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Original Article

Stability studies of isothiocyanates and nitriles in aqueous media

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Abstract

This is the first report to assess the stability of authentic commercial isothiocyanates and nitriles incubated anaerobically at 37 °C over 24 hrs in various aqueous solutions including nutrient broths with/without the presence of bacterial cells, and to assess the stability of these isothiocyanates in different buffers of pH 7.0 (Tris-Cl, phosphate buffer saline, and citrate phosphate buffer) without bacterial cells. The presence of bacterial cells contributed to accelerating decline of both isothiocyanates and nitriles in nutrient broths with greater effects on isothiocyanates as analysed by GC-MS. Allyl nitrile was most unstable in nutrient broths and allylisothiocyanate was most unstable with cell incubation whereas erucin and allylisothiocyanate were most unstable without cells. The decline of isothiocyanates was more rapid in buffers than in deionized water and allylisothiocyanate was the most unstable in most buffers. Our results present a possible explanation for the detection of the less than expected levels of isothiocyanate production upon glucosinolate metabolism in media or buffers.

Keywords: glucosinolate, nitrile, isothiocyanate, bacterial cells, buffers

1. Introduction

Glucosinolates (GSLs) are hydrolyzed by myrosinase (a thioglucosideglucohydrolase; EC 3.2.1.147) to produce isothiocyanates (ITCs), many of which are implicated in chemopreventive effects, and/or nitriles (NITs), with less information on their bioactivities (Zhang *et al.*, 2007). Myrosinase can be found in various organisms; Cruciferous plants (Rask *et al.*, 2000), specialist aphid (Jones *et al.*, 2002), certain fungi (Ohtsuru and Hata, 1973; Sakorn *et al.*, 1999) and certain bacteria (Cheng *et al.*, 2008; Elfoul *et al.*, 2001; Rabot *et al.*, 2005). In several reports, ITC productions from bacterial metabolisms of GSL substrates in culture broths declined over a time course and the total percentage product of both ITC and NIT formation from each GSL metabolism never reached 100% (Krul *et al.*, 2002; Mullaney *et al.*, 2013; Luang-In *et al.*, 2014). It has been suggested that there might be other metabolite products that were not detected or that ITCs may be further metabolized by bacteria (Llanos-Palop *et al.*, 1995; Combourieu *et al.*, 2001). However, little knowledge is available on the stability of each ITC and NIT in culture media and different buffered conditions. Thus the aims of this study were (1) to investigate the stability of ITC/NIT in aqueous solutions i.e. in nutrient broths and buffers and (2) to test whether bacterial cells (*E. coli* VL8 used in this work) have an impact on the decline of these compounds and (3) to discuss the possible loss of these compounds in the studied solutions.

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2. Materials and Methods

2.1 Preparation of authentic ITC/NIT standards

Authentic standards, phenethylisothiocyanate (PITC), phenethyl nitrile (PNIT), benzylisothiocyanate (BITC), benzyl nitrile (BNIT), allylisothiocyanate (AITC), allyl nitrile (ANIT), erucin (ERN), erucin nitrile (ERN NIT), and iberverin (IBV) were purchased from Sigma-Aldrich Co (Dorset, UK). Each ITC/NIT was weighed in a clear glass 1.5 mL vial and dissolved in absolute ethanol to make 10 mM stock solution.

2.2 Stability of ITC/NIT standards in nutrient broths

E. coli VL8, isolated from the previous work (Luang-In *et al.*, 2014), was sub-cultured in a corresponding 5 mL nutrient broth overnight at 37°C anaerobically in an anaerobic generation system using a 2.5 L Anaerogen jar supplied with an AnaeroGen sachet (Oxoid, UK). The following day, 100 µL of overnight culture (OD_{600nm} = 0.6) was sub-cultured into 900 µL fresh nutrient broth containing 1 mM ITC or NIT standard. The control samples included 1 mL nutrient broths containing 1 mM ITC or NIT standard without *E. coli* VL8 cells. Biological triplicates were incubated for each time interval; 0, 2, 4, 6, 8, 10, and 18 or 24 hrs at 37°C in an anaerobic jar. The AnaeroGen sachet was replenished at each time interval to maintain an effective anaerobic environment. At each time interval, each sample (1 mL) was centrifuged at 16,100g for 5 min. Clear supernatants were prepared and subjected to GC-MS analysis for identification of ITC/NIT.

2.3 Stability of ITC standards in buffers

Different buffers containing each authentic ITC standard without *E. coli* VL8 cells were anaerobically incubated at 37°C in an anaerobic jar over a time course of 24 h. Three types of buffers; (i) 0.1 M citrate phosphate buffer pH 7.0, (ii) 0.1 M phosphate buffer saline (PBS) buffer pH 7.0 and (iii) 0.1 M Tris-Cl buffer pH 7.0 were tested in this work. Deionized water was used as a control. At each time interval, each sample (1 mL) was centrifuged at 16,100 g for 5 min. Clear supernatants were prepared and subjected to GC-MS analysis for the identification of ITCs.

2.4 Sample preparation for GC-MS analysis

A clear supernatant (1 mL) was mixed with the same volume of dichloromethane (DCM) on a vortex followed by centrifugation at 16,100 g for 5 min. The solution was separated into two layers. The lower layer was transferred to a new 2.0 mL Eppendorf and 0.5 g magnesium sulphate was added to remove water residues. The mixture was mixed well and centrifuged at 16,100 g for 10 min, clear supernatant was then transferred to a vial for GC-MS analysis.

2.5 GC-MS analytical conditions to detect ITC/NIT products

A Hewlett Packard 6890 series system connected to a Hewlett Packard 5973 mass selective detector was used for the GC-MS analysis. Two capillary columns (30 m x 0.25 mm i.d.; film thickness, 0.25 µm), Restek 200MS (crossbond trifluoropropyl methylpolysiloxane) for allyl nitrile (ANIT) and Agilent HP-5MS (5% Phenylmethylsiloxane) for other ITCs/NITs were used with helium as the carrier gas (split mode, 25:1; splitter inlet pressure, 40 kPa). For Restek 200MS column the temperature was held constant at 50°C for 4 min and for Agilent HP-5MS column the temperature was kept at 50°C for 5 min and ramped up to 150°C at the rate of 4°C/min for 25 min and then to 250°C at the rate of 4°C/min for 15 min with the flow at 1 mL/min, average velocity of 36 cm/s, pressure at 7.56 kPa and injection volume of 1 µL. Mass spectra were obtained by electron ionization (EI) over a range of 50-550 atomic mass units. Ion source temperature was 230°C, and the electron multiplier voltage was 70.1 eV. Mass spectra and GC-MS chromatograms of all ITCs/NITs tested were shown in Table 1 and Figure 1. ITCs/NITs were quantified using calibration curves of external standard method as previously described (Luang-In *et al.*, 2014).

Table 1. Mass spectral (MS) data of authentic ITC/NIT standards

ITC/NIT	MS spectra data m/z	T_R (min)[a]
Allyl-ITC (AITC)	99 (M^+), 72, 65	6.9
Allyl-NIT (ANIT)	67 (M^+), 52	2.8
Phenethyl-ITC (PITC)	163 (M^+), 105, 91, 77, 72	24.7
Phenethyl-NIT (PNIT)	131 (M^+), 91, 62	18.6
Benzyl-ITC (BITC)	149 (M^+), 91, 65	22.0
Benzyl-NIT (BNIT)	117 (M^+), 91, 62	15.5
Iberverin (IBV)	147 (M^+), 126, 101, 72, 61	20.6
Erucin (ERN)	161 (M^+), 115, 72, 61	23.8
Erucin nitrile (ERN NIT)	129 (M^+), 87, 61, 55	17.4

[a]Retention time at which ITC/NIT was detected by GC-MS analysis.

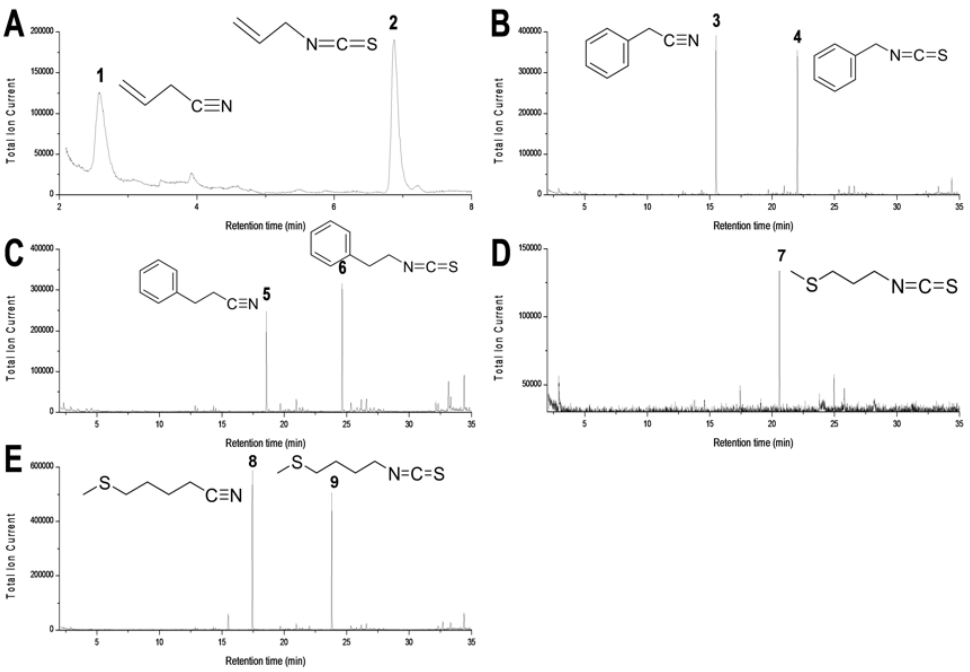

Figure 1. GC-MS chromatograms of authentic ITC/NIT standards. (A) ANIT 1, at 2.83 min and AITC 2, at 6.98 min. (B) BNIT 3, at 15.5 min and BITC 4, at 22.0 min. (C) PNIT 5, at 18.6 min and PITC 6, at 24.7 min. (D) IBV 7, at 20.6 min. (E) ERN NIT 8, at 17.4 min and ERN 9, at 23.8 min.

2.6 Statistical analysis

Significant differences in the types of ITCs in the same treatments were determined by One-Way ANOVA analysis, Post Hoc Multiple Comparison, Duncan's test at the significance level of 0.05 using SPSS software. Significant differences of ITCs/NITs between incubations with cells and without cells were determined by unpaired t-test.

3. Results and Discussion

3.1 Presence of bacterial cells accelerated decline of ITC/NIT

In this work, all ITCs were unstable in aqueous solutions tested. The levels of all ITC standards tested including AITC, BITC, PITC, IBV, and ERN declined sharply from 1.0 mM (at time 0 hrs) to 0.2-0.4 mM within 8 hrs in nutrient broths without bacterial cells (Figure 2A). These results suggest that ITCs had short lives in nutrient broths. That was because nutrient broth is composed of yeast extract, beef extract, peptone and NaCl. The first three ingredients contain amino acids, peptides with nucleophilic property such as -OH, -SH and -NH2 groups exhibiting some degrees of binding ability to ITCs (Cejpek *et al.*, 2000; Liu and Yang, 2010). The pH values were not recorded in this study, however our preliminary results from related work showed that changes in pH from 6.5 to 5.8 occurred in samples with GSLs and *E. coli* cultures from 0 to 8 h and ITC production increased till after 8 hrs the pH values remained fairly constant and ITC production started to gradually decline, but NIT production did not decline much (data not shown). From these results changes in pH values from incubations for 24 hrs did not affect ITC/NIT stability. When bacterial cells were added to those ITC standards in nutrient broths, the decline of ITCs levels was faster (from 1 mM to 0.1-0.3 mM within 8 hrs) (Figure 2B). Our results are similar to the previous reports stating that sulforaphane as another type of ITC was lost at 24 hrs incubation with bacteria, and almost 50% loss was also found without bacteria (Basten *et al.*, 2002; Lai *et al.*, 2010). It has been proposed that the composition of the media may react with sulforaphane (or any ITCs), and thus depleted ITCs in the culture broths (during bacterial fermentations). In the previous report, after 17 hrs incubation of sinigrin with *L. agilis* R16, a 45% decline in AITC concentration was observed as well as the control incubation of AITC in MRS broth without bacteria (Llanos-Palop *et al.*, 1995). This indicates a spontaneous chemical transformation of AITC, and the chemical nature of any ITC conversion product therefore remains to be investigated. Other ITCs have also been reported for their instability in aqueous solutions. For examples, PITC is unstable in aqueous media and rapidly degraded to phenethylamine at low pH (Negrusz *et al.*, 1998). Our results are in agreement with the previous findings showing that ITCs were unstable in aqueous solutions. The electrophilic character of the functional isothiocyanic group has enabled ITCs to react with some nucleophilic agents including amino, hydroxyl,

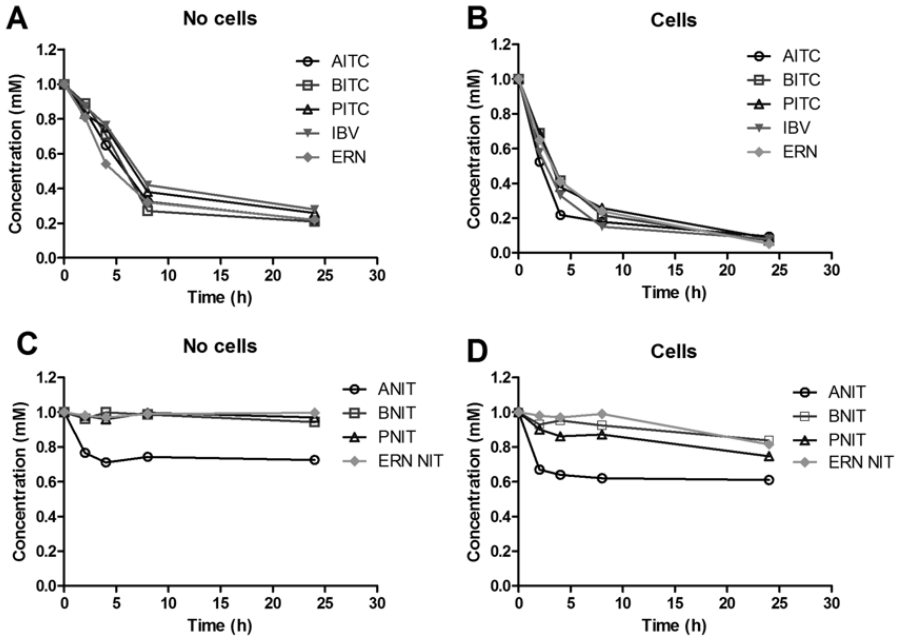

Figure 2. Stability of 1 mM ITC/NIT standards in nutrient broths with/without *E. coli* VL8 cells over 24 hrs. (A) ITC standards without cells. (B) ITC standards with cells. (C) NIT standards without cells. (D) NIT standards with cells. Values are means of triplicates.

thiol, carboxylic acids from small peptides, amino acids and water (Cejpek *et al.*, 1998) and probably flavonols to potentially generate new compounds (Kawakishi and Kaneko, 1987; Cejpek *et al.*, 2000; Luciano *et al.*, 2008). The reactions of AITC with alanine, glycine, and several peptides in model systems have been described (Cejpek *et al.*, 2000). However, it is not clear whether these authentic ITC standards were degraded to other metabolites. Further analysis such as NMR is required to answer that question.

The same test was performed to determine the stability of NIT standards including ANIT, BNIT, PNIT, and ERN NIT in nutrient broths. The levels of these NIT standards in nutrient broths without bacterial cells were rather stable over time, except for ANIT with a decline from 1.0 to 0.7 mM after 2 hrs (Figure 2C). With cells, slight declines of all NITs from 1.0 to 0.8-0.9 mM were observed while ANIT declined sharply from 1.0 to 0.68 mM within 2 hrs (Figure 2D). The fact that NITs are less labile than ITCs (Bheemreddy and Jeffery, 2007) may be a rationale for rather constant NIT levels as they do not contain a highly electrophilic carbon, as is the case for ITCs (-NCS). The time taken to obtain 50% or 25% decline in each ITC or NIT level was shortened by the presence of bacterial cells in nutrient broths (Figure 2). This suggests the possibility of interactions of ITCs or NITs with bacterial cellular components, but with less pronounced effect on NIT levels. There were significant differences in ITCs/NITs incubated with and without cells in nutrient broths at $p<0.05$ with NITs at lesser extent (Table 1). Thus, in this study, the factor influencing ITC/NIT stability includes the presence of bacterial cells in nutrient broth.

3.2 Components in buffers accelerated decline of ITCs

Since NITs were found rather stable in nutrient broths, only ITCs were further studied for their stability in buffered solutions of pH 7.0; 0.1 M citrate phosphate buffer, 0.1 M PBS buffer and 0.1 M Tris-Cl buffer over 24 h. Deionized water was used as a control. It was shown that all ITCs tested in buffered solutions and in deionized water declined over time indicating the instability of ITCs in these solutions (Figure 3). Allyl isothiocyanate declined faster than other compounds. This result is in accordance with the previous report. Combourieu *et al.* (2001) tested the stability of AITC and BITC incubated in the buffer in the absence of cells for 48 hrs at 37°C under the same conditions as those employed with bacterial incubations. Both allylamine and benzylamine were detected by 1D ^{1}H NMR spectroscopy as AITC and BITC declined indicating the high sensitivity of these ITCs to hydrolysis (Combourieu *et al.*, 2001).

In this work, ITCs declined at the fastest rates in the order of the following solutions: citrate phosphate buffer > PBS > Tris-Cl > deionized water. The presence of citrates, phosphates and chlorides in these buffers assumingly may be attributed to faster decline of ITCs when compared to water molecules alone. Although Tris-Cl seems to be a strong nucleophile due to the presence of three hydroxyl groups and one amino group, several factors besides nucleophilic property including steric hindrance, electrical effect and pKa value of the molecules in buffers can also influence how fast molecules in each buffer react with ITCs (Cejpek *et al.*, 2000). In addition, the same paper described that lower pKa

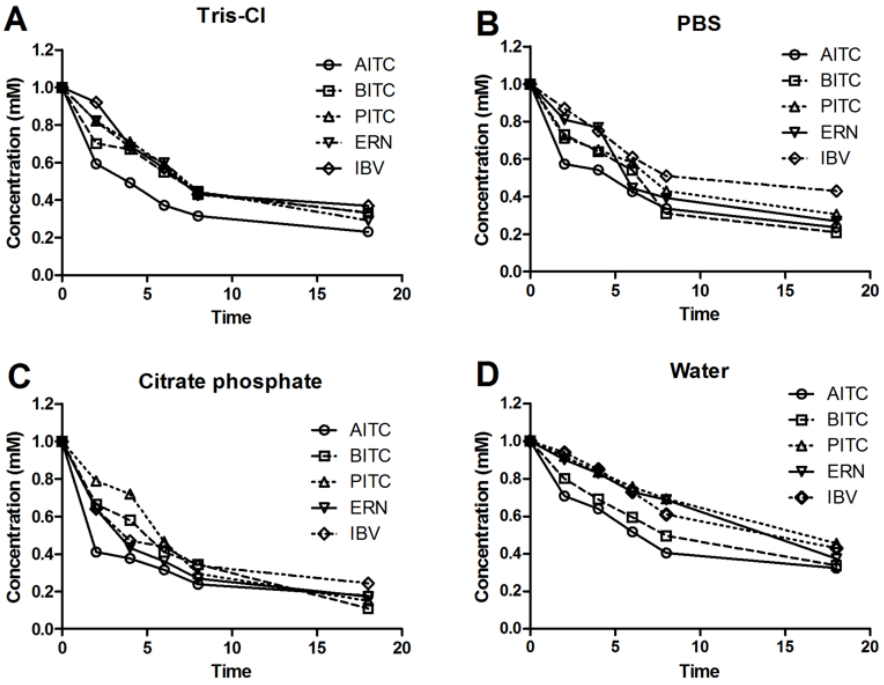

Figure 3. Stability of 1 mM authentic ITC standards in various buffers without *E. coli* VL8 cells over 24 hrs. Several aqueous buffer solutions of 0.1 M and pH 7.0 were used. (A) Tris-Cl. (B) PBS. (C) Citrate phosphate. (D) Distilled water. Values are means of triplicates.

values of molecules led to more rapid reaction with AITC resulting in quicker decline of AITC. It was found that pKa value of citrate phosphate buffer is lower than that of Tris-Cl buffer. Thus, combined effects from these factors may be responsible for our results that faster ITC declination was observed in citrate phosphate buffer > PBS > Tris-Cl > deionized water. There were significant differences in ITCs in different buffers at $p<0.05$ (Table S2), thus the molecules in the buffers affect ITC stability.

To conclude, our results indicate that the ITC instability in the media/buffer is currently underestimated. Thus, the total percentage products obtained from GSL metabolism represented in any previous works may be underestimated. To conclude, scientists should be more cautious about interpreting the results regarding ITCs due to their instability in media and buffers.

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