Short communication

# Determination of indole-3-acetic acid in the Gulf of Gdańsk by high-performance liquid chromatography of its 4-methyl-7-methoxycoumarin derivative

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## Abstract

A modified procedure for the high-performance liquid chromatographic determination of auxin indole-3-acetic acid (IAA) in the marine environment is described. 4-(Bromomethyl)-7-methoxycoumarin (BrMmc) was used as a fluorescence labelling reagent for chromatographic analysis of IAA. The conversion of IAA into a coumaryl ester (IAA-Mmc) was carried out in the presence of potassium carbonate and with crown ether as a catalyst. The detection limit for the ester was about 20 fmol per injection. The new procedure was applied to the analysis of seawater and marine sediment samples collected from the Gulf of Gdańsk. For the first time, gas chromatography–mass spectrometry was used to confirm the presence of IAA in marine sediment.

*Keywords:* Derivatization, LC; Marine sediments; Water analysis; Indoleacetic acid; Auxins

## 1. Introduction

Indole-3-acetic acid (IAA) is well known as a naturally occurring auxin that, by itself and in interaction with other phytohormones, plays a crucial role in the regulation of growth and development of higher plants [1]. Sixty years ago, auxin was isolated and identified for the first time and since then it has been found in many plant species, including marine algae [2,3]. The first tentative identification of auxin in the sea, based on bioassay and paper chromatography, was carried out by Bentley [4] in Scottish coastal water. The presence of an auxin-like compound (19 pmol/l) was thought to be connected with

metabolic activity of planktonic organisms. Maruyama et al. [5] traced the presence of IAA in Japanese coastal water (12–220 pmol/l) and in interstitial water from marine sediments (0.1 nmol/l–1 mmol/l) by means of high-performance liquid chromatography (HPLC) and a highly specific enzyme-linked immunosorbent assay (ELISA). The bacterial origin of the isolated compound was considered. Mazur and Homme [6] identified IAA in water from the north of the Adriatic Sea and identification was based on gas chromatography–mass spectrometry (GC–MS).

Research carried out so far has not pointed out one specific source of auxin in the marine environment, although phytoplankton blooms as well as microbial decay of organic matter are taken into account.

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Further experiments should determine the contribution of individual groups of marine organisms or certain biogeochemical processes to IAA production. However, this requires the use of a highly sensitive assay method as the amount of IAA in the marine environment is very low.

A reversed-phase HPLC method is used for the routine detection, tentative identification and quantitative analyses of plant hormones [7]. The examined samples can be analyzed in either a derivatized or non-derivatized form and monitored with UV, electrochemical or fluorescence detection. In the case of naturally fluorescent IAA, fluorescence detection has often been chosen for the HPLC analysis. However, quite rapid elution of IAA with most columns and mobile phase systems, and the fact that when operating at $\lambda_{max}$ (excitation 280 nm and emission 350 nm) most indoles can also be detected, make the identification and quantification of auxin in environmental samples difficult. Better resolution and selectivity can be attained using a precolumn derivatization reaction. In the case of carboxylic acids, 4-(bromomethyl)-7-methoxycoumarin (BrMmc) is one of the most commonly used fluorescence labelling reagents [8]. Thanks to high molar absorptivity, this compound may be monitored using a UV detector as well. The fluorescence detection of BrMmc derivatives was applied to the analysis of some gibberellins [9]. In this work, the 4-methyl-7-methoxycoumarin ester of IAA has been synthesized. The use of the new derivative in the HPLC analysis of trace amounts of IAA in seawater and sediments collected from the Gulf of Gdańsk has been examined.

# 2. Experimental

## 2.1. Sampling and preliminary extraction

Samples of seawater and marine sediment were collected during spring algal bloom at off-shore stations of the Gulf of Gdańsk. Samples (10 l) of surface seawater were immediately passed through a Whatman glass microfibre filter GF/F. Approximately 150 g of the superficial sediment (about 0–5 cm) were suspended in 1 l of distilled water. After continuous stirring for 8 h, the sample was cen-

trifuged at 4000 g for 20 min and the liquid phase was removed. The residue was resuspended twice in 0.5 l of distilled water, stirred for 1 h and centrifuged again. The obtained supernatants were combined and subjected to the same procedure as that used for the seawater samples.

## 2.2. Reagents

All solvents were purchased from POCh (Gliwice, Poland) and were distilled prior to use. BrMmc was from Aldrich (Milwaukee, WI, USA), IAA was from Fluka (Buchs, Switzerland) and 18-crown-6 ether was from Merck (Darmstadt, Germany). Double glass-distilled water was used throughout. Amberlite XAD-4 was purchased from Serva (Heidelberg, Germany) and extracted in a Soxhlet apparatus with acetone and methanol (12 h each). A 5-g amount of XAD-4 was transferred to a glass column and rinsed with 25 ml of distilled water. Silica gel 60, for column chromatography, was purchased from Machery-Nagel (Düren, Germany). The stock solutions of IAA (1 μmol/l), BrMmc (10 μmol/l) and 18-crown-6 (10 μmol/l) were prepared in acetonitrile. Approximately 10 mg of finely powdered anhydrous potassium carbonate ($K_2CO_3$) and 3 μl of water were added to 1.0 ml of the crown ether solution. The mixture was sonicated for 15 min and passed through a 0.2-μm Millex-FG$_{13}$ filter (Millipore). In order to minimize the degradation process, all stock solutions were stored in the dark at 4°C.

## 2.3. Synthesis of the reference compound

A 200-μl aliquot of IAA stock solution was placed in a 1-ml reaction vial and evaporated to dryness under a stream of nitrogen gas. Aliquots (100 μl) of the crown ether and BrMmc stock solutions were added to the residue. The reaction mixture was allowed to stand in the dark for 20 min at 60°C and then the acetonitrile was evaporated under a stream of nitrogen. The residue was dissolved in 250 μl of n-hexane–ethyl acetate (2:1, v/v). The excess BrMmc and other by-products generated during the derivatization reaction were removed by purification on a silica gel 60 column (3.5×1 cm I.D.). The interfering substances were washed out with 25 ml of n-hexane–ethyl acetate (5:1, v/v) and then the auxin

coumaryl ester was eluted with 25 ml of n-hexane–ethyl acetate (3:1, v/v). The main fraction was evaporated in vacuo at 35°C, redissolved in 1.0 ml of acetonitrile and analyzed using the HPLC system. In order to determine the recovery of IAA–Mmc, the purification procedure on the silica gel column was repeated five times for five individual derivatives. The electron impact (EI) mass spectrum of the obtained reference compound was recorded with an AMD-604 mass spectrometer (AMD-Intectra, Germany) at 70 eV, an ion source temperature of 220°C and a direct inlet temperature of 173°C. A solution containing 1 μmol/l of IAA–Mmc was prepared for quantitative studies of the derivatization and purification procedures. Serial dilution of the standard solution was used to construct a calibration graph using HPLC analysis.

## 2.4. Analytical procedure

Seawater and water extracted from sediments were adjusted to pH 7 with 6 M HCl and passed through an Amberlite XAD-4 column (4.5×1.4 cm I.D.) at a flow-rate of 3 ml/min. The resin was washed with 25 ml of distilled water and then the sorbed substances were eluted with 25 ml of methanol. The alcohol fraction from XAD-4 was taken to dryness in vacuo at 30°C. The residue was dissolved in 500 μl of acetonitrile and derivatized with 100 μl of the 18-crown-6 solution and 100 μl of the BrMmc solution. The derivatization and purification procedures were carried out in the same way as for the reference compound.

Both the reference compound and the derivatized samples were analyzed with a chromatographic system consisting of Hewlett-Packard Series 1050 chromatographic pump, a Model 7125 Rheodyne injector with a 20-μl loop and a Hewlett-Packard Series 1046 programmable spectrofluorimeter linked to a Hewlett-Packard Series 3394 integrator. Separation was performed on a LiChrospher 100 RP-8 column (250×4 mm I.D.; 5 μm packing; Merck). Either a linear gradient was run from 50 to 2% water in methanol over 30 min or isocratic elution with water–methanol (35:65, v/v) was used. The flow-rate was 0.5 ml/min. The excitation and fluorescence emission wavelengths were experimentally adjusted to 312 and 399 nm, respectively.

A GC–MS system (Micromass, Winsford, UK) was used in the electron impact mode (70 eV) for identification of IAA in marine sediment. A fraction of the organic compounds extracted from marine sediment by a liquid–solid extraction was freeze-dried and silylated with a mixture of N,O-bis-(trimethylsilyl)acetamide and trimethylchlorosilane (15:85, v/v) at 80°C for 30 min. A 20-m capillary column, type SPB-1, with a film thickness of 1 μm and an I.D. of 0.25 mm was used. The helium carrier gas flow-rate was 0.5 ml/min. Injection took place at 200°C; the oven temperature was 150°C at injection and then was increased by 4°C/min to 250°C. The temperature of the ion source was 200°C and the emission current was 200 μA. The recovery of IAA from marine sediment was determined as follows. A 1.0-ml volume of a 1 mmol/l solution of IAA was placed in a glass vessel. After solvent evaporation under a stream of nitrogen, 1 l of distilled water and 150 g of marine sediment were added. The mixture was stirred in the dark for 8 h and the extraction process was carried out as described in Section 2.1 and Section 2.4.

## 3. Results and discussion

### 3.1. Derivatization and purification procedures

BrMmc has been used as a fluorescence labelling reagent in the HPLC analyses of many biologically active compounds and its application was extended to IAA here. It was found that in a manner analogous to the procedure described for fatty acids [10,11], the carboxyl group of IAA reacts with BrMmc and that the corresponding ester is formed, even under mild conditions. Reaction of the potassium salt of IAA with BrMmc was facilitated by the addition of crown ether as a solid–liquid phase transfer catalyst and acetonitrile was chosen as the best solvent [12]. Under the described derivatization conditions, formation of the auxin coumaryl ester IAA-Mmc was completed within 20 min. Quantitative studies at a concentration of 1 μmol/l IAA were carried out by multiple derivatization (n=5) followed by HPLC. The reaction yield was found to be 80.0% when at least a five-fold excess of the reagent over the amount of acid was used. HPLC analyses of the

obtained derivatives gave peak areas with a relative standard deviation of 2.3%. When refrigerated, the resulting compound turned out to be stable, even after several months. The scheme for the reaction of BrMmc with IAA is shown in Fig. 1.

As a result of the required surplus of the reagent, the reaction mixture contains a considerable amount of by-products that interferes with the HPLC analysis. To overcome this problem, silica gel chromatography was used. This purification step eliminated nearly 90% of the impurity peaks while the recovery of IAA–Mmc was 89.8% (S.D.=3.5; $n=5$). The stopped-flow scanning of excitation and fluorescence emission spectra of the auxin coumaryl ester gave optimum wavelengths at 312 and 399 nm, respectively, when water–methanol (30:70, v/v) was used as the mobile phase.

The limit of IAA–Mmc detection was determined to be about 20 fmol for a 20-µl injection and at a signal-to-noise ratio of 3. A calibration graph of peak area versus amount of IAA–Mmc was plotted and can be described by the following equation:

$$y = -0.0358 + 0.2386x,$$

where $y$ stands for the peak area and $x$ for the amount of IAA-Mmc injected onto the chromatographic column. The regression coefficient was 0.991. Over the examined range from 0.02 to 20 ng of IAA–Mmc, the response of the fluorescence

detector was found to be linear. The obtained improvement in selectivity and chromatographic properties constitutes a substantial advantage in the fluorescence detection of auxin as the coumaryl ester (IAA-Mmc). The UV detector, which is approximately $10^3$ fold less sensitive than the fluorescence detector, when operated at 312 nm, gave a peak area that was twenty-times bigger than that obtained for non-derivatized IAA. Additionally, a direct inlet high-resolution MS analysis was carried out to confirm the IAA-Mmc structure. The mass spectrum of the derivative (Fig. 2) is characterized by a molecular ion peak at $m/z$ 363 and a base peak at $m/z$ 130, which is thought to be the protonated quinolinium or methyl-3-indole ion [13]. The metastable ions spectra gave the fragmentation pathway of the examined ester. It was found that the auxin fragment yields ions at $m/z$ 174, 130, 103 and 77, whereas the coumaryl fragment yields ions at $m/z$ 190, 162 and 147.

## 3.2. Analytical application

The reliability of the proposed method of IAA determination was examined in experiments with samples of seawater and sediment collected from the Gulf of Gdańsk during the spring algal bloom. Isolation and purification steps that often lead to the breakdown of IAA were improved by the application of a very efficient adsorbent, XAD-4 column [14]. The IAA recovery from samples of seawater, determined by comparing the peak area of a known amount of auxin before and after elution from XAD-4, was 99.0±0.8%. However, in the case of sediment, recovery of IAA was only 27.7±2.5%. It was found that further extraction of IAA with distilled water did not improve the recovery. Presumably the low recovery was due to IAA sorption on sediment particles or to compound degradation. HPLC analyses of derivatized organic substances extracted from surface seawater and pore water from marine sediments showed the presence of a compound with a fluorescence emission spectrum and a retention time that were identical to those of authentic auxin coumaryl ester. The identity of the peak was confirmed by co-chromatography with standard IAA-Mmc (Fig. 3).

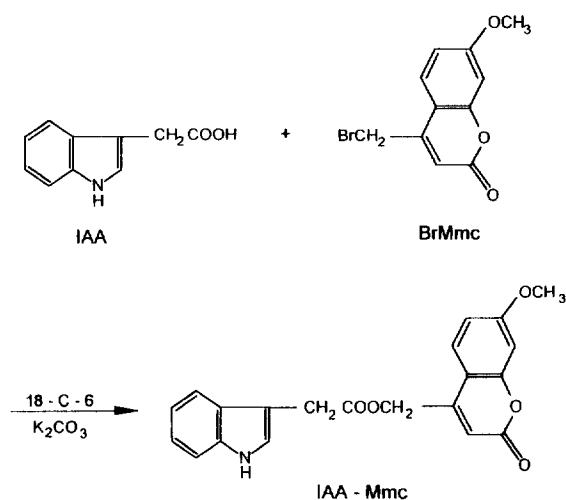The presence of IAA in marine sediment was



Fig. 1. Scheme for the reaction course of indole-3-acetic acid (IAA) with 4-(bromomethyl)-7-methoxycoumarin (BrMmc).
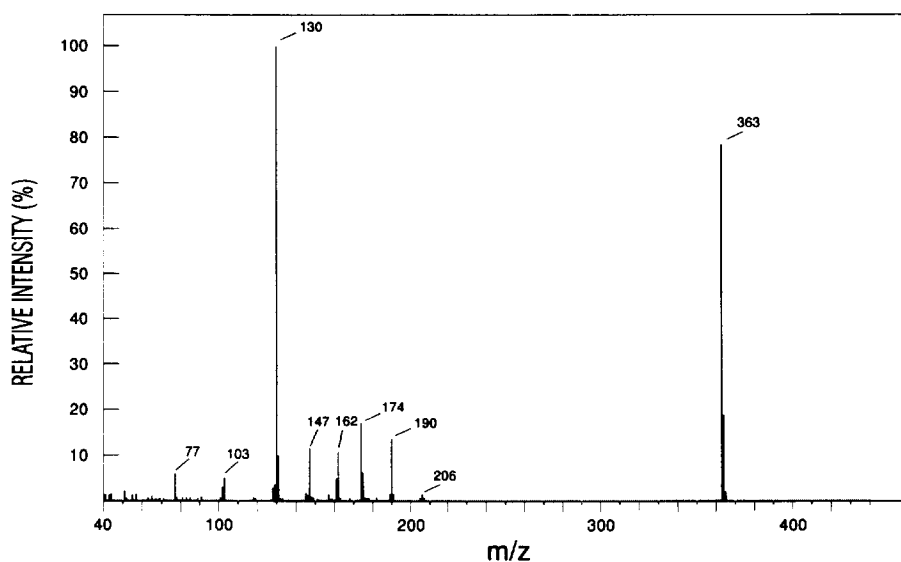
Fig. 2. Electron-impact mass spectrum of indole-acetic coumaryl ester (IAA–Mmc).
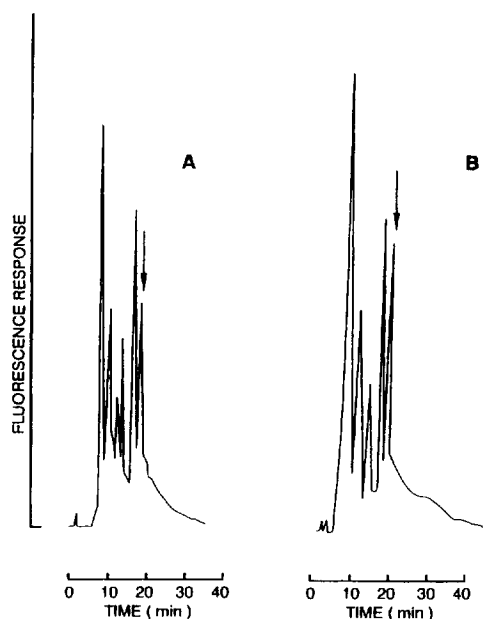


Fig. 3. Chromatogram of an extract of marine sediment that was treated with BrMmc (A); chromatogram of the same sample on addition of IAA–Mmc standard (B). LiChrospher 100 RP-8 column (250×4 mm, 5 μm), 30 min linear gradient from 50 to 2% water in methanol.

proved by a GC–MS method. The obtained total ion current of the trimethylsilyl (TMS) derivative gave a retention time peak corresponding to that of TMS–IAA. At this retention time, the EI mass spectrum of the silylated compound from sediment showed major ions at $m/z$ 319, 202 and 73. At the same retention time, the same ion peaks were present in an EI spectrum of the silylated standard. Moreover, the relative intensities of the corresponding peaks from the derivatized standard and sediment extract spectra were in good agreement.

The concentrations of auxin in samples of surface seawater collected during the spring algal bloom ranged from 25 to 150 pmol/l, whereas in sediments they ranged from 2.5 to 6.3 nmol/kg. These results are in a good agreement with those published by Maruyama et al. [5] and suggest that, apart from living algae, it is the processes proceeding in marine sediments that significantly contribute to IAA production. Maruyama et al. [5] proved that not only terrestrial bacteria but also some strains of bacteria living in marine sediments are able to convert tryptophan to IAA. The results presented above, obtained using the new sensitive method of IAA determination, and those results previously obtained by Bentley [4], Maruyama et al. [5] and Mazur and Homme [6] lead us to conclude that there is a common occurrence of IAA in eutrophicated regions.

## Acknowledgments

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