Commission Regulation (EEC) No 2568/91 of 11 July 1991 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis

[F1ANNEX X U.K.

DETERMINATION OF FATTY ACID METHYL ESTERS BY GAS CHROMATOGRAPHY

Textual Amendments

F1 Substituted by Commission Implementing Regulation (EU) 2015/1833 of 12 October 2015 amending Regulation (EEC) No 2568/91 on the characteristics of olive oil and olive-residue oil and on the relevant methods of analysis.

1. SCOPE U.K.

This Annex gives guidance on the gas chromatographic determination of free and bound fatty acids in vegetable fats and oils following their conversion into fatty acid methyl esters (FAME).

The bound fatty acids of the triacylglycerols (TAGs) and, depending on the esterification method, the free fatty acids (FFA), are converted into fatty acid methyl esters (FAME), which are determined by capillary gas chromatography.

The method described in this Annex allows the determination of FAME from C_{12} to C_{24}, including saturated, cis- and transmonounsaturated and cis- and trans-polyunsaturated fatty acid methyl esters.

2. PRINCIPLE U.K.

Gas chromatography (GC) is used for the quantitative analysis of FAME. The FAME are prepared according to Part A. They are then injected into and vaporised within the injector. The separation of FAME is performed on analytical columns of specific polarity and length. A Flame Ionisation Detector (FID) is used for the detection of the FAME. The conditions of analysis are given in Part B.

Hydrogen or helium may be used as the carrier gas (mobile phase) in the gas chromatography of FAME with FID. Hydrogen speeds up separation and gives sharper peaks. The stationary phase is a microscopic layer of a thin liquid film on an inert solid surface made of fused silica.

As they pass through the capillary column the volatilised compounds being analysed interact with the stationary phase coating the inner surface of the column. Due to this different interaction of different compounds, they elute at a different time, which is called the retention time of the compound for a given set of analysis parameters. The comparison of the retention times is used for the identification of the different compounds.

PART A U.K.

PREPARATION OF THE FATTY ACID METHYL ESTERS FROM OLIVE OIL AND OLIVE-POMACE OIL

1. SCOPE U.K.

This part specifies the preparation of the methyl esters of fatty acids. It includes methods for preparing fatty acid methyl esters from olive and olive-pomace oils.

2. FIELD OF APPLICATION U.K.

The preparation of the fatty acid methyl esters from olive oils and olive-pomace oils are performed by transesterification with methanolic solution of potassium hydroxide at room temperature. The necessity of purification of the sample prior to the trans-esterification depends on the sample's free fatty acids content and the analytical parameter to be determined, it can be chosen according to the following table:

Category of oil	**Method**
Virgin olive oil with acidity ≤ 2,0 %	1. Fatty acids 2. *trans*-Fatty acids 3. ΔECN42 (after purification with silica-gel SPE)
Refined olive oil	
Olive oil composed of refined olive oil and virgin olive oils	
Refined olive pomace oil	
Olive pomace oil	
Virgin olive oil with acidity > 2,0 % Crude olive pomace oil	1. Fatty acids (after purification with silica-gel SPE) 2. *trans*-Fatty acids (after purification with silica-gel SPE) 3. ΔECN42 (after purification with silica-gel SPE)

3. METHODOLOGY U.K.

3.1. **Trans-esterification with methanolic solution of potassium hydroxide at room temperature** U.K.

3.1.1. *Principle* U.K.

Methyl esters are formed by trans-esterification with methanolic potassium hydroxide as an intermediate stage before saponification takes place.

3.1.2. *Reagents* U.K.

3.1.2.1. Methanol containing not more than 0,5 % (m/m) water.

3.1.2.2. Hexane, chromatographic quality.

3.1.2.3. Heptane, chromatographic quality.

3.1.2.4. Diethyl ether, stabilised for analysis.

3.1.2.5. Acetone, chromatographic quality.

3.1.2.6. Elution solvent for purifying the oil by column/SPE chromatography, mixture hexane/diethyl ether 87/13 (v/v).

3.1.2.7. Potassium hydroxide, approximately 2M methanolic solution: dissolve 11,2 g of potassium hydroxide in 100 ml of methanol.

3.1.2.8. Silica gel cartridges, 1 g (6 ml), for solid phase extraction.

3.1.3. *Apparatus* U.K.

3.1.3.1. Screw-top test tubes (5 ml volume) with cap fitted with a PTFE joint.

3.1.3.2. Graduated or automatic pipettes, 2 ml and 0,2 ml.

3.1.4. *Purification of oil samples* U.K.

When necessary, the samples will be purified by passing the oil through a silica gel solid-phase extraction cartridge. A silica gel cartridge (3.1.2.8) is placed in a vacuum elution apparatus and washed with 6 ml of hexane (3.1.2.2); washing is performed without vacuum. Then a solution of the oil (0,12 g approximately) in 0,5 ml of hexane (3.1.2.2) is loaded onto the column. The solution is pulled down and then eluted with 10 ml of hexane/diethyl ether (87:13 v/v) (3.1.2.6). The combined eluates are homogenised and divided in two similar volumes. An aliquot is evaporated to dryness in a rotary evaporator under reduced pressure at room temperature. The residue is dissolved in 1 ml of heptane and the solution is ready for fatty acid analysis by GC. The second aliquot is evaporated and the residue is dissolved in 1 ml of acetone for triglyceride analysis by HPLC, if necessary.

3.1.5. *Procedure* U.K.

In a 5 ml screw-top test tube (3.1.3.1) weigh approximately 0,1 g of the oil sample. Add 2 ml of heptane (3.1.2.2), and shake. Add 0,2 ml of the methanolic potassium hydroxide solution (3.1.2.7), put on the cap fitted with a PTFE joint, tighten the cap, and shake vigorously for 30 seconds. Leave to stratify until the upper solution becomes clear. Decant the upper layer containing the methyl esters. The heptane solution is ready for injection into the gas chromatograph. It is advisable to keep the solution in the refrigerator until gas chromatographic analysis. Storage of the solution for more than 12 hours is not recommended.

PART B U.K.

ANALYSIS OF FATTY ACID METHYL ESTERS BY GAS CHROMATOGRAPHY

1. SCOPE U.K.

This part gives general guidance for the application of capillary column gas chromatography to determine the qualitative and quantitative composition of a mixture of fatty acid methyl esters obtained in accordance with the method specified in Part A.

The part is not applicable to polymerised fatty acids.

2. REAGENTS U.K.

2.1. **Carrier gas** U.K.

Inert gas (helium or hydrogen), thoroughly dried and with an oxygen content of less than 10 mg/kg.

Note 1: Hydrogen can double the speed of analysis but is hazardous. Safety devices are available.

2.2. **Auxiliary gases** U.K.

2.2.1. Hydrogen (purity ≥ 99,9 %), free from organic impurities.

2.2.2. Air or oxygen, free from organic impurities.

2.2.3. Nitrogen (purity > 99 %).

2.3. **Reference standard** U.K.

Mixture of methyl esters of pure fatty acids, or the methyl esters of a fat of known composition, preferably similar to that of the fatty matter to be analysed. Cis and trans isomers of octadecenoic, octadecadienoic and octadecatrienoic methyl esters are useful for the identification of trans isomers of unsaturated acids.

Care should be taken to prevent the oxidation of polyunsaturated fatty acids.

3. APPARATUS U.K.

The instructions given are for the usual equipment used for gas chromatography, employing capillary columns and a flame-ionisation detector.

3.1. **Gas chromatograph** U.K.

The gas chromatograph shall comprise the following elements.

3.1.1. *Injection system* U.K.

Use an injection system with capillary columns, in which case the injection system should be specially designed for use with such columns. It may be of the split type or the splitless on-column injector type.

3.1.2. *Oven* U.K.

The oven shall be capable of heating the capillary column to a temperature of at least 260 °C and of maintaining the desired temperature to within 0,1 °C. The last requirement is particularly important when a fused silica tube is used.

The use of temperature-programmed heating is recommended in all cases, and in particular for fatty acids with less than 16 carbon atoms.

3.1.3. *Capillary column* U.K.

3.1.3.1. Tube, made of a material inert to the substances to be analysed (usually glass or fused silica). The internal diameter shall be between 0,20 to 0,32 mm. The internal surface shall undergo an appropriate treatment (e.g. surface preparation, inactivation) before receiving the stationary phase coating. A length of 60 m is sufficient for fatty acid and cis and trans isomers of fatty acids.

3.1.3.2. Stationary phase, polar polysiloxane (cyanopropylsilicone) bonded (cross-linked) columns are suitable.

Note 2: There is a risk that polar polysiloxanes may give rise to difficulties in the identification and separation of linolenic acid and C_{20} acids.

The coatings shall be thin, i.e. 0,1 to 0,2 µm.

3.1.3.3. Assembly and conditioning of the column U.K.

Observe the normal precautions for assembling capillary columns, i.e. arrangement of the column in the oven (support), choice and assembly of joints (leak tightness), positioning of the ends of the column in the injector and the detector (reduction of dead-spaces). Place the column under a flow of carrier gas (e.g. 0,3 bar (30 kPa) for a column of length 25 m and internal diameter 0,3 mm).

Condition the column by temperature programming of the oven at 3 °C/min from ambient temperature to a temperature 10 °C below the decomposition limit of the stationary phase. Maintain the oven at this temperature for one hour until stabilisation of the baseline. Return it to 180 °C to work under isothermal conditions.

Note 3: Suitably pre-conditioned columns are available commercially.

3.1.4. *Flame ionisation detector and converter-amplifier* U.K.

3.2. **Syringe** U.K.

The syringe shall have a maximum capacity of 10 µl, graduated in 0,1 µl divisions.

3.3. **Data acquisition system** U.K.

Data acquisition system connected online with the detectors and employed with a software program suitable for peak integration and normalisation.

4. PROCEDURE U.K.

The operations described in 4.1 to 4.3 are for the use of a flame-ionisation detector.

4.1. **Test conditions** U.K.

4.1.1. *Selection of optimum operating conditions for capillary columns* U.K.

Owing to the efficiency and permeability of capillary columns, the separation of the constituents and the duration of the analysis are largely dependent on the flow-rate of the carrier gas in the column. It will therefore be necessary to optimise the operating conditions by adjusting this parameter (or simply column head loss) depending on whether the aim is to improve separation or speed up analysis.

The following conditions have proved to be suitable for the separation of FAMEs (C_4 to C_{26}). Examples of chromatograms are shown in Appendix B:

Injector temperature:	250 °C
Detector temperature:	250 °C
Oven temperature:	165 °C (8 min) to 210 °C at 2 °C/min
Carrier gas hydrogen:	column head pressure, 179 kPa
Total flow:	154,0 ml/min;
Split ratio:	1:100
Injection volume:	1 µl

4.1.2. *Determination of the resolution (see Appendix A)* U.K.

Calculate the resolution, R, of two neighbouring peaks I and II, using the formula:

$R = 2 \times ((d_{dr(II)} - d_{r(I)})/(\omega_{(I)} + \omega_{(II)}))$ or $R = 2 \times ((t_{r(II)} - t_{r(I)})/(\omega_{(I)} + \omega_{(II)}))$ (USP) (United States Pharmacopeia),

or

$R = 1{,}18 \times ((t_{r(II)} - t_{r(I)})/(\omega_{0,5(I)} + \omega_{0,5(II)}))$ (EP, BP, JP, DAB), (JP (Japanese Pharmacopeia), EP (Pharmacopée Européenne), BP (British Pharmacopeia))

where:

$d_{r(I)}$ is the retention distance of peak I;

$d_{r(II)}$ is the retention distance of peak II;

$t_{r(I)}$ is the retention time of peak I;

$t_{r(II)}$ is the retention time of peak II;

$\omega_{(I)}$ is the width of the base of peak I;

$\omega_{(II)}$ is the width of the base of peak II;

$\omega_{0,5}$ is the peak width of the specified compound, at mid-height of the peak;

If $\omega_{(I)} \approx \omega_{(II)}$, calculate R using the following formulas:

$R = (d_{r(II)} - d_{r(I)})/\omega = (d_{r(II)} - d_{r(I)})/4\sigma$

where:

σ is the standard deviation (see Appendix A, Figure 1).

If the distance dr between the two peaks $d_{r(II)}$ - $d_{r(I)}$ is equal to 4σ, the resolution factor R = 1.

If two peaks are not separated completely, the tangents to the inflection points of the two peaks intersect at point C. In order to completely separate the two peaks, the distance between the two peaks must be equal to:

$d_{r(II)}$ - $d_{r(I)} = 6\ \sigma$ from where $R = 1{,}5$ (see Appendix A, Figure 3).

5. EXPRESSION OF RESULTS U.K.

5.1. **Qualitative analysis** U.K.

Identify the methyl ester peaks of the sample from the chromatogram in Appendix B, figure 1, if necessary by interpolation, or by comparison with those of the methyl esters reference mixtures (as indicated at point 2.3).

5.2. **Quantitative analysis** U.K.

5.2.1. *Determination of the composition* U.K.

Calculate the mass fraction w_i of the individual fatty acid methyl esters, expressed as a percentage by mass of methyl esters, as follows:

5.2.2. *Method of calculation* U.K.

5.2.2.1. General case U.K.

Calculate the content of a given component i, expressed as a percentage by mass of methyl esters, by determining the percentage represented by the area of the corresponding peak relative to the sum of the areas of all the peaks, using the following formula:

$w_i = (A_i/\Sigma A) \times 100$

where:

A_i is the area under the peak of the individual fatty acid methyl ester *i*;

ΣA is the sum of the areas under all the peaks of all the individual fatty acid methyl esters.

The results are expressed to two decimal places.

Note 4: For fats and oils, the mass fraction of the fatty acid methyl esters is equal to the mass fraction of the triacylglycerols in grams per 100 g. For cases in which this assumption is not allowed, see 5.2.2.2.

5.2.2.2. Use of correction factors U.K.

In certain cases, for example in the presence of fatty acids with less than eight carbon atoms or of acids with secondary groups, the areas shall be corrected with specific correction factors (Fci). These factors shall be determined for each single instrument. For this purpose suitable reference materials with certified fatty acid composition in the corresponding range shall be used.

Note 5: These correction factors are not identical to the theoretical FID correction factors, which are given in Appendix A, as they also include the performance of the injection system etc. However, in the case of bigger differences, the whole system should be checked for performance.

For this reference mixture, the mass percentage of the FAME *i* is given by the formula:

$w_i = (m_i / \Sigma m) \times 100$

where

m_i is the mass of the FAME **i** in the reference mixture;

Σm is the total of the masses of the various components as FAMEs of the reference mixture.

From the chromatogram of the reference mixture, calculate the percentage by area for the FAME *i* as follows:

$w_i = (A_i/\Sigma A) \times 100$

where:

A_i is the area of the FAME *i* in the reference mixture;

ΣA is the sum of all the areas of all the FAMEs of the reference mixture.

The correction factor F_c is then

$F_c = (m_i \times \Sigma A)/(A_i/\Sigma m)$

For the sample, the percentage by mass of each FAME *i* is:

$w_i = (F_i \times A_i)/\Sigma (F_i \times A_i)$

The results are expressed to two decimal places.

Note 6: The calculated value corresponds to the percentage of mass of the individual fatty acid calculated as triacylglycerols per 100 g fat.

5.2.2.3. Use of an internal standard U.K.

In certain analyses (for example where not all of the fatty acids are quantified, such as when acids with four and six carbons are present alongside acids with 16 and 18 carbons, or when it is necessary to determine the absolute amount of a fatty acid in a sample) it is necessary to use an Internal Standard. Fatty acids with 5, 15 or 17 carbons are frequently used. The correction factor (if any) for the Internal Standard should be determined.

The percentage by mass of component i, expressed as methyl esters, is then given by the formula:

$w_i = (m_{IS} \times F_i \times A_i)/(m \times F_{IS} \times A_{IS})$

where:

A_i is the area the FAME *i*;

A_{IS} is the area of the internal standard;

F_i is the correction factor of the fatty acid *i*, expressed as FAME;

F_{IS} is the correction factor of the internal standard;

m is the mass of the test portion, in milligrams

m_{IS} is the mass of the internal standard, in milligrams.

The results are expressed to two decimal places.

6. TEST REPORT U.K.

The test report shall specify the methods used for the preparation of the methyl esters and for the gas chromatographic analysis. It shall also mention all operating details not specified in this Standard Method, or regarded as optional, together with details of any incidents which may have influenced the results.

The test report shall include all the information necessary for complete identification of the sample.

7. PRECISION U.K.

7.1. **Results of interlaboratory test** U.K.

Details of an interlaboratory test on the precision of the method are set out in Annex C to standard IOC/T.20/Doc. No 33. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

7.2. **Repeatability** U.K.

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 r given in tables 1 to 14 in Annex C to standard IOC/T.20/Doc. No 33.

7.3. **Reproducibility** U.K.

The absolute difference between two 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 R given in tables 1 to 14 in Annex C to standard IOC/T.20/Doc. No 33.

Appendix A U.K.

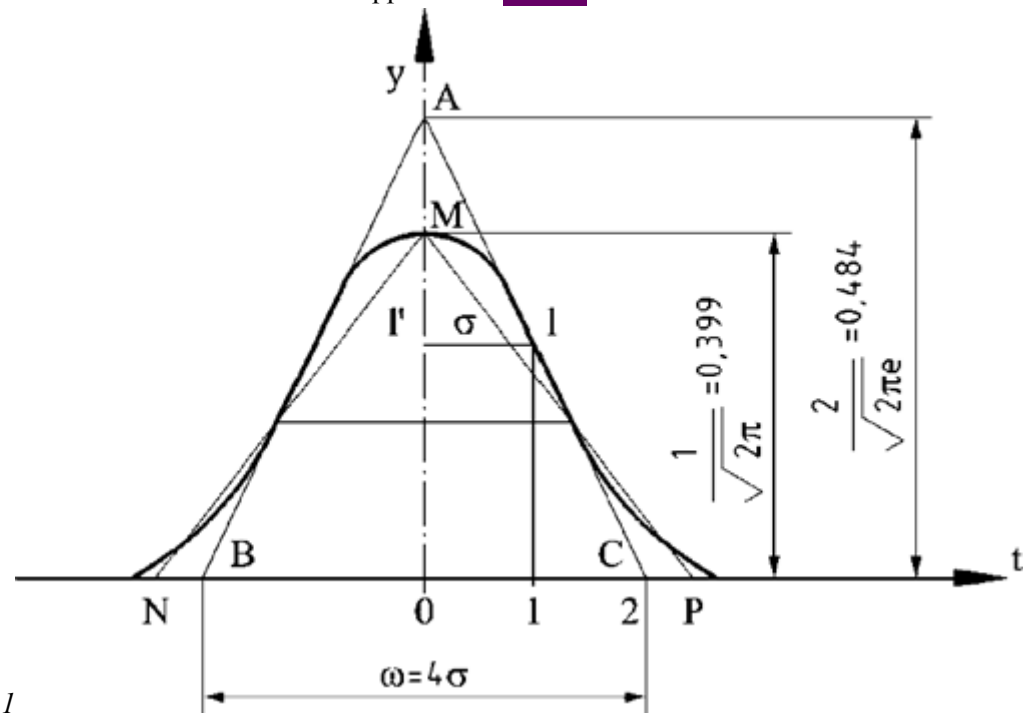
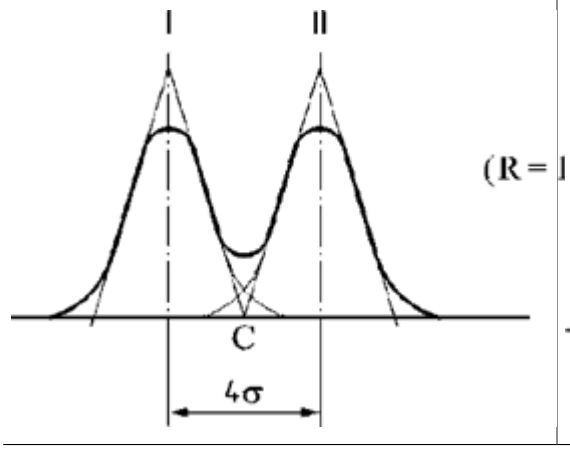
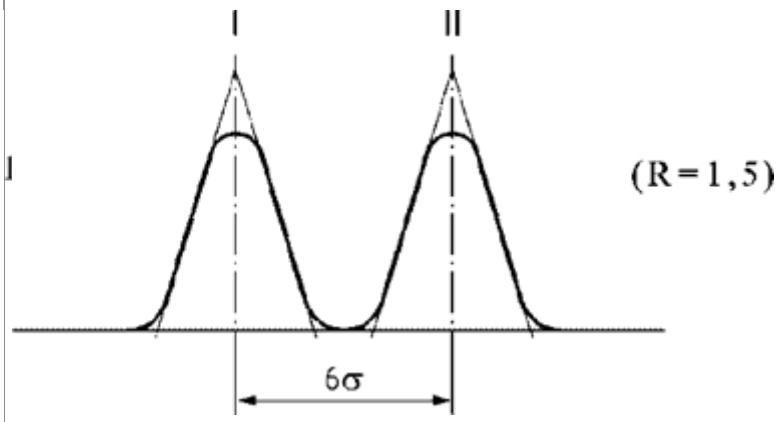


Figure 1

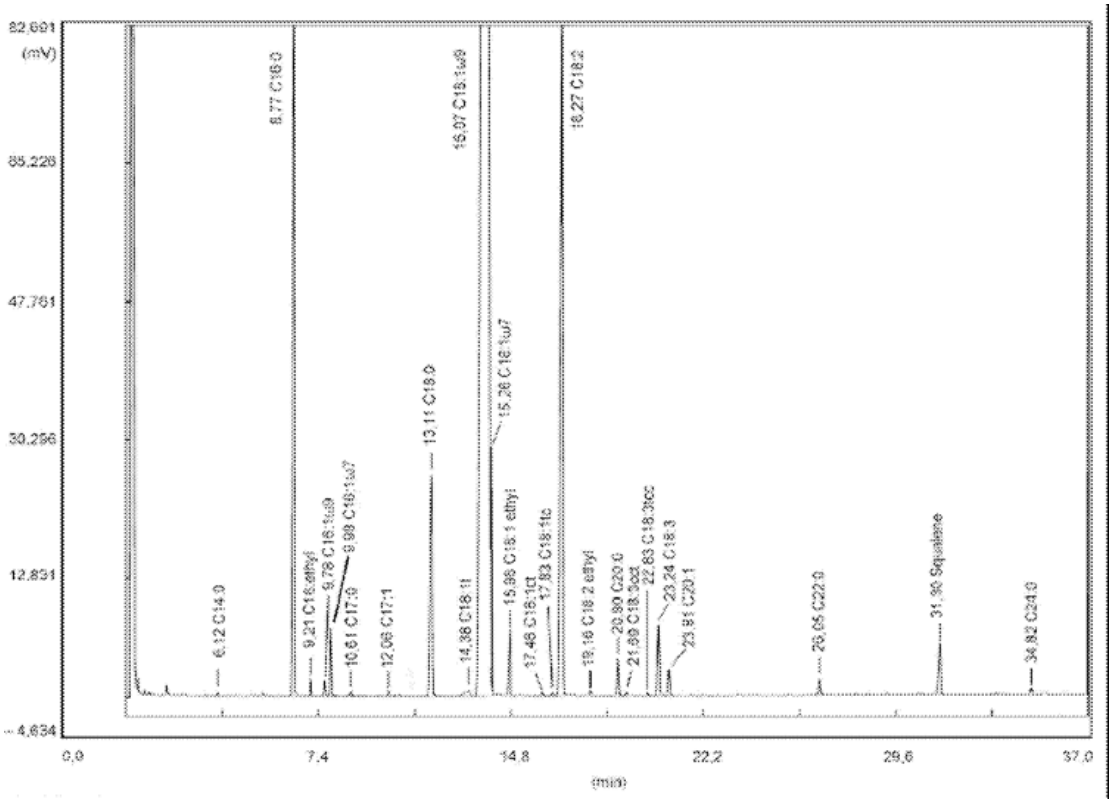
$\omega_{0,5}$ width at half height of the triangle (ABC) and b width at half height of the triangle (NPM).

Figure 2	*Figure 3*
	

Appendix B U.K.

Figure 1

Gas chromatographic profile obtained by the cold methylation method from olive-pomace oil



The chromatographic peaks correspond to the methyl and ethyl esters except where otherwise indicated.**]**

Status:
Point in time view as at 31/12/2020.

Changes to legislation:
There are outstanding changes not yet made to Commission Regulation (EEC) No 2568/91. Any changes that have already been made to the legislation appear in the content and are referenced with annotations.