Artificial Preservatives in Pet Food: BHT, BHA and Ethoxyquin Extraction by QuEChERS Methodology and Analysis by LC-MS/MS

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Introduction

Pet food safety and quality issues have grabbed the headlines across the globe. This highlights challenges and opportunities for manufacturers to assess their products and meet customer expectations for pet safety. Preservatives in pet food are one aspect of pet food quality that may also be related to pet food safety, particularly in the case where the preservatives may be linked to long-term issues such as cancer, or where these preservatives may be implicated in allergic reactions in pets.

This study focuses on the development of a simple, effective method for extracting three preservatives from pet foods: butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and ethoxyquin. Typically used to retard oxidation and rancidification of fats added to pet food, the allowable limits for these compounds vary depending on geography, both within the United States and globally. Ethoxyquin, for example, is only allowed up to 150 ppm in animal feed under the US FDA requirements (1). Using an optimized QuEChERS (**Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged, **S**afe) sample prep method (2) combined with liquid chromatography and tandem mass spectrometry (LC-MS/MS), the resulting method provides a reliable means of identifying and quantifying these preservatives in finished pet foods.

	BHT	BHA	Ethoxyquin
Molecular Structure			
Log P	5.32	3.09	4.1
pKa	12.23	8.9	4.56
Molecular Weight	220	180	217

Experimental

For the purposes of this study, the sample preparation method was optimized for recoveries of BHA, BHT and ethoxyquin by evaluating both the extraction/partitioning and dSPE steps of the QuEChERS methodology. Chromatography and mass spectrometry conditions were optimized for the compounds, and propyl paraben was used as the internal standard (Figure 1).

The extraction/partition method for the QuEChERS used a mixture of extraction salts developed for EN QuEChERS methods. The correct dispersive SPE mixture needed to be determined experimentally based on recoveries of the preservatives.

Experimental, cont.

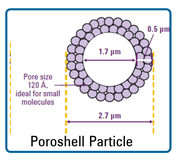
QuEChERS Sample Preparation Method:
The QuEChERS method described here is suitable for use with either wet or dry pet food. Adding the QuEChERS extraction salts to the mixed sample is recommended over using salts pre-measured in the tubes.

Extraction/Partitioning
- Add 2 g pet food (dry or wet) to 50 mL centrifuge tube
- Add 2 ceramic homogenizers
- Add 8 mL water, then vortex 1 min at 2500 rpm
- Add 10 mL ACN, then vortex 1 min at 2500 rpm
- Add QuEChERS EN extraction salts: 4 g MgSO4, 1 g NaCl, 1 g NaCitrate, 0.5 g disodium citrate sesquihydrate (p/n: 5982-5650)
- Shake vigorously 1 min, then centrifuge at 5000 rpm for 5 min

dSPE – Optimized Method
- Transfer 6 mL of extract to d-SPE tube containing 25 mg PSA, 2.5 mg GCB, 15 mg MgSO4 (p/n: 5982-5221)
- Vortex 1 min at 2500 rpm, then centrifuge at 5000 rpm for 5 min
- Transfer 700 µL of upper organic layer to a new tube for evaporation under N_2
- Reconstitute in 700 µL ACN:Water (1:4)
- Filter sample, using a 0.45 µm PTFE filter (p/n: 5185-5836 or 5190-1415) and transfer to autosampler vial for analysis

LC-MS/MS Conditions:
An Agilent LC-triple quadrupole mass spectrometer was used for this analysis. An Agilent Poroshell 120 SB-C18 LC column was selected because the large pore size frits are compatible with the complex pet food matrix, and are more resistant to clogging that would increase back pressure. The mass spectrometer method required optimization of the acquisition parameters, because BHA and BHT are poorly ionized (Figure 2).

LC Conditions: 1200 LC
Poroshell 120 SB-C18, 2.7 µm, 2.1 x 50 mm
Mobile Phase A: Water (0.1% FA)
B: ACN (0.1% FA)
Time 0.1 25% B
4.0 90% B
4.5 90% B
4.6 25% B

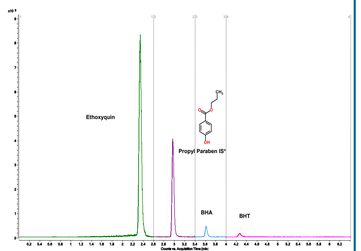


Poroshell Particle

MS/MS Conditions:
Agilent 6460 Triple Quadrupole Mass Spectrometer
Gas Temp: 300 °C
Gas Flow: 10 L/min
Nebulizer: 40 psi
Sheath Gas Temp: 350 °C
Sheath Gas Flow: 12 L/min
Capillary: 3500 V, Positive
Nozzle Voltage: 500 V

Results and Discussion

Figure 1. Standard Mix with Internal Standard at 100 ppb



Initially, the LC conditions and mass spec transitions were optimized using unextracted standards plus the propyl paraben internal standard (IS) at 100 ppb (Figure 1). Two transitions were acquired for each compound, and the first transition was used for quantitation while the second was used for qualitative confirmation. Each compound was acquired in a separate segment (Figure 2).

After establishing the analytical conditions, the extraction method was optimized. Several dSPE combinations were tried, using extracted pet foods. Finding a "blank" pet food sample was very difficult due to the ubiquity of one or all of the target preservatives. Both pre- and post-extraction spiked pet food samples were used for the study, which was a useful approach for assessing extraction performance.

Table 1. MRM Transitions, EMV and Time Segments for Preservatives Studied

Compound	Time Segment	EMV	MRM Channels (m/z)	Dwell	Fragmentor (V)	CE (V)
Ethoxyquin	1	300	218 > 174 218 > 160	6 6	30 30	27 31
Propyl Paraben IS*	2	500	181 > 139 181 > 120.9	20 20	57 57	3 15
BHA	3	1000	181.1 > 166 181.1 > 138	100 20	89 89	11 19
BHT	4	0	221.2 > 165 221.2 > 41.1	6 6	84 84	8 27

As shown in Figure 2a, when the dispersive mixture did not contain GCB, the recovery of BHA was dramatically reduced. By selecting a dSPE mixture that included end-capped C18, PSA and GCB, the recovery of BHA was significantly improved (Figure 2b.

Figure2b. Post-Spiked 100 ppb, d-SPE with PSA + C18EC + GCB

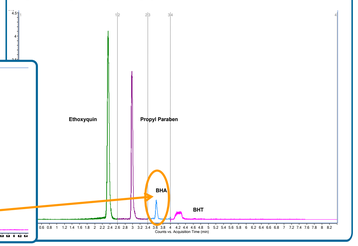
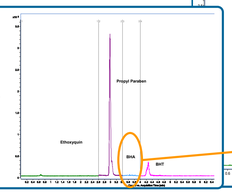


Figure 2a. Post-Spiked 100 ppb, dSPE with PSA + C18EC, no GCB



Results and Discussion, cont.

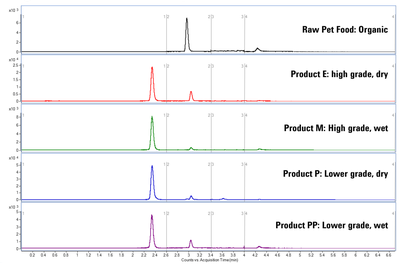
"Blank" pet food was used with pre- and post-extraction spiking of the preservatives and internal standards, and samples were prepared at 200, 400, 600, 800, and 1000 ppb. These concentrations are relatively high in terms of typical contaminant analysis, in part because the limit for ethoxyquin in animal feed in the US is set to less than 150 ppm.

The precision and relative recoveries for BHA, BHT and ethoxyquin are shown in Table 1. These results demonstrate that the sample preparation method and analytical conditions provide the necessary precision and extraction capabilities for reliable quantification of these preservatives.

Table 2. Precision and Accuracy for Preservatives Studied

Compound	400 ppb Accuracy (%)	400 ppb RSD (%)	600 ppb Accuracy (%)	600 ppb RSD (%)	800 ppb Accuracy (%)	800 ppb RSD (%)
Ethoxyquin	64	4	57	6.5	55	5.4
BHA	101	1.5	100	3.6	100	1.0
BHT	103	7.6	110	6.5	130	6.9

Figure 3. Incurred Sample Results – Commercial Pet Foods



To demonstrate the effectiveness of the method, five commercially available pet food samples were analyzed using the optimized QuEChERS sample preparation technique. The results of theses analyses are shown in Figure 3. The raw, organic pet food contained some BHT and no ethoxyquin, whereas the other products contained high levels of ethoxyquin, and lower concentrations of BHA and/or BHT.

Conclusions

- The described Agilent QuEChERS sample prep method is suitable for the extraction of artificial preservatives in pet food
- A Poroshell 120 SB-C18 provides suitable peak shape and resolution and is compatible with pet food matrix
- LC-MS/MS analysis of artificial preservatives in pet food provides the required precision and accuracy for confident identification and quantification of these preservatives

1) United States Code of Federal Regulations (CFR), Title 21, Parts 573.380 and 573.400, and established tolerances are in Part 172.140
2) M. Anastassiades et al., J of AOAC Int., 2003, 86(2) 412–31