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Automated Salting-out Assisted Liquid-Liquid Extraction and Determination of Bisphenol A in Beverage Samples using a Robotic Autosampler and LC-MS/MS Platform

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Sample Preparation, LC-MS/MS, High Throughput Lab Automation, Bisphenol A, Food Safety

Abstract

Bisphenol A (BPA) is a widely produced chemical used as a precursor in the formation of plastics (polycarbonate) and coatings for food and beverage containers (epoxy resin coatings)[1]. Bisphenol A is classified as an endocrine disruptor since it mimics the actions of hormones. Since bisphenol A can migrate from the packaging material into beverages, the presence of bisphenol A in ready-to-consume beverages has the potential to pose a health risk. With the wide variety of beverage types on the market, sample preparation could constitute a bottleneck to the reliable analytical methods and high throughput analysis needed for quantification of bisphenol A.

Here we show that a robotic sampler can be used to automate the extraction and determination of bisphenol A from various beverage samples. Automating the entire extraction and subsequent analysis by LC-MS/MS provides high throughput analysis for bisphenol A in beverage samples. Using the GERSTEL MultiPurpose Sampler MPS robotic^PRO, syringe transfer of all liquids involved in the liquid-liquid extraction as well as the controlled mixing and centrifugation of the samples for defined periods was performed. The resulting extracts from the automated extractions were then introduced into the Agilent Ultivo triple quadrupole LC/MS system for detection and quantification.

Introduction

Liquid-liquid extraction (LLE) is used to separate compounds having different solubilities in two immiscible liquids. Typically, a non-polar solvent like hexane is mixed with an aqueous sample matrix. However, adjustment of pH (very high or very low) is commonly required to enhance recoveries of polar compounds.

Enhanced recoveries of polar compounds without the need for pH adjustment can be achieved by using more polar solvents like acetonitrile for extraction, however, these solvents are water miscible and do not form a solvent bilayer.

This problem can be solved by introducing an inorganic salt to the aqueous sample before mixing, this causes a separation of the solvent from the sample and forms a two-phase or bilayer system after extraction. This "salting-out" technique enables the use of more polar organic solvents to perform the liquid-liquid extraction and has become known as salting-out assisted liquid-liquid extraction (SALLE). In addition, since the extraction solvent (in this case acetonitrile) is also compatible with LC-MS/MS system, the final extract can be injected into the analytical system without the need for evaporation and solvent exchange.

As a result of this study, we were able to show that an automated SALLE method using the GERSTEL MPS robotic^PRO sampler could successfully be used to extract bisphenol A from a variety of beverage samples. Bisphenol A isolated from the beverage samples using the automated extraction procedure was introduced to an Agilent Technologies 1260 HPLC coupled with an Agilent Ultivo Triple Quadrupole Mass Spectrometer with Jet stream electro-

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spray source. Accuracy data for bisphenol A extracted QC samples averaged 99.9% (range: 99.6% - 102%) and precision data averaged 2.32% RSD (range: 1.42% - 3.22%).

Experimental

Materials

Unlabeled bisphenol A was purchased from Cambridge Isotope Laboratories, Inc. An intermediate analyte stock solution was prepared by combining the analyte stock solution with acetonitrile, resulting in appropriate concentrations for the method evaluation for bisphenol A.

The deuterated analogue, d_{16}-bisphenol A, was purchased from Cambridge Isotope Laboratories, Inc. An internal standard stock solution containing the deuterated internal standard was prepared in acetonitrile at a concentration of 1000 µg/mL. A working internal standard solution was prepared in acetonitrile at a concentration of 10 µg/mL.

Calibration standard and QC samples were prepared by making appropriate dilutions of the combined intermediate analyte stock solutions using water to reach the concentrations listed in table 1. Calibration standards were prepared using a dilution ratio strategy from the high concentration sample of 1:2:5:2:5:2:5:2:5. The high, middle, and low QC samples were prepared using a dilution ratio strategy from the high concentration sample of 1:10:10. Table 1 lists the concentrations for the highest calibration standard and the limit of quantitation found during this study.

Table 1: Mass spectrometer acquisition parameters.

Compound Name	Precursor Ion [m/z]	Product Ion [m/z]		Fragmentation Voltage [V]		CE [V]		Ret Time [min]	High Std Conc. [ng/mL]	LOQ [ng/mL]
d_{16}-BPA	241.1	223.2	142.1	110	110	20	30	1.77	-	-
BPA[1]	227	212	133	110	110	20	30	1.84	1000	0.100

1 - Internal Standard d16-BPA

Soda (both diet and regular), orange juice (both with and without pulp), iced tea, iced coffee with cream, and a vitamin fortified protein drink were purchased from a local market. Samples were prepared in triplicate by pipetting 100 µL of a 1000 ng/mL bisphenol A intermediate stock solution into 4 mL of each respective plastic bottled beverage sample. The soda samples were degassed by sonication for 30 minutes prior to use. Blanks (both with and without internal standard) were extracted along with the triplicate recovery samples for each matrix evaluated.

All other reagents and solvents used were reagent grade.

Instrumentation

All automated Prep Sequences were performed using a MPS robotic[PRO] sampler with the GERSTEL quickMIX and CF-200 centrifuge options as shown in figure 1. All analyses were performed using an Agilent 1260 HPLC with an Agilent Zorbax RRHD, Eclipse Plus C18 column, (2.1 x 50 mm, 1.8 µm) and an Agilent Ultivo Triple Quadrupole Mass Spectrometer with Jet stream electrospray source. Sample injections were made using the GERSTEL LC-MS tool into a 6 port (0.25 mm) Cheminert C2V injection valve outfitted with a 5 µL stainless steel sample loop.

Figure 1: MPS robotic[PRO] sampler with GERSTEL quickMIX and CF-200 centrifuge options.

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Beverage Sample Pretreatment

1. Pipette 4 mL of beverage sample into a 10 mL autosampler vial containing 1 g of sodium chloride.

2. Pipette 20 µL of a 10 µg/mL working internal standard into each sample and cap with a magnetically transportable cap.

Automated MPS Prep Sequence for Bisphenol A Beverage Samples

1. The MPS adds 4 mL of 1% acetic acid in acetonitrile to each vial.

2. The MPS pulse mixes each vial for 15 seconds at 1500 rpm a total of 5 times.

3. The MPS mixes each vial for an additional 5 minutes at 1500 rpm.

4. The MPS centrifuges each vial for 10 minutes at 3000 rpm.

5. The MPS adds 600 µL of water to a 2 mL autosampler vial.

6. The MPS transfers 400 µL of the organic supernatant from the extraction into a 2 mL autosampler vial.

Automated MPS Sample Introduction

1. Using the GERSTEL LCMS Tool, the MPS injects the extract into the LC/MS system.

LC Method Parameters

| Mobile phase | A – 1 mM ammonium fluoride in water |
	B – acetonitrile
LC gradient	isocratic, (60:40) A: B,
	flow rate=0.3 mL/min
Run time	10 minutes
Injection volume	5.0 µL (loop over-fill technique)
Column temperature	30 °C

Mass Spectrometer Parameters

Operation	electrospray negative mode
Gas temperature	250 °C
Gas flow (N_2)	5 L/min
Nebulizer pressure	45 psi
Sheath gas flow (N_2)	11 L/min
Sheath gas temperature	350 °C
Capillary voltage	4000 V
Nozzle voltage	500 V
Delta EMV	0 V

The mass spectrometer acquisition parameters are shown in Table 1 with qualifier ions.

Results and Discussion

Figure 2 shows representative mass chromatograms for bisphenol A and the deuterated internal standard obtained from an extracted spiked soda sample.
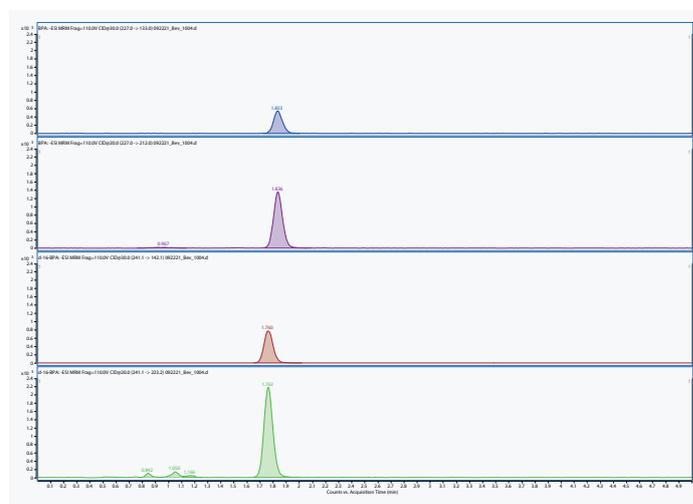
Figure 2: Representative mass chromatograms for extracted low QC sample.

The lower limit of quantitation for this method was found to be 0.100 ng/mL as shown in table 1 which is five times lower than the AOAC Standard Method Performance Requirements [1]. A representative calibration curve is shown in Figure 3. Regression analysis for bisphenol A within this method resulted in a R^2 value of 0.997.
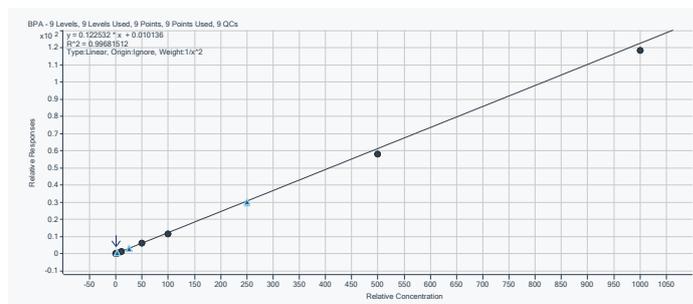
Figure 3: Representative calibration curve for extracted BPA standards.

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The accuracy and precision of the method were evaluated for bisphenol A using QC samples at high, middle, and low concentrations. Table 2 shows the resulting accuracy and precision data for bisphenol A. Accuracy data averaged 99.9% (range: 99.6% - 102%) and precision data averaged 2.32% RSD (range: 1.42% - 3.22%) for bisphenol A.

Table 2: QC sample % accuracy and % precision results.

QC Level	Final Conc. [ng/mL]	Accuracy [%]
2.5 ng/mL - 1	2.49	99.6
2.5 ng/mL - 2	2.60	104
2.5 ng/mL - 3	2.59	103
mean	2.56	102
SD	0.0598	2.39
% CV	2.34	2.34
25 ng/mL - 1	24.9	99.5
25 ng/mL - 2	25.7	103
25 ng/mL - 3	24.1	96.4
mean	24.9	99.6
SD	0.801	3.21
% CV	3.22	3.22
250 ng/mL - 1	244	97.5
250 ng/mL - 2	241	96.6
250 ng/mL - 3	248	99.3
mean	244	97.8
SD	3.47	1.39
% CV	1.42	1.42

Representative mass chromatograms from extracted blank samples of diet soda (A), orange juice with pulp (B), tea (C), and vitamin fortified protein beverage (D) along with an extracted reagent blank (E) and an extracted 1 ng/mL bisphenol A standard in water (F) are shown in figures 4 A-F. None of the samples evaluated in this study were found to have bisphenol A.
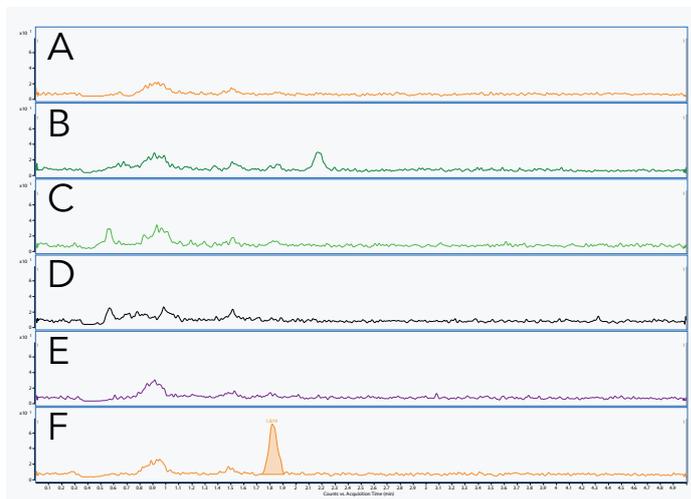
Figure 4: Resulting extracted blank beverage samples (A) diet soda, (B) OJ with pulp, (C) tea, (D) protein drink, (E) extracted reagent blank, and (F) extracted 1 ng/mL standard in water.

To assess the recovery of bisphenol A from extracted beverage samples, the beverage sample recovery replicates were extracted using the automated SALLE procedure and the resulting peak areas were then compared to those from three replicate injections of a spiked recovery standard having concentrations equivalent to the extracted bisphenol A samples. The recovery results of bisphenol A when extracted from the various beverage samples are shown in table 3. Together with the data from the extracted QC samples, this data shows that the MPS robotic[PRO] sampler can be used for extraction of bisphenol A from a variety of beverage samples combined with sample introduction and LC-MS determination in one automated system.

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Table 3: Recovery results from extracted spiked beverage samples.

Name	BPA Peak Area	Int Std. Peak Area
neat Rec 1	7612	11553
neat Rec 2	6705	10883
neat Rec 3	6628	10685
mean	6982	11040
SD	547	455
% CV	7.84	4.12
Tea Rec 1	6011	9983
Tea Rec 2	6327	10503
Tea Rec 3	6377	10559
mean	6238	10348
SD	198	318
% CV	3.18	3.07
% Recovery	89.4	93.7
OJ no pulp Rec 1	8842	11947
OJ no pulp Rec 2	9274	11876
OJ no pulp Rec 3	9096	11688
mean	9071	11837
SD	217	134
% CV	2.40	1.13
% Recovery	130	107
OJ Pulp Rec 1	8728	12239
OJ Pulp Rec 2	8616	11842
OJ Pulp Rec 3	8699	11859
mean	8681	11980
SD	58	225
% CV	0.671	1.88
% Recovery	124	109
Soda Rec 1	6630	10617
Soda Rec 2	7063	10842
Soda Rec 3	7977	12256
mean	7223	11238
SD	688	889
% CV	9.52	7.91
% Recovery	109	105
Diet Soda Rec 1	6318	9796
Diet Soda Rec 2	6449	9860
Diet Soda Rec 3	6320	9778
mean	6362	9811
SD	75.5	43.0
% CV	1.19	0.439
% Recovery	96.0	91.8

Coffee Rec 1	7858	11799
Coffee Rec 2	8505	13436
Coffee Rec 3	9099	13969
mean	8488	13068
SD	621	1131
% CV	7.31	8.65
% Recovery	128	122
Protein Drink Rec 1	8360	12965
Protein Drink Rec 2	6926	10872
Protein Drink Rec 3	8798	13566
mean	8028	12467
SD	979	1414
% CV	12.2	11.3
% Recovery	121	117

Conclusions

As a result of this study, we were able to show:

■ Bisphenol A from various beverage samples can be successfully extracted using an automated salting-out assisted liquid-liquid extraction method and determined using the Agilent Ultivo Triple Quadrupole LC/MS system

■ This method was readily automated using the GERSTEL MPS robotic^{PRO} sampler.

■ A linear calibration curve resulting in a R^2 value of 0.997 was achieved for bisphenol A.

■ The automated SALLE-LC-MS/MS method proved to be accurate and precise. Accuracy data averaged 99.9% (range: 99.6% - 102%) and precision data averaged 2.32% RSD (range: 1.42% - 3.22%) for bisphenol A extracted QC samples.

■ The recovery of bisphenol A when extracted from beverage samples averaged 114% (range: 89.4% - 130%).

References

[1] S. Li, J. Shippar, and K. Mastovska, **2019** "Determination of Bisphenol A (BPA in Commercially Packaged Ready-to-Consume Carbonated and Noncarbonated Water and Nonalcoholic Beverages: A Single-Laboratory Validation Study, First Action 2017.15", J. AOAC Int., 102:605.