Improved methods for analysis of fats and fatty acids—Guarantees for quality and quantity

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Overview

- Fatty acid composition of feedstuffs
- National Renderer's Assoc. quality standards for fats
- Analysis—Soxhlet vs GLC
- Fatty acid extraction/methylation
- GLC profiles of feedstuffs
- Conclusions

Some definitions

- Total fatty acids
  - All fatty acids in a feedstuff, independent of chemical form
  - “Fatty acid content”
- Unesterified fatty acids
  - Often called “free fatty acids”
- FAME
  - Fatty acid methyl esters, the most common derivative of fatty acids used for GLC
- Fatty acid profile
  - The pattern of fatty acids in a fat, expressed as a percentage of total fatty acids or as mg/g of fatty acids

Lipid composition (% of total oil) of crude soybean oil

Triacylglycerol	95 – 97
Phosphatides	1.5 – 2.5
Unsaponifiable matter	1.6
Sterols	0.33
Tocopherols	0.15 – 0.21
Hydrocarbons	0.014
Unesterified fatty acids	0.3 – 0.7

Pryde, 1980

Content and composition of ether extract from forage leaves

	% of DM	% of EE
Ether Extract	5.3	100
Fatty Acids	2.3	43
Non-Fatty Acid		
Galactose	0.41	8
Glycerol	0.46	9
Chlorophyll	0.23	4
Waxes	0.9	17
Other non-sap	1.0	19

Palmquist and Jenkins, 1980

Ether extract and fatty acids in forages

Forage	Ether Extract (% of DM)	Fatty Acid [1] (% of EE)
Alfalfa	3.1	65.1
Rye grass	4.2	64.4
White clover	2.8	69.6
Corn Silage	2.7	66.2

[1] Determined by one-step extraction/methylation

Ether extract and fatty acids in cereals

Source	Ether Extract (% of DM)	Fatty Acids[1] (% of EE)
Barley	2.1	91.2
Corn	4.1	98.6
Oats	6.3	80.3
Wheat	1.9	95.8
Commercial dairy feed	9.8	67.8

[1] Determined by one-step extraction/methylation

Ether extract and fatty acids in protein supplements

Source	Ether Extract (% of DM)	Fatty Acid[1] (% of EE)
Canola meal	5.6	87.9
Distillers dried grains	11.6	79.2
Fishmeal	9.0	57.8
Meat/bone meal	12.6	60.5
Soybean meal	2.4	131.2

[1] Determined by one-step extraction/methylation

Fatty acid content and composition (% of total FA) of some feedstuffs

Feedstuff	FA,% of DM	16:0	18:0	18:1	18:2	18:3
Barley	1.6	27.6	1.5	20.5	43.3	4.3
Corn	3.2	16.3	2.6	30.9	47.8	2.3
Dehy Alfalfa	1.4	28.5	3.8	6.5	18.4	39.0
Ryegrass	3 - 7	11.9	1.0	2.2	14.6	68.2
Cottonseed	18.6	25.3	2.8	17.1	53.2	0.1

Palmquist,1988

Major fatty acids (% of total FA) of menhaden oil

14:0	16:0	16:1	18:0	18:1n-9	20:5n-3	22:6n-3
10.5	21.5	14.2	3.4	10.3	15.1	6.5

Ackman, 1982

Rendered fats

- Uses
  - Livestock, pet and fish feeds
  - Industrial chemicals
  - Soaps, personal care products
  - Edible tallow, lard
  - Biofuels

National Renderers Assoc.

Quality standards for trading feed fats

- Standards apply to titer, % unesterified fatty acids (FFA), color, and MIU (moisture, impurities and usaponifiable material)
  - Though important for establishing trading standards, **our concern at this point is only the procedure for extraction and preparation of the oil for determining total fatty acid content**

Titer and MIU

- Titer is a measure of the solidification point of a fat after it has been saponified and the soaps reacidulated to free fatty acids. Determined by melting the fatty acids, and while slowly cooling, measuring the congealing temperature in degrees centigrade.
  - < 40C = grease
  - > 40C = tallow
- MIU
  - Impurities include protein, bone, hair, plastic
  - Unsaponifiables
    - Sterols, waxes, pigments, polymerized oxidation products
    - Not absorbable or utilizable for energy by animals
    - Not measured by GLC procedures

National Renderers Assoc.

Moisture and unsaponifiable matter in feed fat raw material

Material	Moisture (mg/g)		Unsaponifiable matter (mg/g)	
	Mean	SD	Mean	SD
Tallow	5.7	5.8	2	8
Crude soybean oil	1.4	0.7	5	2
Palm acid oil	2.8	2.3	15	4
Palm fatty acid dist.	--	--	23	11
Recycled veg. oil	13.6	14.8	7	4
Fish acid oil	11.7	8.8	21	5

Edmunds,1990

Table a: American Fats and Oils Association specifications for Tallows and Greases

Grades	Specifications				
	TITER Min °C	FFA max	FAC max	R&B max	MIU
1) Edible tallow	41.0	0.75	3	none	*
2) Lard (edible)	38.0	0.50	**	none	*
3) Top white tallow	41.0	2	5	0.5	1
4) All beef packer tallow	42.0	2	none	0.5	1
5) Extra fancy tallow	41.0	3	5	none	1
6) Fancy tallow	40.5	4	7	none	1
7) Bleachable fancy tallow	40.5	4	none	1.5	1
8) Prime tallow	40.5	6	13-11E	none	1
9) Special tallow	40.0	10	21	none	1
10) No 2 tallow	40.0	35	none	none	2
11) A tallow	39.0	15	39 2	none	2
12) Choice white grease	36.0	4	13-11E	none	1
13) Yellow grease	***	***	39	none	2

* *moisture maximum 0.20%. Insoluble impurities maximum 0.05%*

** *Lovibond color 5 1/4 inch cell - max 1.5 red. Lard peroxide value 4.0 meq/kg max*

*** *Titer minimum and FFA maximum, when required, to be negotiated between buyer and seller on a contract by contract basis*

www.renderers.org

Chemical data for feed fats

Table d: Chemical Data of Feed Grade Fats: Average values

Fat Source	°C Titer	% MIU*	Max% FFA**	Iodine Value	U/S***	% Fatty Acids		
						Sat.	Unsat.	Linoleic
FGF - for all feeds	29 -45	2 - 4	40	40 - 100	1.0 – 3.0	25-50	50-75	4-40
FGF – for milk replacers	38-41	1	5	47	1.0	50	50	4
All-beef tallow	38-43	1	5	47	1.0	50	50	4
All-pork fat	32-37	2	15	68	1.6	38	62	12
All-poultry fat	28-33	2	15	85	2.6	28	72	20
Acidulated veg soapstock	28-35	4 - 6	70	32	4.1	20	80	2
Palm Oil	28-36	2	5	53	1.4	42	58	10

*MIU =moisture, insolubles and unsaponifiables
**FFA = free fatty acids
***U/S = unsaturate:saturate ratio

FGF, feed grade fats

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The ether extract method

- The product is defined by the method
- The product is nutritionally non-uniform
- Provides minimum information about the quality of the feedstuff
- Has low precision
- Is archaic (dates from 1860)

Crude fat or ether extract-AOAC

- Sample
  - 2 g, dry
  - Pre-extract with water if large amounts of water-soluble materials are present
- Extract in Soxhlet with dry diethyl ether
  - 4 hr @ condensation rate of 5-6 drops/sec, or
  - 16 hr @ 2-3 drops/sec
- Evaporate ether, cool, weigh

AOAC 920.39

Acidified ether extract-AOAC

- Required for extruded feeds and some high calcium feeds to extract insoluble soaps
- Sample
  - 2 g, dried
  - 2 ml EtOH
  - 10 ml 8 N HCl
  - 30 - 40 min @ 70 - 80°C, with shaking
  - Wash with ether, filter
  - Evaporate ether, weigh residue

AOAC 954.02

Fat content by Soxhlet: effects of acid and quantitation method

Method	Hay	Corn silage	Hay/grain mix	Hay/high fat grain
Non-acidified:		(mg/g of dry sample)		
Soxhlet-GLC*	5.50	21.25	7.84	13.75
Soxhlet-gravim.	26.38	28.66	26.39	38.83
Acidified:		(mg/g of dry sample)		
Soxhlet-GLC*	13.20	38.60	17.89	33.87
Soxhlet-gravim.	32.37	53.00	37.56	54.51

*Fatty acid content

Sukhija and Palmquist, 1988

Fat content by Soxhlet: effects of acid and quantitation method, cont'd

Fraction/method	Hay	Corn silage	Hay/grain mix	Hay/high fat grain
Non-Fatty Acid		(mg/g of dry sample)		
Non-Acidified	20.88	7.41	18.55	25.08
Acidified	19.17	14.40	19.69	20.64
Fatty Acid		(% of EE)		
Non-Acidified	20.85	74.15	29.71	35.44
Acidified	40.78	72.83	47.63	62.14

Sukhija and Palmquist, 1988

Repeatability of some analyses for fat quality in fat supplements

	Total Fatty Acids	Unsapon-ifiable	Oxidized F.A.	Moisture
No. repeats	10	10	10	10
Mean (mg/g)	364	19.7	8.6	6.2
SD	2.5	2.4	1.54	6
CV (%)	0.7	12.2	17.9	97

Edmunds, 1990

GLC as an alternative to ether extract

- Simple, rapid, one-step quantitative extraction
- High precision
- Quantity and quality (fatty acid profile) in one analysis
  - Oxidized fatty acids (unavailable) are not analyzed

Issues for development of AAFCO-approved fatty acid analysis for feedstuffs

- Analysis of FAME uses standard AOAC methods
- Need to agree on extraction/methylation procedures
  - Should be simple
  - Must be quantitative

Key references

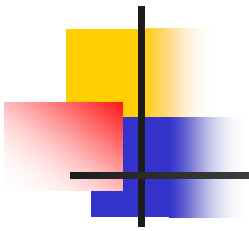
- Outen et al., J. Sci. Food Agric. 27:419-425, 1976
- Sukhija and Palmquist, J. Agric. Food Chem. 36:1202-1206, 1988
- Palmquist and Jenkins, J. Anim. Sci. 81:3250-3254, 2003
- Kramer et al., Lipids 32:1219-1228, 1997
- Carrapiso and Garcia, Lipids 35:1167-1177, 2000
- Hansen, S.L. 2008. Personal communication
- Jenkins, T.C. 2009. Personal communication
- Kraft, Preseault, and Lock. 2009. Personal communication
- **Christie http://www.lipidlibrary.co.uk/analysis.html**

Tissue extraction solvents

- Folch
  - $CHCl_3$:MeOH (2:1)
- Bligh and Dyer
  - $CHCl_3$:MeOH (1:2)
- Radin
  - Hexane:Isopropanol (3:2) -- Low toxicity, low cost, convenient, fewer contaminants
  - Lipid layer is on top
- Supercritical fluid
  - Incomplete extraction

Carrapiso and Garcia, 2000

Tissue extraction procedures—solvents for one step extraction



- Toluene, tetrahydrofuran
  - Effective
- Not recommended
  - Benzene—toxicity issues
  - Chloroform---artefacts
- Methyl-*tert*-butyl ether
  - Good amphiphilic properties
- Toluene + acetone (Kraft et al)
  - Effective, with lower toxicity
  - Acetone forms artefacts with alkaline methylation

Quantifying fatty acids—choosing the internal standard

- Commonly, odd-chain fatty acids
  - Must not occur in the sample
    - if it occurs in the sample, amount will be underestimated
  - Must be separable and identifiable from sample FA
  - Available and economical
- C17:0 and C19:0
  - Commonly-used, C17:0 is found in many fat sources, C19:0 in some
  - C21 and C23 reported in some studies
  - C13:0 and C13:1 becoming commonly used

Esterifying samples

- Acid-catalyzed
  - Esterifies all fatty acids
  - May cause isomerization of conjugated bonds
  - High concentration or high temperature may oxidize unsaturated fatty acids
- Base-catalyzed
  - Transesterifies only -- does not esterify “free” fatty acids
  - Milder than acid – does not cause migration or isomerization of double bonds

Characteristics of the catalyzing medium

	Acidic	Basic
Temperature	High	Ambient
Time	Min.-hours	Sec.-min.
Esterifying power	Medium-high	No
Transester. power	Low	High
Risk of saponification	Low	High
Water interference	Low	High

Carrapiso and Garcia, 2000

Methylation catalysts--Christie

- Acid catalysts
  - $BF_{3,}$ 14% in methanol
    - Fast, use discouraged by Christie—artefacts, etc.
  - Acetyl-Cl, 10% in methanol
    - Slower, best all around catalyst
    - Sulfuric acid, 2% in methanol
      - Equal to acetyl-Cl
- Base catalysts
  - Na methoxide, 0.5M in methanol
    - Fast, preferred
    - Use for milk fat

Choice of esterifying reagent

- Longer chain length of the derivatizing agent improves FID efficiency
  - Methyl group yields low theoretical efficiency
  - Isopropyl esters – Wolff and Fabien (1989)
  - Ethyl, propyl, butyl esters – Ulberth, et al (1999)
  - See Christie, *lipidlibrary*, for more information

Extraction/methylation of feedstuffs—Hansen method

- Pipet internal standard (IS) into round flat-bottom flask
  - Evaporate solvent
- Add weighed sample, boiling chips, HCl-MeOH
  - Attach flask to condensor
  - Reflux 15 min after boiling begins
  - Add NaOH/MeOH
  - Reflux 15 min
  - Add BF_3/MeOH
  - Reflux 5 min

Extraction/methylation of feedstuffs-Hansen method, cont'd

- Add solvent to boiling flask
- Remove and cool
- Add sat. NaCl to the neck of flask
- Cap and shake
- Allow layers to separate, transfer organic layer to autosampler vial containing $NaSO_4$
- Cap, vortex
- Ready for GC

Reaction conditions for one-step extraction/methylation of feedstuffs

- Dry sample containing 10-50 mg fatty acid in a 20mm x 150mm tube with teflon lined cap
- Add organic solvent containing 4 mg internal standard
- Add 3 ml 5% acetyl-Cl in methanol or 2% methanolic sulfuric acid
- Incubate at 70 - 90C for 2 hr, or at 50C overnight (preferred)
- Cool, add 1 ml hexane
- Add 10 ml 6% K_2CO_3, mix, centrifuge
- Transfer solvent layer to a GC vial
- Add a small amount of charcoal + sodium sulfate
- Cap, ready for GLC

Reaction conditions for one-step extraction/methylation of highly unsaturated plant, animal and fish products

- Prepare samples and follow procedures for other feedstuffs
- Incubate at 50C overnight
  - Milder conditions minimize loss of highly unsaturated fatty acids
- Continue as for other feedstuffs

Conditions for one-step methylation of milk fat and milk products

- Set up as for feedstuffs; 10 – 50mg FA
- Add 4 mg internal standard in solvent
- Add 2 ml of 0.5M sodium methoxide in methanol
- Cap tightly and vortex lightly
- Incubate at 50°C for 10 minutes
- Remove and cool for 5 minutes.
- Add 3 ml of 5% methanolic HCl. Recap tightly and vortex.
- Incubate at 80°C for 10 minutes.
- Cool, add K_2CO_3, prepare for GLC analysis

Summary--FA analysis of feedstuffs made simple

- Lipids of most common feedstuffs contain a limited number of fatty acids
  - Cereals, forages, oilseeds, commercial fat supplements
- Feedstuffs with complex lipid profiles
  - Fish and other animal products
  - Dairy products
  - Fermentation products
- Simple and complex sources require different extraction and GC conditions

Summary--FA analysis of feedstuffs made simple, cont'd

- Common feedstuffs
  - Extract and methylate with one-step acid-catalyzed reaction
  - Analyze on a 30 meter polar capillary column
- Dairy products
  - Extract and methylate with a single tube acid/alkaline catalyzed reaction
  - Analyze on a 100 meter polar capillary column
- Highly unsaturated fatty acid products
  - As for other feedstuffs, incubated at lower temperature (50C overnight), analysis on 100 meter polar column

Methylation artefacts

- Methyl levulinate
  - Levulinic acid is produced during high temperature acid hydrolysis of samples containing sugar (such as pelleted or steam-treated feeds)
    - Becomes methylated
  - Elutes on most GLC chromatograms near methyl 13:0
- BHT
  - This commonly-used antioxidant elutes with methyl 14:0 on most polar GLC chromatograms

Recommended Method Performance Characteristics for GLC analysis of feed lipids

	Target Concentration	Accuracy, %		Repeatability, % (CV_r)		Reproducibility, % (CV_R)	
Fatty Acids	Operational Range (%)[1]	at 2x LOQ	at midrange	at 2x LOQ	at midrange	at 2x LOQ	at midrange
Palmitic Acid 16:0	0.01 – 50	85 - 110	95 - 102	< 8	< 3	< 16	< 6
*Eicosapentaenoic Acid 20:5n-3 (EPA)	0.01 – 15	85 – 110	95 – 102	< 8	< 3	< 16	< 6
Vaccenic Acid 18:1*trans*-11	0.01 – 10	85 – 110	92 – 105	< 8	< 3	< 16	< 6

* Analyzed only in samples of animal origin

[1] Note that operational range values are listed as % of total fatty acids.

Performance limits prepared by Aaron Price, Special Project Chemist, Canadian Food Inspection Agency, Ottawa, Ontario, Canada

Reportable fatty acids in feedstuffs

*4:0 butyric
*6:0 caproic
8:0 caprylic
10:0 capric
12:0 lauric
14:0 myristic
14:1 n-5 myristoleic
15:0 pentadecanoic
16:0 palmitic
16:1 palmitoleic
17:0 heptadecanoic
18:0 stearic
18:1 *cis*-9 oleic
18:1 *trans*-9 elaidic
18:1 *trans*-11 vaccenic
18:2 n-6 linoleic
*18:2 *cis*-9, *trans*-11 rumenic (CLA)
18:3 n-6 *gamma*-linolenic acid (GLA)
18:3 n-3 linolenic
18:4 n-3 stearidonic
20:0 eicosanoic

* Animal products only

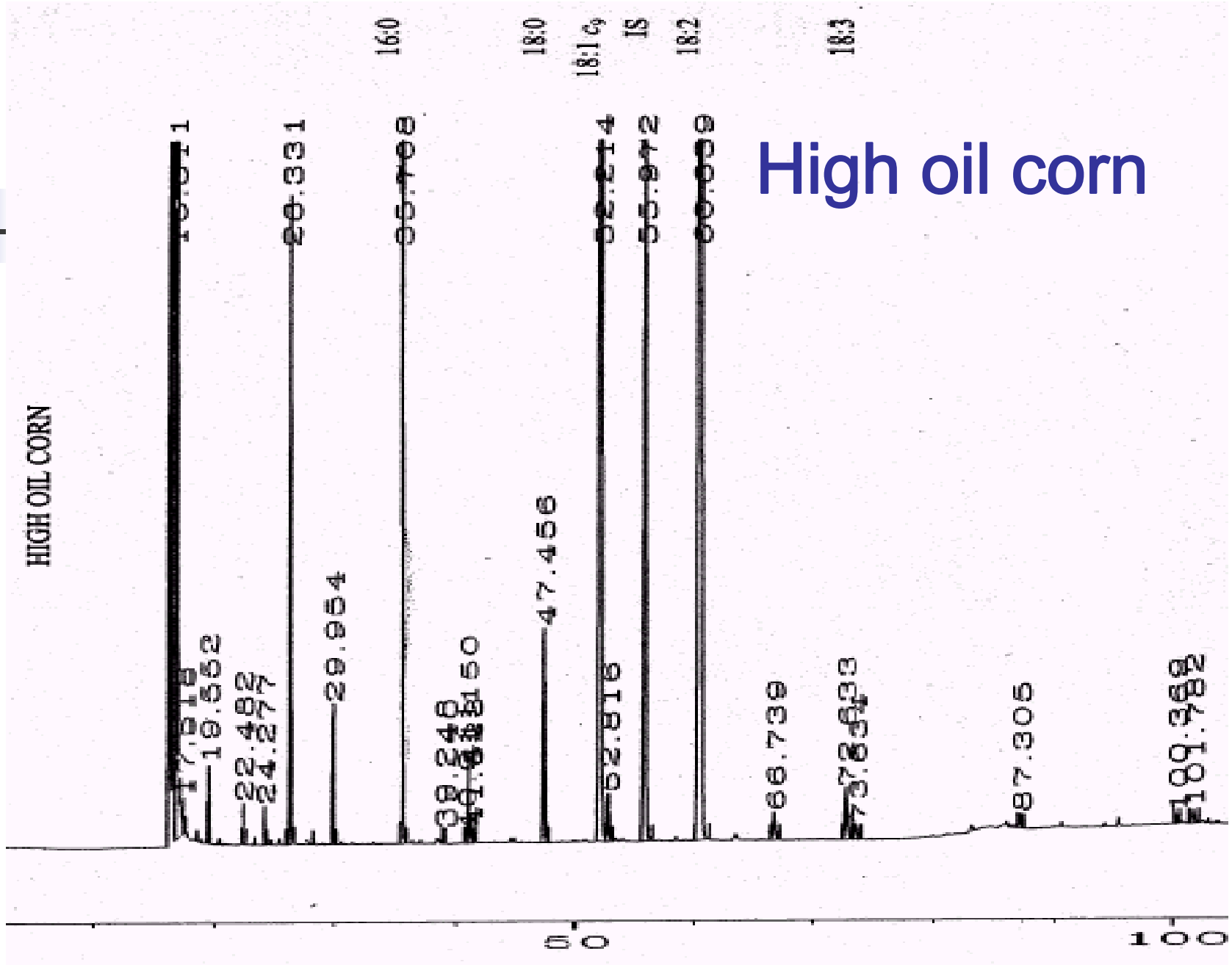
Reportable fatty acids in feedstuffs, cont'd

20:3 n-6 dihomo*gamma* linolenic acid (DGLA
*20:4 n-6 arachidonic (AA)
*20:5 n-3 eicosapentaenoic acid (EPA)
22:0 behenic
22:1 n-9 erucic
*22:5 n-3 docosapentaenoic acid (DPA)
*22:6 n-3 docosahexaenoic acid (DHA)

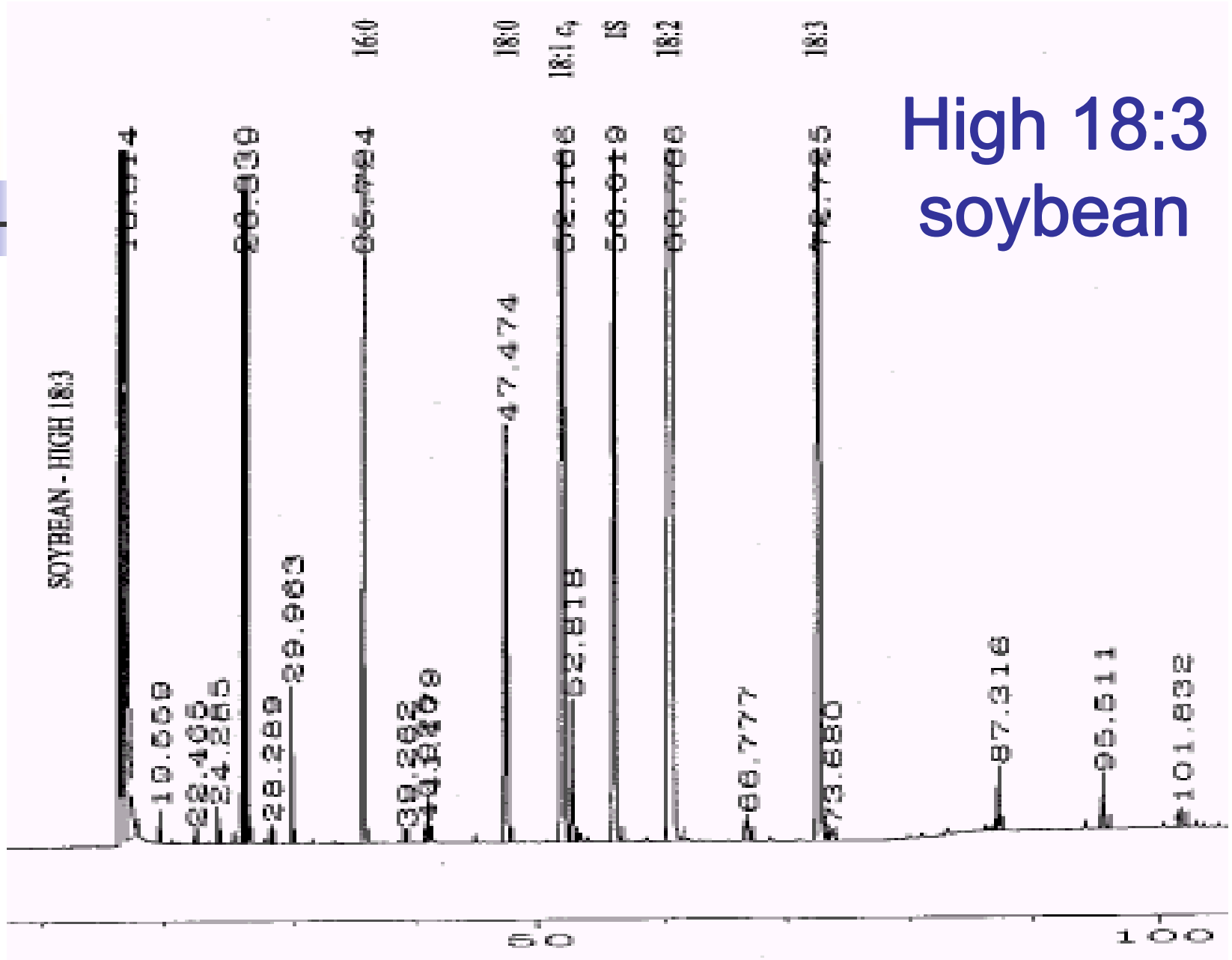
* Animal products only

GLC fatty acid profiles from different feedstuffs/ingredients

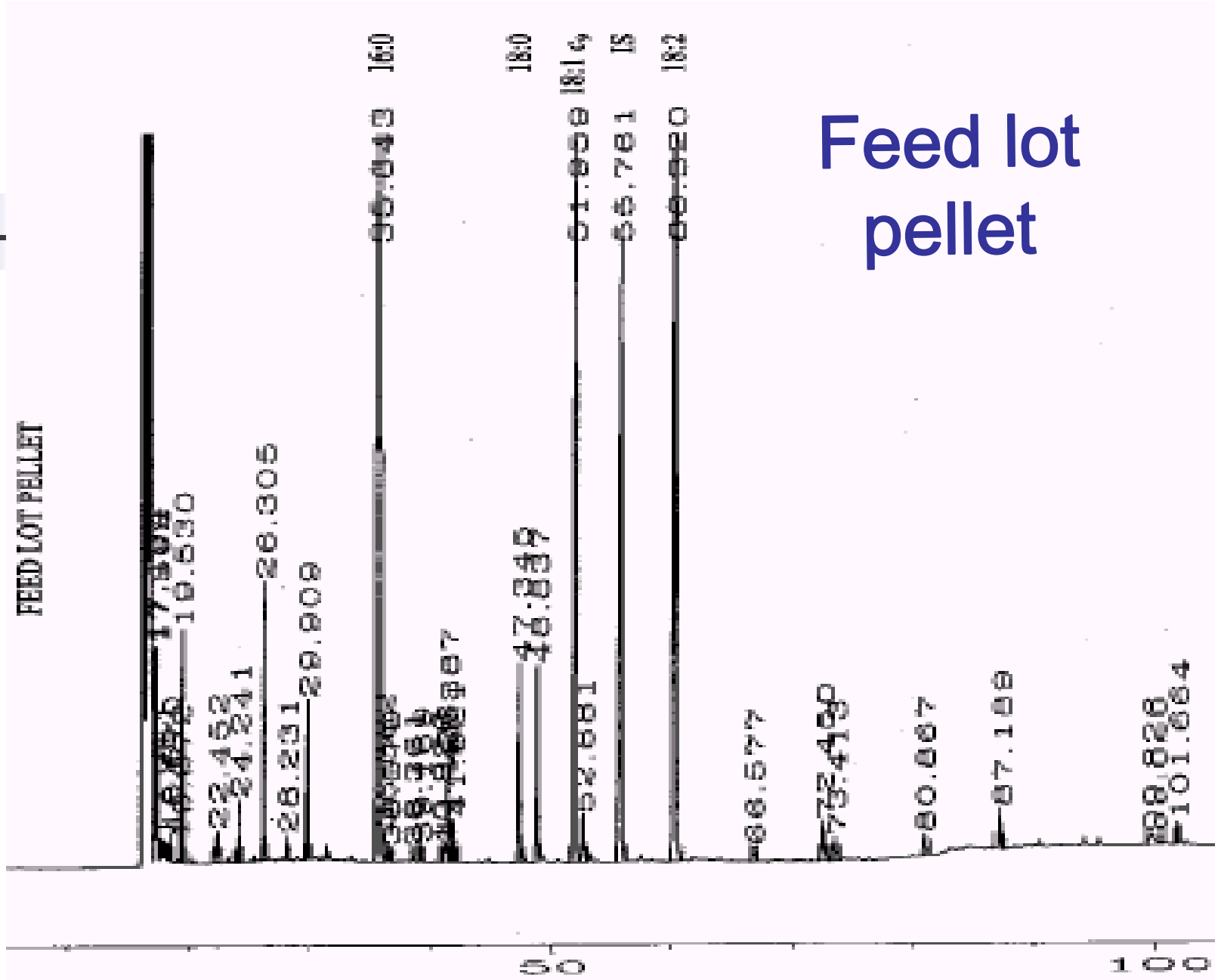
High oil corn



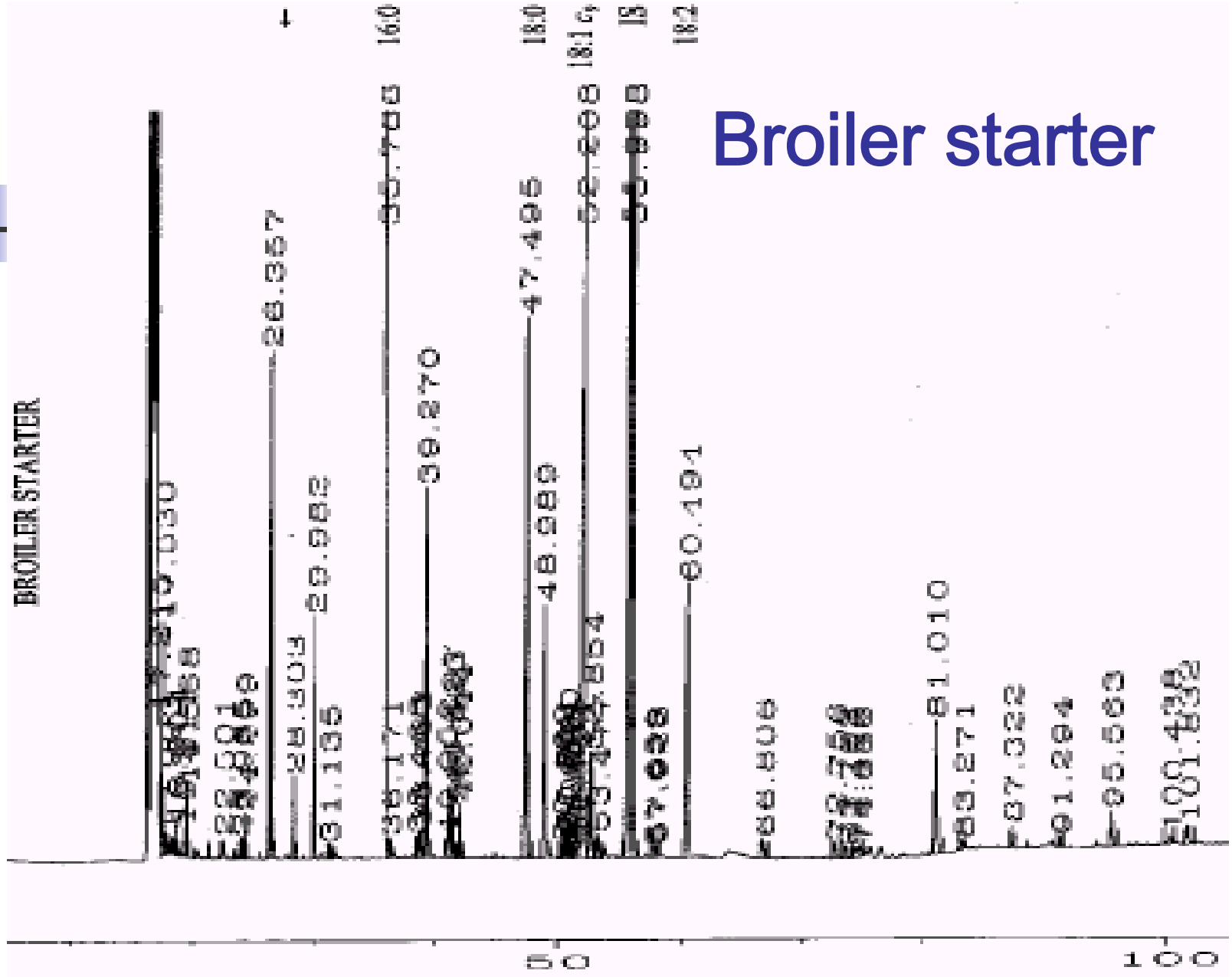
High 18:3 soybean

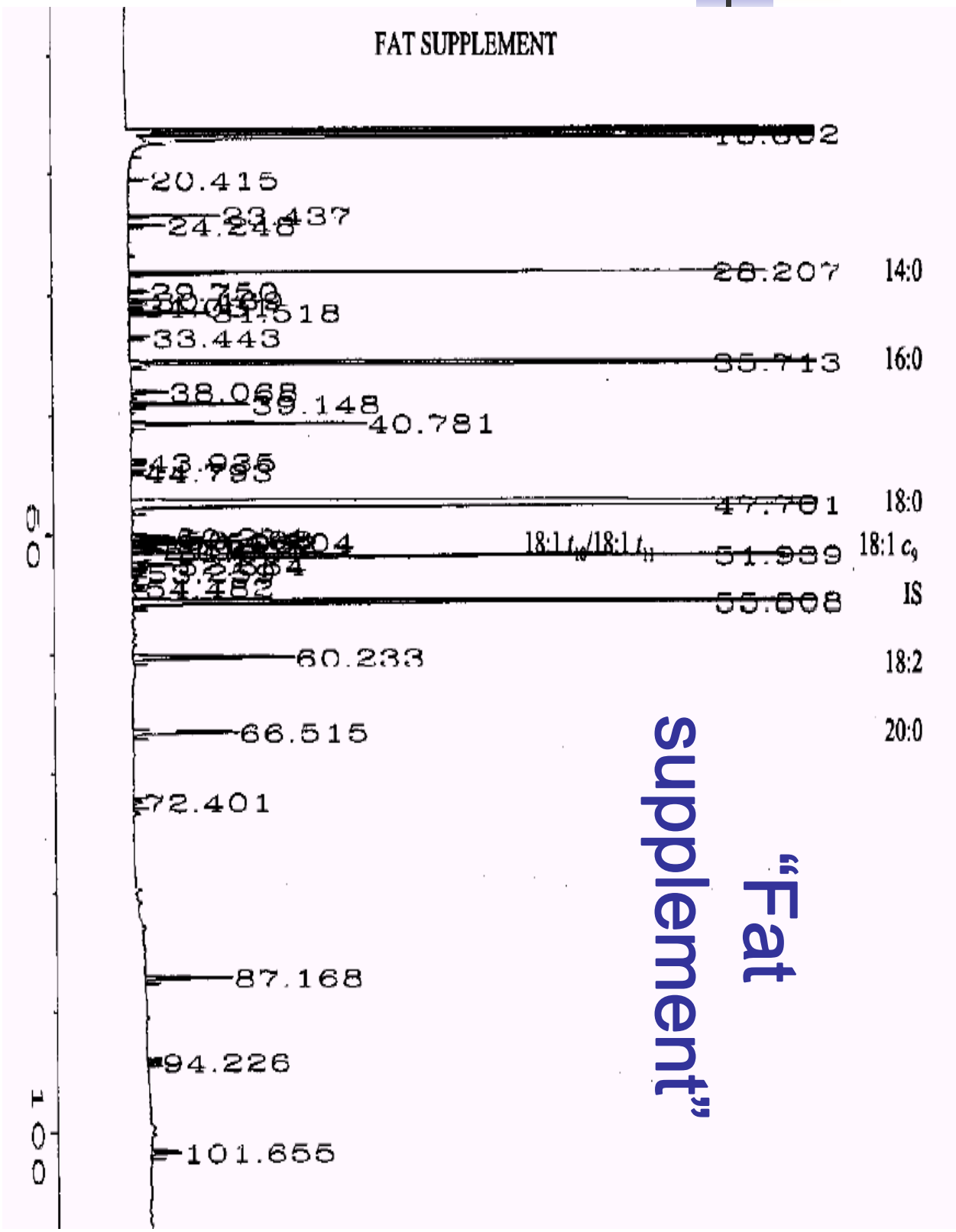


Feed lot pellet



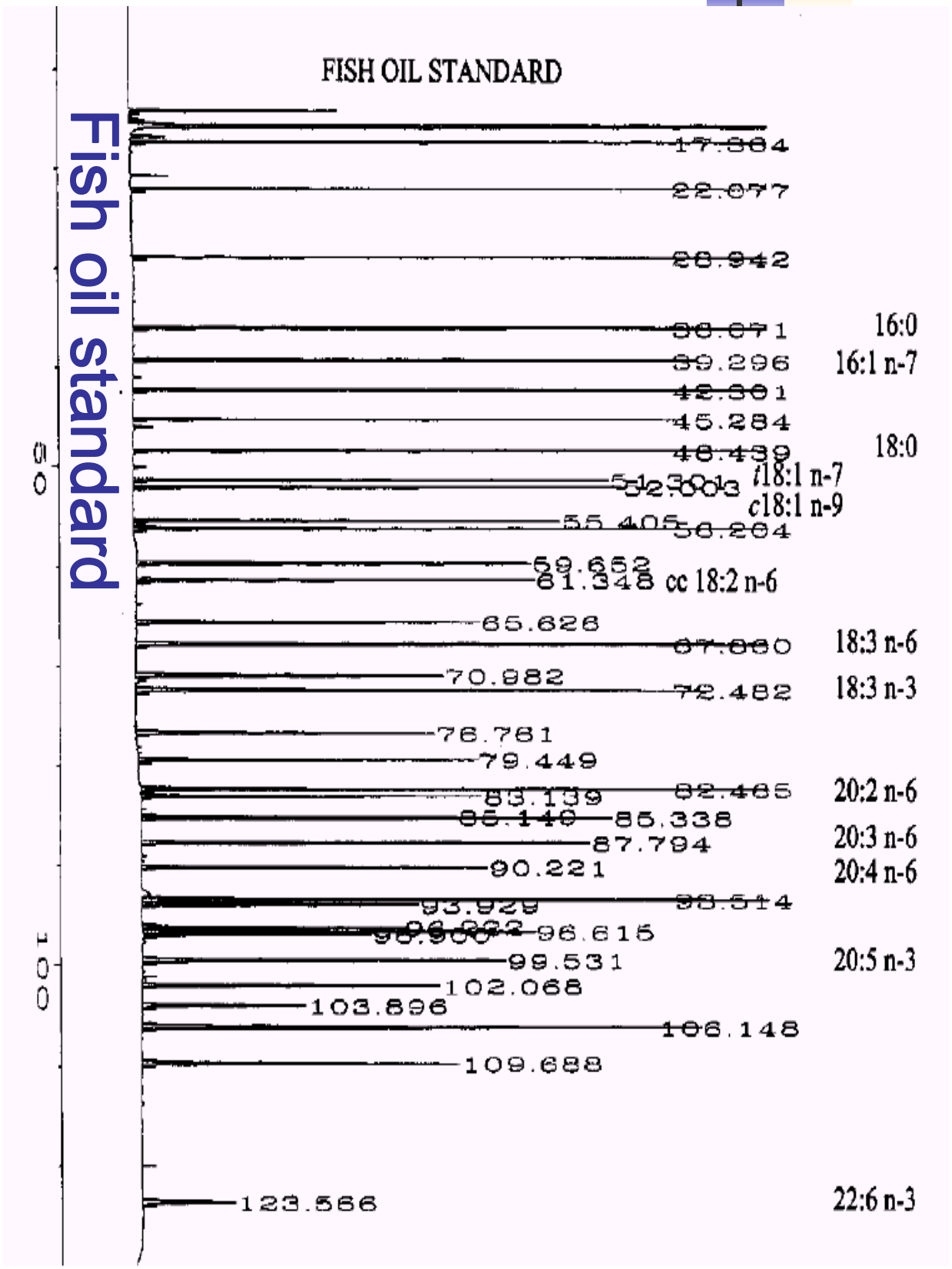
Broiler starter



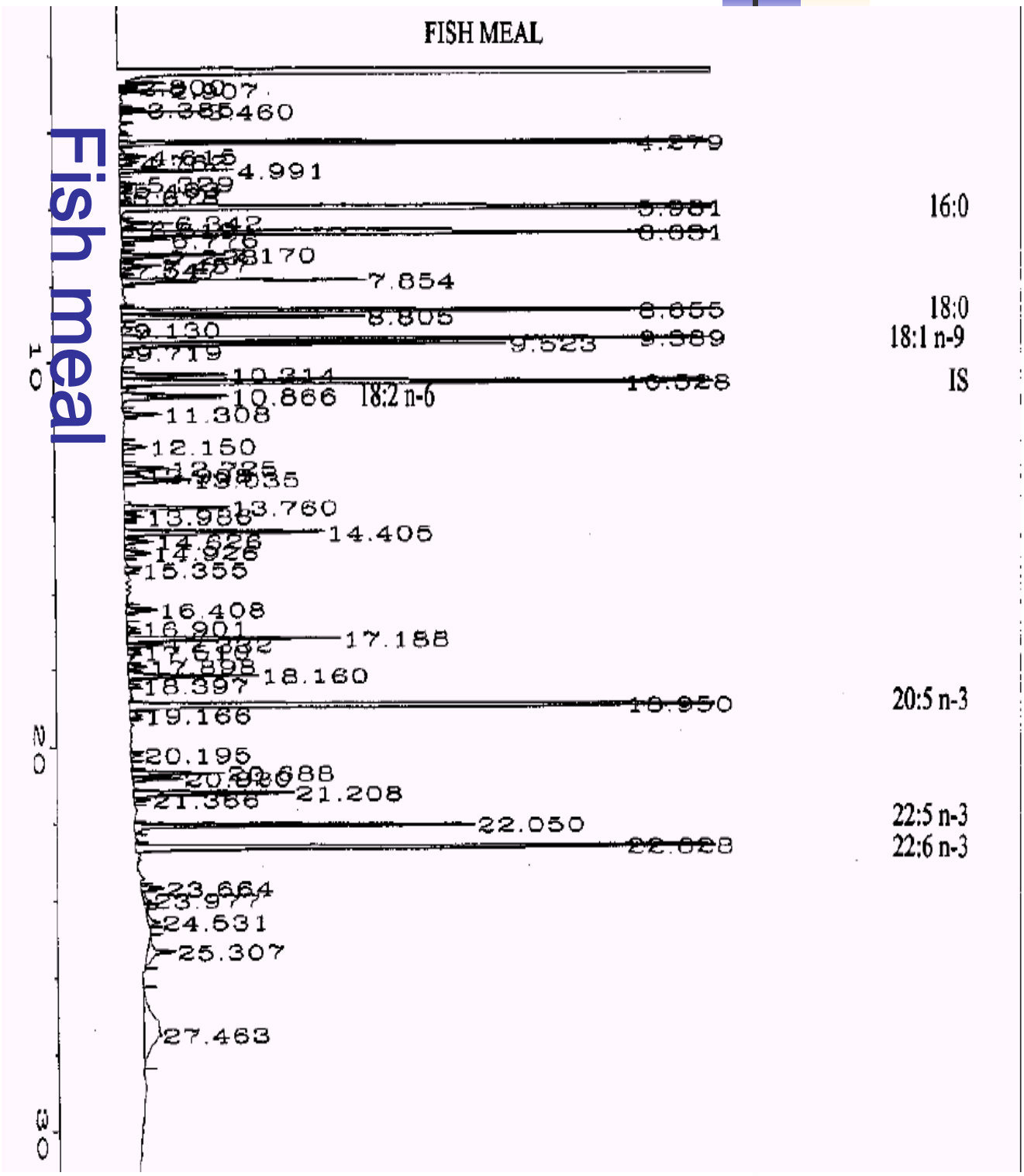


"Fat supplement"

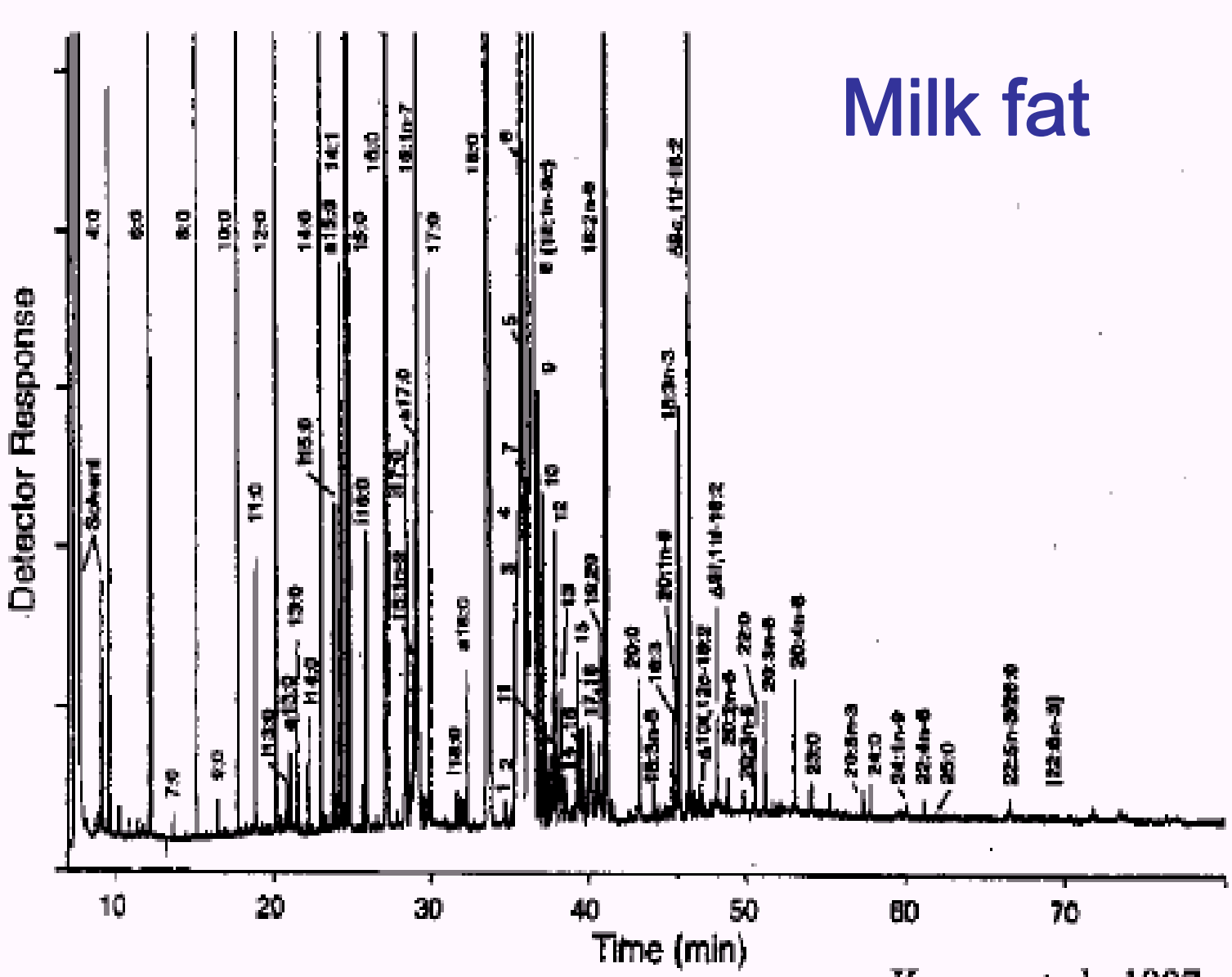
Fish oil standard



Fish meal

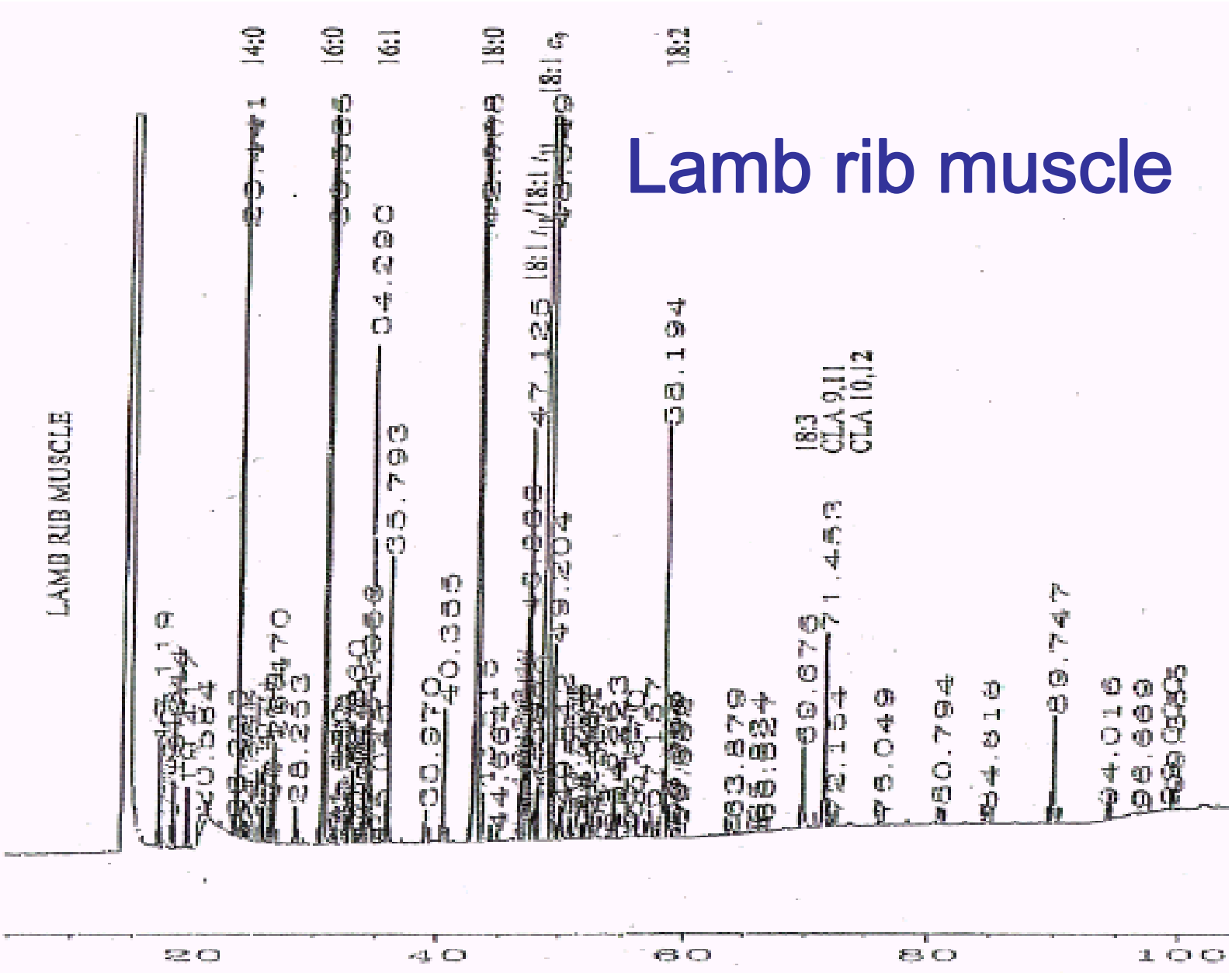

FISH MEAL
4.991
7.854
8.805
9.523
10.866
18:2 n-6
11.308
12.150
13.760
14.405
15.355
16.408
17.188
18.160
19.166
20.195
21.208
22.050
24.631
25.307
27.463
16:0
18:0
18:1 n-9
IS
20:5 n-3
22:5 n-3
22:6 n-3

Milk fat

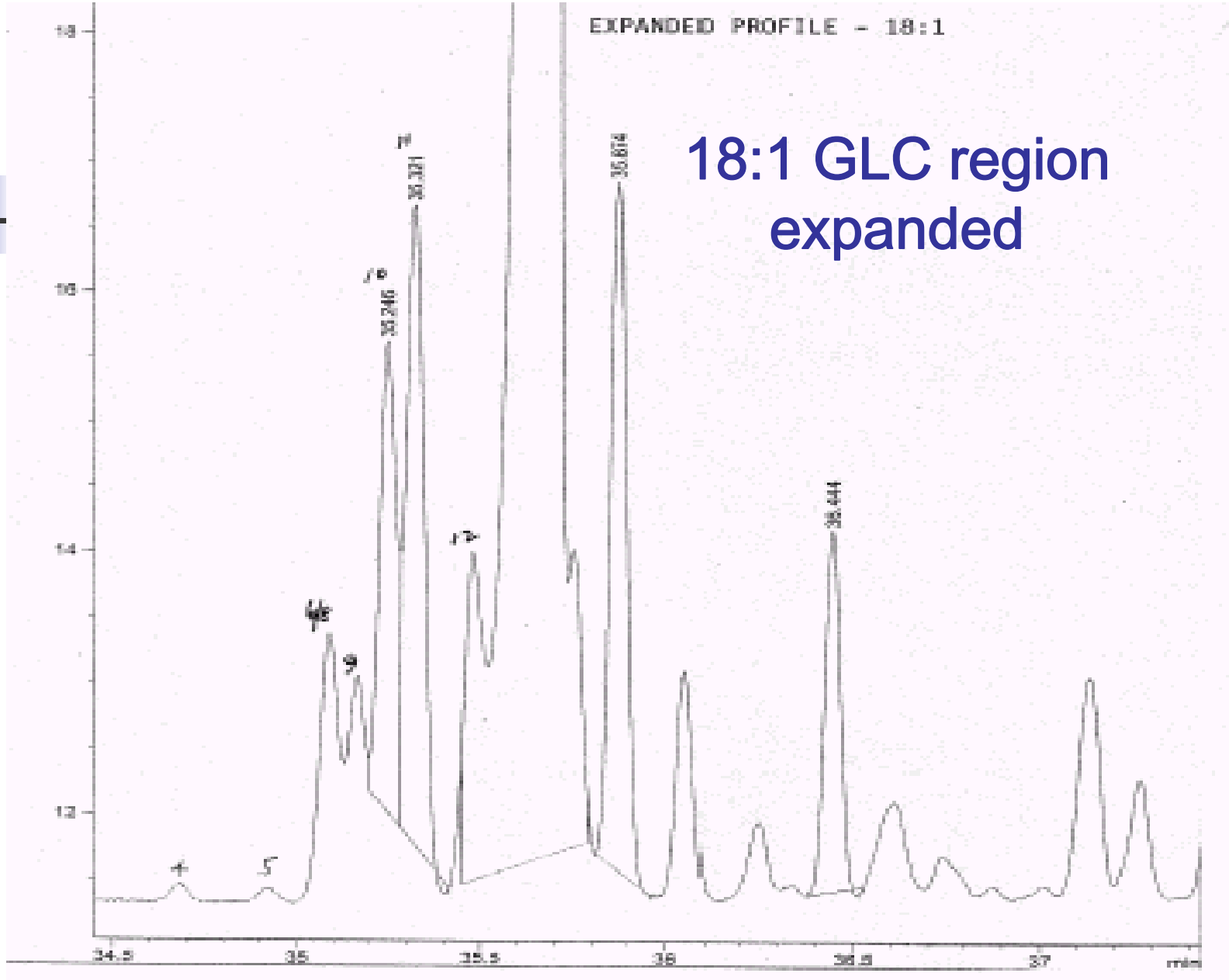


Kramer et al., 1997

Lamb rib muscle



18:1 GLC region expanded



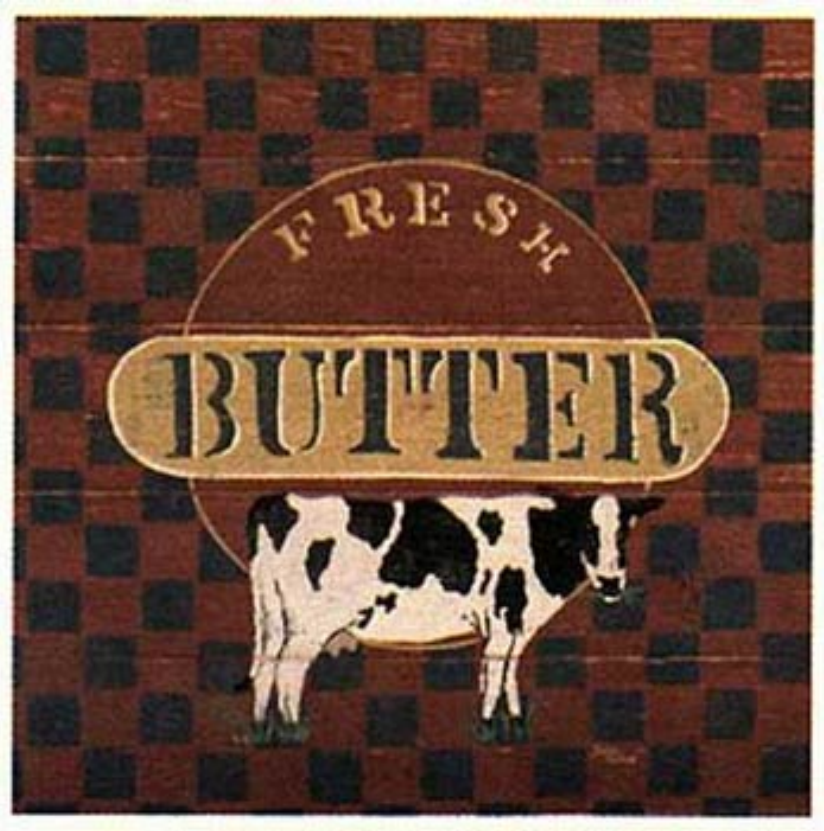
Conclusions

- The classical ether extraction procedure continues to be the method used by industry. It is inadequate because:
  - The object (EE) is defined by the method, not as a chemically-identifiable fraction
  - Incompletely extracts lipid
  - Extracts non-nutritive ether soluble material
  - Procedures as practiced are not standardized
  - Provides minimum information about the sample

Conclusions, cont'd

- Analysis of total fatty acids is recommended:
  - Fatty acids are completely extracted and analyzed
  - The procedure is rapid and specific
  - The result is an unambiguous nutritionally-uniform fraction

Thank you !!!



Questions ???