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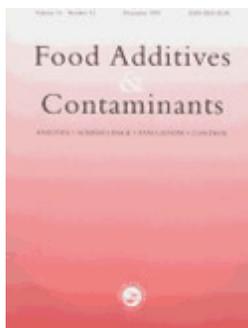
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Consideration of fluorescence properties for the direct determination of erythrosine in saffron in the presence of other synthetic dyes

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Direct selective detection of erythrosine in saffron in the presence of other synthetic dyes considers its fluorescence at 532 nm excitation / 548 nm emission. Saffron pre-treatment was according to the ISO 3632-2 trade standard test methods. On account of calculated quantum yield values none of the yellow dyes is expected to interfere. Among red ones, reservations about allura red AC, azorubine and red 2G were not verified by experimentation signifying excellent method specificity. Detection and quantification limits (0.56 and 1.70 nM) were of the same magnitude to those reported in the literature after chromatographic separation of erythrosine. The percentage recovery from spiked saffron samples ranging from 63 - 141 was acceptable for residue levels in foods. Matrix effect from crocins (saffron pigments) was evidenced only at a lower spiking level (0.02 mg kg⁻¹). The minimum required performance limit (MRPL) value was 0.04 mg kg⁻¹ indicating that the method is appropriate for determining traces of erythrosine in saffron. Our approach offers improved sensitivity (by three orders of magnitude) and specificity than the direct spectrophotometric detection of certain synthetic dyes in saffron and deserves attention by the ISO Technical Committee for "Herbs, culinary spices and condiments"

Keywords *saffron; erythrosine, fluorimetry; derivative spectrometry; food colour analysis*

Introduction

Since 2003, in international transactions, certification of saffron authentication according to the ISO 3632-1, 2 trade standards includes verification of absence of synthetic water-soluble acid dyes. The recommended analytical procedure in the version issued in 2003 proposed retention of these potential adulterants on polyamide columns or solid phase extraction (SPE) cartridges after pH adjustment of aqueous solutions of the test samples at ~2.5; removal of endogenous pigments, i.e. crocetin glycosides (crocins) by successive washing with water, methanol, acetone, and once more with methanol before elution of the bound synthetic dyes with a mixture of methanol: ammonia solution. After solvent evaporation the dyes, re-dissolved in methanol, were separated by different chromatographic protocols. TLC systems were proposed as screening procedures for all of the tested dyes. Ion-pair RP-HPLC protocols were then used as confirmatory procedures. The latter proved to be effective for azo-dyes but not for erythrosine (2',4',5',7'-tetraiodo-3',6'-dihydroxy-spiro[3H-isobenzofuran-1,9'-xanthen]-3-one disodium salt, E127, FD&C Red No 3, Food Red 14) due to the weak acidic character of the latter (Kiseleva et al., 2003). As a result the recovery of erythrosine was lower than that reported for the other dyes. The recently issued revised ISO 3632-2 version (2010) does not address the above mentioned analytical limitations regarding erythrosine. On the contrary, this dye is excluded from the list of synthetic dyes that can be detected using the methods therein, not because such an adulteration is of less importance but because the recommended protocols cannot cover its determination sufficiently.

To our knowledge, publications on the detection of synthetic dyes in saffron are rather limited. Lozano et al. (1999) tested tartrazine, ponceau S and methyl orange after addition to saffron extracts at a rather high level (2 % w/w). The synthetic dyes were separated from crocins on a RP-HPLC column (C₁₈) by gradient elution with methanol-water (20 - 70 % in 50 min). Subsequently Zougagh et al. (2005) used an automated extraction system to pre-concentrate tartrazine, ponceau 4R and methyl orange on a cotton column from which the tested dyes were then eluted with a dilute ammonia solution. Identification at selected wavelengths was by means of an on-line photometer whereas quantification was achieved using partial least squares regression analysis. Another screening procedure was reported at that time for the detection of eleven synthetic dyes in saffron. It involved removal of crocins by acid hydrolysis

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4 prior to pre-concentration of the dyes on polyamide cartridges and direct detection by
5 means of derivative UV-Vis spectrometry (Zalacain et al., 2005). Under the sample
6 pre-treatment conditions (pH 0.1, 90 °C) all of the tested compounds including those
7 referred in the relative ISO specification plus allura red AC, naphthol yellow and red
8 2G were stable except for erythrosine. Our experimentation with the ion-pair RP-
9 HPLC protocols described in ISO 3632-2 (2003) using a fluorimetric detector instead
10 of DAD did not improve quantification limit as the acid ion-pair environment
11 quenched its fluorescence severely (Ordoudi et al., 2010).
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17 The analytical limitations described above along with legislative restrictions
18 on the uses of erythrosine because of its low ADI value (0-0.1 mg/kg body
19 weight/day) motivated us to focus on the direct selective detection of this additive in
20 mixture with yellow or red synthetic dyes utilizing its fluorescence properties. The
21 results were compared with those obtained by using derivative UV-Vis spectrometry
22 as the identification means in conjunction to the same sample preparation process.
23 Our effort deserves attention by the ISO Technical Committee for “Herbs, culinary
24 spices and condiments” responsible for ISO 3632-1 and 2 revisions.
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33 **Materials and methods**

34 *Reagents and materials*

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37 Authentic saffron samples were donated by Saffron Cooperative of Kozani
38 (Greece). Amaranth (80%, CI 16185), allura red AC (80 %, CI 16035), erythrosine
39 (ER) (90%, CI 45430:2), naphthol yellow (99%, CI 10315), orange II (85%, CI
40 15510), ponceau 4R (75%, CI 16255), quinoline yellow (95%, CI 47005), sunset
41 yellow FCF (90%, CI 15985) and tartrazine (85%, CI 19140) were supplied by
42 Sigma-Aldrich Chemie (Steinheim, Germany). Azorubine (85%, CI 14720) and red
43 2G (60%, CI 18050) were from Aldrich (Steinheim, Germany). Rocelline (99 %, CI
44 15620) was from Acros Organics (USA). All chemicals were of analytical reagent
45 grade. Polyamide SPE cartridges (Chromabond® 200-PA, 40–80 mm, 3 mL/200 mg)
46 were from Macherey-Nagel (Düren, Germany). PTFE filters (11 mm, 0.45 mm) were
47 from Millipore (Bedford, MA, USA).
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Sample preparation

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Stock and working dye solutions were prepared in methanol according to the ISO 3632-2 test method for water-soluble acid synthetic dyes (2003; 2010). Details for the preparation of spiked samples are given below: saffron powder (0.5 g) was weighed into a glass centrifuge tube to which water (10 mL, 60 °C) was added. The mixture was vortexed (1 min), kept in the dark for 10-12 min, vortexed again (1 min) and finally centrifuged (4080 × g, 10 min). The supernatant was spiked with the synthetic dye (10, 25, 62.5, 87.5, 112.5 µL from 1 µM solution) and the mixture was homogenized. The pH of the mixture was adjusted to ~2.5 by addition of glacial acetic acid (2 mL). The extract was transferred onto a polyamide SPE cartridge preconditioned with 1 mL of water. The cartridge was successively washed with water (10 x 3 mL), methanol (10 x 3 mL), acetone (10 x 3 mL) and finally methanol¹ (10 x 3 mL) under vacuum (15 mm Hg) and the eluates were discarded. The bound colourants were then eluted using a mixture of methanol: ammonia solution, 95:5, v/v (1 x 5 mL) in a heart-shaped flask and the solvent was evaporated at room temperature under vacuum. The residue was re-dissolved in 0.5 mL methanol and vortexed for 1 min. The extraction and purification procedure was carried out in triplicate.

Fluorimetric examination

An aliquot of the methanol extract was transferred into a volumetric flask and the volume was made up with methanol (dilution factor = 10 or 100). Fluorescence excitation (exc) maximum was found to be at 532 nm. The corresponding emission (em) maximum was then recorded at 548 nm on a Shimadzu RF-1501 spectrofluorimeter (Kyoto, Japan) using the PC-1501 software facilities of the instrument. Scan speed was 2775 nm min⁻¹ and instrument response time was automatically set. The system was thermostated at 25 °C *via* an outer water-circulating bath. Spectra were corrected for background as described by Papoti and Tsimidou (2009). For each sample, measurements were made in triplicate.

Method validation

Intra-laboratory validation of the fluorimetric method was performed according to the Decision 2002/657/EC. Parameters such as method linearity, limit of

¹ The use of acidic methanol (recommended in the 2010 version of ISO 3632-2) was avoided as it affects erythrosine stability

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detection (LOD, calculated as $3.3 s/b$, where s , the standard deviation of the intercept value and b , the slope of the calibration graph), limit of quantification (LOQ, calculated as $10s/b$), intra and inter day precision, specificity and minimum required performance limit (MRPL)² were examined. These parameters were checked using standard solutions of ER or mixtures with other synthetic dyes. Recovery studies were performed using saffron samples spiked with five levels of ER. For each level, triplicate measurements were obtained.

Spectrophotometric examination

An aliquot of the methanol extract (0.1 mL) was placed in a quartz micro cell (1x 1x 0.1 cm) from Starna Brand (Essex, Germany) and visible spectra were then recorded on a Shimadzu UV 1601 spectrophotometer equipped with the UV-1601PC software (v. 3.9, Shimadzu). The system was thermostated at 25 °C via an outer water-circulating bath. Second order spectra derivatives were calculated according to the procedure described by Zalacain et al. (2005). For each sample, measurements were made in triplicate.

Statistical analysis

Statistical comparisons of the mean values were performed by one-way analysis of variance (ANOVA), followed by the multiple Duncan test ($p < 0.05$ confidence level) using the SPSS 14.0 software (SPSS Inc., Chicago, IL).

Results and Discussion

Background work

The spectral characteristics of ER as well as those of the other water soluble acid dyes referred in the relevant ISO methodology were tested in methanol so that through calculation of molar absorption coefficients (ϵ) at λ_{\max} of ER (Fery-Forgues and Lavabre 1999), to estimate the respective quantum yield (Φ_{F532}) values not available in literature. Absorption maxima, molar absorption coefficients, excitation and emission maxima as well as quantum yields of the tested compounds at 532 nm in methanol are shown in Table 1. The first interesting extrapolation from these data

² *Minimum Required Performance Limit: the minimum content of an analyte in a sample, which at least has to be detected and confirmed.*

concerned the visible properties of all of the analytes. Azorubine, amaranth, ponceau 4R, red 2G, allura red AC and rocelline, with λ_{max} greater than 500 nm, exhibited ϵ_{532} values that were four to six-fold lower than that of ER. As a consequence, to achieve a certain absorption value at 532 nm the required amount of extractable ER is expected to be many folds lower than that of any other tested dye. Concerning the estimated Φ_{F532} value of ER in methanol, it was in good agreement with that reported in strongly alkaline phosphate buffer (Ryvolova et al. 2007). The corresponding quantum yield values of yellow dyes (tartrazine, quinoline yellow, sunset yellow FCF, naphthol yellow and orange II) that absorb light in the region below 500 nm were found negligible. None of these compounds is expected to interfere with fluorescence detection at 532 nm (ex) due to the lack of a fluorescein moiety in their structure. The Φ_{F532} values of azorubine, amaranth, ponceau 4R, red 2G, allura red AC and rocelline were three to four orders of magnitude lower than that of ER pointing out a rather weak fluorescence at 532 nm. Based on the quantum yield values of Table 1, allura red AC, azorubine and red 2G could be considered as the only potential interferences so that these dyes were used for further experimentation toward the selective detection of ER in saffron.

Method development and validation procedure

Linearity and sensitivity. The linearity of fluorescence response for ER was tested in the range 1 to 22.5 nM. The regression equation was $y = a + bx$ ($R^2 = 0.993$), where $a = 861.4 \pm 171.0$ and $b = 1008.7 \pm 12.6$. These results demonstrate the linearity of the fluorescence response in the assessed range. LOD was found to be 0.56 nM and LOQ was 1.70 nM. It should be noted that our data are of the same order of magnitude with the respective ones found for this dye by other researchers using other techniques (e.g. Miniotti et al. 2006; Ryvolova et al. 2007; Rongjie 2008).

Precision. Measurement precision was satisfactory for the determination of ER at nM levels with regard to performance criteria for quantitative methods adopted in the Decision 2002/657/EC. Intra-day precision was checked by the same analyst on three random days. Obtained percent coefficient of variation (% CV) values of pooled data for 7.5, 12.5, and 22.5 nM of the analyte were 8.4%, 4.8% and 3.1%, respectively ($n = 9$). The inter-day precision was also examined at different levels; % CV values were below 8.4 % showing that the procedure is reproducible.

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5 *Specificity.* Taking into account the results of our background work, specificity of the
6 fluorimetric method was assessed for binary mixtures of ER with allura red AC,
7 azorubine or red 2G at different molar ratios (1:50, 1:10, 1:5, 1:1, mol/mol).
8 Fluorescence response of allura red AC, azorubine and red 2G at all working
9 concentrations (12.5 - 625 nM) was found to be close to instrumental noise (data not
10 shown). Our findings did not verify dependence on concentration that could be
11 attributed to the low range of concentrations used. Such dependence was observed by
12 Ryvolova et al. (2007) for azorubine and red 2G at much higher concentrations ($10 \times$
13 10^4 - 120×10^4 nM). The present data imply that fluorescence response of ER is not
14 expected to be influenced by the presence of allura red AC, azorubine or red 2G as
15 suggested in the background work.
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Indeed, calculated values for erythrosine content in 1:1 binary mixtures (Table
2) showed acceptable deviations, expressed as percentage (positive or negative)
within the range 5-22.5 nM. A possible interference due to the co-presence of red 2G
was observed only at the lowest tested concentration (5 nM). The presence of the
second analyte did not affect significantly quantification of ER within the 95 %
confidence limits of the regression equation in line with suggestions for specificity
recommended in the Decision 2002/657/EC.

Accuracy and matrix effect. Recovery studies were then conducted for standard ER
solutions as well as for spiked saffron samples to evaluate matrix effect and overall
accuracy of the fluorimetric method. Analyses were carried out on two different days
by two different analysts for one spiking level corresponding to the mid of the
fluorescence regression line (12.5 nM standard solution or 1 mg kg^{-1} saffron). These
data are shown in Table 3. The recovery of ER from standard solutions was 61 – 73.5
% in accordance with that reported by Kirschbaum et al. (2006) for the recovery rates
of standard ER solution (0.28 mM) retained on various types of polyamide
adsorbents. These authors had noticed that the performance of the adsorbent may vary
depending on the manufacturing process or/and lot-to-lot variability of the polymeric
material. In our study, for the same lot of polyamide and one spiking level, the matrix
effect to both mean recovery values and measurement precision was obvious. To
further examine the effect of endogenous saffron pigments on the determination of

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3 ER, recovery studies were performed before and after spiking a saffron sample with
4 very low levels of the dye (*ca.* 0.02 – 0.2 mg kg⁻¹ saffron). The calculated values are
5 presented in Table 4. Recovery rates from spiked saffron samples ranged from 62.9 ±
6 6.6 % (for 0.2 mg kg⁻¹ saffron) to 141.0 ± 26.7 % (for 0.04 mg kg⁻¹ saffron). These
7 values are close to the minimum acceptable ones according to the Codex Alimentarius
8 requirements for residue recovery in foods (80-110 %). Analysis of samples
9 containing ER at 0.02 mg kg⁻¹ resulted in overestimation of recovered amounts (227.8
10 ± 15.6 %). Based on all abovementioned results the MRPL value for ER was 0.04 mg
11 kg⁻¹. Further efforts to detect the dye at lower levels are out of the aim of the study,
12 which is the control of saffron adulteration with this particular dye.
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25 ***Comparison with other direct detection protocols.***

26 An inherent advantage of our proposal with regard to the screening method by
27 Zalacain et al. (2005) is the avoidance of harsh sample pretreatment conditions, which
28 eliminate crocins but also decompose erythrosine. Replacement of UV-Vis
29 spectrometry by fluorimetry also proved to be advantageous as sensitivity, specificity
30 and accuracy of the former were inferior. Indeed, after spectrophotometric
31 examination of standard ER solutions and linear regression analysis [a five-point
32 regression line (2.5 to 12.5 µM) was described by the equation $y = a + bx$ ($R^2 =$
33 0.999), where $a = 0.043 \pm 0.005$ and $b = 0.085 \pm 0.001$], the LOD and LOQ values
34 were found to be 0.19 µM and 0.57 µM, respectively. Upon findings sensitivity of the
35 spectrophotometric determination was 3 x 10³ – fold lower than that evidenced by
36 means of fluorimetry. Additionally, evaluation of calculated second derivative spectra
37 data of both ER and its binary mixtures (1:50, 1:10, 1:5, 1:1, mol/mol) with allura red
38 AC, azorubine or red 2G (C=12.5 µM) did not assist specificity. In particular, the
39 characteristic wavelengths corresponding to minimum and maximum Y-ordinate
40 values in the derivative spectrum of each binary mixture (Figure 1) were found almost
41 identical to those of standard ER. Only when the latter was present at a much lower
42 level than that of the second analyte (e.g. 1:50, mol/mol) a shift in λ_{\min} was observed.
43 The accuracy of the spectrophotometric method examined at one level of addition (1
44 mg kg⁻¹) was 98.9 ± 19.2 % for the standard solution and 57.2 ± 25.8 % for the spiked
45 sample. The corresponding % CV values indicated poor repeatability (% CV = 19.4
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3 and 45.1, respectively), worse than those recorded for fluorescence (see Table 3).
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5 Matrix effect due to visible spectra overlapping was pronounced.
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8 9 **Conclusion**

10 Summarizing current and previous findings it can be suggested that ‘complete
11 absence’ of ER in saffron can be certified by the direct fluorimetric approach. The
12 proposed procedure offers high selectivity and sensitivity (MRPL=0.04 mg kg⁻¹)
13 indicating that it is fit for the determination of traces of erythrosine in this precious
14 spice in the presence of other synthetic dyes. Our method deserves attention by the
15 ISO Technical Committee for “Herbs, culinary spices and condiments”
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25 **Acknowledgement:**

26 Mrs. K. Tsiogka, undergraduate student, is acknowledged for her participation in the
27 recovery study. We also thank the unknown reviewers for useful remarks.
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Figure Captions

Figure 1. Second derivative visible spectra of ER solutions and the corresponding binary mixtures (1:50, 1:10, 1:5, 1:1, mol/mol) with (a) allura red AC, (b) azorubine or (c) red 2G (C=12.5 μ M).

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Table 1. Absorption maxima, molar absorption coefficients, excitation and emission maxima and quantum yields of the tested dyes in methanol at 532 nm.

E value	Dye	λ_{\max}^a (nm)	$\epsilon_{\lambda_{\max}}$ (10^4 $L mol^{-1}cm^{-1}$)	ϵ_{532} (10^4 $L mol^{-1}cm^{-1}$)	λ_{exc} (nm)	λ_{em} (nm)	Φ_{F532}^b ($\times 10^4$)
E102	Tartrazine	426	1.73	0.00			-
E104	Quinoline Yellow	426	1.88	0.00			-
E110	Sunset Yellow FCF	480	1.60	0.00			-
E122	Azorubine	516	1.85	1.65			2.63
E123	Amaranth	520	2.12	2.04			0.84
E124	Ponceau 4R	510	1.85	1.61			0.47
E127	Erythrosine	532	9.06	9.06	532	548	1058.90
E128	Red 2G	534	2.02	1.99			1.87
E129	Allura red	506	2.18	1.96	474	551	2.91
-	Naphthol Yellow	428	1.47	0.35×10^{-4}			-
-	Orange II	480	1.78	0.44×10^{-4}			-
-	Rocelline	509	1.47	1.27	329	408	0.32

^a Methanol solutions (4×10^{-5} M, 25 °C); ^b F_{532} : area under curve at 532 nm excitation/548 nm emission; Rhodamine 6G was used as standard.

Table 2. Percent deviation of erythrosine content* for 1:1 (mol/mol) binary mixtures with allura red, azorubine or red 2G

Concentration (nmol L ⁻¹)	% Deviation (positive or negative)		
	Mixture with		
	allura red AC	azorubine	red 2G
5.0	+12.7	- 3.2	+46.4
7.5	+ 6.2	- 6.1	+16.4
12.5	- 1.4	- 8.7	- 3.2
17.5	+ 9.7	-13.4	-11.5
22.5	- 8.2	+ 6.7	- 5.6

*from the calibration curve based on calculation of area under curve at 532 nm excitation/548 nm emission (y=861.4 + 1008.7x, R²=0.993)

Table 3. Accuracy of the fluorimetric method in the mid of the fluorescence regression line (12.5 nM standard solution or 1 mg kg⁻¹ saffron)^a.

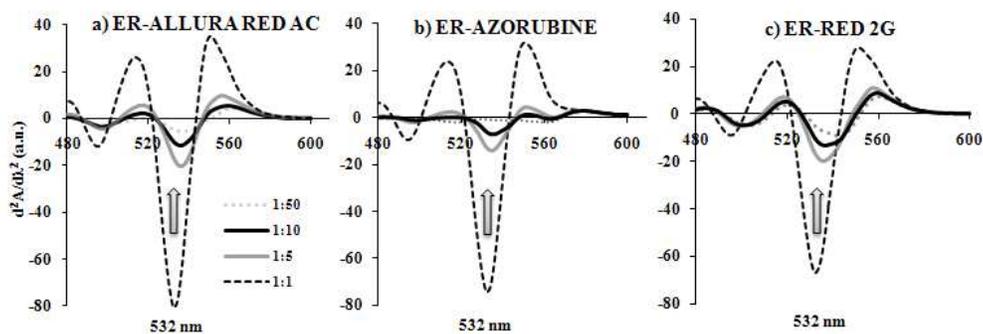
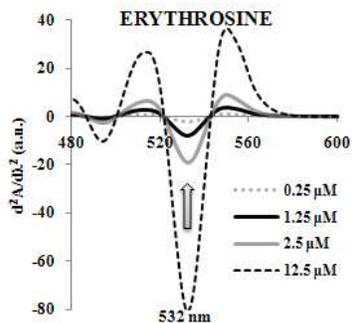
	% Recovery of ER	
	Standard solution	Spiked saffron sample
Day 1 Analyst 1 ^b	67.6 ± 5.9 (CV= 8.8%)	78.6 ±14.3 (CV=18.2%)
Day 2 Analyst 2 ^c	65.4 ± 3.8 (CV= 5.7%)	62.9 ±10.4 (CV=16.6%)

^afrom the calibration curve based on calculation of area under curve at 532 nm excitation/548 nm emission; ^b $y = 861.4 + 1008.7x$, $R^2=0.993$; ^c $y = 1453.3 + 846.0x$, $R^2=0.990$; Mean value ± standard deviation ($n = 3$)

Table 4. Recovery rates of ER at very low spiking levels*

Spiking level (mg kg ⁻¹)	% Recovery of ER
0.02	227.8 ± 15.6 (CV= 6.9%)
0.04	141.0 ± 26.7 (CV= 18.9%)
0.11	71.7 ± 11.3 (CV=15.7%)
0.15	67.4 ± 0.7 (CV=1.1%)
0.20	62.9 ± 6.6 (CV=10.5%)

* from the calibration curve based on calculation of area under curve at 532 nm excitation/548 nm emission ($y = 861.4 + 1008.7x$, $R^2=0.993$); Mean value ± standard deviation ($n = 3$)



Second derivative visible spectra of ER solutions and the corresponding binary mixtures (1:50, 1:10, 1:5, 1:1, mol/mol) with (a) allura red AC, (b) azorubine or (c) red 2G ($C=12.5 \mu\text{M}$) 204x140mm (96 x 96 DPI)