



## Liquid Chromatography/ Mass Spectrometry

**Author:**

Wilhad M. Reuter

PerkinElmer, Inc.  
Shelton, CT

# Analysis of Quaternary Ammonium Compounds (QACs) as Possible Disinfectant Residues in Milk by LC-TOF

### Introduction

Quaternary ammonium compounds (QACs) have the basic structure  $NR_4^+$ . Those possessing R groups with long alkyl chains are

known to be especially effective as antimicrobial agents and particularly useful for the disinfection of containers and surfaces. This is particularly relevant in the milk industry, as QACs are typically used to disinfect the insides of tanks used for transporting milk from farms to processing plants. If significant QAC residues are left behind after tank disinfection, these compounds may leach into the milk and, ultimately, may get into the store-bought milk supplies at levels compromising personal health. Recent data points to nearly 12% of all monitored milk to be tainted with QACs.<sup>1</sup>

The primary QACs that may be found in milk are benzyldimethyldodecylammonium chloride (BAC 12), benzyldimethyltetradecylammonium chloride (BAC 14), benzyldimethylhexadecyl ammonium chloride (BAC 16) and didecylmethylammonium chloride (DDAC). Their chemical structures and expected parent masses in solution are shown in Figure 1.

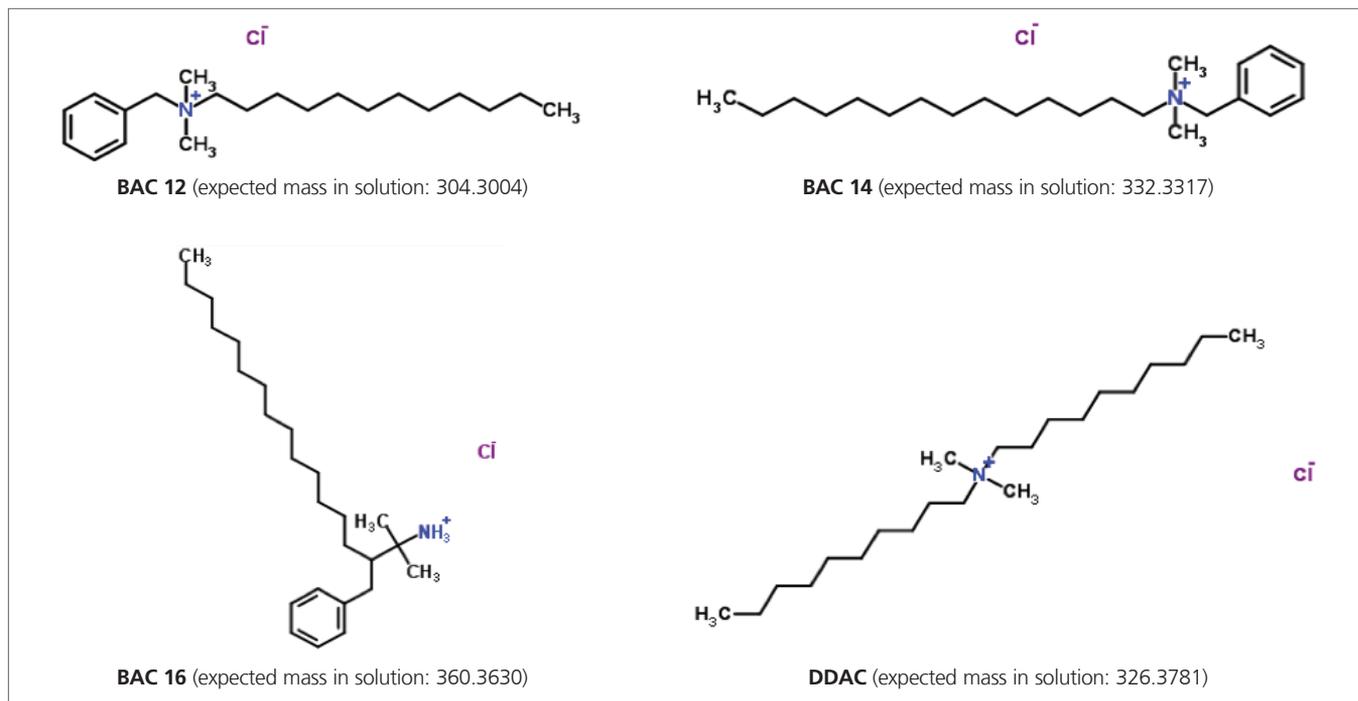


Figure 1. Chemical structures and expected masses of the four quaternary ammonium compounds (QACs) analyzed.

Regarding safety and regulations, the European Union Reference Laboratory (EURL) has taken the following position:

“Because no specific maximum limit for residues of DDAC and BAC was established under EU Regulation No. 396/2005, the general residue limit of 0.01 mg/kg applies.

In October 2012, the Standing Committee on the Food Chain and Animal Health (SCoFAH) endorsed Guidelines on measures to be taken regarding the presence of DDAC<sup>2</sup> and BAC<sup>3</sup> in or on food and feed. It was recommended that EU Member States carry out investigations on the possible causes of BAC/DDAC contamination and to put in place a monitoring program to get an overview of the BAC and DDAC levels in all food and feed of plant and animal origin. Considering that the current default MRLs for DDAC and BAC (of 0.01 mg/kg) are not a health standard, a temporary enforcement level of 0.5 mg/kg was agreed upon. As no specific residue definition was defined, there is still uncertainty as to how residues are to be expressed. Based on the first results of the monitoring program a lower enforcement level for QACs is under discussion.”<sup>4</sup>

Considering the above, this application note presents an LC-TOF (time-of-flight) method for the analysis of the four most common QACs that may be found in milk (a rather complex matrix), with relatively little sample preparation. This method takes advantage of the inherent mass accuracy and high resolution afforded by TOF detection for specificity and component identification. Method conditions and performance data, including linearity and repeatability, are presented.

## Experimental

### Hardware/Software

For all chromatographic separations, a PerkinElmer Flexar UHPLC System was used, including the Flexar FX-20 pump, FX autosampler, column oven and AxION 2 TOF MS detector.

All instrument control, analysis and data processing was performed using the PerkinElmer Chromera® CDS software platform.

### Method Parameters

The HPLC method parameters are shown in Table 1.

Table 1. HPLC Method Parameters.

HPLC Conditions																															
Column:	PerkinElmer Brownlee™ 2.7- $\mu$ m 2.1 x 50-mm C18 (Part# N9308402)																														
Mobile Phase:	Solvent A: 0.1% formic acid in water Solvent B: 0.1% formic acid in acetonitrile Solvent Program: <table border="1"> <thead> <tr> <th>Step</th> <th>Time</th> <th>Flow Rate (mL/min)</th> <th>%A</th> <th>%B</th> <th>Curve</th> </tr> </thead> <tbody> <tr> <td>0 (Equil.)</td> <td>4.0</td> <td>0.4</td> <td>70</td> <td>30</td> <td>1</td> </tr> <tr> <td>1</td> <td>1.5</td> <td>0.4</td> <td>50</td> <td>50</td> <td>1</td> </tr> <tr> <td>2</td> <td>2.0</td> <td>0.4</td> <td>0</td> <td>100</td> <td>1</td> </tr> <tr> <td>3</td> <td>0.5</td> <td>0.4</td> <td>0</td> <td>100</td> <td>1</td> </tr> </tbody> </table>	Step	Time	Flow Rate (mL/min)	%A	%B	Curve	0 (Equil.)	4.0	0.4	70	30	1	1	1.5	0.4	50	50	1	2	2.0	0.4	0	100	1	3	0.5	0.4	0	100	1
Step	Time	Flow Rate (mL/min)	%A	%B	Curve																										
0 (Equil.)	4.0	0.4	70	30	1																										
1	1.5	0.4	50	50	1																										
2	2.0	0.4	0	100	1																										
3	0.5	0.4	0	100	1																										
Analysis Time:	4 min.																														
Flow Rate:	0.4 mL/min. (maximum pressure: ~3900 psi)																														
Oven Temp.:	25 °C																														
Detection:	Perkin Elmer AxION 2 TOF MS; positive ion mode; mass range: 100-1250 MW Ions 118.0862 and 922.0098 were used as lock mass calibrants to compensate for any mass drift during acquisition; spectral rate: 5 spectra/sec MW channels (EICs): BAC 12: 304.300; BAC 14: 332.332; BAC 16: 360.363; DDAC: 326.378																														
Injection Volume:	2 $\mu$ L																														

### Solvents, Standards and Samples

All solvents used were HPLC grade and filtered via 0.45- $\mu$ m filters.

All QAC standards were purchased from Sigma-Aldrich (St Louis, MO USA), including the following: benzyldimethyldodecylammonium chloride (BAC 12), benzyldimethyltetradecylammonium chloride (BAC 14), benzyldimethylhexadecyl ammonium chloride (BAC 16), and dicyldimethylammonium chloride (DDAC).

A 10-ppm stock standard solution was prepared by dissolving 10 mg of each of the four QAC components into 1 liter of 50:50 acetonitrile/water. This stock solution was then used to prepare 1-ppm to 0.05-ppm standard solutions by serial dilution.

The analyzed product was a store-bought container of whole milk. Using a 1-L solvent bottle, a spiked sample was prepared by spiking 10 mg of each standard component into 1 liter of milk. Both this spiked sample and an unspiked liter of milk were then placed on a magnetic stirring plate and stirred for 30 minutes at 30 °C. Both samples were then capped and refrigerated until further use.

When ready for analysis, after allowing both samples to equilibrate to room temperature, 20 mL of each sample was transferred to a separate 50-mL polypropylene centrifuge tube. 20 mL of acetonitrile was then added to each tube, causing the

proteinaceous ingredients in the milk to precipitate (“protein crash”). Both samples were then centrifuged at 8000 rpm for five minutes. After centrifugation, 2 mL of each supernatant was filtered through a 0.45 µm filter, transferred into a 2-mL sample vial and subsequently injected.

Prior to injection, all calibrants were also filtered through 0.45-µm filters, removing any small particles.

## Results and Discussion

Figure 2 shows the chromatographic separation of the 0.5-ppm QAC standard, separating the four QAC compounds in under 3.5 minutes. All displayed chromatograms consist of the overlays of four EICs (extracted ion chromatograms), each corresponding to the expected mass of the individual QAC components.

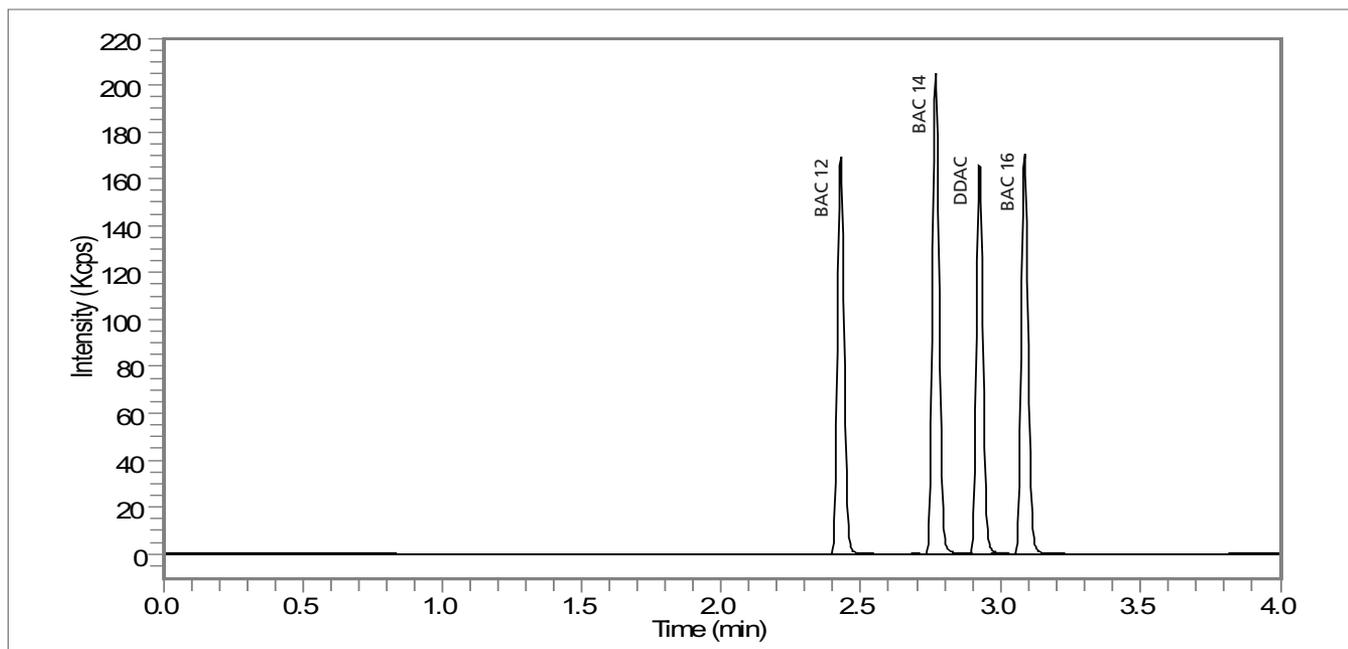


Figure 2. Chromatogram of 0.5-ppm QAC standard; EIC channels: BAC 12: 304.300; BAC 14: 332.332; BAC 16: 360.363; DDAC: 326.378

Figure 3 shows the overlay of 10 replicate injections of a QAC standard mix, demonstrating exceptional reproducibility. The retention time (RT) %RSDs ranged from 0.09 to 0.16.

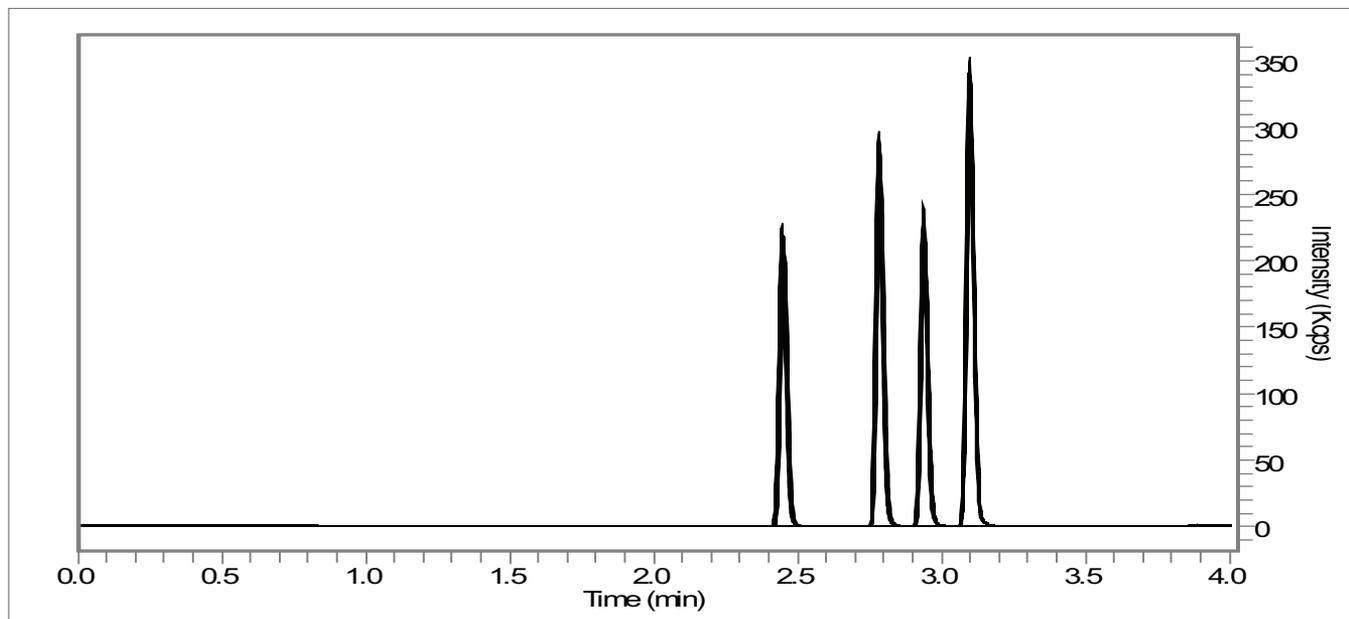


Figure 3. Overlaid chromatograms of 10 replicates of a preliminary standard mix.

Figure 4 shows the calibration plots over a concentration range of 0.05 to 1 ppm, with all four QAC components having quadratic fit coefficients  $> 0.99$  ( $n = 3$  at each level).

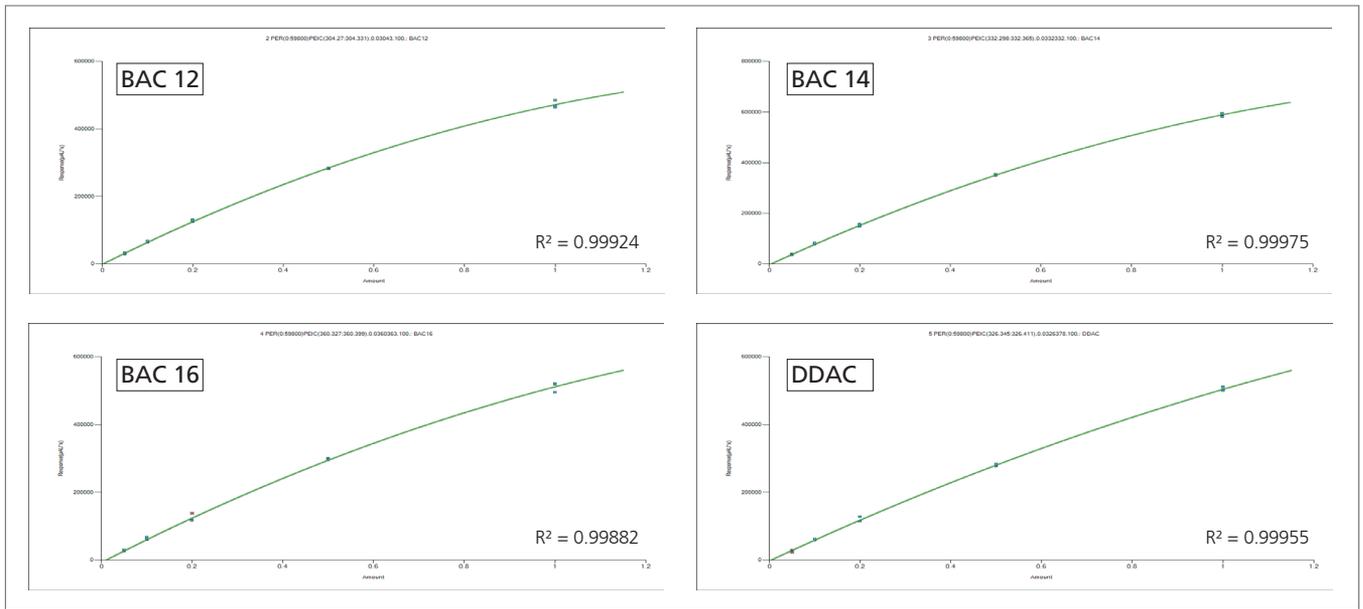


Figure 4. Plots of 5-level calibration set for all four QACs.

Figure 5 shows the averaged MS spectra for all four QAC components, highlighting the exceptional mass accuracy that was achieved using the integrated lock mass option. These were based on the expected exact masses for each component in solution.

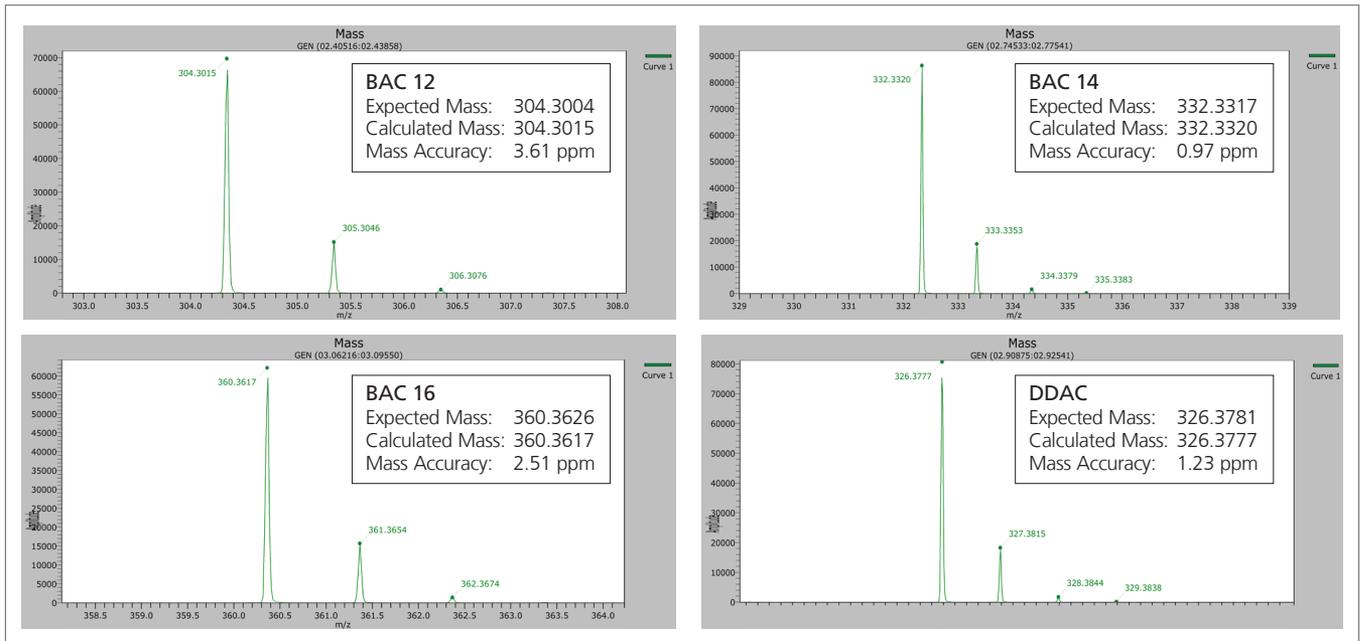


Figure 5. Averaged MS spectra showing the mass accuracy achieved for each of the four QAC components.

The identity of the QACs was further confirmed with the help of elemental composition matching via AxION EC ID software. Per example shown in Figure 6, the accurate mass and isotope information for DDAC was entered into the software and searched against a selected database, in this case, PubChem. The search resulted in an elemental composition that perfectly matched DDAC.

Following the liquid-liquid extraction procedure described earlier, an extracted sample of whole milk and the same whole milk previously spiked with of 1-ppm QAC were analyzed. The overlaid chromatograms (EICs) of both extracts are shown in Figure 7.

The quantitative results for the spiked milk extract are shown in Table 2. Considering that the liquid-liquid extraction procedure involved a 1:1 sample dilution, the normalized amounts were 0.984, 0.730, 0.852 and 0.952 ppm, for BAC 12, BAC 14, BAC 16 and DDAC, respectively. Further refinements in the extraction procedure may very well be able to improve on this recovery.

Also, in Figure 7, the expanded view of the four components shows a small amount of QACs in the unspiked milk extract. However, as shown in Figure 8, these amounts are very low and are similar to levels observed for the 70% acetonitrile (ACN) blank injections. Follow-up testing revealed this small amount of QAC background to be due to slight column carryover. As this level is far below the maximum allowable 0.5 mg/kg (ppm) regulation QAC level set in the EU (the only global region that has any regulations for this thus far), this background was considered insignificant and quite acceptable.

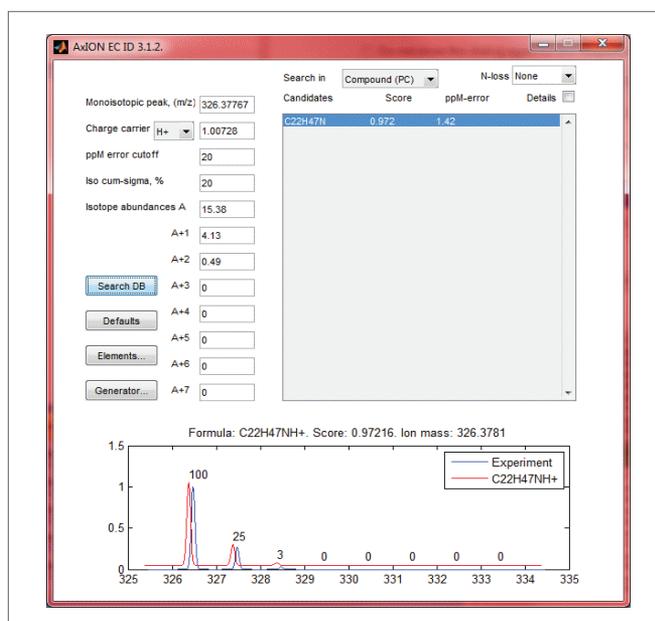


Figure 6. Example of a database search for the formula confirmation of DDAC.

Table 2. Calculated/normalized amounts for QACs in 1 ppm-spiked whole milk extract.

Component	Calculated Amount (ppm)	Normalized Amount (ppm) – considering 1:1 dilution	% Recovery
BAC 12	0.492	0.984	98%
BAC 14	0.365	0.730	73%
BAC 16	0.426	0.852	85%
DDAC	0.476	0.952	95%

\* All samples were run in duplicate, using averaged values

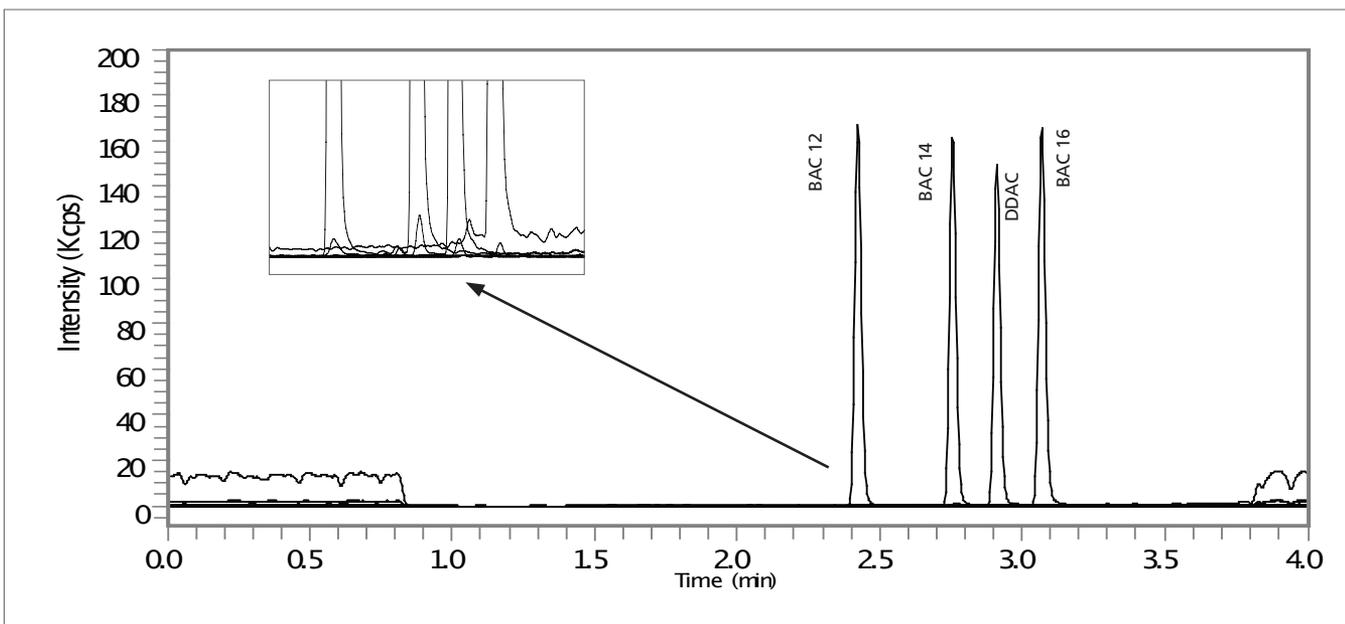


Figure 7. Chromatograms (EICs) of 1 ppm-spiked whole milk extract (four large peaks) overlaid with that of the unspiked milk extract. Due to the very low levels, the EICs of the unspiked milk extract can only be seen in the expanded view.

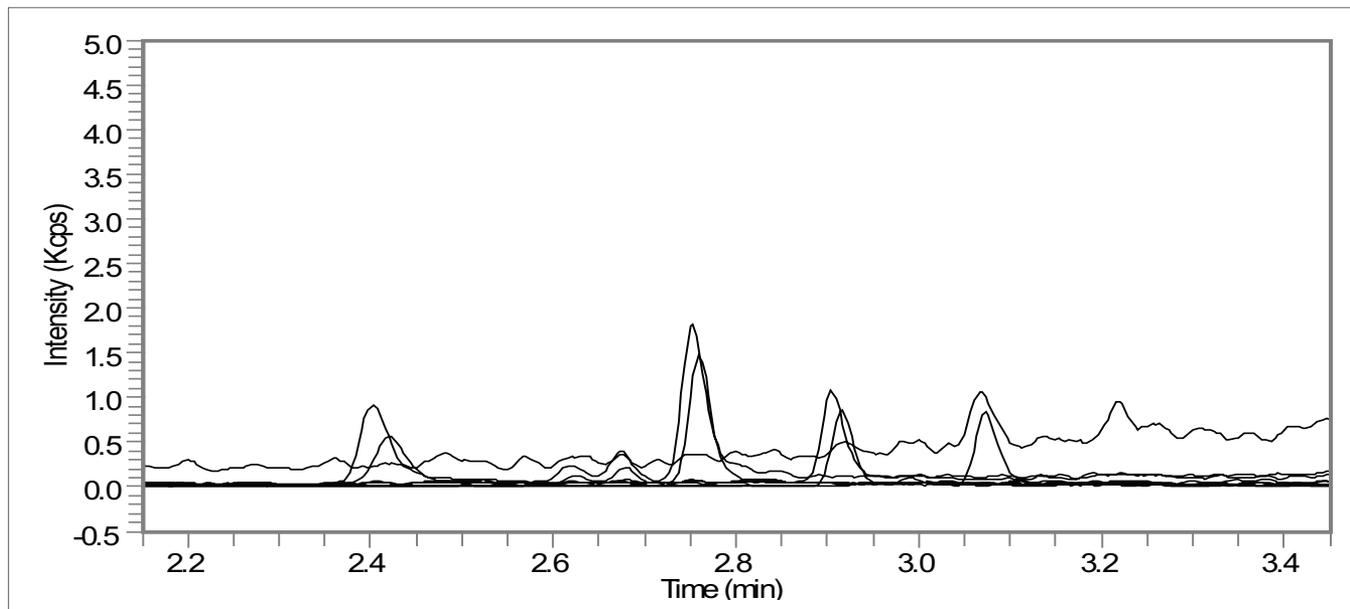


Figure 8. Chromatogram (EICs) of un-spiked milk extract overlaid with 70% ACN/water blank injection.

## Conclusion

With rising health concerns and the large quantities of milk that are consumed, it is important to have reliable procedures for the monitoring of possible unhealthy contaminants in dairy products. With this in mind, this work demonstrated the fast/effective chromatographic separation for the quantitative analysis of four quaternary ammonium compounds (QACs) in milk by LC-TOF. The results exhibited exceptional reproducibility with more than adequate sensitivity for monitoring down to current regulated levels. In addition, by using a TOF detector, the combination of averaged MS spectra, mass accuracy checks and database search results allowed for the definitive identification/confirmation of the four QAC components.

## References

1. <http://www.efsa.europa.eu/de/supporting/doc/483e.pdf>
2. European Commission, 2012a. Guidelines as regards measures to be taken as regards the presence of Didecyl Dimethyl Ammonium Chloride (DDAC) in or on food and feed agreed by the Standing Committee of the Food Chain and Animal Health (SCoFAH) on July 13, 2012 and modified on October 5, 2012.
3. European Commission, 2012b. Guidelines as regards measures to be taken as regards the presence of Benzalkonium Chloride (BAC) in or on food and feed agreed by the Standing Committee of the Food Chain and Animal Health (SCoFAH) on July 25, 2012 and modified on October 5, 2012.
4. EU Reference Laboratory for Pesticides Requiring Single Residue Methods, CVUA Stuttgart, Schaflandstr. 3/2, 70736 Fellbach, Germany.