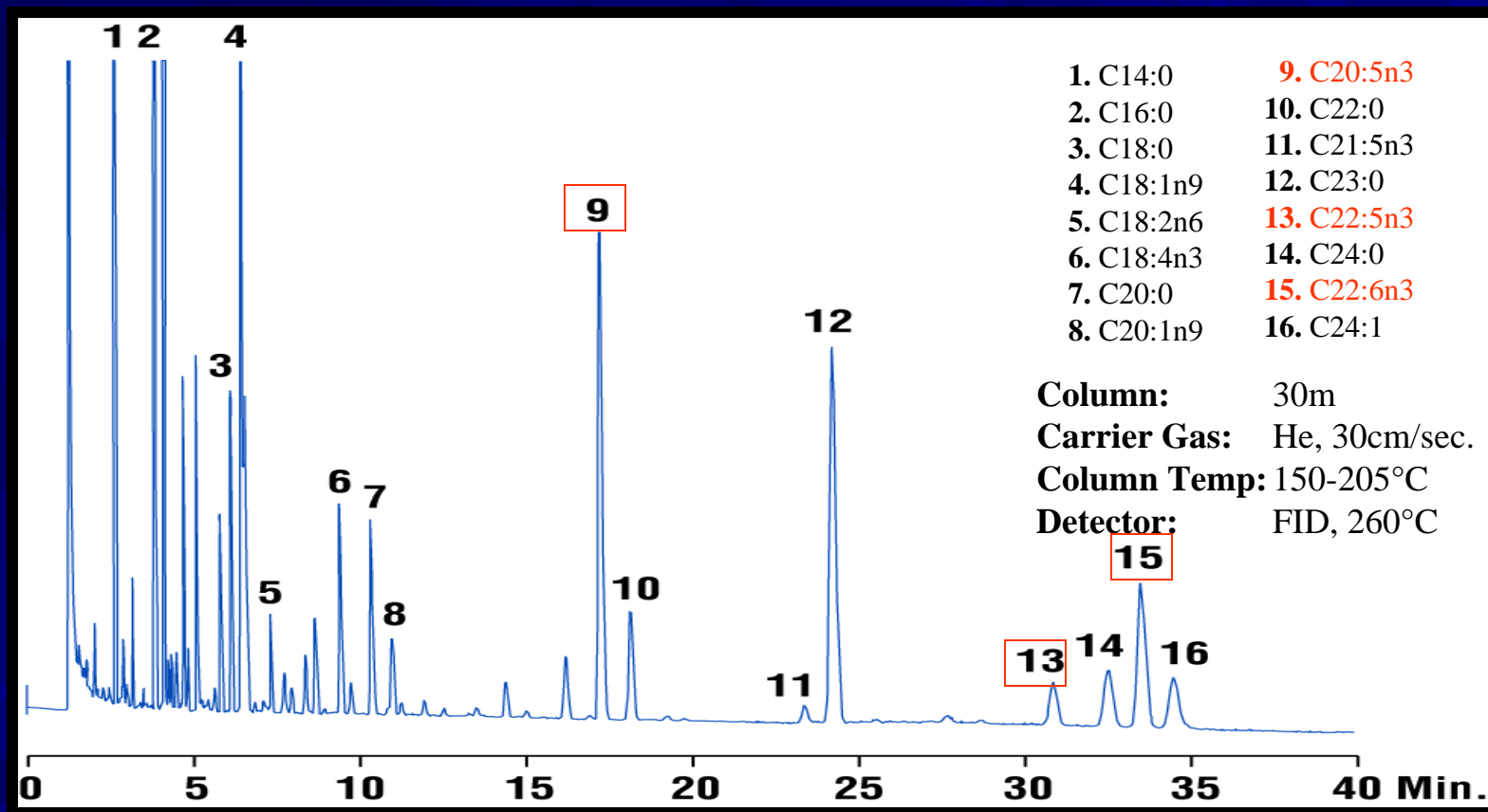
- **Verification of LC/MS ESI Technology in characterization fatty acid content in embryos**
- **Determination of the effect of broodstock diet on egg quality: 15 brood pairs (5 replicate pairs per treatment) rotated through 3 treatment diets – 4 months on each diet, with each 5 replicate pairs receiving each treatment diet.**
- **Determine variation of fatty acid content in single eggs from a clutch**

Gas Chromatography

Standard Technique – requires fatty acid methyl ester formation – separation essentially on boiling point with FID or Mass Spec detection



Common Technique



Retention Time: Note 34 minutes before emergence of C22:6n3 (DHA). Can we process samples quicker?

HPLC and ESI Mass Spectrometry



Waters HPLC and Mass Spectrometer

Operated with Mass Lynx Software

HPLC: Waters Alliance: XTerra C₁₈ Reverse

Phase column - 50mm length

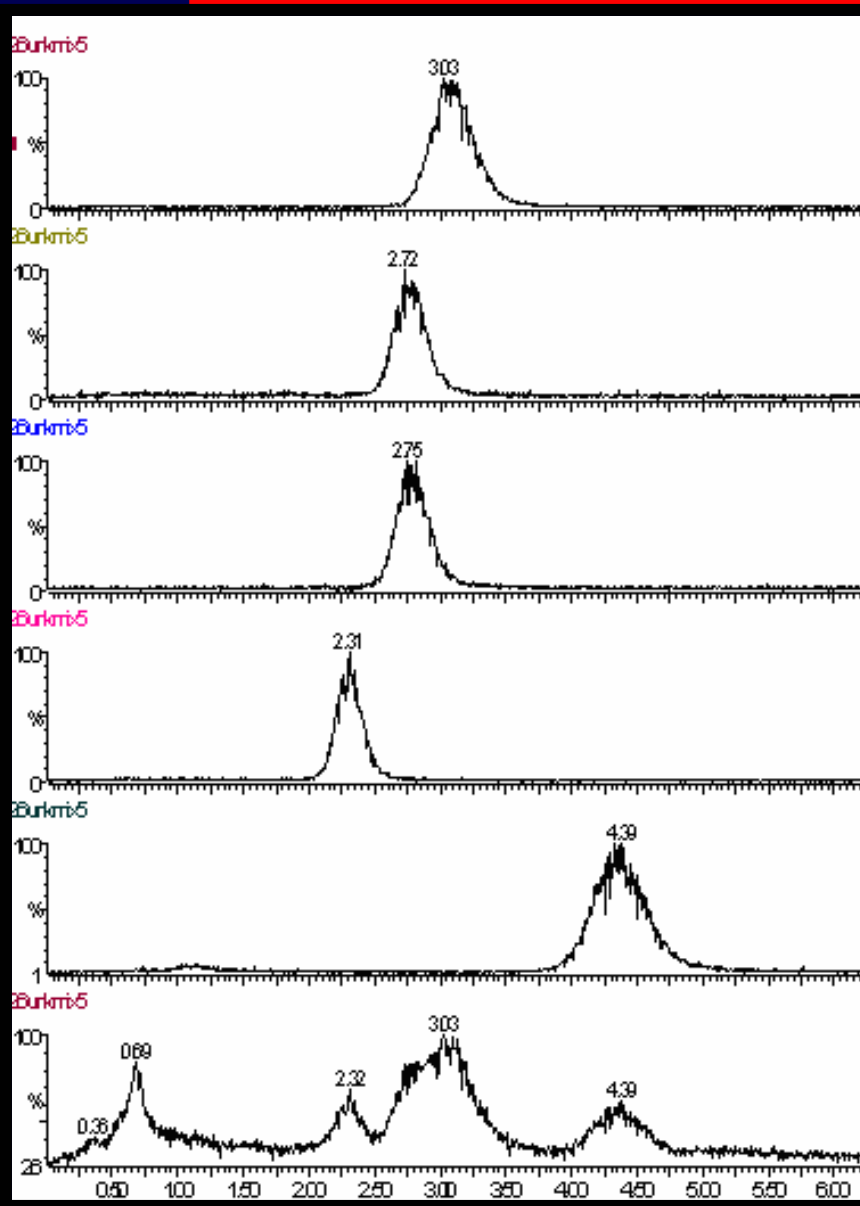
- 3.5 μ m diameter silica bead

Isocratic Solvent Flow (90% MeOH, 10% Water)

Mass Spec: Waters Micromass ZQ

Electrospray Ionization (ESI).

Mixed Standards



Docosapentaenoic Acid (DPA) 22:5n-6

Docosahexaenoic Acid (DHA) 22:6n-3

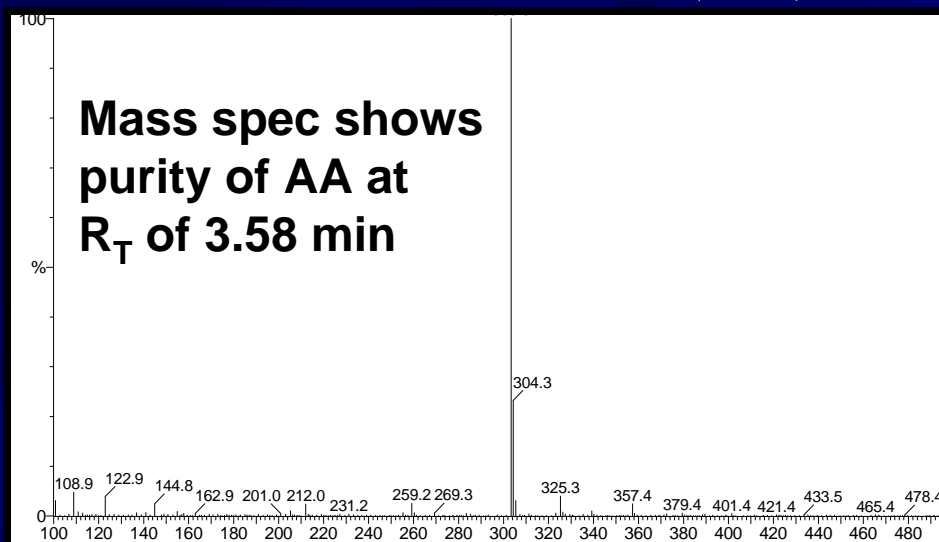
Arachidonic Acid (AA) 20:4n-6

Eicosapentaenoic Acid (EPA) 20:5n-3

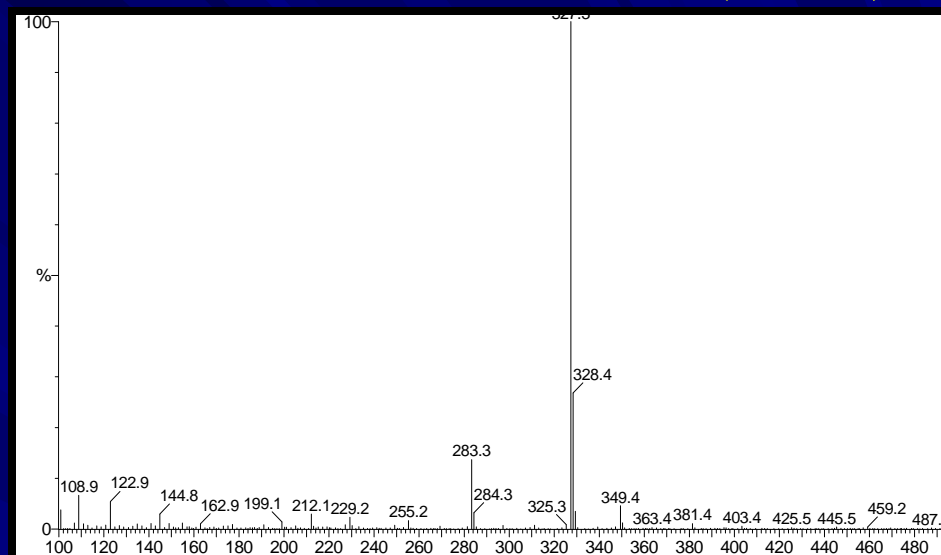
Heptadecanoate (internal standard)

**Note retention times are
TIC < 5 minutes, not 34 minutes**

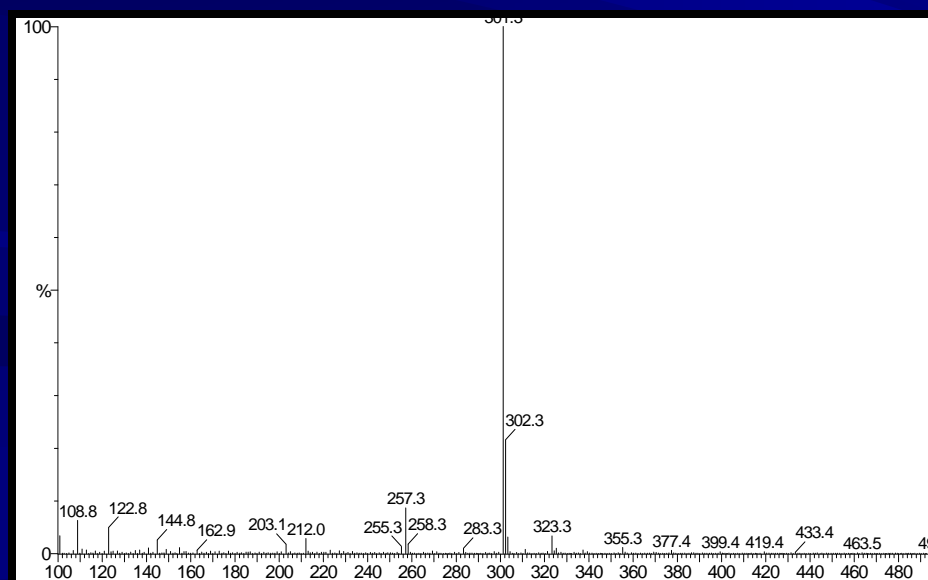
Arachidonic Acid (AA)



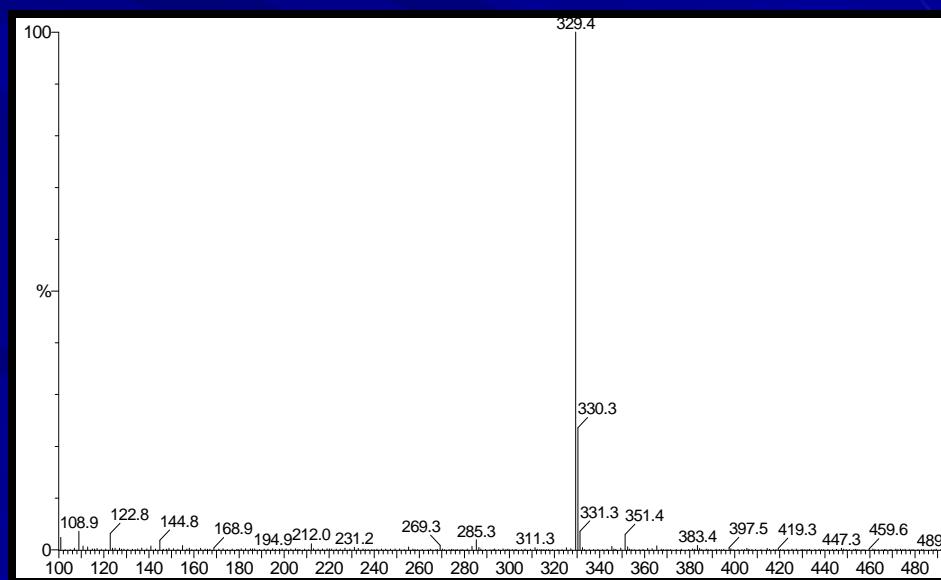
Docosahexaenoic Acid (DHA)



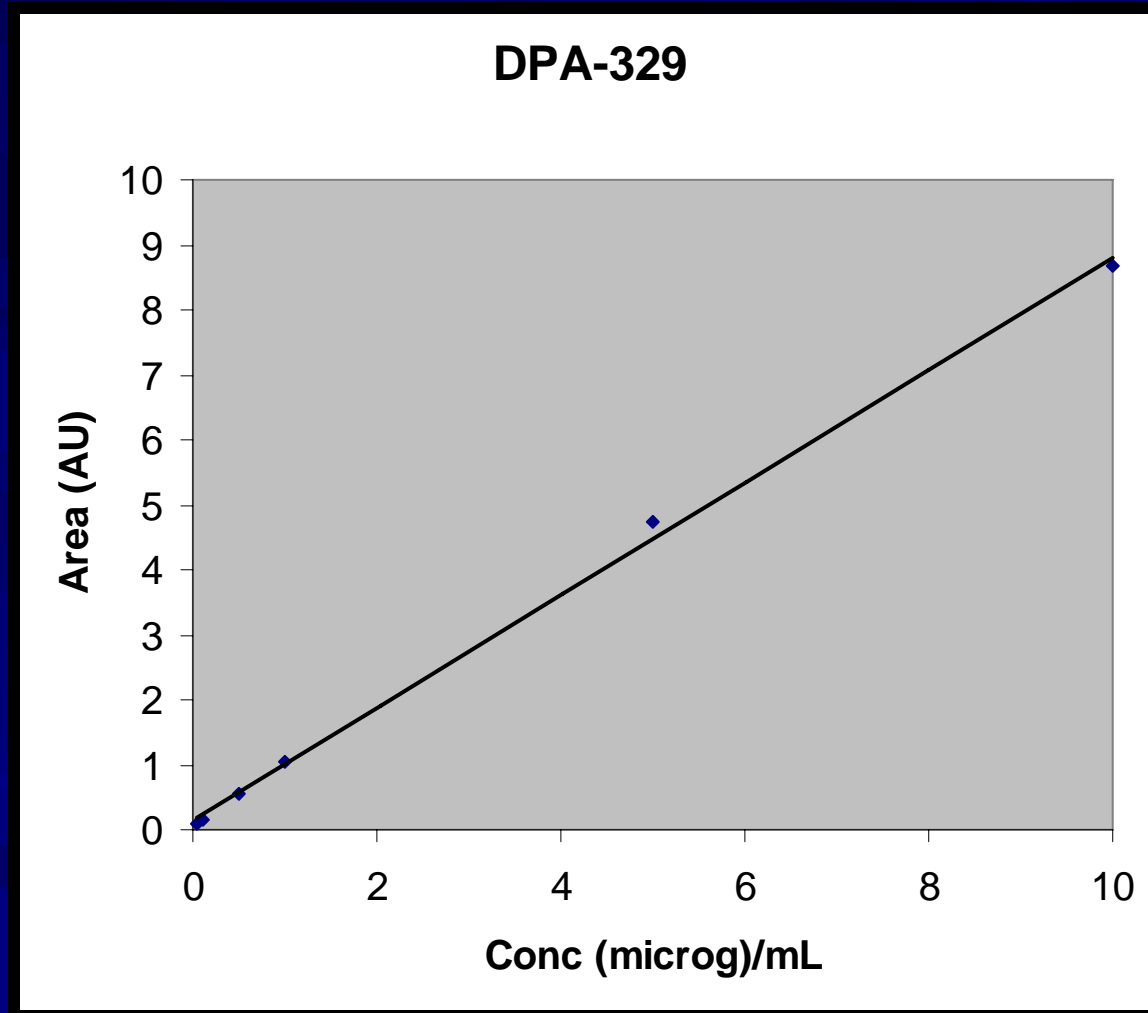
Eicosapentaenoic Acid (EPA)



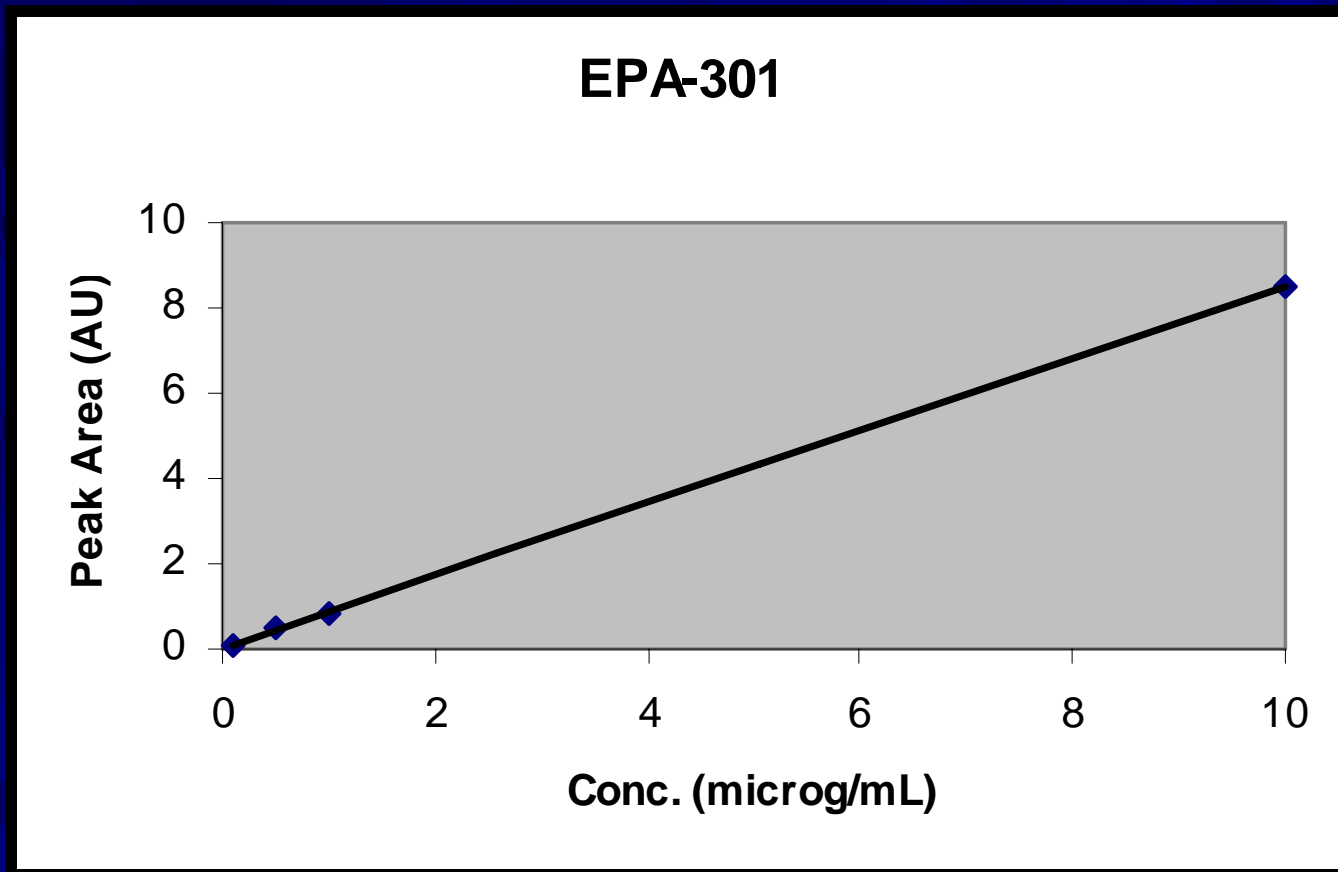
Docosapentaenoic Acid (DPA)



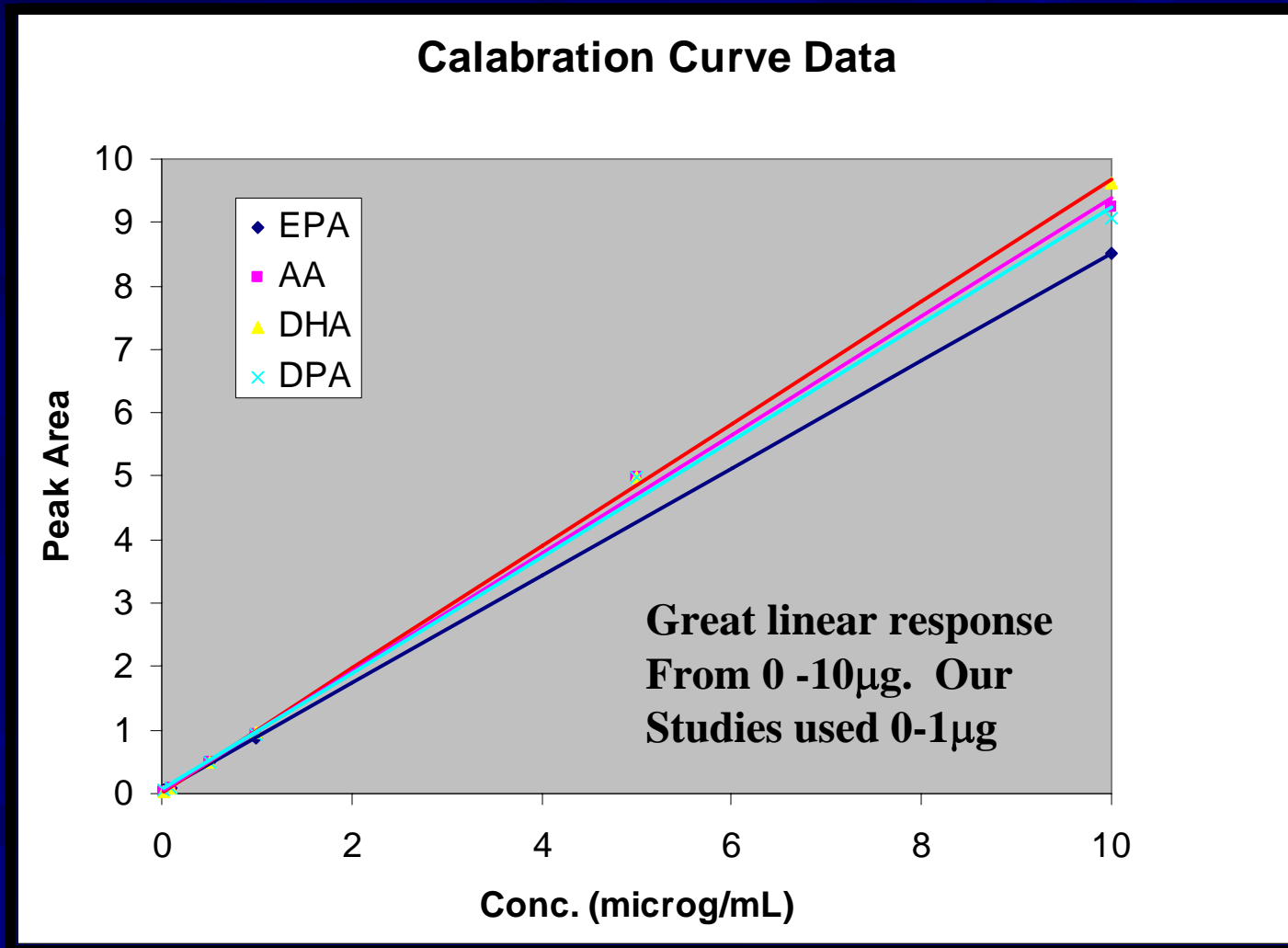
Calibration Curve Data



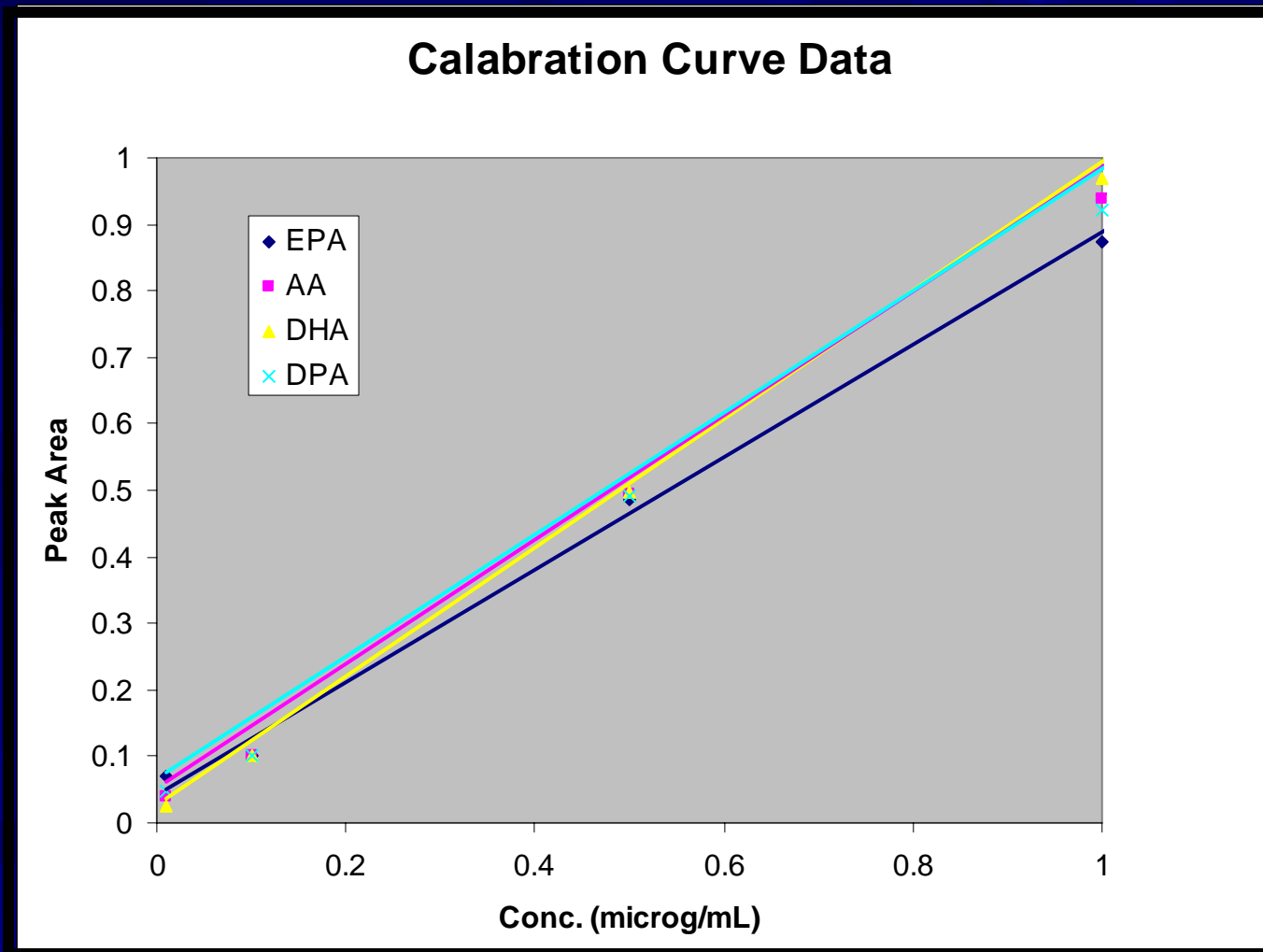
Calibration Curve Data



Combined Calibration Curve Data



Combined Calibration Curve Data



Proof of Method

Artemia Investigation



Matthew R. Palmtag, Cynthia Faulk and G.J.Holt
University of Texas Marine Science Institute

Enriched *Artemia* Feeding Study

Artemia fed live algae *Isochrysis galbana*
Fatty acid composition compared to a previous
study using GC/MS

Palmtag, Faulk and Holt in press

Holt's Methodology

•Tissue Grinding

Extraction into chloroform methanol solution (2:1)

•Saponification

Methanolic base hydrolysis 70°C (15 mins) [not 30 mins classical]

•Esterfication

BF₃ catalyzed esterfication 70°C (2 mins) [not 15 mins classical] back extraction into hexane

•HPLC Preparation

Dried under nitrogen flow and resuspended in LC/MS grade methanol with standard (C₁₇ heptadecanoate)

Enriched Artemia Feeding Study

Artemia fed live algae *Isochrysis galbana*

% Total Fatty Acids

GC Analysis

EPA/AA

3.0 ± 0.2

LCMS Analysis

EPA/AA

3.09 ± 0.2

Clownfish diet study – each 5 brood pair group was rotated through all treatments for 4 months on each treatment

Five Brood Pairs



Five Brood Pairs

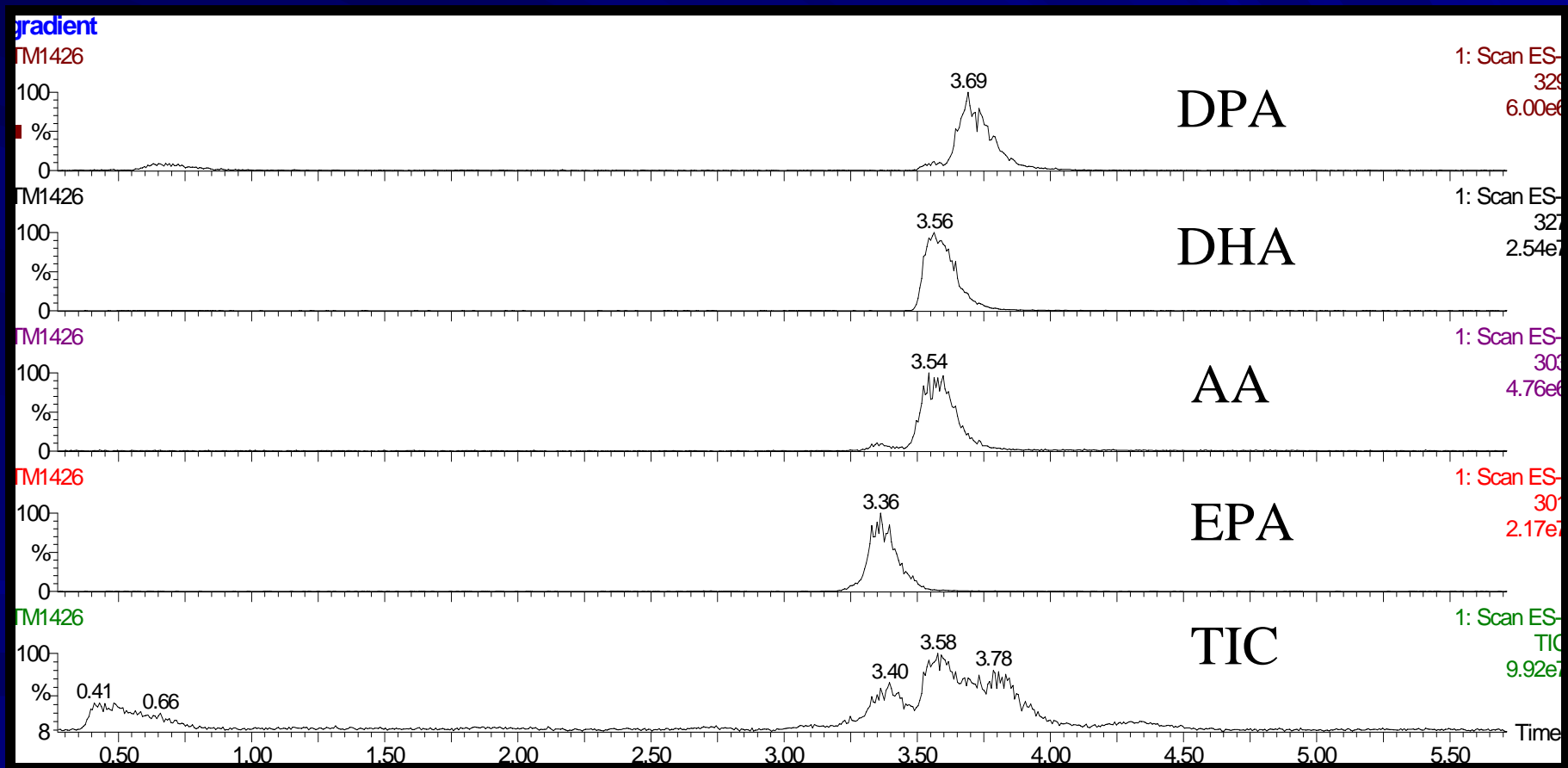


Five Brood Pairs



Number of spawns, hatch rate, survival to 30 days
Determination of fatty acid content in eggs

Tetra Marine Flake



Food Fatty Acid Content

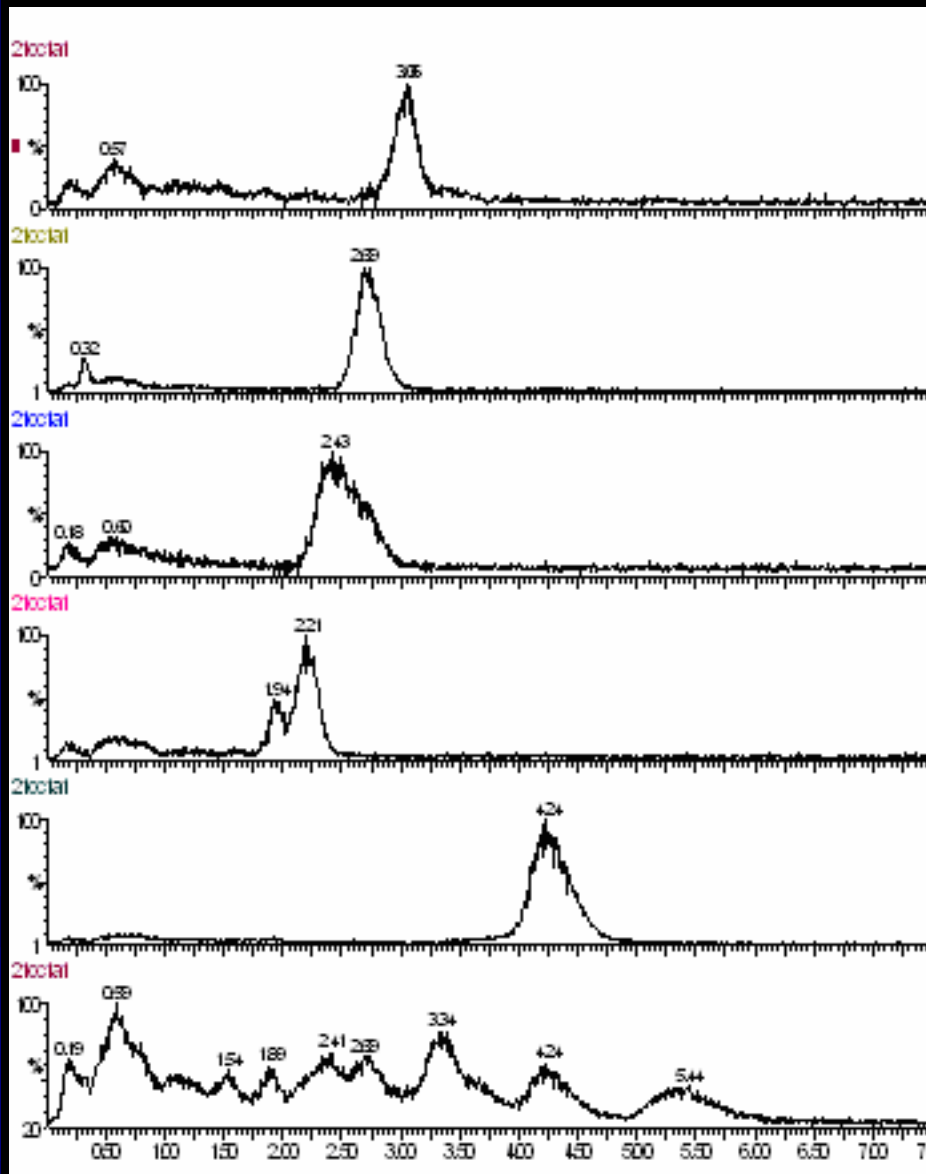
	EPA	AA	DHA	DPA
Formula 1	1	0.140	0.678	0.119
Gelly Belly	1	0.222	0.494	0.141
Tetra Marine Flake	1	0.243	1.335	0.236

Spawns

- Picture taken of nest – analyzed with NIH Image J to determine counts and size distribution of eggs
- Sample of 20 eggs collected, weighed, and analyzed for fatty acids
- Remaining eggs were returned to hatch tank for determination of hatch rate.



Eggs from Formula One Diet



Docosapentaenoic Acid (DPA) 22:5n-6

Docosahexaenoic Acid (DHA) 22:6n-3

Arachidonic Acid (AA) 20:4n-6

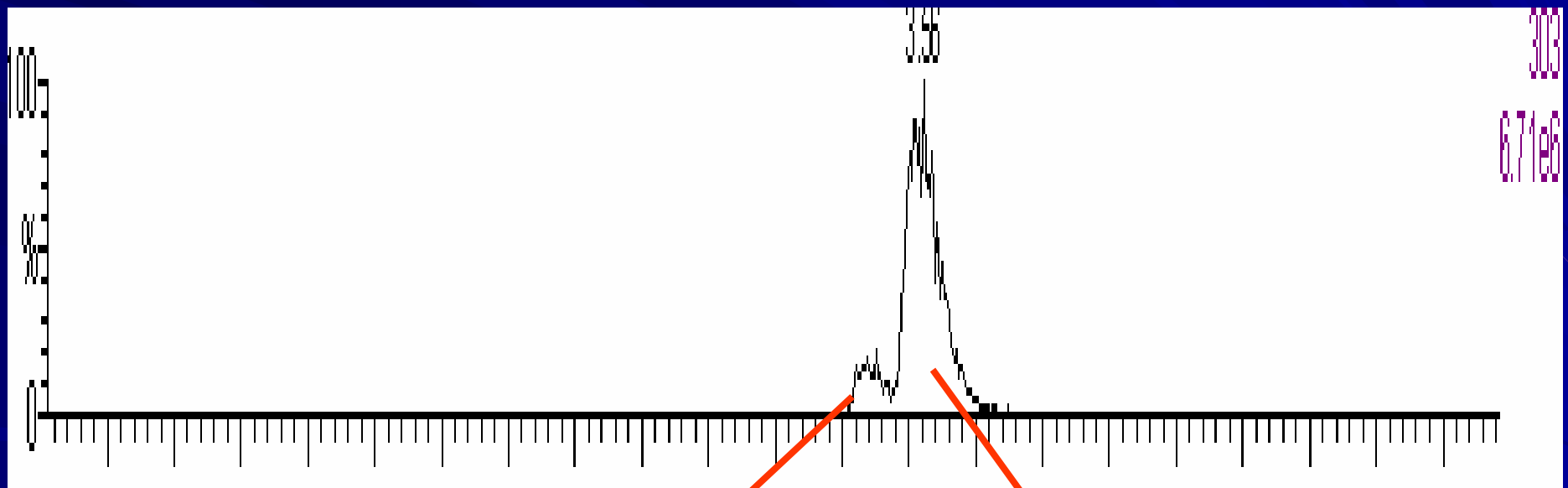
Eicosapentaenoic Acid (EPA) 20:5n-3

Heptadecanoate (internal standard)

TIC

Cis-Trans Isomerization

Arachidonic Acid (AA)



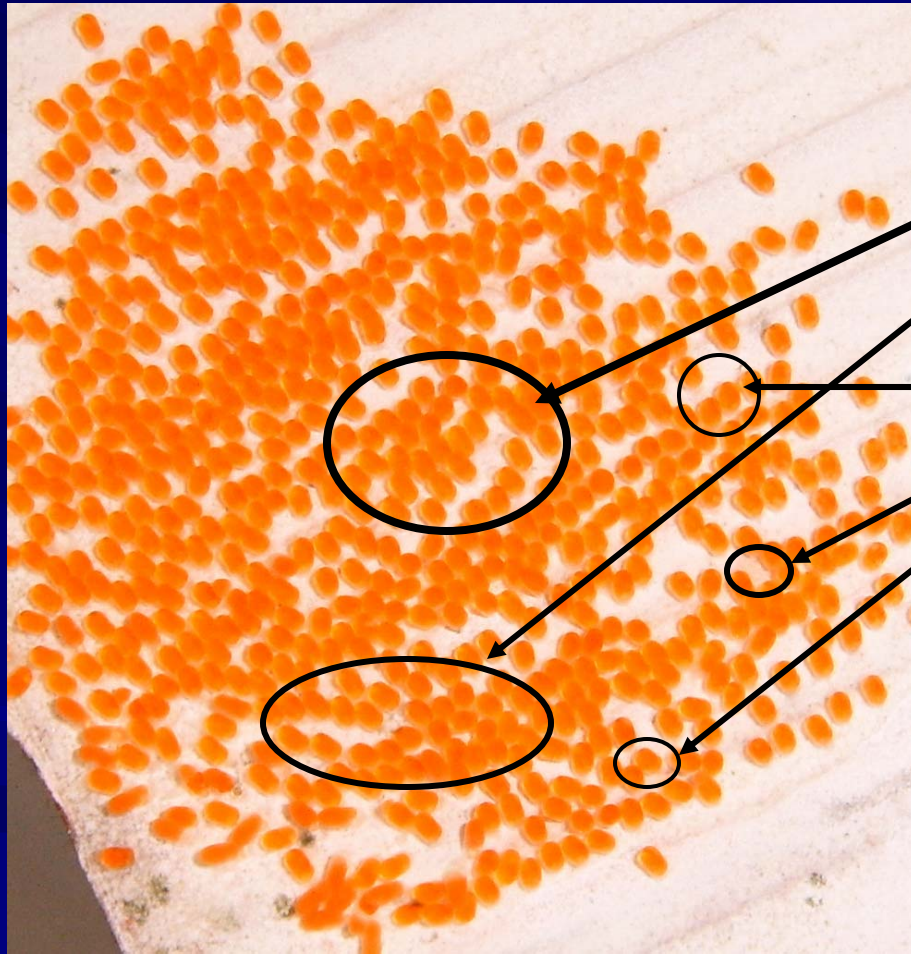
Trans

Cis

Diet Study Results

- EPA content in eggs was significantly different for brood stock fed on TMF
- AA, DHA and DPA did not vary significantly with the three diets
- On average, of the 4 fatty acids studied in eggs DHA (56%) and EPA (28%)
- TMF gave smallest clutch number but largest size eggs with poor hatch survival quality
- Breeding pairs fed the same diet did not show distinct differences in accumulation of fatty acids in eggs

Determination of variation of fatty acid content in a clutch



Twenty Eggs

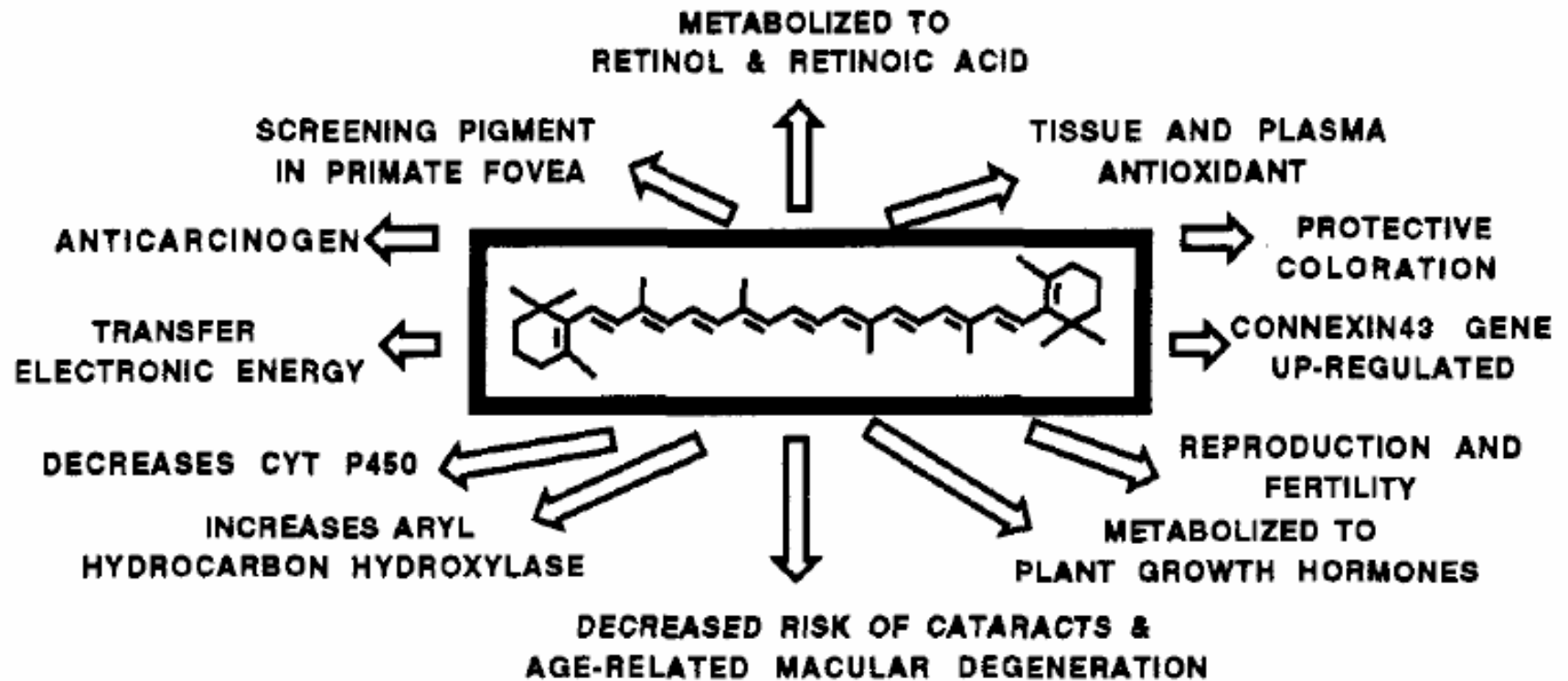
Two Egg Selection

Fatty Acid Content Variation with Sample Size

$\mu\text{g/ml}$

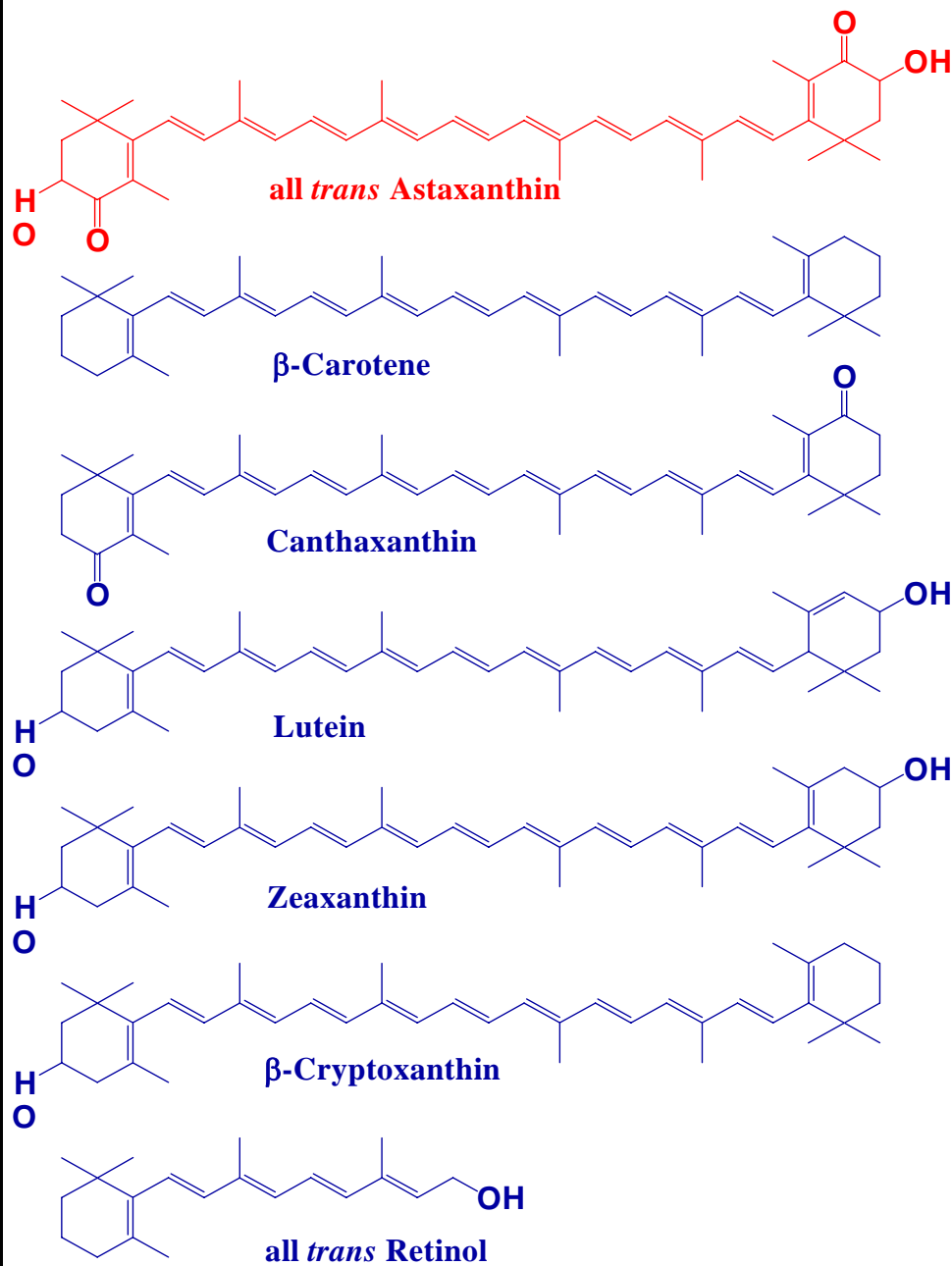
	EPA	AA	DHA	DPA
AV 20 eggs Std. Dev.	0.14 $\pm.004$	0.05 $\pm.001$	0.32 $\pm.042$	0.06 $\pm.009$
AV 2 eggs Std. Dev.	0.10 $\pm.028$	0.03 $\pm.01$	0.21 $\pm.063$	0.05 $\pm.019$

Carotenoids Biological Activity



Background

- **Carotenoids are essential nutrients important in maintaining good health in marine fish**
- **Diets supplemented with astaxanthin result in higher survival rates in atlantic salmon juveniles and in goldfish**
- **Astaxanthin has been shown to be the key determinant of color in clownfish**



**Important
active carotenoids
determined
to have
biological activity**

Juvenile Experiment Diet

- **Tetra Marine Flake**
- **Wardley Total Color Marine[®] Gourmet Flake Blend**
- **Ocean Nutrition[™] Formula**
- **Cyclop-eeze[®]**

		Tetra Marine Flake	Wardley Flake	Formula One Flake	Cyclop-eeze Flake
Food Content	Protein	44.0%	46.0%	54.6%	50.0%
	Fat	8.0%	5.0%	11.8%	35.0%
	Fiber	2.0%	4.0%	0.5%	~
	Moisture	6.0%	9.0%	7.7%	~
	Ash	~	~	5.8%	3.5%



Feeding

- 4 diet treatments
- 6 replicates per treatment with 30 fish per replicate
- Tanks stocked with fish at 30 dph
- All fish fed the same standard larval diet until 30 dph
 - Rotifers
 - Enriched Artemia
 - Golden Pearl Artemia



Digital Pixel Analysis

- **Photo set-up frame**
 - Kodak – CX 7530 Digital Camera**
 - Camera frame**
 - Scale**
 - Petri dish**
 - Ruler**



Pixel Analysis

- **Color**

 - Photoshop CS**

- **Length**

 - NIH Image J Software**

- **Weight**

 - Taken at same time as pictures**

- **Flake foods**



Photometry

- **Acetone extraction**
- **UV/Vis Analysis @450nm**
- **Determination of concentration from an average extinction coefficient for carotenoid mixture ($130058 \text{ M}^{-1}\text{cm}^{-1}$)**
- **Flake Foods**
- **Poor astaxanthin diet fish**
- **Rich astaxanthin diet fish**

Waters HPLC and Mass Spectrometer

Operated with Mass Lynx Software

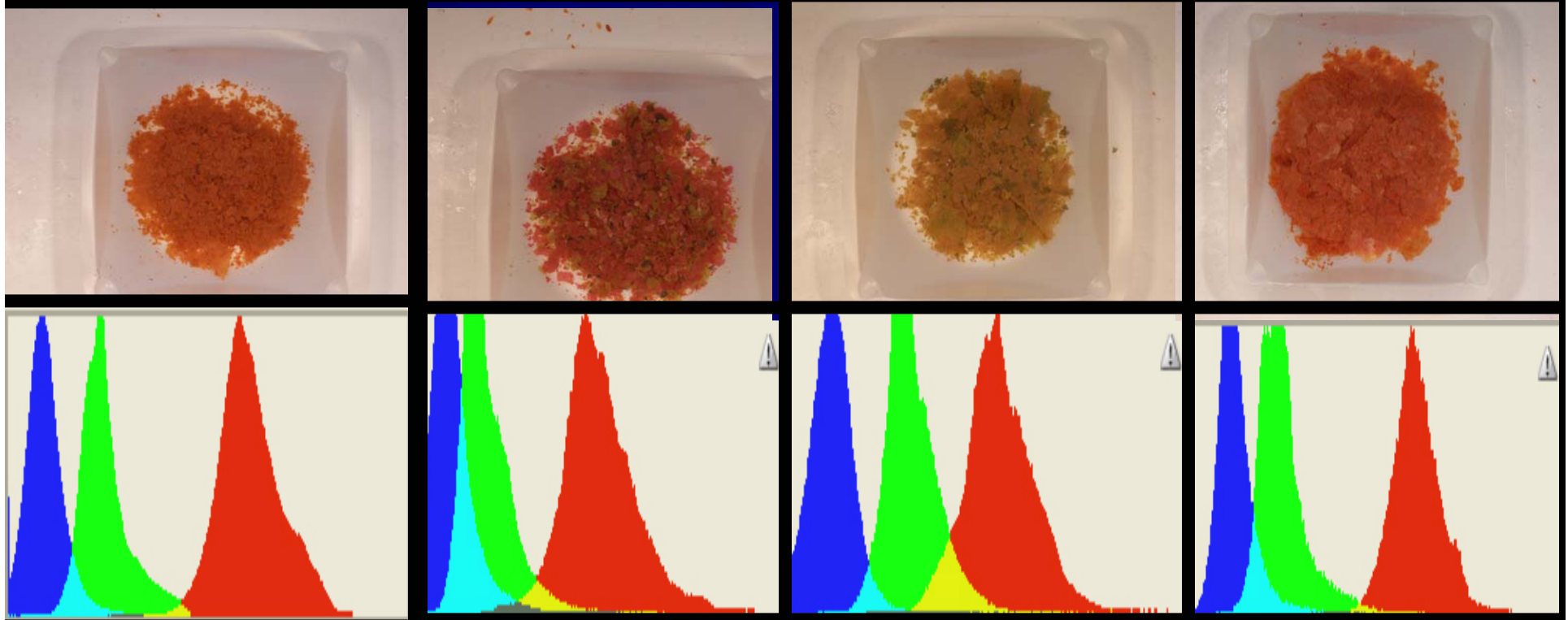
HPLC: Waters Alliance: XTerra C₁₈ Reverse Phase column - 50mm length - 3.5 μ m diameter silica bead – Gradient Solvent Flow 25 min. run

(Initial MTBE 5% 90%MeOH, 5% Water to MTBE 60% 35%MeOH, 5% Water)

Mass Spec: Waters Micromass ZQ

Electrospray Ionization (ESI).

Digital Pixel Analysis of Feeds



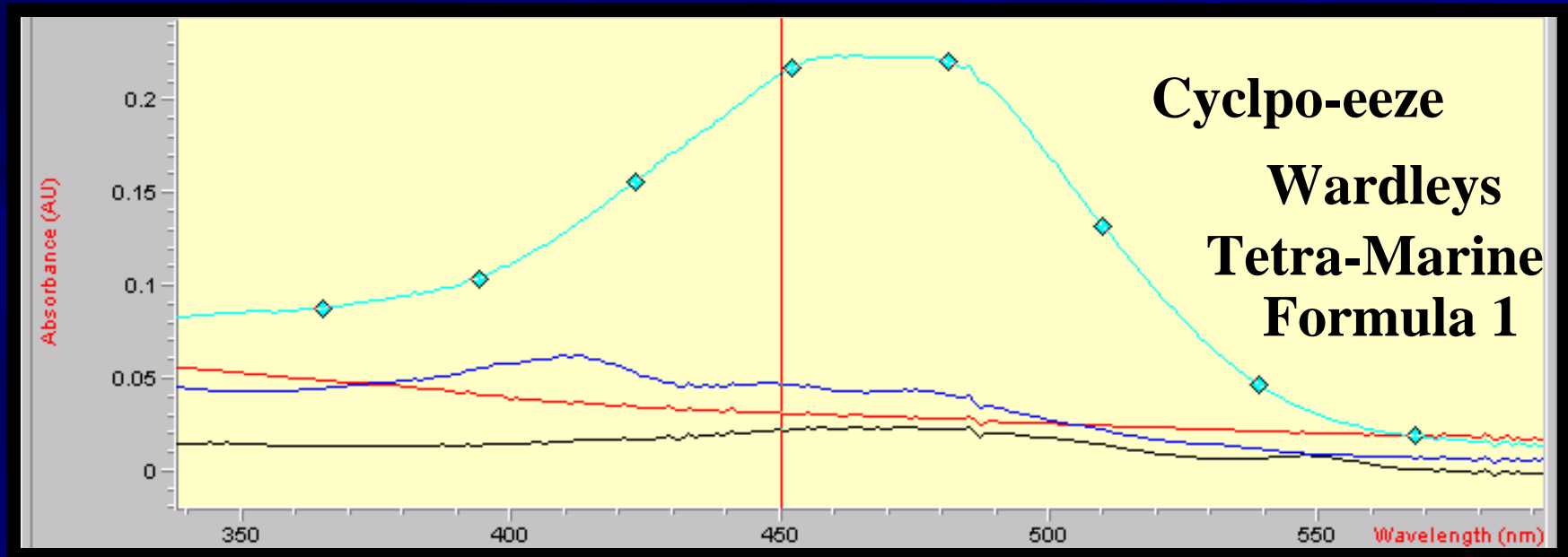
F1 **Tetra Marine** **Wardleys** **Cyclop-eeze**

Histogram

Significance of red green and blue Percentage of red pigment

Area of curve

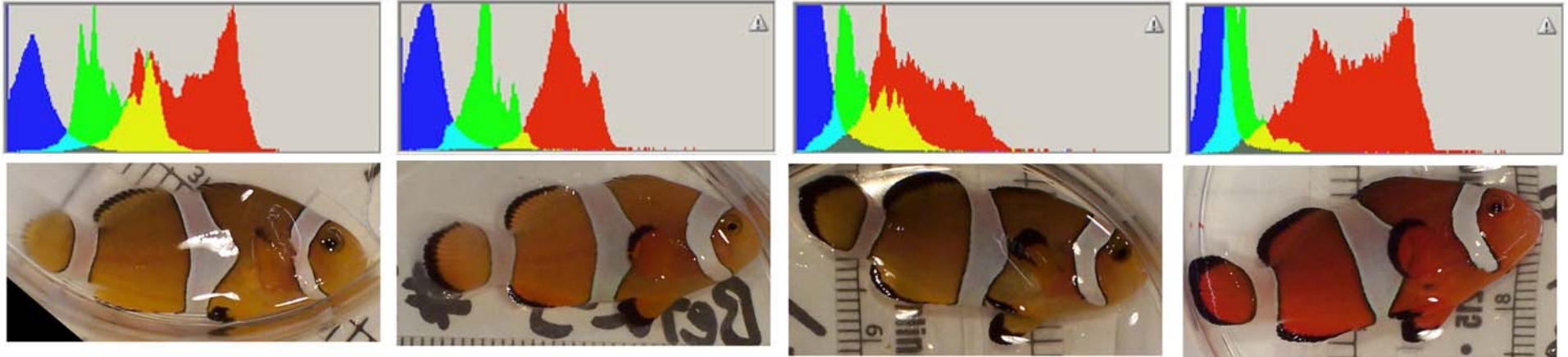
UV/Visible Spectra



Relative Concentrations of Carotenoids Determine @450nm

Cyclop-eeze	0.95mg/g
Wardleys	0.20mg/g
Tetra-marine	0.14mg/g
Formula 1	0.09mg/g

Pixel Diet Study



Tetra Marine

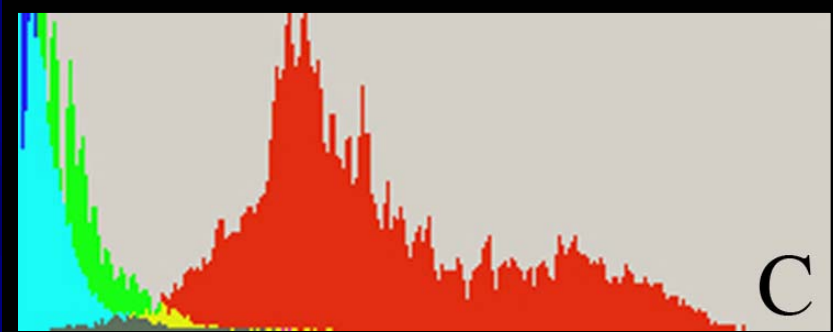
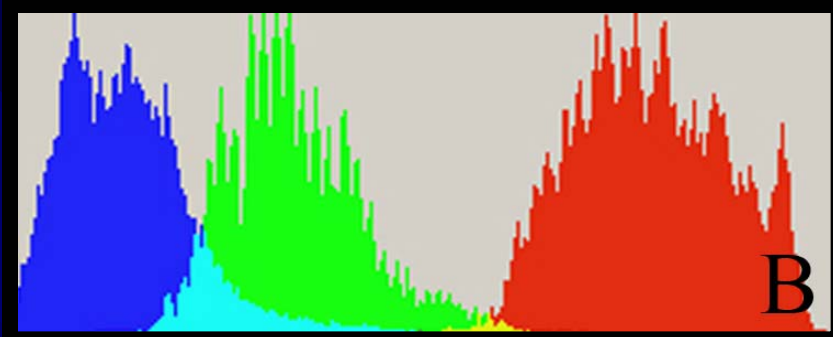
Wardley's

Formula One

Cyclop-eeze

● Histogram

- Significance of red green and blue
 - Area of curve
 - Location of colors
- Percentage of red pigment



Bottom left fish Cyclop-eeze (astaxanthin rich) diet showing intense red coloration. Histogram of bottom left fish showing high amount of red pixels (61.59%).

**Top right fish (astaxanthin low) diet showing dull coloration
Histogram of top right fish showing relatively low amount of red pixels (40.88%).**

Photometry Results

A. Ocellaris Skin Carotenoid Content

Acetone extraction

UV/Vis Analysis @450nm



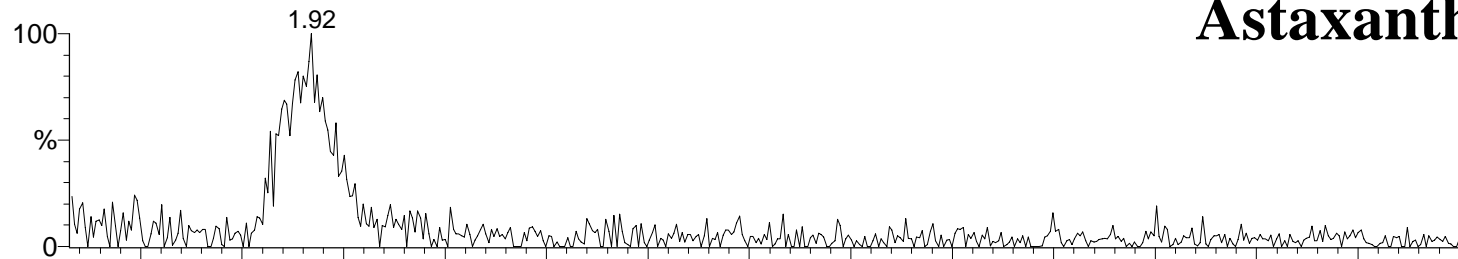
Astaxanthin poor feed
Carotenoid Content
Dull Color 4.0 $\mu\text{g}/\text{cm}^2$

Astaxanthin rich feed
Carotenoid Content
Bright Color 10.4 $\mu\text{g}/\text{cm}^2$

Cyclop-eeze

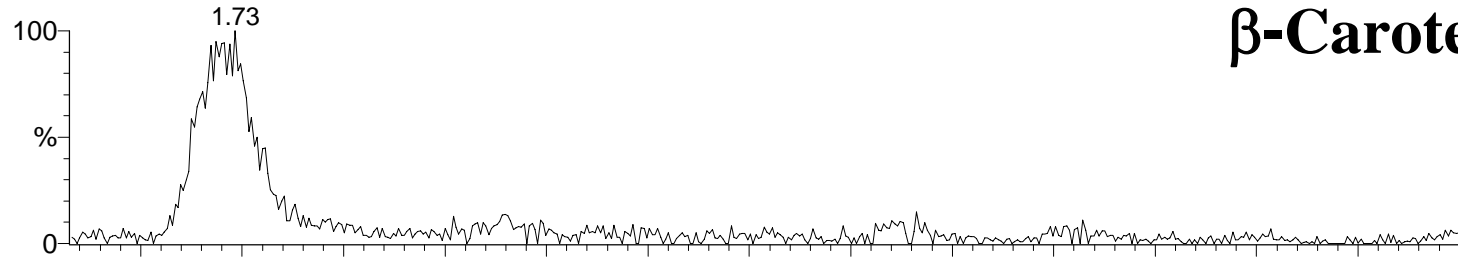
10microliters

720food1



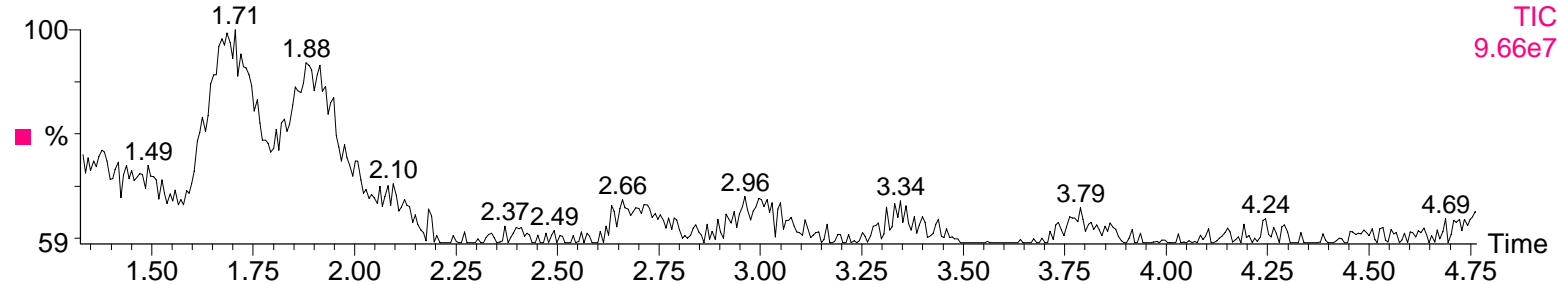
Astaxanthin

720food1



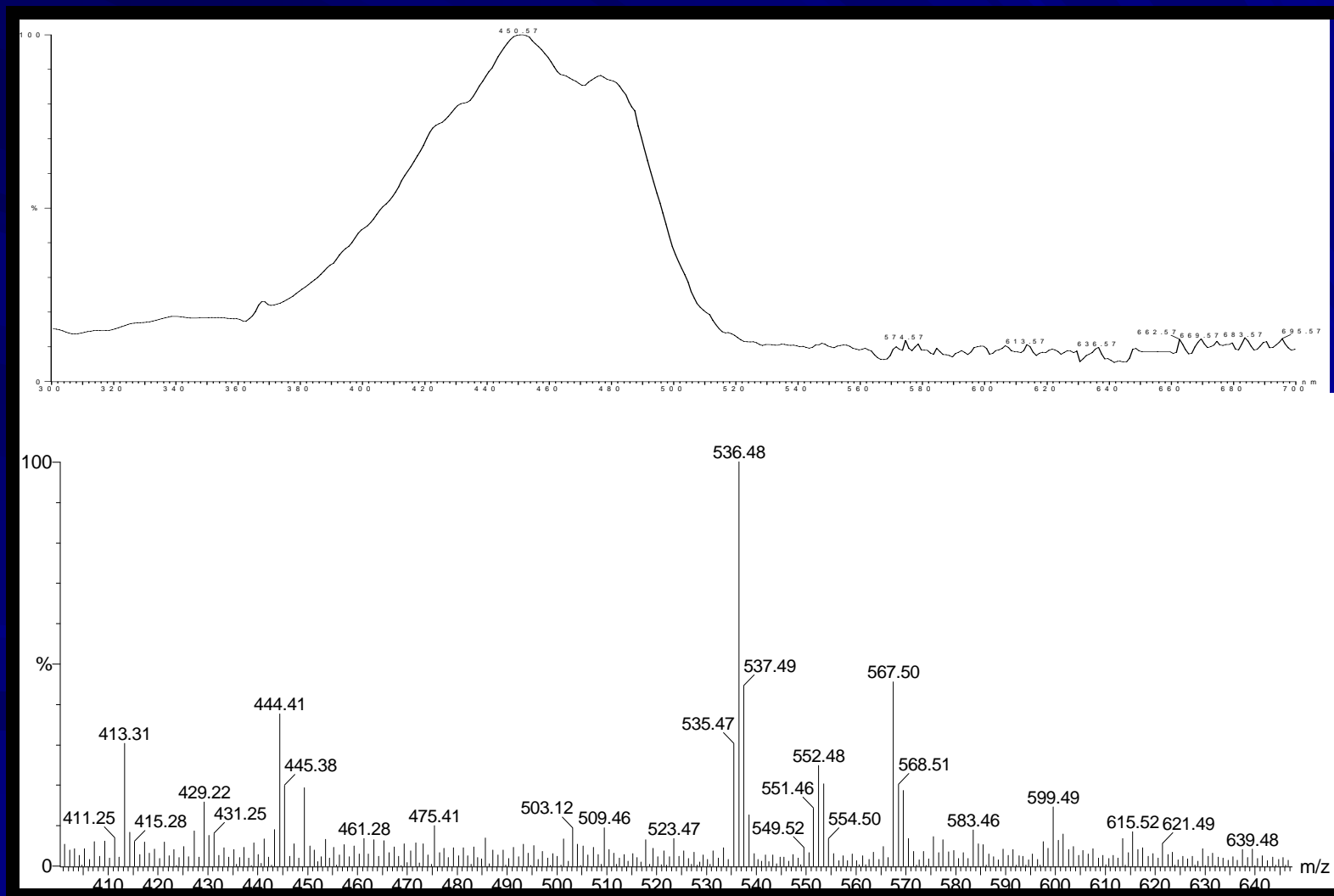
β -Carotene

720food1



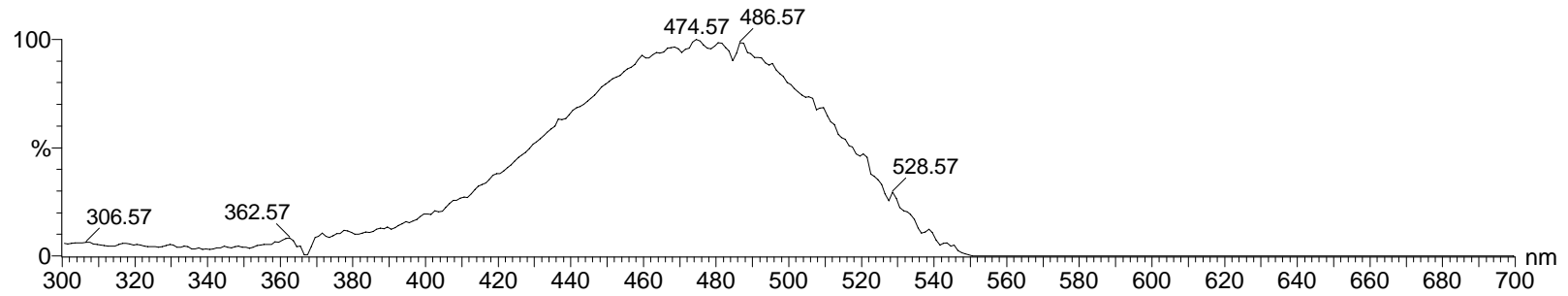
1: Scan ES+
TIC
9.66e7

UV/Vis Mass Spectrum β -Carotene

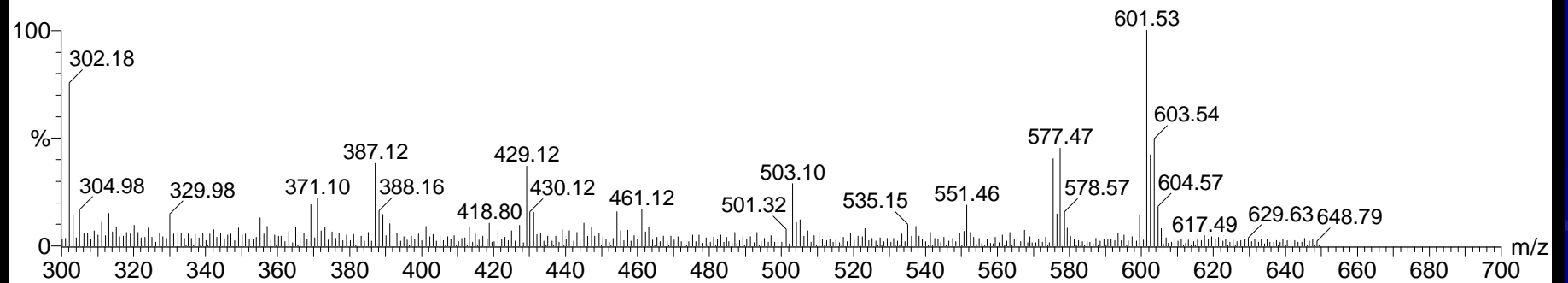


Astaxanthine

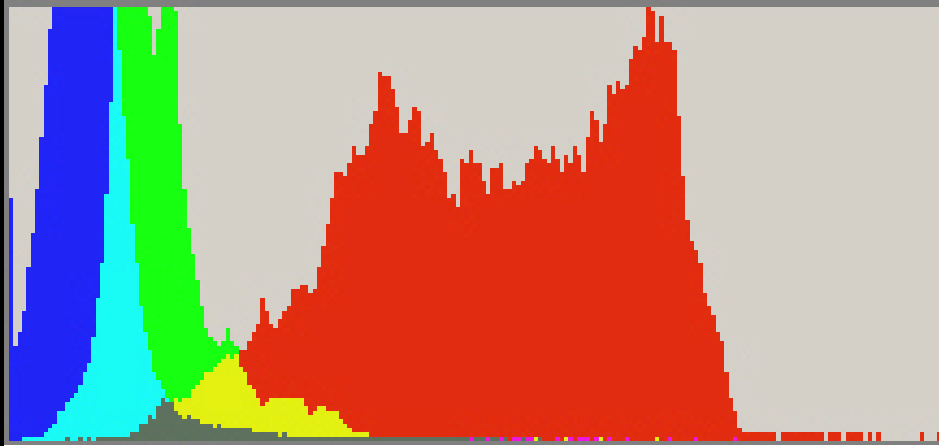
10microliters



10microliters



Cyclop-eeze



Cyclop-eeze showed the best results

In color, length, and weight

No differences in survival

In diet switch trials fish became more colorful.

Funding

- NRAC Grant
- Waters Corporation
- Lead Scientist
Michael Balogh



Rogue's Gallery

