Direct Determination of the Thiobarbituric Acid Value in Trichloracetic Acid Extracts of Fish as a Measure of Oxidative Rancidity*

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The possibilities of the direct determination of the thiobarbituric acid (TBA) value in trichloracetic acid (TCA) extracts instead of distillates were evaluated. Tests carried out on herring (Clupea harengus L.), redfish (Sebastes marinus L.), cod (Gadus morhua L.), plaice (Pleuronectes platessa L.) and spurdog (Squalus acanthias L.) of varying degree of oxidation gave 95.3 % recovery in the teleost species but only 75.2 % in the elasmobranch spurdog, due to the urea present. With the distillation technique, recoveries were lower and averaged 66.1 % for teleosts and 43.0 % for spurdog. Addition of 0.1 % of both propyl gallate and EDTA to the TCA solution lowered TBA values by 25 to 59 %. When fish samples were submitted to a 4 hours' forced oxidation period, TBA values increased to a greater extent with the direct extraction procedure than with the distillation method.

Über die direkte Bestimmung der Thiobarbitursäurezahl in Trichloressigsäure-Extrakten von Fisch als Maß für die oxydative Ranzigkeit

Die Möglichkeiten der direkten Bestimmung der Thiobarbitursäure (TBA)-Zahl in Trichloressigsäure (TCA)-Extrakten anstatt in den Destillaten wurde untersucht. Die an Hering (Clupea harengus L.), Rotbarsch (Sebastes marinus L.), Kabeljau (Gadus morhua L.), Scholle (Pleuronectes platessa L.) und Dornhai (Squalus acanthias L.) mit unterschiedlichen Oxydationsgraden ausgeführten Tests ergaben 95.3 % Analysenausbeute bei den Teleost-Spezies, dagegen nur 75.2 % bei dem elasmobranchen Dornhai; im letztgenannten Falle wegen des vorhandenen Harnstoffs. Nach den Destillationsmethoden waren die entsprechenden Ausbeuten niedriger; sie betrugen durchschnittlich 66.1 % bei den Teleosten und 43.0 % beim Dornhai. Zusätze von jeweils 0.1 % Propylgallat und EDTA zur TCA-Lösung verringerte die TBA-Zahlen um 25 bis 59 %. Setzte man die Fischproben einer 4stündigen Oxydation aus, so erhöhten sich die TBA-Zahlen in stärkerem Maße bei der direkten Extraktionsmethode als bei der Destillationsmethode.

Détermination directe de l'indice d'acide thiobarbiturique dans des extraits trichloracétiques de poisson en tant que mesure de la rancidité oxydative

On a étudié les possibilités de détermination directe de l'indice d'acide thiobarbiturique (TBA) dans des extraits trichloracétiques (TCA) au lieu des distillats. Les essais effectués sur Clupea harengus L., Sebastes marinus L., Gadus morhua L., Pleuronectes platessa L. et Squalus acanthias L., d'un degré d'oxydation inégal, ont fourni des rendements analytiques de 95.3 % chez les poissons téléostéens et de 75.2 % seulement dans le cas de Squalus acanthias L., en raison de l'urée présente. Les méthodes de distillation ont fourni en moyenne 66.1 % et 43 %, respectivement. Des additions de gallate de propyle (0.1 %) et EDTA (0.1 %) à la solution TCA ont réduit les indices TBA de 25 à 59 %. Une oxydation pendent 4 heures des échantillons de poisson augmente les indices TBA plus fortement dans la méthode directe que dans la méthode de distillation.

О прямом определении тиобарбитуровокислотного числа в трихлоруксуснокислых экстрактах рыбы как мере окислительной прогорклости.

Исследовались возможности прямого определения тиобарбитуровокислотного (ТВК) числа в трихлоруксуснокислых (ТХУК) экстрактах вместо определения в дистиллятах. Пробы со сельдью (Clupea harengus L.), морским окунем (Sebastes marinus L.), треской (Gadus morhua L.) камбалой (Pleuronectes platessa L.) и акулой (Squalus acanthias L.) различной степени окисления показали, что аналитический выход для истинных костистых рыб достигает 95.3%, но только 75.2% у акулы, в последнем случае из-за присутствия мочевины. Методами дистилляции достигались более низкие выходы: в среднем 66.1% для истинных костистых рыб и 43.0% для акулы. Добавка 0.1% пропилгаллата и 0.1% ЭДТА к ТХУК-раствору снижает ТВК-число на 25—59%. Когда рыбные пробы подвергаются четырехчасовому окислению, ТВК-числа при прямом методе экстракции повышаются более сильно чем при дистилляционном методе.

The thiobarbituric acid (TBA) test is now extensively used as a measure of oxidative rancidity in plant and animal tissues, oils, fats and fat-containing foods. Malonaldehyde is the principal compound in oxidized lipids that reacts with TBA to give the red pigment on which the test is based. In fishery products, the method of *B. Tarladgis* et al.[1] is generally employed. It involves separation of the TBA-reactive substances from the fish muscle by distillation before the TBA-reagent is added.

In a later publication, *B. Tarladgis* et al.[2] proposed a direct method for meat, bread and potato products. The samples are blended with distilled water and the TBA-reaction is carried out on the clear filtrate. With fish, clear water extracts are difficult to obtain; when using a deproteinizing agent, however, filtrates are clear. Moreover, the elimination of proteins, some of which have been shown to interfere with the TBA reaction[3,4] should not have adverse effects. Other possible interfering substances are oxygen, peroxides, carbohydrates, carbonyl compounds[5,6], iron salts[7,8], hematin compounds and certain free amino acids[9]. In this connection, it should be emphasized that the advantage of the distillation method consists in the removal of malonaldehyde from interfering substances. However, it has been shown that fat oxidation with production of aldehydes from non-rancid material can occur during distillation owing to heating, which affects results[1]. Moreover, pH of sample influences distillation of aldehydes and should

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[1] *B. Tarladgis, B. Watts* and *M. Younathan*, J. Amer. Oil Chemists' Soc. **37**, 44 [1960].

[2] *B. Tarladgis, A. Pearson* and *L. Dugan* jr., J. Sci. Food Agric. **15**, 602 [1964].

[3] *D. Crawford, T. Yu* and *R. Sinnhuber*, J. Food Sci. **32**, 332 [1967].

[4] *H. Buttkus*, J. Food Sci. **32**, 432 [1967].

[5] *T. Yu* and *R. Sinnhuber*, Food Technol. **11**, 104 [1957].

[6] *T. Yu* and *R. Sinnhuber*, J. Amer. Oil Chemists' Soc. **41**, 540 [1964].

[7] *C. Castell, B. Moore* and *W. Neal*, J. Fisheries Res. Board Canada **23**, 737 [1966].

[8] *C. Castell* and *G. Boyce*, J. Fisheries Res. Board Canada **23**, 1587 [1966].

[9] *C. Castell* and *D. Bishop*, J. Fisheries Res. Board Canada **26**, 2299 [1969].

be brought to 1.5 quite carefully[1]. Difficulties were encountered in our laboratory during this operation with some fish species (e. g. cod). Hence, it seemed worthwhile to study the possibilities of a direct, rapid extraction procedure for determining the TBA-value.

Recovery tests with added malonaldehyde were carried out on trichloracetic acid-extracts of cod, herring, redfish, plaice and spurdog in order to estimate possible interferences.

C. Castell et al.[7,8] obtained better results with the method of *B. Tarladgis* et al.[1] when propyl gallate (PG) and EDTA were added before distillation. It reduced fat oxidation during the test itself (PG) and the effect of iron salts as prooxidants (EDTA). The influence of both reagents in the direct extraction procedure was also tested.

Finally, the sensitivity of both the original distillation procedure and the extraction method was investigated.

Experimental

Fish

Cod *(Gadus morhua L)*, redfish *(Sebastes marinus L)*, herring *(Clupea harengus L)*, plaice *(Pleuronectes platessa L)* and spurdog *(Squalus acanthias L)* kept for 6 to 24 months in frozen condition and having varying degrees of rancidity were used. Only white muscles were taken.

TBA tests

Distillation procedure: According to *B. Tarladgis* et al.[1] but using *N. Antonacopoulo's* still[10]; 10 g of blended fish was distilled for 10 min. giving 100 ml of distillate. In some experiments, 0.5 % of both propyl gallate and EDTA (calculated on sample weight) were added[7].

Extraction procedure: In the final method, 20 g of fish was homogenized with 100 ml of 7.5 % trichloracetic acid solution (TCA) for one minute in a Waring blendor and filtered.

TBA reaction: 5 ml of TBA reagent (0.02 M 2-thiobarbituric acid in distilled water) were added to 5 ml of distillate or filtrate in test tubes with screw caps; they were placed for 40 min. in a boiling water bath. Absorbence was read at 538 nm after cooling in tap water; a *Hitachi-Perkin-Elmer* model 139 was employed. In the extraction method, the original TCA extract was used as a blank. Blank values ($E_{538\,nm}^{1\,cm}$) measured against pure TCA solution varied from 0.010 to 0.030 according to fish species.

Recovery tests: were carried out with 1,1,3,3,-tetra-ethoxypropane (TEP) which is hydrolyzed to malonaldehyde. In the distillation procedure, 10 ml of $6 \cdot 10^{-5}$ M TEP were added before distillation; in the extraction method a solution of $6 \cdot 10^{-6}$ M in TCA was used.

Results and discussion

Deproteinizing solutions

Solutions of magnesium sulfate (60 %) and zinc sulfate (40 %) gave slightly turbid filtrates and low reproducibilities with TBA reagent. Phosphotungstic acid in the same concentration (20 %) as used for determination of total volatile acids, which are an index of fish spoilage[11], gave more promising results but the reproducibility of blank values caused difficulties. This reagent was not investigated any further.

Finally, TCA appeared to be a suitable deproteinizing reagent and was chosen for further experiments.

[10] Z. Lebensmittel-Unters. u. -Forsch. **113**, 113 [1960].
[11] Official Methods of the AOAC, 9th Ed., p. 236 [1960].

Recovery of malonaldehyde

Results of recovery tests carried out on 20 different samples of fish with both the extraction and the distillation procedures are mentioned in Table 1. Highest recoveries were obtained with the TCA extraction procedure. Distillation gave lower values but these compared favourably with data reported by *B. Tarladgis* et al.[1], viz 66 to 70 % for both samples and pure TEP solutions. In the latter case, however, an average recovery of 93.0 % was obtained with *N. Antonacopoulos'* still[10].

Table 1

Recovery tests (in %) with the distillation and extraction procedures (standard deviation in brackets)

Fish species	TEP added to TCA solution	TEP added before distillation	TEP added to distillate
Herring	95.0 (2.6)	64.1 (2.9)	85.2 (2.1)
Redfish	96.4 (3.5)	66.2 (4.2)	76.0 (4.6)
Cod	94.5 (2.7)	69.0 (3.2)	86.8 (3.5)
Plaice	95.4 (2.3)	65.3 (2.8)	83.5 (2.5)
Spurdog	75.2 (3.8)	43.0 (3.4)	66.2 (3.7)

It should be noted that even on the distillate, recoveries were lower than on the TCA extract. This indicates that some compounds interfering with the TBA reaction are distilled over.

Spurdog gave significantly lower recoveries. As this elasmobranch fish is richer in nitrogenous extractives than teleosts (33 to 38 % against 9 to 18 %)[12], the possible influence of the most important compounds was investigated. The concentrations chosen were based on a previous study carried out on the nitrogenous extractives of the spurdog[13]. All substances gave negligible blanks with the TBA reagent. From results reported in Table 2 it appears that only urea had a marked influence on the TBA reaction. Additional tests showed this influence to be directly proportional to the amount of urea. It is very likely that this compound is responsible for the lower recoveries both with the extraction and the distillation techniques mentioned in Table 1. However, this cannot explain the lower recoveries in the distillate. The lower recovery of malonaldehyde in spurdog (and probably other elasmobranchs) should be taken into account when calculating the final TBA value of a given sample (mg malonaldehyde per 1000 g of fish).

[12] *J. Shewan*, in: Fish as Food, Vol. 1, Ed. by *G. Borgstrøm*, Academic Press, New York, p. 487 [1961].
[13] *W. Vyncke*, Marine Biology **6**, 248 [1970].

Table 2

Influence of nitrogenous extractives on the TBA reaction

Compound	Concentration [mg/100 ml TCA-extract]	Relative absorbence * [%]
Urea	400	72.0
Trimethylamine oxide N	40	98.1
Trimethylamine N	5	99.3
Ammonia N	10	99.2
Creatine	150	101.0
Creatinine	20	103.0
Taurine	70	99.1
Sarcosine	25	102.6
Proline	30	101.7
Hydroxyproline	5	99.4
Cysteine	5	98.0
Cystine	5	100.3
α-alanine	20	100.1
β-alanine	5	101.2
Arginine	5	101.2
Aspartic acid	5	98.9
Phenylalanine	5	99.5
Lysine	5	99.4
Serine	5	101.4
Threonine	10	101.6
Valine	5	100.9
Tyrosine	5	101.8
Histidine	5	101.2
Glutamic acid	5	98.2
Glycine	20	103.0
Leucine	15	99.6
Betaine	70	99.1
Choline	3	97.8

* A solution of 6.10^{-6} M TEP in TCA = 100 %

With teleost fish, the TCA extraction method gave an average recovery of 95.3 %, which can be considered as satisfactory. Although fish of different degrees of rancidity were used, standard deviations were rather low. Moreover, application of *H. Hartley's* test[14] showed variances between the five fish species not to be significantly different. This would indicate that the TBA reaction in the TCA extract is not markedly influenced by the degree of oxidation of the fish.

Effect of propyl gallate (PG) and EDTA

For practical reasons, concentrations were expressed as w/v %. PG and EDTA (disodium salt) gave negligible blanks and did not interfere with the TBA reaction when added to pure TEP solutions. Preliminary experiments with different fish showed that amounts of 0.1 % of both PG and EDTA were sufficient.

Results are reported in Table 3. Propyl gallate and EDTA lowered TBA values markedly but maximum effects were obtained when both compounds were added to the TCA extract. The influence of PG and EDTA was less pronounced with herring than with the four other fish species.

The high standard deviations indicate that all fish samples did not have the same prooxidant capacity and were not influenced to the same extent by the antioxidant and the chelating agent.

Recovery tests on the other hand showed the TBA reaction itself not to be influenced by PG or EDTA. Results were practically identical with those obtained on TCA solutions without these reagents (see Table 1). Standard deviations were low and did not differ significantly. As already found in previous experiments, spurdog showed low recoveries (ca. 75 %) which were not inproved by the addition of PG and/or EDTA.

The presence of the antioxidant and the chelating agent in the TCA extracts did not give completely the same results as reported by *C. Castell* and *G. Boyce*[8] for the distillation method. These authors found practically

Table 3

Effect of 0.1 % propyl gallate (PG) and 0.1 % EDTA in the TCA-extract on the TBA test (in %; standard deviation in brackets)

Fish species	+ PG [a]	+ PG + TEP [b]	+ EDTA [a]	+ EDTA + TEP [b]	+ PG + EDTA [a]	+ PG + EDTA + TEP [b]
Herring	77.5 (15.6)	94.4 (3.2)	80.4 (18.4)	95.2 (3.0)	75.2 (17.4)	96.0 (2.8)
Redfish	45.2 (10.3)	96.8 (4.0)	45.7 (8.5)	95.9 (3.8)	41.2 (10.7)	95.6 (4.1)
Cod	63.0 (22.1)	94.2 (2.7)	61.4 (12.1)	93.8 (3.2)	52.6 (22.6)	94.7 (3.0)
Plaice	55.4 (10.9)	96.0 (2.5)	61.7 (7.2)	95.5 (2.8)	46.0 (6.1)	95.1 (3.1)
Spurdog	71.0 (12.0)	76.1 (4.3)	77.2 (9.7)	74.7 (3.9)	66.0 (8.5)	75.6 (4.0)

[a] TBA value without PG or EDTA = 100 %
[b] % recovery of TEP

[14] Biometrika 37, 308 [1950].

no difference for the white muscle of lean fish when both reagents were omitted. On the other hand, they obtained marked differences for red muscles of lean fish and for fatty fish species. This discrepancy is discussed in the following series of tests.

Sensitivity of TCA extraction and distillation methods

In order to estimate and compare the sensitivities of both extraction and distillation techniques to changes in degree of oxidation of the fish muscles, a series of model experiments was carried out.

Finely ground fish samples were spread in thin layers on polyethylene plates which were placed into a well ventilated fume hood for 4 hrs. TBA tests were made before and after this oxidation period. PG and EDTA were added before distillation.

The loss of weight due to drying (7 to 20 %) was taken into account. All tests were repeated ten times with fish samples of different degrees of oxidation. Significance of differences between both methods was tested statistically by the method of paired comparisons[15]. Results are reported in Table 4.

Table 4

Increase in TBA values after 4 hrs. oxidation with distillation and extraction procedures (value before oxidation = 100 %; standard deviations in brackets)

Fish species	Increase in TBA value [%]		Significance of difference [a]
	Distillation	Extraction	
Herring	63.3 (7.1)	73.5 (8.4)	*
Redfish	1.2 (5.4)	24.0 (6.9)	**
Cod	65.1 (15.0)	74.2 (17.8)	—
Plaice	2.4 (9.5)	19.5 (8.6)	**
Spurdog	95.0 (26.2)	104.3 (29.8)	—

[a] not significant
* significant at the 95 % level
** significant at the 99 % level

The average increase in TBA values was higher for all fish species with the TCA extraction procedure. Only with cod and spurdog differences were not statistically significant, probably owing to the high standard deviations.

As to be expected, all samples were not affected to the same extent by the forced oxidation. Redfish and plaice showed lower increases in TBA values and it should be emphasized that practically the same values were obtained with the distillation method after the 4 hours' oxidation period. This suggests that the extraction procedure is more sensitive to small increases in degree of oxidation. Owing to this different behaviour, it was not surprising that correlation between results of both methods, although still satisfactory, was lower than expected: the correlation coefficient calculated on 100 paired data was 0.854.

Measurements were also made on the same TCA extracts, but without PG or EDTA. The increase in absorbence (average values of 3 tests) before and after the 4 hours' oxidation period is mentioned in Table 5.

Table 5

Increase in absorbence ($E_{538\ nm}^{1\ cm}$) after 4 hrs. oxidation in TCA extracts with and without PG and EDTA

Fish species	Without PG and EDTA			With PG and EDTA		
	Before	After	Difference	Before	After	Difference
Herring	0.474	0.743	0.269	0.380	0.645	0.265
Redfish	0.465	0.505	0.040	0.211	0.254	0.043
Cod	0.080	0.112	0.032	0.047	0.077	0.030
Plaice	0.301	0.342	0.041	0.152	0.187	0.035
Spurdog	0.263	0.438	0.175	0.173	0.342	0.169

Although the initial values of the extracts without PG or EDTA were higher, as already found in previous experiments, the average increase in absorbence was practically the same for both extracts. This means that the same amount of erroneous malonaldehyde is formed in the sample, independently of the degree of oxidation. As lipids in most fish tissues rapidly oxidize when in contact with air, it is very likely that the higher amounts of malonaldehyde found in the TCA extracts when no PG or EDTA were added, were formed mainly during blending and filtering of the sample, during which an appreciable amount of air is incorporated into the solution.

Conclusions

Recovery tests with added malonaldehyde and model experiments with forced oxidation indicate that the TCA extraction procedure can be used for determining the TBA value in fish. An antioxidant (PG) and a chelating agent (EDTA) should be added in any case in order to avoid erroneously formed monaldehyde or other TBA reactive substances during blending and filtering of the sample.

The greatest advantages of this direct method are its speed and simplicity.

[15] *E. Bauer*, A Statistical Manual for Chemists, Academic Press, New York, p. 43 [1960].