AUTOMATED EXTRACTION OF ANTHRAQUINONE IN DRY MATRICES AND REAL SAMPLE MONITORING

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Overview

Anthraquinone (9,10-dioxoanthracene) is an aromatic organic compound of risk to human health and to the environment. It can be found in food due to its formation and adsorption during **combustion processes**, which are **used to dry vegetables** like tea, camomile or coffee. The European Commission recommends its control at low concentrations, since these vegetables' consumption is high [1].

The **objectives** of this study are (i) to evaluate the difficulties associated with the currently used **multiresidue methods** for food within the European Union; (ii) to develop an automated extraction method that allows the control of anthraquinone at very **low concentrations** –below the current maximum residue limit (MRL) of 20 µg/kg for tea, coffee, herbal infusions and cocoa [2]–; and (iii) to perform a **sampling study** of potentially contaminated dry vegetables.

Methods

Table 1. Automated pressurized liquid extraction method development parameters using the EDGE instrument.

Method (AMXX)	Solvent	Volume (mL)	Bubbling time (s)	Hold time (s)	T (° C)	Rinse step	Rinse volume (mL)	Total solvent (mL)	Dilution factor (V/m)	Clean-up (dSPE)	LOQ (µg/kg)	Rec. (%) (n = 3)
AM01	AcN	10	-	120	40	No	-	10	2.50	-	20	46
AM02	AcN	10	-	120	40	No	-	10	2.50	PSA	20	49
AM03	AcN	10	-	120	40	No	-	10	2.50	PSA, FA	20	54
AM04	AcOEt	10	-	120	40	No	-	10	2.50	-	20	27
AM05	AcOEt	10	-	120	40	No	-	10	2.50	PSA	20	29
AM06	AcOEt	10	-	120	40	No	-	10	2.50	PSA, FA	20	28
AM07	AcN	10	60	60	40	No	-	10	2.50	-	> 20	-
AM08	AcN	10	90	60	40	No	-	10	2.50	-	20	31
AM09	AcN	5	60	60	40	Yes	5	10	2.50	-	20	41
AM10	AcN	10	-	90	40	Yes	5	15	3.75	-	10	78
AM11	AcN	10	30	90	40	Yes	5	15	3.75	-	20	57
AM12	AcN	10	-	150	40	Yes	5	15	3.75	-	7.5	101

AcN = acetonitrile; *AcOEt* = ethyl acetate; *FA* = formic acid; *PSA* = primary secondary amine
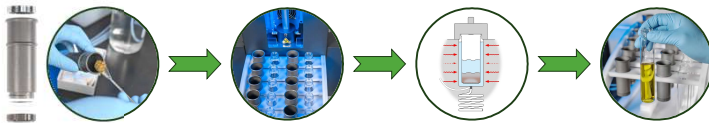
Automated sample extraction with pressurized liquid extraction was performed with the aid of an EDGE instrument. The amount of Earl Grey tea used in every method was 4 g. The limit of quantitation (LOQ) was determined by spiking three tea aliquots with anthraquinone (AQ) at three concentration levels (7.5, 10 and 20 µg/kg) (**Table 1**).

Deuterated anthraquinone (AQ-D_8) was employed as a means to correct any matrix effects that could arise from the studied methods, and to evaluate its use as a surrogate standard for AQ. The spiked levels of AQ-D_8 were also 7.5, 10 and 20 µg/kg.

All analyses were carried out using gas chromatography coupled to tandem mass spectrometry (GC-QqQ-MS/MS) (**Figure 1**).

Sample extraction

EDGE automated extraction system

GC parameters

Agilent Intuvo 9000 system

2 planar columns
(15 m × 0.25 mm × 0.25 µm)
Splitless injection
Total runtime 12.4 min
Injection volume 1 µL

MS parameters

Agilent 7010B Triple Quad

Ionization mode: EI
Acquisition mode: dMRM

AQ transitions:
208-152; 180-152; 208-180
AQ-D_8 transitions:
216-160; 188-160; 216-188

Figure 1. Sample extraction and GC-QqQ-MS/MS analysis.

Results

An already established multiresidue method including a sample hydration step [3] was tested, providing poor results. With the hypothesis that sample hydration was causing the coextraction of interfering matrix compounds in the analysis of AQ, automated pressurised liquid extraction was studied (**Table 1**).

First, AcN was determined to be the most appropriate extraction solvent, as it provided higher recovery values than AcOEt. Moreover, the use of a clean-up step including PSA was found to be unnecessary (**AM01-AM06**). A bubbling step to increase sample and solvent mixing was tested, but it was deemed counterproductive (**AM07-AM09**). Furthermore, a rinse step was also evaluated, and while it increased recovery values, it also meant a greater dilution factor. In the end, increasing the hold time (time the sample within the solvent) and using a rinse step were the crucial steps in the obtention of a low LOQ (7.5 µg/kg) and a 101 % recovery value (**AM12**).

The coextracted natural compounds which affect AQ's transition 180.0 -> 152.0 have been tentatively identified as the methylxanthines **caffeine** and **theobromine** with the aid of an Agilent 7890A GC instrument coupled to an Agilent 7250 GC/Q-TOF high-resolution accurate mass spectrometer.

The developed automated method has also been validated for **cocoa**, **coffee** and other **dry herbs**, at the same LOQ.

Tea samples

AQ detections 48%
Blank samples 52%

All samples

AQ detections 32%
Blank samples 68%

A sampling study was undertaken using **AM12**, during which AQ was detected in 32 % of the analysed samples (48 % if only tea samples are considered) at levels between the LOQ and the MRL.

Figure 3. XIC of caffeine (brown), theobromine (orange) and anthraquinone (green) (*top*). Mass spectrum at 10 min (*bottom*).

Multiresidue method [3]

2 g tea
+
4 mL H$_2$O (*wait 30 min*)
+
10 mL AcN
+
200 µL IS (25 mg/L)

Shake 7 min

4 g MgSO$_4$, 1 g NaCl, 1 g Na$_3$Citrate·2 H$_2$O, 0.5 g Na$_2$HCitrate·1.5 H$_2$O

Shake 7 min
Centrifuge 5 min

dSPE: 3 mL extract + 150 mg CaCl$_2$ + 150 mg PSA

Vortex 30 s
Centrifuge 5 min

Transfer supernatant into 4 mL vial + add 30 µL formic acid (5 %)

Evaporate 50 µL of the extract and dissolve in AcOEt

ANALYSIS

Method AM12

4 g tea

Cover with sand or Q-Screen

10 mL AcN

Hold 150 s at 40 ºC

5 mL AcN (rinse)

Evaporate 50 µL of the extract and dissolve in AcOEt

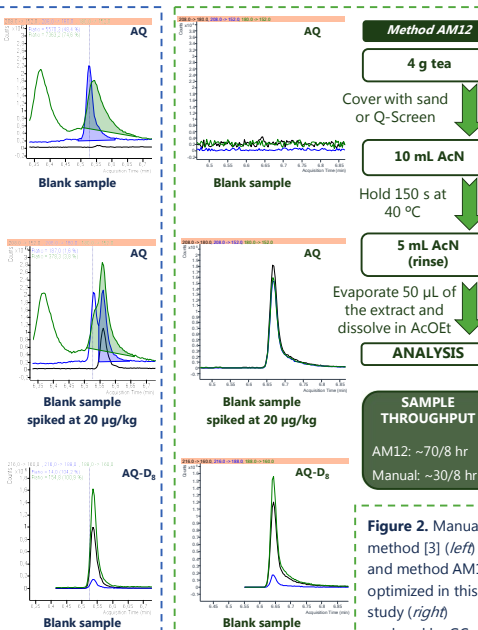ANALYSIS

SAMPLE THROUGHPUT

AM12: ~70/8 hr
Manual: ~30/8 hr

Figure 2. Manual method [3] (*left*) and method AM12, optimized in this study (*right*) analysed by GC-QqQ-MS/MS.
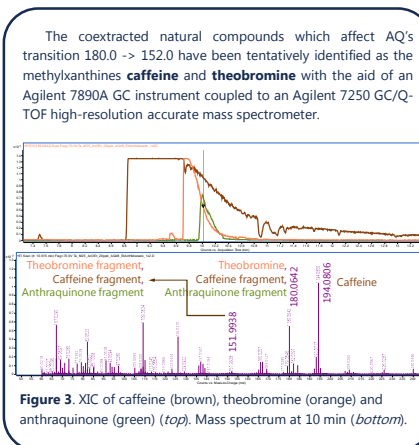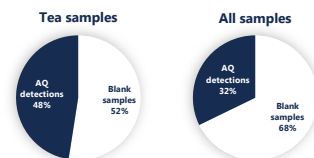
Conclusions

- Using an automated extraction method based on **pressurized liquid extraction** avoids the coextraction of matrix interferences since it **does not require a hydration step**.

- **Caffeine** and **theobromine** have been tentatively identified as the **main coextracted interferences** in manual extraction methods with a hydration step.

- The new **limit of quantitation is 7.5 µg/kg**, significantly lower than the current maximum residue level stablished by the European Commission.

- A sampling study of dry vegetables has detected **anthraquinone in 32 % of the samples** at levels **below the current maximum residue limit**, showcasing its better performance compared to manual extraction methods.

- Automated sample extraction instruments –including those based on pressurized liquid extraction– represent a very promising step forward in sample treatment and analysis, and can offer a **higher sample throughput** than manual extraction methods.

References

[1] European Food Safety Authority. The 2016 European Union report on pesticide residues in food. *EFSA Journal* **2018**, *16* (7).
[2] Commission Regulation (EU) No 1146/2014 of 23 October 2014 amending Annexes II, III, IV and V to Regulation (EC) No 396/2005 of the European Parliament and of the Council as regards maximum residue levels for anthraquinone, benfluralin, bentazone, bromoxynil, chlorothalonil, famoxadone, imazamox, methyl bromide, propanil and sulphuric acid in or on certain products. Official Journal of the European Union L308/3, 29.10.2014:1-58.
[3] Lozano, A.; Rajski, Ł.; Belmonte-Valles, N.; Uclés, A.; Uclés, S.; Mezcua, M.; Fernández-Alba, A. R. Pesticide residue analysis in teas and chamomile by liquid chromatography tandem mass spectrometry using a modified QuEChERS method: Validation and pilot survey in real samples. *J. Chrom. A* **2012**, *1268*, 109-122.