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Journal of Chromatography A, 1062 (2005) 285–289
JOURNAL OF CHROMATOGRAPHY A
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Short communication

Liquid chromatography determination of residue levels on apples treated with cetylpyridinium chloride

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Received 19 March 2004; received in revised form 2 November 2004; accepted 11 November 2004
Available online 8 December 2004

Abstract

Cetylpyridinium chloride (CPC) has been found to be effective in reducing microbial contamination in apples. A sensitive and specific HPLC method was developed to determine CPC residues in apples treated with CPC. This method involves ion exchange solid-phase extraction, and the use of stearylpyridinium chloride (SPC) as internal standard. Limit of quantitation, was 0.5 μg/ml of CPC for the apple ethanolic extracts. The observed residues in apple (2.35–4.35 μg/g of apple) were lower than those previously reported for chicken and beef. The method is specific, sensitive, reproducible and accurate.

Keywords: Cetylpyridinium chloride; Apples; Residue levels

1. Introduction

The incidence of food poisoning in humans by *Escherichia coli* O157:H7 is increasingly of concern. This microorganism was first recognized as a cause of food-borne illness in a 1982 outbreak, which was traced to contaminated hamburgers [1]. Since then, many other outbreaks of *E. coli* O157:H7 infections have been associated with contaminated hamburgers [2], dry-cured salami [3], mayonnaise [4], drinking water [5], and unpasteurized commercial apple juice [6]. Apple juice contamination has been traced to apples that had contact with soil and possibly animal feces [7–10].

Previously we have established that cetylpyridinium chloride (CPC, Fig. 1) is effective in reducing bacteria in poultry [11–13] and beef [14] and apples [15]. The U.S. Food and Drug Administration has recently approved the use of CPC to reduce bacterial contamination on poultry. It is in this context that we have explored the potential of CPC as a chemical rinse to reduce bacteria from contaminated apples. In order to establish the practicality of the use of chemical rinses for microbial decontamination of a particular food, it is necessary to assess the chemical residues produced by the treatment.

Recently we reported an HPLC method for measuring the CPC residues that is applicable to beef and chicken carcasses [14,16], where a description of methods for CPC analysis in different matrix was reviewed. Since then, another assay methodology has been described [17] but its suitability and validation on biosurfaces remains to be determined. Additionally, CPC residue analysis of treated vegetables has been performed, but validation of the method was not reported [18].

From the evaluation of CPC residues in beef and chicken tissues [14,16] it is evident that CPC residues levels are strongly influenced by the nature of the treated food surface or tissue. In particular, it has been demonstrated that CPC binds to proteins [19] and fats [14]. Fruits are very rich in carbohydrates and very low in proteins and fat [20,21]. Thus, in apples

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doi:10.1016/j.chroma.2004.11.039

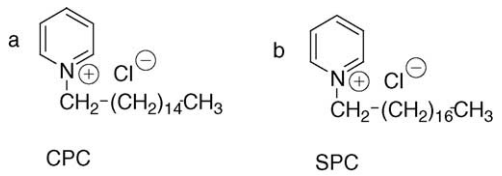

Fig. 1. Chemical structures of (a) cetylpyridinium chloride and (b) stearylpyridinium chloride.

we were anticipating lower residue levels than those observed in beef or chicken. Therefore, it became necessary to develop a more sensitive analytical method than previously available.

During the application of our method to measure CPC residues on apples, significant interference from the matrix was found for both CPC and the internal standard peaks. Thus in the present study we have developed a method which includes a solid-phase extraction step using a carboxylic acid, cationic exchange cartridge. Under the conditions presented, this method selectively separates CPC from apple extract. In addition, the method detects this compound with increased sensitivity.

2. Experimental

2.1. Chemicals and materials

CPC (Fig. 1a) was purchased from Zeeland Chemicals, Inc. (Zeeland, MI). Stearylpyridinium chloride (SPC; Fig. 1b) was synthesized according to the literature [22], and its purity and identity was confirmed by melting point, NMR and elemental analysis data (theoretical: 71.56%C, 11.49%H, 3.63%N, found 71.83%C, 11.51%H, 3.65%N). Tetramethylammonium hydroxide pentahydrate (TMAHP) was obtained from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade methanol and water were purchased from Fisher Scientific (Pittsburgh, PA, USA). Deionized water was obtained from a Milli-Q50 unit (Millipore Corp., Bedford, MA). Alcohol USP (95% ethanol and 5% water) was obtained from Aaper Alcohol Co. (Shelbyville, KY).

Concentrated HCl and trisodium phosphate were analytical reagent grade. Presample preparation was carried out in a 500 mg carboxylic acid (CBA), cationic exchange Bond Elut cartridge from Varian (Harbor City, CA) which was used in conjunction with a Visiprep 24-port model vacuum manifold (Supelco, Bellefonte, PA). CBA cartridges were preconditioned before use by successive treatment with 2.5 ml of methanol, 0.2 mol/l trisodium phosphate, and deionized water.

Granny Smith apples, weighing between 110 and 150 g, were purchased from a grocery store. Arkansas Black apples, weighing between 140 and 200 g were purchased from a local farmer. The latter apples were not waxed or processed. Shaking for the apple extractions was performed using an Orbit Environ-Shaker (Lab-Line Instruments Inc., Melrose Park, IL).

2.2. HPLC conditions

HPLC analyses were performed using a Waters Corp. (Milford, MA) system consisting of Waters 600E Multisolvent Delivery System, Waters 490E Programmable Multiwavelength Detector, Waters 746 Data Module, and Waters 600E System Controller. The column (Alltima cyano, 250 mm × 4.6 mm, 5 μm) and the guard cartridge (Alltima cyano, 7.5 mm × 4.6 mm, 5 μm) were obtained from Alltech Associates (Deerfield, IL). Mobile phase was buffer:methanol (29:71) mixture. The buffer was a TMAHP (0.008 M) in aqueous acetic acid (0.14 M), pH 3.6. Ultraviolet absorbance detection was at 260 nm. Each run was completed within 16 min. The flow rate was 1 ml/min and the injection volume was 20 μl.

2.3. Ethanol extraction

Arkansas Black and Granny Smith apples were weighed and individually placed in polyethylene bags (12 in. × 20 in.), extracted with 95% ethanol (2 ml ethanol/g of apple) at 60 °C for 1 h, 150 rpm. After the bags were cooled to room temperature, the ethanol extract was collected and stored at −20 °C for analysis.

2.4. Assay

For the analysis, aliquots (10 ml) of apple ethanol extract were spiked with 90 μg (30 μl) of ethanolic SPC as internal standard. The spiked solutions were applied to a 500 mg CBA. After application of the test solutions, the cartridges were washed with 2.5 ml of methanol, and eluted with 2.0 ml of methanol containing 2% HCl, and collected in a glass vial. Twenty microlitres of the samples were injected in to HPLC for CPC quantitation.

2.5. Calibration

Calibration of the method was performed using blank extract of Granny Smith waxed apples obtained by extraction with 95% ethanol (2 ml ethanol/g of apple, 60 °C, 1 h, shaking at 150 rpm). Aliquots (10 ml) of the blank extract were spiked with 50 μl of ethanolic CPC standard to give concentrations 0.5, 1, 2, 4, 8, 16, and 32 μg/ml. Ethanolic SPC (30 μl, 90 μg) was added to each sample. The samples were analyzed as described in Section 2.4 above. A calibration curve was obtained by performing weighted linear regression of CPC/SPC peak-area ratio versus CPC concentration.

2.6. Recovery of ethanol extraction

Aqueous solutions (100 μl) containing CPC (0.212 and 7.48 mg) were applied to the surface of waxed Granny Smith apples using a micro-syringe. After the applied solutions dried out, the apples ($n = 5$) were extracted with 95% of ethanol (2 ml ethanol/g of apple, 60 °C, 1 h, shaking at

150 rpm). The amount of CPC was measured as described in Section 2.4 above. The extraction recovery was calculated as the ratio of measured CPC to added CPC.

2.7. Ion exchange solid-phase recovery

Ten-millilitre aliquots of the blank extract were spiked with 50 μl of ethanolic CPC standards to give concentrations 0.6 and 30 μg/ml. Samples were treated and analyzed for CPC in the same way as described in Section 2.4 except that the internal standard was added after ion exchange SPE. Twenty microlitres of the solution were injected on the HPLC system. The recovery was calculated as the ratio of measured CPC to added CPC.

2.8. Assay application

Arkansas Black ($n = 5$) and Granny Smith ($n = 5$) apples were weighed and individually placed in a polyethylene bag (12 in. × 20 in.), and treated for 3 min with 0.4 ml of CPC solution per gram of apple. The solutions tested contain 0, 2 or 4 mg/ml of CPC in deionized water. After treatment, apples were rinsed with tap water (0.8 ml/g of apple, pH 7.3, 25 °C), placed individually in polyethylene bags (7 in. × 11.5 in.), and extracted with 95% ethanol. After the bags were cooled to room temperature, the ethanol extract was collected and stored at −20 °C for analysis. The residual CPC levels in the apples were analyzed as described above (Section 2.4).

3. Results and discussion

3.1. Optimization of experimental conditions

The HPLC method developed uses an internal standard to reduce analytical errors due to variations in such factors as detector sensitivity and injection volume. We have previously used dodecylpyridinium chloride (DPC) as internal standard for measuring CPC on beef and chicken carcasses [14,16]. However, there is significant interference to both CPC and DPC from apple extractives. In a effort to increase the specificity of the method we have introduced a solid-phase extraction step using a carboxylic acid, cationic exchange cartridge, which under proper conditions can selectively separate CPC from apple extract.

Unfortunately, even with the use of the ion exchange cartridge there was interference with the DPC peak. The fact that the interfering peaks appeared to be less lipophilic than CPC suggested the used of a more lipophilic compound as internal standard. SPC, which has two more methylene carbons than CPC, was found to be an appropriate internal standard. Under the conditions used, the retention times were about 7.9 and 9.5 min for CPC and SPC, respectively. There is no significant interference from apple extract at either of these two times (Fig. 2). In addition, it was possible to reduce flow rate from 2 to 1 ml/min, causing a reduction in back pressure.

3.2. Assay validation: linearity and precision

A seven-point calibration curve was obtained over the CPC concentration range 0.5–32 μg/ml of ethanolic extract, equivalent to 1.0–64 μg/g of apple. Linearity was satisfactory as shown by the high correlation coefficient ($r = 0.9999987 \pm 0.0169$; R.S.D. = 1.69%). Weighted linear regression produced the equation $y = 0.12565x - 0.0059$, where x is the CPC concentration and y is the peak-area ratio (CPC/SPC). The standard deviation for the slope and the intercept were 0.0010 and 0.0011, respectively. This corresponded to R.S.D. of 0.796 and 18.6%, respectively.

The error and the coefficient of variation (CV) were within 11% for both intra- and inter-assay, which was less than the maximum acceptable limit, 15% (Tables 1 and 2). [23]

3.3. Limit of quantitation

Solid-phase extraction allows baseline separation from any interfering peaks. Moreover, the high efficiency of this technique maximizes sensitivity [24].

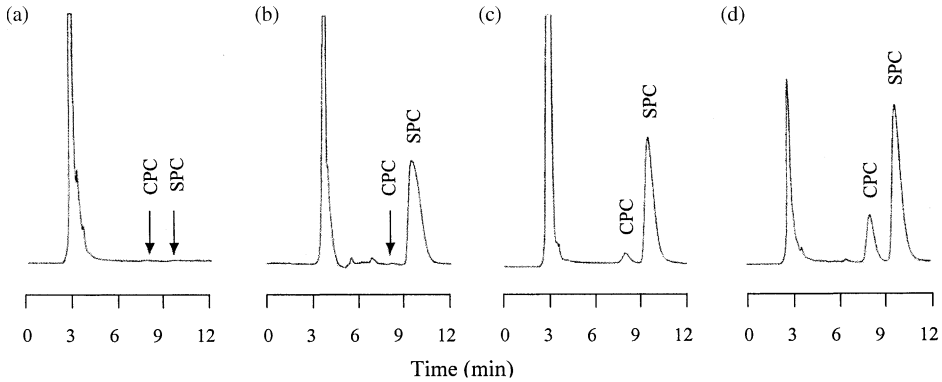

Fig. 2. High performance liquid chromatograms of (a) blank extract, (b) blank extract spiked with SPC at 90 μg/ml, (c) blank extract spiked with SPC and CPC at 90 and 0.5 μg/ml, respectively, and (d) extract of a CPC-treated apple spiked with SPC at 90 μg/ml (measured CPC concentration: 1.80 μg/ml in the extract or 3.58 μg/g in the apple).

Table 1
Intra-day assay precision and accuracy for the determination of cetylpyridinium chloride in spiked apple extract ($n = 5$)

Spiked concentration (μg/ml)	Measured concentration (mean ± S.D.) (μg/ml)	Coefficient of variation (%)	Relative error (%)
0.61	0.62 ± 0.05	8.51	2.34
15.0	14.7 ± 0.50	3.38	2.20
30.0	28.5 ± 0.96	3.39	5.11

Table 2
Inter-day assay precision and accuracy for the determination of cetylpyridinium chloride in spiked apple extract ($n = 5$)

Spiked concentration (μg/ml)	Measured concentration (mean ± S.D.) (μg/ml)	Coefficient of variation (%)	Relative error (%)
0.61	0.66 ± 0.07	11.1	8.19
15.0	15.0 ± 0.69	4.38	5.24
30.0	29.6 ± 2.26	7.65	1.28

In the present method, use of an ion exchange cartridge resulted in an increase in sensitivity. The limit of quantitation, defined as the lowest concentration that can be determined with acceptable precision (CV < 20%) and accuracy (error < 20%) [25], was 0.5 μg/ml for the apple ethanolic extracts, representing 1.0 μg/g of apple.

Incidentally, the limit of detection was 0.026 μg/ml.

3.4. Recovery of ethanol extraction and ion exchange SPE

The ethanol extraction method was very efficient in recovering CPC from the apple surface. Likewise, the solid-phase extraction procedure had high recovery of CPC. When apples ($n = 5$) were treated with 0.212 and 7.48 mg of CPC, the recoveries were 106 ± 14 and 99.9 ± 4.88% respectively. These high extraction recoveries suggest that applied CPC remains on the surface of the apples, where it is accessible to extraction. Ion exchange SPE recoveries were 103 ± 6.94, and 97.0 ± 3.85% for CPC concentrations of 0.6 and 30 μg/ml in ethanol extracts, respectively.

3.5. Assay application

The method was applied to evaluate the residue level on Granny Smith (waxed) and Arkansas Black (non-waxed) apples treated with aqueous solution of CPC. When Granny Smith apples were treated with aqueous CPC solution of 2 and 4 mg/ml, the residue levels were 4.35 ± 1.34 and 4.33 ± 0.53 μg/g of apple, respectively. For non-waxed apples, the respective residue levels were 3.21 ± 0.60 and 2.35 ± 0.18 μg/g of apple when treated with the above concentrations of aqueous CPC.

According to these results, an apple weighing 150 g treated with CPC under the conditions proposed would have a residue of this compound between 0.35 and 0.65 mg. Interestingly, even though CPC's antimicrobial efficacy varied greatly between waxed and non-waxed apples and was influenced by the CPC concentration used, the residue level changed very little over the range of concentrations of applied CPC.

4. Conclusion

An HPLC assay for determination of CPC residual levels on apples has been developed. The assay employs 95% ethanol for the efficient extraction of CPC from apples. The interferences from apple extractives are well separated from CPC and the internal standard SPC by ion exchange solid-phase extraction and then HPLC. The method is specific, sensitive, reproducible and accurate.

Acknowledgments

This research was supported by grants from the Arkansas Science and Technology Authority, USDA/CSREES through the Food Safety Consortium, and USDA/NRICGP (grant number: 9802739).

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