8.3.3 Add, using a micropipette (5.5), 0,05 ml of ammonium thiocyanate solution (4.3) and mix.

8.3.4 Transfer the test sample blank mixture to a photometer cell (5.7). Close the cell with a cap and allow it stand for 10 min to obtain equilibrium in the mixture. Measure the extinction (E'_0) of the sample blank against the methanol/1-decanol/*n*-hexane mixture (4.1).

8.3.5 Correct the obtained test sample blank extinction (E'_0) in 8.3.4 for the differences in mass of the test portions of the test sample blank and of the test sample by using the following equation:

$$E_0 = E'_0 \times \frac{m}{m_0}$$

where

E_0 is the numerical value of the corrected sample blank extinction;

E'_0 is the numerical value of the sample blank extinction (8.3.4);

m_0 is the mass of the test sample blank (8.3.1);

m is the mass of the test sample (8.4.1).

8.4 Test portion

8.4.1 Weigh, to the nearest 1 mg, approximately 0,33 g of prepared test sample (see 7.2 or 7.3) in a test tube (5.8).

8.4.2 Without any delay, add, using the dispenser (5.3), 9,60 ml of methanol/1-decanol/*n*-hexane mixture (4.1) to the test portion in the tube. Mix gently to dissolve the sample fat.

8.4.3 Add, using a micropipette (5.5), 0,05 ml of ammonium thiocyanate solution (4.3) to the mixture in the tube and mix.

8.4.4 Add, using a micropipette (5.5), 0,05 ml of iron(II) chloride solution (4.2) to the mixture in the tube and mix again.

8.4.5 Transfer the test portion mixture to a photometer cell (5.7) Close the cell with a cap and let it stand for 10 min to obtain equilibrium in the mixture. Measure the extinction (E_2) of the test portion against the methanol/1-decanol/*n*-hexane mixture (4.1).

8.4.6 The procedures described in 8.3 and 8.4 may be performed in one run directly by using a single photometer cell of suitable size. Proceed as described in 8.3.1 to 8.3.3 inclusive. Measure the test blank extinction, E_0, against the methanol/1-decanol/*n*-hexane mixture as in 8.3.4. Then proceed as described in 8.4.4 by adding and mixing 0,05 ml of iron(II) chloride solution (4.2) directly to the photometer cell and measure the sample extinction (E_2) as in 8.4.5.

8.5 Extinction coefficient of the red iron(III) complex

Add, using the dispenser device (5.4), 0,5 ml, 1,0 ml, 1,5 ml and 2,0 ml of iron(III) chloride standard solution (4.4), respectively, to four test tubes (5.8) to obtain a series of solutions containing 5 µg, 10 µg, 15 µg and 20 µg of Fe^{3+} respectively.

Add, using the dispenser device (5.3), 9,4 ml, 8,9 ml, 8,4 ml and 7,9 ml of methanol/1-decanol/*n*-hexane mixture (4.1), respectively, to the four tubes to obtain a 9,9 ml mixture in each tube.

Add, using the micropipettes (5.5), 0,05 ml of ammonium thiocyanate solution (4.3) and 0,05 ml of hydrochloric acid solution II (4.6) to each of the four tubes and mix.

Transfer the obtained reaction mixtures to photometer cells (5.7). Close the cells with caps and allow them to stand for 10 min to obtain equilibrium in the mixture. Measure the extinction (E_{Fe}) of each cell against the methanol/1-decanol/*n*-hexane mixture.

Using the obtained extinction data with the associated mass (in micrograms) of Fe^{3+}, calculate by means of the appropriate statistical formulae the linear regression equation as follows:

$$Y = a + bX$$

where

Y is the numerical value of the extinction, E_{Fe}, obtained in the cell (tube);

X is the mass of Fe^{3+} in the cell (tube);

a is the numerical value of the intercept of the regression;

b is the numerical value of the extinction (regression) coefficient of the red iron(III) complex that has to be used in the calculation of the mass of Fe^{3+} (see 9.1.2).

The standard deviation, $s_{y,x}$, of the regression equation shall be less than 0,010 extinction units. If the above requirement is not fulfilled, check the photometric procedure, the glassware and the reagents. Correct the procedure or replace what is necessary.

NOTE The regression equation can be extended to 50 µg of Fe without loss of its linearity (see Annex A).

9 Calculation and expression of results

9.1 Calculation

9.1.1 Calculation of extinction

Calculate the extinction, E, attributable to the red iron(III) complex, by using the following equation:

$$E = E_2 - (E_0 + E_1)$$

where

E_2 is the numerical value of the sample extinction measured as described in 8.4.5;

E_0 is the numerical value of corrected sample blank extinction calculated in 8.3.5;

E_1 is the numerical value of the reagent blank extinction measured as described in 8.2.4.

9.1.2 Calculation of the mass of Fe^{3+}

Calculate the mass of Fe^{3+}, m_c, expressed in micrograms, by using the following equation:

$$m_c = \frac{E}{b}$$

where

E is the numerical value of the extinction, attributable to the red iron(III) complex, calculated in 9.1.1;

b is the numerical value of the extinction coefficient of the red iron(III) complex, calculated in 8.5.

9.1.3 Calculation of the peroxide value

Calculate the peroxide value of the fat, PV, expressed as millimoles of oxygen per kilogram, by using the following equation:

$$PV = \frac{0,5\, m_c}{55,84\, m}$$

where

m_c is the mass of Fe^{3+}, in micrograms, calculated in 9.1.2;

m is the mass, in grams, of the test portion (8.4.1);

55,84 is the atomic mass of Fe^{3+} needed to express the results in millimoles.

9.2 Expression of test results

Do not round intermediate values. Express the final results to two decimal places.

10 Precision

10.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases be greater than 0,03 mmol of oxygen per kilogram.

10.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than 0,07 mmol of oxygen per kilogram.

11 Test report

The test report shall specify:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, together with reference to this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

e) the test result(s) obtained, and, if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

Summary of the procedure and examples of calculations

Table A.1 shows a scheme for the procedure. Tables A.2 and A.3 give examples of calculations.

Table A.1 — Scheme of the procedure

	Determination of the extinction coefficient									
	Test sample	Test blank	Reagent blank	Standard procedure (µg Fe)				Extended procedure [a] (µg Fe)		
				5	10	15	20	30	40	50
Test portion (7.2/7.3), g	± 0,33	± 0,33	—	—	—	—	—	—	—	—
Reagent solutions										
Fe(III) (4.4), ml				0,50	1,00	1,50	2,00	3,00	4,00	5,00
Mixture (4.1), ml	9,60	9,60	9,90	9,40	8,90	8,40	7,90	6,90	5,90	4,90
NH_4SCN (4.3), ml	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
Fe(II) (4.2), ml	0,05	—	0,05	—	—	—	—	—	—	—
HCl (4.6), ml	—	—	—	0,05	0,05	0,05	0,05	0,05	0,05	0,05
Measurement										
E_c (near 500 nm)	E_2	E'_0	E_1	E_5	E_{10}	E_{15}	E_{20}	E_{30}	E_{40}	E_{50}
[a] See Note in 8.5.										

Table A.2 — Calculation of the extinction coefficient of the red iron(III) complex (8.5)

X µg Fe(III)	Y $E_{Fe(III)}$
5	0,141
10	0,283
15	0,423
20	0,562

EXAMPLE $E_{Fe(III)}$ = 0,001 5 + 0,028 1 µg Fe(III), which finally leads to an extinction coefficient of 0,028 1 extinction units per microgram of Fe.

Table A.3 — Calculation of peroxide value (Clause 9)

Sample number	Measurement					Calculation			
	Test sample (m) g	Test blank (m_0) g	E'_0	E_1	E_2	E	E_0	m_c µg	PV mmol/kg
	0,290 9	0,290 8	0,019	0,020	0,204 0	0,165 0	0,019 0	5,880	0,18
	0,291 5	0,291 0	0,037	0,020	0,094 0	0,036 9	0,037 1	1,316	0,04

Annex B
(informative)

Interlaboratory trial

An international collaborative trial involving eleven laboratories from seven countries was carried out on two different samples of eight anhydrous milk fat samples. The test was organized by Agriculture Research Centre (CRA) in Belgium.

The results obtained were subjected to statistical analysis in accordance with ISO 5725-1 and ISO 5725-2 to give the precision data shown in Table B.1.

Table B.1 — Results of interlaboratory tests

	Anhydrous milk fat								
	1	2	3	4	5	6	7	8	Mean
No. of laboratories retained [a] after eliminating outliers	9	9	9	9	9	9	8 [b]	8 [b]	
Mean value [c]	1,049	0,699	0,607	0,445	0,308	0,271	0,913	0,690	0,618
Repeatability standard deviation, s_r [c]	0,040	0,021	0,024	0,015	0,014	0,011	0,015	0,017	0,022
Coefficient of variation of repeatability [d]	3,77	2,94	3,92	3,47	4,47	4,08	1,62	2,40	3,56
Repeatability limit, r (= 2,8 s_r) [c]	0,111	0,058	0,067	0,043	0,038	0,031	0,041	0,046	0,06
Reproducibility standard deviation, s_R [c]	0,061	0,052	0,041	0,035	0,042	0,030	0,058	0,050	0,047
Coefficient of variation of reproducibility [d]	5,82	7,38	6,83	7,83	13,62	11,01	6,34	7,20	7,61
Reproducibility limit R (= 2,8 s_R) [c]	0,171	0,144	0,116	0,098	0,117	0,084	0,162	0,139	0,13

[a] The results of two laboratories were discarded for difficulties with the method or too high reagent blank values. So only the results of the remaining nine laboratories were taken into account in the statistical evaluation.

[b] Outlier removed.

[c] Values are expressed as milli-equivalent of oxygen per kilogram. For expressing them in millimoles (= SI unit), divide them by 2.

[d] Values are expressed as percentage (%).

Annex C
(informative)

Comparison trial

C.1 Reason for implementation of methanol/1-decanol/*n*-hexane mixture

For ecological reasons, the chloroform/methanol mixture (with a volume fraction ratio of 7:3) used in ISO 3976:1977 and IDF 74A:1991 has been replaced by a methanol/1-decanol/*n*-hexane mixture (with a volume fraction ratio of 3:2:1).

NOTE Due to the bad smell of the latter, however, some countries still have preference for using the chloroform/methanol mixture as long as this is allowed.

C.2 Comparison of results obtain with the old and new reagent

For comparison purposes, some performance characteristics of both solvent mixtures are given in Figure C.1 and Table C.1.

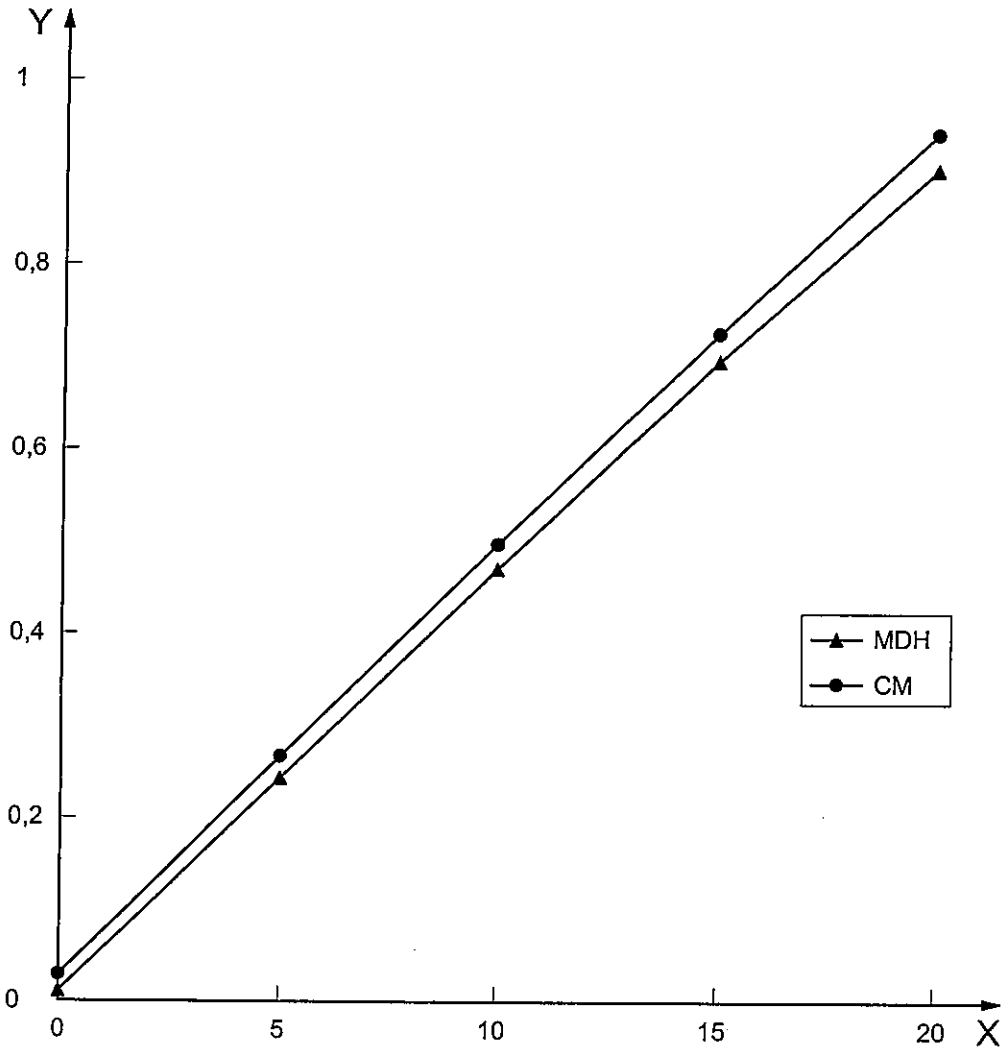
Figure C.1 shows the calibration curves determined by using the method as described in ISO 3976:1977 or IDF 74A:1991, using as reagent either the chloroform/methanol (CM) mixture or its replacement the methanol/1-decanol/*n*-hexane (MDH) mixture.

Table C.1 shows the peroxide values, expressed in milli-equivalents of oxygen per kilogram, of 26 milk fat samples determined by using the method as described in ISO 3976:1977 or IDF 74A:1991 using as reagent either the chloroform/methanol (CM) mixture or its replacement the methanol/1-decanol/*n*-hexane (MDH) mixture.

C.3 Summary

The mean peroxide value of the 26 milk fat samples obtained with either of the solvent mixtures shows no significant difference.

The repeatability, the sensitivity of the method, the linearity of the calibration curves and the absorption of the blank obtained by either of the solvent mixtures are also comparable.



Key

X mass of Fe^{3+}, µg

Y absorption

Figure C.1 — Calibration curve of chloroform/methanol (CM) and methanol/1-decanol/*n*-hexane (MDH)

Table C.1 — Peroxide values of 26 milk fat samples using chloroform/methanol (7:3) (CM) or methanol/1-decanol/*n*-hexane (3:2:1) (MDH) as method solvent

Sample	Solvent CM			Solvent MDH		
	Peroxide values (meq/kg) [a]			Peroxide values (meq/kg)		
	Result 1	Result 2	Mean	Result 1	Result 2	Mean
1	0,036	0,030	0,033	0,036	0,028	0,032
2	0,384	0,395	0,390	0,361	0,376	0,369
3	0,100	0,101	0,101	0,102	0,102	0,102
4	0,027	0,027	0,027	0,038	0,037	0,038
5	0,061	0,092	0,077	0,058	0,052	0,055
6	0,072	0,072	0,072	0,069	0,075	0,072
7	0,078	0,080	0,079	0,068	0,071	0,070
8	0,096	0,086	0,091	0,091	0,091	0,091
9	0,080	0,083	0,082	0,137	0,100	0,119
10	0,104	0,082	0,093	0,101	0,104	0,103
11	0,037	0,034	0,036	0,048	0,055	0,052
12	0,070	0,070	0,070	0,088	0,077	0,083
13	0,375	0,379	0,377	0,339	0,342	0,341
14	0,052	0,058	0,055	0,045	0,030	0,038
15	0,041	0,039	0,040	0,035	0,034	0,035
16	0,040	0,030	0,035	0,020	0,034	0,027
17	0,060	0,045	0,053	0,054	0,050	0,052
18	0,062	0,043	0,053	0,040	0,041	0,041
19	0,031	0,038	0,035	0,057	0,050	0,054
20	0,054	0,052	0,053	0,062	0,064	0,063
21	0,037	0,046	0,042	0,034	0,039	0,037
22	0,062	0,065	0,064	0,056	0,059	0,058
23	0,147	0,142	0,145	0,105	0,119	0,112
24	0,098	0,076	0,087	0,059	0,054	0,057
25	0,034	0,018	0,026	0,033	0,035	0,034
26	0,059	0,050	0,055	0,071	0,065	0,068
Mean			0,087			0,084
Repeatability			0,023			0,020

[a] Values are expressed as milli-equivalents of oxygen per kilogram. For expressing them in mmoles (SI unit) they should be divided by 2.

Bibliography

[1] ISO 707 | IDF 50, *Milk and milk products — Guidance on sampling*

[2] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[3] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

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[5] AOAC 920.160, *Determination of peroxide values*

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