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3-Chlorotyrosine formation in ready-to-eat vegetables due to hypochlorite treatment and its dietary exposure and risk assessment

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ABSTRACT

Washing of iceberg lettuce with HOCl solutions in concentrations ranging from 1.41 to 141 mg/L resulted in 0.69 to 2.05 μg 3-chlorotyrosine/g vegetable. As also six commercial ready-to-eat iceberg lettuces from different producers contained 3-chlorotyrosine from 1.00 to 2.24 μg/g vegetable, a total of 122 ready-to-eat vegetable samples purchased in Belgian supermarkets were further screened for their 3-chlorotyrosine content. 3-chlorotyrosine was detected above the detection limit (0.19 μg/g sample) in 97, 24 and 14% of the lettuce mixes, vegetable mixes and frozen vegetables, respectively. In combination with consumption data of ready-to-eat vegetables by Belgian and Spanish consumers, a quantitative exposure assessment was performed, exemplifying a lower and higher ready-to-eat vegetables consuming population. Exposure to 3-chlorotyrosine from the frozen vegetables and vegetable mixes was lower compared to the lettuce mixes due to the combination of lower contamination and lower consumption. 3-chlorotyrosine exposure via lettuce mixes could be considered as a public health concern, especially in higher consuming populations represented by the Spanish population, with 17% of consumers (>4.2 million people) and 8.5% of the total population (>2,6 million people) exceeding the threshold of toxicological concern.

1. Introduction

In recent years, consumption of ready-to-eat (RTE) vegetables has continuously increased in Europe and the US as a result of consumer demand for fresh, healthy, convenient and easy-to-prepare foods (Ulrike, Werner, & Karin, 2016; Weng et al., 2016). Some of the microbial pathogens associated with vegetables include *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes*, *Shigella* spp. and Norovirus (FAO/WHO, 2008b). The 2011 WHO-Guidelines established a guideline value of ≥0.5 mg/L up to 5 mg/L of free chlorine ($HOCl/OCl^-$, after at least 30 min contact time at pH < 8.0) for effective disinfection of water (WHO, 2011). In order to reduce microbial contamination on vegetables, chlorine can be added to wash water as a decontamination agent. When the bacterial load of food is to be reduced during washing, higher concentrations are needed because chlorine is reacting with the organic material, thus lowering the actual concentrations present (Van Haute, Sampers, Holvoet, & Uyttendaele, 2013). Chlorine solutions (50–200 mg HOCl/L, pH < 8) prepared from sodium hypochlorite (NaOCl) are the most used decontamination agent for washing RTE vegetable, due to their competitive prices, as well as easy application and efficiency, with a typical reduction of <2 logs CFU/g of the bacterial load present on vegetables (Van Haute et al., 2013; Gomez-Lopez, 2012).

However, there is a concern about the environmental and human health risks associated with the possible formation of disinfection by-products (DBPs), including trihalomethanes (THMs) and haloacetic acids (HAAs) from chlorine when applied on foods or water in contact with foods, due to interaction of chlorine with organic material. Although those two major groups of carbonaceous DBPs were intensively studied during water treatment, their interactions with food matrices

Abbreviations: BW, body weight; DBP, disinfection by-product; HAAs, haloacetic acids; HOCl, hypochlorous acid; LOD, limit of detection; NaOCl, sodium hypochlorite; OCl^-, hypochlorite ion; RTE, ready-to-eat; SD, standard deviation; THMs, trihalomethanes; TTC, threshold of toxicological concern.

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were much less investigated (Cardador & Gallego, 2012; FAO/WHO, 2008a; McDowell et al., 2007). Several studies determined the THMs formation when vegetables were washed in chlorinated water, such as fresh-cut butterhead lettuce (up to 50 mg/L HOCl), baby spinach leaf (2–4 mg/L HOCl), carrot (200 mg/L HOCl) (Klaiber, Baur, Wolf, Hammes, & Carle, 2005; Gomez-Lopez, Marin, Medina-Martinez, Gil, & Allende, 2013; Van Haute et al., 2013). These researchers collectively suggested that under those experimental conditions, chlorine sanitizers would not cause any risk of disinfection by-products because THMs formation was negligible. Nevertheless, partially because of the possible generation of DBPs, the use of chlorine, as a decontamination agent in RTE vegetable washing, is prohibited in some European countries such as Germany, Switzerland, the Netherlands, Denmark and Belgium (non-exhaustive list). In those countries, chlorine can only be applied in low concentrations to disinfect the wash water (up to 0.25 mg/L HOCl) but not to decontaminate the vegetables (Rico, Martin-Diana, Barat, & Barry-Ryan, 2007; Artes, Gomez, Aguayo, Escalona, & Artes-Hernandez, 2009). In Spain however, chlorine use is allowed during the RTE vegetable washing process up to recommended concentration amounting 100 mg/L HOCl (pH 6.5, 1 min) (Allende, Selma, Lopez-Galvez, Villaescusa, & Gil, 2008).

It is described that 3-chlorotyrosine is formed when hypochlorous acid (HOCl) reacts with tyrosine residues in proteins (Domigan, Charlton, Duncan, Winterbourn, & Kettle, 1995; Kettle, 1996). Moreover, it is known that myeloperoxidase, present in neutrophils, catalyses the formation of HOCl from the Cl^- ion and H_2O_2. Thus HOCl converts tyrosine in both the free and bound forms into chlorinated tyrosine, also in physiological conditions. Thus 3-chlorotyrosine serves as a useful biomarker of protein damage by myeloperoxidase and has been observed in a variety of pathological processes such as atherosclerosis, glomerulonephritis, cystic fibrosis, rheumatoid arthritis, asthma, and Alzheimer's disease (Bergt et al., 2004; Buss et al., 2003; Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2006; Davies, Fu, Wang, & Dean, 1999; Robaszkiewicz, Bartosz, & Soszynski, 2011).

The potential use of 3-chlorotyrosine as an indicator for the treatment of fish fillets with hypochlorite has recently been reported (Bao Loan, Devlieghere, Van Hoeke, & De Meulenaer, 2015). It should be emphasized that this was the first study to introduce a reliable marker to detect the use of chlorine disinfectants in foods. Accordingly, the present study aimed to extend the previous work by studying the formation (based on lab simulations of the washing process) and occurrence of 3-chlorotyrosine in RTE vegetables (based on a screening of commercially RTE vegetables, cold and frozen on the Belgian market). In addition, the dietary exposure via RTE vegetable consumption for Belgian and Spanish populations, exemplifying Northern European lower RTE vegetable consumption and Mediterranean higher consumption patterns, was estimated. An evaluation of the potential risk was performed, comparing the calculated exposures with the threshold of toxicological concern (TTC).

2. Materials and methods

2.1. Chemicals

Pyridine and trifluoroacetic acid were of analytical grade and obtained from Merck (Darmstadt, Germany). All other chemicals and reagents were of analytical or HPLC grade and obtained from Sigma Aldrich (Bornem, Belgium) and Chemlab (Zedelgem, Belgium).

2.2. NaOCl treatments of iceberg lettuce

The concentration of stock NaOCl solution was determined by an iodometric titration, and then the appropriate working solutions (1.41 to 141 mg HOCl/L) were made. The pH of each NaOCl solution was adjusted to 6.00 with hydrochloric acid. Iceberg lettuce (*Lactuca sativa* var. *capitata* L.) was purchased from a local supermarket (Delhaize, Ghent, Belgium). Both the outer leaves and the inner core were removed, and the vegetable was cut in 1 cm shreds by a sharp knife. A 100 g of cut iceberg lettuce was immersed in 1 L of NaOCl solution at room temperature and shaken at 150 rpm for 5 min on an orbital shaker (Ika, Staufen, Germany). Afterwards, the lettuce was rinsed in tap water for 1 min and centrifuged for 1 min in a manual kitchen centrifuge (Zyliss, Bern, Switzerland). For each NaOCl treatment point, three independent experiments were conducted.

2.3. Sampling plan for ready-to-eat vegetables

A total of 122 vegetable samples were purchased between June 2014 and November 2014 and analysed immediately after sample collection. RTE vegetables were divided into three groups, including frozen vegetable, fresh vegetable mix and fresh lettuce mix. The samples including brand named and private label products, were purchased from five supermarket chains located in Ghent, Belgium and were analysed on the day of purchase.

2.4. Determination of total 3-chlorotyrosine

The internal standard 3-[$^{13}C_6$] chlorotyrosine was obtained by chlorinating L-[$^{13}C_6$] tyrosine with NaOCl and purifying via reverse-phase HPLC (Thermo Electron Corporation, Waltham, MA, USA), with monitoring of the absorbance at 276 nm (Hazen, Crowley, Mueller, & Heinecke, 1997). Then, the total 3-chlorotyrosine of ground vegetable was analysed by a stable isotope assay using Agilent 5975 GC/MSD (Agilent Technologies, Germany) as described earlier (Bao Loan et al., 2015). Briefly, a known amount of internal standard (1.8 μg/mL) was added to all the oxidized samples before hydrolysis with 12 N hydrochloric acid (24 h at 110 °C). The amino acid fraction was isolated using cation-exchange column chromatography (DOWEX 50WX8, 200–400 mesh) and dried under nitrogen. Amino acids were dissolved in water:ethanol:pyridine (60:32:8 v:v:v) and derivatized with ethyl chloroformate. The samples were extracted with dichloromethane containing 2% ethyl chloroformate. An aliquot was taken from the dichloromethane layer, dried over Na_2SO_4 and injected to an Agilent 5975 GC/MSD (Agilent Technologies, Germany). The concentrations of 3-chlorotyrosine were calculated from the observed ion current ratios from *m/z* 226/232 and 298/304 for 3-chlorotyrosine and its isotopic labelled internal standard respectively. Validation experiments showed that the optimized method presented good linearity ($R^2 > 0.997$) in a range from 0 to 100 μg 3-chlorotyrosine/mL (equal to 0–72 μg 3-chlorotyrosine/g sample), satisfactory precision (CV < 4.2%), and high recovery (96–110%). The detection limit (LOD) proved to amount 0.19 μg 3-chlorotyrosine/g sample and the limit of quantification (LOQ) was 0.38 μg 3-chlorotyrosine/g sample.

2.5. Consumption data

Fresh vegetable consumption data were obtained from the survey conducted from 2010 to 2011 in two European countries, Belgium and Spain (Jacxsens et al., 2015). These two countries represent Northern versus Southern Europe and have two distinct eating cultures. Daily intake was collected from 556 respondents for Spain and 1522 for Belgium, aged between 18 and 65 years. The usual or daily intake of each fresh vegetable consumed per day (expressed as g consumed/day) was calculated for each respondent by multiplying the frequency of consumption with the portion size of that commodity. The outcome was divided with the reported average body weight (BW) of 60 kg (Jacxsens et al., 2015). The consumption dataset (g ready-to-eat vegetables/kg BW/day) from this study was applied for the further exposure calculations of RTE vegetable mix and RTE lettuce mix.