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# Development of a sensitive fluorescent derivatization reagent 1,2-benzo-3,4-dihydrocarbazole-9-ethoxy-carbonylhydrazine and its application for determination of aldehydes from alcoholic beverage using high-performance liquid chromatography with fluorescence detection and enhance mass spectrometric identification

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**Abstract**

A pre-column derivatization method for the sensitive determination of aldehydes using the tagging reagent 1,2-benzo-3,4-dihydrocarbazole-9-ethoxy-carbonylhydazine (BCEC) followed by high-performance liquid chromatography with fluorescence detection and enhance mass spectrometric identification has been developed. The chromophore of fluoren-9-methoxy-carbonylhydrazine (Fmoc-hydrazine) reagent was replaced by 1,2-benzo-3,4-dihydrocarbazole functional group, which resulted in a sensitive fluorescence derivatizing agent BCEC. BCEC can easily and quickly label aldehydes. The maximum excitation (333 nm) and emission (390 nm) wavelengths were essential no change for all the aldehyde derivatives. The fluorescence intensity was substantially affected by the solvents, being higher in organic than protic solvents. Derivatives are sufficiently stable to be efficiently analyzed by high-performance liquid chromatography. The derivatives showed an intense protonated molecular ion corresponding $m/z$ $[M + (CH_2)_n - 1]^+$ ($M$: molecular mass of BCEC, $n$: corresponding aldehyde carbon atom numbers) under positive-ion mode. The collision-induced dissociation of protonated molecular ion formed products at $m/z = 245.7.0$, $m/z = 263.7$ and $m/z = 217.7$, and corresponding the cleavage of $-CH_2-OCO$, $-CH_2O-CO$ and $N-CH_2CH_2$ bonds, respectively. Studies on derivatization demonstrated excellent derivative yields in the presence of trichloroacetic acid (TCA) catalyst. Maximal yields close to 100% were observed with a 10- to 15-fold molar reagent excess. Separation of the derivatized aldehydes has been optimized on ZORBAX Eclipse XDB-$C_8$ column with aqueous acetonitrile in conjunction with a binary gradient elution. Excellent linear responses were observed at the concentration range of 0.08–16.65 μmol/L with coefficients of >0.9999. Estimated detection limits for the aldehydes, obtained by successive dilution of a derivatized standard solution containing 16.65 μmol/L of each aldehyde (at a signal-to-noise ratio = 3:1), are from 3.75 to 16.65 fmol.


*Keywords:* Column liquid chromatography; Aldehydes; 1,2-Benzo-3,4-dihydrocarbazole-9-ethoxy-carbonylhydrazine (BCEC); Mass spectrometry

## 1. Introduction

A reliable and economic monitoring of organic toxic substances is crucial in the field of environmental analysis and industrial chemistry. Aldehydes such as formaldehyde (FA), propionaldehyde (PAL), *n*-butyraldehyde (nBAL), *iso*-butyraldehyde (iBAL), and 2-ethylhexanal (EHAL) are acknowledged to be important organic compounds, which are present in many ways, such as industrial operations, environmental atmospheres, alcoholic beverages and aqueous [1]. The quantitative analysis of them is very important for environmental pollution control, industrial

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applications and consumers' health. The common method is gas chromatography, where carbonyl compounds in exhaust gases or aqueous samples are determined as their derivatives by UV–vis or fluorescence spectrometry. Several gas chromatographic (GC) methods have been described for the determination of aldehydes in water or air. Classically 2,4-dinitrophenylhydrazine (DNPH) has been used for derivatization despite the low volatility of these derivatives [2]. The most commonly used method in recent years has been reversed-phase HPLC where aldehyde compounds in exhaust gases and environmental samples are determined as their 2,4-dinitrophenylhydrazine derivatives by UV detection [3–5]. Hydrazones are separated by HPLC and monitored using a UV–vis-detector at a wavelength between 340 and 380 nm, depending on the absorption maximum of the relevant hydrazone [6]. Sampling of carbonyl compounds in air can be performed using solutions of DNPH in impingers [7] or solid sorbents coated with DNPH, including test tubes for pumped sampling and passive sampling devices [8]. The quantitative analysis without derivatization has been described for formaldehyde, propionaldehyde, and 2,3-bis (hydroxymethyl)butyraldehyde (BHBAL) among other compounds in synthesis mixtures by reversed-phase HPLC using a refractive index detector [9]. Fluorescence detection is the method of choice in numerous determinations of target materials at their real-life levels. The recent upsurge of interest in this area has resulted in many new fluorescence labelling reagents, as documented by the review literature [10]. A number of such reagents, e.g., 6,7-dimethoxy-1-methyl-2-oxo-1,2-dihydroquinoxalin-3-yl-propionohydrazide (DMEQ-hydrazine) [11], fluoren-9-yl-methoxycarbonylhydrazine (Fmoc-hydrazine) [12], anthracenecarboxylic acid hydrazides [13], 4-hydrazino-7-subtituted-benzoxadiazoles [14] and 5-hydrazino-*N*,*N*-dimethylnaphthalene-1-sulfonamide (Dns-hydrazine) [15–16] have been developed for the determination of carbonyl compounds such as aldehydes and ketones by high-performance liquid. Many of the reagents for the labeling of aldehydes and ketones possess a hydrazine group ($-NHNH_2$) as the reactive site. These reagents have been applied to the determination of carbonyl compounds at trace levels. In recent years, new reagents such as, 5-amino-4-sulfanylphthalhydrazide (ASPH) [17] and 2-aminooxy-*N*-[3-(5-dimethylamino)-naphthalene-1-sulfonylamino]propyl acetamide [18] were used in the preparation of absorbing or fluorescence derivatives. To overcome the oxidation of DNPH with nitrogen dioxide or ozone above-mentioned, *N*-methyl-2,4-dinitrophenylhydrazine (MDNPH) [19] and *N*-methyl-4-hydrazine-7-nitrobenzofurazan [20] were developed as derivatization reagents with reduced interferences, because the reaction of MDNPH with both nitrogen dioxide and ozone led to only one defined product, *N*-methyl-2,4-dinitroaniline (MDNA). More recently, a dual-signaling fluorescence reagent 3, 3′-5,5′-tetramethyl-*N*-(9-anthrylmethyl)-benzidine for optical sensing of aliphatic aldehydes and carbonyl compounds has been developed [21]. However, reliable derivatization procedures based on novel labelling reagents that offer high sensitivity, high selectivity and good fluorescence properties with good stabilities of the reagents and the derivatives are still needed.

Fmoc-hydrazine prepared by FMOC with hydrazine is developed to act as carbonyl reaction functional group for labelling of aldehydes. The reagent is not optimized regarding chromophoric properties for quantitative spectrophotometric determination. The combination of a very sensitive function group such as hydrazine and a strong absorption moiety in the molecule will result in an attractive reagent. In our previous studies [22–24], we described the synthesis of some fluorescence tagging agents and the application for common amino compound analysis. On the basis of the fluorescence characteristics of carbazole moiety as our previously described [25], we synthesized a novel fluorescence reagent 1,2-benzo-3,4-dihydrocarbazole-9-ethoxy-carbonylhydrazine (BCEC) that the chromophore of fluoren functional group was replaced by 1,2-benzo-3,4-dihydrocarbazole resulting in a strong fluorescence. BCEC has been found to be easily accessible and very stable in its crystal state. In this study, the optimal derivatization conditions such as catalyst, reaction time and solvent system are investigated. Linearity, detection limits, and precision of the whole procedure were also determined. To the best of our knowledge, this is the first time that BCEC fluorescent probe with its application for the determination of aldehydes has been reported.

## 2. Experimental

### 2.1. Instrumentation

Experiments were performed using a LC/MSD-Trap-SL electrospray ion trap liquid chromatography/mass spectrometry (1100 Series LC/MSD Trap, a complete LC/MS/MS). All the HPLC system devices were from the HP 1100 series and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), a fluorescence detector (FLD) (model G1321A), and a diode array detector (DAD) (model G1315A). Ion source type, atmospheric pressure chemical ionization (APCI); dry gas temperature, 350 °C; nebulizer pressure, 60.0 psi; dry gas flow, 5.0 L/min; APCI Vap Temp 450 °C; corona current (nA) 4000 (pos); capillary voltage 3500 V. Derivatives were separated on ZORBAX Eclipse XDB-$C_8$ column (150 mm × 4.6 mm 5 μM, Agilent). The HPLC system was controlled by HP Chemstation software. The mass spectrometer from Bruker Daltonik (Bremen, Germany) was equipped with an atmospheric pressure chemical ionization (APCI). The mass spectrometer system was controlled by Esquire-LC NT software, Version 4.1. A 65-min gradient elution (A: 55% acetonitrile consisting of 30 mM ammonium formate, pH 3.5; B: 100% acetonitrile) was selected for the separation of aldehyde derivatives. Fluorescence excitation

and emission spectra were obtained at a 650-10 S fluorescence spectrophotometer (Hitachi). Excitation and emission bandpass are both set at 10 nm. The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfiled, IL)

### 2.2. Chemicals

All aldehydes standards were purchased from Sigma Co (St. Louis, MO). HPLC grade acetonitrile (spectroscopically pure acetonitrile) was purchased from Yucheng Chemical Reagent Co. (Shandong Province, China). Formic acid was analytical grade from Shanghai Chemical Reagent Co. Water was purified on a Milli-Q system (Millipore, Bedford, MA). The standard trichloroacetic acid (TCA, catalyst) for derivatization reaction at concentrations of 1.0% (v/v) was prepared by dilution the corresponding stock solutions (10%, v/v) of trichloroacetic acid with anhydrous acetonitrile prepared by distilling the dried HPLC grade acetonitrile with $P_2O_5$.

### 2.3. Preparation of standard solutions

The derivatizing reagent solution ($1.0 \times 10^{-3}$ mol/L) was prepared by dissolving 3.21 mg 1,2-benzo-3,4-dihydrocarbazole-9-ethoxy-carbonylhydrazine in 10 mL of anhydrous acetonitrile prepared by distilling the dried HPLC grade acetonitrile with $P_2O_5$. Individual stock solutions of the aldehydes ($1.0 \times 10^{-2}$ mol/L) were prepared in acetonitrile, and if necessary, tetrahydropyrane was added until the compound dissolved. The standard aldehydes for HPLC analysis at individual concentrations of $1.0 \times 10^{-4}$ mol/L were prepared by dilution the corresponding stock solutions ($1.0 \times 10^{-2}$ mol/L) of each aldehyde with acetonitrile. When not in use, all standards were stored at 4 °C in a refrigerator.

### 2.4. Synthesis of derivatization reagent (BCEC)

Syntheses of 1,2-benzo-3,4-dihydrocarbazole and 1,2-benzo-3,4-dihydrocarbazole-9-ethanol were described as our previously reported [26].

#### 2.4.1. Preparation of 1,2-benzo-3,4-dihydrocarbazolecarbazole-9-ethyl chloroformate (BCEOC)

To a solution containing 15 g solid phosgene and 100 mL dichloromethane (0 °C) in a 500-mL of round-bottom flask, a mixture of 1,2-benzo-3,4-dihydrocarbazole-9-ethanol (25 g) and pyridine (2 g catalyst) in 150 mL of dichloromethane solution was added dropwise within 2 h with stirring. After stirring at 0 °C for 4 h, the contents were kept at ambient temperature for another 6 h period with vigorous stirring, then the solution was concentrated by a rotary evaporator. The residue was extracted four times with warm ether; the combined ether layers were concentrated in vacuum to yield a white crystal. The crude products were recrystallized twice from ether to give the white crystal 26.5 g (85.5%), mp 105–105.7 °C. Found, C 69.3, H 4.12, N 4.34, Cl 11.2; Calculated, C 69.9, H 4.90, N 4.29, Cl 11.0; IR (KBr), 1772.8 (–C=O); 1461.6 (C–H); 1372.9, 1346.0 (C–H); 1202.4, 1128, 848.9, 739.1.

#### 2.4.2. Preparation of 1,2-benzo-3,4-dihydrocarbazolecarbazole-9-ethoxy-carbonylhydrazine (BCEC)

To a solution containing 3.26 g 1,2-benzo-3,4-dihydrocarbazolecarbazole-9-ethyl chloroformate (BCEOC) and 10 mL dichloromethane (0 °C) in a 50-mL of round-bottom flask, a mixture of anhydrous hydrazine (0.35 g) and pyridine (1.0 g catalyst) in 15 mL of dichloromethane solution was added dropwise within 30 min with stirring. After stirring at 0 °C for 1 h, the contents were kept at ambient temperature for another 1 h period with vigorous stirring, then the solution was concentrated by a rotary evaporator. The residue was recrystallized twice from acetonitrile to give the white crystal 3.0 g (93.3%), mp 180.7–181 °C. Found, C 71.01, H 5.90, N 13.21; Calculated, C 71.03, H 5.92, N 13.08; IR (KBr) 3336.7 ($-NH_2$), 1691.5 (–C=O); 1461.6 (C–H); 1513.7, 1289.6, 1196.3; 1059.4, 738.6. $m/z$:$[M+H]^+$ 322 (APCI source at positive mode).

### 2.5. High-performance liquid chromatography

HPLC separation of BCEC derivatives was carried out by ZORBAX Eclipse-XDB-$C_8$ column with a binary gradient elution, using the following linear gradient: Eluent A was 55% of acetonitrile consisting of 30 mM formic acid buffer (pH 3.5); B was acetonitrile (100%). During conditioning of the column and prior to injection, the mobile phase composition was 95% A and 5 B%. The percentage of mobile was changed as follows after injection: 5–10% (B) from 0 to 5 min; 10–30% (B) from 5 to 10 min; 30–45% (B) from 10 to 15 min; 45–65% (B) from 15 to 20 min; 65–100% (B) from 20 to 30 min; 100% (B) from 30 to 40 min. The flow rate was constant at 1.0 mL/min and the column temperature was set at 35 °C. The fluorescence excitation and emission wavelengths were set at $\lambda_{ex}$ 333 and $\lambda_{em}$ 390 nm, respectively.

### 2.6. LC/APCI/MS procedure

Prior to its use, the instrument was checked to meet the sensitivity defined by the manufacturer. The FLD were calibrated and tested using the FLD diagnosis procedure of the ChemStation software for HP1100 system. The HP1100 LC/MSD-SL was calibrated with APCI tuning solution obtained from Agilent Technology (Palo Alto, CA). The mass spectrometer was calibrated so that mass accuracy specification and sensitivity were achieved over the entire mass range.

### 2.7. Derivatization procedure

The BCEC-aldehydes derivatization proceeded in acetonitrile solution in the presence of trichloroacetic acid catalyst. A 20–30 μL of aldehydes in water–acetonitrile (8:2, v/v) (or

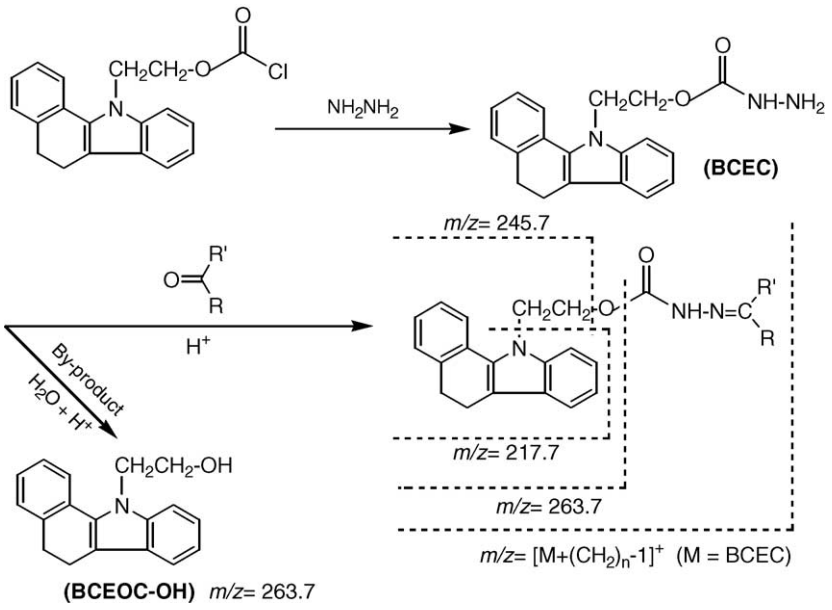


Fig. 1. Derivatization scheme of 1,2-benzo-3,4-dihydrocarbazole-9-ethoxy-carbonylhydrazine (BCEC) with aldehydes in the presence of trichloroacetic acidic catalyst and corresponding mass breakage mode of its derivatives.

60–80 μL alcoholic beverage) was added into a vial (1.0 mL), and then successively added 20 μL of 1.0% TCA acetonitrile solution and 60 μL of BCEC acetonitrile solution. The vial was then sealed and the mixture was heated at 60 °C for 30 min in a thermostatic water-bath, and the reaction solution was cooled in ice-water to stop the reaction. An aliquot (10 μL) of the derivatization solution was injected to HPLC. The reagent blanks without aldehydes were also treated in the same manner. The derivatization process is shown in Fig. 1.

### 2.8. Quantitative analysis

Quantitative conversion of the aldehydes from the alcoholic beverages to their BCEC-hydrazones is guaranteed by using a large excess of BCEC. All aldehydes were quantified in the alcoholic matrices using the external standard method with detection at 390 nm. The calibration curves for each aldehyde-BCEC were obtained by linear regression plotting peak area versus concentration.

### 2.9. Fluorescence procedure

A thermostated curette holder connected to a circulator controlled the temperature of measuring cell. The fluorescence intensity of the BCEC in aqueous or non-aqueous solvent was investigated at different temperatures. The steady-state fluorescence intensity of 1,2-benzo-3,4-dihydrocarbazole-9-ethoxy-carbonylhydrazine was also investigated at different temperatures. The temperature was tested in 15 °C increments from 15 to 75 °C. Stock standard solution ($1.0 \times 10^{-5}$ mol/L) of the reagent (BCEC) was prepared in acetonitrile. For studies of the effects of organic solvents, and temperature on the fluorescence properties, 50 μL portions of the stock standard solution was added to 2 mL of the organic solvents or aqueous solutions. The fluorescence spectra and fluorescence intensities were recorder by a 650-10 S fluorescence spectrophotometer within 5 min equilibrium in measuring cell after preparation of the solutions.

## 3. Results and discussion

The objective of this study was to develop a highly sensitive labelling reagent that can be used to analyze trace concentrations of aldehydes from natural environmental matrixes by fluorescence detection and enhance atmospheric pressure chemical ionization detection. To improve the APCI detection sensitivity, we sought to introduce a highly ionizable group into the labelling agent core molecule and significantly enhanced the ionization of corresponding derivative molecules. The main challenge of the present work was to test the feasibility of labelling agent in a variety of conditions.

### 3.1. Fluorescence and ultraviolet absorption of BCEC

The excitation and emission spectra of BCEC and its derivatives were collected using the scanning mode of the fluorescence detector. Maximum fluorescence responses were achieved at the excitation wavelength of 333 nm and emission wavelength of 390 nm. Its derivatives and corresponding degradation by-products show no remarkable difference relative to that of BCEC under the same operating conditions. The fluorescence emission intensity in methanol (100%) was 2.1% stronger than that in acetonitrile (100%). The excitation and emission wavelengths in acetonitrile–water solution were

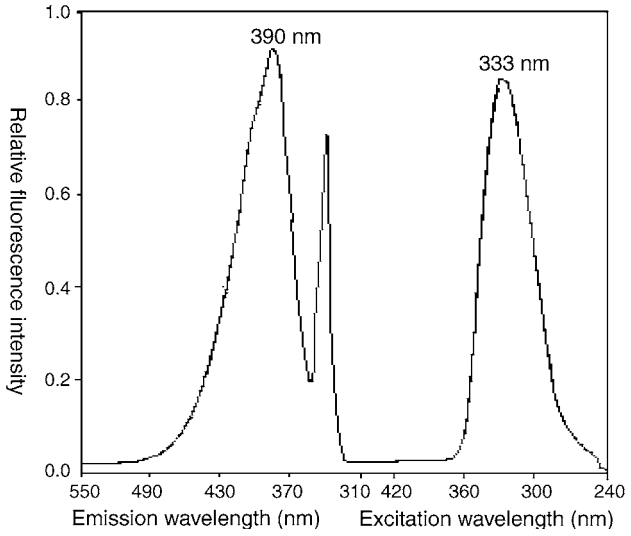


Fig. 2. The fluorescence spectrum of BCEC in 50% of acetonitrile solution (BCEC concentration at $1.7 \times 10^{-6}$ mol/L).

no obvious difference relative to that in methanol–water solution. The fluorescence excitation and emission spectra are shown in Fig. 2.

UV–vis spectra of BCEC were investigated in acetonitrile solution. The absorption wavelengths of BCEC were obtained with the scanning range of 200–400 nm. Maximum ultraviolet absorption responses were observed at the wavelengths of 249 and 320 nm, respectively. The molar absorption coefficients ($\varepsilon$) are $3.2 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ (249 nm) and $2.6 \times 10^4$ L mol$^{-1}$ cm$^{-1}$ (320 nm), respectively.

### 3.2. Stabilities of reagent (BCEC) and its derivatives

In this study, the synthesized BCEC in our laboratory has the similar structure to that of DBD-ProCZ [6] and Fmoc-hydrazine [12]. Anhydrous acetonitrile solution of BCEC was stored at 4 °C for 2 weeks in a refrigerator, the derivatization yields for aldehydes showed no obvious difference. The labeled aldehydes stored at 4 °C in a refrigerator, the corresponding derivative for further HPLC analysis at least 48 h with normalized peak areas varying <2.4%.

### 3.3. Enhance atmospheric pressure chemical ionization (APCI) responses

Recent advances in the development of atmospheric pressure chemical ionization (APCI) mass spectrometry have been allowed for the specific detection of a wide array of biochemicals at low concentrations within endogenous matrixes [27]. However, direct analysis of aldehydes from biochemicals by HPLC with current APCI sources has been traditionally difficulty due to their particular physicochemical properties, i.e., high volatility and polarity, and poor resolution (sample concentration $<1.0 \times 10^{-6}$ mol/L, $C_1$–$C_5$ aldehydes could not detect due to poor resolution and lower detection responses) using atmospheric pressure chemical ionization at positive-ion mode. In view of these shortcomings, the derivatization with fluorescence detection or enhance mass spectrometric detection has been extensively employed for the determination of trace amount of aldehydes. To enhance detection sensitivity, we sought to introduce a highly ionizable group into the labelling reagent molecule using a derivatization reaction. Our simulation of the ionization efficiency for a number of feasible derivatives indicated that the addition of a 1,2-benzo-3,4-dihydrocarbazole functional group in labeling agent molecule, which bearing a weak basic nitrogen in molecular core structure, should enhance the ionization of BCEC-aldehydes significantly under acidic conditions (for example, mobile phases containing 30 mM formic acid buffer, pH < 3.5). At the same time, an acidic mobile phase will also be useful for the separation of BCEC-aldehyde derivatives with HPLC.

The ionization and fragmentation of the isolated BCEC-aldehyde derivatives was studied by mass spectrometry with atmospheric pressure chemical ionization (APCI). As expected, the BCEC-aldehydes produced an intense molecular ion peak at $m/z$ $[M + (CH_2)_n - 1]^+$ under positive-ion mode ($M$: molecular mass of BCEC) (see Fig. 1 and Table 1). This is probably, in part, due to the fact that BCEC molecule, in which its *n–(* conjugation system is dramatically augmented due to introducing a 1,2-benzo-3,4-dihydrocarbazole functional group, forms more stable molecular ion by the weak basic nitrogen atom from molecular core structure and resulting in high ionization of BCEC-aldehyde derivatives with APCI at positive-ion mode. We also observed that the collision-induced dissociation of molecular ion generates some intense fragment ions at $m/z$ = 245.7.0, $m/z$ = 263.7 and $m/z$ = 217.7, and corresponding the cleavage of –$CH_2$–OCO, –$CH_2$O–CO and N–$CH_2CH_2$ bonds, respectively.

### 3.4. Effects of temperature and solvent on fluorescence properties of BCEC

The steady-state fluorescence intensity of BCEC in 50% methanol, 50% acetonitrile and aqueous solution was, respectively, investigated at different temperatures. The temperature was tested in 15 °C increments from 15 to 75 °C.

Table 1
LC/MS/MS for aldehyde derivative

| Compound | $[M + H]^+$ | Fragment ion[a] |
|---|---|---|
| BCEC | 321.7 | 245.7 (L), 217.6 (S) |
| Formaldehyde | 333.7 | 263.7 (m), 245.7 (L), 217.6 (S) |
| Acetaldehyde | 347.7 | 263.7 (m), 245.6 (L), 216.7 (S) |
| Propanal | 361.7 | 263.7 (m), 245.7 (L), 216.6 (S) |
| Butanal | 375.7 | 263.7 (m), 245.6 (L), 217.6 (S) |
| Pentanal | 389.8 | 263.7 (m), 245.7 (L), 216.7 (S) |
| Hexanal | 403.8 | 263.7 (m), 245.7 (L), 216.6 (S) |
| Heptanal | 417.8 | 263.7 (m), 245.6 (L), 217.6 (S) |
| Octanal | 431.8 | 263.7 (m), 245.7 (L), 217.6 (S) |
| Nonaldehyde | 445.8 | 263.7 (m), 245.7 (L), 217.7 (S) |
| Decanal | 459.8 | 263.7 (m), 245.7 (L), 217.7 (S) |

[a] S, M and L stand for relative intensities; S: small; M: middle; L: large.

Table 2
Effects of temperature and solvent on fluorescence intensity

| Solvent | Relative intensity (°C) | | | | | Stability energy (kJ/mol) | Regression coefficient ($R$) |
|---|---|---|---|---|---|---|---|
| | 15 | 30 | 45 | 60 | 75 | | |
| Group 1 | | | | | | | |
| Methanol | 68 | 61 | 56 | 52 | [b] | 4.767 | 0.9986 |
| Ethanol | 76.2 | 66.8 | 53.4 | 45 | [b] | 9.626 | 0.9934 |
| 1-Propanol | 82.4 | 79.9 | 76.9 | 73.8 | 75.2 | 2.001 | 0.9973 |
| 1-Butanol | 84 | 79.5 | 74.4 | 71.5 | 68.6 | 2.882 | 0.9982 |
| 1-Pentanol | 90.2 | 83.2 | 78.8 | 74.2 | 70.6 | 3.247 | 0.9988 |
| Group 2 | | | | | | | |
| Formic acid | 8.8 | 7.0 | 5.1 | 4.0 | 3.5 | 13.598 | 0.9964 |
| Acetic acid | 92.7 | 64.5 | 46.1 | 33 | 26.1 | 18.029 | 0.9996 |
| Propionic acid | 94.2 | 67.9 | 44.3 | 32.8 | 27.5 | 17.997 | 0.9961 |
| Butyric acid | 106 | 77.8 | 72.6 | 43.2 | 33.2 | 16.289 | 0.9982 |
| Pentoic acid | 37 | 26.5 | 19 | 13.8 | 9.9 | 18.438 | 0.9986 |
| Group 3 | | | | | | | |
| Water | 55.4 | 47.3 | 38.8 | 34.6 | 29.5 | 8.765 | 0.9971 |
| 50% Acetonitrile | 79.8 | 59.2 | 47 | 36.8 | [b] | 13.654 | 0.9992 |
| 50% Methanol | 87.2 | 67 | 50 | 42 | [b] | 13.303 | 0.9975 |

| Solvent[a] | Emission (nm) | Relative intensity |
|---|---|---|
| Water | 390 | 47.3 |
| Dimethylsulfoxide | 390 | 82.9 |
| Methanol | 384 | 61 |
| Acetonitrile | 385 | 68.5 |
| Acetone | 385 | 19.0 |
| Benzene | 385 | 61.1 |
| Ethyl acetate | 385 | 73.0 |

[a] Under the same measuring conditions.
[b] The data were not determined due to volatilization of solvents.

The results indicate that the emission intensities decrease with increasing temperature. Possibly due to the loss of excited-state energy through hydrogen bonding or due to the protonation in strong hydrogen-bonding solvents leading to a corresponding quench in emission intensity. A representative kinetic analysis of fluorescence intensity revealed a linear correlation between $\ln I_{em}$ and $1/T$ ($I_{em}$: relative fluorescence intensity; $T$: thermodynamic temperature). The slopes of the working curves show the emission stabilization energies were 8.765 kJ/mol ($R^2 = 0.9971$, in water), 13.654 kJ/mol ($R^2 = 0.9992$, in 50% acetonitrile solution) and 13.303 kJ/mol ($R^2 = 0.9975$, in 50% methanol solution). In this study, BCEC is thermally stable and exhibits no significant decomposition over the temperature ranges investigated. A kinetic analysis of fluorescence intensity of BCEC at different temperature in various non-aqueous solvents from 15 to 75 °C in 15 °C increments also led to a linear correlation between $\ln I_{em}$ and $1/T$. The slopes of the working curves for emission stabilization energies are shown in Table 2. View the situation as a whole, with solvents from group 1, the fluorescence intensity of BCEC decreases with the increasing temperature, such a decrease is almost completely reversible when the temperature is returned from 75 to 15 °C. In addition, at the same temperature, the fluorescence intensity of BCEC in alcoholic solvents increases with increasing carbon atom numbers of alcohols, it is probably due to the fact that hydrogen bond acting forces between BCEC and various alcoholic molecules decrease with increasing the carbon number of alcoholic molecules (in other words, the viscosity of alcoholic molecules increase with increasing carbon numbers). With solvents from group 2, BCEC fluorescence intensity is also largely decreased with increasing temperature, but still excellently reversible when temperature is returned from 75 to 15 °C. At the same temperature, the fluorescence intensity of BCEC in organic acid solvents increases with increasing carbon numbers exception being pentoic acid. It is probably due to the fact that BCEC molecule in pentoic acid solvent exhibits relative low solubility resulting in low responses. A large decrease in emission intensity in formic acid is observed, it is probably due to the fact that relatively strong formic acid causes partial protonation by the weak basic nitrogen atom from BCEC molecular core structure and results in the dramatically quenching in fluorescence intensity. The increasing temperature and solvent polarity show little effect on the emission spectra, in most cases, the maximum emission remains unchanged with the exception being water and dimethylsulfoxide with emission wavelength at 390 nm.

To be confirmed the ionization of weak basic nitrogen atom from BCEC molecular core structure, the effects of

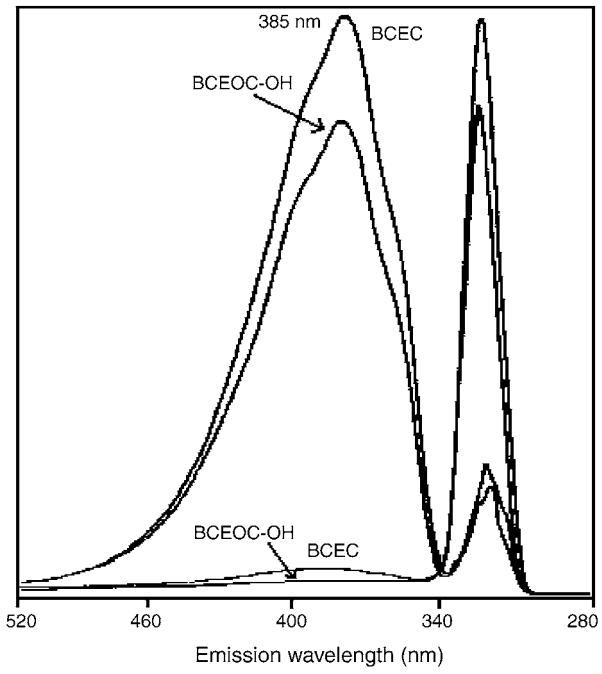


Fig. 3. Effects of solvents on the emission spectra of BCEC and BCEOC-OH conditions: concentration of BCEC and BCEOC-OH at 0.14 μmol/L, temperature at 30 °C; scan ranges at 280–520 nm; two curves at the top are the emission spectra of BCEC and BCEOC-OH in 50% methanol solution; two curves at the end of figure are the emission spectra of BCEC and BCEOC-OH in formic acid solvent.

formic acid on the fluorescence intensity of BCEC and BCEOC-OH were investigated. As can be seen from Fig. 3, the emission intensities of BCEC and BCEOC-OH in 50% methanol solution are remarkably stronger than that in formic acid solvent. An almost complete quench in fluorescence to BCEC and BCEOC-OH in formic acid solvent was observed. This is obviously due to the fact that hydrogen ion from formic acid molecule reacts with the weak basic nitrogen atom to form stable molecular ion, and the $n$–$\pi$ conjugation system of molecular core structure is dramatically decreased and results in the quench in fluorescence responses. The quench in fluorescence to BCEC and BCEOC-OH exhibits the similar results. As essentially the same fluorescence properties were observed for the BCEOC-OH, BCEC and its derivatives, it is proved that the quench in fluorescence does not come from the hydrazine group of BCEC molecule. It is likely that the fluorescence is due to the fluorophore of the 1,2-benzo-3,4-dihydrocarbazole functional group. Clearly, the introduction of a 1,2-benzo-3,4-dihydrocarbazole moiety results in high ionization of BCEC as well as its derivatives with atmospheric pressure chemical ionization (APCI) detection. The enhancement MS/APCI responses should be converged on the weak basic nitrogen atom from BCEC molecular core structure as illustrated in text above-mentioned. This is crucial for the qualitative identification of trace levels of aldehyde components from biological and natural environmental samples by APCI detection.

### 3.5. Effect of BCEC concentration on derivatization

As expected, the molecular structure of 1,2-benzo-3,4-dihydrocarbazole-9-ethoxy-carbonylhydrazine (BCEC) that it plays the same hydrazine reaction with aldehydes as do of DBD-ProCZ and Fmoc-hydrazine previously reported [6,12]. Derivatization of BCEC with aldehydes can be achieved within 10 min at 60 °C in the presence of trichloroacetic acid catalyst. The effect of BCEC concentrations on the derivatization yields was investigated for aldehyde derivatives. The concentration of BCEC is critical for the labelling reaction, the fluorescence intensity of BCEC-derivatives increases with increasing amounts of BCEC concentration. Constant fluorescence intensity was achieved with the addition of 10- to 15-fold molar reagent excess to total molar aldehydes, increasing the excess of reagent beyond this level had no significant effect on yields. With less than a 10-fold molar excess of derivatization agent, the derivatization of aldehydes was incomplete and remarkably resulted in low detection responses. A slight side reaction is that the reagent (BCEC) reacts partially with water in the presence of acidic catalyst to give itself hydrolysis product (BCEOC-OH, $m/z$ = 263.7). In fact, the addition of >15-fold molar reagent excess to total molar aldehydes, the presences of small water in reaction medium do not affect on the derivatization yields. The yields were almost the same in the presence of 5–10% of water in derivatization solution. The presence of a small amount of BCEOC-OH does not interfere with the separation of other aldehyde derivatives by the adjusting of elution composition of mobile phase.

Another interesting aspect regarding the derivatization of ketone hydrazones, the derivatization conditions of ketone such as acetone and 2-butanone exhibited significant difference compared to those of aldehydes mentioned above. A significantly longer reaction time for the preparation of ketone hydrazones was observed. This is probably due to the fact that the formation of a ketone hydrazone with BECE exhibits lower reactivity compared to those of aldehydes. In most cases, the complete formation of a ketone hydrazone with reagent BECE needs about 2 h. These differences show very clearly that BECE for detection aldehydes can be a very useful reagent for the elimination of the interference of ketone derivatives under the proposed derivatization conditions as described in text.

### 3.6. Effects of catalyst concentration, solvents and temperature on derivatization

The derivatization reactions of carbonyl compounds with hydrazines are usually accelerated in the presence of an acidic catalyst to give the corresponding hydrazone derivatives. Several types of acidic catalysts were evaluated in this study for optimal derivatization, including acetic acid, hydrochloric acid, trichloroacetic acid and trifluoroacetic acid, and the catalyst amounts are from 0.05% to 5.0% (v/v). The results indicated that trichloroacetic acid was found to be the best

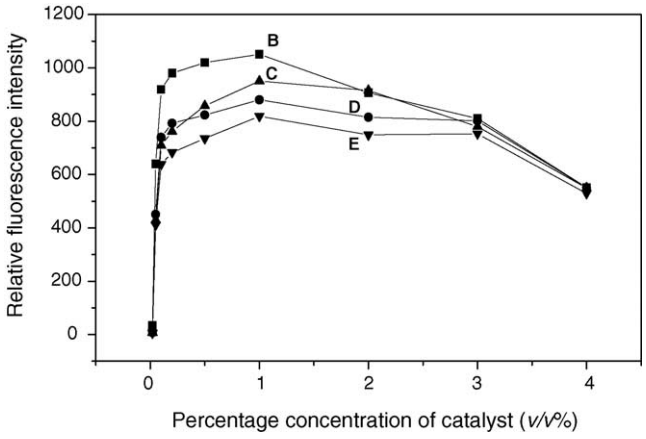


Fig. 4. Effect of percentage concentration of trichloroacetic acid catalyst on derivatization, B: propanal; C: pentanal; D: hexanal; E: decanal.

choice. With respect to the concentration, a small amount of trichloroacetic acid provided quantitative yields of the derivatives, the detection responses for representative aldehydes including propanal, pentanal, hexanal and decanal increase with consecutively increasing amount trichloroacetic acid from 0.05% to 1.0% (v/v). As illustrated in Fig. 4, the maximum detection responses were observed with ca.1.0% of trichloroacetic acid. With the concentration of trichloroacetic acid >1.0%, a remarkable decrease in detection response with several unknown disturbance peaks for the derivatized aldehydes was observed. This is probably due to the fact that the derivatization with high concentration of trichloroacetic acid causes some side-reactions and results in the low detection responses. No reaction was observed without catalysts.

Several types of solvents were also evaluated in this study for the optimum derivatization, including DMSO, DMF, THF, 1,4-dioxane, methanol and acetonitrile. Acetonitrile was used as the derivatization co-solvent in preference to other solvents as it possessed maximum detection responses (here, BCEC exhibits high fluorescence responses in DMSO and ethyl acetate solvents, however, both of them are not suitable for the preparation of mobile phase components). With DMF solvent, the lowest detection responses for derivatization were observed, it was probably due to the fact that the trace levels of basic components from DMF solvent such as methylamine and dimethylamine reacted with the trichloroacetic acid to reduce the catalytic activity of catalyst. Other solvents such as THF, DMSO, 1,4-dioxane and methanol were not adequate to achieve satisfactory derivatization yields. In this study, the results converged at acetonitrile as the optimal derivatization solvent.

The effect of temperature on derivatization was tested in acetonitrile solvent containing 1.0% trichloroacetic acid. The results indicated that detection responses remarkably increases with increasing temperature from 25 to 60 °C. With reaction temperature at 25 °C, the complete derivatization could be achieved after 60 min. With further higher temperature >65 °C, the complete derivatization was obtained with reaction time <5 min. However, a slight decrease in detection response was observed. This is probably due to the fact that higher derivatization temperature results in some side-reactions. Taking both short derivatization time and low side-reaction into consideration, working temperature is set at 60 °C. Complete derivatization could be achieved after 10 min at this condition. In most cases, the yields were not affected when the derivatization time was over 10 min.

### 3.7. HPLC separation for derivatized aldehydes

Several gradient programs were investigated to ensure satisfactory HPLC separation within the shortest time. For the complete separation of all aldehyde derivatives, a binary gradient consisting of acetonitrile and 30 mmol/L of a mixture of water/formaic acid/ammonia water was used (the used high concentration buffer was prepared as follows: 18.86 mL formic acid was mixed with 17 mL ammonia water, pH ≈ 3.5). The prepared formic acid buffers was used instead of borate buffer to control mobile phase pH during HPLC separation in order to reduce to a minimum from the contamination of metal ions to ionization chamber of mass spectrometer. The resolution of formaldehyde and acetaldehyde derivatives can be significantly affected by pH of mobile phase. The constant pH (pH > 4.0) will be unable to fully suit the separation for the early eluted aldehyde derivatives with good resolution. To achieve optimal separation, the choice of pH value of mobile phase A was tested on Hypersil $C_{18}$ column. Separation of the derivatized aldehyde standards can be accomplished at acidic condition with pH 3.0–3.7. With pH < 3.7, most of the aldehyde derivatives were resolved with a good baseline resolution. In comparison with the acidic conditions (pH < 3.7), operation at pH > 4.0 resulted in obvious decrease in resolution for formaldehyde and acetaldehyde derivatives. With more basic mobile phase conditions (pH > 5.0), formaldehyde and acetaldehyde derivatives were partially co-eluted. At the same time, the protolysis efficiency of BCEC core molecule was remarkably decreased. After further experiments, it was found that the pH value of mobile phase A was adjusted to pH 3.5, a complete baseline resolution for all aldehyde derivatives could be achieved with high ionization efficiency within the shortest time as suggested elution program above-mentioned. The separation of a standard mixture containing all of ten aldehyde derivatives on ZORBAX Eclipse XDB-$C_8$ column is shown in Fig. 5.

### 3.8. Analytical recovery and reproducibility

The recoveries were determined from values obtained following actual analysis of the alcoholic beverage as calculated from the calibration graph constructed by using the performed aldehyde derivatives. In two identical alcoholic beverages, a known amount of the 10 aldehydes was added. The samples were derivatized with BCEC as described in text and the analyses were carried out in duplicate. The experimentally found recoveries are between 89 ± 2.6%

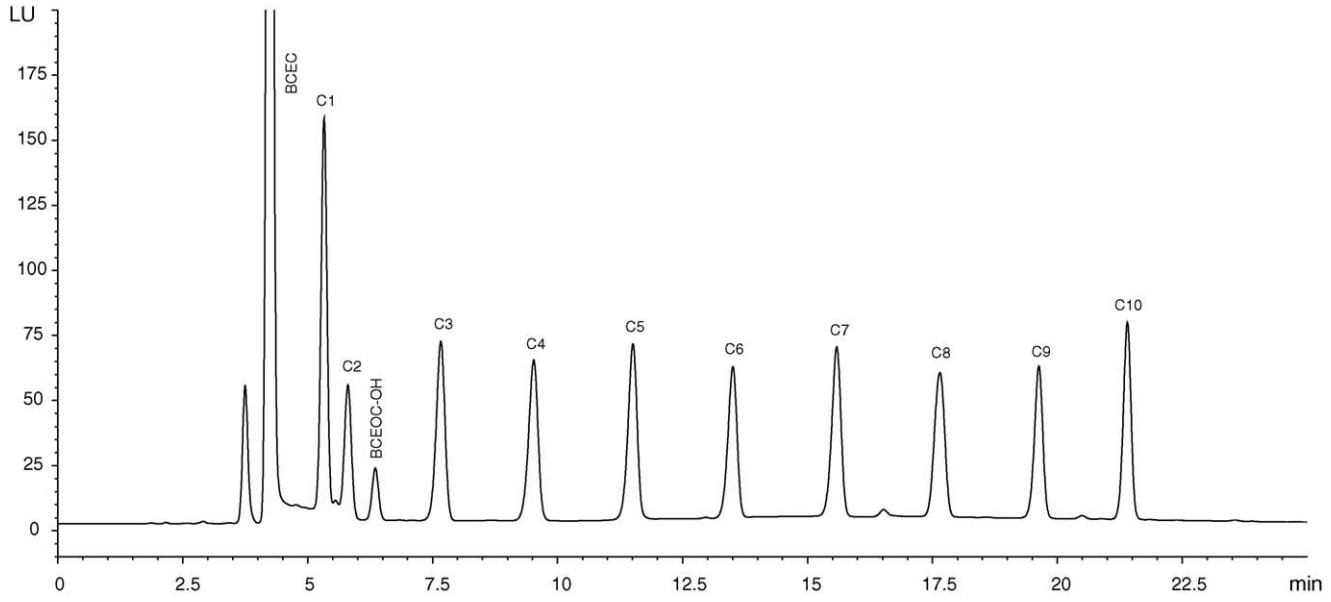


Fig. 5. HPLC chromatogram of the 1,2-benzo-3,4-dihydrocarbazole-9-ethoxy-carbonylhydazine (BCEC) for aldehyde standard derivatives, injected amount 20.8 pmol. Column temperature is set at 35 °C; excitation and emission wavelengths $\lambda_{ex}$ 333 nm, $\lambda_{em}$ 390 nm; column 150 mm × 4.6 mm ZORBAX Eclipse-XDB-$C_8$ (5 μm); flow rate = 1.0 mL $min^{-1}$, C1: formaldehyde; C2: acetaldehyde; C3: propanal; C4: butanal; C5: pentanal; C6: hexanal; C7: heptanal; C8: octanal; C9: nonaldehyde; C10: decanal, BCEOC-OH (1,2-benzo-3,4-dihydrocarbazole-9-ethanol).

(heptanal) and 126 ± 4% (formaldehyde). High recovery for formaldehyde is probably due to the introduction of a small amount of formadehyde from solvents. The reproducibility of the method was demonstrated by the determination known concentrations of 10 aldehydes (concentration from 10–100 ng/mL) in quintuplicate on three separate (consecutive) days. The standard deviations of actual determined values were between 3.2% and 6.7%.

### 3.9. Detection limits and linearity for derivatized aldehydes

Detection limits are an important consideration when the components of biological matrices are analyzed, particularly they are present at low or trace concentrations. Estimated detection limits for the aldehydes were obtained by successive dilution of a derivatized standard solution containing 16.65 μmol/L of each aldehyde in acetonitrile. The detection limits for each derivatized aldehyde (at a signal-to-noise ratio = 3:1) are from 0.375 to 1.665 nmol/L (Table 3). The linearities were established over a 2000-fold concentration range with analysis of serial dilutions of the standard solution ranging from 0.008 to 16.65 μmol/L for aldehydes. All of the aldehydes were found to give excellent linear responses in this range investigated, with correlation coefficients of >0.9999 (see Table 3). The linear relationships for further higher concentrations are not tested due to all peaks with large overrun. The proposed method for the trace determination of aldehydes offers much lower detection limits compared to those of methods using labeling reagents reported by other research groups [6,28,29]. The much lower detection limit can be directly attributed to the removal of impurities present in the reagents and blank water. These impurities can be eliminated by purifying the reagent and will give a further increase in sensitivity.

### 3.10. Application

Detailed knowledge of the chemical composition of alcoholic beverages is very important for quality control and for evaluating the effects on consumers' health. The toxicity associated with aldehydes, especially, formaldehyde and acetaldehyde, is well known and their presence in alcoholic beverages is quite often related to nausea, vomiting, sweating, confusion, and rapid heartbeat and hangover headaches. The

Table 3
Detection limits, linearity and correlation coefficients for derivatized aldehydes (concentration range from $8.0 \times 10^{-9}$ to $1.665 \times 10^{-5}$ mol/L; injected 10 μL, corresponding actual injected amount range from 0.08 to 166.5 pmol)

| Aldehyde | $Y = A + BX$ | $R$ | Detection limit (nmol/L) |
|---|---|---|---|
| Formaldehyde | $Y = 10.81 + 15.73X$ | 0.9999 | 0.375 |
| Acetaldehyde | $Y = -0.46 + 38.31X$ | 0.9999 | 0.495 |
| Propanal | $Y = -7.12 + 60.74X$ | 0.9999 | 1.403 |
| Butanal | $Y = -7.47 + 63.21X$ | 0.9999 | 1.493 |
| Pentanal | $Y = -5.93 + 63.62X$ | 0.9999 | 1.332 |
| Hexanal | $Y = -6.11 + 56.60X$ | 0.9999 | 1.665 |
| Heptanal | $Y = -3.54 + 95.19X$ | 0.9999 | 0.783 |
| Octanal | $Y = -3.59 + 66.15X$ | 0.9999 | 1.050 |
| Nonaldehyde | $Y = -0.47 + 85.61X$ | 0.9999 | 0.740 |
| Decanal | $Y = -3.55 + 65.18X$ | 0.9999 | 0.924 |

$Y$: peak area; $X$: actual injected amount (pmol).

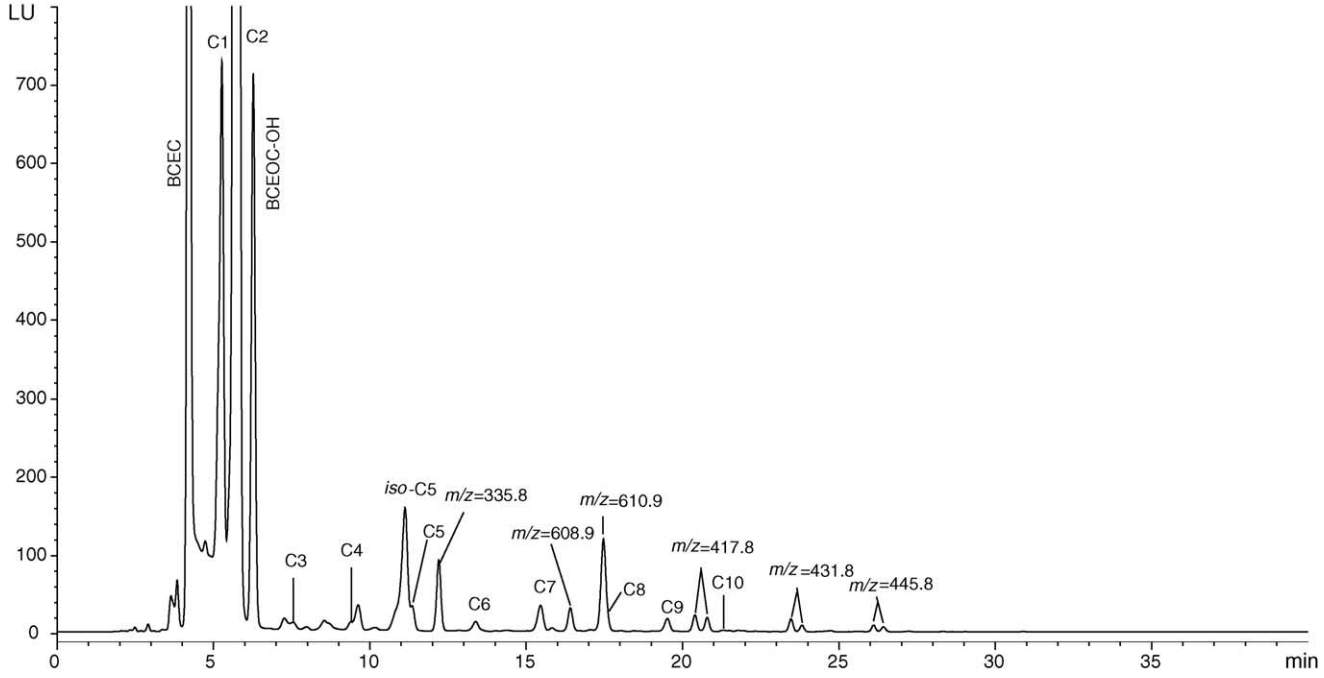


Fig. 6. HPLC chromatogram of BCEC-aldehydes from alcoholic beverage column temperature is set at 35 °C; excitation and emission wavelengths $\lambda_{ex}$ 333 nm, $\lambda_{em}$ 390 nm; column: 150 mm × 4.6 mm ZORBAX Eclipse-XDB-$C_8$ (5 μm); flow rate = 1.0 mL $min^{-1}$, C1: formaldehyde; C2: acetaldehyde; C3: propanal; C4: butanal; *iso*-C5: *iso*-pentanal; C5: pentanal; C6: hexanal; C7: heptanal; C8: octanal; C9: nonaldehyde; C10: decanal, BCEoC-OH (1,2-benzo-3,4-dihydrocarbazole-9-ethanol, a by-product from the hydrolysis of BCEC); The molecular structure corresponding peaks at: *m*/*z* = 335.8; *m*/*z* = 608.9; *m*/*z* = 610.9; *m*/*z* = 417.8 and *m*/*z* = 445.8 are not identified.

chromatogram for the analysis of aldehydes from alcoholic beverage samples with fluorescence detection is shown in Fig. 6. As can be seen from Fig. 6, the content of the ten relevant aldehydes in the alcoholic beverages exhibits significant difference. The major aldehydes in alcoholic beverages are formaldehyde and acetaldehyde. The concentration ranges, expressed in μg/mL, the compositional data are shown in Table 4. The amount of formaldehyde and acetaldehyde in the alcoholic beverages is significantly higher compared to that of other aldehydes. The developed method in this study shows good correlation in comparison with DBD-ProCZ previously reported by Toyo'oka and Liu [6]. Toyo'oka et al. was unable to detect the components for $C_1$–$C_3$ aldehydes. Our results represent an improvement, one of the most attractive features is the introduction of a highly ionizable functional group 1,2-benzo-3,4-dihydrocarbazole into the labelling reagent molecule and allow high sensitive mass spectrometric qualitative identification of trace levels of aldehyde derivatives. At the same time, a complete baseline resolution including $C_1$–$C_3$ aldehydes was achieved in conjunction with a convenient binary gradient elution with satisfactory results.

Table 4
Compositional analysis of free aldehydes alcoholic beverages

| Aldehyde[a] | Kongfu Jia (μg/mL) | Identification by MS/MS[b] | Heitu Di (μg/mL) | Identification by MS/MS[b] |
|---|---|---|---|---|
| Formaldehyde | 3.08 | Yes | 8.20 | Yes |
| Acetaldehyde | 9.06 | Yes | 14.37 | Yes |
| Propanal | 0.03 | No | 0.07 | No |
| Butanal | 0.03 | Yes | 0.20 | Yes |
| *iso*-Pentanal | 0.63 | Yes | 0.82 | Yes |
| Pentanal | 0.07 | Yes | 0.12 | Yes |
| Hexanal | 0.07 | Yes | 0.08 | Yes |
| Heptanal | 0.10 | Yes | 0.21 | Yes |
| Octanal | 0.08 | Yes | 0.10 | Yes |
| Nonaldehyde | 0.07 | Yes | 0.11 | Yes |
| Decanal | 0.02 | Yes | 0.03 | Yes |

[a] Aldehydes were quantified in the alcoholic beverages using the external standard method.
[b] Yes: the corresponding components were directly identified by online post-column mass spectrometric analysis. No: the qualitative of corresponding components was performed by retention value.

## 4. Conclusions

In the present study, we designed and synthesized a new environment-sensitive fluorescence probe that is capable of labeling aldehydes with superior properties compared to currently employed reagents, including rapid, convenient derivatization, excellent sensitivity, stability and derivatization yields. Complete derivatization in the presence of acidic catalyst at 60 °C takes not more than 10 min. The improved performance of the reagent BCEC for the quantitative analysis of aldehydes has been demonstrated in details. The present work has shown that derivatization of aldehydes with BCEC is quantitative. The principal merits of this work is the introduction of a highly ionizable functional group into the labelling reagent molecule and allow high sensitive mass spectrometric qualitative identification of trace levels of aldehyde after derivatization. Detection limits are in the femtomole range. The HPLC separation for the derivatized aldehydes showed good repeatability.

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