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WHO Melting-Point Reference Substances

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Batches of 13 highly purified chemicals, intended for use as reference substances in the calibration of apparatus for melting-point determinations, have been subjected to a collaborative assay by 15 laboratories in 13 countries. All the laboratories performed melting-point determinations by the capillary methods described in the proposed text for the second edition of the Pharmacopoea Internationalis *and some, in addition, carried out determinations by the microscope hot stage (Kofler) method, using both the "going-through" and the "equilibrium" technique.*

Statistical analysis of the data obtained by the capillary method showed that the within-laboratory variation was small and that the between-laboratory variation, though constituting the greatest part of the whole variance, was not such as to warrant the exclusion of any laboratory from the evaluation of the results. The average values of the melting-points obtained by the laboratories can therefore be used as constants for the substances in question, which have accordingly been established as WHO Melting-Point Reference Substances and included in the WHO collection of authentic chemical substances.

As to the microscope hot stage method, analysis of the results indicated that the values obtained by the "going-through" technique did not differ significantly from those obtained by the capillary method, but the values obtained by the "equilibrium" technique were mostly significantly lower.

At the meeting of the WHO Expert Committee on the International Pharmacopoeia in October 1957 it was suggested that a study should be made of the inclusion of international reference substances suitable for checking melting-points in the collection of authentic chemical substances.[2] The WHO Centre for Authentic Chemical Substances, Apotekens kontrollaboratorium, Stockholm, was authorized to propose the organization of the work and, in a report from that laboratory in 1959,[3] it was suggested that a set of 13 substances, which had been found to be suitable for the purpose, should be adopted as melting-point reference standards.

The WHO Expert Committee on Specifications for Pharmaceutical Preparations agreed at its meeting in November 1959 [4] that the work should be carried out along the lines suggested by the Apotekens kontrollaboratorium. In a preliminary report [5] an account was given of the procuring of the substances and also details about the organization of the collaborative testing. The present paper is the final report prepared after completion of the collaborative study.

THE REFERENCE SUBSTANCES

The WHO set of Melting-Point Reference Substances contains the following 13 substances:

Substance	*Melting-temperature*
1. Azobenzene	69°C
2. Vanillin	83°C
3. Benzil	96°C
4. Acetanilide	116°C
5. Phenacetin	136°C
6. Benzanilide	165°C
7. Sulfanilamide	166°C
8. Salophen	192°C
9. Sulfapyridine	193°C
10. Dicyandiamide	210°C
11. Saccharin	229°C
12. Caffeine	237°C
13. Phenolphthalein	263°C

[1] WHO Centre for Authentic Chemical Substances, Apotekens kontrollaboratorium, Stockholm, Sweden.

[2] Unpublished working document WHO/Pharm/330.

[3] Unpublished working document WHO/Pharm/371.

[4] Unpublished working document WHO/Pharm/237.

[5] Unpublished working document WHO/Pharm/385.

Substances 2, 4, 5, 7, 9 and 12 are those recommended in the first edition of the *Pharmacopoea Internationalis* (Ph. I.) as suitable for the purpose of checking the accuracy of different melting-point methods. These six substances also constitute the Melting Point Reference Standards of the *United States Pharmacopeia* (USP). Substances 1, 3, 4, 5, 6, 8, 10, 11 and 13 are recommended by Kofler et al. (1954) for calibrating purposes and for determining eutectic melting-temperatures with the microscope hot stage and the hot bar. Substances 8 and 10 are actually included in the WHO set primarily for the purpose of determining eutectic melting-temperatures.

The substances were purchased after examination of about 35 samples from 9 different suppliers. In most cases it was possible to obtain substances of sufficient purity from commercially available sources. Only substance 6, benzanilide, and substance 11, saccharin, were subjected to further purification.

The substances were assayed and checked for chemical purity at the Apotekens kontrollaboratorium, and the results are shown in Table 1.

Samples of each substance were taken from four different parts of the container and analysed with respect to the melting-range. In no case was a significant difference, indicating lack of homogeneity of the material, observed. The four samples of each substance were then carefully mixed to make gross samples, from which subsamples were taken for distribution to the participants in the international collaborative assay.

THE INTERNATIONAL COLLABORATIVE ASSAY

The names and addresses of the laboratories that took part in the international collaborative assay are listed in the Annex (see page 187). Throughout this report the participating laboratories are referred to by arbitrary numbers, which have no connexion with the order of listing of the laboratories in the Annex.

Together with the samples of the 13 substances, the following general directions for the assay were sent to the participants:

" Principally the melting-point determinations should be carried out according to the capillary method proposed for the second edition of the Ph. I. (a copy of the relevant text is included). If other apparatus or methods than those outlined in the Ph. I. are used, a short description of the essential parts of the apparatus (method) should be given together with the results.

For all the substances the melting-range, as defined in the Ph. I., should be determined and for each substance at least two determinations should be made. The following data should be reported:

1. Uncorrected temperature readings.
2. Corrections applied:
   (*a*) correction for deviation of the standard thermometer;
   (*b*) emergent-stem correction.
3. Melting-range in corrected temperatures.

If possible, it would be of very great value if you could also carry out melting-point determinations on these substances with a microscope hot stage. For such determinations we suggest that the following data are reported:

1. Melting-range from " going-through " determinations and the approximate rate of temperature rise at the melting-temperature.

2. Observed micro melting-point, using the " equilibrium " technique as described, for instance, by Kofler et al. (1954) in *Thermo-Mikro-Methoden*, pages 17 and 18."

CAPILLARY METHOD

Statistical analysis and results

The temperature at which the substances begin to collapse as well as the temperature at which they are completely molten were determined, in accordance with the text of the Ph. I. (2nd ed.), by all the laboratories except one, which only determined the melting-temperatures.

In most laboratories the melting-point apparatus was constructed in accordance with the directions in the Ph. I. (2nd ed.). In three laboratories, electrically heated blocks of aluminium or copper were used. Since the Ph. I. accepts this type of apparatus, provided that it gives the same accuracy in the determinations as the prescribed type, it has been possible to use the results from these three laboratories too.

One of the participants distributed the substances between six different laboratories in his country. Most of these laboratories determined the melting-points of only 5 substances. To facilitate the

TABLE 1

RESULTS OF ASSAY AND CHECKING OF CHEMICAL PURITY

Substance	Assay (Method)	Purity
Azobenzene	100.1 % (Kjeldahl)	Residue on ignition: nil
Vanillin	100.3 % (Non-aqueous titration with lithium methoxide)	Conforms to purity tests in draft monograph of *Pharmacopoea Nordica*
Benzil	100.8 % (Ketone determination with hydroxylamine)	Residue on ignition: nil
Acetanilide	99.9 % (Non-aqueous titration with perchloric acid)	Conforms to purity tests in draft monograph of *Pharmacopoea Nordica*
Phenacetin	100.2 % (Refluxing with hydrochloric acid; non-aqueous titration with perchloric acid)	Conforms to purity tests in draft monograph of *Pharmacopoea Nordica*
Benzanilide	99.1 % (Kjeldahl)	Residue on ignition: nil
Sulfanilamide	100.5 % (Bromometric)	Conforms to purity tests in draft monograph of *Pharmacopoea Nordica*
Salophen	99.2 % (Kjeldahl)	Residue on ignition: nil
Sulfapyridine	100.7 % (Potentiometric, with nitrite)	Conforms to purity tests in *USP XV*
Dicyandiamide	99.7 % (Kjeldahl)	Residue on ignition: nil
Saccharin	99.3 % (Acidimetric)	Conforms to purity tests in *USP XVI*
Caffeine	100.6 % (Non-aqueous titration with perchloric acid)	Conforms to purity tests in draft monograph of *Pharmacopoea Nordica*
Phenolphthalein	100.3 % (Iodometric)	Conforms to purity tests in draft monograph of *Pharmacopoea Nordica*

statistical evaluation in this investigation the results from these laboratories have been summarized in two groups. As the type of apparatus and the procedure of determination was the same in all laboratories, this manipulation was considered justified.

The results from one laboratory have not been included because they were received after the statistical analysis had been finished. The results from this laboratory, however, agree well with those from the others and their inclusion in the statistical analysis would not have changed the conclusions reached in this study.

The statistical evaluation has been carried out according to the model designed by Mandel (1959) and by Mandel & Lashof (1959) The " collapse " points and melting-points have been dealt with separately. Tables 2 and 3 show the averages obtained by each laboratory for each substance (after correction for the deviation of the standard thermometer and for the emergent stem). Each such average makes one " cell ". The tables therefore consist of 13×14 and 13×15 " cells ". The average for each substance and laboratory and the standard deviations and ranges for each substance have been calculated. The averages of the within-cell standard deviations have been calculated according to Tippet (1925). The average of all determinations in all laboratories has also been computed—i.e., the " grand " average ($\bar{x}$).

If the within-cell standard deviation shows an order-of-magnitude relationship with the temperature measurements, the cell averages have to be adjusted accordingly. It can be seen from Tables 2 and 3, however, that the within-cell standard deviations do not show any relationship of this kind. Therefore, the following calculations can be carried out on the original cell averages.

The purpose of the evaluation is not only to show the discrepancies between the laboratories but also to give numerical expression to the variations within the laboratories. This last-mentioned within-laboratory variability, η, consists broadly speaking of two components, one of them, ω, due to the normal replication error when making more than one determination and the other, λ, due to, for example, different accuracy in the calibration of the scale of an instrument. With regard to the variations between laboratories, see the accompanying figure.

From the figure it is seen that an ideal laboratory ought to have a line with the ordinate of the centroid $M=C$ and with the slope $\beta_i=1$. (Under these conditions the line must pass through the origin.)

TABLE 2

"COLLAPSE" POINT DATA, AVERAGES, STANDARD DEVIATIONS AND RANGES

Substance / Lab.	1	2	3	4	5	6	7	8	9	10	11	12	13	Average
1	67.4	81.6	94.7	114.2	134.6	163.1	164.0	190.9	191.1	209.0	227.5	235.0	261.0	164.2
2	68.7	82.9	95.6	114.6	135.1	164.1	164.9	191.6	191.5	209.0	227.7	236.7	263.5	165.1
3	67.9	81.6	94.6	114.6	135.0	164.2	165.0	188.6	191.5	208.3	228.4	235.3	262.4	164.4
4	68.0	81.7	95.2	115.4	135.7	164.5	165.5	192.5	192.2	210.2	228.4	237.1	263.8	165.4
5	67.9	81.8	94.0	113.9	134.2	163.1	164.2	191.0	190.5	208.2	226.5	235.5	261.1	164.0
6	67.8	82.0	94.5	114.4	134.9	164.0	165.0	192.2	191.6	209.0	227.8	236.5	262.4	164.8
7	68.0	80.0	93.5	114.5	134.0	163.0	163.0	189.5	191.0	206.5	228.0	236.5	262.0	163.8
8	67.4	82.1	95.1	114.5	135.7	163.0	165.3	191.4	192.4	209.9	228.8	236.8	261.8	164.9
9	67.5	82.9	96.0	115.4	136.3	164.8	166.8	191.9	194.8	210.9	227.3	235.2	261.7	165.5
10	67.3	80.9	93.8	114.0	132.5	162.1	164.1	188.6	191.1	208.6	226.6	233.7	259.3	163.3
11	67.3	81.6	94.6	114.1	134.3	162.9	164.4	188.1	191.2	209.2	224.6	235.4	258.1	163.5
12	68.0	82.1	95.8	114.5	134.8	164.0	165.0	191.8	192.2	210.0	226.7	236.4	261.5	164.8
13	68.2	81.6	95.2	114.5	134.7	163.7	165.1	192.1	191.5	209.8	228.1	237.0	262.4	164.9
14	67.7	81.5	94.7	113.5	133.7	162.3	163.4	190.5	190.5	207.6	224.4	234.2	259.7	163.4
15	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Average	67.8	81.7	94.8	114.4	134.7	163.5	164.7	190.8	191.7	209.0	227.2	235.8	261.5	164.4 = $\bar{x}$
Standard deviation	0.4	0.7	0.7	0.5	0.8	0.8	0.9	1.5	1.1	1.1	1.3	1.1	1.6	
Range	67.3–68.7	80.0–82.9	93.5–95.8	113.5–115.4	132.5–136.3	162.1–164.8	163.0–166.8	188.1–192.5	190.5–194.8	206.5–210.9	224.4–228.8	233.7–237.1	258.1–263.8	
Average of the within-cell standard deviation	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.5	0.3	0.2	

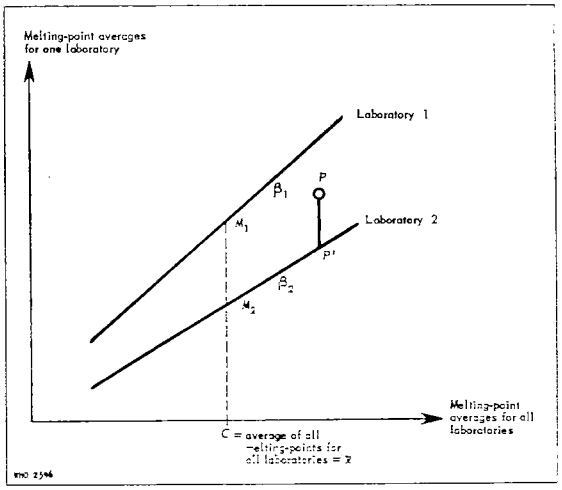
TABLE 3

MELTING-POINT DATA, AVERAGES, STANDARD DEVIATIONS AND RANGES

Substance / Lab.	1	2	3	4	5	6	7	8	9	10	11	12	13	Average
1	68.2	82.6	96.0	115.2	136.1	164.4	165.4	192.2	192.0	210.8	229.3	236.5	262.5	165.5
2	69.6	83.6	96.5	115.7	136.1	164.7	165.7	192.2	192.4	209.9	229.4	237.1	264.9	166.0
3	69.1	82.7	95.4	115.8	135.8	164.7	165.5	189.5	192.1	209.2	229.6	236.6	263.0	165.3
4	68.7	82.6	96.3	116.3	136.6	165.2	166.4	193.3	193.0	210.9	230.2	237.9	264.6	166.3
5	68.6	82.8	95.5	115.4	135.6	164.6	165.9	192.3	191.7	209.2	228.3	236.6	262.6	165.3
6	68.6	82.4	95.5	115.3	135.8	164.6	165.9	192.8	192.3	210.0	229.3	237.2	263.2	165.6
7	69.0	83.0	95.0	116.0	135.0	164.0	165.0	190.5	192.0	208.0	229.0	237.0	263.5	165.2
8	68.2	83.1	95.7	115.3	135.9	164.2	165.9	192.2	193.2	210.7	229.4	237.6	262.6	165.7
9	68.5	84.9	98.0	117.1	137.5	166.8	167.8	194.9	195.8	211.9	229.8	237.2	263.7	167.2
10	68.8	82.9	95.8	116.0	135.0	163.6	165.6	190.6	193.1	210.1	230.1	235.7	261.8	165.3
11	69.7	83.4	97.1	116.6	136.5	165.5	166.5	191.2	192.5	210.5	228.8	237.4	260.7	165.9
12	68.7	82.5	96.7	116.1	135.9	165.0	166.7	193.2	193.2	211.0	229.6	237.5	263.9	166.2
13	68.8	82.5	96.1	115.4	135.9	164.9	165.8	193.5	192.4	211.1	229.6	238.0	263.2	165.9
14	69.1	82.5	95.6	114.9	135.2	163.5	164.7	191.5	191.2	208.9	227.5	235.4	260.7	164.7
15	68.5	83.0	95.5	115.0	136.0	165.0	165.5	192.0	193.0	211.0	230.0	237.5	264.5	165.9
Average	68.8	83.0	96.0	115.7	135.9	164.7	165.9	192.1	192.7	210.2	229.3	237.0	263.0	165.7 = $\bar{x}$
Standard deviation	0.4	0.6	0.8	0.6	0.6	0.8	0.7	1.4	1.1	1.0	0.7	0.7	1.3	
Range	68.2–69.7	82.4–84.9	95.0–98.0	114.9–117.1	135.0–137.5	163.5–166.8	164.7–167.8	189.5–194.9	191.2–195.8	208.0–211.9	227.5–230.2	235.4–238.0	260.7–264.9	
Average of the within-cell standard deviation	0.2	0.1	0.2	0.1	0.3	0.2	0.3	0.3	0.2	0.3	0.3	0.2	0.3	

Each line in the figure is characterized by a location parameter μ_i (in this case M_1C and M_2C) and by its slope (β_1 and β_2). This means that the between-laboratory variability consists of two components, one characterized by μ and one by β. This last component may also be separated into two parts, the first one related to the correlation between μ and β and the second to that part of the variability of β that is unrelated to μ, hereafter called δ.

COMPONENTS OF VARIABILITY BETWEEN AND WITHIN TWO LABORATORIES



The departure of an experimental point obtained by laboratory 2 from its response line $PP' = \eta$ is the same as the above-mentioned within-laboratory variability consisting of ω and λ.

Thus, according to Mandel's model, the variance $V(y)$ of a "collapse" point or melting-point value may be considered as being composed of a number of components, namely, $V(\omega)$, $V(\lambda)$, $V(\mu)$ and $V(\delta)$. Each of these variances has been calculated in the manner described below.

When deriving the response line for each laboratory, the least-square formulae for linear regression were used. The following quantities were first computed:

$$X = (\Sigma x_j^2) - b\bar{x}^2 \quad \ldots\ldots (1)$$

$$Z_i = (\Sigma z_{ij}^2) - b\mu_i^2 \quad \ldots\ldots (2)$$

$$P_i = (\Sigma x_j z_{ij}) - b\mu_i\bar{x} \quad \ldots\ldots (3)$$

where x_j = average of the melting-points for substance j
b = number of substances
$\bar{x}$ = "grand" average
z_{ij} = melting-point of a substance in cell "ij"
μ_i = average of the melting-points obtained by laboratory i

TABLE 4

VALUES OF X, Z_i AND P_i OBTAINED FROM EQUATIONS (1), (2) AND (3)

Lab.	The values from Table 2 give:			The values from Table 3 give:		
	X	Z_i	P_i	X	Z_i	P_i
1	46 431.14	46 069.77	46 249.60	46 544.50	46 453.49	46 498.25
2		46 253.56	46 340.92		46 439.04	46 489.78
3		46 424.76	46 423.63		46 311.73	46 424.61
4		47 055.54	46 741.42		47 151.23	46 846.55
5		46 089.95	46 259.37		46 341.40	46 442.05
6		46 601.19	46 515.11		46 831.69	46 687.36
7		46 768.53	46 593.14		46 153.98	46 345.13
8		46 921.53	46 674.29		46 726.77	46 634.68
9		46 170.22	46 294.81		46 510.91	46 521.65
10		45 763.31	46 093.00		46 237.36	46 387.75
11		45 414.45	45 914.54		45 382.47	45 958.25
12		46 365.00	46 396.90		46 686.12	46 614.49
13		46 829.82	46 629.11		47 103.61	46 822.12
14		45 225.49	45 823.75		45 423.44	45 980.52
15		—	—		47 141.72	46 840.96

The calculated values of X, Z_i and P_i are shown in Table 4.

The slope of the response line for laboratory $i(=\beta_i)$ was then obtained by using the following formula:

$$\beta_i = \frac{P_i}{X} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (4)$$

The centroids M_1C, M_2C etc. $(=\mu_i)$ have already been computed in Tables 2 and 3, but they are also given in Table 5. This table also shows the values of V_i (η)—i.e., the within-laboratory variance consisting of the two components $V(\omega)$ and $V(\lambda)$—computed by the formula:

$$V_i(\eta) = \frac{a}{a-1} \cdot \frac{1}{b-2} \left(Z_i - \frac{P_i^2}{X}\right) \quad \ldots\ldots\ldots (5)$$

where a = number of laboratories
b = number of substances

At this point in the calculations it is of value to study the results obtained. The average of β ought to be 1.000. As can be seen from Table 5 the averages obtained are close to this value and the differences are negligible. If V_i (η) for any laboratory is much greater than the average, it is advisable to calculate the individual estimates of η, in order to detect discrepant individual points. In such cases, the laboratory may be omitted from the evaluation, but then it is necessary to recalculate the values of β_i and V_i (η) for the remaining laboratories; μ_i will be the same, but the over-all average $\bar{x}$ must also be recomputed. In the present data the differences are so small that no omission was considered necessary. Observe that $V_i(\eta)$ only shows the extent of deviation of the experimental points found by one laboratory from that laboratory's response line and not from the response line for an "ideal" laboratory.

The average of $V_i(\eta)$ was then used to give the two components $V(\omega)$ and $V(\lambda)$. The formula used was:

$$V(\lambda) = V(\eta) - 1/n \cdot V(\omega) \quad \ldots\ldots\ldots\ldots (6)$$

where n = number of determinations within a cell
$V(\eta)$ = average of $V_i(\eta)$

$V(\omega)$ was calculated according to Mandel's method (*Technometrics*, 1959, vol. 1, p. 256), using the values for within-cell standard deviation in Tables 2 and 3. $V(\omega)$ then becomes 0.058 for the "collapse" points and 0.053 for the melting-points. Generally, the average of three determinations has been given within the cells. Therefore the values of $V(\lambda)$ will be, respectively:

$$0.468 - \frac{0.058}{3} = 0.449 \text{ and } 0.335 - \frac{0.053}{3} = 0.317.$$

TABLE 5

PARAMETERS OF THE STRAIGHT LINES CORRESPONDING TO THE VARIOUS LABORATORIES

Lab.	β_i	μ_i	V_i (η)
	"Collapse" points		
1	0.996	164.2	0.098
2	0.998	165.1	0.262
3	1.000	164.4	0.846
4	1.007	165.4	0.173
5	0.996	164.0	0.167
6	1.002	164.8	0.192
7	1.003	163.8	1.255
8	1.005	164.9	0.276
9	0.997	165.5	1.110
10	0.993	163.3	0.586
11	0.989	163.5	1.053
12	0.999	164.8	0.226
13	1.004	164.9	0.186
14	0.987	163.4	0.115
Average	0.998	164.4	0.468
	Melting-points		
1	0.999	165.5	0.141
2	0.999	166.0	0.372
3	0.997	165.3	0.654
4	1.006	166.3	0.065
5	0.998	165.3	0.154
6	1.003	165.6	0.101
7	0.996	165.2	0.718
8	1.002	165.7	0.169
9	1.000	167.2	1.179
10	0.997	165.3	0.569
11	0.987	165.9	0.301
12	1.002	166.2	0.150
13	1.006	165.9	0.216
14	0.989	164.7	0.007
15	1.006	165.9	0.236
Average	0.999	165.7	0.335

The next step was to calculate $V(\mu)$, $V(\beta)$ and $V(\delta)$, i.e., the between-laboratory variances. An analysis of the variance was carried out according to the usual procedure on the data presented in Tables 2 and 3. The results are shown in Tables 6 and 7, which were drawn up on the following basis:

Sources of variation	*Degrees of freedom*	*Sums of squares*	*Mean squares*	*Ratio of variances*
Between laboratories	$a-1$	S_L	M_L	F
Between substances	$b-1$	S_M	M_M	
Error	$(a-1)(b-1)$	S_{LM}	M_{LM}	
Total	$ab-1$	S_T		

From tables of the F distribution — see, for example, Bauer (1960) — it is seen that in this case $F=2.1$ at a 99% significance level. The value of 12.7 obtained (Table 6) therefore means that, with regard to the determination of the " collapse " points, there is a significant difference between the laboratories, with a probability of being wrong less than once in one hundred times.

TABLE 6
ANALYSIS OF VARIANCE OF DATA FOR THE " COLLAPSE " POINTS

Sources of variation	Degrees of freedom	Sums of squares	Mean squares	Ratio of variances
Between laboratories	13	93.63	7.202	12.7
Between substances	12	647 994.72	54 000	
Error	156	88.46	0.567	
Total	181	648 176.81		

TABLE 7
ANALYSIS OF VARIANCE OF DATA FOR THE MELTING-POINTS

Sources of variation	Degrees of freedom	Sums of squares	Mean squares	Ratio of variances
Between laboratories	14	64.43	4.602	10.6
Between substances	12	697 218.49	58 102	
Error	168	72.63	0.432	
Total	194	697 355.55		

From Table 7 it is obvious that there is the same significant difference between the laboratories in respect of the determination of the melting-points. But in both cases the level of significance was not considered to be high enough to justify the exclusion of any laboratory.

The values of the mean squares were used for computing $V(\mu)$ and $V(\beta)$, as follows:

$$V(\mu) = \frac{M_L - V(\eta)}{b} \quad \ldots\ldots\ldots\ldots\ldots\ldots (7)$$

$$V\beta = \frac{a(M_{LM} - V(\eta)}{M_M - V(\eta)} \quad \ldots\ldots\ldots\ldots\ldots\ldots (8)$$

For the " collapse " points the following values were obtained:

$V(\mu) = 0.518 \qquad V(\beta) = 0.0000257$

The corresponding values for the melting-points were:

$V(\mu) = 0.328 \qquad V(\beta) = 0.0000250$

Formulae (9) and (10) were used for calculating $V(\delta)$:

$$V(\delta) = V(\beta) - \alpha^2 V(\mu) \quad \ldots\ldots\ldots\ldots\ldots\ldots (9)$$

$$\alpha = \frac{a\,b\,(\Sigma\mu_i P_i - \bar{x}\Sigma P_i)}{S_L S_M} \quad \ldots\ldots\ldots\ldots\ldots\ldots (10)$$

Since the equations yielded negative values in both cases, the variance of δ was then taken to be zero (Table 8).

$V(\lambda)$ is, as pointed out earlier, a measure of the variations due to different accuracy in the calibration of the instrument scales. This variance is independent of the number of measurements, unlike $V(\omega)$, which is affected by replication because it is the replication error. It is very difficult to get the same melting-point on repeat determinations. This means that a better result is obtained with an increasing number of determinations. But replication is useful only to the point of making $V(\omega)/n$ small with respect to $V(\lambda)$. Table 8 shows that the replication error is small within the laboratories. There would

TABLE 8
NUMERICAL VALUES OF THE DIFFERENT TYPES OF VARIANCE

	$V(\lambda)$	$V(\omega)$	$V(\omega)/n$	$V(\delta)$	$V(\mu)$	$V(\beta)$
" Collapse " points	0.449	0.058	0.019	0	0.518	0.0000257
Melting-points	0.317	0.053	0.018	0	0.328	0.0000250

be nothing to gain with more determinations per substance, since, as can also be seen in Table 8, $V(\lambda)$ is higher than $V(\omega)$. This indicates inaccurate calibration of the thermometers used. Another and more probable explanation is that some of the laboratories estimated their thermometer readings to the nearest half-degree and these estimations may have given results which were too high in one part of the thermometer scale and too low in another. (It is not always possible to recognize these approximate readings in Tables 2 and 3 because the emergent-stem correction in some cases is made with an accuracy of 0.1°.)

Table 9 shows the relative importance of the various sources of variability for some of the " collapse " points and melting-points. The equation used for this breakdown of the total variability of any value of y is:

$$V(y)=V(\omega)+V(\lambda)+[1+a(y-\bar{x})]^2 . V(\mu)+(y-\bar{x})^2 . V(\delta) \quad (11)$$

The values obtained confirm the conclusions made above concerning the sources of the total variability of the " collapse " points and melting-points. The most important source was μ, i.e., the variability between laboratories.

The values of μ and β in Table 5 show that the agreement between laboratories was fairly good. μ and β are, as stated above, parameters in the equation for a straight line. An ideal laboratory will have a straight line passing through the origin at an angle of 45°. If laboratories with such values of β as those shown by lab. 11 and 14 are eliminated, there will be little or no change in the average of the " collapse " points or the melting-points. Moreover, it is intended to give the melting-points of the reference substances to the nearest whole number of degrees. The same applies to the average value of β, which ought to be 1.000 and was, as Table 5 shows, 0.998 for the " collapse " points and 0.999 for the melting-points. These values can both be accepted. $V(\mu)$ was also low and that will enable us to take all values of μ as they are. The discrepancies between the individual values of μ and β are perhaps due to the use of different kinds of apparatus (all types used, however, are permitted by the Ph. I.) and to the different accuracy of calibration of the thermometers. The method of treatment (pulverization and drying) before the determination is certainly of some importance, as is the speed of heating during the determination (if, for example, one laboratory heats at the rate of 0.5° per minute and another at 1.5° per minute, differences will arise, because it has been shown that temperature equilibrium between the outside and the inside of the capillary tube is not reached until about 30 seconds have passed).[1]

$V(\delta)$ is that component of the between-laboratory variance which is associated with that part of β which is not correlated with μ (the greatest component of $V(\beta)$ is usually the one correlated with $V(\mu)$). $V(\delta)$ was in this case zero, which is to be looked upon as an advantage.

Finally, for all the substances but one, the standard deviation values (Tables 2 and 3) were surprisingly low (the exception was salophen, a substance that will be used only for determining eutectic melting-temperatures).

As a final conclusion we can state that the averages of the different melting-temperatures obtained in the collaborative assay by the capillary method can be used as constants for the WHO collection of Melting-Point Reference Substances.

TABLE 9

RELATIVE IMPORTANCE OF THE DIFFERENT SOURCES OF VARIATION

Substance	Average	Sources of variation			
		Within laboratories		Between laboratories	
		$V(\omega)$	$V(\lambda)$	$V(\mu)$	$V(\varepsilon)$
" Collapse " points					
1	67.8	0.00	0.04	0.96	0
4	114.4	0.02	0.17	0.81	0
9	191.7	0.01	0.11	0.88	0
13	261.5	0.00	0.02	0.98	0
Melting-points					
1	68.8	0.00	0.01	0.99	0
4	115.7	0.01	0.05	0.94	0
9	192.7	0.01	0.06	0.93	0
13	263.0	0.00	0.01	0.99	0

MICROSCOPE HOT STAGE METHOD

Some of the laboratories also determined the " collapse " points and melting-points according to Kofler's microscope hot stage method. The results from nine laboratories that made determinations by the "going-through" technique are shown in Table 10, together with the averages, standard deviations and

[1] Unpublished working document WHO/Pharm./Ed. Sec. 63 Add.1.

TABLE

"COLLAPSE" POINTS AND MELTING-POINTS

Substance / Lab.	1		2		3		4		5		6	
1	68.3-69.3		82.0-83.0		94.8-95.8		114.3-115.3		134.8-135.5		163.0-164.0	
2	69.8-70.2		82.3-83.3		96.2-96.5		115.8-116.2		134.4-136.0		164.3-164.6	
3	68.3-68.6		81.6-82.3		93.9-95.4		114.7-116.1		134.5-135.2		164.6-164.8	
4	68.1-68.4		81.0-81.6		94.9-95.2		114.4-114.8		134.9-135.3		163.7-163.9	
5	68.0-69.0		82.5-83.0		94.5-95.0		115.0-116.0		134.5-135.0		162.8-163.0	
6	67.9-68.9		82.6-83.2		96.2-96.6		115.2-116.0		135.5-136.0		167.2-167.7	
7	*x*-68.6		*x*-83.0		*x*-95.2		*x*		*x*		*x*	
8	*x*		81.0-81.5		*x*		114.5-115.5		*x*		164.0-165.0	
9	66.0-67.5		80.5-82.0		93.5-95.0		113.0-113.5		134.0-134.5		*x*-163.0	
Average	68.1	68.8	81.7	82.5	94.9	95.6	114.6	115.4	134.7	135.4	164.2	164.5
Standard deviation	1.1	0.8	0.8	0.7	1.0	0.7	0.8	0.7	0.4	0.5	1.5	1.5
Ranges (R_l and R_u)	66.0-69.8	67.5-70.2	80.5-82.6	81.5-83.3	93.5-96.2	95.0-96.6	113.0-115.8	113.5-116.2	134.0-135.5	134.5-136.0	162.8-167.2	163.0-167.7

[a] R_l = lower range ("collapse" points); R_u = upper range (melting-points); *x* = not determined.

ranges. Seven laboratories determined the melting-points according to the "equilibrium" technique; these results can be seen in Table 11. It is not good statistical practice to calculate averages and standard deviations on such a small number of values as in Tables 10 and 11, but as a means of obtaining an idea about the differences between the methods it is justifiable. When comparing the standard deviations of the melting-points shown in Tables 3, 10 and 11 (see Table 12), it is obvious that the standard deviation was generally greater with the "going-through" technique than with the capillary method or the "equilibrium" technique and that the last-mentioned technique on the whole gave the lowest values for the standard deviation. This confirms our theoretical conclusions about the three methods with respect to the different rates of heating, etc.

From a comparison of the average melting-points obtained by the "going-through" method (complete melting) and by the capillary method, it can be seen that the latter method generally gave higher melting-points. This might have been due to the shorter time of heating applied in the Ph. I. (capillary) method. In order to test this hypothesis, the melting-temperatures of some of the substances were determined by the capillary method but with varying heating-times. It was found, however, that there was no significant difference between the melting-temperatures obtained with the longer heating-times and those obtained with the heating-time of about 5 minutes used in the Ph. I. method. Another possible explanation of the differences might lie in the calibration of the thermometers of the microscope hot stages. Such calibration is accomplished by means of certain test substances with "known" melting-temperatures. This results in what might be termed a "circular definition", since the melting-temperatures obtained with microscope hot stages are dependent on how the melting-points of the test substances were originally determined and laid down. So far as we know, no details of the method of determination have been published.

In order to find out whether the differences between the melting-points obtained by the capillary and "going-through" methods were significant, a modified *t*-test was carried out by the method of

10

OBTAINED BY THE "GOING-THROUGH" TECHNIQUE [a]

7		8		9		10		11		12		13	
164.3-165.5		188.0-190.0		189.5-191.3		208.3-210.0		226.8-228.0		235.0-236.3		257.8-259.8	
167.5-168.0		188.0-189.8		191.4-192.0		209.6-211.2		228.3-229.4		239.2-239.7		260.7-265.2	
165.3-166.3		189.0-190.8		192.2-193.2		209.2-211.7		227.9-229.9		sublimated		262.3-265.9	
163.9-164.4		188.0-190.2		190.3-190.8		209.6-210.6		227.6-228.5		234.9-235.0		*x*	
165.0-166.0		190.0-193.0		*x*-193.0		210.0-210.5		sublimated		sublimated		sublimated	
167.5-168.1		190.8-193.0		192.8-193.5		209.9-211.6		229.0-230.3		*x*-238.0		262.8-264.0	
x-165.2		*x*		*x*-194.0		*x*-210.0		*x*-228.0		*x*		*x*-260.5	
x		*x*		*x*		210.0-211.5		*x*		*x*		*x*	
163.0-164.0		188.0-189.0		191.0-191.5		209.0-210.0		226.5-227.3		234.0-235.5		259.5-262.5	
165.2	165.9	188.8	190.8	191.2	192.4	209.5	210.8	227.7	228.8	235.8	236.9	260.6	263.0
1.5	1.5	1.2	1.6	1.2	1.2	0.6	0.9	0.9	1.1	2.3	1.9	2.1	2.5
163.0-167.5	164.0-168.1	188.0-190.8	189.0-193.0	189.5-192.8	190.8-194.0	208.3-210.0	210.0-211.7	226.5-229.0	227.3-230.3	234.0-239.2	235.0-239.7	257.8-262.8	259.8 265.9

TABLE 11

MELTING-POINTS OBTAINED BY THE "EQUILIBRIUM" TECHNIQUE [a]

Substance / Lab.	1	2	3	4	5	6	7	8	9	10	11	12	13
1	69.0	82.8	95.0	114.6	135.5	163.5	164.8	189.0	190.0	208.8	227.5	236.0	259.3
2	69.3	82.9	96.0	115.4	136.0	164.9	165.6	192.5	193.1	211.6	231.0	subl.	266.2
3	68.5	81.8	95.3	115.0	135.0	163.4	164.9	189.3	190.8	210.0	229.9	235.5	261.5
4	68.2	81.5	94.9	114.8	135.2	162.5	163.0	188.2	188.0	209.5	228.0	236.0	261.0
5	*x*	81.6	96.2	115.6	135.3	163.0	165.1	191.1	191.6	209.7	229.0	237.5	261.9
6	*x*	*x*	*x*	117.0	136.0	164.6	*x*	190.0	*x*	*x*	*x*	*x*	*x*
7	66.5	80.0	94.0	112.5	133.5	162.5	162.5	187.0	189.0	208.0	226.5	234.5	260.5
Average	68.3	81.8	95.2	115.0	135.2	163.5	164.3	189.6	190.4	209.6	228.7	235.9	261.7
Standard deviation	0.5	0.5	0.4	0.6	0.4	0.4	0.6	0.7	0.8	0.5	0.7	0.5	1.1
Range	66.5-69.3	80.0-82.9	94.0-96.2	112.5-117.0	133.5-136.0	162.5-164.9	162.5-165.6	187.0-192.5	188.0-193.1	208.0-211.6	226.5-231.0	234.5-237.5	259.3-266.2

[a] *x* = not determined.

TABLE 12
STANDARD DEVIATION VALUES FROM TABLES 3, 10 AND 11

Substance	1	2	3	4	5	6	7	8	9	10	11	12	13
Capillary method	0.4	0.6	0.8	0.6	0.6	0.8	0.7	1.4	1.1	1.0	0.7	0.7	1.3
" Going-through " method	0.8	0.7	0.7	0.7	0.5	1.5	1.5	1.6	1.2	0.9	1.1	1.9	2.5
" Equilibrium " method	0.5	0.5	0.4	0.6	0.4	0.4	0.6	0.7	0.8	0.5	0.7	0.5	1.1

Moore (1957). This test is based on the distribution of the following quantity:

$$M = \frac{X_j - Y_j}{R_x + R_y} \qquad \ldots\ldots\ldots\ldots\ldots\ldots (12)$$

where X_j = average of the melting-points of substance j determined by the capillary method

Y_j = average of the melting-points of substance j determined by the " going-through " method

R_x = range of the melting-point values obtained with the capillary method (=" Range " in Table 3)

R_y = range of the melting-point values obtained with the " going-through " method (=R_u in Table 10)

The calculated values of M are shown in Table 13.

From Table 13 it can be seen that the null hypothesis is acceptable in 12 cases out of 13, and very nearly acceptable in the 13th case. This means that all the differences in melting-points between the capillary and the " going-through " methods can be considered as due to chance alone.

The corresponding values of M obtained in a comparison of the averages of the capillary melting-points with those of the " equilibrium " points are presented in Table 14.

It can be seen from Table 14 that the capillary melting-points are mostly significantly higher than the " equilibrium " ones, which means they are two different constants applicable to the substances. The " equilibrium " points, however, agree fairly well with the values given for the test substances supplied with the microscope hot stages, so that the WHO set of Melting-Point Reference Substances can also be used for determining " equilibrium " points by the microscope technique.

TABLE 13
VALUES OF M OBTAINED IN A COMPARISON OF THE CAPILLARY AND THE " GOING-THROUGH " METHODS USING EQUATION (12)

Substance	1	2	3	4	5	6	7	8	9	10	11	12	13
X_j	68.8	83.0	96.0	115.7	135.9	164.7	165.9	192.1	192.7	210.2	229.3	237.0	263.0
Y_j	68.8	82.5	95.6	115.4	135.4	164.5	165.9	190.8	192.4	210.8	228.8	236.9	263.0
R_x	1.5	2.5	3.0	2.2	2.5	3.3	3.1	5.4	4.6	3.9	2.7	2.6	4.2
R_y	2.7	1.3	1.6	2.7	1.5	4.7	4.1	4.0	3.2	1.7	3.0	4.7	6.1
M	0	0.11	0.08	0.06	0.12	0.02	0	0.13	0.03	−0.10	0.09	0.01	0
Acceptance of the null hypothesis at the probability level 0.05	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes

TABLE 14

VALUES OF *M* OBTAINED IN A COMPARISON OF THE CAPILLARY AND THE "EQUILIBRIUM" METHODS USING EQUATION (12)

Substance	1	2	3	4	5	6	7	8	9	10	11	12	13
X_j	68.8	83.0	96.0	115.7	135.9	164.7	165.9	192.1	192.7	210.2	229.3	237.0	263.0
Y_j	68.3	81.8	95.2	115.0	135.2	163.5	164.3	189.6	190.4	209.6	228.7	235.9	261.7
R_x	1.5	2.5	3.0	2.2	2.5	3.3	3.1	5.4	4.6	3.9	2.7	2.6	4.2
R_y	2.8	2.9	2.2	4.5	2.5	2.4	3.1	5.5	5.1	3.6	4.5	3.0	6.9
M	0.12	0.22	0.15	0.10	0.14	0.21	0.25	0.22	0.23	0.08	0.08	0.19	0.12
Acceptance of the null hypothesis at the probability level 0.05	Yes	No	No	Yes	No	No	No	No	No	Yes	Yes	No	Yes

Annex

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RÉSUMÉ

Dans le cadre de l'activité du Centre OMS des substances de références, à Stockholm, un essai comparatif de substances de référence pour la détermination des températures de fusion a été organisé. Il a porté sur 13 substances, dont les points de fusion ont été déterminés dans 15 laboratoires de 13 pays, soit par la méthode du tube capillaire, soit par celle du microscope à platine chauffante.

L'analyse statistique des résultats obtenus par la première méthode montra que la moyenne des divers laboratoires pouvait servir de constante pour chacune des substances en question. Celles-ci ont été établies comme substances de références OMS pour la détermination des points de fusion, et feront partie de la collection des substances chimiques de référence.

La méthode du microscope à platine chauffante a donné des résultats analogues à ceux de la précédente, par la technique de la fusion complète *(going-through)*, mais de valeur inférieure par la technique de l'« équilibre ».

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