Quality control of choline as a dietary supplement by high performance liquid chromatography coupled to a charged aerosol detector

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Goal

To develop a single method with direct detection of choline salts and impurities without the need for derivatization.

Application benefits

- Direct and simultaneous detection of choline, its counterions, and impurities
- Suitable for assay of choline content and impurities and identification of counterions in commercially available dietary supplements
- Method compatible for both charged aerosol detection and mass spectrometry for determination of impurities

Introduction

Choline is an essential nutrient involved in cell membrane integrity, lipid transport and metabolism, neurotransmission, and many other functions.[1] The main food sources of choline are animal-based products, beans, and nuts, which contain various forms including free choline, phosphocholine, and phosphatidylcholine.[2] Choline deficiency can lead to muscle damage, liver damage, and nonalcoholic fatty liver disease.[2] To ensure nutritional adequacy, the use of dietary supplements containing choline (e.g., choline bitartrate, choline chloride, choline citrate, phosphatidylcholine, and lecithin) is common.[3] Since the quality of dietary supplements can vary greatly, laboratory testing of raw materials and final products is often needed to ensure safety and effectiveness.

Traditionally, high performance liquid chromatography (HPLC) with ultraviolet (UV) detection is used for the analysis of choline salts. As choline salts respond poorly to this approach, precolumn derivatization with reagents containing a strong chromophore is required. However, this approach is limited: derivatization may interfere with the counter ions and may miss impurities that do not react.[4]

In this study, HPLC with a charged aerosol detector (CAD) was used for measurement of content and purity of choline bitartrate, choline citrate, and choline chloride (see Figure 1 for structures). A mixed-mode column operated in HILIC mode with an ammonium acetate buffered mobile phase and acetonitrile-water gradient was used. This approach enabled the separation of choline, its counterions, and several impurities. CAD provided direct detection of these analytes, thus avoiding the need for derivatization used in older methods. Several impurities were identified based on retention time comparison to individual standards or by mass spectrometry and include Na^+, K^+, Cl^- and O-(2-hydroxyethyl)choline (CAS 41830-55-1) (see Figure 1 for structure). Choline content was measured over the range of 0.08 to 0.12 mg/mL with precision ≤3.0% RSD, recovery from 96.7 to 100.8%, and correlation coefficient (r^2) for linear regression of > 0.997.

This HPLC-CAD method provides advantages over derivatization techniques by allowing more comprehensive measurement of sample composition using a simplified approach. This method is recommended for the analysis of choline citrate in dietary supplements.[4]

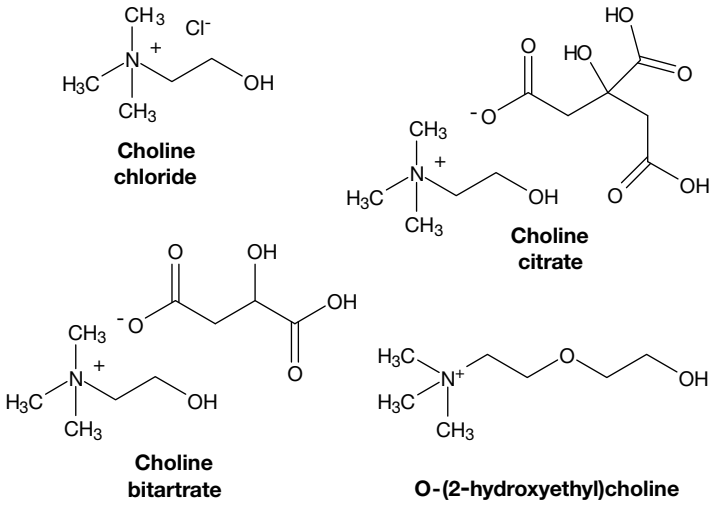

Figure 1. Structures of choline salts and the impurity O-(2-hydroxyethyl)choline

Experimental

Reagents and standards

Chemical name	Part number
Deionized water, 18.2 MΩ·cm resistivity or higher—from a Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System	50136149
Fisher Scientific™ acetonitrile, Optima™ LC/MS grade	A955-212
Fisher Scientific™ ammonium acetate, LC/MS grade	A114-50
Fisher Scientific™ acetic acid, LC/MS grade	A113-50
Fisher Scientific™ sodium chloride (>99.9%)	S/3165/53
Choline bitartrate, choline chloride, and choline citrate were purchased from reputable vendors	

Equipment

Item name	Part number
Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer	14-955-152
Thermo Scientific™ Orion™ 3 Star pH Benchtop Meter	13-644-928
Thermo Scientific™ Finpipette™ F1 Variable Volume Single-Channel Pipettes: 100-1000 µL, 10–100 µL, 1–10 µL	4641100N 4641070N 4641030N
Thermo Scientific™ PP Crimp/Snap Top autosampler vials	C401114
Thermo Scientific™ 11 mm autosampler vial crimp caps (Chlorobutyl, PTFE)	11568150

Mobile phase preparation

Prepare an 80.4 mM ammonium acetate buffer by adding 6.2 g of ammonium acetate to 900 mL deionized water and adjust the pH to 4.7 with glacial acetic acid. Bring the volume to 1.0 L in a volumetric flask. This buffer is used for mobile phase and diluent preparation. Note: pH electrodes can be a significant source of contamination causing higher baseline noise and baseline artifacts. For best results, it is recommended to determine the volume of glacial acetic acid needed to adjust the pH and then use only volumetric and gravimetric techniques to prepare buffers used for diluent and mobile phase. This avoids contamination from the pH electrode and typically provides more reproducible retention times between batches.

For mobile phase A, combine 200 mL of the prepared buffer with 800 mL of acetonitrile and mix thoroughly. For mobile phase B, combine 200 mL of the prepared buffer with 300 mL deionized water and 500 mL acetonitrile and mix thoroughly.

Standard and sample preparation

The diluent for standard and sample preparation was prepared as 80.4 mM ammonium acetate buffer/acetonitrile (30/70, v/v).

Standard solutions of choline bitartrate, choline chloride, and choline citrate were prepared from commercially available standards in diluent. Standard solutions of 0.08 mg/mL, 0.10 mg/mL, and 0.12 mg/mL were used for assay of content and for identification while a 2.0 µg/mL standard solution was used for impurities.

Samples were prepared at 0.10 mg/mL for assay of choline content and at 2.0 mg/mL for impurities. Assay method validation was done based on choline citrate with solutions of 0.08, 0.10, 0.12 mg/mL for calibration and accuracy/precision determination. Instrument precision was calculated as %RSD from six consecutive injections of 0.10 mg/mL solution.

A solution containing related salt counter ions was prepared as 0.10 mg/mL sodium chloride.

Instrumentation

Chromatographic separation was performed on a Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system. Analytes were detected using a charged aerosol detector. An optional single quadrupole mass spectrometer was used to help identify impurities and artifacts.

Module	Part Number
Thermo Scientific™ Vanquish™ System Base Flex	VF-S01-A
Thermo Scientific™ Vanquish™ Quaternary Pump	VF-P20-A
Thermo Scientific™ Vanquish™ Split Sampler HT	VH-A10-A
Thermo Scientific™ Vanquish™ Column Compartment H	VH-C10-A-03
Thermo Scientific™ Vanquish™ Charged Aerosol Detector F	VF-D20-A
Thermo Scientific™ ISQ™ EM Single Quadrupole Mass Detector*	ISQEM-ESI

*For some experiments, a post-column tee was used to split the flow 1:1 between the CAD and the single quadrupole mass detector.

Data processing and software

Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS), version 7.2.9 was used for data acquisition and processing.

Table 1. Chromatographic conditions

Parameter	Setting
Column	MilliporeSigma™ SeQuant™ ZIC™—pHILIC, 4.6 × 150 mm, 5 µm (P/N 1.50461.0001)
Mobile phase A	80.4 mM ammonium acetate buffer pH 4.7 / acetonitrile (20/80, v/v)
Mobile phase B	80.4 mM ammonium acetate buffer pH 4.7 / water / acetonitrile (20/30/50, v/v/v)
Injection volume	10 µL
Flow rate	0.5 mL/min
Gradient	See Table 2
Column temperature	30 °C still air mode, 30 °C active preheater
Autosampler temperature	10 °C
CAD conditions	Data collection rate: 10 Hz; Filter: 3.6 s; Evap T: 35 °C; PFV: 1.00
MS conditions	Vaporizer temp.: 144 °C; Ion transfer tube: 300 °C; Source voltage: 3000 V (positive), -2000 V (negative); Sheath gas: 32.3 psig; Aux gas: 3.6 psig; Sweep gas: 0.5 psig
MS full scan, positive	Time: 0–35 min; Spectrum type: centroid; Mass range: 50–400 *m/z*; Dwell time: 0.1 s; Source CID voltage: 15 V
MS SIM, positive	Time: 0–35 min; SIM mass: 148.2 *m/z*; SIM width: 0.1 amu; Dwell time: 0.1 s; Source CID voltage: 15 V
MS full scan, negative	Time: 0–35 min; Spectrum type: centroid; Mass range: 50–400 *m/z*; Dwell time: 0.1 s; Source CID voltage: 15 V

Table 2. Gradient conditions

Time [min]	%A	%B
0	90	10
3	90	10
20	0	100
22	0	100
26	85	15
28	90	10
35	90	10

Results and discussion

Choline, its counterions, and impurities were separated using a mixed-mode column containing a zwitterionic stationary phase using gradient elution (Table 2). Using this approach analytes are resolved by both HILIC and ion exchange mechanisms enabling the simultaneous separation of cations, anions, and neutral analytes. Click here for further information about mixed-mode columns.

The charged aerosol detector is a universal detector and, under isocratic conditions, shows uniform response for all non-volatile analytes, independent of their chemical structure. For ionic solutes like choline and its counterions, CAD response is based on the salt formed between the analyte and oppositely charged mobile phase additives. In this method, choline is measured as its acetate salt while citrate, bitartrate, and chloride are measured as their ammonium salts.

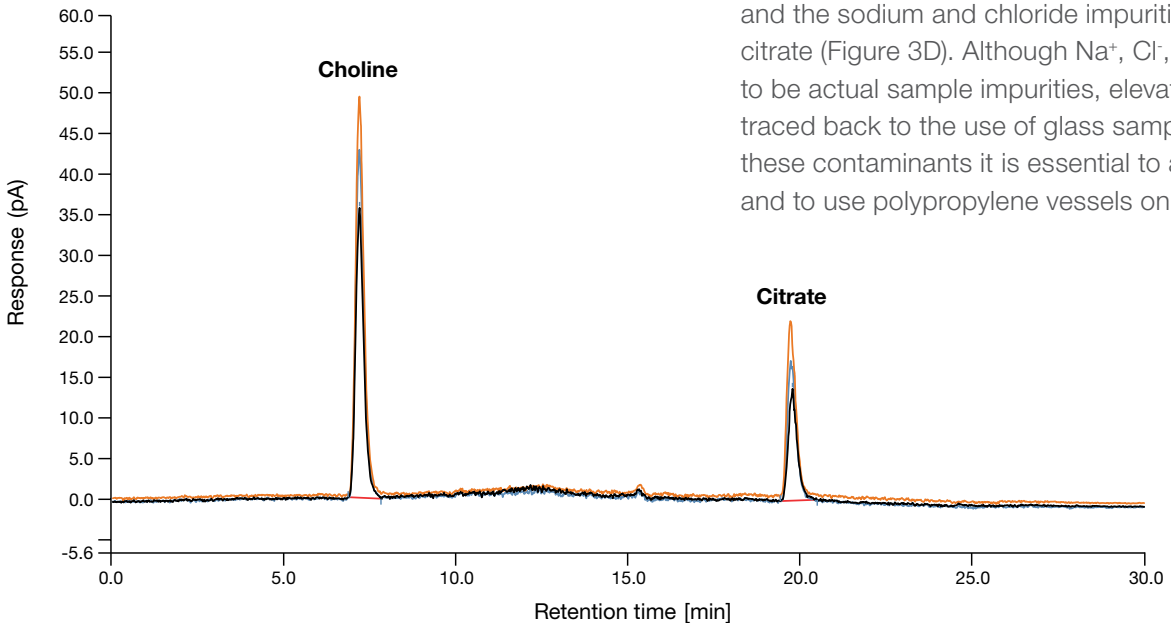

Figure 2. Chromatogram of choline citrate (black = 0.08 mg/mL; blue = 0.10 mg/mL; orange = 0.12 mg/mL)

Choline citrate method

Figure 2 shows the separation of choline and its counterion citrate at three different concentration levels (0.08, 0.10, and 0.12 mg/mL). Choline was detected as its acetate salt, while citrate was detected as its ammonium salt. Both peaks were well separated and easily quantified.

The validation data for choline citrate analysis are presented in Table 3. The precision was <2% on two separate days. Linearity (0.08, 0.10, 0.12 mg/mL) and recovery (at 80, 100, and 120% of the content specification) were based on linear least squares regression.

Table 3. Validation data for choline citrate on precision, linearity, and recovery

Choline citrate				
Parameter	Choline		Citrate	
	Day 1	Day 2	Day 1	Day 2
Precision 0.10 mg/mL (% RSD, n = 6)	1.83	1.23	1.33	1.76
Linearity (r^2)	0.99997	0.99784	0.99778	0.99918
Recovery (%) at level	**Day 1**	**Day 2**	**Day 1**	**Day 2**
80	98.3	100.2	94.2	105.5
100	100.8	99.4	98.1	102.2
120	99.1	96.7	99.8	98.7

Measurement of other choline salts and impurities

The method can be used to evaluate other choline salts (e.g., choline chloride and choline bitartrate) and impurities (Figure 3), for example, the sodium impurity found in choline tartrate (Figure 3B) and choline chloride (Figure 3C) and the sodium and chloride impurities found in choline citrate (Figure 3D). Although Na^+, Cl^-, and possibly K^+, seem to be actual sample impurities, elevated levels can also be traced back to the use of glass sample vials. To minimize these contaminants it is essential to avoid the use of glass and to use polypropylene vessels only.[4]

An impurity found in choline citrate samples (RT ~6.0 min) was further studied using a single-quadrupole mass detector (Figure 4). Based on its mass spectrum, the impurity was determined to be O-(2-hydroxyethyl)choline, a known by-product formed during the production of choline hydroxide.

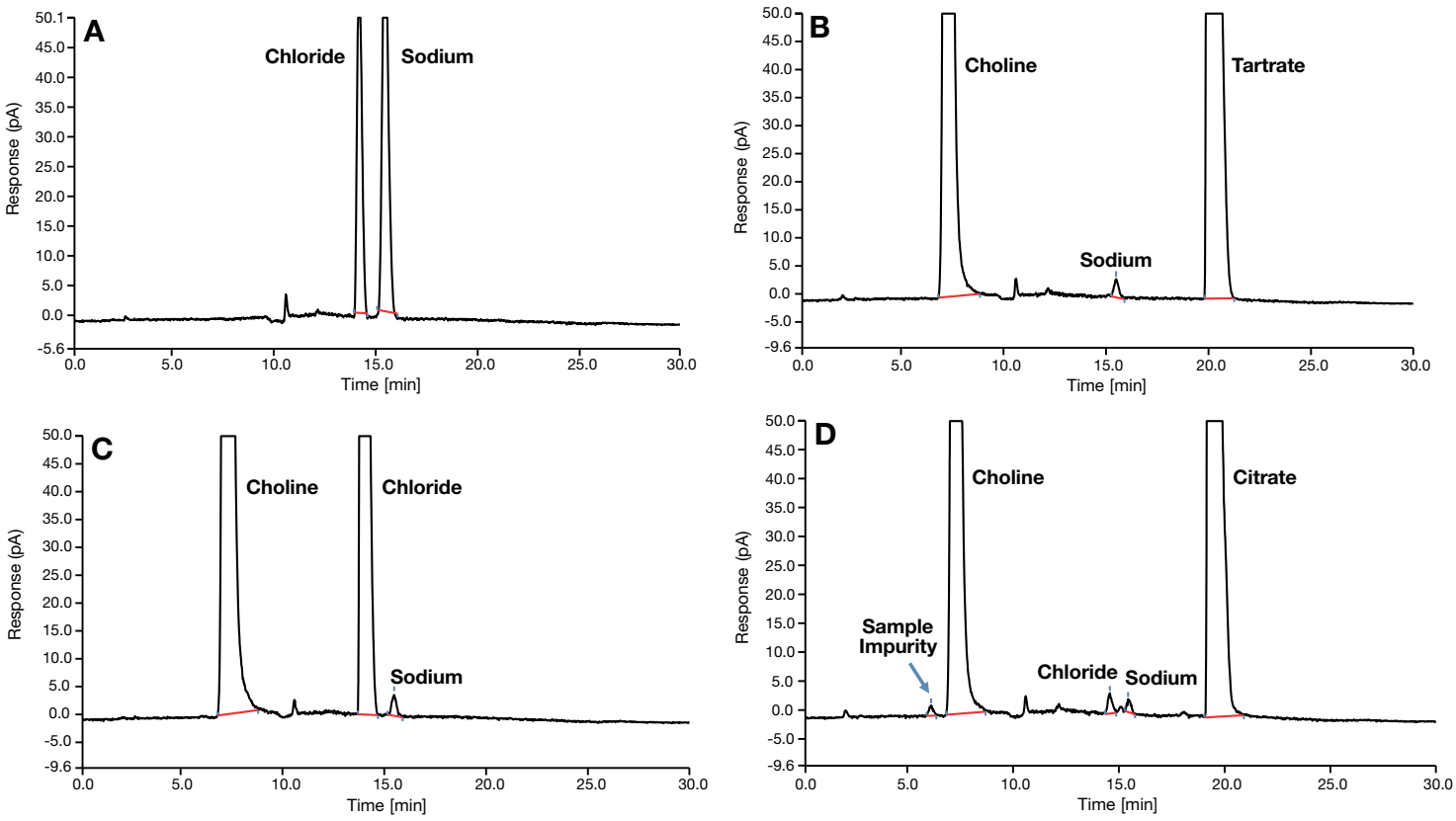

Figure 3. (A) Chromatogram of sodium chloride (0.1 mg/mL)—sodium detected as its acetate salt, chloride as its ammonium salt; (B) analysis of choline bitartrate (2.0 mg/mL) showing sodium impurity; (C) analysis of choline chloride (2.0 mg/mL) showing sodium impurity; (D) analysis of choline citrate (2.0 mg/mL) showing sodium, chloride, and a choline-related sample impurity (eluting at 6.0 min)

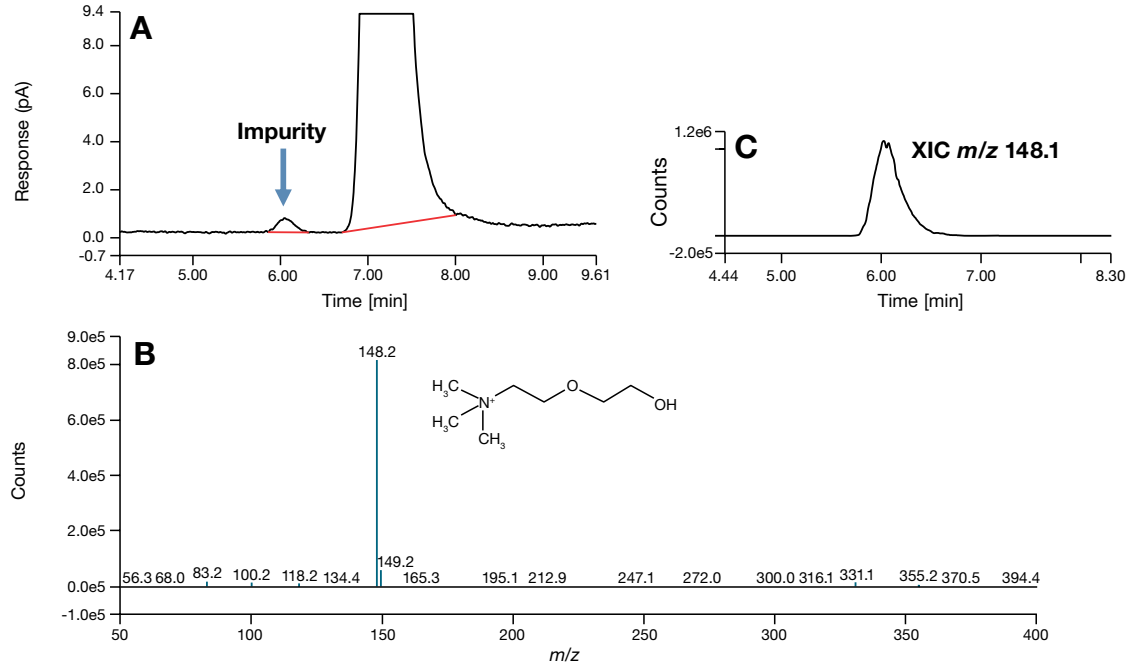

Figure 4. (A) CAD chromatogram of choline citrate (2.0 mg/mL) showing an impurity at RT ~6 min; (B) mass spectrum at the peak apex of the impurity peak at RT ~6 min; (C) extracted ion chromatogram (XIC) of *m/z* 148.1

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Conclusion

- Mixed-mode chromatography with charged aerosol detection enables the simultaneous analysis of anions and cations, and was used to evaluate choline bitartrate, choline chloride and choline citrate samples
- The low ng sensitivity of CAD allows control of the O-(2-hydroxyethyl)choline impurity and individual unspecified impurities within acceptance criteria of not more than (NMT) 0.1%.[4]
- Use of MS in parallel with CAD facilitated the structural identification of choline-related impurity O-(2-hydroxyethyl)choline in commercial samples.

References

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