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Optimization and ruggedness testing of the determination of residues of carbadox and metabolites in products of animal origin

Stability studies in animal tissues

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ABSTRACT

A method developed for the determination of residues of carbadox and its metabolites in swine tissues using high-performance liquid chromatography with on-line precolumn enrichment and postcolumn derivatization with UV–VIS detection was optimized and the applicability of the method was extended to plasma and eggs. With the optimized method, more than twenty samples per person per day can be analysed. In the matrices investigated, the observed limit of determination for carbadox is 0.5–1 μg/kg and for desoxy-carbadox 0.5–2 μg/kg. The mean recovery for desoxy-carbadox in kidney, muscle and liver as established by two laboratories over a 2-month period is 95% (relative standard deviation = 14%, $N = 37$, 10 μg/kg). In other matrices the recoveries are between 83 and 91%. The recovery for carbadox is 70–80% in muscle, plasma and eggs. The method has been used routinely in pharmacokinetic and surveillance studies.

Stability studies of kidney and liver samples spiked with carbadox showed that carbadox is rapidly decomposed (*in vitro* metabolism). After storage for about 1 h at 4°C, more than 50% of the added amount is converted by reduction to desoxy-carbadox. In contrast, carbadox is stable in eggs and muscle under spiking conditions and during storage at −20°C. Desoxy-carbadox is stable during spiking and storage at −20°C in eggs and muscle. In kidney and liver, its stability was good under spiking conditions but could not be proved unequivocally during storage.

INTRODUCTION

High-performance liquid chromatographic (HPLC) [1–4], polarographic [5–7], thin-layer chromatographic (TLC) [8] and gas chromatographic (GC) [9] methods

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have been described for the determination of residues of the growth-promoting and chemotherapeutic agent carbadox (CBX) and its metabolites in various matrices. In a previous study, a method [10] was developed for the trace analysis of residues of carbadox in swine tissues. Therefore, the sample clean-up, chromatographic conditions and detection parameters were chosen according to the demands of this polar compound. After the method had been developed it was used in stability studies of reference materials and preliminary pharmacokinetic experiments with swine. The results of these studies showed that the toxic metabolite desoxycarbadox (desoxy-CBX) is the predominant residue in the target substrates liver and kidney. Further, it became evident that the spiking procedure for liver and kidney was very critical. These matrices markedly influenced the analytical recovery of CBX. Finally, the elution profiles of CBX and desoxy-CBX from the clean-up column were occasionally abnormal. Therefore, it was decided to optimize the method for the routine determination of desoxy-CBX, and further to investigate whether the scope of the method could be broadened to the analysis of other matrices, *i.e.*, egg and plasma.

EXPERIMENTAL

The experimental conditions were identical with those described earlier [10] with the exception that a Gilson 231-401 auto-injector (Gilson Medical Electronics, Villiers le Bel, France) was used instead of a Rheodyne Model 7125 injector. In Table I, a summary of the optimized analytical procedure is presented.

RESULTS AND DISCUSSION

Alumina/Florisil clean-up

In the original procedure [10], the first 10 ml of the total of 20 ml of column eluate were collected. This choice was mainly based on the speed of analysis. During routine analysis, however, it was observed that the desoxy-CBX concentrations found in

TABLE I

SUMMARY OF THE OPTIMIZED ANALYTICAL PROCEDURE

Parameter/step	Value/conditions	Parameter/step	Value/conditions
Sample size	10 g	Flushing time	20 min
Extraction	40 ml acetonitrile–methanol (1:1)	Back-flushing time	5 min
Alumina–Florisil clean-up	Take 10 ml of mixed total eluate	Eluent	Acetonitrile–0.01 *M* sodium acetate (pH 6) (14:86)
Evaporate to	0.9–1.1 ml	Eluent flow-rate	0.5 ml/min
Dilute to	4 ml with water	Analytical column	100 × 3 mm I.D., 5 μm Chromspher-C_{18}
Partition with	2 ml isooctane		
Inject	1 ml into the LC system	Derivatization reagent	0.5 *M* NaOH (0.23 ml/min)
Enrichment-flush water flow	0.3 ml/min	Reaction coil	2 m × 0.5 mm I.D., knitted PTFE
Enrichment column	10 × 2.1 mm I.D., Sep-Pak C_{18}	Detection	390 nm, 0.001 a.u.f.s.

practice samples and the analytical recoveries for desoxy-CBX in spiked (10 μg/kg) liver, muscle and kidney samples were about 10% higher in the second 10-ml eluate fraction. Occasionally, even greater differences were observed for CBX in muscle samples (Table II). The latter result was at variance with the earlier observations [10] for CBX which indicated a slightly increased concentration in the first 10 ml. The irreproducible differences between the results obtained for the two eluate fractions are probably caused by adsorption of the analytes to the alumina. To circumvent this problem, the total eluate was collected and 10 ml were taken for further analysis. Table II gives some data on the recoveries obtained when 10 ml of the total eluate are used.

Enrichment column

In the original procedure [10], the LC enrichment column used was a 60 × 4.6 mm I.D. stainless-steel cartridge filled with 37–50-μm Bondapak C_{18}/Corasil material. These dimensions were necessary to retain CBX and its metabolites adequately. A drawback of this type of column was the occasional peak broadening resulting from the variation in homogeneity of the dry-packed column. Therefore, the possibility of using a small-sized enrichment column was investigated. On the basis of results obtained in other research programmes in our department, Sep-Pak C_{18} with a particle size of 55–105 μm [11], as present in commercially available off-line solid-phase extraction cartridges (Millipore), was tested. A 10 × 2.1 mm I.D. enrichment column, fitted with 20-μm screens, was slurry packed with this material. After successful preliminary experiments, the original and modified enrichment procedures were compared by carrying out routine analysis of 29 liver and kidney samples obtained from a pharmacokinetic study with CBX. For each sample the desoxy-CBX concentration was determined based on measurement of peak areas. The results were statistically evaluated by a Student *t*-test. The calculated *t*-value (1.68) was well below the critical value for a double-sided 95% confidence interval (2.05), showing that the

TABLE II

COMPARISON OF AVERAGE ANALYTICAL RECOVERIES (%) OBTAINED WITH SPIKED SAMPLES (10 μg/kg) USING DIFFERENT COLUMN ELUATE FRACTIONS[a] FROM THE ALUMINA/FLORISIL COLUMN ($n=4$)

Tissue	Eluate fraction[b]	Average recovery		R.S.D. %
		Analyte	%	
Muscle	A	CBX	59	7.9
		Desoxy-CBX	89	8.9
	B	CBX	86	0.6
		Desoxy-CBX	100	3.5
	T	CBX	70	3.2
		Desoxy-CBX	91	5.6
Kidney	A	Desoxy-CBX	88	18.0
	B	Desoxy-CBX	98	8.0
Liver	A	Desoxy-CBX	91	6.9
	B	Desoxy-CBX	99	17.0

[a] The final procedure in Table I was used.

[b] A = first 10 ml of eluate; B = second 10 ml of eluate; T = 10 ml of the combined total eluate.

results obtained with the two enrichment columns were not significantly different.

The average peak width at half-height obtained with the 10-mm Sep-Pak C_{18} column was slightly smaller and much more reproducible than that obtained with the 60-mm Bondapak C_{18}/Corasil column, *viz.*, 0.24 mm ± 9% *versus* 0.25 mm ± 23%. It can therefore be concluded that the Sep-Pak C_{18} material present in the 10-mm column, which is only 3% of the amount of Bondapak C_{18}/Corasil in the 60 × 4.6 mm I.D. column, retains CBX and its metabolites very efficiently in the presence of a biological matrix. To ensure that the low analytical recovery of CBX in muscle (see Table II) was not due to breakthrough on the 10-mm enrichment column, a number of 10 μg/kg spiked samples were also analysed using a 60 × 3 mm I.D. enrichment column filled with Sep-Pak material. The recovery results obtained were fully comparable.

The low cost of the packing material and the ease of packing the cartridge allow the daily replacement of the 10 × 2.1 mm I.D. Sep-Pak C_{18} column during routine analysis, *i.e.*, after about 20 samples.

Chromatography and detection

Chromatography. The chromatographic cycle as described earlier [10] takes about 55 min per sample. Owing to the technical features of the autosampler used, more than 10 min are required for a reliable injection of 2 ml of sample extract. Considering the large number of samples that have to be analysed in pharmacokinetic or monitoring studies, it is desirable to speed up the procedure. Chromatograms obtained with the original method [10] showed that the chromatographic separation between CBX and its metabolites, and also the separation from matrix components, are not very critical when a Chromspher C_{18} column (200 × 3 mm I.D.) (Chrompack) was used as the analytical column. Therefore, the length of the column was reduced to 100 mm. To maintain the separation of the N^1- and N^4-monooxy-CBX metabolites the eluent composition was modified slightly (*viz.*, the acetonitrile content was lowered from 15% to 14%). Fig. 1 shows chromatograms obtained for standard solutions with the two chromatographic systems.

The time required for the analytical separation after back-flushing from the enrichment column has now been reduced from 40 to 25 min. By applying a concurrent operation of the processes of enrichment and separation, as is shown in Fig. 2, the total analysis time per sample (except the first sample) was reduced to 31 min.

An alternative for speeding up the procedure is to connect the enrichment column directly with the valve of the auto-injector, omitting the sample loop. The special properties of the injector used and the small dimensions of the concentration column allow the sample injection to the enrichment column and its use to flush the enrichment column. Injection directly onto the enrichment column makes it possible to use a higher injection speed; as a result, the same volume can be injected in a shorter time with the same reliability. Further, only one solvent delivery system is necessary. With this procedure also a chromatographic cycle of about 31 min is possible.

Injection volume and detection. The injection volume was reduced from 2 to 1 ml, in order to be able to inject each extract in duplicate and to improve the performance of the enrichment column. To compensate for decreased sensitivity, the choice of detection wavelength was evaluated. The peak heights of standard solutions of CBX and its relevant metabolites were measured at four different wavelengths. Table III

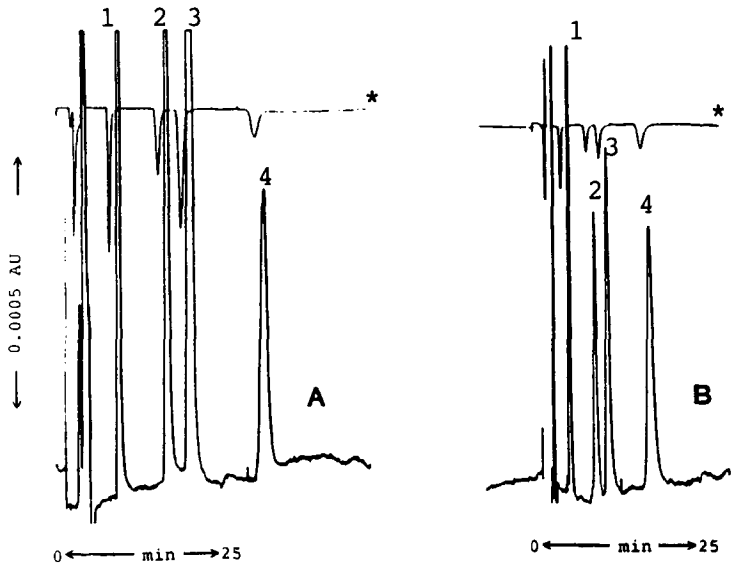

Fig. 1. LC separation of standard solutions of CBX and its metabolites under the conditions of (A) the original chromatographic system [10] and (B) the optimized conditions (Table I). Peaks: 1 = CBX; 2 = N^4-monoxy-CBX; 3 = N^1-monoxy-CBX; 4 = desoxy-CBX. The traces marked with asterisks were recorded at a 10-fold lower sensitivity.

shows the responses obtained. The system noise was found to be constant over the wavelength range investigated. From the data, it can be concluded that the 390-nm response for desoxy-CBX is more than double that at 420 nm. The responses of CBX and N^4-monoxy-CBX are also higher, although not so pronounced. The analysis of a number of blank muscle, liver and kidney samples, processed according to the modified procedure and detected at 390 nm, revealed no increase in matrix interferences in the chromatograms (see Fig. 3). Consequently, the decrease in sensitivity of desoxy-CBX resulting from the injection of only 1 ml was fully compensated by changing the detection wavelength. None of the compounds listed in Table IV interfered in the determination of CBX and desoxy-CBX.

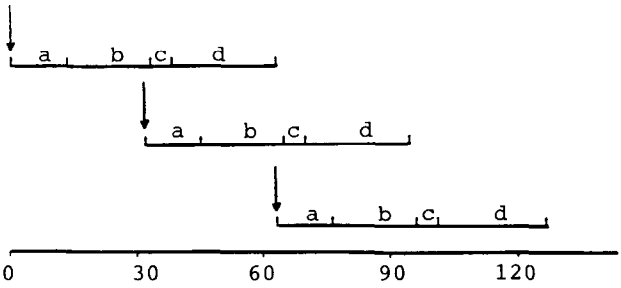

Fig. 2. Timetable showing the concurrent processes of (a) injection of sample, (b) enrichment and flushing of sample, (c) backflushing to the analytical column and (d) chromatographic separation; three sample cycles are shown.

TABLE III

UV–VIS RESPONSE (PEAK HEIGHT) AT FOUR WAVELENGTHS OF CBX AND ITS METABOLITES AFTER LC SEPARATION AND POSTCOLUMN REACTION

Wavelength (nm)	Peak height (cm)			
	CBX	N^4-Monoxy-CBX	N^1-Monoxy-CBX	Desoxy-CBX
380	26.0	10.2	12.6	10.5
390	25.0	11.9	14.1	10.7
400	23.5	11.8	16.1	8.4
420	21.5	10.2	17.9	4.2

Application in routine analysis

Swine tissues. The modified method was routinely used in a pharmacokinetic study with swine. More than 200 muscle, kidney and liver samples of carbadox-treated swine were analysed. The samples were processed at the Central Veterinary Institute (CDI) and the residue levels were determined at the RIKILT laboratories, using the column-switching LC procedure.

Results were calculated by use of a calibration graph. This graph proved to be linear over the range from 1 to at least 100 ng/ml of desoxy-CBX ($r^2 = 1.0000$) for peak area and peak height. Peak areas are routinely used for the calculations. The concentration of 100 ng/ml corresponds with a concentration of 190 μg/kg in tissues, which is more than the maximum level found for desoxy-CBX in liver and kidney from carbadox-treated animals after zero days withdrawal time. With each series of analysis, blank samples and samples spiked with desoxy-CBX (10 μg/kg) were analysed. The mean recovery for desoxy-CBX in the liver, kidney and muscle samples was 95% [relative standard deviation (R.S.D.) = 17%, $n = 17$, 10 μg/kg]. In the same period a number of spiked samples were analysed at the RIKILT laboratories by technicians having more experience with the method. The recovery found was also 95%, but the reproducibility was better, *viz.*, R.S.D. = 11%, $n = 20$. Recoveries found for tissue samples spiked at 10 and 100 μg/kg desoxy-CBX were comparable.

TABLE IV

VETERINARY DRUGS THAT DID NOT INTERFERE WITH THE DETERMINATION OF CBX AND DESOXY-CBX

Standard solutions corresponding to a tissue concentration of 100 μg/kg were injected into the HPLC system.

Chloramphenicol	Furaltadone	Nitrofurantoin	Sulphanilamide
Chlorotetracycline	Furazolidone	Nitrofurazone	Sulphadimethoxine
Clopidol	Fenbendazole	Nitrovin	Sulphadoxine
Dapsone	Furnicozone	Olaquindox	Sulphamerazine
Decoquinate	Halofuginone	Oxytetracycline	Sulphamethazine
Dimetridazole	Ipronidazole	Pyrantel tartrate	Sulphamethoxazole
Dinitolmide	Methylbenzoquate	Robenidine	Sulphaquinoxaline
Doxycycline	Nicarbazine	Ronidazole	Tetracycline
Ethopabate	Nifursol	Sulphadiazine	Thiophanate
			Trimethoprim

The limit of determination for desoxy-CBX in liver, kidney and muscle, calculated as the mean of the measured content of indepedent representative blank samples ($n=20$) plus six times the standard deviation of the mean [12], was 2 μg/kg.

Plasma. In the course of the pharmacokinetic study, a number of plasma samples were analysed. The procedure developed for swine tissue proved to be applicable unchanged to heparinized plasma. The recoveries for CBX and its reduced metabolites were high and reproducible. This is particularly important for CBX because it was found to be the major residue present in plasma. Table V shows the results of spiking experiments.

Eggs. The modified method was also applied to egg samples spiked with CBX and desoxy-CBX. When 10 g of homogenized whole egg, egg white or egg yolk were processed, very clean chromatograms and high, reproducible recoveries were obtained, as shown in Table V and Fig. 3. The limit of determination was about 0.5 μg/kg for both CBX and desoxy-CBX.

Stability of CBX and desoxy-CBX in animal products

During routine analysis, large series of liver and kidney samples had to be analysed and spiked samples were sometimes left to stand for a longer time before starting the extraction. These samples showed low CBX recoveries and interferences were observed in the chromatograms. A series of experiments were performed to investigate the stability of CBX after addition to liver and kidney (10–100 μg/kg). Homogenized bulk samples were spiked with CBX and stored at 4°C. Starting after 30 min, at regular intervals, 10-g aliquots were taken from the samples and extracted. The concentrations of CBX and desoxy-CBX were determined in each sub-sample while the concentrations of the monoxy metabolites could only be determined because only semi-quantitative standards were available.

Fig. 4 shows a typical example of the concentration profile that is obtained for kidney. On the basis of these experiments, it can be concluded that CBX is rapidly transformed *in vitro* to its reduced metabolites. First, the monoxy metabolites are formed, but after about 1 h most of the added amount of CBX is converted to desoxy-CBX. At these points in time less then 10% of the added amount was still present as CBX The results also lead to the conclusion that it is very unlikely that residues of CBX will be found in real liver and kidney samples and also that desoxy-CBX is the marker residue. The nature of the enzymes responsible for the *in vitro* metabolism of CBX in liver and kidney is not known yet.

TABLE V

AVERAGE RECOVERIES (%) OF CBX AND ITS METABOLITES IN BIOLOGICAL MATRICES

Matrix[a]	n	CBX	N^4-Monoxy-CBX	N^1-Monoxy-CBX	Desoxy-CBX
Plasma	5	81 ± 7	91 ± 5	93 ± 7	83 ± 7
Egg	8	81 ± 7	N.D.[b]	N.D.[b]	91 ± 5
Muscle	8	72 ± 3	80 ± 5	84 ± 7	91 ± 6
Liver/kidney/muscle	37				95 ± 14

[a] Spiking concentrations: 5–10 μg/kg (swine plasma), 5 μg/kg (eggs) and 10 μg/kg (muscle, liver and kidney).

[b] Not determined.

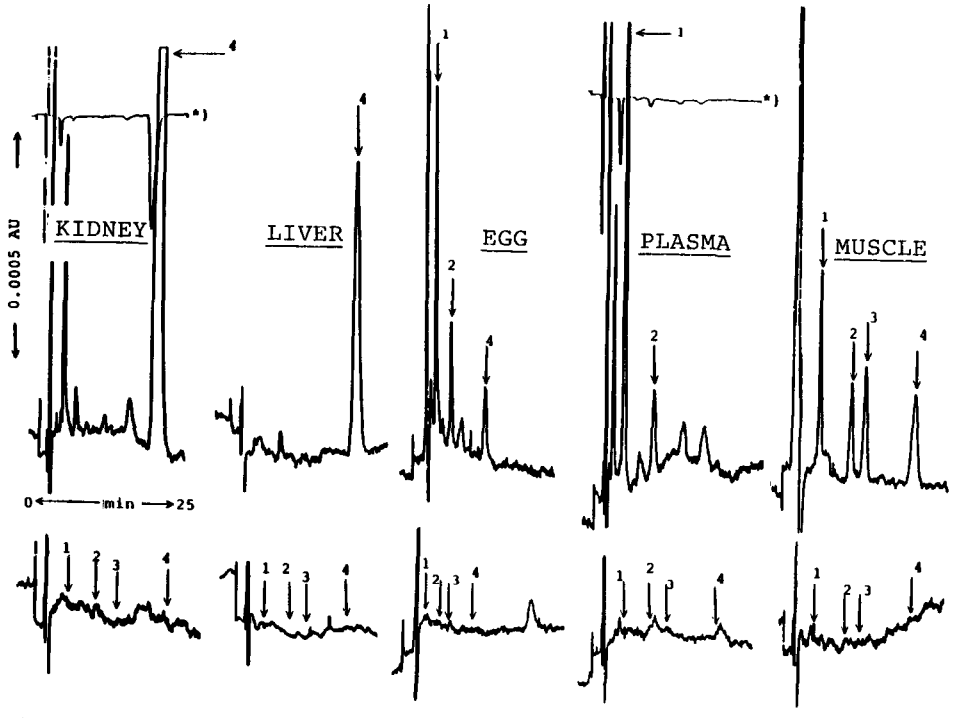

Fig. 3. Typical chromatograms obtained with the modified method under the conditions given in Table I. The lower chromatograms are blanks, the upper traces are for real or spiked samples of kidney (70 μg/kg desoxy-CBX), liver (14 μg/kg desoxy-CBX), egg (6 μg/kg CBX and 2 μg/kg desoxy-CBX), plasma (23 μg/kg CBX) and muscle (5 μg/kg spike of CBX and its metabolites). Peaks as in Fig. 1. The traces marked with asterisks, were recorded at a 10-fold lower sensitivity.

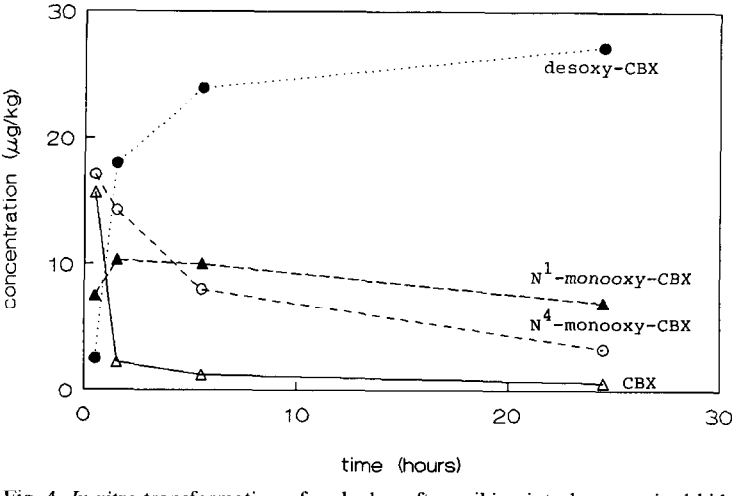

Fig. 4. *In vitro* transformation of carbadox after spiking into homogenized kidney tissue (50 μg/kg) and storage at 4°C. The concentrations of the monoxy metabolites are only estimates.

In contrast with the situation for liver and kidney, CBX proved to be stable under spiking conditions (< 10% decrease after storage of the spiked sample for 1 h at room temperature) in muscle and egg. In eggs that had been stored at −20°C for 6 months, no decrease in CBX concentration was observed ($n = 4$, 2–6 μg/kg). Similar results were found for muscle samples stored at −20°C for 2 months ($n = 4$, 10 μg/kg).

Desoxy-CBX was stable in eggs and swine muscle under the conditions mentioned for CBX and in liver and kidney under spiking conditions. In another experiment, kidney samples spiked with desoxy-CBX (5 μg/kg) were stable for 2 months at −20°C. In the future, additional storage stability studies will be of interest.

CONCLUSIONS

The procedure originally developed for the determination of residues of, mainly, CBX was modified to be routinely applicable to the determination of the major metabolite desoxy-CBX in swine liver, kidney, muscle and plasma and in eggs and to the determination of CBX in the latter three matrices. The concurrent operation of trace enrichment and separation in the column-switching LC procedure and the optimized LC parameters reduced the cycle time from 55 to 31 min. The ruggedness and reproducibility were improved by optimizing the dimensions of the enrichment column and its packing material, and also the off-line column purification. The overall recoveries for CBX in muscle, plasma and egg are 70–80%, with R.S.D. values of about 7%. For desoxy-CBX, the overall recoveries are 83–95% in all the matrices investigated. The corresponding R.S.D.s are between 5 and 17%, depending on the matrix and/or the conditions under which the data were obtained. The limit of determination for CBX is 1 μg/kg in muscle tissue and 0.5 μg/kg in plasma and egg. For desoxy-CBX the values are 2 μg/kg in muscle, kidney and liver and 0.5 μg/kg in eggs.

CBX rapidly decomposes (*in vitro* metabolism) to, mainly, its carcinogenic metabolite desoxy-CBX, when it is added to liver and kidney tissue. Therefore, desoxy-CBX can be considered as the marker residue in these matrices and it is very unlikely that CBX will be found in real samples. CBX has good stability in frozen muscle tissue and in eggs. In all the matrices investigated, desoxy-CBX was stable under the spiking conditions (1 h at room temperature).

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