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Short communication

High-performance liquid chromatographic separation of carminic acid, α- and β-bixin, and α- and β-norbixin, and the determination of carminic acid in foods

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Abstract

During a study of natural food colours, a simple and reliable high-performance liquid chromatography system was developed for use with cochineal and annato. An isocratic mobile phase, consisting of methanol and 6% aqueous acetic acid, resolved bixin and norbixin, while a gradient system was used to separate carminic acid and the annato compounds. The carminic acid contents of cochineal extract, carmine and carmine hydrosoluble were determined using an isocratic mobile phase (40:60, v/v). The detection limit for carminic acid in the various products was approximately 100 ng/g. Carminic acid was determined quantitatively in fruit beverages, yogurt and candies. It was demonstrated that, because of decomposition, carminic acid was not suitable for use in candies when manufacturing temperatures above 100°C were required. Most membrane filters are not suitable for use with cochineal solutions, but a cellulose membrane filter did not adsorb carminic acid and was used successfully to remove impurities from water-based cochineal products and food extracts containing carminic acid.

Keywords: Food analysis; Carminic acid; Bixins; Norbixins; Annatto; Dye

1. Introduction

Throughout the world, the use of natural-type food colours continues to increase. Many consumers believe, even without valid proof, that natural colours are less harmful and therefore more acceptable than synthetic dyes. In Canada, both synthetic and natural colours are permitted in foods.

Cochineal extract [C.I. (1956) 75470; C.I. Natural Red 4; EEC No. E 120] is the common name of the colorant obtained when the dried bodies of the female *Coccus cacti (Dactylopius coccus* Costa) insect, a variety of shield louse, are first extracted with aqueous alcohol then the alcohol is removed. The colouring principle of the extract is carminic acid, an hydroxyanthraquinone linked to a glucose unit, comprising approximately 10% of cochineal and 2–4% of its extract. Carmine is the aluminum or calcium–aluminum lake on an aluminum hydroxide substrate of the colouring principle (chiefly carminic acid) obtained by the aqueous extraction of cochineal. Carmine is normally 50% or more carminic acid. Both colorants produce pink shades in candy, confections, chewing gum, concentrated fruit juice (except orange), lobster paste, smoked fish, liqueurs and alcoholic cordials, soft drinks, yogurt, ice cream,

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tomato catsup, baked goods, jams, jellies, eye shadow, rouge and pill coatings.

Annatto [C.I. (1956) 75120; C.I. Natural Orange 4; EEC No. E 160b] is the common name of the colorant produced from the seeds of the tropical tree, *Bixa orellana L.* The seeds are coated with resin containing the oil-soluble carotenoid *cis*-bixin (α-bixin) as its primary pigment. *cis*-Bixin, on heating in solution, is partially converted into the *trans* isomer (β-bixin) and yellow degradation products. The temperature and duration of the heating process regulates the red/yellow balance. Hydrolysis of the methyl ester group yields the dicarboxylic acid, norbixin, which is soluble in aqueous alkaline solutions. In foods, the hue of annatto is affected by pH. During the past one hundred years, annatto has been used primarily to colour high fat dairy products such as butter and cheese, but today it is used also in a wide range of products such as cereals, snack foods, coffee creamers, ice cream and seasonings.

In 1993, the Canadian Health Protection Branch (HPB) decided to conduct comprehensive analytical studies on the three most widely used natural colours, β-carotene, annatto and cochineal (carminic acid). First, annatto was examined and the results of that study were published recently [1]. Next, a study of cochineal was conducted. Various spectrophotometric and high-performance liquid chromatography (HPLC) methods were assessed, and several procedures for determining carminic acid in foods were tested. During initial HPLC research, it was observed that none of the methods investigated [2–5] met our requirements fully. Since annatto and carminic could appear singly or in combination in a food product, it was hoped to find a simple and reliable single system, suitable for *both* colours. Since none was available, one was developed, and is described below.

2. Experimental

2.1. Chemicals and reagents

Commercial samples of carminic acid and the four annatto compounds were provided by Warner-Jenkinson (Canada) and by Pointing (Canada). A stock solution was prepared for each individual compound, and concentrations were verified using a Cary (Varian, Sunnyvale, CA, USA) 219 spectrophotometer.

A 0.1067-g portion of cochineal/carmine powder (Pointing; 61.9% carminic acid) was transferred to 30 ml of 2 *M* HCl, boiled gently to dissolve the powder, cooled, then the solution was transferred quantitatively to a 1000-ml volumetric flask and diluted to volume with distilled water, providing a stock solution containing 66 μg of carminic acid/ml (1.32 μg/20-μl injection). A few particles (impurities) remained in the flask, so the solution was filtered using a Sartorius (Mississauga, Canada) RC15 Minisart filter (see Section 3.3) before chromatography. The concentration of carminic acid, verified spectrophotometrically, was not affected.

A stock solution containing 0.2189 g of norbixin powder (28.4% norbixin) and 0.2 g of NaOH, dissolved in 100 ml of water, was prepared. In order to obtain a concentration suitable for HPLC analysis, a 1.25-ml aliquot was diluted to 100 ml. The concentration of norbixin was 156 ng/20-μl injection. In addition, a stock solution of bixin was prepared by combining a 0.622-g portion of bixin powder (83.9% bixin), 0.1 g of NaOH and 100 ml of methanol in a flask, producing a concentration of 521.9 μg bixin/ml. The solution was mixed well, then allowed to sit overnight. The flask was shaken again before HPLC analysis.

2.2. Chromatography

The LC system consisted of two Beckman (Mississauga, Canada) Model 110B pumps set at a flow-rate of 1.0 ml/min, a Rheodyne (Cotati, CA, USA) 7725 syringe-loading injection port with a 20-μl loop, a Kratos (Ramsey, NJ, USA) Spectroflow 783 variable-wavelength detector set at a wavelength of 493 or 526 nm and a range of 0.05–1.0 AUFS, a Spectra-Physics (San Jose, CA, USA) SP4270 integrator, and a Supelco (Bellefonte, CA, USA) LC-18 (5 μm) column (250 × 4.6 mm I.D.). An IBM PS/2 computer system, loaded with Beckman Gold software and connected to a Beckman 406 analog interface, was utilized as a system controller.

The mobile phases (filtered and degassed) consisted of methanol–6% aqueous acetic acid at ratios of 40:60 (v/v) (pH ca. 2.8) (mobile phase A) and 90:10 (v/v) (mobile phase B). For food extracts

containing carminic acid, only mobile phase A was required. A gradient system was used to separate annatto and carminic acid. At the time of injection the gradient was programmed from 0 to 90% mobile phase B in 2 min and held. The column was conditioned using mobile phase A (40:60, v/v) for 30 min daily, before samples were injected. At the end of the day, the column was washed well with ca. 50 ml of water followed by 30 ml of methanol.

3. Results and discussion

3.1. HPLC

Using HPLC, several combinations of cochineal, norbixin and bixin were tested until a suitable mixture was produced. Due to the various pH values of the solutions and to the different solubilities, normally these colours are not combined. Before injection, it was necessary to thoroughly mix the composite standards. A solution containing 1.26 μg of carminic acid, 355 ng of norbixin and 199 ng of bixin per 20-μl injection was suitable for chromatography.

An isocratic mobile phase consisting of methanol and 6% aqueous acetic acid resolved bixin and norbixin, but a gradient system was required in order to separate carminic acid and the annatto compounds. Fig. 1 shows a chromatogram of carminic acid, α- and β-norbixin and α- and β-bixin. All compounds were readily resolved. The annatto peaks were slightly broad, but were reproducible.

The isocratic mobile phase (40:60, v/v) was used also with a number of cochineal products, such as cochineal extract, carmine, liquid carmine and carmine hydrosoluble, in order to determine the carminic acid content, and was shown to be suitable for use with all of the above. Before chromatography, each product was examined spectrophotometrically.

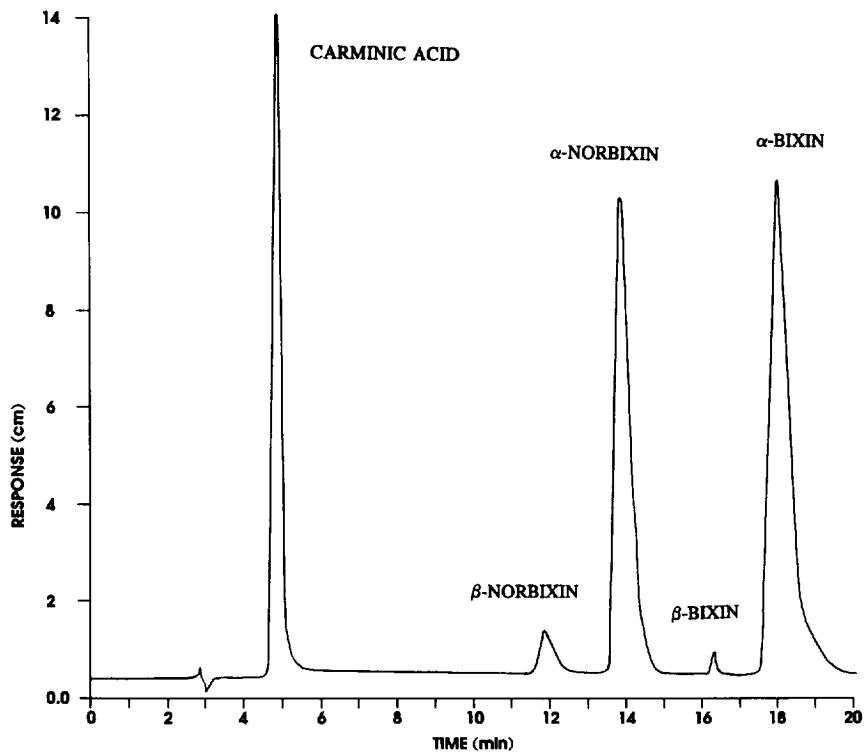

Fig. 1. Gradient chromatogram of carminic acid (1.26 μg), α- and β-norbixin (355 ng), and α- and β-bixin (199 ng). Initial mobile phase, methanol–6% aqueous acetic acid (40:60, v/v). At T_{inj} the gradient was programmed to 85:15 (v/v) in 2 min then held. Detector wavelength, 493 nm; Detector range, 1.0 AUFS.

The wavelength where maximum absorbance occurred was determined, and then utilized for HPLC. For example, a wavelength of 493 nm was suitable for carmine powder while 526 nm was selected for cochineal products used in water-based systems. The detection limit for carminic acid in the various products was determined to be approximately 100 ng/g.

3.2. Foods

During the study, food items such as fruit beverages and candies were extracted and the carminic acid content was determined quantitatively using HPLC and the methanol–acetic acid mobile phase. In Canada, permitted synthetic colours, such as amaranth, are used when a manufacturer wishes to produce a red shade in foods and few items are coloured with carminic acid. However, a colour manufacturer reported that one brand of fruit juice beverages contained carminic acid, so four different flavours were purchased for analysis. A solid-phase extraction (SPE) technique, reported previously [5], using Sep-Pak C_{18} cartridges (Waters, Milford, MA, USA) and tetra-*n*-butylammonium phosphate (TBAP) paired-ion reagent was tested. Solutions ($n=4$) containing citric acid, sugar and water were spiked with known amounts of carminic acid (20–200 μg/g), extracted by SPE, and analyzed using HPLC and mobile phase A. Recoveries ranged from 97–99%. The same procedure was then applied to the fruit beverages. Internal standards were used for confirmation. Only one of the four beverages, a raspberry-lemon flavour, was found to contain carminic acid (152 ± 3 μg/g)(see Fig. 2).

Since no hard candies containing carminic acid were available commercially, they were prepared in this laboratory. Hard candy is often manufactured at a temperature of ca. 150°C, but due to the low melting point of carminic acid (136°C), a range of temperatures from 100 to 135°C was selected. Recoveries ranged from 60% at 101°C ($n=2$) to less than 20% at 135°C ($n=2$). Using the same ingredients at room temperature (23°C), it was demonstrated that the method recovered 97 ± 2% of the carminic acid that was present. Therefore, it would appear that, due to poor *price per colour unit* values because of decomposition, carminic acid is not suitable for use in candies when manufacturing temperatures above 100°C are required.

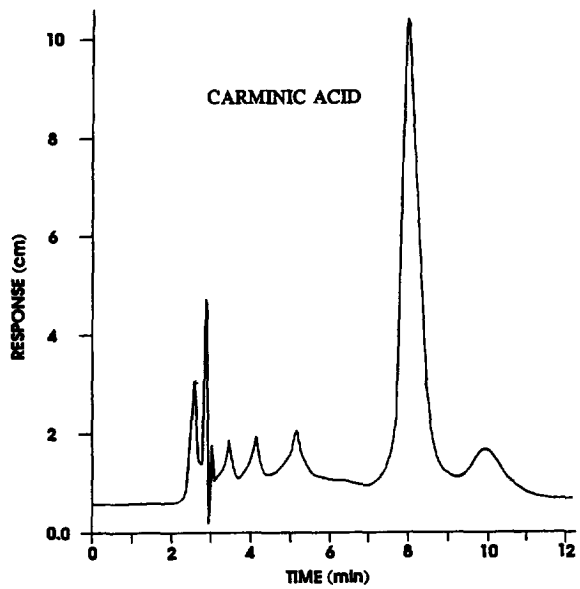

Fig. 2. HPLC chromatogram of carminic acid (3.04 μg) recovered from a beverage (extracted using SPE). Mobile phase, methanol–6% aqueous acetic acid (40:60, v/v). Detector wavelength, 493 nm; Detector range, 0.2 AUFS.

In addition, a method for the determination of carminic acid in yogurt [2] was tested. The extraction procedure ($n=5$) produced acceptable results, although recoveries (76–83%) were generally 5–10% below the reported values. Yogurts coloured with carminic acid were not available locally, so spiked specimens were used. For example, a commercial sample of uncoloured, plain yogurt was spiked with 140 μg/g of carminic acid, mixed thoroughly and extracted using the referenced procedure. The concentration of carminic acid was determined both spectrophotometrically (Cary 219 spectrophotometer) and chromatographically (using HPLC and mobile phase A). The average recovery from three extractions was 80 ± 2.1%.

The HPLC system [using a mobile phase of acetonitrile–1.19 *M* formic acid (19:81, v/v)], described in the same manuscript [2], did not perform as expected. The low pH of the mobile phase (pH 1.65), below the limit (pH 2) recommended for reversed-phase columns, caused rapid deterioration of the C_{18} column and led to leaks in the pumps. The

methanol–acetic acid mobile phase worked well with the yogurt extracts and, since the pH (2.4) was higher, the risk of damage to the column and pumps was eliminated.

3.3. Filters

It was reported previously by Jalón et al. [2] and verified in our laboratory that most membrane filters should not be used with cochineal solutions because they adsorb carminic acid. However, during our study, it was determined that the Sartorius Minisart RC15 (polypropylene housing, regenerated cellulose membrane, hydrophilic, 0.45 μm pore size) filter was very effective when used with water-based cochineal products. It was tested ($n=8$) using stock solutions of known concentration (0.5–200 μg/g) as well as with extracts from commercial food products containing carminic acid and no noticeable reduction in concentration occurred. Fig. 3 shows a chromatogram of carminic acid extracted from yogurt, filtered and then chromatographed using the methanol–acetic acid mobile phase. The concentration of carminic acid in that sample was determined to be 112 μg/g, 80% of the amount added. Detection limits of carminic acid in foods such as yogurt were ca. 500 ng/g, which is somewhat higher than the 100 ng/g limits determined for other food colours such as annatto or the synthetic dyes.

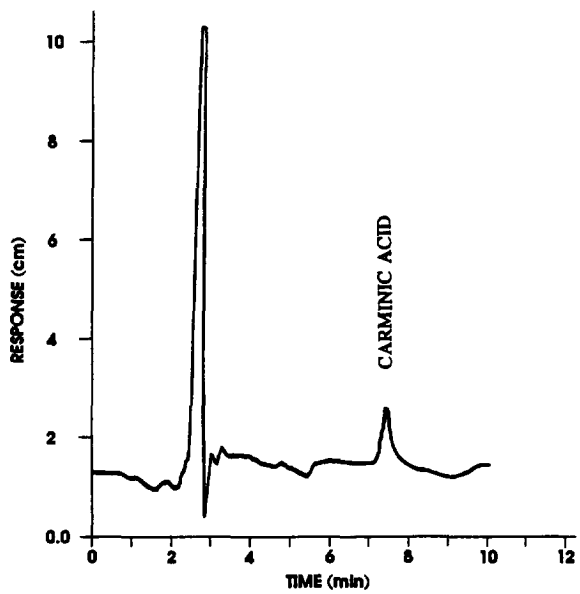

Fig. 3. HPLC chromatogram of carminic acid (156 ng/20-μl injection) recovered from a yogurt (extracted using enzymatic digestion). Mobile phase, methanol–6% aqueous acetic acid (40:60, v/v). Detector wavelength, 493 nm; Detector range, 0.08 AUFS.

4. Conclusions

A simple and reliable HPLC system suitable for both carminic acid and annatto has been developed. The system has been tested successfully with food products such as fruit beverages, yogurt and candies. In addition, it was demonstrated that RC15 filters are suitable for use with carminic acid and that an extraction procedure for carminic acid in yogurt [2] worked as described.

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References

[1] F.E. Lancaster and J.F. Lawrence, Food Add. Contam., 12 (1995) 9.
[2] M. Jalón, M.J. Peña and J.C. Rivas, J. Assoc. Off. Anal. Chem., 27 (1989) 231.
[3] S. Yamada, N. Noda, J. Hayakawa, E. Mikami and K. Uno, Eisei Kagaku, 31 (1985) 179.
[4] D.M. Marmion, Handbook of U.S. Colorants, Foods Drugs, Cosmetics and Medical Devices, 3rd ed., Wiley and Sons, (1991).
[5] F.E. Lancaster and J.F. Lawrence, J. Chromatogr., 210 (1981) 168.