Determination of volatile basic nitrogen in fish: a third collaborative study by the West European Fish Technologists' Association (WEFTA)

Nicolaus Antonacopoulos [1] and Wilfried Vyncke [2]

[1] Federal Research Centre for Fisheries, Institute for Biochemistry and Technology, Palmaille 9, D-2000 Hamburg 50, Federal Republic of Germany
[2] Ministry of Agriculture, Fisheries Research Station, Ankerstraat 1, B-8400 Oostende, Belgium

Bestimmung des flüchtigen Basenstickstoffs in Fisch: 3. Ringversuch der WEFTA (Vereinigung westeuropäischer Fischtechnologen)

Zusammenfassung. Am 3. WEFTA Ringversuch beteiligten sich 13 Laboratorien, 11 davon benutzten die als gemeinsame Methode vorgesehene Direktdestillation von Fisch unter Zusatz von Magnesiumoxid (MgO) als mildem Alkalisierungsmittel (Antona-Methode). 2 Labors verwendeten ausschließlich, 6 Labors zusätzlich Haus-Methoden; in 5 von diesen ist eine saure Extraktion mit Trichloressigsäure (TCA) oder Perchlorsäure (PCA) der Destillation unter Zusatz von Natriumhydroxid (NaOH) vorgeschaltet (Typ Codex-Methode). Als Probenmaterial dienten Scholle und Hering (3 Frischegrade). Die Ergebnisse des 3. Ringversuches bestätigten im Prinzip die Befunde des 2. Ringversuches, bei der ein Extraktionsverfahren als gemeinsame Methode benutzt wurde. Beide Methodenvarianten (Antona und Codex) weisen ähnliche systematische Fehler auf. Die mittlere Wiederfindungsrate mit der Antona-Methode, bezogen auf Basiswerte des Testmaterials, betrug 94,7 ± 9,4% bei Schollen und 91,6 ± 8,8% bei Heringen, die Wiederfindung von zugesetztem Ammoniumsulfat war mit 94,1 und 88,7% vergleichbar. Ergänzende Untersuchungen zeigten, daß bei Alkalitäten > pH 11 sekundäres Ammoniak gebildet wird. MgO erwies sich dabei gegenüber NaOH als schonender. Um Schwankungen von TVB-N-Resultaten möglichst auszuschließen – eingeschlossen der bei beiden Methoden unvermeidbaren Blindwerte in frischem Fisch – ist eine detaillierte alle Schritte umfassende Arbeitsvorschrift als wesentliche Voraussetzung für die Standardisierung erforderlich: Ein Entwurf ist als Anhang beigefügt. Die TVB-N-Methode wird für Belange der laufenden Qualitätsüberwachung als geeignet beurteilt, wobei der Direktdestillation wegen der schnellen und wirtschaftlichen Durchsetzbarkeit großer Probenserien der Vorzug gegeben wird. Aus Extrakten sind im Zweifelsfalle zur Beweissicherung zusätzliche Bestimmungen weiterer Amine, z. B. Trimethylamin, möglich.

Offprint requests to: N. Antonacopoulos

Summary. Thirteen laboratories participated in the third WEFTA exercise on determination of volatile base nitrogen (TVB-N) in fish. Plaice and herring, each at three stages of freshness, were distributed. Eleven laboratories applied the direct distillation of fish after addition of magnesium oxide (MgO) (Antona method). Six laboratories carried out analysis by their own methods also, while two laboratories used their own methods only. In five of these own methods, extracts with trichloroacetic or perchloric acid made alkaline with sodium hydroxide (NaOH) were distilled. The results of the third exercise confirm in principle the findings of the second trial, in which participants used an extraction procedure (Codex method). Both Antona and Codex methods show similar systematic errors. The mean recoveries of TVB-N by the Antona method, related to reference values of the test materials, were 94.7 ± 9.4% for plaice and 91.6 ± 8.8% for herring: the mean recoveries of added ammonium sulphate were similar, 94.1% and 88.7%, respectively. Supplementary tests indicated that a pH greater than 11 during distillation promotes secondary generation of ammonia; MgO had an advantage over NaOH in this respect. In order to reduce variations in TVB-N results, a detailed description of all steps of the method is an essential precondition for standardisation: a draft is appended. The TVB-N method is judged to be suitable as a standard method for routine quality control. Being quick and relatively inexpensive to carry out, the direct distillation procedure is preferred for large numbers of samples; extraction methods permit parallel determination of other amines, such as trimethylamine, where confirmation is required.

Z Lebensm Unters Forsch (1989) 189:309–316

1 Introduction

The determination of total volatile basic nitrogen (TVB-N) is, because of its simplicity, a widely employed quality assessment method for wet fish. There are a number of methods in current use: all involve making fish or an extract of fish alkaline, allowing the bases to volatilize, mostly by distillation, and collecting them for measurement by titration. A disadvantage of all TVB-N methods is a more or less high blank value, caused by deamination of nitrogenous compounds in the fish or in the fish extract during the alkaline volatilization step. This is the reason for the wide range of proposed methods, most of which involve different extraction procedures for minimizing blank values, instead of the original direct distillation of fish [1, 2]. As extraction agents, aqueous solutions of trichloroacetic acid (CCl_3CO_2H) or perchloric acid ($HClO_4$) are in common use.

2 First and second collaborative studies

The aim of the WEFTA collaborative studies is to select a standardized method that will allow comparable results from different laboratories. In the first and second WEFTA studies, which were undertaken in 1983 and 1984 among 9 and 6 European laboratories respectively, the so-called Codex method was used as a common method in comparison with "home" methods. The Codex method, which was proposed in 1968 by Canada to the FAO/WHO Codex Committee on Fish and Fishery Products [3], involves distillation of 25 ml CCl_3CO_2H fish extract with 6 ml 10% NaOH solution. As "home" methods six different procedures were applied, which are in use in the nine laboratories: three labs carried out direct distillation of fish with magnesium oxide (MgO) according to Antonacopoulos [4, 5], one used an aqueous extract with MgO; two reacted acid extracts with NaOH solutions and three applied the Conway microdiffusion technique [6] to samples extracted with water or CCl_3CO_2H. As sample materials cod and mackerel of different degrees of freshness were used. The results of both collaborative exercises, published by Vyncke et al. [7], showed important systematic errors between participating laboratories both with the Codex and with the home methods. Better comparability was obtained when pure solutions of ammonia, dimethylamine and trimethylamine which are the main components of TVB, were subjected to the same methods.

3 Third collaborative study

The WEFTA Analytical Working Group at its 1985 meeting concluded, on the basis of the first two studies, that the tested methods are either inadequately described or very sensitive to uncontrolled factors encountered in the various laboratories. It was recommended that the methodology should be scrutinized further, for example on the need for a more detailed description of the distillation apparatus and distillation procedure. As the extraction with acid solutions showed no significant advantage over the more simple and economic direct distillation of fish with MgO, which supplied fairly comparable results, a third collaborative study was agreed, using this technique, the so-called Antona method, with fewer analytical steps as common method.

The Hamburg laboratory prepared a detailed working description and adequate samples: from plaice, to avoid the possible generation of dimethylamine during frozen storage, and from herring as a fatty fish, each in three stages of freshness, including hidden duplicates and a sample containing an added known amount of a volatile base. Additionally, home methods could be used.

Eleven laboratories participated in the third exercise with the "Antona method": Icelandic Fisheries Laboratories, Reykjavik (Iceland); Ifremer, Nantes (France), Technical Research Centre of Finland, Espoo (Finland), Torry Research Station, Aberdeen (Scotland, UK), Rijksstation voor Zeevisserij, Oostende (Belgium); from the Federal Republic of Germany: Bundesforschungsanstalt für Fischerei, Hamburg [2], Veterinäruntersuchungsämter of Bremerhaven, Cuxhaven and Hamburg and the Nordsee-Zentrallabor, Bremerhaven. Two laboratories used home methods only: Instituto del Frio, Madrid (Spain), Irish Sea Fisheries Board, Dublin (Ireland); six of the above-mentioned laboratories also used home methods, in two cases more than one method. The order of the laboratories listed above is not identical with the order in the tables.

3.1 Material

Fillets of plaice (*Pleuronectes platessa*) and herring (*Clupea harengus*), purchased from the Hamburg fish market (A-quality) were used directly or after periods of ice storage, to obtain three degrees of freshness: good (A), fair (B), and poor (C) quality. Sample lots were thoroughly mixed in a cutter and, during continuous stirring, subsamples were weighed, placed in closeable plastic bags and immediately frozen to preserve the TVB content. As reference base,

Table 1. 3rd WEFTA Collaborative Study – TVB–N contents of the test materials

Code	Fish	Quality	TVB–N (mg/100 g)	N added (mg/100 g)
P1	Plaice	A	16.4±0.2	–
P2		A, spiked	28.2±0.3	11.8
P3		B	30.3±0.4	–
P4		C	53.8±0.3	–
P5		C, duplicate	53,8±0.3	–
H1	Herring	A	24.5±0.1	–
H2		A, spiked	32.9±0.2	8.4
H3		B	30.8±0.2	–
H4		B, duplicate	30.8±0.2	–
H5		C	56.8±1.0	–

a known amount of ammonium sulfate ($(NH_4)_2SO_4$) was added under the same conditions to one A-quality lot of each fish. Each participating laboratory received 10 coded samples, spiked samples and hidden duplicates included. The TVB-N contents of the samples, measured by six-fold determination immediately after homogenization, are shown in Table 1.

3.2 Analyses

3.2.1 Antona method. After thawing, the pre-weighed well-homogenized fish sample (10 g) is suspended with a small amount of water and quantitatively transferred into the reaction vessel of the Antona steam distillation unit [8]. After addition of 2 g MgO and 2–3 drops antifoam emulsion, the vessel is inserted into the preheated steam generator and the distillation is started immediately (steam flow approx. 10 ml/min). The distillate is collected in the receiver, containing 10 ml boric acid (3%) and 8 drops Tashiro indicator, filled up to approx. 100 ml with distilled water. Distillation time is exactly 12 min: 10 min with condenser outlet immersed, 2 min above the surface of the distillate. The distillate is titrated with 0.1 M hydrochloric or 0.05 M sulphuric acid until the grey neutral point is reached. (A detailed method description is given in an Addendum.)

3.2.2 Home methods. Five laboratories distilled acid extracts made alkaline with NaOH. One used the Conway microdiffussion method; four used semiautomatic or automatic distillation units for the direct distillation of fish with MgO: of these, two used Tecator units and two the Büchi 315 unit, specially modified for this purpose [9].

3.2.3 Statistical methods. TVB-N determinations were reported in duplicate. Statistical processing of the data was carried out according to Youden and Steiner [10] in the same manner as described in the publication of the former two collaborative tests [7].

3.3 Results and discussion

The TVB-N values obtained with the Antona method by the participating laboratories are listed in Table 2.

Table 2. Averages and differences between duplicates of TVB–N determination of plaice (P) and herring (H) samples with the Antona method (mg N/100 g)

Lab.	Average (difference) of TVB–N (mg/100 g)									
	P1	P2	P3	P4	P5	H1	H2	H3	H4	H5
A	15.0 (0.3)	28.0 (0.4)	29.9 (0.7)	52.7 (0.5)	56.5 (2.5)	23.5 (0.8)	31.2 (1.0)	27.9 (0.7)	29.4 (1.0)	55.5 (1.0)
B	14.1 (0.8)	24.5 (0.9)	28.1 (0.5)	51.6 (0.6)	52.2 (0.3)	22.9 (1.3)	29.1 (0.6)	29.9 (0.3)	26.5 (0)	51.6 (1.8)
C	10.5 (0.7)	20.1 (2.4)	23.3 (0.5)	51.4 (0.9)	50.8 (2.2)	17.4 (2.8)	26.3 (0)	24.1 (1.0)	23.0 (2.1)	51.0 (0.5)
D	15.4 (0.3)	26.1 (1.0)	29.2 (1.6)	53.7 (–)	55.3 (1.2)	22.6 (1.0)	30.7 (0)	28.8 (0.3)	26.9 (0.5)	54.4 (0.5)
E	11.2 (2.4)	21.7 (0.8)	21.3 (2.2)	51.8 (7.1)[a]	53.8 (2.6)	18.2 (2.2)	22.9 (1.5)	22.2 (1.0)	22.7 (3.1)	49.2 (6.7)[a]
F	14.0 (0.2)	25.4 (0.1)	25.8 (1.2)	51.0 (0.3)	54.8 (0)	24.2 (0.1)	30.0 (0)	29.2 (0.1)	28.4 (0.8)	54.4 (0.5)
G	10.4 (0.1)	20.1 (6.4)	17.7 (3.4)	41.9 (8.9)	48.1 (0.4)	11.1 (0.1)	14.7 (0.2)	16.4 (1.6)	14.4 (0.1)	26.9 (1.2)
H	15.1 (0.7)	26.9 (4.4)	30.2 (0.9)	52.2 (0.7)	54.5 (0.8)	24.6 (0.9)	31.4 (0.1)	27.9 (0.2)	27.8 (0)	55.3 (1.8)
K	17.9 (0.6)	29.2 (1.5)	34.2 (1.5)	56.7 (1.5)	58.9 (2.6)	26.1 (0.2)	37.6 (1.0)	31.2 (0.2)	29.3 (0.4)	55.9 (1.7)
L	13.6 (0.8)	24.8 (1.5)	27.3 (0.3)	51.1 (1.5)	53.3 (1.1)	22.2 (0.1)	30.2 (0)	26.2 (0.2)	27.8 (0.3)	52.1 (1.0)
M	17.9 (2.0)	29.0 (1.2)	35.2 (3.8)	59.6 (1.1)	57.6 (1.0)	26.8 (2.7)	33.6 (0.3)	31.5 (0.8)	31.7 (1.6)	52.4 (4.4)
$\bar{x}$	14.5	25.6	28.5	53.3	54.8	22.8	30.3	27.9	27.4	53.6
Tm	16.4±0.2	28.2±0.3	30.3±0.4		53.8±0.4	24.5±0.1	32.9±0.2		30.8±0.2	56.8±1.0

Sample P1 was spiked with 11.8 mg N/100 g and sample H1 with 8.4 mg N/100 g; (P4–P5) and (H3–H4) are hidden duplicates. Value for lab. G were rejected by Dixon's test for systematic error [a] Rejected by Cochran's test: outliers (large difference between duplicates) *Tm* Test materials, measured before distribution by six fold estimation by Hamburg Lab, using Antona method

Table 3. Statistical summary of collaborative results for TVB–N determination with the Antona method

Parameter	Value for									
	P1	P2	P3	P4	P5	H1	H2	H3	H4	H5
n	20	20	20	16	20	20	20	20	20	18
$\bar{x}$	14.47	25.57	28.45	53.28	54.77	22.85	30.30	27.89	27.35	53.62
s_r	0.59	1.30	1.17	0.69	1.20	1.10	0.49	0.41	0.97	1.32
s_b	2.38	2.83	4.27	3.11	2.29	2.94	3.91	2.97	2.70	1.60
s_d	2.45	3.11	4.43	3.19	2.59	3.14	3.94	3.00	2.87	2.08
V_r	4.08	5.01	4.11	1.30	2.20	4.81	1.62	1.47	3.55	2.46
V_b	16.45	11.06	15.01	5.84	4.18	12.87	12.90	10.64	9.87	2.98
V_d	16.93	12.17	15.57	5.99	4.73	13.74	13.00	10.76	10.49	3.88
r	1.67	3.62	3.31	1.95	3.40	3.11	1.39	1.16	2.75	3.74
R	6.93	8.80	12.54	9.03	7.33	8.89	11.15	8.49	8.12	5.89
F	34.01 ***	10.05 ***	27.63 ***	41.18 ***	8.26 **	15.31 ***	130.84 ***	103.58 ***	16.60 ***	3.93 *

Table 2 for description of samples; n = number of results representing each sample (P4: lab D excluded as no duplicate analysis available); s_r = random error (precision standard deviation); s_b = systematic error (between laboratory variability); s_d = combined error (reproducibility standard deviation); V_r = relative standard deviation (repeatability coefficient variation); V_b = relative standard deviation (between laboratory coefficient of variation), V_d = relative standard deviation (reproducibility coefficient of variation); r = Repeatability; R = reproducibility (ISO 5725) $P(F)<0.05$*; $P(F)<0.01$**; $P(F)<0.001$***

3.3.1 Differences within and between laboratories

The F-test (Table 3) indicated that the variance component due to collaborators was significantly different from zero for all samples analysed by the Antona method. This means that the between-laboratory systematic error (s_b) was highly significant. This was also the case in the previous trials where the Codex method was the one used by all participants.

There was no difference in average s_b between the plaice (3.06) and the herring samples (2.92). This was also found in the previous trials using the Codex method for cod, a lean fish, and mackerel, a fatty fish. Moreover, the s_b values of the present study were not significantly different from those in the previous trials, indicating that the systematic errors were of the same order of magnitude for both methods.

The precision standard deviations (s_r) were not significantly different for plaice and herring, with root-mean square values of 1.03 and 0.93 respectively. In the previous study an average value of 1.07 was found for cod by the Codex method not significantly different from the present values. For mackerel however the root-mean-square was significantly lower (0.44), probably due to a different fat content and texture.

The TVB-N values obtained for the hidden duplicates (P4 + 5 and H3 + 4) were subjected to an analysis of variance with two factors (laboratories and duplicates). No significant differences were found, confirming the previous calculations on the precision standard error. The Antona method has a good reproducibility.

3.3.2 Recoveries of TVB-N added to fish

No outliers were detected with Dixon's test. Both averages (94.1 and 88.7%) were significantly different from 100%, stressing again the presence of a systematic error (Table 4).

Table 4. Recovery of TVB–N added to fish as $(NH_4)_2SO_4$ with the Antona method

Lab.	Recovery in sample			
	P2–P1		H2–H1	
	(mg)	(%)	(mg)	(%)
A	13.0	110.1	7.7	91.7
B	10.4	88.1	6.2	73.8
C	9.6	81.4	8.9	106.0
D	10.7	90.7	8.1	96.4
E	10.5	89.0	4.7	56.0
F	11.4	96.6	5.8	69.0
H	11.8	100.0	6.8	81.0
K	11.3	95.8	11.5	136.9
L	11.2	94.9	8.0	95.2
M	11.1	94.1	6.8	81.0
$\bar{x}$	11.1	94.1	7.5	88.7
s	0.9	7.7	1.9	22.4
V (%)	8.2	8.2	25.3	25.3

Table 2 for description of samples. Added TVB–N to P2 = 11.8 mg; H2 = 8.4 mg

The standard deviation for plaice (7.7) was significantly lower than for herring (22.4). Owing to this difference a modified t-test according to Satterthwaite [11] was applied to test the difference between both mean recovery values. No significant difference was found, allowing the calculation of an average recovery: 91.4%.

3.3.3 Recovery of TVB-N referred to initial values

No outliers were detected with Dixon's test (Table 5). The average recovery was 94.6% for plaice. This value

Table 5. Recovery of TVB–N by Antona method, related to reference values of test materials

Lab.	Recovery from sample (%)											
	P1	P2	P3	P4	P5	Mean	H1	H2	H3	H4	H5	Mean
A	91.5	99.3	98.7	98.0	105.0	98.5	95.9	94.8	90.6	95.5	97.7	94.5
B	86.0	86.9	92.7	95.9	97.0	91.7	93.5	88.4	97.1	86.0	90.8	91.2
C	64.0	71.3	76.9	95.5	94.4	80.4	71.0	79.9	78.2	74.7	89.8	78.7
D	93.9	92.6	96.4	99.8	102.8	97.1	92.2	93.3	93.5	87.3	95.8	92.4
E	68.3	77.0	70.3	96.3	100.0	82.4	74.3	69.6	72.1	73.7	86.3	75.3
F	85.4	90.1	85.1	94.8	101.4	91.4	98.8	91.2	94.8	92.2	95.8	94.6
H	92.1	93.4	99.7	97.0	101.3	96.7	100.4	95.4	90.6	90.3	97.4	94.8
K	109.1	103.5	112.9	105.4	109.5	108.1	106.5	114.3	101.3	95.1	98.4	103.1
L	82.9	87.9	90.1	95.0	99.1	910	90.6	91.8	85.1	90.3	91.7	89.9
M	109.1	102.8	116.2	110.8	107.1	109.2	109.4	102.1	102.3	102.9	92.3	101.8
$\bar{x}$	88.2	90.5	93.9	98.9	101.8	94.7	93.3	92.1	90.6	88.8	93.7	91.6
s	14.7	10.4	14.4	5.3	4.6	9.4	12.4	11.9	9.7	9.0	3.9	8.8
V (%)	16.7	11.5	15.3	5.3	4.5	9.9	13.3	13.0	10.7	10.2	4.2	9.6

Table 2 for description of samples. Means are overall mean recoveries for plaice (P) and herring (H)

Table 6. Recovery of TVB–N with home methods and recovery of TVB–N added to fish as $(NH_4)_2SO_4$ with home methods, related to reference values of the test materials

Lab	Method	Overall mean recovery		Recovery of added TVB–N	
		P1 to P5	H1 to H5	P2–P1	H2–H1
L	MgO + Büchi mod.	98.3 ± 2.4	97.8 ± 2.7	92.4	100.0
M1		104.9 ± 2.5	100.6 ± 1.8	96.6	104.8
N1	MgO + Tecator 10 min	121.6 ± 4.8	111.8 ± 5.2	104.2	110.7
N2	6 min	100.7 ± 4.2	92.0 ± 6.9	83.1	54.8
B	200 s	74.6 ± 19.2	84.8 ± 30.1	105.1	88.1
D	CCl_3CO_2H extract Codex	93.1 ± 3.7	99.1 ± 7.9	90.7	115.5
M2		101.7 ± 5.3	100.6 ± 1.8	96.6	106.0
N3		91.9 ± 0.7	99.9 ± 10.3	94.4	96.4
O		125.2 ± 15.1	92.2 ± 7.2	83.8	132.1
G	$HClO_4$ extract	86.7 ± 12.8	83.1 ± 6.1	100.0	113.1
C	Conway microdiffusion	81.0 ± 14.5	88.1 ± 3.3	97.5	109.5

See Table 2 for description of samples. TVB–N was measured with Antona method for reference values. Home methods are ordered according to type of separation. The Büchi modification used direct distillation according to the Antona method. The Tecator used direct distillation without steam regulation. The $HClO_4$ extract was distilled in the Büchi

was not significantly different from 100%. For herring, an average recovery of 91.7% was found, which was significantly different from 100%. Fatty fish species apparently have a lower recovery when reference values are taken for comparison.

3.3.4 Home methods (Table 6)

The recoveries by laboratories using home methods include an acid extraction step (last six in Table 6), are quite similar to those with the direct Antona method (Tables 4 and 5).

The first 5 in Table 6 show results of the direct distillation of fish and MgO with semiautomatic distillation units instead of the Antona unit. The results for the first two indicate better recovery and uniformity by use of the Büchi 315 unit, which was modified for the TVB-N determination, confirming findings by Antonacopoulos [12]. Related results with Tecator units (N_2, N_2 and B) demonstrate by the much higher variations of the yields the importance of a reduced steam flow and adjusted distillation conditions: 200 s is too short; increased values after 10 min may indicate too rigorous conditions.

3.3.5 General statistical conclusions

From the point of view of precision (within laboratory reproducibility) and between-laboratories variations (systematic error), the Antona method and the previously tested Codex method are quite similar. The Antona method however involves fewer analytical steps and hence is less laborious and time-consuming.

4 Final considerations and recommendations

The WEFTA collaborative studies have confirmed in principle earlier findings by Vyncke [1], from comparative studies by WEFTA members [13–15] and from other working groups [16, 17], that extraction methods have no significant advantage over the direct distillation of fish, other than lower initial values.

A critical review of the various studies and supplementary investigations, recently carried out by Antonacopoulos [18] demonstrated that the principal reason for variation of results between proposed methods is the different alkalization conditions: the higher the pH during distillation, the more additional ammonia is formed by secondary deamination of nitrogenous compounds. The resulting higher TVB-N values are not related to spoilage. This occurs also in acid extracts; the amount of extractable and deaminable N compounds increases with continuing spoilage. Comparative tests confirmed the superiority of magnesium oxide (MgO) as a mild alkalization agent compared with sodium hydroxide (NaOH) [16, 18].

Considering these findings, both procedures, direct distillation of fish and distillation of extracts, are in principle suitable for standardization. For purposes of routine quality control, however, direct distillation has advantages over methods requiring additional extraction step: compared with direct distillation the costs are substantially higher, because (a) the time needed is nearly doubled (17 min to 30 min): indeed, the filtration of a magnesium sulfate extract, which is proposed by Botta et al. [17], needs more than 1 h; (b) the number of reagents required is higher (7 cf. 5), and (c) the amount of equipment needed is greater (8 cf. 4).

Further possible disadvantages of extraction methods are (a) trichloracetic and perchlor acids, when routinely used for a large number of samples, may be a health risk for the staff and a risk to the environment; (b) if a 50-ml extract is used (in comparison with 10 g fish), an increased volume due to initial heating of the sample could result in incomplete transfer of TVB-N.

Therefore the additional extraction step can be recommended only in particular cases when confirmation may be needed, since acid extracts can be stored for a limited period to allow parallel determinations on further, more specific, indicators of spoilage, such as dimethylamine and trimethylamine, hypoxanthine and other biogenic amines; these however, involve much more instrumental effort (GC, HPLC). Equally, frozen fish samples can also be stored for such confirmatory tests.

Based on the existing comprehensive studies, the findings of various other authors and the results of recent supplementary investigations and related proposals for additional improvements of the method presented by Antonacopoulos [18], the WEFTA Working Group, at its meeting in April 1988 in Ijmuiden (NL), came to the following conclusions.

a) The TVB-N method is a routine method, which should be used to provide information only on the later stages of spoilage of wet fish, i.e. whether the fish is fit or unfit for human consumption. The method is only suitable for confirmation of sensory assessments.

b) The identification of the early stages of freshness is not possible with TVB-N values, because of the high initial values. For this purpose the trimethylamine estimation, e.g. by the existing AOAC standard method [19], is distinctly superior, because practically no trimethylamine blank values are found in the first 3 to 5 days of ice storage. This method includes an extraction step, and needs additionally a spectrophotometer or a gas chromatograph (GC): this more expensive and sensitive technique is less suitable for common routine control.

c) Considering the need for a method that is simple, quick and economic, to ensure its wide acceptance and use in practice, the TVB-N determination by direct distillation of fish is suitable as a standard method for assessing the marketability of wet fish by official or voluntary quality control action.

d) For confirmation (in case of doubt) and for research purposes an alternative standard TVB-N method including an extraction step should be available.

e) In the light of existing knowledge about the scope and limitations of the TVB-N method, further collaborative studies were felt not to be necessary. Rather the elaboration of a working manual, including detailed prescription of all essential parameters such as preparation of samples and equipment, the critical alkalization step, the time and volume of distillation and the titration step, seems sufficient to obtain further reduction in standard deviations between repeat determinations, between operators and between laboratories. Further improvements seem not to be probable.

f) The Hamburg laboratory was asked, in the light of its results and proposals: (i) to carry out a storage test to compare the direct distillation technique and the distillation of perchloric and/or trichloroacetic acid extracts under mild alkaline conditions, preferably with MgO; (ii) to calculate a possible conversion factor between the TVB-N results after direct or extract distillation respectively; (iii) finally to formulate a corresponding working procedure as a model for a TVB-N Standard Method. A corresponding draft of such a method, derived from the TVB-N review paper of Antonacopoulos [18] is attached.

Acknowledgement. The authors wish to thank Dr. Angus Aitken (Torry Research Station, Aberdeen, Scotland) for the thorough revision of the manuscript and his useful advice.

References

1. Vyncke W (1971) Comparison of two methods for determining volatile basic nitrogen. FAO Fisheries Report No. 115, pp 44–47
2. Vyncke W (1983) Determination of total volatile bases in fish: a review of methology. Report of the Fisheries Research Station, Oostende, Belgium No. 193-BV/34
3. FAO/WHO (1968) Method for the determination of total volatile basic nitrogen (TVB) in fish muscle. Presented to Codex Committee on Fish and Fishery Products, 3rd Session, Bergen (N) as Codex Fish 1/7
4. Antonacopoulos N (1968) In: Acker I (ed) Handbuch der Lebensmittelchemie, Bd III/2. Springer, Berlin, pp 1493–1501
5. Antonacopoulos N (1973) In: Ludorff/Meyer: Fische und Fischerzeugnisse. Paul Parey, Berlin Hamburg, S 179–182, 224–228
6. Conway EJ (1947) Microdiffusion analysis. Van Nostrand, New York, see also: Pearson D, Muslemuddin M (1971) J Assoc Publ Anal 9:28
7. Vyncke W, Luten J, Brünner K, Moermans Renaat (1987) Determination of total volatile bases in fish: a collaborative study by the WEFTA. Z Lebensm Unters Forsch 184:110–114
8. Antonacopoulos N (1960) Verbesserte Apparatur zur quantitativen Destillation wasserdampfflüchtiger Stoffe. Z Lebensm Unters Forsch 113:113–116
9. Antonacopoulos N (1985) BÜCHI-destillation unit 315, modified for TVB-N determination. BÜCHI GmbH, Esslingen
10. Youden W, Steiner E (1975) Statistical manual of the AOAC, AOAC Arlington, VA, USA
11. Satterthwaite F (1980) In: Snedecor G, Cochran W, Statistical methods, 7th Edition, The Iowa State College Press, Ames, Iowa, USA, PP 97/98, 228/229, 254, 325
12. Antonacopoulos N (1985) Determination of total volatile bases (TVB-N) – Comparison of separation techniques. Private communication to the 8th meeting of the WEFTA Analytical Working Group, Nantes

13. Ruiter A (1972) Determination of total volatile base (TVB) content in fish. Report No. V-4, Institute for Fisheries Products TNO (NL) – Private Communication to the 3rd WEFTA meeting, Hamburg 1973
14. Howgate P (1973) Torry Research Station, Aberdeen (UK): Some observations on the determination of total volatile bases. Private Communication to the 3rd WEFTA meeting, Hamburg
15. Antonacopoulos N (1973) Comparison of 3 methods for determining total volatile bases (TVB-N). Private Communication to the 3rd WEFTA meeting, Hamburg
16. Oehlenschläger J (1988) Inf Fischw 35:31–34
17. Botta JR, Lauder JT, Lewer MA (1984) J Food Sci 49:734–736, 750; see also: Method No. 10, Total volatile base. In: Recommended laboratory methods for assessment of fish quality. Canadian Technical Report of Fisheries and Aquatic Sciences No. 148 (1986)
18. Antonacopoulos N (1989) Zur Eignung der TVB-N-Methodik zur Frischegrad-Bestimmung bei Seefischen. Arbeiten aus dem Institut für Biochemie und Technologie der Bundesforschungsanstalt für Fischerei, Hamburg, Nr. 5/1989
19a. Horwitz W (ed) (1980) Official Methods of the Association of Analytical Chemists (AOAC). Method No. 18.031–18.033: Trimethylamine Nitrogen. Washington DC: AOAC, 13th ed.; see also JAOAC 54:725 (1971)
19b. Horwitz W (1971) J Assoc Oil Am Chem 54:725

Received April 17, 1989

Addendum

Determination of total volatile basic nitrogen (TVB-N) in marine fish

1 *Principle*

The volatile basic nitrogen content is liberated by addition of magnesium oxide (MgO), a weak alkali, to the thoroughly homogenized fish, followed by steam distillation. The volatile bases are absorbed in boric acid solution, and determined by titration with 0.1 N acid. Alternatively an extract of fish with 0.6 N perchloric acid is used for confirmation in cases of doubt.

Because this is a standard method the defined working and distillation conditions must be well adhered to, in order to keep deamination at a constant level.

2 *Equipment*

2.1 Balance, accuracy 0.05 g or better
2.2 Steam distillation unit, e.g., either
2.2.1 Antona apparatus [9] consisting of 2-l round-bottom flask with glass side arm and stop cock (steam generator), reaction vessel insert, connecting tube and coil condenser with extended outlet or an appropriate projection from the condenser, electric heating mantle for the 2-l round bottom flask, receiver support (height adjustable), or
2.2.2 Büchi distillation unit model 315-special with insulating mantle for the reaction vessels, control valve for steam regulation and special condenser
or a related unit with adjustable reduced steam flow
2.3 Mincer, homogenizer/blender (e.g., top-drive blender)
2.4 Weighing dishes, preferably with spout (c 50 ml content)
2.5 Powder funnel, diameter at top 10 cm, at bottom 2 cm
2.6 300-ml broad-necked Erlenmeyer flask, graduated, as distillate receiver
2.7 10- or 25-ml burette for the 0.1 N acid
2.8 Funnel, diameter 15 cm with fast filtering fluted filter papers
2.9 25-ml pipettes (the latter two items are only for the alternative extraction method)

3 *Reagents*

3.1 Water, distilled or deionized
3.2 Magnesium oxide, reagent grade
3.3 Silicone antifoam emulsion
3.4 Approximately 3% aqueous boric acid solution
3.5 0.1 N hydrochloric or sulphuric acid
3.6 Tashiro-indicator mixture (methyl red and methylene blue)
3.7 Perchloric acid, 0.6 N (6%) (for alternative method only)

4 Preparation of the distillation unit

4.1 Before analysing samples, carry out a blind distillation of 200 ml water into the receiver (put 50 ml water into the reaction vessel and 100 ml water into the receiver), to avoid TVB-N losses in the first distillates.
4.2 Adjust the distillate flow to 10 ml/min; check the distillate amount occasionally.
4.3 Pipette approx. 10 ml of the boric acid solution into the graduated Erlenmeyer flask (receiver), add approx. 8 drops of the Tashiro indicator and fill up with distilled water to 100 ml. Place the flask on the receiver support, so that the outlet of the condenser is immersed.

5 *Preparation of samples*

Take a fish flesh sample of at least 100 g (preferably a total fillet) and homogenize thoroughly with a mincer and/or blender Investigate immediately, within 1 h in chilled storage, otherwise quick freeze the minced sample (e.g., in a closed container/plastic bag) and store at – 18° C or lower for a limited period.

From frozen fish, e.g., fillet blocks, cut a 100–200 g sample of approximately 2 cm thickness, place it in a water-tight plastic bag and thaw, e.g., by immersing the bag in a gently stirred water bath at about 20° C but not more than 25° C; thawing takes approximately 15 min. Homogenize the total sample including thaw drip.

6 *Separation of TVB-N*

6.1 Directly from fish flesh
6.1.1 Weigh 10.0 ± 0.1 g from the well homogenized fish flesh sample into a suitably sized flat dish. After addition of a small amount of water disperse the sample with a glass rod. Transfer it by means of a powder funnel quantitatively into the reaction vessel. Rinse with a small amount of water to ensure that as far as possible the sample lies on the bottom of the vessel. Shake to ensure proper dispersion and avoid clotting during distillation.
6.2 From extracts (alternative method)
6.2.1 Weigh 20.0 ± 0.1 g of the homogenized fish flesh sample into a suitable beaker, add 80 ml 0.6 M perchloric acid, homogenize for 1–2 min by use of a homogenizer (2.3), then filter through fluted paper. (The extract can be stored at 2–6° C up to 7 days.)
6.2.2 Pipet 25 ml of the extract into the reaction vessel.
6.3 Add to the sample in the reaction vessel 2–3 g magnesium oxide (e.g., with a measuring spoon) and 2–3 drops of the silicone antifoam emulsion.
6.4 Insert the reaction vessel immediately into the pre-heated steam generator and connect with the bridge to the condenser at once.
6.5 Antona unit: bring the water in the round-bottom flask (approx. 1 l) to boiling point with the stopcock open in order to reduce dilution by condensation, which would retard the process of separation: close the cock when boiling starts.

6.6 Distill for 10 min with the outlet tube from the condenser immersed, and 2 min with it above the surface. (Lower the support with the receiver).

6.7 When distillation is completed

6.7.1 Open the cock in the steam generator (Antona unit) or switch off distillation (Büchi unit).

6.7.2 Rinse the condenser outlet with a small amount of distilled water and then remove the receiver for titration.

6.8 Preparation of the units for the following sample

6.8.1 Remove the reaction vessel while it is still hot, empty and rinse the reaction vessel well with water, also rinse condenser and connecting tube of the Antona unit a little. It is advantageous to operate with two reaction vessels alternately for preparation and distillation.

6.8.2 Antona unit: after each distillation, with glass stopcock open, fill up the steam generator with hot water and preheat near to the boiling point, before the next reaction vessel is inserted. Grease the joints well to avoid sticking.

7 *Determination of TVB-N*

7.1 Titration. Titrate the distillate containing the volatile basic nitrogen against the 0.1 N acid (from a burette) until the neutral point is reached (the colour changes from green to red-violet and is grey at the neutral point).

7.2 Calculation. Volume ml 0.1 N acid $\times 14 =$ mg TVB-N/100 g or expressed more exactly: ml 0.1 N $\times 1.4 \times 100$/sample mass in g (if 25 ml extract is used, sample mass in calculation is 25% of original mass extracted).