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Ecotoxicological evaluation of caffeine and its derivatives from a simulated chlorination step

Armando Zarrelli [a,*], Marina DellaGreca [a], Maria Rosaria Iesce [a], Margherita Lavorgna [b], Fabio Temussi [a], Luigi Schiavone [b], Emma Criscuolo [b], Alfredo Parrella [b], Lucio Previtera [a], Marina Isidori [b]

[a] Udr Napoli 4 Consortium INCA, Dipartimento di Scienze Chimiche, Università Federico II di Napoli, Via Cintia 4, I-80126 Naples, Italy
[b] Dipartimento di Scienze e Tecnologie Ambientali, Biologiche e Farmaceutiche, Seconda Università di Napoli, Via Vivaldi 43, I-81100 Caserta, Italy

HIGHLIGHTS

- Processes of chlorination in the treatment of raw water
- STP chlorination of caffeine
- 8-Chlorocaffeine, the most toxic compound in the long term on rotifers
- *N*,*N*′-dimethylurea toxic to algae
- Antigenotoxicity of caffeine and two derivatives

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ABSTRACT

Caffeine is ubiquitous in surface and ground waters and it has been proposed as a marker of the anthropogenic pressure on the environment. Sewage treatment plants based on active sludges seem to be not very efficient in its complete removal from effluents while additional disinfection treatments by chlorination are able to do it. In a simulation of the chlorination step herein we report that caffeine is transformed in six by-products: 8-chlorocaffeine, 1,3-dimethyl-5-azabarbituric acid, *N*,*N*′-dimethylparabanic acid, *N*,*N*′-dimethyloxalamide, *N*-methylurea and *N*,*N*′-dimethylurea. The ecotoxicity of caffeine and identified compounds was evaluated on the rotifer *Brachionus calyciflorus* and the alga *Pseudokirchneriella subcapitata* to assess acute and chronic toxicity, while SOS Chromotest and Ames Test were used to detect the genotoxic potential of the investigated compounds. Moreover, we assessed the possible antigenotoxic effect of the selected compounds using SOS Chromotest after co-incubation with the standard genotoxin, 4-nitroquinoline 1-oxide. Chronic exposure to these compounds caused inhibition of growth population on the rotifer while the algae seemed to be unaffected. Results indicated that caffeine (**1**), *N*,*N*′-dimethyloxamide (**4**) and *N*,*N*′-dimethylparabanic acid (**5**) reduced β-galactosidase activity in comparison with positive control, both at 1 and 5 mg/L of 4-NQNO with a good dose–response.

1. Introduction

Caffeine is the main alkaloid of coffee plants and it is also present in tea leaves, in cacao pods and in about other 60 species as the ilex plant. A world release of about 100 million kg of caffeine (ICO) is estimated.

As result, caffeine, and in some cases its metabolite paraxanthine, has been found worldwide in surface and ground waters that is why caffeine is the most commonly proposed anthropogenic marker in surface waters and is a life-style compound just as nicotine (Buerge et al., 2003).

Caffeine has been found in waters of Wascana Creek (Waiser et al., 2011), Ontario and Lake Erie in Canada (Metcalfe et al., 2003), Llobregat and its tributaries (Huerta-Fontela et al., 2007, 2008) in ground waters near Barcelona, Spain (Teijon et al., 2010; Albaiges et al., 1986), and 72 surface waters in the United States (Focazio et al., 2008; Kolpin et al., 2004).

The presence of caffeine in water bodies reflects the fact that this compound is not completely removed in many sewage treatment plants. Martinez-Bueno et al. (2011), in a study run on a plant located in the southeast of Spain, have detected mean concentrations of caffeine and its metabolite paraxanthine in the influent (67.1 and 49.7 μg/L) and in the effluent (16.7 and 11.4 μg/L); concentrations of 135 μg/L of caffeine in the influent and 52 μg/L in the effluent (Yuo et al., 1999) have been found in the Lanzhou plant in China. The presence of caffeine has also been reported in the effluents of plants of the towns of Cantabria, Almeria (Gomez et al., 2010) as well as in the Zagreb plant in Croatia (Grung et al., 2007) and the Montreal plant in Canada (Blaise et al., 2006).

Caffeine removal is incomplete in sewage treatment plants which use conventional treatments based on activated biological sludges

* Corresponding author.
E-mail address: zarrelli@unina.it (A. Zarrelli).

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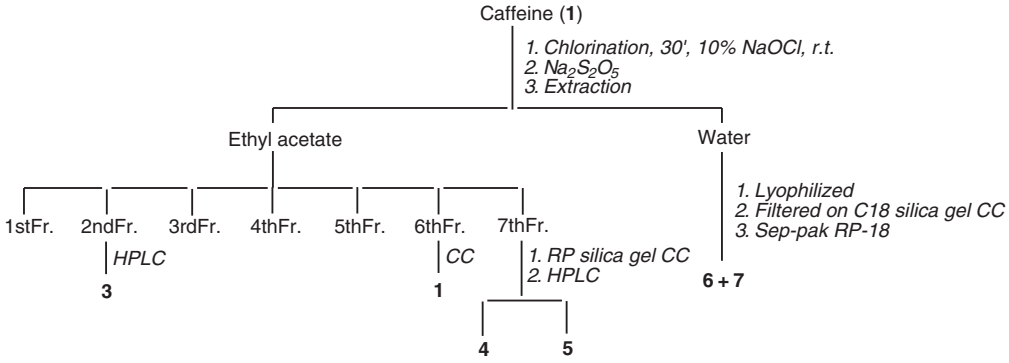

Scheme 1. Isolation of the different identified compounds.

(Yang et al., 2011). On the contrary, high or complete removal of caffeine has been reported by Huerta-Fontela et al. (2008), Stackelber et al. (2004) and Boleda et al. (2011) when chlorination is used as additional treatment in drinking water treatment plants. However Glassmeyer and Shoemaker (2005) reported no apparent change in caffeine even after 48 h of contact with hypochlorite via benchtop experiments. Sodium hypochlorite is the most widely used disinfectant because of its efficiency and cheapness. Despite these advantages, hypochlorite can react with organic matter to yield a wide variety of by-products (Christman et al., 1983; Helmer, 1999; Zarrelli et al., 2012; DellaGreca et al., 2009) that have been associated with adverse health effects (Clark et al., 2001; Nieuwenhuijsen et al., 2000; Nakamura et al., 2008; Sekizawa and Onodera, 2010).

Caffeine (**1**) chlorination by hypochlorite has been already investigated by Gould and Hay (1982) and Gould and Richards (1984). The authors studied the kinetics of the reaction in the pH range 5–9 and identified some by-products by GC–MS analysis. The authors concluded that the reaction is a quite slow process and that the reaction rate is strongly pH-dependent and it is influenced by caffeine — hypochlorite ratio more than the absolute concentration of reactants.

Some studies report the toxicological effects of caffeine on different freshwater species and results show that caffeine does not seem to be a threat for the aquatic environment at least in short term exposure due to the high concentrations required to determine a significant effect (Calleja et al., 1994; Moore et al., 2008). However, the continuous introduction of caffeine may cause subtle effects acting as a pseudo-persistent pollutant for its continuous release in the environment and little is known about its chronic effects (OECD, 2002). Furthermore, several studies reported the genotoxic potential and mutagenic potential of caffeine on animal models and results are inconsistent and inconclusive (Choundhury and Palo, 2004) even if caffeine showed antigenotoxic activity towards known genotoxins (Woziwodzka et al., 2011).

Then, for the worldwide presence of caffeine in the aquatic systems, it is important to evaluate the environmental impact of caffeine transformation products, since only the ecotoxicity of one by-product, *N,N′*-dimethylurea, is known (OECD, 2003). Therefore, the main aim of this work was to test caffeine and its derivatives obtained by a reaction between caffeine and sodium hypochlorite mimicking the chlorination step. We investigated the acute and chronic toxicities of caffeine and its six transformation products on organisms from two levels of the freshwater aquatic chain, the rotifer *Brachionus calyciflorus* and the alga *Pseudokirchneriella subcapitata*. Furthermore, the possible mutagenesis and genotoxicity of these compounds were performed using the Ames Test on *Salmonella typhimurium* and the SOS Chromotest on *Escherichia coli* PQ37, respectively, to detect point mutations and the induction of SOS DNA repair system. The SOS Chromotest on caffeine and some derivatives, previously co-incubated with the standard genotoxin 4-nitroquinoline 1-oxide, was performed to establish the possible antigenotoxic effect.

2. Material and methods

2.1. Apparatus

HPLC was performed on a Shimadzu LC-10AD by using UV–VIS detector Shimadzu RID-10A. A semipreparative HPLC was performed using a RP18 (LiChrospher 10 μm, 250 × 10 mm i.d., Merck) column with a flow rate of 1.2 mL min^{-1}. Column chromatography (CC) was carried out on Merck Kieselgel 60 (230–400 mesh). Electronic impact mass spectra (EI-MS) were obtained with a QP-5050A (Shimadzu) EI 70 eV spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian INOVA-500 NMR instrument (^{1}H at 499.6 MHz and ^{13}C at 125.62 MHz), referenced with deuterated solvents ($CDCl_3$ or CD_3OD) at 25 °C. Proton-detected heteronuclear correlations were measured using a gradient heteronuclear single-quantum coherence (HSQC), optimized for $^{1}J_{HC} = 155$ Hz, a gradient heteronuclear multiple bond coherence (HMBC), optimized for $^{n}J_{HC} = 8$ Hz.

2.2. Chlorination procedure and product isolation

Caffeine **1** (1 g) dissolved in MilliQ water (1 L) was treated for 30 min with 10% hypochlorite (molar ratio 1:6; concentration spectroscopically determined λ_{max} 292 nm, ε 350 $dm^3\,mol^{-1}\,cm^{-1}$) at room temperature. The pH of the solution, measured by a pH-meter at 5 min intervals, rises from the initial pH 6.8 to 9.3, after 5 min, and it remained at this value in the residue time. After 30 min, the reaction was quenched by sodium sulfite excess and lyophilized. The residue was distributed between ethyl acetate and water.

The ethyl acetate fraction (689 mg) was chromatographed on silica gel CC using a gradient of dichloromethane:acetone, to give seven fractions (Scheme 1). The 2nd (67 mg), eluted with dichloromethane, was purified by HPLC using a reversed phase column and eluting with 2:1:7 methanol:acetonitrile:water, to give compound **3** (15 mg). The 4th (59 mg), eluted with 99:1 dichloromethane:acetone, contained

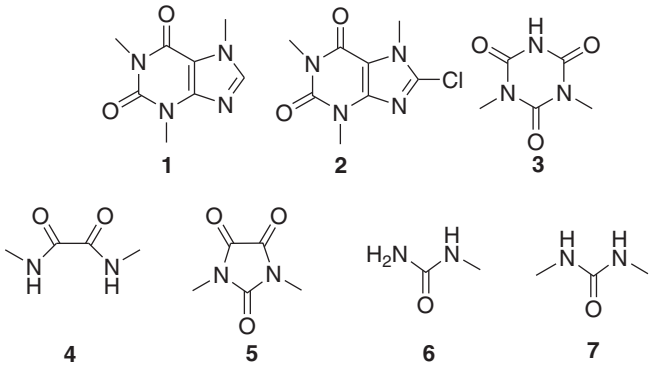

Fig. 1.