Extraction, Derivatization, and Analysis of Fatty Acid Methyl Ester (FAME) in Tissue Homogenates and Blubber by ASE and Gas Chromatography

Short Version

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Method description for FAME Analysis

Application and Methodology

This method is based on the microscale recovery procedure for total lipids by accelerated solvent extraction (ASE) developed by Eric D. Dodds and the ASET Lab and is published in Dodds et al. (2004, 2005a, 2005b) and by Beck et al. (2007). It provides a means for the determination of 70 fatty acids (FA) in tissues and lipids (Figure 1, Table). This is accomplished by a solvent extraction driven by high temperature and pressure in an inert atmosphere of nitrogen. The analytes are derivatized into free fatty acids (FA) by a base catalyzed reaction and esterified to form fatty acid methyl esters (FAMEs) in an acid catalyzed reaction using the Lewis Acid boron trifloride in methanol. The analytes are injected on a gas chromatography (GC) system for separation, and are subsequently detected by a flame ionization detector (FID). Analyte identity is verified by an additional injection on a GC where the analytes are separated and identified by a mass spectrometric detector (MSD). The used calibration standards are listed in Table I (See appendix).

Preparation of solutions:

*Individual Stock Standards***:** Individual stock standard solution is prepared for each compound in a concentration of 10.0 mg/mL in hexane.
*Internal Standard Solution (IS)***:** A stock internal standard solution is prepared to a concentration of 1 mg/mL from 10.0 mg of henicosanoic acid methyl ester (C21:0).
Surrogate Standard Solution (SS): A stock surrogate standard solution is prepared to a concentration of 1 mg/mL from 10.0 mg of trinonadecanoin (Tri C19:0).
*Stock Calibration Standard Mix***:** The stock calibration standard mix is prepared in two separate groups to minimize co-elution and prevent overloading the column. The calibration groups are from stock standard solutions and diluted to a final volume of 5.0 mL. Table II, and Table III (See appendix) give the concentrations of the calibration solution, surrogate and internal standard.

Extraction procedure and transesterification

Samples are stored at or below – 60 ºC until ready for extraction to minimize oxidation and degradation of samples. Samples are thawed at room temperature and weighted out on an analytical balance to 0.01 mg then mixed with ~1g hydromatrix (Varian, Walnut Creek, CA) and added to the assembled 11 ml ASE cell. The samples are extracted with 65% chloroform and 35% methanol at 2000 psi pressure and 100 ºC for 10 minutes. Approximately 40ml of a 0.7% NaCl saline solution is added to the crude extract to remove methanol and water soluble components of the extract. The aqueous layer is siphoned off and the organic phase is dried by pouring it through a sintered glass funnel containing several grams of anhydrous sodium sulfate, followed by thorough rinsing of the funnel with chloroform. The pooled dried extract is concentrated to approximately 1 mL under a stream of nitrogen using a TurboVap apparatus (Zymark, Hopkinton, MA) set at a bath temperature of 35°C and remaining solvent is evaporated by impinging with nitrogen from the extraction concentrate. Two milligrams of the recovered lipids are reconstituted in 1 mL of 0.5 M methanolic KOH and hydrolyzed at 80°C for 1 hour and 1 mL of fresh 10% BF3 in methanol is added. Transesterification is performed at 100°C for 20 min. After transesterification, 2 mL DI H_2O and 1 mL hexane are added to the sample to quench the reaction. The recovered organic phase is pooled and spiked with the methyl ester of 21:0 to a final concentration of 25.0 µg/mL.

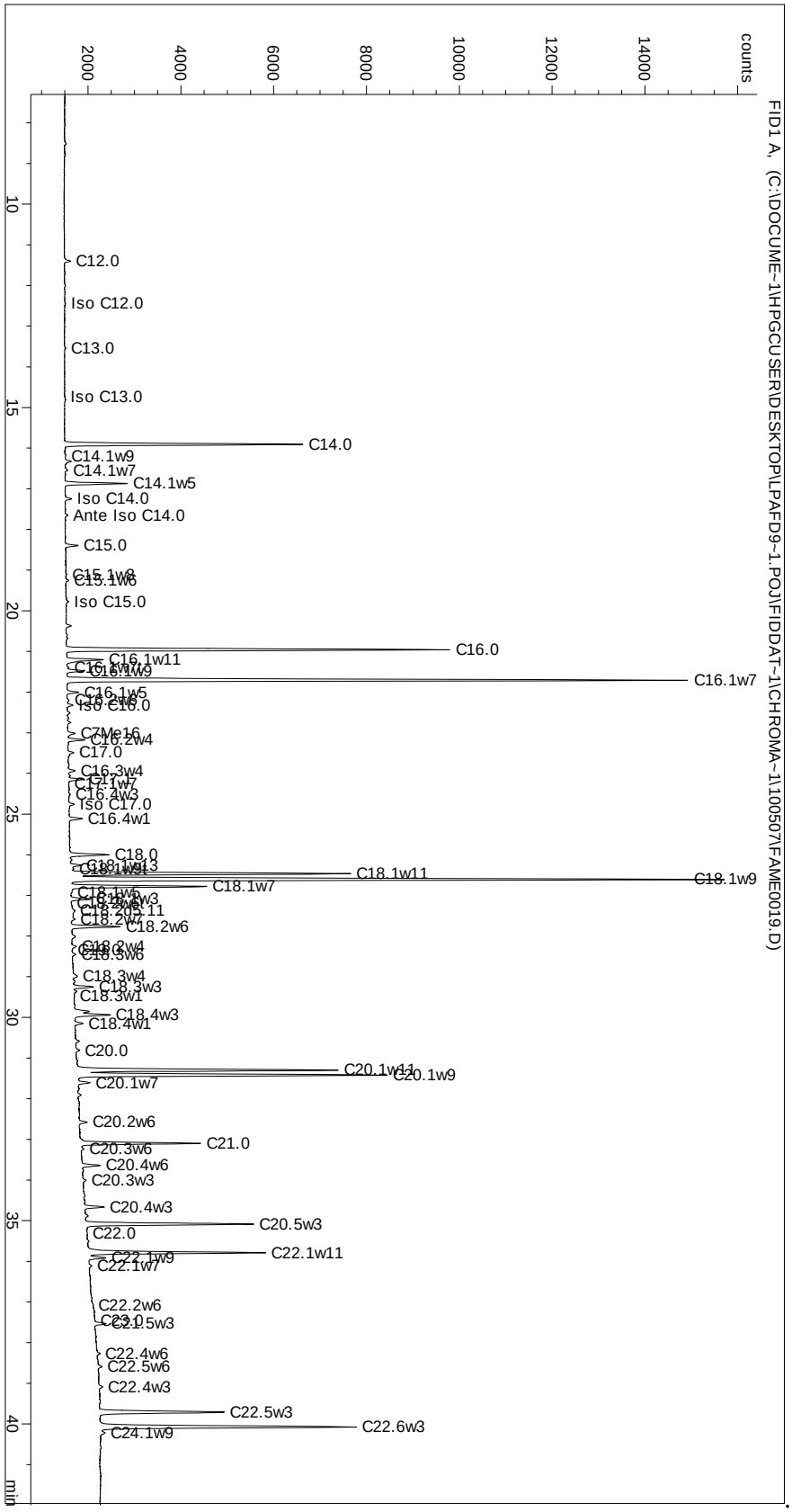

Figure I. CC-FID Chromatogram of Harbor Seal Blubber

Sample Analysis

Surrogate Standard (SS): Prior to extraction 50µL of SS, 1mg/ml trinonadecanoin in hexane, is added to each unknown sample and blank solution. For each sample run, the surrogate must be recovered with a tolerance of no greater than ± 30.0% of the actual value. Should any surrogate recoveries fail, the sample must be reanalyzed. SS is not used for tissue analysis due to its high amount of lipids.

GC-FID analysis: The samples are analyzed on an GC FID (HP 5890 Series II Plus system, Hewlett-Packard, Palo Alto, CA) using 60m DB-23 column with I.D. 0.25mm and 0.25µm film thickness. Helium is used as carrier gas with 1 mL/min constant flow compensation, split ratio is 20:1, injection temperature of 300ºC and temperature range from 125 to 240 ºC at 3 º/min. Total runtime is 40 min, with acquisition from 8 to 40 min.

GC MS analysis: 10% of samples are measured with GC-MS (Varian CP-3800 GC equipped with a Varian Saturn 2200 MS) to confirm peaks. The same measurement conditions as for GC-FID are used. The mass range is 50-400 m/z, scan time 0.5 sec/scan and emission current of 10uA, Acquisition is from 8 to 40 min. Compound retention times are listed in Table IV (See Appendix).

Quality Control

Laboratory Method Blank (MB): At least one MB sample is extracted and analyzed with every 23 samples or sample set (all samples extracted within a 24-hour period), which ever is greater. The MB is to be extracted and analyzed as an unknown sample. The MB value of each analyte must be less than the calculated limit of detection. Samples with analyte concentrations with greater than 10X the detected level in the MB may be reported.

Continuing Calibration Verification (CCV): For each group of no more than twenty samples analyzed, a second source continuing calibration verification (CCV) is analyzed (consisting of 0.5mg/ml Supelco37 FAME Standard in hexane as shown in Table I, fortified to a concentration of 25mg/ml Henicosanoic acid methyl ester). In order to validate each bracketed group of analysis results, each CCV analyte must be determined with a tolerance of no greater than ± 20.0% of actual value. Should any CCV fail, the bracketed sample must be reanalyzed. In the event of multiple QC failures, instrument recalibration is indicated.

Determination of Limit of Detection (LOD): Prior to the analysis of unknown samples, the LOD must be determined for each analyte. The LOD is calculated from the calibration curves using the equation:

$$LOD = \frac{3.3 \times S_{xy}}{m}$$

where (S_{xy}) is the standard deviation of the x-intercept and (m) is the slope. All analytes detected below the LOD are reported as <LOD. Typical LOD for FAME analysis in ASET laboratory are listed in Table V (See appendix).

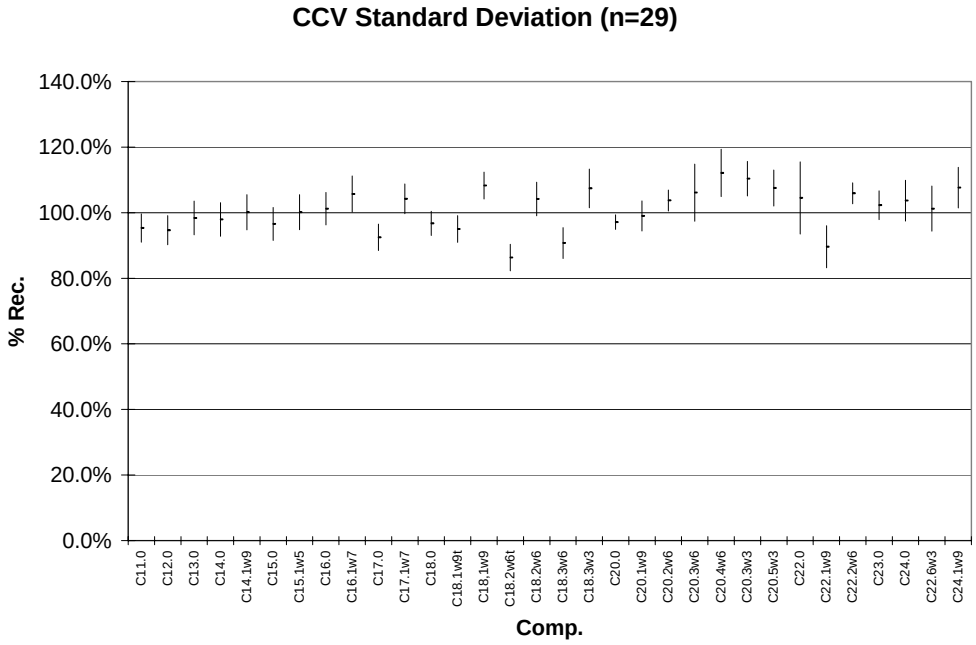

Figure II. Standard deviations of CCV compounds (Supelco37 FAME Standard) collected over six months (n=29)

References

Beck, C.A., Rea, L.D., Iverson, S.J., Kennish, J.M., Pitcher, K.W., and Fadely, B.S. (2007): Blubber fatty acid profiles reveal regional, seasonal, age-class and sex differences in the diet of young Steller sea lions in Alaska. *Marine Ecol. Prog. Ser.*, 338, 269-280.

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Appendix

Table I. FAME Calibration Standards.

Nu-Chek	Designator	Scientific Name	Common Name
N-12-M	C12:0	Lauric acid methyl ester	Methyl Laurate
N-13-M	C13:0	Tridecanoic acid methyl ester	Methyl Tridecanoate
N-14-M	C14:0	Tetradecanoic acid methyl ester	Methyl Myristate
U-36-M	C14:1w5	cis-9-Tetradecenoic acid methyl ester	Methyl Myristoleate
N-15-M	C15:0	Pentadecanoic acid methyl ester	Methyl Pentadecanoate
N-16-M	C16:0	Hexadecanoic acid methyl ester	Methyl Palmitate
U-41-M	C16:1w7t	trans-9-Hexadecenoic acid methyl ester	Methyl Palmitelaidate
U-40-M	C16:1w7	cis-9-Hexadecenoic acid methyl ester	Methyl Palmitoleate
N-17-M	C17:0	Heptadecanoic acid methyl ester	Methyl Heptadecanoate
U-42-M	C17:1w7	cis-10-Hexadecenoic acid methyl ester	Methyl 10-Heptadecenoate
N-18-M	C18:0	Octadecanoic acid methyl ester	Methyl Stearate
U-47-M	C18:1w9t	trans-9-Octadecenoic acid methyl ester	Methyl Elaidate
U-46-M	C18:1w9	cis-9-Octadecenoic acid methyl ester	Methyl Oleate
U-48-M	C18:1w7	cis-11-Octadecenoic acid methyl ester	Methyl Vaccenate
U-60-M	C18:2w6t	trans-9,12-Octadecadienoic acid methyl ester	Methyl Linolelaidate
U-59-M	C18:2w6	cis-9,12-Octadecadienoic acid methyl ester	Methyl Linoleate
U-63-M	C18:3w6	cis-6,9,12-Octadecatrienoic acid methyl ester	Methyl Gamma Linolenate
U-62-M	C18:3w3	cis-9,12,15-Octadecatrienoic acid methyl ester	Methyl Linolenate
N-19-M	C19:0	Nonadecanoic acid methyl ester	Methyl Nonadecanoate
N-20-M	C20:0	Arachidic acid methyl ester	Methyl Arachidate
U-65-M	C20:1w12	cis-12-Eicosenoic acid methyl ester	Methyl 8-Eicosenoate
U-66-M	C20:1w9	cis-11-Eicosenoic acid methyl ester	Methyl 11-Eicosenoate
U-68-M	C20:2w6	cis-11,14-Eicosadienoic acid methyl ester	Methyl 11, 14 Eicosadienoate
U-70-M	C20:3w3	cis-11,14,17-Eicosatrienoic acid methyl ester	Methyl 11, 14, 17-Eicosatrenoate
N-21-M	C21:0	Henicosanoic acid methyl ester	Methyl Heneicosanoate
U-69-M	C20:3w6	cis-8, 11, 14-Eicosatrienoic acid Methyl ester	Methyl Homogamma Linolenate
U-71-M	C20:4w6	cis-5,8,11,14-Eicosatetraenoic acid methyl ester	MethylArachidonate
U-99-M	C20:5w3	cis-5,8,11,14,17-Eicosapentaenoic acid methyl ester	Methyl Eicosapentaenoate
N-22-M	C22:0	Docosanoic acid methyl ester	Methyl Behenate
U-79-M	C22:1w9	cis-13-Docosenoic acid methyl ester	MethylErucate
U-81-M	C22:2w6	cis-13,16-Docosadienoic acid methyl ester	Methyl Docosadienoate
N-23-M	C23:0	Tricosanoic acid methyl ester	Methyl Tricosanoate
U-83-M	C22:4w6	cis-7,10,13,16-Docosatetraenoic acid methyl ester	Methyl Docosatetraenoate
N-24-M	C24:0	Tetracosanoic acid methyl ester	Methyl Lignocerate
U-101-M	C22:5w3	cis-7,10,13,16,19-Docosapentaenoic acid methyl ester	Methyl Docosapentaenoate
U-88-M	C24:1w9	cis-15-Tetracosenoic acid methyl ester	Methyl Nervonate
U-84-M	C22:6w3	cis-4,7,10,13,16,19-Docosahexaenoic acid methyl ester	Methyl Docosahexaenoate

Table II. Concentration of analyte, Surrogate, and Internal Standard in Group 1.

Compound Type	Compound	Concentration (µg/mL) at Level:					
		1	2	3	4	5	6
Analyte	C14.1w5	0.25	0.50	2.50	5.00	12.50	25.00
	C16.1w7	1.00	2.00	10.00	20.00	50.00	100.00
	C17.1w7	0.25	0.50	2.50	5.00	12.50	25.00
	C18.1w9	3.00	6.00	30.00	60.00	150.00	300.00
	C18.1w7	1.50	3.00	15.00	30.00	75.00	150.00
	C18.2w6	0.50	1.00	5.00	10.00	25.00	50.00
	C20.1w11	0.25	0.50	2.50	5.00	12.50	25.00
	C20.2w6	0.25	0.50	2.50	5.00	12.50	25.00
	C20.3w6	0.25	0.50	2.50	5.00	12.50	25.00
	C20.3w3	0.25	0.50	2.50	5.00	12.50	25.00
	C20.5w3	1.50	3.00	15.00	30.00	75.00	150.00
	C22.2w6	0.25	0.50	2.50	5.00	12.50	25.00
	C23.0	0.25	0.50	2.50	5.00	12.50	25.00
	C22.4w6	0.25	0.50	2.50	5.00	12.50	25.00
	C22.5w3	1.00	2.00	10.00	20.00	50.00	100.00
	C24.1w9	0.25	0.50	2.50	5.00	12.50	25.00
Surrogate	C19.0	0.50	1.00	5.00	10.00	25.00	50.00
Internal Standard	C21.0	25.00	25.00	25.00	25.00	25.00	25.00

Table III. Concentration of analyte, Surrogate, and Internal Standard in Group 2.

Compound Type	Compound	Concentration (µg/mL) at Level:					
		1	2	3	4	5	6
Analyte	C12.0	0.25	0.50	2.50	5.00	12.50	25.00
	C13.0	0.25	0.50	2.50	5.00	12.50	25.00
	C14.0	0.50	1.00	5.00	10.00	25.00	50.00
	C15.0	0.25	0.50	2.50	5.00	12.50	25.00
	C16.0	2.50	5.00	25.00	50.00	125.00	250.00
	C16.1w7t	0.25	0.50	2.50	5.00	12.50	25.00
	C17.0	0.25	0.50	2.50	5.00	12.50	25.00
	C18.0	0.75	1.50	7.50	15.00	37.50	75.00
	C18.1w9t	0.25	0.50	2.50	5.00	12.50	25.00
	C18.2w6t	0.25	0.50	2.50	5.00	12.50	25.00
	C18.3w6	0.25	0.50	2.50	5.00	12.50	25.00
	C18.3w3	0.25	0.50	2.50	5.00	12.50	25.00
	C20.0	0.25	0.50	2.50	5.00	12.50	25.00
	C20.1w9	0.50	1.00	5.00	10.00	25.00	50.00
	C20.4w6	1.00	2.00	10.00	20.00	50.00	100.00
	C22.0	0.25	0.50	2.50	5.00	12.50	25.00
	C22.1w9	0.50	1.00	5.00	10.00	25.00	50.00
	C24.0	0.25	0.50	2.50	5.00	12.50	25.00
	C22.6w3	1.50	3.00	15.00	30.00	75.00	150.00
Internal Standard	C21.0	25.00	25.00	25.00	25.00	25.00	25.00

Table IV. Compound and corresponding expected retention times.

#	Retention Time	Compound		#	Retention Time	Compound
1	8.12	C11.0		**41**	23.77	C18.1w5
2	9.73	C12.0		**42**	24.08	C18.1w3
3	10.73	Iso C12.0		**43**	24.12	C18.2w6t
4	11.62	C13.0		**44**	24.29	C18.2d5,11
5	12.76	Iso C13.0		**45**	24.48	C18.2w7
6	13.76	C14.0		**46**	24.65	C18.2w6
7	13.96	C14.1w9		**47**	25.06	C18.2w4
8	14.27	C14.1w7		**48**	25.14	C19.0 (SS)
9	14.57	C14.1w5		**49**	25.21	C18.3w6
10	14.97	Iso C14.0		**50**	25.70	C18.3w4
11	15.33	Ante Iso C14.0		**51**	25.93	C18.3w3
12	16.01	C15.0		**52**	26.22	C18.3w1
13	16.61	C15.1w8		**53**	26.53	C18.4w3
14	16.73	C15.1w6		**54**	26.72	C18.4w1
15	16.89	C15.1w5		**55**	27.66	C20.0
16	17.29	Iso C15.0		**56**	28.00	C20.1w11
17	18.45	C16.0		**57**	28.13	C20.1w9
18	18.57	C16.1w11		**58**	28.30	C20.1w7
19	18.74	C16.1w7t		**59**	29.12	C20.2w6
20	18.82	C16.1w9		**60**	29.87	C21.0 (IS)
21	19.08	C16.1w7		**61**	29.68	C20.3w6
22	19.30	C16.1w5		**62**	30.01	C20.4w6
23	19.50	C16.2w6		**63**	30.41	C20.3w3
24	19.66	Iso C16.0		**64**	30.97	C20.4w3
25	20.27	C7Me16		**65**	31.32	C20.5w3
26	20.30	C16.2w4		**66**	31.99	C22.0
27	20.74	C17.0		**67**	32.26	C22.1w11
28	20.96	C16.3w4		**68**	32.38	C22.1w9
29	21.27	C17.1		**69**	32.56	C22.1w7
30	21.36	C17.1w7		**70**	33.43	C22.2w6
31	21.34	C16.3w1		**71**	33.62	C21.5w3
32	21.48	C16.4w3		**72**	33.99	C23.0
33	21.97	Iso C17.0		**73**	34.37	C22.4w6
34	22.02	C16.4w1		**74**	34.64	C22.5w6
35	23.23	C18.0		**75**	35.14	C22.4w3
36	23.35	C18.1w13		**76**	35.98	C24.0
37	23.21	C18.1w9t		**77**	35.63	C22.5w3
38	23.50	C18.1w11		**78**	35.91	C22.6w3
39	23.72	C18.1w9		**79**	36.38	C24.1w9
40	23.85	C18.1w7				

Table V. Calculated Limits of Detection from Calibration Curves.

Compound	LOD (ug/ml)	From Cal. Compound (If from different Compound)
C11.0	0.1	C12.0
C12.0	0.1	
Iso C12.0	0.1	C13.0
C13.0	0.1	
Iso C13.0	0.1	C13.0
C14.0	0.2	
C14.1w9	0.1	C14.1w5
C14.1w7	0.1	C14.1w5
C14.1w5	0.1	
Iso C14.0	0.2	C15.0
Ante Iso C14.0	0.2	C15.0
C15.0	0.2	
C15.1w8	0.1	C14.1w5
C15.1w6	0.1	C14.1w5
C15.1w5	0.1	C14.1w5
Iso C15.0	0.2	C15.0
C16.0	0.9	
C16.1w11	0.1	C16.1w7
C16.1w7t	0.2	
C16.1w9	0.1	C16.1w7
C16.1w7	0.9	C18.1w9
C16.1w5	0.1	C16.1w7
C16.2w6	0.2	C18.2w6
Iso C16.0	0.1	C17.0
C7Me16	0.1	C16.1w7
C16.2w4	0.2	C18.2w6
C17.0	0.1	
C16.3w4	0.1	C18.3w3
C17.1	0.1	C17.1w7
C17.1w7	0.1	
C16.3w1	0.1	C18.3w3
C16.4w3	0.8	C20.4w6
Iso C17.0	0.1	C17.0
C16.4w1	0.8	C20.4w6
C18.0	0.2	
C18.1w13	0.6	C18.1w7
C18.1w9t	0.4	
C18.1w11	0.6	C18.1w7
C18.1w9	0.9	
C18.1w7	0.6	
C18.1w5	0.6	C18.1w7
C18.1w3	0.6	C18.1w7
C18.2w6t	0.1	
C18.2d5,11	0.2	C18.2w6
C18.2w7	0.2	C18.2w6

C18.2w6	0.2	
C18.2w4	0.2	C18.2w6
C19.0	0.7	
C18.3w6	0.2	
C18.3w4	0.1	C18.3w3
C18.3w3	0.1	
C18.3w1	0.1	C18.3w3
C18.4w3	0.8	C20.4w6
C18.4w1	0.8	C20.4w6
C20.0	0.2	
C20.1w11	0.2	
C20.1w9	0.1	
C20.1w7	0.1	C20.1w9
C20.2w6	0.2	
C20.3w6	0.1	C20.3w3
C20.4w6	0.8	
C20.3w3	0.1	
C20.4w3	0.8	C20.4w6
C20.5w3	1.8	
C22.0	0.6	
C22.1w11	1.5	C22.1w9
C22.1w9	1.5	
C22.1w7	1.5	C22.1w9
C22.2w6	0.4	
C23.0	0.4	
C21.5w3	1.8	C22.5w3
C22.4w6	0.4	
C22.5w6	1.8	C22.5w3
C22.4w3	0.4	C22.4w6
C24.0	0.4	
C22.5w3	1.8	
C22.6w3	2.0	
C24.1w9	0.3	

