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Development of Novel Methods for The Determination of Synthetic Colorants

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Content:

- A Brief Look at Colorants
  - What are Food Colorants
  - Classification
  - Why do we use?
  - Legislations and Limitations
- Objectives
- Methodology
- Experimental Studies
- Results
- Conclusion

A Brief Look at Food Colorants

What are food colorants??

- Food colorants are an important class of food additives attracting the attention of consumers, and give the first impression about the taste and quality of a food product.
- Color in one form or another has been added to our foods for centuries. It is known that Egyptians colored candy and wine dating back to 400 BC.

A Brief Look at Food Colorants

Synthetic Food Colorants

✓Any artificially synthesized substance, pigment or dye for coloring foods

✓High Stability to light, oxygen and pH

✓Color uniformity

✓Low microbiological contamination

✓Relatively lower production costs (Alves et al. 2008)

✖ Some life-threatening risks (Kapadia et al. 1998; Eigenmann and Haenggeli 2007)

Natural Food Colorants

✓ manufactured by extracting from natural substances

✓ no limitation for the quantity

✖ low stability

✖ high cost

A Brief Look at Food Colorants

Why do we use??

- ☛ to intensify the actual color of foods
- ☛ to obtain color stability in mass production
- ☛ to regain the lost color of a food after some food process
- ☛ coloring some types of food such as confectionary which are actually colorless

A Brief Look at Food Colorants

Legislations

- EU COMISSION

(30 June 1994 on colors for use in foodstuffs)

- World Health Organization

- US Food and Drug Administration

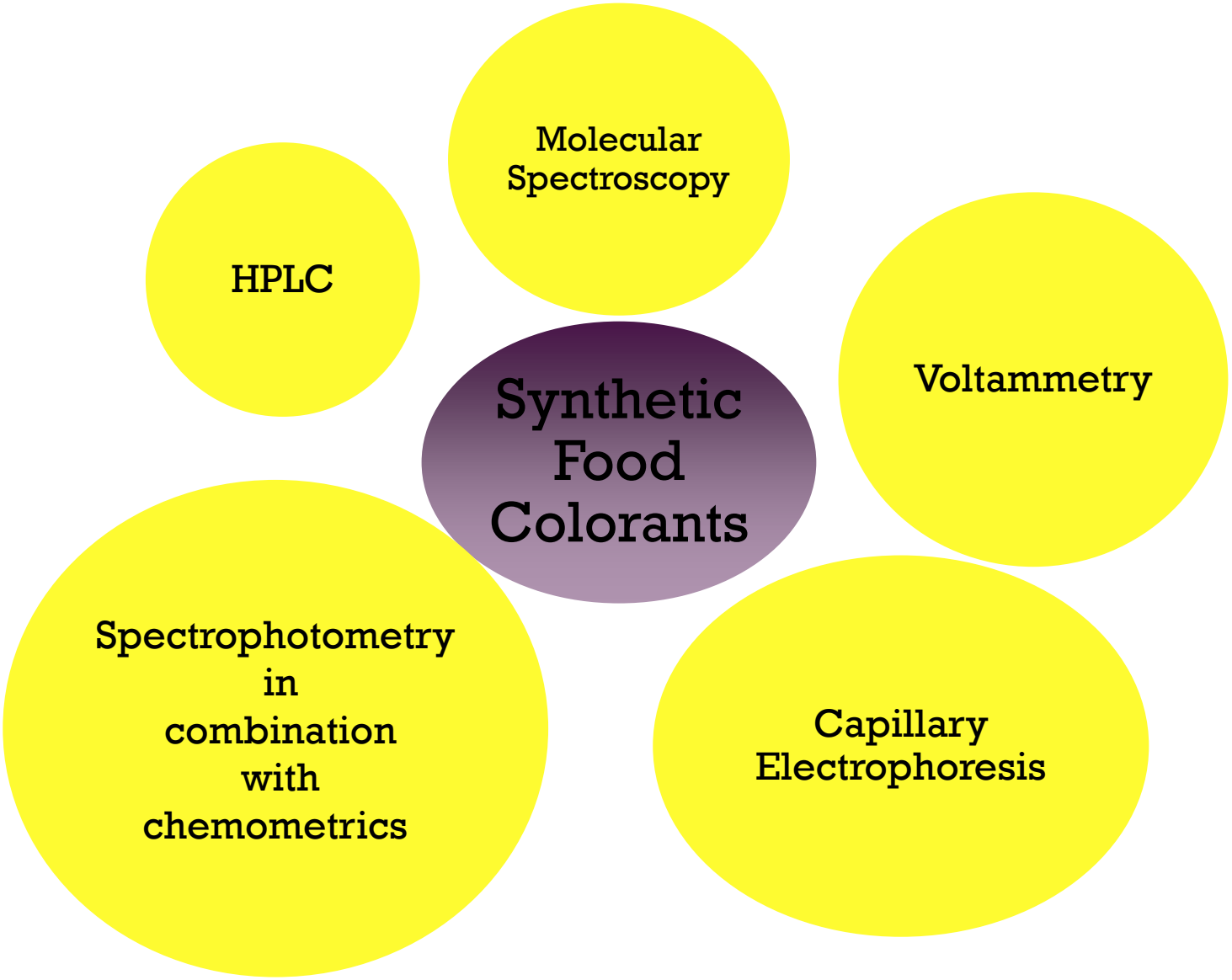
A Brief Look at Food Colorants Legislations

E Number	Name of Colorant	E Number	Name of Colorant
E 100	Curcumin	E 155	Brown HT
E 101	Riboflavine	E 160	Carotenoids
E 102	Tartrazine	E 163	Anthocyanines
E 132	Indigo Carmine	E 124	Ponceau 4R
E 133	Brilliant Blue	E 170	Calcium Carbonate
E 141	Chlorophylls	E 171	Titaniumdioxide
E 142	Green	E 173	Alluminum
E 150	Amonnium Caramel	E 174	Silver
E 151	Brilliant Black	E 175	Gold
E 153	Carbon	E 180	Litolrubin BK
E 154	Brown FK	E 110	Sunset Yellow

Objectives

- Developing and investigating novel methods for the determination of synthetic food colorants
- Analyzing synthetic colorant content of food products
- Providing food control by informing consumers about the limitations of these substances
- Aiming the issues above, adapting in-vitro antioxidant assay CUPRAC for the determination of synthetic food colorants
- Correlation of proposed method results with HPLC findings
- Combination of in-vitro antioxidant assays with HPLC technique - application of online HPLC-CUPRAC technique

Methodology
In Literature



Methodology

CUPRAC(Cupric ion Reducing Antioxidant Capacity)

- The CUPRAC method is a simple and versatile antioxidant capacity assay useful for a wide variety of polyphenols, including phenolic acids, hydroxycinnamic acids, flavonoids, carotenoids,anthocyanins, as well as for thiols, synthetic antioxidants, and vitamins C and E.

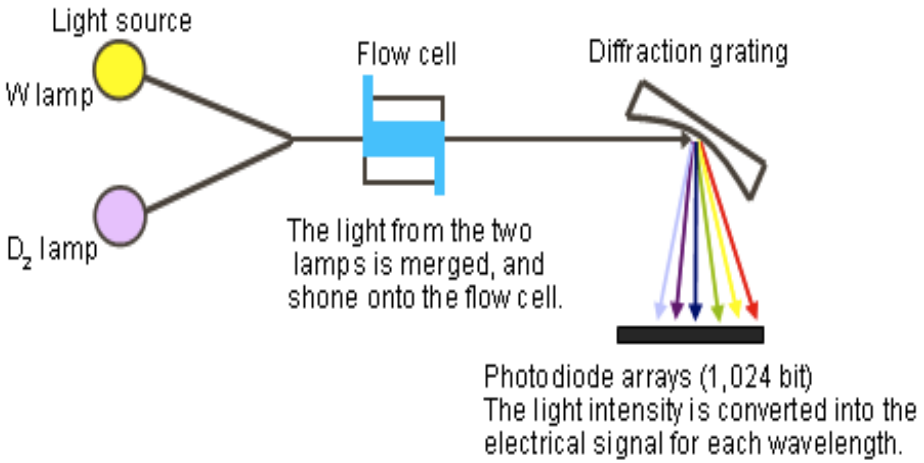
- The chromogenic oxidizing reagent bis(neocuproine)copper(II) cation (Cu(II)-Nc) is used as an outer-sphere electrontransfer agent and by reduction of this reagent with antioxidants, bis(neocuproine) copper(I) cation (Cu(I)-Nc) is formed.

$n\mathrm{Cu(Nc)_2}^{2+}$ + *n*-electron reductant (AO) $n\mathrm{Cu(Nc)_2}^{+}$ *n*-electron oxidized product + $n\ \mathrm{H}^{+}$

R. Apak, K. Guclu, M. OzyUrek, S. E. Karademir and M. Altun, Free Radical Res., 2005, 39, 949–961.
R. Apak, K. Guclu, M. Ozyurek, S. E. Karademir and E. Ercag, Int. J. Food Sci. Nutr., 2006, 57, 292–304.

Methodology

HPLC-PDA Technique



- Most preferred methods for the determination of synthetic food colorants are still chromatographic techniques coupled with ultraviolet (UV) or diode array detectors (Serdar and Knezevic 2009; Culzoni et al. 2009; Kirschbaum et al. 2006)
- There are two main problems with the use of single-wavelength UV detectors.

⌘ Various UV–visible (UV–Vis) spectra with different maximum absorbance wavelengths ➔long seperation time

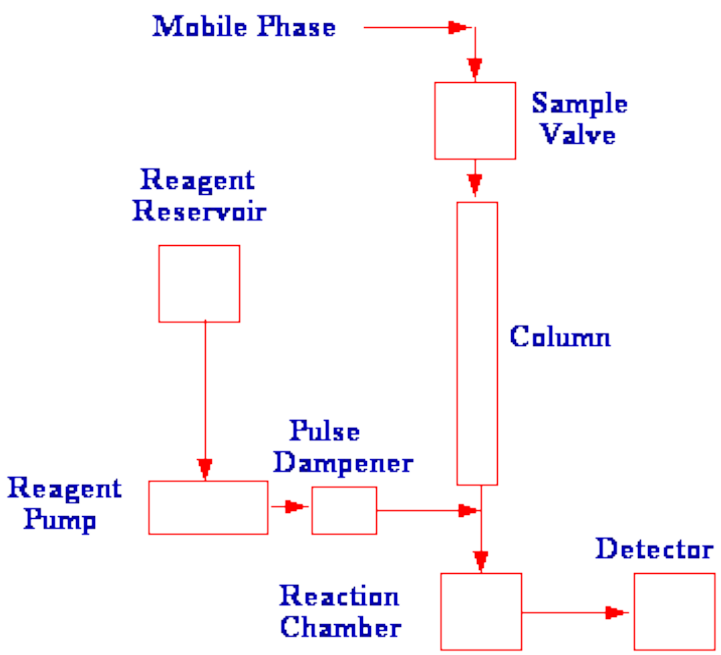
⌘ Possible overlap of colorant peaks or the presence of other organic compounds such as flavors in the sample.

☺ Both problems can be solved in the case of DADs. All dyes can be detected near to their maximum wavelength with the aid of multisignal detection capability, and peak identity can be easily confirmed.

Methodology

On-Line HPLC Derivatization Techniques

- Post-column derivatization involves the modification of the chromatographic system to allow the reaction to take place prior to entering the detector by inserting a post column reactor between the column and the detector.



The post-column reactor is required to fulfill the following functions:

1)Provide a source of reagent and a means of mixing it efficiently with the column eluent.

2)Ensure the reaction is complete before the derivatized product enters the detector.

3)Minimize the dispersion that takes place in the reactor so that the integrity of the separation achieved by the column is maintained.

Experimental Studies

Color index numbers, European codes, names and molecular formulas of analyzed synthetic colorants

Color Index (CI) Numbers	E Codes	Name of Colorants	Molecular Formula
19140	E 102	Tartrazine	$C_{16}H_9N_4Na_3O_9S_2$
15985	E 110	Sunset Yellow	$C_{16}H_{10}N_2Na_2O_7S_2$
73015	E 132	Indigo Carmine	$C_{16}H_8N_2Na_2O_8S_2$
45430	E 127	Erytrsosine	$C_{20}H_6I_4Na_2O_5$
16255	E 124	Ponceau 4R	$C_{20}H_{11}N_2Na_3O_{10}S_3$

Experimental Studies

Preparation of Standard and Sample Solutions

Tartrazine, sunset yellow, indigo carmine, erythrosine, ponceau4R

- Weighed as 100 mg and diluted to 100 mL
- Kept in ultrasonic bath for 30 min to achieve complete homogenization

Powder beverage samples (orange, lemon, rosehip) were purchased from local market

- Weighed as 100 mg and diluted to 50 mL
- Kept in ultrasonic bath for 30 min to achieve complete homogenization

The colorant standart and sample solutions were injected to the chromatographic system after filtering through 0.45μm disposable syringe filters. Spectrophotometric CUPRAC procedure was applied.

Experimental Studies

Spectrophotometric Assays of Total Antioxidant Capacity Applied to the Determination of Total Colorant Content

CUPRAC

1 mL Cu(II) + 1 mL Nc + 1 mL buffer solution (ammonium acetate solution)

+

x mL colorant solution

+

(1.1 - x) mL H_2O; total volume of 4.1 mL,

Let the mixture stand for 30 min at room temperature and

measure the absorbance at 450 nm

(Apak et al.2004,2005)

TCC (g/ 100 g)=

(Absorbance value / $\varepsilon_{ponceau\ 4R}$) × (total volume/ sample volume) × $Mw_{\ ponceau\ 4R}$ (g/mol) / amount of sample(g) × 100

+ Experimental Studies

Chromatografic Methods

Chromatographic Separation

✔ In order to achieve full resolution of all colorants, a variety of gradient elution programs were tested, using different mobile phases and changing retention times. But in all optimization experiments, the flow rate and injection volume were kept constant as 1 ml min^{-1} and 20 μL, respectively.

t (min)	A (%)	B (%)
0	100	0
2	100	0
20	45	55
30	0	100
32	0	100
33	100	0
35	100	0

Table 2. Optimized gradient elution program for the separation of colorants by HPLC-PDA
(A: 0.13 M ammonium acetate solution, B: HPLC grade methanol)

$$TCC_{HPLC} = \Sigma C_i PECC_i \times [\text{Total sample amount (L)} / \text{sample amount (g)}] \times 100$$

F. A. Olgun, B. Demirata, R. Apak,Food Anal. Methods,2012,5: 1335-1341

Experimental Studies
Chromatografic Methods

Chromatographic Conditions

- Reversed phase C18 column system
- Gradient elution program

(with mobile phase A: 0.13M ammonium acetate solution, B: methanol)

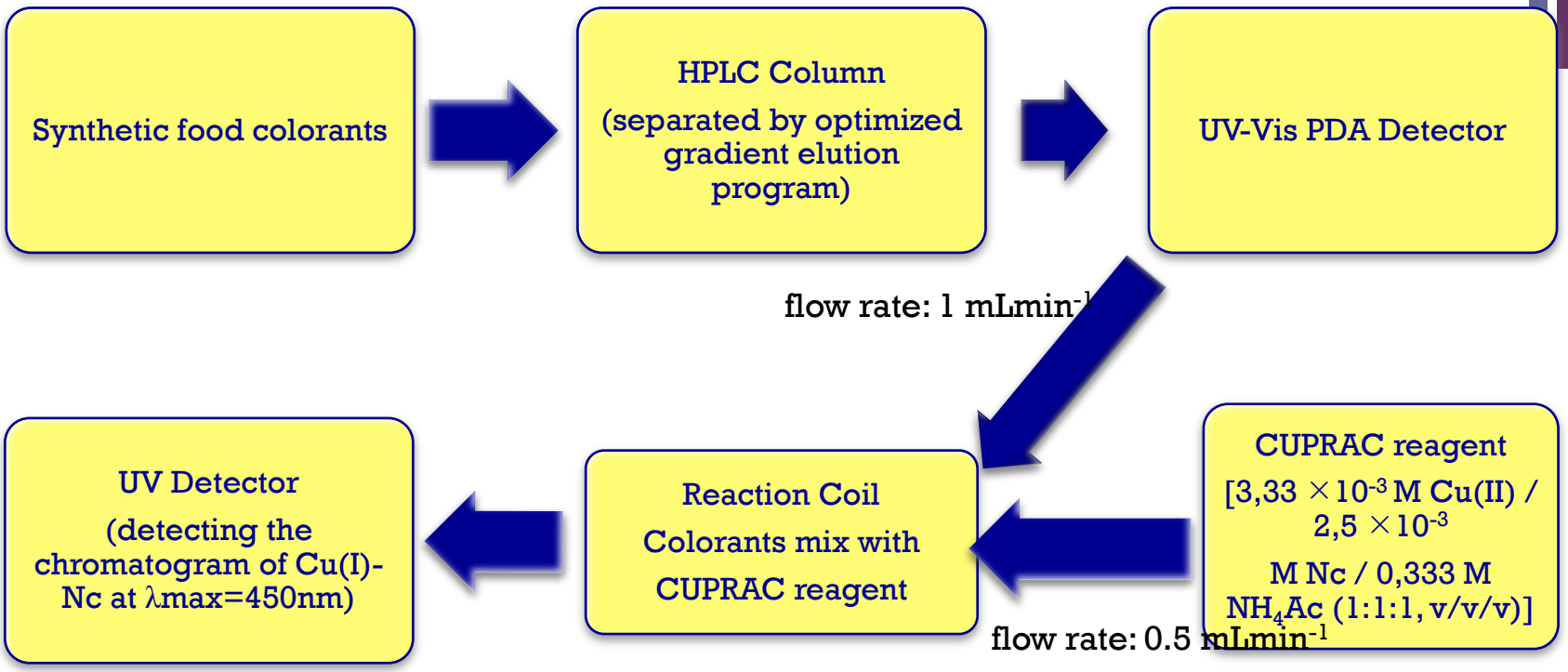
- Flow rate: 1mLmin^{-1}
- Injection volume: 20μL
- Detection: PDA detector monitoring each colorant at its own appropriate wavelength

(λ_{max} was chosen as 485 nm for mutual evaluation of colorants)

F. A. Olgun, B. Demirata, R. Apak,Food Anal. Methods,2012,5: 1335-1341

Experimental Studies

On-line HPLC CUPRAC Assay



On-line HPLC-CUPRAC method assayed by Celik et. al. (2010) was applied directly to synthetic food colorants separating with the related gradient elution program. Colorants were let to react with CUPRAC reagent in a time period of 1 minute.

$$TCC_{HPLC\text{-}CUPRAC} = (\Sigma yi / slope) \times Total\ volume\ (L) / sample\ amount\ (g)$$

S. E. Celik, M. Ozyurek, K. Guclu and R. Apak, Anal. Chim. Acta, 2010, 674, 79–88.

Results

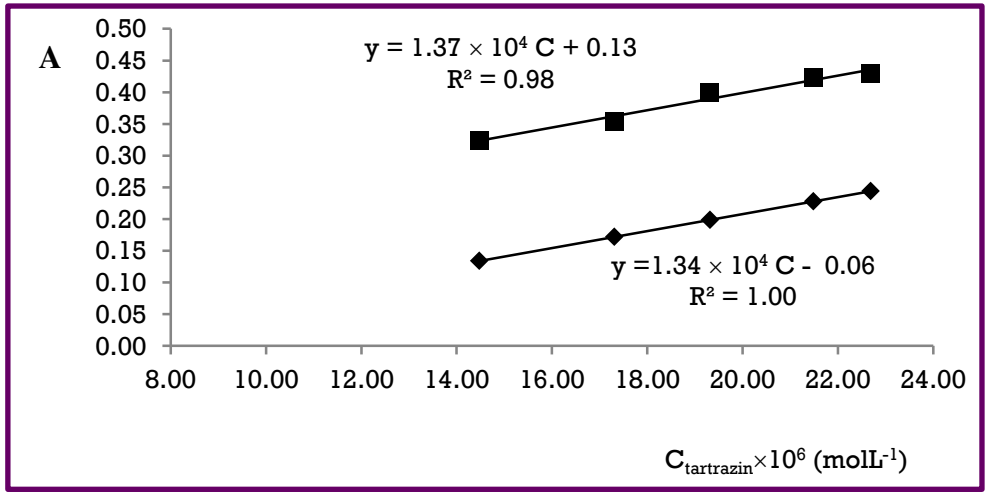
CUPRAC Assay of Total Antioxidant Capacity Applied to the Determination of Synthetic Food Colorants

The indirect molar absorptivities and linear concentration ranges of the tested colorants with respect to the CUPRAC method (N=5)

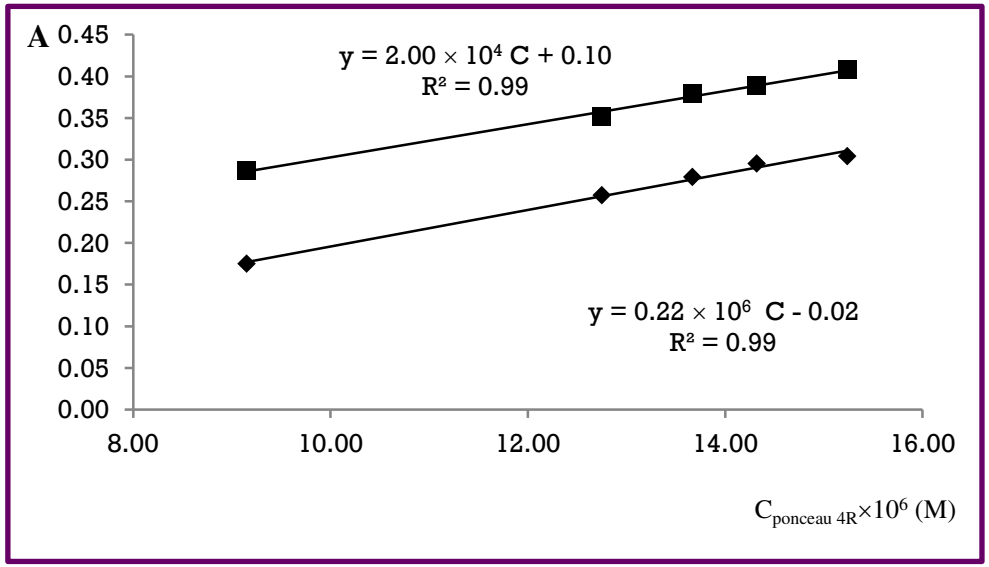
Name of colorants	ε ($L mol^{-1} cm^{-1}$)	Working ranges ($\times 10^{-5}$ M)	Calibration equations	LOD (M)	LOQ (M)	PECC
Ponceau 4R (E124)	2.24×10^4	0.48 – 3.90	$A = (2.24 \pm 0.36) \times 10^4 \times C_{PONCEAU\ 4R} - (0.0227 \pm 0.0073)$, $R = 0.9990$	0.11×10^{-6}	0.36×10^{-6}	1.00
Tartrazine (E102)	1.34×10^4	0.58 – 4.64	$A = (1.34 \pm 0.12) \times 10^4 \times C_{TARTRAZINE} - (0.0060 \pm 0.0003)$, $R = 0.9945$	1.48×10^{-6}	4.93×10^{-6}	0.60
Erythrosine (E127)	5.20×10^3	0.10 – 0.38	$A = (5.20 \pm 0.31) \times 10^3 \times C_{ERYTHROSINE} + (0.0087 \pm 0.0020)$, $R = 0.9996$	0.21×10^{-6}	0.70×10^{-6}	2.32
Sunset Yellow (E110)	1.49×10^4	0.56 – 4.74	$A = (1.49 \pm 0.06) \times 10^4 \times C_{SUNSETYELLOW} + (0.0151 \pm 0.0041)$, $R = 0.9996$	1.80×10^{-6}	6.00×10^{-6}	0.67
Indigo carmine (E132)	1.03×10^4	0.11 – 0.91	$A = (1.03 \pm 0.11) \times 10^4 \times C_{INDIGOCARMINEO} + (0.0829 \pm 0.0588)$, $R = 0.9912$	1.91×10^{-6}	6.38×10^{-6}	0.46

+ Results

CUPRAC Assay of Total Antioxidant Capacity Applied to the Determination of Synthetic Food Colorants



The interaction of orange powder beverage with tartrazine (◆:1mL 10^{-2}M $CuCl_2$ + 1mL 7.5×10^{-3}M Nc +1mL 1M NH_4Ac+ tartrazine; ■: 1mL 10^{-2}M $CuCl_2$ + 1mL 7.5×10^{-3}M Nc +1mL 1M NH_4Ac+ tartrazine + orange powder beverage)



The interaction of rosehip powder beverage with ponceau 4R (◆:1mL 10^{-2}M $CuCl_2$ + 1mL 7.5×10^{-3}M Nc +1mL 1M NH_4Ac+ ponceau 4R; ■: 1mL 10^{-2}M $CuCl_2$ + 1mL 7.5×10^{-3}M Nc +1mL 1M NH_4Ac+ ponceau 4R + rosehip powder beverage)

+ Results

CUPRAC Assay of Total Antioxidant Capacity Applied to the Determination of Synthetic Food Colorants

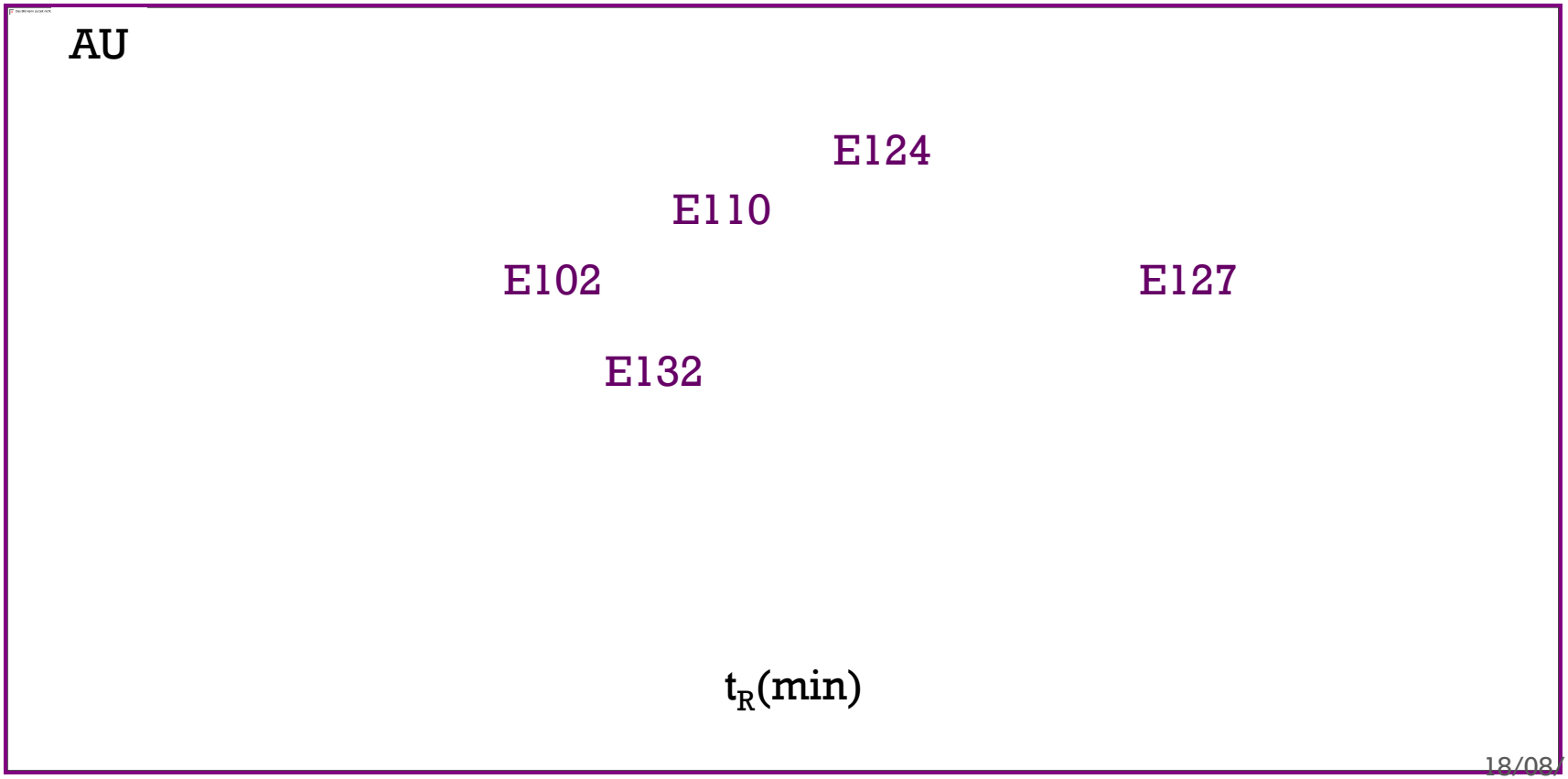
Relative Standard Deviation % and Recovery % of Synthetic Food Colorants added to powder beverage samples

Colorant addition to powder beverages	TCC (calculated with PECC coefficients) (M)	Concentration added (M)	Concentration expected (M)	Concentration found (M)	Recovery (%)
Tartrazine addition to orange powder beverage	6.82×10^{-6}	17.31×10^{-6}	24.13×10^{-6}	26.86×10^{-6}	111.0
		21.49×10^{-6}	28.31×10^{-6}	32.01×10^{-6}	113.0
Ponceau 4R addition to rosehip powder beverage	4.58×10^{-6}	12.76×10^{-6}	18.23×10^{-6}	16.72×10^{-6}	91.7
		14.32×10^{-6}	19.79×10^{-6}	18.38×10^{-6}	92.9

Results

Determination of Synthetic Food Colorants by HPLC-PDA

The chromatogram of standard colorant mixture solution (Colorant mixture consists of 1: E102, 2: E132, 3: E110, 4: E124, 5: E127, respectively. Flow rate: 1mLmin-1; λ_{max}: 485nm)



Results

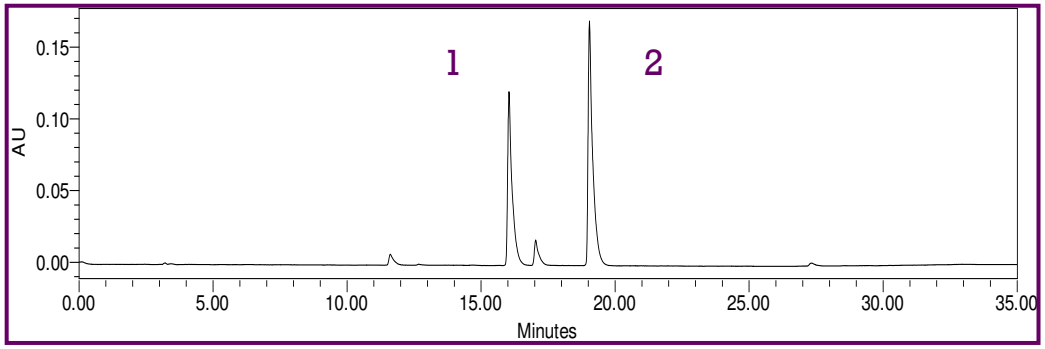
Determination of Synthetic Food Colorants by HPLC-PDA

Retention times (t_R(min)), linear ranges, calibration equations, regression coefficients, LOD and LOQ values of the tested colorants with respect to HPLC-PDA technique

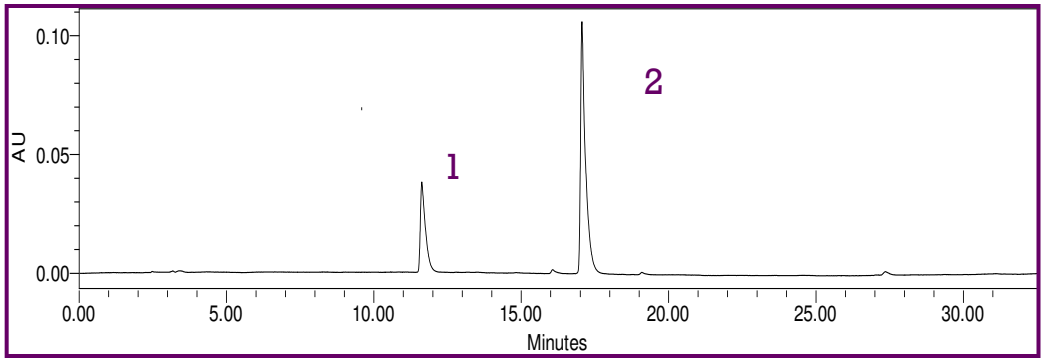
Name of colorants	λ_{max} (nm)	t_R(min)	Working ranges (M)	Calibration Equation A= mC+n (A: Peak Area)	R^2	LOD (M)	LOQ (M)
Ponceau 4R (E124)	508	17.43 ± 0.05	8.27×10^{-6} – 8.27×10^{-5}	A= (8.0 ± 0.69) × 10^{10} C + (1.07 ± 2.50) × 10^{5}	0.9935	7.56×10^{-6}	25.21×10^{-6}
Tartrazine (E102)	427	14.58 ± 0.03	9.20×10^{-6}- 9.20×10^{-5}	A= (2.06 ± 0.16) × 10^{11} C + (1.11 ± 128.5) × 10^{5}	0.9997	2.02×10^{-6}	6.74×10^{-6}
Erythrosine (E127)	528	27.39 ± 0.03	5.68×10^{-6} – 5.68×10^{-5}	A= (3.39 ± 0.05) × 10^{11} C - (1.06 ± 1.17) ×10^{5}	0.9998	8.64×10^{-7}	28.89×10^{-7}
Sunset Yellow (E110)	482	16.41 ± 0.04	1.11×10^{-5} – 1.11×10^{-3}	A= (6.60 ± 0.61) × 10^{10} C + (1.25 ± 2.94) × 10^{5}	0.9995	1.06×10^{-6}	3.55×10^{-6}
Indigo carmine (E132)	608	13.53 ± 0.03	1.91×10^{-6} – 1.91×10^{-5}	A= (2.12 ± 0.18) × 10^{10} C + (7.02 ± 17.02) × 10^{3}	0.9994	1.47×10^{-6}	4.87×10^{-6}

Results

Determination of Synthetic Food Colorants by HPLC-PDA



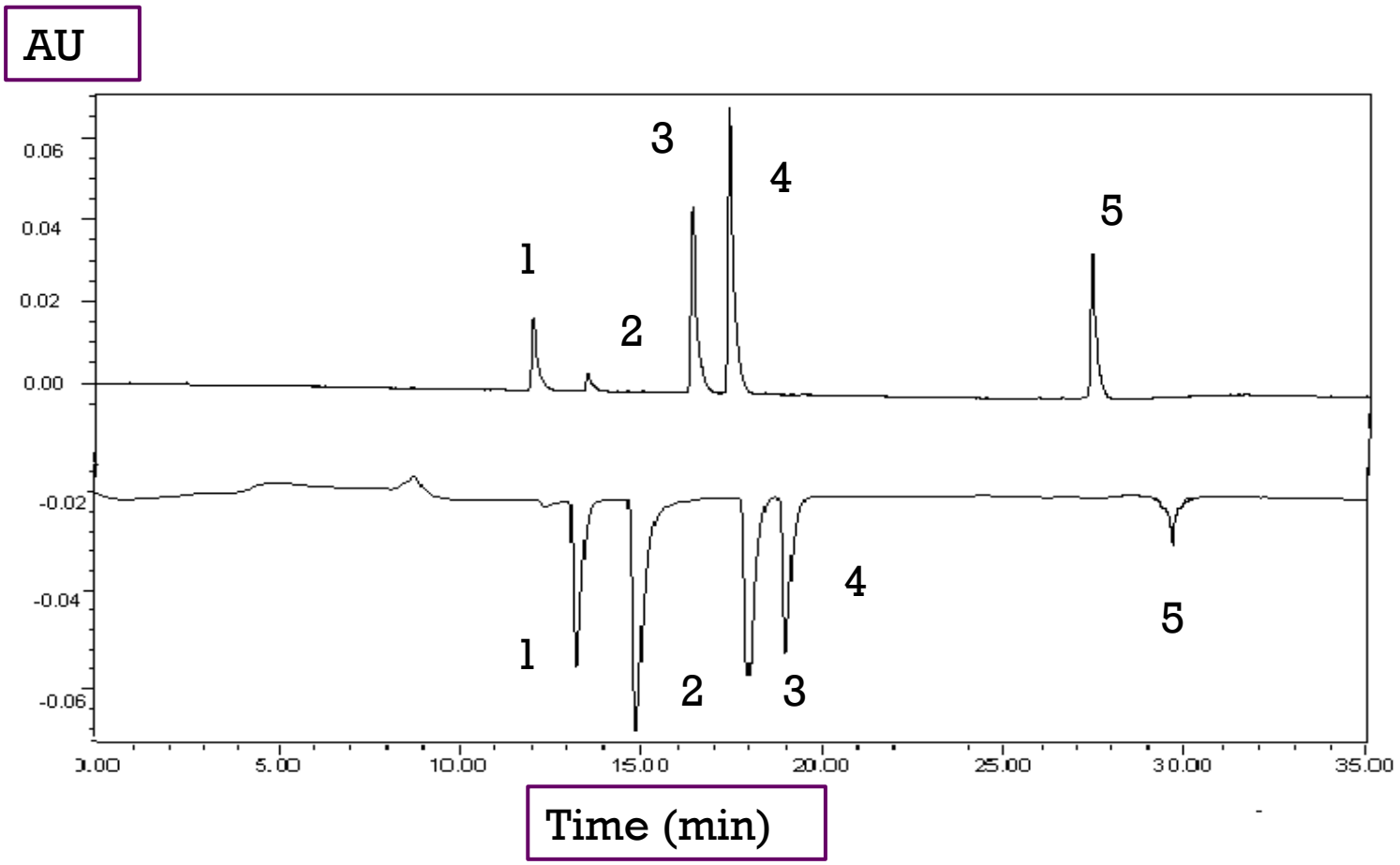
The chromatogram of rosehip powder beverage monitored at λmax: 485nm (1: sunset yellow, 2: ponceau4R)



The chromatogram of orange powder beverage monitored at λmax: 485nm (1: tartrazine, 2: ponceau4R)

Results

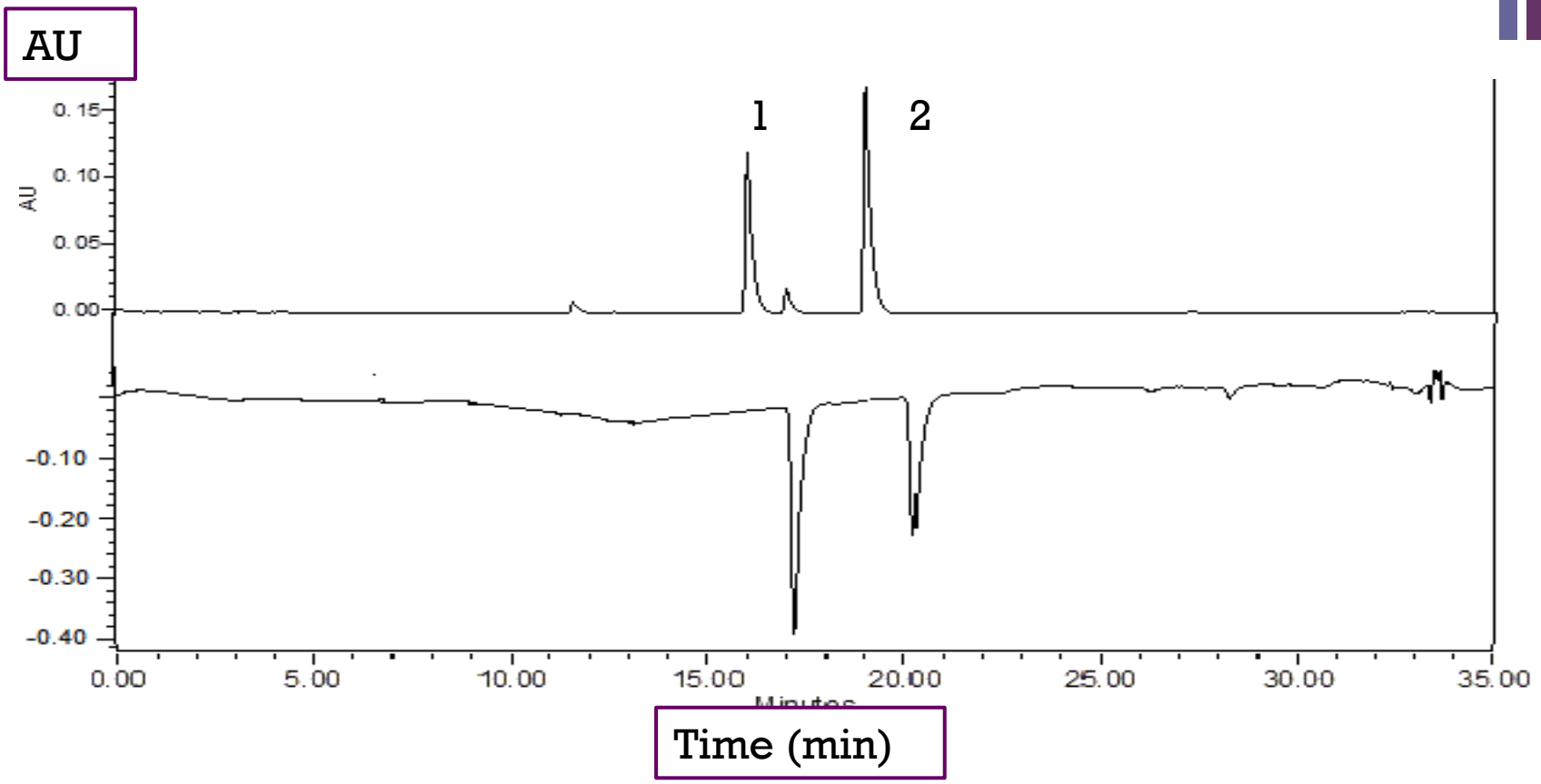
Determination of Synthetic Food Colorants by on-line HPLC-CUPRAC Method



The chromatogram of synthetic mixture of synthetic food colorants mixture solution (consists of 1: E102, 2: E132, 3: E110, 4: E124, 5: E127), at 485 nm and 450 nm respectively.

Results

Determination of Synthetic Food Colorants by on-line HPLC-CUPRAC Method



The chromatogram of rosehip powder beverage sample solution monitored at 485 nm and 450 nm, respectively. (Peak 1: sunset yellow; Peak 2: ponceau 4R)

Results

Determination of Synthetic Food Colorants by on-line HPLC-CUPRAC Method

Retention times (t_R(min)), linear ranges, calibration equations, regression coefficients, LOD and LOQ values of the tested colorants with respect to on-line HPLC-CUPRAC method

Name of colorant	t_R (min)	Calibration equation A=mC(M)+n	R^2	working range (× 10^{-6} M)	LOD (M)	LOQ (M)
Tartrazine (E102)	12.81 ± 0.15	A= (7,27 ± 1,31) × 10^{11} C- (1,51 ± 2,49) × 10^5	0.9937	0.46 – 3.71	4.17 × 10^{-7}	13.90 × 10^{-5}
Sunset Yellow (E110)	17.17 ± 0.08	A= (6.88 ± 0.85) × 10^{10} C – (9.92 ± 18.88) × 10^4	0.9959	5.70 – 41,20	3.72 × 10^{-6}	12.40 × 10^{-6}
Erythrosine (E127)	27.39 ± 0.03	A= (1.25 ± 0.27) × 10^{11} C – (1.14 ± 2.73) × 10^4	0.9990	0.26 – 2.10	0.27 × 10^{-7}	0.90 × 10^{-7}
Indigo Carmine (E132)	14.34 ± 0.03	A= (7.71 ± 2.89) × 10^{10} C – (4.73 ± 10.68) × 10^5	0.9953	8.95 – 71.60	1.39 × 10^{-6}	2.33 × 10^{-6}
Ponceau 4R (E124)	17.43 ± 0.05	A= (8.19 ± 2.08) × 10^{10} C – (3.97 ± 4.84) × 10^5	0.9978	5.65 – 45.20	3.00 × 10^{-6}	10.00 × 10^{-6}

Results

Determination of Synthetic Food Colorants by on-line HPLC-CUPRAC Method

Relative Standard Deviation and Recovery % of Synthetic Food Colorants added to powder beverage samples with respect to on-line HPLC-CUPRAC Method

Colorant addition to powder beverage samplse	Colorant content (M)	Added concentration (M)	Expected concentration (M)	Found Concentration (M)	Recovery (%)
Tartrazine addition to orange powder beverage	Tartrazine in orange powder beverage (M) 1.02×10^{-6}	0.93×10^{-6}	1.95×10^{-6}	2.24×10^{-6}	114.8
		1.85×10^{-6}	2.87×10^{-6}	3.00×10^{-6}	104.5
Ponceau 4R addition to rosehip powder beverage	Ponceau 4R in rosehip powder beverage (M) 9.77×10^{-6}	1.13×10^{-6}	10.90×10^{-6}	10.12×10^{-6}	92.80
		2.26×10^{-6}	12.03×10^{-6}	10.73×10^{-6}	90.12

Results

Comparison of retention times (tR), limit of detection values (LOD) and limit of qualification values (LOQ) of tested colorants, with respect to HPLC-PDA technique and on-line HPLC-CUPRAC method

Name of Colorant	t_R (HPLC-PDA)	t_R (on-line HPLC-CUPRAC)	LOD (HPLC-PDA; 485 nm)	LOD (on-line HPLC-CUPRAC, 485nm)
Ponceau 4R	17.43±0.05	18.16±0.03	7.56×10^{-6}	2.80×10^{-6}
Tartrazine	12.03±0.08	12.81±0.15	2.02×10^{-6}	4.17×10^{-7}
Erythrosine	27.39±0.03	28.27±0.06	8.64×10^{-7}	2.70×10^{-8}
Sunset Yellow	16.41±0.04	17.17±0.08	1.06×10^{-6}	3.72×10^{-7}
Indigo Carmine	13.53±0.03	14.34±0.11	1.42×10^{-6}	1.39×10^{-6}

The Analyses of Powder Beverage Samples

Colorant contents of powder beverage samples found by HPLC and on-line HPLC-CUPRAC assays (N=5)

Name of colorant	Orange powder beverage (g/100g powder beverage)		Rosehip powder beverage (g/100g powder beverage)	
	HPLC-PDA (485 nm)	On-line HPLC-CUPRAC (450 nm)	HPLC-PDA (485 nm)	On-line HPLC-CUPRAC (450 nm)
Tartrazine (E102)	0,61±0,02	0,45±0,03	0,29±0,03	0,28±0,04
Sunset Yellow (E110)				
Erythrosine (E127)	0,07±0,01	0,04±0,01	-	-
Indigo Carmine (E132)	-	-	-	-
Ponceau 4R (E124)	-	-	0,24±0,02	0,22±0,01

The Analyses of Powder Beverage Samples

Total colorant contents of powder beverage samples found by HPLC (with CUPRAC calculations) and on-line HPLC-CUPRAC assays (N=5)

Name of sample	CUPRAC g Ponceau 4R/ 100 g	HPLC (CUPRAC calculations) g Ponceau 4R/ 100 g	On-line HPLC-CUPRAC g Ponceau 4R/ 100 g
Orange powder beverage	0.85±0.01	0.65±0.01	0.76±0.02
Rosehip powder beverage	0.68±0.02	0.51±0.01	0.49±0.01
Statistical Analysis with two-way ANOVA Test	**CUPRAC, HPLC (CUPRAC calculated) and on-line HPLC-CUPRAC**	**P= 0.05; $F_{experimental}$ = 8.04; $F_{critical}$= 18.51; $F_{experimental} < F_{critical}$**	

Conclusions

- In this study, determination of five synthetic food colorants was investigated using spectrophotometric and chromatographic methods.

- By adapting the novel spectrophotometric CUPRAC assay of total antioxidant capacity to the determination of total food colorant content, certain beverage samples were easily and accurately analyzed. The total colorant content was found at low reagent and instrumentation costs with the use of a UV–vis spectrophotometer easily found in a conventional laboratory equipped with simple instruments.

Conclusions

- HPLC analysis of colorants was performed with two different techniques.

1) In conventional HPLC method, PDA detector system was used to monitor each colorant at its maximum absorbance wavelength. The selected mobile phase for the gradient elution program enabled the shortening of total analysis time when compared to other chromatographic methods. Furthermore, since acetonitrile as the conventional solvent was not used in the eluent, solution costs were minimized.

2) On-line HPLC-CUPRAC was adapted for the determination of synthetic food colorants. Optimized gradient elution program was used for the separation of colorants. Retention time periods were increased due to additional installation for derivatization coil. However, LOD values were decreased with on-line HPLC-CUPRAC method.

Conclusions

- Two-way ANOVA test results were calculated for CUPRAC, HPLC (with CUPRAC calculations) ve on-line HPLC-CUPRAC ($P= 0,05$; $F_{experimental} = 0,26$; $F_{critical} = 18,51$; $F_{experimental} < F_{critical}$) with 95% confidence levels.


THANK YOU...