

## Determination of quaternary ammonium compound residues in fruits and vegetables by QuEChERS following LC-MS/MS analysis

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### Abstract

A simple and fast method to determine residues of quaternary ammonium compounds (QAC) in fruit and vegetable samples is presented. The analytes were extracted applying the frequently used QuEChERS method. Analysis was performed by liquid chromatography-electrospray tandem mass spectrometry (LC-ESI-MS/MS) in the positive mode using a triple quadrupole instrument. The method was validated for the following representative matrices: cucumber (high water content), raisin (high sugar content), lemon (sour) and wheat (dry) at 0.1 mg kg<sup>-1</sup> level. The method validation resulted in satisfying recoveries and relative standard deviations (RSDs) for all QAC. In cucumber, raisin, lemon and wheat matrices, recoveries ranged between 88 and 104% and RSDs ranged between 1.1 and 9.8%. All analytes were regularly detected at very low level (<0.005 mg kg<sup>-1</sup>) in blank sample material and even when only solvent was injected. Therefore, the reporting limit was set to 10 µg kg<sup>-1</sup>. Our results demonstrate that determining QAC can easily be integrated in routinely conducted QuEChERS extraction followed by LC-MS/MS analysis. In a final step we proved the method's performance analyzing conventional and organic grown samples from the common market.

### Introduction

Quaternary ammonium compounds (QAC) are surface active substances containing a quaternary cationic nitrogen atom, substituted by alkyl chains of varying length. The important representatives benzalkonium chlorides (BAC) and didecylidimethylammonium chloride (DDAC) are shown in figure 1. QAC are enriched in cell membranes of living organisms and can impair cell membrane functions [1]. Due to these characteristics, QAC are used as biocides [2], pesticides [3, 4], disinfectants [5, 6], wood preservatives [7, 8] and additives for technical applications [9], furthermore as ingredients in human and veterinary

medicinal products and cosmetics [9]. Additionally, plant protection products like plant strengtheners can contain QAC not as active ingredients but as additives. The authorization of such plant strengtheners has meanwhile been withdrawn within Germany.

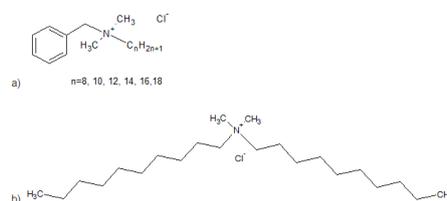


Fig. 1 (a) Benzalkonium chloride (BAC) with chain length of n=10, 12, 14, 16  
(b) Didecylidimethylammonium chloride (DDAC)

The use of QAC in plant protection products or biocides were expected not to result in any residues in food and no maximum residue levels (MRLs) have been fixed. Based on the broad application spectrum of QAC food and feed can probably be cross-contaminated due to contact with contaminated surfaces, containers and transport equipment.

Some QAC have been listed in Commission Regulation (EC) 1112/2002 [10] of the European Union to be assessed as active substances for pesticide use. With Commission Decision 2004/129/EC [11] most of the QAC have not been included in Annex I of Commission Regulation 91/414 of the EU which is now repealed by Commission Regulation (EC) 1107/2009. Therefore, QAC are not authorized for the use in plant protection products within the EU. Only DDAC is approved within Commission Regulation (EC) 1107/2009 [12] as an active substance for plant protection products and authorizations are in place in Spain, Belgium and France [9]. As no specific MRLs are established within Commission Regulation (EC) 396/2005 [13] the default MRL of 0.01 mg kg<sup>-1</sup> according to Art (1) (b) of Regulation (EC) 396/2005 is in force for

residues of QAC in food and feed items listed in Annex I of that Regulation, no matter if the residue is caused by application of biocides or plant protection products. Recent analyses carried out by food businesses and regulatory authorities in Germany have detected unexpected residues of BAC and DDAC high above the statutory MRL of 0.01 mg kg<sup>-1</sup>. It is assumed that disinfectant use is the primary source of these residues – caused by disinfection of water used for food washing and irrigation or by treatment of surfaces with biocide products. In July 2012, the Standing Committee on the Food Chain and Animal Health (SCFCAH) thereupon decided that food and feed of plant and animal origin with a level of DDAC as well as BAC higher than 0.5 mg kg<sup>-1</sup> should not be placed on the market and be withdrawn from the market and disposed of safely [14, 15].

Amongst other things, the length and branching of the alkyl chains contribute to the QAC's toxicity. The German Federal Institute for Consumer Protection (BfR) has derived the acute reference dose (ARfD) and acceptable daily intake (ADI) for DDAC and BAC of 0.1 mg per kg body weight per day [16, 17]. Quaternary ammonium compounds are only considered acutely toxic in few cases. Toxic effects on aquatic organisms have been reported [9], whereas in reproductive toxicity studies on DDAC, neither adverse effects on the reproductive parameters, nor any indication of teratogenic properties were observed [18]. However, QAC increase the permeability of membranes by damaging the protective water lipid membranes of the outer skin and can thus have an effect on the absorption level of other toxic substances.

Several approaches for the analysis of quaternary ammonium compounds are described in literature. There are different types of methods for QAC determination in varying matrices, e.g. enzyme-linked immunosorbent assay [19], photometry [20], liquid chromatography [8] as well as ion

chromatography [21] and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) [22]. A growing number of methods have been reported using liquid chromatography coupled to mass spectrometry with quadrupole [23-26] and TOF [27, 28] detection.

Only small investigations have been reported for the determination of QAC applying the frequently used QuEChERS (short for Quick Easy Cheap Effective Rugged and Safe) [28, 29] method. QuEChERS permits a simple and fast extraction procedure and provides high recoveries for a broad spectrum of pesticides. Aim of this work was to include the determination of QAC into a multiresidue scheme applying QuEChERS extraction. Analysis was performed applying liquid chromatography coupled with triple quadrupole mass spectrometry detection (LC-MS/MS).

## Experimental

### Chemicals and standards

Acetonitrile and methanol of gradient grade were purchased from Merck (Darmstadt, Germany). Water for liquid chromatography was deionized in the laboratory using a Direct-Q 3 UV Ultrapure Water Purification System (Billerica, MA, USA). Dry ice for sample comminution was delivered in blocks by a local provider, stored at -80°C until use and was tested not to contain any of the analysed QAC at relevant levels. Ammonium formate was purchased from Sigma-Aldrich (Steinheim, Germany). Magnesium sulfate anhydrous grit, trisodium citrate tribasic dihydrate and disodium hydrogen citrate sesquihydrate all used for phase separation after QuEChERS extraction were from Sigma-Aldrich (Steinheim, Germany). Sodium chloride was purchased from Merck (Darmstadt, Germany).

The analytical pesticide standards DDAC and the internal standard substance chlorpyrifos D10 (purity > 98%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany), BAC 10, 12 and 16 (purity > 99.9%) were purchased from Sigma-Aldrich (Steinheim, Germany) and BAC 14 (purity > 98 %) was purchased from Fluka (Buchs, Germany). All standards were stored at -18°C. Stock solutions of 1 mg mL<sup>-1</sup>, considering standard purity, were prepared in acetonitrile. For the internal standard chlorpyrifos D10 a working solution of 20 µg mL<sup>-1</sup> was prepared in acetonitrile. All solutions were stored in the dark at 4°C.

### Samples and commodities

Different fruit and vegetable samples of different origins were collected in the routine

laboratory for pesticide analysis in fruits and vegetables located at the Chemisches und Veterinäruntersuchungsamt (CVUA) Stuttgart. Representative parts of the fresh blank samples were pre-chopped and deep-frozen at -18°C overnight after arriving in the routine pesticide laboratory. The pre-chopped samples were subsequently milled with dry ice and stored at -18°C until extraction.

Dried fruits (e.g. raisins) were mixed with cold water at a ratio of 1:1.7, homogenized with the addition of dry ice and the homogenates were deep-frozen at -18°C until use.

Dry samples (e.g. wheat) were grinded thoroughly without adding water and were stored at room temperature until extraction.

### Apparatus

Frozen samples were chopped with dry ice using a Prime Cut UM5 universal machine from Stephan Machinery GmbH (Hameln, Germany). Dry samples were ground at room temperature using a Grindomix GM 200 knife mill by Retsch (Haan, Germany). The automatic shaking machine Geno Grinder 2010 (SPEX Sample Prep, Metuchen, USA) was used for QuEChERS automated extraction. The centrifuge Rotana 460 by Hettich (Tuttlingen, Germany), appropriate to the centrifuge tubes employed in the procedure and capable of achieving 4000×g, was used. Electronic pipettes applicable for volumes of 10 - 100 µL and 100 - 1000 µL, respectively, and manual pipettes applicable for volumes of 1 - 10 mL were from Eppendorf (Hamburg, Germany). Analytical balances capable of weighing to 0.1 mg or to 0.01 g were from Mettler-Toledo (Greifensee, Switzerland). The Fortuna Optifix 10 mL Universal Dispenser was used to add solvent to the samples. The automatic dispenser system Opus (20 - 50 mL; Ex 20°C; Hirschmann Laborgeräte, Eberstadt, Germany) was used for dilution of the working solutions.

50-mL PP (114×28 mm) single-use tubes with screw caps for the sample extraction were from Sarstedt (Nümbrecht, Germany). 1.5-mL LC autosampler vials and 20-mL screw-cap vials were from Ziemer GmbH (Mannheim, Germany). Stackable plastic cups for the storage of pre-weighed partitioning salt mixture were from Juro-Labs (Henfenfeld, Germany). Salt mixtures for phase separation were pre-weighed using a rotary sample divider from Fritsch (Idar-Oberstein, Germany).

An Agilent 1100 HPLC system (Agilent, Waldbronn, Germany), consisting of a quaternary pump, a standard autosampler and a column oven, combined with the mass

spectrometer API 4000 (ABSCIEX, Darmstadt, Germany), run in ESI positive mode, was used for analysis of the QuEChERS extracts. The samples were injected on a Phenomenex Synergi Hydro RP 2.1 x 150 mm column with 4 µm particle size (Phenomenex, Aschaffenburg, Germany).

### QuEChERS extraction

For the QuEChERS method 10 ± 0.1 g (5 g in case of dry matrices and 13.5 g in case of frozen homogenates from dried fruits) of the prepared frozen samples were weighed into 50-mL PP tubes. 10 mL of acetonitrile followed by 100 µL of the internal standard working solution of chlorpyrifos D10 were added to the samples. The tube was closed and shaken by a mechanical shaker for one minute as a first extraction step. The second extraction step subsequently followed including the addition of a pre-weighed partitioning salt mixture (4 g magnesium sulfate anhydrous grit, 1 g sodium chloride, 0.5 g disodium hydrogen citrate sesquihydrate, 1 g trisodium citrate dihydrate) and shaking for 1 min by hand or mechanical shaker. The tube was centrifuged for 5 min at 3500 x g and the upper acetonitrile phase was transferred to vials and directly applied to LC-MS/MS measurement without any further cleanup.

### LC-MS/MS analysis

Mobile phase A consisted of 5 mM ammonium formate in purified water, mobile phase B was 5 mM ammonium formate in methanol. A gradient program was used starting with a flow rate of 0.1 mL min<sup>-1</sup> and 100% of mobile phase A (0%B) at injection time, gradually changing to 30% A (70% B) over 3 min and to 15% A (85% B) until 6 min after injection. Then, the flow was set to 0.3 mL min<sup>-1</sup> and mobile phase composition was shifted to 10% A (90% B) over 3 min. This mobile phase composition was kept for 11.5 min and was then shifted back to starting conditions within 0.5 min. The conditions were maintained for 11 min for column equilibration. The injection volume was 3 µL, automatically diluted with 10 µL of mobile phase A during injection procedure. The column temperature was set to 40°C. ESI interface in positive mode was applied, the MS/MS detection was performed in the multiple reaction monitoring (MRM) mode. The ion spray voltage was 5500 V, the nebulizer gas and the turbo gas were synthetic air, both at 60 psi, and the curtain gas was nitrogen at 30 psi, the source temperature was 500°C.

Two precursor-productions were monitored for each compound in the multiple reaction

monitoring mode (MRM). The first transition was used for quantification, while the second transition served as a quality control.

### Method validation

The method was validated at 0.1 mg kg<sup>-1</sup> level for the following representative matrices: cucumber (high water content), raisin (high sugar content), lemon (sour) and wheat (dry). Blank samples of cucumber, raisin, lemon and wheat were spiked with a mixture of BAC 10, 12, 14, 16 and DDAC (n=5) following QuEChERS extraction and LC-MS/MS analysis. All blank samples were proved not to contain QAC in relevant amounts right before validation. We applied matrix matched calibration standards at concentrations representing 60 and 120% of the validation level for each matrix and analyte. Quantification was carried out via calculating the ratio between analyte peak area and ISTD peak area for both, calibration standards and recovery samples. We then calculated the concentration of the recovery samples from the known concentration of the calibration standard.

### Results and discussion

Each compound could be detected in LC-MS/MS with high sensitivity. The analytes eluted as well-defined and narrow shaped peaks, BAC 10, 12, 14 and 16 at retention times of 11.09, 11.91, 13.03, and 14.42 min respectively, DDAC eluted at 13.17 min. Tab. 1 gives an overview of the compounds' MRM transitions, MS conditions and retention times. Typical LC-MS/MS chromatograms are shown in Fig. 2 and Fig. 3.

Limits of Quantification (LOQ), defined as the lowest concentration showing a signal to noise ratio >10, were well below 10 µg kg<sup>-1</sup> for all analytes. However, we detected small peaks of all QAC in blank samples as well as in organic solvents. Small amounts of QAC seem to be always present, which can be ascribed to the fact that QAC are commonly used in a broad spectrum of cleaning agents and disinfectants and can also be present in indoor air [21] or house dust [1]. The positive findings of QAC in blank samples and also in solvents remained problematic. Although the LOQs were well below 10 µg kg<sup>-1</sup>, our Reporting Limit was set to 10 µg kg<sup>-1</sup>.

The method validation resulted in satisfying recoveries and relative standard deviations (RSDs) for all QAC. In cucumber, raisin, lemon and wheat matrices, recoveries ranged between 88 and 104% and RSDs ranged between 1.1 and 9.8%. The average recoveries and RSDs for all applied matrices are shown in Tab. 2 and Tab. 3.

Additional recovery data was obtained within the framework of an on-going validation. BAC 12 showed recoveries of 103-124% in mango (2x) and orange (1x) samples and DDAC showed recoveries of 87-120% in banana (3x), parsley (2x), bean (2x), (rocket (1x), mushroom (1x), grape (1x) and blueberry (1x) samples. More data can also be obtained at the EURL Datapool [30].

Since May 2012 a total of 1058 fruit and vegetable samples have been analysed for QAC residues.

19 out of 556 fruit samples (3.4%) were found to contain residues of QAC, thereof 13 samples with amounts exceeding the default MRL of 0.01 mg kg<sup>-1</sup>. The maximum amount detected was 0.3 mg kg<sup>-1</sup> DDAC in a mandarin sample. 13 out of 502 vegetable samples (2.6%) were found to contain

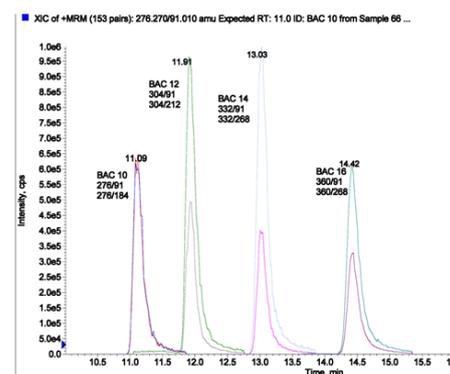


Fig. 2 LC-MS/MS chromatogram of BAC 10, 12, 14, 16

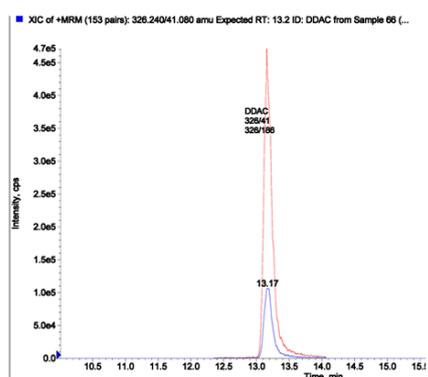


Fig. 3 LC-MS/MS chromatogram of DDAC

Tab. 1 Properties of the studied compounds including MS operating conditions

	Empirical formula	Retention time (min)	First Transition	DP	CE	CXP	Second Transition	DP	CE	CXP
BAC 10	C <sub>19</sub> H <sub>34</sub> CIN	11.09	276 → 184	55	27	10	276 → 91	55	37	36
BAC 12	C <sub>21</sub> H <sub>38</sub> CIN	11.91	304 → 212	91	29	10	304 → 91	91	37	16
BAC 14	C <sub>23</sub> H <sub>42</sub> CIN	13.03	332 → 240	83	31	10	332 → 91	83	59	8
BAC 16	C <sub>25</sub> H <sub>46</sub> CIN	14.42	360 → 268	78	33	12	360 → 91	78	67	10
DDAC	C <sub>22</sub> H <sub>48</sub> CIN	13.17	326 → 186	61	39	12	326 → 41	61	93	6
Chlorpyrifos D10 (ISTD)	C <sub>9</sub> HCl <sub>3</sub> D <sub>10</sub> NO <sub>3</sub> PS	12.50	360 → 199	66	23	12	-	-	-	-

residues of QAC, thereof 13 samples with amounts exceeding the default MRL of 0.01 mg kg<sup>-1</sup> and a maximum amount of 0.92 mg kg<sup>-1</sup> detected in a parsley sample. Residue above the MRL were found for DDAC mostly (18 samples), but in 9 samples residues above the MRL were found for BAC. An overview of QAC residues in the tested samples is given in Tab. 4.

Conventional as well as organic fruits and vegetables were affected by positive findings and exceeding MRLs. Residues of QAC were found in different food commodities coming from different countries of origin. Therefore a broader use of products containing QAC seems to be in place. A source of residues of QAC might be the use of biocides in food processing such as washing and packaging. Another source especially of residues of DDAC seems to be the use of plant strengthener products containing DDAC.

Tab. 2 Mean recoveries in % at 0.1 mg kg<sup>-1</sup>

	BAC 10	BAC 12	BAC 14	BAC 16	DDAC
Cucumber	98	101	100	95	96
Raisin	101	104	98	97	98
Lemon	99	97	97	100	97
Wheat	98	92	88	88	96

Tab. 3 RSDs in % at 0.1 mg kg<sup>-1</sup> (n=5)

	BAC 10	BAC 12	BAC 14	BAC 16	DDAC
Cucumber	4.2	3.6	4.3	2.2	3.7
Raisin	4.4	1.6	2.0	1.9	2.9
Lemon	3.6	1.1	2.8	1.4	2.3
Wheat	5.6	1.6	3.2	5.0	9.8

Therefore, the product "Vi-Care" was recently deleted from the official list of plant strengtheners by the German food safety authority Federal Office of Consumer Protection and Food Safety (BVL). The

product is not allowed to be placed on the market in Germany anymore.

## Conclusion

We presented a simple and fast method to determine residues of quaternary ammonium compounds (QAC) in fruit and vegetable samples. The samples were extracted due to the QuEChERS method and QAC were analyzed by LC-MS/MS. All compounds showed satisfying recoveries and RSDs while method validation. A remaining problem was that QAC could be detected in blank samples and even after the injection of solvents. As a consequence, we set our reporting limit to 10  $\mu\text{g kg}^{-1}$ . We could show that QAC can simply be included into a multiresidue scheme applying QuEChERS extraction followed by LC-MS/MS detection.

Residues of the QAC compounds DDAC and BAC have actually been found in a broad variety of fruits and vegetables exceeding the default MRL of 0.01  $\text{mg kg}^{-1}$  in many cases. Conventional and organic produce was affected likewise. The residues might be caused by using QAC containing disinfection products in food processing like washing and packaging but also by applying plant strengtheners containing QAC. To avoid exceeding MRLs QAC containing products should neither be used in cultivation nor in food processing.

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Tab. 4 Residues of QAC in naturally grown fruit and vegetable samples

Commodity	No. of samples	With residues	With residues (%)	Without residues
Berry fruits	255	2	0,8	253
Stone fruits	116	4	3,4	112
Exotic fruits	96	8	8,3	88
Citrus fruits	40	5	12,5	35
Pome fruits	49	-	-	49
Leguminous plants	62	6	9,7	56
Leafy vegetables	131	4	3,1	127
Fruiting vegetables	165	1	0,6	164
Sprout vegetables	66	-	-	66
Root vegetables	28	-	-	28
Cabbage	9	-	-	9
Cucurbit	41	2	4,9	39
Total	1058	32	Ø 3,0	1026

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