LIPIDS ANALYSIS IS A SIGNIFICANT, OFTEN UNRECOGNIZED SOURCE OF UNCERTAINTY IN POPS RESULTS FOR HUMAN BLOOD

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Abstract
The use of lipid adjustments for the reporting of POPs such as PCBs and PCDD/DF in human blood samples is common practice. Small variations in lipid results can result in large variations in the lipid normalized POPs results. The most common methods of total lipids (TL) determination are gravimetric and enzymatic. Although gravimetric methodologies can yield results that are comparable to enzymatic methods, they are much more tedious, less amenable to automation, require the use of larger amounts of solvents and are very susceptible to the effects of small, seemingly minor variations in the methodology. In addition the use of human blood standard reference materials for the validation of gravimetric techniques has been very limited. During our work on a number of different studies, we have found that there has generally been a lack of recognition of the uncertainty contributed by the lipid analysis step. In this paper we present data which indicates that gravimetric techniques being used were less precise and exhibited a low bias compared to enzymatic methods. In addition, TL values from enzymatic methods may be underestimated due to issues with the summation formula that is used. We present some recommendations on how to handle these issues.

Introduction
Many persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-p-dioxin/dibenzofurans (PCDD/DF) are lipophilic in nature. Reporting the concentration results for these compounds in human serum on a lipid adjusted basis eliminates fluctuations in levels due to post prandial effects etc. [1]. Expressing the results for these compounds on a lipid adjusted basis may also permit comparison of different matrices (e.g. serum vs. plasma vs whole blood) in humans. The use of lipid adjustment is considered to offer some way of comparing different sample groups, although there are still numerous issues which must be considered [2].

Most laboratory lipid determinations in other biological matrices (fish, plants etc.) are based on the gravimetric method of Folch [3] or modifications to this method [4]. Gravimetric methodologies have been criticized as being flawed for some applications and are susceptible to errors with human blood due to issues such as the polarity of the solvent. Gravimetric methods have also been reported to be unreliable for sample sizes of less than 5 g. For human blood, enzymatic summation methods are also used. Most laboratories utilizing enzymatic methods report TL based on "long form" or "short form" equations[1]. Recently several alternative summation formulas have also been proposed which may also impact the total lipids number produced. Comparisons carried out by the US Centers for Disease Control (CDC) between gravimetric and enzymatic summation methodologies indicates that, under properly controlled conditions, both methods can provide comparable data[5]. However the availability of automated enzymatic methods and the reduction in labor, laboratory equipment, solvents, space, and typical errors attendant to performing the gravimetric methodologies has resulted in extensive (nearly universal) use of the enzymatic methods in human clinical laboratories. All lipids results for the NHANES data are produced using the enzymatic summation method[2]

Since the calculation of lipid adjustment involves dividing the POPs result, on a wet weight basis, by a small number (TL<0.01%), small variations in the lipid values will result in larger variations in the lipid adjusted concentrations reported [2]. Therefore the impact of method used to determine the TL number needs to be considered, so as to allow for scientifically credible comparisons and decisions to be made.

Materials and Methods
We have analysed two sets of data where both enzymatic and gravimetric analysis were both performed on the

same human serum samples. The Arctic Monitoring and Assessment Program (AMAP) sponsors the "Ring Test" for laboratories three times per year for certain POPs [6]and has been including lipids since at least 2004. We analysed the TL data for the last 10 rounds (2004-1,-2, -3; 2005-1,-2,-3; 2006-1,2,-3; 2007-1) of the Ring Test on a combined and stratified by methodology basis to determine if there were any statistical differences. In the other data set, from human serum of 10 persons in USA, lipids determined by an environmental laboratory using gravimetric methodology, were compared with enzymatic method results produced using several different summation equations. All data were analysed using standard statistical software (SAS version 9.1.3).

Results and Discussion

The data in table 1 summarizes the mean values relative to the assigned values on the following basis: all data, all data for which the methodology was identified, gravimetric methods and enzymatic methods. Figure 1shows a plot of data for the participating laboratories relative to the assigned value. It is clear from this data that 1) there is a difference between laboratories using enzymatic and gravimetric methods, 2) gravimetric methods exhibit a low bias relative to the assigned value, and 3) gravimetric methods exhibit a much larger variability than the enzymatic methods, especially within certain laboratories. The results from this data can be used by the individual laboratory to determine their measurement uncertainty (MU) for the lipid analysis component, and its contribution to the overall MU for the lipid normalized value of any POPs. While determination of MU is a requirement of ISO 17025 we believe it is simply good scientific practice.

Table 2 contains a summary of the total lipids for the same group using gravimetric and enzymatic methods, and different summation equations for the enzymatic results. Table 3 summarizes the differences observed for the Gravimetric compared the enzymatic results using the Rylander equation or the "short form" equation from Phillips. The data in Table 3 indicates that the gravimetric method used by Lab A was biased low in the Range of 30 to 45%. This means that even in the event of perfect agreement on POPs measured on a wet weight basis, these data would be biased high on a lipid normalized basis. In the event these data were attempted to be compared to reference values such as those in the National Exposure Report (NER)[7] they could be considered misleadingly high.

For gravimetric methods it appears that many laboratories have not been able to fully validate their method due a lack of reference materials [8]. However enzymatic methods have been using control serum samples [9]and there at least 2 SRMs available from NIST with certified values for lipids [10,11].

We have several recommendations to address this issue. For the enzymatic methods the determination of total lipids using the “short form” equation, at a minimum is recommended. For gravimetric methods laboratories must provide initial and ongoing objective evidence of method performance using SRMs and QC check samples. All laboratories should determine the contribution of the lipid analysis step to the measurement uncertainty for POPs analysis.
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Table 1: Stastical Summary of AMAP Ring Test Total Lipids Results 2004-2007

	All Data	All Data with Method Info	Gravimetric Only	Enzymatic only	No Method Identified
Mean (% Difference)	-0.02	-0.17	-4.66	6.99	0.54
Std Dev	16.53	16.44	18.28	9.32	16.95
95% CI	1.70	1.90	2.69	1.73	3.84
CV	-80327.18	-9844.58	-392.50	133.29	3128.85
N	363.00	288.00	177.00	111.00	75.00
Median	0.00	0.00	-5.66	7.12	0.00
Min	-63.64	-63.64	-63.64	-20.48	-54.29
Max	60.38	60.38	60.38	29.04	53.97
Range	124.01	124.01	124.01	49.52	108.25

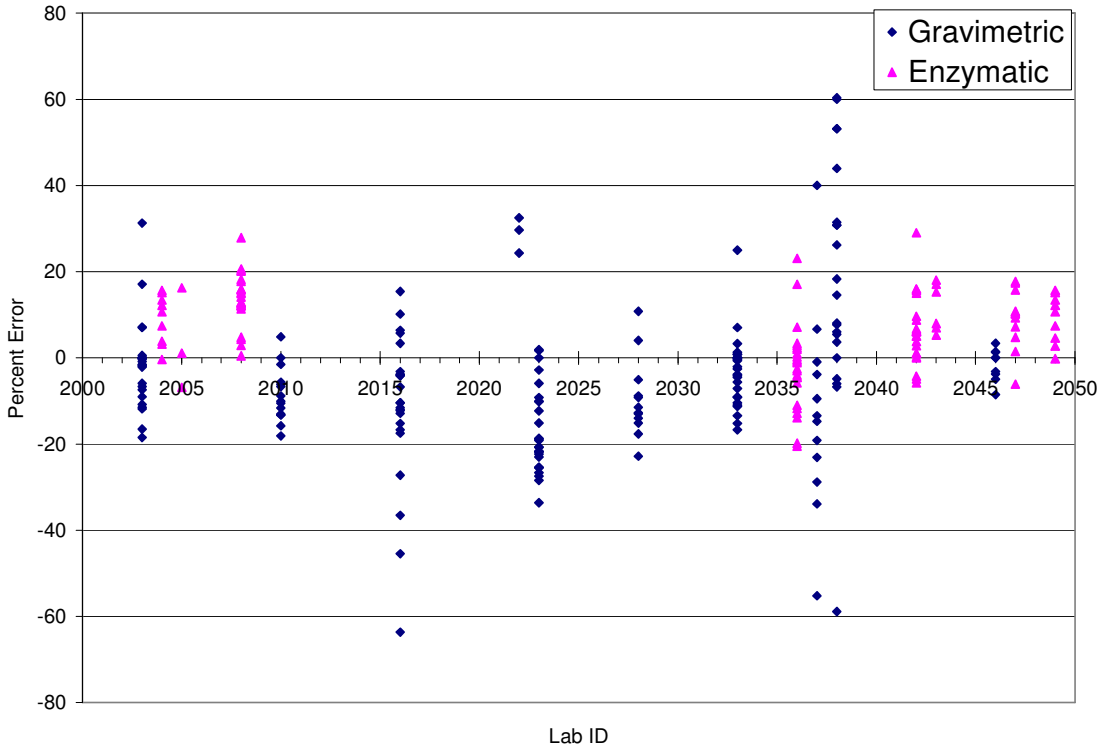

Figure 1: gravimetric and enzymatic methodology results for AMAP, round-robin samples

Table 2: TL Data for a Study Group

Person ID	Lab A % Lipids (gravimetric)	Lab A Total lipids (mg/ml) Gravimetric	Lab B Cholesterol (mg/dl) enzymatic	Lab. B Triglycerides (mg/dI) enzymatic	Lab. B Total lipids (mg/ml) Rylander	Lab B Philips short form
1	0.405	4.05	258	104	5.61	7.5196
2	0 342	3.42	193	102	4.74	6.0241
3	0.351	3.51	170	146	5.00	5.942
4	0.305	3.05	146	82	3.86	4.7572
5	0.393	3.93	214	153	5.67	7.0108
6	0.249	2.49	207	103	4.93	6.3519
7	0.449	4.49	242	186	6.46	7.9764
8	0.268	2.68	182	29	3.64	5.0444
9	0.288	2.88	148	49	3.46	4.4726
10	0.371	3.71	236	74	4.93	6.7202
normal range Value Used	0.4-0.9		100-199	0-149		

Table 3:Summary of Differences Between Gravimetric vs Enzymatic Methods and Summation Equation (for Table 2)

	Lab A Gravimetric as % of Rylander	Lab A Gravimetric as % of Phillips Short Form
average	71.50	55.81
min	50.51	39.20
max	83.24	64.39
range	32.73	25.19
median	72.17	56.17
sdev	8.595318539	7.005346166
RSD	12.02141131	12.55233982
N	10	10