Appendix A

Determination of Retinoids, Vitamin E and Carotenoids in Eggs from Black-capped Chickadees from the Regions of Anchorage, AK and Mat-Su Valley, AK Collected in 2001.

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Prepared for: Colleen M. Handel
Research Wildlife Biologist
Alaska Biological Science Center
1011 E. Tudor Road
Anchorage, AK 99503

Prepared by: _____
Jeffrey J. Whyte
Biochemistry and Physiology Branch

_____
Donald E. Tillitt
Branch Chief, Biochemistry and Physiology Branch

U.S. Geological Survey
Columbia Environmental Research Center
4200 E. New Haven Road
Columbia, Missouri 65201

SUBJECT:

Results from the determination of retinoids (3,4-dehydroretinol, all-trans-retinol, all-trans-retinal, and all-trans-retinol-palmitate), vitamin E (alpha-tocopherol), and carotenoids (astaxanthin, canthaxanthin, and beta-carotene) in black-capped chickadees (*Poecile atricapillus*) from the regions of Anchorage and Mat-Su Valley, Alaska collected in 2001.

INTRODUCTION:

Retinoids (vitamin A) is a general term applied to a group of isoprenoid compounds originated either from animal products, the retinoids, or from plant isoprenoid pigments, the carotenoids. These compounds are involved in biochemical pathways which involve diverse biological functions (Collins & Mao 1999). Sufficient levels of retinoids are essential for (i) vision, (ii) effective reproduction, (iii) the growth and development of young, (iv) nerve function, (v) epithelial function, and (vi) an effective immune response in mammals and birds (Thompson, 1976). Imbalances in retinoid homeostasis have been hypothesized as part of the mechanism of action of dioxin-like chemicals, due to their capacity to reduce vitamin A stores following exposure in different groups of vertebrates, including birds (Martinovic et al. 2003).

This report summarizes the findings of the measurement of retinoids, a form of vitamin E (alpha-tocopherol), and carotenoids in black-capped chickadees (*Poecile atricapillus*) from the regions of Anchorage and Mat-Su Valley, Alaska collected in 2001.

MATERIALS AND METHODS:

Eggs shipped from the Alaska Biological Science Center, Anchorage, AK in 1.5 mL cryotubes were stored at –80ºC until extraction and chromatographic analysis.

Chickadee eggs were analyzed for selected retinoids, carotenoids, and vitamin E based on the method recently described by Carvalho (2002). Individual eggs were used in the analysis of retinoids and carotenoids. Analytical procedures were performed under yellow fluorescent lights, and on ice. Each individual chickadee egg was weighed, and then 15% of this egg mass was placed in a separate tube. This egg fraction was homogenized in 2 mL of deionized water using a Omni TH homogenizer. The homogenate was split into 2 mL cryotubes for retinoid and carotenoid analysis. An internal standard mixture of retinol acetate and alpha-tocopherol-acetate was added to the retinoid samples for recovery estimates. A stock solution of Beta-Apo-8'-carotenal was used as the internal standard for recovery estimates in carotenoid samples. All samples were vortexed, and extracted with a 3:2 (v/v) ethyl acetate:hexane (both HPLC grade) solvent mixture 3 times. Samples were then centrifuged at 7500 rpm in a microcentrifuge (Fisher Scientific, Pittsburgh, model 16KM) and the upper organic phase was carefully transferred to a 1.5 mL amber cryo-tube. All samples were evaporated to dryness under a stream of dry N_2, and stored at –80ºC until chromatographic analysis. Each homogenate

from a single egg was analyzed in duplicate for each analyte. In addition, one sample was analyzed in triplicate (i.e. three portions from a sample).

Separate chromatographic procedures were used for retinoids and vitamin E, and another for carotenoids. Chromatographic peaks were identified by comparing retention times of the different analytes. The standards used during retinoid and vitamin E analysis were 3,4-dehydroretinol (gift from F. Hoffman-La Roche Ltd.), all-trans-retinol 95% pure (Sigma), all-trans-retinal 98% pure (Sigma), retinol-acetate (internal standard), the vitamin E alpha-tocopherol 95% pure (Sigma), the internal standard alpha-tocopherol-acetate 96% pure (Sigma), and all-trans-retinol-palmitate 1,800,000 USP units/g (Sigma). The standards for carotenoid analyses were: astaxanthin 99% pure (Sigma), canthaxanthin (gift from F. Hoffman-La Roche Ltd.), the internal standard beta-apo-8'-carotenal 99% pure (Sigma) and beta-carotene 95% pure (Sigma).

The HPLC system (Agilent Technologies, Wilmington, mode 1100) consisted of a binary pump, a variable wavelength UV absorbance detector and an autosampler. A reverse-phase Adsorbosphere C18 column (4.6 mm internal diameter, 150 mm length) equipped with a column guard (Altech Associates, Deerfield, IL) was employed for all analyses. The detector was set at 325 nm for the retinoids, 292 nm for alpha-tocopherols, and 474 nm for carotenoids. Flow rate was 1.0mL.min-1 during all analytical procedures. Both the retinoid and carotenoid analysis employed the same basic solvent mixtures, composed of methanol:water:ethyl-acetate 90:10:0 (mixture A) and methanol:water:ethyl-acetate 29:1:70 (mixture B). Retinoids were eluted with an isocratic flow during the first 10 min with 100%A:0%B. At 10 min the flow was changed to a linear elution gradient starting at 100%A:0%B and changing to 0%A: 100%B over 21.2 min. Each retinoid run lasted 30 min, with 4 min reequilibration time between samples. Carotenoids were eluted with a linear elution gradient starting with 80%A: 20%B, and changing to 0%A: 100%B after 19 min. Each carotenoid analysis lasted 20 min, with 4 min reequilibration time between samples. Recovery rates were 73 ± 12% (mean ± standard deviation) for carotenoids, 58 ± 21% (mean ± standard deviation) for smaller retinoids, and 74 ± 11% (mean ± standard deviation) for longer chain vitamin E and retinol-palmitate. Results were corrected based on the recovery of these internal standards. Concentrations are reported per gram egg.

The limits of detection (LOD) and quantitation (LOQ) for the method were calculated as described by Keith et al. (1983). These parameters were calculated using the daily assay method blanks. The lowest definable area of a peak for each analyte (based on a signal-to-noise ratio of two) was determined for each method blank. The LOD was defined to be equal to the mean concentration (n = 5 per method) for the lowest definable peak area plus 3 times the standard deviation of the mean associated with that peak area. The LOD was defined to be equal to the mean concentration for the lowest definable peak area plus 3 times the standard deviation of the mean associated with that peak area. These measures were used to evaluate the sample data results and to determine whether they were detectable or measurable above that of the background. Detection limits were very consistent among blanks and thus, the mean detection limit was used for analyses. Mean detection limits for each analyte method are as follows: 3,4-dehydroretinol, retinol, and retinal (0.519 ± .000627 ng); alpha-tocopherol (49.9 ± 0.487 ng); retinol-palmitate (0.168

± 0.022 ng); astaxanthin (2.41 ± 0.098 ng); canthaxanthin (0.908 ± 0.00340 ng); and beta-carotene (0.010 ± 0.00130 ng). Note that detection limits are for the raw chromatogram values measured, and not the egg-mass corrected concentrations reported in Tables 2 and 3.

QUALITY ASSURANCE AND QUALITY CONTROL:

The objective of the quality assurance plan of this study was to assure that the extraction and biochemical analyses were accurate and precise measurements of the samples collected in the field portion of this study. The general scheme included replication of assayed samples during each extraction, comparison of calibration against known standards, proper maintenance and calibration of equipment, proper documentation at all steps of sample processing and other considerations of Good Laboratory Practice (GLP). The specific aspects of the QA plan related to the retinoid and carotenoid extraction and evaluation procedures are given below.

All experimental information was recorded in bound laboratory notebooks and electronic copies maintained in a separate, secured area (network drive backup). Instrumental printouts and computer generated data tables were uniquely labeled and cross-referenced to the project notebook. Hard copies of computerized data files were maintained in a project notebook. Computer files were copied to a network storage area and by archived tape back up. All equipment used in this study was routinely inspected and preventative maintenance performed. A logbook was kept for each instrument to document its use, performance and maintenance.

Replication and subsequent performance checks were performed at different stages of the retinoid/carotenoid determination procedure. During the extraction, one sample was randomly selected to be extracted in triplicate (i.e. three portions from the sample). Five point standard curves for each analyte and internal standard were prepared each week. In addition, two retinoid and carotenoid standards were analyzed in conjunction with each HPLC analysis of extracted chickadee egg samples to verify analyte retention times. Sample calibration standard curves for each analyte are displayed in Figures 1 through 8. The sources and lot numbers of the retinol, retinyl acetate and retinyl palmitate used as standards were recorded in the laboratory notebook. Data summaries for retinoid, vitamin E, and carotenoid standard curves have been included with this report to demonstrate the consistency of the detector response with quantity of analyte over the time course of the study. In addition to allowing quantitative determination of analyte present, the weekly standard curves also provided a performance check of the operating system.

DATA ANALYSIS:

In order to verify the consistency and consequently the reliability of the sample data as a whole, the weekly standard curve data for each analyte were recorded and monitored over time. In each case a mean and standard deviation were calculated for each standard curve (Table 1). Standard curve slopes and r-squared were very consistent among weeks. Y-intercept values were more variable (data not shown), but did not influence estimates of analyte concentrations in samples. Values are reported to three significant digits.

Table 1. Mean, standard deviation, and coefficient of variation of parameters for weekly standard curves of retinoid and carotenoid analytes (*n* = 3 for each analyte).

Standard Curve	Slope mean (S.D.) CV%	y-intercept mean (S.D.) CV%	r-squared mean (S.D.) CV%
3,4-dehydroretinol	307000 (9350) 3%	-89300 (28400) 32%	0.999 (0.000192) 0%
all-trans-retinol	485000 (21700) 4%	-133000 (75700) 57%	0.999 (0.000688) 0%
all-trans-retinal	119000 (10100) 8%	-49800 (20200) 41%	0.999 (0.00106) 0%
alpha-tocopherol	3200 (57.7) 2%	-132000 (40800) 31%	0.998 (0.00143) 0%
alpha-tocopherol acetate (int)	241000 (13300) 6%	-79400 (30700) 39%	0.999 (0.00223) 0%
all-trans-retinol-palmitate	221000 (26800) 12%	-40900 (62400) 153%	0.996 (0.00721) 1%
astaxanthin	130000 (10000) 8%	-48000 (24800) 52%	0.999 (0.000162) 0%
canthaxanthin	147000 (8730) 6%	-57900 (11500) 20%	0.999 (0.000135) 0%
beta-apo-8'-carotenal (int)	247000 (11900) 5%	-110000 (47500) 43%	0.999 (0.000119) 0%
beta-carotene	107000 (5590) 5%	29400 (29100) 99%	0.998 (0.00229) 0%

Estimates of the efficiency of recovery based on internal standards are reported in the data tables. Due to solvent changes during extraction, only 14% of the extracted sample was analyzed by HPLC. It was necessary to adjust the measured retinoid, vitamin E, and carotenoid values accordingly. This was done as indicated in equation 1.

$$\text{Analyte content in egg} = \frac{\text{(mass of analyte measured)}}{(0.14 * \text{mass of egg extracted})} \quad [1]$$

RESULTS AND DISCUSSION:

The error associated with the slopes measured for the routine standard curves were relatively small, ranging from 1% to 12%. This suggests that the sensitivity of the detector, instrumental response and standard solutions were inherently unchanged through out the time course of the measurements. The error associated with the y-intercepts for the composite standard curves were significant, ranging from 20% to 153%. This is why we run standards on a regular basis. This variability may reflect the weekly variability in environmental and instrumental conditions as opposed to anything inherent in the detection method or the system. Sample chromatograms for retinoid and carotenoid analyses are displayed in Figures 9 through 12. Duplicate procedural extraction blanks measured with each HPLC run revealed that no analytes were present in the extraction reagents or introduced during the extraction procedure.

Data for retinoids, vitamin E, and carotenoids determined in the chickadee eggs are reported in Tables 2 and 3 along with their associated estimated recoveries of retinol acetate, alpha-tocopherol acetate, and beta-apo-8'-carotenal. For the analytes measured, greater than 85% of the duplicate samples had an associated coefficient of variance of less than 20%. Egg samples examined in triplicate had coefficients of variation less than 15% for the majority of analytes (all-trans-retinol-palmitate had a higher coefficient of variation at 37%).

For all samples, 3,4-dehydroretinol was below the limit-of-detection in the eggs analyzed. This is expected, because 3,4-dehydroretinol is found mainly in fish, although terrestrial vertebrates can obtain it through fish-based diets (Kakela et al. 2003). Retinoids stored in the avian egg are essential for normal development. All-trans retinol concentrations were the highest of the retinoids in the chickadee eggs, ranging from 491 to 9250 ng/g egg (Table 2). This is supported in developmental studies of other avian species. For example, the vitamin A content in freshly-laid eggs of normal domestic hens was analyzed to be approximately 80% retinol (Parrish et al. 1951). The mean chickadee egg concentration of the antioxidant, alpha-tocopherol, was 85.2 µg/g egg. This is only slightly higher than normally reported values for hen eggs (Chen et al. 1998). For carotenoids, both astaxanthin and canthaxanthin concentrations in eggs were below the method limit-of-detection, while the mean beta-carotene concentration was 638 ng/g egg. Other studies have reported beta-carotene as the major carotenoid present in bird eggs (Surai et al. 2001), but the reason for the absence of astaxanthin and canthaxanthin in the chickadee samples is unknown (no literature reference values found for comparison).

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Appendix 1 – Sample calibration curves and chromatograms for analytes measured.

Figure 1 - 3,4-Dehydroretinol

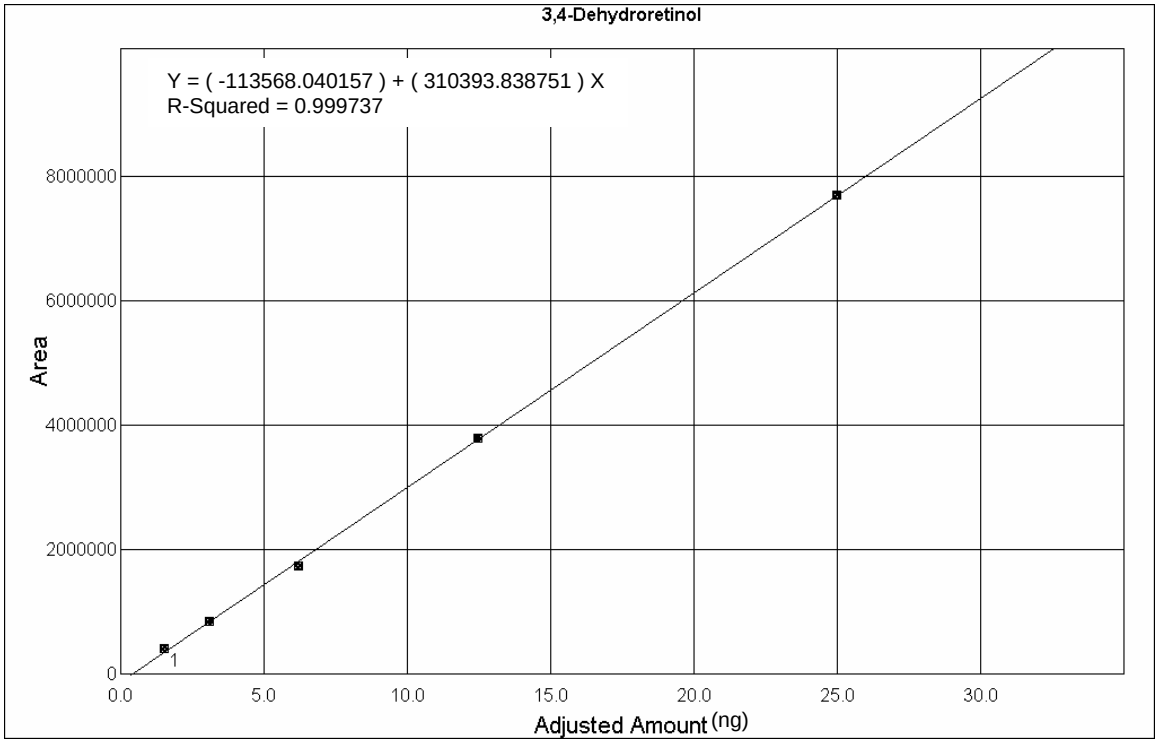

Figure 2 - All-trans-retinol

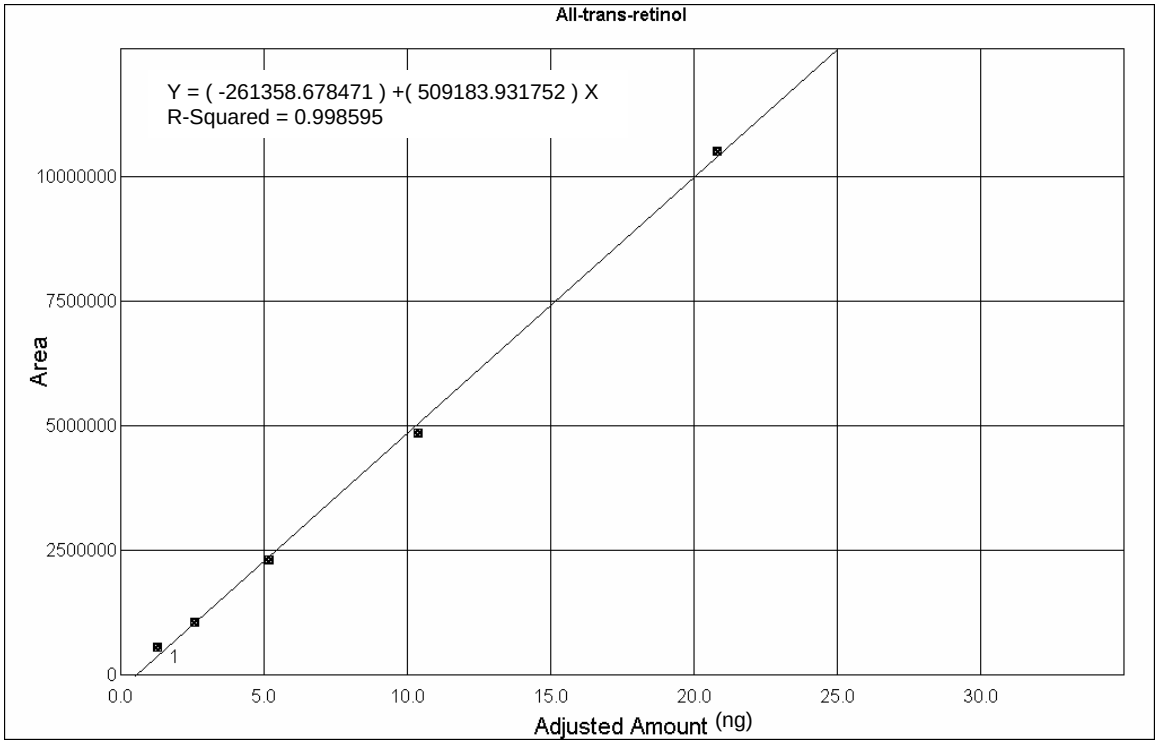

Figure 3 - All-trans-retinal

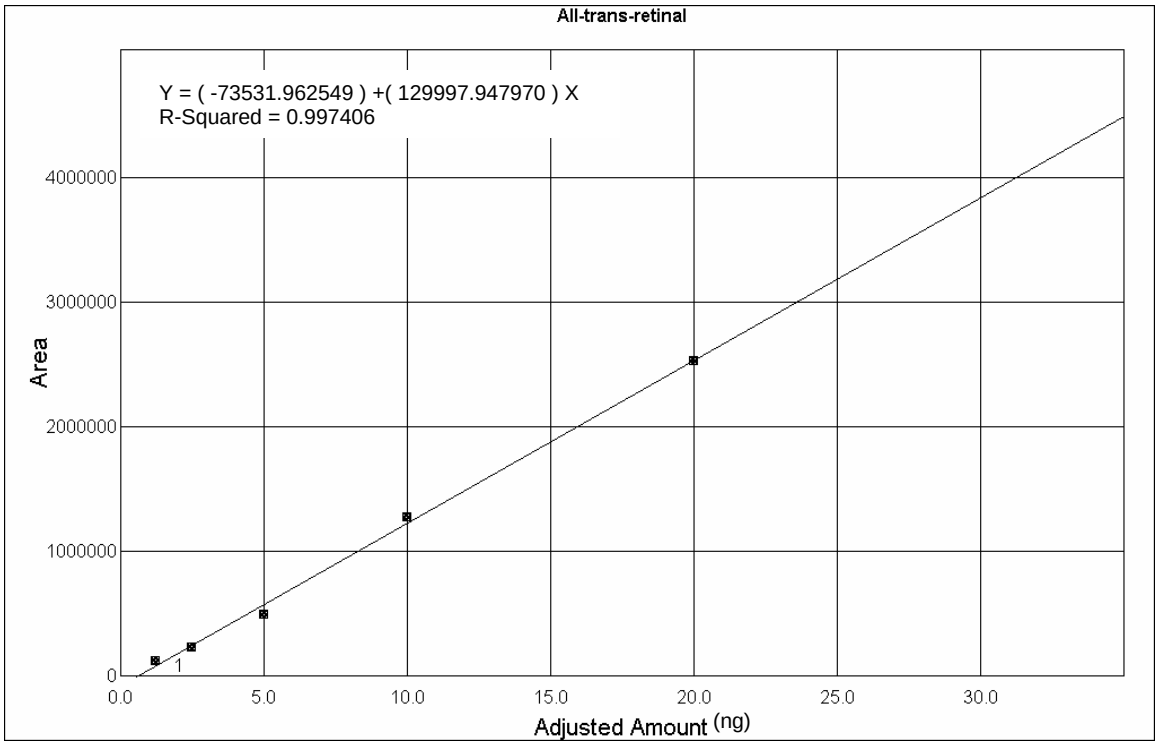

Figure 4 - Alpha-tocopherol

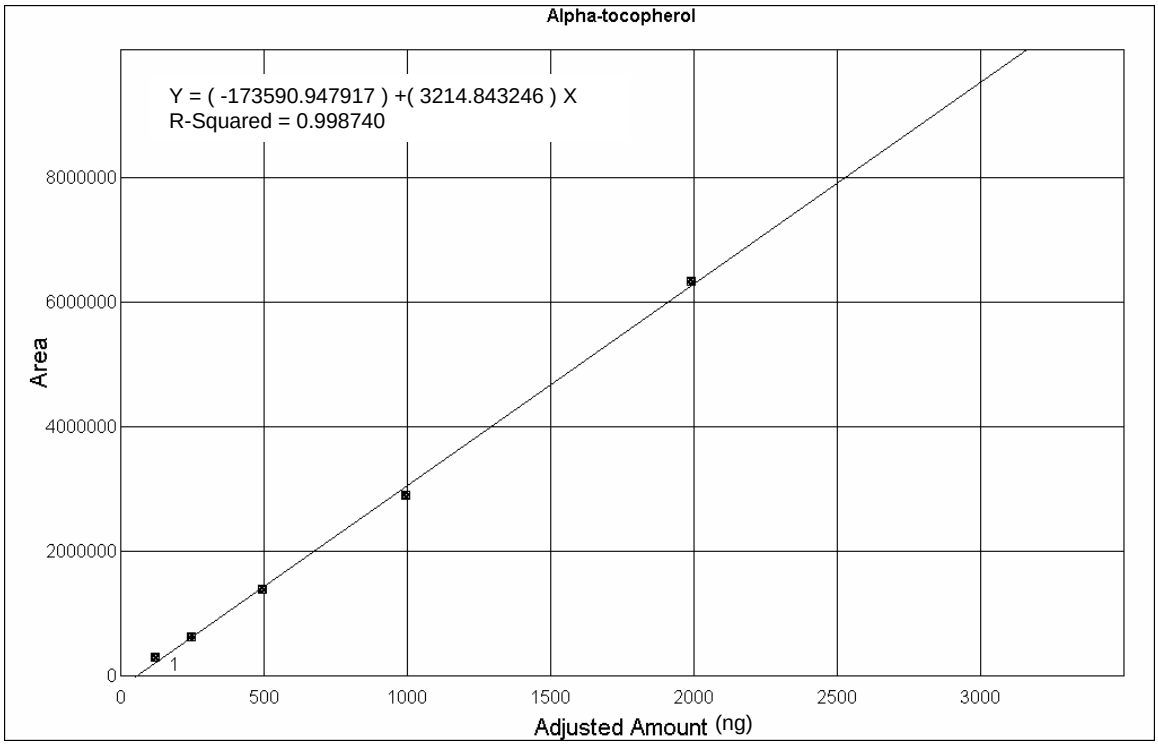

Figure 5 - Retinol-palmitate

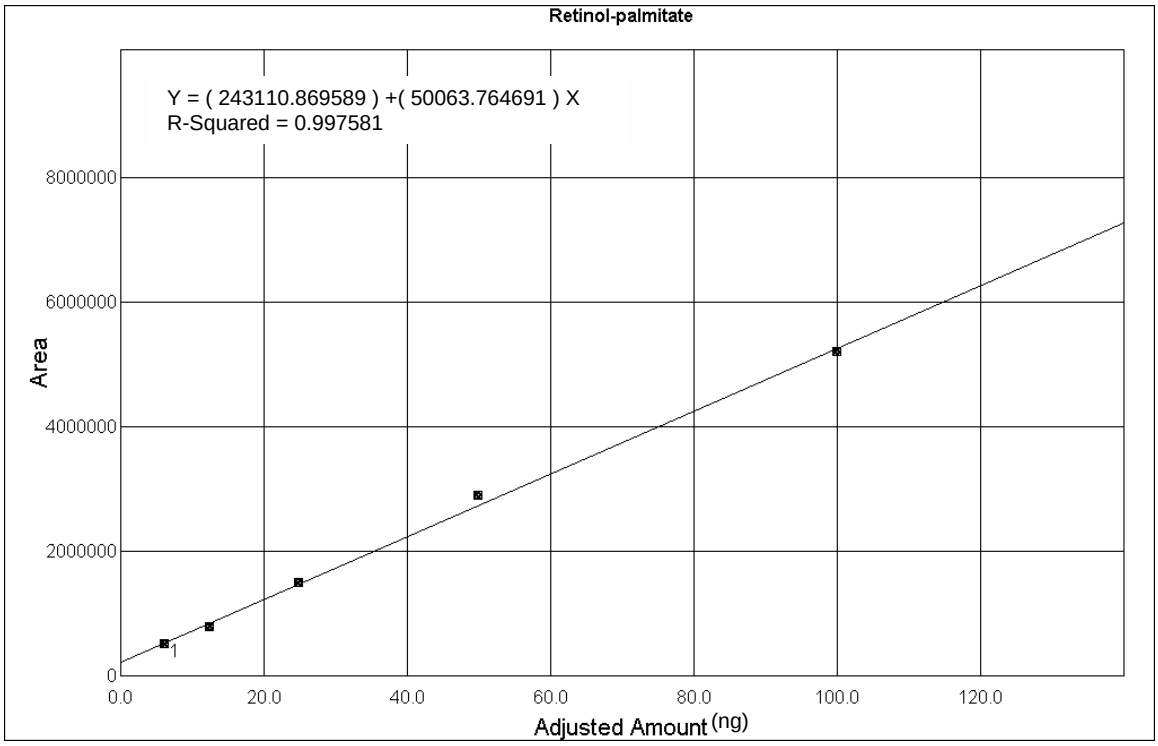

Figure 6 - Astaxanthin

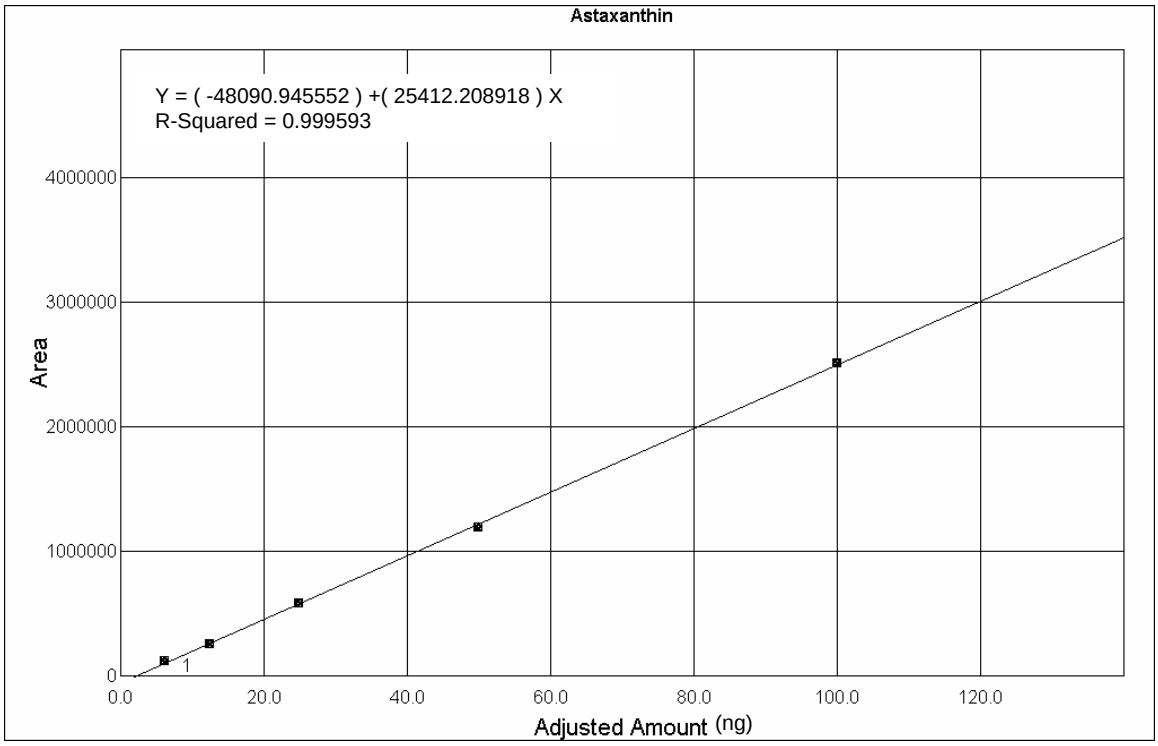

Figure 7 - Canthaxanthin

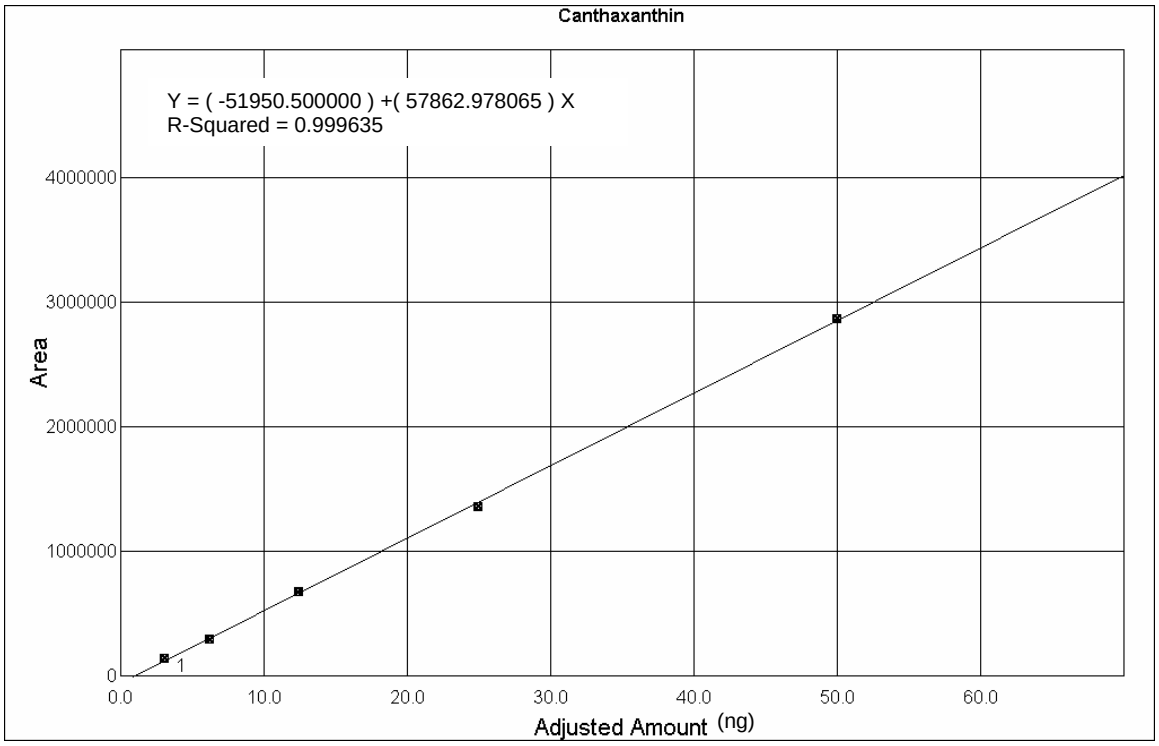

Figure 8 - Beta-carotene

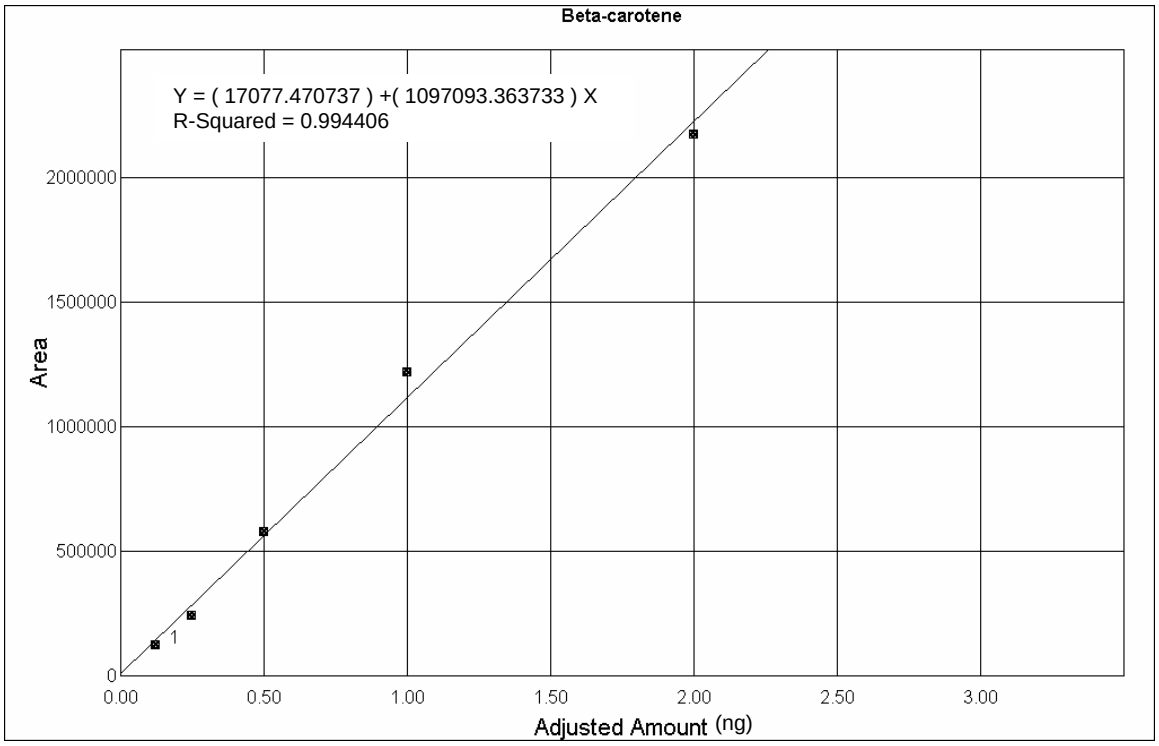

Figure 9 - Sample Chromatogram
Retinoid Calibration Standards

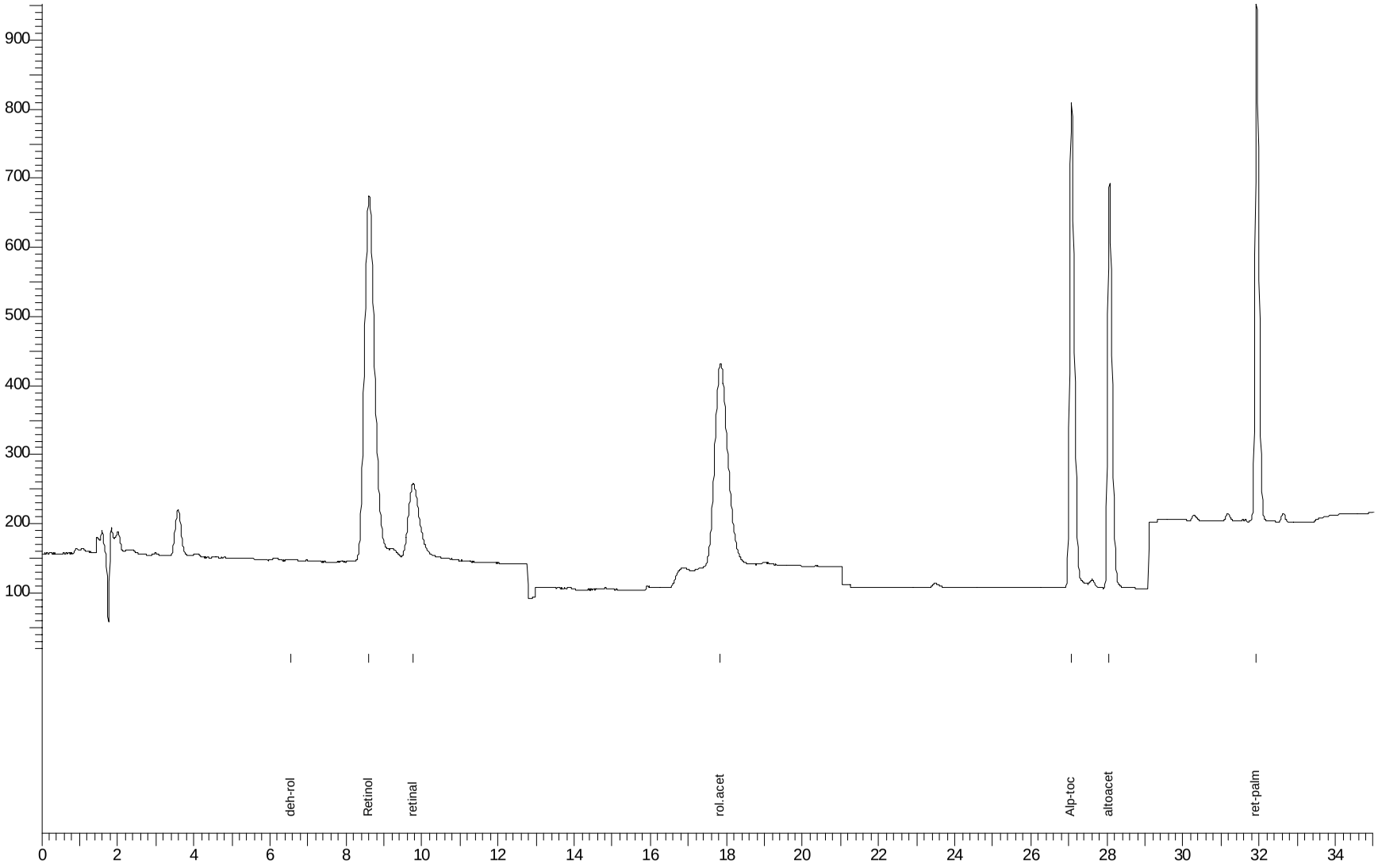

Figure 10 - Sample Chromatogram
Retinoid Analysis – Black-capped Chickadee Sample 4 (Replicate 1)

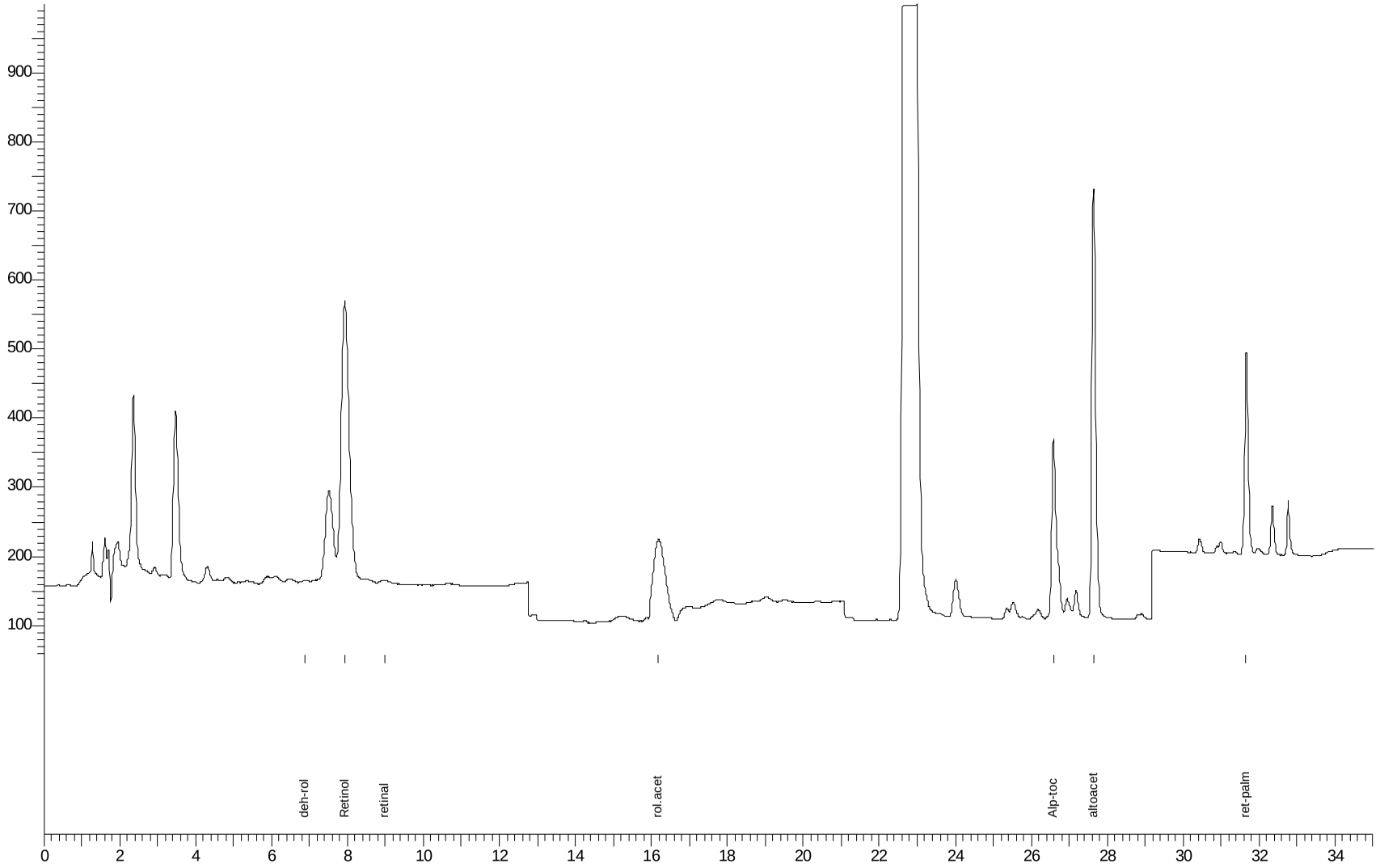

Figure 11 - Sample Chromatogram
Carotenoid Calibration Standards

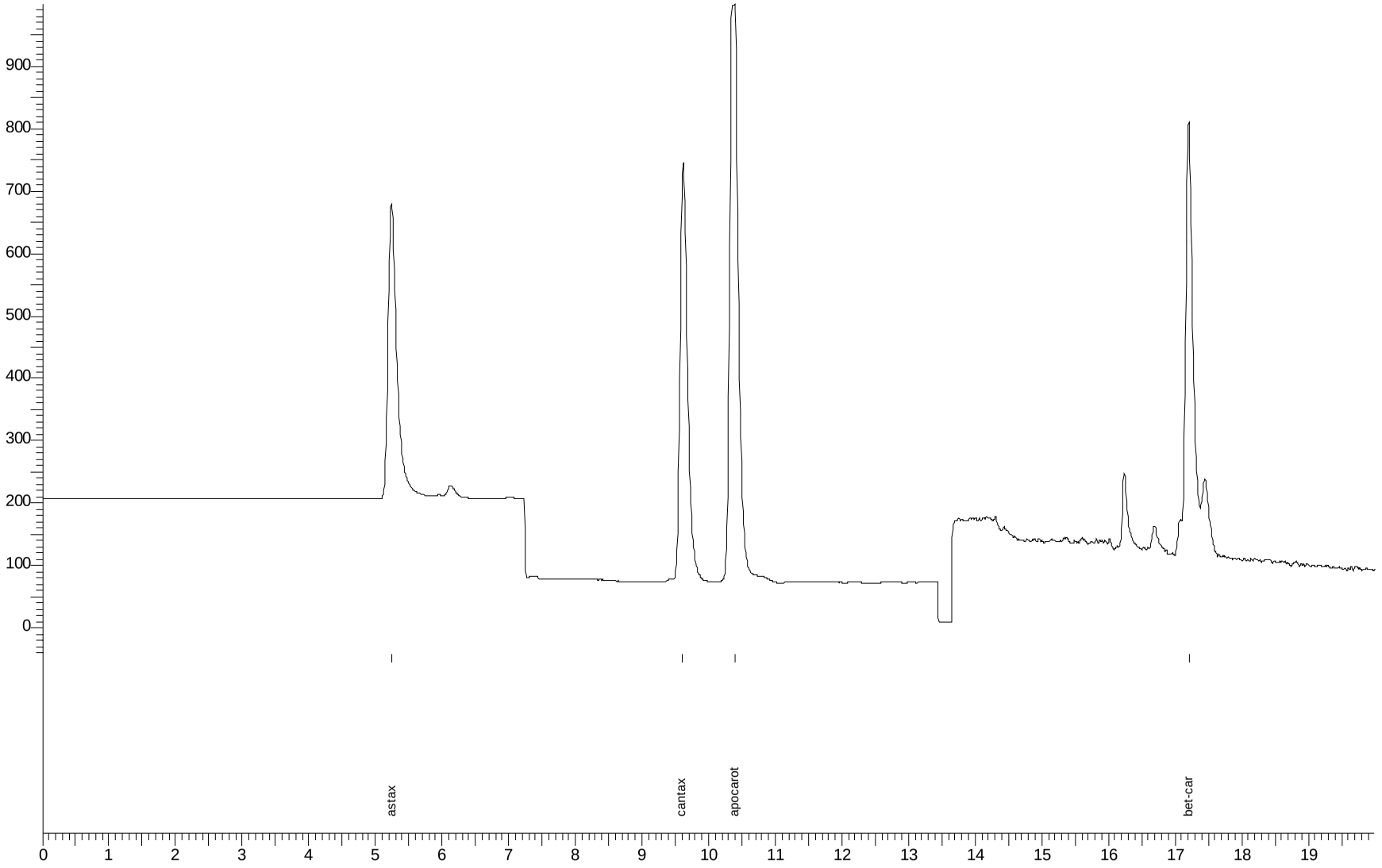

Figure 12 - Sample Chromatogram
Carotenoid Analysis - Black-capped Chickadee Sample 1 (Replicate 1)

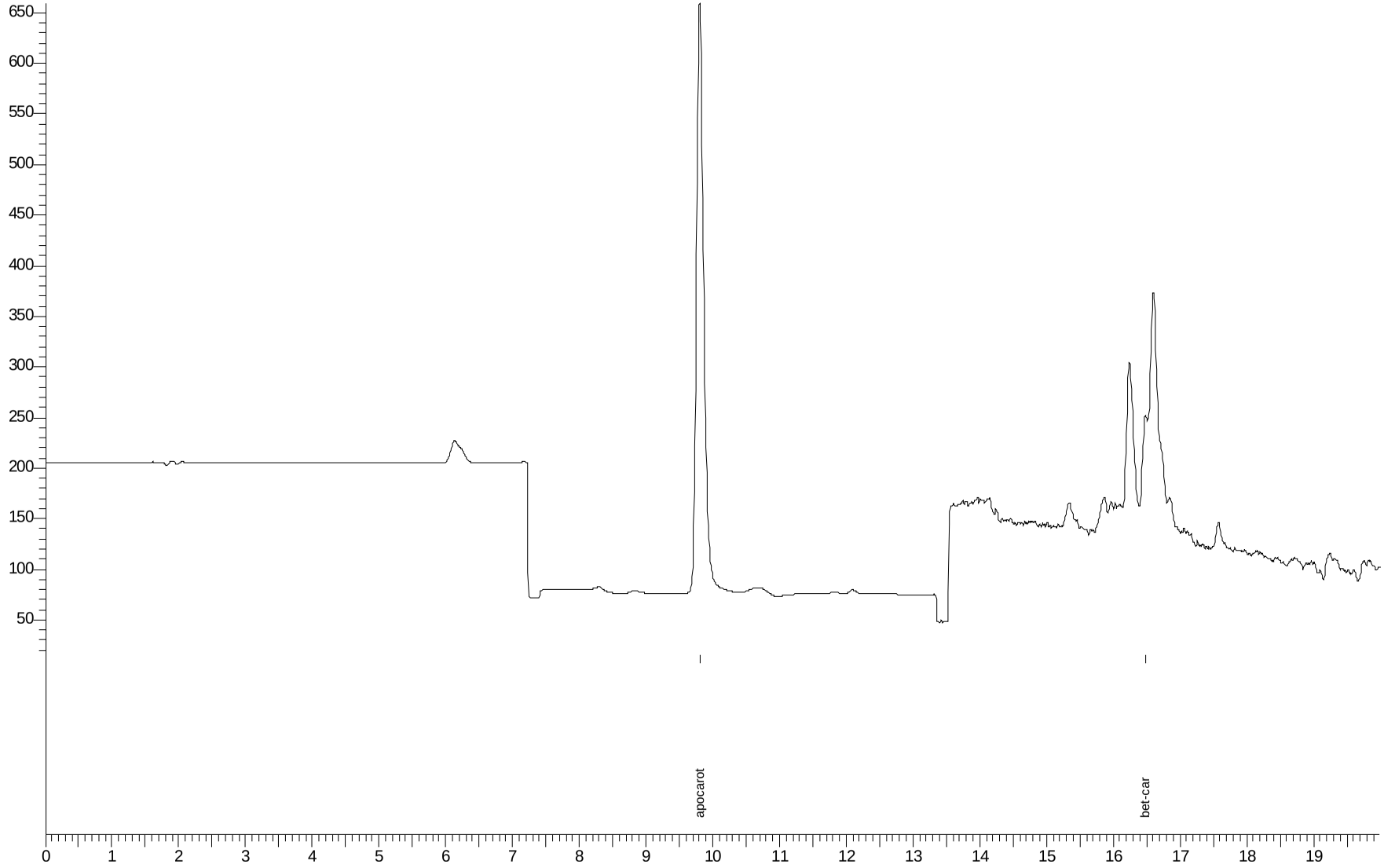

Table 2. Retinoid vitamins and vitamin E concentrations in black-capped chickadee eggs collected from the Anchorage, AK and Mat-Su Valley, AK. Values shown are corrected for percent recovery of internal standard. All egg homogenate samples were measured in duplicate. Procedural triplicate samples are highlighted in bold.

Sample Number	Sample Code	retinol acetate % recovery	3,4-dehydroretinol (ng/g egg)	S.D.	all-trans-retinol (ng/g egg)	S.D.	all-trans-retinal (ng/g egg)	S.D.	acetate % recovery	alpha-tocopherol (ug/g egg)	S.D.	retinol-palmitate (ug/g egg)	S.D.
1	1TEEL02	93%	13.5	(2.03)[1]	491	(38.7)	52.5	(7.66)	73%	64.7	(0.942)	4.29	(0.267)
2	1STER01	54%	25	(1.19)[1]	759	(10.3)	240	(38.4)	87%	55.3	(0.901)	1.79	(0.0209)
3	1SIMA01	68%	19.9	(2.14)[1]	1190	(27.8)	19.9	(2.14)[1]	68%	77.1	(1.5)	1.97	(0.0607)
4	1RACR05	64%	22.4	(4.97)[1]	1110	(140)	108	(5.14)	80%	58.2	(0.461)	2.33	(0.143)
5	1PTWO10	36%	49.1	(19.1)[1]	1640	(236)	391	(49.8)	67%	51.3	(0.209)	2.92	(0.166)
6	1PTWO07	39%	56.7	(9.57)[1]	3340	(1370)	403	(157)	67%	34.7	(4.89)	4.81	(0.247)
7	1PTWO04	44%	28.3	(5.14)[1]	940	(171)	177	(32.3)	78%	61.1	(0.322)	3.22	(0.0576)
8	1POPO02	42%	35	(3.82)[1]	1060	(29.4)	255	(29.4)	82%	77.5	(7.72)	3.66	(0.148)
9	1POMA05	47%	34.8	(1.67)[1]	1970	(181)	183	(25.6)	61%	136	(3.18)	4.73	(0.0238)
10	1PAJO11	72%	30.3	(2.31)[1]	2130	(62.9)	130	(14)	78%	96.9	(1.35)	4.7	(0.129)
11	1MUPA05	97%	13.1	(0.346)[1]	516	(37)	58.5	(1.09)	82%	42.5	(0.106)	4.81	(0.0778)
12	1LAMT11	60%	24.7	(1.63)[1]	1270	(30.4)	97.6	(10.8)	78%	65.2	(0.694)	3.7	(0.0137)
13	1KULE01	41%	30.6	(2.49)[1]	1240	(1.4)	127	(14.8)	79%	59.4	(0.547)	3.49	(0.0722)
14	1KIPA16	39%	35.3	(0.0997)[1]	1340	(12.1)	35.3	(0.0997)	76%	70.4	(2.31)	1.1	(0.104)
15	1KIPA05	32%	46.9	(2.75)[1]	3250	(80.9)	894	(55.5)	38%	140	(4.4)	1.48	(0.121)
16	1JOTR01	59%	23.8	(4.59)[1]	925	(62.4)	113	(8.3)	78%	42.6	(3.39)	4.06	(0.0372)
17	1HIPA01	90%	25.2	(16.5)[1]	620	(63.6)	61.5	(0.813)	76%	36.1	(2.61)	3.01	(0.0828)
18	1HANS03	55%	24.3	(1.59)[1]	1850	(67.1)	90.4	(5.45)	82%	94.7	(2.19)	3.55	(0.18)
19	1DEWH01	70%	19.1	(1.03)[1]	911	(0.261)	77.3	(9.18)	83%	65.5	(0.828)	4.39	(0.157)
20	1CACR06	53%	37.7	(2.01)[1]	2530	(121)	231	(34)	78%	122	(1.58)	8.97	(0.367)
21	1CACR04	91%	16.3	(2.65)[1]	1070	(69.8)	90.4	(31.7)	70%	64.2	(1.34)	3.97	(0.436)
22	1BRUD01	51%	27.1	(2.26)[1]	1760	(137)	131	(3.97)	74%	67.2	(0.504)	3.43	(0.106)
23	1BLUM04	51%	72.3	(3.37)[1]	9250	(26.1)	459	(35.6)	69%	417	(7.3)	13.3	(0.312)
24A*	1BISS03 (rep 1)	51%	25.5	(2.61)[1]	1710	(105)	180	(2.86)	82%	39.2	(0.875)	3.69	(0.125)
24B*	1BISS03 (rep 2)	51%	25.7	(5.11)[1]	1800	(352)	195	(35.1)	62%	49.9	(2.07)	1.75	(0.0683)
24C*	1BISS03 (rep 3)	51%	25.7	(2.25)[1]	1820	(94.1)	156	(7.37)	76%	45.6	(0.311)	3.73	(0.222)
		Mean	30.7	(14)	1790	(1760)	192	(190)		85.2	(76.3)	4.03	(2.51)
			min	max	min	max	min	max		min	max	min	max
		Range	13.1	72.3	491	9250	19.9	894		34.7	417	1.1	13.3

[1] Below method limit-of-detection (LOD). One-half LOD used for calculation purposes.

* Overall mean was calculated using the mean of the procedural triplicate samples, not the individual triplicate values.

Table 3. Carotenoid vitamin concentrations in black-capped chickadee eggs collected from the Anchorage, AK and Mat-Su Valley, AK. Values shown are corrected for percent recovery of internal standard. All egg homogenate samples were measured in duplicate. Procedural triplicate samples are highlighted in bold.

Sample Number	Sample Code	beta-apo-8'-carotenal % recovery	astaxanthin (ng/g egg)	S.D.	canthaxanthin (ng/g egg)	S.D.	beta-carotene (ng/g egg)	S.D.
1	1TEEL02	79%	74.9	(18.6) [1]	28.2	(6.99) [1]	26	(17.3)
2	1STER01	89%	69.8	(1.29) [1]	26.3	(0.486) [1]	29.1	(2.78)
3	1SIMA01	66%	94	(7.47) [1]	35.4	(2.82) [1]	69.7	(9.61)
4	1RACR05	63%	103	(4.94) [1]	38.9	(1.86) [1]	51.1	(0.58)
5	1PTWO10	89%	85.9	(4.74) [1]	32.4	(1.79) [1]	709	(59.1)
6	1PTWO07	71%	92.8	(2.46) [1]	35	(0.927) [1]	414	(16.9)
7	1PTWO04	87%	65.6	(4.26) [1]	24.7	(1.61) [1]	308	(42.3)
8	1POPO02	66%	102	(7.18) [1]	38.6	(2.7) [1]	320	(49.1)
9	1POMA05	65%	116	(8.89) [1]	43.7	(3.35) [1]	1680	(272)
10	1PAJO11	46%	221	(29.6) [1]	83.1	(11.1) [1]	887	(11.5)
11	1MUPA05	90%	65.9	(6.84) [1]	24.8	(2.58) [1]	192	(47.5)
12	1LAMT11	68%	101	(2.35) [1]	38.1	(0.884) [1]	738	(752)
13	1KULE01	63%	93	(5.56) [1]	35	(2.1) [1]	752	(130)
14	1KIPA16	79%	83.1	(20.5) [1]	31.3	(7.73) [1]	320	(55.3)
15	1KIPA05	68%	101	(5.49) [1]	38.1	(2.07) [1]	1230	(26.5)
16	1JOTR01	64%	99.8	(1.48) [1]	37.6	(0.557) [1]	367	(59.2)
17	1HIPA01	68%	89.1	(3.92) [1]	33.6	(1.48) [1]	422	(82.7)
18	1HANS03	68%	91.4	(7.19) [1]	34.4	(2.71) [1]	223	(18.7)
19	1DEWH01	64%	97.3	(2.12) [1]	36.7	(0.801) [1]	995	(65.9)
20	1CACR06	78%	118	(4.77) [1]	44.3	(1.8) [1]	1210	(93.4)
21	1CACR04	100%	68.3	(5.45) [1]	25.7	(2.05) [1]	509	(75.4)
22	1BRUD01	76%	82.8	(4.24) [1]	31.2	(1.6) [1]	438	(30.4)
23	1BLUM04	70%	244	(18.2) [1]	92.1	(6.86) [1]	2280	(88.5)
24A*	1BISS03 (rep 1)	67%	89.9	(7.55) [1]	33.9	(2.85) [1]	988	(175)
24B*	1BISS03 (rep 2)	75%	80.5	(2.66) [1]	30.3	(1) [1]	1150	(13.4)
24C*	1BISS03 (rep 3)	70%	86.4	(6.08) [1]	32.6	(2.29) [1]	1260	(48.6)
		Mean	102	(42.8)	38.4	(16.1)	638	(561)
			min	max	min	max	min	max
		Range	65.6	244	24.7	92.1	26	2280

[1] Below method limit-of-detection (LOD). One-half LOD used for calculation purposes.

* Overall mean was calculated using the mean of the procedural triplicate samples, not the individual triplicate values.

Appendix B

A Report to the

United States Fish and Wildlife Service

On

An Investigation of DNA Damage

In Black-capped Chickadees.

By

International EcoGen Inc.

Contact: Michael Easton, Ph.D.
Phone: 604 - 986 - 2400
E-mail: michael_easton@intl-ecogen.com
2015 McLallen Court
North Vancouver
British Columbia
CANADA V7P 3H6

July 22nd, 1999.

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SUMMARY

The flow cytometry analysis of the black-capped chickadee blood indicated that the beak-deformed birds had a highly significant amount of DNA damage compared to the normal bird samples (X^2 = 152.4, 1 df, P=0). In addition, the normal Palmer samples that were kept in snow for 4 hours may have suffered degradation, since their CV DIF values varied significantly from the normal bird samples that were frozen immediately on dry ice (X^2 = 34.8, 1 df, P=0). It is suggested that additional statistical analyses be carried out to further understanding about the causation of the observed DNA damage.

Introduction

This study examines the possibility that the chickadee population with beak deformities in the Anchorage region also show evidence of genotoxicity in the form of clastogenic damage. The latter is defined as the breakage and subsequent rearrangement of chromosomal material. This rearrangement involves either reattaching to another chromosome (translocation) or remaining as a separate entity. At cell division, these separate entities are lost from the nucleus and may becomed enveloped in a membrane to form a micronucleus. The end result is that the subsequent daughter cells receive an unequal amount of parental DNA. This change in DNA content is then perpetuated and increased in the general cell (i.e. red blood cell) population by further divisions of these corrupted cell lines until the terminal division which ends in the formation of functional somatic cells such as blood cells. Thus the cell incidence of the initial clastogenic damage is multiplied many times before the damaged cells are released to the general cell population of the tissue (i.e. peripheral blood circulation). The function of these cells may be impaired as a consequence of the loss (or gain) of genetic material. This loss of function likely impacts the animal's fitness. In addition genotoxicity of the blood may be an indicator of genotoxic activity in cells from other tissues.

Flow cytometry can be used to determine the net amount of DNA in each cell to a very high degree of accuracy. In examining a population of about 10,000 cells from an individual animal, a very clear measurement of the degree of variation of DNA content within this population of cells can be determined. This value is called the coefficient of variation (CV) and is computed by dividing the standard deviation by the mean. A CV value significantly higher than the control or reference value indicates a significant degree of DNA damage has occurred in the cells of that tissue. This increase in variation was shown to be dose dependent (Otto et al, 1981; Easton et al, 1997). The mechanisms for producing such a state may or may not involve direct acting mutagens. Some genotoxins such as the heavy metals mercury, lead, arsenic and cadmium act by blocking the DNA repair capability of the cell, thus enabling normal breakage events to go unrepaired.

Flow cytometry has been used to detect genotoxic damage in field populations of fish (Easton, 1997; Lingenfelser *et al*, 1997), birds (Custer *et al*, 1994; George *et al.*, 1991), turtles (Lamb *et al.*, 1991; Bickham *et al.*, 1988) frogs (Lowcock *et al*, 1997) and mice (McBee and Bickham, 1988) where contaminated sites were compared with reference sites. Results have been verified by other cytogenetic methods (Bickham *et al.*, 1992; McBee and Bickham, 1988).

Methods

Populations Sampled

Samples of chickadees were collected from 4 locations (see Figure 1 and Table 1), Anchorage (4 sites), Trapper Creek (1 site), Talkeetna (1 site) and Palmer (1 site). In all 44 samples were collected. There were two questions of interest:

1. Did the abnormal cross-billed group also have DNA damage?
2. Did the samples stored in snow for 4 hours differ qualitatively from the normal population?

To answer the above questions the samples were divided into 3 groups regardless of location. These groups consisted of:

- Birds with nornal beaks (normal) - 22 samples;
- Birds with deformed cross-beaks (abnormal) - 8 samples;
- Normal birds whose blood sample was stored in snow for 4 hours prior to freezing (snow) - 11 samples.

Flow Cytometry

All samples were stored at -80^{o}C until used. The 44 samples were processed as a single batch. This ensured that all staining times and length of exposed cells to staining reagents were kept constant. The samples were all run on an Epic Elite Flow Cytometer (Coulter Corp.) using an argon laser (488 nanometers).

The instrument was aligned prior to the running of a specimen batch first using DNA Check Beads (Coulter Corp) followed by human lymphocytes and chicken erythrocyte nuclei (CENs, BioSure Controls) as external biological controls for the staining protocol and to fine tune the alignment and ensure stability of the instrument. The mean channels for DNA content for the chickadee blood and the human lymphocytes was established in the instrument's most sensitive range and maintained throughout for all 44 specimens.

The samples were run on the cytometer at a rate of about 150 cells per second, the data being collected as both Histogram and Listmode files in a double gated environment to ensure only nuclei were being measured. The subsequent data was analyzed with Elite Software (Coulter Corp.) to produce the mean channel and full peak CV values used in the subsequent data analysis.

All cells were stained using a modified whole cell method (Clevenger *et al*, 1985). The frozen chickadee red blood cells (CRBCs) were thawed rapidly at 37^{o}C and then placed on ice. They were washed twice in phosphate buffered saline (PBS) and the cells were counted in order to adjust the numbers to the optimum level

for the staining protocol ($2x10^6$ cells per ml). A known volume of standard human lymphocytes was then added to the CRBCs as an internal control. Separate tubes of CENs and human lymphocytes were stained at the same time as external controls.

The cells were fixed in 1.0 ml 0.5% paraformaldehyde for 10 minutes at 4°C. the cells were then centrifuged and the supernatant removed. Membrane perforation was achieved by the addition of 1.0 ml 0.1% Triton X-100 (Phoenix Flow Systems) for 3 minutes at 4°C followed by centrifugation and removal of the supernatant. The RNA, which, if present, would take up the fluorescent dye and result in a false DNA reading, was removed by the addition of 0.1 ml of 1.0 mg/ml Rnase and incubation for 20 minutes at 37°C. The cells were then centrifuged and the supernatant removed. The cells were then resuspended in 1.0 ml of 50 mg/ml propidium iodide (PI), a fluorescent stain that is proportionately bound to the nuclear DNA. The samples were allowed to stand for 1 hour in total darkness at 4°C. The PI is excited to fluoresce at 488 nanometers. Just prior to running on the flow cytometer, the cells from each specimen were passed through a fine insulin syringe and then filtered through a 47 micron screen (Phoenix Flow Systems) to remove any cell debris and any unbroken cell clumps.

Statistical Analysis

Recently, Misra and Easton (1999) have developed the only statistically valid protocol for analyzing CV values from flow cytometry data. Since the CV value is itself a summary statistic derived from the division of the standard deviation by the mean, an approach was required that recognized this unique feature of the collection of CV values that comprised the data set. A computer program was written by R.K. Misra to utilize the statistically well recognized weighted least squares procedure (i.e. see Johnson and Wichern, 1988) for analyzing the CV values. This procedure increases the sensitivity of the flow cytometry analysis by at least an order of magnitude in comparison to the inappropriate ANOVA and non-parametric methods formerly used.

The CV values from the chickadees were standardized by subtraction from the human lymphocyte CV internal control values. This value is referred to as DIF. The DIF value is the parameter that was analyzed using the least squares weighted procedure and is the value represented in the following tables and figures. The flow cytometry data summary for all samples analyzed is presented in APPENDIX 1.

Results

A density plot of the DIF values are shown in Figure 1, the larger the DIF value the greater is the amount of clastogenic damage. Subsequent analysis for outliers (Hoaglin *et al*, 1983) using probability plots (Figure 2) identified one anomalous data point from the abnormal treatment group which could abnormally skew the analysis in favour of a greater difference. This data point was subsequently removed before applying the least squares weighted analysis.

FIGURE 1. A jitter plot showing the distribution of the flow cytometry results for each group. The data points were jittered in order to show their actual density relationship without overlap of identical values. The data point far to the right in the abnormal group was treated as an outlier and not utilized in the weighted least squares analysis.

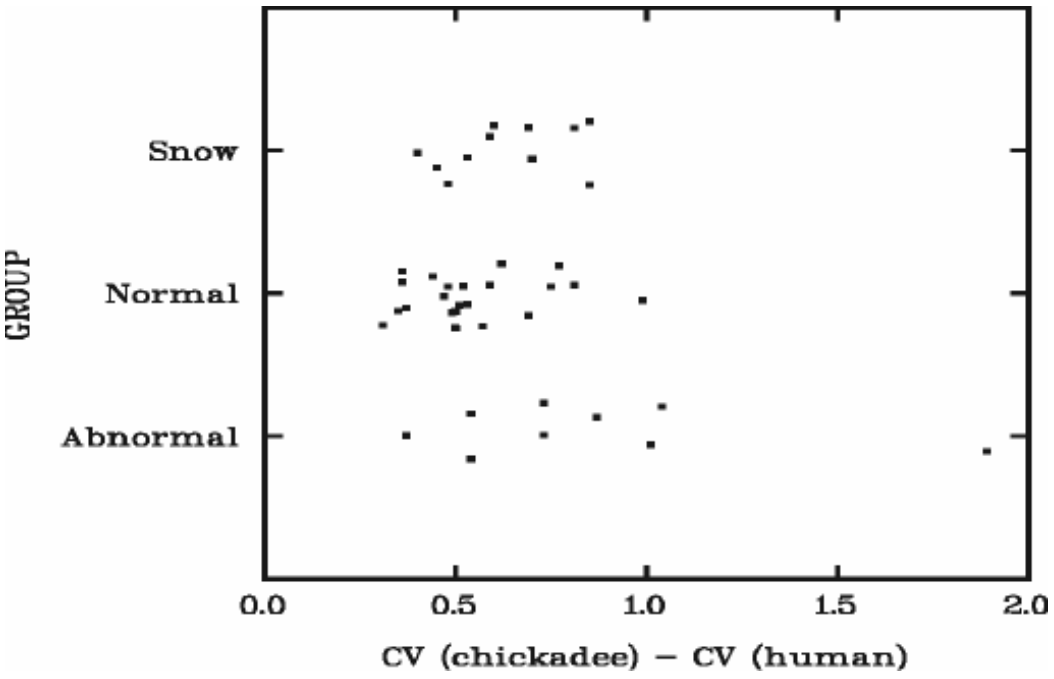
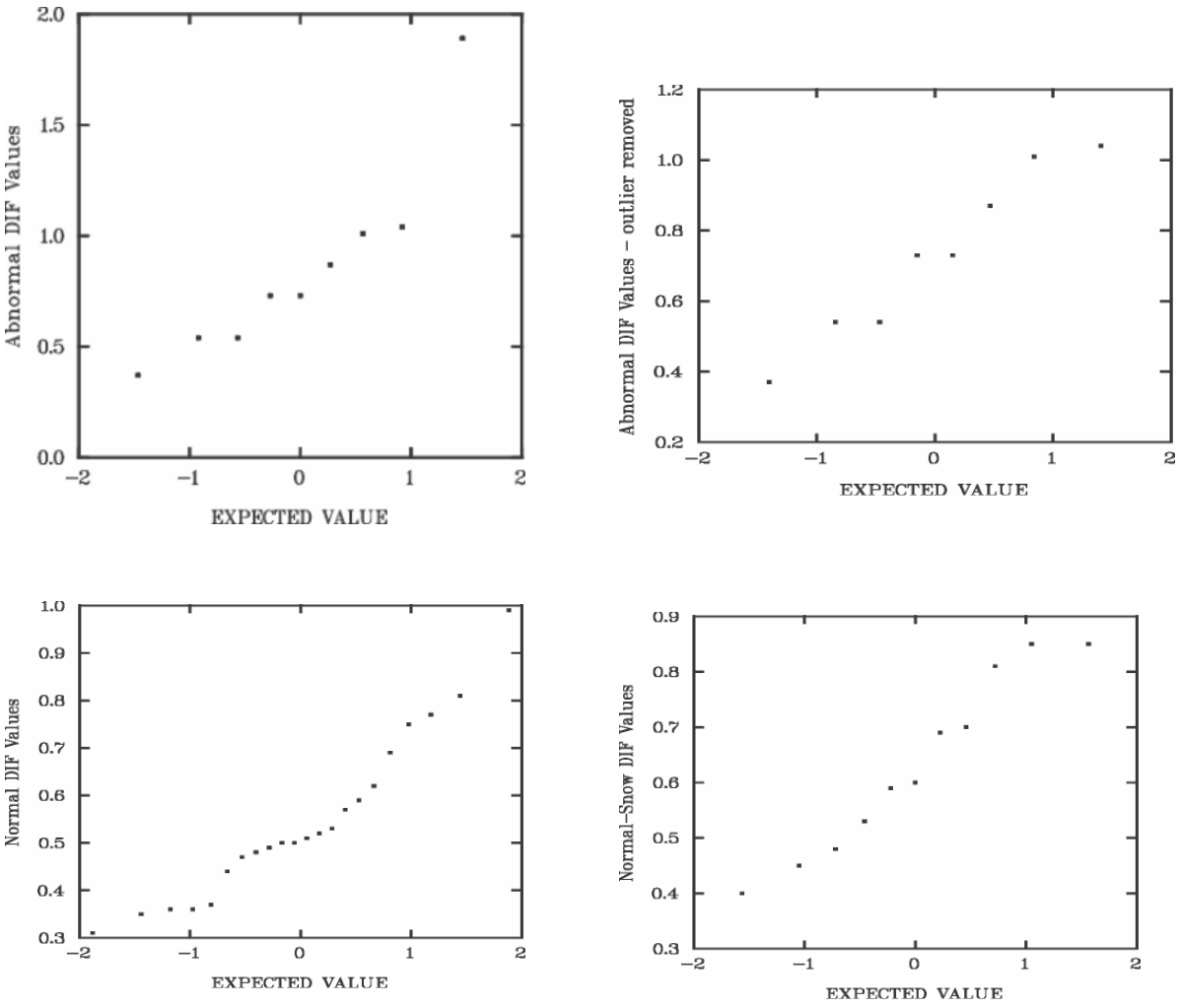


FIGURE 2. The Probability plots of the DIF data by group to isolate outlying data points for exclusion from the subsequent analysis.



The results of the weighted analysis (Table 1) indicates that there are highly significant differences between the treatment groups (X^2 =155.4 with 2 degrees of freedom). A comparison of the individual treatments demonstrates that the abnormal birds show a very high degree of red blood cell DNA damage (X^2 = 152.4, P=0) relative to the normal reference group. In addition there is a highly significant difference between the normal samples that were frozen immediately upon collection and those that were kept in snow for 4 hours prior to freezing (X^2 = 34.8, P=0). The snow samples were still significantly different than those of the abnormal birds, but the Chi-square value has diminished (X^2 = 37.6 vs X^2 =

152.4). When all the normal birds (snow + normals) are combined, the resulting Chi-square value is still highly significant ($X^2 = 101$, P=0).

TABLE 1. Results of the weighted analysis from the Alaska chickadee flow cytometry data.

Treatment	**N**	**Mean CV DIF**
Abnormal (A)	8	0.72875
Normal (N)	22	0.54455
Snow-normal (SN)	11	0.63182

Source	**Chi-square**	**DF**	**Probability**
Between treatments	155.4	2	0.00000000
A vs N	152.4	1	0.00000000
A vs SN	34.8	1	0.00000007
N vs SN	37.6	1	0.00000002
A vs N+SN	101.0	1	0.00000000

Discussion

The genetic damage found in the abnormal chickadee populations are the first reported for this type of phenomenon. Given the range of observed DNA damage in the beak-deformed chickadees, there may be variation in:

- genetic resistance to the clastogenic action of the unknown chemicals;
- dose received of the genetically active material.

The main source of variation could be determined by a regression analysis of the multifactoral quantitative chemical analysis of individual birds with the individual CV DIF values. A strong relationship with one or more chemicals would indicate a dose effect, while a non-significant relationship may indicate:

- differential genetic resistance
- or the principal clastogenic material was not effectively measured because
  - the material has been excreted from the body
  - or is still present, but not yet identified.

The difference in CV of the snow samples from the normal samples may either be:

- a function of the sample collection protocol (i.e. 4 hours in snow before freezing)

- or a function of a real difference in genotoxic effect between the Tattlow site at Palmer, Alaska and the normal samples that came from several sites in Anchorage.

The confounding of these parameters may be cleared up by the regression analysis of chemical contaminant information (or possibly another indicator of exposure such as MFO induction) collected separately for each bird and the CV DIF values for the non-snow samples.

If a relationship exists:

- then the relationship would be expected to differ between non-snow and snow samples when the collection procedure was responsible for the difference in observed CV values;
- the relationship would not be expected to differ between non-snow and snow samples when the observed genotoxic effect at the Tattlow site was real.

We can do these analyses if the appropriate chemical analysis data is available.

Implications of Genetic Damage to Blood Cells

DNA damage is its own significant endpoint.

1. The exact direct negative physiological event that may be triggered by this damage is unknown, but can be determined through further research. Perhaps the red blood cells do not have the normal variety of haemoglobins or the oxygen binding properties have been damaged which would affect flying stamina and possibly the ability to withstand cold temperature. In addition immunocompetence may be reduced because of damage to the lymphocytes.
2. One of the main consequences of mutagenicity on somatic cells is the greatly increased risk of cancer in the bird. Clastogenic activity has been shown to be closely associated with cancer incidence (IPCEMC, 1988).

3. All the above activities and others ultimately have a direct effect on the fitness of the individual and set the stage for natural selection to bring about a genetic adaptation to the genotoxic stress. The very act of responding genetically to the contaminants, is in itself a form of genetic damage.

   Selection response by its very nature is a form of controlled change in the genetic structure of the population whereby formerly rare genes become common and common genes become rarer and rare genes may be completely lost from the population. This latter event especially can reduce the population's genetic flexibility to respond to new population stressors in the future. These phenomenon have been studied in vertebrate populations using fish because of cost, convenience and generation time. Laboratory studies with fathead minnows (Diamond et al, 1995a) and mosquitofish (Boyd and Ferguson, 1964; Angus, 1983) have clearly shown that animals have the ability

to genetically respond by selection to chemical pollutants. This has been corroborated by field studies with Killifish (Weiss and Weiss, 1984) and mosquitofish (Angus, 1983). Recent work on the genetic structure of selected and unselected fish populations have shown the significant changes in gene frequency occur within these populations (Diamond et al, 1995b; Theodorakis and Shugart, 1995).

Recommendations

1. Additional statistical analysis using a multiple regression approach to relate contaminant and effect if data from individual birds is available. This analysis will require a special method to eliminate the colinearity problem with correlated contaminant values and to take advantage of the unique features of the DNA damage estimator, the coefficient of variation DIF value. Dr. R.K. Misra knows this problem well and its solution.

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APPENDIX 1 The data used in the statistical analysis.

		Black Capped Chickadee Blood				Human Blood (internal control)				
Sample	**Group**	# of Cells	Mean	S.D.	C.V.	# of Cells	Mean	S.D.	C.V.	**Difference (DIF)**
1	Abnormal	12390	200.9	4.2	2.10	4596	559.1	9.7	1.73	0.37
2	Normal	9843	195.5	4.4	2.23	5611	545.8	9.4	1.72	0.51
3	Abnormal	11637	195.1	4.4	2.24	2724	551.8	9.4	1.70	0.54
6	Normal	8947	190.1	5.0	2.64	4383	541.1	11.7	2.16	0.48
8	Abnormal	13836	174.6	5.5	3.12	1185	504.0	10.6	2.11	1.01
9	Normal	10011	194.6	5.3	2.72	3620	542.3	12.0	2.22	0.50
11	Normal	11191	189.7	5.8	3.04	2827	533.0	13.4	2.51	0.53
12	Abnormal	25943	204.1	4.8	2.33	857	562.2	8.2	1.46	0.87
14	Abnormal	12486	199.6	4.4	2.23	1503	556.3	8.3	1.50	0.73
15	Abnormal	14903	195.0	4.1	2.08	1042	550.7	7.4	1.35	0.73
16	Abnormal	24083	179.9	5.0	2.76	1744	522.2	9.0	1.72	1.04
17	Normal	10513	187.3	5.5	2.92	4292	535.6	11.3	2.11	0.81
18	Normal	15508	183.1	3.6	1.99	673	527.4	7.2	1.37	0.62
19	Abnormal	9909	194.6	7.6	3.91	2531	543.9	11.0	2.02	1.89
20	Normal	7449	171.5	5.5	3.19	2695	501.2	11.0	2.20	0.99
21	Normal	9871	206.5	5.8	2.81	2423	551.3	13.5	2.45	0.36
23	Snow	13247	195.5	4.2	2.12	1363	547.4	8.3	1.52	0.60
24	Snow	9688	187.4	4.6	2.47	1223	533.7	9.5	1.78	0.69
25	Snow	12021	195.9	5.4	2.77	2605	544.7	10.7	1.96	0.81
26	Snow	9064	188.9	5.9	3.13	3046	533.6	12.2	2.28	0.85
27	Snow	11469	192.1	6.1	3.17	1862	543.6	13.4	2.47	0.70
28	Snow	9937	211.7	5.7	2.71	2212	574.0	10.7	1.86	0.85
29	Snow	5803	197.9	5.3	2.66	2884	546.0	11.9	2.18	0.48
30	Snow	11762	203.8	5.5	2.70	2468	562.0	12.2	2.17	0.53
31	Snow	15763	210.6	4.4	2.09	1088	571.8	9.7	1.69	0.40
32	Snow	13305	202.9	5.0	2.45	1646	552.3	10.3	1.86	0.59
33	Snow	8024	194.3	5.0	2.59	2409	545.5	11.7	2.14	0.45
34	Normal	11079	186.5	4.9	2.60	1416	537.8	11.5	2.13	0.47
36	Normal	5325	196.9	7.1	3.63	4348	548.5	16.1	2.94	0.69
37	Normal	13215	198.9	4.7	2.34	826	555.2	10.1	1.82	0.52
38	Normal	2480	187.4	6.7	3.59	4562	521.3	16.2	3.10	0.49
39	Normal	2251	203.7	6.8	3.34	5178	552.0	16.5	2.99	0.35
40	Normal	4883	180.8	6.5	3.59	3360	514.4	14.6	2.84	0.75
41	Normal	6387	190.8	5.8	3.05	3566	534.6	14.6	2.74	0.31
42	Normal	24175	191.7	5.4	2.84	1296	536.9	12.1	2.25	0.59
43	Normal	10964	197.8	4.9	2.48	1686	554.5	11.0	1.98	0.50
44	Normal	9739	187.3	5.9	3.13	2980	521.5	14.4	2.76	0.37
45	Normal	8214	192.1	5.8	3.04	2685	537.9	14.4	2.68	0.36
46	Abnormal	14902	196.2	4.5	2.31	1204	546.7	9.7	1.77	0.54
47	Normal	12434	191.9	4.1	2.12	1156	539.5	8.4	1.55	0.57
48	Normal	26484	202.9	4.2	2.07	1712	555.3	9.1	1.63	0.44
49	Normal	18425	191.1	4.3	2.23	1366	539.4	7.9	1.46	0.77