Food Chemistry 234 (2017) 408–415

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Facile and sensitive determination of N-nitrosamines in food samples by high-performance liquid chromatography via combining fluorescent labeling with dispersive liquid-liquid microextraction

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ARTICLE INFO

Article history:
Received 16 January 2016
Received in revised form 9 January 2017
Accepted 5 May 2017
Available online 6 May 2017

Keywords:
N-nitrosamines
Dispersive liquid–liquid microextraction
High-performance liquid chromatography
Fluorescent labeling

ABSTRACT

The intake of N-nitrosamines (NAs) from foodstuffs is considered to be an important influence factor for several cancers. But the rapid and sensitive screening of NAs remains a challenge in the field of food safety. Inspired by that, a sensitive and rapid method was demonstrated for determination of five NAs (Nitrosopyrrolidine, Nitrosodimethylamine, Nitrosodiethylamine, Nitrosodipropylamine and Nitrosodibutylamine) using dispersive liquid–liquid microextraction (DLLME) followed by high-performance liquid chromatography with fluorescence detection (HPLC–FLD). The NAs were firstly denitrosated and labeled by 2-(11H-benzo[a]carbazol-11-yl) ethyl carbonochloridate (BCEC-Cl) and finally enriched by DLLME. Furthermore, the main DLLME conditions were optimized systematically. Under the optimal conditions, satisfactory limits of detection (LODs) were obtained with a range of 0.01–0.07 ng g^{-1}, which were significantly lower than the reported methods. The developed method showed many merits including rapidity, simplicity, high sensitivity and excellent selectivity, which shows a broad prospect in food safety analysis.

1. Introduction

Recently, food safety has attracted rapidly growing attentions in the world due to potential threat to public health (Röhr, Lüddecke, Drusch, Müller, & Alvensleben, 2005; Safety, 2011). N-nitrosamines (NAs) as one kind of the most important toxic substances have been proven to be carcinogenic and mutagenic for humans, which can result in a series of diseases such as gastric, colorectal and esophageal cancer (Lee et al., 2006; Winter et al., 2007). Generally, humans are exposed to NAs that are mainly from various foodstuffs containing marine fish (Bulushi, Poole, Deeth, & Dykes, 2009), sausage (De Mey et al., 2014), cured meat (Hsu, Arcot, & Lee, 2009), vegetable oil (Fiddler, Pensabene, & Kimoto, 1981), cheese (Dellisanti, Cerutti, & Airoldi, 1996), drinking water (Charrois, Boyd, Froese, & Hrudey, 2007), beer (Yurchenko & Mölder, 2005) and so on. Their formation has been confirmed by the nitrosation reaction of a nitrosating reagent derived from either nitrites or nitrogen oxide with the N-containing substances (Yurchenko & Mölder, 2005). Thus, the use of nitrites and nitrates applied as preservatives is also strictly controlled in meat industry (Honikel, 2008). Moreover, the United States Environmental Protection Agency Integrated Risk Information System has classified 8 NAs as probable human carcinogens. And the tolerance level of human exposure to NAs has also been strictly set in different countries (Andrade, Reyes, & Rath, 2005; Ventanas & Ruiz, 2006). Thus, the sensitive and facile determination of trace NAs is of significant importance.

To date, various analytical methods for NAs detection have been demonstrated such as gas chromatography (GC) coupled with thermal energy analysis (TEA) or mass spectrometry (MS) (Huang, Chen, Fu, & Ding, 2013; McDonald, Harden, Nghiem, & Khan, 2012; Ramírez et al., 2012), liquid chromatography coupled with mass spectrometry (LC–MS) (Plumlee, López-Mesas, Heidlberger, Ishida, & Reinhard, 2008; Wagner, Finkel, Fossett, & Gillman, 2005), capillary electrophoresis (CE) (Yang et al., 2011) and so on. Although each of these methods displayed different character-

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http://dx.doi.org/10.1016/j.foodchem.2017.05.032
0308-8146/

istics for NAs screening, these approaches usually suffered from more or less drawbacks in practical application. For example, due to the limited versatility and relatively high cost for GC–TEA, this detector was not available in most laboratories (Campillo, Viñas, Martínez-Castillo, & Hernández-Córdoba, 2011). In addition, because of the low-molecular-weight of NAs, the signal suffered significantly suppression effect in the range of MS background noise. And the expensive isotope internal standard and complex instrument operation were necessary for MS based methods, which also limited its routine use in common analytical laboratories (Kodamatani et al., 2009). As a mature and versatile technique providing excellent selectivity and good repeatability, HPLC coupled with FLD is a good choice. It is well-known that NAs has no detectable fluorescent signal, so fluorescence derivatization may become an effective procedure to improve the detection sensitivity. In this analytical strategy, firstly, it was necessary to accomplish the denitrosation to produce secondary amines (You, Fan, Lao, Ou, & Zhu, 1999). Subsequently, the generated secondary amines were derived by a fluorescence labeling reagent. In the present study, a novel fluorescence derivatization reagent named 2-(11H-benzo[a]carbazol-11-yl) ethyl carbonochloridate (BCEC-Cl) was employed to label the secondary amines. BCEC-Cl was prepared in our previous studies (You et al., 2007), which possessed excellent fluorescent sensitivity and high reactive selectivity, and provided less byproducts in reaction process. Thus, BCEC-Cl can be employed for the rapid, sufficient and sensitive NAs determination.

Furthermore, the sample complexity and extremely low concentration of target compounds in foods increased the difficulty of extraction and placed more requires on the enrichment techniques. To overcome these difficulties, many sample pretreatment techniques, including liquid-liquid extraction (LLE) (Song et al., 2006) and solid-phase extraction (SPE) (Vega-Morales, Sosa-Ferrera, & Santana-Rodríguez, 2010), had been applied to purify and extract the target analytes. However, there were many disadvantages during their application such as time-consuming, laborious, specialized apparatus to automate, and solvents-consuming, which significantly restricted their applications for general usage. As an alternative method, dispersive liquid–liquid microextraction (DLLME) has drawn increasing attentions with many advantages including rapidity, simplicity, slightly matrix effect, significant timesaving, low consumption of chemical reagents, and high enrichment factor (Bidari, Ganjali, Norouzi, Hosseini, & Assadi, 2011; Li, Liu et al., 2015; Li, Lu et al., 2015). Furthermore, in this method, hydrophobic structure was endowed to NAs after fluorescent derivatization, which could increase their solubility in extraction solvent and be more conducive to DLLME. The optimization of main experimental parameters of DLLME was investigated by Box–Behnken design (BBD) of response surface methodology (RSM). Based on the optimal results, target analytes were successfully enriched in just 1.25 min, which showed the excellent properties of quickness and high efficiency.

In present study, a novel strategy for NAs screening has been developed via combining the advantages of fluorescent labeling with DLLME. To obtain the optimal extraction efficiency, several vital parameters affecting DLLME had been optimized systematically by RSM. Under the optimized conditions, the accuracy and precision of the method were evaluated, and it had been successfully applied to the determination of NAs in six food samples.

2. Materials and methods

2.1. Instruments

NAs analysis was performed using Agilent 1260 HPLC systems coupled on-line to a fluorescence detector. The further structural identification for derivatives was realized by an ion-trap mass spectrometer (Agilent Corp., Waldbronn, Germany) equipped with an atmospheric pressure chemical ionization (APCI) source. Chromatographic separation was performed on a reversed-phase ZORBAX SB-C_{18} (4.6 mm × 150 mm, 5 μm) column. A Xiangzhi TGL16 M high-speed refrigerated centrifuge was equipped for DLLME. Solutions were fully mixed by an ultrasonic cleaner (KQ3200E, Kunshan Ultrasonic Instrument, Jiangsu, China) and a VX-200 vortex mixer (Labnet, USA).

2.2. Reagents

All NAs standards including Nitrosopyrrolidine (NPYR), Nitrosodimethylamine (NDMA), Nitrosodiethylamine (NDEA), Nitrosodipropylamine (NDPA) and Nitrosodibutylamine (NDBA) were purchased from Sigma Corporation (St Louis, MO, USA). Chemical structures of the targeted analytes in this study are showed in Fig. S1. BCEC-Cl was synthesized in our laboratory (You et al., 2007). HPLC grade acetonitrile, ethanol, chloroform, methanol and acetone were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Hydrobromic acid, acetic acid and dichloromethane were supplied by Fuyu Chemical Reagent Co. (Tianjin, China). Other reagents of analytical grade were obtained from Jining Chemical Reagent (Jining, Shandong Province, China). Water was purified by a Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Chromatographic parameters

Derivatives separation was performed on a reverse-phase ZORBAX SB-C_{18} (4.6 mm × 150 mm, 5 μm) column by using linear gradient elution mode. A fluorescence detector (model G1321B, Agilent, USA) was used, and detection wavelength was set at λex/λem = 279/380 nm, respectively. The eluent A was water containing 5% ACN and eluent B was 100% ACN. The gradient elution program was as follows: 0 min = 50% B, 25 min = 100% B and 30 min = 100% B. The flow rate was constant at 1 mL min^{-1} and the column temperature was kept at 30 °C. All solvents were filtered with a 0.2-μm nylon membrane filter (Alltech, Deerfield, IL, USA) before use.

2.4. Preparation of standard solutions

BCEC-Cl solution (1.0×10^{-3} mol L^{-1}) was prepared by dissolving 3.2 mg BCEC-Cl into 10 mL acetonitrile. Individual stock solution of five NAs including NPYR, NDMA, NDEA, NDPA and NDBA (1.0×10^{-2} mol/L) was prepared by dissolving the corresponding NAs in methanol. The denitrosation reagent solution was consisted of acetic acid and hydrobromic acid at 5:1 (V/V). All reagent solutions were stored at 4 °C in a refrigerator until use.

2.5. Sample Preparation

All foodstuffs were purchased from local supermarkets in Qufu (Shandong province, China) including pickle, cured sausage, cooked sauce beef, beer, salted duck egg and fried horse mackerel. All samples were treated as follows: the prepared sample (2 g or 2 mL) was measured in centrifuge tube and then mixed with 8 mL of dichloromethane. The mixture was fully mixed in an ultrasonic cleaner for 30 min and then centrifuged at 12,000 rpm for 5 min. The supernatants were collected into a vial, evaporated to dryness by nitrogen-blow, and then re-dissolved by 0.2 mL methanol. The vial was sealed and reserved at 4 °C until analysis.