

## Study on endocrine disruptors levels in raw milk from cow's farms: Risk assessment

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### Abstract

Diet represents the primary route for human exposure to bisphenol A (BPA). As endocrine disruptor (ED), BPA has raised concerns about its adverse effects on human health. Therefore, EFSA recommended a tolerable daily intake (t-TDI) of 4 µg/kg bw/day and the EU Regulation n. 2018/213 fixed a specific migration limit (SML) of 0.05 mg/kg for BPA in food from plastic materials intended to come in contact with food. BPA could be present in milk due to environmental contamination, and also as a result of the migration from contact materials used during milking and storage. Considering the widespread consumption of milk and milk products, the contamination of dairy products is a matter of public health concern. The aim of the study was to investigate the BPA contamination levels of raw cow's milk from two farms located in Campania region, Italy. The milk samples (n=22), weekly collected from the cooling tank, were analyzed using liquid chromatography with fluorescence detection. In raw milk from both farms, preliminary results showed the occurrence of BPA levels lower than the SML limit, ranging from not detected to 2.34 µg/L. The consumer exposure, calculated considering a hypothetical raw milk consumption and three possible scenarios, was below the t-TDI. Despite the low levels of exposure through milk consumption, low doses can have lasting effects during human development. Thus, new approaches, methods, and plans should be applied to monitor ED contamination, such as BPA and other pollutants, and to assure milk safety.

### Introduction

Many chemical compounds, recognized as endocrine disruptors (EDs), are capable of mimicking the effect of natural hor-

mones, interacting with their receptors and inducing the activation of the same physiological pathways in the living organisms (US EPA, 2000). The environmental presence of EDs is mainly linked to anthropogenic activities, and the diet represents the primary route for human exposure. Complex mixtures of EDs can enter the food chain and accumulate in living organisms higher up the food chain such as humans (Diamanti-Kandarakis *et al.*, 2009).

Among the different ED compounds, Bisphenol A (BPA), 2,2-bis(4-hydroxyphenyl) propane, is an industrial chemical widely used in food packaging (Tzatzarakis *et al.*, 2017). BPA tends to migrate from can containers into foods, especially at elevated temperatures, and may be detected in human biological matrices, including serum, urine, amniotic fluid, follicular fluid, placental tissue, breast milk and umbilical cord blood (Cirillo *et al.*, 2015; EFSA 2015; Siddique *et al.*, 2016). It is able to interfere with the endocrine system inducing negative impacts on sexual development, growth, stress response, insulin production, sexually dimorphic behavior, reproduction and fetal development, and immune functions (Cirillo *et al.*, 2015; Legler *et al.*, 2015; Bansal *et al.*, 2018). Therefore, a temporary tolerable daily intake (t-TDI) of 4 µg/kg bw/day was recommended for BPA by EFSA. In addition, the EU Regulation No. 2018/213 fixed a specific migration limit (SML) of 0.05 mg/kg in food from plastic materials intended to come in contact with food, while the EU Regulation No. 321/2011 imposed not to use BPA in the manufacture of baby bottles (European Commission, 2011; EFSA, 2015; European Commission, 2018). As chemical hazard, BPA may enter the dairy supply chain through various sources, as environmental contamination, uptake, and accumulation by animals (Grumetto *et al.*, 2013). Being fat-soluble, it may be stored in adipose tissue, secreted in cow's milk fat and accumulated in fat dairy products (Georgescu and Georgescu, 2013). In addition, BPA may be introduced in milk at the farm during milking from plastic parts of the milking machines, through the transfer from bulk milk to storage tanks, or also at the dairy company during thermal treatments and packaging. In fact, the diffusion coefficient of plastic components increases as temperature increases, and the BPA migration tends to increase when extreme temperature fluctuations (for example, from freezer temperatures to ultra-high temperatures) occur during the milk treatments (Tehrany and Desobry, 2004; Danaher and Jordan, 2013; Galloway, 2015; Mercogliano and Santonicola, 2018).

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Key words: Raw milk, Bisphenol A, Risk assessment.

Contributions: the authors contributed equally.

Conflict of interest: the authors declare no potential conflict of interest.

Funding: none.

Received for publication: 3 July 2018.

Revision received: 12 October 2018.

Accepted for publication: 16 October 2018.

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Italian Journal of Food Safety 2018; 7:7668

doi:10.4081/ijfs.2018.7668

Considering the widespread consumption of milk products by infants, children, and adults, the occurrence of quantifiable levels of BPA represents a matter of public health concern (Niu *et al.*, 2017). Several studies reported the occurrence of BPA levels in dietary milk, but data on the occurrence in raw milk at the farm are scarce, and further information on the contamination pathways in milk is needed (EFSA 2015). The aim of the research was: i) to study the occurrence of BPA, as ED, in raw milk, and ii) to evaluate the risk assessment considering different scenarios of dietary exposure through a hypothetical consumption of raw milk at the farm.

## Materials and Methods

### Reagents and materials

BPA standard (minimum purity of 99%) was purchased from Sigma-Aldrich (Dorset, UK). Methanol (for high-performance liquid chromatography, HPLC), acetonitrile (for HPLC), and water plus (for HPLC) were provided by Carlo Erba (Milan, Italy). The SPE cartridge (Chromabond C18, Macherey-Nagel, Duran, Germany) were purchased from Delchimica (Naples, Italy).

Stock solution (0.1 mg/mL) of BPA was prepared in acetonitrile, as solvent, and stored at 4°C. The analyses were carried out

using a Jasco HPLC apparatus equipped with a 20- $\mu$ L loop, and a Jasco quaternary pump 2089 *plus*, combined with a Jasco fluorescence detector 821-*Fp* (HPLC/FD). A Synergi column 4  $\mu$ m Fusion-RP 80 Å (250 by 4.60 mm inside diameter; Phenomenex, Torrance, CA) was used.

### Sample collection

Two cow farms indicated as A and B and located in Irpinia, a district of the Apennine Mountains in Campania region (Italy), participated in this survey. Irpinia is a territory largely mountainous with valleys where environmental-friendly agriculture is promoted. The farms were chosen with fields >30 km away from any major source of contamination. During summer, the cows Brown Swiss (n.77 of farm A and n.67 of farm B; average 5 years old; producing milk with fat content of 4.5%) were grazing outside in pastures, while at night and during winter inside the stables. The milking process occurred mechanically twice a day in the stables, and every 2-3 days the cooling tank of the farms was emptied by a milk collecting company that delivered the milk to the dairy factories. A total number of 22 raw milk samples was weekly collected in glass containers from the cooling tank of each farm, and then refrigerated at  $\pm 4^{\circ}\text{C}$  until the time of analysis.

### BPA analysis in raw milk

The sample preparation was performed, according to Grumetto *et al.* (2013), by adding 2.5 mL of milk to 7.5 mL of deionized water. Then this mixture was sonicated for 30 minutes at room temperature in an ultrasonic apparatus (40 kHz; Branson ultrasonic 2210, Branson Ultrasonics, Danbury, CT), and loaded into an SPE cartridge, previously conditioned with 10.0 mL of acetonitrile and equilibrated with 10.0 mL of deionized water. The cartridges were then washed with 20.0 mL of water, and two different solutions of water and methanol (80:20 and 60:40, vol/vol) under vacuum. Finally, the BPA retained in the cartridge was eluted with 10.0 mL of acetonitrile, and the eluate was collected in an amber vial before the HPLC analysis. Each sample was analyzed in duplicate.

### Instrumental parameters

BPA detection was performed through HPLC/FD. The mobile phase consisted of acetonitrile-water (70:30, vol/vol), and the flow rate was set at 0.9 mL/min (isocratic run). The fluorometric detection was carried out at an excitation wavelength of 273 nm and an emission wavelength of 300 nm.

BPA was identified based on retention time, and the quantification was performed using an external standard method. A calibration curve was obtained by injecting standard solutions of BPA at concentrations in a range of 0.03-100  $\mu\text{g/L}$ . The LOD and the LOQ were 0.01 and 0.03  $\mu\text{g/kg}$ , respectively. The correlation coefficient ( $r$ ) between peak area and BPA concentrations was 0.9969.

### BPA daily intake assessment

In order to evaluate the potential risks related to the occurrence of BPA levels detected at farms A and B, in our study a hypothetical consumption of raw milk collected from the cooling tank was considered.

According to Cirillo *et al.* (2015), BPA daily intake was evaluated considering a low (100 mL/die), and high milk consumption (500 mL/die), based on the average milk consumption (200 mL/die) for an Italian consumer (Piccinelli *et al.*, 2011). These data were correlated to BPA levels detected in raw milk samples. Three possible exposure scenarios were considered: a best case (minimum BPA concentration), a medium case (average BPA concentrations), and a worst case (maximum BPA concentration). To measure the BPA daily intake ( $\mu\text{g/kg bw/day}$ ) the following formula was applied (Cirillo *et al.*, 2015):

$$\text{Daily intake} = \frac{C * dIR}{bw}$$

where  $C$  is the BPA concentration (minimum, maximum and mean concentrations) ( $\mu\text{g/Kg}$ ) found across sample units of analyzed raw cow's milk,  $dIR$  (daily Ingestion Rate) is the daily consumption of milk (low, high and mean consumption), while  $bw$  is the corresponding average body weight (Kg 62) of Italian consumers.

## Results

### BPA concentrations in raw milk

Except for one sample from farm A, quantifiable BPA levels, below the SML value, were detected in raw milk samples at the farms (Table 1).

### BPA daily intake

The levels of exposure through the consumption of the raw milk samples showed a BPA daily intake below the t-TDI (Table 2).

## Discussion

### BPA contamination of raw milk

The study showed the occurrence of quantifiable BPA levels in raw cow's milk from two farms located in Campania region. To the best of our knowledge, there was not any similar study on the BPA levels in raw cow's milk at the farm (Mercogliano and Santonicola, 2018).

The cow's exposure through the environmental sources and the ingestion of polluted feed could be potential contamination pathways inducing the BPA excretion through the milk (Carnevali *et al.*, 2017). Lower BPA concentrations were detected in raw milk from farm A than farm B. This finding suggests that, as environmental

**Table 1. Concentrations of BPA (min-max and mean values) in raw milk.**

Milk samples from the cooling tank, N		Range, $\mu\text{g/L}$	Mean, $\mu\text{g/L}$
Farm A	11	ND-1.465	0.685
Farm B	11	0.032-2.340	0.892

**Table 2. BPA daily intake based on the daily milk consumption and contamination levels of raw milk.**

Daily milk consumption	BPA concentrations		
	Best case: Minimum BPA concentration	Medium case: Mean BPA concentrations	Worst case: Maximum BPA concentration
		Farm A	Farm B
Low (100 mL/die)	0.00003	0.001	0.0014
Mean (200 mL/die)	0.0002	0.002	0.0028
High (500 mL/die)	0.001	0.005	0.007

source, the geographic area, where the milk was produced, was characterized by low levels of contamination, and may not have played an important role in the BPA levels along the milk chain. According to Cirillo *et al.* (2015), BPA migration from plasticized contact materials (e.g. milk tubes, and sealants used in the milking machine) and from the contact materials of the cooling tank may have contributed to contamination levels detected in raw milk from both farms. In particular, rubber and PVC milk tube (short milk, air, and pulse tubes) and other plastic accessories (e.g. pulsators) were used as components of milking machine and milk delivery lines of the two farms. Therefore, the mechanical milking and the transfer of milk in the cooling tank might be identified as contamination sources (Danaher and Jordan, 2013; Chege and Ndungu, 2016).

Variable BPA contamination levels may be detected in raw, commercial milk and cheese. Bibliographic data reported higher BPA levels in commercial milk (0.38-5.47 µg/kg, and 14.0-521.0 µg/kg) and cheese (2.24 µg/kg) (Molina-Garcia *et al.*, 2012; Grumetto *et al.*, 2013; Włodarczyk, 2015) than those detected in our study in raw milk. The different contamination levels in raw, commercial milk and cheese could be related to factors, such as the quality of the materials in contact with milk during production at the farm, and the treatment conditions and packaging materials used at the dairy company (Liu *et al.*, 2008; Grumetto *et al.*, 2013). These data suggest that the contamination levels of raw milk could further change during production and packaging of the commercial milk.

The occurrence of BPA in a widely consumed food such as milk represents a food safety concern, also at low levels (Muncke, 2009; Kolatorova *et al.*, 2017). Therefore, to promote dairy safety, monitoring programs risk-based and focused on the most relevant chemical hazards, including EDs, should be applied into the milk chain

(Santonicola and Mercogliano, 2016; European Commission, 2017; Santonicola *et al.*, 2017a; Santonicola *et al.*, 2017b; van Asselt *et al.*, 2017).

### BPA human exposure

Although EDs may be absorbed through inhalation and dermal exposure, one consistent source of exposure is represented by the consumption of contaminated food (Carnevali *et al.*, 2017). Adults may be affected mostly through the consumption of canned food (50%), while infants through the infant formula consumption (25-37%) (Bemrah *et al.*, 2014; EFSA, 2015).

A comprehensive and quantitative risk assessment involves the evaluation of the daily consumption of food and the levels of the contaminant. In our study, the risk assessment based on a hypothetical consumption of raw milk from the cooling tank showed exposure levels below the t-TDI (EFSA, 2015). Nevertheless, considering the average BPA concentrations (0.685 and 0.892 µg/L, respectively for farms A and B) and mean daily milk consumption (200 mL/die), in respect to the medium scenario the consumer might be exposed to higher BPA levels in almost 45% (farm A) and 36% (farm B) of samples (Figure 1).

If BPA exposure through milk and dairy products is considered, the age of the consumers is one of the most important factors (Muncke, 2009). It should be considered that BPA also at low-dose may have lasting effects during infant development, and the exposure of a typical Italian consumer may be higher than the reported values because of other contaminated food items. Many studies have reported the effects induced by the exposure to a specific ED, but more attention should be also focused on the potential effects of mixtures of different substances and the synergic effect on human health related to the occurrence of BPA, and other different EDs in milk (EFSA, 2015; Carnevali *et al.*, 2017; Kolatorova *et al.*, 2017).

## Conclusions

The study investigated the occurrence of BPA levels in raw milk at the farm, and BPA dietary exposure, through a hypothetical consumption of raw milk from the cooling tank. BPA has become ubiquitous in the environment as a result of its high production. Global distribution of BPA levels in effluent discharges, surface waters, sewage sludge, sediments, soils, air, and wildlife may influence the contamination of the food chain. As potential contamination pathway of milk chain, environmental sources could have influenced cow's exposure and the excretion of quantifiable levels of BPA in raw milk. The concentrations detected in raw milk from both farms, although slightly lower in milk from farm A, suggest that the geographic area, where the milk was produced, was characterized by low contamination levels. BPA migration from plasticized contact materials during the mechanical milking and the transfer of milk in the cooling tank may have contributed to raw milk contamination, as additional contamination source.

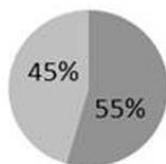
The BPA exposure was below the t-TDI also if the maximum BPA concentration and high daily milk consumption were considered. Nevertheless, the adverse effects of BPA on human health also at low doses, and the synergistic effect of mixtures of BPA and other chemicals should be highlighted. In fact, mixtures of EDs and degradation products of single compounds are frequently involved in the exposure. Therefore, the BPA levels along the milk chain must be routinely monitored, and evaluated from the perspective that milk represents a continuous low-level exposure to different EDs. The application of monitoring programs risk-based and focused on the most relevant chemical hazards in the dairy supply chain might promote food safety.

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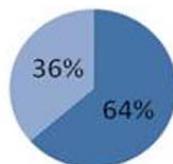
### Farm A: Medium case

■ A >0.137µg/200 mL ■ A <0.137µg/200 mL



### Farm B: Medium case

■ B >0.178 µg/200mL ■ B <0.178 µg/200mL



**Figure 1. Distribution of contamination levels (%) in raw milk compared to medium case scenario and mean daily milk consumption.**

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