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Determination of putrescine and cadaverine in seafood (finfish and shellfish) by liquid chromatography using pyrene excimer fluorescence

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Abstract

A liquid chromatography (LC) method is described for the easy determination of the biogenic diamines putrescine (PUT) and cadaverine (CAD) in canned tuna, frozen tuna loin, fresh mahimahi fillet, frozen raw shrimp, cooked lump crabmeat, and fresh and cold-smoked salmon. The method is also a useful screen for histamine (HTA). The method involves homogenization of fish tissue, extraction of biogenic amines into borate-trichloroacetic acid solution, centrifugation, and derivatization of supernatant with 1-pyrenebutanoic acid succinimidyl ester. The derivatized diamine species allow for the intramolecular excimer fluorescence of the pyrene moiety at a higher emission wavelength than is possible for the endogenous tissue monoamines, thus providing visual specificity of detection. All seafood species were fortified with 0.5, 1.0, 5.0, 10.0, and 15.0 μg/g (ppm) of PUT and CAD. Determination was based on standard graphs for PUT and CAD using peak areas with standard solutions equivalent to 0.375, 1.0, 5.0, 10.0, and 20.0 ppm in tissue. A set of five matrix controls (unfortified seafood tissue) were also analyzed; endogenous PUT was found in all samples except the canned tuna, and CAD found only in the shrimp, crab, and cold-smoked salmon. The background amines were thus subtracted prior to determining spike recovery. The intra-assay average recoveries ranged from 71 to 94% across species and spike levels.

Keywords: Putrescine; Cadaverine; Histamine; Tuna; Mahimahi; Shrimp; Salmon; Crab; Biogenic amines; 1-Pyrenebutanoic acid succinimidyl ester; Derivatization; LC; Pyrene

1. Introduction

Decomposition of seafood products not only renders product unpalatable and often hazardous to one's health, but it also contributes to economic waste and loss. In Scombroid species, such as tuna and mahimahi, histamine (HTA) is typically taken as the chemical marker of hazardous decomposition. However, histamine is not always present in every "decomposed" sample, and it may be that the alkyl diamines putrescine (PUT) and cadaverine (CAD) (Fig. 1) – which give a decomposed piece of seafood its distinct putrid smell – are better markers of decomposition, and may even potentiate the deleterious health effects of consuming HTA-spoiled seafood [1].

Currently, organoleptic (sensory) analysis can detect the presence of PUT and CAD, but not of HTA (unless the analyst is highly allergic and senses HTA through an allergic response, which is of course not a preferred method). Therefore, seafood samples are regularly analyzed by chemical means for the presence of HTA and hence "spoilage." Trace levels of histamine are common in tuna and mahimahi samples, and up to 50 ppm are permitted in marketable product [2]. At the time of this writing, chemical analyses for PUT or CAD are not routinely performed because the evidence is not completely clear on the role they play in food *poisoning*, despite their known role in food *decomposition*. Until a limit is set, PUT and CAD may be chemically monitored only for their correlation with or support for sensory findings.

Liquid chromatographic analysis of biogenic amines has traditionally focused around visualization of poorly stable

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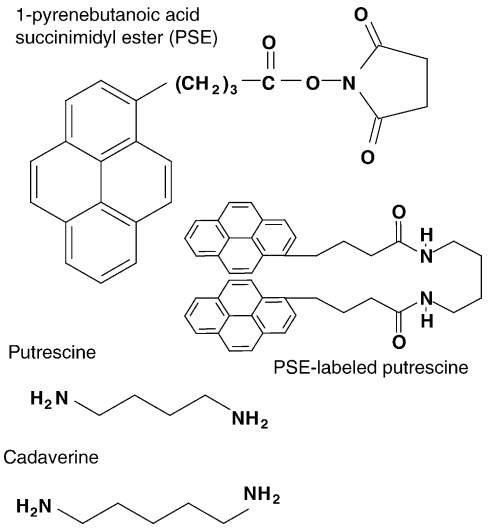

Fig. 1. Structures of biogenic diamines putrescine (PUT) and cadaverine (CAD) and the fluorescent derivatizing agent 1-pyrenebutanoic acid succinimidyl ester (PSE). Note how a PSE-labeled diamine allows for close arrangement of the PSE moiety hence affording an excimer effect.

o-phthaldialdehyde (OPT) derivatives following complex clean-ups [3–8] or other fluorescent derivatives [8–11]. Chemical methodology for determination of PUT and CAD in tuna and mahimahi relies on the AOAC Official Method for PUT and CAD using gas chromatography based on the work of Rodgers and Staruszkiewicz [12–14]. Although this method has been a mainstay for years, an alternative method is needed to provide simplicity for use in a high-throughput environment. We present here an LC method that includes a single simple extraction step prior to derivatization. Because of the sensitivity and specificity of our derivatization agent, clean-up and concentration of the extract is not needed, providing for a very rapid and simple analysis using common analytical equipment.

The key to our method is the use of 1-pyrenebutanoic acid succinimidyl ester (PSE) (Fig. 1) for derivatizing amines with a fluorescent moiety. Such a reagent has historically been used as a DNA probe [15]; but more recently Japan's Fukuoka University pharmaceutical sciences group has utilized this reagent for determining polyamines in clinical samples to monitor disease states [16–23]. They take advantage of the excimer fluorescence generated by the pyrene moiety attached to neighboring primary or secondary amine sites on the molecule of interest. Excimer fluorescence is a notable shifting of the emission to a significantly longer wavelength when two (or more) pyrene moieties lie in a favorably close position to promote intramolecular excitement. The endogenous biogenic monoamines do not exhibit an excimer effect and thus are "invisible" when viewed at the excimer's emission wavelength. To the best of our knowledge, at the time of this writing the method presented here is the first report of using PSE to visualize amines in a food matrix.

2. Experimental

Mention of brand or firm name does not constitute an endorsement by the US Food and Drug Administration over others of a similar nature not mentioned.

2.1. Reagents

(a) *Methanol* (*MeOH*) *and acetonitrile (ACN)*: High-purity, LC, or residue grade.
(b) *Sodium borate decahydrate* (*borax*), *trichloroacetic acid* (*TCA*), *sodium hydroxide pellets, ammonium acetate*—Reagent grade.
(c) *Deionized water* (*DI*): Suitable for LC usage.
(d) *Putrescine*: Putrescine dihydrochloride (1,4-diaminobutane) (Cat. No. 100450, ICN Biochemicals, Costa Mesa, CA).
(e) *Cadaverine*: 1,5-Diaminopentane (Cat. No. 101181, ICN Biochemicals, Costa Mesa, CA).
(f) *1-Pyrenebutanoic acid succinimidyl ester* (*PSE*): CAS # 114932-60-4 (Cat. No. P130, Molecular Probes, Eugene, OR; or Cat. No. 457078 [1-pyrenebutyric acid *N*-hydroxysuccinimide ester], Aldrich Chemical, Milwaukee, WI). Avoid breathing dust and handle standard and PSE-containing solutions using appropriate personal protective gear. Dispose of PSE-containing persistent waste in accordance with your lab's procedures.
(g) *Borate buffer* (*0.05 M, naturally pH 9.2*): For 1 L, weigh 19.2 grams of borax, take up to 1 L with DI water.
(h) *Extraction solution* (*0.05 borate, 7.5%, w/v TCA*): For 1 L, weigh 19.2 grams of borax and 74.8 grams of TCA, take up to 1 L with DI water. Handle standard and TCA-containing solutions using appropriate personal protective gear. Dispose of TCA-containing persistent waste in accordance with your lab's procedures.
(i) *PSE derivatization solution*: 5 mmol PSE in ACN. Weigh 19.25 mg PSE into a 10 mL volumetric flask (or multiples thereof) and fill to mark with ACN. (If using Molecular Probes product, assume the labeled container weight of 100 mg is accurate and quantitatively transfer solubilized contents to 50 mL volumetric container to make ca. 5 mmol solution.) Divide and place portions in suitably labeled amber vials for freezer storage until use. Bulk storage recommended at −20 °C or below (stable for at least a year at −80 °C), whereas a vial in current use may be stored when not in use at −5 °C for at least 2 weeks.
(j) *Sodium hydroxide solution* (*5N*): For 250 mL, weigh 50.2 grams of sodium hydroxide pellets, combine with DI water to make 250 mL of solution.
(k) *Mobile solvent A*: For 1 L combine 600 mL of ACN with 400 mL of MeOH.
(l) *Mobile solvent B*: Ammonium acetate buffer (25 mmol). For 1 L, weigh 1.92 grams of ammonium acetate, take up to 1 L with DI water.

(m) *Mobile phase*: Set the LC instrument to mix 80% of solvent A (organic) with 20% of solvent B (aqueous).

2.2. Equipment

(a) *LC system*: Agilent 1100: binary pump with autosampler, thermostatted column compartment, and fluorescence detector (Agilent Technologies, Wilmington, DE). Conditions: flow 1 mL/min, column oven 30 °C, excitation 345 nm, emission 475 nm, photomultiplier tube (PMT) gain 10, 20 μL injection.
(b) *Analytical column*: Hypersil, 250 mm length × 4.6 mm i.d. × 5 μm particle size (Cat. No. 006-0152-EO, Phenomenex, Torrance, CA).
(c) *Reacti-vials and heating block*: Clear glass to hold 0.3 mL (Cat. No. 3-3291) with Teflon-silicone cap liners (Cat. No. 27155) with suitable heating block (Supelco, Bellefonte, PA).
(d) *pH indicator strips*: ColorpHast pH 0-14 range (Cat. No. 9590, EM Reagents, Gibbstown, NJ).
(e) *Syringe filters*: Acrodisc polypropylene 13 mm × 0.2 μm filters (Cat. No. 4554, Pall/Gelman Sciences, Ann Arbor, MI).
(f) *General equipment*: Vortex mixer; food processor; polypropylene, conical, screw-cap graduated centrifuge tubes in 50 mL and 15 mL sizes; centrifuge (must be capable of holding specified 50 mL tubes and running at 3500 × *g*); micropipettors and appropriate tips; HPLC vials with micro-volume inserts; 1 mL polypropylene syringe barrels; Parafilm; aspiration device made with trap flasks and a stopper fitted with narrow tubing with disposable pipettor tip on end.

2.3. Preparation of standard solutions

(a) *Putrescine primary stock standard solution* (*1 mg/mL*): Made by weighing 90.0 mg of putrescine dihydrochloride and diluting to 50 mL with DI water.
(b) *Cadaverine primary stock standard solution* (*1 mg/Ml*): Made by weighing 50.0 mg of cadaverine and diluting to 50 mL with DI water.
(c) *Intermediate standard (IS) solution* (*10 μg/mL*): Made by pipetting 100 μL of each stock standard solution into a 10 mL volumetric flask and diluting to the mark with borate buffer.
(d) *Spiking standard solution* (*200 μg/mL*): Made by pipetting 2.0 mL of each stock solution into a 10 mL volumetric flask and diluting to the mark with DI water.

Store (a)–(d) under refrigeration and minimize excessive light exposure. The stock solutions are stable for 1 month or longer.

Five different linearity/calibration standards were made according to Table 1 using adjustable pipettors. Aliquots of IS and borate buffer were combined in small test tubes and covered with Parafilm until derivatization.

Table 1
Preparation of linearity/calibration standards

Parameter	Standards				
Solution concentration equivalent to ppm in tissue[a]	0.375	1.0	5.0	10.0	15.0
Solution concentration (μg/mL in flask)[a]	0.15	0.4	2.0	4.0	8.0
Volume of IS (μL)[b]	15	40	200	400	800
Volume of borate solution (μL)[b]	985	960	800	600	200

[a] Equality of solution concentration in the flask to that in a tissue sample can be described by the following logic: Spike 4 μg PUT/CAD into 4 g tuna = 1 ppm. Or extract 4 μg PUT/CAD into 10 mL of solution gives 0.4 μg/mL in that solution.
[b] Using appropriate micropipettors, combine the listed volumes in an LC injection vial to make a standard solution of the given concentration (presented as both ppm tissue equivalent and direct solution concentration).

2.4. Sample preparation

Approximately 0.5 kg each of canned chunk light tuna in water, fresh-frozen tuna loin (CO-treated), fresh mahimahi fillet (bloodline still red), raw frozen headless shell-on black tiger shrimp, canned cooked lump crabmeat, fresh Atlantic salmon fillet, and cold-smoked "Scottish" salmon were purchased from a local grocer. The tuna and mahimahi samples passed a sensory exam by a qualified organoleptic analyst resident in our laboratory (verbal data), whereas the other samples were not organoleptically tested prior to chemical analysis. The canned tuna was drained of excess water and the meat was homogenized in a food processor. The vacuum package of frozen tuna loin was thawed under cool running water, opened, the excess juices drained, and the meat homogenized in a food processor. Mahimahi fillet was skinned, chunked, and then homogenized in a food processor. Because the mahimahi was neither cooked nor partially frozen (in other words, not already partly denatured) when homogenized, it was not possible to grind the product as ultra finely as it was with the other products analyzed due to clumping of tissue. The shrimp were de-iced by briefly placing under cool running water and then patted dry. The shells were removed, and the partially frozen meat homogenized in a food processor. The canned crabmeat was drained of excess water and the meat was homogenized in a food processor. The fresh skinless salmon fillet and cold smoked salmon slices were simply chunked and then homogenized in a food processor. The cooked crab and canned tuna homogenized to the finest paste, followed by the salmons and fresh tuna. Because shrimp is rather sticky when raw, it took several pulses of the food processor (with scraping of the sides of the bowl) to reach an acceptable paste state. Processing not more than 0.5 kg of shrimp at a time, and keeping it partially frozen, allowed a homogenous mixture to be achieved without the aid of grinding with dry ice. (Although samples processed with dry ice are perfectly acceptable to use for this analysis.) Immediately following processing, a series of 50 mL polypropylene centrifuge tubes were filled with 4.0 ± 0.1 g aliquots of each

seafood homogenate, capped, and were stored at either −4° for use within a few days or at −80 °C for later use.

2.5. Sample extraction

To a thawed 4.0 g aliquot of fish homogenate in a 50 mL centrifuge tube was added 8 mL of borate–TCA Extraction Solution. The tube was capped and vortexed for ca. 30 s. The tube was centrifuged at room temperature for 5 min at 3500 × *g*. The supernatant was decanted into a fresh 15 mL graduated centrifuge tube. (Some floating debris may decant too—its presence does not affect the analysis.) To this were added 600 μL of 5N NaOH solution, and the pH of the representative control sample (not all samples) checked with a pH indicator strip. If the pH was below pH9, borate buffer would be used on the next step; otherwise the borate-TCA extraction solution would be used. Additional borate or borate-TCA solution, as appropriate, was added to take the volume to the 10 mL mark (rarely is the plain borate solution required). The final pH (of the representative sample) was checked with a pH indicator strip to read within the target range for a robust reaction of between pH 8 and 11. The tube was centrifuged for 5 min at 3500 × *g*. For fatty cold-smoked salmon replicates, the extract was briefly shaken with 1.0 mL of added heptane prior to centrifugation, and then after centrifugation the hexane layer was aspirated off the top, including any solid floating bits.

2.6. Sample derivatization

A 50 μL aliquot of the sample extract (a pipettor was used to reach below the surface and past any floating particulates) was placed in a Reacti-vial, and 200 μL of 5 mmol PSE solution were added. The vial was capped and heated for 15 min at 55 °C in a heating block. The vial was then removed and cooled, and the mixture was filtered through a 0.2 μm polypropylene syringe filter attached to a 1 mL syringe barrel into an amber HPLC vial fitted with a micro-volume insert, ready for analysis and injection of 20 μL.

2.7. Method design and validation

Individual 4.0 g samples were fortified to contain 0.5, 1.0, 5.0, 10.0, and 15.0 μg/g each of PUT and CAD per g of tuna by using 10, 20, 100, 200, and 300 μL of 200 μg/mL spiking solution, respectively. Using a single batch of canned tuna homogenate, five replicates of each of five fortification levels were analyzed. With this same batch, five control (or blank) unspiked replicates were also analyzed to provide a population of 30 samples ($n = 30$). An additional 30 samples of tuna loin homogenate were also analyzed, as well as 30 samples each of mahimahi, shrimp, crab, fresh salmon, and cold-smoked salmon homogenates.

For each day of analysis, the linearity (or calibration) standards were injected, and the 10 ppm equivalent standard was also injected five times in succession as a measure of system suitability. In lieu of a wash sequence to eliminate late-eluting peaks between matrix samples, the chromatographic run was extended past the retention time of CAD with the organic phase ramped up to 90% and then brought back down to 80% to equilibrate for the next run (all within 30 min.).

Calibration graphs were constructed based on PUT and CAD peak areas of the linearity standards of 0.15, 0.4, 2.0, 4.0, and 8.0 μg/mL in respective actual solution (equivalent to 0.375, 1.0, 5.0, 10.0, and 20.0 ppm in tissue). PUT and CAD in the samples were quantitated against these graphs.

3. Results and discussion

3.1. Extraction

The aqueous extraction solution uses TCA to deproteinate the tissue and borate to help buffer against the TCA when in a later step NaOH is added to accurately achieve a target pH range. This aqueous TCA extractant solution was chosen over using organic ACN because it extracted less extraneous material, and also conveniently provided both the aqueous and alkaline components needed for the derivatization reaction. Borate was chosen as the buffer species for the extraction solution and the diluting solution used in the preparation of the standards because its natural pH in solution is ca. 9.2; hence no pH adjustment is needed on the part of the analyst, and the pH is already in the correct range for optimal derivatization. We also found that the borate ion, versus the commonly used phosphate ion, minimized the occurrence of PSE by-products.

During extraction of tuna, the supernatant of canned tuna may dislodge particulates from the tube wall that will be collected during the decantation step and float around in the liquid. The floating particulates will not hurt the subsequent chemistry, and a pipettor tip can easily be inserted around this floating layer to gather the needed aliquot. The supernatant of a raw fish will likely be slightly more acidic than that of canned tuna. So when NaOH and additional extraction solution are added, the final pH of the two samples may be different (but both within the specified target range). The cold-smoked salmon supernatant gave the most troublesome floating layer of any species tried, consisting of mainly oil, which was then difficult to cleanly pass a pipettor tip around. Hence, we found that for cold-smoked salmon a heptane wash can aid in sample-handling. All shellfish and salmon benefited from a second centrifugation after the alkalizing step to push down fine particulates and clarify the liquid from which the derivative aliquot is subsequently taken. We did not do this in our original tuna and mahimahi runs, but the other species were more awkward and ease of subsequent analyst handling benefited from a more stringent clean-up at the noted step.

We noticed that PUT and CAD are very forgiving of any changes and manipulations, and tend to give high recoveries and strong responses under a range of parameter choices. We originally started development of this method with the goal

of including HTA for quantitative determination, and perhaps also spermidine (SPD) and spermine (SPM). SPD and SPM tended to have low recoveries, and the former also tended to show as two peaks (quite far apart) with PSE derivatization (possibly the di-pyrene substituted and the tri-pyrene substituted species). HTA, due to its imidazole ring, is very sensitive to reaction conditions and the presence of matrix components during the derivatization reaction, resulting in less than full response under the given conditions. What was best for PUT and CAD was not necessarily best for HTA, and vice versa. We decided to optimize and make immediately available the method for PUT and CAD, and continue working on optimizing chemistries for a multi-amine pyrene-derivative LC method including quantization for HTA (although this method can be used as is for screening at or above the action level).

3.2. Derivatization

The thoroughness of the PSE derivatization is influenced by several factors, and our final parameters reflect balancing these factors. First, there must be enough PSE present to react with all the amines that are present in a fish tissue sample. This is simple stoichiometry. However, we don't ever know how many molecules of amines are present, so we have to provide a reasonable excess of PSE. Second, the PSE concentration per volume of reaction solution needs to be adequate to induce a critical number of molecular collisions and hence cause the reaction to proceed. There may be enough PSE present for a molar reaction, but if it is so diluted in reaction solvent, the reaction rate is decreased. Third, the reaction environment needs to be at an optimal pH. Initial studies using phosphate buffer from pH 5 to 12 indicated that the reaction proceeds at a pH above ca. 7–8 (data not shown). Further evaluation with 0.05 M borate buffer on 5 μg/mL standards (12.5 ppm equivalent in fish) revealed the reaction is robust from pH 8 up to 11, with RSDs across the four pH values of 1.8% and 2.05 for PUT and CAD, respectively. Fourth, heat and/or time are needed to drive the reaction to completeness. Because PSE is such a reactive molecule, not much extra time under heat is needed. We found 15 min (±5 min) to be a reasonable time for reaction at a medium heat of 55 °C. Although slightly higher area counts (∼3.5% increase) were obtained at 5 min reaction times, the reaction mixture contained undesirable, non-interfering, side products, which were significantly decreased by extending the reaction time above 10 min. From 10 to 40 min reaction times, the reaction is robust with RSDs across the seven time points of 1.0 and 2.5% for PUT and CAD, respectively. Fifth, there shouldn't be so much excess PSE leftover after reacting the amines that copious amounts of by-products are formed. When this happens, the reagent by-product peaks on the chromatogram increase. This can be seen in the chromatograms for the standards where no matrix components are present to use up excess PSE resulting in a couple of noticeable (but not interfering) peaks (Figs. 2 and 3).

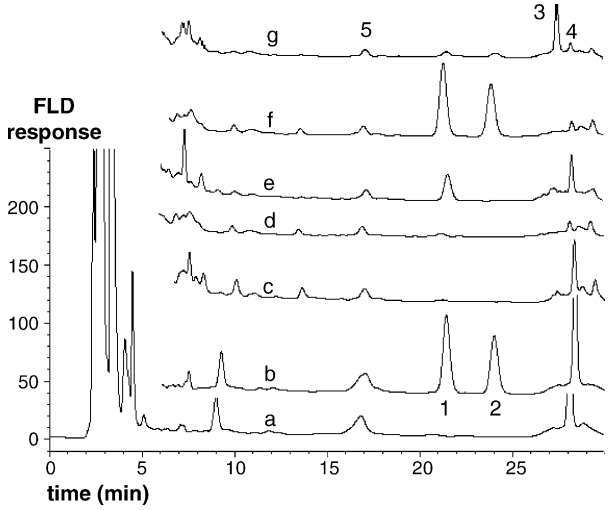

Fig. 2. Chromatograms of: (a) reagent blank (solvents and reagents taken through the method without matrix); (b) 5 ppm standard PUT and CAD; (c) canned tuna blank tissue; (d) tuna loin blank tissue; (e) mahimahi blank tissue; (f) 5 ppm fortified tuna loin; and (g) a different tuna loin fortified with 50 ppm HTA. Overlays are offset, but each at same scale of $y = 250$ fluorescence units and $x = 30$ min. Peaks: 1, PUT; 2, CAD; 3, HTA; 4 and 5, reagent peaks (larger when matrix not present). Sample size of 4 g fish tissue.

For this study we obtained PSE from two common sources, which vary considerably in price. A batch of PSE reagent is made up in ACN at 5 mmol working concentration, divided into smaller useful portions placed in amber screw-cap vials,

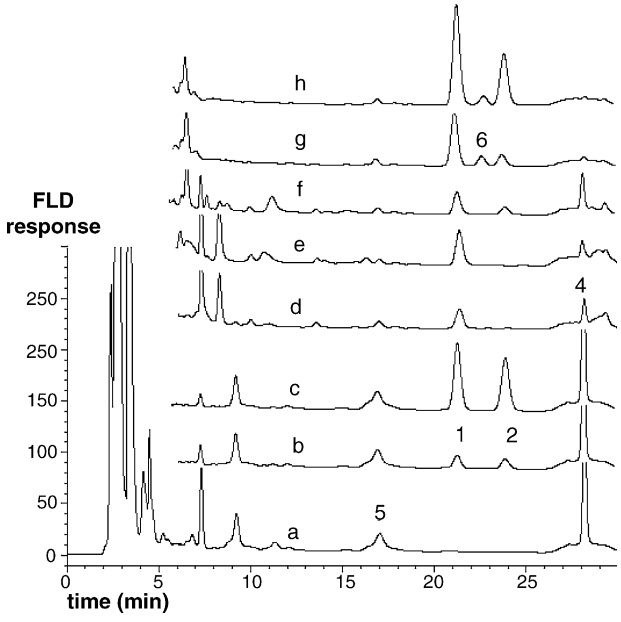

Fig. 3. Chromatograms of: (a) reagent blank; (b) 1 ppm standard PUT and CAD; (c) 5 ppm standard PUT and CAD; (d) fresh salmon blank tissue; (e) cold-smoked salmon blank tissue; (f) cooked crabmeat blank tissue; (g) shrimp blank tissue; and (h) shrimp fortified with 5 ppm each of PUT and CAD. Overlays are offset, but each at same scale of $y = 250$ fluorescence units and $x = 30$ min. Peaks: 1, PUT; 2, CAD; 4 and 5, reagent peaks (larger when matrix not present); 6, shrimp matrix. Sample size of 4 g shellfish/fish tissue.

and stored frozen until use. It is not recommended to make a more concentrated PSE stock solution, because at higher millimolar concentrations (e.g. 50 mmol), PSE is only partially soluble in ACN and requires the addition of DMSO to the solvent (which incidentally is amenable with the reaction).

Besides its ease of use, PSE is a desirable fluorescence agent because of its extremely long fluorescence lifetime (reported > 100 ns) [30] and ability to form excited-state dimers (excimers) to shift and strengthen the fluorescence signal. The normal emission fluorescence (seen with monomers) is typically 360–420 nm, while the excimer fluorescence (seem with dimers) is in the range of 450–520 nm [16]. This difference of almost 100 nm allows for visual selection of derivatized species. For our purposes in terms of monitoring food safety, the amines that we are interested in are diamines and polyamines, which when derivatized allow the intramolecular excitation to occur. The endogenous biogenic monoamines do not exhibit an excimer effect and thus are "invisible" when viewed at the excimer's emission wavelength. Furthermore, the excimer energy is more intense, and allows for greater sensitivity over normal fluorescence. The excimer effect is also possible when any two pyrene moieties share intramolecular excitement, be it only a pair, two out of three, or two pairs of pyrene attached to a target molecule, or simply in excess in solution (therefore reagent peaks – typically early-eluting – are significant in any chromatogram using a similar method).

3.3. Chromatography

When using excimer fluorescence where only the biogenic polyamines are visible, it is possible to baseline separate the decomposition amines PUT, CAD, HTA, SPD, and SPM. However, this entails a chromatographic run of 15–35 min (depending on column) to elute the peaks of interest and separate the analytes from reagent peaks. Matrix does not add significantly to the extraneous peaks under the reported conditions—most stray peaks are due to the reagents. The chromatography of the shellfish and pigmented finfish (Fig. 3) performs just as well as the chromatography for tuna and mahimahi (Fig. 2). However, the only anomaly is a small matrix peak in shrimp samples, in between the time of PUT and CAD, which under our conditions separates well. When present, all species tested here contained endogenous PUT (except canned tuna) within the range of the standard curve (tuna loin just below), but only the two shellfish species and cold-smoked salmon contained detectable CAD.

To eliminate unseen late-eluting peaks between matrix samples, it is advisable to extend the chromatographic run past the retention time of CAD, ramp up the organic phase to 90% (between time = 22–23 min, hold until $t = 25$) and then bring it back down to 80% (by $t = 26$) to equilibrate (until $t = 30$) for the next run.

We started with a 150 mm × 4.6 mm id × 5 μm Zorbax C8 column which separated all 5 above biogenic amines when using 25% 25 mmol NH_4OAc and 75% organic (55 ACN:45 MeOH). We noticed that borate buffer in the reaction mix gave an improved chromatogram over phosphate buffer by minimizing by-products. We switched to a 250 mm × 4.6 mm × 5 μm Hypersil C18 column with 80% organic (now 60 ACN:40 MeOH) after we decided to concentrate on PUT and CAD. The longer length was meant to better separate PUT and CAD from a reagent peak that always fell right in-between and partially overlapped the two analytes. The C18 column not only spaced out PUT and CAD as theorized, but its different selectivity dramatically separated the problematic reagent peaks far away from PUT and CAD, leaving a long span of uninterrupted baseline ideal for delineating the peaks of interest. SPD and SPM are no longer present on the current chromatogram. HTA now comes out near the end of the chromatogram during the "wash" gradient, just ca. 0.5 min before a reagent peak. As mentioned above, HTA gives a less than full chromatographic response (for a combination of reasons), estimated at one-forth of expected peak size. However, this peak is still big enough to easily see at or above the regulatory limit of 50 ppm (Fig. 2) and can be used to corroborate high PUT and CAD readings with the presence of illness-inducing quantities of HTA. We are continuing research to optimize the separation and detection of all five amine species.

ACN in the mobile phase gave the best shape with the shortest retention, but did not separate the interfering reagent peaks. Methanol alone separated the analytes, but gave too long a retention time for such a high percentage of organic phase, so we mixed the two solvents for optimal chromatography. The aqueous portion of the mobile phase needed to be non-acidic or at least very slightly alkaline. The commonly used phosphate buffer was prone to salting, and the organic buffers TRIS, glycine, and imidazole, having their own available amines, would interfere with the stability of the derivatives (only TRIS was tested). Ammonium acetate, with a native pH of ca. 7, was found to work. The nature of the ammonium ion apparently does not interfere with the PSE derivative.

Fluorescence detection of the pyrene-substituted amines reaches a maximum at ca. 350 units high (which is thousands of area counts) on our instrument, which when choosing all our parameters, is a bit above 20 ppm for PUT. CAD has a slightly lower fluorescence response, so its upper limit a slightly more. The longer alkyl chain of CAD probably doesn't permit the pyrene substituents to twist as closely together as with PUT, hence giving less excimer energy. HTA has an even lower excimer fluorescence intensity, so its determinable range would be the mid ppm range, which corresponds to the actual range of interest. For the amines, when the upper visible limit is exceeded, the peak apex for an analyte is seen to somewhat flatten (while getting wider at the base with increasing additions), but not be truly, squarely truncated. If an unknown sample ever gives peaks that reach up near 350–400 units high with a less than perfect apex (at least with our system), it would be recommended to derivatize a portion of fish extract appropriately diluted with buffer

(or alternately dilute a portion of the final extract from the HPLC vial with ACN), and back calculate to fit the standard graph.

3.4. Quantitation

The correlation coefficient (r) values of all the calibration graphs were greater than 0.9997 (most days at 1.0000) and naturally passed almost through the origin. The system suitability relative standard deviations (RSDs) were all less than 2.1%. The reagent blanks (one run with each matrix set) showed no interfering peaks in the time range for PUT and CAD (Figs. 2 and 3). We preformed this study in two parts, first with the species tuna and mahimahi, which come under the most commercial scrutiny for decomposition, directly followed by the shellfish and salmon species.

Study of Tables 2 and 3 can reveal trends in data distortion of spiked samples due to background subtraction at the lower two levels, and between species depending on how much endogenous PUT and CAD are present.

Fresh and cooked fishes (salmon, tuna, and mahimahi) performed the best, followed by the cooked crab and cold-smoked salmon, and lastly the shrimp. CAD showed tighter numbers than PUT, but this is because the samples had lesser or no endogenous CAD present to affect the statistics. The higher the background PUT (or CAD), the farther off the numbers were for spike levels below or near the endogenous level. The last two points are directly a result of the common effects of disparate number size on statistics. The species that have the highest control sample RSDs may then see this affect the RSDs of the background-subtracted spiked sample peak areas. In "real world" market survey use there

Table 2
Results for putrescine and cadaverine in canned tuna, tuna loin, and mahimahi

Parameter	Spike level[a]					Intra-assay average
	0.5 ppm	1.0 ppm	5.0 ppm	10.0 ppm	15.0 ppm	
Canned tuna (Control tissue average: PUT 0 ppm; CAD 0 ppm)						
Putrescine						
RSD of uncorrected peak[b] (%)	4.7	5.9	4.7	3.0	2.2	4.1
Average found (ppm)	0.46	0.87	4.2	8.15	12.54	NA
Average recovery (%)	91.3	86.6	84.0	81.5	83.6	85.4
RSD of recovery (%)	5.6	6.5	4.8	3.0	2.2	4.4
Cadaverine						
RSD of uncorrected peak[b] (%)	3.8	2.6	4.6	2.9	2.4	3.3
Average found (ppm)	0.41	0.84	4.13	8.07	12.40	NA
Average recovery (%)	81.9	83.8	82.6	80.7	82.6	82.3
RSD of recovery (%)	3.9	2.6	4.6	2.9	2.4	3.3
Tuna loin (control tissue average: PUT 0.1 ppm, RSD 9.8%; CAD 0 ppm)						
Putrescine						
RSD of uncorrected peak[b] (%)	11.2	7.3	6.7	6.6	3.8	7.1
Average found (ppm)	0.33	0.74	4.45	8.52	13.03	NA
Average recovery (%)	65.4	74.5	88.9	85.2	86.9	80.18
RSD of recovery (%)	22.6	10.1	7.2	6.9	3.9	10.1
Cadaverine						
RSD of uncorrected peak[b] (%)	8.9	10.4	6.3	6.7	3.5	7.2
Average found (ppm)	0.45	0.87	4.46	8.46	12.90	NA
Average recovery (%)	89.2	86.8	89.3	84.6	86.0	87.18
RSD of recovery (%)	8.2	10.0	6.2	6.7	3.5	6.9
Fresh mahimahi (control tissue average: PUT 1.7 ppm, RSD 7.8%; CAD 0 ppm)						
Putrescine						
RSD of uncorrected peak[b] (%)	4.9	1.7	4.4	3.7	4.9	3.9
Average found (ppm)	0.26	0.97	3.70	8.41	11.81	NA
Average recovery, %	52.3[c]	96.6	74.0	84.1	78.7	83.35[d]
RSD of recovery, %	44.3[c]	5.0	6.7	4.5	5.8	5.5[d]
Cadaverine						
RSD of uncorrected peak[b] (%)	4.7	2.1	2.6	3.9	4.7	3.6
Average found (ppm)	0.57	0.97	3.74	8.40	12.10	NA
Average recovery (%)	113.4[c]	97.3	74.7	84.0	80.7	84.2[d]
RSD of recovery (%)	4.9	2.1	2.6	3.9	4.7	3.3[d]

[a] Five replicate extracts at each of the five levels were analyzed.
[b] %RSD of the uncorrected "raw" peak areas; shows precision and repeatability.
[c] Numbers skewed to negative or extreme due to relative size of background subtraction.
[d] Calculated for levels 1.0–15.0 ppm only.

Table 3
Results for putrescine and cadaverine in raw tiger shrimp, cooked crabmeat, fresh Atlantic salmon, and cold-smoked Scottish salmon

Parameter	Spike level[a]					Intra-assay average
	0.5 ppm	1.0 ppm	5.0 ppm	10.0 ppm	15.0 ppm	
Raw shrimp (control tissue average: PUT 3.8 ppm, RSD 14.2%; CAD 0.9 ppm, RSD 14.9%)						
Putrescine						
RSD of uncorrected peak[b] (%)	3.9	6.1	8.9	9.2	4.3	6.5
Average found (ppm)	–[c]	0.52[c]	3.36	8.94	11.91	NA
Average recovery (%)	–[c]	52[c]	67	89	79	78[d]
RSD of recovery (%)	–[c]	54.4[c]	22.1	12.7	5.7	13.5[d]
Cadaverine						
RSD of uncorrected peak[b] (%)	3.3	5.3	9.7	9.5	3.9	6.3
Average found (ppm)	0.24[c]	1.7[c]	5.10	9.31	12.95	NA
Average recovery (%)	49[c]	169[c]	102	93	86	94[d]
RSD of recovery (%)	18.7	5.5	9.9	9.6	3.9	7.8[d]
Cooked lump crabmeat (control tissue average: PUT 1.5 ppm, RSD 4.0%; CAD 0.6 ppm, RSD 5.0%)						
Putrescine						
RSD of uncorrected peak[b] (%)	3.9	3.7	5.5	5.5	4.0	4.5
Average found (ppm)	0.47	0.91	3.47	8.41	12.68	NA
Average recovery (%)	94	91	69	84	85	79[d]
RSD of recovery (%)	17.9	9.7	7.9	6.5	4.5	6.3[d]
Cadaverine						
RSD of uncorrected peak[b] (%)	5.2	3.5	6.8	5.8	3.8	5.0
Average found (ppm)	0.41	1.45[c]	3.99	3.02	13.26	NA
Average recovery (%)	83	145[c]	80	90	88	86[d]
RSD of recovery (%)	14.0	3.5	6.8	5.8	3.8	5.5[d]
Fresh salmon (control tissue average: PUT 1.5 ppm, RSD 2.4%; CAD 0 ppm)						
Putrescine						
RSD of uncorrected peak[b] (%)	1.1	3.1	3.5	3.4	5.3	3.3
Average found (ppm)	0.32	0.78	4.07	8.68	12.89	NA
Average recovery (%)	64	78	81	87	86	79
RSD of recovery (%)	6.8	9.8	5.1	4.0	6.0	6.3
Cadaverine						
RSD of uncorrected peak[b] (%)	4.8	3.6	3.7	3.6	5.4	4.2
Average found (ppm)	0.35	0.77	4.13	8.70	13.02	NA
Average recovery (%)	69	77	83	87	87	81
RSD of recovery (%)	6.3	4.2	3.8	3.6	5.4	4.7
Cold smoked salmon (control tissue average: PUT 2.6 ppm, RSD 8.5%; CAD 0 ppm)						
Putrescine						
RSD of uncorrected peak[b] (%)	4.2	4.6	5.6	5.4	6.6	5.3
Average found (ppm)	0.50	0.59	4.10	7.38	11.84	NA
Average recovery (%)	100	59	82	74	79	79
RSD of recovery (%)	27.0	27.4	9.6	7.2	8.0	15.8
Cadaverine						
RSD of uncorrected peak[b] (%)	5.2	3.7	5.5	4.7	6.6	5.1
Average found (ppm)	0.30	0.68	3.97	7.06	11.43	NA
Average recovery (%)	61	68	79	71	76	71
RSD of recovery (%)	5.3	3.7	5.5	4.7	6.6	5.2

[a] Five replicate extracts at each of the five levels were analyzed.
[b] %RSD of the uncorrected "raw" peak areas; shows precision and repeatability.
[c] Numbers skewed to negative or extreme due to relative size of background subtraction.
[d] Calculated for levels 5.0–15.0 ppm only.

would be no background subtraction performed on unknown samples (hence, one would compare uncorrected peak area counts, which show good RSDs); and only the spiked samples used for quality assurance purposes would use background subtraction—and then, likely only against a spike of a more significant level than 0.5 or 1.0 ppm, where endogenous PUT or CAD would have minimal effect.

To illustrate, at the 0.5 ppm fortification level in all species, the intra-assay RSD ranged from 1.1 to 11.2% for PUT and 3.3–8.9% for CAD for uncorrected peak area counts

(with similar numbers for the 1.0 ppm level), indicating precision of analyst extraction using this method. However, the endogenous background PUT (1.5–3.8 ppm) was well above the amount of fortified PUT in all species except tuna, and once that large peak area was subtracted from each fortified sample, the standard deviation became significant enough to cause the RSD of the average absolute recovery to increase dramatically. The smaller amounts of endogenous CAD caused a similar, yet lesser effect, as would be expected. Each of the sets of replicates (per each of five spike levels per each species) was run concurrently with one unspiked control extract whose endogenous PUT and CAD values were used for background subtraction. The RSDs of endogenous PUT in the five total control samples per species were all good to acceptable: 2.4% for fresh salmon, 4.0% for cooked crabmeat, 7.8% for mahimahi, 8.5% for cold-smoked salmon, 9.8% for tuna loin, and 14.2% for raw shrimp.

In tuna loin, the peak area of the natural PUT was smaller than the peak area of the lowest standard, so the natural concentration could only be estimated if needed. (The samples of the shellfish and salmon species all had significant endogenous PUT or CAD, thus the lower limit of the method was not fully experienced with all species tested, but is believed to be comparable.) Because the baseline was relatively flat at this time range, the LC's integrator was able to integrate this little peak and assign it an area and an estimated concentration of ca. 0.15 ppm (given the excellent linearity of the calibration graph, this estimate is likely accurate). This implies that the limit of detection (LOD) for PUT and CAD could be as low as ca. 0.10 ppm. However, we recommend for typical testing lab use that the useful LOD be 0.25 ppm; that the LOQ to be equivalent to no less than the lowest calibration standard (reporting anything less as a trace); and that the lowest standard be at least 0.5 ppm. Quantitation of values less than that have little meaning versus sensory determination of decomposition.

The lowest level of interest for PUT and CAD is down in the ones to tens ppm range. Sensory analysis is believed to possibly detect them just under 1 ppm, whereas spoiled fish can typically reach as high as several ppm to around 20 ppm [24–28]. Rare cases of 30–80 ppm have been known [29]. As performed here, this method only measured as low as 0.375 ppm even though a strong fluorescent moiety is used. The strength of PSE allowed us to make the extraction very simple and minimize labor and bench time with one extraction and no concentration steps, yet still being able to see PUT and CAD down to minimum levels of interest.

4. Conclusion

The present method provides a very user-friendly, fast, and simple means to quantitatively determine low ppm levels of the primary decomposition diamines PUT and CAD that contribute to odors of spoilage in seafood, and to provide an easy way to correlate sensory findings with hard numbers—in a sense corroborating the expert nose. It can also screen for the presence of HTA near 50 ppm and above. With a single extraction, no concentration, and an easy derivatization, we believe that this method will prove to be very useful for quality monitoring. This method is shown compatible with multiple finfish and shellfish species.

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