Vol 10, Special Issue 1, 2018

INTERNATIONAL JOURNAL OF APPLIED PHARMACEUTICS

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

ISSN - 0975-7058

Research Article

ANALYSIS OF SYNTHETIC RED DYES IN RED SPINACH SAMPLES (*AMARANTHUS TRICOLOR* L.)

DOLVIN NOVITASARI, RANI SAURIASARI*, MARYATI KURNIADI

Department of Faculty of Pharmacy, Universitas Indonesia, Depok, 16424, Indonesia. Email: rani@farmasi.ui.ac.id

Received: 16 June 2018, Revised and Accepted: 22 October 2018

ABSTRACT

Objective: The purpose of this study was to determine whether synthetic red dyes are present in red spinach samples.

Methods: The presence of the dyes Ponceau 4R, Carmoisine, Rhodamine B, Amaranth was determined in red spinach using reactions, followed by paper chromatography and thin-layer chromatography (TLC)-densitometry. In paper chromatography analyses, analytes were eluted using n-butanol–ethanol–water (4:5:5) and isobutanol–ethanol–water (3:2:4), and in TLC-densitometry, analytes were eluted with n-butanol–ethanol–water (3:7:1).

Results: No synthetic red dyes were found in the seven red spinach samples.

Conclusion: The synthetic dyes Rhodamin B, amaranth, Ponceau 4R, and Karmoisin were not found as contaminants of red spinach.

Keywords: Amaranth, Karmoisin, Paper chromatography, Ponceau 4R, Red spinach, Rhodamin B, Thin-layer chromatography-densitometry, thin-layer chromatography.

 DOI: http://dx.doi.org/10.22159/ijap.2018.v10s1.04

INTRODUCTION

Producers and retailers often manipulate foodstuffs to increase attractiveness to consumer to consumers often manipulate foodstuffs. Food additives are defined as substances that are not normally present in foods and have no nutritional value. Such additives are often intentionally used for technological purposes during manufacture, processing, preparation, treatment, packaging, and storage [1] and are added to improve food characters and qualities [2]. Food additives have diverse uses such as sweeteners, flavorings, preservatives, antioxidants, flavors, emulsifiers, nutrients, and dyes [3].

Food colorings are additives that are regulated by the Law of the Republic of Indonesia No. 7 (1996) concerning food. Chapter II of the second part of Article 10 paragraph 1 states that in foods for distribution, it is illegal to add anything that is prohibited or exceeds the maximum limit set. In the Regulation of the Minister of Health of the Republic of Indonesia No. 239/Menkes/Per/V/85 and Decree of the Directorate General of POM of the Ministry of Health of the Republic of Indonesia No. 003861/C/SK/II/90 concerning the amendment of the attachment to the Regulation of the Minister of Health of the Republic of Indonesia No.239/Menkes/Per/V/85, there are 35 types of dyes that are declared as hazardous substances and their use in food is prohibited.

The addition of harmful dyes to foods and beverages also follows ignorance, of which dyes are allowed in food and which are not. Community members are often unaware that textile dyes such as aromatic amines released by azo dyes can cause long-term health problems including cancer [4,5]. Vegetables are critical dietary components for the maintenance of human health; however, some manipulations of marketed vegetables can be questionable. In particular, Indonesia investigation report on June 17, 2012, reported some color manipulations in vegetables. Specifically, some vegetable retailers soak vegetables in large tubs with dye to enhance their appearances.

Based on data from Rimanews Indonesian internet newspaper dated September 21, 2012, the Citarum river in South Bandung is supplied from industrial wastewater, especially from the textile industry that uses color dyes. Not all industrial wastewater flows to downstream areas, and some industrial chemicals have been found in the mud of flood areas. Since river water is used to cultivate vegetables and other plants, chemical compounds can be absorbed into the roots and leaves of plants. These vegetables and plants may affect the health of people who consume them and can be dangerous because dyes are generally stable and refractory to biodegradation.

Boiling of red spinach produces a stew of strong red color, leading to suspicious of red dye contents. Therefore, to know whether it is natural color or not, we determined the synthetic red dye contents of red spinach samples from traditional and modern markets in Depok.

MATERIALS AND METHODS

Materials

Experiments were performed using a CAMAG thin-layer chromatography (TLC) scanner 3, ultraviolet (UV) lamps, 10×10- and 20×20- cm CAMAG chromatographic vessels, 2-μL capillary pipes, analytical scales, glassware, rubber balloons, electric stoves, water baths, plate drops, spatulas, stirrer bars, refrigerators, heating ovens, and a vapor plate.

Standard solutions of the edible red dyes Ponceau 4R (Cl. 16255; Dynemic Products Ltd.) and Carmoisine (CI No. 14720; Dynemic Products Ltd.) were purchased from PT Tanduk Air Mas. The toxic red dyes Rhodamin B (Cl 45170) and amaranth (Cl 16185) were purchased from BPOM (Drug and Food Control Agency).

Chemicals used for the color reactions included concentrated sulfuric acid p.a (Merck), concentrated hydrochloric acid p.a (Merck), n-butanol p.a (Merck), glacial acetic acid p.a (Mallinckrodt), and chloroform (Merck). Ethyl methyl ketone p.a, ammonia p.a, ethanol p.a, isobutanol, and aquadest were purchased from Merck.

Red spinach samples were purchased from modern and traditional markets in Depok. Other materials included sheep wool from a goat retailer at Cisalak Depok market, universal indicator, Whatman paper No.1, filter paper, silica gel 60 F254 (Merck), and aluminum plates of 20×20 cm.

Methods

Preliminary optimization of eluent types and compositions using dye standards

Reagent solutions were prepared in 70% ethanol solution and 10% ammonia. 1 L solutions of 70% ethanol were prepared by mixing 701 mL of 99.9% ethanol with 1000 mL with distilled water. 10 mL solutions of 10% ammonia were prepared by diluting 4.0-mL aliquots of 25% ammonia solution with 10 mL with distilled water.

Preliminary tests were performed to determine the types and compositions of selected eluents using standard dyes. Subsequently, eluents were optimized for paper chromatography and TLC-densitometry. Dye standards were weighed, and 2.0 mg samples were dissolved in 5.0-ml aliquots of 70% ethanol. Solutions were then stored in glass bottles, and up to 2.0-µL aliquots were spotted onto Whatman No.1 paper and incubated at room temperature until solvents evaporated. Tested eluents for paper chromatography included ethyl methyl ketone–acetone–water (7:3:3) [5], ethanol–n-butanol–water (4:5:5) [5], n-butanol–glacial acetic acid–water (4:5:1) [5], and isobutanol–ethanol–water (3:2:2) [5]. TLC-densitometry was conducted using ethanol–n-butanol–water (3:6) and ethanol–n-butanol–water (3:7:1) mixtures as eluents [5]. Standard solutions of dyes were bottled and 2.0-µL aliquots were placed on TLC plates. Elusion was performed using the eluents mentioned above, and TLC papers and plates were then lifted and dried and the resulting spots were observed.

Two eluents were chosen for paper chromatography and one was selected for TLC-densitometry. The eluent compositions ethanol–n-butanol–water (4:5:5) and (20:25:15) and isobutanol–ethanol–water (3:2:4) and (3:2:2) were then tested in paper chromatography analyses, and the eluent compositions ethanol–n-butanol–water (4:5:7), (3:7:1), and (3:6:1) were compared in TLC-densitometry analyses. Standard dye solutions were placed as 2.0-µL droplets onto chromatographic paper and TLC plates with 1-cm spotting points from the bottom edge. All dye spots were spaced at 1 cm and were allowed to migrate until solvents had completely evaporated. Dye components were eluted up to 8 cm from spotting points using the eluents described above. After elution, TLC papers and plates were lifted and dried and the resulting spots were observed.

To determine the maximum wavelengths of standards, dye solutions were prepared by dissolving 25.3 mg of 4R Ponceau, 25.2 mg of Carmoisine, 25.3 mg of Rhodamin, and 25.2 mg of amaranth in 70% ethanol to concentrations of 2–200 ppm and then measuring absorbance spectra at visible light wavelengths of 380–780 nm.

The construction of calibration curve

To generate the calibration curves and perform linearity tests, 25.3 mg of Ponceau 4R standard was added to a 25 mL measuring flask and was diluted to volume in 70% ethanol, and the solution was then diluted to 30.36, 40.48, 50.60, 60.72, 80.96, and 91.08 ppm. Similarly, 25.2 mg of Carmoisine was added to a 25 mL measuring flask and was diluted to volume in 70% ethanol, and this solution was then diluted to 30.24, 40.32, 50.40, 60.49, 80.60, and 100.8 ppm. Up to 25.2 mg of ammonium, stock solution was added to a 25-mL measuring flask and was diluted to volume with 70% ethanol. Dilutions were then made to concentrations of 10.08, 20.16, 30.24, 40.32, 70.56, and 80.64 ppm. To generate the Rhodamin B standards, 25.3 mg of Rhodamin B standard compound was added to a 25-mL measuring flask and was diluted to volume with 70% ethanol. The resulting solution was then diluted to 20.24, 30.36, 40.48, 50.6, 80.96, and 91.08 ppm. All solutions were then spotted onto TLC plates at 2 µL per spot at 1 cm from the bottom edge and 1 cm from each other spot, and were then eluted over 8 cm using ethanol–n-butanol–water (3:7:1). Following elution, spots were scanned with a TLC scanner at the maximum wavelength for each standard. The resulting absorption values were plotted against concentrations, and correlation coefficients of calibration curves were calculated.

Limit of detection and selectivity test

Detection limits of each dye standard were determined using respective calibration curves and were expressed in ng. To perform selectivity tests, standards were added to approximately 4-mL samples of red spinach extracts that did not contain the dye. Subsequently, 1-mL aliquots were mixed into isolates and 100-ppm solutions were then spotted in 2-µL aliquots onto chromatographic paper at 1 cm from the bottom edge and other spots, and were finally eluted over 8 cm using ethanol–n-butanol–water (4:5:5). Chromatographic papers were then removed and dried and spots were observed. Finally, we calculated the Rf values of each spot and compared these with Rf values of standards.

Method of sample isolation

To isolate synthetic dyes from samples, a total of seven red spinach specimens were purchased from Mekarsari (the largest fruit garden in Indoensia), traditional, and modern markets in Depok and surrounding areas. Samples were stored at room temperature and were protected from light. Wool samples were washed thoroughly with detergent, were dried in the sun, and were then soaked in chloroform and dried again [6].

Samples of approximately 20 g of spinach were placed in 100-mL volumetric flasks with water and were soaked with 25 mL of ethanol. The resulting mixtures were then acidified using an aqueous hydrochloric acid solution to a pH of about 3, and 2 g of clean fat-free white fleece was then added. After heating in a water bath for 15 min stirring, wool samples were absorbed and were then rinsed several times using cold water before placing in another flask with 10 mL of 10% ammonium hydroxide solution. Finally, samples were reheated in the water bath with frequent stirring until the solution became colored [7].

To distinguish between natural and synthetic dyes in wool fleece, colored solutions from wool treatments were acidified again using hydrochloric acid and new wool fibers were added. Synthetic substances can bind fibers to color them, whereas natural dyestuffs cannot recolor fibers, likely reflecting chemical damage. Dyes were isolated in 70% ethanol for both TLC and paper chromatography analyses.

Identification of the dyes in red spinach samples

To identify dyes in samples, qualitative color analyses were performed using color reactions with an isolated dye. Reaction yields for sample dyes were compared with those of standard dyes using concentrated sulfuric acid, concentrated hydrochloric acid, and 10% ammonia [6]. Qualitative analyses were also performed using paper chromatography. In these experiments, 2.0-mg dye standards were carefully weighed and dissolved in 5 mL of 70% ethanol. Rhodamine B samples of 1.0 mg were dissolved in 10.0 mL of 70% ethanol, and standard dye solutions were compared with extracted sample solutions on the same chromatographic papers as described above, with a spotting volume of 2 µL, 1-cm spaces between spots and the bottom edge, and elution with ethanol–n-butanol–water (4:5:5) and isobutanol–ethanol–water (3:2:4) over 8 cm. After elution, chromatographic papers were removed from the apparatus and were dried, and spots were then observed. Calculated Rf values for each spot were compared with those of standards. As qualitative analyses, we performed experiments using TLC-densitometry. Spots of sample and suitable compound dye solutions were applied to TLC plates and were eluted in a vessel that was saturated with selected eluents. The chromatographic plate was then dried and the resulting spots were observed visually and under UV light. Dye contents of samples were then determined as Rf values of spots relative to those of standards. TLC plates with selected mobile phases were also analyzed using a TLC scanner, and absorption spectra and maximum wavelengths of sample and standard spots were quantified using TLC-densitometry. Finally, dye standards and samples were spotted onto chromatographic paper adjacent to each other and were eluted under optimized conditions. After drying chromatographs, standard and samples were compared directly at known absorption maxima using the TLC scanner.

RESULTS AND DISCUSSION

Preliminary optimization of eluent types and compositions using dye standards

In preliminary paper chromatography experiments with dye standards, various eluent compositions were compared and ethanol–n-butanol–

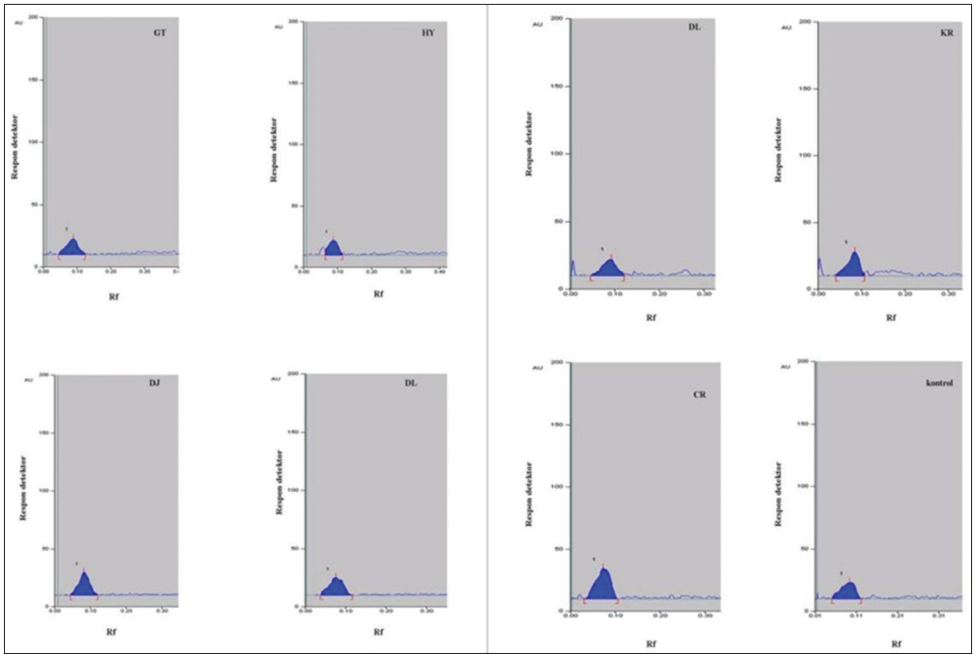

Fig. 1: Differences of pH and temperature between experiments performed on different days

water and isobutanol–ethanol–water at ratios of 4:5:5 and 3:2:4, respectively, were selected. These elution conditions resulted in sufficient separation of Rf ranges between standard dyes, and ease of elution and good stability. In TLC experiments, elution with ethanol–n-butanol–water at the ratio of 3:7:1 produced good separation of the Rf ranges of standards, although with long elution times of about 1 h.

Calibration curves

Linear regression analysis of Ponceau 4R calibration curves gave a correlation coefficient of r=0.99945 with the equation y=989.2x+26.11. Similarly, the calibration curve of Carmoisine was described by y=424.5x+33.16 and had an r=0.99908. Calibration curves for Rhodamine B and amaranth were linear with r=0.99905 and 0.99911 and equations y=1330x+33.6 and y=631.9x+25.85, respectively.

Limits of detection

Limits of detection for Ponceau 4R, Carmoisine, Rhodamine B, and amaranth were 5.2097, 6.9743, 8.6662, and 7.7823 ng, respectively.

Selectivity tests

Selectivity tests were performed to confirm that determinations of dye contents were not affected by other components of the sample matrix. These experiments showed that single chromatographic spots contained chlorophyll, anthocyanin, and the added standard dye. This separation technique exploited differing polarities of test substances and met the requirements of selectivity.

Isolation of samples

The present method for isolating dyes from wool is commonly used [8,9]. Since wool is amphoteric, it can be colored by acidic or alkaline dyes, and extraction can be used to identify synthetic and natural dyes [9]. After stripping, natural dyes fail to recolor fibers, and none of the seven samples of red spinach contained dyes that could restore color to fibers after stripping. These observations warranted subsequent experiments using paper chromatography and TLC-densitometry.

Identification of the dyes in red spinach samples

Qualitative analysis of color reactions

Color tests were performed using concentrated sulfuric acid, concentrated hydrochloric acid, and 10% ammonia as described previously [6]. Color comparisons of standards and samples showed mainly purple and red in both. Therefore, further, identification is required to distinguish synthetic from natural dyes in vegetable samples.

Qualitative paper chromatography analyses

In paper chromatography tests using the eluents ethanol–n-butanol–water at 4:5:5 and isobutanol–ethanol–water at 3:2:4, dye contents were clearly separated and showed clear differences in spotting colors and Rf values of the seven samples and four standards. Critically, these data indicate that the seven red spinach samples do not contain synthetic dyes.

Qualitative analyses with TLC-densitometry

To confirm the results of paper chromatography, we analyzed dye contents of samples and standards using TLC. After eluting with ethanol–n-butanol–water at a ratio of 3:7:1, no synthetic dyes were identified in any of the seven vegetable samples. Densitometric analyses of scanned TLC images also showed differing Rf values and absorption spectra for samples and standards. However, Rf values can be affected by vessel saturation and analysis conditions of pH and temperature, which can lead to differences between experiments performed on different days as shown in Fig. 1.

Taken together, the present experiments are evidence that no synthetic dyes were present in our red spinach samples. Further, analyses using the present optimized methods with more samples are required to discourage the application of synthetic dyes to vegetables by retailers, ignorant, or otherwise.

CONCLUSION

Herein, we eluted dyes in paper chromatography analyses using ethanol–n-butanol–water at the ratio of 4:5:5 and isobutanol–ethanol–water at the ratio of 3:2:4. In TLC analyses, analytes were eluted with ethanol–n-butanol–water at the ratio of 3:7:1. The synthetic dyes Rhodamine B, amaranth, Ponceau 4R, and Carmoisine were not found as contaminants of red spinach.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Cahyadi W. Analysis and Health Aspects of Food Additives. 2nd ed. Jakarta: Penerbit Bumi Aksara; 2008. p. 61-5.

2. Mudjajanto ES, Pertanyaan H. Eddy Setyo Mudjajanto. Food and Nutrition Technology Observer. Bogor: Harian Pagi Jurnal Bogor; 2009.
3. Baliwati YF, Khosman A, Dwiriana CM. Introduction to Food and Nutrition. Jakarta: Penerbit Penebar Swadaya; 2004. p. 90-1.
4. Neumann HG. Aromatic amines: Mechanisms of carcinogenesis and implications for risk assessment. Front Biosci (Landmark Ed) 2010;15:1119-30.
5. Brüschweiler BJ, Merlot C. Azo dyes in clothing textiles can be cleaved into a series of mutagenic aromatic amines which are not regulated yet. Regul Toxicol Pharmacol 2017;88:214-26.
6. Mahindru SN. Food Additives: Characteristics, Detection and Estimation. New Delhi: Tata McGraw-Hill Publishing Company; 2000. p. 91-105.
7. National Standardization Agency. Methods for Food Coloring Testing. Jakarta: National Standardization Agency; 1992.
8. Ozdemir H. Dyeing properties of natural dyes extracted from the junipers leaves (*J. excelsa* Bieb. and *J. oxycedrus* L.). J Nat Fiber 2017;14:134-42.
9. Ghouila H, Meksi N, Haddar W, Mhenni MF, Jannet HB. Extraction, identification and dyeing studies of isosalipurposide, a natural chalcone dye from *Acacia cyanophylla* flowers on wool. Industr Crops Prod 2012;35:31-6.