Simple and rapid quantification of brominated vegetable oil in commercial soft drinks by LC-MS.

Priyanka Chitranshi, and Gonçalo Gamboa da Costa*

Division of Biochemical Toxicology, National Center for Toxicological Research, US Food and Drug Administration, Jefferson, AR 72079, USA

*To whom correspondence should be addressed:

Email: goncalo.gamboa@fda.hhs.gov

Phone: +1 (870) 543-7400

Fax: +1 (870) 543 7136

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Abstract

We report here a simple and rapid method for the quantification of brominated vegetable oil (BVO) in soft drinks based upon liquid chromatography-electrospray ionization mass spectrometry. Unlike previously reported methods, this novel method does not require hydrolysis, extraction or derivatization steps, but rather a simple “dilute and shoot” sample preparation. The quantification is conducted by mass spectrometry in selected ion recording mode and a single point standard addition procedure. The method was validated in the range of 5 to 25 $\mu\text{g/mL}$ BVO, encompassing the legal limit of 15 $\mu\text{g/mL}$ established by the US FDA for fruit-flavored beverages in the US market. The method was characterized by excellent intra- and inter-assay accuracy (97.3-103.4%) and very low imprecision [0.5-3.6% (RSD)]. The direct nature of the quantification, simplicity, and excellent statistical performance of this methodology constitute clear advantages in relation to previously published methods for the analysis of BVO in soft drinks.

1. Introduction

Brominated vegetable oil (BVO; CAS 8016-94-2) is a food additive used in certain fruit-flavored beverages as a density stabilizer for citrus oil emulsions. BVO, prepared by reaction of liquid bromine with unsaturated vegetable oils, presents a relatively high density (*ca.* 1.3 g/cm³). A mixture of the appropriate amount of (relatively low density) citrus oils and BVO to match the density of the beverage facilitates the preparation of stable citrus-based emulsions in certain brands of soft drinks. In 1970, the US Food and Drug Administration (FDA) established a limit of 15 ~~ppm~~ µg/mL for BVO in certain commercial beverages (CFR, 2015).

A range of analytical methodologies has been proposed for the analysis of BVO. These include gas chromatography with flame ionization detection (GC-FID) (Chadha, 1986; Conacher, 1969; Lawrence, 1983), GC coupled with mass spectrometry (GC-MS) (Bendig, 2012; Bendig, 2013), liquid chromatography with ultraviolet detection (LC-UV) (Lawrence, 1987), and bromide ion detection approaches (Turner, 1972; Yousef, 2012). All these methods are indirect methods for the identification and quantification of BVO and involve the chemical degradation of the triglyceride structures to derivatized brominated fatty acids or to bromide ions. These approaches are thus characterized by laborious and often complex sample preparation procedures and/or relatively lengthy chromatographic procedures (Conacher, 1969; Turner, 1972; Bendig, 2012). The complexity of the sample preparation and the indirect nature of the BVO identification and quantification may reduce the robustness of these analytical methods. For example, Lowry *et al.* (Lowry, 1981) indicated that in the official Association of Analytical Communities (AOAC) GC-based method for the quantification of BVO the trans-esterification time and competing reactions that may occur in the GC analysis may affect the reproducibility of the experiment.

More recently, the direct analysis of BVO in soft drinks was demonstrated using supercritical fluid chromatography coupled with mass spectrometry (SFC)-MS. In this method, BVO was extracted from the sample and was analyzed directly without any derivatization (Yang, 2014); however, this method is limited to analytical labs that have such uncommon instrumentation.

In contrast with SFC-MS, liquid chromatography coupled with mass spectrometry (LC-MS) is becoming increasingly prevalent in the analytical laboratory setting. In order to expand the range of analytical methodologies for the analysis of BVO in soft drinks, we report here the development and validation of a novel and facile, but highly accurate and precise, method based upon a “dilute and shoot” approach for sample preparation followed by the direct quantification of BVO by high performance liquid chromatography coupled to electrospray ionization mass spectrometry (HPLC-ESI-MS).

2. Materials and methods

2.1 Chemicals

BVO was obtained from Spectrum Chemical (New Brunswick, NJ, USA). Soft drinks containing and not containing BVO were purchased from local grocery stores. LC/MS-grade solvents and buffers were purchased from Fisher Scientific (Pittsburgh, PA).

2.2 – Sample preparation

To a set of five standard autosampler vials containing 950 μ L of 10% water in acetone (unspiked samples) and a set of five autosampler vials containing 950 μ L of 10% water in acetone and 750 ng BVO (spiked samples) were added 50 μ L of soft drink sample per vial. The vials were briefly vortexed, centrifuged, and directly analyzed in the LC-MS system. In order to ensure optimal precision and accuracy, all pipetting operations were conducted using an eVol XR digital analytical syringe (Trajan Scientific Americas Inc., Austin, TX).

2.3 Liquid chromatography-mass spectrometry

All experiments were conducted on a Waters Acquity UPLC system coupled to a Waters Premier XE tandem mass spectrometer (Waters Corporation, Milford, MA) operated in positive electrospray mode. The data acquisition and analysis were conducted using Waters MassLynx version 4.1.

A standard of commercial BVO was dissolved in a chloroform:isopropanol solution (1:1) and directly infused into the mass spectrometer to optimize the instrument parameters for selected ion recording (SIR) centered at m/z 1,700. The optimized instrumental parameters were as follows: capillary voltage 1.0 kV, cone voltage 70 V, source temperature 120 °C, desolvation temperature 450 °C, desolvation gas flow 1000 L/hr, and cone gas flow 50 L/hr. These same settings were used to obtain full scans in the mass range m/z 1000-1950.

The chromatographic separation was performed on a Kinetex Core Shell C-18 column (50 x 2.1 mm, 100 Å, 2.6 µm column (Phenomenex, Torrance, CA)) protected with a guard cartridge packed with C18 media (SecurityGuard ULTRA, Phenomenex, Torrance, CA). Mobile phases consisted of (A) 25 mM ammonium formate in 60:40 water:isopropanol (v/v), and (B) 25 mM ammonium formate in 10:10:80 water:isopropanol:n-butanol (v/v/v). The gradient profile was 20 to 100% B in 2.5 min, and remained at 100% B for 0.3 min, followed by equilibration at initial conditions for a total run time of 5.5 minutes. The flow rate was 300 µL/min, the column temperature was kept at 60 °C, and the injection volume was 2 µL. The autosampler temperature was maintained at 20 °C for the duration of the analyses.

2.4 – Calculations

The concentration of BVO in the samples analyzed as outlined above is derived taking into consideration the increased integration of the BVO peak at m/z 1,700 in the samples spiked with 15 ~~ppm~~ µg/mL equivalent of BVO versus the integration of the unspiked samples. This concentration can be conveniently calculated using the equation below (Equation 1), where C is the concentration of BVO in the beverage in ~~ppm~~ µg/mL, $SPK \pm SDS$ are the mean and standard deviation of the integration of the BVO peaks in the five independent spiked samples, and $UNS \pm SDU$ are the mean and standard deviation of the integration of the BVO peaks in the five independent unspiked samples.

$$C = \frac{15 \times UNS}{SPK - UNS}$$

Equation 1- Calculation of the concentration of BVO in the beverage

The standard deviation (SD) associated with the analysis is determined following standard rules for the propagation of uncertainty through mathematical operations on the equation above, as follows (Equation 2):

$$SD = 15 \times \frac{\sqrt{\left(\frac{SDS}{SPK}\right)^2 + \left(\frac{SDU}{UNS}\right)^2}}{\left(\frac{SPK}{UNS} - 1\right) \times \left(1 - \frac{UNS}{SPK}\right)}$$

Equation 2 – Calculation of the standard deviation in the determination of the concentration of BVO in the beverage

2.4 Method validation

The linearity of response of the LC-MS system was ascertained by linear regression analysis of the integration of the m/z 1,700 peaks stemming from synthetic samples prepared with a commercial BVO-free citrus soft drink matrix spiked with BVO to afford a final sample concentration of 0.125, 0.25, 0.5, 1, 1.5, 2, and 2.5 $\mu\text{g/mL}$ BVO (equivalent to 2.5 – 50 ppm $\mu\text{g/mL}$ in the soft drink; $n=5$ per concentration). ~~The intra- and inter-assay accuracy of the method was evaluated by comparing the mean experimental and nominal concentrations of synthetic soft drink samples spiked at the concentrations of 5, 15, and 25 ppm ($n=5$ per concentration) on two separate days.~~ The intra- and inter-assay accuracy of the method was evaluated by comparing the mean experimental and nominal concentrations of synthetic soft drinks carefully prepared to contain the concentrations of 5, 15, and 25 $\mu\text{g/mL}$ BVO on two separate days. On each day aliquots of each synthetic soft drink were used to prepare 15 individual spiked samples, and 15 individual unspiked samples, and each sample thus prepared was analyzed by LC-MS as outlined in Section 2.3. The precision of the method in each of these days was estimated by the coefficient of variation (%RSD) obtained in each day. Although the method was validated only in the range of 5-25 ppm $\mu\text{g/mL}$ BVO, the estimated

limit of detection of the method, based upon a signal to noise ratio of 3:1 was experimentally determined to be approximately 0.02 ~~ppm~~ µg/mL.

3. Results and discussion

3.1 LC-MS method development

The development of a novel method for the direct quantification of BVO in soft drinks by HPLC-ESI-MS posed a number of practical challenges. BVO is comprised of a complex mixture of high molecular weight triglycerides that vary in their brominated fatty acid composition. Due to its low polarity, BVO is very highly retained in most chromatographic systems, and does not easily undergo ionization in LC-MS interfaces (electrospray or atmospheric pressure chemical ionization). Furthermore, the somewhat variable composition of soft drinks, and thus the analytical matrix, presented an extra challenge for a method that aimed to simplify the sample preparation to the maximum extent possible. While we initially contemplated the synthesis of a (commercially unavailable) isotope-labeled internal standard that could obviate a number of these challenges, we decided not to follow this route in order to keep the analytical procedure as simple as possible. We have thus implemented a standard addition method, capable of addressing matrix effects, and the expected day-to-day variability in the absolute response of the HPLC-ESI-MS instrumentation. Considering the legal limit imposed by the FDA for BVO in soft drinks (15 ~~ppm~~ µg/mL), the method was validated in the range of 5-25 ~~ppm~~ µg/mL, and the sample spiking was conducted at 15 ~~ppm~~ µg/mL.

As indicated above, BVO is extremely non-polar, and it soon became clear that the use of “standard” reversed-phase chromatographic conditions was not appropriate and that only the use of unconventional eluents like isopropanol and n-butanol (McIntyre, 2008) enabled a proper elution of the analyte. However, these solvents are characterized by a high viscosity, which initially led to unreasonably high back-pressures. This problem was obviated by the use of a column based on superficially porous particles operated at a relatively high temperature (60 °C). Initial concerns about the durability of the column under these elution conditions

proved unfounded, and no appreciable loss of chromatographic resolution was observed after an estimated 800 analytical runs. Finally, the addition of a low concentration of ammonium formate to the eluent enabled simultaneously a good chromatographic resolution (Figure 1), and a substantial enhancement of the signal by ammoniation of the brominated triglycerides and detection of the ammoniated adduct $(M+NH_4)^+$ (McIntyre, 2008). Under these conditions a full scan analysis revealed the detection of a number of clusters of ions whose centroids evidenced a sequential increase of 2 bromine atoms in an ammoniated C_{18} fatty acid-based triacylglyceride structure (TAG) as follows: m/z 1,067 (TAG-Br₂), m/z 1,225 (TAG-Br₄), m/z 1,383 (TAG-Br₆), m/z 1,541 (TAG-Br₈), m/z 1,700 (TAG-Br₁₀), and m/z 1,858 (TAG-Br₁₂) (Figure 2). ~~The SIR profile of these ions obtained upon analysis of commercial soft drinks containing BVO was identical to the profile of the commercial BVO standard, an essential determination to guarantee the validity of this standard addition method (Supplementary Figures S1a and S1b).~~ Considering that the analytical method reported herein relies on a standard addition procedure, it was of utmost importance to ascertain if the composition of the brominated vegetable oil used in the spiking experiments was identical to that used by the vendors in the preparation of their commercial beverages. An analysis of the beverages and the brominated vegetable oil revealed that the intensity profile of each of the brominated triglyceride ions indicated above (TAG-Br₂ to TAG-Br₁₂) was identical in all beverages and brominated vegetable oil (Supplementary Figures S1a and S1b), demonstrating the adequacy of the standard used in the preparation of the spiked samples. As expected, the ion chromatograms of samples stemming from soft drinks not containing BVO were entirely devoid of these ions (data not shown). SIR analyses conducted with each of these high-molecular-weight BVO ions indicated that the ion at m/z 1,700 offered the best compromise between signal-to-noise ratio and precision in the integration, as assessed by the RSD of replicate injections of a beverage sample, and was thus chosen for method development and validation. While the use of a tandem mass spectral technique, like single reaction monitoring (SRM), would have probably enabled a more sensitive analysis of BVO, we opted to develop the method using SIR in order to enable the implementation of the methodology in laboratories where only single quadrupole mass spectrometers may be available.

The linearity of response of the HPLC-ESI-MS system was assessed by linear regression in the range of 2.5-50 ~~ppm~~ µg/mL BVO (Figure 3). The excellent linearity ($R^2 = 0.999$) demonstrated in this concentration range enabled the development and validation of a one-point standard addition method in the range of 5 – 25 ~~ppm~~ µg/mL, at a spiking level of 15 ~~ppm~~ µg/mL. This approach (versus a multiple point standard addition approach) allowed for a simpler experimental procedure while enabling a good number of replicate samples (n=5) within each analysis, an approach that has been advocated provided that linear response is well demonstrated in the working range (Ellison & Thompson, 2008).

~~The method was validated with regard to its intra- and inter-assay precision and accuracy by analyzing beverages with a BVO content of 5, 15, and 20 ppm on two separate days. These samples were prepared by spiking a commercially obtained citrus soft drink devoid of BVO with the appropriate levels of BVO. On each day three independent beverage samples were analyzed for each BVO concentration. As outlined in Section 2.2, each beverage sample afforded five spiked and five unspiked analytic samples.~~ The method was validated with regard to its intra- and inter-assay precision and accuracy by analyzing beverages with a BVO content of 5, 15, and 25 µg/mL on two separate days. These beverages were prepared by spiking a commercially obtained citrus soft drink devoid of BVO with the appropriate levels of BVO. On each day 15 independently spiked samples and 15 unspiked samples were prepared for each BVO concentration and analyzed by LC-MS, for a total of 45 spiked samples and 45 unspiked samples in each of the two days. These analyses revealed excellent intra- and inter-assay statistical performance of the method (Table 1) with accuracies ranging between 97.3-103.4% and precision errors under 3.6%. It should be noted that a crucial element to achieve this level of statistical performance was the consistent use of appropriately calibrated digital analytical syringes in all sample preparation procedures. There were no observable matrix effects noted in the analysis of samples, probably due to the considerable dilution that the samples undergo in their preparation (20-fold dilution) and the very high retention of BVO, thus, enabling an early elution of interfering matrix components.

3.3 Analysis of commercial soft drinks

The newly validated method was applied to the analysis of ten soft drinks purchased in the local market, whose labels indicated the presence of BVO in their composition. The beverage selection included brands recognized as having a large market share in the US, and less well known brands associated with local market chains. The samples were analyzed independently on two separate days to ascertain the precision of the method in a broad range of matrices. Consistent with the findings of the method validation procedure, excellent reproducibility was observed with variations in the determined concentration of BVO between day 2 and day 1 in the range of -4.4 to 2.5% (-1.3% on average). The concentration of BVO in these soft drinks ranged from 9.7-14.7 ~~ppm~~ µg/mL and was thus below the 15 ~~ppm~~ µg/mL limit defined by the US FDA for soft drinks in the US market. The mean concentration of BVO found in soft drinks in this study (12.2 ~~ppm~~ µg/mL) is approximately 50% higher than that reported in a previous study (8 ~~ppm~~ µg/mL) (Bendig, Maier, & Vetter, 2012). It is conceivable that this difference may be explained by the inclusion of different beverages in each study, variations in the formulation of the beverages, or the fact that the previous study conducted an indirect assessment of the concentration of BVO while the current study conducted a direct assessment of this food additive.

4. Conclusions

We have developed and validated a novel methodology for the analysis of BVO in soft drinks. The method was validated in the range of 5-25 ~~ppm~~ µg/mL BVO, a range that is centered in the 15 ~~ppm~~ µg/mL legal limit set by the FDA for fruit-flavored beverages in the US market. In contrast with previously reported methods this new method quantifies BVO directly and not products of its chemical degradation. The methodology affords excellent precision and accuracy while requiring only a “dilute and shoot” sample preparation process and short analyses times. Although the method was developed with the aim of enabling optimal precision and accuracy within the validated range, the estimated limit of detection of BVO (0.02 ~~ppm~~ µg/mL) may also make it appropriate for a straightforward detection of low concentrations of BVO in soft drinks in countries where the use of this food additive is not permitted.

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FIGURES:

Figure 1. SIR chromatogram (m/z 1700) obtained upon analysis of BVO standard.

Figure 2. Full scan (m/z range 1000-1950) ESI-MS analysis of BVO standard, evidencing the detection of clusters of ammoniated brominated triacylglycerides (TAG-Br_n). The mass chromatogram was smoothed for graphical clarity, and the masses indicated for each ion cluster are those of the centroid ascribed by the MassLynx software.

Figure 3. Assessment of the linearity of response of the mass spectrometer in the range of 0.125, 0.25, 0.5, 1, 1.5, 2, and 2.5 µg/mL BVO (equivalent to 2.5 – 50 µg/mL in the soft drink; n=5 per concentration) by linear regression analysis.

TABLES:

Table 1. Intra- and inter- assay accuracy and precision of the analysis of BVO in beverage matrix.

Table 2. Quantification of BVO in commercial soft drinks on two separate days.

Figure 1

Figure 2

Figure 3

Table 1. Intra- and inter- assay accuracy and precision of the analysis of BVO in beverage matrix.

Nominal Concentration of BVO (µg/mL)	Observed Concentration of BVO (µg/mL)	% Accuracy	% RSD
<i>Intra-assay</i>			
5	4.9±0.1*	97.3	2.0
15	15.2±0.5*	101.1	3.6
25	25.2±0.5*	100.9	2.1
<i>Inter-assay</i>			
5	4.8±0.02**	96.9	0.5
15	15.5±0.5**	103.4	3.1
25	25.7±0.6**	102.7	2.5

* Observed concentration data are expressed as mean ± SD of the beverage samples analyzed on day 1 for each concentration (each beverage concentration afforded fifteen spiked and fifteen unspiked analytic samples).

** Observed concentration data are expressed as mean ± SD of the data from day 1 and day 2 of analysis.

Table 2. Quantification of BVO on two separate days in commercial soft drinks whose labels included BVO as an ingredient

Soft Drink	Day 1 ($\mu\text{g/mL}$)*	Day 2 ($\mu\text{g/mL}$)*	% difference between day 2 and day 1
1	12.8 $\pm$ 0.3	12.9 $\pm$ 0.4	-0.2
2	9.7 $\pm$ 0.3	9.9 $\pm$ 0.4	-1.9
3	12.6 $\pm$ 0.3	12.7 $\pm$ 0.5	-0.9
4	14.4 $\pm$ 0.6	15.0 $\pm$ 0.5	-4.4
5	14.0 $\pm$ 0.3	13.8 $\pm$ 0.4	1.6
6	11.6 $\pm$ 0.5	12.0 $\pm$ 0.3	-4.1
7	11.6 $\pm$ 0.3	11.8 $\pm$ 0.3	-2.4
8	11.1 $\pm$ 0.2	11.4 $\pm$ 0.4	-3.1
9	13.3 $\pm$ 0.3	13.0 $\pm$ 0.5	2.5
10	10.0 $\pm$ 0.2	10.1 $\pm$ 0.3	-0.3

* Concentration data are expressed as mean $\pm$ standard deviation of the analysis calculated using Equation 1 and Equation 2, respectively, as outlined in section 2.4.