COLL-CT-2006- Contract Number 030195-2

SAFFIC

Methodologies for Implementing International Standards for Saffron Purity and Quality

SIXTH FRAMEWORK PROGRAMME
HORIZONTAL RESEARCH ACTIVITIES INVOLVING SMES
COLLECTIVE RESEARH

D14 Downloadble proposal of modification of ISO

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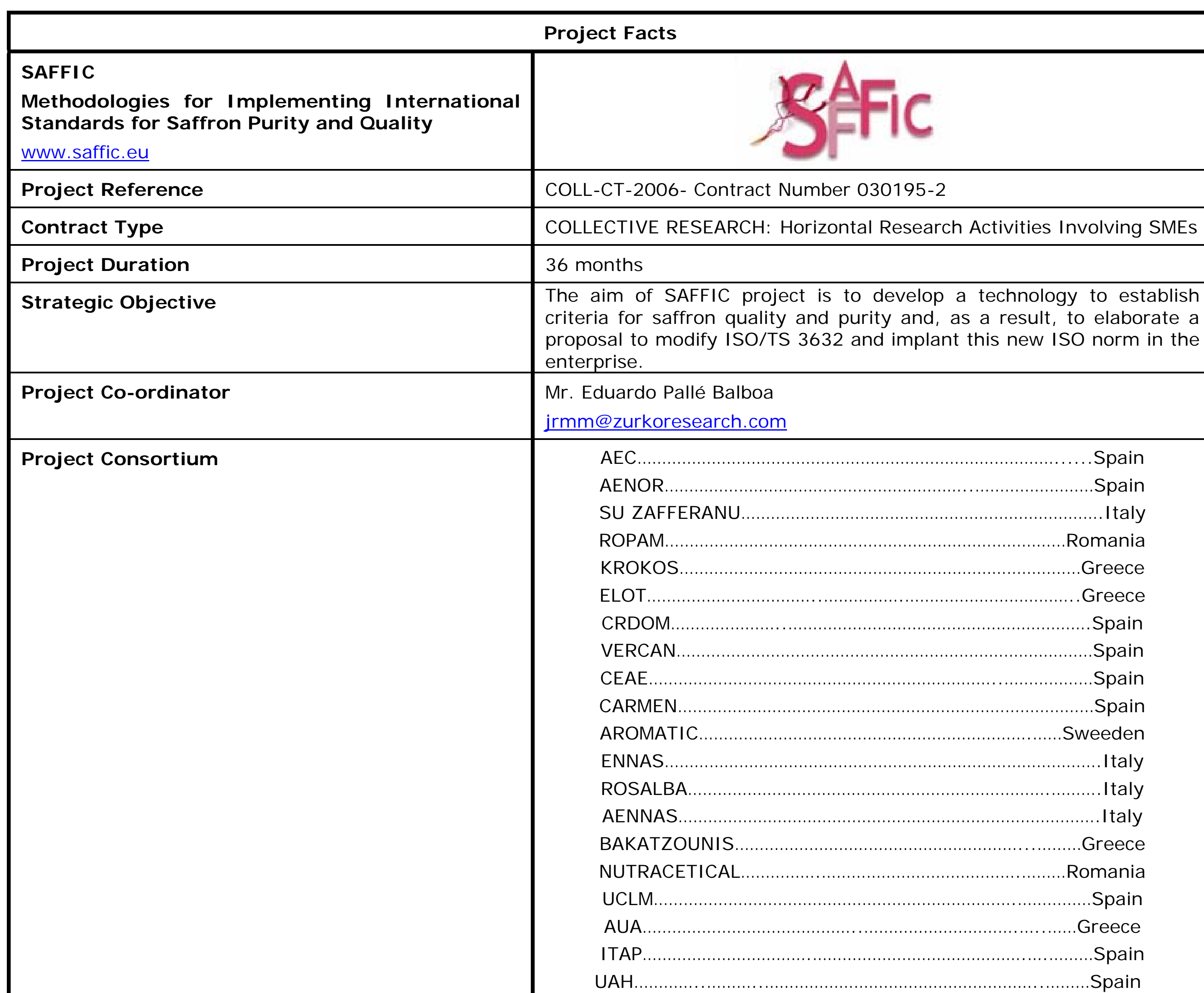

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SAFFIC proposed for modification of ISO/TS 3632-2 —

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SAFFIC proposed for modification of ISO/TS 3632-2 —

17 Determination of picrocrocin from saffron stigmata (Crocus sativus L.)

17.1 Scope

This method determines the picrocrocin content (express as spectrometric absorbance) directly on saffron in powder form provided that the powder conforms to the requirements of subclause of ISO/TS 3632-1, and on saffron filaments and cut filaments after crushing and sieving in accordance with subclause 10.2 of ISO/TS 3632.

17.2 Principle

Determination of picrocrocin content after aqueous extraction and SPE (solid phase extraction) isolation followed by the quantification by UV-Vis spectrometric method.

17.3 Reagents

Unless otherwise indicated, only reagents of recognized analytical grade are used.

17.3.1 Acetonitrile, for analysis**.**

17.3.2 Distilled water, analytical grade.

17.4 Apparatus

Usual laboratory equipment, and, in particular the following:

17.4.1 Analytical balance, capable of weighing to the nearest 0,001 g.

17.4.2 Volumetric flasks, class A, of capacity 10 ml and 100 ml, provided with suitable stoppers.

17.4.3 Magnetic stirrer, suitable for spinning at 1 000 rpm.

17.4.4 Pipette, class A, of capacity 1ml, 2 ml, 5 ml and 10 ml.

17.4.5 Tabletop centrifuge, suitable for 4 000 rpm and 15 ml tubes.

17.4.6 SPE cartridge, silica-based bonded phase with strong hydrophobicity, particle size 55 µm -105 µm, pore size 125 Å, sorbent weight 360 mg (C_{18} Sep-pack Plus[2]).

17.4.7 Vacuum manifold, (optional)

17.4.8 UV-Vis spectrometer, suitable for recording optical density between 200 nm and 700 nm.

17.4.9 Silica cell, with an optical path length of 1 cm.

NOTE C18 Sep-pak Plus is the commercial name of SPE cartridge distributed under reference WAT020515 at Waters, Milford, MA, USA. This information is given for the convenience of users of this part of international standard, and does not constitute an endorsement by ISO of this product.

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17.5 Procedure

17.5.1 Test portion

Weigh 500 mg of the sample, to the nearest 1 mg, in a shoe glass.

17.5.2 Sample extraction

Transfer quantitatively the test portion into a 1 000 ml volumetric flask (17.4.2). Add about 900 ml of distilled water (17.3.2). Stir with a magnetic stirrer (17.4.3) for 1 h, away from light. Remove the magnetic bar. Dilute to the mark with distilled water (17.3.2). Close with a glass stopper (17.4.2) and homogenize. Take an aliquot part with the 10 ml pipette. Transfer to a centrifuge tube (17.4.5) and centrifuge for 5 min at 4 000 rpm.

17.5.3 SPE cartridge conditioned

Prepare the cartridge (17.4.6) using 2 ml of acetonitrile (17.3.1) and then 5 ml of distilled water (17.3.2) at a flow rate of about 5 ml/min. Repeat it twice. Use the optional vacuum manifold (17.4.7) to adjust the solvent flow if necessary.

17.5.4 Picrocrocin isolation and UV determination

Take an aliquot part with the 1 ml pipette (17.4.4) and load it into the SPE cartridge at a flow rate of about 2 ml/min. Wash the cartridge with 10 ml of distilled water and successively elute the picrocrocin using a solution of 12% acetonitrile/water (v/v). Use the optional vacuum manifold to adjust the solvent flow if necessary. Then recover in a volumetric flask the first 10 ml of eluted and measure the absorbance at 250 nm in a 1 cm path length cell in the UV-VIS spectrometer (see Figure 17.1)

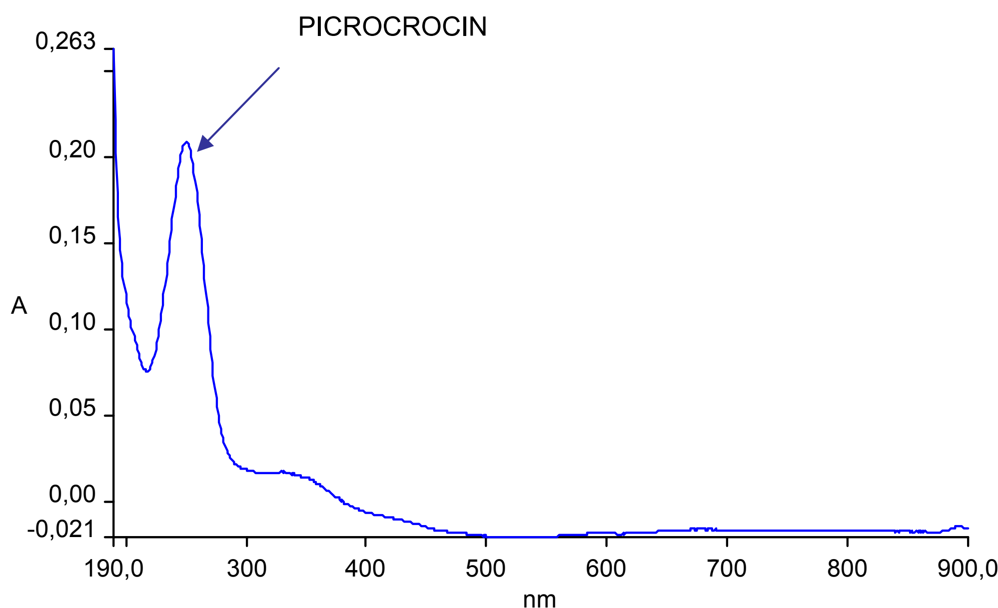

Figure 17.1 – UV-Vis spectrum ranging from 200 nm to 700 nm of an aqueous extract of saffron

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17.6 Expression of results

The results are obtained by direct reading of absorbance $E_{1cm}^{1\%}$ at 250 nm, as follows:

$$E_{1\text{cm}}^{1\%}\,250\,\text{nm} = \frac{\text{D} \times 10.000}{\text{m}\,(100 - \text{H})} \quad (1)$$

Where

D is the specific absorbance;

m is the mass of the saffron sample, in grams;

H is the moisture and volatile content of the sample, expressed as a mass fraction.

17.7 Test report

The test report shall specify the following:

— the method used and the results obtained;

— all information necessary for the complete identification of the sample;

— the moisture content as determined by the method described in Clause 10;

— type of filtration membrane used;

— type of cartridge used;

— all operating details not specified or regarded as optional, as well as any incidents which may have influenced the results.

17.8 Results of an interlaboratory test

An interlaboratory analysis was developed in 2009. In this test three samples of three different categories of saffron were analyzed by 11 laboratories. The following table summarises the results of this test.

	SAMPLE 1	SAMPLE 2	SAMPLE 3
Number of participant laboratories	11	11	11
Number of laboratories retained after eliminating outliers	10	10	10
Means	**39,638**	**37,039**	**35,925**
Standard deviation, s	1,1556	1,4379	1,3228
Repeatibility standard deviation , s_r	1,156	1,438	1,323
Repeatibility relative standard deviation, RSD_r	**2,92%**	**3,88%**	**3,68%**
Repeatibility limit, r	**3,236**	**4,026**	**3,704**

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Reproducibility standard deviation, s_R	3,331	2,666	2,471
Reproducibility relative standard deviation, RDR_R	8,40%	7,20%	6,88%
Reproducibility relative standard deviation, R	9,326	7,465	6,918

18 Determination of safranal

18.1 Scope

This method determines safranal content in saffron obtained from *Crocus sativus L.* flowers.

It is applicable to saffron in both the following forms:

-whole and cut filaments;

-powder.

18.2 Principle

The variation in optical density between 200 nm and 700 nm of an organic extract of saffron is recorded at ambient temperature.

18.3 Reagents

Unless otherwise specified, use only reagents of recognized analytical grade.

18.3.1 Chloroform, boiling point 61,2 ºC at 103,3 kPa (760 mmHg), density 20/20 = 1,489.

18.3.2 Safranal standard, technical grade, assay ca. 75% (GC) (Cas Nº: 116-26-7. Fluka)

18.4 Apparatus

Usual laboratory equipments, and in particular the following.

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18.4.1 Spectrometer, suitable for recording optical density in the ultraviolet band between 200 nm and 700 nm.

18.4.2 Silica cell, with an optical path length of 1 cm.

18.4.3 Ultrasound water bath, with 300 mm × 150 mm × 150 mm internal dimensions, at the fixed frequency of 35 kHz. The temperature of the sonicated water bath should be at 25 ^{o}C.

18.4.4 Round bottom flasks, class A, made of anti-actinic glass, of capacity 10 ml.

18.4.5 Plastic syringes, of capacity 5 ml.

18.4.6 Syringe filters, made of polyester, size 25 mm, porosity 0.45 μm, and resistance to chloroform.

18.4.7 Volumetric flasks, class A, in anti-actinic glass, of capacity 5 ml.

18.4.8 Pipette, class A of capacity 20 ml.

18.5 Procedure

18.5.1 Test portion

Weigh 100 mg of the sample, to the nearest 1 mg, in a shoe glass.

18.5.2 Calibration curve

Establish a calibration curve for a series of safranal standards solutions in chloroform (n=7) as a function of safranal's absorbance at 310 nm ($A_{safranal}$ 310 nm) and safranal's content. Use chloroform as solvent. Recommended concentration range 0,01 to 0,1 mg/5 mL of chloroform. Make the absorbance measurements in triplicate.

The concentration range that the method is valid for the determination of safranal by UV-Vis spectrophotometry is 0.01 mg safranal/ 5 ml chloroform to 0.1 mg safranal/ 5 ml chloroform.

18.5.3 Determination

Transfer quantitatively the test portion into the 10 ml round bottom flask (18.5.4). Add using the pipette 4 ml of chloroform. Close with a stopper.

Place the flask into the ultrasound water bath (18.5.3) and sonicate for 15 min. The initial temperature should be 20 ± 1 ^{o}C and at the end of the extraction the temperature should not exceed 25 ^{o}C. Finally the flask should be placed over the same ultrasound source in the ultrasound water bath.

Place the organic extract into the plastic syringe (18.5.5) and filter it through the syringe filter (18.5.6) so as to obtain a clear extract.

Place the filtrate into the 5 ml volumetric flask (18.5.7). Dilute to the mark with the chloroform. Close with a stopper and homogenize.

Adjust the spectrometer (18.5.1) and record the variation in absorbance of the filtered organic extract between 200 nm and 700 nm using chloroform as the reference liquid. Measure the absorbance at 310 nm in the 1 cm path length cell.

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An example of the UV-Vis profile is given in Fig. 1.

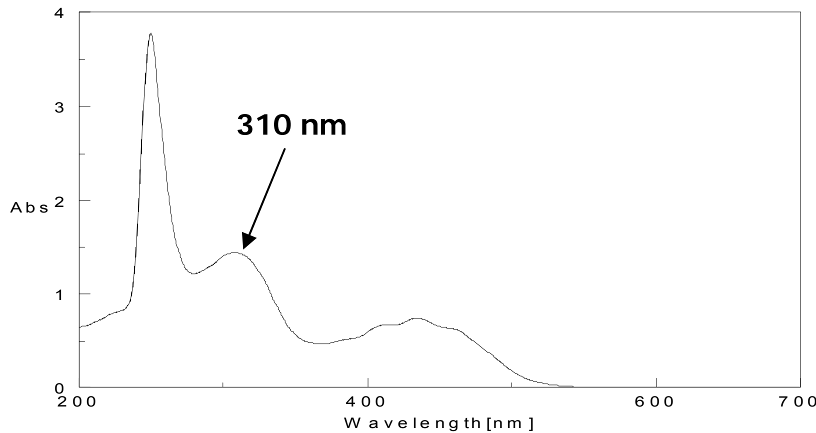

Figure 1 — UV-Vis spectrum ranging from 200 nm to 700 nm of an organic (Chloroform) extract of saffron - (maximum absorbance)

18.6 Expression of results

18.6.1 Determination of absorbance

The absorbance , $E_{1cm}^{1\%}$, is obtaining by direct reading at 310 nm as follows:

$$E_{1cm}^{1\%}\, 310\ \mathrm{nm} = \frac{\mathrm{D} \times 5}{\mathrm{m}\ (100 - \mathrm{H})} \times \mathrm{fd}$$

Where

D is the specific absorbance;

fd: dilution factor

m is the mass of the saffron sample, in grams;

H is the moisture and volatile content of the sample, measured as specified in clause 10 and expressed as a mass fraction.

18.6.2 Results of an interlaboratory test

An interlaboratory analysis was developed in 2009. In this test three samples of three different categories of saffron were analyzed by 10 laboratories. The following table summarises the results of this test.

	SAMPLE 1	SAMPLE 2	SAMPLE 3
Number of participant laboratories	**10**	**10**	**10**
Number of laboratories retained after eliminating outliers	**9**	**9**	**9**
Means	**1,047**	**1,531**	**2,170**
Standard deviation, s	**0,0752**	**0,0789**	**0,1099**
Repeatibility standard deviation , s_r	**0,075**	**0,079**	**0,110**

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Repeatability relative standard deviation, RSD_r	7,18%	5,15%	5,06%
Repeatability limit, r	0,210	0,221	0,308
Reproducibility standard deviation, s_R	0,226	0,250	0,314
Reproducibility relative standard deviation, RDR_R	21,56%	16,31%	14,45%
Reproducibility relative standard deviation, R	0,632	0,699	0,878

18.6.3 Determination of safranal content

Using the calibration curve or the calibration empirical equation determines the content of safranal.

The results are expressed as mg of safranal per Kg of saffron.

18.6.4 Results of an interlaboratory test

An interlaboratory analysis was developed in 2009. In this test three samples of three different categories of saffron were analyzed by 11 laboratories. The following table summarises the results of this test.

	SAMPLE 1	SAMPLE 2	SAMPLE 3
Number of participant laboratories	11	11	11
Number of laboratories retained after eliminating outliers	10	10	10
Means	1591,534	2224,297	3190,783
Standard deviation, s	117,12	127,53	157,12
Repeatibility standard deviation , s_r	117,117	127,531	157,115
Repeatibility relative standard deviation, RSD_r	7,36%	5,73%	4,92%
Repeatibility limit, r	327,928	357,088	439,922
Reproducibility standard deviation, s_R	348,333	527,505	805,564
Reproducibility relative standard deviation, RDR_R	21,89%	23,72%	25,25%
Reproducibility relative standard deviation, R	975,331	1477,014	2255,579

18.7 Test report

The test report shall specify the following:

- the method used and the results obtained;
- all information necessary for the complete identification of the sample;

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- the moisture content as determined by the method described in Clause 10;
- type of filtration membrane used;
- type of cartridge used;
- all operating details not specified or regarded as optional, as well as any incidents which may have influenced the results.

19 Detection of non polar colorants and pigments by high-performance liquid chromatography

19.1 Scope

The HPLC technique is used not only to carry out the chromatographic characterisation of adulterants but also to look for chemical biomarkers that are useful to detect frauds.

This method is applicable to saffron filaments, cut filaments and saffron powder. It allows detection of the presence of non- polar colorants and pigments.

19.2 Principle

Non- polar colorants and pigments are identified by reverse phase high-performance liquid chromatography with detection by a diode array.

19.3 Reagents

Unless otherwise indicated, use only reagents of recognised analytical grade.

19.3.1 Bis-distilled Water for HPLC analysis

19.3.2 Methanol, for HPLC, Gradient Grade

19.3.3 Acetonitrile, for HPLC, Gradient Grade

19.3.4 Chloroform, for HPLC analysis

19.4 Apparatus

19.4.1 Glass Solvent filter degasser

19.4.2 Erlenmeyer flask of 1 l capacity

19.4.3 Membrane filters, with 0,45 µm pores

19.4.4 Analytical balance, Max 101g d=0,1 mg

19.4.5 Adjustable volume analytical pipette 10 µl -100

19.4.6 Adjustable volume analytical pipette 100 µl -1 000 µl

19.4.7 Microsyringe for HPLC 100 µl

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19.4.8 Beakers of 5 ml, 10 ml

19.4.9 One mark volumetric flasks of capacity 10 ml, 20 ml

19.4.10 Spatulas

19.4.11 Pasteur pipettes

19.4.12 Blue tips (1 000 µl), Yellow tips (100 µl)

19.4.13 Glass vials 25 ml

19.4.14 Centrifuge tubes of 30 ml capacity with cap

19.4.15 Vortex Mixer

19.4.16 Ultrasound bath

19.4.17 Nitrogen for evaporation

19.4.18 Syringe filters, 25 mm, 0,45 µm PTFE

19.4.19 10 ml syringes

19.4.20 High-performance liquid chromatograph equipped with a binary pump and a diode array detector which enables measurements of between 200 nm-700 nm, (set at 504, 516, 536, 480 nm) a syringe-loading injection valve with 20 µL injection loop or equivalent and a data system.

19.4.21 Chromatography column for HPLC, type C18

— Material: stainless steel

— Length: 25 cm

— Interior diameter: 4 mm

— Stationary phase: granulated silica of HPLC quality, octadecyl hydrophobic monomeric grafting with granulometric particle dimensions of 5 µm and a pore diameter of 100 Å

NOTE In this Technical Specification, the chromatographic conditions and composition of the mobile phase have been established on the basis of a Nucleosil 100.5. C18 HD Macherey Nagel column. The use of another of column may require certain modifications to the mobile phase and chromatographic conditions.

19.5 Preparation of solutions

19.5.1 Mobile phase A Acetonitrile/Methanol/Water (45:45:10), (% v/v)

In a one litre Erlenmeyer flask (19.4.2) add 450 ml of Acetonitrile, 450 ml of Methanol and 100 ml of bis-distilled water. Homogenize and filter the mixture with 0,45 µm membrane filters (19.4.3) in a glass solvent degasser (19.4.1)

19.5.2 Mobile phase B Chloroform

Add 1000 ml of Chloroform in a one litre Erlenmeyer flask. Filter with membrane filters 0,45 µm (19.4.3) in a glass solvent degasser (19.4.1)

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19.5.3 Non-polar colorants and pigments stock solutions, corresponding to 1g or 0.1g of colorant or pigment per litre of adequate solvent were prepared as it is sited to the following table.

Each stock solution was prepared separately by weighting the appropriate amount of the standard material, transferring into a volumetric flask and diluting to volume with the appropriate solvent as shown in the following table.

STOCK SOLUTIONS				
COLORANT OR PIGMENT	**Weight of colorant or pigment**	**Solvent**	**Volume of solvent**	**Concentration of colorant or pigment in the stock solution**
	(mg)		**(ml)**	**(mg/ml)**
Sudan I	20	Acetonitrile	20	1
Sudan II	20	Chloroform	20	1
Sudan III	20	Chloroform	20	1
Sudan Red 7B	20	Chloroform	20	1
Sudan IV	20	Chloroform	20	1
Sudan Red B	20	Chloroform	20	1
trans-b-apo-8-carotenal	20	Chloroform	20	1

In one 10 ml beaker (19.4.9) dissolve respectively 20 mg of Sudan I in acetonitrile. Transfer to a series of four 20 ml volumetric flasks (19.4.9). Make up to the mark and stir. The solution contains 1 g of colorant per litre of acetonitrile (19.3.3).

In a series of seven 10 ml beakers (19.4.8) dissolve respectively 20 mg Sudan II, Sudan III, Sudan Red 7B, Sudan IV, Sudan Red B, trans-b-apo-8-carotenal in chloroform. Transfer to a series of seven 20 ml volumetric flasks (19.4.9). Make up to the mark and stir. Each solution contains 1 g of colorant per litre of chloroform (19.3.4).

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19.5.4 Non-polar colorants and pigments working solutions, corresponding to 10 mg of colorant or pigment per litre of acetonitrile (19.3.3).

WORKING SOLUTIONS					
COLORANT OR PIGMENT	**Concentration of initial working solution (mg/ml)**	**Volume of initial working solution (ml)**	**Solvent (ml)**	**Volume of solvent**	**Concentration of colorant or pigment in the working solution (µg/ml)**
Sudan I	1	0,1	Acetonitrile	10	10
Sudan II	1	0,1	Acetonitrile	10	10
Sudan III	1	0,1	Acetonitrile	10	10
Sudan Red 7B	1	0,1	Acetonitrile	10	10
Sudan IV	1	0,1	Acetonitrile	10	10
Sudan Red B	1	0,1	Acetonitrile	10	10
trans-b-apo-8-carotenal	1	0,1	Acetonitrile	10	10

In a series of eight 10 ml volumetric flasks (19.4.9) add 0,1 ml of stock solution of Sudan I, Sudan II, Sudan III, Sudan Red 7B, , Sudan IV, Sudan Red B, trans-b-apo-8-carotenal using a pipette (19.4.5). Make up to the mark and stir.

NOTE These solutions are used to record individually the retention times according to the procedure written in 19.6.3, 19.6.4.

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19.5.5 Non-polar colorants and pigments mixed working solution, corresponding to a mixture of colorants and pigments with 40 mg of colorant or pigment per litre of acetonitrile (19.3.3).

MIXED WORKING SOLUTIONS					
COLORANT OR PIGMENT	**Concentration of initial working solution (mg/ml)**	**Volume of working solution (ml)**	**Solvent**	**Volume of solvent (ml)**	**Concentration of mixture of colorants or pigments in the mixed working solution (µg/ml)**
Sudan I	1	0,4	Acetonitrile	10	40
Sudan II	1	0,4	Acetonitrile	10	40
Sudan III	1	0,4	Acetonitrile	10	40
Sudan Red 7B	1	0,4	Acetonitrile	10	40
Sudan IV	1	0,4	Acetonitrile	10	40
Sudan Red B	1	0,4	Acetonitrile	10	40
trans-b-apo-8-carotenal	1	0,4	Acetonitrile	10	40

In a 10 ml volumetric flask (19.4.9) add 0,4 ml of stock solution of Sudan I, Sudan II, Sudan III, Sudan Red 7B, Sudan IV, Sudan Red B, trans-b-apo-8-carotenal and b-carotene and 4 ml of stock solution of capsanthin using a pipette (19.4.5). Make up to the mark and stir.

19.5.6 Non-polar colorants and pigments calibration solutions, corresponding to a mixture of colorants and pigments with concentrations ranging from 0,15 µg to 4 µg of colorant or pigment per millilitre of acetonitrile (19.3.3).

CALIBRATION SOLUTIONS						
Initial Solution	**Concentration of initial solution (µg/ml)**	**Volume of initial solution (ml)**	**Solvent**	**Volume of solvent (ml)**	**Final Concentration of the calibration solution (µg/ml)**	**Label**
MIXED	40	1	Acetonitrile	10	4	CAL 1
MIXED	40	0,75	Acetonitrile	10	3	CAL 2
MIXED	40	0,375	Acetonitrile	10	1,5	CAL 3
MIXED	40	0,25	Acetonitrile	10	1	CAL 4
CAL 1	4	0,75	Acetonitrile	10	0,3	CAL 5

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CAL 1	4	0,375	Acetonitrile	10	0,15	CAL 6

19.5.7 Non-polar colorants and pigments recovery solutions, corresponding to a mixture of colorants and pigments with concentrations ranking from 0,3 µg to 2,5 µg of colorant or pigment per millilitre of acetonitrile (19.3.3).

19.6 Procedure

19.6.1 Test portion

Take a test portion of about 100 mg from the powder obtained for saffron in filaments and cut filaments and for saffron in powder form.

19.6.2 Extraction of non-polar colorants

Add the test portion into a centrifuge tube (19.4.14). Add 4 ml of chloroform and vortex mix (19.4.15) for 1 min. Place in an ultrasound bath for 15 min and filter through a 0,45 µ syringe filter (19.4.18). Wash the centrifuge tube with another 1 ml of chloroform. Evaporate the chloroform extract to dryness under nitrogen flow (19.4.17). Reconstitute in 90 µl of chloroform followed by 210 µl of Mobile Phase A solution (19.5.1).

Due to the light sensitivity of the carotenoids and their degradation with time reported in bibliography, care should be taken to avoid exposure to direct light by covering the samples with aluminium foil and also by analysing the samples at the same day as the extraction. Samples should be kept in a refrigerator until analysis and the reconstitution of the extracted samples should be made right before injection.

19.6.3 HPLC analysis

19.6.3.1 Setting the apparatus

Set the chromatograph (19.5.20) and adjust as follows:

— Flow rate of the mobile phase (19.5.1, 19.5.2): 1,5 ml/min

— Temperature of the column (19.4.21): ambient

19.6.4 Analysis

Use the gradient technique as follows:

Time	Solvent A	Solvent B	Flow Rate
(min)	(%)	(%)	(ml/min)
0	98	2	1,5
2,5	98	2	1,5
16	73	27	1,5
25	73	27	1,5
25,1	98	2	1,5
30	98	2	1,5

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Once the mobile phase A (19.5.1) has been adjusted to the properties of the column and equilibrium has been reached, inject 20 µL of a system suitability test standard solution followed by a blank solvent to detect any carry over effects and then an identical volume of the reconstituted extract obtained in 19.6.2.

The “system suitability test” is a standard solution at a medium concentration of the calibration curve used to determine the instrument performance. As a “system suitability test” a calibrator or a recovery standard could be used (p.e. CAL 4 or REC 2).

Prepare a calibration curve whenever chromatographic conditions change. Inject 20 µL of each calibration solution into the HPLC apparatus and plot peak area values of each colorant against their masses in µg/mL.

If the content of colorants in the samples falls outside the calibration range, appropriate dilution shall be performed. In this case calculation shall be reconsidered accordingly.

19.7 Interpretation and expression of the results

19.7.1 Interpretation

Identify any artificial colorants in the extract by comparing the retention time and the peak spectra of the relevant peak in the sample with the peak of the standard substance in the chromatogram.

Structural confirmation is carried out by scan mode from 200 nm to 700 nm.

19.7.2 Determination

To carry out the determination by the external standard method, integrate the peak area and use the calibration curve equation.

19.7.3 Calculation

Read of from the calibration curve, the amount in µg of colorant per mL of test solution injected into the HPLC column.

Calculate the mass concentration of colorant in milligrams per kilogram using the equation:

$$C = (A * V * 1000)/W$$

Where A= the amount in µg of colorant per mL of test solution

V= is the reconstitution volume in mL (0.3 mL)

W= is the weighted amount of sample in mg (100 mg)

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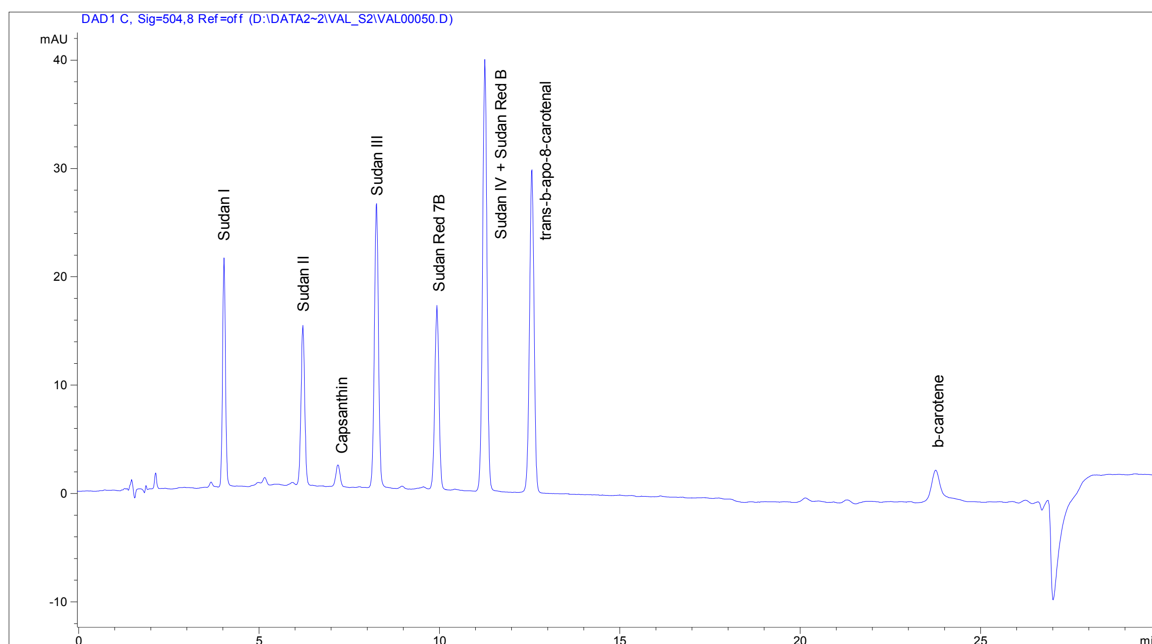

Figure 2 — Indicative chromatogram of a standard solution of non polar colorants at 504nm

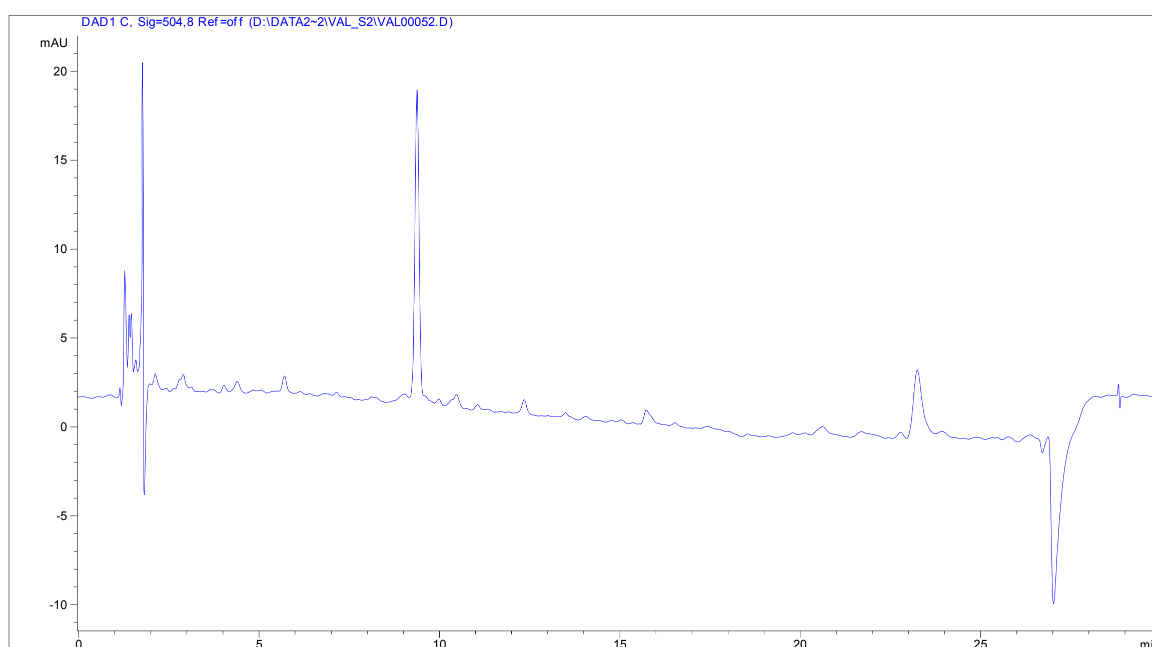

Figure 3 — Indicative chromatogram of a blank saffron sample at 504nm

19.7.4 Results of an interlaboratory test

An interlaboratory analysis was developed in 2009. In this test three samples of three different categories of saffron were analyzed by different laboratories. The following table summarises the results of this test.

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SUDAN I	SAMPLE 1	SAMPLE 2	SAMPLE 3
Number of participant laboratories	9	9	9
Number of laboratories retained after eliminating outliers	8	8	8
Means	0,658	3,448	6,486
Standard deviation, s	0,1081	0,3927	0,5139
Repeatability standard deviation , s_r	0,108	0,393	0,514
Repeatability relative standard deviation, RSD_r	16,42%	11,39%	7,92%
Repeatability limit, r	0,303	1,099	1,439
Reproducibility standard deviation, s_R	0,603	0,789	1,065
Reproducibility relative standard deviation, RDR_R	91,53%	22,87%	16,43%
Reproducibility relative standard deviation, R	1,687	2,208	2,983

SUDAN II	SAMPLE 1	SAMPLE 2	SAMPLE 3
Number of participant laboratories	9	9	9
Number of laboratories retained after eliminating outliers	8	8	8
Means	0,615	3,918	7,545
Standard deviation, s	0,1290	0,3521	0,4402
Repeatability standard deviation , s_r	0,129	0,352	0,440
Repeatability relative standard deviation, RSD_r	20,97%	8,99%	5,83%
Repeatability limit, r	0,361	0,986	1,233
Reproducibility standard deviation, s_R	0,591	0,805	1,320
Reproducibility relative standard deviation, RDR_R	96,02%	20,55%	17,50%
Reproducibility relative standard deviation, R	1,654	2,254	3,697

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SUDAN III	SAMPLE 1	SAMPLE 2	SAMPLE 3
Number of participant laboratories	9	9	9
Number of laboratories retained after eliminating outliers	8	7	9
Means	0,582	4,450	8,124
Standard deviation, s	0,1013	0,3381	0,6836
Repeatibility standard deviation , s_r	0,101	0,338	0,684
Repeatibility relative standard deviation, RSD_r	17,39%	7,60%	8,41%
Repeatibility limit, r	0,284	0,947	1,914
Reproducibility standard deviation, s_R	0,562	0,450	1,556
Reproducibility relative standard deviation, RDR_R	96,48%	10,10%	19,15%
Reproducibility relative standard deviation, R	1,573	1,259	4,356

SUDAN IV	SAMPLE 1	SAMPLE 2	SAMPLE 3
Number of participant laboratories	9	9	9
Number of laboratories retained after eliminating outliers	9	7	8
Means	0,666	4,511	8,158
Standard deviation, s	0,1015	0,3468	0,4700
Repeatibility standard deviation , s_r	0,101	0,347	0,470
Repeatibility relative standard deviation, RSD_r	15,23%	7,69%	5,76%
Repeatibility limit, r	0,284	0,971	1,316
Reproducibility standard deviation, s_R	0,565	0,543	1,457
Reproducibility relative standard deviation, RDR_R	84,80%	12,03%	17,86%
Reproducibility relative standard deviation, R	1,581	1,519	4,079

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SUDAN RED 7B	SAMPLE 1	SAMPLE 2	SAMPLE 3
Number of participant laboratories	8	8	8
Number of laboratories retained after eliminating outliers	7	7	7
Means	0,534	4,703	9,401
Standard deviation, s	0,1723	0,4232	0,6936
Repeatability standard deviation , s_r	0,172	0,423	0,694
Repeatability relative standard deviation, RSD_r	32,28%	9,00%	7,38%
Repeatability limit, r	0,482	1,185	1,942
Reproducibility standard deviation, s_R	0,518	0,923	1,642
Reproducibility relative standard deviation, RDR_R	97,11%	19,62%	17,46%
Reproducibility relative standard deviation, R	1,451	2,584	4,596

SUDAN RED B	SAMPLE 1	SAMPLE 2	SAMPLE 3
Number of participant laboratories	9	9	9
Number of laboratories retained after eliminating outliers	8	8	8
Means	0,507	3,712	7,745
Standard deviation, s	0,0580	0,3191	0,6073
Repeatability standard deviation , s_r	0,058	0,319	0,607
Repeatability relative standard deviation, RSD_r	11,45%	8,60%	7,84%
Repeatability limit, r	0,162	0,893	1,700
Reproducibility standard deviation, s_R	0,572	1,012	1,759
Reproducibility relative standard deviation, RDR_R	112,88%	27,27%	22,72%
Reproducibility relative standard deviation, R	1,602	2,834	4,926

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TRANS-B-APO-8-CAROTENAL	SAMPLE 1	SAMPLE 2	SAMPLE 3
Number of participant laboratories	7	7	7
Number of laboratories retained after eliminating outliers	6	6	6
Means	0,620	3,945	7,694
Standard deviation, s	0,0854	0,4696	0,9301
Repeatibility standard deviation , s_r	0,085	0,470	0,930
Repeatibility relative standard deviation, RSD_r	13,77%	11,90%	12,09%
Repeatibility limit, r	0,239	1,315	2,604
Reproducibility standard deviation, s_R	0,540	0,834	1,628
Reproducibility relative standard deviation, RDR_R	87,01%	21,13%	21,15%
Reproducibility relative standard deviation, R	1,511	2,334	4,557

20 Detection of polar colorants and pigments of high-perfomance liquid chromatography

20.1 Scope

The HPLC technique is used not only to carry out the chromatographic characterisation of adulterants but also to look for chemical biomarkers that are useful to detect frauds.

This method is applicable to saffron filaments, cut filaments and saffron powder. It allows detection of the presence of polar colorants and pigments.

20.2 Terms and definitions

All words with a specific meaning have to be defined

20.3 Principle

Polar colorants and pigments are extracted and after a solid phase extraction clean up procedure are identified by reverse phase high-performance liquid chromatography with detection by a diode array.

20.4 Reagents

Unless otherwise indicated, use only reagents of recognised analytical grade.

20.4.1 Bis-distilled water for HPLC

20.4.2 Acetonitrile, HPLC Gradient Grade

20.4.3 Methanol, HPLC Gradient Grade

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20.4.4 Ethanol, Gradient grade for HPLC

20.4.5 Acetic Acid 100% for HPLC

20.4.6 Formic Acid 98% for HPLC

20.4.7 Acetone for HPLC

20.4.8 Ammonia 25% for HPLC

20.5 Apparatus

20.5.1 Glass Solvent filter degasser

20.5.2 Erlenmeyer flask of 1 litre capacity

20.5.3 Membranes filters, with 0.45 µm pores

20.5.4 Analytical balance, Max 101g d=0,1mg

20.5.5 Adjustable volume analytical pipette 10-100µL

20.5.6 Adjustable volume analytical pipette 100-1000µL

20.5.7 Microsyringe for HPLC 100µL

20.5.8 Beakers of 10, 100 ml

20.5.9 One mark volumetric flasks of capacity 10, 20, 100, 250 ml

20.5.10 Spatulas

20.5.11 Pasteur pipettes

20.5.12 Blue tips (1000µL), Yellow tips (100µL)

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20.5.13 Glass vials 25 ml

20.5.14 Oven

20.5.15 Centrifuge tubes with cap 30 ml

20.5.16 Graduated glass pipettes 10 ml

20.5.17 Centrifuge

20.5.18 Vortex mixer

20.5.19 pH-metric strips

20.5.20 SPE vacuum system

20.5.21 Round-bottom flasks 10 ml

20.5.22 Rotary evaporator

20.5.23 SPE columns C18, 3 mL capacity, octadecyl hydrophobic, (end capping) particle size 55 µm -105 µm, pore size 125 Å, sorbent weight 500 mg

NOTE C18 BakerBond is the commercial name of SPE cartridge distributed under reference WAT020515 at Waters, Milford, MA, USA. This information is given for the convenience of users of this part of international standard, and does not constitute an endorsement by ISO of this product.

20.5.24 High-performance liquid chromatograph equipped with a binary pump and a diode array detector which enables measurements of between 200 nm-700 nm (set 274, 440, 480 nm) a syringe-loading injection valve with 20 µL injection loop or equivalent and a data system.

20.5.25 Chromatography column for HPLC, type C18:

- Material: stainless steel
- Length: 25 cm
- Interior diameter: 4 mm
- Stationary phase: granulated silica of HPLC quality, octadecyl hydrophobic monomeric grafting with granulometric particle dimensions of 5 µm and a pore diameter of 100 Å

NOTE In this Technical Specification, the chromatographic conditions and composition of the mobile phase have been established on the basis of a Nucleosil 100.5. C18 HD Macherey Nagel column. The use of another of column may require certain modifications to the mobile phase and chromatographic conditions.

20.6 Preparation of solutions

20.6.1 Solvent of 0.02N Acetic Acid

Add 0.29 ml of Acetic acid (20.4.5) to a 250 ml volumetric flask, make up to the mark with bis- distilled water and stir.

20.6.2 Solvent for working solutions of Methanol: Water 50:50 (% v/v)

Add 50 ml of Methanol to a beaker and add 50 ml bis- distilled Water

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20.6.3 Mobile phase A : 1% Acetic acid in Water HPLC

20.6.4 Mobile phase B : 1% Acetic acid in Acetonitrile HPLC

20.6.5 Elution Solvent. Add 5 ml of ammonia 25% to a 100 ml test specimen. Add 95 ml methanol.

20.6.6 Polar colorants and pigments stock solutions, corresponding to 1 g or 0.1 g of colorant or pigment per litre of adequate solvent as it is situed to the following table.

STOCK SOLUTIONS					
No	**NAME**	**Weight**	**Solvent**	**Volume**	**Concentration**
		(mg)		**(ml)**	**(mg/ml)**
1	Alizarin	20	Methanol (hot)	20	1
2	Alizarin Red S	20	Water	20	1
3	Carminic Acid	20	Water	20	1
4	Chrysoidine G	20	Water	20	1
5	Crocin	20	Water	20	1
6	Curcumin	20	Ethanol	20	1
7	Riboflavin-5-monophosphate Sodium Salt Hydrate	10	Acetic acid 0.02N	100	0,1

Each stock solution was prepared separately by weighting the appropriate amount of the standard material, transferring into a volumetric flask and diluting to volume with the appropriate solvent as shown in the following table.

In a 10 ml beaker (20.5.8) dissolve respectively 20 mg of Alizarin in hot methanol (20.4.3). Transfer to a 20 ml volumetric flask (20.5.9). Cool, make up to the mark and stir. The solution contains 1 g of colorant per litre of methanol.

In a series of four 10 ml beakers (20.5.8) dissolve respectively 20 mg Alizarin Red S, Carminic Acid, Chrysoidine G and Crocin in water (20.4.1). Transfer to a series of four 20 ml volumetric flasks (20.5.9). Make up to the mark and stir. Each solution contains 1 g of colorant per litre of water.

In a series of two 10 ml beakers (20.5.8) dissolve 10 mg of Riboflavin-5-monophosphate Sodium Salt Hydrate in acetic acid 0.02 N (20.6.1). Transfer to a series of two 100 ml volumetric flasks (20.5.9). Make up to the mark and stir. Each solution contains 0.1 g of colorant per litre of acetic acid 0.02 N.

20.6.7 Polar colorants and pigments working solutions, corresponding to 10 mg of colorant or pigment per litre of a mixture of water/ methanol 50:50 (v/v) solution (20.6.2).

WORKING SOLUTIONS						
No	**NAME**	**Concentration**	**Volume**	**Solvent**	**Volume**	**Concentration**
		C1 (mg/ml)	**V1 (ml)**		**V2 (ml)**	**C2 (µg/ml)**
1	Alizarin	1	0,1	MeOH/Water	10	10

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WORKING SOLUTIONS						
No	NAME	Concentration	Volume	Solvent	Volume	Concentration
		C1 (mg/ml)	V1 (ml)		V2 (ml)	C2 (µg/ml)
2	Alizarin Red S	1	0,1	MeOH/Water	10	10
3	Carminic Acid	1	0.1	MeOH/Water	10	10
4	Chrysoidine G	1	0,1	MeOH/Water	10	10
5	Crocin	1	0,1	MeOH/Water	10	10
6	Curcumin	1	0,1	MeOH/Water	10	10
7	Riboflavin-5-monophosphate Sodium Salt Hydrate	0,1	1,0	MeOH/Water	10	10

In a series of six 10 ml volumetric flasks (20.5.9) add 0,1 ml of stock solution Alizarin, Alizarin Red S, Carminic Acid, Chrysoidine G, Crocin and Curcumin using a pipette (20.5.5). Make up to the mark and stir.

In a series of three 10 ml volumetric flasks (20.5.9) add 1 ml of stock solution of Bixin, Riboflavin 10% and Riboflavin-5-monophosphate Sodium Salt Hydrate using a pipette (20.5.6). Make up to the mark and stir.

NOTE These solutions are used to record individually the retention times according to the procedure written in 20.7.4.

20.6.8 Polar colorants and pigments mixed working solution, corresponding to a mixture of colorants and pigments with 20 mg of colorant or pigment per litre of a mixture of water/ methanol 50:50 (% v/v) solution (20.6.2).

MIXED WORKING SOLUTION						
No	NAME	Concentration	Volume	Solvent	Volume	Concentration
		C1 (mg/ml)	V1 (ml)		V2 (ml)	C2 (µg/ml)
1	Alizarin	1	0,5	MeOH/Water	25	20
2	Alizarin Red S	1	0,5	MeOH/Water	25	20
3	Carminic Acid	1	0,5	MeOH/Water	25	20
4	Chrysoidine G	1	0,5	MeOH/Water	25	20
5	Crocin	1	0,5	MeOH/Water	25	20
6	Curcumin	1	0,5	MeOH/Water	25	20
7	Riboflavin-5-monophosphate Sodium Salt Hydrate	0.1	5	MeOH/Water	25	20

In a 20 ml volumetric flask (20.5.9) add 0,5 ml of stock solution of Alizarin, Alizarin Red S, Carminic Acid, Chrysoidine G, Crocin and Curcumin and 5 ml of stock solution of Riboflavin-5-monophosphate Sodium Salt Hydrate using a pipette (20.5.5, 20.5.6). Make up to the mark and stir.

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20.6.9 Non-polar colorants and pigments calibration solutions, corresponding to a mixture of colorants and pigments with concentrations ranging from 0,5 µg to 12 µg of colorant or pigment per millilitre of a mixture of water/ methanol 50:50 (% v/v) solution (20.6.2).

CALIBRATION SOLUTIONS						
Initial Solution	**Concentration C1 (µg/ml)**	**Volume V1 (ml)**	**Solvent**	**Volume V2 (ml)**	**Final Concentration C2 (µg/ml)**	**Label**
MIXED	20	6	MeOH/Water	10	12	CAL 1
MIXED	20	5	MeOH/Water	10	10	CAL 2
MIXED	20	2,5	MeOH/Water	10	5	CAL 3
MIXED	20	1,5	MeOH/Water	10	3	CAL 4
MIXED	20	0,5	MeOH/Water	10	1	CAL 5
MIXED	20	0,25	MeOH/Water	10	0,5	CAL 5

20.6.10 Non-polar colorants and pigments recovery solutions, corresponding to a mixture of colorants and pigments with concentrations ranking from 1 µg to 7,5 µg of colorant or pigment per millilitre of a mixture of water/ methanol 50:50 (% v/v) solution (20.6.2).

20.7 Procedure

20.7.1 Test portion

Take a test portion of about 500 mg from the powder obtained for saffron in filaments and cut filaments and for saffron in powder form.

20.7.2 Extraction of polar colorants

Add the test portion into a centrifuge tube (20.5.15). Add 10 ml of water at about 60 ºC and stir. Wait for between 10 min and 12 min, stir thoroughly, then centrifuge and acidify with 250 µl formic acid.

NOTE It is essential that the supernatant should be free of any particles of saffron so that the solid-phase extraction used in the following step does not become clogged. Therefore, the centrifuging step should repeated if necessary.

20.7.3 Purification of sample

Rince the column with 1 ml of water. Using a pipette, add the totality of the supernatant to the column. Leave the solution to drip through. Successively wash with water, methanol, and finally acetone, in volumes such that the solvent emerging at the end of the wash is colorless. In the case of a persistent color extract after the last wash, the sequence of washes may be carried out again. After the water wash, verify that the pH is neutral.

NOTE The washing stage is considerably quicker if a vacuum system is used since it facilitates the use of larger volumes (about 30 ml of each)

Elute bound colorants with about 5 ml of the elution solvent and recover the colored elute in a heart-shaped flask. Evaporate to dryness on a rotary evaporator at ambient temperature. Take up the residue with 500µl of methanol using a micropipette (20.5.6).

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20.7.4 HPLC analysis

20.7.4.1 Setting the apparatus

Set the chromatograph (20.5.24) and adjust as follows:

Flow rate of the mobile phase (20.6.3, 20.6.4): 1 ml/min

Temperature of the column (20.5.25): ambient

20.7.4.2 Analysis

Gradient technique

Time	Solvent A	Solvent B	Flow Rate
(min)	(%)	(%)	(ml/min)
0	80	20	1
4,5	80	20	1
20	40	60	1
30	5	95	1
30,1	80	20	1
35	80	20	1

Once the mobile phase A (20.6.3) has been adjusted to the properties of the column and equilibrium has been reached, inject 20 µL of a system suitability test standard solution followed by a blank solvent to detect any carry over effects and then an identical volume of the reconstituted extract obtained in 20.7.3

The “system suitability test” is a standard solution at a medium concentration of the calibration curve used to determine the instrument performance. As a “system suitability test” a calibrator or a recovery standard could be used (p.e. CAL 4 or REC 2).

Prepare a calibration curve whenever chromatographic conditions change. Inject 20 µL of each calibration solution into the HPLC apparatus and plot peak area values of each colorant against their masses in µg/mL.

If the content of colorants in the samples falls outside the calibration range, appropriate dilution shall be performed. In this case calculation shall be reconsidered accordingly.

20.8 Interpretation and expression of the results

20.8.1 Identification

Identify any artificial colorants in the extract by comparing the retention time and the peak spectra of the relevant peak in the sample with the peak of the standard substance in the chromatogram.

Structural confirmation is carried out by scan mode from 200 nm to 700 nm.

20.8.2 Determination

To carry out the determination by the external standard method, integrate the peak area and use the calibration curve equation.

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20.8.3 Calculation

Read of from the calibration curve, the amount in µg of colorant per mL of test solution injected into the HPLC column.

Calculate the mass concentration of colorant in milligrams per kilogram using the equation:

$$C = (A * V * 1000)/W$$

Where A= the amount in µg of colorant per mL of test solution

V= is the reconstitution volume in mL (0.5 mL)

W= is the weighted amount of sample in mg (500 mg)

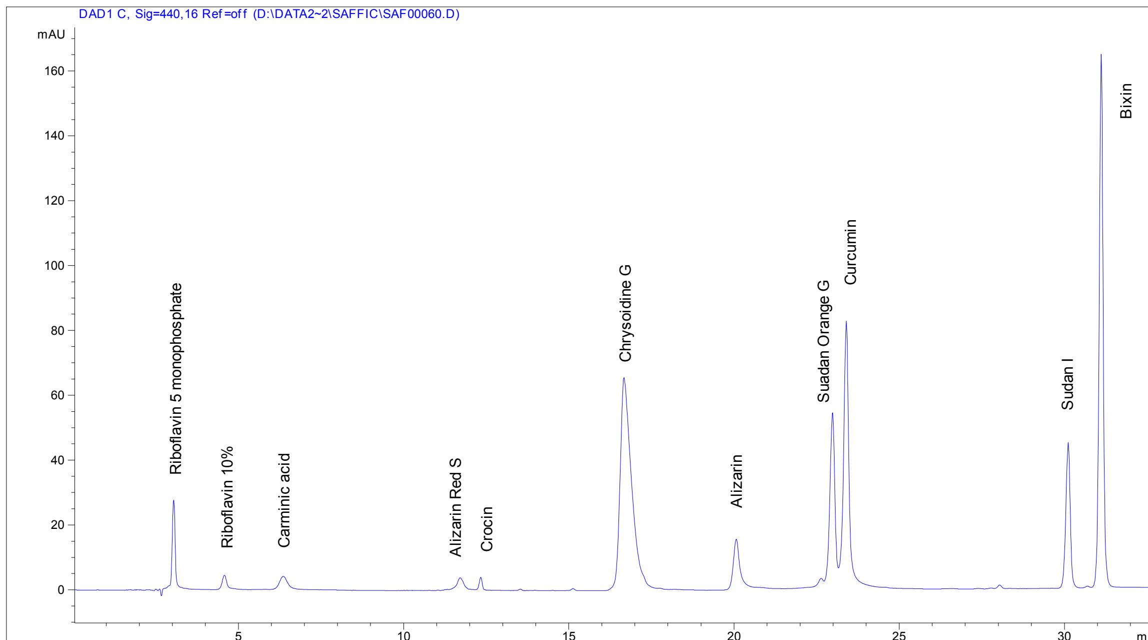

Figure 4 — Indicative chromatogram of a standard solution of polar colorants at 440nm

21 Determination of presence of *Salmonella* in saffron by PCR

Introduction

This method allows the determination of presence or absence of *Salmonella* sp. in 5 g of saffron. The 5 g sample is obtained after an effective homogenisation process in a ball mill from a 25 g sample, used for all the analytical determinations.

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NOTE: If PCR detection is positive, a second sample of 5 g shall be analysed by microbiological culture methods to confirm or discard the presence of viable *Salmonella*. Alternatively, the culture method can be used directly. PCR detection allows saving time in the result is negative. (See Clause 23)

21.1 Scope

This method determines the presence or absence of *Salmonella* in saffron samples by polymerase chain reaction (PCR).

21.2 Normative references

ISO 6579: 2002, Microbiology of food and animal feeding stuffs. Horizontal method for the detection of Salmonella spp

21.3 Principle

A sample of saffron is suspended in a pre-enrichment broth (1:10 w/v) and aliquots are used for the remaing microbiological analysis (*Escherichia coli, Bacillus cereus Clostridium perfringens* and *Enterobacteriaeae* plate counts). After the pre-enrichment, this culture is used for PCR detection of *Salmonella*.

21.4 Reagents

Unless otherwise specified use reagents of analytical grade and specially the follow:

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21.4.1 Buffered peptone water: Non-selective pre-enrichment medium according to ISO 6579:2002.

21.4.2 TE buffer: 10 mM Tris-HCl (pH 8,0), 0,1 mM EDTA. pH is adjusted with HCl.

21.4.3 TAE buffer: 4 mM Tris-Acetate (pH 8,0), 0,1 mM EDTA. pH is adjusted with acetic acid.

21.4.4 Gel-loading buffer: 0,1 % Bromophenol blue, sucrose 50%, SDS 0,1% (w/v) in 50 mM EDTA.

21.4.5 PCR primers: Oligonucleotides purified by HPLC or PSF with the following sequence: invAF (5'-CGGTGGTTTTAAGCGTACTCTT-3') and invAR (5'-CGAATATGCTCCACAAGGTTA-3'). They are designed to amplify a 796 base pair fragment of the *invA* gene, specific of pathogenic *Salmonella* (Fratamico, P.M., 2003. *Molecular and Cellular Probes*, 17:215-221).

21.5 Apparatus

21.5.1 Incubators for bacterial culture, set to 27 ºC and 43 ºC.

21.5.2 Automatic pipettes, covering the 0,5 *µl* -100 *µl* range, with sterile tips; for PCR reactions tips must include a filter to avoid cross-contamination.

21.5.3 Ball mill with stainless steel balls and dispensable or sterilizable cups (E.g.: IKA Ultra-Turrax® ball mill)

21.5.4 Thermocycler for PCR reactions.

21.5.5 PCR cabinet with UV light to avoid cross-contamination with DNA.

21.5.6 Micro-centrifuge for eppendorf tubes. Maximun force: 14,000g - 15,000g.

21.5.7 Disposable filter devices with 10 µm pore filter in eppendorf centrifuge tubes (E.g.: Whatman VectaSpin Micro™).

21.5.8 Chromatographic mini-columns for DNA purification (E.g.: GENECLEAN® Turbo for PCR, Qbiogene).

21.5.9 Horizontal gel electrophoresis system.

21.5.10 Constant voltage power supply.

21.5.11 UV-transilluminator (254 nm or 300 nm).

21.6 Procedure

21.6.1 Preparation of the samples

Approximately 5 g of saffron (weighted with 0,01 g of precision) are taken from a sample previously homogenised in a small ball mill for 50 s at max. rpm. Homogenised samples are diluted 1:10 (w/v) in pre-enrichment broth. After 10 min at room temperature, suspension is mixed in vortex and three aliquots of 1 ml were taken from this suspension and used for *E. coli*, *Bacillus cereus* and *Clostridium perfringens* counts. The remaining suspension is incubated 16 h ± 2 h at 37 ºC ± 1 ºC for pre-enrichment.

21.6.2 DNA extraction and purification

After pre-enrichment, 500 µL of the culture are placed in an eppendorf tube with a 10 µm pore diameter filter and centrifuged 3 min, 14,000g at room temperature, in order to eliminate saffron debris. This step is essential to avoid interferences with Taq-polymerase.

Filter and supernatant are discarded and 100 *µ*l of TE buffer were added; after mixing in vortex the samples are incubated 10 min at 100 ºC in a heat block for bacterial lysis.

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After 2 min at room temperature, samples are centrifuged 3 min, 14,000g at room temperature.

50 *µ*l of the supernatant are transferred to a chromatographic mini-column for DNA purification and processed following the manufacturer instruction. This step is also essential to ensure reproducibility along different saffron samples.

21.6.3 PCR amplification

Every PCR reaction is prepared in a thermocycler tube or multiwell plate by mixing:

13,5 *µ*l ultrapure, DNAse free water (molecular biology grade)

2,5 *µ*l Taq-polymerase buffer (according to the Taq-polymerase supplier recommendations)

0,8 *µ*l dNTPs containing 200 *µ*M of each deoxynucleotide triphosphate

1 *µ*l invAF primer

1 *µ*l invAR primer

1,25 *µ*l Taq-polymerase, containing 0,1-0,5 units (according to the supplier recommendations)

5 *µ*l of the DNA extracted and purified from the sample

A negative control, with 5 *µ*l of water instead of DNA solution, must be included. Preparation of PCR reactions should be done in a cabinet with UV light to inactivate DNA between different analyses. A positive control, 5 *µ*l of DNA solution extracted with the same protocol from a *Salmonella* colony, must be used for validation of the method, but not included in the reaction to avoid a possible cross-contamination, resulting in false-positives.

Reactions are carried out in a PCR thermocycler with the following programme:

Number of cycles:	Time (min)	Temperature (ºC)	Purpose
1	5	94	Denaturation
35	1	94	Denaturation
	1	55.6	Annealing
	1	72	Extension
1	3	72	Final extension

21.6.4 Product detection

Prepare an agarose gel with 1,5 % agarose (molecular biology grade) in TAE buffer, melt, and pour in an electrophoresis device.

Five µl of each amplification product is added to a volume of loading buffer, according to the size of the agarose gel, and charged in the gel well.

One well at least should be charged with an appropriate DNA size marker (DNA ladder).

Electrophoresis is carried in TAE buffer at 100 V until the dye of the loading buffer approaches to the end of the gel.

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Gel is examined over a UV trans-illuminator, and the size of the amplification products deduced from the comparison with the DNA ladder. The size of the DNA bands should be of about 800 base pair. Presence of a DNA band on this characteristic indicates a positive detection. Absence of DNA bands indicates a negative result. Otherwise an unspecific amplification can be suspected.

21.7 Expression of the results

The result shall be expressed as “presence” or “absence” of *Salmonella* in 5 g of the sample.

22 Determination of presence of *Salmonella* in saffron by culture

This method allows the determination of presence or absence of viable *Salmonella* in 5 g of saffron, and must be used a confirmation of the presence of viable bacteria if PCR detection (21) is positive, because PCR can detect dead bacteria. This method can also be used directly as an alternative to the PCR detection.

22.1 Scope

This method determines the presence or absence of viable *Salmonella* in saffron samples by selective enrichment, isolation and identification.

22.2 Normative references

ISO 6579: 2002, Microbiology of food and animal feeding stuffs. Horizontal method for the detection of Salmonella spp

22.3 Principle

A sample of saffron is suspended in a pre-enrichment broth (1:10 w/v) and aliquots are used for microbiological analysis (*Escherichia coli, Bacillus cereus, Clostridium perfringens* and *Enterobacteriaeae* plate counts). After pre-enrichment, this culture is used for detection of *Salmonella*.

22.4 Reagents

22.4.1 Non-selective pre-enrichment medium: Buffered peptone water according to ISO 6579:2002.

22.4.2 First selective enrichment medium for *Salmonella:* Rappaport-Vassiliadis Soy (RSV) broth according to ISO 6579:2002.

22.4.3 First selective enrichment medium for *Salmonella:* Muller-Kauffman Tetrationate Novobiocine (MKTTn) broth according to ISO 6579:2002.

22.4.4 Xilose-Lysine-Desoxicolate (XLD) and any other selective media, complementary to XLD, suitable for the isolement of lactose-positive *Salmonella* as well as *Salmonella* Typhy and *Salmonella* Paratyphi according to ISO 6579:2002.

22.4.5 Other media and sera for biochemical and serological identification of *Salmonella* as described in ISO 6579:2002.

22.5 Apparatus

22.5.1 Incubators for bacterial culture set to 27 ºC ± 1 ºC and 43 ºC ± 1 ºC.

22.6 Procedure

22.6.1 Preparation of the samples

Preparation of the samples and pre-enrichment culture is done as specified for the PCR assay (21.6.1)

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22.6.2 Culture and identification

After pre-enrichment, 1 mLlaliquots of the culture are inoculated in 9 ml of RSV broth and in 9 ml of MKTTn broth. These media are incubated 24 h ± 3 h; the RSV broth at 41,5 ºC ± 1 ºC and the MKTTn broth at 37 ºC ± 1 ºC (Never surpasses 42.5 ºC).

After incubation, samples taken from RSV and MKTTn broths are inoculated with bacteriological loop onto plates of XLD medium and of the second selective medium for *Salmonella* (as indicated in 21.5.4). These plates are incubated 24 h ± 3 h; those of XLD medium at 37 ºC ± 1 ºC and those of the other medium at the temperature recommended by the manufacturer.

Suspicious colonies grown in any of the selective plates are identified in accordance to ISO 6579:2002.

22.7 Expression of the results

The result shall be expressed as “presence” or “absence” of viable *Salmonella* in 5 g of the sample.

Table X presents as an example model of report of results template.

	PCR		**CULTURE**	
SAMPLE	ABSENCE	PRESENCE	ABSENCE	PRESENCE
PCR1(1)				
PCR2(1)				
PCR3(1)				
PCR4(1)				

22.8 Comparative results of an interlaboratory test

The following table summarises the results of an interlaboratory test carried out in 2009

Method	**PCR**	**Culture (ISO 6579)**
PCR1 (5×10^6)		
Repeatability [%]	77.8%	100.0%
Standard error of repeatability [SDr]	0.67	0.0%
Standard error of laboratories [SD(L)=SQRT(SDb×2–(SDr×2)/n)]	0.55	0.0%
Standard error of reproducibility [SD(R)=SQRT(SD(L)×2+SDr×2)]	0.87	0.0%
PCR2 (5×10^3)		
Repeatability [%]	77.8%	88.9%
Standard error of Repeatability [SDr]	0.67	0.47
Standard error of laboratories [SD(L)=SQRT(SDb×2–(SDr×2)/n)]	0.73	0.62
Standard error of Reproducibility [SD(R)=SQRT(SD(L)×2+SDr×2)]	0.99	0.78
PCR3 (5×10^2)		
Repeatability [%]	87.5%	88.9%
Standard error of repeatability [SDr]	0.50	0.47
Standard error of laboratories [SD(L)=SQRT(SDb×2–(SDr×2)/n)]	0.65	0.62

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Standard error of reproducibility [SD(R)=SQRT(SD(L)×2+SDr×2)]	0.82	0.78

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