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Clémence Moitessier, Khémesse Kital, Pierre-Edouard Danjou, Francine Cazier-Dennin. 4-Methoxy-ortho-phthalaldehyde: a promising derivatizing agent for the fluorimetric evaluation of histamine in seafood. Talanta Open, 2020, 2, pp.100014. 10.1016/j.talo.2020.100014. hal-02942358

HAL Id: hal-02942358

https://hal.science/hal-02942358

Submitted on 17 Sep 2020

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Full Length Article

4-Methoxy-*ortho*-phthalaldehyde: a promising derivatizing agent for the fluorimetric evaluation of histamine in seafood



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ARTICLE INFO

Keywords: Biogenic amines Histamine fluorimetric analysis Molecular fluorescence spectroscopy 4-methoxy-ortho-phthalaldehyde

ABSTRACT

Histamine, the molecule associated with food poisoning, is nowadays considered as a reliable marker of seafood freshness and therefore its rapid and easy quantification appears critical. Existing method like HPLC or direct fluorimetric analysis employing *ortho*-phthalaldehyde (OPA) can be time-consuming and tedious. To overcome this problem, we propose in this paper to evaluate the application potential of 4-methoxy-*ortho*-phthalaldehyde (MO-OPA) for the rapid and easy evaluation of histamine in seafood samples. For this purpose, the histamine complexation conditions were studied by molecular fluorescence spectroscopy using MO-OPA as derivatization agent and then compared with the reference compounds: OPA. MOPA proved to be superior to OPA for histamine detection in terms of linearity range $(1.10^{-6}-1.10^{-4} \text{ mol.L}^{-1}, \text{R}^2 > 0.99, \text{ n} = 10)$ and detection limit $(9.5.10^{-7} \text{ mol.L}^{-1})$. The optimal conditions were next applied to the analysis of histamine in a mixture of biogenic amines standards and finally on real samples of yellowfin tuna of known histamine concentration.

Introduction

Food safety and quality are increasingly at the center of preoccupation for consumers as well as health agencies. Consequently, a permanent evaluation of the freshness and toxicity index of foods is required and involves in particular the characterization of spoilage markers such as biogenic amines (BAs). In food, these non-volatile nitrogenous organic bases result from the decarboxylation of their amino acids precursors generated by pathogenic microorganisms as well as by maturation and fermentation processes. [1,2] While BAs are essential components in the process aiming to regulate physiological functions in the human body, they can also pose a serious risk to the consumers health due to their relatively low toxic threshold. [3-5] Detection and quantification studies of BAs are extensively illustrated in the literature, particularly those addressing histamine (HA). Indeed, the presence of HA in seafood is regulated by health authorities like the European Commission [6] or the U.S. Food and Drug Administration (FDA) [7] since it is responsible for histamine intoxication. Currently, other BAs such as putrescine and cadaverine are also studied considering that they can potentiate the toxic effect of HA. [8] As a result of its toxicity and regulation, HA analysis techniques are widely documented, including colorimetric, [9-11] enzymatic [12,13], electrochemical [14-16] and chromatographic methods [17-25]. Fluorescence spectroscopy

analysis is also a commonly employed technique [26-28] especially the one developed by the AOAC which is currently approved by the FDA [7,29,30]. Since HA does not contain any fluorophore moiety, a derivatization procedure is crucial in order to allow an analysis by molecular fluorescence spectroscopy. Several derivatizing agents have been employed and the most commonly used ones are dansyl chloride, [31,32] fluorescamine, [33,34] 2,3-naphthalenedicarboxaldehyde [35,36] and ortho-phthalaldehyde (OPA). [29,37-39] Employed since 1959, [40] the OPA takes the lead over other reagents since it enables the detection of BA at low concentrations and without HPLC separation. The selectivity over other aminated compounds is achieved through the specific excitation and emission wavelengths of each BA/OPA complexes. [41] Nevertheless, OPA derivatization leads to poorly stable compounds. The addition of thiol reagents such as 2-mercaptoethanol, [42] 3-mercaptopropionic acid [42,43] or N-acetylcysteine [42,43] was proposed in order to create less unstable OPA/thiol/HA complexes, to extend the pH range required for the formation of compounds as well as to facilitate its applicability. With a similar aim, we propose here to study the behavior of HA fluorescent analysis of a new derivatization agent possessing a methoxy group directly linked to the aromatic core of the OPA: 4-methoxy-ortho-phthalaldehyde (MO-OPA) (Fig. 1). As far as literature is concerned, derivatives based on an OPA core have scarcely been used as derivatization agents and were never applied to BAs

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Fig. 1. The chemical structures of ortho-phthalaldehyde and 4-methoxy-ortho-phthalaldehyde (left) and biogenic amines (right) used in this study.

analysis. In a recent report, Zhang et all. [44,45] proposed a synthetic route to MO-OPA and 4,5-dimethoxy-ortho-phthalaldehyde to achieve a fluorescence probe for ammonium traces detection with the successful development of a hand-held portable fluorometer. They report that the addition of an electron-donating group allows to increase the electron density of the aromatic core leading to an enhancement of the fluorescence intensity. Moreover, it enables faster reaction with analytes at room temperature. [44] In this paper, we take advantage of our precedent expertise on MO-OPA synthesis [46] to evaluate its efficiency on HA fluorescent analysis. To fulfill this goal, the complexation conditions of HA with MO-OPA as well as the molecular fluorescent parameters were investigated and then properties of MO-OPA as derivatization agent were compared with standard OPA. These optimal conditions were next applied to the fluorimetric analysis of HA with MO-OPA in a mixture of BAs standards and finally on real yellowfin tuna (Thunnus albacares) samples.

Experimental section

Reagents

All the chemicals were purchased from Acros Organic or Sigma-Aldrich and were used as received without further purification unless specified. Biogenic amines were purchased as: Agmatine sulfate salt (\geq 97%), cadaverine dihydrochloride (\geq 99%), histamine dihydrochloride (\geq 99%), putrescine dihydrochloride (\geq 99%), spermidine trihydrochloride (\geq 99%), *ortho*-phthalaldehyde (\geq 99%; recrystallized from n-hexane). 4-methoxy-*ortho*-phthalaldehyde (MO-OPA) was synthesized according to the procedure of Moitessier *et al.* [46]

HPLC grade water was used throughout the study for the preparation of analytical solutions. The pH was adjusted using sodium hydroxide 1M or hydrochloric acid 1% aqueous solutions.

Tuna samples

Two doped canned samples of yellowfin tuna (*Thunnus albacares*) were provided by the French Agency for Food, Environmental and occupational Health & Safety (ANSES) with known final concentration of HA at 15.79 ± 2.53 mg/kg and 148.64 ± 7.99 mg/kg respectively.

Apparatus

UV-Vis measurements were performed at 20 $^{\circ}$ C on a Varian Cary 100 spectrophotometer. A 100-OS 10 mm pathlength optiglass cuvette was used. Fluorescence measurements were performed at 20 $^{\circ}$ C on a Varian Cary Eclipse spectrofluorimeter. A 100-QS 10 mm pathlength quartz fluorescence cuvette was used.

Procedures

Solution preparation

Stock solutions of HA $(1.10^{-2}~\text{mol.L}^{-1})$ and derivatization agent (OPA or MO-OPA; $1.10^{-2}~\text{mol.L}^{-1})$ were prepared by dissolving the compounds in water. Serial dilutions were performed to obtain working standard solutions. All solutions were protected against light and stored at 4°C prior to use unless specified. The shelf-life of the solutions must not exceed one week. [29] BAs and derivatization agent were mixed during the final dilutions prior to fluorimetric analysis. The solutions were degassed in an ultrasonic bath during their preparation and before the recording of molecular fluorescence spectra.

Analytical measurements

For the fluorimetric method, the fluorescence intensity was monitored at the fixed analytical excitation (λ_{ex}) and emission (λ_{em}) wavelengths of the complexes. Linear calibration curves were obtained at these λ_{ex} and λ_{em} values by measuring the spectra height signal. All fluorescence measurements were corrected for the solvent signal with the appropriate blank.

Fish samples preparation

Two tuna cans of known level of HA (15.79 \pm 2.53 mg/kg and 148.64 \pm 7.99 mg/kg) were processed according to a modified procedure based on Bjornsdottir–Butter et al. [30] work. Briefly, methanol (75% v/v; 50 mL) was added to 10 g of tuna and the mixture was vigorously stirred during 30 min. The sample was then filtered over fritted glass, evaporated and put back in solution in HPLC grade water. The aqueous sample was transferred into a 50 mL volumetric flask and completed with water. 1 mL of the resulting solution was diluted with water into a 10 mL volumetric flask containing 1 mL of MO-OPA aqueous solution (1.10 $^{-3}$ mol.L $^{-1}$; finale concentration 1.10 $^{-4}$ mol.L $^{-1}$). This solution was stored at 4 °C in the dark pending the analysis by molecular fluorescence spectroscopy.

Results and discussion

Determination of optimal complexation parameters

An iterative process was used to understand the experimental parameters (pH, stoichiometry, kinetics) that influences the formation of fluorescent complexes between HA and MO-OPA. Based on our experience and taking into account that Oyelakin *et al.* indicate that the of OPA/PU complexes fluorescence emission is optimal only after 24 h, [47] two conditions were evaluated through this study: the first one at t=15 min at 20 °C and the second one after 24 h at 4 °C in the dark.

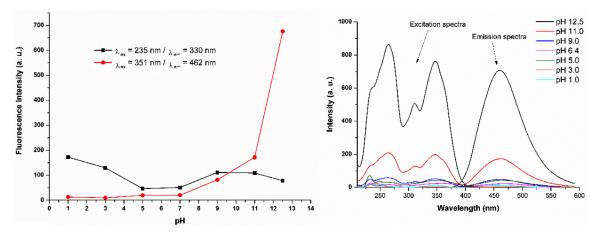


Fig. 2. Maximum emission intensity of the MO-OPA/HA complexes at 330 nm ($\lambda_{\rm ex}$ = 235 nm) and at 462 nm ($\lambda_{\rm ex}$ = 351 nm) as a function of pH (left). Excitation ($\lambda_{\rm em}$ = 462 nm) and emission ($\lambda_{\rm ex}$ = 351 nm) spectra of MO-OPA/HA complexes as a function of pH (right). Conditions: [MO-OPA] = 4.10⁻⁵ mol.L⁻¹; [HA]= 8.10⁻⁶ mol.L⁻¹; T= 20°C; t= 15 min

pH influence

In order to achieve the maximum fluorescence intensity for the MO-OPA/HA complexes, it was essential to determine the optimal excitation wavelength as a function of pH. For OPA/HA complexes, the literature indicates that excitation and emission wavelengths differ according to pH and that a basic pH (greater than 11.5) allows a higher fluorescent emission intensity compared to an acidic medium. [41,48] In this study, the same findings were obtained for OPA complexes (see supplementary data Fig. S1). A UV-spectra of MO-OPA/HA complexes was recorded in water and present two maximal absorption peaks at 235 nm and 351 nm. Excitation of the complexes at 235 nm lead to a moderate fluorescence intensity at 330 nm while excitation at 351 nm lead to a strong fluorescence emission centered around 462 nm in basic medium.

The study of the influence of the pH on the fluorescence intensity was carried out by modulating the pH of a solution containing MO-OPA and HA and recording the maximal intensity for each excitation/emission pair previously determined. Results are presented in Fig. 2 and clearly indicate that MO-OPA operate as his parent molecule OPA by displaying a maximal fluorescence intensity at basic pH. Therefore, a pH of 12.5 was fixed for the remainder of this study.

Stoichiometry

To get a better insight on the possible stoichiometry of the complexes, the molecular fluorescence intensity of the MO-OPA/HA complexes were recorded at fixed HA concentration by adding incremental amount of MO-OPA. From those experiments, one can observe three linear domains (Fig. 3). The first one was characterized by a MO-OPA/HA ratio (R) less than or equal to 1 which was attributed to the predominant formation of a 1:1 complex (1 molecule of OPA with 1 molecule of HA). The second linear range with R comprised between 1 and 3 was attributed to the main formation of 2:1 complex while the last linear range with R above 3 was attributed to the formation of higher order complexes. It is important to mention here that, at the current state of knowledge, the exact structures of OPA/BAs complexes are still not known with certainty even if mechanisms were proposed in the 1980s for OPA/HA complexes [37,38].

Kinetics

In our experience, OPA/HA complexes have proven to be unstable and their analysis required a long stabilization period (about 25 h at 4 °C) to reach maximum intensity (see supplementary data Fig. S2) as well as a stable fluorescence emission wavelength (see supplementary data Fig. S3). It therefore seemed interesting to study the kinetics of formation of the MO-OPA/HA complexes and to compare it with the

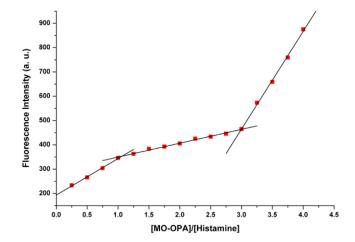
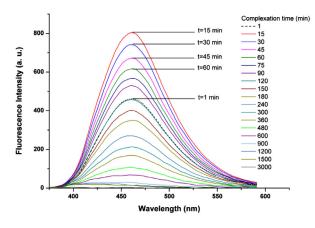


Fig. 3. Maximum fluorescence emission intensity ($\lambda_{ex} = 351$ nm; $\lambda_{em} = 462$ nm;) as a function of MO-OPA/HA ratio. Conditions: [HA] = 8.10^{-6} mol.L⁻¹; T= 20° C; t= 15 min; pH= 12.5.

standard derivatization reagent OPA. The maximum fluorescence intensity and associated wavelength were measured when MO-OPA and HA were brought into contact (t = 1 min) and then every 15 min for 50 h (Fig. 4, some points were omitted for clarity). For this purpose, and for the entire duration of the analysis, the samples were either temperature-controlled at 20 °C or stored at 4 °C between each spectrum recording. Remarkably, the maximum emission wavelength did not evolve over time at 4 °C as is the case for the OPA (see supplementary data Fig. 3) and remained stable around 462 nm. Delightfully, fluorescence intensity obtained during 50 h at 4 °C were stable and identical to those obtained after 15 min at 20 °C (Fig. 4) which is not the case for OPA (see supplementary data Fig. S2 and Fig. S3). Fortunately, this stability allows us to avoid the timekeeping of manipulations and in doing so, to analyze larger numbers of samples compared to the 6-10 samples recommended by the AOAC, which constitutes a real improvement over known methads.

Limits of detection (LOD), quantification (LOQ) and Linearity

To assess the applicability of MO-OPA for HA sensing, limits of detection and quantification as well as linearity range were evaluated. The LOD was assessed by evaluating the fluorescence response of a range of five solutions with concentrations from $1.00.\ 10^{-7}\ \text{mol.L}^{-1}$ to $1.00.\ 10^{-6}\ \text{mol.L}^{-1}$. It was found that at 9. 5. $10^{-7}\ \text{mol.L}^{-1}$, the spectrum



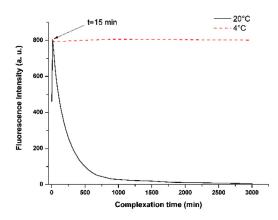


Fig. 4. Fluorescence emission spectra (λ_{ex} = 351nm) of MO-OPA/HA complexes as a function of time at 20°C (left). Evolution of the maximum emission intensity of MO-OPA/HA complexes as a function of time at 4°C and 20°C (right). Conditions: [MO-OPA] = [HA] = 8.10^{-6} mol.L⁻¹; pH= 12.5.

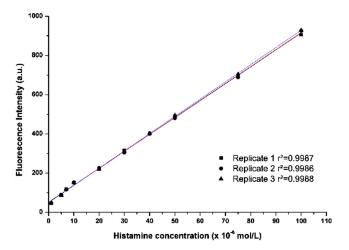


Fig. 5. Fluorescence emission intensity (λ_{ex} = 351 nm) of MO-OPA/HA complexes as a function of histamine concentration. Conditions: [MO-OPA] = 5 [HA]; T= 20°C; t= 15 min; pH= 12.5

presents some irregularities incompatible with quantification but was significantly above baseline. Agreeably, the molecular fluorescence intensity revealed to be linear between 1.10^{-6} and 1.10^{-4} mol.L⁻¹ (Fig. 5) with coefficients of determination greater than 0.99 (n=10). Pleasingly, this range is significantly improved by about one order of magnitude compared to that of the OPA which, in our hands, has been found to be between 5.10^{-6} and 5.10^{-5} mol.L⁻¹ (see supplementary data Fig. S4).

Evaluation of other BAs interferences

HA is not the only BA produced during fish alteration and therefore evaluation of interferences from other BAs must be conducted. To this end, 4 common BAs were selected to challenge the method. Cadaverine (CA) and putrescine (PU) were selected since they are listed as potentiators of HA effects. [8] Agmatine (AG) and HA were already known to interfere during analyses with OPA [49] and spermidine (SP) was examined to assess the impact of a secondary aliphatic amine. The robustness of HA quantification was evaluated in harsh condition i.e. with BAs amounts similar or even higher to the HA amount and without purification on column to avoid extra handling. [30]

Interferences of BAs on the molecular fluorescence intensity of MO-OPA/HA complexes was firstly evaluated individually (data not shown) then collectively with the following conditions: pH= 12.5, complexation time = 15 min at 20 °C, $\lambda_{\rm ex}$ = 351 nm and $\lambda_{\rm em}$ = 462 nm. Advantageously CA, PU and SP does not induce any significant change on the molecular fluorescence intensity, alone or collectively, even at concentration two

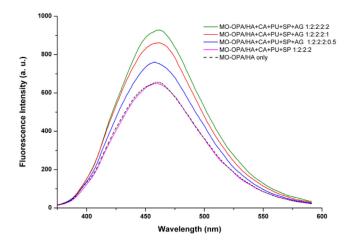


Fig. 6. Evaluation of interfering biogenic amines (CA, PU, SP and AG) on the fluorescence emission intensity of MO-OPA/HA complexes ($\lambda_{\rm ex}$ = 351nm). Conditions: [MO-OPA] = 10^{-4} mol.L⁻¹; [HA]= 8.10^{-6} mol.L⁻¹; T= 20° C; t= 15 min; pH= 12.5.

times greater than HA (Fig. 6). It was attributed to an absence of fluorescence emission of MO-OPA/BAs complexes at an excitation wavelength of 351 nm. However, as for analysis with OPA, the presence of AG induces a noteworthy fluorescence enhancement up to 33% for HA and AG in equimolar proportions since MO-OPA/AG complexes proved to be slightly fluorescent at this excitation wavelength. This enhancement rises to 39 % when OPA is used in place of MO-OPA.

Histamine analysis in fish sample

To estimate the potential of MO-OPA for the analysis of real samples, HA analysis was performed in duplicate on two doped canned samples of yellowfin tuna (*Thunnus albacares*) provided by the French Agency for Food, Environmental and occupational Health & Safety (ANSES) with known final concentration of HA at 15.79 ± 2.53 mg/kg and 148.64 ± 7.99 mg/kg respectively. After opening the sealed can, the tuna meat is extracted with methanol according to a modified protocol described by Bjornsdottir-Butter *et al.* [30] then solutions were analyzed by molecular fluorescence spectroscopy in two replicate according to previously optimized parameters with both, MO-OPA (Fig. 7) and OPA (see supplementary data Fig. S5) as derivatizing agent. For real samples, fluorescence intensities were plotted against known concentration of HA.

In both cases fluorometric analysis lead to an overestimation of HA amount but, interestingly enough, this overestimation is less marked

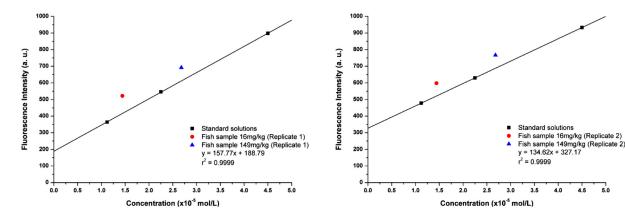


Fig. 7. Fluorescence analysis of real tuna samples in the presence of MO-OPA. Replicate 1 (left) and replicate 2 (right).

Table 1Comparative table of mass fractions obtained from the maximum molecular fluorescence intensities of the OPA/HA and MO-OPA/HA complexes.

| Derivatizing agent | Sample 1 ^a | | Sample 2 ^b | |
|--------------------|--------------------------|--------------------------|----------------------------|----------------------------|
| | Replicate 1 | Replicate 2 | Replicate 1 | Replicate 2 |
| MO-OPA OPA | 21.1 mg/kg 22.0 mg/kg | 20.1 mg/kg 21.4 mg/kg | 159.5 mg/kg 166.2 mg/kg | 163.4 mg/kg 170.4 mg/kg |

 $^{^{\}rm a}$ sample doped at 15.79 \pm 2.53 mg/kg.

when using MO-OPA as evidenced by results summarized in Table 1. MO-OPA proved to be more accurate than its parent OPA and therefore could be a great tool in the future to estimate the degree of freshness of fishery products.

Conclusion

To conclude, we have successfully studied the application potential of MO-OPA for the fluorometric analysis of HA in seafood. Performance of MO-OPA was compared to the reference compounds OPA and it was demonstrated that MO-OPA possesses several advantages. Indeed, it has an extended linear range of work of about one order of magnitude compared to the standard OPA. In addition to that, limits of detection and quantification of HA were found to be about 5 times lower than with OPA. The time required to reach the maximum molecular fluorescence intensity has proven to be very fast (15 min) and stable for more than two days for solution kept at 4°C. This outstanding rapidity and stability allow for the analysis of samples without timekeeping operations. Those points constitute a major improvement over the existing derivatization method. Those advantages coupled with the low interferences of other BAs on HA analysis by MO-OPA have permitted the analysis of HA with a slightly lower overestimation than with the use of OPA. MO-OPA therefore appears to be a first-choice candidate for rapid and easy molecular fluorescence evaluation of Ha in fishery products.

Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

Pole Metropolitain Cote d'Opale (PMCO) is gratefully acknowledged for financial support. French Agency for Food, Environmental and occupational Health & Safety (ANSES) (Dr. Guillaume Duflos and Mrs Gaëlle Hannequin) is acknowledged for discussion and the generous gift of spiked tuna. The authors also would like to warmly thanks Pr. David Landy for fruitful discussion about molecular fluorescence spectroscopy.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.talo.2020.100014.

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