Performance of the method EN 12396-1 - with the introduction of minor modifications - *versus* the criteria of SANCO 12571/2013 for quantitative determinations

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1. INTRODUCTION

Dithiocarbamates (DTCs), non-systemic fungicides, are an important group of pesticides still used for plant protection. In general, due to their low solubility in water and in the common organic solvents and also to their low stability in the presence of plant matrix, DTCs are not amenable to multi-residue methods. Although the European Union regulation (Commission Directive 2007/57/EC) has established some individual values of MRL to certain DTC (such us thiram, ziram and propineb), the content expressed as CS_2 released during the hydrolysis, continues to be required on the Monitoring of Pesticides Residues in Products of Plant Origin, in all member-states. Carbon disulfide released from DTCs upon decomposition can be determined following different analytical methodologies [1] being LC-MS^n very promising in the determination of "intact" DTCs (non decomposed). Nonetheless, the spectrophotometric method based on EN 12396-1:1998 standard [2] continues to be widely used in routine test laboratories for the quantification of total dithiocarbamates (DTCs) in vegetable food, expressed as disulfide carbon released during the decomposition by acidic hot-hydrolysis followed by distillation and quantification by Molecular Absorption Spectrophotometry, applied in the range of 50-250 µg CS_2 /25 mL. During the colour development, two copper(II) complexes are formed with CS_2 and diethanolamine (1:1 and 1:2 complexes, Cu:CS_2), varying their concentration ratios with the total concentration of CS_2. Consequently, linearity is observed in relatively narrow ranges of CS_2 concentration. The procedure described on this European Standard leads to unsatisfactory limits of quantification (LoQ) for several commodities, since it corresponds to 5-10 times their Maximum Residue Limits (MRL) set up in the European Union [3], taking values as low as 0.05 mg/kg. Therefore, in an attempt to reduce the LoQ, two major modifications were introduced to the method: decreasing the lower calibration level down to one order of magnitude and using spectrophotometric cells with 2 cm of pathway, instead of 1 cm. The document SANCO 12571/2013 [4] is focused on the determination of pesticide residues in food by mass spectrometry coupled to chromatography techniques but the same criteria of quantitative validation were here used to evaluate the performance of the present spectrophotometric method. The assessment of linearity, working range, intermediate precision, trueness, selectivity, sensitivity, limit of quantification and uncertainty were based on results of different commodities, spiked with thiram at several concentration levels.

2. EXPERIMENTAL

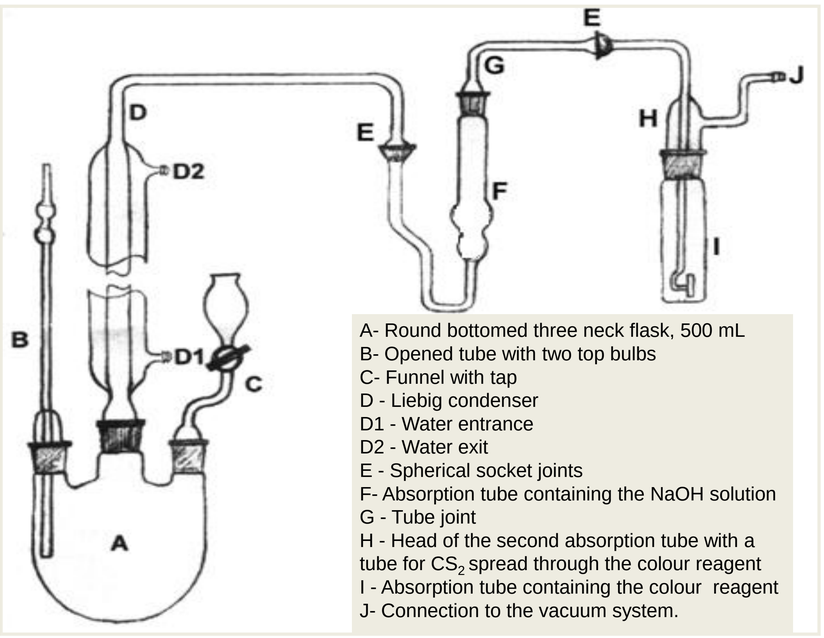
The experimental procedure was based, in general, on the European Standard EN 12396-1, with the introduction of the following changes:

- use of spectrophotometric cells with 2 cm of pathway;
- decreasing of the calibration range, namely the lowest calibration level down to 5 µg CS_2 /25 mL.

In this work, half quantities of Sn^{2+} and HCl (conc.) solutions were used in the digestion of the samples.

DIGESTION AND DESTILLATION OF SAMPLES

A glass apparatus composed by several glass components, manually assembled according to **Figure 1** was used to carry out the digestion of the sample (in flask A), the distillation and the complexation of the produced CS_2 by a solution of Cu(II)-diethanolamine contained in Tube I. A batch of four apparatus was used, connected to a vacuum system (J). The NaOH solution (100 g/L) retains the H_2S (an interference).



A- Round bottomed three neck flask, 500 mL
B- Opened tube with two top bulbs
C- Funnel with tap
D - Liebig condenser
D1 - Water entrance
D2 - Water exit
E - Spherical socket joints
F- Absorption tube containing the NaOH solution
G - Tube joint
H - Head of the second absorption tube with a tube for CS_2 spread through the colour reagent
I - Absorption tube containing the colour reagent
J- Connection to the vacuum system.

Figure 1 - Apparatus for digestion of dithiocarbamates, distillation of the CS_2 and its absorption in a copper-diethanolamine solution.

PREPARATION OF THE COLOUR REAGENT

The colour reagent was used for the preparation of the calibration standards, Quality Control Standard and as the "absorption" solution after digestion/distillation of the samples (Tube I - Figure 1).

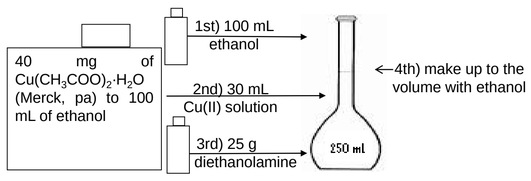


Figure 2 - Preparation of the colour reagent

DAILY PREPARATION OF CS_2 SOLUTIONS

The "pure standard" CS_2 (Merck, 99.9 % of purity) was used for the daily preparation of the stock solution and the following two diluted solutions. The less concentrated solution (*ca.* 50 µg/mL or 25 µg/mL) was used to prepare the calibration standards. (Figure 3)

Quality Control Standards (QCS) - these solutions of CS_2 were prepared also daily and independently from those used in the calibrations.

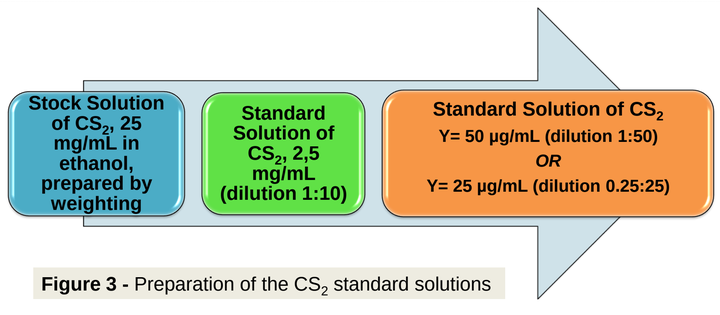
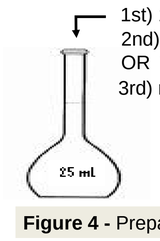


Figure 3 - Preparation of the CS_2 standard solutions

DAILY PREPARATION OF CALIBRATION AND QUALITY CONTROL STANDARDS



1st) 15 mL development solution
2nd) X mL CS_2 solution, Y=50 or 25 µg/mL
OR Y=25 µg/mL
3rd) make up to the volume with ethanol

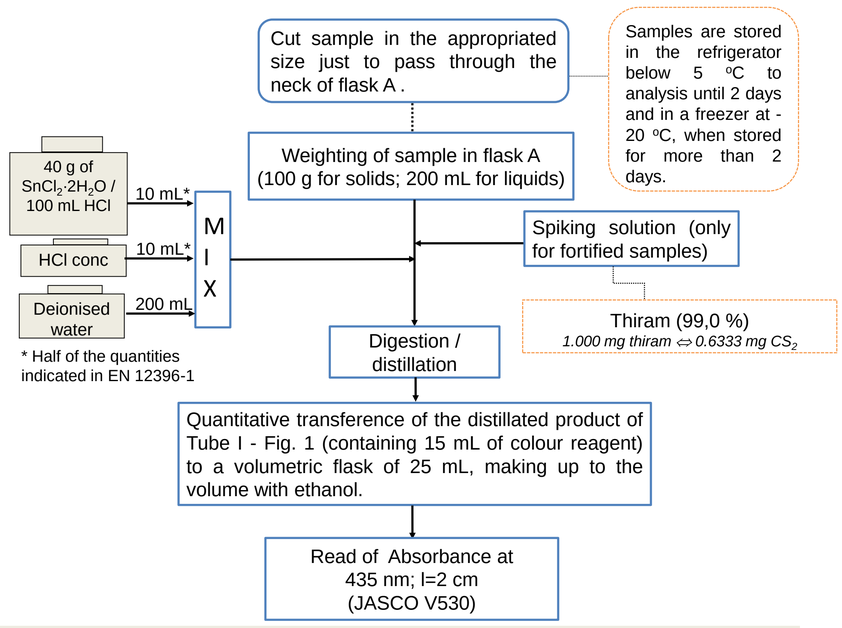
Y= 50 µg/mL	
Calibration Standard µg CS2 / 25 mL	X= Vpip (mL)
10	0,20
20	0,40
30	0,60
40	0,80
50	1,00
100	2,00
150	3,00
200	4,00
250	5,00

Y= 25 µg/mL	
Calibration Standard µg CS2 / 25 mL	X= Vpip (mL)
5	0,20
10	0,40
15	0,60
20	0,80
25	1,00
45	1,80
50	2,00

Figure 4 - Preparation of calibration standards and QCS

Depending on the **calibration range**, the CS_2 solution of 50 or 25 µg/mL was selected to prepare the calibration standards. All the calibration curves were defined with 5 levels.
The **Quality Control Standards** (QCS) were used to control the calibration curve, a concentration around the middle of the calibration curve having been selected.

ANALYSIS OF THE SAMPLES



* Half of the quantities indicated in EN 12396-1

Figure 5 - General procedure for the decomposition of DTCs of samples and its quantification, expressed as the concentration of its degradation product (CS_2)

SELECTED SAMPLES

Selected vegetable matrixes were analysed, corresponding to groups of Document SANCO 12571/2013 [4]:

- **High water content:** apple, broccolis, cabbage, carrot, cucumber, green beans, lettuce, peach, pear, potato, spinach, tomato
- **High acidic content and high water content:** Grapes, lemon, orange, kiwi, strawberry
- **High starch and/or protein content and low water and fat content:** lentils, rice, wheat flour

Red and white wine were also tested.

3. RESULTS AND DISCUSSION

QUALITY CONTROL STANDARDS (QCS)

The **average recovery** obtained since 2010 (n=189; several levels of concentration) was **100 %** with a **RSD of 7.6 %**. The criterion expressed on document SANCO 12571/2013 for the QCS analysed in routine corresponds to recoveries from **70 to 130 %**. In this set of studies, values varied from 77.8 % to 123.5 %, all of the results being acceptable.

SAMPLES FORTIFIED WITH THIRAM

The results obtained for samples spiked with thiram, for each level of fortification, are presented in **Table 1**.

Table 1 - Average recoveries, standard deviation (SD), relative standard deviation (RSD) and average relative error for samples of different matrices spiked with thiram.

Level (mg CS_2 / 25 mL)*	Concentration (mg CS_2 / mL)*	Concentration (mg CS_2 / mL)*	Concentration - solid samples (mg CS_2/kg) **	n	Average recovery (%)	SD (%)	RSD (%)	Average Relative Error (%)
5	0.20	0.20	0.05	43	106.9	20.4	19.1	+6.9
10	0.40	0.40	0.10	17	92.3	17.5	18.9	-7.7
25	1.00	1.00	0.25	20	89.3	17.4	19.5	-10.7
50	2.00	2.00	0.50	17	85.7	15.1	17.7	-14.3
125	5.00	5.00	1.25	14	85.8	12.5	14.6	-14.2
250	10.0	10.0	2.50	14	84.0	17.2	20.5	-16.0

* Total volume after colour development ** For wine, the value is half of the indicated and units are mg/L (200 mL of sample was used for liquids, instead of 100 g for solids)

➢The obtained recoveries tend to be below 100 %, what may be partially justified by heating problems.

➢Gonranka et al [1] referred that it was shown that for thiram temperatures of digestion below 80 ºC favor the side products COS and H_2S, decreasing the yielding of CS_2,

➢The use of heating mantles having 200 W of potency instead of the recommended (on EN 12396-1) 450 W could promoted a slower heating producing side compounds.

➢Trapping the H_2S by the NaOH in Tube F (Fig. 1) avoids its complexation by copper(II) and therefore the elimination of the interference in absorbance readings but if the origin is the dithiocarbamates a lower yield of CS_2 will be obtained.

➢The incomplete decomposition of DTC during the acidic hydrolysis also contributes to lower concentrations of CS_2.

METHOD'S PERFORMANCE

LINEARITY AND WORKING RANGE

The linearity was validated based on the visual observation of the calibration points, residual plot and Pearson s correlation coefficient (r) (r_{min}=0,9698, but almost were above 0,990). Ranges below 50 µg CS_2/25 mL were the most relevant since the EN 12396-1 only covers the upper range. An example is shown **Figs 6A** and **6B**.

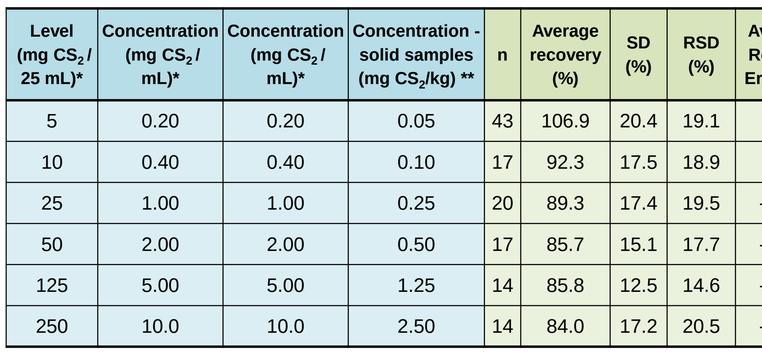


Figure 6 - (A) Plot of Absorbance=f(µg CS_2 /25 mL) corresponding to the data of Table 2, with representation of the trend line and its statistic parameters (calculated by Least Square Method) ; (B) plot of residuals.

The working range was always restricted to concentrations varying a maximum of 5-10 times (example 5-50 or 50-250 µg CS_2/25 mL).

Sensibility

In general it was observed that sensibility (slope, *ca.* 10^{-3}) was inferior for lower concentration of CS_2 (range until 50 µg CS_2/ 25 mL) being consistent with the lower extinction coefficient (at 435 nm) for complexes 1:1. Since variations of 0,002-0,003 units of absorbance in Molecular Absorption Spectrophotometry are usual, this sensibility is not very satisfactory.

Selectivity

Some commodities (such as cabbage and spinach) have phytogenic compounds producing CS_2 under the conditions of the analysis. Then, the method is not very selective and can lead to false positives. In present studies, some positive values were detected in "blank samples" for example for spinach, cabbage and kiwi, the source of which was impossible to determine.

Trueness and intermediate precision

In Table 1, data corresponding to the performed recovery tests are present. Few outliers, detected by Grubbs' test [5], were excluded.
According to SANCO, the **trueness ("acccuracy"** in document SANCO) and **intermediate precision** are acceptable (average recoveries of 84-107 % and maximum RSD of 20 %), complying with the criterion of being in the range 70 % to 120 % and being ≤ 20 %, respectively, for all range of concentrations.
Using only the more recent data (and then less information) RSD decrease significantly, except for the level of 5 µg CS_2 /25 mL.
To achieve a more rigorous assessment of the measurement trueness, the laboratory has participated in **proficiency tests** (Table 3), the results having been very satisfactory (|z-score| <3).

Table 3 - z-score values corresponding to the participation in proficiency tests.

Identification of proficiency test	Matrix	Obtained value (mg CS_2/kg)	Expected value (mg CS_2/kg)	z-score	Conclusion
EUPT-2010	Rice flour	0,60	0,65	+0,30	Satisfactory
EUPT-2011	Apple puree	0,12	0,25	-2,2	Satisfactory but questionable
EUPT-2012	Lentils	0,59	0,62	-0,17	Satisfactory

In the case of EUPT-2010 such so negative z-score (-2.2) likely corresponds to an incomplete digestion/distillation or other losses in the system. An extra tube was attached to tube I (Figure 1), also containing colour reagent, and a null absorbance was read for a spiked sample.

Trueness (bias)

Trueness was evaluated by the *t-test* [5] using most recent data, from 2012 to 2014, and only for the level of 5 µg CS_2/25 mL (n=33) no relevant systematic effects were observed.

Limit of Quantification

For this method of DTCs determination, the **Limit of Quantification** (LOQ) in mg CS_2/kg (or mg CS_2/L) depends on the weight (or volume) of sample and the low calibration level used in the calibration curve. The minimum LOQ established was 5 µg CS_2/25 mL.
Then, the LOQ expressed in mg CS_2/kg is 0.05 for 100 g of solid test samples and 0.025 mg CS_2/L for 200 mL of liquid test samples (wines).

UNCERTAINTY EVALUATION

The uncertainties were evaluated using the "top-down" approach [6,7], considering the following components, for the period 2010-2014 (Table 4) .

Precision
- RSD of spiked samples

Trueness (bias)
- Average recovery of spiked samples
- Purity of the "pure standard" of fortification (thiram)
- Fortifying solutions preparation

Additional components
- Working standard solutions preparation
- Purity of the "Pure Standards" of CS_2
- Weighing of sample

Table 4 - Uncertainty estimation per concentration level, based on "top-down" approach. Expanded uncertainty values correspond to a confidence level of 95 % **(A)** data from 2010 to 2014 **(B)** data from 2012 to 2014

A

Concentration solid samples (mg CS_2/kg) *	n	Relative Uncertainty (components)			Expanded Relative Uncertainty ** (%)	Expanded Absolut Uncertainty (mg CS_2/kg)
		Trueness (%)	Precision (%)	Additional factors (%)		
0.05	43	5.9	19.1	0.036	40	0.020
0.10	17	3.3	18.9	0.036	41	0.041
0.25	20	2.0	19.5	0.036	41	0.10
0.50	17	1.8	17.7	0.036	38	0.19
1.25	14	1.8	14.6	0.036	32	0.40
2.50	14	1.9	20.5	0.036	45	1.1

B

Concentration solid samples (mg CS_2/kg) *	n	Relative Uncertainty (components)			Expanded Relative Uncertainty ** (%)	Expanded Absolut Uncertainty (mg CS_2/kg)
		Trueness (%)	Precision (%)	Additional factors (%)		
0.05	33	5.9	20.3	0.036	42	0.021
0.10	12	3.4	14.4	0.036	32	0.032
0.25	14	2.0	11.7	0.036	26	0.06
0.50	6	1.9	6.7	0.036	18	0.09
1.25	11	1.5	3.4	0.036	8	0.10
2.50	7	2.0	9.3	0.036	23	0.6

* for liquid samples the value should be multiplied by 1/2 and the unit is mg/L
** For n≥30 a covering factor of 2 was applied and for n<30, the t-student was considered (for a level of confidence of 95 %).

Comparing data from A and B (Table 4) the decreasing of the value of the component "Precision" and consequent effect on "Expanded Uncertainty" is notorious (mainly for the last 3 levels), meaning that precision improved with the experience of the analyst (in spite of these data correspond to a low n).

4. CONCLUSIONS

➢By this method, the validation parameters ***trueness*** (accuracy in SANCO document) and ***precision*** fulfill the requirements expressed on the document SANCO 12571/2013 (average recoveries of 70-120 % and RSD ≤ 20 %, respectively).

➢The **LoQ** of this method complies to the above requirements of trueness and precision and is adequate to assess complaince with MRL of kiwi, spinach, rice and wheat flour.

➢ Parameters like ***precision***, improved with the time, except for the level corresponding to 0.05 mg/kg likely due to the low signal: noise ratios and lower linearity in the lower limit of the calibration curve.

➢The trueness was evaluated by the participation in three proficiency tests, with acceptable results.

➢ The **expanded uncertainty** is satisfactory face to document SANCO 12571/2013 and the value established by EFSA (50 %) to check violations to the MRL.

➢The compliance of the performance parameters with the requirements of document SANCO was proved.

5. REFERENCES

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