Collaborative Test of Determination of Iodine Value in Fish Oils. 1. Reaction Time and Carbon Tetrachloride or Cyclohexane as Solvents

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ABSTRACT: Twenty-two laboratories participated in a collaborative test to determine the iodine value (IV) of eight samples of fish oil (four with IV < 150, four with IV > 150) with either carbon tetrachloride (AOCS Official Method Cd 1-25) or cyclohexane (AOCS Recommended Practice Cd 1b-87) as solvent and either 1 or 2 h of reaction time. Laboratories received coded duplicate samples (hidden duplicates) and carried out duplicate determinations on each oil by each solvent–time combination (open duplicates). Replacing carbon tetrachloride with cyclohexane resulted in a lower IV ($P < 0.001$). The decrease averaged 1.6 IV units for low-IV oils and 3.8 IV units for high-IV oils; this difference in response of 2.2 IV units between low- and high-IV oils was significant ($P < 0.001$). Increasing the reaction time had a relatively small effect (0.34 ± 0.18). There was no interaction of reaction time with solvent or oil type. Cyclohexane caused emulsions, which made it difficult to titrate residual iodine and thus increased the variability of the determination. The repeatability standard deviations (s_r), based on hidden duplicates, for 1-h reaction time with carbon tetrachloride and cyclohexane were 2.17 and 3.35, respectively. The corresponding reproducibility standard deviations were 2.73 and 4.53.

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KEY WORDS: Carbon tetrachloride, collaborative test, cyclohexane, fish oil, iodine value.

American Oil Chemists' Society (AOCS) methods are widely used for contract purposes in the trading of oils and fats. The traditional method (1) for determining iodine value (IV: AOCS Official Method Cd 1-25, corrected 1991) prescribes the solvent carbon tetrachloride. In a number of countries, this solvent is now banned for use in laboratories because of its carcinogenic properties. Consequently, this method of analysis has been modified to use first cyclohexane (2) (AOCS Recommended Practice Cd 1b-87, revised 1990), and more recently, cyclohexane–acetic acid (3) (AOCS Recommended Practice Cd 1d-92) as solvents.

In addition, both the traditional and the two newer methods specify that the reaction time with Wijs solution should be either 1.0 or 2.0 h, depending on the iodine value of the sample: IV less than 150, 1.0 h; IV equal to or greater than 150, 2.0 h. Unfortunately, many fish oils have values close to 150, necessitating that the analyst guess an expected IV to choose the correct time, and if this choice proves to be wrong, to repeat the analysis. These times are also specified in ISO (4), IUPAC (5), and AOAC (6) methods. Earlier versions of the Wijs method specified a reaction time of 30 min with a note that a longer time may be necessary for high-IV oils. A preliminary collaborative study of two fish oils, representing low and high IV, in two laboratories established that, while 30 min was definitely inadequate, there was no consistent difference between 1- or 2-h reaction times (A.P. Bimbo and S. Thorisson, unpublished data). Berner (7) reported a small study of four fish oils in which carbon tetrachloride and cyclohexane were compared as solvents (reaction time unspecified) and concluded that there may be concern about the use of cyclohexane for samples with an IV greater than 100, especially for fish oils. Berner (8) gave a preliminary summary of an ISO/IUPAC collaborative study to compare carbon tetrachloride and cyclohexane–acetic acid with 1-h reaction time (except for fish oil and tung oil where the time was not stated but is presumed to be 2 h in expectation of values in excess of 150) and concluded that they produced excellent agreement. However, the one fish oil used was atypical, with a low IV of 109. Firestone (6) gave the same results as method performance data in support of the new cyclohexane–acetic acid method, specifying the use of 1.0 or 2.0 h depending on the IV of the sample.

The purposes of the collaborative test on IV were: (i) to assess whether the final results obtained on various commer-

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cial fish oils were the same with either carbon tetrachloride or cyclohexane as solvent, and, if not, to measure the difference, and (ii) to see whether the use of different reaction times, depending on IV, was justified with the range found in normal commercial fish oils.

The list of participating laboratories is recognized in the Acknowledgments section. But this list does not correspond to the laboratory numbering in the Tables.

EXPERIMENTAL PROCEDURES

The work reported here was part of an international collaborative study, organized by the International Fishmeal and Oil Manufacturers Association (IFOMA). Twenty-five laboratories participated in the collaborative test.

Each participating laboratory received from the distribution center in the USA eight samples of oil and was asked to analyze each sample in duplicate (open duplicates). Each sample consisted of 57 g in a sealed amber glass bottle. Each laboratory was asked to analyze the eight samples with carbon tetrachloride (Cd 1-25) and cyclohexane (Cd 1b-87) as solvent, and for both methods to use reaction times of 1.0 and 2.0 h. Detailed protocols for methods Cd 1-25 and Cd 1b-87 were sent to all laboratories. For Cd 1-25, the following changes were made to the revised 1991 method: procedure item 2 allowed an alternative use of a weighing bottle and stated that the carbon tetrachloride was added after weighing the sample; procedure item 3 specified the use of 1.0 or 2.0 h without reference to expected IV and stated that the flasks were to be stored in the dark at 25 ± 5ºC; Table 1 sample weights for IV ≥ 80 were corrected (values for 150 and 100% excess are reversed). For Cd 1b-87, procedure 1 omitted the optional use of an oven at 100°C while filtering because this would enhance oxidation; similarly, procedure 2 of equilibration to 68–71°C before weighing was omitted; Table 1 sample weights for IV ≥ 80 were corrected; procedure 6 was changed to use two blanks; procedure 7 was changed to specify that sample and blank flasks were stored in the dark at 25 ± 5ºC for 1.0 or 2.0 h. Laboratories were asked to keep the samples in a freezer and in the dark before and between analyses. Unknown to recipient laboratories, each received only four samples of oil but in hidden duplicate.

The sample distribution center obtained eight primary samples of fish oil, four selected to be low in IV (<150) and four selected to be high in IV (>150). The low-IV oils were: 1, sand eel; 2, herring; 3, capelin; and 4, menhaden stearine. The high-IV oils were: 5, mackerel; 6, anchovy; 7, pilchard plus menhaden (blend); and 8, menhaden.

The eight primary oils were distributed to participating laboratories (two low- and two high-IV oils to each) according to a statistical pattern designed to give overall balance to comparisons between the oils. Each pair of laboratories represented a complete set of the eight oils. Had all 31 laboratories, initially contacted, responded with successful results, there would have been four or five comparisons within a laboratory of each low-IV oil with every other low-IV oil, and similarly four or five comparisons within a laboratory of each high-IV oil with every other high-IV oil. Because of the irregular return of the completed forms by the laboratories, the balance finally achieved averaged 3.8 (SD ± 1.17) comparisons within laboratory for low-IV oils and 3.8 (SD ± 0.75) comparisons within laboratory for high-IV oils. Each of these comparisons is based on the mean of two determinations (two hidden duplicates using the first reported value of the open duplicates).

RESULTS

Initial screening of data for outliers. Three laboratories returned incomplete data and were omitted from the analysis. The remaining data were scrutinized for obvious errors, checked with the supplying laboratory, and corrected where appropriate. For each of the eight oil samples in each laboratory, mean values (average of open duplicates × two solvents × two times), together with the Solvent, Time, and Solvent × Time Interaction Effects for each oil in each laboratory were calculated. Histograms of the 176 paired comparisons are given in Figure 1 for the Solvent Effect, in Figure 2 for the Time Effect, and in Figure 3 for the Solvent × Time Interaction Effect. All histograms indicate the occurrence of a few extreme values that might be regarded as anomalous outliers. The distribution of the Solvent Effect was skewed, there being relatively larger differences where carbon tetrachloride returned the higher determination. The distribution of the Time Effect was fairly symmetrical, as was the distribution of the Interaction Effect.

A principal component analysis using the data and four parameters of mean IV and the three effects shown in Table 1 was carried out, with the result shown in Figure 4. Principal component analysis is a statistical technique for compressing the maximum amount of information, contained in several (this study, four) variates, into fewer, such as two, dimensions to display the relative position and proximity of the individuals (laboratories). Figure 4 indicates that laboratories 2, 4, 9,

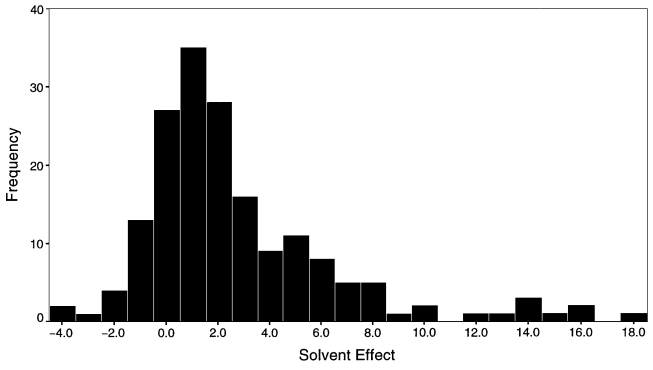

FIG. 1. Histogram of the frequency distribution of the Solvent Effect (Cd 1-25 – Cd 1b-87) on iodine value in eight samples analyzed in 22 laboratories.

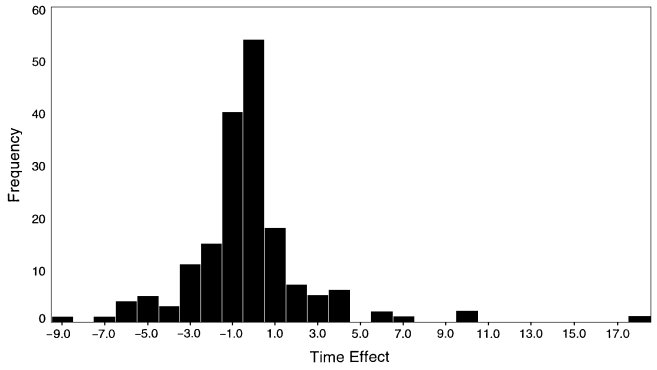

FIG. 2. Histogram of the frequency distribution of the Time Effect (1 h – 2 h) on iodine value in eight samples analyzed in 22 laboratories.

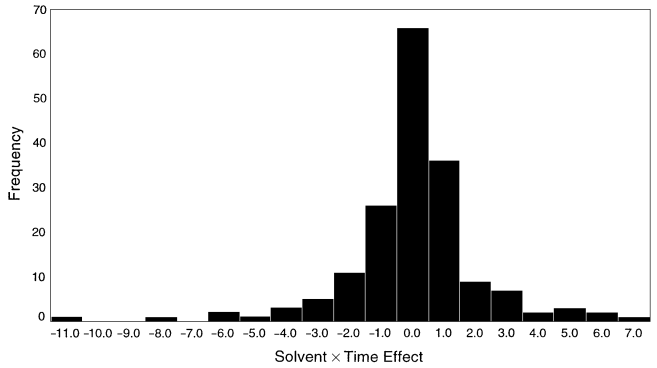

FIG. 3. Histogram of the frequency distribution of the interaction of Solvent by time effect on iodine value in eight samples analyzed in 22 laboratories.

and 18 were rather removed from the grouping of the other laboratories. Inspection of Table 1 shows that laboratories 2 and 4 had large Solvent Effects, laboratory 9 had a large positive Time Effect, i.e., values after 2-h of reaction were less than after 1 h, and laboratory 18 had a large Interaction Effect. Laboratory 2 had large Solvent Effects for seven out of the eight oils; laboratory 4 similarly had large Solvent Effects for six oils. Laboratory 9 had one large positive Time Effect value for oil No. 5, but also five other values were high and positive. Laboratory 18 had one large Interaction Effect for oil No. 8 because of low values in both open duplicates with cyclohexane at 2 h, compared with both cyclohexane at 1 h and carbon tetrachloride at 1 or 2 h.

A principal component analysis was also carried out on the standard deviations of a single determination (see Table 2), based on the differences between hidden duplicate oils for each of the four analyses (2 solvents × 2 reaction times), with the result shown in Figure 5. This identifies laboratory 13, in addition to laboratories 2 and 9, as having a consistently greater variation between the hidden duplicates in all four procedures.

Analysis of variance was carried out on all data and also after omitting laboratories 2, 4, 9, 18, and 13 as outliers in terms of one or more values or because of greater variability. Omitting the outlier laboratories did not change the overall conclusions, so the data presented are based on all laboratories without exclusion of any individual data points. Where omitting the outlier laboratories resulted in a change in statistical significance, this is indicated.

Main treatment effects. Table 1 gives the least-square

TABLE 1
The Estimated Mean Iodine Value, Averaged over Solvent and Time, Together with the Solvent Effect [Cd 1-25 (carbon tetrachloride) minus Cd 1b-87 (cyclohexane)], Time Effect (1 h minus 2 h), and the Interaction Effect of Solvent with Time for each Laboratory[a]

Laboratory code	Mean value	Solvent effect	Time effect	Interaction
4	148.17	8.347	−1.554	0.658
19	149.90	4.389	−1.501	0.324
16	151.45	2.295	−1.278	1.242
13	151.58	0.617	−0.506	−0.002
2	151.60	8.346	1.044	−1.458
10	152.51	3.440	0.394	1.256
17	152.58	4.826	−0.023	−0.332
6	152.73	4.194	−0.151	1.142
18	152.98	3.844	1.497	−2.191
7	153.05	1.338	−0.172	−0.078
24	153.27	5.030	−0.322	0.287
11	153.75	3.902	−0.657	0.086
15	153.88	2.466	−0.703	−0.409
21	153.99	1.003	−0.966	0.203
1	155.02	−0.370	−2.240	−0.549
5	155.12	1.360	0.368	−0.354
9	155.44	3.453	5.011	−0.020
3	155.50	0.173	−1.231	−0.399
12	155.83	1.124	−0.859	−0.432
20	156.02	0.720	−2.896	1.442
8	156.50	−1.066	−1.272	−0.166
14	156.78	0.587	0.535	−0.072

[a]The laboratories are ranked by the mean iodine value.

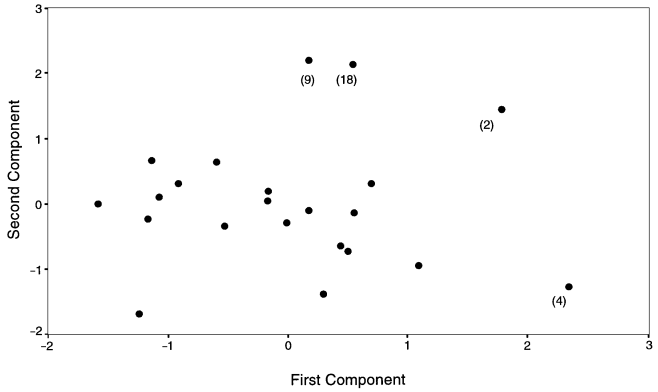

FIG. 4. Plot of the first two components in a principal component analysis of the distribution of laboratories in terms of their mean iodine value. Solvent, Time, and Solvent by Time (interaction) Effects displayed in Table 1. The laboratory numbers have been appended for the outliers.

means of IV for the 22 laboratories, together with the Solvent Effect (the difference calculated as Cd 1-25 minus Cd 1b-87) and the Time (1 h minus 2 h) and Solvent × Time Interaction Effects. The laboratories have been ranked in order of their mean IV. These mean values have been adjusted for differences between the different samples analyzed, and the values therefore reflect the true differences between the laboratories. Although it would be unwise to carry out multiple comparisons among the laboratories, a guide to the importance of the differences between these means is given by their standard error of approximately 1.14.

Method Cd 1-25 (carbon tetrachloride) gave significantly ($P < 0.001$) higher values than method Cd 1b-87 (cyclohexane). The mean Solvent Effect with its standard error was 2.7 ± 0.16. For individual laboratories, the Solvent Effect varied from −1.1 to +8.3 (Table 1). The mean values for all eight oils are shown in Table 3. The size of the solvent effect differed ($P < 0.001$) between oils, with the greatest effect for the oils selected as high-IV (oils 5 to 8). On average, the Solvent

TABLE 2
Standard Deviations of Single Determinations, as Calculated from the "Hidden Duplicates," Together with the Within-Laboratory and Between-Laboratory Components of Variance, and Pooled Estimates of Repeatability and Reproducibility[a]

	Cd 1-25 (carbon tetrachloride)		Cd 1b-87 (cyclohexane)	
Laboratory code	1 h	2 h	1 h	2 h
10	0.29	0.84	2.48	3.21
12	0.32	3.66	2.34	2.18
7	0.40	0.72	0.36	1.00
11	0.47	1.01	3.91	3.89
4	0.80	1.08	5.13	3.68
24	0.82	0.66	1.12	1.08
17	0.94	0.87	3.06	2.76
15	0.96	0.75	0.79	0.50
5	0.97	0.76	1.55	0.89
16	0.99	1.09	4.94	2.15
6	0.99	1.31	6.60	2.04
3	1.24	0.56	1.20	1.26
19	1.40	2.41	1.86	1.36
21	1.49	0.91	0.31	0.92
20	1.67	0.68	0.80	0.16
8	1.85	0.86	0.71	1.31
1	2.00	1.48	0.66	1.17
18	2.01	0.60	3.52	6.21
2	3.08	2.97	7.16	5.81
14	3.22	1.06	2.03	1.27
9	4.95	4.99	2.15	3.85
13	5.70	5.52	5.67	5.30
Components of variance				
Within Laboratory	4.70	4.46	11.19	8.52
Between Laboratories	2.75	2.17	9.33	9.13
Repeatability standard deviation (s_r)	2.17	2.11	3.35	2.92
Relative repeatability standard deviation (RSD_r)	1.40	1.36	2.20	1.90
Reproducibility standard deviation (s_R)	2.73	2.57	4.53	4.20
Relative reproducibility standard deviation (RSD_R)	1.76	1.66	2.98	2.75

[a]Laboratories are in rank order of their precision of determination of IV by Cd 1-25 after 1 h.

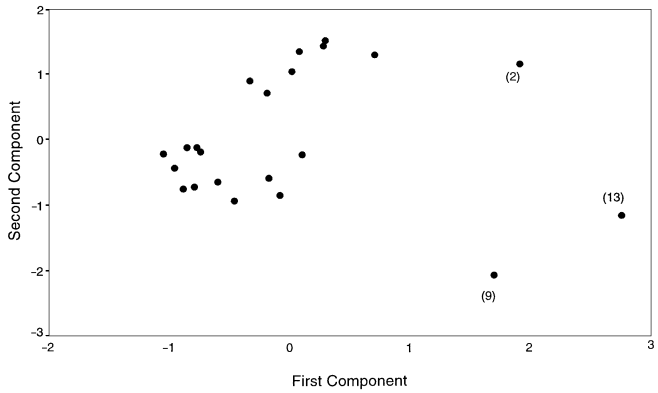

FIG. 5. Plot of the first principal component (x axis) against the second principal component (y axis), where the original variables were the within-laboratory errors for iodine value displayed in Table 3. The laboratory numbers have been appended for the outliers.

Effect was 2.2 ± 0.34 units greater with the high-IV than with the low-IV oils ($P < 0.001$). The biggest difference between solvents (+6.4) was seen with oil 8, the menhaden oil, although this was not the oil with the highest IV. Excluding the five outlier laboratories reduced the mean difference due to solvent to 2.1 ± 0.16, and the difference in Solvent Effect between high- and low-IV oils to 1.4 ± 0.34, but both differences were still significant at $P < 0.001$.

Increasing reaction time from 1 to 2 h increased the IV on average (mean of both solvents) by 0.34 ± 0.18 IV units. This effect, although small, bordered on being significant, and when the outlying laboratories were omitted, the effect became significant (0.80 ± 0.139, $P < 0.001$). There was no significant Solvent × Time Interaction Effect (mean, 0.008 ± 0.154). Differences due to reaction time (averaged over both solvents) did not vary in any systematic way with increasing IV of the samples (Table 3). Inspection of the treatment combination means in Table 3 further illustrates that increasing reaction time with cyclohexane does not increase the value for high-IV oils, where the solvent effect was greatest, up to that obtained with carbon tetrachloride for 1 h, the mean value for the latter being 3.6 IV units greater than that obtained with cyclohexane for 2 h.

Repeatability and reproducibility. Estimates of the standard deviations of single determinations in each laboratory, as computed from the "open duplicates," were calculated. Compared with the standard deviations based on the hidden duplicates (Table 2), many of these figures were low, up to a factor of one-tenth, and they cannot be regarded as reliable estimates of experimental error. The pooled repeatability standard deviations from the open duplicates were 1.05, 1.07, 1.46, and 1.68 for Cd 1-25 after 1 and 2 h and Cd 1b-87 after 1 and 2 h, respectively.

The standard deviations of single determinations in each laboratory, as estimated from the "hidden duplicates," are given in Table 2. The laboratories are listed approximately in rank order of the precision of determination of IV. This Table also gives the pooled estimate of the within-laboratory variance (s_e^2), the repeatability standard deviation (s_r), repeatability relative standard deviation (RSD_r), the between-laboratory component of variance (s_L^2), the reproducibilty standard deviation (s_R) of a single determination at a randomly chosen laboratory $(s_e^2 + s_L^2)^{0.5}$, and the relative reproducibility standard deviation (RSD_R), calculated as $100(s_R$/mean value of the determination).

The within-laboratory variances were on average 2.15 times greater for cyclohexane than for carbon tetrachloride, but there were considerable differences between laboratories, and those laboratories in the top part of the table that achieved

TABLE 3
Mean Iodine Value for Each Oil and Each Solvent and Time Combination, Together with Estimates of the Solvent Effect (Cd 1-25 minus Cd 1-897b), the Time Effect (1 h minus 2 h), and the Solvent X Time Interaction

Oil	Mean					Treatment effects		
	Cd 1-25		Cd 1b-87					
	1 h	2 h	1 h	2 h	Mean	Solvent	Time	Interaction
Low:								
1	137.8	138.1	135.3	136.4	136.9	2.13	–0.69	0.40
2	117.8	118.3	116.1	116.5	117.2	1.72	–0.45	–0.08
3	123.7	123.9	122.7	123.0	123.4	0.94	–0.22	0.03
4	137.2	137.2	135.0	135.8	136.3	1.79	–0.41	0.38
High:								
5	168.2	168.0	165.1	165.2	166.6	2.95	0.07	0.12
6	194.1	194.6	191.1	192.0	193.0	2.84	–0.66	0.29
7	185.1	186.6	183.0	182.6	184.3	3.04	–0.55	–0.99
8	173.8	173.7	167.5	167.2	170.6	6.41	0.20	–0.10
Mean	154.7	155.1	152.0	152.3	153.5	2.73	–0.34	0.008
SEM						0.158	0.184	0.157
P value for mean effect						<0.001	0.067	N.S.[a]
High vs. Low						2.17	0.21	–0.35
SEM						0.338	0.394	0.337
P value for difference between high and low oils						<0.001	N.S.	N.S.

[a]N.S., not significant.

the best precision were able to achieve similar low variabilities with both solvents. The between-laboratory component of variance was similar to or smaller than the within-laboratory component, but again it was 3.75 times greater with cyclohexane. When these estimates of variance were combined and expressed as RSD_R, the value for cyclohexane (average for 1 h and 2 h) was 1.67 times greater than that for carbon tetrachloride. There appears to be a measure of correlation between the standard deviations of each laboratory for the two solvents. Kendall's Rank Correlation statistic was estimated as 0.32 ($P < 0.05$). In particular, several laboratories returned fairly high errors for both solvents.

DISCUSSION

The new method (Cd 1b-87) that prescribes cyclohexane gave an IV that was 2.7 units lower when averaged over all eight primary oils. However, the difference was greater with oils selected as having an IV >150, namely 3.8, compared with oils of IV < 150, where the difference was 1.6. Omitting five outlier laboratories reduced these differences to 2.8 units for oils with IV > 150 and 1.4 units for oils with IV < 150. A number of laboratories observed virtually no difference between the two methods (a mean difference over the eight oils of less than one unit in either direction; see Table 1 for Solvent Effect and Table 2 for standard deviations). A difference of 5.7 units, with cyclohexane giving the lower result, was observed in a small study with four fish oils with IV in the range 165 to 185 (4).

Ten participants noted that the endpoint of the titration was more difficult to determine with cyclohexane as solvent. The samples formed an emulsion, so more vigorous shaking and a longer time were needed for complete titration. Two laboratories reported that this led to a greater chance of overshooting the endpoint, and consequently to a lower calculated IV. The greater variability and generally lower IV values with cyclohexane as the sole solvent suggest that the problem is due to difficulty in determining the endpoint, with a tendency to overtitrate to ensure completion. Where laboratories take great care, as evidenced by low standard deviation from the hidden duplicates in both methods (good repeatability), comparable values can be obtained by both methods. However, one participant noted that a 1:1 mix of cyclohexane/glacial acetic acid works better than cylohexane alone. Consequently, a subsequent collaborative trial to determine bias, repeatability, and reproducibility of 1:1 cyclohexane/glacial acetic acid, compared with carbon tetrachloride, was conducted (9) to confirm this observation.

Although it was possible to detect that increasing the reaction time from 1 to 2 h increased the IV for both solvents, the increase at 0.3 to 0.8 units is negligible compared with variability of even the best method. For example, using the RSD_R of 1.76% for carbon tetrachloride, the 95% confidence limits for an oil of IV 150 analyzed once in a randomly selected laboratory are 144.8–155.2. For most purposes, 1 h reaction time would suffice.

Open duplicates agreed much more closely than the hidden duplicates, resulting in false low estimates of within-laboratory variances. Not only can the true within-laboratory variation only be determined in collaborative trials by analyzing hidden replicates but also laboratories routinely should not place reliance on agreement of duplicates run side by side. A better procedure would be to analyze a series of samples once, and then to repeat the analysis of the series on a second occasion as independently as possible.

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