Development of a pair of differential H/D isotope-coded derivatization reagents \(d_0/d_3-4-(1\text{-methyl-1H-phenanthro}[9,10-d]\text{imidazol-2-yl})\) phenlamine and its application for determination of aldehydes in selected aquatic products by liquid chromatography–tandem mass spectrometry

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**A B S T R A C T**

A new pair of derivatization reagents, \(d_0-4-(1\text{-methyl-1H-phenanthro}[9,10-d]\text{imidazol-2-yl})\)phenlamine \((d_0\text{-MPIA})\) and \(d_3-4-(1\text{-methyl-1H-phenanthro}[9,10-d]\text{imidazol-2-yl})\)phenlamine \((d_3\text{-MPIA})\) have been designed and synthesized. It was successfully used to label aliphatic aldehydes and the aldehyde derivatives were analyzed by high-performance liquid chromatography–tandem mass spectrometry \((\text{HPLC–MS/MS})\). The new isotope-coded reagents could easily label aldehydes under acidic conditions in the presence of NaCNBH₃. The target derivatives exhibited intense \([M+H]^+\) and regular product ions with electrospray ionization source in positive mode. The \(d_0/d_3\text{-MPIA}\)-aldehydes were monitored by the transitions of \([M+H]^+\) \(\rightarrow m/z\) 322 and \([M+H]^+\) \(\rightarrow m/z\) 165, and the obtained detection limits were in the range of 0.18–15.9 pg/mL at signal to noise ratio of 3. The global isotope internal standard technology was employed for quantification analysis with \(d_3\text{-MPIA}\)-aldehyde as internal standard for corresponding \(d_0\text{-MPIA}\)-aldehyde. Excellent linear responses for relative quantification were observed in the range of 1/10–10/1 with coefficients > 0.998. The developed method has been applied to the quantification of aliphatic aldehydes in selected aquatic products with RSD < 3.6% and recoveries > 85.2%.

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1. Introduction

Aliphatic aldehydes as secondary products from the lipid oxidation of the polyunsaturated fatty acid or the enzymatic process, widely exist in beverage, food, and fluids from biological origin [1,2]. These compounds at a trace amount may contribute to the fresh aroma of various foods, but higher concentration may facilitate food rancidity and release off-flavor. Furthermore, low-molecular aldehydes are typical irritants for eye, nose, and throat [3,4], and also have carcinogenic and toxic effects on biological tissues [5–8]. Recently much more attention is paid to aliphatic aldehydes as they have implication with the pathogenesis of various diseases such as heart attack, cancer, atherosclerosis and fatty streaks [9–11]. Additionally, they are considered as potential marker for oxidative stress and metabolic status [1], which have been proposed to estimate cancer status and aging [12], or metric of fatty acid oxidation [13]. Hence, it is critical to establish a method with high selectivity and sensitivity for the analysis of aldehydes.

Due to the lack of a chromophore as well as its high volatility and reactivity, it is difficult to analyze the aldehydes by high-performance liquid chromatography (HPLC) with optic detectors. Therefore chemical derivatization is often employed to increase detection sensitivity and improve selectivity. Until now, several ultraviolet or fluorescent derivatization reagents for aldehydes have been reported such as fluoren-9-yl-methoxycarbonylhydrazine (Flmoc-hydrazine) [14], 5-hydrazine-NN-dimethylaminophthalene-1-sulfonamide (Dns-hydrazine) [15], 4-(2-carbazoylpyrrolidin-1-yl)-7-(NN-dimethylamino- sulfonyl)-2,1,3-benzoxadiazole (DBD-ProCZ) [16], 2,4-dinitrophenylhydrazine (DNPH) [17–19]. However, adequate chromatographic separations are generally needed with ultraviolet or fluorescence detector, which hinders the establishment of rapid analysis methods. High-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) is a highly selective method in selected ion monitoring and in multiple reactions monitoring mode.

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Only the signal of interest is acquired, leaving out the information about the occurrence of all the other compounds. Rapid analysis can be achieved without the need of adequate chromatographic separation. With HPLC–MS/MS having these characteristics of high selectivity, sensitivity, and throughput, it is not surprising that this technology is being increasingly used in the pharmaceutical industry, clinical research, forensic analysis, environmental science, metabolomics studies and so on [11,20,21]. Unfortunately, the matrix effect (the phenomena of signal suppression or enhancement caused by co-eluted components) often seriously plagued in HPLC–MS/MS analysis. In fact, matrix effect is becoming a major threat in the successful application of HPLC–MS/MS, reducing the typical advantages of mass spectrometric detection in terms of selectivity and specificity [22,23]. Luckily, a method of using isotope internal standards based on HPLC–MS/MS can be employed to overcome this problem. Nevertheless, it is worthwhile to notice that isotope labeled standards are expensive and not always available, especially in the analysis of large numbers of analytes. Isotope-coded derivatization as an alternative approach to introduce mass tag to every analyte is being used, since raised by Aebersold and co-workers [24–26]. The heavy labeled and light labeled standards were mixed at defined ratio, and the mixture was analyzed by HPLC–MS/MS. On the basis of the mixed ratio and MS signal intensities ratio of ion pairs, the content of analytes in sample can be calculated.

Among the developed labeling reagents, DNPH is the most commonly used labeling reagent to detect aldehydes. Its corresponding isotope-code reagents [d3]-DNPH [27] and [15N4]-DNPH [28] have been developed for HPLC–MS/MS analysis of aldehydes. However, there are some inevitable shortcomings when DNPH is used as a derivatization reagent for aldehydes. Primarily, DNPH derivatization products exist as two isomers, which are subject to matrix effect in the complex matrix. For example formaldehyde and trimethyamine are possibly produced from the decomposition of trimethylamine oxide in meat [2]. Furthermore, hydrazine reagents are flammable, irritant to nerves, skin, and respiratory tract and unstable as they should be prepared just before analysis [30]. Therefore, it is a challenging work to exploit novel, convenient and practical isotope-coded derivatization reagents for aldehydes.

In this study, a pair of novel isotope-coded derivatization reagents, d0-4-(1-methyl-1H-phenanthro[9,10-d]imidazol-2-yl)phenlamine (d0-MPIA) and d4-4-(1-methyl-1H-phenanthro[9,10-d]-imidazol-2-yl)phenlamine (d4-MPIA) were developed. The preparation of d0-MPIA and d4-MPIA was simple and cost-efficient. The aldehydes were derivatized by d0/d4-MPIA through a reductive amination procedure, and the obtained secondary amine derivatives did not exhibit isomerization. The derivatization reaction was proceeding in a weak acid medium (pH 5.7) with few undesirable products. A simple, sensitive and selective isotope-coded strategy based on d0/d4-MPIA derivatization was developed for determination of aliphatic aldehydes. The d0-MPIA and d4-MPIA labeled derivatives were mixed at defined ratio and analyzed by HPLC–MS/MS, and the linearity and feasibility of relative quantification were fully validated. The aldehyde from aquatic products were sensitively determined and accurately quantified by HPLC–MS/MS with a global isotope internal standard technology.

2. Experimental

2.1. Chemicals and reagents

d0-MPIA and d4-MPIA were synthesized in our laboratory. Phenanthrenequinone, p-nitrobenzaldehyde, iodomethane (CH3I), d3-iodomethane (CD3I) and sodium cyanoborohydride (NaCNBH3) were purchased from J&K Chemical (Shanghai, China). Aldehyde standards were purchased from Aladdin (Shanghai, China) in the highest quality available and stocked in the dark at room temperature. HPLC grade acetonitrile (ACN) was purchased from Sigma-Aldrich (USA). Water was purified on a Milli-Q system (Millipore, Bedford, MA). Unless otherwise specified, all reagents used were analytical grade.

2.2. Synthesis of isotope-coded MPIA

MPIA was synthesized by three steps, namely annulations, methylation and reduction. The scheme of synthesis routes is shown in Fig. 1.

2.2.1. 2-(4-nitro-phenyl)-1H-phenanthro[9,10-d]imidazole

Glacial acetic acid (100 mL), p-nitrobenzaldehyde (1 g) and phenanthrenequinone (1 g) were full mixed in a flask (200 mL). The mixture was heated and refluxed with stirring at 90 C for 3 h. After cooling to room temperature, the solution was poured into the water (300 mL). The precipitated solid was recovered by filtration, washed with water and dried at room temperature for 48 h. Then the crude product was recrystallized three times with EtOH (95%).

2.2.2. 1-(d0/d4)-methyl-2-(4-nitro-phenyl)-1H-phenanthro[9,10-d]imidazole

2-(4-nitro-phenyl)-1H-phenanthro[9,10-d]imidazole (0.5 g), K2CO3 (3 g) and DMF (20 mL) were mixed in 100 mL round-bottom-flask. CH3I or CD3I (2 mL) dissolved in 10 mL DMF was added dropwise.

Fig. 1. The synthesis routes of d0-MPIA and d4-MPIA and derivatization scheme of MPIA with aldehydes (X=H or X=D).
into round-bottom flask within 0.5 h and the mixture of the flask was continuously stirred for 4 h at room temperature (20 °C). After cooling, the products were recovered by filtration, washed with water (200 mL), and dried at 60 °C for 5 h. A yellow crystal (about 0.4 g, purity, 95%) with sufficient purity for further synthetic manipulations was recovered.

2.2.3. d_{0}/d_{3}-4-(1-methyl-1H-phenanthro[9,10-d]imidazole-2-yl)-phenylamine

SnCl₂ - 2H₂O (10 g), EtOH (100%, 15 mL) and HCl (5 mL) were mixed in a 100-mL round-bottom flask. When the solution became clear and transparent, 1-(d₂)-methyl-2-(4-nitro-phenyl)-1H-phenanthro[9,10-d]imidazole or 1-(d₃)-methyl-2-(4-nitro-phenyl)-1H-phenanthro[9,10-d]imidazole (0.4 g) was added into the solution and the contents of the flask were heated with stirring for 3-4 h. After cooling, the pH of the solution was adjusted to 8 by adding aqueous NaOH and then the solution was extracted four times with ethyl acetate. The organic extract was gathered and evaporated to dryness. A light yellow crystal of d₀-MPIA or d₃-MPIA was obtained. The crude products were recrystallized twice with EtOH (95%) to give the light yellow crystal. Finally, semi-preparation chromatography (Waters 600E) was used to purify the target reagents. The purity of d₀-MPIA or d₃-MPIA evaluated by HPLC with fluorescence detection was > 99% for both compounds.

2.3. Preparation of standard solutions and samples

Stock solutions of d₀-MPIA or d₃-MPIA (2.5 × 10⁻³ mol/L) and aldehydes standard (1 × 10⁻³ mol/L) were prepared by HPLC-grade acetonitrile. All further working solutions with different concentrations were prepared by diluting corresponding stock solutions with acetonitrile. NaCNBH₃ (0.75 mg/mL) was prepared in 25 mL of acetonitrile. When not in use, all the solutions were stored in refrigerator at 4 °C.

The selected aquatic products (shredded squid, dried sea steak and prawn) were obtained from local market. Muscle tissues of samples were homogenized in a blender and preserved in the refrigerator until used. Sample preparation was according to Stafig et al. [31] with slight changes. Aliphatic aldehydes were extracted from aquatic product by adding 4 mL ethanol to 1 g sample in a centrifuge tub. After sonication for 10 min, the mixture was centrifuged at 4 °C (3500 rpm for 20 min). The supernatant was collected and used for derivatization directly.

2.4. Derivatization procedure

The derivatization reaction was proceeding in the presence of ammonium acetate buffer (pH 5.7) for 3 h at room temperature. The optimized condition was used: 140 μL d₀-MPIA (or d₃-MPIA), 30 μL NaCNBH₃, 50 μL ammonium acetate buffer and 20 μL defined concentration of aldehyde standards or real sample supernatant. The derivatization reaction is shown in Fig. 1.

2.5. HPLC–MS/MS analysis

The analysis was performed on the Agilent 1290 series HPLC system, equipped with an online degasser, a quaternary pump, an autosampler, and a thermostated column compartment. The injection volume was 0.5 μL at a constant flow rate 0.2 mL/min. An Agilent ZORBAX RRHD Eclipse C18 (2.1 × 50 mm, 1.8 μm) column was used at 30 °C. Eluent A was acetonitrile (100%) and eluent B was water–acetonitrile–formic acid (95% H₂O+5% acetonitrile+0.1% formic acid, v:v:v). The gradient conditions: initial = 30% A and 70% B, 4 min = 76% A and 24% B, 5 min = 100% A, then maintained 100% A for 5 min.

Mass spectrometry was conducted on an Agilent 6460 triple quadrupole mass spectrometer equipped with an electrospray (ESI) source system. The optimal mass spectrometer conditions were as follows: drying gas temperature 300 °C; drying gas flow rate 9 L/min; nebulizer gas pressure 40 psi; sheath gas temp 250 °C; sheath gas flow 8 L/min and capillary voltage 3.5 kV. The collision-induced dissociation energy (CV) and fragmentor (FV) were also optimized for the corresponding target compound. The data was acquired with MRM in the positive ion mode.

2.6. Evaluation of the isotope-coded strategy for relative quantification

According to Dai et al. [32], the feasibility of the stable isotope-coded strategy for relative quantification was evaluated in two ways: (1) the defined concentration of aldehyde standards (5 × 10⁻⁴ mol/L, 2.5 × 10⁻⁴ mol/L, and 1.25 × 10⁻⁴ mol/L) were individually labeled by d₀-MPIA. The d₃-MPIA was used to label aldehyde standards (2.5 × 10⁻⁴ mol/L). The d₂-MPIA aldehyde derivatives (light labeled) were mixed with d₃-MPIA aldehyde derivatives (heavily labeled) at an equal volume, respectively. The mixtures containing 1:2, 1:1, and 2:1 d₃-MPIA/d₀-MPIA aldehyde derivatives were analyzed by HPLC–MS/MS in triplicate to investigate the MS signal intensity ratios of d₃-MPIA derivatives and d₀-MPIA derivatives; (2) the aldehyde standards (1 × 10⁻³ mol/L) were labeled by d₀-MPIA and d₃-MPIA respectively. After the derivatization, the d₀–d₂-MPIA aldehyde derivatives were mixed at different volume ratios (10:1, 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10) and the mixtures were analyzed by HPLC–MS/MS.

2.7. Quantitative strategy for real sample

After derivatization, the d₀-MPIA-aldehyde derivatives at various concentration levels and d₃-MPIA-aldehyde standards derivatives were mixed at defined ratio, and the mixture was analyzed by HPLC–MS/MS in MRM mode. The relative quantification was achieved by plotting MS/MS signal intensity ratio of light/heavy ion pairs vs. molar ratio of d₀-d₂-MPIA-aldehyde. The mixture of d₀-MPIA labeled sample and d₀-MPIA labeled aldehyde standards was analyzed by HPLC–MS/MS. Thus, the content of aldehyde in real sample was calculated based on quantitative equations.

3. Results and discussion

3.1. Optimization of derivatization conditions

In order to obtain the highest derivatization efficiency for aldehydes, the derivatization conditions were investigated. The low reaction temperature was important because of the high reactivity and volatility of the aldehyde. Room temperature was used easily and could decrease its volatility. The single-factor experiment was employed for optimizing the derivatization parameters, including MPIA concentration, NaCNBH₃ amount and derivatization time.

3.1.1. Effect of MPIA concentration

The concentration of derivatizing reagent plays a vital role in the derivatization. Sufficient reagent was necessary to ensure the complete derivatization and obtain accurate quantitative analysis. In this study, the effects of MPIA concentration on the derivatization yields were investigated in detail. The results indicate that the signal intensity of derivatives increase with the amounts of derivatizing reagent. The constant intensity was observed when the 7-fold molar excess reagents were used, and further excess of reagent beyond this level has no significant effect on the
derivatization yields. Therefore, 7-fold molar excess MPIA to total molar aldehydes was adopted for the subsequent derivatization.

3.1.2. Effect of NaCNBH₃ amount
The amount of NaCNBH₃ (0.75 mg/mL) used in the derivatization was also investigated in the range of 10–50 μL. It was found that the largest peak areas appeared at NaCNBH₃ amount of 30 μL. However, when the NaCNBH₃ amount exceeded 40 μL, an obvious low response and some uncertain chromatogram peaks were observed. This should attribute to the fact that the excess amount of NaCNBH₃ consumed tagging reagent, thus the analytes react inadequately and some undesirable products were produced. Therefore, the optimum NaCNBH₃ amount was determined as 30 μL.

3.1.3. Effect of derivatization time
Derivatization time could also affect the derivatization efficiency significantly, and the effect of the derivatization time was tested from 90 to 300 min. Long time could accelerate the derivatization reaction. However, when the reaction time was longer than 180 min, the peak area had no obvious change. It indicated that the derivatization reaction has completed. Therefore 180 min was chosen as the reaction time.

3.2. HPLC separation and MS/MS monitoring
In our experiment, the aliphatic aldehydes were transformed to secondary amine derivatives by the derivatization with MPIA. The Agilent ZORBAX RRHD Eclipse column, a full end-capped C18 column, was employed to eliminate the peak trailing phenomenon. The mobiles containing formic acid (0.1%) were used to enhance intensity of mass spectrometry signals in positive mode. Ideally, the light and heavy coded analytes are expected to co-elute during chromatographic separation. However, some deuterium isotope derivatives may show some isotopic effect, which means the H/D isotopically coded analytes are partially or completely separated on reversed-phase liquid chromatography. Therefore, a fast gradient elution was used to decrease the difference in retention time between the deuterium labeled species and their hydrogen counterparts. Under the conditions described in Section 2, the obtained chromatograms of MPIA-aldehyde derivatives are shown in Fig. 2, and the retention times are listed in Table 1. As can be seen in Fig. 2 and Table 1, the

![Fig. 2. Extracted ion chromatogram of d₀-MPIA and d₅-MPIA labeled eight aldehyde standards derivatives.](image-url)
Table 1
Production ion and specific dissociation reaction condition used in MRM mode for aldehyde derivatives. The underline indicate the product ions for quantitative analysis.

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Retention time (min)</th>
<th>[M + H]^+</th>
<th>Production ion</th>
<th>Fragmentor (v)</th>
<th>CV (v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d0-MPIA-propanal</td>
<td>2.82</td>
<td>366.2</td>
<td>322.2, 165.2</td>
<td>175</td>
<td>40</td>
</tr>
<tr>
<td>d3-MPIA-propanal</td>
<td>2.81</td>
<td>369.2</td>
<td>322.2, 165.2</td>
<td>175</td>
<td>40</td>
</tr>
<tr>
<td>d0-MPIA-butanal</td>
<td>3.13</td>
<td>380.2</td>
<td>322.2, 165.2</td>
<td>175</td>
<td>40</td>
</tr>
<tr>
<td>d3-MPIA-butanal</td>
<td>3.12</td>
<td>383.2</td>
<td>322.2, 165.2</td>
<td>175</td>
<td>40</td>
</tr>
<tr>
<td>d0-MPIA-pentanal</td>
<td>3.45</td>
<td>394.2</td>
<td>322.2, 165.2</td>
<td>175</td>
<td>40</td>
</tr>
<tr>
<td>d3-MPIA-pentanal</td>
<td>3.44</td>
<td>397.2</td>
<td>322.2, 165.2</td>
<td>175</td>
<td>40</td>
</tr>
<tr>
<td>d0-MPIA-hexanal</td>
<td>3.79</td>
<td>408.2</td>
<td>322.2, 165.2</td>
<td>180</td>
<td>40</td>
</tr>
<tr>
<td>d3-MPIA-hexanal</td>
<td>3.77</td>
<td>411.2</td>
<td>322.2, 165.2</td>
<td>180</td>
<td>40</td>
</tr>
<tr>
<td>d0-MPIA-heptanal</td>
<td>4.12</td>
<td>422.2</td>
<td>322.2, 165.2</td>
<td>180</td>
<td>40</td>
</tr>
<tr>
<td>d3-MPIA-heptanal</td>
<td>4.11</td>
<td>425.2</td>
<td>322.2, 165.2</td>
<td>180</td>
<td>40</td>
</tr>
<tr>
<td>d0-MPIA-octanal</td>
<td>4.42</td>
<td>436.2</td>
<td>322.2, 165.2</td>
<td>180</td>
<td>47</td>
</tr>
<tr>
<td>d3-MPIA-octanal</td>
<td>4.41</td>
<td>439.2</td>
<td>322.2, 165.2</td>
<td>180</td>
<td>47</td>
</tr>
<tr>
<td>d0-MPIA-nonanal</td>
<td>4.72</td>
<td>450.2</td>
<td>322.2, 165.2</td>
<td>185</td>
<td>50</td>
</tr>
<tr>
<td>d3-MPIA-nonanal</td>
<td>4.71</td>
<td>453.2</td>
<td>322.2, 165.2</td>
<td>185</td>
<td>50</td>
</tr>
<tr>
<td>d0-MPIA-decanal</td>
<td>5.00</td>
<td>464.2</td>
<td>322.2, 165.2</td>
<td>185</td>
<td>50</td>
</tr>
<tr>
<td>d3-MPIA-decanal</td>
<td>4.98</td>
<td>467.2</td>
<td>322.2, 165.2</td>
<td>185</td>
<td>50</td>
</tr>
</tbody>
</table>

Fig. 3. MS/MS cleavage mode of MPIA derivatived butanal (A), typical product ions MS/MS spectra of d3-MPIA-butanal (B) and d0-MPIA-butanal (C).
The MPIA-aldehyde derivatives are completely eluted with satisfactory peak shape in 6 min, and the retention difference between d0/d3-MPIA-aldehydes are about 0.01–0.02 min. The MPIA-aldehyde derivatives gave abundant [M+H]^+ in MS and regular cleaving fragments in MS/MS, the representative MS/MS and cleavage mode for d0-MPIA-butanal and d3-MPIA-butanal are described in Fig. 3. As observed in Fig. 3, the production ion at m/z 322.2 is from the cleavage of NHCH2-CH3 bond along with loss of methyl group, while m/z 165.2 from the breakage of phenanthrene. In the following experiments, the ion at m/z 322.2 was used for quantitative analysis, and the ion at m/z 165.2 was employed to confirm the identification. The MRM parameters for quantitative transitions ([M+H]^+ → m/z 322.2) and qualitative transitions ([M+H]^+ → m/z 165.2) were systematically optimized, and the obtained FV and CV values are shown in Table 1. As can be seen in Table 1, the optimal FV and CV are about 180 V and 45 V, respectively.

3.3. Analytical characteristics

The method validation was accomplished by the d0-MPIA labeling with different concentration aldehydes standards under the optimized derivatization conditions. The linearity, detection and quantitation limits, precision and stability were studied and the results are summarized in Table 2.

The calibration curve was established for each aldehyde using linear regression by plotting peak area versus concentration. As can be seen in Table 2, the correlation coefficient (R^2) shows superior values higher than 0.99. Limits of detection (LODs) and limits of quantitation (LOQs) were determined at signal-to-noise ratio 3 and 10, respectively. The LODs range from 0.18–15.9 pg/mL, while LOQs range from 0.74 to 50.9 pg/mL. It indicates that the proposed method is sufficiently sensitive for the determination of trace amount aliphatic aldehyde in food samples.

The relative deviations (RSDs) of peak areas for intra-day and inter-day variations were used to evaluate the precision of the proposed method. The intra-day variation was tested by running in triplicate the same standards mixture for six replicates within 1 day, and the inter-day variation was determined by running aldehyde standards derivatives at the same level with three replicates on three different days. As can be seen in Table 2, the intra-day RSDs for the tested standard are in the range of 1.6–2.8%, while the inter-day RSDs are between 2.7% and 3.9%.

The stability of MPIA was also investigated. An acetonitrile solution of MPIA was hermetically stored at room temperature for 1 month without obvious change in derivatization yields for aldehyde compared with the newly prepared MPIA. In addition, the stability of aldehyde derivatives was also evaluated. The aldehyde derivatives were placed 0, 4, 8, 12, 24 and 48 h in refrigerator at 4 °C and analyzed by HPLC–MS/MS. No significant change in the peak areas and retention time was observed when compared with the previous analysis. As expected, the MPIA and aldehyde derivatives are stable enough for the following HPLC–MS/MS analysis.

3.4. Stable isotope-based quantification

The feasibility of the stable isotope-coded strategy for relative quantification was evaluated according to Dai et al [32]. The application of the methodology is shown in Fig. 4 to butanal. The results obtained for the remaining aldehydes is available in supplementary Figs. S1–S7. In Fig. 4A, the ion pairs of the d3-MPIA and d0-MPIA aldehyde derivatives are found in one HPLC–MS/MS run, and the difference of elution time between heavy and light forms is only 0.01 min. While d1-MPIA-butanal shows an m/z = 383.2, the d0-MPIA-butanal is determined to have an m/z = 380.2 (see Fig. 4B). The difference in m/z value is attributed to the replacement of three hydrogen atoms by three deuterium atoms. In Fig. 4C, the MS signal intensity ratio of ion pairs is approximately 0.5 (ranging from 0.49 to 0.50, average = 0.50), 1 (ranging from 1.02 to 1.05, average = 1.03) and 2 (ranging from 1.97 to 2.03, average = 2.00) for aldehydes when the H/D = 0.5, H/D = 1 and H/D = 2. In Fig. 4D, the MS signal intensity ratios of the ion pairs in the 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, and 1:10 mixed by d0-MPIA-aldehyde and d1-MPIA-aldehyde shows a superior linear regression. The superior linear regression indicates that accurate relative quantification data can be obtained using the developed stable isotope-coded strategy.

3.5. Real sample analysis

In order to apply the developed HPLC–MS/MS protocol, different aquatic product matrices including shredded squid, dried sea shrimp, prawn and fish steak, were selected to detect aliphatic aldehydes. The supernatant of aquatic product was labeled by d0-MPIA, while defined concentration aldehyde standards were labeled by d3-MPIA. After that, the light labeled sample and heavy labeled standards were mixed at volume ratio of 3:1. The mixture was diluted to 500 µL by acetonitrile and analyzed by HPLC–MS/MS in triplicate. The contents of different aldehydes in real sample are summarized in Table 3. A typical chromatogram of aldehyde from shredded squid is shown in Fig. 5, and chromatograms for other samples are shown in Supporting informations Figs. S8–S10. As can be seen in Table 3, propanal, hexanal and nonanal are the most abundant compounds in these matrices except prawn. The proper levels of aldehyde standards with nearly concentration in real matrices were spiked to estimate the recoveries, and satisfactory recoveries in the range of 85.2–96.7% were obtained from all samples.

3.6. Comparison with the reported DNPH

DNPH has been widely applied in the HPLC analysis of aldehyde compounds in various samples [31,33–39]. And the d0/d3-DNPH...
and $^{15}$N$_0$/15N$_4$-DNPH have also been developed for HPLC–MS analysis of aldehydes [27,28]. In order to evaluate MPIA further, it is compared with DNPH in terms of derivatization conditions, enhancement for sensitivity and availability of isotope-coded analog. The derivatization conditions and LODs of reported methods are listed in Table 4.

3.6.1. Comparison of the derivatization conditions

The most popular derivatization of aldehydes with DNPH is based on the reaction of forming hydrazones. Concretely, aldehydes and DNPH are incubated in acidic medium at suitable temperature for a period of time [28,31–39]. As indicated in Table 4, the derivatization time much depends on the reaction temperature. Erhard Schulte’s group and Robert Andreoli’s group reported that the derivatizing at ambient temperature need at least 1 h [34,35]. There are also some researchers performing this derivatization at room temperature for 12–24 h [36–38]. The derivatization can be accelerated by heating the reaction mixture. Stafiej et al. have reported that the derivatization was accomplished within 35 min at 75 °C [31]. Sanches-Silva et al. have also been trying to adopt 40 °C for 45 min [33].

The derivatization of aldehydes with MPIA is based on the reductive amination reaction. Specifically, aldehydes and MPIA are incubated in acidic medium in presence of NaCNBH$_3$. The derivatization time of reductive amination is also closely related to the adopted temperature, which has already been elaborated by Eggink et al. [40]. The derivatization with MPIA can be accomplished within 0.5 h under 60 °C. Nevertheless, the low reaction

![Fig. 4. Results of relative quantification based on H/D stable isotope MPIA labeling: extracted ion chromatogram of light and heavy labeled aldehyde (A); mass spectrum of the ion pair of light and heavy labeled aldehyde (B); MS intensity ratios of light/heavy labeled aldehyde with the amount of 1:2, 1:1, and 2:1 (C); and ion pairs of light/heavy labeled aldehyde in 10:1, 5:1, 2:1, 1:2, 1:5 and 1:10 mixed solution showed a good linear regression (D).]
temperature is important for the derivatization of aldehyde because of the volatility. So we adopted the room temperature with a long time. As also can be seen from Table 4, the room temperature is the most suitable for the derivatization of aldehydes.

Another point to note is the reductant used in derivatization procedure. Usually, the DNPH derivatizations are performed with no reductant, and the obtained hydrazone are separated by means of HPLC-UV or HPLC-MS. However, this DNPH derivatization method may cause an analytical problem as DNPH hydrazones have both E- and Z-stereoisomers due to the C=\(\text{N}\) double bond [29]. To our knowledge, only Uchiyama et al. have tried to transform the C=\(\text{N}\) double bond into a \(\text{C}\rightarrow\text{N}\) single bond by an additional reductive amination procedure. They employed 2-picoline borane to reduce the DNPH hydrazones, which proved to be effective to overcome analytical errors caused by E- and Z-geometrical isomers [36]. In this work, the MPIA derivatizations are performed under acidic conditions in presence of NaCNBH\(_3\), and the resulted derivatives are secondary amines. The formed \(\text{C}\rightarrow\text{N}\) single bond could rotate freely, which can effectively avoid isomerization phenomena.

### 3.6.2. Comparison of the enhancement of MS signal

It has been reviewed that DNPH can significantly improve the MS response signals [20]. As indicated in Table 4, the reported methods with DNPH give the low LODs at ng/mL or even ng/L level [28,33,35-39]. The method with MPIA resulted in much lower LODs than those using DNPH. Therefore, the MPIA also exhibits significant enhancement of MS signals for aldehydes derivatives, and MPIA can be used as a signal sensitizing derivatization reagent for determination of aldehydes at trace level. The high sensitivity of MPIA derivatives is probably attributed to two major factors. On one hand, the lone-pairs on imidazole nitrogen-atom had a strong proton affinity, which made it easy to give [M+H]\(^+\)

![Fig. 5. Chromatograms of aldehyde derivatives from shendai sample mixed with aldehyde standards.](image-url)
in positive mode. On the other hand, the hydrophobic character that derivatization confers to the analytes, allow MPIA derivatives to be eluted in a much higher percentage of organic mobile-phase during the reversed phase HPLC gradient runs. The ionization desolvation process became much more efficient at a higher percentage of organic solvent.

3.6.3. Comparison of the availability of isotopic labeled analogs

DNPH is a commercial reagent, but its iso-coded analogs are expensive and difficult to prepare. For $d_0/d_3$-DNPH, the isotope signature ($d_0/d_3$) is introduced through the initial material $d_0/d_3$-chlorobenzene, and two electrophilic substitutions with nitric acid and $^{15}$N0/$^{15}$N2-hydrazine as reactants [28]. On the contrary, the preparation of $d_0/d_3$-MPIA is simple and economical. The isotope signature ($d_0/d_3$) could be easily assembled by methylation with low-cost $d_0/d_3$-iodomethane, and the target molecules could be obtained after a following efficient reduction. The facile preparation made $d_0/d_3$-MPIA more economical and practical than $d_0/d_3$-DNPH or $^{15}$N0/$^{15}$N2-DNPH. In view of cost advantage of heavy isotope raw materials, the $d_0/d_3$-MPIA was expected to be a pair of cheap and practical iso-coded derivatization reagents.

In short, the reported reagents, $d_0/d_3$-MPIA exhibited three important advantages over those iso-coded DNPH: (1) easy-handled derivatization procedure; (2) higher sensitivity; and (3) facile and economical preparation.

4. Conclusions

This work describes a convenient isotopic internal standard strategy based on iso-coded MPIA for the quantitative analysis of aliphatic aldehydes with high selectivity and sensitivity. Aliphatic aldehydes were converted to phenanthroimidazol derivatives by derivatization with MPIA in the presence of NaCNBH3. The aldehyde derivatives were accurately quantified by HPLC–MS/MS with a global iso-coded internal standard technology. The developed method was sensitive with LODs at pictogram level, and the developed method was successfully applied to determine aliphatic aldehydes in aquatic products. Regarding sensitivity, selectivity and simplicity, the developed method has a high potential in the analysis of aliphatic aldehydes in other complex matrices, such as beverages, foods, body fluids and so on.

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Appendix A. Supplementary material

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References