

FOOD STANDARDS AGENCY INFORMATION BULLETIN ON METHODS OF ANALYSIS AND SAMPLING FOR FOODSTUFFS

This Bulletin is issued by the Food Standards Agency to Public Analysts, to other analysts working in the food sector and to others with an interest in the sector. Its principal purpose is to act as an electronic consultation forum on methods of analysis and sampling for foodstuffs proposed for inclusion in EU Regulations and Directives, or on topics to be discussed in the organisation such as the Codex Alimentarius Commission. Other topics, e.g. forthcoming collaborative trials to validate specific methods of analysis, will be covered from time to time.

This Bulletin may be regarded as the successor to the MAFF Information Bulletin for Public Analysts on EEC Methods of Analysis and Sampling for Foodstuffs. ***However, unlike that Bulletin, it will only be issued in electronic form.*** It will be issued in pdf format downloadable from the FSA Website.

It should be regarded as somewhat less formal than the previous Bulletin. Comments are invited on any items included in the Bulletin, but only *via* a conventional email approach rather than through a Bulletin Board approach. It is hoped that this will not only elicit comments but also develop discussion between recipients; comments will therefore be copied to all Bulletin recipients.

Any general enquiries or comments regarding the Bulletin should be addressed to Roger Wood at the address below.

Food Standards Agency
Institute of Food Research,
Norwich Research Park,
Colney,
Norwich,
NR4 7QA

Tel: + 44 (0) 1603 255231
Fax: + 44 (0) 1603 507723

E-mail: roger.wood@foodstandards.gsi.gov.uk

The FSA website address is: www.food.gov.uk

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Cumulative Index for the Food Standards Agency Information Bulletin on Methods of Analysis and Sampling for Foodstuffs

See separate index.

1. Research Website

This is to inform you that research material for Program E01 has been published on the website at:

<http://www.food.gov.uk/science/research/researchinfo/supportingresearch/methodsofanalysisresearch/e01projlist/>

2. Collaborative Trial 135 - Determination of the Fat Content of Butter, Edible Oil Emulsions and Spreadable Fats using a “Direct” Method.

Information on the above collaborative trial is given in this Bulletin. In particular information on the trial format, the method of analysis used and the results obtained are given.

The method will probably be adopted by IDF and ISO as the reference method in this area.

3. The Detection Of Sudan I, II, III And IV In Palm Oil By Thin Layer Chromatography

Information on a method for the above determination is included in this Bulletin.

Comments on any of the above would be appreciated.

Thank you.

COLLABORATIVE TRIAL 135 - DIRECT DETERMINATION OF THE FAT CONTENT OF BUTTER, EDIBLE OIL EMULSIONS AND SPREADABLE FATS

1. Trial Details

- 1.1 Fifteen laboratories took part in the study. The fat content was measured using the given method.
- 1.2 Participants received twelve samples comprising: butter; edible-oil emulsions; and spreadable fats, of differing fat content (Table 1) and were requested to analyse each sample once.
- 1.3 Data was submitted to the Food Standards Agency who undertook statistical analysis

2. STATISTICAL ANALYSIS OF RESULTS

- 2.1. The trial results were examined for evidence of individual systematic error ($p < 0.01$) using Cochran's and Grubbs' test progressively, by procedures described in the internationally agreed Protocol for the Design, Conduct and Interpretation of Collaborative Studies⁽¹⁾.

2.2. Repeatability and Reproducibility

- 2.2.1. Calculations for repeatability r and Reproducibility R as defined by that protocol⁽¹⁾ were carried out on those results remaining after removal of outliers. These are given in Tables 2 to 7 and summarised in Table 8.

2.3. Horwitz Predicted Precision Parameters

- 2.3.1. There is often no validated reference or statutory method with which to compare precision criteria when assessing a new method. In such cases it is useful to compare the precision data obtained from a collaborative trial with predicted acceptable levels of precision. These levels, as predicted by the Horwitz equation, give an indication as to whether the method is sufficiently precise for the level of analyte being measured⁽²⁾.

- 2.3.2. The Horwitz predicted value is calculated from the Horwitz equation⁽²⁾:

$$RSD_R = 2^{(1-0.5\log C)}$$

- 2.3.3. C is the measured concentration of analyte expressed as a decimal, i.e. % = 0.01.

2.4. Horrat Value (H_o)

- 2.4.1. The Horrat⁽³⁾ value gives a comparison of the actual precision measured with the precision predicted by the Horwitz equation for a method measuring at that particular level of analyte. It is calculated as follows:

$$H_o = RSR_R(\text{measured})/RSD_R(\text{Horwitz})$$

- 2.4.2. A Ho_R value of 1 usually indicates satisfactory inter-laboratory precision, while a value of >2 indicates unsatisfactory precision i.e. one that is too variable for most analytical purposes or where the variation obtained is greater than that expected for the type of method employed.
- 2.4.3. Similarly Ho_r is calculated, and used to assess intra-laboratory precision, using the approximation $RSD_r(\text{Horwitz}) = 0.66 \times RSD_R(\text{Horwitz})$. This assumes the approximation $r = 0.66R$. The Horrat values calculated from the results of this trial are given in Tables 2 to 7 and summarised in Table 8.

3. DISCUSSION

- 3.1. The data for the percentage (m/m) fat content in the butter, edible oil emulsions and spreadable fat test materials is good where the mean values determined compare well with the nominated product values.
- 3.2. For all the samples analysed, Horrat values are < 2 indicating inter-laboratory precision to be satisfactory.
- 3.3. Of the test materials used, the analysis of Sainsburys Butter for fat content yielded the lowest RSD_R with 0.278%. The analysis of Flora Diet for fat content yielded the highest RSD_R with 1.284%. Figure 1 shows a graph of $RSD_R(\%)$ plotted against percentage fat content (m/m). It can be seen that there is a relationship between obtained RSD_R and sample fat content (% m/m) where the higher the fat content the lower the observed RSD_R . Data obtained during the collaborative trial of ISO CD17189.2 is also plotted on the graph where a similar relationship is evident but where the obtained RSD_{RS} are lower than those obtained in this trial.

4. CONCLUSION

- 4.1. The data for the analysis of fat content of butter, edible oil emulsions and spreadable fat test materials using method 135 produced satisfactory inter-laboratory precision.

5. REFERENCES

- [1] "Protocol for the Design, Conduct and Interpretation of Collaborative Studies" (Ed) Horwitz W, Pure and Applied Chemistry, 1988, 60 (6), 855-864.
- [2] Evaluation of methods used for regulation of foods and drugs, Horwitz W, Analytical Chemistry, 1982, 57, 67A-76A.
- [3] "Precision Parameters of Standard Methods of Analysis of Dairy Products", Peeler J T, Horwitz W and Albert R. JAOAC, 1989, 72, (5) 784-806.

FIGURE 1.

RELATIONSHIP BETWEEN PERCENTAGE FAT CONTENT (M/M) AND $RSD_R\%$ FOR TRIAL DATA OBTAINED USING METHOD 135 AND DRAFT ISO/IDF METHOD

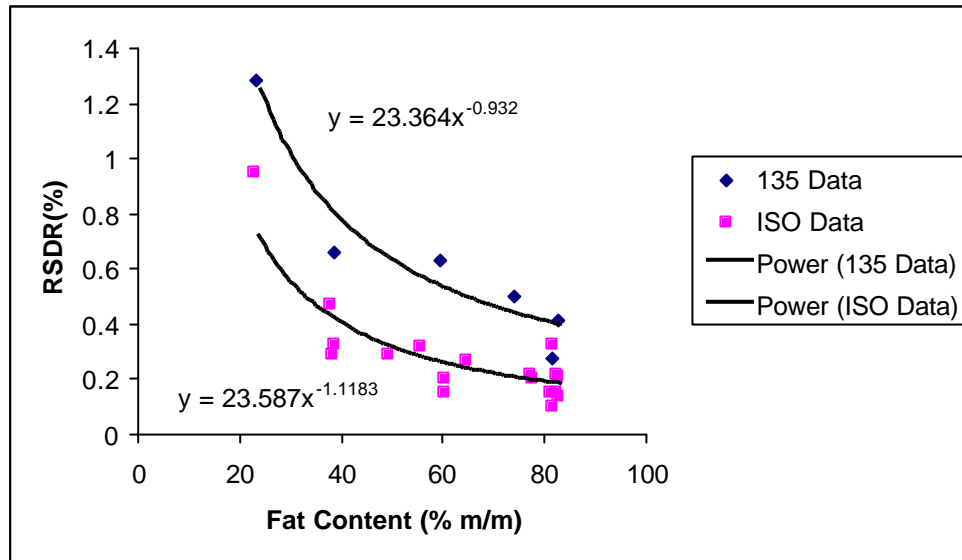


TABLE 1.
SUMMARY OF SAMPLES USED IN THE COLLABORATIVE TRIAL

Sample Code	Sample Type	Sample Description	Fat Content (% m/m)
B	Blind Duplicates	Diet low fat spread	23
F			
C	Blind Duplicates	Half fat butter	40
J			
A	Blind Duplicates	Olive oil based fat spread	59
K			
D	Blind Duplicates	Fat spread	72
M			
E	Blind Duplicates	Butter	81
H			
G	Blind Duplicates	Margarine	82
L			

TABLE 2.**SUMMARY OF CALCULATED STATISTICAL PARAMETERS FOR FAT CONTENT (% M/M) OF DIET LOW FAT SPEAD TEST MATERIAL**

Laboratory	Sample B	Sample F
1	23.50	23.30
2	23.68	23.73
3	23.13	22.81
4	23.20	23.41
5	23.35	23.42
6	23.89	23.85
7 [C]	23.42	24.73
8	22.69	22.86
10	23.37	23.43
11	23.13	23.68
12	23.17	23.59
13	23.22	23.67
14	23.45	23.30
15	23.18	23.57
16	23.55	23.55
Overall Mean	23.38	
r	0.551	
s _r	0.197	
RSD _r	0.842	
Ho _r	0.512	
R	0.841	
s _R	0.300	
RSD _R	1.284	
Ho _R	0.516	

TABLE 3.**SUMMARY OF CALCULATED STATISTICAL PARAMETERS FOR FAT CONTENT (% M/M) OF HALF FAT BUTTER TEST MATERIAL**

Laboratory	Sample C	Sample J
1	38.40	38.20
2	38.58	38.73
3	38.00	38.48
4	38.65	38.69
5	38.61	38.56
6	38.79	38.79
7 [C]	38.38	39.98
8	38.31	38.36
10	38.58	38.66
11	38.46	38.70
12	38.72	39.25
13	38.26	38.45
14	38.79	38.60
15	38.16	38.73
16	38.84	38.62
Overall Mean	38.57	
r	0.552	
s _r	0.197	
RSD _r	0.512	
Ho _r	0.336	
R	0.709	
s _R	0.253	
RSD _R	0.657	
Ho _R	0.284	

TABLE 4.**SUMMARY OF CALCULATED STATISTICAL PARAMETERS FOR FAT CONTENT (% M/M) OF OLIVE OIL BASED SPREAD TEST MATERIAL**

Laboratory	Sample A	Sample K
1	59.10	59.60
2	59.82	59.70
3	59.46	59.38
4	59.32	58.53
5	59.70	59.85
6	59.92	60.43
7	60.23	58.85
8	59.07	59.79
10	59.70	59.53
11	59.39	59.66
12	59.73	59.90
13	59.46	59.48
14	59.73	59.24
15	59.82	59.60
16	59.67	59.59
Overall Mean	59.58	
r	1.026	
s _r	0.366	
RSD _r	0.615	
Ho _r	0.431	
R	1.057	
s _R	0.378	
RSD _R	0.634	
Ho _R	0.293	

TABLE 5.**SUMMARY OF CALCULATED STATISTICAL PARAMETERS FOR FAT CONTENT (% M/M) OF FAT SPREAD TEST MATERIAL**

Laboratory	Sample D	Sample M
1	73.80	73.30
2	74.06	73.93
3	74.21	74.34
4	73.87	73.23
5	74.14	74.50
6	74.31	74.40
7	73.48	73.40
8	73.64	73.52
10	74.03	74.09
11	74.01	73.85
12	73.35	74.15
13	73.86	74.02
14	74.18	73.96
15	74.65	73.83
16	74.35	74.27
Overall Mean	73.96	
r	0.771	
s _r	0.275	
RSD _r	0.372	
Ho _r	0.269	
R	1.041	
s _R	0.372	
RSD _R	0.503	
Ho _R	0.240	

TABLE 6.**SUMMARY OF CALCULATED STATISTICAL PARAMETERS FOR FAT CONTENT (% M/M) OF BUTTER TEST MATERIAL**

Laboratory	Sample E	Sample H
1	81.20	81.40
2	81.73	81.63
3	81.81	81.79
4	81.56	81.18
5	81.74	81.70
6	81.77	81.84
7 [C]	81.46	80.66
8	81.25	81.37
10	81.61	81.64
11	81.79	81.71
12	81.72	81.80
13	81.52	81.65
14	81.64	81.62
15 [C]	69.57	81.34
16	82.19	81.86
Overall Mean	81.64	
r	0.327	
s _r	0.117	
RSD _r	0.143	
Ho _r	0.105	
R	0.636	
s _R	0.227	
RSD _R	0.278	
Ho _R	0.135	

TABLE 7.**SUMMARY OF CALCULATED STATISTICAL PARAMETERS FOR FAT CONTENT (% M/M) OF MARGARINE TEST MATERIAL**

Laboratory	Sample G	Sample L
1	81.90	82.70
2	82.82	82.77
3	82.59	82.73
4	82.81	81.62
5 [C]	15.56	82.90
6	82.65	82.97
7	82.01	82.54
8	82.52	82.25
10	82.85	82.75
11	82.90	83.09
12	82.91	82.90
13	82.89	82.74
14	82.79	82.76
15	82.79	82.76
16	83.10	82.69
Overall Mean	82.67	
r	0.881	
s _r	0.315	
RSD _r	0.381	
Ho _r	0.280	
R	0.961	
s _R	0.343	
RSD _R	0.415	
Ho _R	0.202	

TABLE 8.

SUMMARY OF CALCULATED STATISTICAL PARAMETERS FOR FAT CONTENT IN BUTTER, EDIBLE OIL EMULSIONS AND SPREADABLE FAT TEST MATERIALS USING METHOD 135

Test Materials	Diet low fat spread	Half Fat Butter	Olive oil based fat spread	Fat Spread	Butter	Margarine
<i>Mean</i>	23.38	38.57	59.58	73.96	81.64	82.67
r	0.551	0.552	1.026	0.771	0.327	0.881
S_r	0.197	0.197	0.366	0.275	0.117	0.315
RSD_r (%)	0.842	0.512	0.615	0.372	0.143	0.381
Ho_r	0.512	0.336	0.431	0.269	0.105	0.280
R	0.841	0.709	1.057	1.041	0.636	0.961
S_R	0.300	0.253	0.378	0.372	0.227	0.343
RSD_R (%)	1.284	0.657	0.634	0.503	0.278	0.415
Ho_R	0.516	0.284	0.293	0.240	0.135	0.202
n	14	14	15	15	13	14

TABLE 9.

KEY TO TABLES 2-8

[C]	Outlier identified using Cochran's test.
[G]	Outlier identified using Grubbs test.
[NC]	No results calculated.
n	Number of laboratories whose data were used in the statistical calculation, excluding outliers.
r	Repeatability (within laboratory variation). The value below which the absolute difference between two single test results obtained with the same method on identical test material under the same conditions may be expected to lie with 95% probability.
S_r	The standard deviation of the repeatability.
RSD_r	The relative standard deviation of the repeatability ($S_r \times 100/\text{MEAN}$).
Ho_r	The HORRAT value for repeatability is the observed RSD_r divided by the RSD_r value estimated from the Horwitz equation using the assumption $r = 0.66R$.
R	Reproducibility (between lab variation). The value below which the absolute difference between two single test results obtained with the same method on the identical test material under different conditions may be expected to lie with 95% probability.
S_R	The standard deviation of the reproducibility.
RSD_R	The relative standard deviation of the reproducibility ($S_R \times 100/\text{MEAN}$).
Ho_R	The HORRAT value for reproducibility is the observed RSD_R value divided by the RSD_R value calculated from the Horwitz equation.

COLLABORATIVE TRIAL 135: DETERMINATION OF FAT CONTENT IN BUTTER, EDIBLE OIL EMULSIONS AND SPREADABLE FATS

The use of this method may involve hazardous materials, operations and equipment. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to establish safety and health practices and determine the applicability of regulatory limitations prior to use.

1. SCOPE

This protocol specifies a method for the determination of the fat content of butter, edible oil emulsions and spreadable fats (margarine, vegetable oil spreads, dairy spreads and blended spreads).

NOTE Edible oil emulsions are defined as products having the same constituents as butter, but a composition that does not meet the Codex definition for butter.

2. DEFINITION

For the purposes of this protocol, the following definition shall apply:

- 2.1. Fat Content: the mass fraction of substances determined by the procedure specified in this protocol.

NOTE The fat content is expressed as mass fraction, in percent.

3. PRINCIPLE

Fat is extracted from the test portion using a specified solvent. The solvent/fat phase is separated from the water phase and transferred quantitatively to a fat-collecting vessel. The solvent is removed by distillation or evaporation and the mass of substances extracted is determined.

4. REAGENTS

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralised water or water of equivalent purity.

The reagents shall leave no appreciable residue upon evaporation when the determination is carried out by the method specified (see 7.1.2).

- 4.1 **Petroleum ether**, with any boiling range between 30°C and 60°C, or, as equivalent, **hexane** ($\text{CH}_3[\text{CH}_2]_4\text{CH}_3$), with a boiling point of 69°C and complying with the requirements for the solvent blank test (7.1.2).
- 4.2 **Ethanol ($\text{C}_2\text{H}_5\text{OH}$)**, concentration of at least 94% (volume fraction).
- 4.3 **Congo-red solution**. Dissolve in about 50 ml of water 1 g of Congo-red in a 100 ml one-mark volumetric flask. Make up to the mark with water.

NOTE The use of this solution, which helps the analyst to better see the interface between the solvent layer and the aqueous layer is optional (see 7.2). Other aqueous colour indicators may be used provided they do not affect the fat result.

5. APPARATUS

WARNING - As the determination involves the use of volatile flammable solvents, electrical apparatus employed shall comply with legislation relating to the hazards in using such solvents.

Usual laboratory equipment and, in particular, the following.

5.1 Analytical balance, capable of weighing to the nearest 1 mg, with a readability of 0.1 mg.

5.2 Drying oven, electrically heated, ventilated, thermostatically controlled, capable of being maintained at a temperature of $102^{\circ}\text{C} \pm 2^{\circ}\text{C}$ throughout its working space. The oven shall be fitted with a suitable thermometer.

5.3 Desiccator, containing a suitable drying agent, for example, freshly dried silica gel with hygrometric indicator.

NOTE If the method is used purely to obtain a routine result, that is, where high accuracy and precision are not required, then the fat-collecting vessels may be cooled to the temperature of the weighing room on the laboratory bench protected from dust.

5.4 Fat-collecting vessels, such as boiling glass flasks with ground necks, of capacities 125 ml, or metal dishes.

NOTE When using metal dishes, it is recommended that dishes with relatively high walls (e.g. 6 cm) be used to reduce the risk of fat loss through splashing of the solvent during solvent transfer from the centrifuge tube to the fat-collecting vessel, or vigorous boiling during the evaporation of the solvent.

5.5 Boiling aids, fat free, of non-porous porcelain or silicon carbide (optional when metal dishes are used).

5.6 Tongs, made of metal, **or cotton gloves**, for holding the fat-collecting vessels (5.4).

5.7 Leak-proof centrifuge tubes, with screw cap, of capacity 50 ml, of plastic being resistant to the solvent (4.1) for at least the duration of the test.

NOTE Tubes having a large opening (e.g. 25 mm to 35 mm) are preferred to facilitate the addition of the test portion.

5.8 Vortex mixer

5.9 Centrifuge, capable of holding the leak-proof centrifuge tubes (5.7) and capable of producing a radial acceleration of 50 *g* to 100 *g* at the outer ends of the tubes.

NOTE The use of the centrifuge is optional but recommended (see 7.4).

5.10 Apparatus for quantitative transfer of the solvent/fat phase, such as a 5 ml automatic pipette or other suitable liquid-transfer apparatus.

5.11 Distillation or evaporation apparatus (e.g. steam bath), for distilling or evaporating the solvent from the fat-collecting vessels (see 7.8).

5.12 Solvent dispenser or measuring cylinders, of capacities 10 ml and 20 ml.

6 PROCEDURE

6.1 Preparation of test sample

- 6.1.1** Warm the test sample in the original unopened container to a temperature at which the sample will be soft enough to facilitate thorough mixing to a homogeneous state (either by a mechanical shaker or by hand) without any rupture of the emulsion. The temperature of mixing should typically be between 24°C and 28°C, and should normally not exceed 35°C (butter and edible oil emulsions) or 30°C (spreadable fats).
- 6.1.2** Where applicable, cool the test sample to ambient temperature while mixing until cooling is complete. As soon as possible after cooling, open the sample container and stir briefly for no longer than 10 s with a suitable device, e.g. a spoon or spatula, before weighing.

6.2 Blank tests

6.2.1 Method blank test

Simultaneously with the determination of the test portion (7.3), carry out a blank test using the same procedure for the preparation of the fat-collecting vessel (6.3) and the determination (7), but without weighing of the test portion (7.1) and the addition of the Congo-red solution (*i.e.* add solvents only).

6.2.2 Solvent blank test

To test the quality of the solvent (4.1), evaporate 60 ml of the solvent from an empty fat-collecting vessel, prepared as specified in 6.3. Additionally, use an empty fat-collecting vessel, prepared as specified in 6.3, for mass control purposes. The solvent shall leave no residue greater than 1.0 mg (see A.1). Replace or redistill unsatisfactory solvent.

6.3 Preparation of the fat-collecting vessel

- 6.3.1** Dry the empty fat-collecting vessel (5.4) with a few boiling aids (5.5) in the drying oven (5.2), set at 102°C, for at least 30 min.
- 6.3.2** Allow the fat-collecting vessel to cool in the desiccator (5.3) to the temperature of the weighing room. Weigh the fat-collecting vessel to the nearest 0.1 mg.

NOTE The length of the cooling time will depend on the number of fat-collecting vessels and the size of the desiccator used. Ensure that the length of the cooling time used for the empty fat-collecting vessel is practically the same as that for the fat-collecting vessel containing the extracted fat.

7. DETERMINATION

- 7.1** Weigh 4 g to 6 g (butter and edible oil emulsions), or 1 g to 2 g (spreadable fats), of the test sample (6.1.2) into a centrifuge tube (5.7) and record the weight to the nearest 0.1 mg. For butter and edible oil emulsions, if gravity separation of the phases is used (see 7.4), weigh 2 g to 3 g of the test sample into the centrifuge tube.
- 7.2** Add 20 ml of petroleum ether (4.1) and one drop of the Congo-red solution. Firmly screw the cap on the centrifuge tube.

NOTE 1 The number of extractions and the volume of solvent required for the various extractions depend on the type of product and the means used to separate the phases (table 1).

NOTE 2 The use of the Congo-red solution is optional, but is particularly useful for some spreadable fats which give a transparent serum phase.

7.3 Mix the contents of the centrifuge tube by using the vortex mixer (5.8) until all lumps of sample have been dissolved.

7.4 Centrifuge the tube until a clear petroleum ether phase is obtained. If a suitable centrifuge is not available, allow the two phases to separate under gravity until the petroleum ether phase is clear and distinctly separated from the serum phase.

NOTE The centrifuge speed depends on the type of centrifuge; a clear petroleum ether layer should typically be obtained within 3 min to 5 min at 50 *g* to 100 *g*. Ensure that adequate safety precautions are taken when centrifuging ether-containing tubes.

For spreadable fats, add 2 ml of ethanol if, after separation of the phases, a cloudy solvent phase or a persistent emulsion is obtained. Again mix the contents of the tube and again centrifuge the tube.

7.5 Unscrew the cap and check for evidence of leakage of the centrifuge tube by inspecting the outside of the rim of the tube for fat. Repeat the analysis when there is evidence of fat loss.

Transfer as much as possible of the petroleum ether phase quantitatively to the corresponding fat-collecting vessel without withdrawing some of the aqueous phase. Perform the solvent transfer over a fume bench or in a fume hood.

Do not immerse the pipette tip too deeply into the petroleum ether phase. Always place the tip just below the surface and move the tip down as the petroleum ether is removed. This technique will greatly reduce the quantity of fat remaining on the outside of the tip.

Avoid cross-contamination of fat from one sample to the next. If an automatic pipette is used for solvent transfer, a different tip (numbered if necessary) must be used for each fat-collecting vessel. Upon completion of the transfer there may be some residual fat left on the outside of the tip. Place the tip in a position that avoids loss of this fat (e.g. place it horizontally on a rack or, alternatively, rest the tip at an angle in the corresponding fat-collecting vessel, but not in the collected petroleum ether extracts).

NOTE 1 At the first extraction stage, the petroleum ether phase has a relatively high concentration of fat. Even small losses of the petroleum ether phase during transfer from the tube to the fat-collecting vessel can significantly affect (lower) the fat result.

NOTE 2 Rubber seals in pipettes used for transferring petroleum ether may deteriorate. Check that the pipette can be used for transferring solvent, or dedicate the pipette to this method only.

7.6 Perform a second extraction by adding fresh petroleum ether (table 1) to the tube while rinsing the inside of the pipette tip. Use some of this volume to also rinse the outside lower end of the tip. Replace the screw cap, vortex for 15 s and centrifuge as described in 7.4. Repeat the petroleum ether transfer as described in 7.5 adding the petroleum ether phase to the previous extract.

7.7 Perform a third extraction using fresh petroleum ether as described in 7.6.

If gravity separation of the phases is used, perform a fourth extraction using fresh petroleum ether (table 1).

- 7.8** Remove the solvent as completely as possible from the fat-collecting vessel by using the distillation or evaporation apparatus (5.11).

NOTE Ensure that adequate safety precautions are taken and that the risk of fire is eliminated.

- 7.9** Place the fat-collecting vessel containing the fat in the drying oven (5.2) for 30 min at 102°C. Cool in the desiccator (see note of 6.3.2) and weigh. Repeat the oven drying and cooling process until the difference in mass between two consecutive weighings of the fat-collecting vessel, corrected for the blank, does not exceed 1.0 mg or until the mass increases. Use the lowest mass for the calculation.

NOTE The drying time may depend on the type of fat-collecting vessel used. For metal dishes, 30 min is usually sufficient, but for glass flasks a drying time of 1 h may be more suitable (to reduce the number of drying and cooling cycles).

If more than one analysis is performed, weigh the method blank vessel in parallel to the sample fat-collecting vessels until the last fat-collecting vessel attains constant mass. If the fat-collecting vessels need a different number of weighings to attain constant mass, use for the calculation of the fat content the blank value that corresponds to the lowest mass for the fat-collecting vessel (*i.e.* different blank values may have to be used when the fat-collecting vessels need a different number of weighings to attain constant mass).

Table 1— Number of extractions and volume of solvent (ml) to be used per extraction for centrifugation separation and gravity separation of the phases for different types of product

Product	Centrifugation separation			Gravity separation			
	Number of extractions			Number of extractions			
	1	2	3	1	2	3	4
Butter; edible oil emulsions	20	10	10	20	20	10	10
Spreadable fats	20	20	20	20	20	20	20

NOTE Reducing the volumes of solvent below those shown in the table may result in an incomplete fat recovery.

8 CALCULATION AND EXPRESSION OF RESULTS

8.1 Calculation

Calculate the fat content of the sample using the following equation:

$$w_f = \frac{(m_1 - m_2) - (m_3 - m_4)}{m_0} \times 100 \%$$

where:

- w_f is the percentage by mass of the fat content in the sample;
- m_0 is the mass of the test portion (7.1), in grams;
- m_1 is the mass of the fat-collecting vessel and extracted matter, determined in 7.9, in grams;
- m_2 is the mass of the prepared fat-collecting vessel (6.3.2), in grams;
- m_3 is the mass of the fat-collecting vessel used in the blank test and any extracted matter, determined in 7.9, in grams;
- m_4 is the mass of the fat-collecting vessel used in the blank test (6.3.2), in grams;

8.2 Expression of results

Express the test result to two decimal places.

8.3 Precision

Details of interlaboratory tests in accordance with ISO 5725-1/2 [3, 4] on the precision of the method are presented in annex B.

The values for repeatability and reproducibility limits are expressed for the 95% probability level and may not be applicable to concentration ranges and matrices other than those given.

8.4 Repeatability

The absolute difference between two independent single test results, obtained using 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:

For butter and edible oil emulsions: 0.23%;

For spreadable fats (tentative): 0.27% (result of pilot study).

8.5 Reproducibility

The absolute difference between two independent single test results, obtained using 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

For butter and edible oil emulsions: 0.45%;

For spreadable fats (tentative): 0.44% (result of pilot study).

8.6 Test report

The test report shall specify:

- all the information required for the complete identification of the sample;
- the sampling method used, if known;
- the test method used, together with reference to this protocol;
- all operating details not specified in this protocol, or regarded as optional, together with details of any incident that may have influenced the result(s);
- the test result(s) obtained; or if the repeatability has been checked the final quoted results obtained.

9. SAMPLING

Sampling is not part of the method specified in this protocol. A recommended sampling method is given in ISO 707 [1].

It is important that the laboratory receives a sample, that is truly representative and that has not been damaged or changed during transport or storage.

The laboratory sample should be received in an airtight container. The capacity of the container should be such that one-half to two-thirds is filled by the sample. Store the samples in the closed container until commencing the preparation of the test sample at a temperature of between 5°C and 14°C.

Annex A
(informative)
Notes on procedures

A.1 Blank test to check the solvent (see 6.2.2)

In this blank test, a fat-collecting vessel has to be used for mass control purposes in order that changes in the atmospheric conditions or temperature effects of the fat-collecting vessel will not falsely suggest the presence or absence of non-volatile matter in the solvent. This fat-collecting vessel may be used as a counterweight vessel in the case of a two-pan balance. The criterion for the solvent blank is that the change in apparent mass of the fat-collecting vessel from which the solvent was evaporated, corrected for the apparent change in mass of the fat-collecting vessel for control purposes, shall show no increase greater than 1.0 mg.

Very occasionally, the solvents may contain volatile matter, which is strongly retained in fat. If there are indications of the presence of such substances, carry out blank tests on the solvent using a fat-collecting vessel with about 4 g of anhydrous butterfat. A fat-collecting vessel containing 4 g of anhydrous butterfat has to be used for mass control purposes, so that oxidation of the milkfat, changes in the atmospheric conditions or temperature effects of the fat-collecting vessel will be corrected for. If necessary, redistill the solvent in the presence of 1 g of anhydrous butterfat per 100 ml of solvent. Use the solvent only shortly after redistillation.

Annex B

(informative)

Results of interlaboratory trials

The results obtained were subjected to statistical analysis in accordance with ISO 5725-1/2 [3, 4] to give the precision data shown in tables B.1 and B.2

BUTTER AND EDIBLE FAT EMULSIONS

An international collaborative test involving 12 laboratories was carried out on 8 samples. The levels varied from a mass fraction of 77% to 83% of fat.

Table B.1 - Precision data for butter and edible oil emulsions

Samples	Edible oil emulsion (Russian type butter)	Edible oil emulsion (white sauce blend)	High salt butter	Salted butter	Pastry butter	Unsalted butter	Lactic butter	Unsalted butter
n (labs)*	11	11	11	10	11	11	11	11
m (%)**	77.3	77.5	81.0	81.3	81.4	82.4	82.7	82.7
r (%)**	0.36	0.27	0.11	0.28	0.19	0.20	0.10	0.23
R (%)**	0.48	0.43	0.34	0.75	0.23	0.36	0.34	0.48
RSD _(r) (%)	0.17	0.12	0.05	0.12	0.08	0.09	0.04	0.10
RSD _(R) (%)	0.22	0.20	0.15	0.33	0.10	0.15	0.14	0.21

* Data of one laboratory were discarded in accordance with ISO 5725-2, clause 7.2.5.

** Mass fraction.

SPREADABLE FATS

An international pilot collaborative test involving 6 laboratories was carried out on 10 spreadable fat samples. The levels varied from a mass fraction of 23% to 82% of fat.

Table B.2 - Precision data for spreadable fats (tentative)

Samples	1	2	3	4	5	6	7	8	9	10
n (labs)*	5	5	5	5	5	5	5	5	5	5
M (%)**	23.0	37.8	38.0	38.4	49.3	55.6	60.2	60.2	64.5	82.2
r (%)**	0.21	0.28	0.24	0.17	0.18	0.50	0.34	0.23	0.20	0.24
R (%)**	0.62	0.50	0.31	0.36	0.40	0.50	0.34	0.25	0.49	0.51
RSD _(r) (%)	0.32	0.26	0.22	0.16	0.13	0.32	0.20	0.14	0.11	0.10
RSD _(R) (%)	0.95	0.47	0.29	0.33	0.29	0.32	0.20	0.15	0.27	0.22

* Data of one laboratory were discarded in accordance with ISO 5725-2, clause 7.2.5.

** Mass fraction.

Product identification

1. Vegetable oil spread
2. Dairy spread 1
3. Dairy spread 2
4. Dairy spread 3
5. Vegetable oil spread (sunflower oil based)
6. Vegetable oil spread (canola oil based)
7. Butter-margarine blend
8. Vegetable oil spread (total oil content contained 30% olive oil)
9. Vegetable oil spread (total oil content contained 80% monounsaturated)
10. Butter-margarine blend

Bibliography

- [1]. ISO 707, *Milk and milk products - Guidance on sampling*.
- [2]. ISO 3727-3, *Butter – Determination of moisture, solids-non-fat and fat contents – Part 3: Determination of fat content*.
- [3]. ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*.
- [4]. 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*.
- [5]. Evers, J. M., Crawford, R. A., Wightman, L. M., Beutick, G. J., Contarini, G., Farrington, D. S. *An accurate and rapid method for the direct determination of fat in butter, butter-margarine blends and milkfat spreads*. International Dairy Journal, **9**, 10, 675-682 (1999).
- [6]. Evers, J. M., Crawford, R. A. *Direct determination of the total fat content of butter and edible oil emulsions – An international collaborative study*. International Dairy Journal (in press).
- [7]. Evers, J. M., Wightman, L. M., Crawford, R. A., Contarini, G., Coors, U., Farrington, D. S., Molkentin, J., Nicolas, M. *A precise method to measure the total fat content of spreadable fats*. International Dairy Journal (in press).

**THE DETECTION OF SUDAN I, II, III AND IV IN PALM OIL BY THIN LAYER
CHROMATOGRAPHY**

S Guffogg, P A Brown, S G Stangroom and C A Sutherland

Lincolne Sutton and Wood

70 – 80 Oak Street

Norwich NR3 3AQ

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THE DETECTION OF SUDAN I, II, III AND IV IN PALM OIL BY THIN LAYER CHROMATOGRAPHY

1. **Scope and Field of Application**

- 1.1 The method describes the detection of Sudan I, II, III and IV in palm oil.
- 1.2 The defined Sudans are oil soluble, azo dyestuffs which are genotoxic carcinogens and not permitted in food.

2. **Definition**

Any detected Sudan I, II, III and IV means those compounds exhibiting the same chromatographic properties as their corresponding standard material under the test conditions.

3. **Principle**

The Sudans are extracted into organic solvent, following saponification of the oil. The extract is subjected to Thin Layer Chromatography (TLC) whereby any detected Sudan is identified by virtue of its visual and chromatographic properties relative to similarly applied standard materials.

4. **Health and Safety**

- 4.1 EYE PROTECTION SHOULD NORMALLY BE WORN AT ALL TIMES.
- 4.2 SUDAN I – REFER TO 'MATERIAL SAFETY DATA SHEET'.
- 4.3 SUDAN II – REFER TO 'MATERIAL SAFETY DATA SHEET'.
- 4.4 SUDAN III – REFER TO 'MATERIAL SAFETY DATA SHEET'.
- 4.5 SUDAN IV – REFER TO 'MATERIAL SAFETY DATA SHEET'.
- 4.6 METHANOL IS HIGHLY FLAMMABLE. TOXIC BY INHALATION. KEEP CONTAINER TIGHTLY CLOSED. KEEP AWAY FROM SOURCES OF IGNITION. NO SMOKING. AVOID CONTACT WITH SKIN.
- 4.7 DICHLOROMETHANE IS HARMFUL BY INHALATION AND IF SWALLOWED AND IS IRRITATING TO SKIN. POSSIBLE RISKS OF IRREVERSIBLE EFFECTS. DANGER OF SERIOUS DAMAGE TO HEALTH BY PROLONGED EXPOSURE. DO NOT BREATHE VAPOURS. AVOID CONTACT WITH SKIN AND EYES. WEAR SUITABLE PROTECTIVE CLOTHING.

- 4.8 GLACIAL ACETIC ACID IS HIGHLY FLAMMABLE AND CORROSIVE, CAUSING SEVERE BURNS. DO NOT BREATHE FUMES/VAPOURS. IN CASE OF CONTACT WITH EYES, RINSE IMMEDIATELY WITH PLENTY OF WATER AND SEEK MEDICAL ADVICE.
- 4.9 DIETHYL ETHER IS EXTREMELY FLAMMABLE AND MAY FORM EXPLOSIVE PEROXIDES. USE ONLY IN A DESIGNATED FLAME-FREE AREA.
- 4.10 POTASSIUM HYDROXIDE CAUSES BURNS. WEAR EYE PROTECTION AND GLOVES.
- 4.11 INDUSTRIAL METHYLATED SPIRIT IS HIGHLY FLAMMABLE AND IS HARMFUL IF SWALLOWED. KEEP CONTAINER TIGHTLY CLOSED. KEEP AWAY FROM SOURCES OF IGNITION.
- 4.12 ACETONITRILE IS HIGHLY FLAMMABLE. THE USE OF FLAMMABLE SOLVENTS IS TO BE CARRIED OUT IN A DESIGNATED FLAME-FREE AREA. ACETONITRILE IS TOXIC BY INHALATION OF VAPOUR, IF SWALLOWED AND BY ABSORPTION THROUGH THE SKIN. USE A FUME CUPBOARD WHERE POSSIBLE.

5. Pre-Training Requirements

- 5.1 Use of analytical balance.
- 5.2 Principles and techniques of thin layer chromatography.

6. Reagents

- 6.1 GPR and AR grade reagents are suitable unless otherwise stated. Water should be de-ionised, distilled or of similar quality.
- 6.2 Sudan I: That obtained from Sigma Aldrich (product code 103624) has been found suitable.
- 6.3 Sudan II: That obtained from Acros Organics (product code 190150250) has been found suitable.
- 6.4 Sudan III: That obtained from Acros Organics (product code 419800250) has been found suitable.
- 6.5 Sudan IV: That obtained from Acros Organics (product code 419810250) has been found suitable.
- 6.6 Dichloromethane : HPLC Grade

- 6.7 Methanol : HPLC grade.
- 6.8 Glacial Acetic acid
- 6.9 Diethyl ether
- 6.10 Industrial Methylated Spirits: IMS 99. That supplied by Tennants has been found to be suitable.
- 6.11 Sodium Sulphate (anhydrous)
- 6.12 Acetonitrile: HPLC grade.
- 6.13 Chromatography developing solvent: Mix, in the following proportions:
- | | | |
|-----------------|--------------|---------------------------|
| methanol (6.7): | water (6.1): | glacial acetic acid (6.8) |
| 80: | 15: | 5 |
- 6.14 Sudan I and Sudan II stock solution: Weigh 0.1g Sudan I (6.2) and 0.1g Sudan II (6.3) and respectively transfer to a 100mL volumetric flask with acetonitrile (6.12). Dissolve, with the aid of an ultra sonic bath (7.9) and make to volume with acetonitrile. Mix well. This solution has a nominal concentration of 1000mg/L of each Sudan.
- 6.15 Sudan III and Sudan IV stock solution: Weigh 0.1g Sudan III (6.4) and 0.1g Sudan IV (6.5) transfer to a 100mL volumetric flask and dissolve in 25mL dichloromethane (6.6) with the aid of an ultra sonic bath (7.9). Dilute to volume with acetonitrile. Mix well. This solution has a nominal concentration of 1000mg/L of each Sudan.
- 6.16 Mixed working standard solution: Transfer 1.0mL Sudan I and Sudan II stock solution (6.14) and 1.0mL Sudan III and Sudan IV stock solution (6.15) to a 100mL volumetric flask. Dilute to volume with dichloromethane. Mix well. This solution has a nominal concentration of 10mg/L of each Sudan.
- Once prepared the solution should be protected from the light as far as is practicable e.g. by wrapping in aluminium type foil.
- 6.17 Potassium hydroxide
- 6.18 0.5mol/L alcoholic potassium hydroxide : Dissolve 28g potassium hydroxide (6.17) in about 250mL IMS (6.10) with constant gentle stirring. Cool, transfer to a 1L volumetric flask and dilute to volume with IMS. Mix well.
- 6.19 White solid vegetable oil
- 6.20 Spiked vegetable oil: To 100g melted white vegetable oil (6.19) add 1mL Sudan I and Sudan II stock solution (6.14) and 1mL Sudan III and

Sudan IV stock solution (6.15), thoroughly mix by stirring. Transfer to a suitable container. This fat contains 10mg/kg of each Sudan.

7. Apparatus

7.1 Normal laboratory glassware and apparatus.

N.B Wherever possible glassware is preferable to plastic because of the inherent difficulties encountered in removing residual Sudan compounds from plastic apparatus.

7.2 Drying oven at $102 \pm 2^{\circ}\text{C}$.

7.3 Boiling water bath.

7.4 Analytical balance, capable of weighing to 0.001g.

7.5 Hairdryer.

7.6 Thin Layer Chromatography plates: HPTLC RP8 10cm x 10cm part no. 156542L. Those obtained from VWR International have been found suitable.

7.7 Chromatography tank: Glass covered, glass tank to house a 20cm x 20cm plate.

7.8 Syringe : 10 μL glass barrelled syringe with an angled domed needle for dispensing solutions onto a chromatographic plate (7.6).

7.9 Ultra sonic bath: e.g. Decon containing appropriate fluid.

7.10 Mixer: e.g Hobart with a 60L drum and a paddle stirrer attachment or suitable equivalent capable of mixing large samples (> 5kg).

7.11 Water bath at $40 \pm 2^{\circ}\text{C}$

8. Procedure

8.1 Preparation of test sample

N.B Palm oil melts at about 35°C and is therefore likely to be solid on receipt. It is necessary to prepare a homogenous sample.

8.1.1 For large samples (>5kg) remove the test sample from its container and mix for 30 minutes in a mixer (7.10) using the paddle stirrer.

- 8.1.2 For small samples (< 5kg) place the test sample and container in a water bath at 40°C (7.11) until the oil has completely melted. Pour into a suitable beaker and mix thoroughly.
- 8.2 Saponification.
- 8.2.1 Weigh 2g prepared sample (8.1.1,8.1.2) into a 100mL flat bottomed flask.
- 8.2.2 Add 25mL 0.5mol/L alcoholic potassium hydroxide (6.18).
- 8.2.3 Place on a boiling water bath (7.3), cover with a water filled watch glass and saponify for 30 minutes.
- 8.2.4 Transfer (warm) to a 250mL separator using 50mL water for transference.
- 8.2.5 Carefully add 50mL diethyl ether (6.9) to the fat flask residue and subsequently transfer to the contents of the separator.
- 8.2.6 Stopper and carefully invert the separator, release any generated pressure by cautiously opening the tap. Close the tap and gently shake the contents for 1 minute.
- 8.2.7 Allow the layers to separate and run the lower (aqueous) layer into a second 250mL separator.
- 8.2.8 Extract the aqueous layer with two sequential 25mL of diethyl ether as 8.2.6- 8.2.7, combining the ethers in the original 250mL separator . Any vessels used during the extraction must be rinsed with small (5mL) amounts of diethyl ether and combined in the separator.
- 8.2.9 Gently rinse the combined ethers sequentially with two amounts of 25mL water. Run the water rinsing to waste on each occasion.
- 8.2.10 Filter the combined, rinsed ethers through anhydrous sodium sulphate (6.11) contained in a glass funnel on a plug of cotton wool, into a 250mL flat-bottomed flask.
- 8.2.11 Sequentially rinse the separator with two 5mL amounts of diethyl ether passing each through the anhydrous sodium sulphate and collecting in the flask.
- 8.2.12 Finally rinse the anhydrous sodium sulphate with 5mL diethyl ether also collecting in the flask.
- 8.2.13 Add an anti bumping granule to the ethers and carefully evaporate to low volume on a water bath. DO NOT ALLOW TO EVAPORATE TO DRYNESS.

8.2.14 Transfer the residue to a dry 10mL volumetric flask using small volume rinsings of the flask. Make to volume and mix.

8.3 Thin Layer Chromatography

8.3.1 Prepare a chromatographic plate (7.6) by drawing a pencil base line (care, the plates are easily scored) across the plate 15mm from its bottom edge. Mark another horizontal line, 80mm up from the drawn baseline.

8.3.2 Along the base line mark and label 'spotting' positions as appropriate but they must be at least 10mm apart and at least 5mm in from the plate edge.

8.3.3 Using a syringe (7.8) apply 5 μ L or 10 μ L as appropriate of the mixed working standard solution (6.16) to suitable marked "spotting" positions on the baseline.

8.3.4 Apply 5 μ L, 10 μ L or 25 μ L as appropriate of the test portion extract (8.2.14) to suitable marked "spotting" positions on the base line.

8.3.5 During application of the spots (8.3.3, 8.3.4) it may be necessary to assist drying by use of a hairdryer(7.5).

8.3.6 Prepare a chromatography tank (7.7) by placing ~ 120mL developing solvent (6.13) into the tank.

8.3.7 Carefully lower the prepared plate (8.3.5) into the solvent (aided by tweezers if required) such that the base of the plate rests on the bottom of the tank.

8.3.8 Cover the tank with its glass lid.

8.3.9 Allow the developing solvent to run up the plate to the marked, 80mm line.

8.3.10 Remove the plate from the tank and dry off residual solvent firstly with a hairdryer and to completion by placing the plate in an oven @ 102°C (7.2) for 10 minutes.

8.3.11 Remove the residual solvent from the tank and wipe the inner walls with a clean towel.

8.3.12 Repeat the sequence 8.3.4 – 8.3.9 twice such that the 'spotted' plate runs three times in fresh developing solvent.

8.3.13 Under these chromatographic conditions the four Sudans exhibit the following:

<u>Sudan</u>	<u>colour</u>	<u>Rf</u>
I	orange	0.54
II	pink	0.36
III	red	0.31
IV	purple/red	0.17

8.3.14 Identify the presence of any of the four Sudans by virtue of their Rf value (9) relative to that of the mixed standard solution and their respective colours (8.3.13). See Appendix I.

9. Calculation

9.1 Calculate the Rf value (if required) of the colour spot as follows :

$$Rf = \frac{\text{distance from spotting position to centre of spot}}{\text{distance from spotting position to solvent front}}$$

9.2 It is usually unnecessary to calculate Rf values if the identity of the colour can be assessed by visual inspection of the chromatograms.

10. Expression of Results

Record the identity of the Sudan(s) present in the test sample.

11. Interpretation

Not appropriate

12. Disposal

No specific instruction

13. References

13.1 Stahl Egon Thin Layer Chromatography 2nd Edition.

13.2 VWR International Ltd, Technical Specialist (Peter Wall) Merck House, Poole, Dorset.

13.3 LSW 0033/F/ The Extraction and identification of water-soluble artificial colouring matter in foods by thin layer chromatography.

14. Analytical Quality Assurance

14.1 Performance Characteristics

14.1.1 Limit of detection :

Sudan I and II :10mg/kg

This is based on application of 25 μ L test portion extract and 5 μ L of mixed working standard solution (6.16) to the chromatographic plate.

Sudan III and IV : 5mg/kg

This is based on application of 10 μ L test portion extract and 1 μ L of mixed working standard solution (6.16) to the chromatographic plate.

14.1.2 Bias (WRm and WRs) : not applicable.

14.1.3 Precision (Wp) : not applicable

14.2 Internal Quality Control

14.2.1 Instrument calibration : not applicable

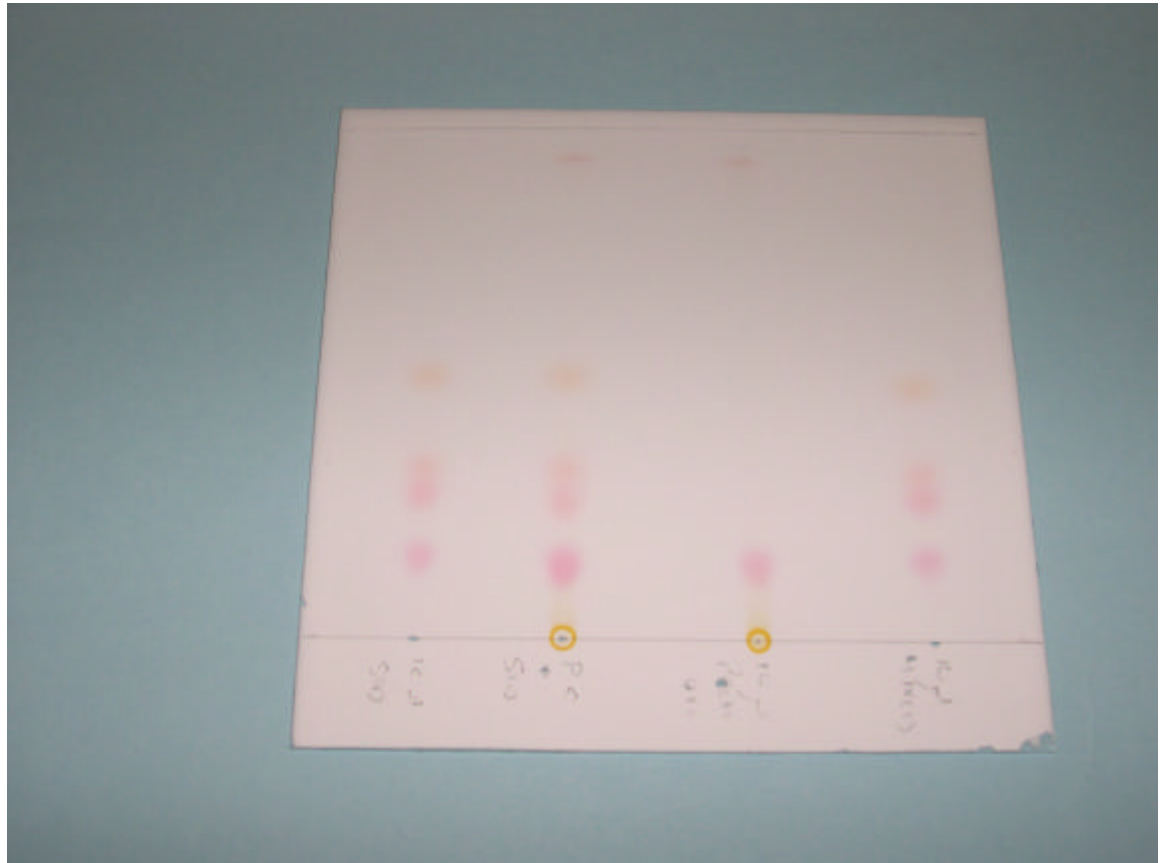
14.2.2 Blank determination : Follow 8.2.1 to 8.2.14 using a palm oil known not to contain any of the Sudans. Application of 25 μ L of the extract (8.2.14) under the chromatographic conditions described in 8.3, will not exhibit any spots corresponding (in respect of Rf and colours) to the four Sudans.

14.2.3 Standard Control : See recovery

14.2.4 Repeatability : not applicable

14.2.5 Recovery check : Follow 8.2.1 to 8.2.14 using 2g of spiked vegetable oil (6.20). Application of 25 μ L of the extract (8.2.14), under the chromatographic conditions specified in 8.3, will exhibit four spots corresponding (in respect of Rf and colour) to the four Sudans.

APPENDIX I: EXAMPLE CHROMATOGRAM OF A PALM OIL SAMPLE CONTAINING SUDAN IV



Mixed Std

Sample
+
Mixed Std

Sample

Mixed Std