WEFTA interlaboratory comparison on nitrogen determination by Kjeldahl digestion in fishery products and standard substances

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An intercomparison exercise on nitrogen determination in fishery samples (Aitken and Smith 1983) and standard substances (Aitken and Smith 1982) by Kjeldahl digestion was performed with 14 participating laboratories from 12 different European countries. 13 out 14 laboratories obtained results which were scattering only little around the mean content calculated. The coefficient of variation of individual laboratories was generally low (»0.5 %). Standard substances with known nitrogen content were successfully analysed (> 98 % of claimed purity found) with good accuracy and precision. The optimal digestion procedure is characterised by a short digestion time (ca. 120 min), a temperature around 430° C and the choose of the appropriate catalyst.

Introduction

The Kjeldahl nitrogen method (Kjeldahl 1883), which was published already in 1883 has, with modifications, been accepted as the standard method for the determination of nitrogen (protein) for decades. After ist publication, many attempts were made to optimize the procedure and to standardize methodology.

In 1982 and 1983 two comparative studies of the Kjeldahl digestion method applied to fishery products have been conducted in the frame of the WEFTA (Western European Fish Technologists' Association) Working Group for Analytical Methods in Fish and Fishery Products. The results of these two studies, in which 14 laboratories participated, however, have only been published in intemal papers (Aitken and Smith 1982, 1983a, 1983b; Anon 1984) because of a number of faults and drawbacks which have occurred during the studies. The main disadvantages of these early studies were: inhomogeneity of samples leading to considerably varying results and remarkable differences in results between laboratories when analyzing standard substances with known nitrogen content which could not be explained. When these studies were conducted only a minority of participants was using automatical digestion apparatuses and distillation/titration devices. Within the last decade most laboratories have changed their equipments, now using modern digestion systems. Some other changes have also taken place: mercury is no longer used as a catalyst and addition of peroxide is no longer in common use.

Summarizing the earlier results, an efficient digestion, indicated by a high overall result ranking was, however, associated, not always significantly, with the following factors:

- use of a fully automatical digestor
- a digestion temperature around 410° C
- a low ration of acid to sulphate
- use of hydrogen peroxide
- a short digestion time

It was not possible to decide whether any one factor was more important, since they are not independent of each other. The nature of the catalyst appeared to have

WEFTA Laborvergleichsuntersuchung zur Stickstoffbestimmung nach Kjeldahl

14 Laboratorien aus 12 europäischen Ländern nahmen an einer Laborvergleichsuntersuchung zur Stickstoffbestimmung in Fischerzeugnissen und Standardsubstanzen nach Kjeldahl teil. 13 Laboratorien erzielten dabei Ergebnisse, die alle in engen Grenzen um die gefundenen Mittelwerte streuten. Der von den einzelnen Teilnehmern erzielte Variationskoeffizient war mit etwa 0,5 % gering. Auch die Standardsubstanzen mit bekanntem Stickstoffgehalt konnten überwiegend mit hinreichender Genauigkeit (98 % der vom Hertsteller angegebenen Gehalte) analysiert werden. Der ideale Kjeldahlaufschluß ist durch kurze Aufschlußzeiten (ca. 120 min), eine Aufschlußtemperatur bei 430° C und durch die Wahl des für die jeweilige Matrix geeigneten Katalysators gekennzeichnet.

Table 1: Description of test samples sent to participants for analysis

Four digit code No.	Identification letter	Description of sample
4321	A	freeze dried, thoroughly ground muscle tissue of ocean perch (*S. mentella*)
7643	B	freeze dried, thoroughly ground muscle tissue of European hake (*M. merluccius*)
1245	C	lean white fish meal, thoroughly ground (Icelandic origin)
3829	D	L-histidine (Riedel de Haën, 39015, 99%, MW 155.16
2463	E	urea (Riedel de Haën, 33247, ≥99.5%, MW 60.06

no influence on the results, despite the advantages of particular catalysts that have been frequently claimed in the literature (e.g. Kane 1984).

The Working Group decided therefore in 1993 to run a new exercise of nitrogen determination by Kjeldahl digestion using both, fish samples and standard substances. It was also decided that no collaborative trial for testing a common, well described method for obtaining method performance data should be performed, but rather an interlaboratory comparison based on the application of „home methods". All participating laboratories should therefore use their standard procedures.

Samples, participants, methods

5 samples (Table 1) were prepared for this exercise, if necessary tested for homogeneity (sample A-C), and coded by a four digit code (for statistical purposes a identification letter (A-D) was used later):

The samples were sent to participants in small hermetically sealed glas jars. Participants received detailed instructions how to handle the samples and on the sample weight to be used. Each participant was asked to carry out 3 and only 3 analyses of each sample, to submit the results rounded to the second decimal place and to describe the method used as his/her „home method" in detail. 14 laboratories (12 WEFTA laboratories and 2 german official laboratories) participated in the interlaboratory comparison (the order of participants below does not corresponded to the order in later sections of this paper):

- Liv Barrat, Fiskeridirektoratet, Strandgaten 229, 5002 Bergen, Norway
- Jonas Bjarnason, Icelandic Fisheries Laboratories, Skulagata 4, 101, Reykjavik, Iceland
- Monique Etienne, IFREMER, Centre de Nantes, Rue de I'lle d'Yeu, 44037 Nantes, France
- Benny Jensen, Technological Laboratory, Ministry of Fisheries, Technical University, Bld. 221, DK-2800 Lyngby, Denmark
- Horst Karl, Bundesforschungsanstalt für Fischerei, Institut für Biochemie und Technologie, Palmaille 9, D-22767 Hamburg, Germany
- Joop Luten, Netherlands Institute for Fisheries Research, Haringkade 1, 1970 AB Ijmuiden, The Netherlands
- Maria Leonor Nunes, Instituto Portugues de Investigacao Maritima, Avenida da Brasilia, 1400 Lisboa, Portugal
- Sean O'Donoghue, Tralee Regional Technical College, Clash, Tralee, Co. Kerry, Ireland
- Alexander H. Ritchie, Ministry of Agriculture, Fisheries and Food, Torry Research Station, 135 Abbey Road, Aberdeen, AB9 8DG, UK
- Margarita Tejada: Instituto del Frio (CSIC), Ciudad Universitaria, 28040 Madrid, Spain
- Wilfried Vyncke, Fisheries Research Station, Ankerstraat 1, B-8400 Ostend, Belgium
- Wilfried Winkelmann, Dr. Specht & Partner, St. Anscharplatz 10, D-20354 Hamburg, Germany
- Franz Winkler, Chemische Landesuntersuchungsanstalt Freiburg, Bissierstr. 5, D- 79114 Freiburg, Germany
- Jan Zalewski, Sea Fisheries Institute, ul. Kollataja, 81-332 Gdynia, Poland

All statistics were calculated with Statistica 5.0, Statsoft Inc., Tulsa, USA.

Results and Discussion

In Table 2 details of the „home methods" used by partners 1-14 are listed.

Table 2: Digestion conditions used in „home methods“ of participating laboratories

Participant	Digestion Temperature (° C)	Catalyst	Sulfuric acid (mL)	Sulphate	total digestion time (min)	Apparatus
1	400	Cu	25	yes	1440	Büchi
2	440	Se	12.5	no	45	Tecator
3	400	Ti/Cu	10	yes	120	self built
4	420	Se	15	yes	50	Kjeltec
5*	400	Se/Cu	20	yes	240	Kjeltec
6	440	Ti/Cu	20	yes	150	Gerhard
7**	450	Se/Cu	20	yes	180	Tecator
8	400	Hg	35	yes	240	self built
9	400	Cu	30	yes	180	Büchi
10	420	Cu	15	yes	90	Gerhardt
11	400	Cu	12	yes	90	Kjeltec
12	420	Se	50	yes	240	Gerhardt
13	365	Cu	25	yes	960	Gerhardt
14	450	Cu	30	yes	150	Büchi

* Participant added 5 mL aqueous hydrogen peroxide (30%)

** Participant used total digestion time of 420 min for fishmeal

Table 3 shows all raw data as delivered by participants. The results presented in the following are based on calculations without data of participant 12. The data provided by this partner had to be ommitted because all data are very much deviating from the means and range of the other 13 partners. It was concluded that this partner must have made a major mistake during analysis. In total 16 data out of 210 were identified as outliers (> 2SD), 92.4 % of all data were used for calculations.

The descriptive statistic of sample A-E is contained in Table 4. The arithmetic means and the medians of individual samples are almost equal, indicating that the values are nearly normal distributed. The theoretical nitrogen content in standard substances (samples D and E) were calculated on the basis of 100% purity and on the minimum content certified by the manufacturer (99 % and 99.5 %, respectively).

The figures in Table 4 show that the minimum nitrogen found in sample D (histidin) corresponds to 90-91 % of theoretical nitrogen content while the maximum equals to 103.6-104.7 %. The corresponding figures for sample E (urea) are: 90-91 % and 104.1-107.2 %. On an average 99.2-99.6 % of the theoretical amount of histidin and 96.9-98.2 % of that of urea were found.

Table 5 gives the mean coefficients of variations for the individual laboratories calculated on the basis of the Cvs of the samples analysed. This CV which is a good measure for the accuracy of results obtained with the same material under the same conditions varies between 0.16 % in Lab. 8 and 2.69 % in Lab. 3 (Lab. 12 is disregarded). Statistical significant differences ($p>0.01$) were found between Lab 3 and Labs. 2, 5, 6, 7, 8, 9, 10, 13 and 14; between Lab 4 and Lab 8, 10; between Lab 5 and Lab 8, 10; between Lab. 6 and 8, 10; between Lab. 7 and 8, 10; between Lab. 8 and 14; between Lab. 10 and 14.

Table 6 shows the mean coefficient of variations calculated for the samples on the basis of the CVs of the individual laboratories. This CV gives a good indication on the homogeneity of the samples and of the difficulty to analyse them. Obviously there were no differences in this respect between the three fish samples (A-C). It seems, however, that the analyses of the standard materials led to a lower CV due the better homogeneity and/or possible less difficulties encountered during analysis. CVs of samples D and E were significantly different ($p>0.01$) from CVs of samples A, B and C.

The results obtained with sample A (Fig. 1) show that the medians of all participants are closely scattering around the mean nitrogen content found (12.69 %) except participant 3.

The nitrogen contents found in sample B (Fig. 2) vary around 13.5 % and 14% nitrogen (mean 13.68 %),

Table 3: Raw data (% nitrogen on a wet weight basis), arithmetic means, standard deviations (SD) and coefficient of variation (CV) of samples A-E, as used in calculations. Figures marked with bold italics (outliers) were removed prior to calculations

Lab.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sample														
A-1	12.20	12.43	12.60	12.54	12.69	12.64	12.08	12.80	12.66	12.83	12.91	***19.60***	13.02	12.65
A-2	12.22	12.44	14.20	12.41	12.76	12.78	12.12	12.73	12.68	12.86	12.14	***18.00***	12.89	12.80
A-2	12.25	12.48	14.14	12.56	12.62	12.66	12.21	12.80	12.70	12.84	12.60	***21.00***	12.89	12.90
mean	12.22	12.45	13.65	12.50	12.69	12.69	12.14	12.78	12.68	12.84	12.55	***19.53***	12.93	12.78
SD	0.02	0.02	0.74	0.07	0.06	0.06	0.05	0.03	0.02	0.01	0.32	***1.23***	0.06	0.10
CV (%)	0.17	0.17	5.43	0.53	0.45	0.49	0.45	0.26	0.13	0.10	2.52	***6.3***	0.47	0.80
B-1	12.51	13.55	14.02	13.34	13.78	13.99	13.02	13.83	13.97	13.84	13.64	***42.9***	13.94	14.18
B-2	13.70	13.75	13.27	13.48	13.65	13.89	13.14	13.86	13.91	13.83	13.76	***62.3***	13.94	14.04
B-3	13.65	13.47	13.04	13.52	13.61	13.92	13.20	13.78	14.03	13.80	13.73	***68.7***	13.94	13.93
mean	13.29	13.59	13.53	13.44	13.68	13.94	13.12	13.82	13.97	13.82	13.71	***57.97***	13.94	14.05
SD	0.55	0.12	0.49	0.08	0.07	0.05	0.07	0.03	0.05	0.02	0.05	***10.97***	0	0.10
CV (%)	4.14	0.89	3.62	0.57	0.53	0.36	0.57	0.24	0.35	0.12	0.37	***18.92***	0	0.73
C-1	9.80	9.86	10.13	9.50	9.88	10.11	9.59	9.88	10.03	10.23	10.89	***14.00***	9.81	10.24
C-2	9.80	9.87	9.52	9.58	9.94	9.96	9.67	9.91	10.21	10.26	10.11	***13.50***	9.88	10.26
C-3	9.80	9.79	10.15	9.62	9.93	9.98	9.67	9.90	10.01	10.25	11.03	***10.70***	9.88	10.08
mean	9.80	9.84	9.93	9.57	9.92	10.02	9.64	9.90	10.08	10.25	10.68	***12.73***	9.86	10.19
SD	0	0.04	0.29	0.05	0.03	0.07	0.04	0.01	0.09	0.01	0.41	***1.45***	0.03	0.08
CV (%)	0	0.36	2.94	0.52	0.26	0.66	0.39	0.12	0.89	0	3.79	***11.40***	0.33	0.79
D-1	27.08	27.12	26.32	26.16	26.74	27.19	26.29	26.88	26.95	27.23	26.55	—	26.69	28.06
D-2	27.35	27.25	24.39	26.16	26.99	27.31	26.30	26.91	26.98	27.16	26.73	—	26.90	27.93
D-3	26.93	27.30	26.65	25.98	26.91	27.30	26.10	—	27.00	27.10	26.43	—	27.18	27.94
mean	27.12	27.22	25.79	26.10	26.88	27.27	26.23	26.90	26.98	27.16	26.57		26.92	27.98
SD	0.17	0.08	1.00	0.08	0.10	0.05	0.09	0.02	0.02	0.05	0.12		0.20	0.06
CV (%)	0.64	0.28	3.87	0.33	0.39	0.12	0.35	0.06	0.08	0.20	0.46		0.75	0.21
E-1	***33.00***	45.92	45.36	44.80	45.62	46.82	44.72	46.20	47.09	46.83	46.43	***139.20***	***32.42***	47.99
E-2	***33.05***	45.59	45.04	45.14	45.72	46.31	44.73	46.20	46.93	46.85	46.80	***142.80***	45.88	46.85
E-3	***33.03***	45.75	44.36	45.99	46.07	46.65	44.64	46.09	47.08	46.87	46.80	***144.60***	45.88	46.83
mean	***33.03***	45.57	44.92	45.31	45.80	46.59	44.70	46.16	47.03	46.85	46.68	***142.2***	45.88	47.22
SD	***0.02***	0.38	0.42	0.50	0.19	0.21	0.04	0.05	0.07	0.02	0.17	***2.24***	0	0.54
CV (%)	***0.06***	0.84	0.93	1.11	0.42	0.46	0.09	0.11	0.16	0.03	0.37	***1.58***	0	1.15

Table 4: Valid N, theoretical nitrogen content in standard substances, arithmetic mean, median, minimum and maximum, standard deviation and coefficient of variation in samples A-D, all participants

Sample	valid N	theor. content (%)	mean	median	minimum	maximum	std. dev.	coeff. var. (%)
A	39		12.69	12.66	12.08	14.20	0.43	3.39
B	39		13.68	13.78	12.51	14.18	0.35	2.53
C	39		9.98	9.91	9.50	11.07	0.32	3.21
D (histidin)	38	26.80-27.07	26.85	26.94	24.39	28.06	0.64	2.38
E (urea)	35	46.39-46.62	46.08	46.09	44.36	47.99	0.86	1.87

Table 5: Mean coefficient of variation for individual laboratories

Laboratory	1	2	3	4	5	6	7	8	9	10	11	12	13	14
samples analysed	4	5	5	5	5	5	5	5	5	5	5	4	5	5
CV (%)	1.24	1.24	2.69	0.49	0.41	0.42	0.37	0.16	0.32	0.09	1.50	9.55	0.31	0.74

Table 6: Mean coefficients of variation for individual samples

Sample	A	B	C	D	E
valid N	13	13	13	13	12
CV (%)	0.92	0.96	0.85	0.60	0.47

Figure 1: Box & Whisker Plot of all results obtained with sample A
freeze dried, thoroughly ground ocean perch muscle

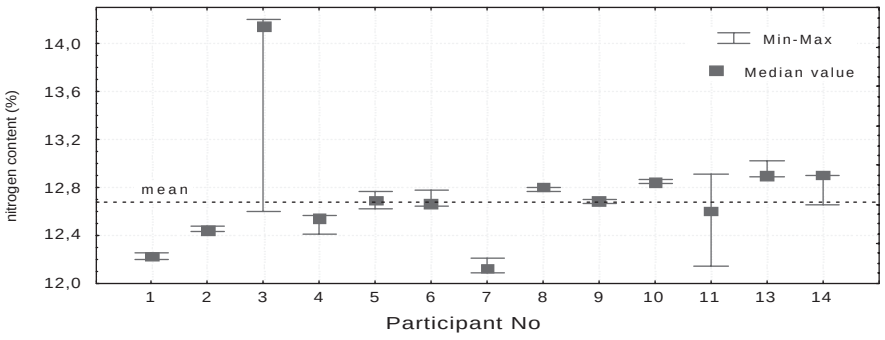


Figure 2: Box & Whisker Plot of all results obtained with sample B
freeze dried, thoroughly ground European hake muscle

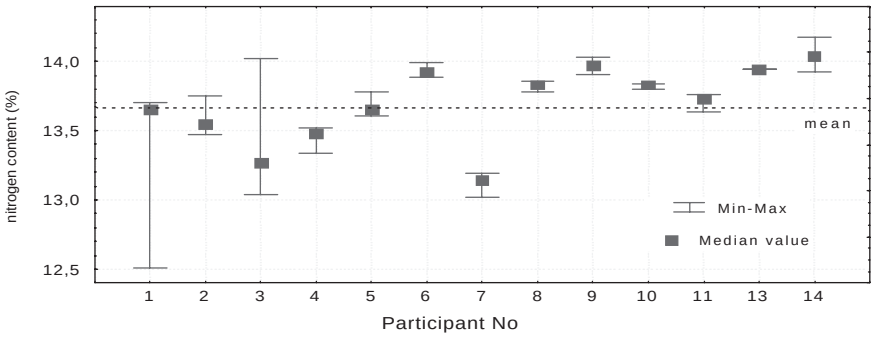


Figure 3: Box & Whisker Plot of all results obtained with sample C
lean white fish meal

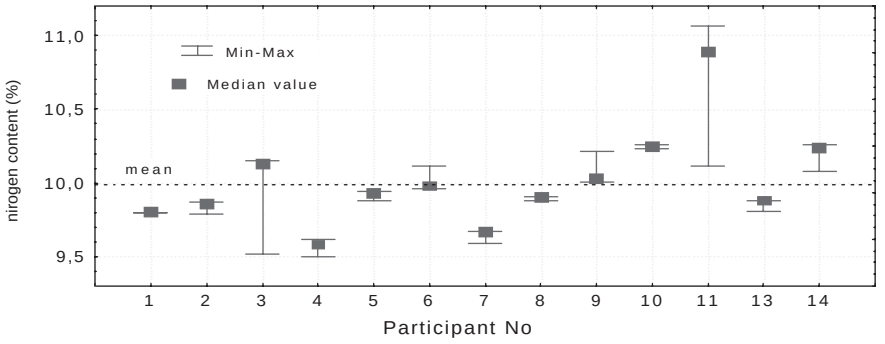
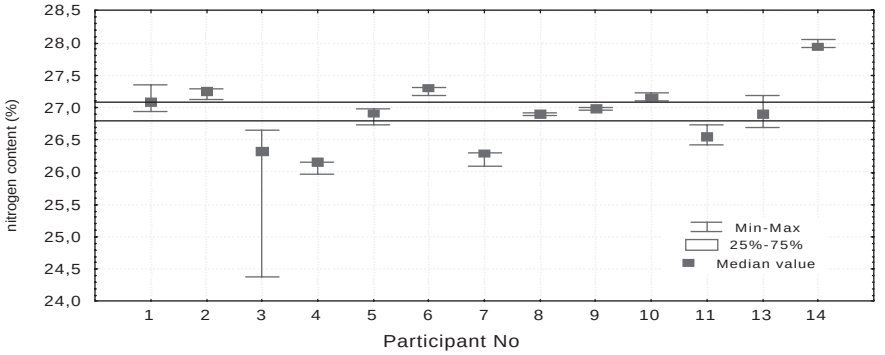


Figure 4: Box & Whisker Plot of all results obtained with sample D, L-histidine

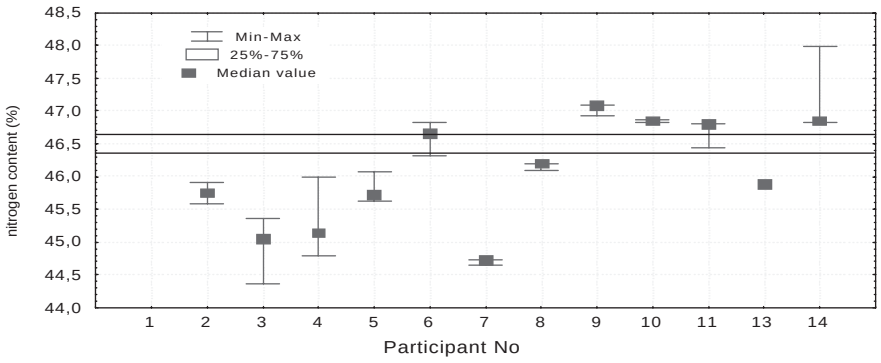


laboratories 3 and 7 found contents below 13.5 %. The results obtained with fish meal (Fig. 3) can be seen to be in a narrow range between 9.5 % and 10.5 % (mean 9.98 %) with the exception of participant 11 who found obviously a too high content (10.9 %).

The results with the first standard sample, L-histidine, as shown in Fig. 4, demonstrate that it was no problem for the participating laboratories to analyze this amino acids nitrogen content. Most values found scatter around the theoretical nitrogen content (upper line 100 % purity, lower line 99 % purity, minimum content as garanteed by the manufacturer), 5 laboratories lying in the range for the theoretical nitrogen content. Almost the same can be stated for the second standard substance, sample D, urea (Fig. 5). The nitrogen contents found by individual laboratories scatter somewhat more around the theortical contents (100 % and 99.5 %, respectively) but no outliers were observed.

The results with urea as a standard substance obtained in this exercise are very similar to those obtained during the second exercise in 1983. In that trial a mean nitrogen content of 46.19 % ± 0.35 % for urea in this 46.08 % ±0.86 % was analysed. The results with L-histidine cannot be compared because in 1982 L-histidine was not used as a standard substance.

Figure 5: Box & Whisker Plot of all results obtained with sample D, urea



As an example in Figure 6 the influence of the catalyst on the amount of nitrogen found in sample C (fish meal) is demonstrated in form of a categorizes box&whisker plot. Laboratories using copper as a catalyst generally found more nitrogen than laboratories using other catalysts. In a comparison between

Figure 6: Effect of catalyst on nitrogen content found in sample C (fish meal)

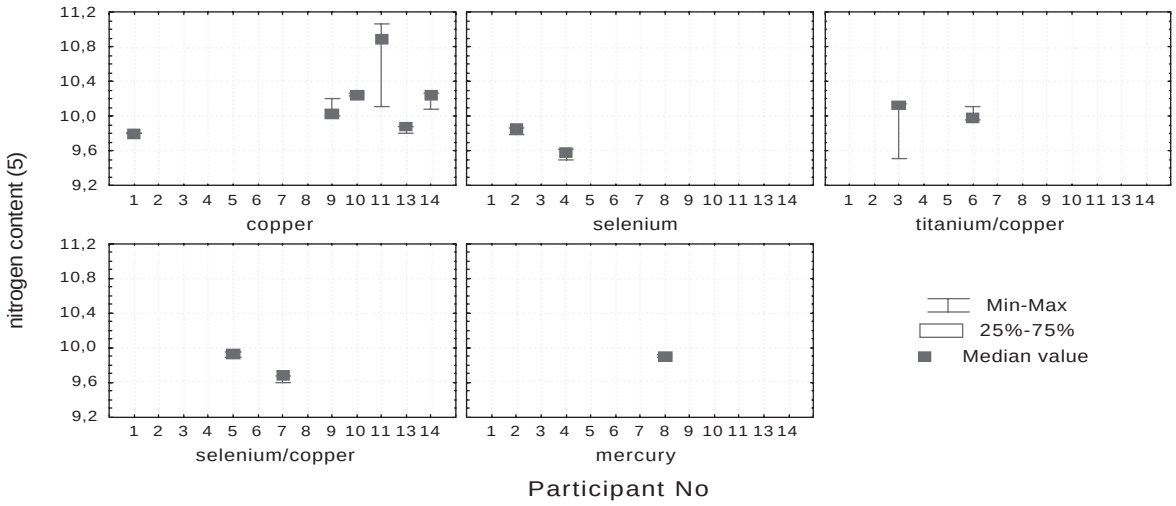
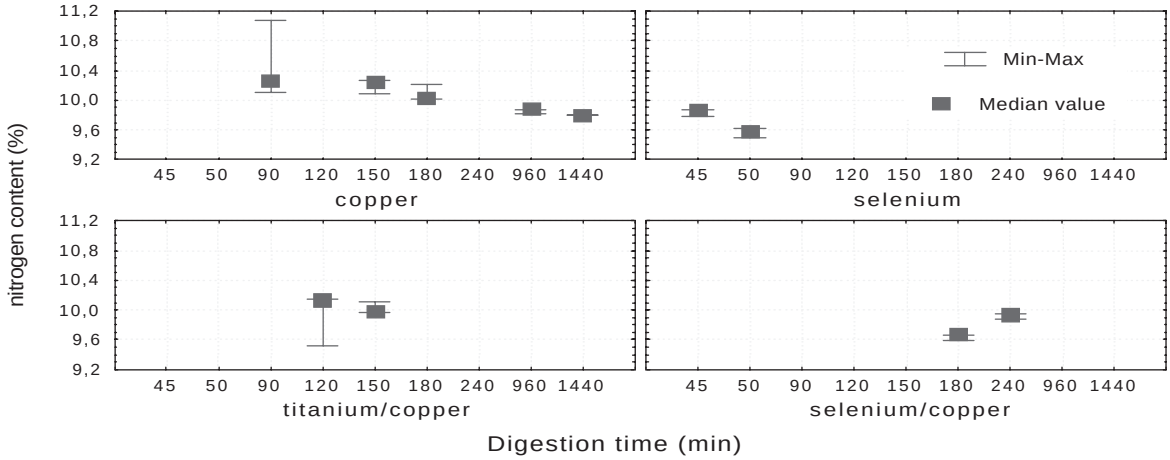
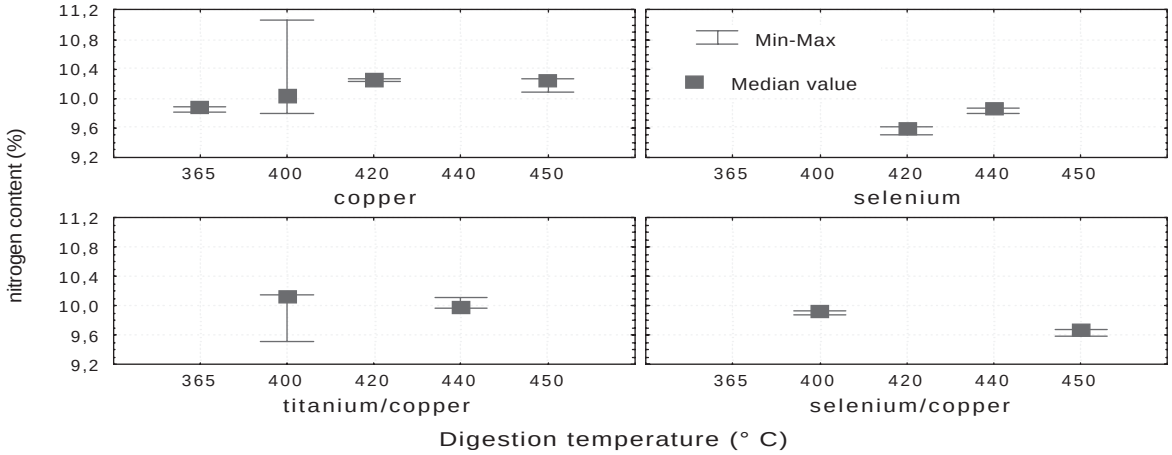


Figure 7: Effect of digestion time on nitrogen content found in sample C (lean white fish meal) using different catalysts



mercury and copper as catalysts in Kjeldahl determination it was reported earlier (Kane 1984) that the means obtained by copper were equivalent or higher as those by mercury. The effect of the catalysts is different from sample to sample. On the basis of all data obtained a categorized evaluation revealed that selenium as catalyst leads to the highest values followed by mercury (1 participant), selenium/copper, copper and titanium copper. This shows that the overall results differ from the results obtained with a single sample (Fig. 6).

Figure 8: Effect of digestion temperature on nitrogen content found in sample C using different catalysts



It seems that the catalyst's efficiency strongly depends on the matrix to be digested.

The effect of digestion time on nitrogen yield is shown in Figure 7. The yield of nitrogen is decreasing with increasing digestion time. 120 min digestion time is sufficient for obtaining optimal yield of nitrogen. It remained unclear why nitrogen yield decreased parallel to increasing digestion time. This phenomenon was already recognised in the earlier exercises performed in 1982/83.The only exeption from this rule was found when using selenium/copper as a catalyst (lower right in Fig. 7).

The digestion temperature (Fig. 8) has no great influence on the nitrogen content found. The temperature range between 400° C and 440° C, however; seems to be offer the optimal temperature for maximum recovery of nitrogen.

Conclusions

From the results presented it can be concluded that the majority (> 90%) of laboratories who had participated in this exercise were able to analyse the nitrogen content of fish samples and standard substances by using their „home methods". The results between laboratories vary only little as indicated by the coefficient of variation which varied between 1.9% and 3.4%. The results obtained with the two standard substances demonstrate that it was also possible for most laboratories to get results close to the theoretical nitrogen content.

Alltogether the results prove that nitrogen determination by Kjeldahl digestion leads to satisfying results. This means also that results analysed by different laboratories can be compared with each other.These findings correspond well with the general findings of the earlier exercises (see first page).

There are indications that the catalyst used has some influence on the results. This influence, however, depends on the nature of the matrix to be analysed. Long digestion times have a negative influence on the yield, and a digestion temperature around 430° C seems to be optimal.

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