Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Green Chromatographic Method for The Determination of Synthetic Azo Dyes in Processed Beef and Chicken Meat Products

Maha S. Abd-Elhafeez[1], Fatma H. Amro[2], Mai A. Fadel[1], Mohamed H. Gaffer[3], Eman Elkhawaga[4], Eman Shukry[4], Pierre E. Mehanny[5] and Fady Sayed Youssef[6*]

[1] *Chemistry, Toxicology and Feed Deficiency Department (Pharmacology and Pyrogen Unit), Animal Health Research Institute, Agriculture Research Center (ARC), 12618 Giza, Egypt.*

[2] *Food Hygiene Department, Animal Health Research Institute, Agriculture Research Center, 12618 Giza, Egypt.*

[3] *Food Hygiene Department, Animal Health Research Institute, Agriculture Research Center of Egypt, Shebin El koom Branch, Egypt.*

[4] *Food Hygiene Department, Animal Health Research Institute, Mansoura branch, Agricultural Research Center, Egypt*

[5] *Biochemistry, Toxicology, Feed Deficiency Department, Animal Health Research Institute, Agricultural Research Center, Dokki, Giza 12618, Egypt.*

[6] *Pharmacology Department, Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt..*

Abstract

FOOD COLORANTS in meat are controlled by European and non-European statutes for food safety concerns. A sensitive and eco-friendly method using High-Performance Liquid Chromatography (HPLC) with a diode-array detector and ammonia-ethanol extraction has been developed to accurately measure seven commonly used sulfonate azo colorants (Amaranth, Erythrosine, Sunset Yellow, Azorubin, Ponceau 4R, Red 2G, and Allura red) in processed meat samples within 10 min. Separation was successful using an Agilent ZORBAX RRHD Eclipse Plus C-18, 2.1 × 100 mm, 1.8 µm column at ambient temperature with a mobile phase consisting of 2.5 mL of Triton X-100 up to 1 L with phosphate buffer solution (0.05 M) at pH 7. The greenness score was measured using the AGREE approach. This highly sensitive and specific method makes it suitable for the analysis of complex matrices. The method demonstrated good analytical performance in terms of sensitivity, selectivity, precision, and accuracy. Recoveries ranging from 92% to 102% were obtained, which met standard analytical requirements. The AGREE score of 0.82 indicates excellent greenness of the HPLC assay. These findings show that the method can effectively be used for conducting commercial meat product confirmation studies.

Keywords: synthetic colorants, meat products, chromatography, GAC.

Introduction

Green analytical chemistry (GAC) first appeared in 2000 [1], attracting interest in environmentally friendly quantitative techniques. Chromatography is a widely used analytical method due to its simplicity, sensitivity, and time saving, making it a crucial component. GAC aspects should guide HPLC method development, considering performance measures like precision, recovery, LOD, LOQ, and linearity. This is a well-established practice [2].

Developing a greener approach in modern analytical chemistry involves integrating green aspects from method development to minimize hazardous substances used in or generated by the method, making it more environmentally safe [3].

The approved number of food colors has been diminished for safety, but they continue to be

*Corresponding author: Fady Sayed Youssef, Email : fadyalsalhany@cu.edu.eg , Tel.: 01094572140
(Received 29 May 2024, accepted 22 August 2024)
DOI: 10.21608/EJVS.2024.293417.2132

commonly abused for their affordability, efficiency, and steadiness. Synthetic colors are closely regulated to minimize toxicity [4]. There is evidence that colorants and their metabolites can be harmful to humans, causing allergies and asthmatic responses, hyperactivity, DNA impairment, and even carcinogenic effects [5]. Various legislative measures have been implemented to confirm food safety and regulate international trade concerning the use of food colorants [6].

Several national regulations govern the use of food coloring in food products. The acceptable daily intake (ADI) and the maximum amount of colorants allowed in certain foods were set by the relevant authorities. Regulation (EC) No 1333/2008 [7] mandates that the European Food Safety Authority (EFSA) conduct a fresh risk assessment for all food additives approved for use in the EU prior to January 20, 2009. In EU, Sunset yellow (E 110); Azorubin (E 122); Amaranth (E 123); Ponceau 4R (E 124); Erythrosine (E 127); Red 2G (E 128), and Allura red (E 129) should be labeled with additional information: 'name or E number of the color(s): can adversely affect activity and attention in children'.

Due to their widespread accessibility, cheapness, and chemical stability, unethical manufacturers can misuse these dyes to enhance the appeal of food products. Analysis of these dyes is crucial for evaluating food quality and safety [8]. For this reason, precise analytical methods are required to monitor dyes in processed foods that are drawn to consumers.

Many analytical methods to determine food colors have been proposed, including, thin-layer chromatography [9], spectrophotometry [10], polarography [11], voltammetry [12], capillary electrophoresis (CE) [13], ion chromatography [14], and high-performance liquid chromatography (HPLC) [15]. Spectrophotometry, polarography, and voltammetry are simple and fast techniques; nevertheless, they lack specificity because they are not suited to defining multiple food dye mixes in the food matrix. CE is characterized by a high resolution and little analysis time; however, fronting or, tailing peaks of a few dyes are noted in the electropherograms.

Numerous quantitative and qualitative techniques have been used to measure the greenness indices of analytical procedures [16,17]. To predict the greenness score, only the "Analytical GREENness (AGREE)" technique considers all 12 principles of GAC [18,19].

Therefore, this study aims to develop a new Green Chromatographic, simple, rapid, and sensitive HPLC method for simultaneous determination of some synthetic Azo dyes in processed beef and chicken meat products following "The International Council for Harmonization (ICH)" Q2-R1 protocols [20].

Material and Method

Ethical statement

Cairo University's Institutional Animal Care and Use Committee approved the current protocol (IACUC number:08072023667).

Reagents and chemical preparations

The azo dye standards (E-110, E-122, E-123, E-124, E-127, E-128, and E-129) were obtained from glpbio.com in Montclair, CA, USA. The mobile phase was prepared by dissolving 2.5 mL of Triton X-100 (Sigma-Aldrich) up to 1 L with phosphate buffer solution (0.05 M) at pH 7. The mixture was purified, before consumption, through a 0.45 µm nylon filter. The critical micellar concentration (CMC) of the surfactant was 0.18% (v/v). The Milli-Q ultrapure water distillation system (Thermo Scientific, Hungary) was used to purify the water.

One hundred and twenty specimens of chicken and beef meat products were collected from markets in Giza, Egypt for analytical purposes. Twenty samples were collected for each type of processed meat product. After collection, the samples were saved at −18 °C ± 2 °C until analysis. Table 1 refers to more information on the samples.

Standard preparation.

The dyes were dried for 6 h at 65°C. The stock solution was prepared by weighing 10 mg of dried dyes in a 10 mL volumetric flask (VF), and the volume was then completed to the mark using 0.05 M ammonium acetate to create a solution with a level of 1000 ppm, which was kept at -20°C for 2 months in amber glass vials.

An intermediate solution with a level of 10 ppm was then prepared by transferring 100 µL of the stock solution into a 10 mL VF and filling it with 0.05 M ammonium acetate, and stored at 4°C in amber glass vials.

Serial dilutions were prepared by diluting 20, 50, 100, 200, 500, 1000, and 2000 µL from the stock solution in 10 mL VF using 0.05 M ammonium acetate to create concentrations of 0.2, 0.5, 1, 2, 5, 10, and 20 ppm [21]. The working solutions were freshly prepared. The quality control (QC) level was 2 ppm.

Sample preparation

A two-gram homogenized sample was combined with 10 mL of ethanol-water-ammonia (80:19:1, V/V/V) extraction mixture and subjected to ultrasonication for 45 min., then centrifuged to remove proteins at 12,000 rpm for 10 minutes at 4°C. Five milliliters of the supernatant was vaporized in a 55°C water bath under a nitrogen stream to dryness.

The residue was redissolved with 1 mL of 50 mM ammonium acetate and then passed through a 0.45 µm nylon filter. Twenty microliters of the extract were injected into the HPLC system.

Chromatographic separation

Separation was conducted using an Agilent 1200 HPLC system with a quaternary pump and a multi-wave detector UV-VIS, all controlled by Chemstation software. The column used for separation was an Agilent ZORBAX RRHD Eclipse Plus C-18, 2.1 × 100 mm, 1.8 µm (Agilent Technologies, Waldbronn, Germany), maintained at ambient temperature (25°C) with a flow rate of 1 mL/min. The volume of injection was 20 µL. Azo dyes were detected at 500 nm [22].

Validation of the developed method

The assay was approved by the ICH regulations [18] and complied with the requirements listed below.

Linearity

Linearity measures a method's ability to produce a response directly proportional to the sample's concentration within a specific range. To determine linearity, seven concentrations, ranging from 0.2 to 20 ppm, were prepared. A mean curve was plotted using the data obtained from each concentration injected into the HPLC thrice. The average area under the peak was then calculated for each level and plotted against the respective concentration. Linear regression analysis was used to derive the regression equation of the standard curve and the determination coefficient (R^2).

Limits of detection and quantification

The lowest analyte level in a sample that can be precisely and reliably identified is known as the Limit of Quantification (LOQ), which is equivalent to the analyte concentration at which the signal-to-noise ratio is 10. Conversely, the lowest quantity that can be identified, or the analyte concentration for which the signal-to-noise ratio is 3, is known as the Limit of Detection (LOD). These parameters were statistically analyzed using the standard deviation of the intercept (S) [20] [LOD= 3.3*S/slope; LOQ= 10*S/slope].

Specificity and Selectivity

The selectivity of the analysis was assessed by examining potential interferences between impurities and the food matrix of the sample extracts and analytes. Blank meat samples were spiked with the studied dyes at the QC level and then analyzed.

Accuracy.

Accuracy in experiments is determined by how closely the experimental values match the true or reference values. To compare the theoretical concentrations, three different concentrations of the standard solution (1/2x, 1x, and 2x of the QC point) were prepared in blank poultry and beef meat. Then, three sets of duplicate spiked samples were prepared, chromatographed, and identified for their concentrations.

Precision

The precision of the technique readings is determined by their level of consistency, which is measured through the relative standard deviation percentage (RSD %). To assess this, six repeats of the QC point were injected into the HPLC after extraction on a constant day for intra-day precision testing and on six different days for inter-day precision. The average concentration was used to calculate the percentage of RSD.

System Suitability Testing parameters

The system suitability testing parameters (SST) of the proposed assay were evaluated by determining the tailing factor (T_f), column effectiveness through the theoretical plates number (N), retention time (T_R), and symmetry (A_s) of six replicates of the QC point.

Greenness estimation

The AGREE methodology, developed by Pena-Pereira et al. [23], was used to predict the greenness index of azo dyes using the HPLC assay. The AGREE scores for the HPLC assay, which ranged from 0.0 to 1.0, were determined by utilizing "AGREE: The Analytical Greenness Calculator (version 0.5, Gdansk University of Technology, Gdansk, Poland, 2020)."

Results and discussion

Chromatographic optimization

To achieve excellent separation, it is crucial to maintain optimal conditions. This involves maximizing the wavelength, flow rate, temperature, and mobile phase components.

A green chromatographic method for measuring dyes was developed using environmentally friendly chemicals. A C18 column, which is widely used in HPLC laboratories worldwide, was chosen as the fixed phase. A surfactant-containing aqueous solution formed the mobile phase. Surfactants are used in green chemistry to replace organic solvents, thereby reducing environmental impact and improving safety [19]. Non-flammable, low-toxicity and biodegradable micellar mobile phases in reversed-phase HPLC offer a green alternative with minimal equipment investment and less hazardous waste. With the initial green approach in mind, all subsequent experiments were carefully planned and executed.

Because the seven Azo dyes have a charge at pH 7 and the C18 group is nonpolar, they have a low propensity for the C18 stationary phase, which makes them very soluble in water. Because these colorants do not interact with the column and elute together, it is not possible to separate them using water alone as a mobile phase. To address this issue, it is recommended to use a non-ionic surfactant as the mobile phase. This technique involves absorbing the surfactant into the stationary phase, thereby improving its polarity and modifying the separation mechanism. In addition, the presence of the surfactant changes the solvent properties, resulting in the production of micelles.

Using a C18 column as the fixed phase and a solution of Triton X-100 surfactant as the mobile phase to separate dyes. The polarity of the stationary phase stabilizes the charges of dye molecules, whereas the 0.25% Triton X-100 solution is chosen for its optimal balance of chromatographic resolution, analytical frequency, sensitivity, and reduced surfactant consumption, resulting in less waste [24].

pH significantly affects colorant separation [25], with poor chromatographic resolution at pH values below 6.5. A pH of 7 improves the resolution and sensitivity, allowing for quantitative analysis. All analyses conducted at this pH gave peaks of better shape and shorter retention times. The buffer used in this study had a low concentration of 0.05 mmol. The low-toxic chemical wastes are neutral, making the method greener, as mentioned by Mishra *et al*. [26].

One crucial factor in the separation of azo dyes is the flow rate of the mobile phase. In this investigation, a flow rate of 1.0 mL/min was chosen because it provided a good balance between analytical frequency and sensitivity. This rate allowed for a quick run time without increasing the pump pressure. Vidotti *et al.* [24] demonstrated that a higher flow rate (2 mL/min) led to lower chromatographic sensitivity and resolution, which supported this decision. Conversely, a lower flow rate (0.25 mL/min) increased the analytical frequency but reduced the sensitivity.

In terms of temperature, the retention time decreases with increasing temperature because of reduced mobile phase viscosity. The ideal temperature is 25°C for peak shape improvement and shortening retention duration. Raising the temperature above 25°C does not affect peak performance.

The DAD detector's scan mode yielded the best UV-Vis results, and the seven dyes had good sensitivity at 500 nm. In a 10-minute run, the chromatogram of the mixed standard solution demonstrated distinct baseline separation of the seven dyes.

The optimal chromatographic conditions are outlined in Table 2. Using these conditions, the peak for azo dyes appeared at retention times (t_R) of 1.494, 4.05, 4.24, 5.58, 6.12, 6.64, and 7.06 min, as shown in Figure 1. Subsequently, this analytical method was validated.

Method validation

Linearity was investigated by investigating mixed preparations of the seven dyes at various concentrations varying from 0.2 to 20 ppm in HPLC. The relationship of linearity was expressed in the regression equation estimation. The results revealed acceptable linearity within the prepared range, with a correlation coefficient (R^2) greater than 0.999. The LOD values ranged from 0.001 to 0.012 ppm (Table 3). The correlation between the measured peak areas and dye concentrations was high, indicating that the developed method is reliable for dye measurement.

In the developed method, solid-phase extraction was not required for sample preparation because of the different chemical characteristics of the seven dyes. In particular, E-127 has a unique chemical structure compared with other dyes [27]. The liquid phase extraction (LPE) pretreatment process exhibited high recovery and selectivity, as shown in Figures 2, 3, and 4, as recommended by [20]. Therefore, this analytical method can accurately quantify a substance and differentiate between the analyte(s) and any impurities that may be present.

Tables 4 and 5 display the high recovery rates attained using the developed method following LPE of spiked beef and chicken meat at the three specified levels. The results indicate that the method is precise because the percentage recovery rates fall within the acceptable criteria established by the ICH [20]. The RSD% for intra-day precision ranged from 0.2% to 0.5% (n=6), whereas the RSD% for inter-day precision ranged from 0.5% to 1.6% (n=6). As demonstrated in Table 6, these results confirm that the assay is highly precise as the obtained RSD% was less than 2% [20].

Table 7 displays the chromatographic parameters for individual peaks of seven azo dyes at a concentration of 2 ppm at 500 nm wavelengths. These parameters include RT (retention time in minutes), T_f (tailing factor), A_S (symmetry factor), and N (numbers of theoretical plates assessed by the tangent method). All chromatographic factors were determined automatically using the ChemStation® software operating an Agilent 1200 chromatograph. These values indicate that the assay is highly stable. All parameters indicate that the chromatographic system is suitable for analyzing these synthetic azo dyes, as recommended by the ICH [20].

This is consistent with GAC tenets, which are a key component of sustainable development. The important components of this theory are the

miniaturization of analytical instruments and the reduction of the time between analysis and obtaining satisfactory results. Solventless extraction methods and the use of substitute solvents are the primary approaches that adhere to the principles of GAC, as stated by Yahaya *et al.* [28].

Applicability of the HPLC-DAD method in commercial samples

The applicability was assessed by analyzing multiple chicken and beef meat product samples. Upon conducting the analysis, it was found that none of the seven analyzed azo dyes were found in any of the examined chicken products and beef burgers. The absence of azo dyes in beef burgers and all chicken products that might pose health risks advises that the manufacturers or suppliers follow regulations and quality control measures.

E 128 and E 124 were each found in 5% (1 out of 20) of beef sausages. Meanwhile, E 129 was detected in 75% (15 out of 20) of beef sausages and 55% (11 out of 20) of beef luncheons (Table 8). The concentrations of these colors were found to be within the maximum permitted levels (25 ppm for E 129 and E 128, 50 ppm for E 124) as set by Regulation (EC) No 1333/2008 [7] and the European Food Safety Authority [29, 30].

Health Hazards of Azo Dyes.

Azo dyes are synthetic colorants that are applied to improve the appearance of meat products. However, their safety is a concern because of potential adverse effects on human health [31,32,33].

Health risks associated with azo dyes include skin irritation, as certain individuals may develop contact dermatitis upon exposure. Allergic reactions can be triggered in sensitive individuals because of these dyes. Furthermore, there is concern regarding the potential carcinogenicity of some azo dyes, with studies hinting at a possible link to cancer, although the evidence remains inconclusive. It is essential to consider these risks when using products containing azo dyes and take necessary precautions to mitigate any adverse health effects.

To promote healthier choices, it is recommended to carefully read food labels, opting for products that contain minimal artificial additives and azo dyes. Practice moderation when consuming processed meat items to reduce the potential health risks associated with these products. In addition, it is advisable to seek guidance from healthcare professionals if you have specific concerns or questions regarding the impact of these additives on your health, ensuring informed decision-making for your well-being.

Greenness estimation.

Several methods have been described for measuring the environmental friendliness of analytical tests [16, 17]. However, the AGREE methodology only studies the 12 principles of GAC for measuring greenness. Therefore, "AGREE" was applied to calculate the greenness scores of the progressed approach. Figure 5 shows the descriptive mark for the AGREE score of the HPLC assay. The AGREE technique assigns an AGREE score of 0 to 1 to each of the GAC principles. GAC principles 3, 4, 6, 9, 10, 11, and 12 received a score of 1. The GAC principle 8 received a score of 0.86. A score of 0.75 was recorded for principle 5. A score of 0.55 was obtained for principle 2. A score of 0.33 was obtained for principles 1 and 7. These results prove the excellent greenness of the developed assay for azo dyes measurement.

Conclusion

A new method using HPLC-DAD and ammonia-ethanol extraction was developed to accurately measure seven frequently used sulfonate synthetic colorants in processed meat samples within 10 min. Separation was successful on the C-18 column using surfactant and phosphate buffer saline at pH 7. The method is highly sensitive and specific and is suitable for analyzing complex matrices. According to the AGREE review, the RP-HPLC technique has an exceptional greenness score, which qualifies for on-site colorant monitoring in various animal products. The method is practical for analyzing real samples and allows for processing large numbers of specimens in a day. Red and yellow dyes may be simultaneously detected in solid matrices using this method, all within a single extraction and chromatographic run.

Acknowledgement

We would like to thank Dr. Elham A. Mobarez, Chief Researcher of the Pharmacology, Toxicology and Pyrogen Unit at the Animal Health Research Institute in Dokki, Giza for her support, valuable advice, and scientific knowledge during the period of our work.

Funding information

No specific grant was provided for this research by governmental, private, or nonprofit funding organizations.

Conflicts of Interest

The authors affirm that there is no conflict of interest between them.

Author contributions

Each author contributed to the aim and design of the study. Fatma H. Amro, Mohamed H. Gaffer, Eman Elkhawaga, and Eman Shukry collected specimens of chicken and beef meat products and conducted sample preparation. Maha, S. Abd-Elhafeez, Fady Sayed Youssef, Pierre E. Mehanny, and Mai A. Fadel developed and validated an eco-friendly HPLC method for Azo dyes analysis. All authors

collaborated on writing, data analysis, revising the manuscript and approved the final revision.

Ethical statement

Cairo University's Institutional Animal Care and Use Committee approved the current protocol (IACUC number: 08072023667).

TABLE 1. **Characterization of collected samples A- F.**

Sample	Characterization
A	Beef burger
B	Beef sausages
C	Beef luncheon
D	Chicken burger
E	Chicken sausages
F	Chicken luncheon

TABLE 2. Optimal chromatographic conditions

Parameters	Optimal condition
Column	Agilent ZORBAX RRHD Eclipse Plus C/18, 2.1 × 100 mm, 1.8 μm.
Elution Mode	Isocratic.
Mobile phase	0.25% Triton X-100 in 50 mmol/L phosphate buffer solution at pH 7.
Temperature	25°C.
Flow rate	1.0 mL/min.
Detector	DAD; the range of wavelength from 300 to 650 nm
Detection UV	500 nm wavelength.
Injection volume	20 μL.

TABLE 3. Linear relationships, R^2, LODs, and LOQs for the seven azo dyes.

Dye	Regression equation	R^2	LOD	LOQ
E-123	Area=183.790493*Amt-8.432876	0.99990	0.009	0.027
E-124	Area=1058.98979*Amt+10.023493	0.99999	0.008	0.025
E-110	Area=150.318208*Amt-7.4308089	0.99998	0.01	0.04
E-129	Area=2510.8707*Amt-21.18519	1.00000	0.001	0.004
E-128	Area=1532.61877*Amt+5.5816908	0.99990	0.005	0.015
E-122	Area=891.66334*Amt-16.541406	0.99999	0.003	0.009
E-127	Area=1828.74038*Amt-15.639692	0.99954	0.012	0.037

TABLE 4. Recovery of the HPLC assay after LPE from spiked blank beef meat (n=3).

	Theoretical conc.	Obtained conc.	Recovery	
	ppm	**ppm**	**%**	**Mean± sd**
	1	0.99±0.03	99	
E-123	2	1.98±0.03	99	99.63±2.1
	4	4.04±0.07	101	
	1	0.94±0.02	94	
E-124	2	1.95±0.04	98	97.78±3.6
	4	4.07±0.04	102	
	1	0.97±0.02	97	
E-110	2	1.98±0.03	98	98.75±2.4
	4	4.04±0.07	102	
	1	0.95±0.02	95	
E-129	2	1.96±0.02	96	96.2±1.7
	4	4.06±0.04	97	

	1	0.97±0.02	97	
E-128	2	1.96±0.02	98	98.0±1.6
	4	3.97±0.07	99	
	1	0.99±0.02	99	
E-122	2	1.98±0.02	99	99.6±1.5
	4	4.03±0.04	101	
	1	1.01±0.02	101	
E-127	2	1.99±0.02	100	100.03±1.5
	4	3.98±0.05	99	

TABLE 5. Recovery of the HPLC assay after LPE from spiked blank chicken meat (n=3).

	Theoretical conc.	**Obtained conc.**	**Recovery**	
	Ppm	**Ppm**	**%**	**Mean± sd**
	1	0.94±0.02	94	
E-123	2	1.95±0.02	98	97.53±3.5
	4	4.05±0.05	101	
	1	0.96±0.02	97	
E-124	2	1.92±0.03	96	97.19±1.6
	4	3.95±0.02	99	
	1	0.92±0.01	92	
E-110	2	1.90±0.01	95	94.44±1.6
	4	3.83±0.01	96	
	1	0.96±0.01	96	
E-129	2	1.93±0.03	96	96.2±1
	4	3.86±0.04	97	
	1	0.94±0.01	94	
E-128	2	1.90±0.01	95	95.6±1.6
	4	3.90±0.02	98	
	1	0.97±0.01	97	
E-122	2	1.90±0.01	95	96.2±1.1
	4	3.88±0.01	97	
	1	0.96±0.01	96	
E-127	2	2.00±0.02	100	98.7±2.3
	4	4.01±0.02	100	

TABLE 6. Intra- and inter-day precision of the HPLC assay for azo dyes separation (n=6).

Dye	**Intra-day precision**		**Inter-day precision**	
	(mean±sd)	**RSD%**	**(mean±sd)**	**RSD%**
E-123	(356± 1)	0.3	(356± 3)	0.9
E-124	(2117± 5)	0.2	(2134± 33)	1.6
E-110	(286± 1)	0.4	(283± 3)	0.9
E-129	(4997± 9)	0.2	(50± 24)	0.5
E-128	(3010± 8)	0.3	(3011± 17)	0.6
E-122	(4446± 9)	0.2	(4480± 42)	0.9
E-127	(359± 2)	0.5	(359± 4)	1.1

TABLE 7. Values for SST (mean± sd; RSD%) of the seven azo dyes measured by HPLC assay.

Dye	RT	N	T_f	A_s
E-123	1.49± 0.01; 0.75	9720± 11; 0.12	1.15± 0.01; 0.95	0.85± 0.02; 2.6
E-124	4.05± 0.01; 0.25	22937± 27; 0.12	0.97± 0.004; 0.44	1.05± 0.02; 1.43
E-110	4.26± 0.04; 0.89	25576± 6; 0.02	0.97± 0.004; 0.41	0.98± 0.002; 0.25
E-129	5.58± 0.02; 0.29	32233± 141; 0.44	0.98± 0.005; 0.47	1.03± 0.004; 0.39
E-128	6.18± 0.08; 1.25	37750± 32; 0.08	0.97± 0.005; 0.52	1.03± 0.02; 1.73
E-122	6.64± 0.004; 0.07	44954± 40; 0.09	0.98± 0.002; 0.22	1.01± 0.01; 1.2
E-127	7.06± 0.004; 0.05	48032± 39; 0.08	0.97± 0.004; 0.4	1.06± 0.01; 1.2
Acceptance criteria	RSD%≤ 1%	N≥ 2000	$T_f \leq 2$	$A_s \geq 1$

TABLE 8. Levels of detected azo dyes in the analyzed samples.

Sample	Detected dye	Incidence (%)	Range (ppm)
	E128	(1/20) 5	4.5
B	E 124	(1/20) 5	0.8
	E129	(15/20) 75	0.9-12
C	E 129	(11/20) 55	2-15

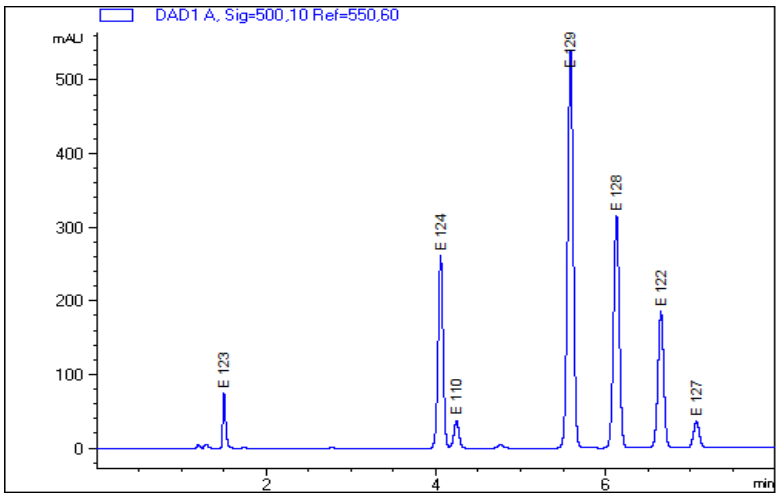

Fig. 1. Chromatogram of the standard working solution at 1 ppm of E-123, E-124, E-110, E129, E128, E-122, and E-127 under the optimum conditions.

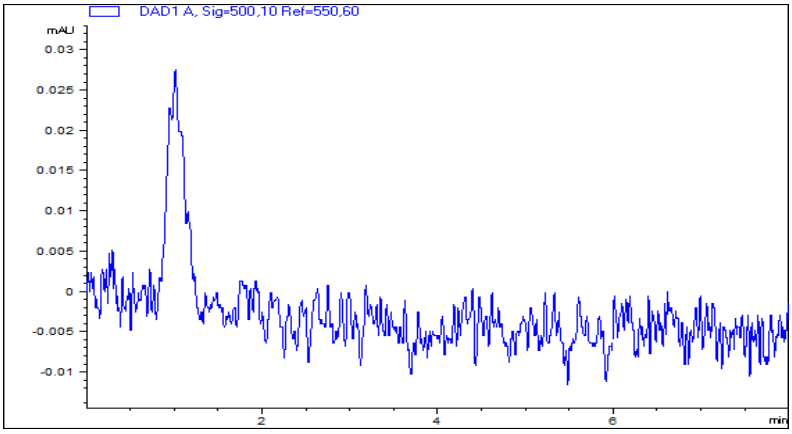

Fig. 2. Chromatogram of blank beef meat after LPE

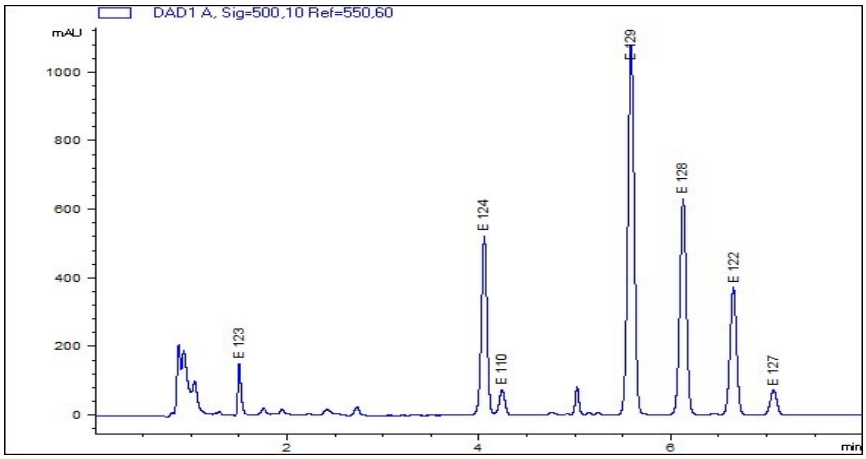

Fig. 3. Chromatogram of fortified blank beef meat after LPE at a concentration of 2 ppm for the seven dyes.

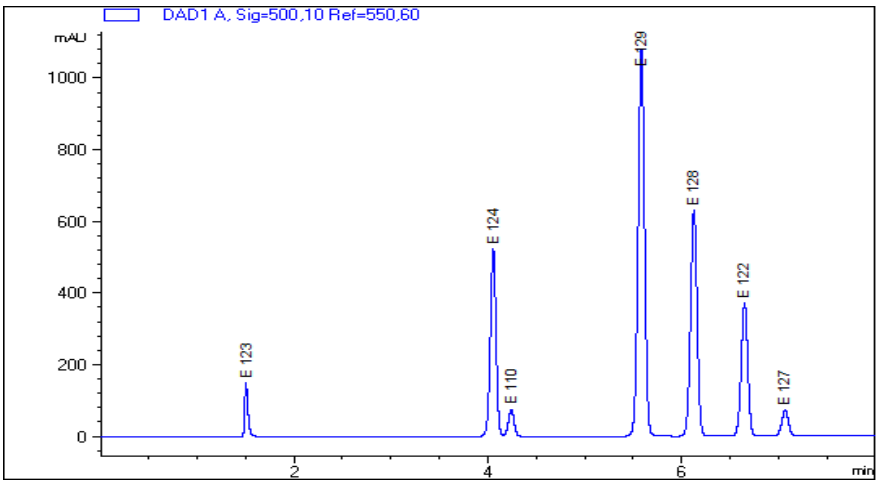

Fig. 4. Chromatogram of a mixed standard preparation containing seven dyes at 2 ppm

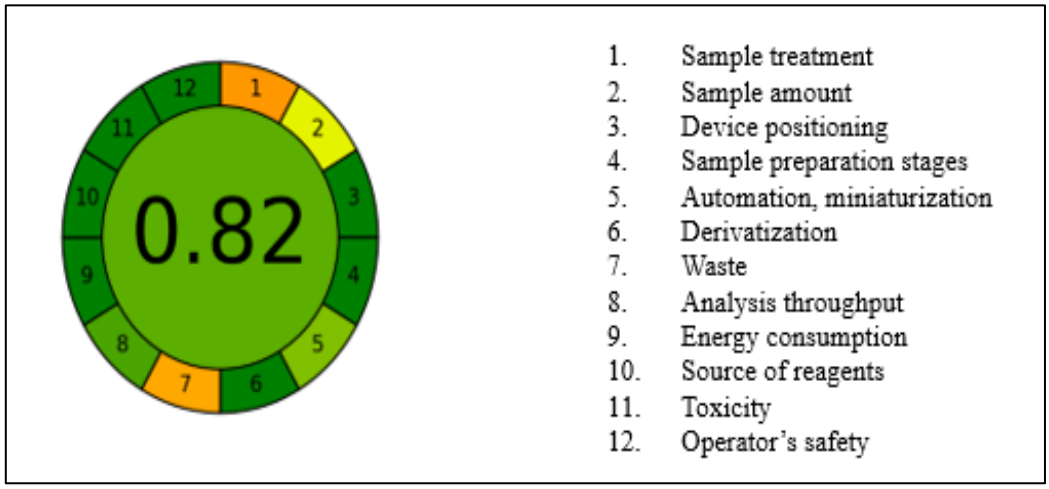

Fig. 5. The descriptive mark for AGREE scores obtained through RP-HPLC using the AGREE calculator

References

1. Armenta, S., Garrigues, S. and de la Guardia, M. Green analytical chemistry. *TrAC Trends in Analytical Chemistry*, **27**(6), 497-511(2008).

2. Ibrahim, F. A., El-Brashy, A. M., El-Awady, M. I. and Abdallah, N. A. Assessment of the greenness of spectrophotometric and micellar liquid chromatographic methods using two approaches: Application to pharmaceutical analysis of hydrochlorothiazide and telmisartan. *Microchemical Journal*, **148**, 197-205 (2019).

3. Elmansi, H. and Belal, F. Development of an Eco-friendly HPLC method for the simultaneous determination of three benzodiazepines using a green mobile phase. *Microchemical Journal*, **145**, 330-336 (2019).

4. Jia, W., Chu, X., Ling, Y., Huang, J., Lin, Y. and Chang, J. Simultaneous determination of dyes in wines by HPLC coupled to quadrupole orbitrap mass spectrometry. *Journal of Separation Science,* **37**,782–791 (2014).

5. Rovina, K., Prabakaran, P. P., Siddiquee, S. and Shaarani, S. M. Methods for the analysis of Sunset YellowFCF (E110) in food and beverage products- a review. *TrAC-Trends in Analytical Chemistry,* **85**, 47–56 (2016).

6. Pettoello-Mantovani, C. and Olivieri, B. Food safety and public health within the frame of the EU legislation. *Global Pediatrics*, **2**, 100020 (2022).

7. Regulation (EC) No. 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. *Official Journal of Europian Union,* **L354**, 16–23 (2008).

8. Rejczak, T. and Tuzimski, T. Application of high-performance liquid chromatography with diode array detector for simultaneous determination of 11 synthetic dyes in selected beverages and foodstuffs. *Food Analytical Methods*, **10**, 3572-3588 (2017).

9. Xu, L. and Liu, S. Forecasting structure of natural products through color formation process by thin layer chromatography. *Food Chemistry*, **334**, 127496 (2021).

10. Behbahani, E. S., Dashtian, K. and Ghaedi, M. Fe/Co-chalcogenide-stabilized Fe3O4 nanoparticles supported MgAl-layered double hydroxide as a new magnetically separable sorbent for the simultaneous spectrophotometric determination of anionic dyes. *Microchemical Journal*, **152**, 104431 (2020).

11. Combeau, S., Chatelut, M. and Vittori, O. Identification and simultaneous determination of Azorubin, Allura red and Ponceau 4R by differential pulse polarography: application to soft drinks. *Talanta*, **56**(1), 115-122 (2002).

12. Ni, Y., Bai, J. and Jin, L. Simultaneous adsorptive voltammetric analysis of mixed colorants by multivariate calibration approach. *Analytica Chimica Acta*, **329**(1-2), 65-72 (1996).

13. Prado, M. A., Boas, L. F. V., Bronze, M. R. and Godoy, H. T. Validation of methodology for simultaneous determination of synthetic dyes in alcoholic beverages by capillary electrophoresis. *Journal of Chromatography A*, **1136**(2), 231-236 (2006).

14. Chen, Q. C., Mou, S. F., Hou, X. P., Riviello, J. M. and Ni, Z. M. Determination of eight synthetic food colorants in drinks by high-performance ion chromatography. *Journal of Chromatography A*, **827**(1), 73-81 (1998).

15. Cheibub, A. M. D. S. S., de Lyra, E. S. B., Alves, B. J., Donagemma, R. A. and Netto, A. D. P. Development and validation of a multipurpose and multicomponent method for the simultaneous determination of six synthetic dyes in different foodstuffs by HPLC-UV-DAD. *Food Chemistry*, **323**, 126811 (2020).

16. Alam, P., Shakeel, F., Alqarni, M. H., Foudah, A. I., Aljarba, T. M., Alam, A., Ghoneim, M. M.; Asdaq, S. M. B., Alshehri, S. and Iqbal, M. Determination of cordycepin using a stability-indicating greener HPTLC method. *Separations*, **10**(38) (2023). https://doi.org/10.3390/separations10010038.

17. Alqarni, M. H., Shakeel, F., Foudah, A. I., Aljarba, T. M., Mahdi, W. A., Bar, F. M. A., Alshehri, S. and Alam, P. A validated, stability-indicating, eco-friendly HPTLC method for the determination of cinnarizine. *Separations*, **10**(138) (2023). https://doi.org/10.3390/separations10020138.

18. Gałuszka, A., Migaszewski, Z. and Namieśnik, J. The 12 principles of green analytical chemistry and the SIGNIFICANCE mnemonic of green analytical practices. *TrAC Trends in Analytical Chemistry*, **50**, 78-84 (2013).

19. Abd-Elhafeez, S. M., Arafa, M. M., Amro, F. H. and Youssef, F. S. Green Analytical Chemistry to Eco-Friendly HPLC Techniques in Pharmaceutical Analysis: A Review. *Egyptian Journal of Veterinary Sciences*, **55**(3), 795-801 (2024).

20. International conference on harmonization (ICH), validation of analytical procedures−text and methodology Q2 (R1). In Proceedings of *the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use*, Geneva, Switzerland, 2005.

21. Hajimahmoodi, M., Afsharimanesh, M., Moghaddam, G., Sadeghi, N., Oveisi, M. R., Jannat, B. and Kanan, H. Determination of eight synthetic dyes in foodstuffs by green liquid chromatography. *Food Additives and Contaminants: Part A*, **30**(5), 780-785 (2013).

22. Pagáčiková, D. and Lehotay, J. Determination of synthetic colors in meat products using high-performance liquid chromatography with photodiode array detector. *Journal of Liquid Chromatography & Related Technologies*, **38**(5), 579-583 (2015).

23. Pena-Pereira, F., Wojnowski, W. and Tobiszewski, M. AGREE—Analytical GREEnness metric approach and software. *Analytical chemistry*, **92**(14), 10076-10082 (2020).

24. Vidotti, E. C., Costa, W. F. and Oliveira, C. C. Development of a green chromatographic method for determination of colorants in food samples. *Talanta*, **68**(3), 516-521 (2006).

25. Dolan, J. W. Back to basics: the role of pH in retention and selectivity. *LC-GC North America*, **35**(1), 22-28 (2017).

26. Mishra, K., Siwal, S. S., Nayaka, S. C., Guan, Z. and Thakur, V. K. Waste-to-chemicals: Green solutions for bioeconomy markets. *Science of the Total Environment*, **887**, 164006 (2023).

27. Zou, T., He, P., Yasen, A. and Li, Z. Determination of seven synthetic dyes in animal feeds and meat by high performance liquid chromatography with diode array and tandem mass detectors. *Food Chemistry,* **138**: 1742–1748 (2013).

28. Yahaya, N., Mohamed, A. H., Miskam, M., Keyon, A. S. A., Loh, S. H., Zain, N. N. M. and Sajid, M. Green analytical chemistry metrics for evaluation of microextraction methods: Fascinating or essential tools in real-world applications? *TrAC Trends in Analytical Chemistry*, **117587** (2024).

29. European Food Safety Authority. Refined exposure assessment for Ponceau 4R (E 124). *EFSA Journal*, **13**(4), 4073 (2015).

30. European Food Safety Authority [EFSA]. Scientific Opinion on there- evaluation of Allura Red (E129) as a food additive. *EFSA Journal*, **7**, 1327–1363(2009).

31. Hazaa, W., Shaltout, F. and El-Shater, M. Identification of some biological hazards in some meat products. *Benha Veterinary Medical Journal*, **37**(1), 27-31 (2019).

32. Saxena, A. and Gupta, S. *Toxicological Impact of Azo Dyes and Their Microbial Degraded Byproducts on Flora and Fauna*. In Innovations in environmental biotechnology, 319-343 (2024).

33. Vitkova, T., Boycheva, V. and Enikova, R. Medical Health Hazards in the Production of Meat and Meat Products. *In Proceedings*, **91**(1), 365, (2024).

الطريقة الكروماتوغرافية الخضراء لتقدير أصباغ الآزو الاصطناعية في منتجات لحوم الأبقار والدواجن المصنعة

مها عبد الحفيظ [1]، فاطمة عمرو [2]، مي فاضل [1]، محمد جعفر [3]، إيمان الخواجة [4]، إيمان شكري [4]، بيير مهني[5] و فادي سيد يوسف [6]

[1] قسم الكيمياء والسموم ونقص الأعلاف (وحدة الصيدلة والبيروجين) - معهد بحوث صحة الحيوان - مركز البحوث الزراعية - 12618 الجيزة - مصر.
[2] قسم صحة الغذاء - معهد بحوث صحة الحيوان - مركز البحوث الزراعية - 12618 الجيزة - مصر.
[3] قسم صحة الغذاء - معهد بحوث صحة الحيوان - مركز البحوث الزراعية المصري - فرع شبين الكوم - مصر.
[4] قسم صحة الغذاء - معهد بحوث صحة الحيوان - فرع المنصورة - مركز البحوث الزراعية - مصر
[5] الكيمياء الحيوية - السموم - قسم نقص الأعلاف - معهد بحوث صحة الحيوان - مركز البحوث الزراعية - الدقي - الجيزة 12618 - مصر.
[6] قسم الأدوية - كلية الطب البيطري - جامعة القاهرة - 12211 الجيزة - مصر.

الملخص

يتم التحكم في ملونات الطعام الموجودة في اللحوم بموجب القوانين الأوروبية وغير الأوروبية لمخاوف تتعلق بسلامة الأغذية. تم تطوير طريقة حساسة وصديقة للبيئة باستخدام تحليل كروماتوجرافي سائل عالي الأداء (HPLC) مع كاشف مصفوفة الصمام الثنائي واستخلاص الأمونيا والإيثانول لقياس سبعة ملونات آزو سلفونات شائعة الاستخدام (أمارانث، إريثروسين، غروب الشمس الأصفر، أزوروبين، بونسو). R4، Red 2G، و Allura red) في عينات اللحوم المصنعة خلال 10 دقائق. كانت عملية الفصل ناجحة باستخدام عمود Agilent ZORBAX RRHD Eclipse Plus C-18، 100 × 2.1 مم، 1.8 ميكرومتر عند درجة الحرارة المحيطة مع طور متحرك يتكون من 2.5 مل من Triton X-100 حتى 1 لتر مع محلول عازل للفوسفات (0.05 م) عند الرقم الهيدروجيني 7. تم قياس درجة الاخضرار باستخدام نهج AGREE. هذه الطريقة شديدة الحساسية والمحددة تجعلها مناسبة لتحليل المصفوفات المعقدة. أظهرت الطريقة أداءً تحليلياً جيداً من حيث الحساسية والانتقائية والدقة. وقد تم الحصول على معدلات استرداد تتراوح بين 92% إلى 102%، وهو ما يلبي المتطلبات التحليلية القياسية. تشير درجة الموافقة البالغة 0.82 إلى قدرة ممتازة لفحص HPLC. تظهر هذه النتائج أنه يمكن استخدام الطريقة بشكل فعال لإجراء دراسات تأكيدية على منتجات اللحوم التجارية.

الكلمات الدالة: الملونات الاصطناعية، منتجات اللحوم، الكروماتوغرافي.