

# Development of a high-performance liquid chromatography method to assess bisphenol F levels in milk

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## Abstract

Bisphenol F (BPF) is a bisphenol A (BPA) analogue. As an endocrine disruptor, BPF shows a similar BPA hormonal activity and greater endocrine effects. To assess BPF levels in milk a selective method based on solvent extraction with acetonitrile, solid-phase extraction (SPE), high-performance liquid chromatography with fluorescence detection (HPLC-FD) system, was developed. The method showed high recovery values (from 97.60 to 107.16%), and good detection and quantification limits (LOD=0.03 µg/L; LOQ=0.1 µg/L). To validate the analytical method, quantitative analyses of n.20 milk samples of whole milk were preliminarily carried out applying a monitoring system based on the control of different stages of pasteurized whole milk processing at a dairy company.

The proposed method is simple, sensitive, and might be suitable to detect BPF residues in milk processing. At the dairy company, the occurrence of BPF levels ranging from <LOQ to 2.956 µg/L was observed. Further analyses and better knowledge about the occurrence, toxicity, and exposure levels of BPF analogue in milk, particularly for vulnerable consumer categories, are needed.

## Introduction

Bisphenol F (BPF) (Bis (4-hydroxyphenyl)methane) is a bisphenol A (BPA) (2,2-Bis(4-hydroxyphenyl)propane) analogue that shows a broad range of industrial applications, particularly as food packaging coating, owing to low viscosity and better resistance to solvents (Usman *et al.*, 2019).

As a result of environmental pollution, bioaccumulation, and leaching from packaging materials bisphenols may enter the human food chain producing endocrine effects (Russo *et al.*, 2019). BPF is an endocrine disruptor (ED) and shows a simi-

lar BPA hormonal activity and critical endocrine effects on metabolism, growth, reproduction, fetal and sexual development, gender behavior, stress response, and insulin production. In addition, BPF contributes through synergistic effects to obesity and diabetes in childhood and immune system impairment (Bansal *et al.*, 2018; Andújar *et al.*, 2019; Dragone *et al.*, 2020). Restrictions on the use of BPA in certain consumer products have been suggested and a specific migration limit (SML) of 0.05 mg/kg into/onto food from varnishes or coatings applied to food contact materials was fixed (Regulation (EU) No 2018/213). Structural BPA analogues, such as BPF and bisphenol S (BPS) (4,4'-sulfonyl bisphenol), as replacements of BPA, are gradually entering the food market (Andújar *et al.*, 2019).

Milk is an important source of exposure to EDs, including bisphenols (Santonicola *et al.*, 2018; Lestido-Cardama *et al.*, 2020). BPA and its analogues may enter the milk chain at the farm during milk production, and due to the leaching from plastic parts, thermal treatments, and packaging conditions at the milk dairy processing plant (Casajuana and Lacorte, 2004).

The potential contamination of BPA analogues in the milk chain is a public health concern. A temporary tolerable daily intake (tTDI) of 4 µg/kg body weight/day for only BPA has been fixed (EFSA, 2015). In a previous study a high-performance liquid chromatography (HPLC) method was applied to whole milk samples at different stages of milk processing (raw milk, pasteurization, and milk packaging) to evaluate the BPA exposure in different age category of consumers. Results showed the occurrence of BPA in all considered steps always below the tTDI level (Mercogliano *et al.*, 2021).

Currently, data on the occurrence and human exposure to BPA analogues through food consumption are scarce (Gonzalez *et al.*, 2020). Considering the similarity between BPF and BPA structure and toxicity, experimental studies suggested that BPF might be not a safe alternative, and both bisphenols should be considered for a more comprehensive risk assessment (Andújar *et al.*, 2019).

Based on the previous method developed to determine BPA levels (Mercogliano *et al.*, 2021), the present study aimed to develop and validate a selective HPLC method to assess levels of the analogue BPF in whole milk during milk processing. To improve the analytical method quantitative analyses on milk samples were preliminarily carried out.

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Key words: Bisphenol F, High-performance liquid chromatography method, Milk.

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

Contributions: The authors contributed equally.

Funding: None.

Ethical approval: Not required (the study was carried on samples of commercial milk).

Received for publication: 14 July 2021.

Revision received: 20 October 2021.

Accepted for publication: 26 October 2021.

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Italian Journal of Food Safety 2021; 10:9975

doi:10.4081/ijfs.2021.9975

## Materials and methods

### Reagents and standards

BPF standard (minimum purity of 99%) was purchased from Sigma-Aldrich (Poole, UK). Methanol and acetonitrile HPLC grade were provided by Carlo Erba (Milan, Italy). The solid-phase extraction (SPE) cartridges (Chromabond C18, Macherey-Nagel, Duran, Germany) were purchased from Delchimica (Naples, Italy).

### Apparatus

A Jasco HPLC apparatus equipped with a Jasco quaternary pump 2089 *plus* combined with a fluorescence detector 821-*Fp* (HPLC/FD) (Jasco, Easton, USA) and a Synergy column 4 µm Fusion-RP 80 Å (250 by 4.60 mm inside diameter; Phenomenex, Torrance, CA) were used for the analyses.

The mobile phase was acetonitrile-water (70:30, vol/vol) at a flow rate of 0.9 mL/min in isocratic mode. The analyses were carried out at room temperature. The fluorescence detector was set at 273 nm excitation and 300 nm wavelength emission. BPF was identified based on the retention time.

### Quality control/quality assurance

To validate the method, the quality parameters of linearity, limit of detection

(LOD) and quantification (LOQ), precision, trueness and selectivity using fortified blank samples were evaluated.

Pasteurized milk in glass bottles obtained from the market, checked not to contain BPF, were used as blank samples. Calibration curve was obtained by external standard calibration method analysis with the blank samples fortified at levels of concentration of BPF standard solutions. LOD and LOQ were calculated based on the signal-to-noise ratio of 3:1 and 10:1, respectively.

Precision was evaluated as repeatability (intraday precision) and intermediate precision (interday precision) by spiked blank samples at two concentrations (10.0 and 50.0 µg/L and expressed as a percentage of Relative Standard Deviation (% RSD). Trueness was evaluated by BPF recovery in spiked blank samples at low (10.0 µg/L) and high concentrations (50.0 µg/L). Selectivity was evaluated by comparing standard and spiked blank samples.

### Milk sampling and BPF extraction method

The whole pasteurized milk chain production at a dairy company was preliminary monitored. Thermal treatment conditions of the milk pasteurization were 71.5°C for 15 s. The fat content was 3.4-3.7% in raw milk, and 3.5% in pasteurized and cardboard packaged milk. A monitoring system based on the control of each stage of milk processing was applied at the following steps: raw milk from the cooling tank (A), raw milk from the storage tank (B), milk at the end of the pasteurization (C), pasteurized milk from the storage tank (D), and cardboard packaged milk (E). Five milk samples were weekly collected for 4 weeks. A total number of n.20 milk samples was analyzed. A quantity of 1.0 mL of milk was mixed with 3.0 mL of deionized water and sonicated for 15 minutes at room temperature in ultrasonic apparatus (40 kHz; Branson ultrasonic 2210, Danbury, CT). Before loading the sample, the SPE cartridge was conditioned with 10.0 mL of acetonitrile and equilibrated with 10.0 mL of deionized water. Then the cartridge was

washed under vacuum with 8.0 mL of water and with 14.0 and 16.0 mL of water and methanol solutions (80:20 and 60:40, vol/vol), respectively.

The analytes were eluted with 10.0 mL of acetonitrile. The extracts were dried under N<sub>2</sub> and then 1.0 mL of acetonitrile was added. Quantitative analysis was performed by HPLC/FD.

## Results

### Linearity, repeatability, detection and quantification limit, recovery, selectivity

Working standard BPF solutions from 0.1 to 100 µg/L were prepared by diluting aliquots of the stock solution (100 mg/L) in acetonitrile. A 50 µl volume of the standard BPF solution was injected into the HPLC system. The BPF retention time was 3.29 min. Calibration curve for concentrations from 0.1 to 100 µg/L versus detector responses (peak area) was obtained with a linear regression program. The coefficient value (R<sup>2</sup>) of the calibration curve was

equal to 0.999 (Figure 1). When BPF was measured consecutively (n. 3 times) in the 10.0 and 50.0 µg/L in fortified blank samples to evaluate the repeatability of the analytical method, the RSD was found to be 4.46 and 1.09%, respectively. Interday precision, as repeatability within-day (n.2), was 14.83 and 5.481 % RSD at high (50.0 µg/L) and low (10.0 µg/L) concentration levels, respectively. The evaluation of instrumental quality parameters resulted in LOD and LOQ values of 0.03 and 0.1 µg/L, respectively. BPF recovery rates for milk are 97.60 to 107.16% at low and high concentration levels. The comparison between standard and blank samples showed the absence of interference peaks.

### Application of extraction and analysis methods

Milk samples collected at the stages A-B-C-D, and E of milk processing were extracted and analyzed as described above. The results showed the lowest concentrations in raw milk from the storage tank (BPF mean value: 0.268 µg/L) and the highest in pasteurized milk samples from the storage tank (BPF mean value: 1.211 µg/L) (Table 1).

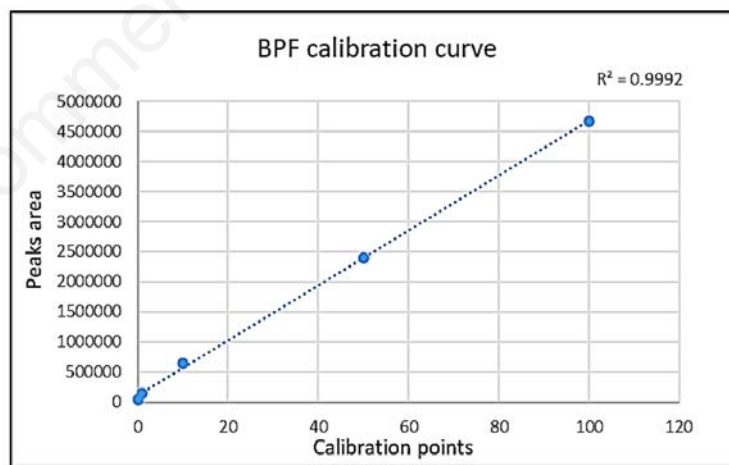


Figure 1. Bisphenol F calibration curve.

Table 1. BPF levels in milk samples collected at different stages of milk processing.

Milk processing stage	N. samples	Bisphenol F, (µg/L)			
		Range	mean	median	
A	Raw milk from the cooling tank	4	0.395-2.956	1.205	0.734
B	Raw milk from the storage tank	4	<LOQ-0.633	0.268	0.219
C	Milk at the end of the pasteurization	4	0.425-0.796	0.541	0.472
D	Pasteurized milk from the storage tank	4	0.412-2.686	1.211	0.872
E	Cardboard packaged milk	4	<LOQ-1.019	0.404	0.299

<LOQ (limit of quantification)

## Discussion

The bisphenol quantification at trace or ultra-trace levels in samples characterized by a complex and variable matrix composition is still challenging. Sample treatment is matrix-dependent and common steps include sample pretreatment, extraction of analytes from the matrix, and cleanup of the extracts to remove interferences (Caballero-Casero *et al.*, 2016; Tuzimski and Szubartowski, 2019). The most critical part of the analysis of milk samples is the cleanup step, because coextracted compounds may inhibit the detection of target compounds through HPLC analyses (Casajuana and Lacorte, 2004). Increasing progress was made during the past years regarding the development of techniques of samples analysis and various methods have been used to extract bisphenols from food-stuff (Tuzimski and Szubartowski, 2019).

In a previous study on the occurrence of BPA in whole milk (Mercogliano *et al.* 2021), fat was removed from the milk samples using 10.0 and 14.0 mL of water and methanol solutions (80:20 and 60:40, vol/vol). To detect BPF levels in milk in the present study fat was removed from the milk samples using a higher volume of water and methanol solutions, and acetonitrile was used as a solvent for the BPF extraction.

The impurities were effectively removed from the samples also through the use of SPE cartridges, and the chromatograms were free of interferences. To detect the BPF, unlike the method of extraction used for BPA levels, milk samples were diluted with water to reduce viscosity and sonicated for emulsion destabilization before the SPE phase. In this way, a better flow rate was achieved during SPE (Caballero-Casero *et al.*, 2016; Mercogliano *et al.* 2021). Sample treatment resulted in good recovery rates, from 97.60 to 107.16%, which fell into the acceptable range of 70-120% suggested by the Codex Alimentarius requirement (Hao *et al.*, 2018).

HPLC equipped with a fluorescence detector for the determination of BPA in milk is commonly used because the system is easy to perform, and its sensitivity is high (Kang and Kondo, 2003). Recently, liquid

chromatography-tandem mass spectrometry has been widely used because it provides high sensitivity for bisphenol analyses (Caballero-Casero *et al.*, 2016). However, the availability of a validated HPLC-FD method can allow more laboratories to monitor bisphenols in milk without using expensive techniques (Grumetto *et al.*, 2013). According to the literature, the proposed HPLC method to detect BPF levels in milk samples showed good performance (Leepipatpiboon *et al.*, 2005; Hao *et al.*, 2018; Xiao *et al.*, 2020). The achieved LOQ (0.1 µg/L) was lower than the specific migration limit of 0.05 mg/kg fixed only for BPA (Regulation (EU) No 2018/213), demonstrating the good sensitivity of the method. Excellent linear regression coefficient (>0.9900) was obtained covering a large concentration range (0.1 to 100 µg/L). Moreover, the method showed good selectivity, while the intra- and the inter-day precision indicated good reproducibility, accuracy, and precision.

The presence of EDs in foods poses health risks for humans. Particularly, cow's milk and dairy products can be sources of soluble EDs in fats because they are comparatively high in lipid fraction (Kang and Kondo, 2003). On the other hand, BPF may be detected in milk and dairy products even when the chemical nature of their packaging does not allow their release (Grumetto *et al.*, 2013; Garcia Ibarra *et al.*, 2019). According to the literature, in the present study, BPF levels were observed in milk samples collected in all monitored stages of milk processing. The contamination levels of raw milk from the storage tank suggest that BPF could already be present during milk production at the farm due to environmental contamination of the areas where the animals are raised (Santonicola *et al.*, 2018). Successively, the contact with plastic materials of the dairy plant or thermic treatments during milk processing might enhance the BPF leaching in the product. BPF can be released even at room and cooling temperature, and the heating during milking can considerably facilitate its release in milk. This suggests that milk pasteurization might have a role in the leaching of BPF (Shao *et al.*, 2007; Teuten *et al.*, 2009).

The information on the contamination

levels of the BPA analogues in milk is still limited. As preliminary data, negligible contamination levels of BPF occurred in raw milk and cardboard packaged milk at the dairy company. The BPF concentrations observed in packaged milk were also below either the SML limit and t-TDI value fixed for BPA. However, the levels in milk samples were similar or, also, lower than those detected in commercial milk and dairy products (Table 2) (Liao and Kannan, 2013). The data was confirmed in a successful study in which the HPLC method above described for BPF was applied to analyze a higher number of samples collected during milk processing (Santonicola *et al.*, 2021). A total number of 84 samples of raw milk from the storage tank, pasteurized milk from the storage tank, and cardboard packaged milk were analysed. Results showed the occurrence of BPF at concentrations (from <LOQ to 2.686 µg/L) below the SML limit and t-TDI value fixed for BPA, and low exposure levels in different consumer age categories (Santonicola *et al.*, 2021).

## Conclusions

The developed HPLC method resulted in a simple, sensitive, and suitable analytical procedure for determining BPF levels during milk processing. The results of the preliminary application of extraction and analysis method of the milk samples collected at different stages of processing of whole milk showed the occurrence of very low levels of the analogue BPF. Particularly, raw milk and pasteurized milk from the storage tank showed relatively higher levels, probably related to the environmental contamination where the animals live and the effects of the thermic treatment at the dairy company, respectively.

Low concentrations and exposure levels of BPF were observed also when the analytical method was afterward applied to a higher number of milk samples (Santonicola *et al.* 2021). Nevertheless, the increasing use of BPA substitutes and the toxicological similarity between BPF and BPA represents a risk to human health because of their potential synergic ED effects on biological systems.

Better knowledge about BPF exposure

**Table 2. Occurrence of BPA analogues in commercial milk and dairy products (µg/kg).**

Sample	N. samples	Country	Bpa	Bpb	Bpf	Bps	References
Milk	68	Italy	14–521	16–67	1–26	-	Grumetto <i>et al.</i> , 2013
Milk; Infant formula; Ice cream; Cheese, yogurt	29	Usa	2.50a	0.01a	0.01a	0.04a	Liao and kannan, 2013
Milk; Infant formula; Cheese, yogurt	17	China	1.40a	Nd	Nd-2.30	Nd-0.11	Liao and kannan, 2014

ND, no detectable. a, mean value.

levels in food is needed to assure food safety, particularly for vulnerable consumer classes. The application of monitoring systems based on the control of each stage of milk processing at the dairy company might represent a useful strategy to control the contamination of the milk chain.

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