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# Liquid chromatographic determination of benzocaine and N-acetylbenzocaine in the edible fillet tissue from rainbow trout

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**Abstract**

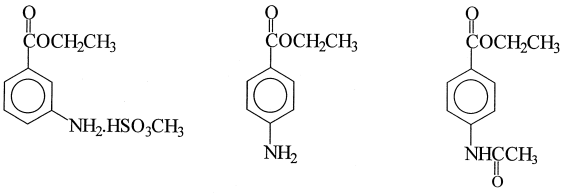
A method was developed for determining benzocaine and N-acetylbenzocaine concentrations in fillet tissue of rainbow trout. The method involves extracting the analytes with acetonitrile, removing lipids or hydrophobic compounds from the extract with hexane, and providing additional clean-up with solid-phase extraction techniques. Analyte concentrations are determined using reversed-phase high-performance liquid chromatographic techniques with an isocratic mobile phase and UV detection. The accuracy (range, 92 to 121%), precision (R.S.D., <14%), and sensitivity (method quantitation limit, <24 ng/g) for each analyte indicate the usefulness of this method for studies characterizing the depletion of benzocaine residues from fish exposed to benzocaine. 

*Keywords:* Fish; Food analysis; Benzocaine; Acetylbenzocaine

## 1. Introduction

Currently, the only approved anesthetic for use on fish in the USA is MS-222 (methanesulfonate salt of ethyl *meta*-aminobenzoate; Finquel; Fig. 1). MS-222 is used extensively in US aquaculture as a general fish anesthetic to reduce mortality and injury of fish during transport, spawning and stocking operations. Despite its wide use in aquaculture, MS-222 has restrictions associated with its use. According to the use label for MS-222, salmonids, ictalurids, escocids, and percids exposed to MS-222 cannot be made available for human consumption for 21 days after exposure. The 21-day withdrawal period is undesirable in many aquaculture situations, therefore the approval of an anesthetic with a shorter withdrawal time is preferred.

Benzocaine (ethyl *para*-aminobenzoate; Fig. 1), a structurally similar compound to MS-222, has been used effectively as a general anesthetic for fish when administered by bath [1–5]. As a general anesthetic




MS-222 Benzocaine N-acetylbenzocaine

Fig. 1. Chemical structure of MS-222 (ethyl *m*-aminobenzoic acid methanesulfonate salt; formula $M_r$, 261.3), benzocaine (ethyl *p*-aminobenzoate; formula $M_r$, 165.2), and N-acetylbenzocaine (ethyl *p*-acetylaminobenzoate; formula $M_r$, 207.2).

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for fish, benzocaine has desirable characteristics similar to those of MS-222 including rapid induction and recovery times. Because of these characteristics and the approved uses of benzocaine in human [6] and veterinary medicine [7,8], benzocaine has potential to be approved by the US Food and Drug Administration (FDA) as a fish anesthetic.

Legal use of benzocaine as an anesthetic for fish depends on approval by the FDA. One aspect of benzocaine that must be characterized before approval is depletion of benzocaine residues in the edible tissues of each fish species proposed for exposure. Characterizing the depletion of an aquaculture drug in edible fish tissue allows the FDA to establish withdrawal times for each fish species after exposure and ensures that the drug concentrations are below permitted concentrations before fish are made available for human consumption.

To conduct residue depletion studies for benzocaine, an analytical method sensitive and specific for benzocaine residues in edible fish fillet tissue is required. Analytical methods for MS-222 in various fish tissue have been reported [9–11] and would presumably determine benzocaine residues in fish tissue. These colorimetric methods, however, lack specificity and sensitivity because they are based on a reaction with a reagent and primary aromatic amines. The objective of this study was to develop an analytical procedure to determine concentrations of benzocaine and a benzocaine metabolite, N-acetylbenzocaine in edible fillets of fish. The suitability of the method for use in residue depletion studies was evaluated by documenting the method's accuracy, precision, and sensitivity with fortified fillets from a freshwater fish species of interest to aquaculture.

## 2. Experimental

### 2.1. Chemicals and reagents

Benzocaine (99.9% assay purity) was obtained from Sigma (St. Louis, MO, USA). N-Acetylbenzocaine was synthesized from benzocaine and acetic anhydride [12]; the purity was 99% as determined by high-performance liquid chromatography (HPLC). Stock solutions of benzocaine and N-acetylbenzocaine were prepared in methanol. Working solutions were prepared by diluting the stock solutions with methanol–water (11:9, v/v). Anhydrous sodium carbonate (J.T. Baker, Phillipsburg, NJ, USA) and anhydrous sodium sulfate (Fisher, Pittsburgh, PA, USA) were of reagent grade. Acetonitrile (Fisher), methanol, and hexanes (J.T. Baker) were of HPLC grade. Water was deionized to a specific resistance of $>17.8$ m$\Omega$ cm with a water purification system (Barnstead E-pure, Dubuque, IA, USA).

### 2.2. High-performance liquid chromatography

The HPLC system was a HP model 1090 Series II HPLC system (Hewlett-Packard, Wilmington, DE, USA). The operation parameters were a flow-rate of 1 ml/min, a mobile phase composition of 55% methanol and 45% water (both solvents helium sparged during use), an injection volume of 10 μl, a column temperature of 40°C, an absorbance setting of 289 nm for benzocaine through the first 7.5 min of a run, then 271 nm for N-acetylbenzocaine thereafter. Separations were performed on a 250×4.6 mm I.D. YMC ODS-A column (YMC, Wilmington, NC, USA) packed with S-5μ (5 μm), 120 D stationary phase. The column was fitted with a 23×4 mm YMC guard column packed with S-5 μ (5 μm), 120 Å $C_{18}$ stationary phase.

### 2.3. Sample preparation

Skin-on fillets were acquired from rainbow trout (*Oncorhynchus mykiss*; estimated total body mass range, 350 to 700 g). The fillets were hardened in a freezer and cut into 1-$cm^2$ sections. The fillet sections were homogenized to a fine powder with dry ice in a Waring stainless steel bar blender (Dynamics Corp. of America, New Hartford, CT, USA) [13]. The fillet–dry ice matrix was poured into a Ziploc freezer bag (Dow Chemical, Indianapolis, IN, USA) and stored at about −20°C overnight (about 18 h). The bag was stored open to allow the dry ice to sublimate from the tissue and the tissue mixed periodically to promote sublimation. After 18 h, the

bag was sealed and the tissue stored frozen at ≤ −70°C until use.

### 2.4. Extraction

Thawed homogenized fillet tissue (10±0.1 g) was weighed in a tared 250-ml glass beaker. To validate the assay, control tissue samples were fortified with less than 0.25 ml of a working solution of the appropriate concentration. Using a stainless steel spatula, the tissue was mixed with 5 g of anhydrous sodium carbonate. After about 15 min, 40 g of anhydrous sodium sulfate was mixed with the tissue until the tissue–salt matrix appeared dry and granular.

A 600×20 mm (I.D.) glass extraction column was prepared by plugging the effluent end with glass wool and connecting a PTFE stopcock and a 6.5-mm glass stopcock extension. The column was clamped vertically to a ring stand support positioned in a fume hood and a 2-cm layer of anhydrous sodium sulfate was poured into the reservoir followed by the tissue–salt matrix. The beaker was dry rinsed with enough anhydrous sodium sulfate to provide a 2-cm layer of sodium sulfate on top of the tissue–salt matrix. The beaker was also rinsed with 200 ml of acetonitrile and the rinse poured into the extraction column. The migration of acetonitrile through the tissue–salt matrix was controlled with the stopcock and stopped when the acetonitrile reached the stopcock. Acetonitrile was allowed to saturate the tissue–salt matrix for 10 min before the flow-rate through the column was adjusted to one drop every 4 s. The acetonitrile was collected in a 300-ml glass evaporation flask. After the flow of acetonitrile through the column stopped, residual acetonitrile was forced through the column with compressed air.

Acetonitrile was evaporated from the 300-ml flask with a Büchi model RE-121 rotary evaporator and Büchi model B-171 vacuum pump (Büchi Labortechnick, Flawil, Switzerland). The water bath temperature was set at 39°C and the vacuum set at 100 mbar. The sample was evaporated to a volume of 5 to 10 ml and quantitatively transferred to a 100-ml glass evaporation flask with two 3-ml rinses of acetonitrile. The sample was extracted twice with 5 ml of hexanes by gently swirling the mixture, allowing the phases to separate, and discarding the hexanes with a pipette. The sample's solvent polarity was modified by adding 10 ml of water and evaporating the sample to about 10 ml using the evaporation parameters previously described.

### 2.5. Solid-phase extraction

A solid-phase extraction (SPE) system (Baker SPE-24G column processor (J.T. Baker), vacuum chamber (J.T. Baker), and vacuum pump) was prepared by attaching a stainless steel needle to an outlet port of the SPE manifold, a stopcock to the manifold head, and a 6-ml, 1000-mg, $C_{18}$ Baker Bond SPE column (J.T. Baker) to the stopcock. The $C_{18}$ column was conditioned with two column volumes of methanol followed by at least two column volumes of water at a flow-rate <5 ml/min while maintaining the solvent level above the column bed. The sample was transferred to the column and aspirated through at a flow-rate <5 ml/min while maintaining the solvent level above the packing. The sample flask was rinsed with 2 ml of a methanol–water (1:5, v/v) solution and the rinse aspirated through the column. The column was dried by aspirating air through for at least 15 s before analytes were eluted from the column into a volumetric flask (5 ml) with three 0.5-ml aliquots of methanol. The flask volume was adjusted with water and the sample thoroughly mixed. A portion of the sample was loaded into a plastic 1-ml tuberculin syringe and forced through a 13-mm, 0.45-μm Acrodisc CR PTFE filter (Gelman Sciences, Ann Arbor, MI, USA) into a glass LC sample vial.

### 2.6. HPLC analysis

Calibration curves were developed with injections of at least five working solutions encompassing the expected concentration range of benzocaine and N-acetylbenzocaine in the extracts. The concentration of benzocaine and N-acetylbenzocaine in an extract was determined from the peak area and the linear regression equation of the calibration curve. The concentration of analytes in a tissue sample was calculated by

$$\frac{AB}{C} = D$$

where $A$ is the analyte concentration in the extract (ng/ml), $B$ is the volume of the extract (ml), and $C$ is the mass of the tissue sample (g). Tissue concentrations of benzocaine and N-acetylbenzocaine ($D$) were reported as ng of analyte per g of tissue (ng/g).

### 2.7. Assay validation

The assay was validated with rainbow trout skin-on fillet samples fortified with benzocaine and N-acetylbenzocaine at nominal concentrations of 16, 50, 500, 5000 and 50 000 ng/g. The method accuracy was calculated as the percent of analyte recovered in the extract from the fortified fillet. Within-day method precision was calculated as the relative standard deviation for each fortification level. Method sensitivity (quantitation limit) for each analyte was calculated as 10$s$ [14] where $s$ was the sample standard deviation (ng/g) for the samples fortified at 16 ng/g. Calculations for determining the method detection limit are based on calculations described in The Code of Federal Regulations [15].

### 2.8. Chemical stability

The stability of benzocaine and N-acetylbenzocaine in the solvent composition of the working solutions and of fillet extracts was evaluated. Working solutions and fillet extracts prepared in clear volumetric glassware were stored on the laboratory bench under ambient laboratory lighting and temperature conditions. Working solutions at nominal benzocaine and N-acetylbenzocaine concentrations of 100, 500, and 5000 ng/ml were analyzed throughout a 37-day period and fillet extracts from tissue fortified at nominal analyte concentrations of 4000 ng/g were analyzed throughout a 6-day period.

## 3. Results

### 3.1. Method accuracy, precision, and sensitivity

The method accuracy for benzocaine in individual samples ranged from 83 to 103% at the 50, 500, 5000 and 50 000 ng/g fortification levels. The overall mean method accuracy for benzocaine was greater than 92% and the method precision ranged from 0.96 to 5.05% among the fortification levels (Table 1).

The method accuracy for N-acetylbenzocaine in individual samples ranged from 85 to 107% at the 50, 500, 5000 and 50 000 ng/g fortification levels. The overall mean method accuracy was greater than 95% and the method precision ranged from 0.96 to 5.57% among the fortification levels (Table 2).

For samples fortified with each analyte at a nominal concentration of 16 ng/g, the mean method accuracy was 106% for benzocaine (Table 1) and 121% for N-acetylbenzocaine (Tables 2). The method precision was 13.4% for benzocaine and 10.6% for N-acetylbenzocaine. The method detection limit determined from the samples fortified at 16 ng/g was 6 ng/g for benzocaine and 6 ng/g for N-acetylbenzocaine and the method quantitation limit was 22 ng/g for benzocaine and 23 ng/g for N-acetylbenzocaine.

Table 1
Accuracy (recovery), precision (relative standard deviation), and sensitivity (method detection and quantitation limit) of a liquid chromatography method to determine benzocaine concentrations in rainbow trout skin-on fillet tissue fortified with benzocaine

| Nominal tissue concentration of benzocaine (ng/g) | $n$ | Recovery (%) | Relative standard deviation (%) | Method detection limit (ng/g) | Method quantitation limit (ng/g) |
|---|---|---|---|---|---|
| 16 | 10 | 106 | 13.4 | 6 | 22 |
| 50 | 10 | 96.1 | 2.86 | | |
| 500 | 10 | 92.2 | 5.05 | | |
| 5000 | 10 | 94.9 | 2.43 | | |
| 50 000 | 10 | 95.5 | 0.96 | | |

Table 2
Accuracy (recovery), precision (relative standard deviation), and sensitivity (method detection and quantitation limit) of a liquid chromatography method to determine N-acetylbenzocaine concentrations in rainbow trout skin-on fillet tissue fortified with N-acetylbenzocaine

| Nominal tissue concentration of N-acetylbenzocaine (ng/g) | *n* | Recovery (%) | Relative standard deviation (%) | Method detection limit (ng/g) | Method quantitation limit (ng/g) |
|---|---|---|---|---|---|
| 16 | 10 | 121 | 10.6 | 6 | 23 |
| 50 | 9 | 97.1 | 3.02 | | |
| 500 | 10 | 96.7 | 5.57 | | |
| 5000 | 10 | 96.9 | 3.07 | | |
| 50 000 | 10 | 95.5 | 0.96 | | |

### *3.2. Chemical stability*

The concentrations of benzocaine and N-acetylbenzocaine prepared in the solvent composition of working solutions did not change by more than 0.9% throughout the 37-day evaluation period. The concentrations of the analytes in the solvent composition of the fillet extract did not change by more than 1.3% throughout the 6-day evaluation period.

## 4. Discussion

During the development of this method, several solvents (acetone, acetonitrile, ethyl acetate, hexane, methanol and methylene chloride) were evaluated as extracting solvents. Although benzocaine and N-acetylbenzocaine were soluble in all solvents evaluated, acetonitrile produced an extract that appeared to have the least amount of potentially interfering compounds and therefore was selected as the extracting solvent.

Initial attempts to clean-up the acetonitrile extract included gel permeation chromatography and a simple solvent–solvent extraction with hexane. Gel permeation chromatography appeared to be efficient at removing potentially interfering compounds, however, the technique was very time consuming. The solvent–solvent extraction with hexane was also effective at removing lipids or hydrophobic compounds while retaining the analytes in the acetonitrile extract, and moreover, the technique reduced the sample processing time by half.

Initially, a normal-phase SPE sorbent, silica gel, was evaluated to concentrate and provide additional clean-up of the extract. In most evaluations, the recovery of each analyte was acceptable, however, on occasion, a sample would result in a low recovery for benzocaine. After several trials to determine the source of error, the inconsistency was surmised to be associated with the inherent differences in prepacked SPE cartridges. Because of the inconsistencies experienced with silica gel, a reversed-phase sorbent, $C_{18}$, was evaluated and found to yield acceptable recovery and precision for each analyte. In addition, samples generated from the $C_{18}$ sorbent resulted in a HPLC chromatograms with little or no interferences at the retention times of the analytes (Fig. 2).

## 5. Conclusion

The objective of this study was to produce a method for determining benzocaine and N-acetylbenzocaine concentrations in rainbow trout fillet tissue. As a result, a method was developed that is simple, accurate, precise, and sensitive for benzocaine and N-acetylbenzocaine in the fillet tissue from at least one salmonid species. The simplicity of the method is demonstrated (use of relatively common techniques) while the accuracy, precision, and sensitivity of the method are seen in the assay validation results. The method uses common and relatively inexpensive reagents, supplies, and instruments that can be purchased through common suppliers of scientific products. One analyst can safely perform the extraction techniques described in this method, as well

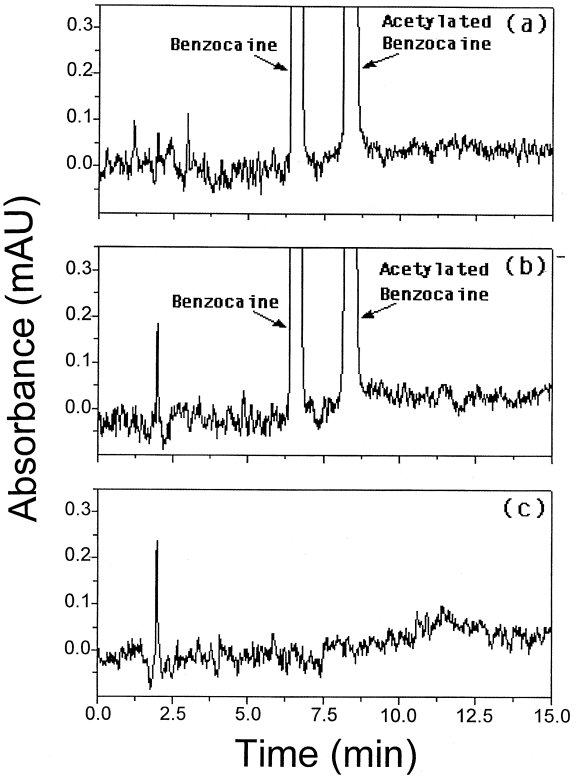


Fig. 2. Isocratic, reversed-phase HPLC absorbance chromatograms of (a) a benzocaine/N-acetylbenzocaine working solution (10 000 ng/ml), (b) an extract from a skin-on rainbow trout fillet tissue sample fortified with benzocaine and N-acetylbenzocaine at a nominal concentration of 50 000 ng/g, and (c) an extract from a control skin-on fillet sample from rainbow trout. Magnified baselines are displayed to show the lack of interfering compounds near the elution time of the analytes. The magnified baselines also show the lack of peak fronting and peak tailing with large concentrations of the analytes.

as the HPLC analysis, on at least 17 samples in a 24-h period.

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