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Multi-dye residue analysis of triarylmethane, xanthene, phenothiazine and phenoxazine dyes in fish tissues by ultra-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Beside the possible illegal use of malachite green in aquaculture, other familiar dyes could also been applied by fraudulent producers due to their antiseptic and antibacterial activity. In this contribution, a new sensitive multi-residue method was developed to determine triarylmethane, xanthene, phenothiazine and phenoxazine dyes in fish by ultra-performance liquid chromatography–tandem mass spectrometry. Samples were extracted with acetonitrile, followed by an oxidation step using 2,3-dichloro-5,6-dicyanobenzoquinone. Further clean-up was performed by tandem solid phase extraction using weak and strong cation exchange cartridges. Extracts were analysed by UPLC-MS^n operating in the positive electrospray ionisation mode (ESI+). The fourteen dyes were separated within only 12 min on a C_{18} BEH column using 1 mM ammonium acetate in water at pH 4.5 and acetonitrile as mobile phases at a flowrate of 0.4 mL min^{-1}. The presented method was validated as defined by the European Union and scientific literature. Good linearity ($R \geq 0.99$ and goodness-of-fit (g) $\leq 10\%$) was achieved over the tested concentration range (0.25–2 ng g^{-1}). Limit of quantification was 0.25 ng g^{-1} for all dyes, with a signal-to-noise ratio of at least 10/1. This is at least 5 to 10 times lower than previous published methods. Limits of detection were all <0.1 ng g^{-1}. Precision and trueness fell within the criteria requested by the EC requirements for this concentration range. Decision limit (CC_α) and detection capability (CC_β) were all <1 and <0.25 ng g^{-1}, respectively. Due to background levels of the xanthene dyes, the two rhodamine dyes could only be determined above 0.75 ng g^{-1}. For these dyes, the method can only be used for screening purposes. To show the applicability of the method, a monitoring study was performed to investigate the occurrence of artificial dyes in wildlife European eel in Flemish rivers

1. Introduction

During the last years, *several* Rapid Alert Reports for Food and Feed (RARFF) were reported by the European Commission (EC) regarding the presence of malachite green (MG) in fishery products. Also another triarylmethane dye, i.e. crystal violet (CV) was mentioned by the RARFF for its unauthorised presence in different aquaculture products [1]. Despite their well known toxicity in humans and fish, these dyes *are still* worldwide used for the treatment of several fungal and parasite infections in aquaculture [2–4]. A remarkable phenomenon is the fact that RARFF reports of MG and CV decreased significantly from 2002 to 2009. For instance, in 2008, only 2 cases were available for MG, while in 2005 almost 50 unauthorised aquaculture products were incurred with MG. The same trend can be seen for CV, comparing 2006 and 2002 [1]. This could indicate that possible replacements of MG and CV could enter the food chain and the environment. Recently, some authors quoted already the importance of brilliant green (BG) as replacement for MG. BG has not been studied yet extensively as MG or CV; but however, structural similarities to MG and CV could indicate

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comparable toxicity to humans and effectively absorption by fish [5]. Also other (structurally) related compounds with possible toxicological implications were covered in residue-analysis of aquaculture products. This could express the growing concern about possible replacement of MG by other basic dyes [6]. In the past, crystal and ethyl violet were used to stamp indelible marks on meat. However, mutagenic properties of these compounds were described [7]. Also the victoria blue derivates (victoria blue B, R and pure blue BO) and methylene blue were known to have good antiseptic properties. Methylene blue (MB) is a photosensitizer and frame shift mutagen and can therefore intercalate in DNA. Its light-induced DNA damage has already been characterized in cellular systems and even in living organisms [8,9]. Xanthene dyes were linked by the Food and Agriculture Organization of the United Nations and scientific literature with the illegal colouring of salmons and fish paste and possible toxicological implications [10,11].

It is worth noting that most all of the above mentioned dyes are free commercially available via the internet and local pet shops for the treatment of ornamental and hobby fish. RARFF reported already the contamination of imported white fish from Vietnam with victoria pure blue BO [1]. Therefore it is obvious that the potential for misuse of these dyes in aquaculture could exist. Also Tarbin et al. mentioned that related dyes in place of MG may go undetected [6].

MG and CV undergo *in vivo* metabolisation to their equivalent colourless leuco-bases. These leuco-forms are formed by an *in vivo* reduction in the liver of treated fish. They have a longer half-life of elimination than their corresponding parent compounds [12,13]. Less scientific literature is available regarding the possible reduction of other dyes to their leuco-forms. Tarbin et al. [6] mentioned that MB could also metabolize into a leuco-form. Thereby, other researchers conducted several studies on the metabolic properties of methylene blue in humans [14–16]. According to these authors, the major metabolite of methylene blue is the leuco metabolite, formed by an acid hydrolysis of the parent molecule. Recently, Hurtaud-Pessel et al. identified the presence of leuco-brilliant green metabolite using high resolution mass spectrometry from an experimental study performed on trout treated with brilliant green [17].

For monitoring programs in the different European countries, the EC has established a 'recommended concentration' (∼minimum required performance level, MRPL) of 2 μg kg^{-1}, expressed as the sum of chromic- and leuco-form for the analysis of MG [18]. On the other hand, no MRPL is available for all other dyes.

Until now, bio-analysis of illegal dyes in aquaculture products is especially focused on the determination of MG, CV, BG and their leuco-metabolites using ultraviolet, fluorimetric or mass spectrometry as detection technique [5,17,42]. Turnipseed et al. developed an analytical methodology for the determination of methylene blue in channel catfish tissues [25]. Also some alternative techniques, like enzyme-linked *immuno*-sorbent assais are available in scientific literature [43,44]. Recently, Tarbin et al. described the multi-residue determination of dyes in fish tissues with tandem mass spectrometry using high volumes of chlorine containing organic solvents for sample extraction [6]. But, in their method the dyes were not determined with the same sensibility as the present method.

In the present study, we described a sensitive and straightforward method for the quantification of dyes in fish tissues by ultra performance liquid chromatography combined with electrospray ionization tandem mass spectrometry. Reduction of the solvent usage speeding up the chromatography by implementing UPLC methods without losses in sensitivity were the points of interest during method development. To prove its applicability, the method was used to *trace* the presence of the dyes in the environment by the analysis of caught European eel. *Anguilla anguilla.*

2. Experimental

2.1. Chemicals, standards and buffer solutions

Malachite green (MG), leuco-malachite green (LMG), crystal violet (CV), leuco-crystal violet (LCV), brilliant green (BG), ethyl violet (EV), azure B (AB), methylene blue (MB), new methylene blue (NMB), nile blue A (NBA), pararosaniline (PR), victoria blue R (VR), victoria blue B (VB), victoria pure blue BO (VBO), rhodamine B (RB) and rhodamine 6G (R6G) were all purchased for Sigma-Aldrich (Bornem, Belgium) (Fig. 1). The internal standards (IS), malachite green-d_5 (MG-d_5) and crystal violet-d_6 (CV-d_6) were both obtained from Witega (Berlin, Germany). Separate stock solutions of all dyes and IS were prepared in methanol at 1 mg mL^{-1}, except for LCV which was prepared in acetonitrile. Two separate working solutions of all dyes and the IS at 10 μg mL^{-1} were prepared by appropriate dilution of the stock solutions in methanol. All stock and working solutions were found to be stable for at least 3 months when stored at 4 °C in the dark. On each analysis day a spiking solution of 20 ng mL^{-1} for the dyes and 200 ng mL^{-1} for IS were prepared by dilution of the respective working solution of 10 μg mL^{-1} with methanol. All spiking solutions were discarded after use. Quality control samples were prepared at 1 ng g^{-1} and analysed at the beginning and the end of the analytical bath to examine post-preparative stability.

Water was of Milli-Q quality (Millipore Corp., Bedford, MA, USA). Solvents used for mobile phase and extraction, i.e. acetonitrile and methanol were of ULC–MS grade (Biosolve, Valkenswaard, the Netherlands). Pentafluoropropionic acid (PFPA) was from Fluka (Bornem, Belgium). Ammonia (39%), disodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$), sodium hydroxide (NaOH), citric acid and sodium acetate were purchased form Merck (Darmstast, Germany). Ammonium acetate, glacial acetic acid and 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) were from Sigma-Aldrich (Bronem, Belgium). Weak cation exchange cartridges (CBA, 3 mL, 500 mg) and strong cation exchange cartridges (SCX, 3 mL, 500 mg) were obtained from Varian (Sint-Katelijne-Waver, Belgium). Whatman Syringe 0.20 μm Filter devices® were from Whatman ('s Hertogenbosch, the Netherlands).

A 0.01 M DDQ solution was prepared by dissolving an appropriate amount in acetonitrile and stored at 4 °C for one month. A 0.005 M *DDQ* solution, to perform sample oxidation reaction, was freshly prepared each week and stored at 4 °C. McIlvain buffer solution at pH 6.5 was prepared by adding 29.65 mL of 0.1 M citric acid to 70.35 mL 0.2 M $Na_2HPO_4{\cdot}2H_2O$. The mixture was adjusted to pH 6.5 with 4 M NaOH.

2.2. Tissue extraction procedure

Two grams of tissue homogenate were transferred into a 50 mL capped propylene tube (Falcon®) and spiked with 25 μL of the IS mixture (200 ng mL^{-1}). After vortex mixing for 15 s, 8 mL of acetonitrile was added followed by 1 g of sodium acetate. The sample tube was well homogenized for 1 min by vortex mixing and rotated for 10 min on a rotary mixture for extraction. After centrifugation (4000 rpm, 10 min, 4 °C), 7 mL of the supernatant was transferred to a clean glass tube. DDQ (1 mL, 0.005 M) was added to the supernatant to allow oxidation reaction. The mixture was left at room temperature in the dark for 30 min and vortex mixed for 5 s every 10 min. After 30 min, the sample was evaporated to dryness under a gentle stream of nitrogen at 50 °C using a Turbovap® LV system (Caliper LifeSciences, Affligem, Belgium). The dry residue was reconstituted in 3 mL of McIlvain buffer pH 6.5/ACN.

For solid phase extraction (SPE), CBA and SCX-SPE cartridges were placed on a vacuum manifold and preconditioned with 3 mL of methanol, 3 mL of water and 3 mL of a McIlvain buffer pH 6.5.