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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 minutes on a C_{18} BEH column using 1mM 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) ≤ 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 0.75ng g^{-1}. For this 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.

Keywords: Residue analysis; dyes; ultra performance liquid chromatography-tandem mass spectrometry; aquaculture products; malachite green; crystal violet

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 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.* (2008) 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.* (2008) 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.* (2011) 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.* (1997) developed an analytical methodology for the determination of methylene blue in channel catfish tissues [28]. Also some alternative techniques, like enzyme-linked immuno-sorbent assais are available in scientific literature [42,43]. Recently, Tarbin *et al.* (2008) 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 wild eel.

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) (Figure 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 Mili-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 ($Na_2HPO_4.2H_2O$), 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, 3mL, 500 mg) and strong cation exchange cartridges (SCX, 3mL, 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 $Na_2HPO_4.2H_2O$. 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 25µL of the IS mixture (200 ng mL^{-1}). After vortex mixing for 15s, 8 mL of acetonitrile was added followed by 1g 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. Additional 4 mL of buffer was added to the SCX cartridge and the CBA cartridge was placed on the SCX-SPE using an adapter. The sample was allowed to pass slowly to the CBA and SCX columns (approximately 0.5 ml min^{-1}). The columns were washed with 3 mL of water and 3mL of acetonitrile. After drying for 5 min, cartridges were disconnected for elution. The CBA cartridge was eluted with 6 mL of a PFPA solution in acetonitrile (3/97, v/v), while the SCX cartridge was eluted with 6 mL of an ammonia 25% solution in methanol (10/90, v/v). The combined eluates were dried under a gentle stream of nitrogen at 50 °C. The dry residue was reconstituted in 250 µL 10 mM ammonium acetate pH 4.5 / acetonitrile (50/50, v/v), poured trough a 0.22 µm filter and a 2 µL aliquot was injected onto the UPLC column.

2.3. Chromatography

The UPLC analysis was performed using an Acquity® sample and solvent manager from Waters (Milford, MA, USA), run by Masslynx software (Version 4.1). Chromatographic separation was achieved using an Acquity UPLC® BEH C_{18} column (2.1 x 100 mm i.d., 1.7 µm) from Waters (Milford, MA, USA). The autosampler and column were kept at 8 and 40 °C, respectively. Mobile phase A consisted of 10mM ammonium acetate pH 4.5 in water, while mobile phase B was acetonitrile. A gradient elution within 12 minutes was run at a constant flow rate of 0.40 mL min^{-1} to separate and elute all compounds as sharp peaks and rinse the column between two successive injections, i.e. a linear gradient from 0-1 min: 10 to 30% B; linear gradient from 1-6.5 min: 30 to 85 B%; 6.6-10 min: 100% B; 10.1-12 min: 10% B.

2.4. MS/MS analysis

The column effluent was interfaced with a XEVO TQ MS® triple quad mass spectrometer (Waters, Milford, MA, USA) operating in the positive electrospray ionization (ESI) MS/MS mode. A divert valve was used to send the eluent to the waste from 0 - 1.8 min and from 7.5 – 12 min. The following tune parameters were used for all compounds: capillary, 2.00 kV; cone 40 V; extractor, 3.00 V; source temperature, 150 °C; desolvation temperature, 550 °C, cone gas flow, 80 L h^{-1}; desolvation gas flow, 1000 L h^{-1}; resolution (LM1, HM1, LM2, HM2), 2.7, 14.9, 2.7, 14.9; ion energy (1, 2), 0.3, 0.7; multiplier 520V; collision gas: argon (Pirani pressure, 7.9 x 10^{-5} mbar); dwell time, 0.003 s. The optimal settings for collision were investigated by direct infusion of a standard solution of all compounds (100 ng mL^{-1}), in combination with the mobile phase, corresponding to a (nearly) 100% fragmentation of the two protonated molecules. The mass spectrometer was operated in the selected reaction

monitoring (SRM) mode. SRM delivers a unique product ion that can be monitored and quantified in a complex biological matrix with a specific cone voltage (V), collision energy (eV) and retention time (min) (Table 1).

2.5. Validation parameters

The presented UPLC-MS/MS method was validated in triplicate on three different days by evaluating a set of parameters which are in compliance with the recommendations as defined by the EU and scientific literature [45-51].

2.5.1. Linearity

Linearity was evaluated at concentrations ranging from 0.25 to 2 ng g^{-1} matrix equivalent. By adding 25 µL, 50 µL, 100 µL, 150 µL and 200 µL of the spiking solution of 20 ng mL^{-1} to 2 g of blank tissue sample, dye concentrations of respectively 0.25, 0.5, 1, 1.5 and 2 ng g^{-1} were obtained. Peak area ratios between dyes and their corresponding internal standard were plotted against their concentration and a regression was performed. For good linearity the correlation coefficient (r) must be ≥ 0.99 and goodness-of-fit coefficient (g) bust be ≤ 10%, as defined by the EU and scientific [45, 46]. Furthermore, to investigate the (non-)linearity of the regression model, applied to quantify, the Mandel's fitting test was used [47]. The test evaluates whether an alternative regression model, i.e. a quadratic model, better fits the data than a line regression model. The peak area were plotted *vs* the concentration. An '*F*-value' was calculated according to the following equation,

$$F = \frac{(n-2)S_{y1}^2 - (n-3)S_{y2}^2}{S_{y2}^2}$$

with S_{y1} and S_{y2} the standard errors of the linear and quadratic regression model, respectively. The '*F*-value' was compared with the tabulated *F*-value, corresponding to the F-distribution with 1 and *n*-3 degrees of freedom and a probability of 99% [47].

2.5.2. Accuracy and precision

Accuracy was expressed as the difference (in %) between the mean found concentration and the spiked concentration of 0.25, 0.5 and 1 ng g^{-1}. As recommended by the 2002/657/EC guideline, all results fell within -50 to +20% [45]. Precision was assessed by analysing fish samples spiked at the concentration levels as for the accuracy evaluation. The relative standard deviation (RSD,%) or coefficient of variation (CV, in %) being the ratio between the standard deviation and the mean found concentration. The obtained value had to fall within 2/3 of the value calculated according the Horwitz equation: RSD_{max} or CV (%) = RSDx2/3, with RSD = $2^{(1-0.5*\log c)}$, and *c* the analyte concentration in g g^{-1}. The RSD or CV should be lower than the RSD_{max} or CV (%) = $2^{(1-0.5*\log c)}$. The between-day precision was evaluated using spiked samples which were prepared and analyzed on different days. The RSD or CV should be lower than the RSD_{max} or CV (%)= $2^{(1-0.5*\log c)}$ [45, 48, 49].

2.5.3. Limit of quantification and limit of detection

The limit of quantification (LOQ) was defined as the lowest level the criteria of accuracy and precision can be fulfilled [49]. The LOQ was established as the lowest point of the calibration curve with a S/N of at least 10 of the last abundant ion [53,55]. The limit of detection (LOD) was defined as the lowest concentration of all dyes which could be detected by the mass spectrometer with reasonable statistical certainty. In our case, it was determined based on the average value found for LOQ samples, and using the S/N = 3/1 criterion [48].

2.5.4. Matrix effect

Matrix effect was investigated by a parallel injection of blank samples spiked after extraction (1 ng g^{-1}), and a reference solution containing the same amount of all dyes. All injections, in matrix as well as in solution, were performed in triplicate. The ratio was made between the average amount of the areas spike / area reference [50].

2.5.5. Specificity

Specificity was evaluated by controlling the presence or absence of peaks at the retention times of all dyes [49]. Known dye-free fish samples were obtained from the local supermarket and had not receive any medication.

2.5.6. Decision limit and detection capability

The decision limit (CC_α) and detection capacity (CC_β) were determined according to the ISO 11843-2 guidelines [51]. Calculation included a weighted regression model assuming that standard deviation linearly depends on the concentration (heteroscedasticity). To determine realistic values, the identification criteria were checked in the chromatograms of all spiked samples obtained during the validation procedure. According the interpretation of the implementation of the Commission Decision 2002/657/EC, practical values of CC_α and CC_β were determined as values where 50% and 95% of the identification criteria were respectively fulfilled [45]. Identification criteria included the relative retention time, the relative abundance of the diagnostic ions and the signal to noise ratio. Relative retention time should always be within the tolerance of ±2.5%, when compared with a standard solution. The

relative abundances of the diagnostic ions and the signal to noise ratios have to fall within the acceptance criteria of the Commission Decision 2002/657/EC [45].

2.6. Applicability of the method

The above-developed method was applied in a monitoring study of synthetic dyes in European eels. Because of the wide use of synthetic dyes in numerous industrial branches, it could be expected that these contaminants end in the environment, plants and animals. To investigate the presence of especially lipophilic compounds, eel is a good bio-indicator [52, 53]. This to generate an overview of the status of dyes in the aquatic environment with their possible origins and to estimate potential threats and health risks of dyes for fish and humans. Ninety-one eels were caught in just as much Flemish rivers, lakes and cannels, randomly selected from 365 sites of the Flemish Eel Pollution Network. Individual European eel *Anguilla anguilla* was selected on the basis of stage and length (40-50cm) to minimize individual variations. Selected eels were measured and weighted at 100 mg accuracy. After being killed by an overdose anesthetizer, skinned and filleted, muscle tissue was taken, weighed again, homogenized and stored in plastic bags at ≤ -20°C pending analysis.

3. Results and discussion

3.1. Optimisation of sample extraction

A multi-residue method for the determination of synthetic dyes with UPLC-MS/MS was developed and validated. Several analytical methodologies for the multi-dye residue analysis were available in scientific literature. However, these methods included only MG, CV, BG and their leuco-metabolites [17-41]. Only two references were found for the determination of MB in aquatic products [19,54]. Andersen *et al.* (2009) included the leuco-metabolite of brilliant green after self-synthesis of the molecule, but were not able to stabilize leuco brilliant green for direct quantitative purposes [5]. These authors assumed that BG is also metabolised into its leuco-metabolites due to the structural similarity compared to MG and CV. Recently, Hurtaud-Pessel *et al.* (2011) identified the presence of leuco-brilliant green in treated trouts (*O. mykiss*), but a custom-made synthesized pure standard of leuco-brilliant green was only stable for 40 h under liquid nitrogen and useless for quantitative purposes [17].

The presented method was developed and optimized with a minimum of extraction solvents. Analytical chemistry is more and more focused on minimization of hazardous wastes like organic solvents [55,56]. Therefore, during method development, efforts were made to minimize the amounts of organic solvents during extraction and to avoid of chlorine containing solvents. Several published methodologies use high amounts of organic solvents per sample analysis (up to 50 mL) [21,29,34,36,37,42] or a dichlorometane-water portioning [5,6,19,22-28,30,31,35]. The current method required only eight milliliters of acetonitrile to extract all dyes from 2 g of fish tissues with a good extraction recovery. With the exception of MG and CV, the leuco-forms of the other dyes of interest were not commercially available as analytical standard. Therefore, an oxidation step with 0.005M DDQ was incorporated in the extraction procedure. The oxidation step with DDQ is a good in situ transformation step to detect all dyes in their chromic form. In scientific literature, no significant differences were showed in residue concentrations of CV about whether or not DDQ oxidation was used during

sample analysis [38]. A further sample clean-up with solid phase extraction was performed to remove the excess of DDQ and to concentrate the extract. In literature, strong cation exchangers were generally used in the extraction of triarylmethane dyes. During preliminary experiments, it was observed that elution step of the solid phase clean-up procedure was the crucial step. AB, MB and NMB were well retained on a strong cation exchange cartridge (SCX), but they could not be eluted with a solution of concentrated ammonia in acetonitrile (up to ~40%). The strong free positively-charged sulphur group of AB, MB and NMB with a pKa ≤1 [57, 58] is most likely responsible for this strong interaction. An alternative elution solvent, 10M NaOH in methanol (20/80, v/v) had to be used in an unreasonably high volume to elute the compounds since it increases beside the pH also the ionic strength. But, the high salt content of elution solvent made it incompatible with UPLC-MS/MS analysis because of large peak broadening and clogging of the heated capillary of the mass spectrometer after only a very few injections. The same phenomenon was observed MCX (=mixed-mode cation exchanger) cartridges. Tabin *et al.* (2008) used a 5M ammonia acetate buffer in water to elute all dyes from the cartridge followed by a further dilution of the eluate with methanol to minimize the matrix effect [6]. During previously experiments, we were unable to elute all dyes with the mentioned buffer solution. Moreover, significant matrix effect was observed resulting in signal suppression, less sensitivity and high limits of quantification. The 5M ammonium acetate buffer was also very viscous and passed very difficult and slow through the different solid phase catridges.

An alternative mechanism was evaluated on a weak cation exchanger (CBA). At pH 6.5, the CBA cartridge was ~100% negatively charged and showed good retention of AB, MB and NMB. An acidic elution was performed to neutralize of the weak ion exchanger sorbent. MB, NMB and AB could be eluted with 1N HCl in acetonitrile (10/90, v/v), but subsequent UPLC-MS/MS analysis resulted in peak broadening due to high ionic strength. Volatile

acids, PFPA and formic acid were tested as alternative. The best result was obtained using 3% PFPA in acetonitrile: almost 100% of the concerning dyes were eluted with 6 mL. The eluate evaporated very quicky and a reconstitution in the mobile phase did not affect chromatographic analysis. The other dyes (MB, PR, MG, NBA, CV, BG, VR, VB, VBO, RB, EV and R6G) with pKa values between 6.90 and 9.36 [58] were not all 100% positively charged at pH 6.5 and only retained for 50% on the CBA cartridge. As a result, a combined solid phase extraction was performed to enhance recoveries and minimizes the loses of the dyes. The CBA was placed above the SCX to capture all dyes of interest. Indeed, all compounds were extracted with a good recovery with an overall limit of quantification of 0.25 ng g^{-1}.

Typical chromatogram of a blank spiked tissue sample at 0.25 ng g^{-1} and a blank chromatogram are show in Figure 2A.

3.2. Method validation

The following performance parameters were evaluated: linearity, limit of detection, precision, recovery, accuracy, specificity, limit of quantification, decision limit and detection capacity. A general overview of the validation results is presented in Table 2.

3.2.1. Linearity

According to the Mandel's fittings test, when the calculated F-value is below the $F_{0.99}$, the quadratic model is not significantly better than a linear model. The $F_{0.99}$ equals 8.6832 for all dyes, except 10.5614 for PR. Based on the obtained '*F*-values' described in Table 2, a

straight-line regression model preferred from a quadratic correlation up to 2 ng g^{-1} with an LOQ of 0.25 ng g^{-1} for all compounds. Moreover, the goodness-of-fit coefficients (g) and correlation coefficients (r) were all below the acceptance criteria of 0.99 and 10%, respectively.

3.2.2. Accuracy and precision

Accuracy, 'within-day' and 'between-day' precision were evaluated on 3 different spike levels for all dyes: 0.25, 0.5, 1 ng g^{-1}. Results are summarized in Table 2. The accuracy for all dyes fell within the ranges of -50 and +20%. The precision, expressed as R.S.D. or CV values, for all levels tested, fell within the maximum R.S.D. or CV values given by the Horwitz equation.

3.2.3. Limit of quantification and limit of detection

The LOQ was set at 0.25 ng g^{-1} for all dyes This is lowest level that could be quantified fulfilling the criteria for accuracy and precision and with a S/N ration of at least 10/1. In our opinion, this is the first method that is able to quantify all this illegal dyes at the level of 0.25 ng g^{-1}. The LOD was determined using the S/N criterion = 3/1 on the above-mentioned samples used for LOQ determination. Figure 2A shows a total ion chromatogram of each dye spiked at 0.25 ng g^{-1}.

3.2.4. Matrix effect

Only for MG and CV the method was free of any matrix effect, likely due to the availability of labelled IS of both compounds. For all other dyes, a significant difference was observed by comparing the mean area of blank samples spiked after extraction and the mean area of an equivalent standard solution at the same concentration (1ng g^{-1}). The ratio *Area spike / Area solution* was ~0.4 for almost all dyes. Nevertheless a big decrease in signal of about 60% in combination with the fish matrix was observed, the method was able to quantify all dyes at the level of 0.25 ng g^{-1} with sufficient sensitivity (Figure 2A).

3.2.5. Specificity and contamination

Blank tissue samples were extracted and analysed using the above mentioned method to investigate specificity. Special attention should be taken in the usage of LC tubing and an injection syringe of peak in the analytical instrument. When red coloured tubing and a peak injection needle were used, relatively background levels of the two rhodamine dyes were observed. Negative control samples and method blanks were found to be positive and contain low levels in the range of ~0.5 to 1 ng g^{-1}. Therefore, precautions should be taken to avoid the usage of red tubing and change it by special black coloured UPLC tubing and use an injection needle of stainless steel, but nevertheless background levels around 1 ng g^{-1} stay present. In literature, the same phenomenon was observed for other dyes. Andersen *et al.* (2009) mentioned the presence of CV background levels around 0.5 ng g^{-1}. Even when special efforts and special precautions to meticulously clean all glassware and laboratory equipment, a minority of the negative control samples during their validation study were found to be positive for CV, although the levels were below 0.1 ng g^{-1} [5]. The authors allocated this phenomenon to the high electrostatic nature of CV which scatters CV across the lab during opening bottles and weighing standards, a well-known phenomenon of dye

analysis Our research didn't expose this phenomenon with CV. Nevertheless, extra precautions were taken when sample tubes were marked during extraction with marker pens, since several of the above mentioned dyes are the major constituents of inks pens [59, 60]. As can be seen from Figure 2B, no significant peaks were detected in the retention time window of the dyes in the present study, except for the xanthene dyes, were significant interferences are present at their retention times.

3.2.4. Decision limit and detection capability

According to the calculated *t*-factor and by comparison with the tabulated *t*, matrix effect was present. Therefore, calibration curves were all made by spiking blank tissue samples. For the analysis of the dyes in fish tissues, CC_α and CC_β based on ISO 11843-2 procedure were all < 1 and < 0.25 ng g^{-1} respectively (Table 2).

4. Monitoring study in eel

A subsequent field study was performed to investigate the occurrence of artificial dyes in wildlife European eel in 91 Flemish rivers. Eel was contaminated by dyes in 77% of the sites (concentration > LOD). Two substances (VR and VB) were detected in one and two samples respectively. Concentrations of all other dyes were always below LOD. MG, LMG, CV, LCV and BG were present in 25-58% of the samples. The highest measured concentration was around 9.5 ng g^{-1} for MG (sum of MG and LMG), five times higher than established MRPL of 2 ng g^{-1} [18]. Dye occurrence was related to distribution of textile and dye production industries. A representative chromatogram (7.96 ng g^{-1} MG + leuco-MG) is shown in Figure 3. The presence of artificial dyes in the eel could give an additional threat to

this critical endangered species. It may be recommended to discourage the consumption of wild eel. Future sampling should also focus on routes of contamination in order to trace the pollution sources. Contaminated eels should be considered as not suitable for consumption, since these dyes are all prohibited for use in aquaculture.

5. Conclusions

A sensitive UPLC-MS/MS method for the quantification of 14 dyes in fish tissues is described. The method involves a liquid-liquid extraction with acetonitrile, followed by an in situ oxidation with DDQ and a tandem solid phase extraction to ensure good recovery of all compounds. The limit of quantification was 0.25 ng g^{-1} for all dyes. The method was successfully applied for the monitoring of synthetic dyes in European eel in order to investigate possible accumulation in organisms and environment in Flanders (Belgium). MG, LMG, CV, LCV and BG were measured in 25-58% of all samples. For MG (expressed as MG + LMG), the highest concentrations were around five times higher than recommended by the authorities. Some questions raised regarding consumption of wild caught eels since all these dyes have potential toxic properties and are prohibited for use in aquaculture.

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FIGURE CAPTIONS

Figure 1. Chemical structure of the triarylmethane, xanthene and phenothiazine dyes.

Figure 2. MS/MS chromatograms of (A) all dyes spiked at 0.25 ng g^{-1} (LOQ level) in fish tissue: RB (rhodamine B), VBO (victoria pure blue BO), VB (victoria blue B), EV (ethyl violet), VR (victoria blue R), BG (brilliant green), CV-d_6 (crystal violet-d_6), CV (crystal violet), R6G (rhodamine 6G), MG-d_5 (malachite green-d_5), MG (malachite green), NMB (new methylene blue), NBA (nile blue A), PR (pararosaniline), MB (methylene blue), AB (azure B).and (B) a blank fish sample.

Figure 3. Incurred eel sample with malachite green (MG), expressed as the sum of MG + leuco-MG, at a concentration of ~7.96 ng g^{-1}.

HEADING OF TABLES

Table 1. Specific MS settings and chromatographic characteristics of the studied dyes.

Table 2. Results of the linearity, trueness, within-day and between-day precision evaluation, decision limit and detection capability and limit of detection.

Table 1. Specific MS settings and chromatographic characteristics of the studied dyes

Compound	Protonated Molecular ion	Fragment ions	Cone Voltage (V)	Collision energy (eV)	Retention Time (min)
Azure B	270.2	254.2 228.0	45 45	35 35	2.05
Methylene Blue	284.2	268.1 252.0	55 55	50 35	3.25
Pararosaniline	288.3	195.0 168.2	45 45	33	2.08
New Methylene Blue	312.0	283.3 254.3	55 55	35 30	3.22
Malachite Green	329.3	313.2 208.4	55 55	35	4.02
Nile Blue A	318.0	274.0 260.0	50 40	40 35	3.57
Crystalviolet	372.2	356.2 340.5	55 55	55 40	4.83
Brilliantgreen	385.5	341.3 297.1	55 55	50 40	5.53
Victoria Blue R	422.3	406.2 393.3	55 55	35 40	5.22
Victoria Blue B	470.5	454.2 349.2	55 55	35 43	5.55
Victoria Pure Blue BO	478.5	433.9 390.2	55 55	60 40	6.52
Rhodamine B	443.3	399.2 355.0	55 55	60 40	4.03
Ethylviolet	456.5	412.5 368.2	55 55	55 45	6.96
Rhodamine 6G	443.3	415.2 341.2	55 55	45 35	4.69
Malachite Green-d_5	334.4	318.2	55	40	4.02
Crystalviolet-d_6	378.4	362.2	55	40	4.83

First fragment ion was used for quantification, the second for identification purpose

Table 2. Results of the linearity, trueness, within-day and between-day precision evaluation, decision limit and detection capability and limit of detection.

	Linearity (0-2 ng g^{-1})	*F*-value	Conc. (ng g^{-1})	Precision within day (RSD or CVr, %)	Trueness (%)	Precision between day (RSD or CVrw, %)	Decision limit (CCα) (ng g^{-1})	Detection capability (CCβ) (ng g^{-1})	Limit of detection (ng g^{-1})
Malachite Green	r= 0.995 g= 5.7%	0.0587	0.25 0.5 1	6.0 3.9 806	-2.4 -1.8 -0.2	10.9 8.9 8.6	<1	<0.25	<0.01
Crystal Violet	r= 0.998 g= 4.9%	1.7409	0.25 0.5 1	3.4 3.5 3.4	+6.0 +8.4 +6.1	3.4 5.9 12.5	<1	<0.25	<0.01
Brilliant Green	r= 0.993 g= 2.7%	0.1145	0.25 0.5 1	12.1 11.1 7.8	-2.0 +3.4 +5.4	12.3 9.9 7.7	<1	<0.25	<0.01
Ethyl Violet	r= 0.999 g= 6.2%	0.5357	0.25 0.5 1	9.8 9.8 8.1	+2.4 +6.7 -3.4	15.4 11.7 11.9	<1	<0.25	<0.01
Azure B	r= 0.999 g= 3.5%	0.0334	0.25 0.5 1	14.1 10.5 14.5	-9.0 -15.9 -11.5	14.1 16.1 14.7	<1	<0.25	<0.01

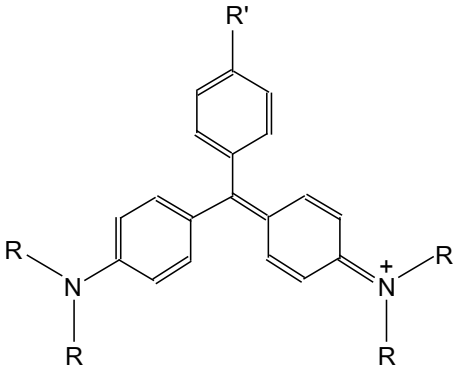
Methylene Blue	r= 0.994 g= 4.7%	0.0723	0.25 0.5 1	8.3 14.0 12.7	+2.1 -3.3 -2.4	10.8 14.3 20.7	<1	<0.25	<0.01
New Methylene Blue	r= 0.994 g= 8.2%	2.1715	0.25 0.5 1	7.3 14.0 6.4	-0.9 -7.1 -0.3	9.7 14.2 20.0	<1	<0.25	<0.01
Nile Blue A	r= 0.995 g= 4.7%	0.3592	0.25 0.5 1	7.5 12.1 10.3	-3.2 -7.8 +0.8	9.9 12.0 20.0	<1	<0.25	<0.01
Pararosaniline	r= 0.995 g= 9.4%	4.2194	0.25 0.5 1	9.0 5.0 12.7	+18.4 -8.5 -31.7	11 9.0 12.7	<1	<0.25	<0.01
Victoria Blue R	r= 0.995 g= 6.2%	0.4753	0.25 0.5 1	9.6 9.3 9.9	-1.1 -2.8 -4.1	9.6 9.4 11.8	<1	<0.25	<0.01
Victoria Blue B	r= 0.995 g= 6.3%	1.6810	0.25 0.5 1	12.9 8.9 9.0	+1.2 -5.3 -5.4	13.9 11.7 9.11	<1	<0.25	<0.01
Victoria Pure Blue BO	r= 0.995 g= 6.1%	0.0457	0.25 0.5 1	11.7 12.0 5.0	+13.9 +5.6 +3.2	13.3 12.0 17.3	<1	<0.25	<0.01

r= correlation coefficient; g= goodness-of-fit coefficient; RSD = relative standard deviation (the ratio between standard deviation and mean found concentration) = CV (coefficient of variation);Trueness = difference between mean found concentration and spiked concentration;
CC_α= decision limit; CC_β= detection capability; The $F_{0.99}$ equals 8.6832 for all dyes, except 10.5614 for pararosaniline

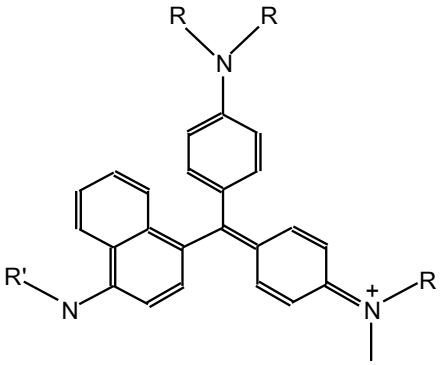
Highlights

- A new UPLC-MS/MS method was developed for the quantification of illegal dyes in fish tissues
- Due to the presence of strong positively charged sulphur groups, attention was paid to the solid phase elution step
- The validation was performed according to standards of European Union (Directive 2002/657/EC) and scientific literature
- The method was able to quantify at 0.25 ng g^{-1}
- Applicability of the method was shown in a subsequent monitoring study of wildlife eel in Flemish rivers

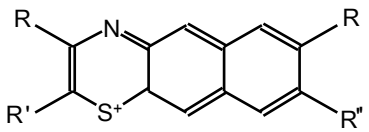
Figure



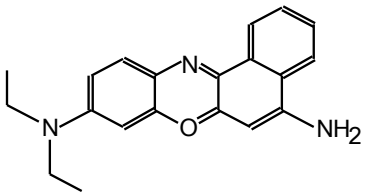
R	R’	Name
CH_3	H	Malachite Green
CH_2-CH_3	H	Brilliant Green
CH_3	$N(-CH_3)_2$	Crystal Violet
CH_2-CH_3	$N(CH_2$-$CH_3)_2$	Ethyl Violet
H	NH_2	Pararosaniline



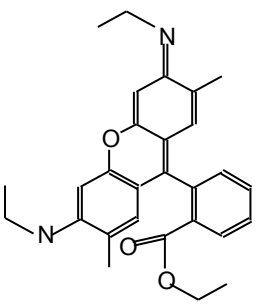
R	R’	Name
CH_3	C_6H_6	Victoria Blue B
CH_3	CH_2-CH_3	Victoria Blue R
CH_2-CH_3	CH_2-CH_3	Victoria Pure Blue BO



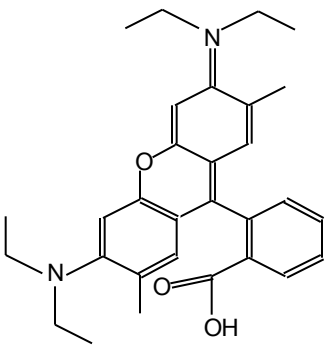
R	R’	R”	Name
H	$N(CH_3)_2$	$N(CH_3)_2$	Methylene Blue
H	$N(CH_3)_2$	$NH(CH_3)$	Azure B
H	$N(CH_2$-$CH_3)_2$	$N(CH_2$-$CH_3)_2$	New Methylene Blue



Nile Blue A



Rhodamine 6G



Rhodamine B

Figure 1.

Figure

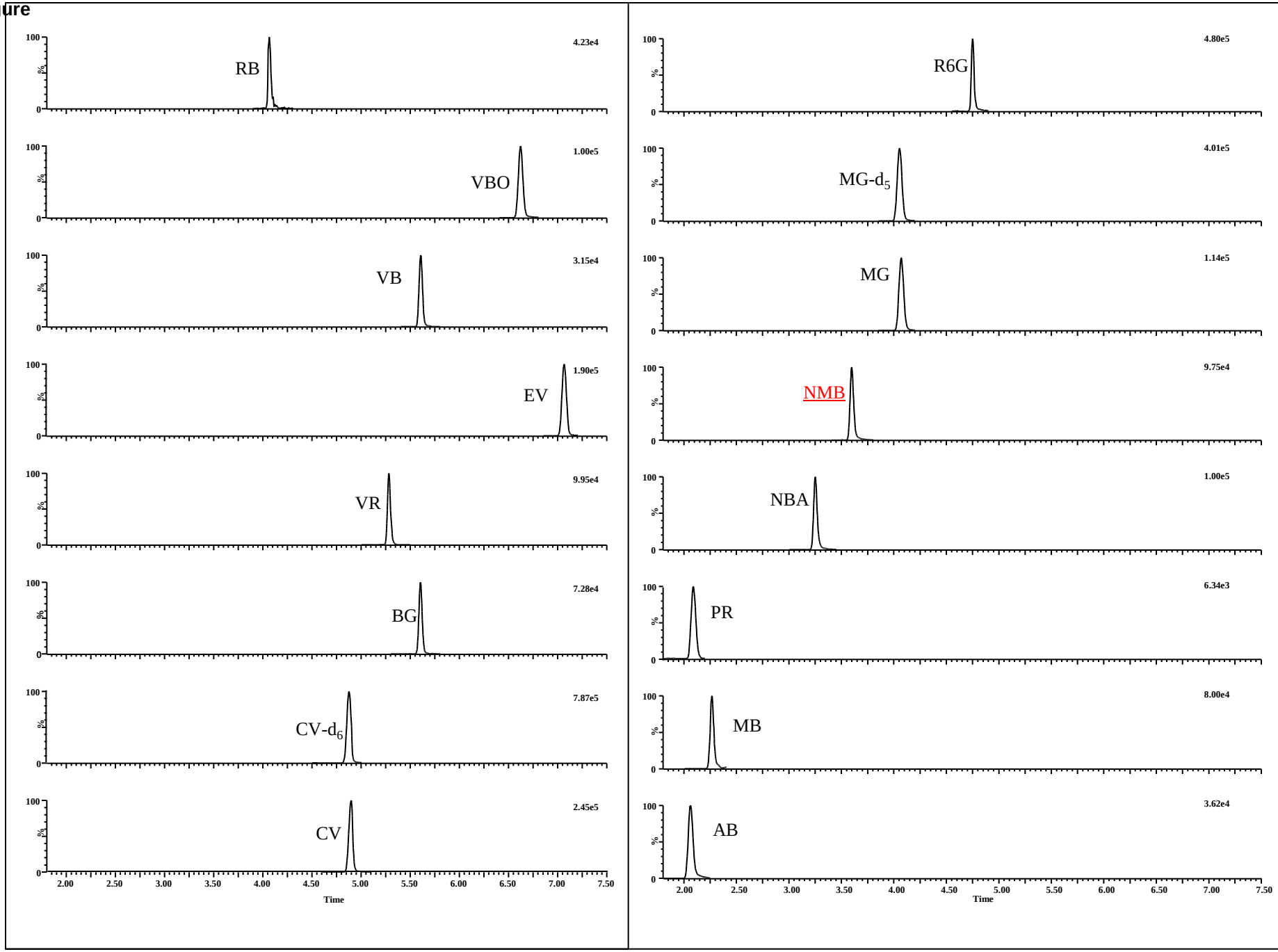


Figure 2-A

Figure

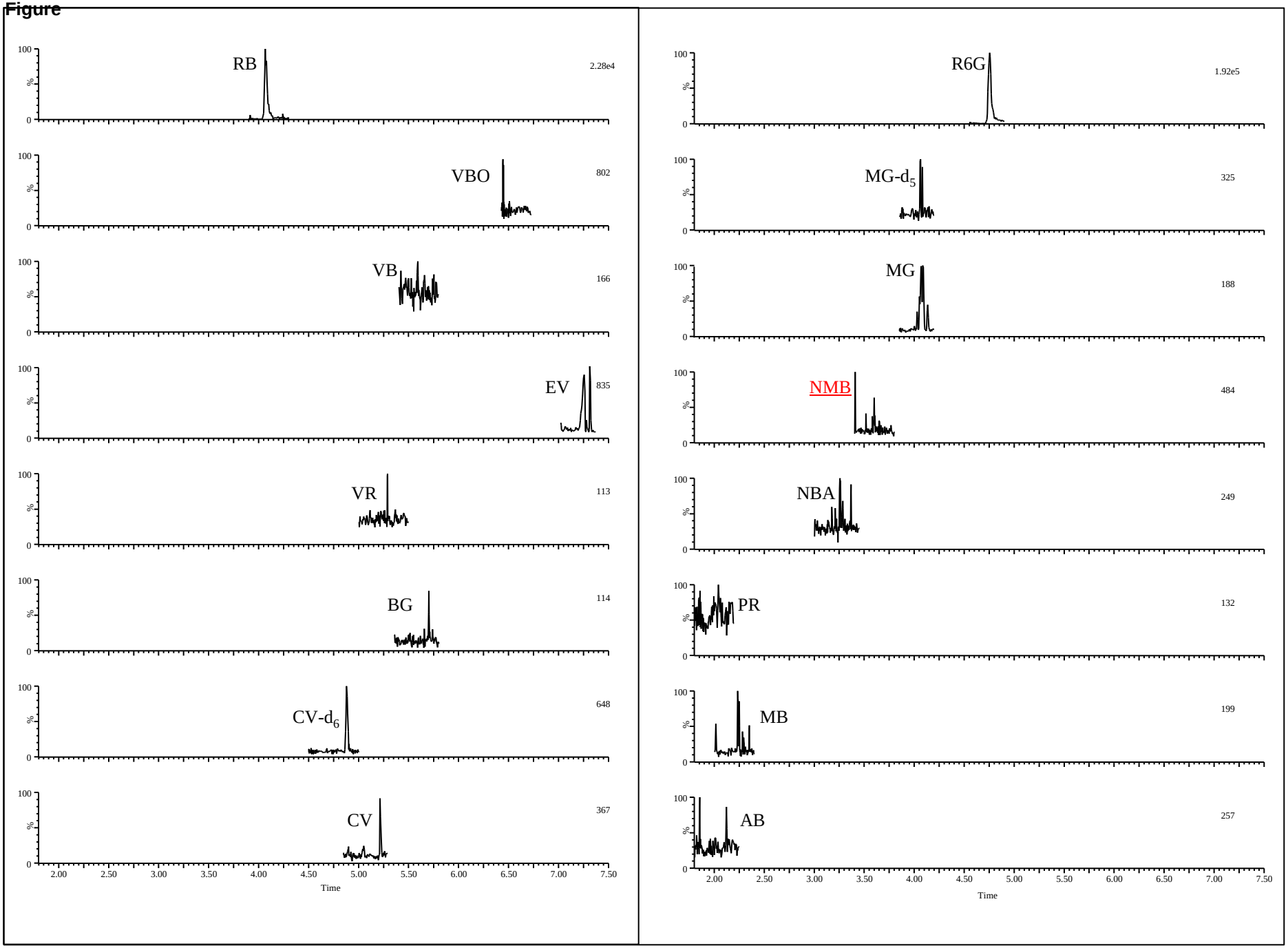


Figure 2-B

Figure

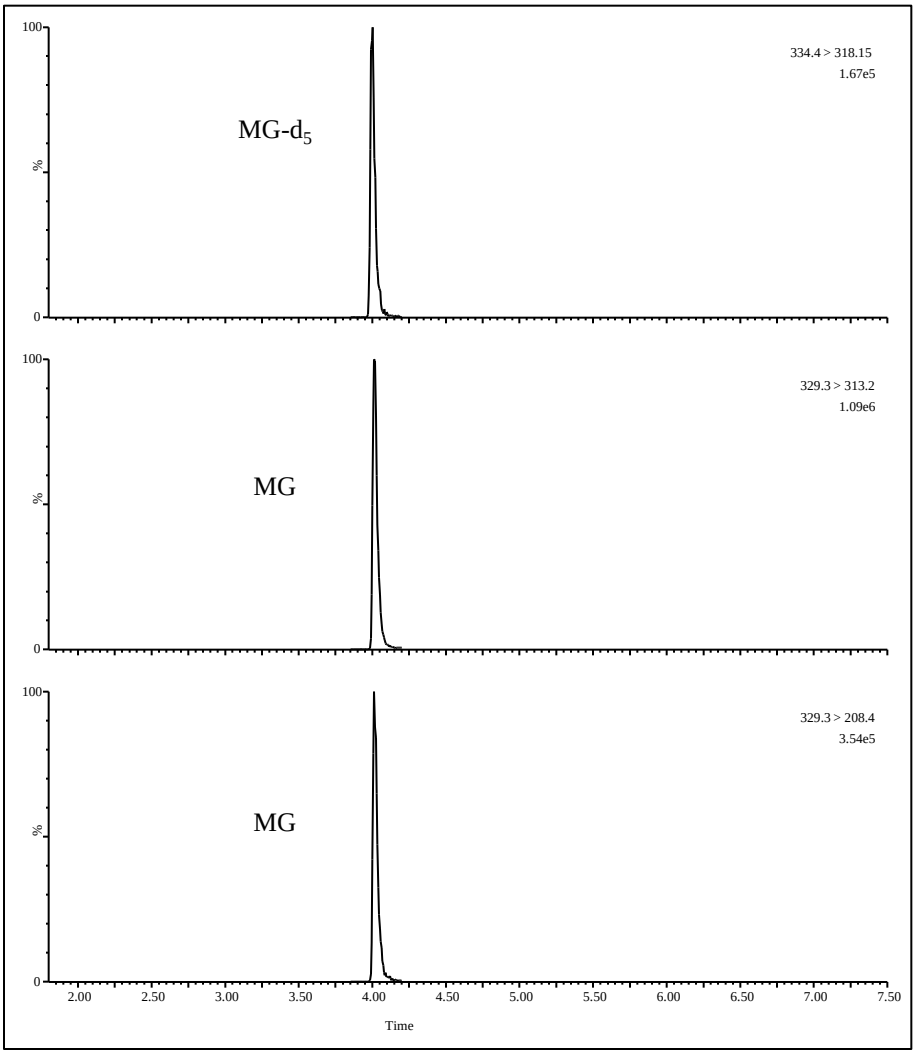


Figure 3