Classification: Biological Sciences; Biochemistry

A skin pigment compound that also functions as "sunglasses" for birds

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Abstract

A prominent ultraviolet (UV)-absorbing compound of unknown identity was discovered in the aqueous humor of birds, believed to be a key UV-filter of the eyes of aves. The compound´s concentration correlates with species´ natural flight altitudes, with the highest concentrations found in goose chamber fluid (up to 10,000 m flight altitude). We present evidence that the compound is 5,6-dihydroxyindole (DHI), based on analysis using liquid chromatography, UV spectroscopy, fluorescence spectroscopy, nuclear magnetic resonance spectroscopy, mass spectrometry and X-ray crystallography. DHI is a precursor of melanin (the key UV-absorbing polymer in biology), produced in the proximity of the chamber fluid. Identification was greatly challenged by the compound´s tendency to polymerize when enriched. DHI may function not only as nature´s UV-filter in the skin, but also as "sunglasses" as well.

Keywords: Bird; Eye; UV; Melanin; 5,6-dihydroxyindole

Significance Statement

Nature provides species with various solutions for protecting the eyes from sun radiation. For example, the eye contains compounds that absorb UV radiation, like ascorbic acid. But what about the more extreme cases like geese, which fly as high as 10,000 meters (above the Himalayas)? In the aqueous humor of birds, an unknown compound provides a major contribution for UV-absorbance. The higher the bird can fly, the more of this compound is present, suggesting its significance to Nature. We have identified this compound as being 5,6-dehydroxyindole. This compound is also a building block of melanin, a skin protector. Hence, using various analytical/spectroscopic techniques, we have unraveled "sunglasses" that evolution has provided aves. It is conceivable that this compound could be used for humans as well, e.g. as a component in both contact lenses and intraocular lenses.

Introduction

The eye is often compared to a camera; it forms an image of the environment on its photoreceptive layer, the retina. The diaphragm of the camera is analogous to the iris of the eye, and the lens focuses the light onto the most central part of the retina, the macula lutea. The light pathway through the eye media consists of cornea, aqueous humor, lens, and vitreous body. One decisive premise to achieve an optimal image is that all components of the media allow visible light (visible radiation, VR: 760-400 nm wavelength) to pass through. Shorter wavelengths (UV-A: 400-320 nm, UV-B: 320-290 nm, and UV-C: 290-200 nm) must be blocked to prevent phototoxic tissue damage, as the effect on biological tissue increases with decreasing wavelength. The most potent UVR is largely absorbed by the ozone layer, minimizing damage on biological structures in general. In fact, only some 5% of all VR/UVR energy striking our planet is in the UV-range, and of this 97% is UV-A. However, it has long been known that this limited dose of actinic UVR reaching the ground is nonetheless able to cause harmful effects on the eye (1), and changes may occur at three different levels, i.e. the cornea (snow blindness/photokeratisis), lens (cataract), and retina (macula degeneration).

However, the human eye has some protection, e.g. the high levels of high molar absorptivity ascorbic acid (AA) present in the aqueous humor and corneal epithelium (30-300 times the serum level, respectively (2, 3)). The function of AA as a UV-absorber in the mammal eye was first proposed by Ringvold (4). This view explains why the AA level in the aqueous humor is high in diurnal and low/zero in nocturnal mammals (5), that the AA content is much higher in corneal epithelium compared to aqueous humor in diurnal mammals and that AA content is higher in corneal epithelium of mammals living in the mountains (such as reindeer) compared to those living close to sea level (6). Furthermore, this hypothesis has also been supported experimentally as artificially increased levels of AA in the aqueous humor of guinea pigs had a protective effect against UV-induced DNA damage to the lens epithelium (7), high aa concentration protects the basal layer of the corneal epithelium by absorbing incident UVR (8), and AA (as well as tocopherol) in the aqueous humor increased the viability of lens epithelial cells after UVR in a dose-dependent manner (9). There is also some evidence that systemic AA supplementation

used prophylactically may reduce the prevalence of hazy vision after photorefractive keratotomy (commercially available excimer lasers for refractive surgery are based on energy output at 213-193 nm wavelength) (10). Other natural products have also been proposed as being viable eye protectors, such as chlorogenic acid that protects the retina when administered prior to exposure of nitric oxide (i.e. free radicals and oxidative stress) (11). Hence, AA seems to act as "sunglasses" for the diurnal mammal eye. It would be natural to believe that the presence of AA would be significant in the aqueous humor of high-flying birds, as these are exposed to vastly more radiation at e.g. 10,000 m altitudes. The aqueous humor is an integral part of the optic pathway of the eye media regardless of whether mammal or bird, and so the observation of low AA content in avian aqueous humor was surprising (12).

What protect the bird's eyes when flying at high altitudes, e.g. above the Himalayas (13)? Several UV-absorbing components (e.g. uric acid, tryptophan and tyrosine) have been proposed as being present in bird aqueous humor. In addition to these, a single, unknown component dominates the UV absorbing profile in goose eye chamber, as observed with liquid chromatography-UV spectroscopy (LC-UV) (14). This compound is less present in lower flying species e.g. duck and owl, and barely visible in chicken, and not present in non-flying species. We hypothesize that this compound "X" has a protective role for the eye of birds and describe here our work in identifying the unknown compound.

Results

Liquid chromatography-UV analysis of highly polar substances in chamber fluid

Capillary liquid chromatography-ultraviolet absorption (LC-UV) 2D plots (**Figure 1**) shows chromophoric molecules (green frame) which were far more prominent in goose chamber fluid (top) compared to e.g. duck (bottom), resembling that observed previously (14). The green frame suggests the presence of multiple compounds. Compound 2 was however seen to form from compound 1 when samples was exposed to air, a clue that compound 1 ("compound X") was sensitive to oxidation. The capillary LC system was coupled with electrospray-mass spectrometry (ESI-MS), a natural combination for gaining structure information of limited samples. Surprisingly, the molecules of interest had signals below

the system´s detection limits, even when using high performance Orbitrap MS instrumentation.

Analysis of enriched material

To obtain improved signal intensities, compound X was enriched with semi-preparative scale LC for subsequent elucidation, using a modified RPLC column variant (ACE AQUA) for increased resolution of the polar analytes (**Figure 2,** 1D view). Instant re-analysis of the isolated compound X revealed a pure peak, but the peak shape would be perturbed in subsequent analyses, again suggesting a reaction chemistry occurring when chromatographed away from the chamber fluid. Moreover, when compound enrichment was attempted spending over 100 eye samples, a trace dark solid was produced. Size exclusion chromatography implied that the enriched material had macromolecular features (**Figure 3**). Hence, the analyte was assumed to form polymeric species/aggregates when concentrated/isolated away from the chamber fluid. When treating with trypsin, no enzymatic cleavage was observed (i.e. presence of tryptic peptides using MS analysis), largely ruling out the presence of a protein. Crystallization procedures were subsequently attempted of isolated material (including developing an in-capillary crystallization procedure). However, only trace amounts of amorphous material were produced, unsuited for X-ray diffraction (XRD) experiments. Nuclear magnetic resonance (NMR) spectroscopy of the isolated material showed protons in the 6-8 ppm range (implying aromatic functionality), but again with vastly insufficient sensitivity for structural analysis.

Analysis of un-separated chamber fluid

The analytes were stable in capped chamber fluid (investigated with samples stored in a freezer, refrigerator, and subjected to freeze-thaw cycles), so analyses of un-chromatographed samples were undertaken. UV analysis confirmed that goose samples had elevated absorbance (**Figure 4, left**). Next, high resolution NMR (800 MHz) of non-chromatographed samples was performed, as this approach can be used for structural elucidation of un-separated molecules. The NMR spectra of un-chromatographed chamber fluid from various bird species did not reveal any prominent aromatic substances in goose samples (**Figure 4, right**), again implying that the analyte must have been mole-wise

present in moderate/trace quantities (while UV experiments demonstrated a substantial molar absorptivity).

Analysis of diluted compound X fractions

Revisiting mass spectrometry with less enriched fractions (to avoid excessive post-chromatography reactions) but using alternative sources to ESI (i.e. chromophore-sensitive atmospheric pressure photo ionization = APPI and atmospheric pressure chemical ionization = APCI), we were able to clearly detect a small molecule substance; in positive mode (generating $M+H^+$ species), an *m/z* = 150.058 species was observed and *m/z* = 133.031 in MS/MS mode, implying a loss of a –OH group when using MS/MS (**Figure 5**). The molecular features suggest the presence of 5,6-dihydoxyindole (DHI (15)), a monomer of the biomacromolecule melanine (16). Compound X featured an identical fluorescence spectrum with tryptophan (which also has indole functionality), further strengthening the hypothesis that this monomer of biomacromolecules was DHI (**Figure 6**).

Discussion

DHI is a building block of melanins, which are key UV-protectors in biology (15). DHI is readily polymerized to melanins, both enzymatically and by autooxidation. The data (obtained via LC-UV, MS, fluorescence spectroscopy, etc.) suggests that DHI is a key UV-absorbant in the eyes of birds, especially species that fly at extraordinary altitudes, e.g. geese. Melanocytes (sites of melanin formation) are in close proximity to the anterior chamber containing the fluids analyzed in this study and is conceivably the source of DHI regarding eye protection. Although our data supports the presence of DHI, we wished to compare our results with a reference material. DHI was purchased from several commercial sources but arrived in autoxidized form (brown-black, similar to that obtained during enrichment of the endogenous compound), making retention time comparisons with the metabolites in the chamber fluid challenging. Attempts were made to de-polymerize the product to release blocks of DHI, using H_2O_2. Although the UV spectra clearly shifted towards the profile of goose eye, and a major peak of the "bleached" product had the retention time of compound X, the practical use of the reference material

was considered to be limited. Therefore, we additionally ruled out that the unknown compound was other absorbing metabolites, e.g. uracil and tryptophan, by comparing LC retention times with reference materials. Structural elucidation was greatly challenged by polymerization occurring during enrichment. For example, it was not possible to create crystals for X-ray diffraction experiments; this has been previously observed with melanin (18). The exact mechanisms of the polymerization have yet to be fully mapped (19). However, it is established that DHI is in equilibrium with a quinonimine (19); the single hydroxyl-group of quinonimine suggests its presence during the APPI-MS experiments. Polymerization does not occur in the native chamber fluid, which is rather natural considering the presence of antioxidants, low concentration, and rapid exchange (20).

Methods

Sample collection/preparation

Goose eyes (*Anserine,* White Italian) were made available to us each year in November, the only time when Holte Gaard (an ecological farm in Drangedal, Norway http://www.holtegard.no/) slaughter geese (for culinary purposes only). A veterinary would inspect the premises and routines the day of slaughter. Immediately after decapitation, aqueous humor was collected at the slaughterhouse through a limbal transcorneal canulation parallel to the iris diaphragm, 0.1-0.3 mL was obtained from each eye. Similar LC-UV were also obtained using wild geese (Grey goose *Anser anser* and Canadian Goose *Branta Canadensis*, donated from hunters). Chicken (*Gallus gallus,* Lomann), duck (Peking duck, *Anatinae*, English hybrid Cherry Waelly) and turkey (*Meleagris gallopavo,* British united turkey) were obtained under similar conditions as with the geese (farms: Prio Rakkestad and Revetal Vestfold, Norway).

Liquid Chromatography and spectroscopy

Capillary LC-UV was performed using a CapLC-UV system (Waters™, Milford, MA). LC analysis was performed with a PerkinElmer (Waltham, MA) series 200 LC pump chromatograph coupled to a Perkin Elmer series 200 autosampler and a Waters™ 486 Tunable Absorbance Detector set to a wavelength of 254 nm. The RP column was an ACE AQ HPLC column (ACE; 250 mm × 10 mm, 5 µm diameter particles, S/N A59810, Advanced

Chromatography Technologies Ltd, Aberdeen, Scotland). The SEC column was a 4.6 × 300 mm TSKgel SuperSW3000 SEC column (Tosoh Corp., Tokyo, Japan).

The mobile phase used for RPLC was an isocratic mixture (99/1, v/v) of water and acetonitrile (with 0.1% addition of formic acid (FA) to both solvents for pH ≈ 2.7), with a flow rate of 5 mL/min. SEC separations were done isocratically with a flow rate of 0.35 mL/min and a mobile phase containing 0.05 M sodium phosphate and 0.3 M NaCl pH adjusted to 7.0. Injection loops of 100 µL and 1 mL were used. Thawed samples were injected without any pre-treatment. The smaller loop was pre-installed in the instrument's autosampler; the bigger one was manually installed using a two-position six-port valve (Model 7000, Rheodyne LLC, Rohnert Park, CA, US). The use of 1 mL loop required manual injections. Chromatograms were obtained with the TotalChrom Navigator software version 6.2.1 (Perkin Elmer, Waltham, MA, US). Evaporation of manually collected fractions was conducted using a Speed-vac™ concentrator (SC110, Savant Instruments Inc., Hicksville, NY, US), in 1.5 mL vials (Thumbs up microtubes, Diversified Biotech, Boston, MA, US), with medium or no heating. Evaporating 1 mL of aqueous solution to complete dryness took about 6 h. Qualitative UV-vis analysis was performed on a Thermo Scientific NanoDrop 2000 UV-vis spectrometer. 2 µL sample were applied on the instrument's pedestal. Type 1 water with 0.1 % FA was analyzed as a blank. The collected data were processed using the Chromeleon Chromatography Studio software (version 7.1.0.898, Dionex Corporation, Sunnyvale, CA, US). For fluorescence analysis an FP-8500 spectrofluorometer from Jasco with a Julabo F25-ED Refrigerated/Heating Circulator was employed. It was equipped with an ETC-815 Peltier thermostated single cell holder (water-cooled) for temperature control. The excitation wavelength was from 200 – 740 nm and emission wavelength from 305 – 750 nm without polarizing filters. Scan speed was 10000, temperature 25 °C. Data collection occurred with Spectra Manager (version 2.13.00). For measurements 350 µL of sample was pipetted into a quartz sample holder. Apart from using aqueous humor, NMR experiments were for all practical purposes performed similarly to that described by Sachse et al (21).

Mass Spectrometry

With a Q-Exactive™ hybrid quadrupole-Orbitrap instrument from Thermo Scientific samples were transferred to mass analyzer using ESI in positive and negative mode with a

spray voltage of 1.5 kV and a capillary temperature of 320 °C. Data were collected in a full scan mode (50 to 500 *m/z* and 150 to 2000 *m/z*). The pump flow was set to 0.02 mL/min (Fusion 100T pump, Chemyx Inc., Stafford, TX, US). For data collection and process control the Xcalibur software (version 3.0.63, Thermo Fisher Scientific Inc.) was used. For analysis with an APCI source, a TSQ Quantiva instrument from Thermo Scientific with built in pump was used. The flow rate of mobile phase was set to 100 µL/min, the capillary temperature to 350 °C, and the collision energy to 30 eV. Samples were directly injected into MS system using a syringe pump, and the data were collected in a full scan mode (mass range 50 – 1500 *m/z*). Xcalibur software was used for data collection and processing. A Bruker maXis II ETD QTOF mass analyzer (Bruker, Billerica, MA, US) was used for APCI/APPI-MS experiments. In these MS analyses, capillary voltage was set to 3.0 kV, heater temperature 450 °C, collision energy 2.0, 5.0, 10.0 and 15.0 eV, mass range 20 – 300 *m/z*. Obtained data were analyzed and processed with Bruker Compass Data Analysis software, version 4.3.

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Figures

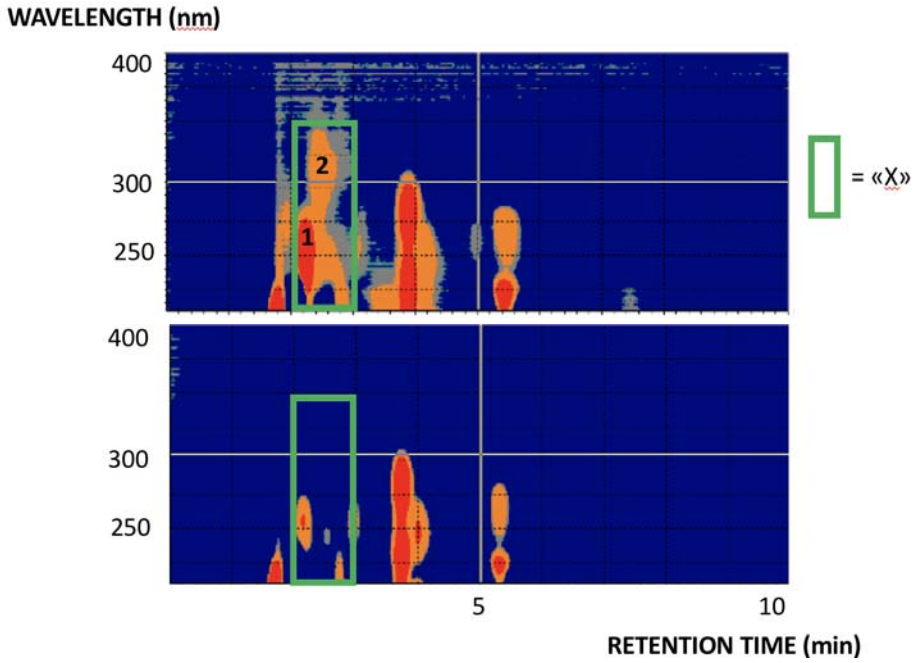

Figure 1. Two dimensional (capillary LC X UV) plots of aqueous humor from goose (top) and duck (bottom). Conditions are described in Materials section (Liquid Chromatography and spectroscopy).

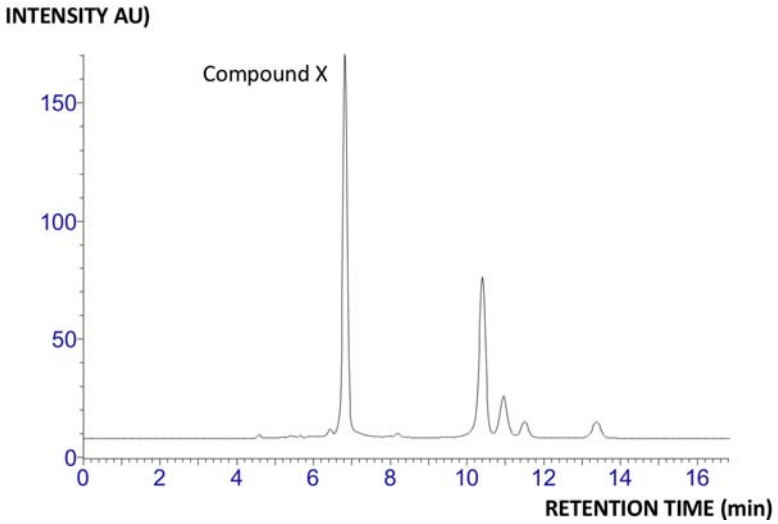

Figure 2. Reversed phase LC chromatography of goose aqueous humor, used for isolation of compound X. Conditions are described in Materials section (Liquid Chromatography and spectroscopy).

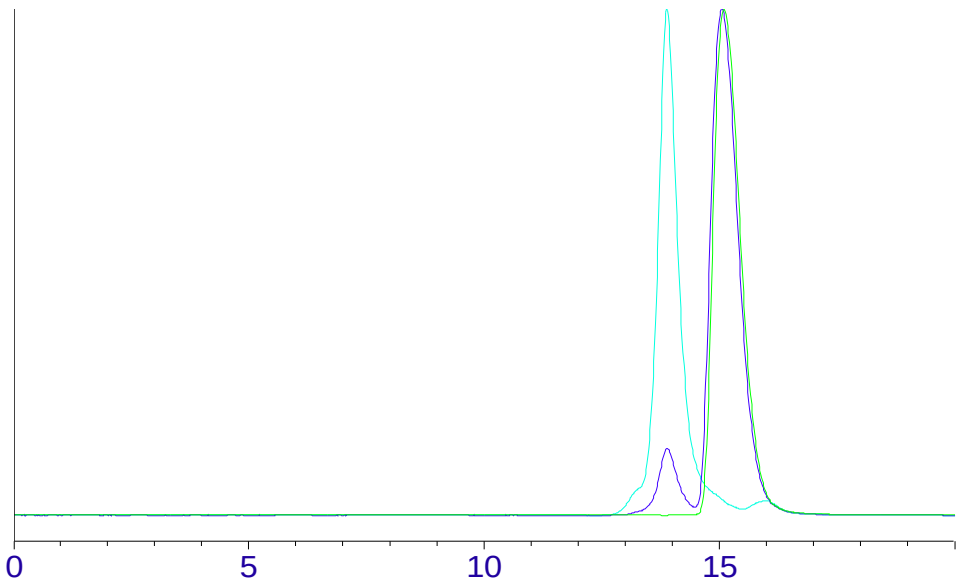

Figure 3. Size exclusion chromatography (SEC) of isolated compound X (light blue trace) and compound X/caffeine mixture (Vt marker, blue trace). Conditions are described in Materials section (Liquid Chromatography and spectroscopy).

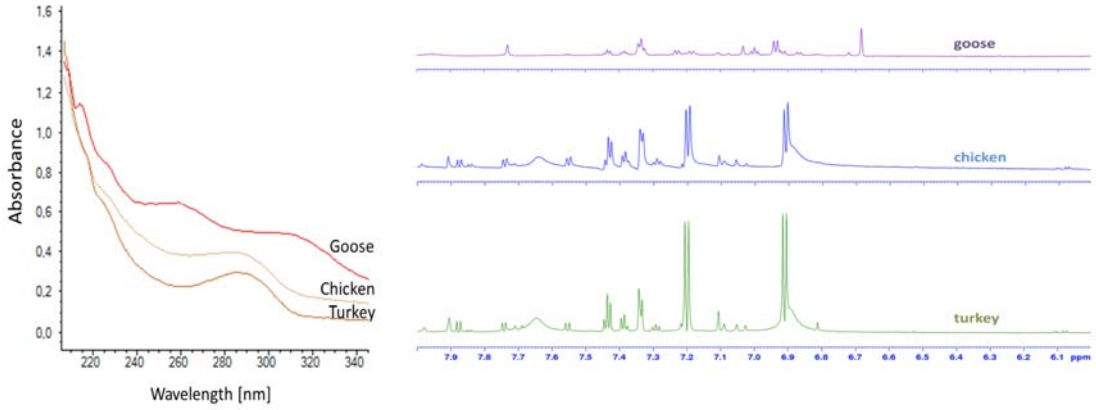

Figure 4. Left: UV absorbance of aqueous humor of goose, chicken and turkey. Right: 800 MHz NMR of the same samples. Conditions are described in Materials section (Liquid Chromatography and spectroscopy).

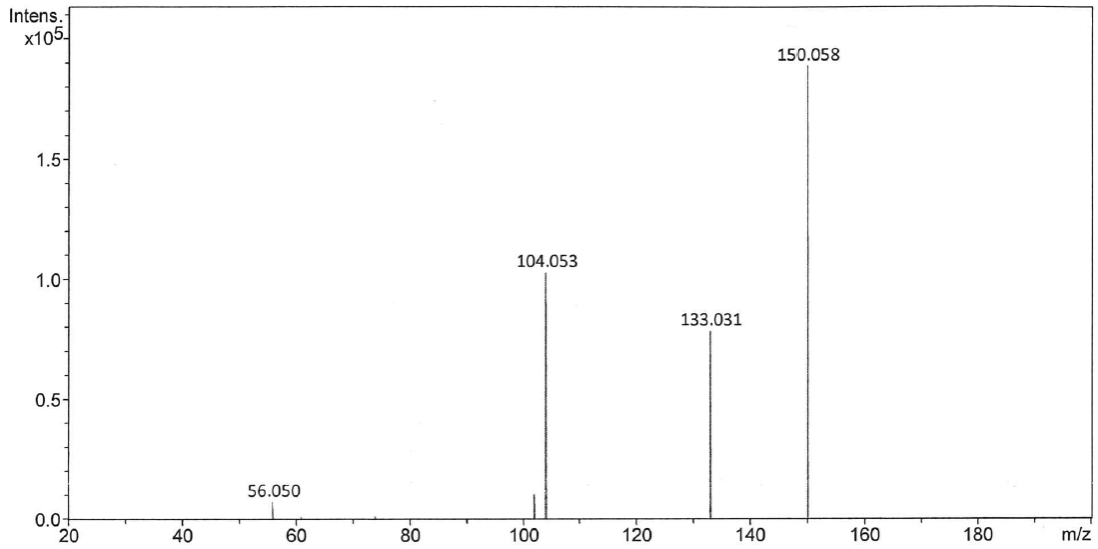

Figure 5. APPI MS spectrum of compound X with direct infusion. Conditions are described in Materials section (Mass spectrometry).

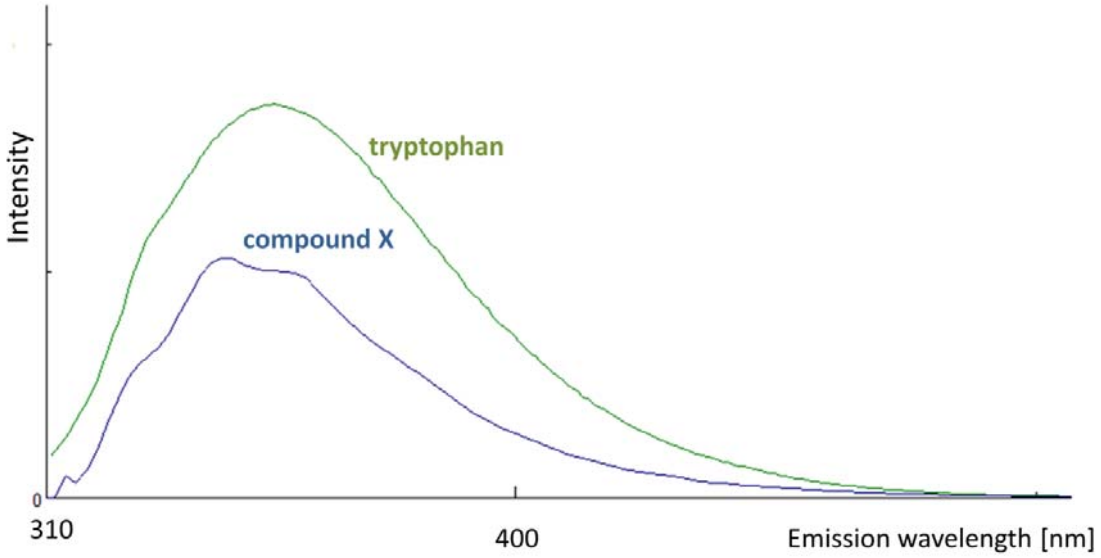

Figure 6. Fluorescence spectra overlay in 2D of compound X and tryptophan. Conditions are described in Materials section (Liquid Chromatography and spectroscopy).