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Analysis of Malachite Green, Gentian Violet and Their Leuco Metabolites in Catfish and Carp by Micellar Electrokinetic Capillary Chromatography

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ABSTRACT

A simple, reproducible and sensitive micellar electrokinetic chromatography (MEKC) method was developed for the separation and determination of malachite green (MG), leucomalachite green (LMG), gentian violet (GV) and leucogentian violet (LGV) from catfish and carp. Field-enhanced sample injection with reverse migrating micelles (FESI-RMM) was used for on-line concentration of the analytes. The buffer contained 50 mM H_3PO_4-NaOH, 140 mM sodium dodecyl sulfate (SDS), 15% acetonitrile and 15% 2-propanol, at the pH of 2.0. The sample solution was diluted with water containing 5 mM SDS and injected for 15 s with -8 kV after injection of 2 s water plug. The effects of concentration of SDS and organic modifier, the sample matrix, the injection time of water plug, the injection voltage and injection time of sample on the separation and stacking efficiency were investigated. Under the optimum conditions, the analytes were well separated and by optimizing the stacking conditions, about 121, 146, 150 and 87 fold improvement in the detection sensitivities were obtained for GV, MG, LMG and LGV, respectively. The instrument detection limits (S/N = 3) of GV, MG, LMG and LGV were 40, 70, 50 and 50 ng/mL, respectively. The recoveries of GV, MG, LMG and LGV in the extracts of catfish were 87.8 - 96.7%, 87.9 - 100.2%, 96.7 - 101.4% and 89.8 - 94.2%, respectively. The GV, MG, LMG and LGV in spiked (1 μg/mL) catfish and carp extracts were successfully determined with satisfactory repeatability and recovery.

Key words: field-enhanced sample injection, reverse migrating micelles, malachite green, leucomalachite green, gentian violet, leucogentian violet

INTRODUCTION

Because of low price and easy to use, malachite green (MG) and crystal violet (CV), the triphenylmethane dyes are commonly used in the prevention and control of fungus diseases such as gill rot and parasitic disease in fish. They have also been used as a disinfectant to extend the survival time of fish in the transport process[1,2]. When absorbed by the fish, MG and CV is rapidly reduced to its non-chromophorous metabolite leucomalachite green (LMG) or leucocrystal violet (LCV)[3]. The LMG and LCV are serious threat to the health of consumers. In view of the dangers of malachite green and crystal violet, many countries have included them in aquaculture prohibited drugs. Canada in 1992 issued a fishing ban on fungicides. In 1993 the U.S. FDA proclaimed malachite green and crystal violet as carcinogenic chemical substances, and MG, LMG, CV and LCV should not be detected in aquatic products. The European Union in June 2002 issued a decree prohibiting the use of malachite green in fisheries. The use of malachite green in aquaculture was also prohibited in May 2002 by Chinese authorities.

Several methods have been reported to determine malachite green, gentian violet or their leuco metabolites, such as thin-layer chromatography (TLC)[4], high performance liquid chromatography (HPLC)[5,6], liquid chromatography mass spectrometry (HPLC-MS)[7-9]. However, TLC method is not accurate for quantitation, and HPLC and HPLC-MS require large amount of organic reagent and tedious operation steps. Recently, owing to its high resolving power, low solvent consumption and simple sample pretreatment, capillary electrophoresis (CE) has been used as an attractive method for food analysis[10-14]. The detection of four structural types of industrial dyes including malachite green (MG) by capillary zone electrophoresis (CZE) was investigated by

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both hydrodynamic and electrokinetic injection[15]. Various dye compounds, including malachite, crystal violet and rhodamine B, were studied by non-aqueous capillary electrophoresis with electrochemical detection[16]. However, these capillary electrophoresis methods have not been applied on actual samples.

Due to its relatively low sensitivity, CE is not the most representative method among analytical separation techniques. On-line solid-phase extraction, membrane preconcentration[17], powerful detectors[18], sample stacking[19-22] have been attampted to increase the detection seneitivity and extend the use of CE for the analysis of analytes in trace amounts. In 1998, Quirino and Terabe developed an on-line concentration technique using field-enhanced sample injection with reverse migrating micelles (FESI-RMM)[23]. Low-pH buffer was used to reduce the electroosmotic flow. The sample is dissolved with the aid of micelles in solution and injected into the capillary using voltage. This concentration technique has been shown to provide more than 100-fold increase in UV detector response with very high plate numbers. Sample stacking with a dynamic pH junction depends on the change in electrophoretic mobility as the charged analytes encounter the pH junction between the sample zone and the background eletrolyte (BGE) zone upon application of voltage. Due to the good result of the sample stacking, the FESI-RMM has been used as an attractive method[24-27].

In this paper, we developed a FESI-RMM method for the simultaneous determination of malachite green, gentian violet and their leuco metabolites. The optimum separation and stacking conditions were achieved by systematically optimizing the concentrations of sodium dodecyl sulfate (SDS) and organic modifier, the sample matrix, the injection time of water plug, the injection voltage and injection time of sample. The MG, LMG, GV and LGV in spiked catfish and carp extracts were successfully determined with satisfactory repeatability and recovery.

MATERIALS AND METHODS

I. *Apparatus and Conditions*

An Agilent HP^{3D} capillary electrophoresis system (Agilent, USA) was used. The applied voltage was held constant at -25 kV. The column was an uncoated 50 μm ID fused-silica capillary of 60 cm and an effective length of 51.5 cm (Yongnian, Hebei Province, China). An oscillation triangular flask (Houde, Shandong Province, China) The temperature of the capillary cartridge during electrophoresis was maintained at 25°C. UV detection was set at 214 nm. Before each use, the capillary was rinsed with 1 M NaOH for 10 min and water for 10 min; it was then conditioned with running electrolyte for 10 min. Between each run, the capillary was rinsed with water and electrolyte for 5 min each. Samples were injected for 15 s with -8 *k*V after injection of 2 s water plugs.

II. *Materials and Reagents*

The catfish and carp were purchased from market of Qingdao, Shandong province, China. Standards of malachite green (MG), leucomalachite green (LMG), gentian violet (GV) and leucogentian violet (LGV) (see Figure 1 for their chemical structures) were obtained from Sigma-Aldrich (Sigma, St. Louis, MO, USA). H_3PO_4, NaOH, sodium dodecyl sulfate, acetonitrile, 2-propanol, neutral alumina and ammonium acetate, etc were of analytical-reagent grade from Beijing Chemical Factory (Beijing, P. R. China). Deionized water was used throughout. All solutions and samples were filtered through a 0.45 μm syringe filter.

The buffer solutions containing H_3PO_4, SDS and organic solvents adjusted to the desired pH with 0.1 M NaOH, were all filtered through a 0.45 μm membrane filter and degassed prior to use. A standard solution of 1500 μg/mL of each analyte was prepared in methanol, filtered, and degassed by the same procedure as used for buffer solutions. The various concentrations of the sample solutions were prepared by appropriate dilution from the stock solution with water containing 5 mM SDS when needed.

III. *Sample Preparation*

The samples were prepared as described[28] with some modifications. 20 g fish meat and four analytes were added into a 250 mL beaker. A 3 mL 0.25 g/mL hydroxylamine hydrochloride, 5 mL 0.05 M p-TSA, and 20 mL 0.1 M ammonium acetate (adjusted to pH 4.5 with glacial acetic acid) were then added. The mixture were homogenized with a homogenizer machine for 5 min, and transferred to a 250 mL flask, then 20 g of neutral alumina for adsorption of the oil was added. Sample was placed in an oscillation triangular flask for about 10 min, transferred to a 50 mL centrifuge tube and then centrifuged at 4000 r/min for 10 min. The supernatant was moved to a 1000 mL separatory funnel. To the centrifuge tube, the remaining sample was shaked with the oscillator for 5 min in 30 mL of acetonitrile. The supernatants were combined to a separatory funnel, its accession to 100 mL of distilled water and 50 mL dichloromethane was then added. A 5 mL diethylene glycol was aslo added to break the emulsion. After vigorous shaking and standing, 50 mL dichloromethane was added to the separatory funnel. The dichloromethane was collected at 60°C using a vacuum rotary evaporator, and then the evaporation flask washed with acetonitrile, set the volume to 1 - 2 mL, filtered using a 0.45 μm cellulose acetate membrane to obtian the sample solution.

RESULTS AND DISCUSSIONS

I. *Optimizing the Separation Conditions*

(I) *Effect of the SDS Concentration*

Experiments were performed with the 50 mM H_3PO_4

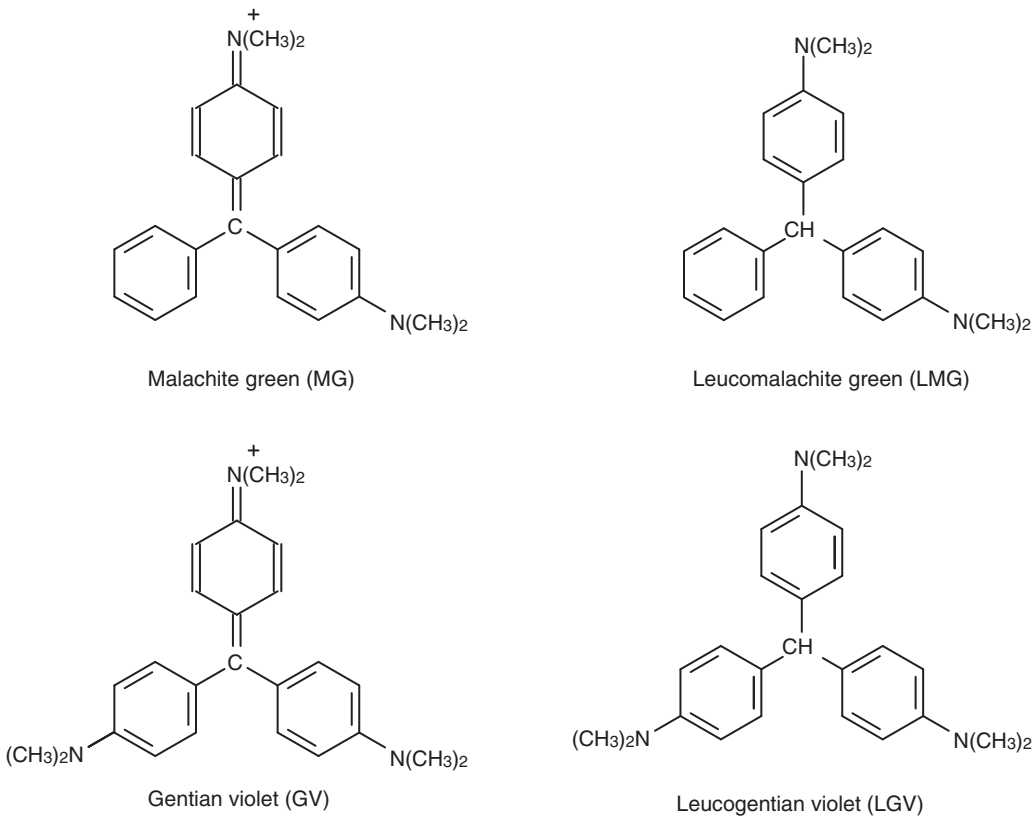

Figure 1. Molecular structures of malachite green (MG), leucomalachite green (LMG), gentian violet (GV) and leucogentian violet (LGV).

(pH 2.0). In FESI-RMM method, the effect of SDS concentration on the separation and stacking of analytes was important. Different SDS concentrations (80, 100, 120, 140 and 160 mM) of the electrolytes were used. The results indicated that with the increased SDS concentrations, the peak areas and peak heights of analytes increased and the migration times decreased. Although the analytes can be completely separated under 160 mM SDS, when the extracts of the catfish and carp were introduced to the capillary, the separation of LGV from other unknown compounds was poor. Thus, taking account of the resolution and analytical time, 140 mM was selected as the prefered SDS concentration for further optimization.

(II) *Effect of Acetonitrile and 2-propanol*

In FESI-RMM method, adding organic solvents, such as acetonitrile or 2-propanol in the buffer can influence the separation selectivity and separation efficiency, because a difference in affinity between micelles and analytes. Figure 2 and 3 show the effect of acetonitrile and 2-propanol on the migration time of three coumarins.

To verify the effect of acetonitrile on migration behavior, the running buffer consisting of 50 mM H_3PO_4, 140 mM SDS, and 15% 2-propanol at pH 2.0 with different acetonitrile concentrations (0 - 20%) were investigated. The results indicated that as the acetonitrile concentration increased, the separation efficiency of the four analytes was improved with increased migration time. Therefore, 15% acetonitrile was selected for the experiment. The 2-propanol concentration was also optimized by adding different concentrations (0, 5, 10, 15 and 20%) of 2-propanol to the electrolyte. The effect of 2-propanol was similar to that of acetonitrile, the baseline separations of four analytes in the standard mixture and real samples were achieved at concentration of 15%.

II. *Optimizing the Stacking Conditions*

(I) *Effect of Sample Matrix on the Peak Area*

In order to investigate the effect of sample matrix on the peak area, the standard mixture solution was diluted with different concentrations (0, 5, 10 and 15 mM) of SDS solution, and the result is shown in Figure 4. As can be seen that the peak areas of GV, MG, LMG and LGV increased and then decreased with the increase of the SDS concentration. The peak areas of GV, MG, LMG and LGV were highest at 5 mM SDS. So, 5 mM SDS was used as sample matrix.

(II) *Effect of the Injection Time of Water Plug on the Stacking Enhancement*

To verify the effect of the injection time of water plug on

the stacking enhancement, experiments were performed at the injection time of water plug from 0 to 6 s. With the injection time of water plug, the peak areas of GV, MG, LMG and LGV increased and then decreased. From 0 to 2 s, the peaks increased with the increase of injection time of water plug. It is because that the water plug provides a higher electric field at the tip of the capillary, which will eventually improve the sample stacking procedure[29]. When the injection time was longer than 2 s, the peak heights began to decrease. The reason is that the water plug was too long, a strong laminar flow generated as a result of the mismatch of EOF velocity in the sample and buffer zones[30]. Therefore, the injection time of water plug was selected as 2 s.

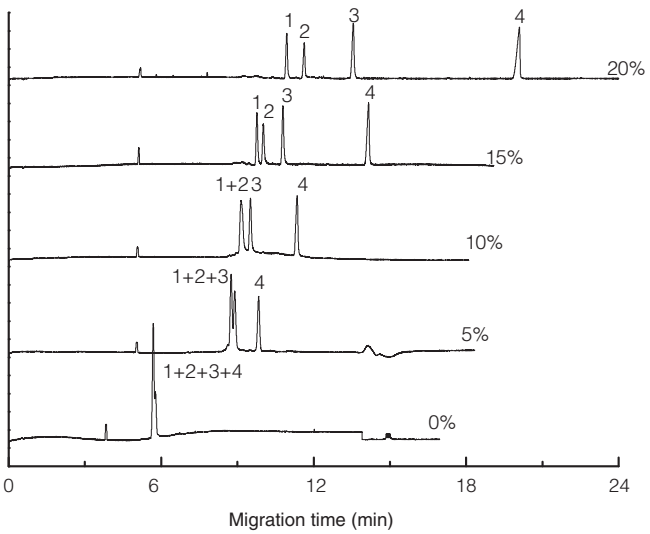

Figure 2. Effect of the acetonitrile concentration on the migration time. 1: GV; 2: MG; 3: LMG; 4: LGV. Analytical conditions: 140 mM SDS.

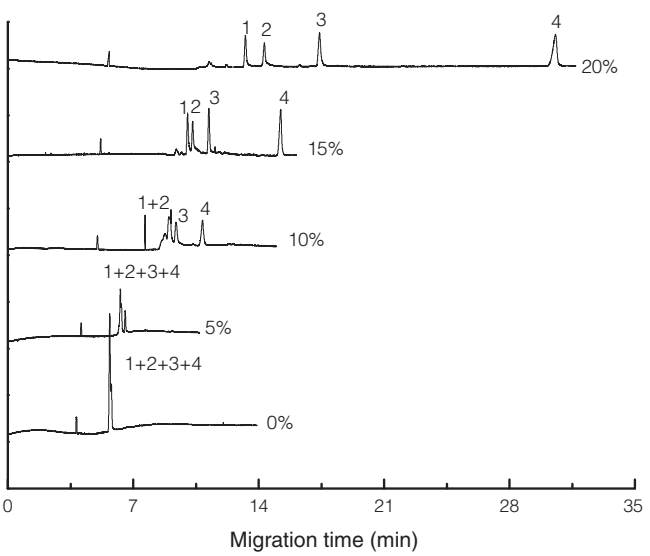

Figure 3. Effect of the 2-propanol concentration on the migration time. 1: GV; 2: MG; 3: LMG; 4: LGV. Analytical conditions: 140 mM SDS, 15% acetonitrile. Other conditions were similar to Figure 2.

(III) *Effect of the Injection Time and Injection Voltage on the Stacking Enhancement*

With the injection time increase from 5 to 20 s, the peak areas of four analytes increased. But when the injection time was longer than 15 s, the four analyte peaks of broadened.

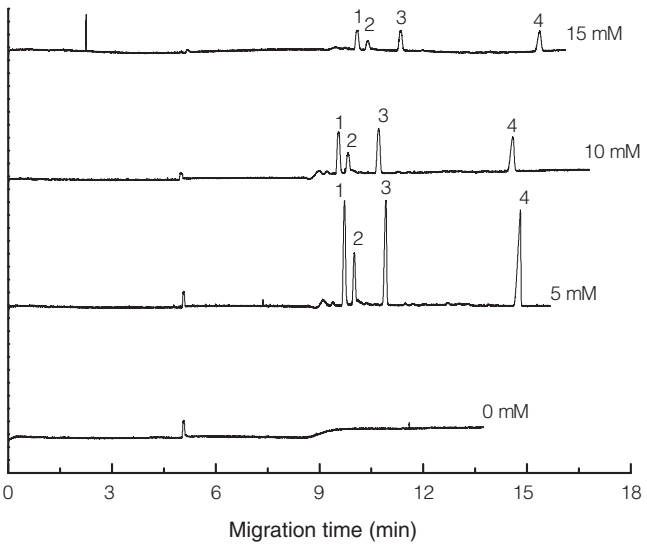

Figure 4. Effect of the SDS concentration on the peak area. 1: GV; 2: MG; 3: LMG; 4: LGV. Analytical conditions: 50 mM H_3PO_4 buffer at pH 2.0. Other conditions were similar to Figure 2.

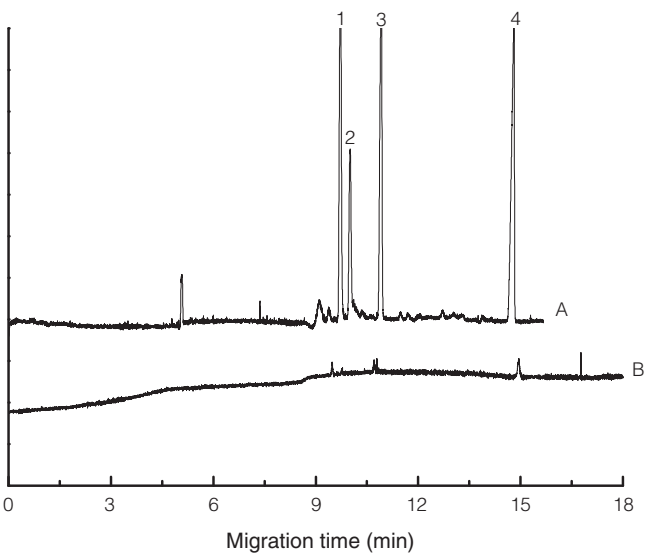

Figure 5. The typical electropherograms of standard mixture of GV, MG, LMG and LGV under stacking condition (A) and normal condition (B). 1: GV; 2: MG; 3: LMG; 4: LGV. Analytical conditions: H_3PO_4 buffer at pH 2.0. Other conditions were similar to Figure 2. (A) The solution standards were diluted with water containing 5 mM SDS and then injected for 15 s with -8 kV after injection of 2 s water plug. The final concentrations of analytes were 2 μg/mL. (B) The solution standards were diluted with water and then injected for 5 s with 50 mbar pressure. The final concentrations of analytes were 10 μg/mL.

The reason is sample overload. Therefore, the injection time was selected as 15 s.

With the injection voltage increasing from -4 to -12 *k*V, the peak areas of four analytes increased and then decreased when higher than -8 *k*V. Therefore, the injection voltage was selected as -8 *k*V.

From the above results, the optimum conditions are an electrolyte containing 50 mM H_3PO_4, 140 mM SDS, 15% acetonitrile and 15% 2-propanol and the buffer pH of 2.0. The sample solution was diluted with water containing 5 mM SDS and injected for 15 s with -8 *k*V after injection of 2 s water plug. The typical electropherograms of standard mixture under the normal and stacking conditions are shown in Figure 5.

III. *Sensitivity Enhancement of the On-Line FESI-RMM Method*

The sensitivity enhancement factor (SEF) in terms of peak height and peak area can be calculated as the following equation: $SE_{height} = H_{stack}/H$ and $SE_{area} = A_{stack}/A$, where the numerator is the peak height or peak area obtained with stacking and the denominator is the peak height or peak area obtained with the usual MEKC. The stacking enhancement factors of the GV, MG, LMG and LGV are listed in Table 1.

Table 1. The stacking enhancement factors under the optimum conditions[a]

Analytes	GV	MG	LMG	LGV
SE_{area}	121	146	150	87
SE_{height}	83	87	90	60

[a] The final concentrations of analytes were 2 μg/mL.

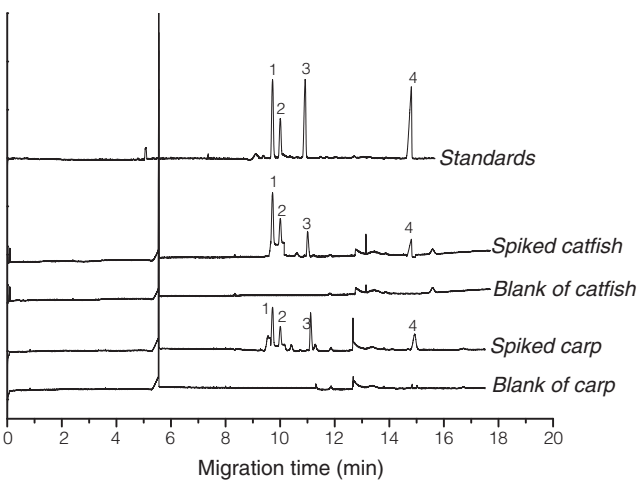

Figure 6. Electrophergrams of the extract of blank and spiked catfish and carp. 1: GV; 2: MG; 3: LMG; 4: LGV. Analytical conditions were similar to Figure 5A. The analytes of 1 μg/mL (final concentration) were added to the catfish and carp.

IV. *Linearity, Reproducibility, Limits of Detection and Recoveries*

Calibration standards [peak area, *y*, vs. concentration, *x* (mg/mL)] were prepared in the range 0.1 - 32 μg/mL for GV, 0.14 - 44 μg/mL for MG, 0.14 - 44 μg/mL for LMG and 0.21 - 68 μg/mL for LGV, respectively. The regression equation of the calibration curves and their correlation coefficients were: GV: y = 83.617x – 42.566 (r = 0.9967); MG: y = 14.4x - 5.3552 (r = 0.9972); LMG: y = 73.96x – 42.429 (r = 0.9970); LGV: y = 136.89x – 179.96 (r = 0.9964). The instrument detection limits (S/N = 3) of GV, MG, LMG and LGV were 40, 70, 50 and 50 ng/mL, respectively.

The method was validated for reproducibility of the migration time and the peak area of the analytes. The relative standard deviations (RSD) of the migration time and the peak area of each analyte was caluculated for six replicate injections. The inter-day RSD (n = 6) of the migration time and the peak area of the analytes were lower than 4.79 and 5.47%, respectively. The intra-day RSD (n = 6) of the migration time and the peak area of the analytes were lower than 5.42 and 6.05%, respectively. Accuracy of the method was determined by adding three different amounts of the analytes (0.2, 4 and 60 μg/mL) to the extracts of catfish. The recoveries of GV, MG, LMG and LGV in the extracts of catfish were 87.8 - 96.7%, 87.9 - 100.2%, 96.7 - 101.4% and 89.8 - 94.2%, respectively.

V. *Determination of the GV, MG, LMG and LGV in Spiked Catfish and Carp*

The acetonitrile solutions of the extracts of catfish and carp spiked with four analytes were injected directly and separated under the optimum condition mentioned above. The electropherograms are shown in Figure 6. Peaks were identified by the addition of standard.

CONCLUSIONS

The results demonstrated that the proposed FESI-RMM method is very suitable for the fast determination of GV, MG, LMG and LGV in catfish and carp. Compared with the conventional MEKC injection method, up to 87 - 150 fold improvement in concentration sensitivity was achieved by using this online pre-concentration method.

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REFERENCES

1. Alderman, D. J. 1985. Malachite green: A review. J. Fish Dis. 8: 289-298.
2. Foster, J. and Woodbury, L. 1936. The use of malachite green as a fish fungicide and antiseptic. Prog. Fish Cult. 18: 7-9.
3. Hajee, C. A. J. 1997. Residues of Mebendazole and Malachite Green in Eel and Trout: Analytical and Phar-Macokinetical Aspects. p. 183.Dissertation at Utrecht University, Utrecht.
4. Edelhaeuser, M. and Klein, E. 1986. Determination of malachite green residues in food fish. Dtsch. Lebensm.-Rundsch. 12: 386-389.
5. Rushing, L. G., and Hansen, E. B. Jr. 1997. Confirmation of malachite green, gentian violet and their leuco analogs in catfish and trout tissue by high-performance liquid chromatography utilizing electrochemistry with ultraviolet-visible diode array detection and fluorescence detection. J. Chromatogr. B 700: 223-231.
6. Rushing, L. G. and Thompson, H. C. Jr. 1997. Simultaneous determination of malachite green, gentian violet and their leuco metabolites in catfish or trout tissue by high-performance liquid chromatography with visible detection. J. Chromatogr. B 688: 325-330.
7. Bergwerff, A. A. and Scherpenisse, P. 2003. Determination of residues of malachite green in aquatic animals. J. Chromatogr. B 788: 351-359.
8. Valle, L., Díaz, C., Zanocco, A. L. and Richter, P. 2005. Determination of the sum of malachite green and leucomalachite green in salmon muscle by liquid chromatography–atmospheric pressure chemical ionisation-mass spectrometry. J. Chromatogra. A 1067: 101-105.
9. Scherpenisse, P. and Bergwerff, A. A. 2005. Determination of residues of malachite green in finfish by liquid chromatography tandem mass spectrometry. Anal. Chim. Acta 529: 173-177.
10. Font, G., Juan-García, A. and Picó, Y. 2007. Pressurized liquid extraction combined with capillary electrophoresis-mass spectrometry as an improved methodology for the determination of sulfonamide residues in meat. J. Chromatogr. A 1159: 233-241.
11. Dossi, N., Toniolo, R., Pizzariello, A., Susmel, S., Perennes, F. and Bontempelli, G. 2007. A capillary electrophoresis microsystem for the rapid in-channel amperometric detection of synthetic dyes in food. J. Electroanal. Chem. 601: 1-7.
12. Ryvolová, M., Táborský, P., Vrábel, P., Krásenský, P. and Preisler, J. 2007. Sensitive determination of erythrosine and other red food colorants using capillary electrophoresis with laser-induced fluorescence detection. J. Chromatogr. A 1141: 206-211.
13. Xing, X. P. and Cao, Y. H. 2007. Determination of 3-chloro-1,2-propanediol in soy sauces by capillary electrophoresis with electrochemical detection. Food Control 18: 167-172.
14. Vickers, P. J., Braybrook, J., Lawrence, P. and Gray, K. 2007. Detecting tartrate additives in foods: evaluating the use of capillary electrophoresis. J. Food Compos. Anal. 20: 252-256.
15. Farry, L., Oxspring, D. A., Smyth, W. F. and Marchant, R. 1997. A study of the effects of injection mode, on-capillary stacking and off-line concentration on the capillary electrophoresis limits of detection for four structural types of industrial dyes. Anal. Chim. Acta 349: 221-229.
16. Matysik, F. M. 1998. Non-aqueous capillary electrophoresis with electrochemical detection. J. Chromatogr. A 802: 349-354.
17. TomLinson, A. J., Benson, L. M., Guzman, N. A. and Naylor, S. 1996. Preconcentration and microreaction technology on-line with capillary electrophoresis. J. Chromatogr. A 744: 3-15.
18. Craig, D. B., Wong, J. C. Y. and Dovichi, N. J. 1996. Detection of attomolar concentrations of alkaline phosphatase by capillary electrophoresis using laser-induced fluorescence detection. Anal. Chem. 68: 697-700.
19. Chien, R. L. and Burgi, D. S. 1992. On-column sample concentration using field amplification in CZE. Anal. Chem. 64: A489- A496.
20. Quirino, J. P. and Terabe, S. 1997. On-line concentration of neutral analytes for micellar electrokinetic chromatography I. Normal stacking mode. J. Chromatogr. A 781: 119-128.
21. Quirino, J. P. and Terabe, S. 1997. On-line concentration of neutral analytes for micellar electrokinetic chromatography: II. Reversed electrode polarity stacking mode. J. Chromatogr. A 791: 255-267.
22. Quirino, J. P. and Terabe, S. 1998. On-line concentration of neutral analytes for micellar electrokinetic chromatography. 3. Stacking with reverse migrating micelles. Anal. Chem. 70: 149-157.
23. Quirino, J. P. and Terabe, S. 1998. On-line concentration of neutral analytes for micellar electrokinetic chromatography. 5. Field-enhanced sample injection with reverse migrating micelles. Anal. Chem. 70: 1893-1901.
24. Wang, S. F., Wu, Y. Q., Ju, Y. G., Chen, X. G., Zheng, W. J. and Hu, Z. D. 2003. On-line concentration by field-enhanced sample injection with reverse migrating micelles in micellar electrokinetic capillary chromatography for the analysis of flavonoids in *Epimedium brevicornum* Maxim. J. Chromatogr. A 1017: 27-34.
25. Wang, S. F., Ye, S. and Cheng, Y. Y. 2006. Separation and on-line concentration of saponins from *Panax notoginseng* by micellar electrokinetic chromatography. J. Chromatogr. A 1109: 279-284.
26. Jiang, T. F., Lv, Z. H., Wang, Y. H. and Yue, M. E. 2010. On-line concentration by field-enhanced sample injection with reverse migrating micelles in micellar electrokinetic capillary chromatography for the analysis of coumarins from traditional Chinese medicine and human serum. Biomed. Chromatogr. 24: 581-587.
27. Jiang, T. F., Lv, Z. H., Wang, Y. H., Yue, M. E. and Peng, J. H. 2010. On-line concentration by field-enhanced

sample injection with reverse migrating micelles in micellar electrokinetic capillary chromatography for the analysis of triterpenoids from traditional Chinese medicine. J. Anal. Chem. 65: 945-950.

28. Bergwerff A. A. and Scherpenisse P. 2003. Determination of residues of malachite green in aquatic animals. J. Chromatogr. B 788: 351-359.
29. Chien, R. L. 1991. Mathematical modeling of field-amplified sample injection in high-performance capillary electrophoresis. Anal. Chem. 63: 2866-2869.
30. Liu, S. H., Li, Q. F., Chen, X. G. and Hu, Z. D. 2002. Field-amplified sample stacking in capillary electrophoresis for on-column concentration of alkaloids in *Sophora fl avescens* Ait. Electrophoresis 23: 3392-3397.