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Determination of Triphenylmethane Dye in seafood by Liquid Chromatography and Tandem Mass Spectrometry

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Abstract

Triphenylmethane dyes (TPMs) are synthetic dyes used for a wide range in industrial applications. Traditionally in aquaculture production TPMs is applied for antibacterial, anti-fungal, anti-parasitic properties due to the low cost. TPMs are mutagenic and carcinogenic to human beings; therefore US and EU have established a law of a zero tolerance of TMPs to be prohibited in agriculture, fisheries and other related industries. TPMS also exist some deficiencies, such as rapid metabolic property to form a reducing metabolite easily in the aquaculture products tissue. In analysis TPMS also have problems of complicated sample pretreatment and analysis instability of instrument. In this paper we develop a new simple method to improve the simplicity, efficiency and accuracy of analysis. The method was developed and validated to analyze the residues of seven triphenylmethane dyes and their metabolites (Malachite green, Leuco malachite green, Crystal violet, Leuco crystal violet, Methylene blue, Methyl Red, Azure B, Nile blue and Victoria Blue) in aquaculture products by using LC/MS/MS. The sample is prepared by a modified QuEChERS technique, which is Quick, Easy, Cheap, Effective, Rugged, and Safe. The optimal extraction and cleanup conditions are needed in experimental design. The validation parameters from the modified method need to meet the requirements of substances in aquaculture products, which establish by regulatory agencies. The results show that nine TPMs can be identified and quantified between 0.5 and 100 ng/mL of concentration and possessed the recovery between 100% and 97.1%(n=15). Under reproducible conditions, RSD had precision between 2.15% and 6.9%. Overall, the finding highlights the impact of simplicity, sensitivity and rapidity as well as the simultaneous determination of analytes in aquatic products

Keywords: Triphenylmethane dyes, QuEChERS, LC/MS/MS

Introduction

Triphenylmethane dyes is a colorless solid, which is soluble in nonpolar organic solvents but not in water. Triphenylmethane (TPM) dyes has the basic skeleton of synthetic dyes called triarylmethane dyes. Many of the triphenylmethane (TPM) dyes are pH indicators, and some display fluorescence [1,3]. Triarylmethanes (TPM) dyes such as malachite green(MG), leucomalachite green(LMG), methyl red(MR),cystal violent(CV), leucocrystalviolent(LCV), methyleneblue(MB), azure b, nile blue(NB) and victoria blue (VB) are potentiallycarcinogenic and mutagenic [3,5]. Triphenylmethane dyes is not a legal veterinary drug in the USA and European Union (EU) [5,6]. Those compounds in seafood products, namely 1.0g/kg(US) and 2.0 g/kg. The leucoform was an in vivo mutagen in trans- genic female viscera [2].

Triphenylmethane (TPM) dyes has been widely used as a topical fungicide and anti- protozoal agent in fish farming throughput the world for several decades. However, the MG and LMG in TPM dyes often use in the aquatic products. The half-life of MG in fish muscle is approximately 70 hours, and LMG persists even longer in fish tissue [7]. According to the European Commission, the determination of MG residues in fish muscle must reach the minimum of required performance limit of 2 mg/kg for the sum of MG and LMG [8]. The Taiwan Food and Drug Administration has also recommended an analytical method for MG and LMG analysis and has set a detection limit of 0.5 mg/kg for both MG and LMG [11]. CV is also known to be effective in the treatment of fungal infections in fish farming. Consequently, analytical methods for CV, MG, and LMG have been reported in the last few years [9,10]. From a practical standpoint, a quick routine procedure with low detection limit and small sample size is desired.

However, TPM dyes are easily metabolized in animal tissues, and previous reports have focused on the determination of TPM dyes in fish tissue by LC/MS/MS [11,12,16]. This was analytic methods can without oxidation reactions and unstable feature [9,10,12]. Analysis of TPM dyes residues in different fish tissue and feed samples is difficult due to the complexity of the matrix and trace concentration levels. Most sample preparation methods are based on the solvent extraction of TPM dyes from fish tissues using McIlvaine's buffer with acetonitrile [13,14]. Liquid/liquid partitioning and a solid phase extraction (SPE) cartridge were used for sample clean-up [15].

The QuEChERS method is now a widely known methodology for the extraction of several classes of drugs, including pesticides and veterinary drugs, from different matrices. This method minimizes the time required for the extraction and cleaning processes and reduces the sample size, costs, required quantities of laboratory glassware, and levels of solvent consumption [9,10]. The aim of this study was to develop a rapid and easy multiresidue analytical method involving the QuEChERS procedure to determine the levels of compounds of TPM dyes and their metabolites in trout, eel, grouper, and tilapia muscles and fish feeds.

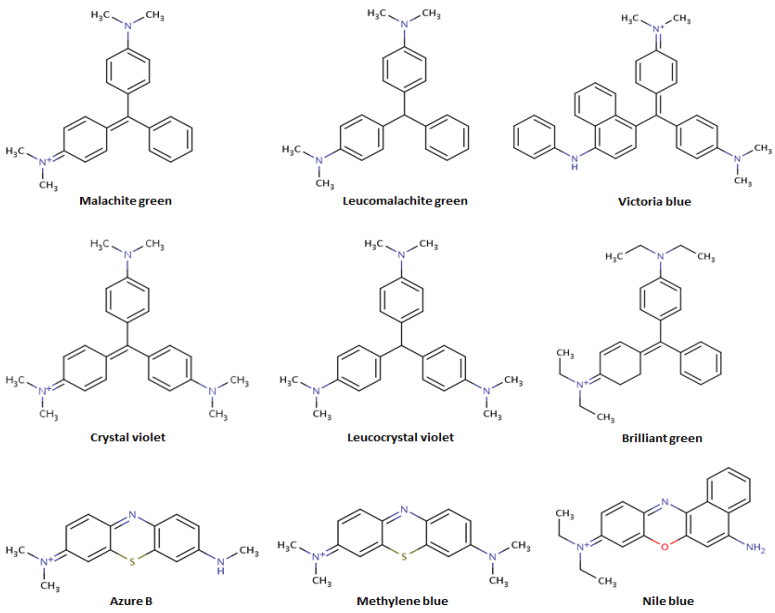

Figure 1. Chemical structures of the TPM dyes in this study.

Methods

Apparatus

The LC/MS/MS system consists of an Agilent Series 1290 Ultra performance liquid chromatography (UPLC) system (Agilent Technologies, Stuttgart, Germany) connected to a Sciex API 5500 plus triple stage quadrupole mass spectrometer (AB Sciex, Chromos, Singapore). LC analysis was performed on a C18 reversed phase column (3.0*100 mm, 1.8μm, ZORBAX Eclipse Plus C18; Agilent) at 40℃. A fast gradient for chromatographic separation of the analytes was performed using water containing 5mM ammonium acetate (mobile phase A; pH=4.5) and LC-grade acetonitrile containing 0.1%

formic acid in Table 1. The flow rate was set at 0.25mL/min. The running time for each sample was set for 15minutes and the injection volume was 5µL. The MS detection system included an ESI source. The ion source block temperature was set at 600℃ and the electrospray capillary voltage was set at + 3500V. The optimal MRM parameters are summarized in Table 1. The flow rate is 0.25 mL/min

Table 1-Gradient program of the mobile phase for UPLC separation of nine TPM dyes.

Time (Min)	A (%) 5mM ammonium acetate in water(pH=4.5)	B (%) 0.1%FA in ACN
0	95	5
2.0	40	60
5.0	40	60
6.0	0	100
9.5	0	100
10	95	5
15	95	5

Table 2- Parameters of MRM conditions of the TPM dyes

Analyte	Quantitation Ion Pair			Qualitative ion pair		
	Q1(m/z)>Q3(m/z)	DP(V)	CE(V)	Q1(m/z)>Q3(m/z)	DP(V)	CE(V)
MG	329>313	100	70	329>208	100	60
LMG	331>239	100	41	329>316	100	29
MB	284..4>268.2	53	44	284.4>240	120	44
MR	270>152	62	68	270>180	62	43
AzB	270>254	80	46	270>228	80	46
CV	372.3>256.2	90	52	372.3>328.2	110	54
LCV	374.4>358.2	110	42	374.4>239	110	40
Nile Blue	318>274	50	53	318>260	90	57
Victoria Blue	422.4>398.1	110	49	422.4>406.4	90	54
MG-d5 IS	334>318	90	53	--	-	-
LMG-d5 IS	334>239	90	43	-		-
LCV-d6 IS	380.2>364.2	110	44	-	-	-

Samples

Samples (muscle of livestock and poultry products) were purchased from local supermarkets and were confirmed to be free of the target drugs. The tissues were homogenized and stored at -20℃ until analysis was begun.

Chemicals, reagents, and solutions

All chemicals were of analytical grade. Acetonitrile, methanol (high-performance liquid chromatography grade) and ammonium acetate by Merck (Darmstadt, Germany). N, N, N', N'-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD; purity 95%) was supplied by Sigma, C18EC was by Agilent. Ammonium formate and sodium chloride were obtained from Merck (Darmstadt, Germany). Ultrapure water (Milli-Q, Millipore Corporation, Bedford, MA, USA) was used to prepare all aqueous solutions. All nine compounds of TPM dyes standards (malachite green, leucomalachite green, methyl red, cystal violent, leucocrystalviolent, methyleneblue (MB), azure b, nile blue and victoria blue) were purchased from Sigma (St. Louis, MO, USA). whose structures are shown in Fig. 1.The internal standards (such as malachite green-d5, leucomalachite Green-d5 lecocrystal Violent-d6) were purchased from TRC. The minimum purity of all standards was 98.0%.

10mM ammonium formate solution was obtained using 0.631 g ammonium formate salt in 1000 mL of water, then adjust to pH=4.5 using formic acid. TMPD solution was obtained by using 50 mg of TMPD and diluting with methanol to 50 ml. The reconstituted solvent consisted of 5mM ammonium acetate solution:acetonitrile (60:40, v/v).

Preparation of standards

Individual stock solutions (1000mg/mL) were prepared by dissolving 10 mg of each compound in 10 mL of acetonitrile. These were stored at-20℃ in brown glass to prevent photo degradation. Mixed standard stock solution and diluting the solution to 10 mL with acetonitrile at concentrations of 100 ng/ml. The mixed standard solutions were stored in amber bottles at -20℃. Three mixed IS solutions were prepared and stored in the same manner.

A working standard solution of ISs at a concentration of 100 ng/mL was determined with subsequent dilutions of

their stock solutions in methanol. When not in use, the working solutions were kept at -20 ℃.

Sample preparation

Aquatic product was sliced from dorsal muscle and homogenized using a high speed blender. Feed sample was first prehomogenized with a large volume blender (up to 1 kg capacity), and then over 50 g was taken and homogenized in a high-speed blender (variable speed up to 10,000 rpm). A well-homogenized fish muscle or feed was accurately weighed (2.0 g) into a 50-mL plastic tube. The tube were adding 6mL of 10mM ammonium formate solution,50μL of TMPD solution ,50μL of ISTD solution and 9mL of acetonitrile. The mix sample solutions were added 2g of sodium chlorite and ceramic homogenizer. **This was vortex for 10 minutes.**

The pellets were separated by centrifuging for 5 minutes at 5000x g and the supernatant was kept. The supernatants were transferred to a new tube containing into 50mg of C18EC. The tubes were shaken vigorously for 3 minute. Following centrifugation at 5000 x g and 10℃ for 10 minutes. The supernatant was evaporated to dryness by a nitrogen blowing concentrator in a water bath at 50℃. The aquatic products were redissolved with 1 mL of 0.1% formic acid in 5mM ammonium acetate solution:acetonitrile (60:40, v/v) respectively. The sample extract was filtered through a 0.22μm Nylon filter into an autosampler vial for the LC-MS/MS analysis.

Method validation

For the method validation, various parameters such as linearity, accuracy, precision, and limits of quantification (LOQs) were evaluated. The peak area of the most intense transition versus the concentration was used to establish the linear regression equation. The linearity of the method was evaluated on the basis of tissue calibration.

Tissue-matched calibration

Tissue -matched calibration curves including fish muscle samples were prepared and used for quantification, respectively. Control samples previously tested and shown to contain no residues were prepared as described in 'General procedure'. Control samples were used for each calibration standard level. pork muscle samples were weighed into 50-mL polypropylene tubes. Blank, calibration curve, and spiked samples. Calibration samples were fortified at levels corresponding to 0.5~100ng/mL. The acceptance criterion was a correlation coefficient $r > 0.995$

Accuracy and precision

The results for accuracy and precision were expressed as the percentage of recovery and the coefficient of variation (CV). Recovery and repeatability were assessed by spiking blank muscle samples at two concentration levels (50and 100ppb) for target analytes in fifteen replicates at each level.

LOQs

LOQs were calculated by analyzing blank samples fortified at 0.5 ppb and defined as the lowest concentration of an analyte for which the signal-to-noise ratio was >10.

Results and Discussion

Method development

The LC/MS/MS method was developed to provide confirmatory data for the analysis of fish muscle samples for TPM dyes- MG, LMG, CV, LCV, MR, MB ,NB, Azure B and VB [1,3,4] . The MS/MS fragmentation conditions were investigated and collision energies were optimized for each individual compound. This yielded four identification points, which provided a suitable confirmatory method in accordance with 2002/657/EC [7]. LC columns and conditions were studied in order to optimize the chromatographic separation in terms of resolution and overall analysis time due to the different

properties of compounds under investigation, ZORBAX Eclipse Plus C18 (3.0*100 mm, 1.8µm; Agilent) using a 5mM ammonium acetate solution and 0.1 %FA in acetonitrile mobile phase was subsequently found to give the most reliable result, good peak shape, and nice resolution. Product ion spectra resulting from collision-induced dissociation were examined and suitable ions selected for multiple reaction monitoring (MRM) schemes (Fig. 2). Numerous LC methods for the determination of TPM dyes- MG,LMG,CV,LCV,MR,MB ,NB ,Azure B and VB . Aquatic products have been proposed. Most of them have applied MS to determine the amount of compounds in samples.

Optimization of sample preparation

Sample preparation plays an important role in analytical methods. Various pretreatment methods have been proposed for monitoring the illegal use of TPM dyes-MG, LMG, CV, LCV, MR, MB, NB, Azure B and VB. Salts and endogenous compounds cannot be fully removed due to the complexity of the biological matrices and the trace levels in real samples, leading to possible matrix effects. In addition, these techniques are time consuming, and the large quantities of organic solvents required, including organic solvent [8,11], may cause environmental pollution. By contrast, the established QuEChERS pretreatment procedure without covering is simple and economical and requires only small amounts of organic solvents. The principle of the QuEChERS method relies on sample cleaning using various dispersive SPE sorbents to analysis [9,10]. To improve efficiency and reduce time consuming sample preparation, the QuEChERS method was developed for the cleaning of target analytes in tissue extracts. The procedure begins with 10mM ammonium formate solution, ISTD solution, TMPD solution and acetonitrile to extraction. While the analytes are transferred to an organic phase, some polar matrix impurities are left in the aqueous layer. A combination of 1g sodium chloride was used as a salting-out agent to partition analyte residues into the acetonitrile layer. C18 enabled satisfactory analyte recovery. The muscle extracts were evaporated under a stream of nitrogen at 40℃ and the final residue was dissolved in 1 mL of 5mM ammonium acetate solution :acetonitrile (60:40, v/v), respectively. The optimized sample preparation protocol enabled a high percentage of recovery for the nine compounds of TPM dyes.

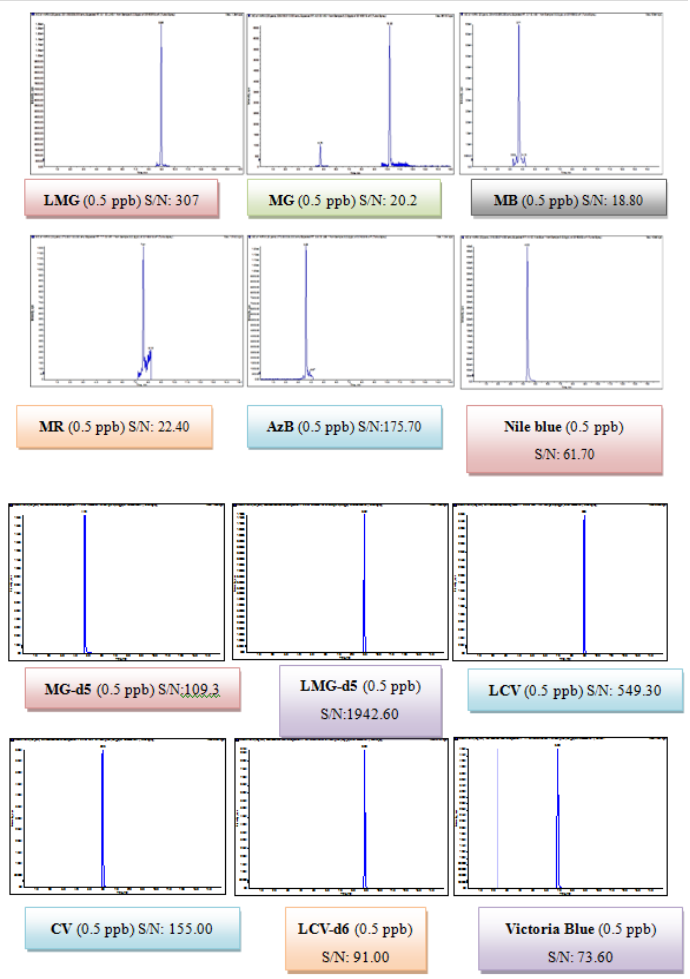

Figure 2- The multiple reactions monitoring (MRM) chromatogram for each of the target analytes in the fish muscle extract spiked at 0.5ppb

Method validation

Linearity

The linearity of the analytical method was validated using the tissue calibration curves for each compound at different concentration levels to prevent matrix effects. The correlation coefficients of the tissue calibration curve are higher than 0.995 for all in muscle sample, which revealed good linearity in the concentration range (0.5~100ng/mL). Table 3 shows R > 0.995 for all nine compounds in fish muscle samples, which revealed good linearity in the concentration range for TPM dyes.

Table.3-Linearity and LOQs of the TPM Dyes

TPM Dyes.	Fish Muscle	
	R	LOQ (ng/mL)
MG	0.9998	0.5
LMG	0.9995	0.5
CV	0.9962	0.5
LCV	0.9999	0.5
MB	0.9996	0.5
MR	0.9993	0.5
Az B	0.9997	0.5
Nile Blue	0.9967	0.5
Victoria Blue	0.9998	0.5

Recovery & Precision

The recovery of seventy-six compounds from pork muscle samples were evaluated at two concentration levels (50 and 100ng/mL) by determining the ratios of the measured and added amounts of the target analyte. The results of the recovery test for the seventy-six compounds in pork muscle samples are listed in Tables3. In muscle samples, the recovery rate of the 1.5 mg/kg and 3.0 mg/kg spiking levels ranged from 60.0% to 120.0% and expressed as the CV. The results are presented in Tables 4. The CV of the muscle samples from 2.0% to 10%.These results showed that the accuracy of this method was satisfactory, respectively (n =15).

LOQ

The LOQ was defined as the concentration at 10 times the signal intensity of noise. The LOQ were to the spiked 0.5 ng/mL in fish muscle sample of nine TPM dyes. The LOQ of this method can achieve to notice methods of taiwan food and drug administration [11]. These results clearly demonstrate the feasibility of the proposed method. The method ensured reliable preparation and precise quantification of nine TPM dyes. This comparison is shown in Fig.2.

Table.4-Recovery rates and CV of the multiclass veterinary drugs from muscle samples

Analyte (n=15)	Fortification concentration level (ng/mL)	CV(%)	Recovery (%)
MG	2.5	2.47	95.1
	5.0	6.84	97.2
LMG	2.5	0	98.0
	5.0	1.02	93.6
MB	2.5	0.42	99.1
	5.0	2.39	96.5
MR	2.5	2.82	91
	5.0	2.23	93.9
AzB	2.5	0.42	105
	5.0	8.02	106
CV	2.5	1.04	109
	5.0	1.25	105
LCV	2.5	1.59	98.1
	5.0	0.2	93.7
Nile Blue	2.5	2.02	107
	5.0	0.42	88.0
Victoria Blue	2.5	1.63	98.9
	5.0	0.47	100

Conclusions

A relatively stable, fast, and selective the QuEChERS extraction procedure and LC/MS/MS method for the

simultaneous determination of TPM dyes- MG, LMG, CV, LCV, MR, MB, NB, Azure B and VB in fish muscle samples was developed. There are few published confirmatory methods for the simultaneous determination of TPM dyes- MG, LMG, CV, LCV, MR, MB, NB ,Azure B and VB in fish muscle samples .This method was validated with blank samples and the extraction procedure was fully optimized. Favorable values of validation parameters such as linearity, recovery, precision, and LOQs were obtained, indicating the suitability of the proposed solvent extraction method for the analysis. The method performed very well in terms of accuracy and stability (n=15). The results of this study were satisfactory for the development of a rugged analytical method.

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