OIV-MA-F1-12 Specific methods for the analysis of grape sugar

Type II method

Determination of meso-inositol, scyllo-inositol and sucrose

1. Principle

Rectified Concentrated Must (RCM) is mainly composed of sugars, polyalcohols and water contained in grapes. All the other organic and mineral components are removed during the rectification process.

Meso-inositol, scyllo-inositol and sucrose are determined through gas chromatography (GC) following silanisation.

The sucrose possibly found in small amounts in the RCM is stable for some months since hydrolysis is greatly slowed down due to the absence of organic and mineral acids, which are removed during the rectification process, to a very low water content, and to a high level of glucose and fructose.

2. Reagents

2.1. Xylitol (CAS no. 87-99-0)

Internal standard: (aqueous solution of at a precisely known concentration of about 10 g/L prepared at the time).

2.2. Meso-inositol ($C_6H_{12}O_6$) (CAS no. 87-89-8)

2.3. Scyllo-inositol ($C_6H_{12}O_6$) (CAS no. 488-59-5)

2.4. Glucose ($C_6H_{12}O_6$), fructose ($C_6H_{12}O_6$), sucrose ($C_{12}H_{22}O_{11}$)

2.5. Bis(trimethylsilyl)trifluoroacetamide - (BSTFA) – ($C_8H_{18}F_3NOSi_2$) (CAS no. 25561-30-2)

Warning: This is a dangerous and inflammable product. Inflammable liquids and vapours may provoke serious skin burns and serious eye lesions. Wear gloves/protective clothing. Protect your eyes/face.

2.6. Trimethylchlorosilane (C_3H_9ClSi) – TMCS – (CAS no. 75-77-4)

Warning: This is a dangerous and inflammable product. Harmful to the skin. Provokes serious skin burns and serious ocular lesions. Toxic when inhaled. May irritate the respiratory tract. Keep away from sources of heat/sparks/free flames /heated surfaces. Do not smoke. Avoid inhaling the vapours. Wear gloves/protective clothing. Protect your eyes/face.

2.7. Pyridine p.a. (C_5H_5N) (CAS no. 110-86-1)

Warning: This is a dangerous and inflammable product. Noxious when inhaled, in contact with the skin and when swallowed.

2.8. Absolute ethanol (C_2H_6O) (CAS no. 64-17-5)

Warning: This is an inflammable product. Liquid and vapours easily inflammable. Keep away from sources of heat/sparks/free flames /heated surfaces. Do not smoke.

2.9. Type 1 Water in conformity with ISO 3696 standard or deionised water with resistivity $\geq$ 18M□ cm

2.10. The silanising reagent is also available in ready to use kits (e.g. HMDS+TMSCl+Pyridine 3:1:9 Supelco cod. 33038)

2.11. Technical gases: nitrogen, hydrogen, helium and air for gas chromatography and for the dehydration phases

3. Apparatus

3.1. Gas chromatograph

3.2. Capillary column able to guarantee a minimum efficiency of N=250,000 plates/column for sucrose at 1 g/L

For example, OV-1 (25 m x 0.30 mm x 0.15 µm) or DB-5 (60 m x 0.25 mm x 0.10 µm).

Operating conditions (only as an example):

carrier gas: pure hydrogen or helium, for gas chromatography,

Hydrogen: Warning: This is an extremely inflammable gas. Store the container in a well-ventilated place and far from flames and sparks. – Do not smoke. Avoid the piling up of electrostatic loads.

carrier gas flow rate: about 2 mL/min,

injector temperature: 250 °C,

temperature of flame ionisation detector (FID): 300 °C,

programming of temperature: 1 minute at 160 °C, 4 °C/minute up to 260 °C, constant temperature of 260 ° C for 15 minutes,

splitter ratio: about 1:20,

auxiliary gases: pure hydrogen and air for gas chromatography

3.3. Integrator

3.4. Microsyringe: 10 µL

3.5. Micropipettes: 10, 100, 400 and 1000 µ

3.6. 2 mL flasks with Teflon stopper

3.7. Oven

3.8. Technical balance, analytical balance able to ensure an accuracy of ± 0.1 mg

3.9. Flasks of 50 and 100 mL

3.10. Dryer

4. Procedure

4.1. Preparation of the sample

In a 50 mL flask, weigh a quantity "p" of rectified concentrated must ranging between 4.9 and 5.1 g, noting the weight of the substance with the precision of ± 0.1 mg.

Then add 1 mL of xylitol standard solution (2.1) and bring to volume with water (2.9).

4.2. Dehydration of the sample

After mixing, 100 µL of solution is taken and placed in a flask (3.6) where it is dried under a gentle stream of nitrogen.

100 µL of absolute ethanol (2.8) may be added to facilitate evaporation.

Note 1: In case a precise dose of sucrose is desired, the diluted solution should be prepared just before the silanisation, in order to limit hydrolysis of the sucrose in the diluted acqueous solution.

Note 2: The repeated measurements of the sucrose content have to be performed on diluted solutions prepared every time before every silanisation.

4.3. Derivatization

The residue is carefully dissolved in 100 µL of pyridine (2.7) and 100 µL of bis (trimethylsilyl)trifluoroacetamide (2.5) and 10 µL of trimethylchlorosilane (2.6) are added. The flask is closed with the Teflon stopper and placed in the oven at 70 °C for 70 minutes.

Take the sealed flasks out of the oven and leave to cool in the dryer in the dark at room temperature for an hour before injecting into the gas chromatograph.

The substance conserved in the sealed flask and kept in the dryer in the dark at room temperature is stable for three days.

Note 3: If working with a silanisation kit, 400 µL of reagent should be used per 100 µL of the dehydrated and diluted sample, as described in 4.1.

Note 4: The silanisation is considered successful if the solution, after a sole phase, has a clear appearance or leaves a slight, white residue. There should not be a dark residue since this would indicate an excess of non-derivatised sugar or an aged silanising substance.

Note 5: Should there be a white suspension, wait until the solid matter deposits on the bottom without centrifuging.

4.4. Gas chromatographic analysis

1 µL is taken with a syringe (3.4) and injected into the chromatograph with the aforementioned splitter.

The chromatogramme should not show a confluence of peaks (sign of a badly performed silanisation), but characteristic peaks as shown in the attached figures. (Fig.9-Fig.12).

4.5. Peak integration criteria

Integrate the gas chromatographic peaks with respect to the horizontal baseline. In case of peaks that are not perfectly resolved, trace the horizontal baseline starting from the deeper valleys that delimit the peak in question. Trace a vertical line downwards starting from the valleys of the peaks up to the baseline to identify the peak area.

Do not use the valley-valley integration method.

An example of the application of these criteria is given in Figure 10 for the internal standard, in Figure 11 for the inositols and in Figure 12 for the sucrose.

Note 6: In case a precise dose of sucrose is desired, it is important to respect the integration criteria explained in paragraph 4.4. and shown in Figure 4.

5. Calculations

5.1. Calculation of response factors

Note: instead of the calculation of response factors a calibration curve can be used

5.1.1. A solution is prepared containing:

- 60 g/L of glucose,
- 60 g/L of fructose,
- 1 g/L of meso-inositol,
- 1 g/L of sucrose,

(weigh 60 g of glucose and 60 g of fructose with precision $\pm$ 1 g; then 1 g of meso-inositol and 1 g of sucrose with precision $\pm$ 0.1 mg) and lastly bring to volume of 1 litre with water.

5.1.2. Silanisation of the reference solution

Carry out the operations described in paragraph 4.1 starting with 5 mL of said solution in place of the 5 g of RMC

Take 5 mL of the solution and proceed as in paragraph 4.

5.1.3. Gaschromatographic response factors

The results for meso-inositol and sucrose with respect to xylitol are calculated from

the chromatogram.

In the case of scyllo-inositol, which has a retention time lying between the last peak of the anomeric form of glucose and the peak for meso-inositol (see Figure 11), use the same response factors achieved for meso-inositol.

Where: $A_{meso-inositol}$ = area of the meso-inositol peak; $A_{sucrose}$= area of the sucrose peak; A_{is}= area of the internal standard peak; $C_{meso-inositol}$ = concentration of meso-inositol in mg/L; $C_{sucrose}$ = concentration of sucrose in mg/L; C_{is} = concentration of internal standard in mg/L, the following formula is true:

$$RF_{meso-inositol} = \frac{A_{meso-inositol}}{A_{is}} \times \frac{C_{is}}{C_{meso-inositol}}$$

$$RF_{sucrose} = \frac{A_{sucrose}}{A_{is}} \times \frac{C_{is}}{C_{sucrose}}$$

The solution for the calculation of the response factors has to be prepared and analysed on the same day (see note 1 of paragraph 4.1).

5.2. Formulation of the results

Meso-inositol, scyllo-inositol and sucrose are expressed in mg/kg of Total Sugars (mg/kg TS) without decimals.

5.2.1. Concentrations expressed in mg/L for the 10% (w/v) solution of RCM (4.1):

$$C_{meso-inositom}\left(\frac{mg}{l}\right) = \frac{C_{is}}{RF_{meso-inositol}} \times \frac{A_{meso-inositol}}{A_{is}}$$

$$C_{sucrose}\left(\frac{mg}{l}\right) = \frac{C_{is}}{RF_{sucrose}} \times \frac{A_{sucrose}}{A_{is}}$$

5.2.2. Concentrations expressed in mg/kg of Total Sugars (mg/kg TS) for the meso-inositol and the scyllo-inositol, and for the sucrose in the RCM.

Indicating with “i” any of the three compounds:

$$CONC_i\left(\frac{mg}{kg\ TS}\right) = C_i \times \frac{5000}{w \times G}$$

Where “w” is the weighed amount in g of the RCM and “G” is the percentage of sugar of the RCM expressed in °Brix [or % (m/m) of sucrose]. The sugar percentage of the RCM sample should be measured using the OIV-MA-AS2-02 method.

6. Characteristics of the method

6.1. Critical points

The method regards the analysis of sugars and polyalcohols found in extremely small quantities in a matrix of glucose and fructose in very high concentrations. It is thus necessary to verify the method's capacity to furnish linear responses in the range of concentrations proposed and that are sufficiently accurate compared with known values.

Furthermore, the method provides for gas chromatographic analysis of the silanised compounds obtained through derivatisation of the sugars. These compounds are sensitive to humidity and tend to deteriorate with time. It is therefore important to verify the adequacy of the instructions regarding conservation and handling of these compounds.

Lastly, sucrose is subject to hydrolysis due to the quantity of residual water in the RCM (from 30 to 45%). However, the low acidity of the matrix and the high concentration of glucose and fructose slow down the hydrolysis process and allow accurate measurements to be carried out. It is therefore important to check that the analysis timeframes are fast compared to the hydrolysis in order to allow repeatable measurements of the sucrose.

6.2. Linearity

A series of six synthetic samples were prepared (including the blank) containing a matrix of glucose and fructose obtained by weighing equal quantities of the two sugars in relation to a 60% (w/w) content of total sugars in the initial RCM.

To five of these synthetic samples, precise amounts in increasing quantities of meso-inositol and sucrose were added so as to achieve the concentrations shown in the following table:

	concentration added	
No.	meso-inositol	sucrose
	(mg/kg TS)	(mg/kg TS)
1	0 (blank)	0 (blank)
2	214.7	427.3
3	420.0	857.7

4	840.2	1675.8
5	1727.0	3338.0
6	2514.0	6719.0

The samples were then diluted (4.1), the diluted solution was dehydrated (4.2), silanised (4.3) and analysed with the GC (4.4-4.5).

The samples were then silanised and analysed using GC. The results were verified in order of linearity, showing on the graph the ratio between the peak areas of the meso-inositol peaks and that of the internal standard (A_{meso}/A_s), and the ratio between the concentration of meso-inositol and of the internal standard (in mg/L), indicated by C_{meso}/C_{is}.The GC analysis was conducted twice and the following data refer to the mean of the two values.

The same treatment was applied to the sucrose as follows

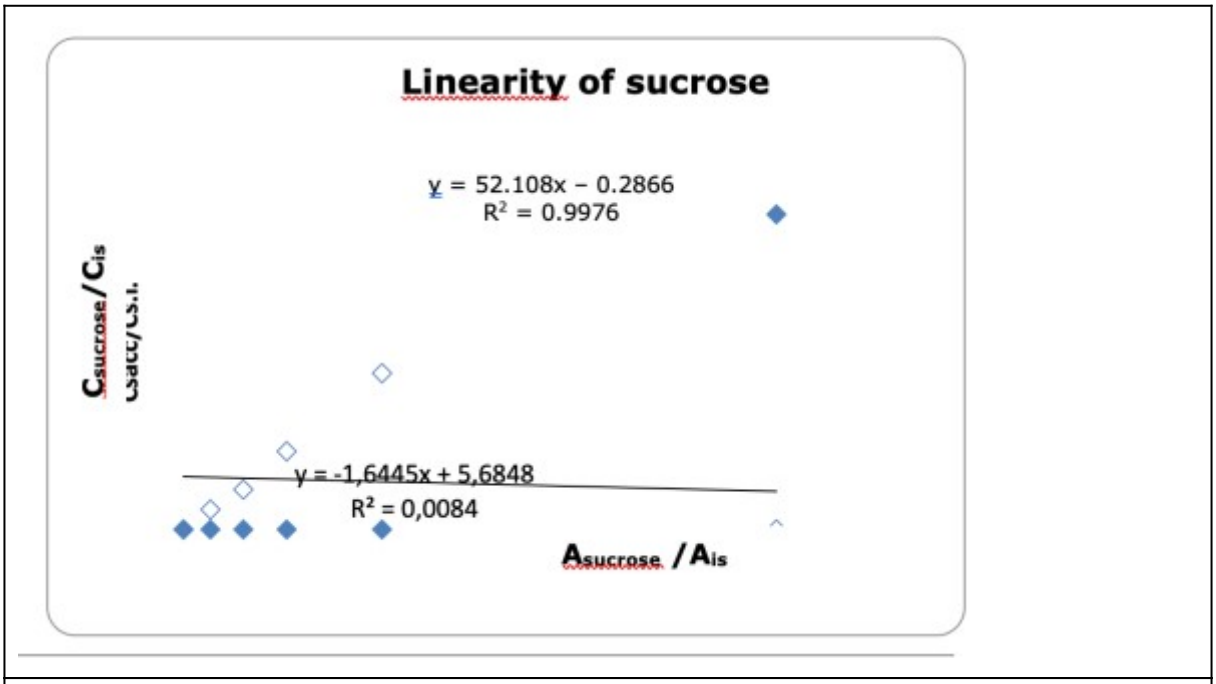

Fig. 1 Linearity of meso-inositol

The linearity of the meso-inositol is highly satisfactory (R>0.998) in the entire range of concentration studied.

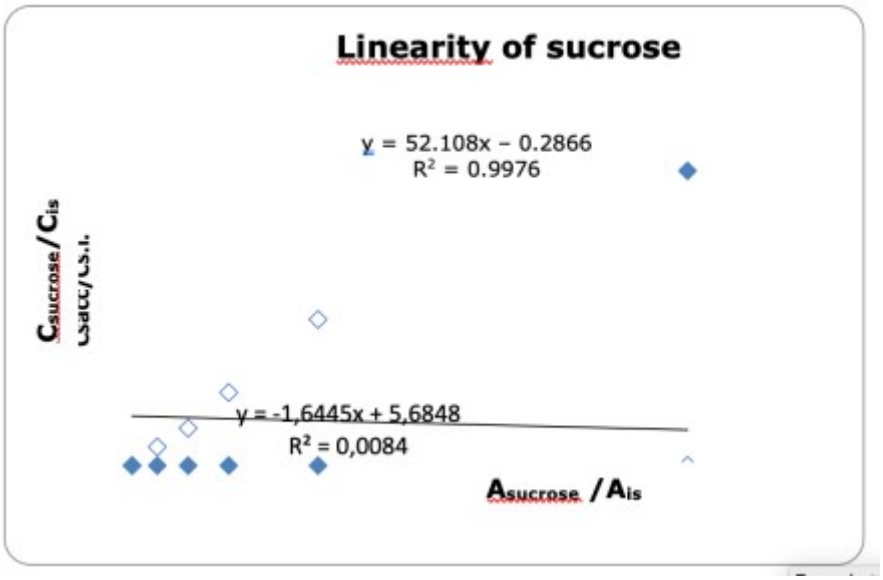

Fig. 2 Linearity of sucrose

In relation to sucrose, the linear relationship hypothesised over the entire range of concentrations studied did not lead to a satisfactory correlation (R=0.967) but, by narrowing down the linearity field to the synthetic sample no. 5, the correlation became comparable to that of meso-inositol (R>0.997).

Following the instructions in par. 5.1, the following response factors were obtained:

RF rel. Meso-inositol/Xylitol I.S.	RF rel. Sucrose/Xylitol I.S.
1.04 ± 0.03 (mean ± □; n=4)	0.36 ± 0.06 (mean ± □; n=4)

6.3. Specificity

The relationship between the added meso-inositol and that determined by GC is linear in the entire measurement range studied, and the slope of the line is very close to one and intercept is very close to zero.

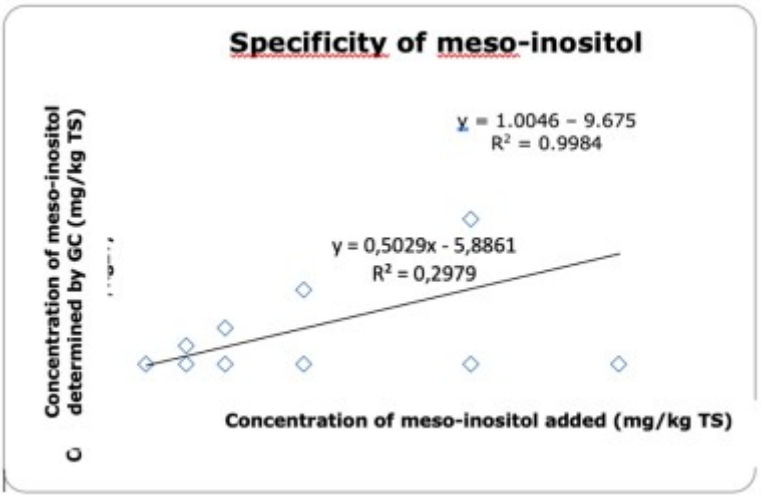

Fig. 3 Specificity of meso-inositol

Also, the recovery is satisfactory at between 95 and 105%, as seen in the following table:

$C_{meso-inositol}$ added (mg/kg TS)	$C_{meso-inositol}$ determined by GC ±□ (n=2) (mg/kg TS)	Recovery (%)
0	0	-
214.7	213 ± 2	99%
420.0	419 ± 3	100%
840.2	852 ± 20	102%
1727.0	1668 ± 214	100%
2514.0	2587 ± 36	99%

As to the specificity of the sucrose, the concentrations obtained from the calculation of the added sucrose and that determined by GC conform and are in a linear relationship with each other, with a slope of one and intercept of almost zero, on the condition, however, that the concentration range is more restricted compared to that studied.

The following graph shows that the linear relationship does not extend up to the last

concentration level of about 6700 mg/kg TS

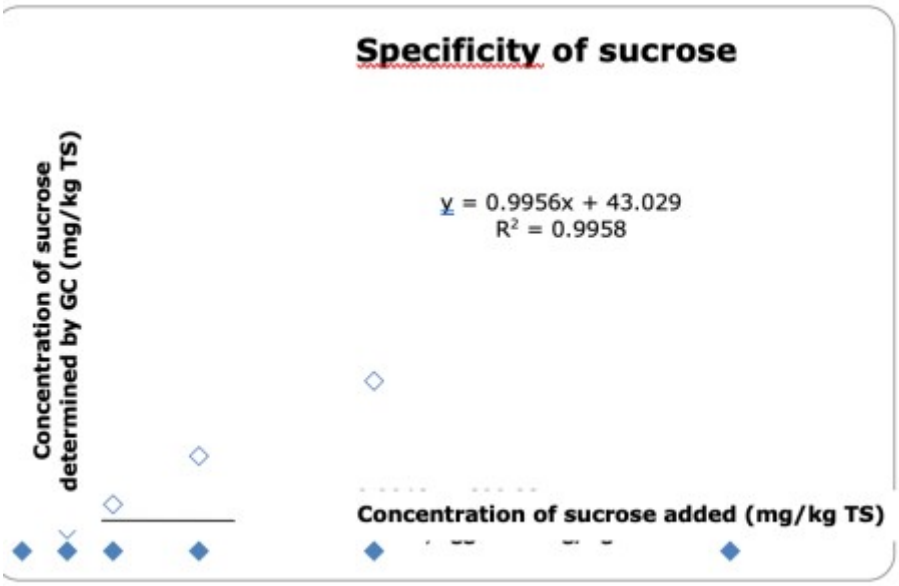

Fig. 4 Specificity of sucrose

Also, the recovery is satisfactory, at between 90 and 110%, excluding the higher concentration levels, as seen in the following table:

$C_{sucrose}$ added (mg/kg TS)	$C_{sucrose}$ determined by GC ± □ (n=2) (mg/kg TS)	Recovery (%)
0	0	-
427.3	423 ± 16	99%
857.7	913 ± 37	107%
1675.8	1852 ± 344	111%
3338.0	3297 ± 284	99%
6719.0	9220 ± 19	137%

6.4. Stability of sucrose in rectified concentrated must

A sample of the RCM with added sucrose was kept at room temperature and analysed at regular time intervals in order to see the incidence of the hydrolysis phenomenon of sucrose in RCM. The results are summarised in the following table:

	t = 0 days	t=9 days	t=53 days
Meso-inositol (mg/kg TS)	2227	2100	2052
Scyllo-inositol (mg/kg TS)	424	430	394
Sucrose (mg/kg TS)	4631	5108	4969

Both the meso-inositol and the scyllo-inositol, as well as the sucrose, do not show significant variations in concentration compared to the initial value up to 53 days after the preparation.

This fact appears to be evident in the following graph

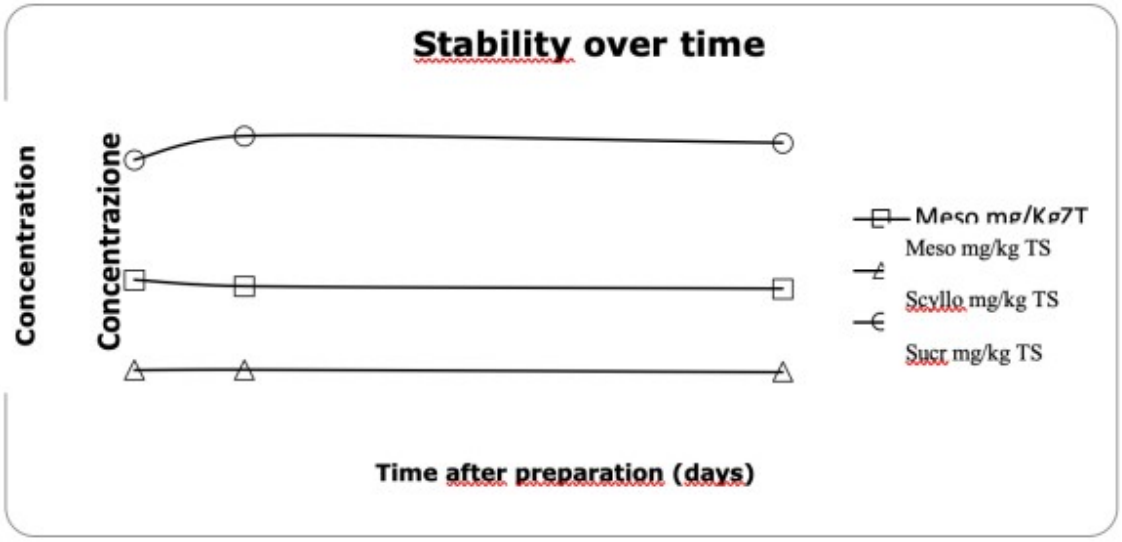

Fig. 5 Stability over time

6.5. Stability of the silanised sample

The silanised product obtained as described in point 4.2 was conserved as described in the same paragraph. At 24-hour intervals the silanised sample was analysed with the gas chromatograph following the procedure set out in point 4.3 and the succeeding points.

The results are described in the following table:

	t=0 mean ± SD (n=3)	t=24 h mean ± SD (n=3)	t=48 h mean ± SD (n=3)	t=72 h mean ± SD (n=3)	t=96 h mean ± SD (n=3)
Meso-inositol (mg/kg TS)	2424 ± 109	2347 ± 44	2358 ± 17	2453 ± 39	2478 ± 15
Scyllo-inositol (mg/kg TS)	261 ± 7	254 ± 2	256 ± 4	257 ± 3	264 ± 1
Sucrose (mg/kg TS)	6233 ± 971	6500 ± 200	6633 ± 58	6733 ± 321	6600 ± 436

There are no significant differences between the results obtained from the same silanised sample up to 4 days after silanisation, adopting the measures for the conservation of the silanised sample described in point 4.2.

This fact is evident in the following graphs:

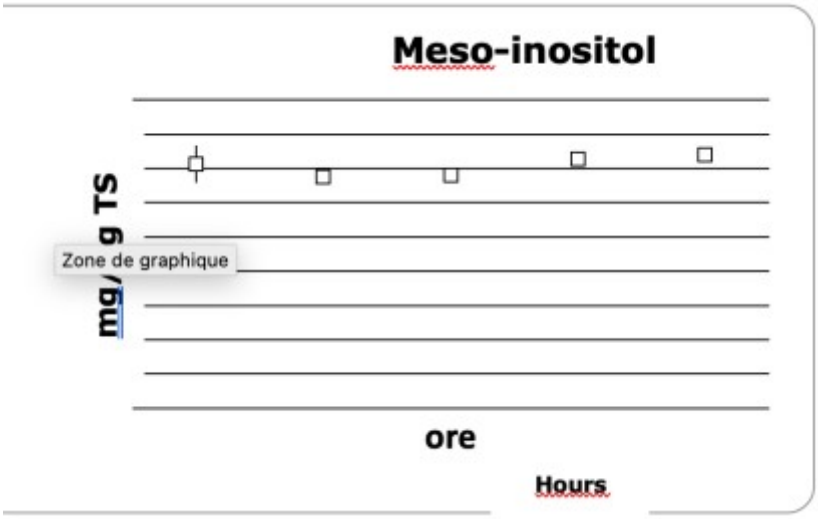

Fig. 6 Stability of Meso-inositol

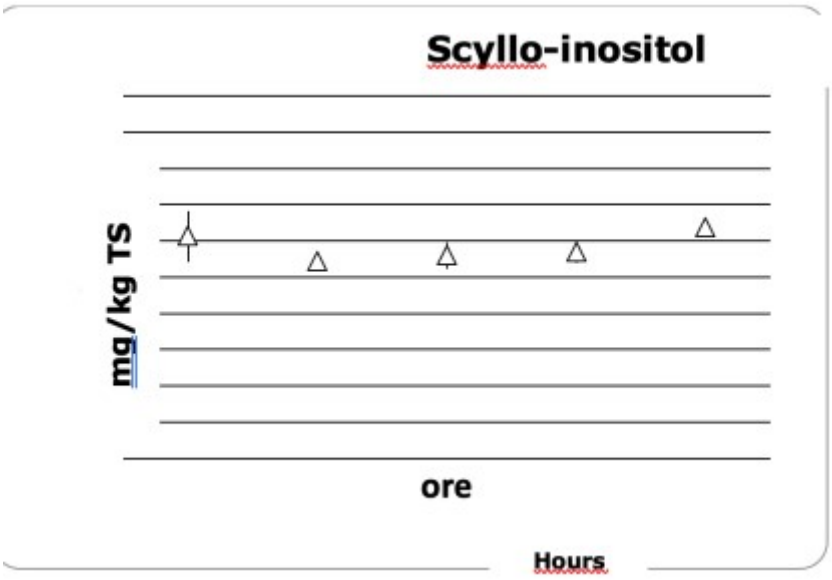

Fig. 7 Stability of Scyllo-inosito

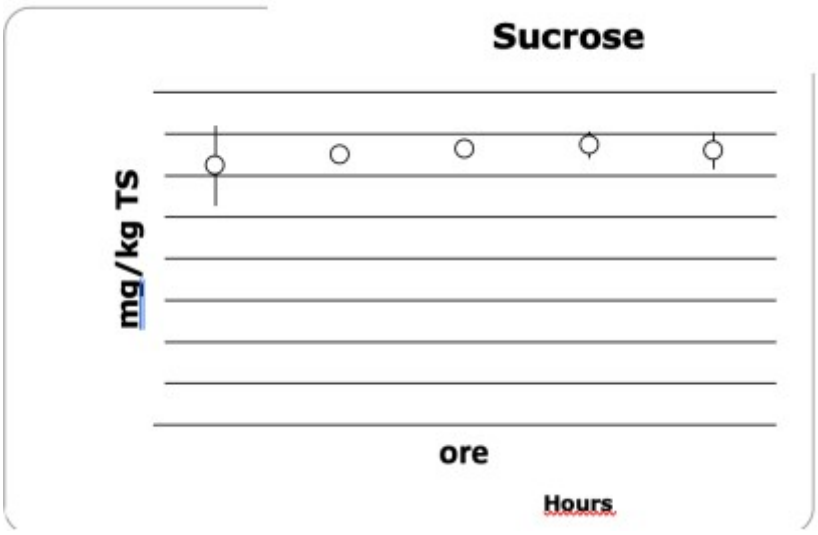

Fig. 8 Stability of Sucrose

6.6. Precision

Precision parameters obtained in the interlaboratory test conducted in April 2014 between 8 Italian laboratories. The Ring Test was performed on a sample with added sucrose at a concentration of 1 g of sucrose / 1 kg RCM

	sucrose	meso-inositol	scyllo-inositol
Number of participating laboratories	8	8	8
Number of accepted test results	23	23	23
Mean values (mg/kg TS)	1665	954	145
Repeatability			
Repeatability standard deviation (Sr)	78	52	11
Relative repeatability standard deviation (%RSD_r)	4.7	5.4	7.3
Repeatability limit (r)	219	146	29
HORRAT r = RSD_r / RSD(R) Horwitz	0.9	1.0	1.0
Reproducibility			
Reproducibility standard deviation (SR)	122	76	19
Relative reproducibility standard deviation (%RSD_R)	7.4	8.0	13
Reproducibility limit (R)	343	213	55
RSD(R) Horwitz %	5.2	5.7	7.6
HORRAT R = RSD_R / RSD(R) Horwitz	1.4	1.4	1.8
S_r/S_R	0.64	0.68	0.58

7. Bibliography

- Versini G., Dalla Serra A. and Margheri G. (1984). Polialcooli e zuccheri minori nei mosti concentrati rettificati. Possibili parametri di genuinità? Vignevini, 11(3), 41-47
- Monetti A., Versini G., Dalpiaz G. and Raniero F. (1996). Sugar adulterations control in concentrated rectified grape musts by finite mixture distribution analysis of the myo-inositol and scyllo-insitol content and the D/H methyl ratio of fermentative ethanol. Journal of Agricultural and Food Chemistry, 44-8: 2194-2210.

Figures

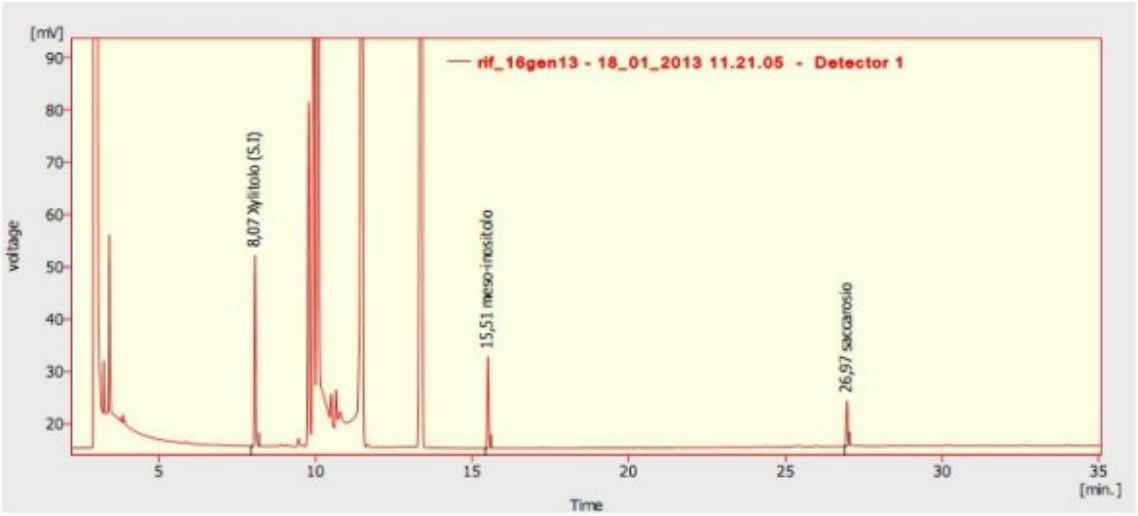

Fig. 9 Chromatogram of the reference solution for the calculation of response factors

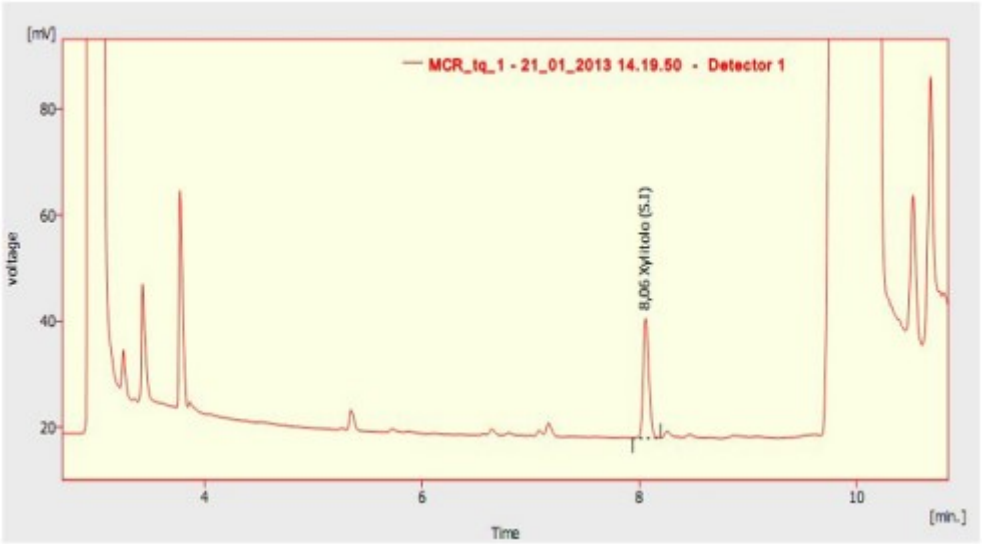

Fig. 10 Chromatogram of an RCM sample – Internal Standard ZONE

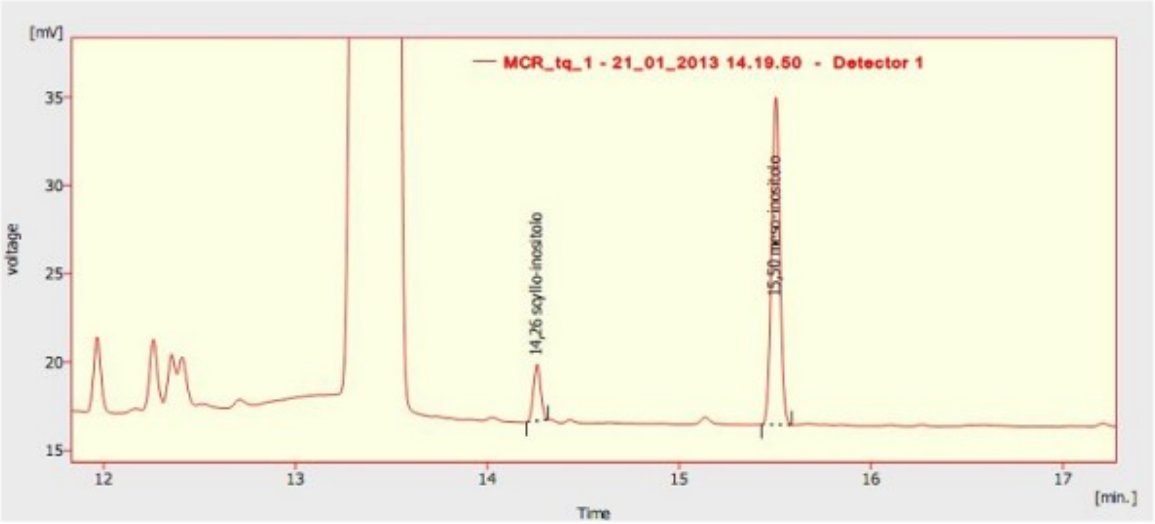

Fig. 11 Chromatogram of an RCM sample - "Inositol" ZONE

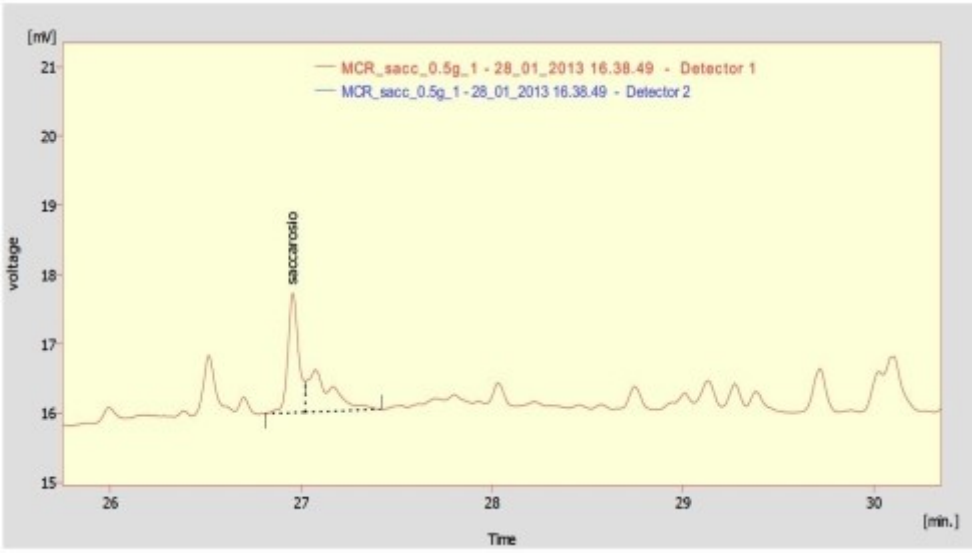

Fig. 12 Chromatogram of an RCM sample –with 0.5 g/kg sucrose added