Bulgarian Journal of Veterinary Medicine (2004), 7, No 3, 189–193

Short communication

SPECTROPHOTOMETRIC DETERMINATION OF THE CARBAMATE INSECTICIDE CARBOFURAN IN BIOLOGICAL SPECIMENS

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Carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) is a broad-spectrum pesticide with a high biological activity against various forage pests (Rangaswamy *et al.*, 1976). Its primary application is for pretreatment of sowing–seeds as well as against soil nematodes (Gupta, 1994). Recently, it was found out that cereal forages in Bulgaria are most commonly contaminated with carbofuran (Vrabcheva *et al.*, 2001; 2002). This is primarily due to non–observance of technological and hygienic rules during its aplication – addition of treated sowing–seeds in forages, non–observance of post treatment quarantine terms during forage storage.

The quantitative determination of carbofuran and carbofuran metabolites residues could be performed via various methods: spectrophotometry (Zanella *et al.*, 1999), gas and liquid chromatography (Cook *et al.*, 1977; Voyksner, 1987; Stahr, 1991), GC/MS (Wan and Harrington, 2000). The majority of those methods however require an expensive equipment and highly qualified personnel, that limit their extensive use for forage control purposes. The spectrophotometric method, described by us, based on the electrophilic reaction of carbofuran and diazoted aniline, is in our opinion the most appropriate means for systemic control of carbofuran residues in forages.

Principle of the method

The method is based upon the reaction between diazoted aniline and carbofuran, resulting in the formation of a yellow compound with peak absorbance at 460 nm. It is suitable for determination of carbofuran in forages, rumen content and waters.

Apparatus

Spectrophotometer (Spekol–11, Carl Zeiss Jena, Germany).

Reagents

- 99.5% aniline (Merck, Germany/ 101621);
- carbofuran (Pestanal, Sigma Aldrich/ 45370);
- diafuran 35CT (350 g/L) – commercial grade carbamate persticide (Mitsubishi, Japan);
- sodium nitrite (Merck, Germany/GR for analysis/106549);
- potassium hydroxyde (Merck, Germany/105021);
- hydrochloric acid (Merck, Germany/100317);
- chloroform (Merck, Germany/ 102445);

- active charcoal (BDH Limited Poole, England/ 33187);
- n–hexane (Merck, Germany/ 110-54-3);
- acetonitrile (Ferak, Germany/ 50 013);
- methanol (Merck, Germany/ 106002).

Preparation of solutions

- 0.005% aniline solution: dissolve 50 mg aniline in sufficient amount of 0.1N hydrochloric acid to obtain 1000 mL;
- 5% sodium nitrite solution: dissolve 5 g sodium nitrite in sufficient amount of distilled water to make 100 mL;
- 0.5N potassium hydroxyde solution: dissolve 28.055 g potassium hydroxide in sufficient amount of distilled water to make up 1000 mL;
- carbofuran standard solutions: prepare solutions of pure carbofuran in graduated tubes with glass stoppers containing 1.0, 5.0, 10.0, 20.0 and 40.0 μg carbofuran/mL methanol (acetonitrile[1]).

Preparation of a standard curve

Pipet 1 mL aliquots of 0.005% aniline solution into 5 separate test-tubes. Then, add 0.5 mL of 5% sodium nitrite to each test-tube as well as 1 mL of each standard solution containing 1.0, 5.0, 10.0, 20.0 and 40.0 μg/mL carbofuran respectively, added dropwise. Shake the mixtures vigourously and then, add 1 mL 0.5N potassium hydroxide to each tube. Shake the latter once again and leave to stand at room temperature for 30 min. Adjust the volume of the obtained yellow solution to 5 mL with methanol (acetonitrile) and measure the absorbance at 460 nm against a blank sample, prepared by substituting the standard carbofuran solution with 1 mL pure methanol (acetonitrile). The colour is stable for 2 hours with peak at min 30. The standard curve is made by plotting the absorbance against the concentration (Fig. 1).

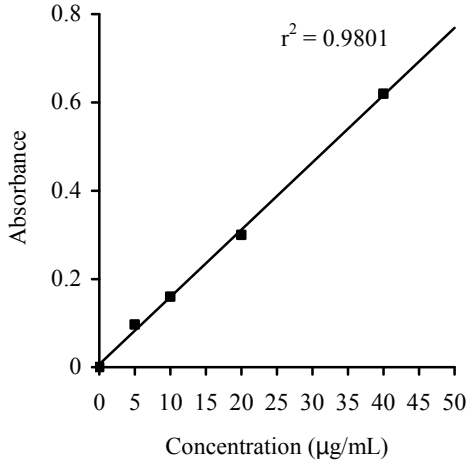

Fig. 1. Standard curve of carbofuran.

Validation of the method

- *Linearity.* It was determined within the concentration range 5–40 μg/mL. Six replicates of each of the following carbofuran concentrations: 5.0, 10.0, 20.0 and 40.0 μg/mL, were analysed. Data were assessed by the least squares linear regression method.
- *Recovery.* Samples of 100 g corn were contaminated with either 5, 10, 17.5, 35.0 or 40.0 μg carbofuran. Six determinations for each concentration were performed. The percentage ratio between real and obtained concentrations was determined.

[1] It was experimentally detected that the standard curve made upon solutions containing from 5 to 40 μg carbofuran/mL methanol coincided with that, made upon solutions containing from 5 to 40 μg carbofuran/mL acetonitrile.

- *Precision (repeatability) and accuracy.* Six consecutive determinations for each of the following carbofuran concentrations: 5.0, 10.0, 17.5, 35.0 and 40.0 μg/100 g forage, were performed. The mean values, the standard deviation and coefficients of variation, were calculated. The accuracy was defined as the percentage difference between mean measured concentrations and the real (added) concentration.
- *Limit of detection (LOD).* It was defined as the least concentration where the presence of carbofuran could be detected. For this purpose, 6 determinations of samples contaminated with either 1.0, 3.0 or 5.0 μg carbofuran /100 g forage were done.
- *Limit of quantification (LOQ).* It was determined as the limit, over which determinations could be performed with a coefficient of variation < 20%. Forage samples contaminated with carbofuran at concentrations 1.0, 3.0, 5.0, 10.0, 20.0, 40.0 and 50.0 μg/100 g sample were tested. Six replicates of each concentration were analysed.
- *Confidence interval of the mean.* It was calculated according to the equation:

$$\mu = \overline{x} \pm \frac{SD.t}{\sqrt{n}}$$

where: $\overline{x}$ = mean value, SD = standard deviation, t = 2.571, n = number of replicates.

Procedure

Put a sample weighing 100 g (forage, stomach or rumen content) into a 300 mL Erlenmeyer flask with a glass stopper. Then, pour 100 mL chloroform, add a little quantity active charcoal and extract the mixture for 30 min on a shaker apparatus. Filter the suspension through a paper filter into a 150 mL beaker and evaporate the filtrate in a water bath at 60–80 ^{o}C. Dilute the dry residue in 20 mL acetonitrile, transfer it into a 125 mL separatory funnel and extract it with 2–3 20-mL n-hexane portions for fat removal. After discarding the hexane layer, transfer the acetonitrile layer into a beaker and evaporate it at a water bath (60–80 ^{o}C). Dilute the dry residue in 2 mL acetonitrile. Use 1 mL of it for the colour reaction, as described under "Preparation of standard curve". In cases when the coloured solution is turbid or emulsified, it should be centrifuged at 2000 rpm for 20 min prior to the measurement of the absorbance.

Calculation

Calculate the concentration of carbofuran in a sample according to the following equation:

$$\text{Carbofuran [μg/100 g]} = F \times A_s \times V_s$$

where: F = factor calculated from the standard curve as the ratio of the concentration and the absorbance of the standard (10 μg/mL), A_s = absorbance of the sample, V_s = sample volume (=1 mL).

Our data showed that the correlation between absorbances and carbofuran concentrations was linear from 5 μg/mL to 40 μg/mL carbofuran (Fig. 1). For higher concentrations, appropriate dilutions must be done as the linearity of the curve disappeared.

The recovery rate of the method varied within 90–99% for concentrations between 5 and 40 μg/mL respectively (Table 1). It was high because the losses due to purifying procedures using silicagel columns are excluded on the account of processing with active charcoal. Compared to the thin-layer chromatography for carbofuran determination, the present

Table 1. Recovery rate in control 100 g forage samples, contaminated with carbofuran

Amount of added carbofuran, μg	Measured concentration, μg/100 g (mean ± SD)	Recovery rate, %
5.0	4.95±0.01	99.00
10.0	9.85±0.02	98.50
17.5	16.34±0.09	93.37
35.5	31.86±0.18	91.00
40.0	36.00±1.63	90.00

Table 2. Precision (repeatability) and accuracy of the method

Actual concentration, μg/100 g	Measured concentration, μg/100 g (mean ± SD)	CV, %	Accuracy, %
5.0	4.95±0.03	0.50	– 1.0
10.0	9.85±0.05	0.50	– 1.5
17.5	16.34±0.23	1.30	– 1.6
35.5	31.86±0.46	1.38	– 10.2
40.0	36.00±1.61	4.26	– 10.0

Table 3. Ninety-five percent confidence interval of the mean value

Theoretical concentration, μg/100 g	μ
5.0	4.95±0.03
10.0	9.85±0.05
17.5	16.34±0.23
35.5	31.86±0.46
40.0	36.00±1.61

method is about 4 times quicker that is valuable with regard to the rapid diagnostics of carbofuran intoxication that develops rapidly (from 15 min to 2 h) and affects a large number of animals.

The precision (repeatability) of our analyses (Table 2) showed coefficients of variation between 0.5% (for 5.0 μg/100 g sample) and 4.26% (for 40.0 μg/100 g sample) suggesting that the precision of the method is satisfactory as it is within –15% ÷ +15%.

The limit of detection was 1 μg/100 g sample (0.01 ppm) and the limit of quantitative determination – 5 μg/100 g or 0.5 ppm. The 95% confidence intervals of arithmetic means for concentrations of 5-40 μg/100 g were $\overline{x} \pm 0.03 \div \overline{x} \pm 1.61$ μg/100 g (Table 3). Those data evidence that the spectrophotometric method could be applied for quantitative determination of carbofuran in contaminated forages in quantities over the maximum allowable limit of 0.05 ppm (Fetvadjieva *et al.*, 1994).

In conclusion, the spectrophotometric method for carbofuran determination in forages based on its interaction with diazoted aniline, described in the present study, was considerable more sensitive than the currently used thin-layer chromatography, that detects amounts not less than 0.2 ppm (Stahr, 1991).

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Paper received 06.10.2003; accepted for publication 15.04.2004

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