Collaborative Test of Determination of Iodine Value in Fish Oils. 2. Carbon Tetrachloride or Cyclohexane/Acetic Acid as Solvents

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ABSTRACT: Nine 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/acetic acid (AOCS Recommended Practice Cd 1d-92) as solvent and 1 h of reaction time. Laboratories received coded duplicate samples (hidden duplicates) and carried out duplicate determinations on each oil by each method (open duplicates). Replacing carbon tetrachloride with cyclohexane/acetic acid resulted in similar mean values for both low- and high-IV oils and similar estimates of repeatability and reproducibility. The repeatability standard deviation (s_r), based on hidden duplicates, with carbon tetrachloride and cyclohexane/acetic acid were 1.71 and 1.55, respectively. The corresponding reproducibility standard deviations were 1.81 and 1.98.
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KEY WORDS: Acetic acid, carbon tetrachloride, collaborative test, cyclohexane, fish oil, iodine value.

American Oil Chemist's 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 a previous collaborative test (4), cyclohexane was shown to give significantly lower values. It was postulated that this was due to difficulty in determining the endpoint of the titration of an emulsion. A preliminary observation indicated that this problem could be avoided by the use of 1:1 cyclohexane/acetic acid as the solvent. The purpose of this new collaborative test was to compare the traditional method with a method that prescribes this solvent mixture and to provide estimates of repeatability and reproducibility.

Both the traditional and the two newer methods specify that the reaction time with Wijs solution should be either 1.0 h 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. These times are also specified in ISO (5), IUPAC (6), and AOAC (7) methods. In the previous study (4), increasing the reaction time from 1 to 2 h had little or negligible effect, irrespective of IV. Consequently, the new test was conducted with 1 h reaction time only. Berner (8) gave a preliminary summary of an ISO/IUPAC collaborative study in which carbon tetrachloride and cyclohexane/acetic acid were compared after 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 (7) gives 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 present study extends the work of Firestone (7) to cover the whole range of values typical of fish oils.

The list of participating laboratories is recognized in the Acknowledgments section.

EXPERIMENTAL PROCEDURES

The work reported here was part of an international collaborative study, organized by the International Fishmeal and Oil Manufacturers Association (IFOMA). Ten laboratories received from the distribution center in the United States eight samples of oil and were asked to analyze each sample in duplicate (open duplicates). Nine laboratories participated in the collaborative test. Laboratories also received a report sheet

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with columns, set out for the two determinations labeled A and B on each sample and by each method. A column for any additional determinations was also set out. 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/acetic acid (Cd 1d-92) as solvents, and for both methods to use a reaction time of 1.0 h only. Detailed protocols for methods Cd 1-25 and Cd 1d-92 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 h 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 1d-92, 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; procedure 5 specified 1 h of reaction time only; Table 1 sample weights for IV 80 were corrected; procedure 9 was changed to use two blanks. Laboratories were asked to keep the samples in a freezer and in the dark before and between analyses. Unknown to the recipient laboratories, each received only four samples of oil but in hidden duplicates.

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 oil samples were the same as those used in the earlier collaborative test (4).

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. The design was such that within a 12-laboratory group every low-IV oil would be compared with every other low-IV oil twice within a laboratory, and similarly for the high-IV oils. Because only seven laboratories submitted acceptable results, the statistical distribution was not complete.

Statistical analysis was carried out in several steps. The data were initially scrutinized for possible gross errors. For each analytical method, the standard deviation for the open duplicates was calculated from the differences between the pairs of values, separately for each laboratory and then combined over all laboratories. From the first reported analysis (A) of the open duplicates, differences between the hidden duplicates were calculated for each laboratory from which the repeatability standard deviations within each laboratory and pooled over laboratories were calculated. This analysis was repeated with the second reported open duplicate (B). From the average of the A and B determinations, the estimated laboratory mean values, corrected for design imbalance in the samples analyzed, were derived from a nonorthogonal analysis of variance by using the algorithm GENSTAT (9). The same type of analysis, but employing in turn the A and B determinations, provided estimates of the between-laboratory standard deviation, i.e., reproducibility. In evaluating error estimates, separate analyses were deemed necessary because of the lack of independence of the A and B determinations. The presence of outlier laboratories was tested by using the Cochran test for a laboratory with a high laboratory variance (hidden duplicates) and the Grubbs test for extreme deviation of laboratory means, according to IUPAC (10). In the latter test, mean values of the four oils, adjusted for differences in the oils, were used instead of carrying out the test on each material one at a time because not all laboratories analyzed the same material. The two methods were compared by computing the differences within laboratory and sample, followed by analysis of variance of these differences. The within-laboratory variances for the two methods were compared by using the Wilcoxon Signed Rank Test (11). The same test was used to examine differences in the errors of determinations of high-IV and low-IV oils.

RESULTS

Initial screening of data for outliers. One laboratory made no return. Two laboratories returned incomplete data and were omitted from the analysis because they could not contribute to the comparison between methods. For laboratory 1, sample 9, the cyclohexane/acetic acid method gave A and B values (open duplicates) that differed by 8 units, but the laboratory supplied an additional replicate that agreed well with the B value. Therefore, these latter two values were used. Similarly, for laboratory 5, sample 10, the cyclohexane/acetic acid method gave A and B values that differed by 16 units, but the laboratory supplied an additional replicate that agreed well with the B value. Therefore, these latter two values were used. No other substitutions were made. No laboratory was rejected as an outlier in either method.

Main treatment effects. There was no significant difference in mean value between the two methods (Table 1). There was no consistent bias of one method over the other from one laboratory to another, and the mean difference (0.13; standard error of difference 0.16) was not significantly different from zero (Table 2). The two methods gave closely similar results for both low- and high-IV oils (Table 3).

Repeatability and reproducibility. As in the previous collaborative study (4), the open duplicates tended to agree more closely than the hidden duplicates, indicating that the second (B) determination was not completely independent of the initial (A) determination. Table 4 indicates that, while the open and hidden standard deviations were similar in some laboratories, other laboratories had 2- to 4-fold higher standard deviations for the hidden duplicates. The preferred estimate of the real within-laboratory variability (repeatability) is then given by the standard deviation based on the first (A) determination of each hidden duplicate. Pooled estimates of repeatability (s_r), repeatability relative standard deviation (RSD_r), the re-

TABLE 1
Mean Iodine Value by Two Methods, Together with Standard Deviations of Single Determinations as Estimated from (i) Open Duplicates, (ii) Hidden Duplicates (repeatability, s_r), (iii) Between Laboratories (reproducibility, s_R)[a]

		Carbon tetrachloride		Cyclohexane/ acetic acid	
Mean determinations		154.9		155.0	
Standard deviations					
Open duplicate		1.08	(0.70)	0.66	(0.43)
Hidden duplicate	A[b]	1.71	(1.10)	1.55	(1.00)
	B[b]	1.01	(0.65)	1.29	(0.83)
Between laboratory	A	1.81	(1.17)	1.98	(1.28)
	B	1.43	(0.92)	1.79	(1.15)

[a]Values in brackets are the standard deviations, expressed as a percentage of the mean values (relative repeatability, RSD_r, and relative reproducibility, RSD_R).
[b]Comparison of results (between hidden duplicates or between laboratories) in the first analysis of the samples (A) and in the second analysis of the same samples (B).

producibility standard deviation (s_R) of a single determination at a randomly chosen laboratory calculated from $(s_r^2 + s_L^2)^{0.5}$, and the relative reproducibility standard deviation (RSD_R), calculated as $100(s_R$/mean value of the determination), are given in Table 1. Estimates of repeatability and reproducibility, based on the B values, tended to be smaller than those based on the A values but not significantly so.

The standard deviations within laboratory (s_r), based on the hidden duplicates, for the high- and low-IV oils, were similar within each method and thus appear to be independent of the absolute value. Expressed as RSD_r, the within-laboratory variability is a little over 1% for low-IV oils and a little under 1% for high-IV oils for both methods (Table 3). There was no difference in repeatability between the two methods, either overall or for the low- and high-IV oils separately.

The between-laboratory standard deviations (s_R) were only a little greater than the within-laboratory variation with a coefficient of variation of 1.2% for the standard method and 1.3% for the new cyclohexane/acetic acid method (from the A values). That there was no difference in reproducibility between the two methods is further illustrated in Table 2, which displays the estimated mean determination for each laboratory, for each of the two analytical methods and corrected for design imbalance in the samples analyzed. These means were derived from the entire database, analyzing the average of the A and B determinations. Differences between the laboratories are similar for the two methods. Table 2 also displays the derived mean difference between the two methods for each laboratory, together with the pooled overall difference and its standard error. The latter was not significantly different from zero.

TABLE 2
Mean Iodine Value by Each Method and the Mean Difference Between Methods for Each Laboratory

Laboratory	Carbon tetrachloride	Cyclohexane/ acetic acid	Mean difference (Cd 1-25 – Cd 1d-92)
1	155.43	154.22	1.21
3	156.02	155.74	0.28
5	154.59	154.87	–0.28
6	154.64	156.61	–0.97
7	154.00	154.29	–0.29
8	154.70	153.56	1.15
10	154.88	156.86	–1.98
Overall			–0.13 ± 0.16

DISCUSSION

The results of this collaborative trial confirm those of another collaborative study of the same two methods, applied to a range of vegetable and animal oils, including one sample of low-IV fish oil (7) in showing that cyclohexane/acetic acid can be used in place of carbon tetrachloride without loss of precision. The repeatability and reproducibility standard deviations reported in that trial for fish oil were 0.5 and 1.1, respectively, for the cyclohexane/acetic acid method, compared with 1.55 and 1.98 in the present study. The corresponding RSD_r and RSD_R were 0.5 and 1.0, compared with 1.00 and 1.28 in the present study.

The previous collaborative test (4) reported that replacement of carbon tetrachloride as the solvent with cyclohexane in the analysis of fish oils for IV gave a figure 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 greater than 150, namely 3.8, compared with oils of IV less than 150 where the difference was 1.6. It was postulated that this difference might be due to the difficulty in determining the endpoint during the color change in the reaction because of emulsion formation in the presence of cyclohexane. It was further postulated that a mixture of cyclohexane and acetic acid might overcome this difficulty.

The results of this collaborative trial show that this is so, and no significant differences were recorded in mean IV between the methods for either low- or high-IV oils or in their repeatability or reproducibility. The similar repeatability of both methods for low- and high-IV oils confirms previous findings that a reaction time of 1 h is sufficient for high-IV oils. Consequently, the modified method with cyclohexane and acetic acid and a 1-h reaction time is recommended for fish oil over the usual range of IV.

The IUPAC (10) protocol for collaborative studies prefers only one analysis on each of two hidden or split levels of each material for the estimation of repeatability but will accept estimates that are based on open duplicates when it is not practical to use the better experimental design. In the present study, the hidden duplicate A values represent the ideal IUPAC case and are the preferred values. Similarly, for between-laboratory standard deviation (reproducibility), the A values are preferred. In the statistical sense, the B values cannot be assumed to be independent or unbiased, but the lower values for repeatability and reproducibility may also represent improved accuracy when the analyst has already had one

TABLE 3
Mean Values and the Hidden Duplicate Standard Deviations, by Method and by Low/High Iodine Value Sample

	Carbon tetrachloride			Cyclohexane/acetic acid		
		A[a]	B[b]		A	B
	Mean	SD (%)	SD (%)	Mean	SD (%)	SD (%)
Low	129.8	1.89 (1.46)	0.89 (0.69)	130.0	1.53 (1.18)	1.21 (0.93)
High	180.0	1.53 (0.85)	1.11 (0.62)	180.1	1.56 (0.87)	1.35 (0.75)

[a]A: first open duplicate analysis.
[b]B: second open duplicate analysis.

TABLE 4
Standard Deviations of Single Determinations as Estimated from (i) Open Duplicates and (ii) Hidden Duplicates for Each Laboratory and Method[a]

	Open duplicate		Hidden duplicate			
			Carbon tetrachloride		Cyclohexane/acetic acid	
Laboratory	Carbon tetrachloride	Cyclohexane/acetic acid	A	B	A	B
1	2.02	0.94	3.14	0.87	2.06	1.12
3	0.48	0.47	1.62	1.71	1.86	2.01
5	0.66	1.21	2.07	1.08	2.69	1.59
6	0.28	0.34	0.35	0.21	0.16	0.19
7	0.84	0.43	0.54	1.00	0.45	0.90
8	0.13	0.16	0.94	0.94	0.17	0.37
10	1.61	0.50	1.58	0.58	1.25	1.66

[a]NB: Comparison between laboratories should be avoided because each analyzed different samples.

preliminary titration to determine the color change. A further corollary is that laboratories routinely should not place reliance on 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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