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Journal of Chromatography B, 670 (1995) 55–62

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Particle beam liquid chromatography–mass spectrometry of triphenylmethane dyes: application to confirmation of malachite green in incurred catfish tissue

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First received 15 November 1994; revised manuscript received 14 March 1995; accepted 15 March 1995

Abstract

Eight triphenylmethane dyes (malachite green, leucomalachite green, gentian violet, leucogentian violet, brilliant green, pentamethyl gentian violet, N′,N′-tetramethyl gentian violet and N′,N″-tetramethyl gentian violet) have been characterized by particle beam liquid chromatography–mass spectrometry. The electron ionization spectra obtained of these dyes by this technique exhibit similar fragmentation, with the formation of phenyl and substituted phenyl radicals, and loss of alkyl groups from the amines. It was observed that the six cationic dyes are reduced in the mass spectrometer source to form the corresponding leuco compounds. This technique was evaluated for the confirmation of malachite green and leucomalachite green in incurred catfish (*Ictalurus punctatus*) muscle tissue.

1. Introduction

Triphenylmethane dyes are used as fungicides in the food industry. For example, gentian violet can be incorporated into chicken feed [1] to prevent mold growth during storage, and malachite green is added to water used in aquaculture [2] to prevent fungal growth which can lead to "white fungus" disease in some fish species. Dyes used in this fashion can manifest themselves as chemical residues in edible tissue. Furthermore, studies have indicated that these compounds can be carcinogenic and teratogenic [3], which makes monitoring of dye residues in some food products crucial. Determinative methods have been developed to quantitate the amount of triphenylmethane dye residues in chicken fat and tissue [4–6] and fish tissue [7–9]. For complete analytical detection, confirmatory methods are critical for the unambiguous identification of suspect residues found in the sample analyzed by the determinative method. Mass spectral analysis is the preferred technique for confirmation of suspect residues due to its inherent specificity and sensitivity.

Mass spectral analysis and evaluations of some triphenylmethane dyes has been reported. The charged, chromic, forms of the dyes have been analyzed by ^{252}Cf plasma desorption [10,11], laser microprobe mass analyzer [12], liquid sec-

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0378-4347/95/$09.50
SSDI 0378-4347(95)00133-6

ondary ion and fission fragment [13], electron ionization and field desorption [14] mass spectrometry. The reduced, leuco forms of the dye are volatile and thermally stable enough to be analyzed by GC–MS [15–17]. Recently, thermospray spectra of these dyes have been published [18,19]. While thermospray analysis is adaptable to existing LC methods and can be used to analyze the parent compounds as well as the volatile metabolites, the spectra are limited to mostly MH^+ and MNH_4^+ ions, limiting this technique's usefulness for confirmation.

Particle beam mass spectral analysis gives mass spectra which are similar to conventional electron ionization spectra, but can also be used for nonvolatile compounds. The azo dyes have been investigated using particle beam, as well as thermospray and electrospray mass spectrometry [20]. Several confirmation methods for nonvolatile animal drugs such as the tetracyclines and ivermectin have been developed using particle beam (PB) LC–MS [21,22]. The purpose of this report is to describe preliminary investigation of triphenylmethane dyes by PB-LC–MS and determine the suitability of this technique for confirming malachite green residues in edible catfish (*Ictalurus punctatus*) muscle tissue.

2. Experimental

2.1. Reagents and apparatus

Malachite green (MG), 99%, was purchased as the oxalate salt from Eastman Kodak (Rochester, NY, USA); leucomalachite green (LMG) was purchased from Sigma (St. Louis, MO, USA); gentian violet (GV) (USP, crystal violet, 96%) and leucogentian violet (LGV) were obtained from Eastman Kodak. The penta and tetra demethylated products of gentian violet were a gift from Dr. J.J. McDonald at National Center for Toxicological Research. Brilliant green can be purchased from Aldrich. All solvents were UV spectrograde–HPLC grade (Burdick & Jackson Laboratories, Muskegon, MI, USA) or equivalent.

The LC–MS instrumentation was a Hewlett-Packard Model 5989A mass spectrometer interfaced with Hewlett-Packard 1090 liquid chromatograph. Data system consisted of Hewlett-Packard Apollo series 400 computer with HP 6000 660S drive and UNIX (Rev. B.07.00) Chemstation software (Hewlett-Packard, Avondale, PA, USA). The HPLC column used was an Ultracarb C_{18} column (150 × 2.1 mm, 5 μm) purchased from Phenomenex (Torrance, CA, USA). The HPLC column was operated at room temperature.

2.2. Chromatography–mass spectrometry

Dyes were chromatographed using the Ultracarb C_{18} column and a mobile phase consisting of 80% acetonitrile and 20% 0.1 *M* ammonium acetate (pH 4.5) at a flow-rate of 0.4 ml/min. Unless stated otherwise, the mass spectrometer source was operated at 250°C; the solvation chamber was set at 45°C. The pressure of helium in the particle beam nebulizer was 40–45 p.s.i. (1 p.s.i. = $6.9 \cdot 10^3$ Pa). Full scan mass spectra were obtained by scanning MS from *m/z* 80–500 at a scan rate of 0.5/s. Selected ion monitoring was used for the confirmation of MG and LMG in catfish using ions at mass to charge 330, 329, 253, 210 and 165 with a dwell time of 50–75 ms/ion.

2.3. Sample preparation and extraction

Samples were prepared according to the method developed for determination of MG and LMG by HPLC–Vis with some modifications [7]. This procedure is modified for samples prepared for particle beam analysis in that the final elution from the propylsulfonic acid solid-phase extraction cartridge is accomplished with three 1 ml portions of 60% ammonium acetate buffer (0.1 *M*, pH 4.5), 40% acetonitrile with 5 m*M* toluenesulfonic acid. Hydroxylamine–methanol solution was not used for the final eluting solvent. The volume of the final sample was also reduced to 2 ml by removing the acetonitrile with N_2-evaporator. An aliquot of 100 μl was manually injected onto the HPLC column.

3. Results and discussion

PB-LC–MS can analyze both the chromic and leuco forms of triphenylmethane dyes. The structures of the dyes used in this study are given in Fig. 1. With this instrument, particle beam and thermospray interfaces were compared to obtain spectra of malachite green (MG) and gentian violet (GV). While analysis by thermospray matched the particle beam interface in response, the thermospray spectra consisted only of MH^+ and MNH_4^+ (data not shown), rendering it less useful as a confirmatory tool.

The electron ionization spectra for MG, GV and their leucometabolites (LMG and LGV) obtained with the particle beam interface are shown in Fig. 2. The spectra of structural analogs of MG and GV are shown in Fig. 3. In general, the fragmentation of these dyes is predictable consisting of the formation of phenyl and substituted phenyl radicals, and loss of alkyl groups from the amines. For example, the most abundant fragment ions in the LMG spectra consisted of 330 $[M]^+$; 329 $[M-1]^+$; 253 $[M-Ph]^+$; $[M-C_6H_4N(CH_3)_2H]^+$; 165 M^{2+}. Doubly charged ions, seen previously in the leuco forms analyzed by GC–MS, also occur in the particle beam electron ionization spectra. In dyes containing a

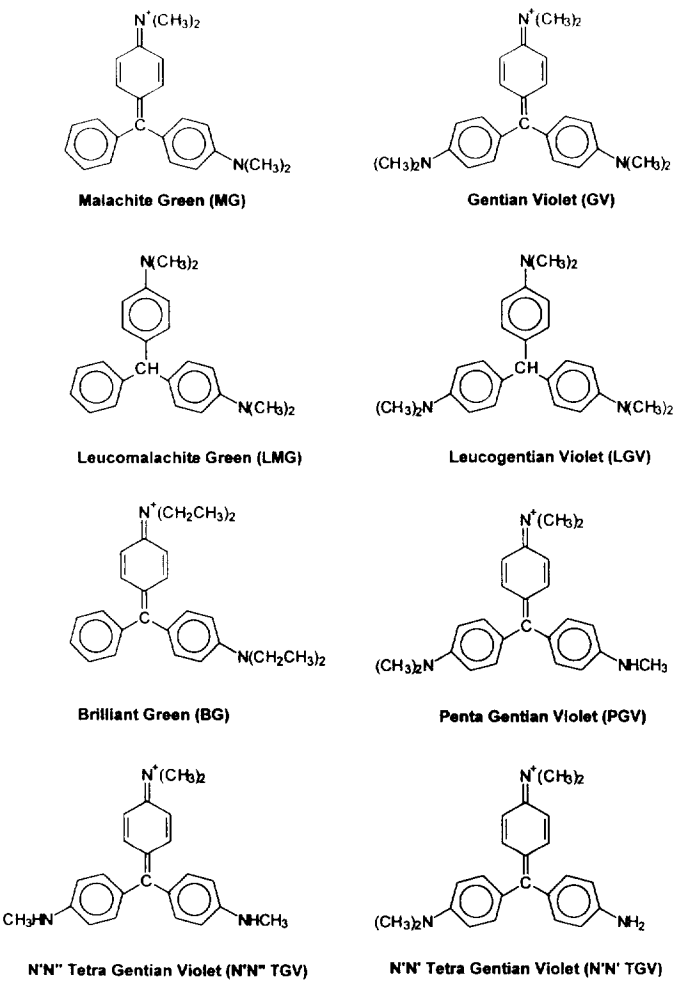


Fig. 1. Structures of triphenylmethane dyes.

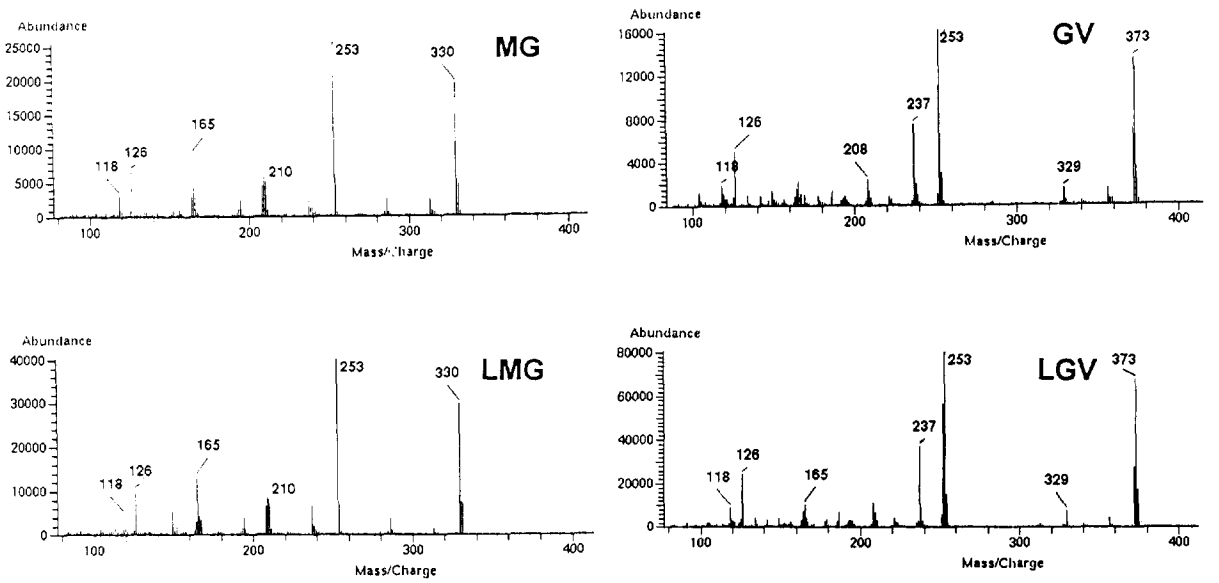
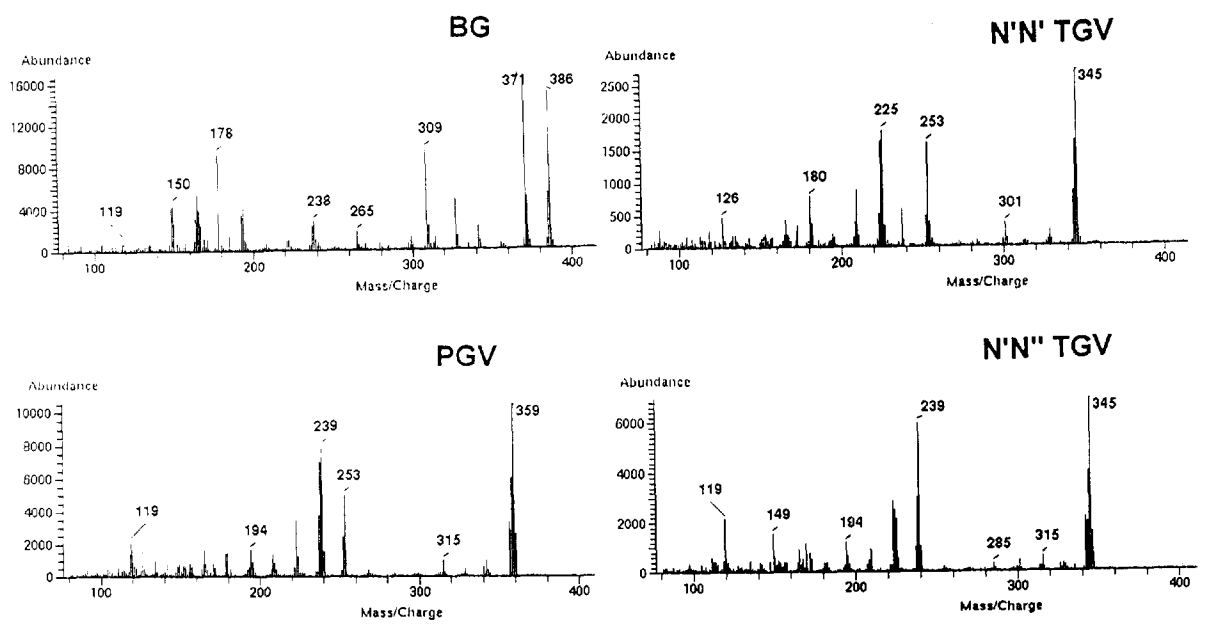


Fig. 2. Full scan mass spectra of dyes obtained by PB-LC–MS (ca. 250 ng each).

primary or secondary amine, i.e. penta and tetra GV, loss of a hydrogen atom can occur to give M − 1 (and M − 2 in the case of N′,N″-tetra GV).

In addition to fragmentation, reduction of the cationic dyes during the analysis leads to large abundances for ions corresponding to MH^+ for each of the six cationic dyes examined. It has been shown previously that triphenylmethane dyes undergo reduction in electron ionization and secondary ion mass spectrometry [13,14].



Several theories for the mechanism of this reduction exist, including thermal reduction on a direct solids probe [14], radicals formed in a liquid matrix, or step-wise protonation and reduction [13]. In this case, it is not known if the reduction is occurring within the particle beam interface, or in the ion source. Evidence suggests the latter, as the relative amounts of M^+/MH^+ depends on source temperature and pressure. For example, when the source pressure is increased by moving the particle beam transfer tube into chemical ionization position where a large opening in the mass spectrometer source is partially blocked, an increase in the ion corresponding to the reduced form is observed in the electron ionization spectrum. Under chemical ionization conditions, using 1 Torr (1 Torr = $1.3 \cdot 10^2$ Pa) methane, the spectra of the cationic dyes are virtually identical to leuco forms. A decrease in source temperature also leads to an increase in the reduced form. Source temperature may be indirectly affecting reduction by changing the source pressure; it has been observed that the ion source pressure increases with decreasing temperature. In any event, these conditions can be controlled to give reproducible spectra for residues which are consistent with those of known standards.

Aside from the reduction of the chromic form in the mass spectrometer source, the triphenylmethane dyes respond well using the particle beam interface. Using selected-ion monitoring, 5 ng of MG and LMG can be measured with a signal-to-noise ratio for the lower abundance ions of >5:1 (through HPLC column). Experiments to determine the linearity of response to these compounds in the particle beam interface was done by bypassing the LC column and injecting varying amounts directly into the particle beam interface using methanol at 0.4 ml/min. By integrating the m/z 253 ion, it was determined that MG and LMG were linear from 5 to 250 ng. For MG, area counts ($/10^6$) vs. ng injected, $m = 0.516 \pm 0.011$, $b = 0.130 \pm 1.04$ ($r = 0.9973$, $n = 7$). For LMG, $m = 0.9337 \pm 0.013$, $b = 0.130 \pm 1.15$ ($r = 0.9990$, $n = 7$).

The extraction procedure developed for the determination of LMG and MG in catfish tissue by HPLC–Vis can be used for the PB-LC–MS method with a few modifications [7]. The final

Table 1
Confirmation data for LMG and MG in catfish

Sample	Retention time (min)	Relative abundance			
		m/z 330	m/z 329	m/z 253	m/z 210
LMG					
Standard	7.98	59	18	100	65
Control		ND	ND	ND	ND
20 ng/g Fort. 1	8.15	63	18	100	67
20 ng/g Fort. 2	8.11	67	17	100	74
50 ng/g Fort. 1	8.16	67	18	100	58
50 ng/g Fort. 2	8.25	67	20	100	72
Incurred	8.05	69	20	100	79
MG					
Standard	3.80	56	26	100	58
Control		ND	ND	ND	ND
20 ng/g Fort. 1	4.06	56	20	100	68
20 ng/g Fort. 2	4.00	70	40	100	70
50 ng/g Fort. 1	4.12	68	28	100	55
50 ng/g Fort. 2	4.20	61	35	100	68
Incurred	4.03	69	34	100	61

Fort. = fortified; ND = not detected.

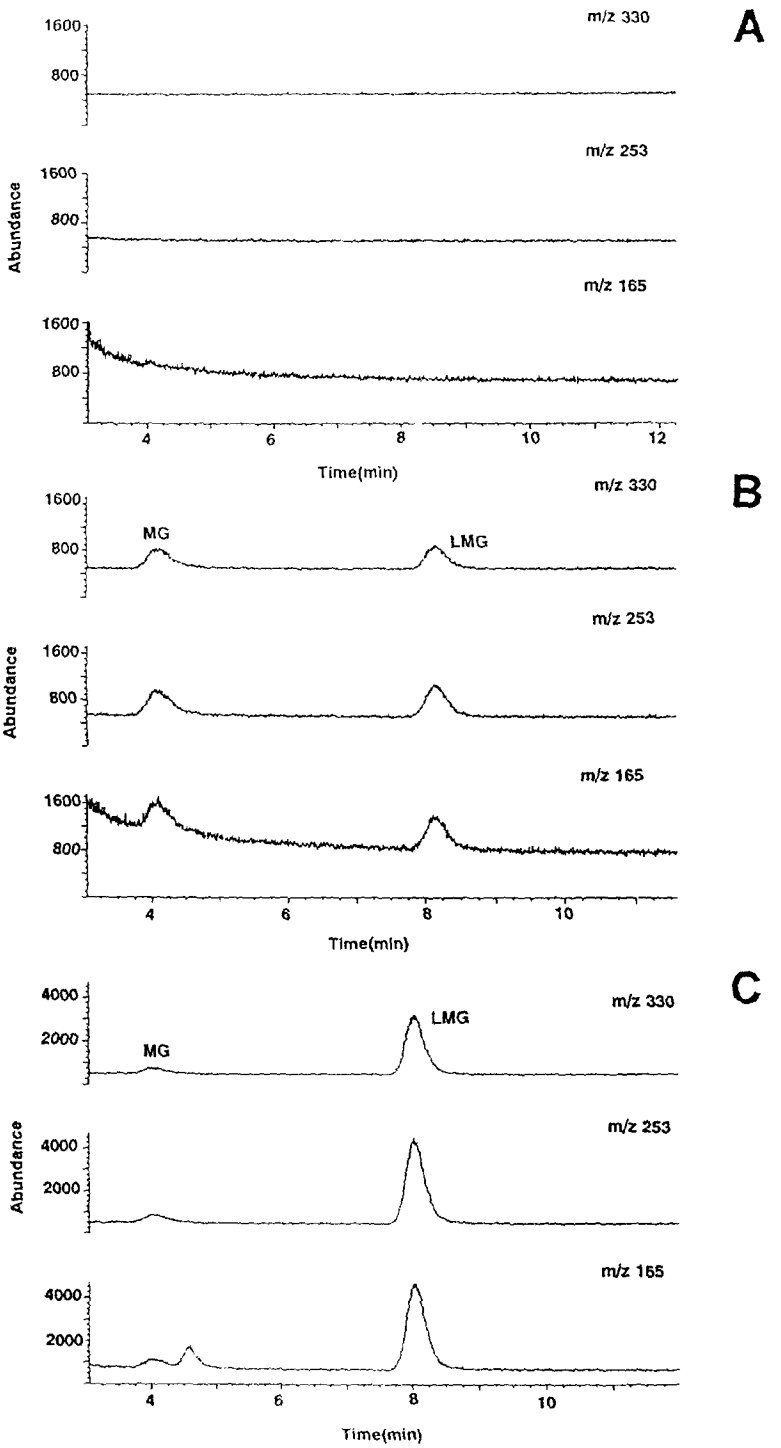


Fig. 4. Particle beam LC–MS ion chromatograms for control catfish tissue (A), catfish tissue fortified with 20 ppb MG and LMG (B), and incurred catfish tissue (C).

cleanup procedure uses a propylsulfonic acid solid-phase extraction column. When preparing samples for PB-LC–MS, the MG residues are eluted from this column with 3 ml of 40% acetonitrile–60% 0.1 *M* ammonium acetate buffer (pH 4.5) instead of the acetonitrile–sodium acetate buffer with hydroxylamine solution that was used in the determinative method. The acetonitrile is then evaporated off and the sample is ready for injection on the PB-LC–MS. This modification in procedure eliminates the nonvolatile buffer and the hydroxylamine, which was found to clog the particle beam interface skimmer cones.

Modifications in the separation protocol of the determinative method were also made to optimize the analysis for the particle beam interface. A semi-micro reversed-phase column with high carbon loading was used to maximize the percent modifier needed to elute compounds from the column as the PB interface performs best at high levels of organic modifier. Ammonium acetate buffer was substituted for the nonvolatile sodium acetate in the chromatographic separation as well. While the counter ion, 5 m*M* toluenesulfonic acid, was used to elute the residues from the solid-phase extraction column it was not added to the mobile phase to reduce possible interferences.

Table 1 shows the retention times and calculated relative abundances for monitored ions of MG and LMG in tissue as compared to standard mixtures. Fig. 4 shows reconstructed ion chromatograms for control, fortified and incurred catfish tissue. For catfish fortified at the 20-ppb level and incurred tissues (live catfish exposed to a bath containing 1.0 μg MG/g water for 1 h [7]) 24 h after exposure, all but one of the LMG ion abundances are within 10% of that observed in the standard. For MG, however, the abundances vary more, 10–15% as compared to the standard. The ion at m/z 165 was also monitored as additional evidence for the drugs, but the relative abundance of this ion was not consistent and is not included in the table. The observation that MG^+ undergoes reduction to LMG in the ion source, as discussed earlier, and the fact that this reduction is very sensitive to source conditions such as pressure and temperature may explain the slightly higher variability in these numbers.

This study has shown the suitability of particle beam analysis for triphenylmethyl dyes; they exhibit high response and linear behavior in the interface. Confirmation of both MG and the reduced metabolite LMG in catfish tissue is an example application of this technique; both residues can be confirmed at the 20 ng/g level. For some confirmation scenarios, it may be more practical to use a GC–MS method to confirm the volatile leuco metabolite [17], which will most likely be the marker residue due to its long residence time in fish tissue. However, if the need arises to monitor both residues, we have demonstrated that PB-LC can be used to identify the nonvolatile chromic as well as the leuco forms of the triphenylmethane dyes. Information obtained from the determinative analysis relative to retention times, lead oxide oxidation reaction of the leuco forms, and extraction protocol coupled with this PB-LC–MS confirmation provides valuable and unambiguous means by which to identify the presence of MG/LMG in catfish tissue.

Acknowledgements

The authors would like to acknowledge Dr. Steve Plakas of the Gulf Coast Seafood Laboratory, Food and Drug Administration for providing control and incurred catfish tissue.

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