Supercritical Fluid Extraction of Atrazine and Other Triazine Herbicides from Fortified and Incurred Eggs

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Triazines are a class of important pre-emergent weed herbicides. Some members of this class of herbicides exhibit carcinogenic and immunotoxicity properties, which make their use controversial in areas where animal feed crops are grown. It is therefore important to determine if triazine residues are transported to animal food products in order to ascertain the extent of human exposure. Most of the current herbicide residue extraction methods are time-consuming and solvent intensive. Supercritical fluid extraction (SFE) using CO_2 has been used as a alternative for other residue extraction methods as a replacement for hazardous organic solvents. In this study, 10 triazines were extracted from eggs fortified at 100 ppb using unmodified supercritical CO_2 at a pressure of 10000 psi and a temperature of 50 °C with off-line collection on a solid phase extraction cartridge containing Florisil. Atrazine recovery averaged 90.4% with an RSD of 3.3%. The other triazines were recovered at mean levels >73%. In a separate feeding study, atrazine and two of its dealkyl metabolites were detected in the egg. The results indicate that SFE is a viable technique for isolating triazine residues from eggs, requiring only 8 mL of solvent for each analysis.

Keywords: *Triazine herbicides; eggs; supercritical fluid extraction; atrazine*

INTRODUCTION

Triazines are a class of chemically similar pre-emergent weed herbicides used extensively worldwide on animal feed crops. Because some members of this class of herbicides exhibit carcinogenic and immunotoxicity properties, their use is controversial. Atrazine, cyanazine, and simazine are currently the most widely used triazines in the United States; however, cyanazine use will be phased out by December 2002 (*Federal Register*, 1996). Because of their hazardous properties, the EPA in 1994 began a Special Review of these triazines to determine their risks to consumers and workers. Therefore, data are needed to determine whether triazine residues are present in the food supply to ascertain the extent of human exposure.

Although most multiresidue methods have been developed for the analysis of triazine residues in soil and water samples, several recent methods have focused on these residues in foods. Pardue (1995) published a solvent extraction method for 19 triazine herbicides and 4 metabolites in corn, apples, celery, milk, silage, and wheat. Other methods include a solid-phase extraction method for atrazine and other pesticides in fruits and vegetables (Kadenczki et al., 1992), a dual solid-phase extraction method for triazine residues in milk (Lagana et al., 1995), and a solvent extraction method for triazine residues in fowl and beef fat (*FSIS Chemistry Laboratory Guidebook*, 1991). However, these residue extraction methods are time-consuming and labor and solvent intensive. Supercritical fluid extraction (SFE), using supercritical carbon dioxide (SC-CO_2), is an alternative to these solvent-intensive isolation procedures, especially for environmental samples. The advantages of SFE over conventional solvent extraction methods include reduction in organic solvent consumption, faster analysis time, and potentially more efficient and selective analyte extractions from complex matrices. There have been several recent publications using SFE to extract triazine residues from soil, sediments, and water, but all of these methods are complicated by the requirement that a cosolvent (modifier) be used in the process (Malone et al., 1997; Barnabas et al., 1994; Alzaga et al., 1996). In contrast to these methods, we have shown that unmodified SC-CO_2 at 10000 psi (680 atm) and temperatures ≥40 °C is effective in extracting compounds of intermediate polarity from eggs (Fiddler et al., 1999; Pensabene et al., 1997, 1999). In this paper, we report on a multiresidue triazine SFE method that is rapid, requires no modifier during the extraction process, and uses significantly less solvent to recover the target analytes than current methods reported for other matrices. We also compared the proposed method to a solvent extraction method for the analysis of atrazine and two of its metabolites in incurred eggs obtained from laying hens.

EXPERIMENTAL PROCEDURES

Materials. Individual samples of prometon, propazine, atrazine, terbuthylazine, simazine, secbumeton, prometryne, ametryn, simetryn, cyanazine, de-ethylatrazine, and deisopropylatrazine were purchased from AccuStandard (New Haven, CT). Hydromatrix (Celite 566, sieved at 30−40 mesh to remove fines; Applied Separations, Allentown, PA), acetone, acetonitrile, dichloromethane (DCM), benzene, hexane, and methanol (Burdick & Jackson Brand, Baxter Health Care, Muskegon, MI) were used without further purification. Florisil

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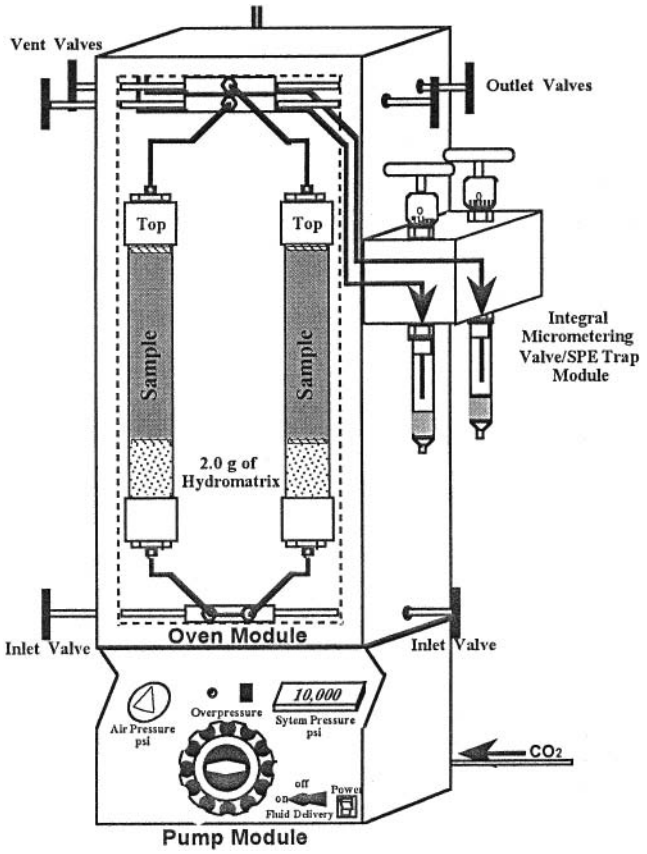

Figure 1. Schematic of the supercritical fluid extractor.

(60−100 mesh; Aldrich Chemical Co., Milwaukee, WI) was washed with acetone, filtered, and dried in a vacuum oven for 4 h at 150 °C and then deactivated with 10% water prior to use. SFC-grade CO_2, without helium headspace (Scott Specialty Gas, Plumsteadville, PA), was used for the SFE extractions. Control eggs were obtained from the FDA, CVM, stored in a −85 °C freezer until needed and then thawed in a 4 °C refrigerator prior to use. In the first feeding study, performed at the FDA, CVM laboratory, five laying hens were given a single daily dose of 20 mg of atrazine (orally by gelatin capsule) on two consecutive days. Eggs were collected on days 2−16. Samples were homogenized, shipped to the ERRC, and then placed in frozen storage at −85 °C until analyzed. In the second feeding study, four different laying hens were similarly dosed with 20, 30, 50, or 80 mg of atrazine daily for five consecutive days. The hen given 50 mg/day produced no eggs, whereas the hen given 30 mg/day produced eggs from day 5 to 11 and on days 15−16. The other two hens produced eggs sporadically during the feeding study.

SFE. Five grams of homogenized whole egg was weighed into a 150 mL beaker and fortified when required with 50 μL of the individual triazine standard (10.0 μg/mL each in MeOH) or with a standard containing all of the triazines. After a 15 min equilibrium period, 7.0 g of Hydromatrix was added to the beaker and the sample was stirred with a glass rod until a dry, free-flowing mixture was obtained. A high-pressure (10000 psi) 24 mL extraction vessel (Keystone Scientific, Bellefonte, PA) was capped at one end (labeled top) to which was added a plug of polypropylene wool (Aldrich Chemical, Milwaukee, WI) and the sample mixture, followed by a plug of polypropylene wool. Finally, 2 g of Hydromatrix was added to the extraction vessel, topped by another plug of polypropylene wool. All of the material added to the extraction vessel was firmly tamped after each addition. The end of the extraction vessel labeled top was fitted into the SFE oven as illustrated in Figure 1.

The extractions were carried out in a two-vessel parallel extractor (Applied Separations Inc.). The description and setup of the extractor are described elsewhere (Maxwell et al., 1992). The system was operated under the following conditions: oven, 50 °C; micrometering valve, 120 °C; extraction pressure, 10000 psi (680 atm); flow rate of expanded gas, 3.0 L/min; total flow, 120 L. The triazines were collected off-line in 6 mL SPE cartridges (Applied Separations Inc.) containing 1.0 g of deactivated Florisil. After completion of the extraction, the SFE cartridge was removed from the extractor and washed with 4 mL of hexane, and then the triazines were eluted with 4 mL of a 10% acetone in benzene solution. Prior to quantitation by gas chromatography (GC), the extract was concentrated to 1.0 mL in a 65 °C water bath under a stream of nitrogen.

Solvent Extraction Procedure. Whole eggs were analyzed using a slight modification to a procedure published previously for triazine residues in fat (*FSIS Chemistry Laboratory Guidebook*, 1991). Briefly, 5.0 g of egg is weighed into a 50 mL glass centrifuge tube, and 30 mL of hexane is added to the sample and shaken for 3 min. The hexane solution is poured through glass wool into a 60 mL separatory funnel using additional hexane as required. Ten milliliters of acetonitrile is added to the separatory funnel, which is then shaken for 1 min. The layers are allowed to separate and the acetonitrile fraction is collected in a 250 mL beaker. This acetonitrile extraction step is repeated twice more. The acetonitrile is removed on a steam bath, and then 5 mL of acetonitrile and 100 mL of water are added to the residue. The solution is transferred to a preconditioned disposable C_{18} extraction column (J. T. Baker Chemical Co., Phillipsburg, NJ), using 5 mL of water. The triazines are eluted from the column with 8 mL of methanol−water (80:20). To this solution is added 60 mL of 0.1 M NaCl and 12 mL of DCM. The mixture is shaken for 1 min, the layers are allowed to separate, DCM is collected in a conical glass tube, and then the extraction is repeated twice more. The pooled DCM is evaporated under a stream of nitrogen at 35 °C, and then 1.0 mL of nonane is added prior to analysis by GC.

GC. Analyses were carried out on a Shimadzu GC-17A GC (Columbia, MD) equipped with a flame thermionic detector (N/P) and a Shimadzu AOC-20i autosampler fitted to the inlet for splitless injection. The detector temperature was set at 300 °C, and the injector inlet was set at 250 °C. A 30 m × 0.25 mm DB-17 (0.25 μm film) capillary column was employed with a He carrier gas flow rate of 35 cm/s. The oven initially was set at 35 °C for 2 min, followed by programmed ramps from 35 to 100 °C at 25 °C/min and from 100 to 250 °C at 5 °C/min, and then held for 6 min. The limit of quantitation was calculated as 1 ppb on the basis of a signal-to-noise ratio >2:1.

RESULTS AND DISCUSSION

The two methods most often used to trap analytes during or after SFE are solvent trapping and one-step sorbent traps. Solvent trapping usually requires removal or concentration of the solvent and additional extract cleanup prior to quantitation. Sorbent traps have been used to facilitate faster cleanup both in-line, in which the analyte is trapped on a sorbent contained inside the extraction vessel, and off-line, where the analyte is collected, after CO_2 decompression, most typically on a sorbent packed in a solid phase extraction cartridge. In SFE, the sorbent used in trapping the analytes is frequently as important as the solubilization and extraction of the analyte. The sorbents used to trap the analytes must be capable of quantitative retention during the decompression process and must also effectively separate the analytes from coextracted contaminants. These characteristics are important when lipids must be removed from the SFE extract, as would be the case for eggs. Because silica gel is the most commonly used sorbent, we evaluated it for both in-line and off-line trapping of the triazines. We found that

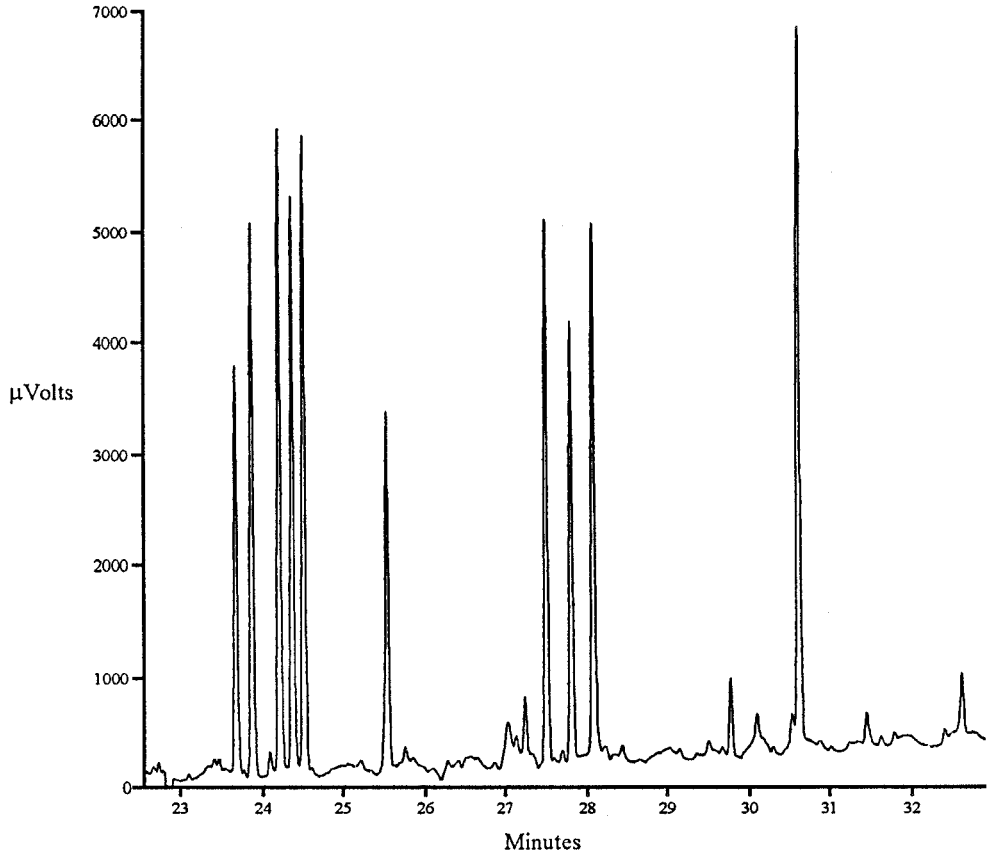

Figure 2. GC-N/P chromatogram of a fortified egg sample; elution order listed in Table 1.

silica gel did not retain all of the triazines in the in-line sorbent trap, and it was found to give inconsistent results when the triazines were collected off-line in an SPE cartridge, the results being dependent on the batch/manufacturer used. Florisil, a synthetic magnesium silicate, commonly used for cleanup of organochlorine pesticides, PCBs, and dioxin-containing extracts, was next evaluated for use as a trap/separation medium for the triazines. Neither activated nor deactivated (10% water) Florisil could be used for in-line trapping because the triazines were not well retained, but when the deactivated form was used to collect the analytes off-line, it proved to be capable of retaining all of the triazines studied, gave consistent batch to batch reproducibility, and resulted in extracts that produced clean chromatograms. Coextracted lipids that were also retained on the Florisil were easily removed with hexane before elution with acetone/benzene.

The off-line Florisil trap was used to recover 10 triazine herbicides added to control egg samples at the 100 ppb level and extracted by SFE using unmodified SC-CO_2 (Table 1). The mean recoveries varied from 73.4 to 90.4% with the recovery of atrazine, the most widely used triazine, being the highest, 90.4 ± 3.3%. These results compare favorably to those reported for other SFE methods that use SC-CO_2 (Janda et al., 1989; Barnabas et al., 1994; Papilloud and Haerdi, 1994; Robertson and Lester, 1995; Alzaga et al., 1996). Most of these applications have been applied to environmental samples, especially soils. Pressures up to 5000 psi and temperatures up to 150 °C were employed, typically using either a cosolvent with concentrations up to 20% methanol or other treatments to primarily help overcome the analyte−matrix interactions. Even with this, the triazine recoveries tended to be variable. Our method did not require a cosolvent. Our use of a pressure at 10000 psi under relatively mild temperature conditions gave SC-CO_2 with sufficient polarity for extracting the triazines, possibly with the help of the lipids in the eggs that could act as a cosolvent. Our results also compare well with those obtained by non-SFE methods used to analyze triazines in milk, fruits, and other agricultural products (Kadenczki et al., 1992; Lagana et al., 1995; Pardue, 1995). A chromatogram (Figure 2) from a fortified egg sample, extracted by SFE, showing the window from 23 to 33 min illustrates the good separation of the triazine peaks and the lack of other interfering components possible by this technique. The triazine elution order is as listed in Table 1.

Table 1. SFE Triazine Recoveries from Fortified Whole Egg

	% recovery at 100 ppb[a]	
herbicide[b]	range	mean ± RSD
prometon	71.0−78.5	75.5 ± 3.0
propazine	71.4−84.9	78.0 ± 6.5
atrazine	86.7−94.4	90.4 ± 3.3
terbuthylazine	83.3−95.8	87.9 ± 5.6
simazine	67.0−81.9	73.4 ± 7.2
secbumeton	68.2−79.3	75.1 ± 4.0
prometryne	85.3−91.6	89.2 ± 3.5
ametryn	71.3−88.5	81.6 ± 7.8
simetryn	80.0−95.5	85.7 ± 6.4
cyanazine	74.7−81.1	79.1 ± 3.4

[a] $N = 8$. [b] Herbicides listed in order of elution on GC.

The next step in the SFE method evaluation process would normally be a comparison with another method, preferably a solvent extraction method that is currently used for the same purpose. However, no solvent extraction methods were found specifically for eggs; as a result, one previously applied to fat samples was

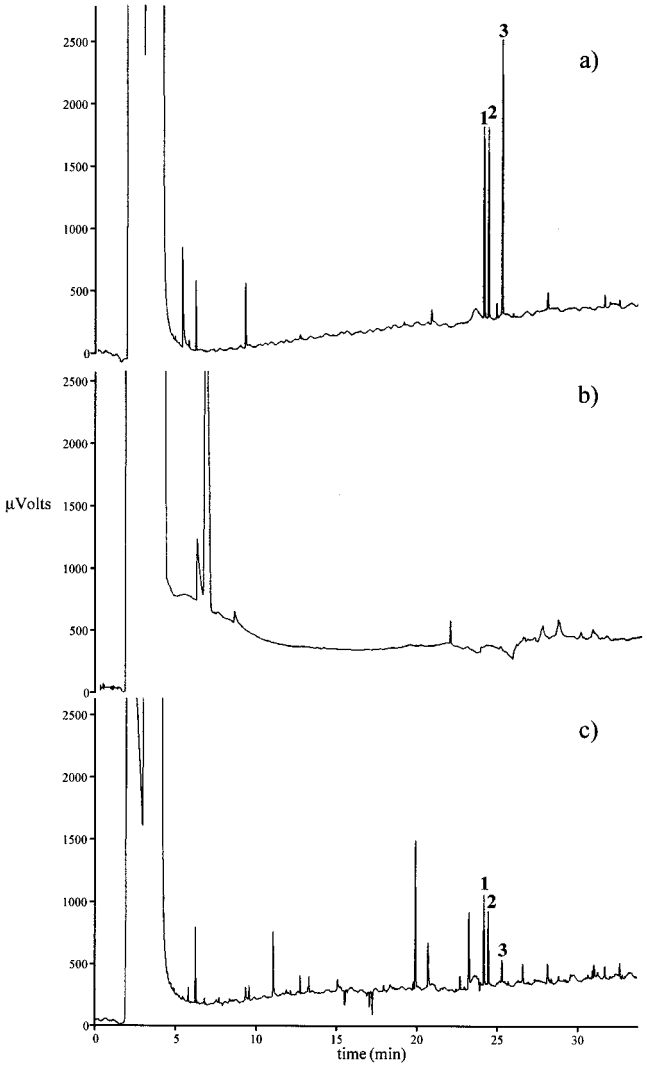

Figure 3. GC-N/P chromatogram of (a) standard with (1) de-ethylatrazine, (2) deisopropylatrazine, and (3) atrazine; (b) control egg; and (c) incurred egg.

selected (*FSIS Chemistry Laboratory Guidebook*, 1991). Because there can be a significant difference in extractability with the same analyte(s) between fortified and normally incurred samples, which can be due to binding characteristics or other factors, a chicken feeding study was conducted to obtain eggs with incurred atrazine. With the goal of obtaining atrazine levels in the egg in the range of 10−150 ppb, egg-laying chickens were given a single daily dose of 20 mg of atrazine for two consecutive days. At this dose, four of the five hens did not consistently produce eggs. Of the one that did, detectable atrazine levels were found only in the eggs obtained on days 3−5 of a 16 day collection period. However, these residual atrazine levels were considered to be too low (3.3−5.7 ppb) to obtain reliable data in which the SFE results could be compared with the solvent method. These results suggested that only a minimal amount of translocation of the atrazine into the egg occurred. Examination of the chromatogram (Figure 3c) showed not only the atrazine peak eluting at 25.2 min but also other peaks located in the same general area, suggesting that other structurally related analytes might be present. Even though the purpose of this study was to analyze for the parent compound, atrazine, we reanalyzed the samples using a mixed standard consisting of atrazine and two of its primary metabolites, de-ethyl- and deisopropylatrazine, to determine whether these compounds were present; they were. In addition to these two metabolites, the other peaks in the chromatogram suggested that additional atrazine-derived metabolites might be present in the incurred sample. This aspect was not pursued further because it was beyond the scope of the present study, which was designed to test only for the parent triazine herbicide, and was already expanded to include the de-ethyl and deisopropyl metabolites. Additional SFE recovery studies for eggs fortified with 100 ppb of the de-ethyl and deisopropyl metabolites found recoveries of 93.4 ± 6.8 and 92.7 ± 5.4%, respectively.

Table 2. SFE versus Solvent Extraction Method: Isolation of Atrazine/Metabolites from Whole Egg[a]

	atrazine[b]		de-ethyl-atrazine[b]		deisopropyl-atrazine[b]	
sample	SFE	solvent	SFE	solvent	SFE	solvent
1	50.0	31.5	250.0	80.0	200.0	54.1
2	108.4	39.7	575.0	101.3	390.0	65.1
3	36.3	27.0	112.5	38.8	78.1	33.5
4	37.5	32.3	108.9	37.5	48.4	34.6
5	22.9	16.0	100.0	24.5	42.1	28.0
6	14.6	12.7	56.7	14.3	18.4	19.1
7	ND[c]	ND	ND	ND	ND	ND

[a] Average of duplicate analyses. [b] ppb. [c] None detected, <1.0 ppb.

The original 20 mg/day, two-day dose level showed that there was more of the de-ethyl- and deisopropylatrazine metabolites in the eggs than atrazine itself. To determine whether we could obtain higher atrazine levels than those available from the first feeding study, four additional laying hens were fed single daily doses of 20, 30, 50, or 80 mg of atrazine for five consecutive days. These dosing levels are significantly higher than would normally be expected in feed. However, the only intent of this study was to obtain atrazine and its metabolites at levels high enough to validate this method. The 50 and 80 mg doses of atrazine caused some of the hens to either reduce the number of eggs produced or stop laying eggs, reflecting the probable toxic properties of this compound. However, a sufficient number of eggs were obtained at the 30 mg of atrazine/day dose level to permit a method comparison. We then analyzed these incurred eggs by both SFE and by a solvent extraction procedure also used for atrazine analysis (*FSIS Chemistry Laboratory Guidebook*, 1991). To obtain a valid comparison between the two isolation procedures, the same GC-N/P detection system was used. The results are shown in Table 2. There was a highly significant ($p < 0.01$) difference between methods of analysis. The overall mean atrazine level was 44.9 ppb (repeatability = 6.5 ppb) for the SFE method compared to 26.6 ppb (13.4 ppb) for the solvent method. This same trend was found for the two metabolites detected; the de-ethylatrazine means were 200.5 ppb (8.8 ppb) by SFE versus 49.4 ppb (3.7 ppb) for solvent extraction, and for deisopropylatrazine, 129.5 ppb by SFE (7.0 ppb) versus 39.1 ppb (2.6 ppb) by solvent extraction. These results demonstrate the improved recoveries for atrazine and its primary metabolites possible by SFE compared to the solvent extraction method.

CONCLUSION

Our results show that 10 triazine herbicides of similar polarity can be effectively recovered from eggs using

SFE with unmodified SC-CO_2 at 680 atm and 50 °C. This is especially apparent for the most commonly used triazine, atrazine, and two of its principal metabolites. Other published work on the application of SFE for atrazine isolation from other matrices indicates the need for up to 20% methanol for the efficient extraction of the same metabolites (Dean, 1996). The proposed SFE method, using off-line analyte trapping on an SPE cartridge containing deactivated Florisil, is superior to the solvent extraction method, is rapid (8−10 samples/day/analyst), uses only a small amount of organic solvent (8 mL), and requires only a minimal amount of sample manipulation and cleanup. This SFE method is suitable for the analysis of triazine residues in eggs.

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