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Development of chromatographic and mass spectrometric methods for the determination of anesthetic drugs and their metabolites in fish tissues: final report for the period April 1, 1993 - March 31, 1994

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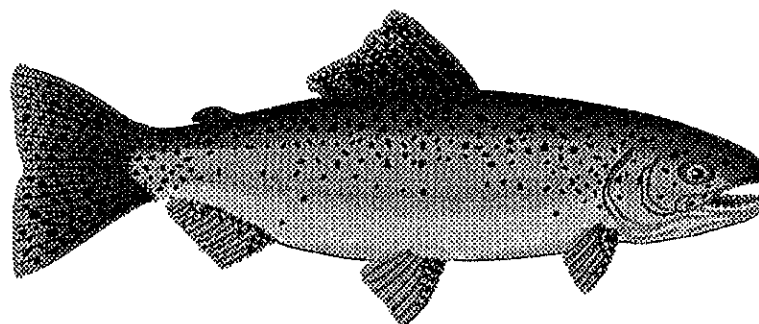# NRC · CNRC

Development of Chromatographic and Mass Spectrometric Methods for the Determination of Anesthetic Residues and Their Metabolites in Fish Tissues

IMB Technical Report 73
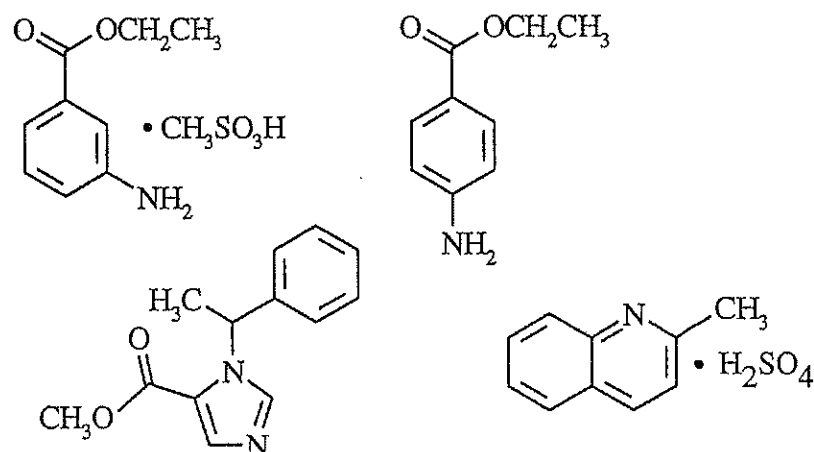
Michael A. Quilliam

January 12, 1995

NRCC No. 38066

Canada

Development of Chromatographic and Mass Spectrometric Methods for the Determination of Anesthetic Drugs and Their Metabolites in Fish Tissues

Final Report for the Period: April 1, 1993 - March 31, 1994

Date: January 12, 1995

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IMB Technical Report 73

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Background

The aquaculture of salmon and other finfish on both the Atlantic and Pacific coasts is in a state of rapid growth and has tremendous economic potential for Canada. An important issue for aquaculturists is that fish raised in close proximity are subject to disease. As in terrestrial farming operations, therapeutic agents are often used for prevention and treatment. In addition, anesthetics are often employed to minimize stress during handling procedures, such as sorting, tagging, spawning and transporting. For consumer protection, it is essential to develop reliable procedures for the analysis of residues of these therapeutants and their metabolites and breakdown products in fish tissues.

For the last four years IMB has conducted research, funded mostly by Health Canada's Bureau of Veterinary Drugs, to develop analytical methods for pharmaceuticals and their metabolites in fish tissues. Research collaborations, with colleagues at Fisheries and Oceans Canada, the University of British Columbia, Simon Fraser University, the University of Prince Edward Island, and Syndel Laboratories Ltd., have resulted in the successful development of methods for the analysis of a variety of drugs. Some of these methods are now used in routine regulatory monitoring of fish products (e.g., oxytetracycline residues).

Another important issue is the fact that very few pharmaceuticals are officially approved for use in aquaculture in Canada, putting Canadian producers at a disadvantage relative to those in other countries such as Chile and Norway. Veterinary drug manufacturers that market drugs for terrestrial farming are keen to introduce new drugs for use in aquaculture but need help in dealing with the greater difficulties associated with approving drug use in fish. Analytical chemistry plays an important role in the approval process, which includes such operations as identification of metabolites, development of analytical methods, and clearance studies to determine appropriate withdrawal times to eliminate residues in tissues prior to slaughter. In the future, we hope to increase the level of collaborations with both the pharmaceutical and aquaculture sectors to develop safe and effective therapeutants for use in Canadian aquaculture. Success in this endeavor will require continued fundamental research on drug metabolism in finfish and the development of modern instrumental analytical methods.

Project Objectives

Several anesthetic drugs have been used in aquaculture. These include: tricaine, benzocaine, metomidate, and quinaldine sulfate (see Scheme 1). Tricaine appears to be the most effective anesthetic and is easily administered as the methanesulfonate salt (MS-222), due to its good aqueous solubility. At the present time, however, none of these drugs are approved for routine use in Canada and special applications must be made to permit their use. Normally, a pharmaceutical company would arrange for the necessary studies required for drug approval, i.e., efficacy studies, identification of metabolites, development of methods of analysis, and clearance studies. Since the anesthetic drugs are

no longer under patent protection, it is up to the aquaculture industry or the government to fund such work.

Scheme 1: Anesthetic drugs used in aquaculture.
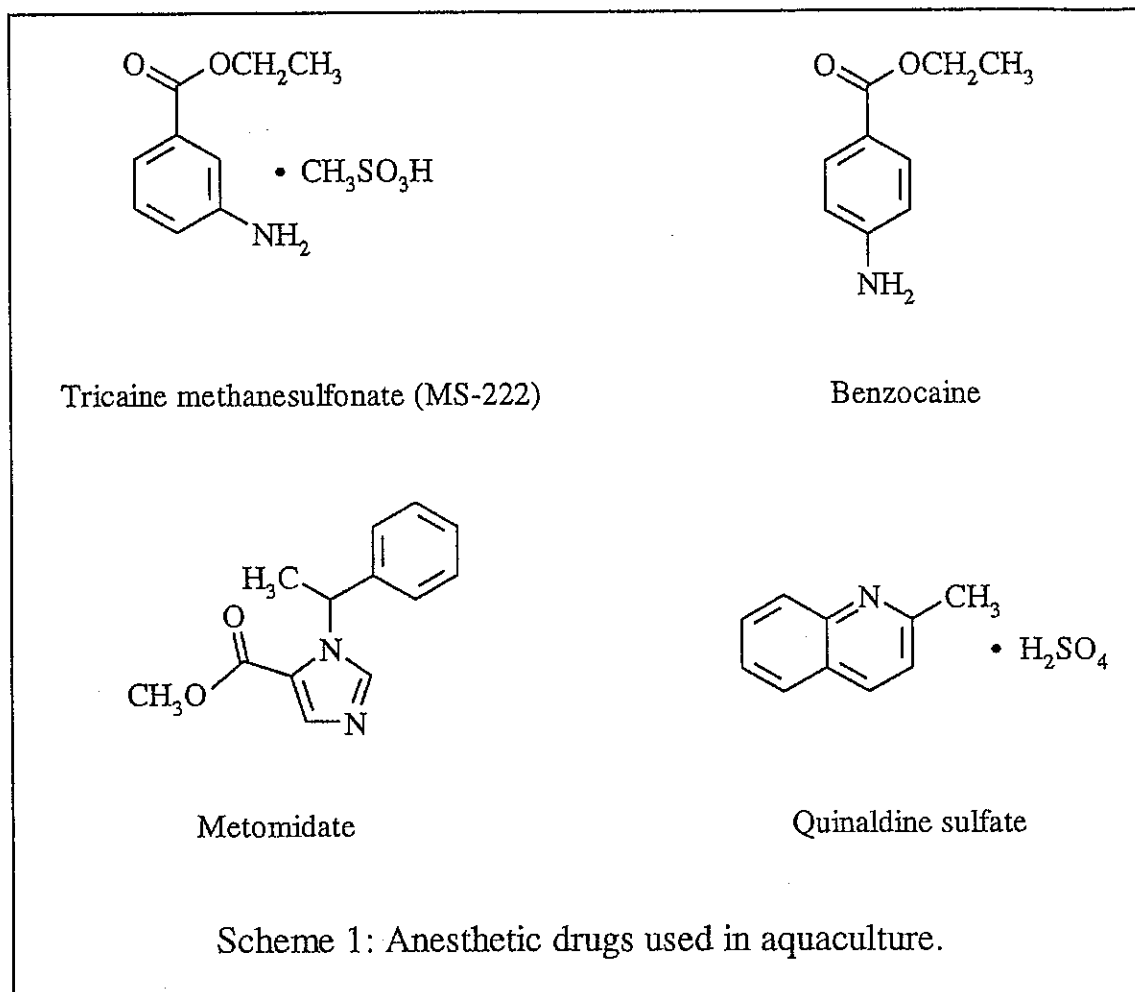
This report reviews progress achieved at the Institute for Marine Biosciences (IMB) on the development of analytical methods for anesthetic drugs in fish. This work was funded in FY 1993-94 by the Bureau of Veterinary Drugs through a Memorandum of Understanding with IMB. The objectives of the project were originally set out in the agreement as follows:

1. to complete development of extraction, cleanup and analysis procedures for anesthetic drugs (tricaine, benzocaine and metomidate) and their metabolites in fish tissues;

2. to perform feeding experiments with salmon in order to identify metabolites of tricaine and metomidate;

3. to perform a depletion study on tricaine in salmon;

4. to continue investigating novel mass spectrometry-based methods of analysis for veterinary drugs and metabolites, including CE-MS/MS and LC-MS/MS;

5. to maintain a bibliographic database on analytical methodologies for veterinary drugs;

6. to provide confirmatory analyses on a few samples (3-5) suspected of containing drug residues, to be provided by HWC to IMB as the need arises; and

7. to publish and transfer such technology to other government and private laboratories, as appropriate.

During the course of the study, it was learned that Dr. Francis Law at Simon Fraser University had been funded by the US-FDA to study the pharmacokinetics of tricaine in trout using radiotracer methods. After discussions with Dr. Yong, it was decided that my group should focus on development of the analytical methods for anesthetic drugs and on identification of the metabolites of tricaine. After Dr. Law's results became available, we would examine them and decide whether it would be necessary to do "cold" pharmacokinetic studies using instrumental analytical methods. In late 1993, Dr. Law contacted me to ask for details of the extraction and liquid chromatography methods that I had developed. As a result, we decided to collaborate on the following aspects of the tricaine study: (a) Dr. Law would test my extraction methods, along with his, on fish fed with radiolabelled tricaine to determine the best method for recovery of metabolites; (b) I would provide Dr. Law with LC separation conditions and synthetic standards of metabolites; and (c) Dr. Law would provide me with various tissues from fish fed with unlabelled tricaine and I would perform analyses to identify the metabolites present.

Literature Review

There are relatively few analytical methods reported in the literature for the determination of the anesthetics in Scheme 1, particularly in fish tissues. Tricaine has been determined at the µg/g level by colorimetry [1] and later the method was improved to allow simultaneous identification of tricaine and its metabolites in fish tissues using thin layer chromatography [2]. Tricaine has been successfully analyzed by GC equipped with a nitrogen-selective alkali flame ionization detector and packed 1 mm i.d. glass column at a detection limit about 150 ng/g in catfish muscle tissue [3]. No papers were found that describe the LC analysis of tricaine or its metabolites. Similarly, very little has been published on the mass spectrometry of this compound. Clearly there is a need to develop more sensitive analysis methods.

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Various studies have used the colorimetric techniques to measure residues of tricaine and some metabolites in salmonids after anesthesia [1-13]. Tricaine is eliminated primarily across the gills [13] and secondarily via the kidney [5]. There appear to be two primary metabolic pathways: hydrolysis of the ester group to the free acid and acetylation of the primary aromatic amine group (see Scheme 2). There is the possibility of formation of N-glucuronide conjugates (and possibly N-glucosides), as well as conjugates of the acidic metabolites with glycine and taurine (see Scheme 3), but these have not been studied thus far. Modern techniques have not been used to study thoroughly the metabolism of tricaine.

Scheme 2: Possible metabolic pathways for tricaine.
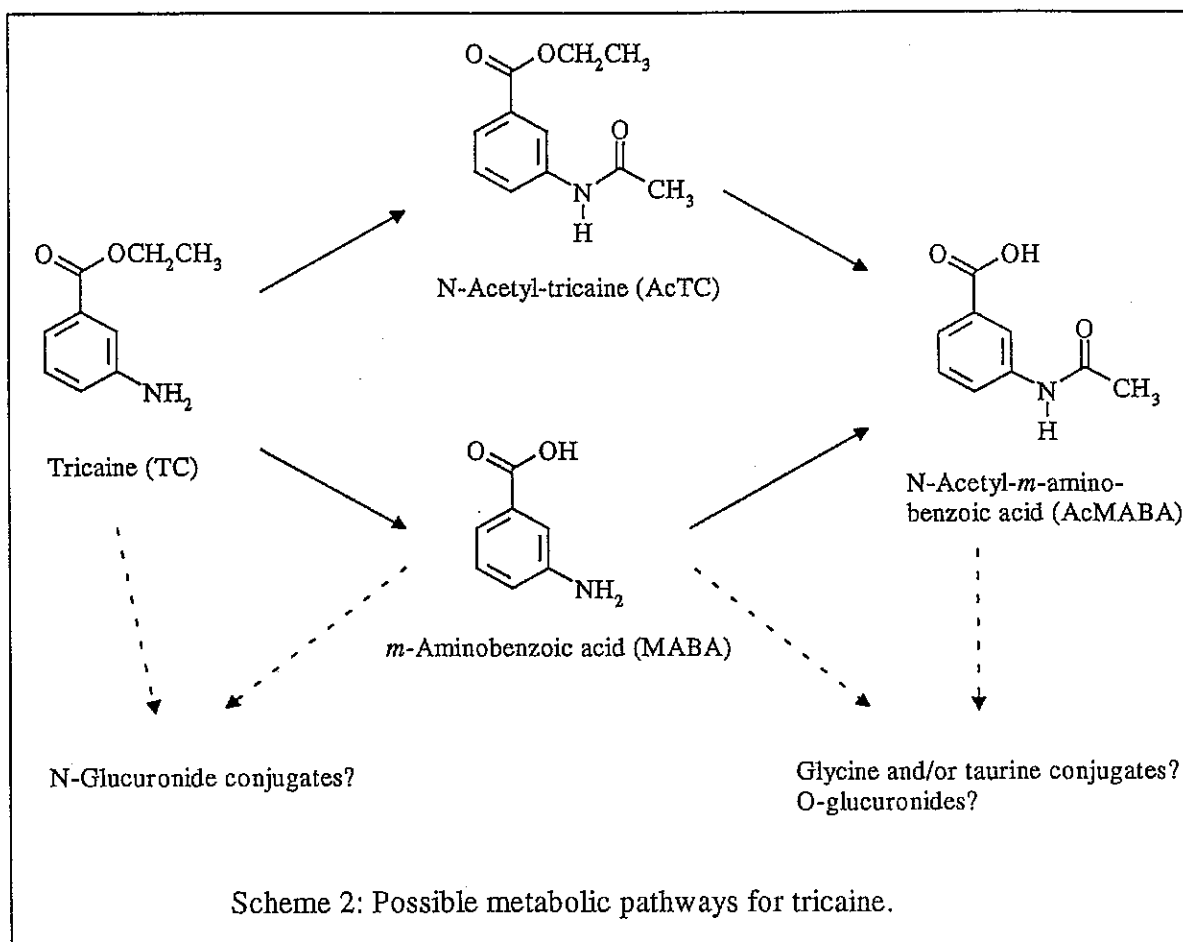
A number of methods have been reported for benzocaine since it is used widely in humans as a local anesthetic in topical solutions (e.g., to avoid local irritation produced by chloramphenicol) as well as part of dermatological preparations used against scabies, pediculosis and skin inflammation. Some LC methods are summarized in Table 1 [14-17]. Gingerich and co-workers have studied the metabolism and elimination of radiolabelled benzocaine by rainbow trout [18].

To our knowledge, no significant publications have appeared on the analysis or metabolism of metomidate. Quinaldine is easily analyzed by a number of methods such as GC and LC, but is not a priority for the present project.
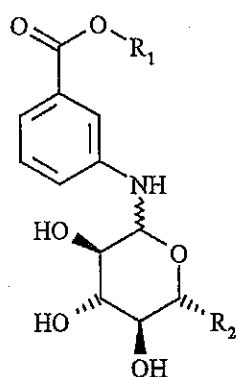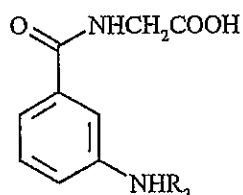
R_1 = Et, R_2 = COOH   N-Glucuronide of TC (TC-GA)

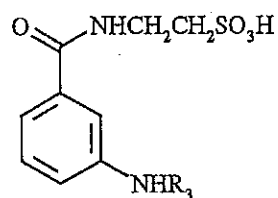R_1 = H, R_2 = COOH   N-Glucuronide of MABA (MABA-GA)

R_1 = Et, R_2 = CH_2OH N-Glucoside of TC (TC-Glu)

R_1 = H, R_2 = CH_2OH N-Glucoside of MABA (MABA-Glu)

R_3 = H   MABA glycine conjugate (MABA-Gly)

R_3 = Ac   AcMABA glycine conjugate (AcMABA-Gly)

R_3 = H   MABA taurine conjugate (MABA-Tau)

R_3 = Ac   AcMABA taurine conjugate (AcMABA-Tau)

Scheme 3: Some possible conjugated metabolites of tricaine.

Table 1. Comparison of published LC methods for benzocaine.

| Column	Mobile Phase	Inj Vol (µL)	UVD λ (nm)	Det. Lim.	Ref.
µ-Porasil (150x3.9mm)	10mM NH_4ClO_4 in methanol	25	270	6 µg/mL	14
µ-Bondapak C18 (300x3.9mm)	35% acetonitrile in water	20	280	na	15
C18 (? brand) (220x4.6mm)	40% acetonitrile in water	50	254	na	16
Ultrabase C18 (250x4.6mm)	40% methanol 50% chloroform 10% water	20	278	4 µg/mL	17

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Results and Discussion

A. Synthesis of Suspected Metabolites and Internal Standards

This project required the acquisition of a number of compounds that could be used as standards for method development, for instrument calibration in quantitative analysis, for use as internal standards, and for confirmation of metabolite identity. Only a few of the compounds shown in Schemes 2 and 3 were commercially available. These included TC, MABA and AcMABA. Other compounds were synthesized in-house by Mr. Bill Hardstaff. Structures were confirmed by UV, MS and NMR.

The N-acetyl derivative of tricaine (AcTC) was prepared by reaction of tricaine with acetic anhydride in pyridine and purified by crystallization. The N-glucuronides and N-glucosides of TC and MABA were prepared by reacting the compounds with a 100-molar excess of D-glucuronic acid or D-glucose, respectively, in water at 40°C overnight. The products were not isolated on a preparative scale.

A search for appropriate internal standards for TC that would be commercially available at first centered on benzocaine (an isomer of TC; see Scheme 1) and the ethyl ester of 2-aminobenzoic acid. Both of these compounds have retention times different from those of TC and its metabolites, but their UV spectra are quite different from that of TC which would mean that a single wavelength would not be suitable for both internal standard and analyte. While this is not a problem with our photodiode array detector, we felt that it was not ideal. Also, benzocaine is one of the anesthetics that the method should be capable of monitoring. Deuterated analogs of TC and its metabolites would be ideal for LC-MS experiments, but would be expensive and not suitable for simple LC analysis. Therefore, we examined a number of homologues of TC including the methyl, *iso*-propyl and *n*-propyl esters of 3-aminobenzoic acid. These were prepared by reaction of 3-aminobenzoic acid in the appropriate anhydrous alcohol saturated with gaseous HCl. The compound that had the best retention time was the methyl ester (Me3ABA). It has a UV spectrum almost identical to that of TC. Me3ABA was purified by liquid-liquid partitioning and crystallization. It appears to perform very well as an internal standard for tricaine. As discussed later, it is not ideal as an internal standard for the metabolites, however.
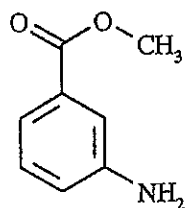
Methyl ester of 3-aminobenzoic acid

(Me3ABA)

B. Extraction and Cleanup Methods

The literature method for the colorimetric measurement of tricaine [1,2] uses a very simple extraction method: homogenization of 1 g tissue with 15 mL water, followed by addition of 4 mL of 15% trichloroacetic acid (TCA) to precipitate proteins, centrifugation and filtration. The GC [3] method uses the same tissue extraction procedure but cleans and concentrates the analytes by extracting a salted, neutralized crude extract with a small volume of benzene/ether (8:2). We have investigated the GC procedure (see previous report to BVD, Jan. 1993) and have found that, in our hands, the extraction yields are rather poor (50-80%) and irreproducible for TC and AcTC, and not at all suitable for the acidic metabolites, MABA and AcMABA. The benzene/ether extract was unsuitable for direct analysis by LC and evaporation of the extract (in order to transfer to aqueous methanol) was found to result in further losses of TC and AcTC.

LC-MS (see later for methodology) was used to survey some different extraction solvents for recovery of the four principal analytes from salmon muscle tissue. The recoveries observed using a single dispersive extraction of 2 g tissue (spiked with 25 µg/g of each analyte) in 18 mL of solvent are reported in Table 2.

Table 2. Recovery (%) of analytes from salmon muscle spiked at the 25 µg/g level.

Analyte	Aq. Acid (3% TCA)	Aq. Acid +Hexane[*]	Aq. MeOH (50%)	Aq. CH_3CN (50%)
MABA	65	43	83	32
Me3ABA	64	47	72	67
TC	69	50	74	77
AcMABA	38	22	98	95
AcTC	28	7	85	92

[*] The crude acid extract was partitioned with 2 x 5 mL hexane to remove lipids.

The acidic extraction is clearly unsuitable for analysis of the complete range of TC metabolites, as it fails to extract the acetylated metabolites. Partitioning of the crude extract with hexane (attempt to remove some of the lipids) resulted in further losses. An additional concern about use of the acid extraction procedure was that conjugated metabolites would be partially or completely hydrolyzed under such conditions. Therefore, organic solvents were examined as a means of precipitating protein and increasing the yield for the more nonpolar metabolites. Aqueous methanol (50%) and aqueous acetonitrile (50%) gave similar results for four of the metabolites (within the expected uncertainty of the experiment) but aqueous acetonitrile gave a poorer yield for MABA.

Based on these results, an extraction procedure was developed that involves homogenization of 2 g of tissue with 2 x 10 mL of 50% aqueous methanol. After high

speed centrifugation, combining supernatants and making to a final volume of 20 mL, the crude extract is filtered and analyzed directly by LC and LC-MS. The recoveries from control muscle tissue spiked at the 2 μg/g level were determined by LC-MS and are reported in Table 3.

Table 3. Recovery of analytes from salmon muscle spiked at the 2 μg/g level, using the aqueous methanol extraction method (n = 5 for samples, n =3 for calibration).

Analyte	Recovery (% ± s.d)
MABA	91 ± 10
Me3ABA	84 ± 4
TC	86 ± 4
AcMABA	99 ± 5
AcTC	98 ± 6

Later analyses of samples of fish exposed to tricaine indicated that aqueous methanol was a suitable solvent for extraction of the conjugated metabolites as well. Further information on extraction efficiency was provided by Dr. F. Law, who tried some of these extraction methods on fish exposed to radiolabelled tricaine. Aqueous methanol was found to be the most suitable solvent, extracting 80-90% of the total radioactivity from most tissues. The remaining activity may be due to metabolites covalently bound to macromolecular components.

A great deal of time was spent trying to develop clean-up methods for elimination of interferences and for preconcentrating the sample. As indicated above, liquid-liquid extraction methods were not satisfactory. Various solid phase extraction cartridges were examined containing strong anion, strong cation, normal phase and reversed phase stationary phases. Due to the wide range of polarities of the various analytes, which include neutral, acidic, basic and amphoteric compounds, it was impossible to achieve quantitative recovery of all analytes, at least into one single fraction. Since it was determined that a crude, filtered aqueous methanol extract did not cause significant damage to the LC column and that LC-MS provided sufficient sensitivity for all experiments, it was decided that no further work would be put into clean-up. Such a procedure would still be desirable for LC analysis because, as shown below, it is not possible to measure low levels of MABA directly in the crude extract with either UV or fluorescence detection and some degree of preconcentration is still required to achieve lower detection limits in LC for all the compounds. To this end, the combined derivatization and clean-up procedure (detailed in Section G) was developed for the measurement of MABA and TC.

C. Capillary Gas Chromatography-Mass Spectrometry (GC-MS)

At first glance, it would appear that GC and GC-MS should be good techniques for the analysis of tricaine residues in fish. Indeed, as mentioned earlier, tricaine has been successfully analyzed by GC equipped with a nitrogen-selective alkali flame ionization detector [3]. However, the detection limit of 150 ng/g (in catfish muscle tissue) was quite poor. The main difficulty in the analysis lies in the cleanup step, which is quite important for protecting the GC column. Since the free base of tricaine is volatile, it is difficult to perform liquid-liquid partitioning with large volumes of solvent which would require evaporative concentration, accompanied by loss of analyte, prior to analysis. Also, many of the expected metabolites cannot be extracted under the same conditions as tricaine and most of them are not suitable for GC analysis without chemical derivatization.

Nevertheless, we examined GC-MS as a technique that might provide better detection limits than GC alone due to the higher selectivity and sensitivity of the MS as detector. We have already presented our work in this area in a previous report to BVD (Jan. 1993), so it will not be repeated here. In summary, we found that with selected ion monitoring and the literature extraction method for GC, it was possible to achieve detection limits of about 0.2 to 2 μg/g for tricaine, benzocaine, metomidate and quinaldine in salmon muscle tissue. However, we experienced recovery problems with the literature extraction method. In addition, response curves were non-linear with non-zero intercepts on the x-axis, suggesting decomposition and/or irreversible adsorption on the column at trace levels. Derivatization (e.g., trifluoroacetylation) of the free amino group of TC would probably result in better quantitation. Due to the difficulties observed and the unsuitability of the technique for analyzing a wide range of compounds, the work on GC-MS was not continued.

D. Liquid Chromatography (LC)

A considerable effort was put into developing a suitable LC analysis method for TC and its metabolites. Various stationary and mobile phases were examined for separation selectivity (i.e., resolution of analytes and matrix peaks) and peak shape. Some columns exhibited severe tailing for the basic compounds. The best stationary phase proved to be Zorbax Rx-C8. This "sterically-shielded" octadecyldi-*iso*-propylsilica sorbent provides excellent chromatographic efficiency (65,000 plates/meter) and symmetrical peak shape for basic compounds. The optimization of the mobile phase was concerned mainly with the ultimate goal of performing combined LC-MS analysis. Sensitivity in LC-MS is enhanced by a volatile, low pH mobile phase. Aqueous methanol or acetonitrile with 0.1% trifluoroacetic acid (TFA) proved useful. In a previous report to BVD (Jan. 1993), we demonstrated that TC, benzocaine and metomidate could be separated with an isocratic separation using 40% methanol/0.1% TFA. Detection limits (expressed as concentration in the final extract) ranged from 13 ng/mL for TC to 56 ng/mL for metomidate using UV absorbance detection (UVD) and a wavelength of 226 nm.

For the tricaine metabolism study, we had to use gradient elution to separate all the compounds in one analysis, as well as to clean the column of any late eluting endogenous compounds from the sample. The best separation selectivity was provided by using acetonitrile as the organic modifier. Figure 1 shows a rapid separation of tricaine and five synthesized expected metabolites, i.e., MABA, AcMABA, TC-Glu, TC-GA and AcTC. Also separated in this analysis are the internal standard, Me3ABA, and low levels of MABA-Glu and MABA-GA (formed when MABA was mixed with the excess glucose and glucuronic acid present in the TC-Glu and TC-GA solutions).

The UV spectra of the various analytes are shown in Figure 2. These were acquired by the photodiode array detector (DAD) during the analysis shown in Figure 1. An acquisition wavelength of 226 nm is clearly the best for sensitivity, but since a DAD can acquire simultaneously at multiple wavelengths, it is possible to use the other bands present in these spectra for confirmation of identity. This is only feasible at higher concentrations, however, because the extinction coefficients at the higher wavelengths are very weak. The retention times and UV spectral data for the various compounds are summarized in Table 4.

The sensitivity of UV detection, even at 226 nm, is not very good for these compounds due to their relatively low extinction coefficients. In addition, detection at such a low wavelength is not very selective, resulting in considerable potential for interference in the case of crude fish extracts. The practical limit of quantitation with LC-UVD is about 1 μg/g in tissue for all metabolites except MABA, which is usually masked by coeluting materials near the solvent front (see below). Therefore, fluorescence detection (FLD) was examined as a way of improving the situation. All the analyte standards were found to be moderately fluorescent; excitation and emission spectra were acquired for all standard compounds and the data are presented in Table 4. Figure 3 shows spectra of three representative compounds: TC, TC-GA and AcTC. The spectra of the different classes of metabolites are quite characteristic.

Figure 4 shows a comparison of the LC-FLD and LC-UVD chromatograms of the same standard mixture shown in Figure 1. The fluorescence chromatogram was acquired with excitation at 232 nm and emission at 436 nm. A relatively low gain setting was used, with the possibility of a further 256-fold increase in sensitivity. Very low baseline drift is observed with the fluorescence detector, as it is insensitive to the refractive index changes occurring during gradient elution. Interestingly, the glucose and glucuronic acid conjugates are 10-fold more fluorescent than the others, making LC-FLD analysis an excellent way to detect trace levels of these conjugates. Since the emission maxima of the conjugated and acetylated metabolites are shifted to lower wavelengths than those of TC and MABA it was not possible to choose a single emission wavelength optimal for each compound. This is mainly a problem for the acetylated compounds. Time programming of the acquisition wavelengths might be a solution. Nevertheless, there is adequate sensitivity for the acetylated compounds using an emission wavelength of 436 nm, since the 359 emission band is quite broad and a large emission slit (and therefore bandwidth) was used. Another solution to the problem is to acquire the emission with a zero-order setting of the emission monochromator and a cut-off filter. Figure 5 shows a comparison of the LC-FLD chromatograms of our routine calibration standard mixture acquired with either 436 nm emission or zero-order emission combined with a 340 nm cut-off filter.

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Under these conditions, both the acetylated and conjugated metabolites are more easily detected than the parent compounds (unfortunately, this technique was not developed until near the end of the project, so all sample analyses shown in this report use an emission at 436 nm). Practical limits of quantitation with LC-FLD are about 200 ng/g in tissue for TC, about 50 ng/g for AcMABA and AcTC, and about 20 ng/g for the conjugated TC metabolites. Detection of MABA and its conjugates is still difficult due to interference from other compounds eluting at the solvent front.

Using the FLD and DAD detectors in series, it is possible to acquire simultaneously the UV and fluorescence chromatograms. This procedure was used for analysis of all samples received from Dr. Law, as will be seen in later figures. It should be noted that slight peak broadening can be observed in UV chromatograms due to the passage of the eluites through the 4 μL flow cell of the FLD prior to the DAD cell.

It should be noted that slight decomposition of the conjugated metabolites was observed during the chromatography, presumably due to the very acidic mobile phase (about pH 2). This manifested itself as a leading edge on the peaks. It was possible to eliminate this by operating at a higher pH (e.g., with 0.1% formic acid, about pH 3) but the separation changed considerably, resulting in coelution of analytes. Since the TFA-based mobile phase proved best for LC-MS analysis, we stayed with it for all analyses. The method used in Figure 1 and 3 has proved to be very robust with good linearity, dynamic range and reproducibility.

Examples of the application to actual fish tissues are given in Figures 6 to 8. These show LC-FLD-UVD analyses of trout tissues provided by Dr. F. Law. Figure 6 presents the data for muscle tissue from a trout exposed to 100 ppm MS-222 for 30 min and then sacrificed and dissected immediately. Tricaine, AcTC and trace levels of TC's glucuronide are visible (at 3.3, 0.1 and 0.1 μg/g, respectively). MABA is also present (0.1 μg/g), as determined later by LC-MS analyses, but cannot be confirmed by this method due to interfering peaks at its retention time that are also observed in control tissues. Figure 7 shows the analysis of a liver sample exposed to 50 ppm MS-222 for 24 hr. It shows much higher levels of all compounds and the fluorescence trace is dominated by the glucuronide of TC. The chromatograms for a control liver tissue are shown in Figure 8. Quantitation of all the samples by the LC method agreed quite well with that achieved by LC-MS, except in cases where there was interference with optical detection (e.g., for MABA). Tissue concentrations will be summarized later in section F.

Table 4: UV and fluorescence spectral data for tricaine and related compounds.

Compound Name/Code	Ret Time (min)	Ultraviolet Absorbance Max (nm)	Rel Int	Fluorescence Excitation Max (nm)	Rel Int	Emission Max (nm)
m-Aminobenzoic acid	2.1	**226**	100	**231**	100	**445**
(MABA)		272	7	247	60	
mw=137		312	1	304	47	
Methyl ester of MABA	4.4	**226**	100	**231**	97	**440**
(Me-3-ABA)		270	7	248	100	
mw=151		312	1	263	46	
Tricaine	6.2	**226**	100	**231**	100	**436**
(TC)		270	7	248	70	
mw=165		312	1	304	18	
N-Acetyl-MABA	6.6	**224**	100	**232**	100	**358**
(AcMABA)		250s	50	248	98	
mw=179		294	5	262	49	
N-Glucoside of TC	7.1	**224**	100	**232**	100	**420**
(TC-Glu)		250s	32	248	50	
mw=227		320	8	314	6	
N-Glucuronide of TC	7.5	**224**	100	**232**	100	**420**
(TC-GA)		250s	32	248	50	
mw=241		320	8	314	6	
Benzocaine	8.5	222	95	232	60	343
(BC)		285	100	252	84	
mw=165				**265**	100	
N-Acetyl-TC	10.6	**224**	100	233	95	359
(AcTC)		250s	50	**249**	100	
mw=207		294	5	261	55	

E. Liquid Chromatography-Mass Spectrometry (LC-MS) Methodology

The recent introduction of atmospheric pressure ionization (API) techniques has revolutionized LC-MS. The unique performance of the SCIEX API-III triple quadrupole mass spectrometer (SCIEX, Thornhill, Ont.), particularly its very high sensitivity, is due mainly to the patented gas curtain interface and cryogenic pumping system. It is very easy to use with a variety of chromatographic systems (wide and narrow bore LC columns, supercritical fluid chromatography and capillary zone electrophoresis) without deterioration of separation efficiency. We have investigated two related API techniques for the LC-MS analysis of anesthetic drugs, viz.: atmospheric pressure chemical ionization (APCI) and ionspray (see Figures 9 and 10).

Ionspray or "nebulizer-assisted electrospray" is achieved by spraying the LC effluent from a needle charged to 5000 V. Ions are produced by a mechanism termed "ion evaporation" from microdroplets (see Figure 10). Ions are sampled through a micron-sized orifice, protected by a counter-current of dry nitrogen, into the MS vacuum chamber. Postcolumn splitting of the effluent is usually used to allow an optimum flow of 30 to 50 mL/min to be directed to the ion source. Ionspray has been used successfully in our laboratory for the analysis of antibiotics in salmon flesh [19,20]. Initial experiments with the anesthetic drugs proved disappointing, however (see previous report to BVD, Jan. 1993). Although protonated molecules, MH^+, as well as abundant acetonitrile adduct ions, $[H+CH_3CN]^+$, were produced for tricaine, benzocaine and metomidate, detection limits (at S/N = 3) were found to be only about 1 ng (injected into the LC). Such high detection limits for nitrogenous compounds are unusual in ionspray LC-MS. The poor sensitivity is in part due to low basicity of aromatic amines and in part due to the background spectrum from the mobile phase being quite high at low mass. Following these initial experiments we turned our attention to the APCI technique. However, later on, we returned to using ionspray (see below) because it proved superior for thermally labile metabolites. With some adjustment of conditions, the sensitivity was improved somewhat and did prove adequate for most samples.

The second API technique tested for LC-MS analysis of the anesthetics was APCI. On the SCIEX instrument, this is accomplished using the heated pneumatic nebulizer (HPN) (see Figure 9). In this system, the LC effluent is nebulized into microdroplets which are then evaporated as they pass through the air in a heated quartz tube. Rapid evaporation of analytes is achieved with minimal thermal decomposition. Gaseous analytes are then subjected to soft chemical ionization by the gaseous mobile phase solvents initiated by a corona discharge at atmospheric pressure. Because the HPN interface accepts liquid flow rates up to 1 mL/min, there is no need for postcolumn splitting.

The APCI mass spectra of TC, benzocaine, Me3ABA and metomidate are presented in Figure 11. It was observed that the appearance of the spectra was very dependent on the potential difference between the orifice plate and the initial AC rods of the MS (see Figure 9). As shown in Figure 11, MH^+ ions dominate the spectra at low potential differences (10V) (spectra on the left), while extensive fragmentation occurs at higher values (30V) (spectra on the right). The fragmentation results from collision-induced dissociation (CID) of the MH^+ ions in the free jet expansion region behind the

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sampling orifice. Increased potential results in more energetic collisions. The presence of fragment ions can be useful for structure elucidation and for confirmation of analyte identity at trace levels when using selected ion monitoring (SIM). However, since extensive fragmentation can also reduce sensitivity, it is important to choose the orifice voltage setting very carefully. One setting may not suit all analytes. This is illustrated in Figure 12, which compares the spectra of TC and AcTC. At a 20 V potential difference, AcTC is extensively fragmented, resulting in a very weak MH$^+$ ion. Another way of generating fragment ion spectra is with tandem mass spectrometry or MS/MS. Ions isolated by one MS are directed into a collision chamber filled with argon gas. The fragment ions created by CID are scanned by a second MS. Figure 13 shows the fragment ion spectra of the MH$^+$ ions of TC and AcTC. Although it provides spectra similar to those in Figure 12, the MS/MS technique is 5- to 10-fold less sensitive than analysis with a single quadrupole and orifice CID. MS/MS does provide greater selectivity and is an excellent method for structure elucidation or confirmation. Moderate sensitivity can be achieved with the selected reaction monitoring mode in which specific fragmentations can be monitored by tuning one MS to the MH$^+$ ion and the second MS to a fragment ion (e.g., m/z 166 → m/z 138 for TC). An application of this technique to an actual sample will be shown in the next section.

The effect of orifice potential on the fragmentation of tricaine and its metabolites has been studied extensively in an effort to find the optimum conditions. Figures 14 and 15 illustrate some of the data with ion intensity plots for TC and AcTC. These plots are useful in selecting the optimum orifice potential for a compromise between sensitivity and production of characteristic ions. With the SCIEX instrument, it should be possible to time program the orifice potential, although this has not been attempted yet.

Figure 16 (top) shows the APCI spectrum of the N-glucuronide of TC. The MH$^+$ ion can be observed along with a large number of other ions, including those due to losses of water molecules and the sugar ring. The N-glucoside spectrum was very similar. In the spectra of both compounds there were a number of ions that were difficult to explain by simple fragmentations. It was suspected that thermal decomposition might be occurring in the heated nebulizer interface. Therefore, the ionspray spectra of the conjugates were examined. Figure 16 (bottom) shows the ionspray spectrum of the N-glucuronide. It is much simpler, presumably due to the fact that the ionspray process is very soft and does not heat the analyte. The APCI-MS/MS spectrum of the N-glucuronide of TC is given in Figure 17. Some of the ions observed in Figure 16 are missing in this spectrum, again supporting the argument that they result from some thermal decomposition in the APCI interface. From the MS/MS spectrum, it is possible to recognize losses of 1, 2 and 3 water molecules due to the three hydroxyls, loss of formic acid from the carboxyl, loss of ethylene from the ethyl ester group, and m/z 166 representing TC itself. Such information is very useful in structure elucidation.

Figure 18 presents the data from an LC-APCI-MS analysis of a 2 μg/mL calibration solution. Mass chromatograms for both MH$^+$ ions and fragment ions (marked with an 'f') are shown. The chromatographic peaks, which correspond to 10 ng of each compound injected on column, are sharp and symmetrical without any significant tailing. Spiking experiments with control salmon muscle tissue were conducted. The aqueous methanol extraction procedure described earlier was used. Recoveries of the spikes were

presented earlier in Table 3. The reproducibility of the entire method including extraction and LC-MS measurements were determined both with and without the Me3ABA internal standard. These results are presented in Table 5. It can be observed that the internal standard does not improve the experimental precision significantly and in fact actually makes it worse for two of the analytes. This is due to the fact that very careful volumetric control was exercised during the experiment and the SCIEX API-III does not suffer from significant drifting of sensitivity over a short time period. On the other hand, the uncertainties associated with integration of two peak areas instead of one result in a higher overall uncertainty. However, if one wished to utilize less careful controls in routine work, the internal standard should be useful in correcting for volumetric errors. It is important to realize that Me3ABA is not the ideal internal standard, especially for the metabolites (see relative recoveries in Table 3). Using this compound to correct for extraction recoveries, rather than just for volumetric errors, would introduce systematic errors. Stable isotope (^{2}H or ^{13}C) labelled compounds would be ideal internal standards but would also be quite expensive to produce.

The LC-APCI-MS technique proved to be very sensitive and selective for TC and its metabolites. Estimates of the detection limits in salmon muscle tissue are presented in Table 6. Also shown are the detection limits for ionspray LC-MS, which are as much as 10-fold lower for some compounds. Applications of LC-MS to actual incurred fish samples are given in the next section.

Table 5. Reproducibility (% RSD) of analyte determination in spiked salmon muscle tissue (2 μg/g level, n=5) using aqueous methanol extraction and LC-APCI-MS.

Analyte	External Calibration	Internal Standardization
MABA	3.8	3.4
Me3ABA (IS)	2.1	---
TC	3.4	3.2
AcMABA	3.9	4.7
AcTC	1.9	2.6

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Table 6. Estimated detection limits (ng/g or ppb) of the two LC-MS methods for TC and its metabolites in muscle tissue.

Analyte	APCI	Ionspray
MABA	25	300
TC	15	80
AcMABA	60	180
AcTC	75	140
TC-GA	100	100

F. Metabolism of Tricaine Determined by LC-MS

All samples received from Dr. Law were analyzed by LC-MS with selected ion monitoring of MH^+ and principal fragment ions. Examples of such an analysis are given in Figures 19 and 20 for muscle tissues from a trout exposed to 20 ppm of MS-222 for 24 hr and from a control trout, respectively. There are only a few interfering compounds (marked with an X) which are resolved chromatographically from the target analytes. MABA, TC, AcMABA and AcTC are easily detected in the exposed sample. Quantitation of all samples was achieved by calibrating the LC-MS with accurate standard solutions. The results of some of our analyses are given in Table 7.

Tissues from the trout exposed to 50 ppm MS-222 for 24 hr were also analyzed by the full scan technique to try to detect any potential metabolites for which we did not have standards, as well as to detect any unexpected metabolites. We analyzed control tissues in order to distinguish metabolites from endogenous compounds that were co-extracted. Figure 21 shows the total ion chromatogram from the full scan analysis of liver tissue. Several peaks (marked with an X) were present in the control liver sample, but it is possible to detect TC and 4 metabolites in the exposed sample's TIC. It was this data that actually detected and identified the N-glucosides of TC and MABA as metabolites, which up to that point had not actually been anticipated. Selected mass chromatograms for this sample, presented in Figure 22, make it easier to see individual compounds. Data sets were also examined for any traces of taurine and glycine conjugates of MABA but nothing was detected. From my experience with analogous compounds, I am confident that these metabolites are not present (at least in significant concentrations).

Figure 23 shows a confirmation of some metabolites in muscle tissue (50 ppm/24 hr) by the selected reaction monitoring technique discussed earlier. The appearance of peaks in the correct ratio and at the correct retention times in all three chromatograms is excellent proof of structure. Many of the samples were also analyzed by ionspray LC-MS, since this technique was better for detecting the conjugated metabolites. Figure 24 shows the ionspray LC-MS mass chromatograms from the analysis of the same trout liver extract analyzed in Figures 21-23.

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Table 7. Concentrations of TC and its metabolites in the tissues of trout exposed to different levels of MS-222. The 100 ppm exposure for 30 min is intended to simulate typical use of the anesthetic in aquaculture operations.

	Concentration in Tissue ($\mu g/g$)				
	MABA	AcMABA	TC	AcTC	TC-GA
Muscle Tissue					
100 ppm/30 min	0.1	nd	3.3	0.1	0.1
20 ppm/24 hr	6.8	2.4	26	3.8	1.6
50 ppm/24 hr	40	7.5	71	3.2	2.2
Kidney					
100 ppm/30 min	3.2	1.4	56	4.0	1.4
20 ppm/24 hr	11	24	34	16	19
50 ppm/24 hr	64	69	79	21	15
Liver					
100 ppm/30 min	0.6	13	10	3.4	3.8
20 ppm/24 hr	9	102	137	25	202
50 ppm/24 hr	42	110	196	20	204
Skin					
100 ppm/30 min	1.9	nd	25	0.2	nd
Blood					
100 ppm/30 min	4.3	0.3	26	0.2	nd
Bile					
100 ppm/30 min	0.7	nd	5	6.3	0.1

nd = not detected

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G. Bratton-Marshall Assay Coupled with LC and LC-MS

The first method developed for tricaine was a colorimetric determination [1,2] based on the Bratton-Marshall assay. This assay was developed in 1939 [21] for the measurement of sulfonamides and was one of the first methods used in drug analysis work. The method utilizes some unique chemistry that is very selective to the aromatic primary amine function. Treatment of an acidic extract with sodium nitrite converts aromatic amines to aryl diazonium ions which are then reacted with the reagent N-1-naphthylethylenediamine dihydrochloride to form a red-colored azo dye. It is a very fast and simple procedure. Scheme 4 illustrates the structures of the reagents and the probable structure of the product with tricaine (R = Et) or MABA (R = H). I have said "probable structure" because as far as I can determine, no one has actually performed a structure elucidation on the product. Of course, azo dye production is a very important industry and the chemistry of such reactions is very well known.

Scheme 4: Colorimetric determination of aryl amines using a diazotization reaction (Bratton-Marshall assay).
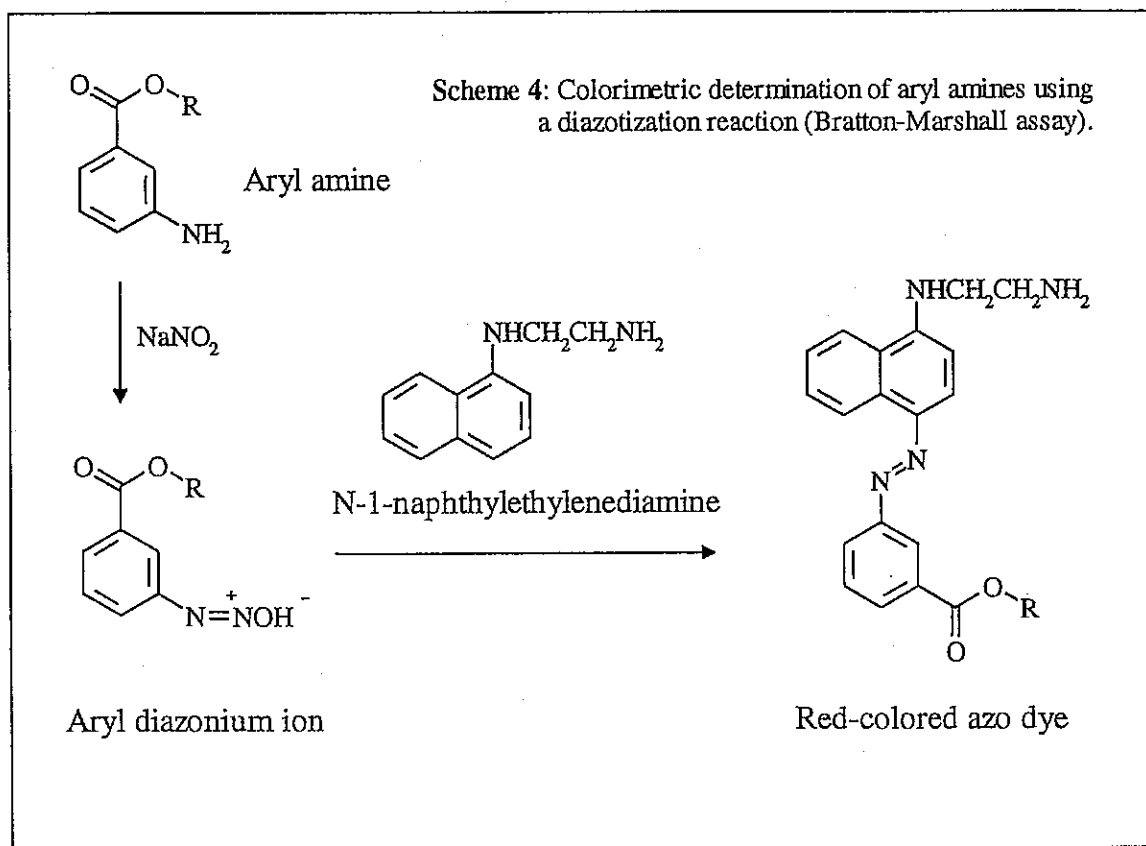
Although the colorimetric assay is relatively insensitive (μg/g detection limits) and suffers from interferences from other aromatic amines (endogenous compounds or other drugs), it occurred to me that coupling this assay with LC might provide a method that could determine not only tricaine, but a number of aromatic amine contaminants such as sulfonamide antibiotics which are also used in aquaculture. It might also eliminate the interferences observed in the colorimetric assay from endogenous amines. The first

important step was to determine if a single product resulted for analytes such as TC and MABA. LC-UVD analysis of the reaction products for both these compounds did give a single red-colored product (λmax = 540 nm). Excellent peak shape and separation was observed using a Vydac 201TP52 stationary phase and an acetonitrile/0.1% TFA mobile phase. Figure 25 shows the gradient LC-UVD analysis of a mixture of aromatic amines, including the two sulfonamides used in Canadian aquaculture, after conversion to Bratton-Marshall azo dye derivatives. Isocratic analysis can be used but the MABA derivative comes close to the solvent front. Each compound gives a single product and all the compounds can be resolved. A preparative scale reaction was used to prepare enough derivative of TC for a proton NMR spectrum. With this and the mass spectrum it was possible to confirm that the structure in Scheme 4 is correct.

Using the published extraction and derivatization procedures coupled with direct analysis of the reaction solution by LC-UVD gave a detection limit of about 0.5 μg/g (ppm) with excellent linearity over the range 1 to 100 μg/g. In order to improve the sensitivity, we decided to use a solid phase extraction (SPE) preconcentration and cleanup step following the reaction. The azo dye products have a high affinity for octadecylsilica stationary phase. We found that it was possible to load a large volume of reaction solution on a C18 SPE cartridge before breakthrough occurred (the red color of the product facilitated visual determination of breakthrough). A maximum safe loading of 0.5 g tissue equivalent was determined. After washing the cartridge with water, it was possible to elute the analytes with 100% methanol which could then be evaporated and the residues dissolved in a small volume (0.2 mL) of mobile phase for injection into the LC. This allowed a detection limit of 15 ng/g (ppb) to be achieved with LC-UVD. Excellent linearity over the range of 50 ng/g to 20 μg/g was observed. Concentrations above 20 μg/g result in detector saturation and should be determined by direct analysis of the reaction solution or dilution of the SPE extract. Figure 26 shows the LC-UVD analysis of a control salmon muscle spiked with 20 μg/g of MABA, Me3ABA and TC. The chromatogram is remarkably clean and shows excellent separation of the analytes.

The recovery and precision of the analysis was tested at two spike levels, 200 ng/g and 20 μg/g. Recovery was determined by performing the derivatization of the same volume of spike solution added to extraction solvent (aqueous TCA). The results are given in Table 8. It can be observed that recoveries are too high, especially for the 20 μg/g spike level. This suggests that there is a matrix effect that helps with either the derivatization or recovery during SPE enrichment. The use of Me3ABA as an internal standard helps to correct for this problem somewhat, but it is apparent that the best way to calibrate the method is to dilute calibrant stock solutions in extracts of control tissue. This will provide accurate determinations of unknown samples. The reproducibility of measurements ranged from 2% to 6% RSD (n = 5; see Table 8).

Figure 27 shows the analysis of muscle tissues from a control trout and from a trout exposed to 100 ppm MS-222 for 30 min. There are no interferences apparent in the chromatogram for the control tissue and there is plenty of sensitivity for MABA and TC (which are at 0.1 and 3.3 μg/g, respectively).

Table 8. Recovery and reproducibility of determination of TC, MABA and benzocaine (BC) in spiked salmon muscle tissue using acidic aqueous extraction, Bratton-Marshall derivatization, solid phase extraction and LC-UVD.

Analyte	200 ng/g (ppb) spike		20 µg/g (ppm) spike	
	% Recovery[a]	Int. Std. Determ.[b]	% Recovery[a]	Int. Std. Determ.[c]
MABA	100 ± 4	204 ± 5 (2.1)	112 ± 3	18.0 ± 0.7 (4.0)
Me3ABA	98 ± 6	----	124 ± 7	----
BC	105 ± 16	214 ± 12 (5.8)	142 ± 17	22.8 ± 1.1 (4.9)
TC	106 ± 14	216 ± 10 (4.8)	138 ± 10	22.2 ± 0.9 (3.8)

[a] % recovery ± 95% CI; n = 5 for samples, n = 3 for calibration standards (no tissue matrix); calculation of 95% CI is based on a propagation of error by calibrant

[b] ng/g ± std. dev. (%RSD), n = 5, quantitation using Me3ABA as internal standard

c µg/g ± std. dev. (%RSD), n = 5, quantitation using Me3ABA as internal standard

An interesting feature of this method is that the red color of the derivative facilitates visual screening for contamination of tissues by aromatic amines. If there is no red colored band on the SPE cartridge, aromatic amines are below the 200 ng/g level. A red color requires LC-UVD analysis for identification or verification of the contaminant identity and for precise quantitation.

A disadvantage of this method is that the acylated and N-conjugated metabolites cannot be measured directly. We explored the possibility of performing a hydrolysis of these metabolites to TC and MABA. This has been used in the colorimetric assay [2]. Determination requires splitting the sample extract and performing two measurements, one without and one with hydrolysis by HCl. The difference in MABA and TC levels should indicate the amount of AcMABA and AcTC levels, respectively. It is likely that the N-glucuronide and N-glucoside conjugates would be hydrolyzed in the initial extraction, although this has not yet been tested. Unfortunately, we found that, under the literature hydrolysis conditions, significant (ca. 40%) de-esterification occurs resulting in partial conversion of TC and AcTC to MABA. This could still be useful if the objective is to determine "total TC residues" in the sample, but it is not useful for metabolism and pharmacokinetic studies where individual metabolites should be measured. It may be that more appropriate hydrolysis conditions can be found, but we have not pursued that at this point. The LC-MS method is clearly the best method from a research perspective for determination of TC and all of its metabolites, requiring no derivatization and only a single measurement. The Bratton-Marshall assay coupled with SPE and LC should still be very useful for regulatory analyses of muscle tissue for TC and MABA because the acylated and conjugated metabolites are only minor components.

When the ionspray mass spectrum of the azo dye derivative of TC was acquired (see Figure 28) it was apparent that ionspray provided very high sensitivity. Therefore, LC-MS was investigated as a tool for trace level determination of aromatic amines and for confirmation of analyte identity. A detection limit of 5 ng/g (ppb) in muscle tissue was found to be possible using ionspray LC-MS. Figure 29 shows the analysis of a control salmon muscle tissue spiked with MABA, Me3ABA, TC and BC at the 40 ng/g level. The derivatization and SPE cleanup seems to provide a very selective and sensitive method for the LC-MS measurement of aromatic amines.

Conclusions

The analysis of anesthetic residues in fish tissues is a significant challenge. Methods are required for both pharmacokinetic studies and regulatory determinations. In this particular study, a method had to be developed that would be capable of measuring TC and its metabolites below 100 ng/g in fish tissues. The metabolites are not easily analyzed by GC and GC-MS due to their polar and thermally-labile nature. No single cleanup step was suitable for the range of metabolites, which include neutral, acidic, basic and amphoteric compounds. Conjugated metabolites are thermally and chemically labile, thus requiring gentle extraction and cool, soft ionization methods for mass spectrometry. The low λ_{max} and ε_{max} of the compounds makes UV detection in LC difficult.

Methodology was developed that uses an aqueous methanol extraction of tissues followed by direct analysis by LC with either combined UVD/FLD detection or mass spectrometric detection with APCI or ionspray ionization. Practical limits of quantitation with LC-FLD were found to be about 200 ng/g in tissue for TC, about 50 ng/g for AcMABA and AcTC, and about 20 ng/g for the conjugated TC metabolites. Detection of MABA and its conjugates is still difficult due to interference from other compounds eluting at the solvent front. LC-APCI-MS provides detection limits ranging from 15 ng/g for TC to 100 ng/g for TC-GA (see Table 6). Ionspray LC-MS is not as sensitive for most of the analytes (e.g., 80 ng/g for TC) but performed better for the conjugated metabolites.

A rapid analysis method based on the chemistry of the Bratton-Marshall assay coupled with SPE and either LC-UVD or LC-MS was developed. It was possible to detect MABA and TC, as well as other aromatic primary amines such as sulfonamides, at levels as low as 15 ng/g (ppb) in muscle tissue with LC-UVD and 5 ng/g using ionspray LC-MS. A hydrolysis step to convert acylated and N-conjugated metabolites to primary amines allows a measurement of total residues, but does not provide accurate concentrations for individual metabolites. Visual screening for positive samples is possible due to the red color of the reaction products.

LC-MS analyses established the principal metabolites of TC to be MABA, AcMABA, AcTC and the N-glucuronides of MABA and TC. Some N-glucoside metabolites of MABA and TC were also observed. No taurine or glycine conjugates could be detected.

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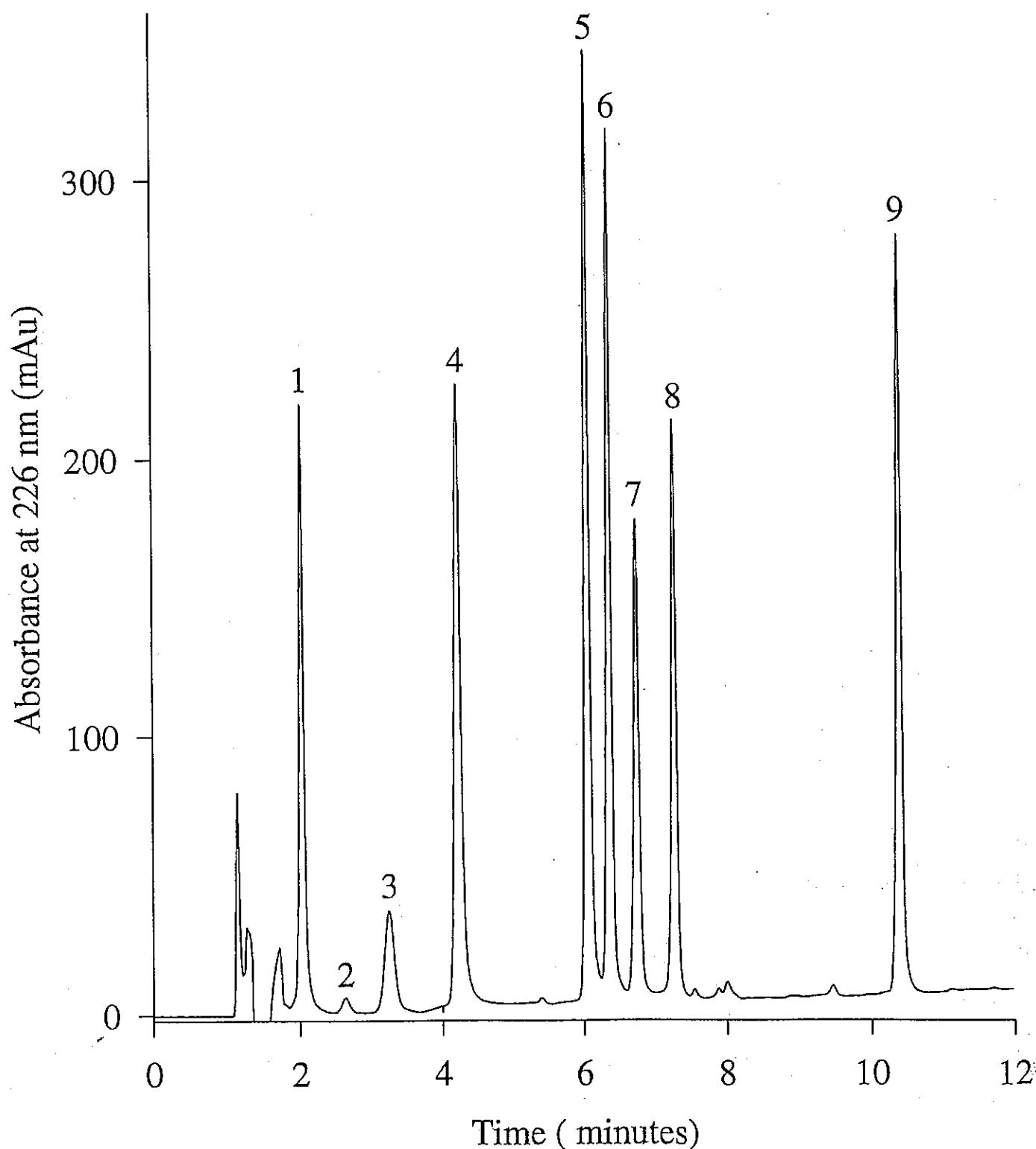
Figure 1: LC-UVD analysis of a mixture of standard compounds. Conditions: gradient elution 10% to 50% (over 10 min) acetonitrile/water /0.1% TFA; 0.3 mL/min flow; 2 x 150 mm Zorbax Rx-C8 column; 3 µL injection volume; detection at 226 nm with 4 nm bandwidth. Compound identities and concentrations: 1 = MABA (46 µg/mL); 2= MABA-Glu; 3 = MABA-GA; 4 = Me3ABA (40 µg/mL); 5 = TC (58 µg/mL); 6 = AcMABA (30 µg/mL); 7 = TC-Glu (ca. 30 µg/mL); 8 = TC-GA (ca. 30 µg/mL); 9 = AcTC (31 µg/mL).
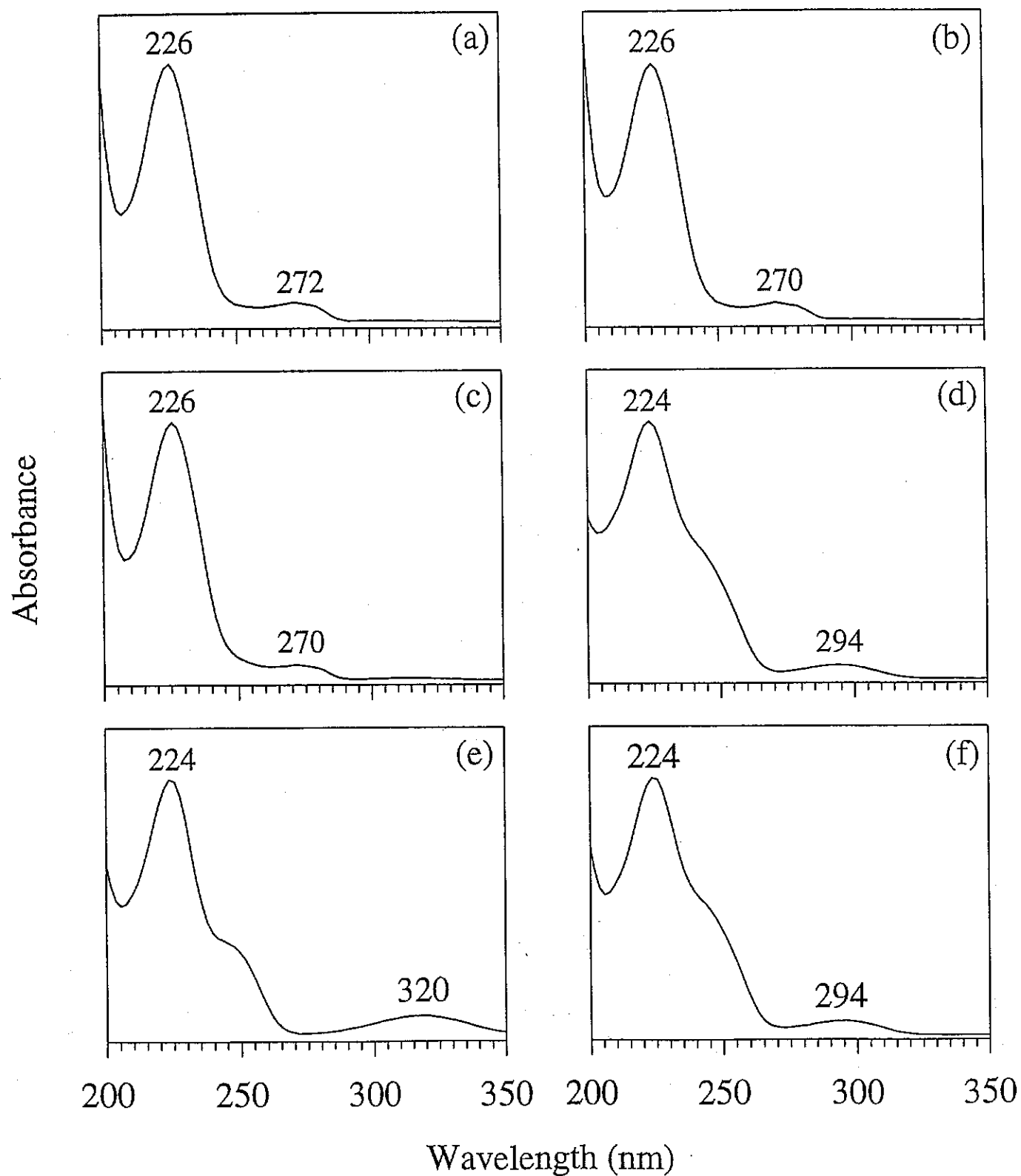
Figure 2: UV spectra acquired during the analysis shown in Figure 1. Compound identities: (a) MABA; (b) Me3ABA; (c) TC; (d) AcMABA; (e) TC-GA; (f) AcTC. The spectrum of TC-Glu (not shown) is identical to that of TC-GA.
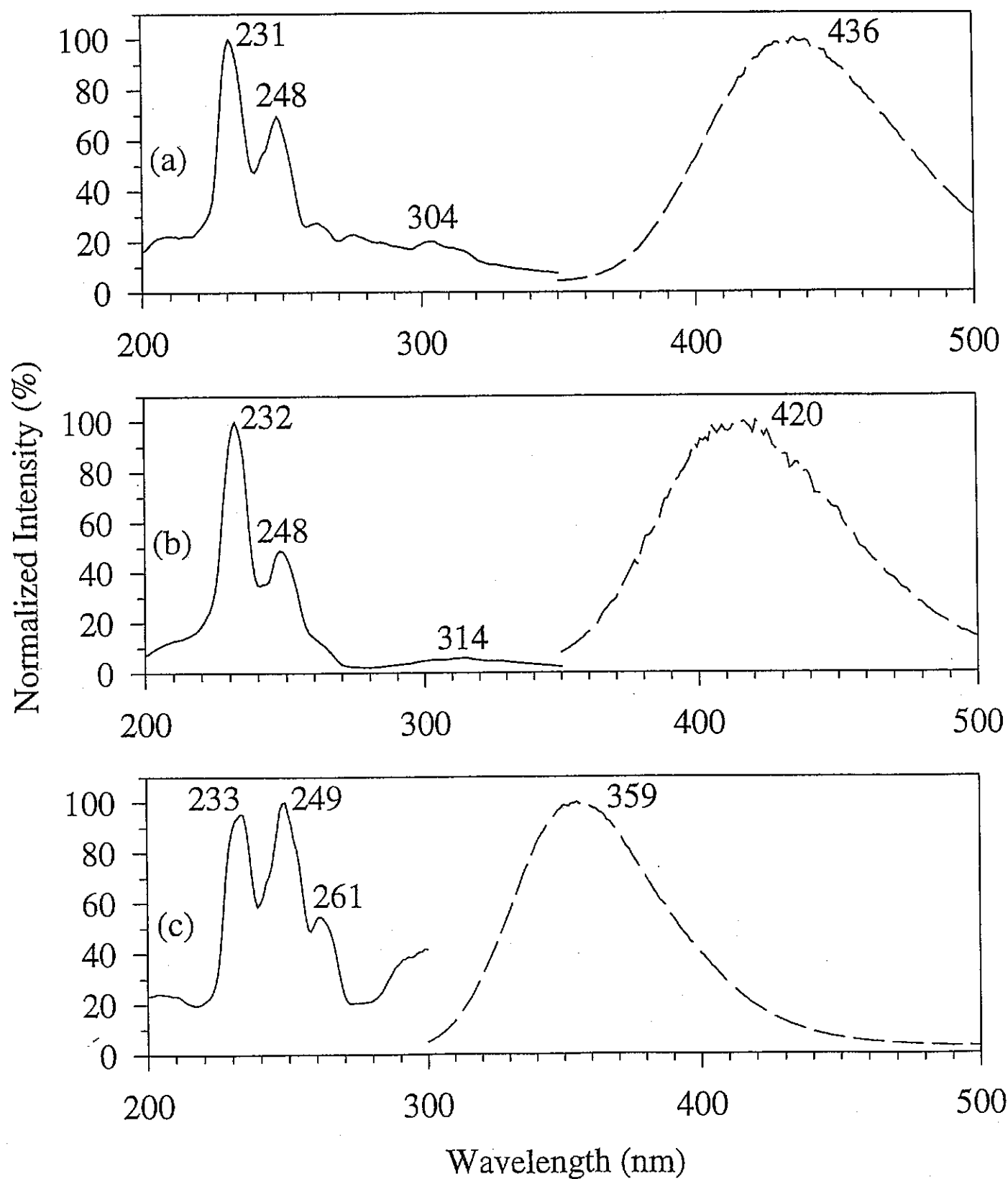
Figure 3: Fluorescence spectra of (a) TC, (b) TC-GA, and (c) AcTC. Excitation spectra are indicated by a solid line and emission spectra by a dotted line.
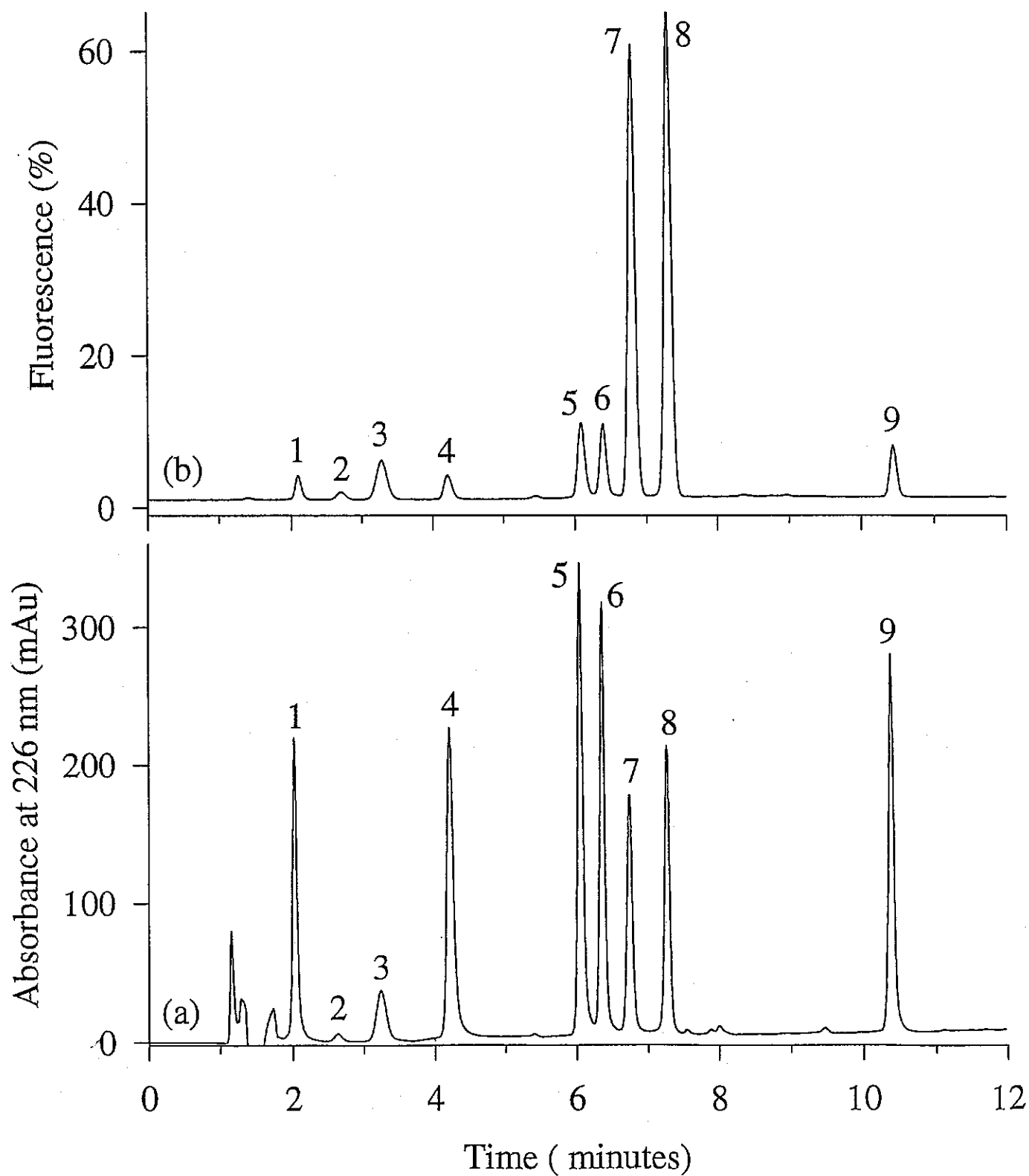
Figure 4: LC analyses of a mixture of standard compounds (same sample as in Figure 1). Conditions: (a) UV detection, same as Figure 1; (b) FLD detection with 232 nm excitation and 436 nm emission. See Figure 1 for peak identities and analyte concentrations.
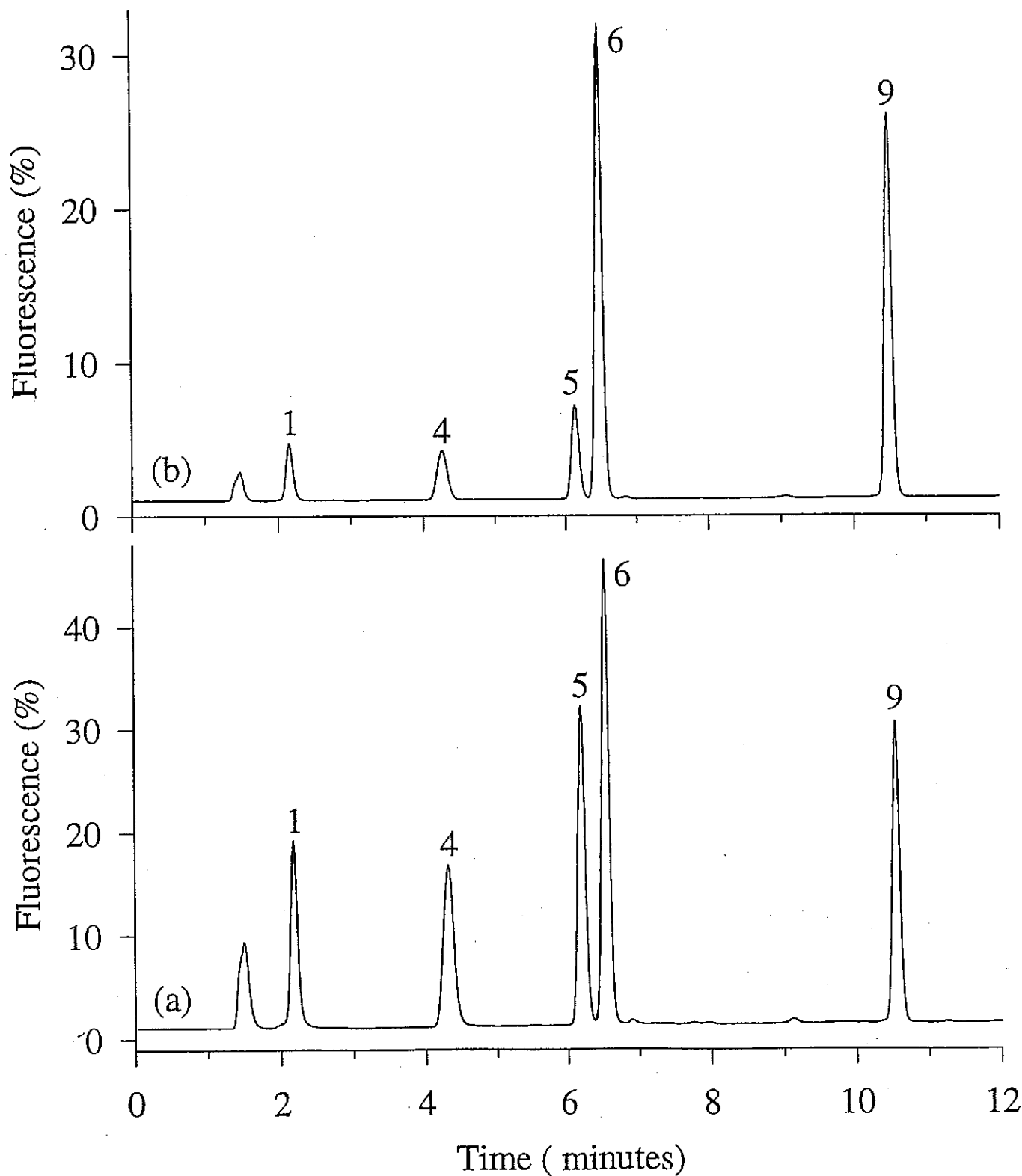
Figure 5: LC-FLD analyses of a mixture of standards. Different emission wavelengths were monitored: (a) 436 nm, gain 2^{13}; (b) zero-order with a 340 nm emission cut-off filter, gain 2^{11}. Other conditions: same as Figure 1 except 5 μL injection. Peak identities and concentrations: 1 = MABA (20 μg/mL); 4 = Me3ABA (16 μg/mL); 5 = TC (20 μg/mL); 6 = AcMABA (10 μg/mL); 9 = AcTC (8 μg/mL).
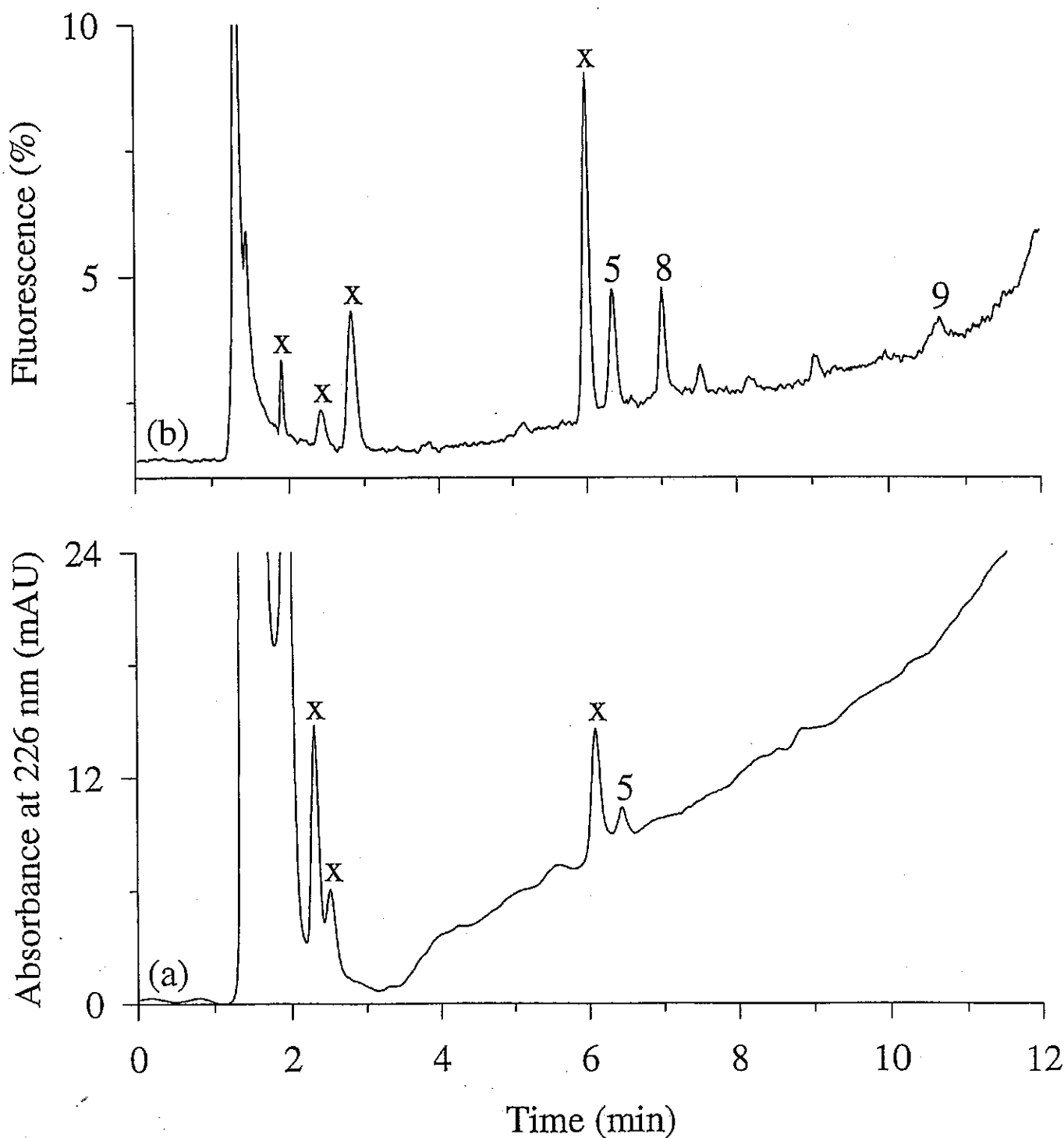
Figure 6: LC-FLD-UVD analysis of muscle tissue from a trout exposed to 100 ppm MS-222 for 30 min and then sacrificed and dissected immediately. Trace (a) shows the UV chromatogram while (b) shows the fluorescence signal. Tricaine (5), AcTC (9) and trace levels of TC-GA (8) are visible (at 3.3, 0.1 and 0.1 µg/g, respectively). MABA (present at 0.1 µg/g according to LC-MS analysis) cannot be detected by this method due to interfering peaks that are present in control tissues. Conditions: same as Figure 3, 5 µL injection. The peaks marked with an X are present in control tissues.
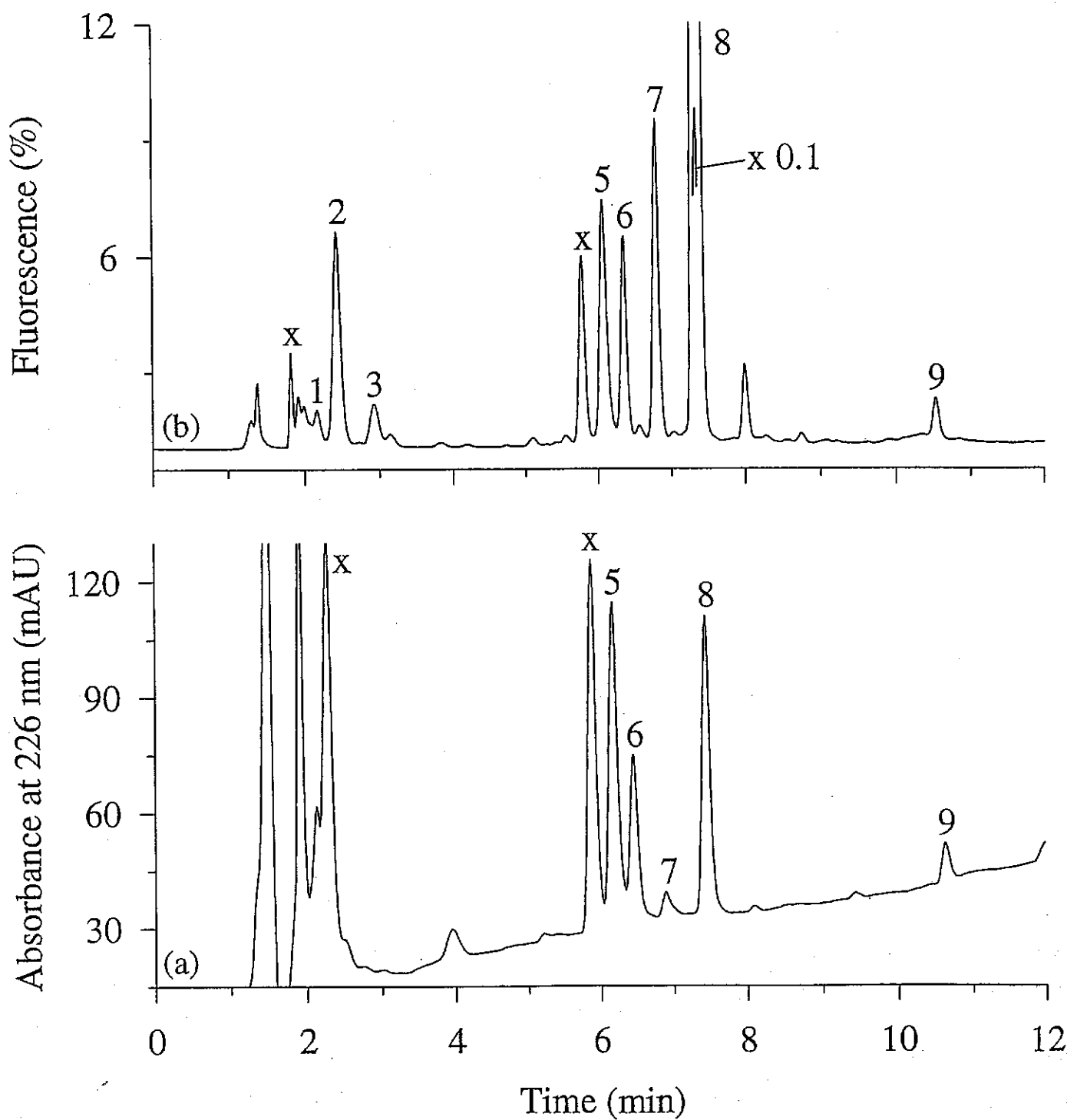
Figure 7: LC-FLD-UVD analysis of liver tissue from a trout exposed to 50 ppm MS-222 for 24 hr: (a) UVD; (b) FLD. Peak identities and measured concentrations in μg/g: 1 =MABA, 42; 2 = MABA-Glu, 5; 3 = MABA-GA, 1; 5 = TC, 196; 6 = AcMABA, 110; 7 = TC-Glu, 15; 8 = TC-GA, 204; 9 = AcTC, 20. The peaks marked with an X are present in control tissues (see Figure 8). Conditions: same as Figure 6.
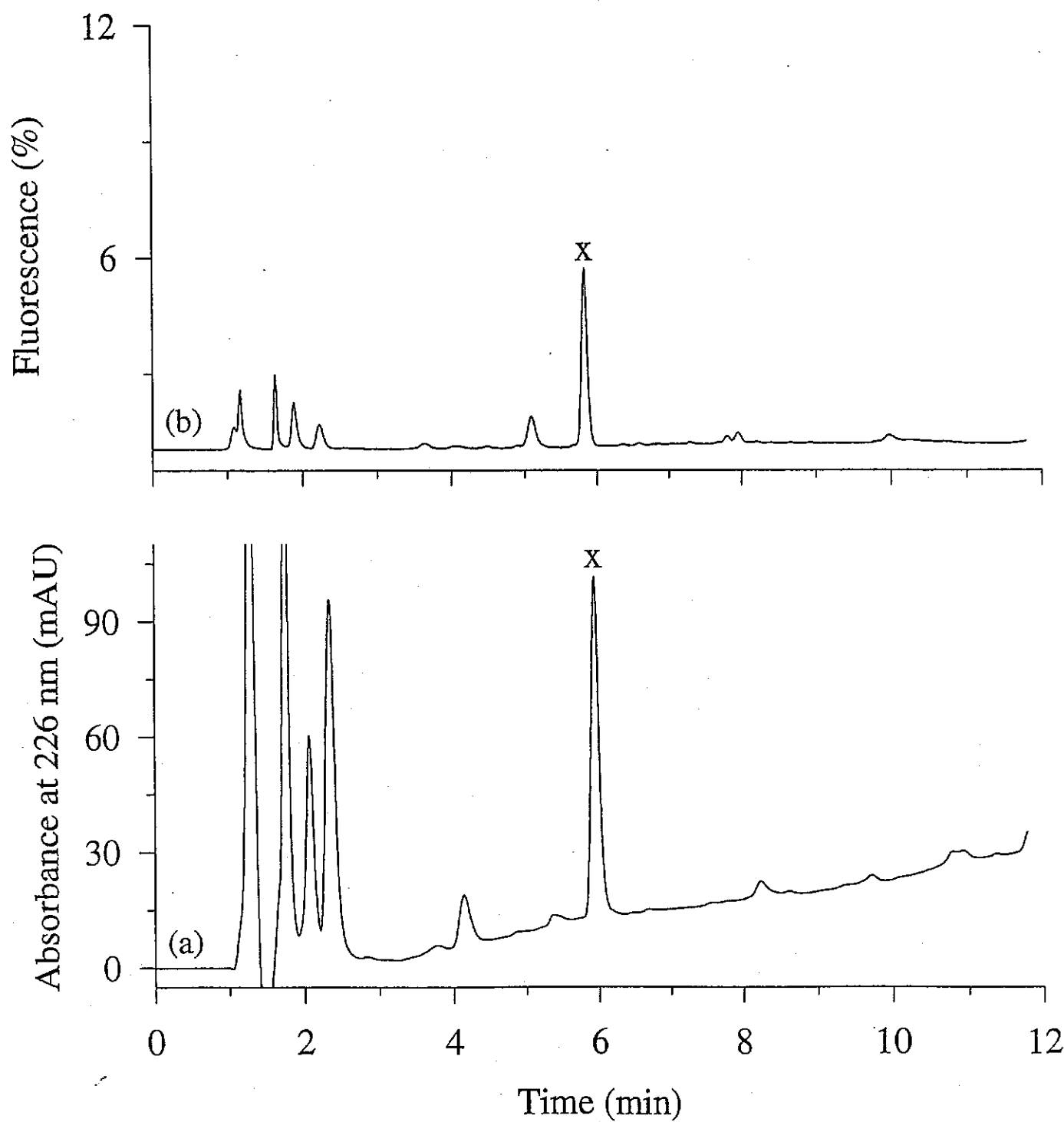
Figure 8: LC-FLD-UVD analysis of liver tissue from a control trout never exposed to MS-222: (a) UVD; (b) FLD. Conditions: same as Figure 6.

Multi-purpose front end

Interface assembly

Front cryoshell

Vacuum chamber

CAD gas inlet

Quadrupole 2

Quadrupole 3

Channel electron multiplier

Computer

AC rods

Quadrupole 1

Rear cryoshell

Sample pump

Cryopump system

Quad power supply sets

Laserwriter printer

Patented SCIEX API interface

N_2

To mass analyzer 10^{-6} Torr

Corona discharge needle

N_2

Nebulizer gas

LC effluent

Make-up gas

Figure 9: Schematic diagram of the SCIEX API-III triple quadrupole mass spectrometer and of the heated nebulizer interface used for LC-MS with APCI.

Patented SCIEX API interface

High voltage

LC effluent →

N_2

N_2

To mass
analyzer
10^{-6} Torr

N_2

Original droplet contains
ions of both polarities
with one being predominant

As the solvent evaporates
the electric field
increases and the ions
move to the surface

At some critical field
value ions are
emitted from the drop

Involatile residue
remains as a
dry particle
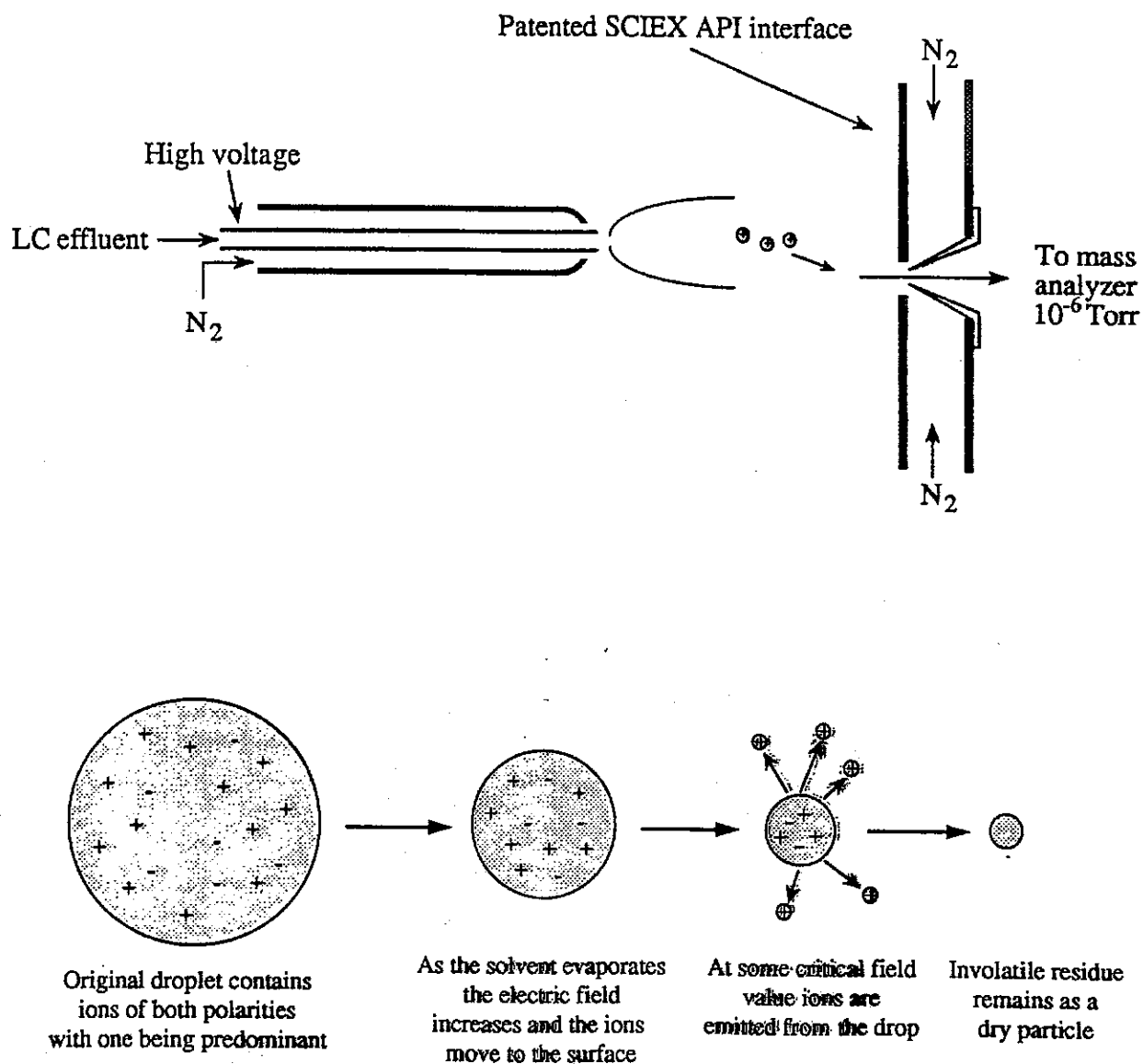
Figure 10: Schematic diagram of the ionspray interface used for LC-MS and an explanation of the ion evaporation process.
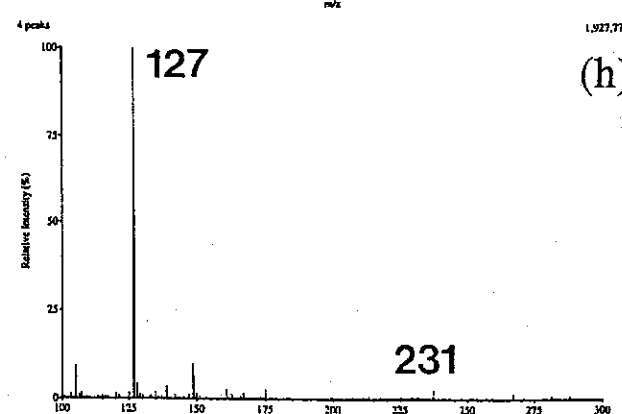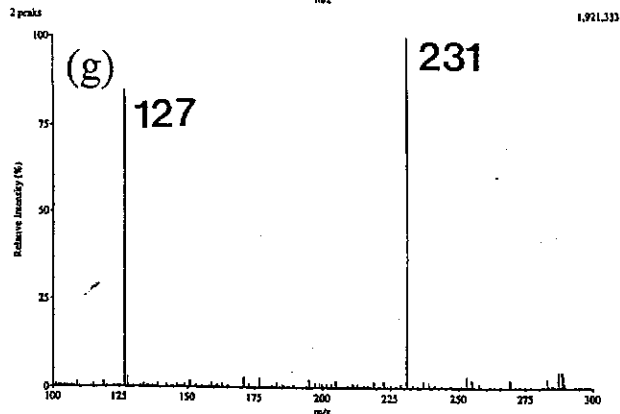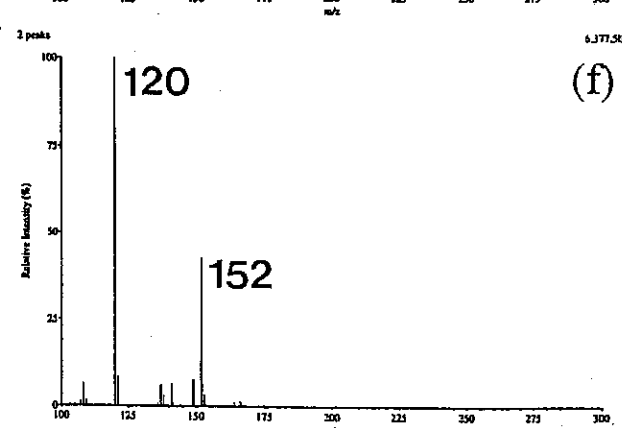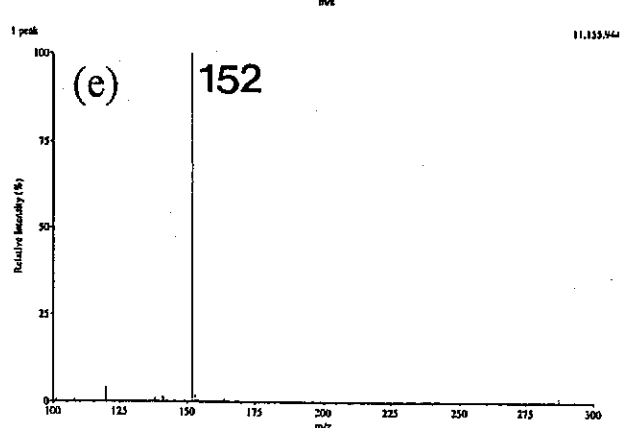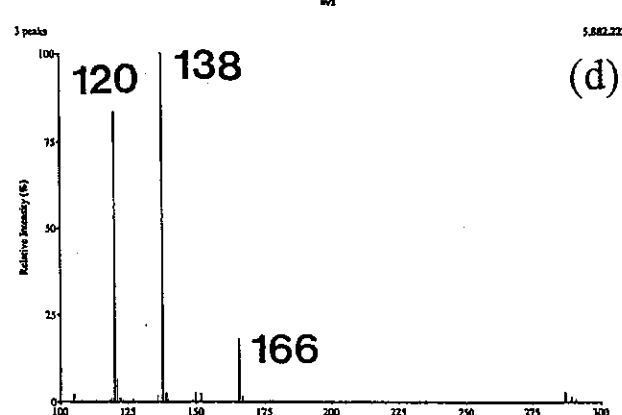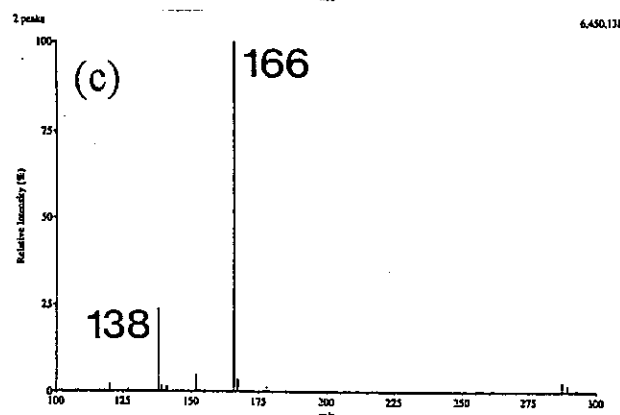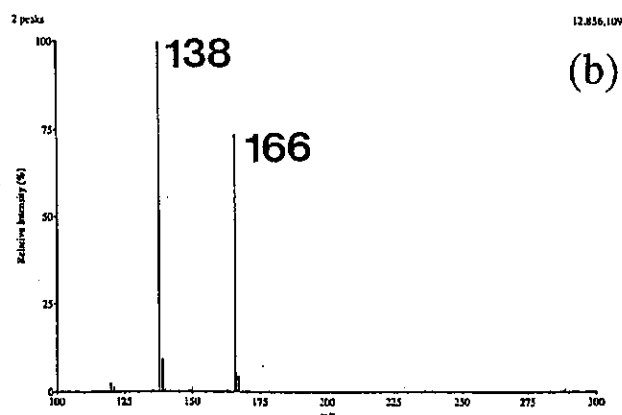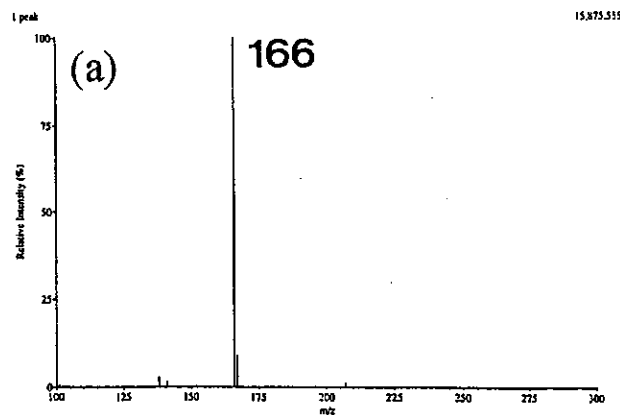
Figure 11: Full scan APCI mass spectra of standards acquired during LC-MS (approximately 40 ng of each injected): (a,b) tricaine; (c,d) benzocaine; (e,f) methyl-3-aminobenzoate; and (g,h) metomidate. Two potential differences between the sampling orifice and the first rf-only quadrupole of the MS were used: 10 V for the spectra on the left; 30 V for the ones on the right.
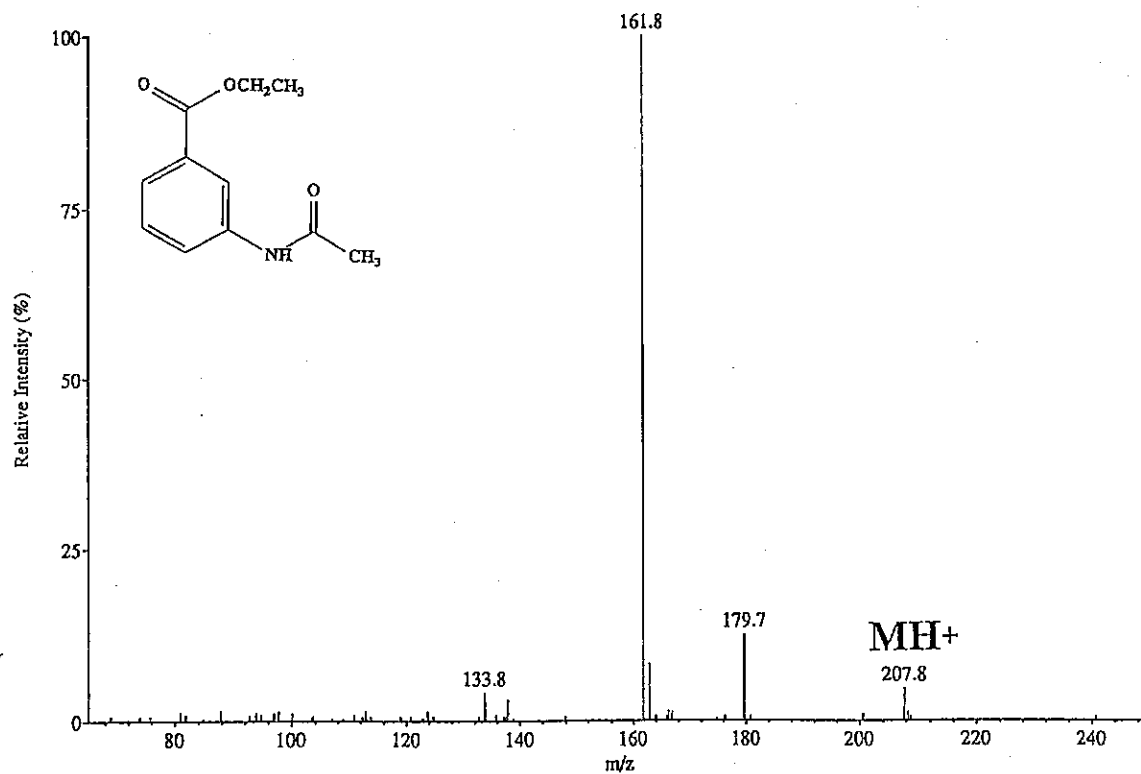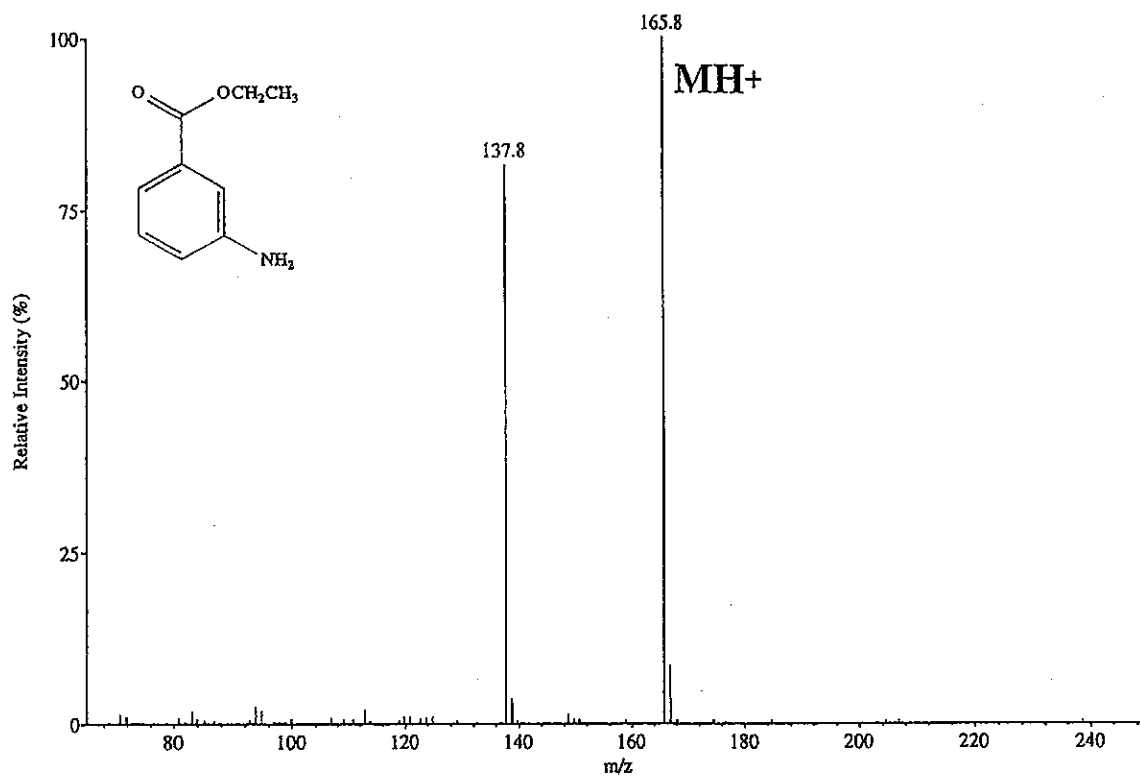
Figure 12: The APCI mass spectra of tricaine (top) and N-acetyltricaine (bottom) acquired during an LC-MS analysis (10 ng of each injected). The voltage difference between the sampling orifice and the initial rf-only quadrupole rods of the MS was 20 V.
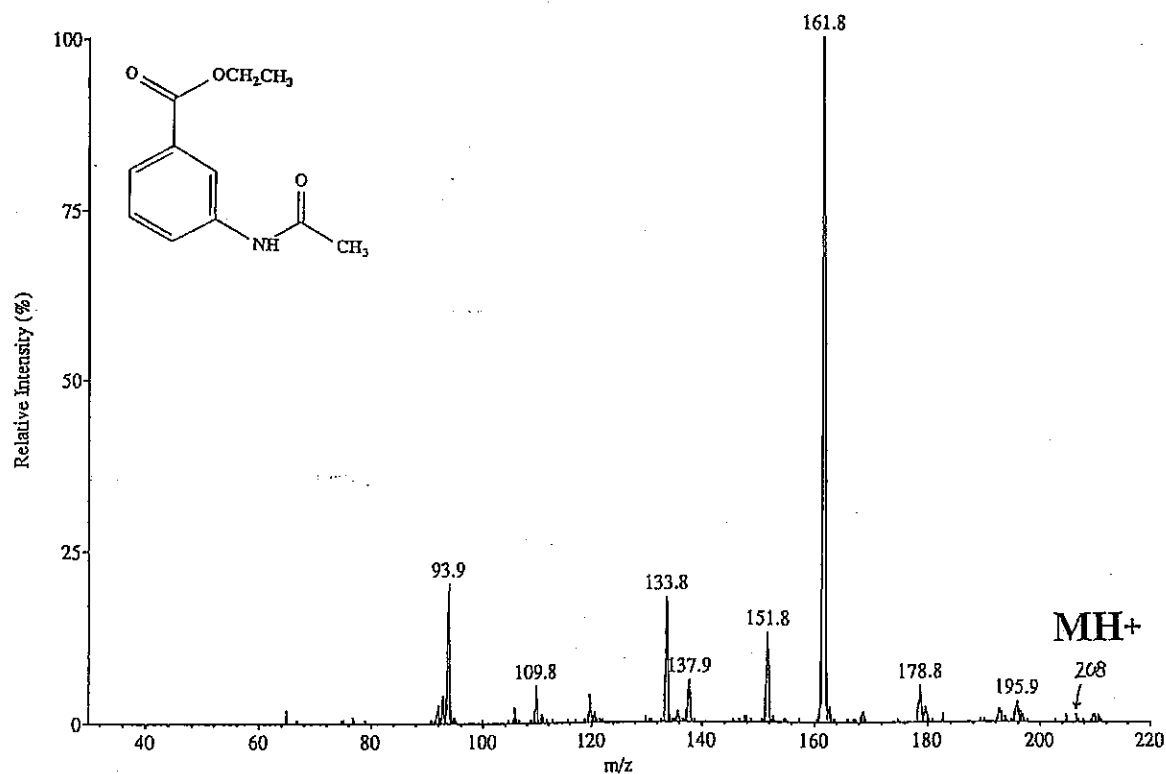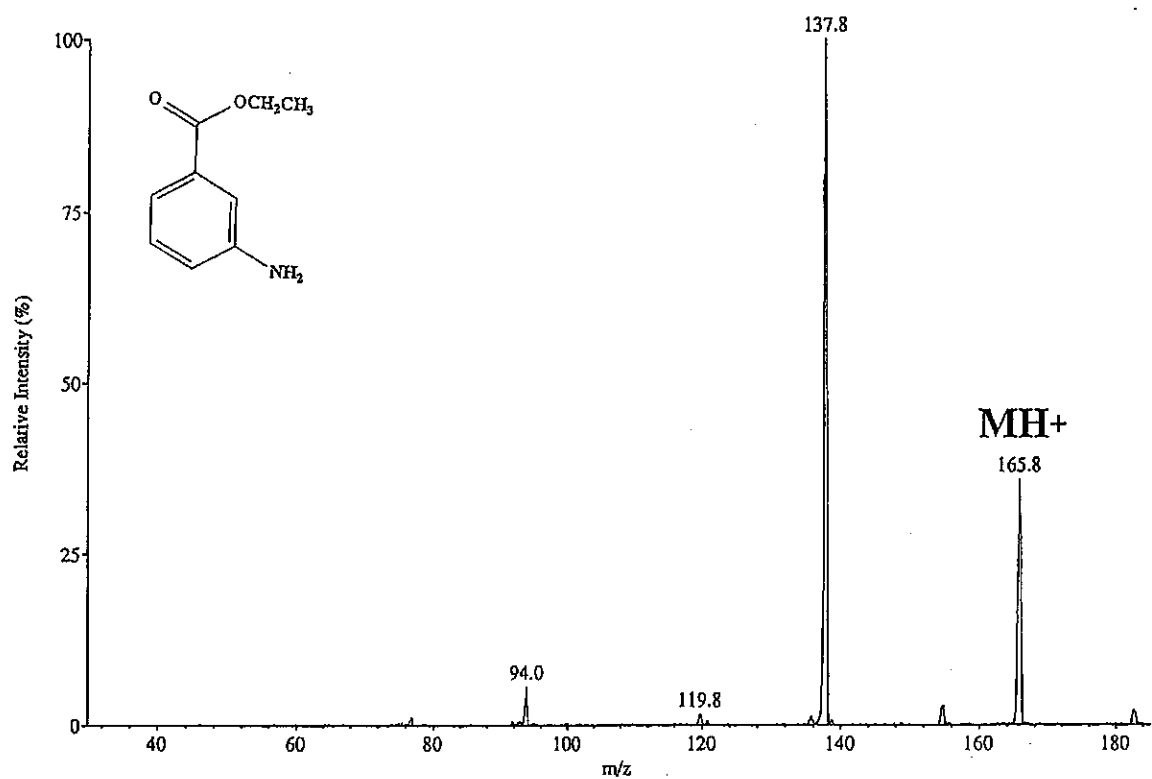
Figure 13: Product ion mass spectra (MS/MS) of the protonated molecules (MH$^+$) of tricaine (top) and N-acetyltricaine (bottom) acquired during an LC-MS analysis (50 ng of each injected). Conditions: APCI; 10 V voltage difference between the sampling orifice and the initial rf-only quadrupole rods; 20 eV collision energy; argon collision gas.
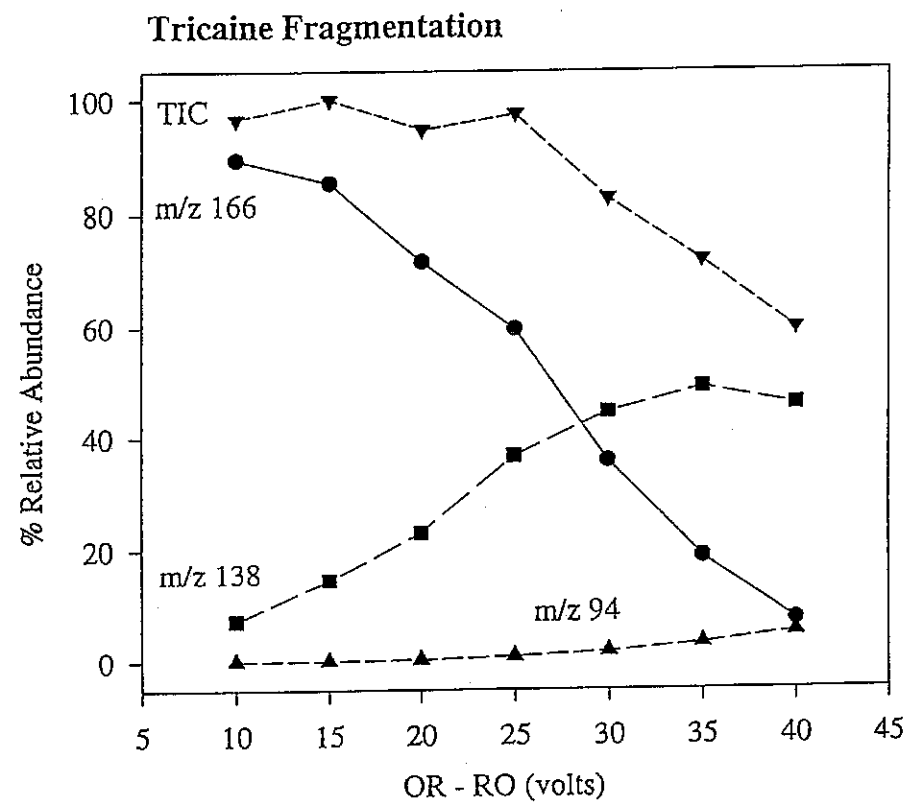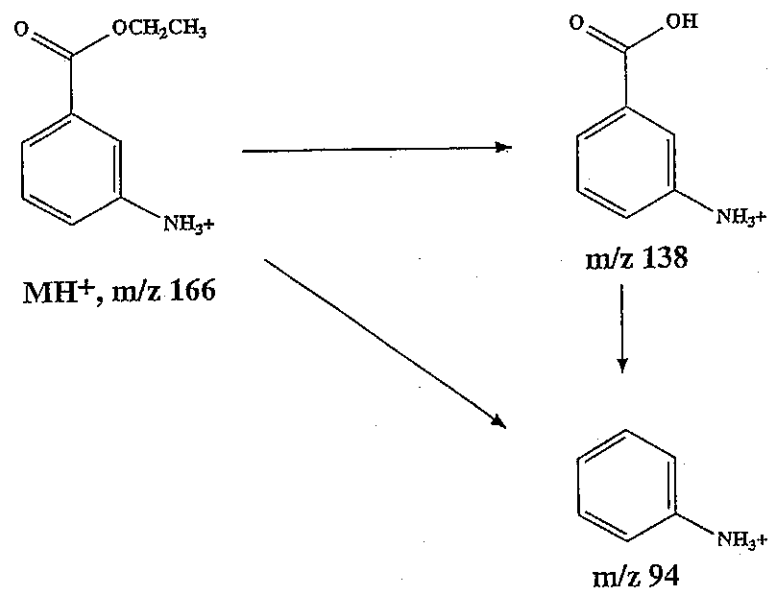
Figure 14: Fragmentation of the MH$^+$ ion of tricaine and the effect of orifice potential on the abundance of the various ions and total ion current (TIC).
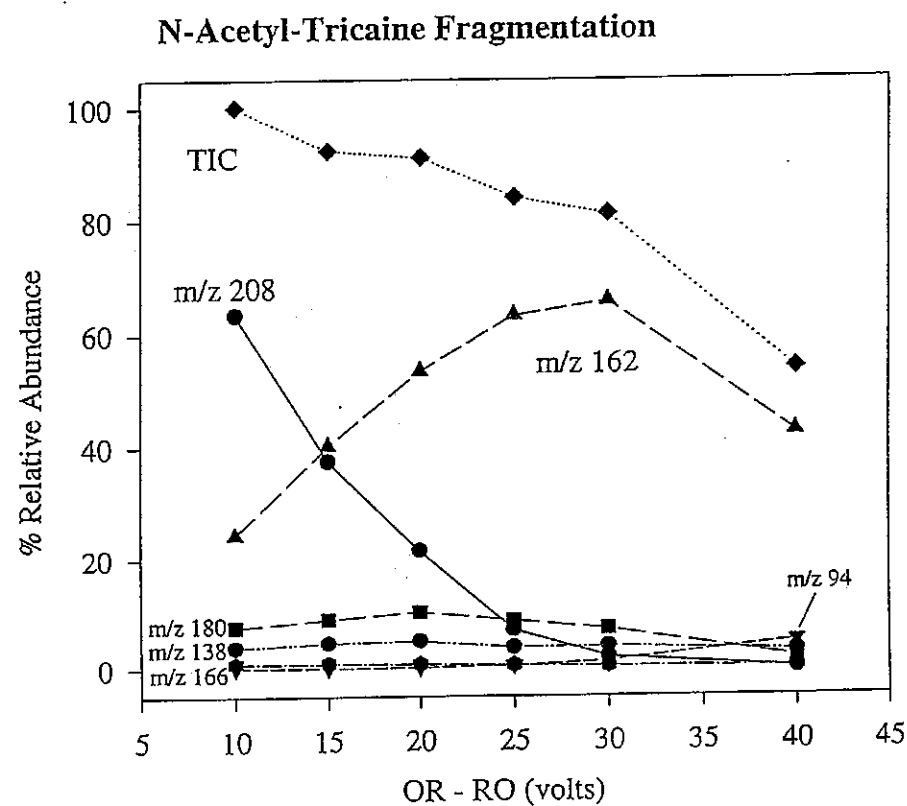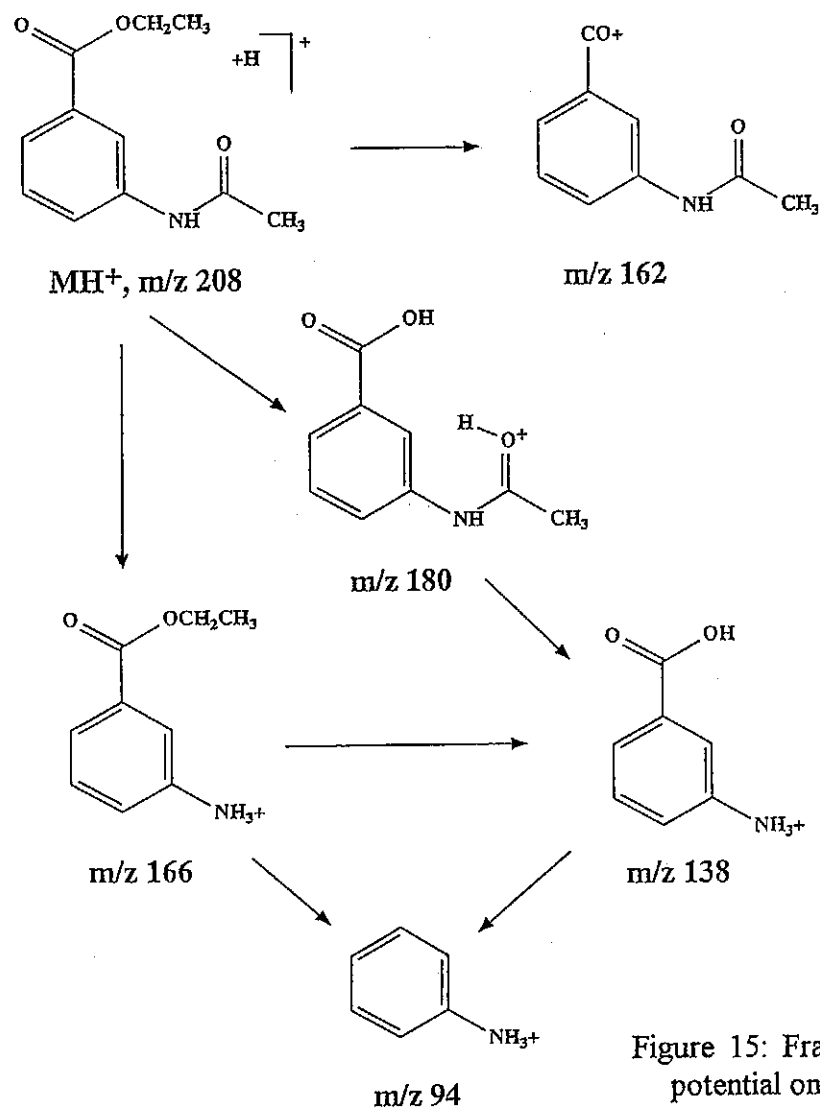
Figure 15: Fragmentation of the MH⁺ ion of N-acetyltricaine and the effect of orifice potential on the abundance of the various ions and total ion current (TIC).
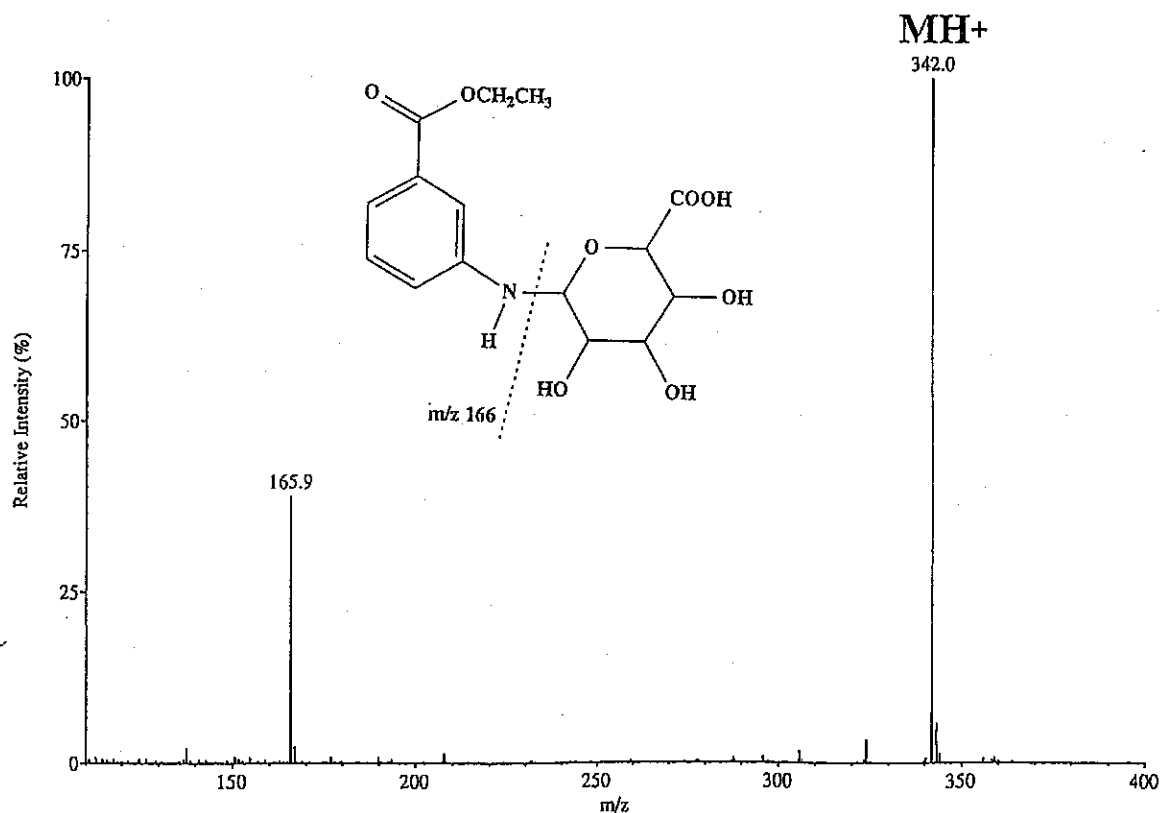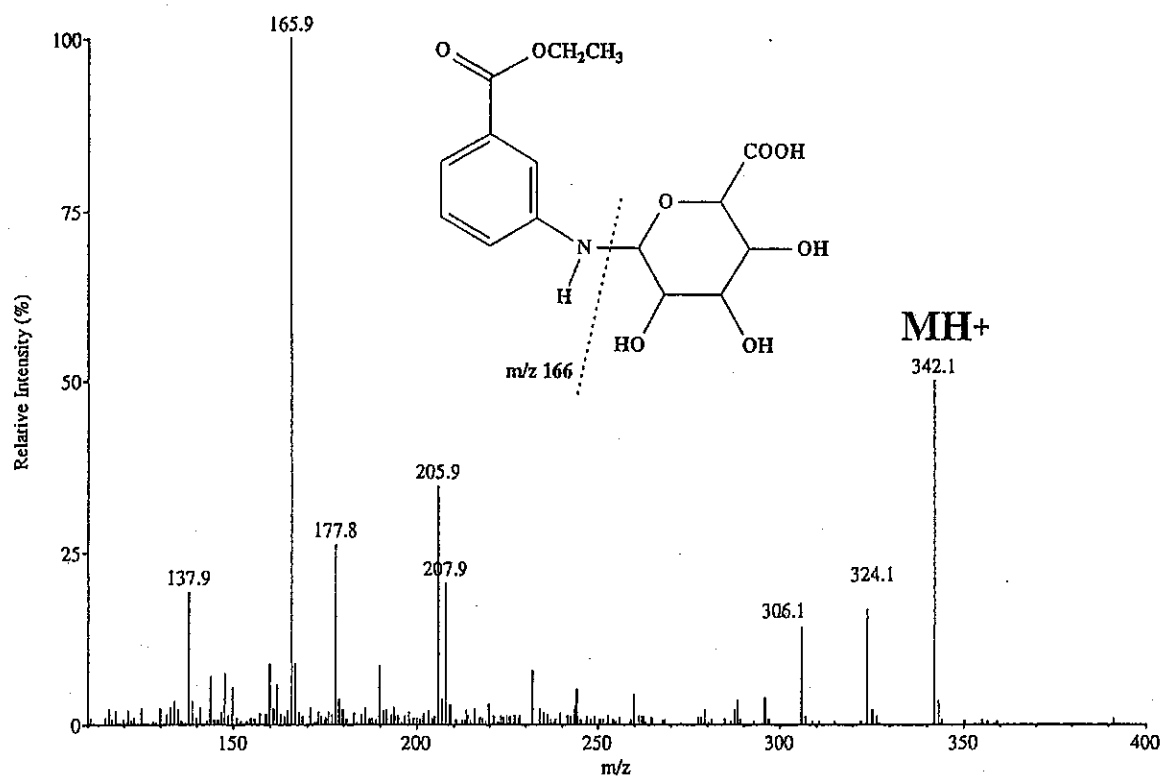
Figure 16: Comparison of the APCI (top) and ion-spray (bottom) mass spectra of the N-glucuronide of tricaine.
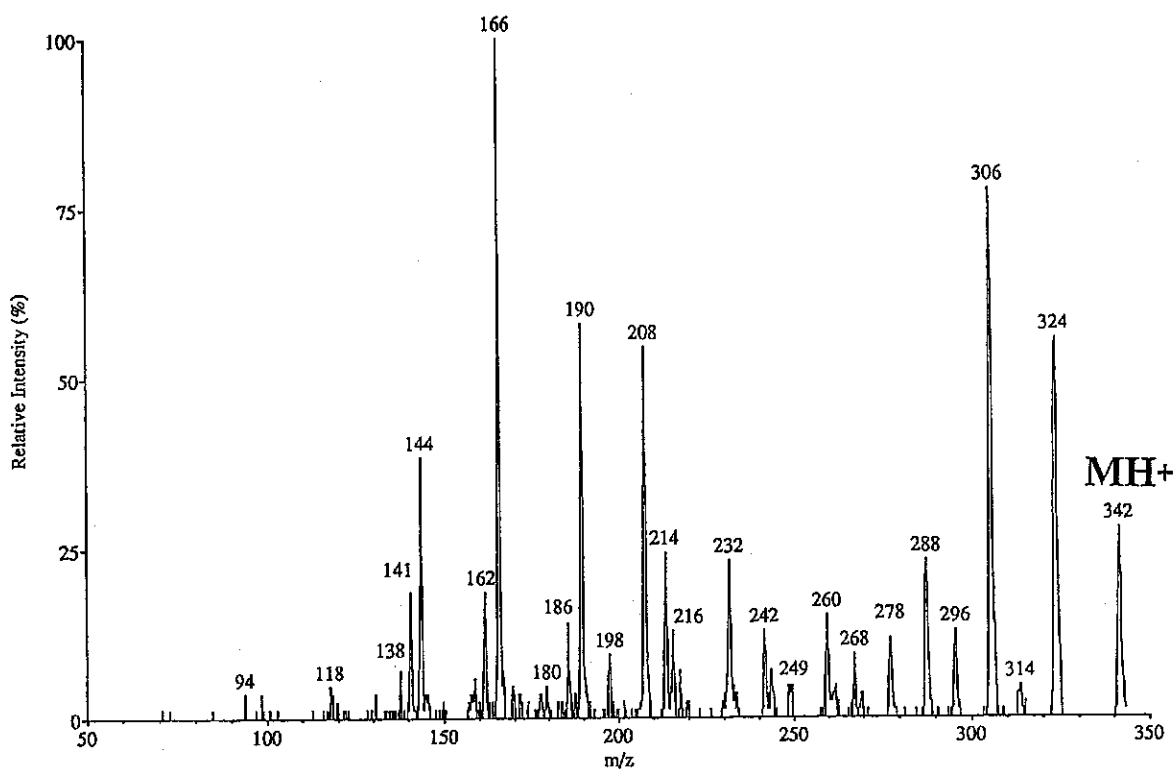
Figure 17: Product ion mass spectrum (MS/MS) of the protonated molecule (MH$^+$) of the N-glucuronide of tricaine acquired during an LC-MS analysis. Conditions: APCI; 10 V voltage difference between the sampling orifice and the initial rf-only quadrupole rods; 20 eV collision energy; argon collision gas.
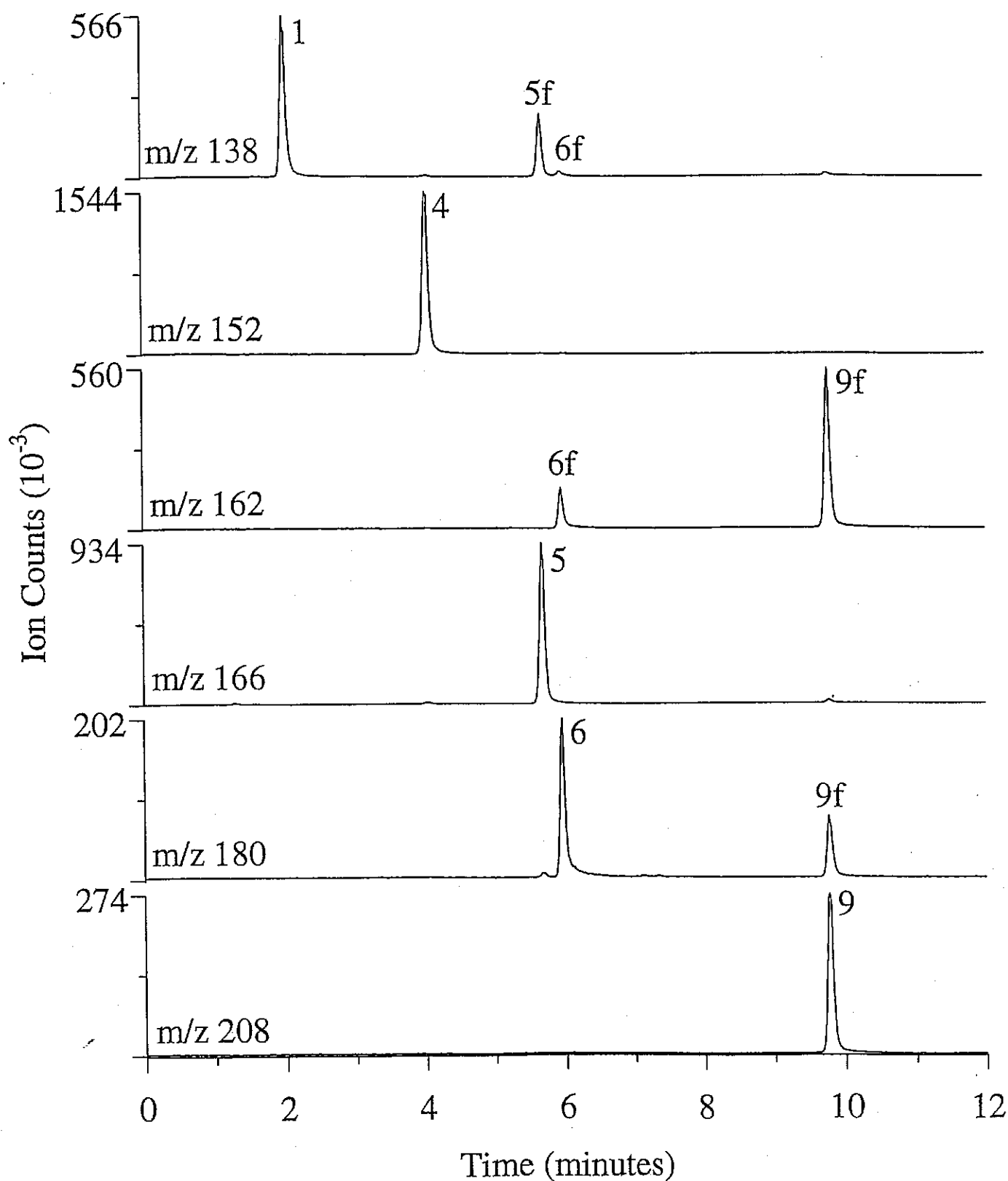
Figure 18: Mass chromatograms from the LC-APCI-MS analysis of a 2 μg/mL calibration solution. Conditions: gradient elution 10% to 50% (over 10 min) acetonitrile/water /0.1% TFA; 0.3 mL/min flow; 2 x 150 mm Zorbax Rx-C8 column; 5 μL injection volume; selected ion monitoring (100 msec dwell time). Compound identities: 1 = MABA; 4 = Me3ABA; 5 = TC; 6 = AcMABA; 9 = AcTC. The peaks marked with an "f" are fragment ions.
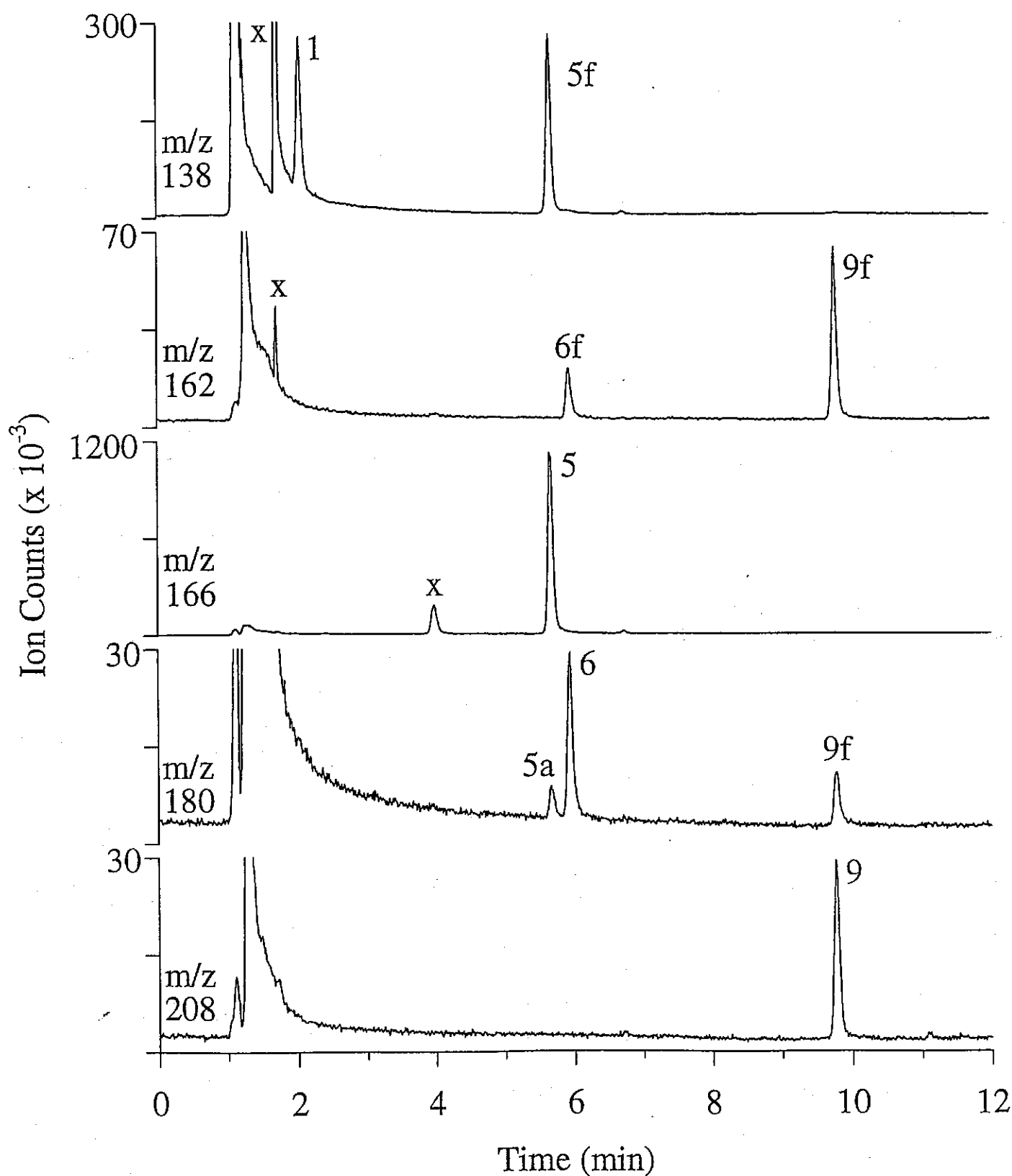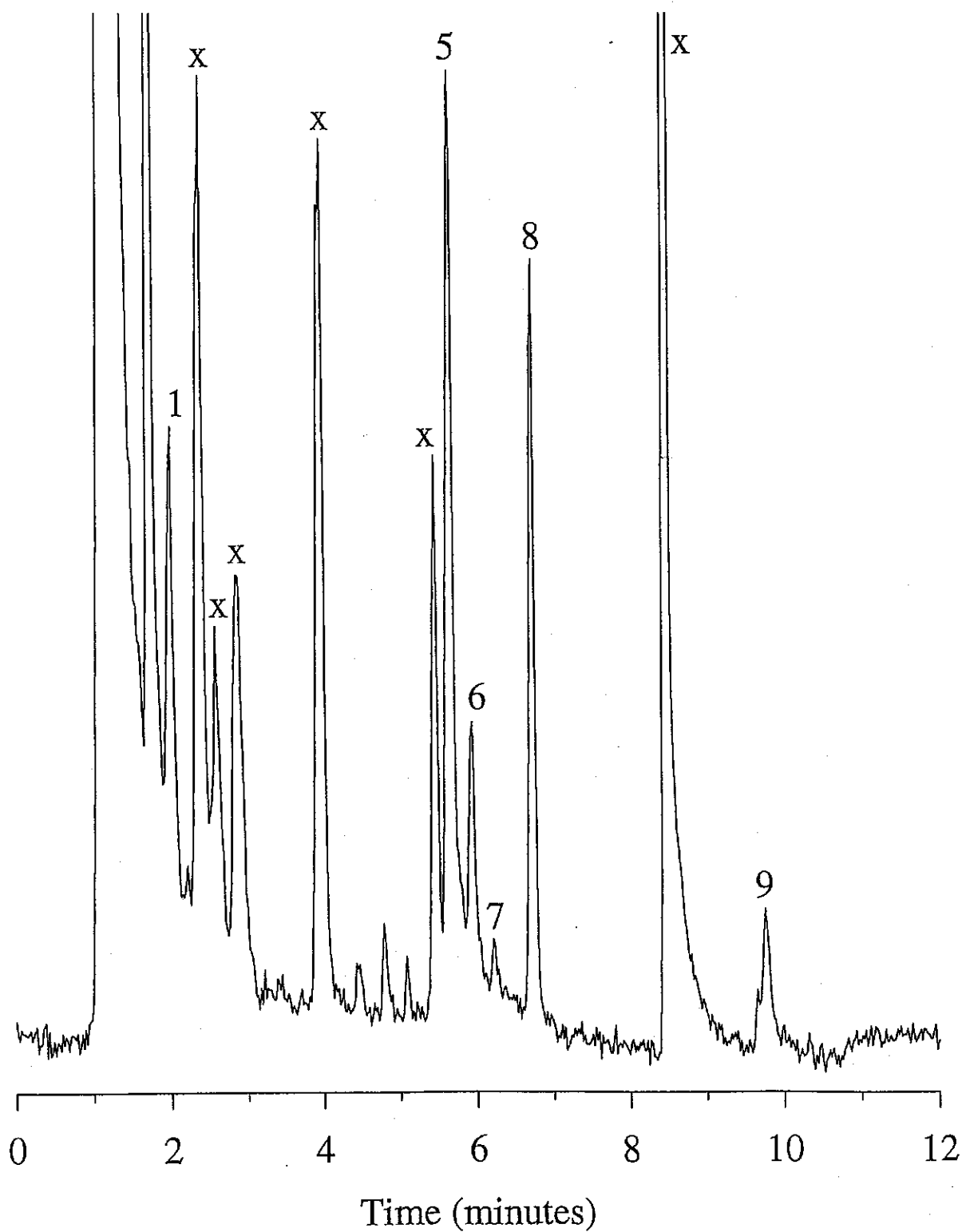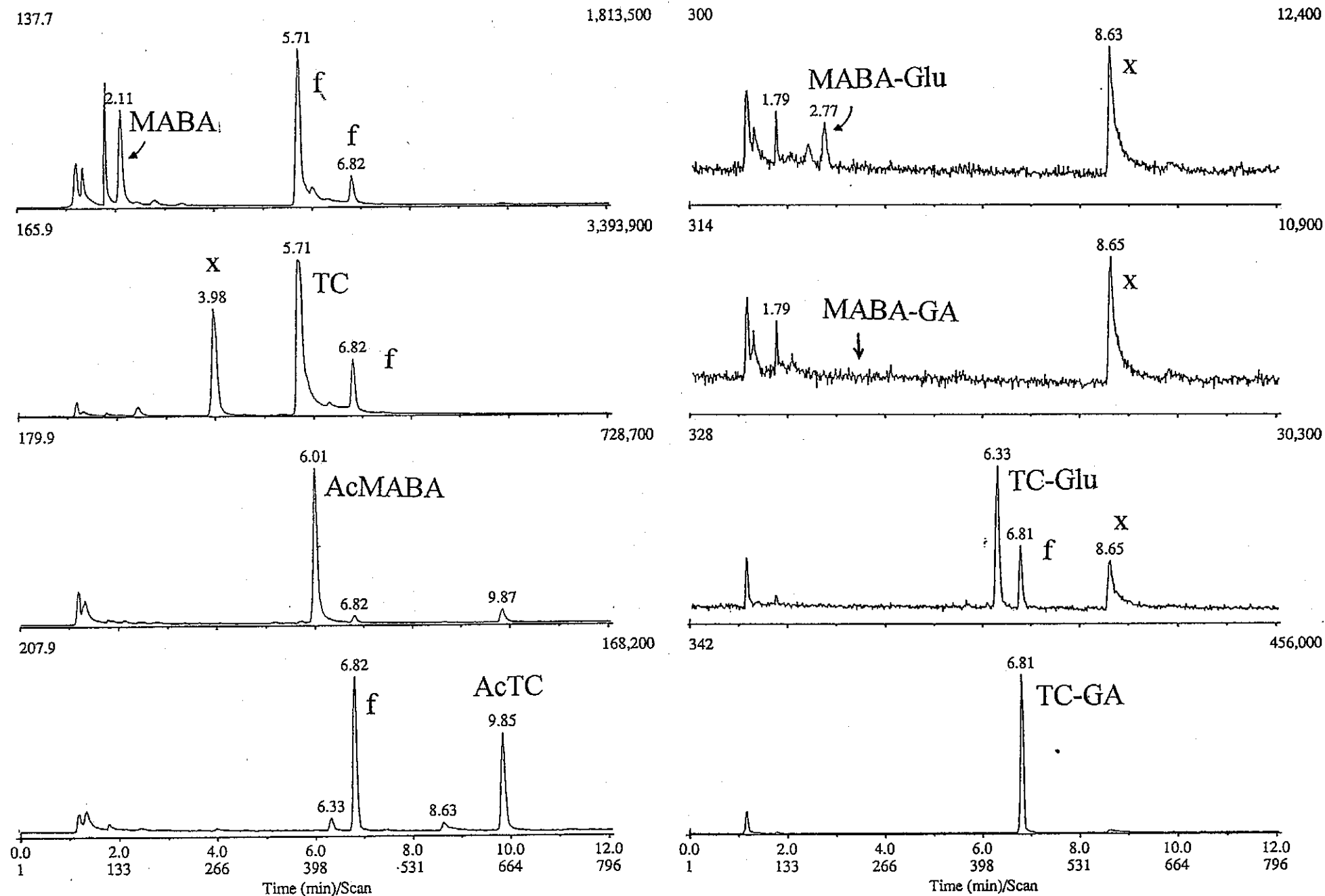
Figure 19: Mass chromatograms from the LC-APCI-MS analysis of an extract of muscle tissue from a trout exposed to 20 ppm MS-222 for 24 hr. Conditions and peak identities same as Figure 18. Peaks marked with an X are present in control tissue (see Figure 20). The peaks marked with "f" and "a" are fragment and adduct ions, respectively.
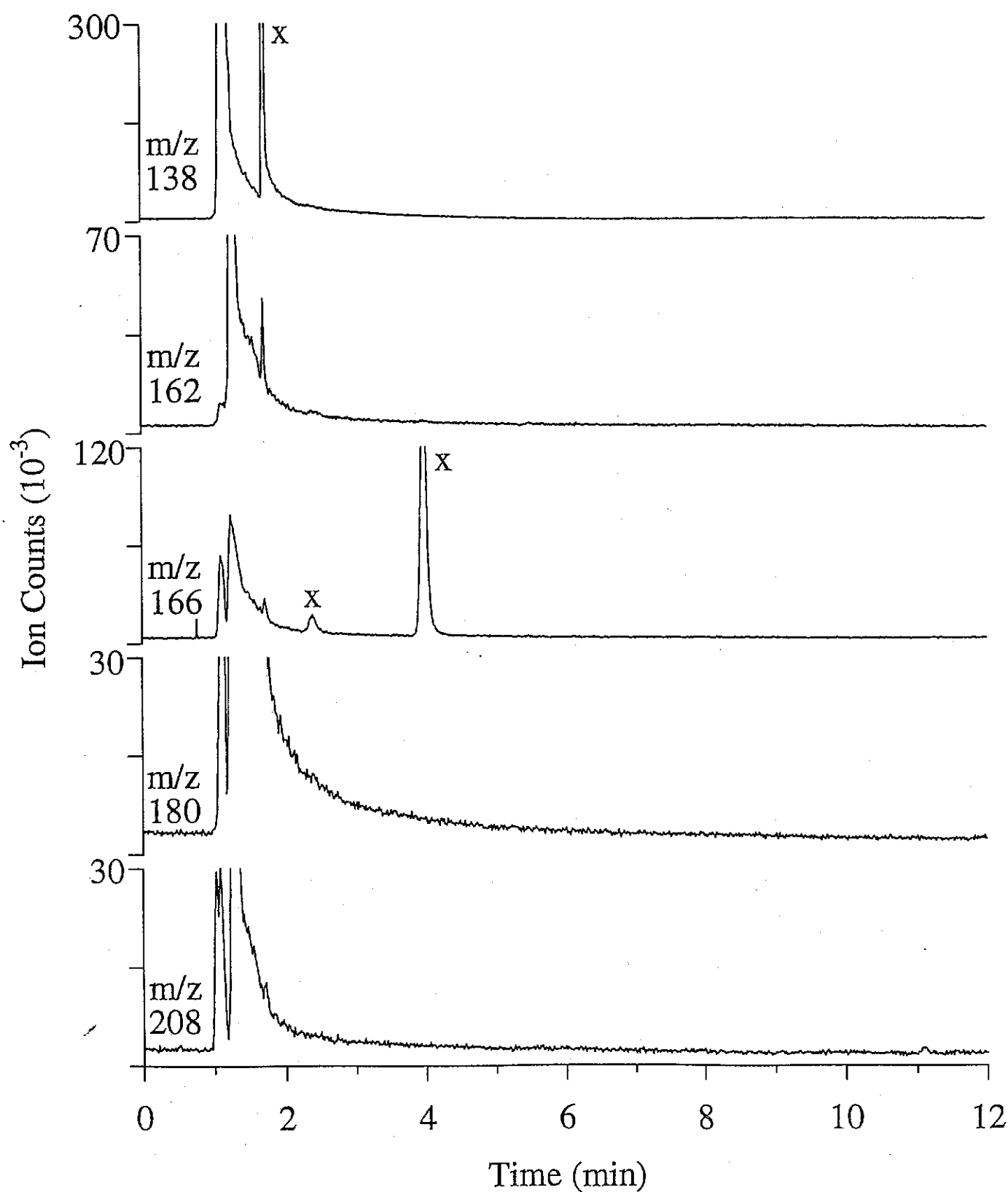
Figure 20: Mass chromatograms from the LC-APCI-MS analysis of an extract of muscle tissue from a control trout never exposed to MS-222. Conditions and peak identities same as Figure 18. Note: there is a 10-fold increase in sensitivity for the m/z 166 trace compared to that in Figure 19.

Figure 21: Total ion current chromatogram from the LC-APCI-MS analysis of an extract of liver tissue from a trout exposed to 50 ppm MS-222 for 24 hr. Conditions: as in Figure 18, except full scan acquisition (110-500 amu, 2 sec).

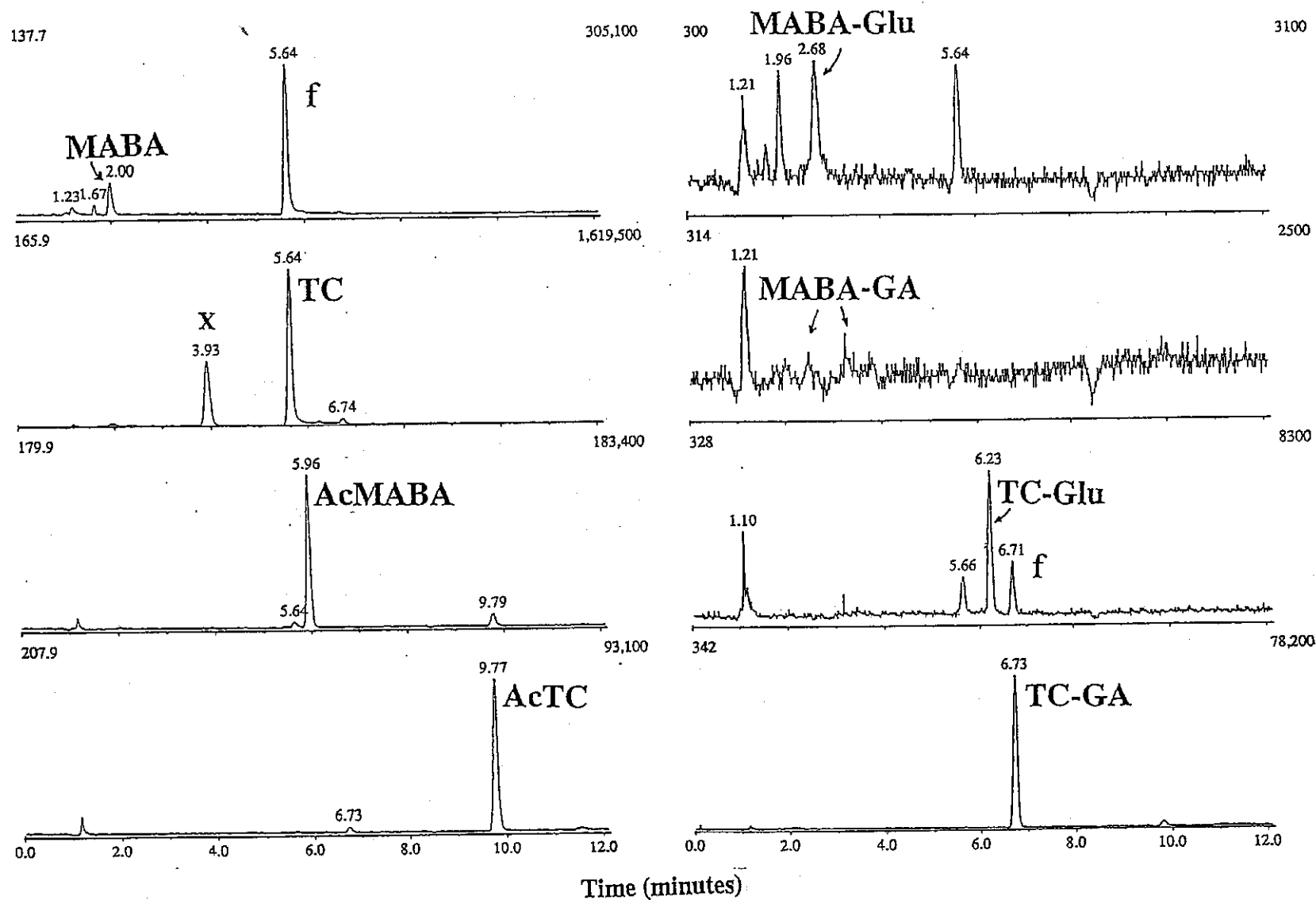Figure 22: Mass chromatograms from the LC-APCI-MS analysis of an extract of liver tissue from a trout exposed to 50 ppm MS-222 for 24 hr. Conditions: as in Figure 18. For each trace, the masses monitored and the scaling in ion counts are given in the upper left-hand and upper right-hand corners, respectively.
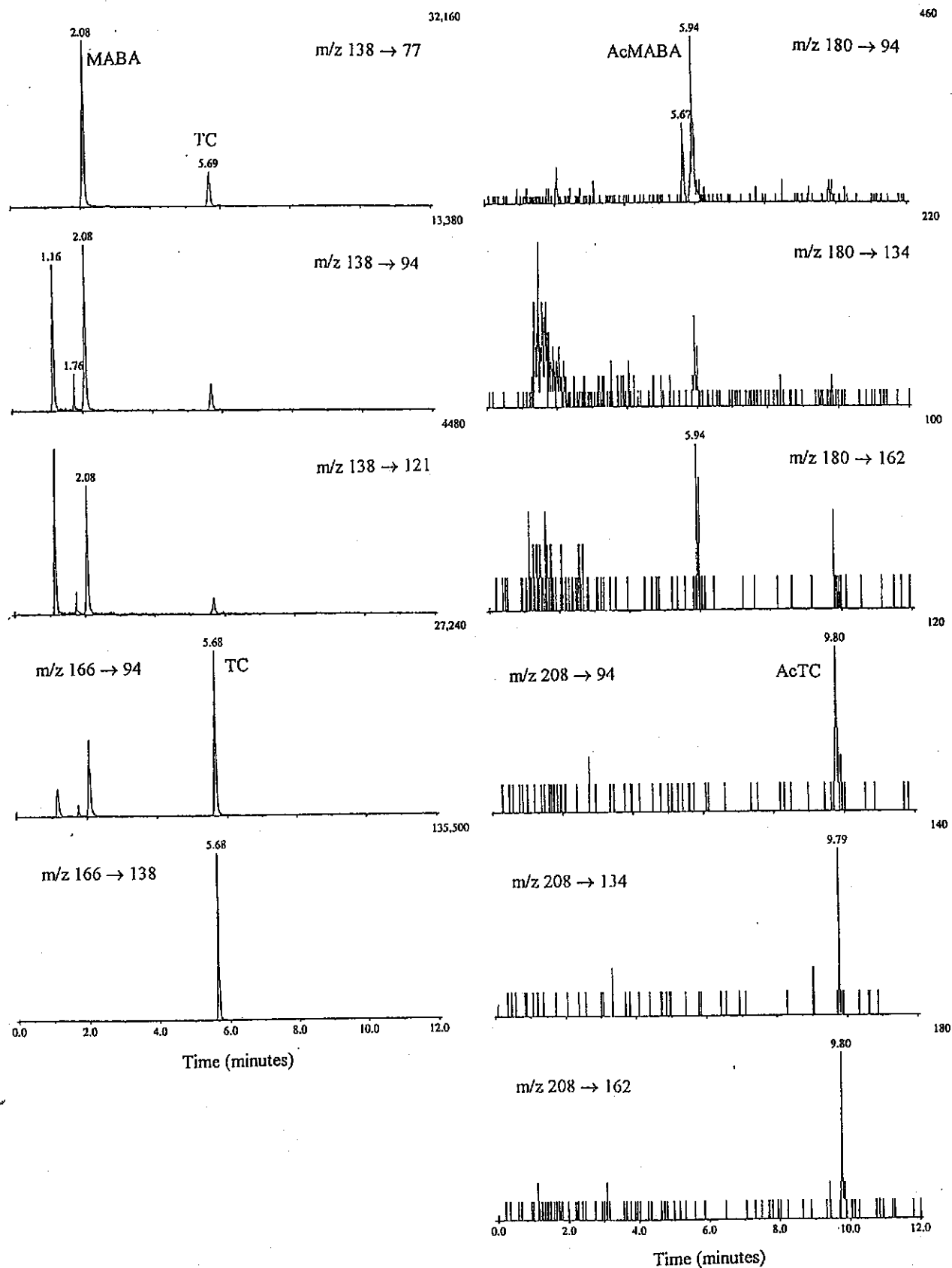
Figure 23: Selected reaction monitoring LC-APCI-MS analysis of an extract of muscle tissue from a trout exposed to 50 ppm MS-222 for 24 hr, showing detection and confirmation of TC and three metabolites.

Figure 24: Mass chromatograms from the LC-ionspray-MS analysis of an extract of liver
tissue from a trout exposed to 50 ppm MS-222 for 24 hr. Conditions: as in Figure 22,
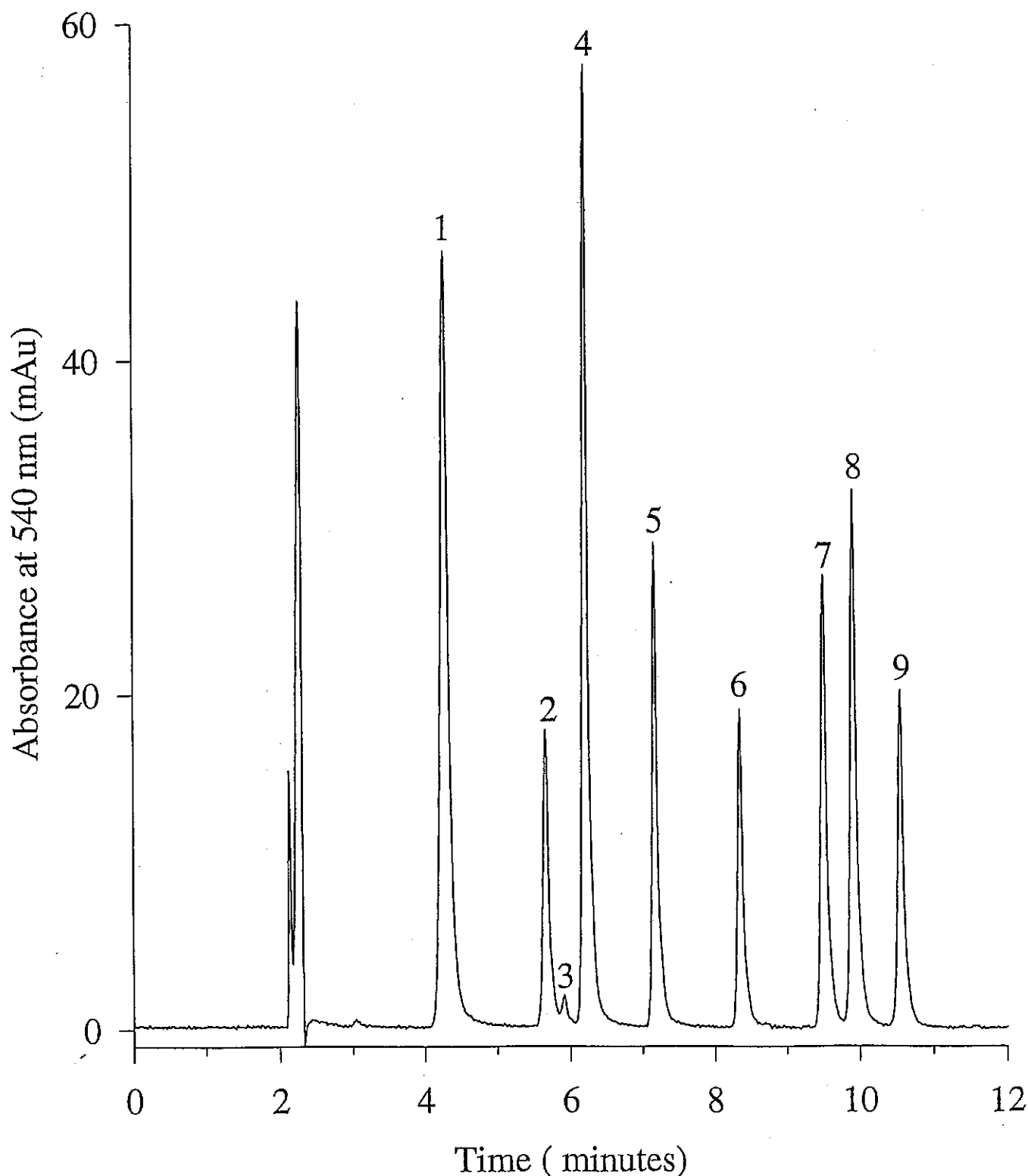except for ionspray interface and a 10:1 (waste:MS) postcolumn split.

Figure 25: LC-UVD analysis of mixture of primary aromatic amines (2-4 µg/mL each)
converted to Bratton-Marshall derivatives: 1 = 4-aminohippuric acid; 2 = sulfadiazine;
3 = impurity from peak 1; 4 = 4-aminobenzoic acid; 5 = 3-aminobenzoic acid (MABA);
6 = sulfadimethoxine; 7 = methyl-3-aminobenzoate (Me3ABA); 8 = benzocaine; 9 =
tricaine (TC). Conditions: gradient elution 20% to 80% (over 15 min)
acetonitrile/water /0.1% TFA; 0.3 mL/min flow; 2 x 250 mm Vydac 201TP52 column;
5 µL injection volume; DAD detection at 540 nm.
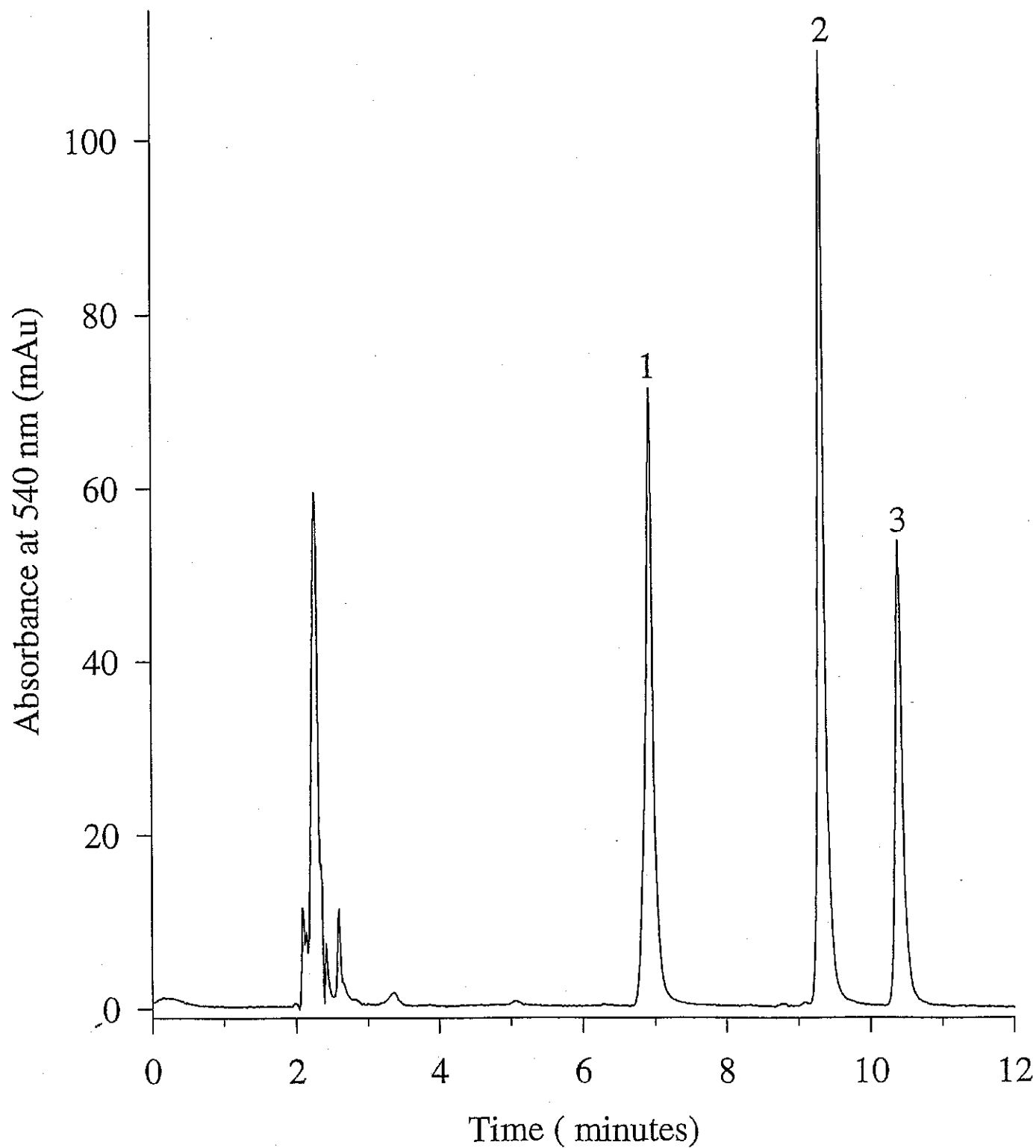
Figure 26: LC-UVD analysis of an extract of control salmon muscle tissue spiked with 20 µg/g of MABA (1), Me3ABA (2) and TC (3), after Bratton-Marshall derivatization and C18-SPE clean-up. Conditions: same as Figure 25.
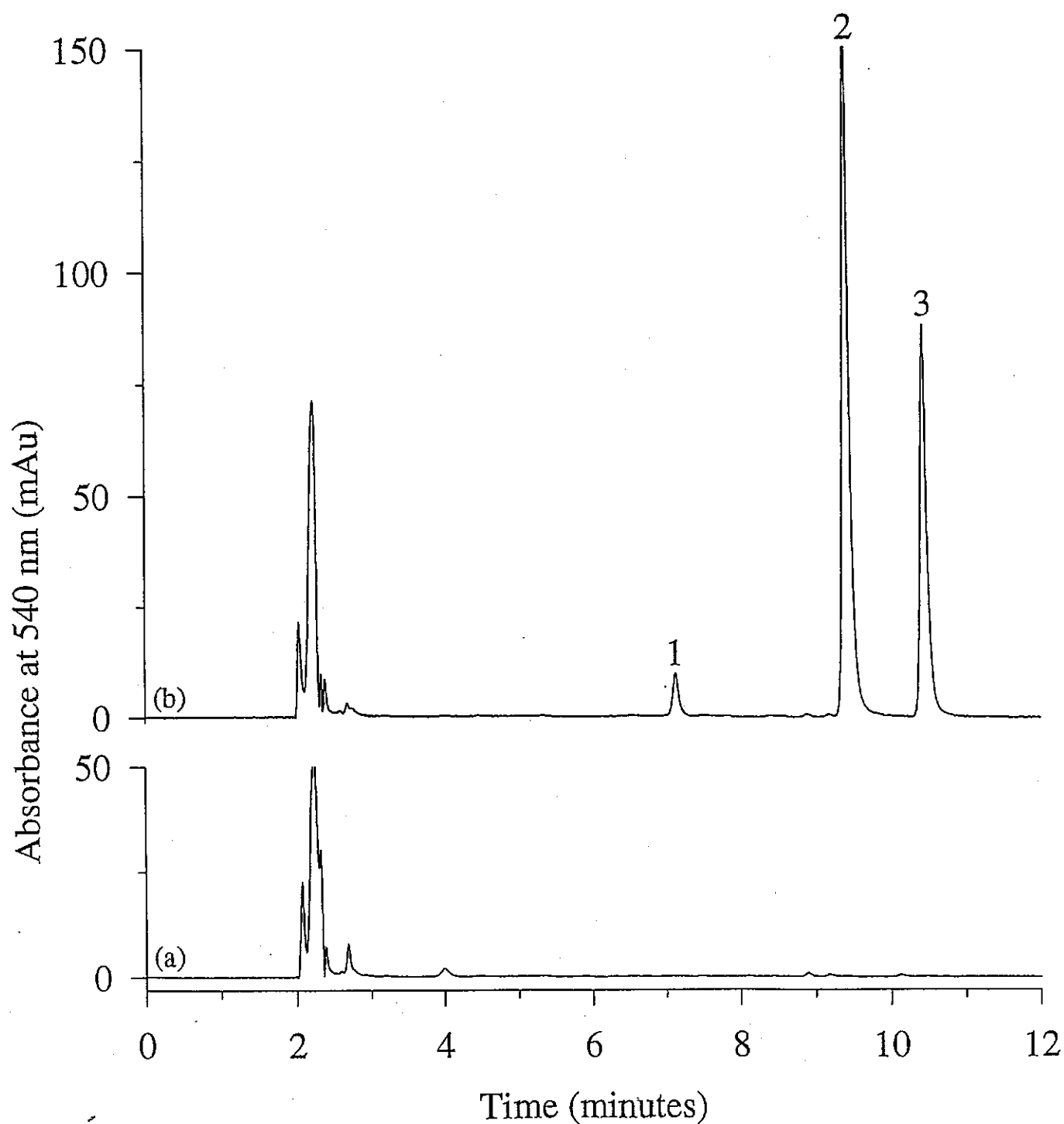
Figure 27: LC-UVD analysis of an extract of muscle tissue from (a) a control trout and (b) a trout exposed to 100 ppm MS-222 for 30 min after Bratton-Marshall derivatization and C18-SPE clean-up. Conditions: same as Figure 25. Peak identities: 1 = MABA; 2 = Me3ABA (internal standard); 3 = TC.
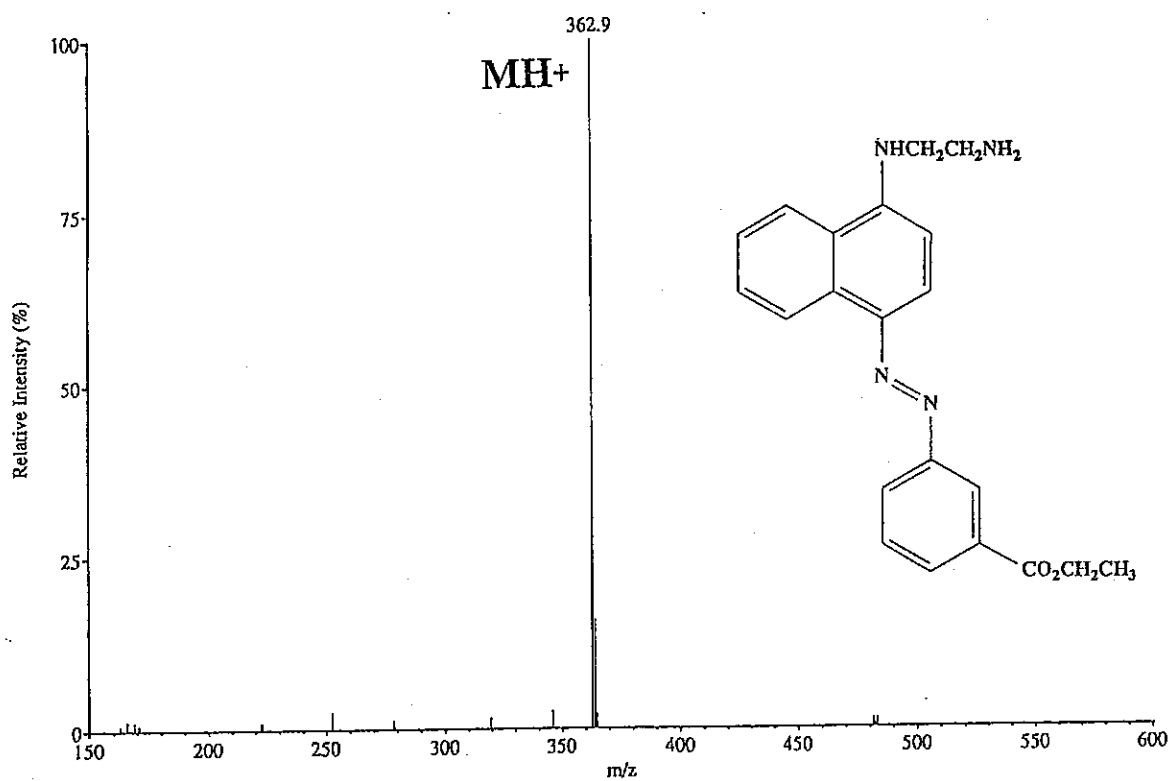
Figure 28: Ionspray mass spectrum of the Bratton-Marshall derivative of tricaine, acquired from an LC-MS analysis of 10 ng of derivative.

2.78

MABA

m/z 335

9133

5.43

Me3ABA (Int Std)

m/z 349

5867

BC
8.61

TC
6.39

m/z 363

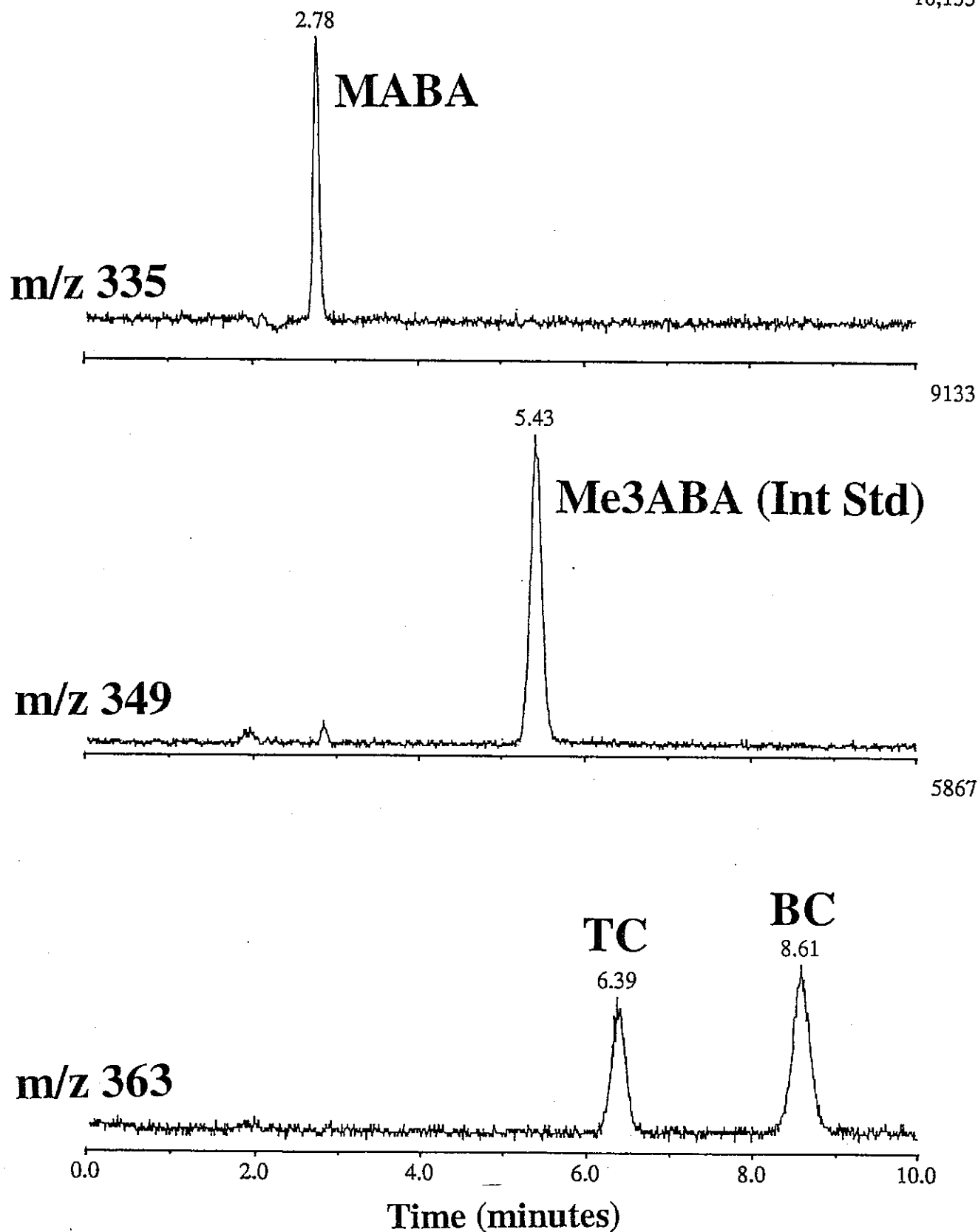| 0.0 | 2.0 | 4.0 | 6.0 | 8.0 | 10.0 |

Time (minutes)

Figure 29: Mass chromatograms from the LC-MS analysis of control salmon muscle tissue spiked with 40 ng/g of MABA, Me3ABA, TC and benzocaine (BC), after Bratton-Marshall derivatization and C18-SPE clean-up. Conditions: isocratic elution with 35% acetonitrile/water /0.1% TFA; 0.3 mL/min flow; 2 x 250 mm Vydac 201TP52 column; 5 μL injection volume; 10:1 (waste:MS) postcolumn split; ionspray interface.