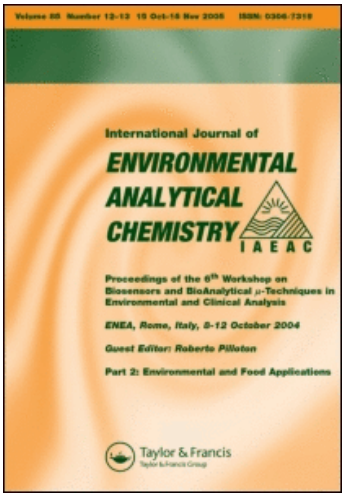

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Determination of dithiocarbamates; improvements to the method described in EN 12396-1 standard

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Determination of dithiocarbamates; improvements to the method described in EN 12396-1 standard

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A procedure to improve the performance and reliability of the method described in European standard EN-12396-1 is presented. A CS_2 working range of 10–50 µg per 25 mL was validated. The quantification limit was lowered from 0.25 mg kg^{-1} to 0.05 mg kg^{-1} (200 g of sample matrix), in agreement with the Maximum Residue Limits for dithiocarbamates (DTCs). The procedure involves the measurement of the average absorbance at 374 nm and 435 nm, to allow for the equilibrium between the two coloured Cupric(II) complexes formed. Two main modifications of the decomposition and distillation apparatus assembly were introduced. One was the stabilization of the colour-forming reagent solution at 0°C to ensure stability of absorbance. The other was the inclusion of a bubbler trap with ethanol, to retain interfering compounds. The possible CS_2 contamination by several types of protective gloves was also studied. Vinyl gloves, made according to the AQL 1.5 – EN 455-1/2 standard, did not interfere with the method.

Keywords: food analysis; dithiocarbamates; carbon disulphide; pesticides; residues

1. Introduction

Dithiocarbamates (DTCs) are an important group of synthetic organosulphur compounds that have been mostly used as pesticides in agriculture. Some products were already in use by the 1930s, and current annual usage amounts to between 25,000 and 35,000 metric tonnes per year [1].

As well as their agricultural use, DTCs are also used in industry as accelerators of rubber vulcanization and as rubber antioxidants. In addition, they are used as slimicides in pulp, paper and sugar production and they are also used in wastewater treatment and as antifoulants for water cooling systems [1].

Efforts have been made to develop analytical methods for dithiocarbamates and their metabolites, to allow their quantification in a simple, rapid, reliable and low reagent-consuming assay [1–5]. However, until now, the most widely used method for routine DTC quantification is the European standard EN-12396-1 for the analysis of non-fatty foods [6]. This method is based on the acidic hydrolysis of these substances, evolution of gas in a specific glass apparatus and collection of the released carbon disulphide (CS_2) in an

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ethanolic solution of diethanolamine and Cu(II); two coloured complexes, with a 1 : 1 and 1 : 2 molar ratio of $Cu^{2+} : CS_2$, are formed as a result of this process. The quantification of the 1 : 2 ($Cu^{2+} : CS_2$) complex is done by spectrophotometry at 435 nm for a concentration range of 50-250 μg CS_2 25 mL^{-1} [7–8]. As well as EN-12396-1, USEPA Method 630 is also based on the same principles [9].

The method uses a single methodology involving fairly toxic and inexpensive reagents and equipment, with results expressed in mg kg^{-1} of carbon disulphide in agreement with the Maximum Residue Limits (MRLs) for DTCs. These results are used in official monitoring of these substances in vegetable matrixes and it is expected that for routine proposes this method will continue to be used for some years, since there are not yet routine methods available for the analysis of dithiocarbamates on an individual basis [10].

In this work we present a modification of the method described in European standard EN-12396-1. The performance and reliability of the method was improved, mainly for DTCs concentrations lower than 50 μg CS_2 25 mL^{-1}. A new CS_2 working range of 10–50 μg per 25 mL was validated, enabling a Limit of Quantification (LOQ) compliant with some established MRLs, at a level of 0.05 mg kg^{-1} of carbon disulphide [11]. The procedure involves the measurement of the average absorbance at 374 nm and 435 nm, to allow for the equilibrium between the two coloured Cupric(II) complexes formed, instead of a single measurement at 435 nm. Further work was carried out in order to ensure the stability of the formed coloured solutions and to improve the reliability of the quantification procedure. To provide it, two main modifications of the decomposition and distillation apparatus assembly were introduced. One was the stabilization of the colour-forming reagent solution at 0°C to ensure stability of absorbance. The other was the inclusion of a bubbler trap with ethanol, to retain interfering compounds and reduce the possibility of matrix interferences using the new concentration range. Finally, since DTC's are frequently used as accelerators in rubber vulcanization [12], several brands of protective gloves were tested for possible interference with this methodology.

2. Experimental

In general, the experimental procedures described in this work followed the European standard EN-12396-1 [6]. Experimental modifications introduced to the standard method will be indicated and the respective results will be presented and discussed.

2.1 *Instrumentation*

Spectrophotometric measurements were performed using a HITACHI U-2800 spectrophometer (Tokyo, Japan). The instrument control and data processing were carried out with a HITACHI UV Solutions Application (Tokyo, Japan). For digestion, BUNSEN 1712, 450 W heating mantles (Madrid, Spain) were used. To generate an air flow through the apparatus, vacuum was applied at the end of the glass apparatus by connecting an HEIDOLPH Rotavac vacuum pump (Schwabach, Germany). The air flow through the glass apparatus was monitored during the analysis with an AALBORG 112-02(S) flow meter (Orangeburg, USA), located between the vacuum pump and the glass

decomposition/distillation apparatus. The glass decomposition/distillation apparatus was provided by M.C. PEIXOTO, Lda (Lisbon, Portugal).

2.2 *Reagents and solutions*

Thiram (Dr. Ehrenstorfer GmbH, 99.0%), carbon disulphide (Merck, 99.9% purity, for calibration standards), copper(II) acetate, diethanolamine, hydrochloric acid 37% (m/v), methanol, sodium hydroxide and tin(II) chloride dihydrate (Merck, analytical grade) and ethanol (Riedel de Haën, 99.8%) were used without further treatment. Distilled water was used for aqueous solution preparation.

The aqueous NaOH solution concentration was $100\,g\,L^{-1}$. The colour-forming reagent solution was prepared with 120 mL of copper(II) acetate solution ($0.4\,g\,L^{-1}$ in ethanol) and 100 g of diethanolamine, made up to 1000 mL with ethanol. The solution of tin(II) chloride/hydrochloric acid was prepared with 20 mL of tin(II) chloride solution ($400\,g\,L^{-1}$ of $SnCl_2$ in concentrated hydrochloric acid), 20 mL of concentrated hydrochloric acid and 200 mL of distilled water. For recovery tests, a standard solution of thiram was prepared with 8.7 mg made up to 100 mL with methanol.

The carbon disulphide stock solution was prepared in two steps; a first solution was made by adding 1 mL of carbon disulphide to a 50 mL volumetric flask containing 40 mL of ethanol, and making it up to the mark with ethanol. The mass of carbon disulphide was accurately measured (±0.01 mg) by difference of weight before and after addition of carbon disulphide. This solution was then diluted to 1 : 500 until it reached a concentration of approximately $50\,\mu g\,mL^{-1}$ of carbon disulphide. This solution was stored at −30°C and used to prepare calibration standards. Calibration solutions were prepared by adding different volumes of CS_2 stock solution to 15 mL of colour-forming reagent solution and making up to 25 mL with ethanol. These calibration solutions were stored at 0 to 5°C for 60 minutes before carrying out spectrophotometric measurements.

2.3 *Sample preparation and analysis*

Samples of vegetable matrices (200 g) were heated with a solution of tin(II) chloride and hydrochloric acid. This treatment decomposes dithiocarbamates, forming carbon disulphide which is distilled and collected in an ethanolic solution of cupric(II) acetate and diethanolamine. Two yellow cupric-*N*,*N*-bis(2-hydroxyethyl)dithiocarbamate complexes with molar ratios of Cu^{2+}/CS_2 1 : 1 and 1 : 2, respectively, are formed and measured by spectrophotometry.

2.3.1 *Decomposition/distillation apparatus preparation*

The decomposition and distillation apparatus was also prepared according to EN-12396-1 with some modifications undertaken as part of the present work. The overall assembly was composed of a heating mantle with a 1 L three-necked flask. The three-necked flask was attached to a gas inlet tube and a charge funnel on the side necks and a condenser on the top neck. Following the condenser, 3 bubbler traps were connected with 10 mL of sodium hydroxide solution in the first one, 25 mL of ethanol in the second (see Section 3.3.1) and 15 mL of colour-forming reagent solution in the third trap.[1] The second and third bubblers were covered with a water/ice mixture (see Section 3.2). A vacuum pump was connected to

the end of the apparatus to keep the air flow at approximately 300 mL min^{-1} and monitored with a flow meter.

2.3.2 *Decomposition and distillation*

Two hundred g of matrix and 240 mL of tin(II) chloride/hydrochloric acid solution were added through the three-necked flask and decomposition was undertaken under reflux conditions for 60 minutes. After this period, the contents of the third bubbler were transferred to a volumetric flask and adjusted to 25 mL with ethanol.

2.3.3 *Spectrophotometric measurements*

The calibration solutions were prepared in two ranges: 10–50 μg of carbon disulphide (introducing an extension to the EN standard concentration range – see Section 3.1) and 50–250 μg of carbon disulphide (25 mL^{-1}).

The spectrophotometric measurements were carried out at 374 and 435 nm in the calibration range of 10–50 μg of carbon disulphide and at 435 nm in the calibration range of 50–250 μg of carbon disulphide. An auto-zero point was made using a blank solution (15 mL of the colour-forming reagent solution made up to 25 mL with ethanol).

The calibration solutions were prepared daily for quality control of slope and abscissa intercept.

2.4 *Protective glove analysis*

Portions (0.3 g) of four brands of commercial protective gloves were tested analogous as samples to quantify the amount of carbon disulphide released. Gloves tested were: proFood, Disposable Nitrile Gloves (Brussels, Belgium); Blue-Comfort, Nitrile Gloves, Latexfrei, Powder free (Thailand); Handsafe TM, Disposable natural Latex gloves, powdered with USP (Malaysia); Star-Premium vinyl examination gloves – AQL 1.5 – EN 455-1/2 (Taipei, Taiwan).

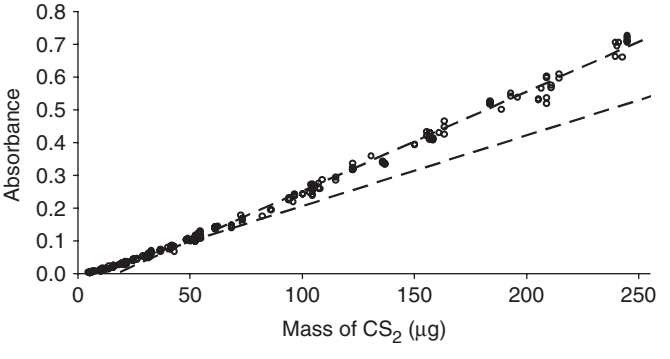

Figure 1. One year data of calibration standards from 10 to 250 μg of CS_2.
Notes: Measurements were performed at 435 nm. The two traced tendency lines represent the change of slope value observed.

3. Results and discussion

3.1 *Low working range*

The linearity of absorbance as a function of CS_2 mass was first studied in the 10–250 μg range. Figure 1 presents the standard calibration data obtained from several measurements made over the course of an entire year. The results clearly show an inflexion at around 50 μg of CS_2. To analyse this behaviour statistically, two calibration curves with five data points each (10, 20, 30, 40 and 50 μg of CS_2 and 50, 100, 150, 200 and 250 μg of CS_2) were prepared. The coefficients and statistical parameters obtained are presented in Table 1. The results show that the two calibration curves had statistically different slope values (**m**). This observation is supported by the variance of the slope parameter ($2.0 < F$) which allowed us to compare the two slope values. This loss of linearity was ascribed to a change in the equilibrium ratio between the two cupric complexes with changing concentration. Figure 2 shows the absorption spectra obtained for different CS_2 concentrations, from 10 to 50 μg. The spectral distribution changed with concentration, indicating that

Table 1. Statistical difference ($\alpha = 0.05$) between slopes from analytical curves, 10–50 μg CS_2 and 50–250 μg CS_2, prepared with the same solution of carbon disulphide and with measurements made at 435 nm.

Calibration curve (10 to 50 μg CS_2)		Calibration curve (50 to 250 μg CS_2)		Parameter S_m	Calibration curve (10 to 50 μg CS_2)	Calibration curve (50 to 250 μg CS_2)
x_i (μg CS_2)	y_i (AU)	x_i (μg CS_2)	y_i (AU)		6.06×10^{-5}	8.63×10^{-5}
10.3	0.010	51.3	0.100	$\mathbf{S_m^2}$	3.67×10^{-9}	7.45×10^{-9}
20.5	0.030	102.6	0.269	$\frac{s^2_{m(50-250)}}{s^2_{m(10-50)}}$	2.0	
30.8	0.051	153.8	0.392	**F value (0.05;3;3)**	9.3	
41.0	0.076	205.1	0.534			
51.3	0.100	256.4	0.702	**m (0.05)**	$(2.2 \pm 0.2) \times 10^{-3}$	$(2.9 \pm 0.3) \times 10^{-3}$

Notes: S_m is the slope standard deviation, x_i and y_i are the coordinated values for calibration curve and AU symbolizes Absorbance Units.

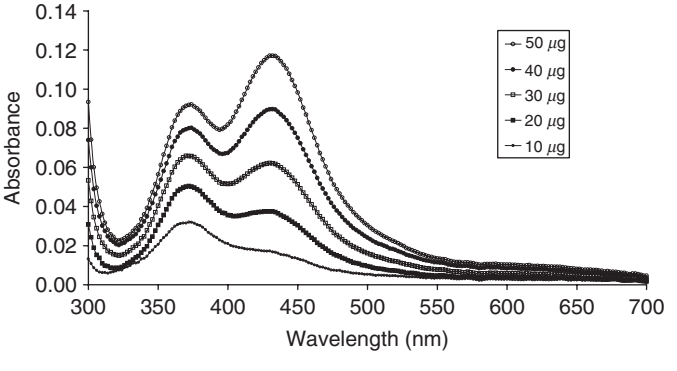

Figure 2. Absorption spectra showing the equilibrium of the 2 coloured complexes within the concentration working range.

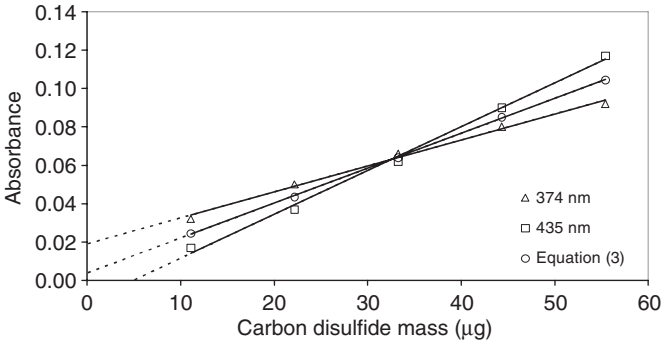

Figure 3. Plot of the calibration curves obtained using absorbance signal measurements at different wavelength conditions ($\alpha = 0.05$).
Notes: 374 nm: $y = (1.3 \times 10^{-3} \pm 0.2 \times 10^{-3})x + (1.9 \times 10^{-2} \pm 0.7 \times 10^{-2})$ ($R^2 = 0.9938$) 435 nm; $y = (2.3 \times 10^{-3} \pm 0.3 \times 10^{-3})x - (1.1 \times 10^{-2} \pm 0.9 \times 10^{-2})$ ($R^2 = 0.9962$) Equation (3): $y = (1.82 \times 10^{-3} \pm 0.05 \times 10^{-3})x + (0.4 \times 10^{-2} \pm 0.2 \times 10^{-2})$ ($R^2 = 0.9997$).

measurements were being made close to the equilibrium of the two complexes (1 : 1 and 1 : 2 molar ratio of Cu^{2+}: CS_2). To allow for this equilibrium effect, the absorbance was measured at 374 and 435 nm, the absorption maxima of each complex.

The absorptions at 374 nm (A_{374}) and at 435 nm (A_{435}) are given by:

$$A_{374} = b\varepsilon_{C_1(374\,nm)}C_1 \tag{1}$$

and

$$A_{435} = b\varepsilon_{C_2(435\,nm)}C_2 \tag{2}$$

respectively, where b is the optical path, ε is the absorption coefficient and C_1 and C_2 are the concentrations of complex 1 and complex 2.

Within this working range, it was experimentally observed that there was a linear correlation between the absorbance average determined for each complex concentration at 374 and 435 nm and the total CS_2 concentration (C_{CS_2}). This correlation can be expressed as follows:

$$\frac{A_{374} + A_{435}}{2} \propto C_{CS_2} \tag{3}$$

Figure 3 and respective analytical equations clearly shows that the fit of the experimental data, using Equation (3) proposed in this work, passes closer to the origin than the other relationships, indicating also a better fit to the statistical least squares regression method [13]. This fact leads to lower limits of detection and quantification estimated with higher statistical confidence, which prevails over the slight lost of sensitivity observed for this concentration range.

The linear relationship (3) was used for quantification and was tested for variance homogeneity and linearity (by visual analysis, from consideration of the correlation coefficient and by comparison with non-linear regression). The results were compared to measurements made only at 374 nm and at 435 nm and are reported in the following sections.

Table 2. Statistical test comparing the regression fitting to calibration data, considering linear and quadratic regression.

Type of regression (signal)	$S_{y(\text{linear})}$	$S_{y(\text{quadratic})}$	PG
374 nm	2.2×10^{-3}	9.8×10^{-17}	1.5×10^{27}
435 nm	2.8×10^{-3}	1.4×10^{-3}	11
Equation (3)	6.1×10^{-4}	4.8×10^{-4}	2.8

Notes: S_y – Residual standard deviation; PG – Calculated test value. Reference Fisher test values: $F(1, 2, 0.05) = 18.9$ and $F(1, 2, 0.01) = 98.5$.

3.1.1 *Linearity*

In order to achieve a better understanding of the copper complex equilibrium effect, the three analytical curves were analysed for linearity, using two different wavelengths (374 and 435 nm) and Equation (3).

To test for linearity, we need first to verify variance homogeneity within the calibration range. For that purpose, 10 independent standard solutions were prepared for the first and last calibration points. A PG result of 1.42 (ratio between the two calibration points variances) showed that variances were not significantly different, since F test values, for 0.05 and 0.01 of significance level, were 3.18 and 5.35, respectively.

For statistical assessment of linearity, the three linear equations were compared with quadratic regression using Equation (4) [14].

$$PG = \frac{(N-2)S^2_{y_{\text{linear}}} - (N-3)S^2_{y_{\text{quadratic}}}}{S^2_{y_{\text{quadratic}}}} \tag{4}$$

The results obtained are presented in Table 2. At 374 nm, the quadratic regression yielded a much lower residual standard deviation which led to the high PG value obtained. At 435 nm and using Equation (3), the linear regression gave a tighter regression. However, using Equation (3) we achieved a PG value of 2.8 indicating the best linearity.

3.1.2 *Limits of detection (LOD) and quantification (LOQ)*

Instrumental quantification limits – LOD and LOQ – were estimated using the residual standard deviation obtained by least squares regression [13]. The mean values of 12 calibration curves were used. The results obtained were 2.8 and 9.4 μg of CS_2 25 mL^{-1}, respectively, equivalent to 0.02 and 0.05 mg kg^{-1} in a 200 g sample matrix. This represents a five times improvement of the previous reference LOQ value of 0.25 mg kg^{-1} given by the European standard EN-12396-1 method.

3.1.3 *Routine quantification*

For routine application, quantification was performed with a linear regression equation defined with data values taken from 12 calibration curves using Equation (5) 10–50 μg of CS_2. These calibration curves were prepared with independent CS_2 solutions and at different times, reflecting intermediate precision conditions for a confidence level of 0.05.

Equation (5) arose from the application of the calibration improvements described and discussed in this paper for:

$$y = (0.0017 \pm 0.0002)x - (0.002 \pm 0.005) \quad (5)$$

The slope and abscissa intercept of this calibration curve was daily validated with control charts and was found to be stable over time.

3.2 *Colour solution stability*

In routine work it was observed that the absorbance of calibration solutions was not stable. To study this behaviour, the stability of absorbance was tested at 374 and 435 nm according to variation in temperature and aspects of the analytical procedure. Absorbance measurements were taken on several standard solutions with a CS_2 amount of 50 µg at times $t = 0$, 10, 20, 30, 40, 50 and 60 minutes, for five different environments: in the bubbler flask at 0 and 25°C (reproducing the formation of complexes in the decomposition and distillation apparatus); and in a volumetric flask at 0, 25 and 40°C (reproducing the preparation of calibration standards). The absorbance measurements were performed in quintuplicate to allow the application of one-way analysis of variance (ANOVA). Results are shown in Figures 4 and 5.

The statistical approach was undergone by the one factor analysis of variance. This test was used to compare, for each environment, the absorbance mean values for each measuring time. The *p*-values obtained vary from 0.14 at 0°C to 1.7×10^{-28} at 40°C (critical *p*-value = 0.05) which confirms the graphical representations showing the influence of temperature environment in the $Cu^{2+}:CS_2$ complex stability.

To ensure the stability of the complex solutions, they must be maintained at about 0°C. To achieve this, the colour solution bubblers were covered with an ice/water mixture and the calibration standard solutions were kept close to 0°C before absorbance measurements.

3.3 *Interference assessment*

3.3.1 *Matrix interferences*

We observed that some vegetable matrices, purchased in the municipal market and different supermarkets, led to positive signal interference at the wavelength of 374 nm (Figure 6). The observation of the absorbance values relation between 374 and 435 nm shows that the interference should not be attributed to the CS_2 eventually produced from the several tested matrices. Actually if this was the case, an absorption band with a maximum at 435 nm, correspondent to the 1 : 2 ($Cu^{2+}:CS_2$) complex, should also be observed. When a bubble trap with 25 mL of ethanol was introduced between the sodium hydroxide and the colour-forming reagent bubbler traps, the interfering substances were retained in the ethanol allowing measurements to be made. This behaviour can be observed in Figure 7 for the onion's matrix, which clearly presents the highest degree of interference.

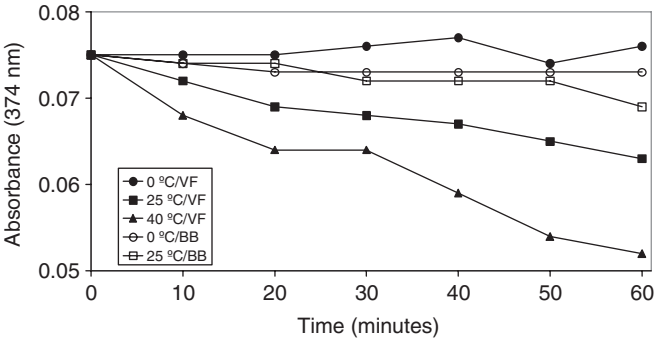

Figure 4. Response stability at 374 nm, at different temperatures and environmental conditions. Notes: VF – Volumetric flask; BB – Bubbler flask.

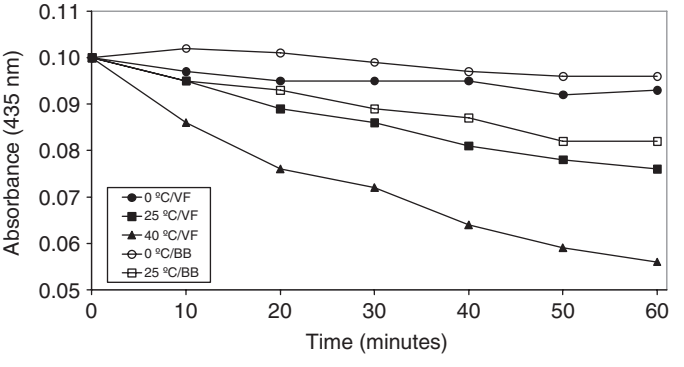

Figure 5. Response stability at 435 nm, at different temperatures and environmental conditions. Notes: VF – Volumetric flask; BB – Bubbler flask.

3.3.2 *Protective glove contamination*

Since rubber materials are likely to interfere with this method [6,12], possible interference from protective gloves used during sample preparation was studied. Star-Premium vinyl exam gloves were the only gloves which did not interfere with the DTC's analytical method (CS_2 level inferior to the detection limit of the method). Blue-Comfort gloves achieved a value of 163 mg kg^{-1} of CS_2 and for the other gloves (Profood and Handsafe Tm), levels were higher than 908 mg kg^{-1} of CS_2.

3.4 *Recovery experiments on vegetable matrices*

To assess the modifications we have proposed to the method described in EN-12396-1, we carried out two recovery tests. The recovery tests were performed using a thiram solution with fortification at two levels – 11 and 33 μg of CS_2, corresponding to values close to the limit of quantification (LOQ) and to the mid-point of the calibration range, respectively. The matrix used was 200 g of onion, considering the high interference previously observed

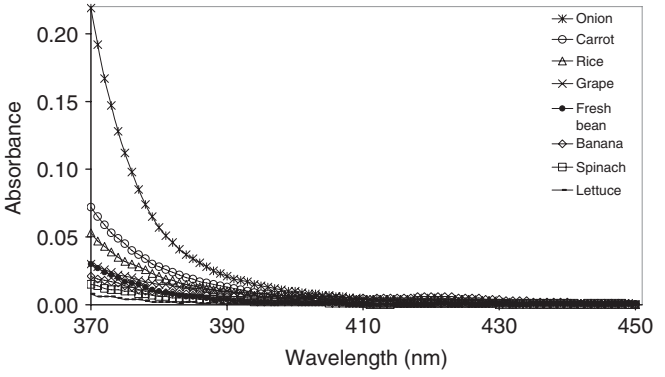

Figure 6. Spectra overlay of colour-forming reagent solutions collected in the analysis of several vegetable matrixes.

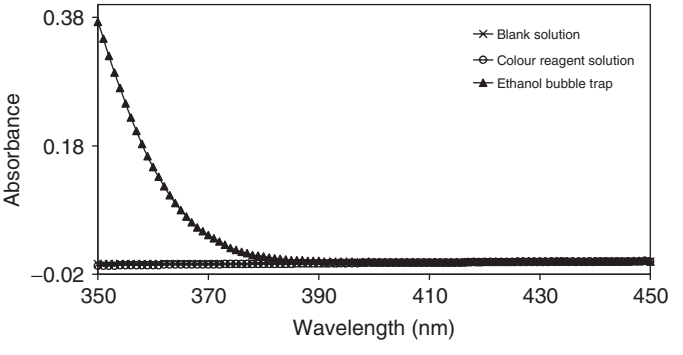

Figure 7. Spectra overlay of blank, colour-forming reagent solution and ethanol bubble trap solutions used to test elimination of onion samples interference effect.

for this matrix. The tests were performed in triplicate and results were calculated within routine conditions, using Equation (5). Recoveries of 109%, with a coefficient of variation of 8.6%, for 11 μg of CS_2 fortification level and 90%, with a coefficient of variation of 10.3%, for 33 μg of CS_2, are in agreement with SANCO guideline criteria [15].

3.5 *Interlaboratory proficiency test*

A test sample provided in the interlaboratory test – TestQual 16 was analysed using the method proposed in this article [16]. The test sample, of marrows treated with an aqueous solution of Afrosan MZ-8 (Mancozeb 80%) and allowed to dry for three hours, was prepared. The sample was then frozen with liquid nitrogen, shipped and sent to participant laboratories. With a test portion of 25.0 g, the result obtained was 1.25 mg kg^{-1} of carbon disulphide, corresponding to c. 31 μg of CS_2. The interlaboratory Z value obtained was 1.0, which is satisfactory. The quantification was performed with Equation (5).

4. Conclusions

The results presented in this article suggest that some changes to the procedure described in the European standard EN-12396-1 are warranted. The LOD and LOQ were reduced to 0.02 and 0.05 mg kg^{-1} of carbon disulphide, respectively, complying with MRLs currently established for this group of pesticides. These limits were achieved using two wavelengths for the absorbance measurements, 374 and 435 nm to allow for the equilibrium reaction which led to the formation of two complexes. To achieve the measurements at 374 nm, it was necessary to introduce a bubble trap with 25 mL of ethanol to remove matrix interfering substances.

We have shown that the coloured solution is unstable at ambient temperature. To avoid this instability, the bubble trap with colour-forming reagent solution was covered with an ice/water mixture and the calibration solutions were stored at around 0°C before measurements. The new calibration range was tested for recovery of thiram with acceptable results at 11 and 33 μg of CS_2. Finally, we have shown that Star-Premium vinyl examination gloves – AQL 1.5 – EN 455-1/2 protective gloves are unlikely to interfere with this method.

Note

1. A fourth bubbler trap with 15 mL more of colour-forming reagent solution (described in the EN-12396-1 methodology) was removed, since routine experimental results show that this additional security trap was not necessary when using the present working range concentrations.

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