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# Determination of Methacholine Chloride and Potential Impurities Using a Reagent-Free Ion Chromatography System

## **INTRODUCTION**

Methacholine chloride [2-(acetyloxy)-N,N,N-trimethyl-1-propanaminium chloride] is a synthetic analogue of the neurotransmitter acetylcholine. Its principal use is as a parasympathomimetic bronchoconstrictor agent to assess bronchial asthma of subjects in respiratory function labs and epidemiological field studies.<sup>1</sup> Methacholine chloride powder is dissolved in 0.9% sodium chloride (with or without the presence of 0.4% phenol used to prevent biological growth) at 16 or 25 g/L.<sup>2,3</sup> After serial dilutions of this initial solution, each methacholine chloride solution is nebulized and inhaled by the subjects, followed by an evaluation of pulmonary function.

The accuracy of this inhalation challenge test depends on the methacholine concentration. However, methacholine has been shown to decompose over time.<sup>4</sup> Methacholine hydrolysis will yield  $\beta$ -methylcholine and acetic acid. Acetylcholine, a possible synthetic impurity, may also be present in the powder. These choline-containing impurities may affect the biological response in an inhalation challenge study.

Methacholine chloride purity and stability have been determined using a colorimetric assay<sup>5</sup> and ion-pairing HPLC.<sup>4,5</sup> Neither of these methods has been

used to determine potential impurities in methacholine chloride solutions. Choline and its analogs have been determined in various matrices using ion chromatography (IC). Carbachol chloride and bethanechol chloride, plus their alkaline decomposition products (choline and 2-hydroxypropyltrimethylammonium chloride, respectively), have been determined in ophthalmic solutions after sample dilution using a Reagent-Free™ Ion Chromatography (RFIC™) system.<sup>6</sup> These cholinergic agents were well resolved from other commonly occurring inorganic cations. The method demonstrated high precision, high recovery, and excellent day-to-day reproducibility. Other application notes showed the feasibility of determining bethanachol and 2-hydroxypropyltrimethylammonium in prescription and non-prescription medications,<sup>7</sup> choline in milk and infant formula,<sup>8</sup> and choline and acetylcholine in a vitamin and mineral formulation.<sup>9</sup> The work shown here demonstrates that an RFIC system can determine methacholine, acetylcholine, and  $\beta$ -methylcholine with good precision and accuracy. Anion IC is used to determine acetate. The first method can be used to assay methacholine, and the combination of both methods can be used to determine methacholine's related substances.

## **EQUIPMENT**

Dionex ICS-2100 system\* comprising:

- Single isocratic pump
- Vacuum degasser
- Eluent generator (for cation analysis)
- High pressure, 6-port injector
- Column heater enclosure
- Conductivity cell detector
- EO Eluent Organizer, including pressure regulator and 2 L plastic bottle

AS Autosampler and 2 mL vial tray

Chromeleon<sup>®</sup> Chromatography Data System (CDS)  
Version 6.8 or 7

Helium or nitrogen, 4.5 grade (99.995%) or better,  
< 5 ppm oxygen (Praxair)

Filter unit, 0.2 µm nylon (Nalgene<sup>®</sup> 90 mm Media-Plus, Nalge Nunc International, [P/N 164-0020] or equivalent nylon filter)

Vacuum pump (Gast Manufacturing Corp.  
[P/N DOA-P104-AA] or equivalent for degassing eluents)

1.5 mL polypropylene injection vials with caps (Vial Kit [Dionex P/N 061696])

\* The application in this note can be run using any Dionex RFIC system.

## **CONSUMABLES**

### **Cation Analysis**

EluGen II MSA Cartridge (Dionex P/N 058902)

CR-CTC II (Dionex P/N 066262)

CSRS<sup>®</sup> 300, 4 mm (Dionex P/N 064556)

IonPac<sup>®</sup> CG17, 4 × 50 mm (Dionex P/N 060560)

IonPac CS17, 4 × 250 mm (Dionex P/N 060557)

### **Anion analysis**

ASRS<sup>®</sup> 300, 4 mm (Dionex P/N 064554)

IonPac AG22, 4 × 50 mm (Dionex P/N 064139)

IonPac AS22, 4 × 250 mm (Dionex P/N 064141)

## **REAGENTS AND STANDARDS**

### **Reagents**

- Deionized water, 18 MΩ-cm resistance or higher
- Sodium chloride, ultrapure (VWR P/N JT4058-1)
- Phenol, SigmaUltra (Sigma P/N P5566)
- Sodium hydroxide, 50% w/w (Thermo Fisher Scientific P/N SS254)
- Hydrochloric acid, Ultrex<sup>®</sup> II (VWR P/N JT6900-5)
- Sodium carbonate concentrate, 0.5 M (Dionex P/N 037162)
- Sodium bicarbonate concentrate, 0.5 M (Dionex P/N 037163) or
- IonPac AS22 eluent concentrate, 100× (Dionex P/N 063965)

### **Standards**

- Sodium acetate, anhydrous (Sigma P/N 71183)
- Methacholine chloride (USP P/N 1396364)
- Acetylcholine chloride (USP P/N 1008501)

## **CONDITIONS**

### **Cations**

- Columns: IonPac CG17, 4 × 50 mm (Dionex P/N 060560)  
IonPac CS17, 4 × 250 mm (Dionex P/N 060557)
- Eluent: 5 mM methanesulfonic acid
- Flow Rate: 1.0 mL/min
- Inj. Volume: 25 µL (full loop)
- Column Temp.: 30 °C
- Detector Temp.: 35 °C
- Detection: Suppressed conductivity, CSRS 300, 4 mm (Dionex P/N 064556), recycle mode, 15 mA suppressor current
- Background: < 1 µS
- Noise: < 0.5 nS/min
- Backpressure: 2500 psi
- Run Time: 26 min

## Anions

Columns:	IonPac AG22 4 × 50 mm (Dionex P/N 064139) IonPac AS22 4 × 250 mm (Dionex P/N 064141)
Eluent:	4.5 mM Sodium carbonate/ 1.4 mM sodium bicarbonate
Flow Rate:	1.2 mL/min
Inj. Volume:	25 µL (full loop)
Column Temp.:	30 °C
Detector Temp.:	35 °C
Detection:	Suppressed conductivity, ASRS 300 4 mm (Dionex P/N 064554), recycle mode, 31 mA suppressor current
Background:	20 to 23 µS
Noise:	< 5 nS/min
Backpressure:	1600 psi
Run Time:	15 min

## ELUENT SOLUTIONS

### Cation Determinations

5 mM methanesulfonic acid (MSA) was generated on-line electrolytically using an Eluent Generator with an MSA EluGen cartridge. Fill the eluent reservoir with degassed deionized water and maintain an inert nitrogen or helium atmosphere of 3 to 5 psi in the eluent reservoir. Alternately, manually prepared MSA may be used. Prepare a 1.0 N stock solution by adding 96.10 g of MSA to a 1 L volumetric flask containing approximately 500 mL of deionized water. Bring to volume with deionized water and mix thoroughly. Prepare 5 mM MSA by diluting 5 mL of the 1 N MSA stock solution to 1 L with degassed deionized water.

### Anion Determinations

The 4.5 mM carbonate/1.4 mM bicarbonate eluent solution was manually prepared by adding 18.90 g of 0.5 M sodium carbonate and 5.88 g of 0.5 M sodium bicarbonate solutions to a 2 L volumetric flask and diluting to volume with degassed deionized water. Alternately, 21.0 g of IonPac AS22 Eluent Concentrate can be added to a 2 L volumetric flask and diluted to

volume with degassed deionized water. It is also possible to generate eluent on-line electrolytically using an Eluent Generator module, Carbonate EluGen cartridge (Dionex P/N 058904), Electrolytic pH Modifier (Dionex P/N 063175) to generate bicarbonate, and a 4 mm Carbonate Mixer Kit (Dionex P/N 061686).

## STANDARDS AND SURROGATE SAMPLE SOLUTIONS

### 1000 mg/L Methacholine Solution

Dissolve 0.500 g of methacholine chloride reference standard in degassed deionized water to make 409.3 g of solution. Store in a high-density polyethylene or polypropylene bottle at 4 °C.

### 1000 mg/L Acetylcholine Solution

Dissolve 0.200 g of acetylcholine chloride reference standard in degassed deionized water to make 160.9 g of solution. Store in a high-density polyethylene or polypropylene bottle at 4 °C.

### 500 mg/L β-Methylcholine Solution

Dilute 0.26 mL of 50% sodium hydroxide solution with 15.8 mL water. Add 33.9 mL of the 1000 mg/L methacholine stock standard to the diluted sodium hydroxide solution. Allow the resulting mixture to react overnight to hydrolyze methacholine to form β-methylcholine and acetate. Acidify the mixture with the minimum volume of 1 N hydrochloric acid needed to lower the pH below 4 (~0.1 mL of HCl/mL of alkaline β-methylcholine solution). Store in a high-density polyethylene or polypropylene bottle at 4 °C.

### 1000 mg/L Acetate Solution

Add 0.1390 g sodium acetate to a 1 L volumetric flask and dilute to volume with degassed deionized water. Store at 4 °C.

### Matrix Stock Solutions for Methacholine Assay

To prepare 0.9% sodium chloride matrix, add 0.900 g of sodium chloride to a 100 mL volumetric flask and dilute to volume with deionized water. To prepare 0.9% sodium chloride–0.4% phenol matrix, add 0.900 g of sodium chloride and 0.400 g of phenol to a 100 mL volumetric flask, then dilute to volume with deionized water. Store at 4 °C.

## Matrix Stock Solutions for Methacholine

### Impurities Determination

Prepare 1 L of 120 mg/L sodium chloride. Immediately before preparing working stock and surrogate sample solutions, mix three parts of the sodium chloride solution with one part of the 1000 mg/L methacholine solution to yield a 250 mg/L methacholine in 90 mg/L sodium chloride solution. Prepare 1 L of 120 mg/L sodium chloride–53 mg/L phenol. Immediately before preparing working stock and surrogate sample solutions, mix 3 parts of the sodium chloride–phenol solution with 1 part of the 1000 mg/L methacholine solution to yield a 250 mg/L methacholine in 90 mg/L sodium chloride–40 mg/L phenol solution. Store matrix solutions at 4 °C.

Make all subsequent dilutions of stock standards gravimetrically when generating standards for calibration and MDL studies and when generating surrogate samples for accuracy and precision studies.

## RESULTS AND DISCUSSION

### Chromatography

To test system suitability for the determination of methacholine and its possible choline-containing impurities in the presence of commonly occurring cations, a chromatographic trace for  $\beta$ -methylcholine, acetylcholine, and methacholine was compared to the chromatogram for a mixture of lithium, sodium, ammonium, potassium, magnesium, and calcium ions. As shown in Figure 1, all of the components are well separated. The large sodium peak is a result of the base-catalyzed hydrolysis of methacholine to form  $\beta$ -methylcholine and acetate. The reaction in 0.1 N NaOH occurs rapidly and was shown to be complete in less than 16 h (Figure 2). After acidification of the reaction products, this mixture was combined with methacholine and acetylcholine standards to create the 3-component sample in Figure 1.

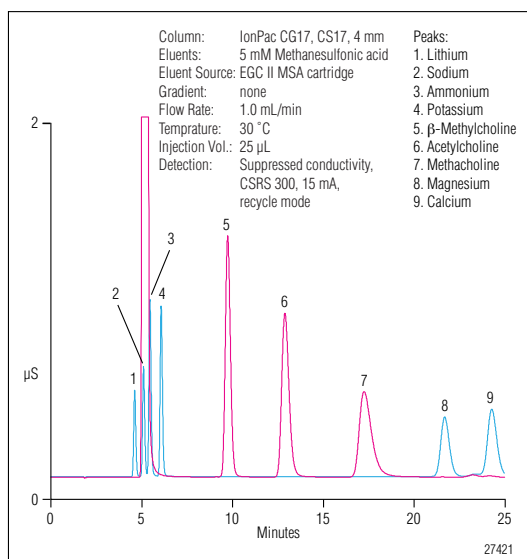


Figure 1. Overlay of  $\beta$ -methylcholine, acetylcholine, and methacholine with a mixed-cation standard.

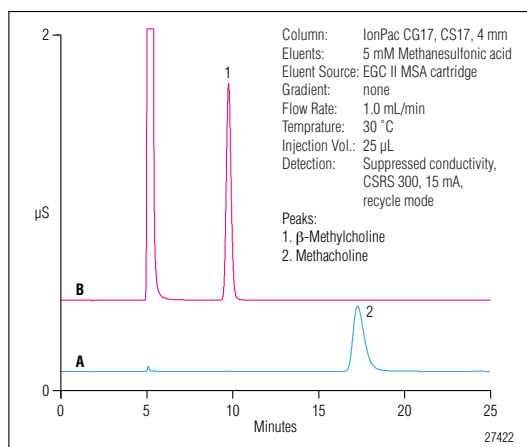


Figure 2. Conversion of methacholine to  $\beta$ -methylcholine in the presence of 0.1 N NaOH. Trace A, time = 0 h; and trace B, time = 16 h.

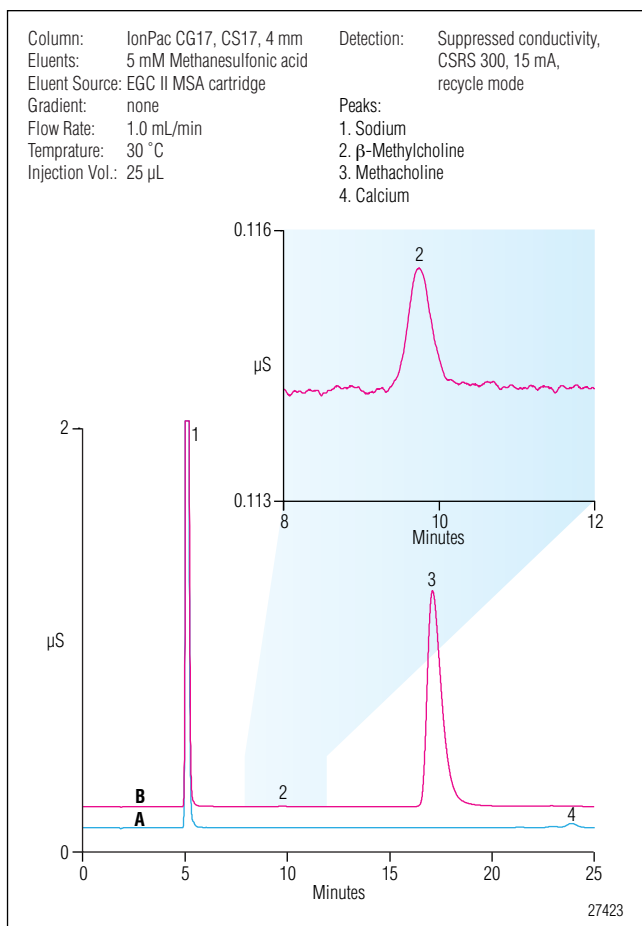


Figure 3. Cations in 1000:1 dilution in 0.9% NaCl + 0.4% phenol matrix. Trace A, matrix blank; trace B, 25 mg/L methacholine in diluted matrix ( $\beta$ -methylcholine conc.  $\sim$  0.1% of methacholine). The inset shows the 8 to 12 min region.

### Methacholine Linearity

The method for methacholine assay includes diluting the initial solution (16 or 25 g/L methacholine in 0.9% sodium chloride with or without 0.4% phenol) 1000-fold with filtered deionized water. Figure 3 shows a chromatogram from a sample in sodium chloride/phenol matrix after 1000-fold dilution.

A trace amount of  $\beta$ -methylcholine, formed from methacholine hydrolysis, can be detected (Figure 3 inset). Calibration data were generated for methacholine over a 5 to 50 mg/L range. Excellent linearity was observed in this calibration range with a correlation coefficient of 0.99995 (Table 1).

### Determination of Linearity and MDL for Possible Methacholine Impurities

Calibration data were collected for  $\beta$ -methylcholine, acetylcholine, and acetate in deionized water. Table 1

Table 1. Calibration Results for Methacholine and Possible Impurities<sup>a</sup>

Analyte	Range (mg/L)	Correlation Coefficient ( $r^2$ )	Offset ( $\mu$ S min)	Slope	RSD
Methacholine	5–50	0.99995	-0.0315	0.0360	0.42
Acetylcholine	0.1–100	0.99991	-0.0159	0.0403	1.6
$\beta$ -Methylcholine	0.1–100	0.99997	-0.0171	0.0503	0.89
Acetate	0.1–50	0.99983	0.0011	0.0148	2.0

<sup>a</sup> Triplicate injections of solutions prepared in deionized water.

shows the calibration results for these three possible impurities. Calibration ranges covered three orders of magnitude. Excellent correlation coefficients ( $>$  0.9998) were observed.

MDL estimates were based on the system blank (no injection). Signal noise was calculated over a four-minute interval centered on the analyte's retention time, converted to analyte concentration using a peak height vs. concentration calibration curve, and multiplied by three to calculate the MDL estimate. Table 2 shows that MDL estimates for  $\beta$ -methylcholine and acetylcholine are at single-digit  $\mu$ g/L levels while the estimate for acetate MDL is 75  $\mu$ g/L.

Table 2. Estimated Method Detection Limits for Possible Methacholine Impurities<sup>a,b</sup>

Analyte	MDL ( $\mu$ g/L)
Acetylcholine	8
$\beta$ -Methylcholine	5
Acetate	75

<sup>a</sup> Noise determined from average of 8 replicates of system (no injection) blanks.

<sup>b</sup> Calculated as 3x noise over 4-min interval centered at analyte RT.

### Recovery of Methacholine and Possible Methacholine Impurities

Recovery and precision studies were performed in simulated matrices. The 1000-fold dilution protocol for methacholine assay is described above. Impurity determination involves a 100-fold dilution with deionized water of 25 g/L methacholine in 0.9% sodium chloride with or without 0.4% phenol. Final matrix composition for possible methacholine impurity determination is 250 mg/L methacholine in 90 mg/L sodium chloride with or without 40 mg/L phenol.

**Table 3. Recoveries of Methacholine and Possible Impurities in Simulated Matrices<sup>a</sup>**

Analyte	Matrix <sup>b</sup>	Spiking Level (mg/L)	Molar % of Methacholine <sup>c</sup>	Recovery (%)	Precision (RSD)
Methacholine	9 mg/L NaCl	10	—	97.2	0.4
		25	—	98.5	0.3
		45	—	99.2	0.2
	9 mg/L NaCl 4 mg/L phenol	10	—	97.2	0.4
		25	—	98.1	0.1
		45	—	99.9	0.2
Acetylcholine	250 mg/L methacholine 90 mg/L NaCl	2.5	1.1	95.0	0.4
		25	11	97.1	0.2
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	2.5	1.1	85.4	0.2
		25	11	97.2	0.2
$\beta$ -Methylcholine*	250 mg/L methacholine 90 mg/L NaCl	2.5	1.4	88.7	0.9
		25	14	98.3	0.1
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	2.5	1.4	84.7	0.3
		25	14	96.7	0.2
Acetate*	250 mg/L methacholine 90 mg/L NaCl	2.5	2.7	88.7	0.9
		25	27	98.1	0.2
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	2.5	2.7	92.4	0.8
		25	27	99.8	0.1

<sup>a</sup>Seven replicates of blanks and spiked matrices.

<sup>b</sup>Diluted 1000-fold for methacholine assay and 100-fold for impurities analysis.

<sup>c</sup>Converting mass ratio to molar ratio based on impurity ion's molecular weight.

\* Measured concentrations of  $\beta$ -Methylcholine and acetate in blank at ~ 0.4 mg/L.

Table 3 summarizes recovery results for methacholine and possible impurities. Methacholine recoveries range from 97 to 100% for the three spiking levels. The three possible impurity analytes were spiked at 2.5 and 25 mg/L in the 100-fold diluted sample matrix. The lower spiking amount corresponds to 1 to 3% of the methacholine present in the matrix (Table 3). Recoveries at 2.5 mg/L spiking range from 85 to 95%. Amounts of  $\beta$ -methylcholine and acetate in the matrix blanks (0.4 mg/L) may be overestimated and contribute to lower than expected recoveries at spiking amounts of 2.5 mg/L. A peak for  $\beta$ -methylcholine is observed in both the matrix blank and acetylcholine-spiked matrix in Figure 4. Recoveries of possible impurities spiked at 25 mg/L ranged from 97 to 100%. Impurity determinations can be performed at amounts approaching 1% of the methacholine in solution.

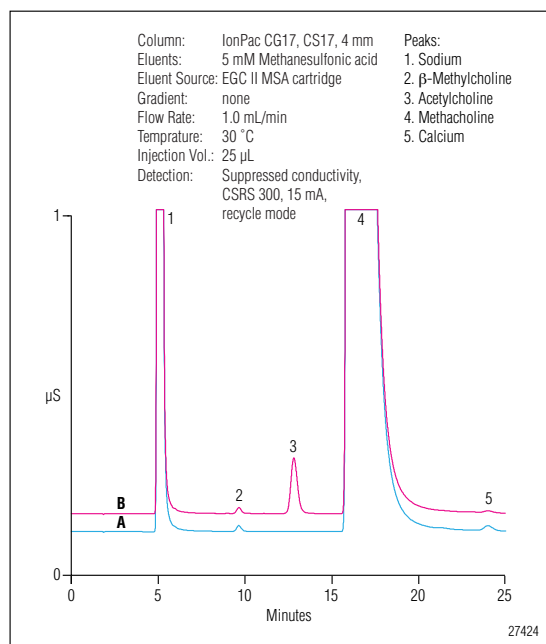


Figure 4. Cations in 100:1 dilution of 25 g/L methacholine + 0.9% NaCl + 0.4% phenol matrix. Trace A, matrix blank; trace B, 2.5 mg/L acetylcholine in diluted matrix.

**Table 4. Retention Time and Peak Area Reproducibilities for Methacholine and Possible Impurities in Simulated Matrices<sup>a</sup>**

Analyte (conc.)	Matrix <sup>b</sup>	Average RT, min (RSD)	Average Peak Area, $\mu$ S min (RSD)
Methacholine (25 mg/L)	9 mg/L NaCl	17.019 (0.040)	0.8190 (0.31)
	9 mg/L NaCl 4 mg/L phenol	17.013 (0.052)	0.8359 (0.40)
Acetylcholine (10 mg/L)	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	12.772 (0.060)	0.3647 (0.45)
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	12.780 (0.047)	0.3634 (0.42)
$\beta$ -Methylcholine (10 mg/L)	250 mg/L methacholine 90 mg/L NaCl	9.642 (0.091)	0.4938 (0.75)
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	9.638 (0.10)	0.4960 (0.70)
Acetate (2.5 mg/L)	250 mg/L methacholine 90 mg/L NaCl	3.249 (0.34)	0.0438 (1.1)
	250 mg/L methacholine 90 mg/L NaCl 40 mg/L phenol	3.248 (0.28)	0.0441 (0.85)

<sup>a</sup> Eight replicates per day in each matrix collected over a five day period.

<sup>b</sup> Samples diluted 1000-fold for methacholine assay and 100-fold for impurities analysis.

### Retention Time and Peak Area Precision

Table 4 summarizes the results of a five-day study of retention time and peak area reproducibilities for methacholine and possible impurities. Eight replicates for each target analyte in each of the two sample matrices were injected daily. No trending in daily averages was observed. Highly reproducible retention time and peak areas were observed for the choline-based analytes. Acetate determination yielded somewhat lower retention time and peak area reproducibility. Lower acetate retention-time reproducibility may be due to use of manually prepared carbonate/bicarbonate eluent, compared to the electrolytically produced eluent for the cation determinations.

### PRECAUTIONS

Methacholine and acetylcholine chlorides are neurotransmitters and can affect several of the body's systems. Contact of the powder with eyes, skin, and respiratory tract causes severe irritation. Wear protective gloves, chemical safety goggles, and a laboratory coat when working with these materials. Methacholine should never be administered orally or by injection because serious toxic reactions can occur. In addition,  $\beta$ -Methylcholine can be irritating to eyes, respiratory system, and skin. To dispose of these materials, contact a licensed waste disposal service. Read the material safety data sheet for these compounds before using them.

Extensive use of the column may cause performance degradation, such as loss of capacity, loss of resolution, shortened retention times, higher noise and background, spurious peaks, and peak tailing. For more information on column troubleshooting, see the *IonPac CS17 Column Product Manual* (Dionex Document No. 031877).

## CONCLUSION

This work presents IC-based methods for the detection and quantification of methacholine and possible impurities in solutions used to assess bronchial asthma of subjects evaluated in respiratory function labs and epidemiological field studies. Methacholine assay involves diluting the original solution (dissolved in 0.9% sodium chloride with or without 0.4% phenol) 1000-fold with deionized water, then analyzing the sample on a cation RFIC system. Acetylcholine and  $\beta$ -methylcholine are determined after a 100-fold dilution of the original solution using the same system. Acetate, another possible impurity, is determined by anion IC. These methods demonstrate high precision and accuracy for both methacholine and possible impurities present in amounts approaching 1%.

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## SUPPLIERS

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