AKADÉMIAI KIADÓ

Acta Chromatographica

33 (2021) 4, 354–360

DOI:
10.1556/1326.2020.00827

ORIGINAL RESEARCH PAPER

Simultaneous determination of carbofuran and 3-hydroxycarbofuran in duck liver by an UPLC-MS/MS

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Received: July 31, 2020 • Accepted: August 18, 2020
Published online: September 25, 2020

ABSTRACT

Carbofuran is a carbamate pesticide, a broad-spectrum, high-efficiency, low-residue, and highly toxic insecticide, acaricide, and nematicide, widely used in agriculture. Carbofuran is most harmful to birds, and birds or insects killed by furan poisoning can be killed by secondary poisoning after being foraged by raptors, small mammals, or reptiles. In this paper, an UPLC-MS/MS method was developed for the determination of carbofuran and its metabolite, 3-hydroxycarbofuran, in duck liver. Liver tissue was first ground into a homogenate and then passed through ethyl acetate liquid-liquid extraction processing samples. Multiple reaction monitoring (MRM) mode was used for quantitative analysis, *m/z* 222.1 → 165.1 for carbofuran, *m/z* 238.1 → 180.9 for 3-hydroxycarbofuran and *m/z* 290.2 → 198.2 for an internal standard. The standard curves of carbofuran and 3-hydroxycarbofuran in duck liver were within a range of 2–2000 ng/g, where the linearity was good, the lower limit of quantification was 2 ng/g. The intra-day precision of carbofuran and 3-hydroxycarbofuran was <14%, and the inter-day precision was <13%, the accuracy range was between 91.8 and 108.9%, the average extraction efficiency was higher than 75.1% with a matrix effect between 93.4 and 107.7%. The developed method was applied to a situation of suspected duck poisoning at a local farm.

KEYWORDS

Carbofuran, metabolite, duck, poisoning, UPLC-MS/MS

INTRODUCTION

Carbofuran, also known as Furadan™, is a broad-spectrum, high-efficiency, low-residue, and highly toxic carbamate pesticide [1, 2]. Carbofuran poisoning is mainly caused by the inhibitory activity of cholinesterase in the body, which makes cholinesterase lose its hydrolytic ability [3, 4]. This mechanism is similar to that of organophosphorus pesticides, but cholinesterase is reversible and less toxic than organophosphorus pesticides [5, 6].

Since the use of pesticides from the middle of the 1900s, the application of pesticides on crops and the development of agricultural productivity have been inextricably linked [1, 7]. Therefore, the research and exploration of pesticides have also attracted the attention of most people [8, 9]. Domestic and wild animal deaths due to carbamate poisoning are increasingly common, and, in many cases, there is a criminal intent. Carbofuran was the most commonly found pesticide in poisoned dead birds from the French Pyrenees (18 cases), followed by aldicarb (3 cases) [10, 11]. In birds, the acute oral LD50 toxicity for carbofuran can be as low as 238 μg/kg bodyweight (b.w.) for the Fulvous whistling-duck [10]. Conventional analytical

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methods include HPLC [12–14], GC-MS [15], and LC-MS/MS [16]. Another method, UPLC-MS/MS, has high sensitivity and good selectivity.

So far, there have been several reports on the detection of carbofuran and its metabolite, 3-hydroxycarbofuran in *vivo* or in *vitro* [10, 17–20]. However, to our best knowledge, none of these documents report on carbofuran and its metabolite, 3-hydroxycarbofuran, in duck liver by UPLC-MS/MS. In this paper, an UPLC-MS/MS method was developed for the determination of carbofuran and its metabolite, 3-hydroxycarbofuran, in duck liver, and applied to duck poisoning cases.

MATERIALS AND METHODS

Chemicals

Carbofuran, 3-hydroxycarbofuran, and diazepam-d5 (internal standard), all purity >98% (Fig. 1), were purchased from Sigma-Aldrich Co. LLC (St. Louis, MO USA). Acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Ultrapure water was prepared by a Millipore Milli-Q purification system (Bedford, MA, USA).

Instruments

The UPLC-MS/MS is composed of an ACQUITY H-Class UPLC and XEVO TQS-micro triple quadrupole mass spectrometer (Waters Corp, Milford, MA, USA).

The column was UPLC BEH C18 (2.1 × 50 mm, 1.7 μm, Waters, USA), and the column temperature was set at 40 °C. The mobile phase consisted of 10 mmol/L ammonium acetate (containing 0.1% formic acid) in water and acetonitrile. The mobile phase gradient was eluted with a flow rate of 0.4 mL/min. From 0 to 0.2 min, acetonitrile was maintained at 15%; from 0.2 to 1.2 min, acetonitrile increased from 15 to 85%; from 1.2 to 2.1 min, acetonitrile remained at 85%; from 2.1 to 2.4 min, acetonitrile was changed from 85 to 15% and; from 2.4 to 4.0 min, acetonitrile stayed at 15%.

Desolvation gas was set at 850 L/h and cone gas was set at 50 L/h. The capillary voltage was set at 1.5 kV, and the desolvation temperature was 450 °C. MRM mode was used for quantitative analysis, *m*/*z* 222.1 → 165.1 for carbofuran (cone voltage 30 V, collision voltage 12 V), *m*/*z* 238.1 → 180.9 for 3-hydroxycarbofuran (cone hole voltage 25 V, collision voltage 12 V) *m*/*z* 290.2 → 198.2 for diazepam-d5 (cone voltage 25 V, collision voltage 30 V), Fig. 2.

Preparation of reference solution

Stock solutions of carbofuran (1.0 mg/mL), 3-hydroxycarbofuran (0.1 mg/mL), and diazepam-d5 (0.1 mg/mL) were prepared with methanol. The stock solution was diluted with methanol to prepare a series of standard working solutions. All were stored at 4 °C.

Standard curve preparation

Blank duck liver tissue was mixed with an appropriate amount of standard working solution to prepare a quasi-curve of whole liver tissue of carbofuran and 3-hydroxycarbofuran, 2, 5, 20, 50, 100, 200, 500, 1,000, and 2,000 ng/g. Quality control (QC) samples (10, 150, and 1,500 ng/g)

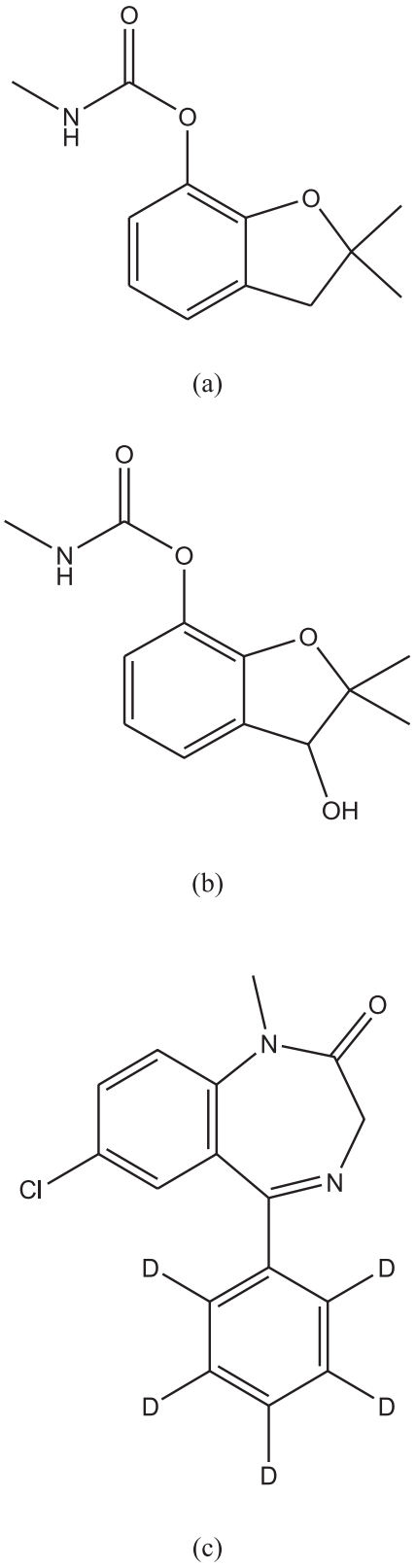

Fig. 1. Chemical structures of carbofuran (a), 3-hydroxycarbofuran (b), and diazepam-d5 (c)

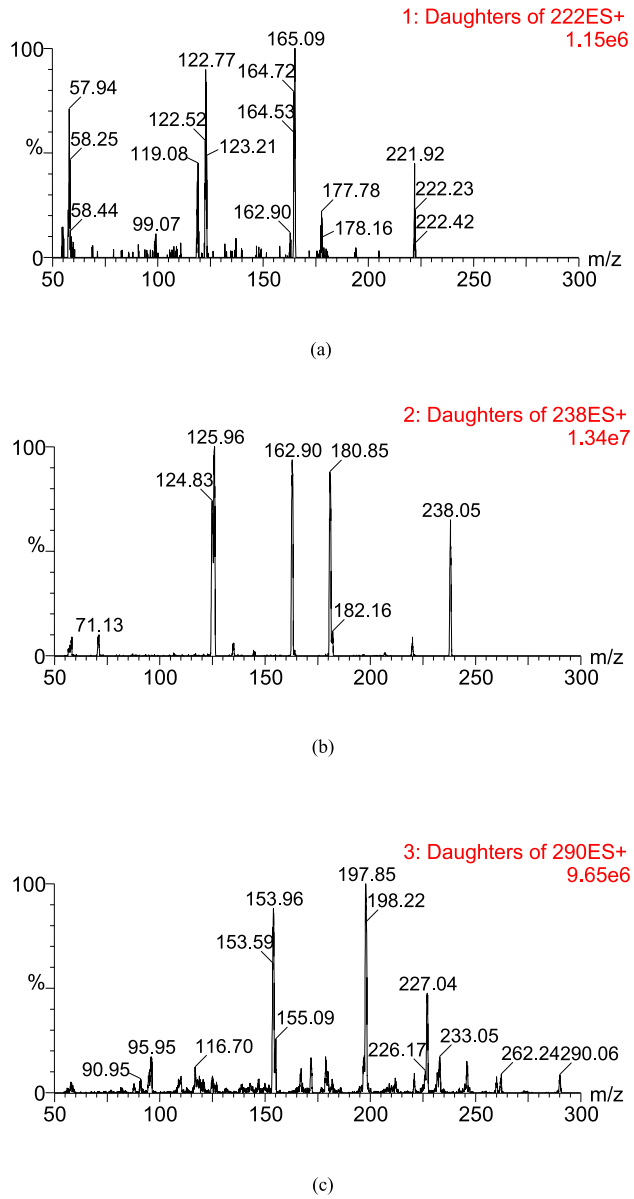

Fig. 2. Mass spectra of carbofuran (a), 3-hydroxycarbofuran (b), and diazepam-d5 (c) in 10 mmol/L ammonium acetate (containing 0.1% formic acid) in water and acetonitrile (1:1, v/v)

were prepared in the same manner as the standard curve. The standard curve samples were treated by ethyl acetate liquid-liquid extraction and analyzed by UPLC-MS/MS.

Sample processing

The liver samples were first thawed. After grinding, 100 mg of accurately weighed liver was placed into a 1.5 mL centrifugation tube, and10 μL of internal standard solution (0.5 μg/mL) was added, 4 mL of ethyl acetate was added, vortexed to mix for 1.0 min, and centrifuged (3,000 rpm, 4 °C) for 10 min; the supernatant (3.5 mL) was then dried under air flow and reconstituted with 100 μL of methanol. After centrifugation, 80 μL of the supernatant was taken into the inner liner of the injection bottle, and 2 μL was injected into UPLC-MS/MS for analysis.

Method validation

A validation method was established in accordance with the US Food and Drug Administration Bioanalysis Method Validation Guidelines [21].

Selectivity. The selectivity of the method was evaluated by analyzing blank liver tissue, blank livers spiked with carbofuran, 3-hydroxycarbofuran, and the internal standard diazepam-d6, and duck liver samples by UPLC-MS/MS.

Linear. The standard curves (2, 5, 20, 50, 100, 200, 500, 1,000, and 2,000 ng/g) of carbofuran and 3-hydroxycarbofuran were in duck liver. Under the same conditions, the peak area of each peak was measured, a standard curve with the peak area against the concentration was drawn, and linearity of the standard curve was evaluated. The precision and accuracy of the lower limit of quantitation should be less than 20% and within 80–120%. The detection limit of the signal-to-noise ratio was three.

Precision and accuracy. The precision and accuracy were evaluated by measuring liver tissue QC samples at three concentration levels in six replicates. Precision was expressed as a relative standard deviation (RSD), and the intra-day and inter-day precisions were determined by measuring QC samples at three concentration levels for three consecutive days. The intra-day and inter-day accuracies were measured between the average value of the three concentration level QC samples and the true value for three consecutive days.

Extraction efficiency and matrix effects. The extraction efficiency was evaluated by comparing the measured peak area of the low, medium, and high concentration QC samples with the corresponding standard peak area. The matrix effect was evaluated by comparing the peak area of the standard solutions of low, medium, and high concentration in the blank liver tissue after sample treatment and the peak area of the corresponding standard solution.

Stability. The stabilities of carbofuran and 3-hydroxycarbofuran in duck liver were investigated by analyzing the QC samples of three low, medium, and high concentration levels placed under three storage conditions. This included long-term stability (−20 °C, 30 days), short-term stability (2 h at room temperature), and freeze-thaw stability (three consecutive freezing and thawing cycles for three days) (−20 °C to room temperature).

RESULTS

Selectivity

The typical UPLC-MS/MS of a blank liver tissue, fortified liver samples with carbofuran, 3-hydroxycarbofuran, and the internal standard, and a liver sample of a poisoned bird, are shown in Fig. 3. The retention times of carbofuran and 3-hydroxycarbofuran and internal standards were 1.99, 1.62,

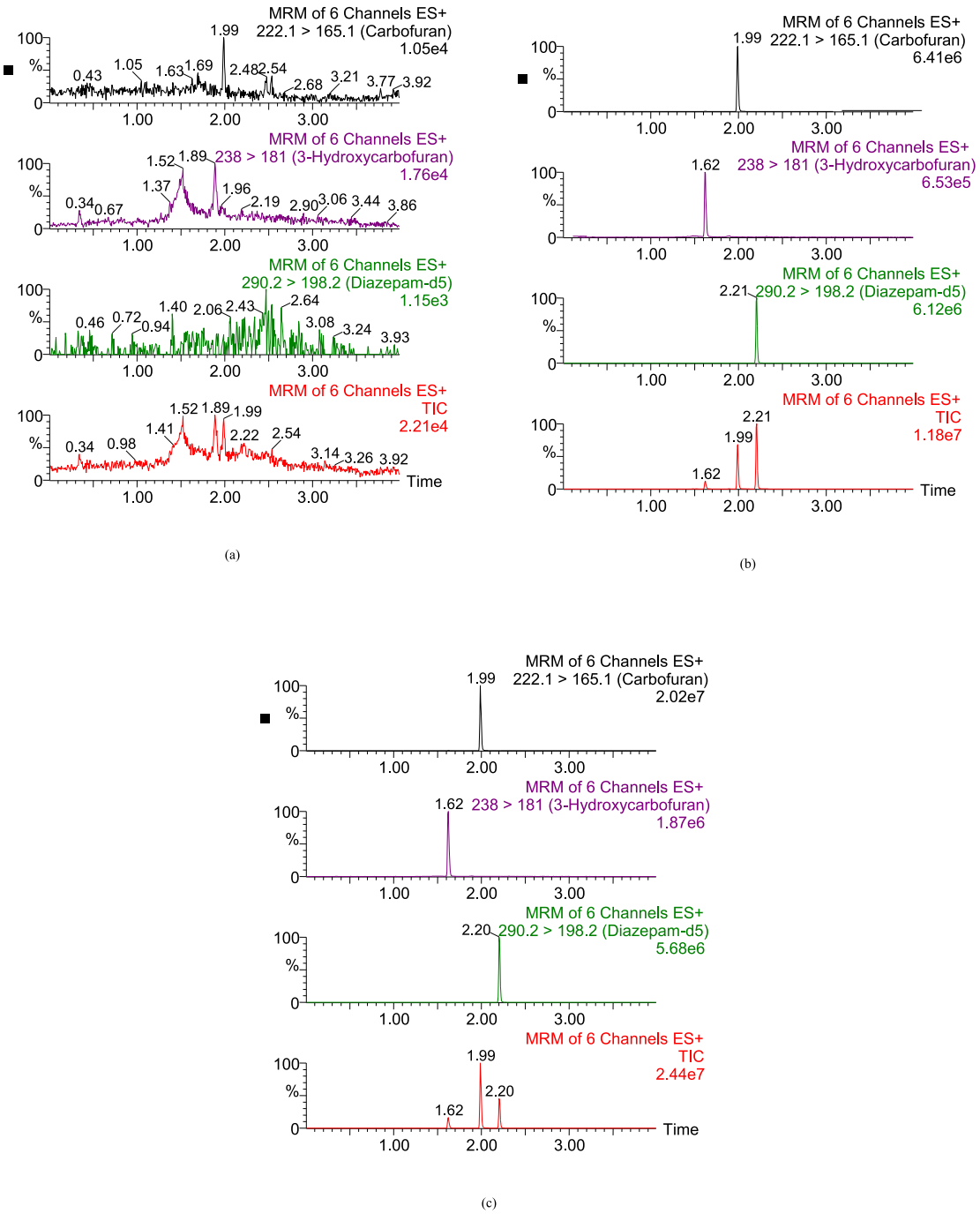

Fig. 3. UPLC-MS/MS chromatograms of carbofuran (t_R = 1.99 min), 3-hydroxycarbofuran (t_R = 1.62 min), and diazepam-d5 (t_R = 2.21 min) in duck liver. a) blank duck liver, b) blank duck liver spiked with carbofuran, 3-hydroxycarbofuran and diazepam-d5, c) a liver sample of poisoned duck.

and 2.21 min, respectively. No obvious co-extractives interfered with the detection.

Standard curve

The standard curves of carbaryl and 3-hydroxycarbofuran in duck liver were in the concentration range of 2–2,000 ng/g. The equations for the standard curves of carbaryl and 3-hydroxycarbofuran are: $Y = (0.0364 \pm 0.0042)\ C + (0.0143 \pm 0.0016)$, $r = 0.9975$; $Y = (0.0503 \pm 0.056)\ C + (0.0216 \pm 0.0018)$, $r = 0.9982$, respectively. Where Y represents the ratio of the peak area ratio of carbaryl and 3-hydroxycarbofuran to the internal standard and C represents the concentration of carbaryl and 3-hydroxycarbofuran in liver tissue. The lower limits of quantitation of carbaryl and 3-hydroxycarbofuran in duck liver are 2 ng/g. The detection limits of carbaryl and 3-hydroxycarbofuran in duck liver are 0.2 ng/g, and the signal-to-noise ratio is 3.

Table 1. Precision, accuracy, extraction efficiency, and matrix effect of carbofuran and 3-hydroxycarbofuran in duck liver (n = 6).

Compound	Concentration (ng/mL)	Accuracy (%)		Precision RSD (%)		Matrix effect	Extraction efficiency
		Intra-day	Inter-day	Intra-day	Inter-day		
Carbofuran	2	96.4	108.9	11.2	12.2	93.4	91.7
	10	98.3	97.0	4.7	11.1	94.5	82.5
	150	106.3	105.2	7.6	9.8	99.0	92.1
	1,500	95.6	97.4	8.5	4.0	91.8	84.1
3-Hydroxycarbofuran	2	109.1	91.8	13.3	11.9	106.7	82.8
	10	98.2	96.4	7.1	8.8	100.1	89.2
	150	94.9	104.4	6.1	10.7	105.9	78.0
	1,500	100.5	96.2	5.1	6.8	107.7	75.1

Precision, accuracy, extraction efficiency, and matrix effects

It can be seen from Table 1 that the intra-day precision RSD of carbaryl and 3-hydroxycarbofuran is less than 14%, the intra-day precision RSD is less than 13%, the accuracy range is between 91.8 and 108.9%, the average extraction efficiency is higher than 75.1%, and the matrix effect is between 93.4 and 107.7%. It showed that the established UPLC-MS/MS method met the analysis of biological samples requirements of carbaryl and 3-hydroxycarbofuran for precision, accuracy, extraction efficiency, and matrix effect.

Stability

For duck liver at room temperature for 2 h, −20 °C for 30 days, and 3 freezing and thawing cycles, the accuracy of carbaryl and 3-hydroxycarbofuran was between 94.9 and 109.6%, and the RSD was within 14% (Table 2). This indicates that carbaryl and 3-hydroxycarbofuran are stable.

Application

A public security bureau entrusted the farm site to test whether the two ducks contained pesticides in the farm and suspected a duck poisoning case. Carbofuran and its metabolite, 3-hydroxycarbofuran, were measured from duck liver tissue. Carbaryl and its metabolite, 3-hydroxycarbofuran, in the liver were then quantified. The concentrations of carbaryl and 3-hydroxycarbofuran in one duck liver were 370 ± 23 and 29 ± 3 ng/g, respectively; the concentrations of carbofuran and 3-hydroxycarbofuran were 480 ± 31 and 56 ± 4 ng/g in the other duck, respectively.

DISCUSSION

Electrosprayed ESI positive and negative electrode selection are often evaluated in methodological studies [22–27]. The experiment found that the ESI positive ion mode was more sensitive than the negative ion mode. The mass spectrometry conditions were optimized, the appropriate cone voltage and collision voltage were selected as the mass spectrometry detection parameters, and the fragment peaks with relatively high fragments were selected as quantitative ion pairs, m/z 222.1 → 165.1 for carbofuran (cone voltage 30 V, collision voltage 12 V), m/z 238.1 → 180.9 for 3-hydroxycarbofuran (cone voltage 25 V, collision voltage 12 V), and m/z 290.2 → 198.2 for internal standard (cone voltage 25 V, collision voltage 30 V), and shown in Fig. 2.

The liquid chromatography conditions should be as far as possible from the separation of endogenous interfering substances from the retention time of carbofuran, 3-hydroxycarbofuran, and the internal standard. This experiment examined methanol or acetonitrile-0.1% formic acid, methanol or acetonitrile-10 mmol/L ammonium acetate buffer solution (containing 0.1% formic acid), and methanol or acetonitrile-10 mmol/L ammonium acetate buffer solution (containing 0.05% ammonia). It was found that the peak shape of the carbofuran and 3-hydroxycarbofuran was poor when methanol was used as the organic phase. Carbofuran and 3-hydroxycarbofuran were more stable under neutral and acidic conditions. Under comprehensive comparison, the gradient elution effect of acetonitrile-10 mmol/L ammonium acetate buffer solution (containing 0.1% formic acid) was the best. The peak shapes of carbofuran and 3-

Table 2. Stability of carbofuran and 3-hydroxycarbofuran in duck liver (n = 6).

Compound	Concentration (ng/mL)	Autosampler ambient		Ambient 2h		−20 °C 30d		Freeze-thaw	
		Accuracy	RSD	Accuracy	RSD	Accuracy	RSD	Accuracy	RSD
Carbofuran	10	102.0	4.9	97.0	6.4	98.2	4.2	107.9	13.8
	150	99.3	3.7	100.9	1.7	97.4	3.8	95.6	5.4
	1,500	98.8	3.8	102.3	2.8	104.3	3.7	97.4	3.2
3-Hydroxycarbofuran	10	100.6	4.9	99.7	9.9	96.7	9.9	109.6	13.8
	150	101.6	3.1	105.1	8.3	102.9	7.8	94.9	2.3
	1,500	97.8	0.9	95.3	2.0	100.5	6.7	95.5	2.4

hydroxycarbofuran were acceptable, and the peak time was appropriate.

It was also an important task to select the internal standard during the method establishment process [28–31]. In this experiment, several compounds including diazepam-d5, carbamazepine, bupivacaine, and lidocaine were compared. It was comprehensively shown that diazepam-d5 had a better peak shape, a stable structure, and the peak time was similar to that of carbofuran and 3-hydroxycarbofuran, and it could meet the correction function of the internal standard of the experiment.

Prior to UPLC-MS/MS analysis, the removal of proteins and potential interferences was a key point for method establishment [32, 33]. Carbofuran and 3-hydroxycarbofuran are soluble in organic solvents such as acetonitrile and ethyl acetate. Liquid-liquid extraction method was selected for pretreatment. In this experiment, the extraction efficiencies of acetonitrile, ethyl acetate, and methanol were compared. The extraction efficiencies of ethyl acetate and acetonitrile (around 80%) were similar, which were better than methanol (around 70%). The protein precipitation effects of acetonitrile and methanol were good, but the methanol extract has many impurities; when the liquid was blown dry in the air stream, ethyl acetate takes a short time, while acetonitrile and methanol take a long time. Taking ethyl acetate as the extraction solvent in comprehensive consideration, the method validation shows that using ethyl acetate for liquid-liquid extraction could obtain satisfactory sensitivity and extraction efficiency.

Carbofuran is a carbamate pesticide, a broad-spectrum, high-efficiency, low-residue, and highly toxic insecticide, acaricide, and nematicide widely used in agriculture [7, 34]. Oral toxicity is highly toxic, and transdermal toxicity is moderately toxic [35]. After entering the human body, carbofuran can inhibit the activity of blood cholinesterase [36]. The acute poisoning caused by it is mainly manifested in significant sweating, fatigue, blurred vision and nausea, and vomiting [37]. In severe cases, pupil dilation and facial and body muscle tremors may occur or render one unconscious [38]. The samples collected by the duck poisoning and public security were representative, the gizzard contents of the duck, and the liver tissue of the duck. All three samples were measured for carbofuran, and 3-hydroxycarbofuran was also measured in the liver tissue, which provided a good basis for the final determination of the cause of poisoning, and also provided a good treatment for future farm poisoning incidents. Through the monitoring basis provided by this laboratory, the public security personnel finally determined that the poisoning was carbofuran. Duck liver tissue was used to measure the metabolite, 3-hydroxycarbofuran, which was also used to further exclude the external contamination of biological samples.

CONCLUSION

In this study, a sensitive, rapid, and selective UPLC-MS/MS method was established for the determination of carbofuran and 3-hydroxycarbofuran in duck liver with a linear range of 2–2,000 ng/g and a detection limit of 0.2 ng/g. UPLC-MS/MS has faster analysis time and higher sensitivity than traditional HPLC. It only takes 4 min to complete the analysis of one liver tissue sample, which could save a lot of time and solvent. We successfully applied this method to the poisoning case of carbofuran.

Conflict of interest statement: The authors declare that there is no conflict of interest regarding the publication of this paper.

Data availability statement: The data used to support the findings of this study are included within the article.

ACKNOWLEDGEMENTS

This work was supported by the Zhejiang Provincial Natural Science Foundation of China (LY19H230001), Wenzhou Science and Technology Bureau (Y20180106).

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