Chemosphere 212 (2018) 393–399

Contents lists available at ScienceDirect

Chemosphere

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

Byproducts of aqueous chlorination of equol and their estrogenic potencies

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HIGHLIGHTS

- Behaviors of equol in the chlorination disinfection process were investigated.
- Chlorination mechanisms of equol were provided.
- Chlorinated equols can elicit similar estrogenic activity to equol.

ARTICLE INFO

Article history:
Received 30 May 2018
Received in revised form
8 August 2018
Accepted 19 August 2018
Available online 20 August 2018

Handling Editor: Maruya

Keywords:
By-product
Chlorination
Chlorinated equols
Estrogenic activity
Drinking water

ABSTRACT

While the phytoestrogen metabolite equol has been reported to exist in surface water, its behavior in drinking water treatment plants remains unrevealed. In this study, eight products including four chlorinated equols (monochloro-equol, dichloro-equol, trichloro-equol, and tetrachloro-equol) were identified in an aqueous chlorinated equol solution by UHPLC-quadrupole-orbitrap-HRMS. Two main pathways of chlorination reaction are proposed: (1) chlorine-substitution reactions on the aromatic ring and subsequent dehydration to form the chlorine-substituted equols, and (2) break-up of the heterocyclic ring with oxygen followed by oxidation of aldehyde to carboxyl. The human estrogen receptor (hER) activating activity for monochloro-equol ($EC_{50} = 3456$ nM) and dichloro-equol ($EC_{50} = 2456$ nM) were slightly stronger than that of equol ($EC_{50} = 3889$ nM). This is the first report on the behavior of equol in drinking water chlorination, which provided an important information on the risk assessment of equol in drinking water.

1. Introduction

Some phenolic chemicals have received attention for their ability to mimic naturally occurring estrogens and interfere with endocrine systems of wildlife and human beings (Krishnan et al., 1993; Routledge and Sumpter, 1996; Miller et al., 2001; Hu and Aizawa, 2003; Kudo et al., 2006; Wang et al., 2016). Of these chemicals, naturally occurring phytoestrogens are currently receiving significant attention due to their multiple health effects (Knight and Eden, 1996; Kuiper et al., 1998). These chemicals are readily available in the diet, particularly in soy products, of which the major class of phytoestrogens is isoflavones. Equol is a bacterial metabolite of phytoestrogen daidzin or daidzein, one of the principal isoflavones in soybeans.(*Glycine max*) (Setchell et al., 2002; Hoerger et al., 2009). Some studies have shown that the estrogenic activity of equol is more potent than its precursors, daidzin and daidzein, and its strong estrogenic activity has been observed in animals (Wang et al., 2016; Setchell et al., 2002; Tang and Adams, 1980). Equol has also been reported to have antiandrogenic property, and could induce intersex incidence in male medaka at environmentally relevant concentrations (Wang et al., 2016). In human, it has been reported that exposure to equol could induce DNA damage in sperm and decrease sperm motility (Anderson et al., 2003; Toshima et al., 2012).

Equol is exclusively a product of intestinal bacterial metabolism,

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https://doi.org/10.1016/j.chemosphere.2018.08.103
0045-6535/

and is relatively stable once formed (Setchell et al., 2002). The transformation from daidzin to equol can occur in humans. It has been estimated that about 25–30% western adults and about 50–80% asian adults are equol producer, which means they excrete equol in urine after taking soy foods (Setchell and Cole, 2006; Setchell et al., 1984; Kelly et al., 1995; Karr et al., 1997; Lampe et al., 1998; Rowland et al., 2000). Notably, this transformation usually occurs in the intestine of domestic animals (Lundh, 1995; Setchell and Clerici, 2010), and excreta of livestock have been reported to be a main source of equol in the aqueous environment since soybean meal is the primary dietary protein source for domestic animals (Hoerger et al., 2009; Furuichi et al., 2006; Yost et al., 2013). The manure of domestic animals is often applied onto crop fields and pastures for its nutrient value, and therefore equol can enter aquatic environments through runoff from pastures or fields. It has been reported that equol occurred with concentrations of 34–121 ng/L in runoff from a pasture containing 43% red clover (*Trifolium pratense*) (Burnison et al., 2003). Due to discharge of livestock waste and runoff from these sources, equol has been detected in rivers throughout Switzerland (0.6–524 ng/L) (Hoerger et al., 2009; Erbs et al., 2007). While these previous papers have highlighted the occurrence of equol in river body, there has been no study on its fate through drinking water treatment processes (Snyder and Trenholm, 2008). It is well-known that phenolic compounds can easily react with hypochlorite, which is widely used as a disinfection agent in drinking water treatment plants (DWTPs), as exemplified by the rapid chlorination of 4-nonylphenol (NP), bisphenol A (BPA), and 17β-estradiol (17β-E2) (Hu and Aizawa, 2003; Hu et al., 2002a, 2002b). Thus, as one of the class of phenolic chemicals, it is of interest to investigate the fate of equol during chlorination, the presence of its potential chlorinated byproducts, as well as whether the byproducts still pose estrogenic activity.

In this study, the products of equol in aqueous chlorination were identified by an ultra-high performance liquid chromatography (UHPLC)-quadrupole-orbitrap- high resolution mass spectrometry (HRMS) method, and two pathways were proposed. Major byproducts, 3 chlorinated equols, monochloro-equol (MCequol), dichloro-equol (DCequol), and trichloro-equol (TCequol), were synthesized, and their estrogenic activities were also determined using a yeast two-hybrid assay.

2. Materials and methods

2.1. Standards and reagents

Equol and the surrogate standard equol-d_4 were purchased from Apollo Scientific Ltd. (Stockport, UK) and Toronto Research Chemicals Inc. (Toronto, Canada), respectively. MCequol, DCequol, and TCequol were synthesized and purified using the methods reported previously (detailed information in the Supplementary Data) (Hu and Aizawa, 2003; Hu et al., 2002a, 2002b; Fan et al., 2013). All commercial and synthesized compounds have a purity of over 95%. Sodium sulfite, sodium thiosulfate, sodium hydroxide, magnesium sulfate, hydrochloric acid (36–38 wt/%), phosphoric acid, and sodium hypochlorite solution (>8% available chlorine) were analytical grade and purchased from Beijing Chemical Works (Beijing, China). Methanol, acetonitrile and ethyl acetate were HPLC grade and purchased from Fisher Chemical Co. (Beijing, China). HPLC-grade water was prepared using a Milli-Q RC apparatus (Millipore, Bedford, MA, USA). Oasis HLB (500 mg, 6 mL) cartridges were purchased from Waters (Waters, Milford, MA, U.S.).

2.2. Computational chemistry

Scigress (ultra version 2.2.0, Fujitsu, USA) was used to obtain optimum geometries and partial atomic charges. The AM1 parameter was used to optimize stable and transition-state structures.

2.3. Chlorination procedures

Chlorination experiment was carried out using previously reported method (Hu et al., 2002b; Fan et al., 2013), and the details are shown in the Supplementary Data.

2.4. UHPLC-quadrupole-orbitrap-HRMS analysis

Identification of chlorinated byproducts of equol was performed by an UltiMate 3000 system (Thermo Fisher Scientific, San Jose, CA, USA) with a quadrupole-orbitrap-high resolution mass spectrometer. Separation of equol and its chlorinated byproducts was achieved by using an Acquity UPLC BEH C18 column (100 × 2.1 mm × 1.7 μm) (Waters, Milford, MA, U.S.) maintained at 40 °C. Mobile phases A and B were ultrapure water and acetonitrile, respectively. A linear gradient was: 0 min, 10% B; 0.5 min, 40% B; 5 min, 75% B; 5.5 min, 98% B; 8 min, 100% B; 9.5 min, 100% B and 11 min, 10% B. The injection volume and the flow rate were 5 μL and 300 μL/min, respectively. Detailed information of mass spectrometer is shown in the Supplementary Data.

2.5. Estrogenic activity of products

In order to assess the human estrogen receptor α (hERα) activating activity for chlorinated equols, 3 major chlorinated equols in the aqueous chlorination experiments (MCequol, DCequol, and TCequol) were synthesized (detailed synthesis process is described in supplementary materials) and dissolved in dimethyl sulfoxide (DMSO). The hERα activating activity of these 3 chlorinated equols together with equol were evaluated using a yeast two-hybrid assay with hERα and coactivator TIF2 according to a method described previously (Hu et al., 2002a). 17β-E2 was used as the positive control.

2.6. Statistical analysis

Thermo Scientific Sieve software (Thermo Fisher Scientific, San Jose, CA, USA) was used to conduct a comprehensive search of byproducts and speed up the process, by simultaneously comparing MS spectra to find differentially expressed feature.

3. Results and discussion

3.1. Identification of byproducts

For the screening of potential byproducts of equol using UHPLC-quadrupole-orbitrap-HRMS analysis, the chromatograms in total ion current (TIC) mode of chlorinated equol solutions at different chlorination time were compared with that before chlorination (Fig. 1). After 10 min of reaction, equol concentration was decreased from 500 to 44.9 μg/L, and meanwhile several new peaks were observed in TIC chromatograms (Fig. 1). Sieve analysis was used to compare TIC chromatograms at 0 min and 10 min and screen possible byproducts. A total of 8 products were screened, and their retention times, elemental compositions, experimental and theoretical exact masses, and relative mass measurement errors (Δm) are listed in Table 1, while Fig. 2(a) and (b) show the chromatograms and mass spectra of all byproducts. All of the eight