Towards green analysis of virgin olive oil phenolic compounds: extraction by a natural deep eutectic solvent and direct spectrophotometric detection

Vito Michele Paradiso*, Antonia Clemente, Carmine Summo, Antonella Pasqualone, Francesco Caponio

University of Bari, Department of Soil, Plant and Food Sciences, Via Amendola 165/a, I-70126, Bari (Italy)

* corresponding author: vito.paradiso@uniba.it, +39 (0)80 544 2272

A. Clemente: antonia.clemente@uniba.it, C. Summo: carmine.summo@uniba.it, A. Pasqualone: antonella.pasqualone@uniba.it, F. Caponio: francesco.caponio@uniba.it

Abstract

The determination of phenolic compounds in extra virgin olive oils (EVOO) by means of rapid, low-cost, environment-free methods would be a desirable achievement. A natural deep eutectic solvent (DES) based on glucose and lactic acid was considered as extraction solvent for phenolic compounds in EVOO. DESs are green solvents characterized by high availability, biodegradability, safety, and low cost. The spectrophotometric characteristics of DES extracts of 65 EVOO samples were related to the total phenolic content of the oils, assessed by methanol-water extraction coupled to the Folin-Ciocalteu assay. A regression model ($n_{calibration}$ = 45, $n_{validation}$ = 20), including the absorbance at two wavelengths (257, 324 nm), was obtained, with an adjusted R^2 = 0.761. Therefore the DES could provide a promising and viable approach for a green screening method of phenolic compounds in EVOO, by means of simple spectrophotometric measurements of extracts, even for on-field analysis (for example in olive mills).

Keywords

Deep eutectic solvents; extra virgin olive oil; phenolic compounds; Folin-Ciocalteu assay; UV spectra

32 **1. Introduction**

33 Natural deep eutectic solvents (DES) are being increasingly considered for green techniques in
34 several fields, such as catalysis, electrochemistry, materials science, extraction of bioactive
35 compounds (Abbott, Boothby, Capper, Davies, & Rasheed, 2004; Hayyan et al., 2012; Martínez,
36 Berbegal, Guillena, & Ramón, 2016; Paiva et al., 2014; Pang et al., 2012; van Osch, Zubeir, van den
37 Bruinhorst, Rocha, & Kroon, 2015). Availability, biodegradability, safety, reusability and low cost
38 are major advantages that are encouraging research on their properties (Dai, van Spronsen,
39 Witkamp, Verpoorte, & Choi, 2013). DES are mixtures of compounds present as metabolites in
40 living cells, and have different physical properties than any of their individual components, due to
41 generation of intermolecular hydrogen bonds (Dai, van Spronsen, et al., 2013; Wei et al., 2015).
42 Among other properties, their ability to solubilize biomolecules is being investigated in order to
43 use them as green solvents for extraction of valuable compounds, such as phenolic compounds
44 (Dai, Witkamp, Verpoorte, & Choi, 2013; García, Rodríguez-Juan, Rodríguez-Gutiérrez, Rios, &
45 Fernández-Bolaños, 2016; Tang, Park, & Row, 2015).

46 Extra virgin olive oil (EVOO) is rich in phenolic compounds, though the concentrations can vary
47 largely depending on several factors such as cultivar, agronomic conditions, extraction technology,
48 storage duration and conditions (Cicerale, Conlan, Sinclair, & Keast, 2009). On the other hand,
49 phenolic compounds play a major role in the overall quality of this highly valuable vegetable oil,
50 affecting its sensory profile as well as its oxidative stability and well-known health properties
51 (Bendini et al., 2007; Cicerale et al., 2009). At present, a widely used method for determining total
52 phenolic compounds is based on the spectrophotometric analysis of water/methanol extracts
53 after colorimetric reaction with the Folin-Ciocalteu reagent (Carrasco-Pancorbo et al., 2005).
54 Research on analytical methods for phenolic compounds in olive oils is ongoing, attempting to
55 improve sensitivity and selectivity and to reduce time and solvents consumption (Alessandri, Ieri,

& Romani, 2014; Fuentes, Báez, Bravo, Cid, & Labra, 2012). Though some DES have been recently tested as extraction solvents for phenolic compounds from EVOO (García et al., 2016), no attempts have been made till now, to the best of our knowledge, to use DES as green solvents in the analytical determination of phenolic compounds in EVOO.

The present research acts in this framework and is aimed to evaluate the spectrophotometric characteristics of EVOO extracts obtained by a DES based on lactic acid and glucose, in order to assess whether it could be considered as a green alternative for a rapid, sustainable, on-field (i.e. directly at oil mills), screening method to evaluate phenolic compounds in EVOO.

2. Materials and methods

2.1. Reagents and samples

Glucose (≥ 99.5%), lactic acid (90%), methanol (≥ 99.8%), and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (Sigma-Aldrich Co. LLC, St. Louis, USA). Hexane (≥ 95.0 %) was purchased from Carlo Erba reagents (Carlo Erba reagents, Milan, Italy). Sodium carbonate was purchased from J.T. Baker (Avantor Performance Materials, Center Valley, USA). All standards were purchased from Sigma Aldrich (Sigma-Aldrich Co. LLC, St. Louis, USA). Sixty-five EVOO samples were obtained from producers and local sellers.

2.2. DES preparation

The DES was obtained by mixing lactic acid, glucose and water (6:1:6 molar ratio, according to Dai et al., 2013, with a slight modification to reduce solvent viscosity), by means of magnetic stirrer at 50 °C for about 90 min, until obtaining a clear solution.

2.3. Preparation of standard solutions

The DES solutions (100 mg/L)) of the following standards was prepared: hydroxybenzoic acid, protocathecuic acid, vanillic acid, tyrosol, *p*-coumaric acid, caffeic acid, apigenin, pinoresinol.

80 *2.4. Extraction and determination of total phenolic compounds (TPC)*

81 Total phenolic compounds of the EVOO samples were extracted and determined according to
82 Caponio et al. (Caponio et al., 2015) Briefly, extraction was carried out on 1 g of oil by adding 1 mL
83 of hexane and 5 mL of methanol/water (70:30 v/v). After vortexing for 10 min and centrifuging at
84 6,000 rpm for 10 min at 4 °C (Beckman Coulter, Fullerton, California, USA), the hydroalcoholic
85 phase was recovered, centrifuged again at 9,000 rpm for 5 min at 4 °C and filtered through nylon
86 filters (pore size 0.45 µm, Sigma-Aldrich, Milan, Italy). Then, 100 µL of extract were mixed with 100
87 µL of Folin-Ciocalteu reagent and, after 4 min, with 800 µL of a 5% (w/v) solution of sodium
88 carbonate. The mixture was then heated in a water bath at 40 °C for 20 min and the total phenol
89 content was determined at 750 nm by an Agilent Cary 60 spectrophotometer (Agilent
90 Technologies, Santa Clara, USA). The total phenolic content was expressed as gallic acid
91 equivalents (mg/kg).

92 *2.5. Extraction with DES*

93 One g of oil was added with 1 ml of hexane and 5 ml of DES. After intense agitation with vortex, a
94 centrifugation was performed for 10 minutes at 6000 rpm. The supernatant was subjected to
95 further centrifugation for 5 minutes at 9000 rpm. The supernatant was then filtered through a
96 0.45 µm nylon filter.

97 *2.6. Acquisition of UV spectra of DES extracts*

98 The DES extracts were analysed in the wavelength range 240-400 nm by means of an Agilent Cary
99 60 spectrophotometer (Agilent Technologies, Santa Clara, USA). The acquisition parameters were
100 the following: 1 cm optical path, 2 nm slit, 60 nm/min scan rate. Pure DES was used for
101 background correction.

102 *2.7. Statistical analysis*

103 Correlation analysis, regression analysis, and principal components analysis were carried out using
104 the software XlStat (Addinsoft SARL, New York, NY, USA).
105
106 **3. Results and discussion**
107 Figure 1 plots the UV spectra of both methanol/water and DES extracts of two different samples of
108 EVOO. Spectra of four independent extracts are represented for each sample.
109 Methanol/water extracts showed typical spectra with a broad peak at 280 nm (Fuentes et al.,
110 2012) related to phenolic compounds, though not significant correlation has been reported with
111 total phenolic compounds content, probably due to other compounds absorbing at that
112 wavelength (Papadopoulos, Triantis, Yannakopoulou, Nikokavoura, & Dimotikali, 2003). DES
113 extracts did not absorb at the lowest wavelengths, apart a small peak at 248 nm. A bigger, sharp,
114 peak of absorbance was observed at 254±1 nm, followed by another wider peak with maximum at
115 277±1 nm. A tail in the spectrum, up to about 380 nm was more or less marked in different oils.
116 Repeatability of extraction (n = 8) is represented in Figure 2, reporting the percent variation
117 coefficient of absorbance plotted versus wavelength. Variability was high at short wavelengths but
118 was below 10% in the range of maximum absorbance and kept at about 5% in the range 252-330
119 nm.
120 Some reference phenolic antioxidants (belonging to benzoic acid derivatives, cinnamic acid
121 derivatives, phenylethylalcohols, flavonoids, lignans) were solubilized in the DES. The spectra of
122 the solutions were acquired and reported in Figure 3. As can be seen, benzoic acid derivatives
123 showed maximum absorbance at about 260 nm and a further peak at about 296 nm when *o*-
124 diphenolic structure was present. The additional double bond in the cinnamic acid derivatives
125 extended the range of absorption, up to about 360 nm in *o*-diphenolic structures.
126 Phenylethylalcohols and lignans, instead, showed a peak absorption at 277 nm. As regards

127 flavonoids, apigenin showed a spectrum with a narrow peak at 266 nm and a broad peak at 340
128 nm. The observed wavelengths of peak absorbance are similar to those typically reported for
129 these compounds also in other solvents (Fuentes et al., 2012; Robbins, 2003).
130 The spectral properties of the DES extracts of EVOO could be therefore the result of combined
131 absorbance of different phenolic antioxidants contained in the extracts. In order to assess whether
132 information about the total content of phenolic antioxidants in EVOO could be obtained by
133 spectral data of DES extracts, a set of 65 oils was analyzed. Table 1 reports the statistical
134 characterization of the sample sets as regards their total phenolic compounds (TPC) content.
135 As a first step, a correlation analysis was carried out between absorbance at different wavelengths
136 in the range 252-370 nm and TPC. The Pearson *r* coefficient was plotted versus wavelength in
137 Figure 4. The highest correlation with TPC (r = 0.870) was found for absorbance at 257 nm,
138 corresponding to the observed maximum absorbance of phenolic acids. Also the wavelengths
139 around 280 nm showed high positive correlations, with a local maximum at 275 nm,
140 corresponding to high absorption observed for several reference compounds. On the other hand,
141 a negative correlation of the absorbance at wavelengths higher than 300 nm was observed, with a
142 minimum at 324 nm, an absorption wavelength related to hydroxycinnamic derivatives and
143 flavonoids, both in the present study and in literature when considering standards in
144 methanol/water (Fuentes et al., 2012). The observed correlations appeared to be promising
145 compared to the correlation coefficients between TPC and the absorbance at 280 nm of hexane
146 dilutions and methanolic extracts of oils (r = 0.6924 and 0.3196, respectively; n = 46) observed by
147 Fuentes et al. (2012). We aimed to gain sufficient information about TPC in EVOO from as few
148 spectral variables as possible, in order to hypothesize a rapid, simple screening method for TPC in
149 EVOO, without the need of chemometric analysis and expensive databases. Therefore, a
150 regression analysis was carried out on the data, after dividing the sample set in two subsets for

151 calibration and validation purposes (*n* = 45 for calibration and *n* = 20 for validation, randomly
152 selected): TPC was considered as a function of absorbance at 257 nm, 275 nm and 324 nm of the
153 DES extracts. Backward removal was applied to select the best model, with a removal threshold of
154 0.1. The obtained regression presented only two absorption wavelengths (257 nm and 324 nm),
155 since absorption at 275 nm was removed from the model. The fit parameters were the following:
156 adjusted R^2= 0.762; root mean square error of calibration (RMSEC) = 64.47; root mean square
157 error of prevision (RMSEP = 68.75); *p*-value of regression < 0.001; sum of squares (SS) of the
158 regression variables, Abs_{257} = 443712.03, Abs_{324} = 35982.46. The results of the regression analysis
159 are reported in Figure 5. Similar values were obtained for RMSEC and RMSEP, and only two
160 samples of the calibration set showed standardized residuals exceeding the threshold value of
161 ±1.96, confirming the robustness of the obtained model. The regression equation was the
162 following:

163 $$\mathrm{TPC}\ (mg\ gallic\ acid/kg\ oil) = 64.6 + 177.4 \times \mathrm{Abs}_{257} - 344.6 \times \mathrm{Abs}_{324}$$

164 The incidence of the different wavelengths in the model could suggest a slightly different
165 selectivity of the DES extraction coupled to direct spectrophotometric analysis respect to
166 water/methanol extraction coupled to Folin-Ciocalteu assay, towards the classes of phenolic
167 compounds. In fact, the model mainly accounted on the absorbance of DES extracts at 257 nm,
168 included with positive coefficient in the model, which was observed in all reference compounds,
169 though being a peak absorption in phenolic acids. The negative coefficient for the absorbance at
170 324 nm pointed out that an overestimation of TPC could be reduced by correcting the contribute
171 due to cinnamic acid derivatives and/or flavonoids.
172 This could be confirmed by literature, since flavonoids were previously reported not to be
173 correlated with the Folin-Ciocalteu spectrophotometric determination of TPC (Alessandri et al.,
174 2014). Moreover, a DES based on glucose (or sucrose) and lactic acid has been reported to be

175 effective in solubilizing cinnamic acids and flavonoids (Dai, van Spronsen, et al., 2013) and
176 extracting them from vegetable matrices (Tang et al., 2015; Wei et al., 2015). Also García et al.
177 (García et al., 2016), while testing several deep eutectic solvents (mainly choline chloride-based)
178 as extraction solvents for phenolic compounds from EVOO, reported different extraction
179 selectivities among the tested solvents.
180

181 **4. Conclusions**

182 The assessment of the content of phenolic compounds in virgin olive oils is of main importance,
183 due to their role in sensory properties, health effects and storage stability. The DES based on
184 glucose and lactic acid could be used as an extraction medium for phenolic compounds of olive
185 oils. The spectroscopic properties of the extracts was related with the total phenol content of the
186 oils, as assessed by the common Folin-Ciocalteu assay carried out on the methanol-water extracts.
187 Therefore, by simply measuring the absorption of the DES extracts at few wavelengths, a
188 screening of the total phenol content of the oils could be performed, reducing significantly the use
189 of hazardous solvents and reagents.
190 Direct spectrophotometric analysis of DES extracts could provide a viable approach for green
191 analysis of phenolic compounds in oils, even for on-field analysis (for example in olive mills).
192

193 **Acknowledgements**

194 The authors thank prof. Lanfranco Conte for his precious suggestions.
195

196 **Conflict of interest**

197 Authors declare no existing conflict of interest
198

199 **References**

200 Abbott, A. P., Boothby, D., Capper, G., Davies, D. L., & Rasheed, R. K. (2004). Deep eutectic
201 solvents formed between choline chloride and carboxylic acids: versatile alternatives to ionic
202 liquids. *Journal of the American Chemical Society*, *126*(29), 9142–7. doi:10.1021/ja048266j

203 Alessandri, S., Ieri, F., & Romani, A. (2014). Minor polar compounds in extra virgin olive oil:
204 correlation between HPLC-DAD-MS and the Folin-Ciocalteu spectrophotometric method.
205 *Journal of Agricultural and Food Chemistry*, *62*(4), 826–35. doi:10.1021/jf403104a

206 Bendini, A., Cerretani, L., Carrasco-Pancorbo, A., Gómez-Caravaca, A. M., Segura-Carretero, A.,
207 Fernández-Gutiérrez, A., & Lercker, G. (2007). Phenolic Molecules in Virgin Olive Oils: a
208 Survey of Their Sensory Properties, Health Effects, Antioxidant Activity and Analytical
209 Methods. An Overview of the Last Decade. *Molecules*, *12*(8), 1679–1719.
210 doi:10.3390/12081679

211 Caponio, F., Squeo, G., Monteleone, J. I., Paradiso, V. M., Pasqualone, A., & Summo, C. (2015). First
212 and second centrifugation of olive paste: Influence of talc addition on yield, chemical
213 composition and volatile compounds of the oils. *LWT - Food Science and Technology*, *64*(1),
214 439–445. doi:10.1016/j.lwt.2015.05.007

215 Carrasco-Pancorbo, A., Cerretani, L., Bendini, A., Segura-Carretero, A., Gallina-Toschi, T., &
216 Fernández-Gutiérrez, A. (2005). Analytical determination of polyphenols in olive oils. *Journal*
217 *of Separation Science*, *28*(9-10), 837–858. doi:10.1002/jssc.200500032

218 Cicerale, S., Conlan, X. a, Sinclair, A. J., & Keast, R. S. J. (2009). Chemistry and health of olive oil
219 phenolics. *Critical Reviews in Food Science and Nutrition*, *49*(3), 218–36.
220 doi:10.1080/10408390701856223

221 Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., & Choi, Y. H. (2013). Natural deep eutectic

solvents as new potential media for green technology. *Analytica Chimica Acta*, *766*, 61–8. doi:10.1016/j.aca.2012.12.019

Dai, Y., Witkamp, G.-J., Verpoorte, R., & Choi, Y. H. (2013). Natural Deep Eutectic Solvents as a New Extraction Media for Phenolic Metabolites in Carthamus tinctorius L. *Analytical Chemistry*, *85*(13), 6272–6278. doi:10.1021/ac400432p

Fuentes, E., Báez, M. E., Bravo, M., Cid, C., & Labra, F. (2012). Determination of Total Phenolic Content in Olive Oil Samples by UV–visible Spectrometry and Multivariate Calibration. *Food Analytical Methods*, *5*(6), 1311–1319. doi:10.1007/s12161-012-9379-5

García, A., Rodríguez-Juan, E., Rodríguez-Gutiérrez, G., Rios, J. J., & Fernández-Bolaños, J. (2016). Extraction of phenolic compounds from virgin olive oil by deep eutectic solvents (DESs). *Food Chemistry*, *197*, 554–561. doi:10.1016/j.foodchem.2015.10.131

Hayyan, A., Mjalli, F. S., Alnashef, I. M., Al-Wahaibi, T., Al-Wahaibi, Y. M., & Hashim, M. A. (2012). Fruit sugar-based deep eutectic solvents and their physical properties. *Thermochimica Acta*, *541*, 70–75. doi:10.1016/j.tca.2012.04.030

Martínez, R., Berbegal, L., Guillena, G., & Ramón, D. J. (2016). Bio-renewable enantioselective aldol reaction in natural deep eutectic solvents. *Green Chem.* doi:10.1039/C5GC02526E

Paiva, A., Craveiro, R., Aroso, I., Martins, M., Reis, R. L., & Duarte, A. R. C. (2014). Natural Deep Eutectic Solvents – Solvents for the 21st Century. *ACS Sustainable Chemistry & Engineering*, *2*(5), 1063–1071. doi:10.1021/sc500096j

Pang, K., Hou, Y., Wu, W., Guo, W., Peng, W., & Marsh, K. N. (2012). Efficient separation of phenols from oils via forming deep eutectic solvents. *Green Chemistry*, *14*(9), 2398. doi:10.1039/c2gc35400d

Papadopoulos, K., Triantis, T., Yannakopoulou, E., Nikokavoura, A., & Dimotikali, D. (2003).

245 Comparative studies on the antioxidant activity of aqueous extracts of olive oils and seed oils
246 using chemiluminescence. *Analytica Chimica Acta*, *494*(1), 41–47. Retrieved from
247 http://www.sciencedirect.com/science/article/pii/S0003267003010134

248 Robbins, R. J. (2003). Phenolic acids in foods: an overview of analytical methodology. *Journal of*
249 *Agricultural and Food Chemistry*, *51*(10), 2866–87. doi:10.1021/jf026182t

250 Tang, B., Park, H. E., & Row, K. H. (2015). Simultaneous Extraction of Flavonoids from
251 Chamaecyparis obtusa Using Deep Eutectic Solvents as Additives of Conventional Extractions
252 Solvents. *Journal of Chromatographic Science*, *53*(5), 836–840.
253 doi:10.1093/chromsci/bmu108

254 van Osch, D. J. G. P., Zubeir, L. F., van den Bruinhorst, A., Rocha, M. A. A., & Kroon, M. C. (2015).
255 Hydrophobic deep eutectic solvents as water-immiscible extractants. *Green Chem.*, *17*(9),
256 4518–4521. doi:10.1039/C5GC01451D

257 Wei, Z.-F., Wang, X.-Q., Peng, X., Wang, W., Zhao, C.-J., Zu, Y.-G., & Fu, Y.-J. (2015). Fast and green
258 extraction and separation of main bioactive flavonoids from Radix Scutellariae. *Industrial*
259 *Crops and Products*, *63*, 175–181. doi:10.1016/j.indcrop.2014.10.013

260

261 **Figure captions**

262 **Figure 1.** UV spectra of methanol/water and DES extracts of two different samples of EVOO.
263 Spectra of two independent extracts are represented for each sample.

264

265 **Figure 2.** Repeatability of DES extraction for three samples of EVOO (n = 8; percent variation
266 coefficient of absorbance plotted versus wavelength).

267

268 **Figure 3.** UV spectra of reference phenolic compounds solubilized in DES (a, hydroxybenzoic acid;
269 b, protocathecuic acid; c, vanillic acid; d, tyrosol; e, *p*-coumaric acid; f, caffeic acid; g, apigenin; h,
270 pinoresinol)

271

272 **Figure 4.** Pearson r coefficient of TPC versus wavelength (n = 65). Reference dashed lines
273 correspond to p = 0.05.

274

275 **Figure 5.** Regression of TPC of sample oils as a function of absorbance of DES extracts at 257 and
276 324 nm: predicted versus observed values (left) and standardized residuals (right).

277

Table 1. Statistical characterization of the sample sets as regards their TPC (ppm).

	n	Mean	SD	Median	Minimum	Maximum
Total	65	248	128	234	45	535
Calibration set	45	265	132	248	79	535
Validation set	20	209	111	168	45	453

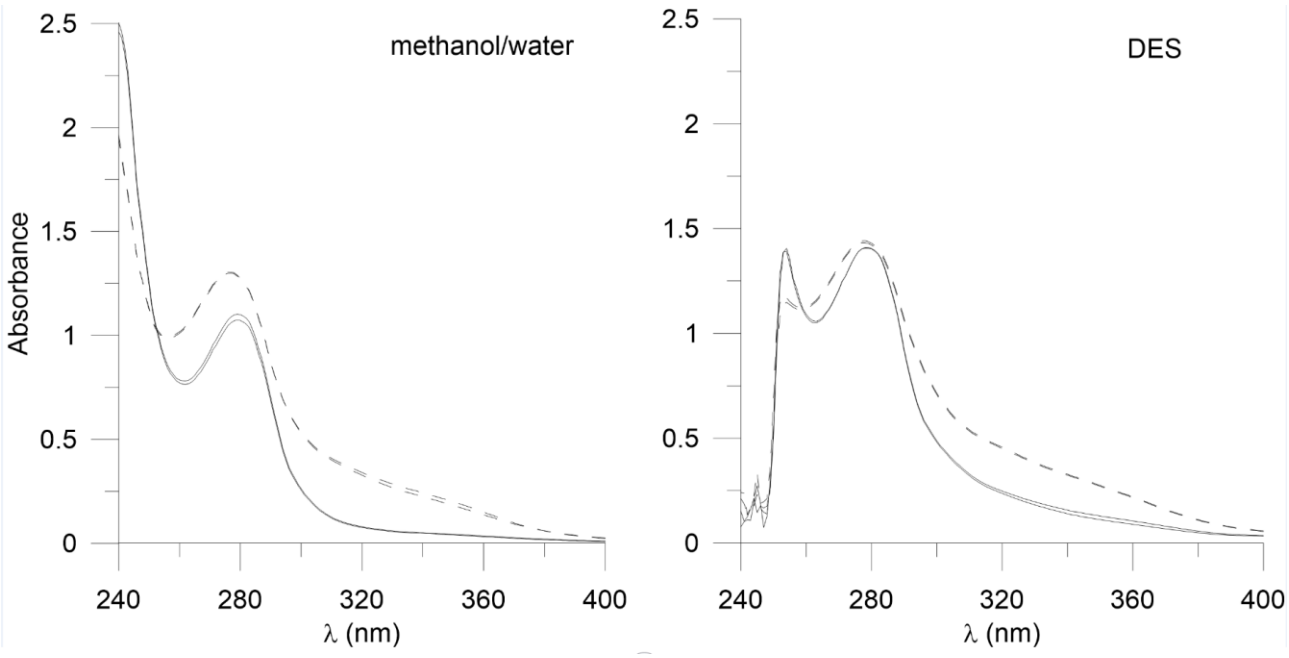

Figure 1 – Paradiso et al.,

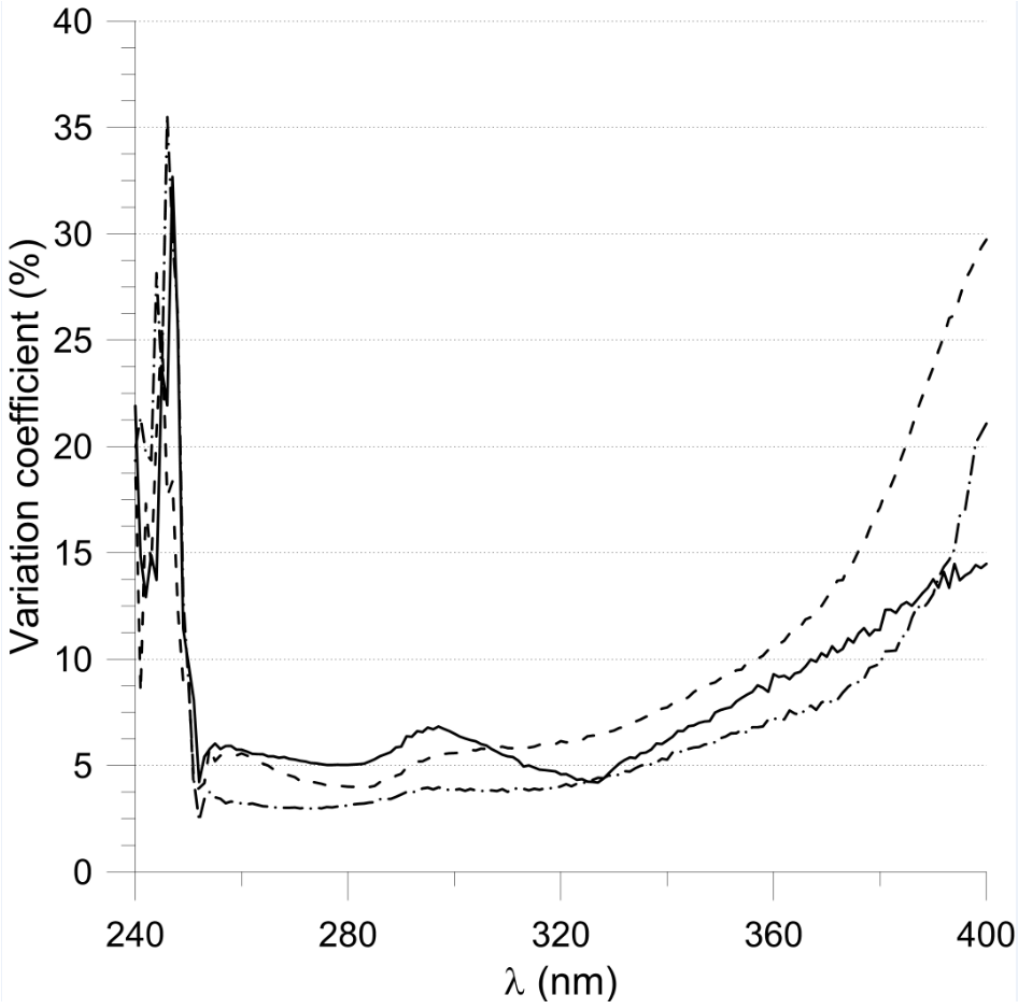

Figure 2 – Paradiso et al.,

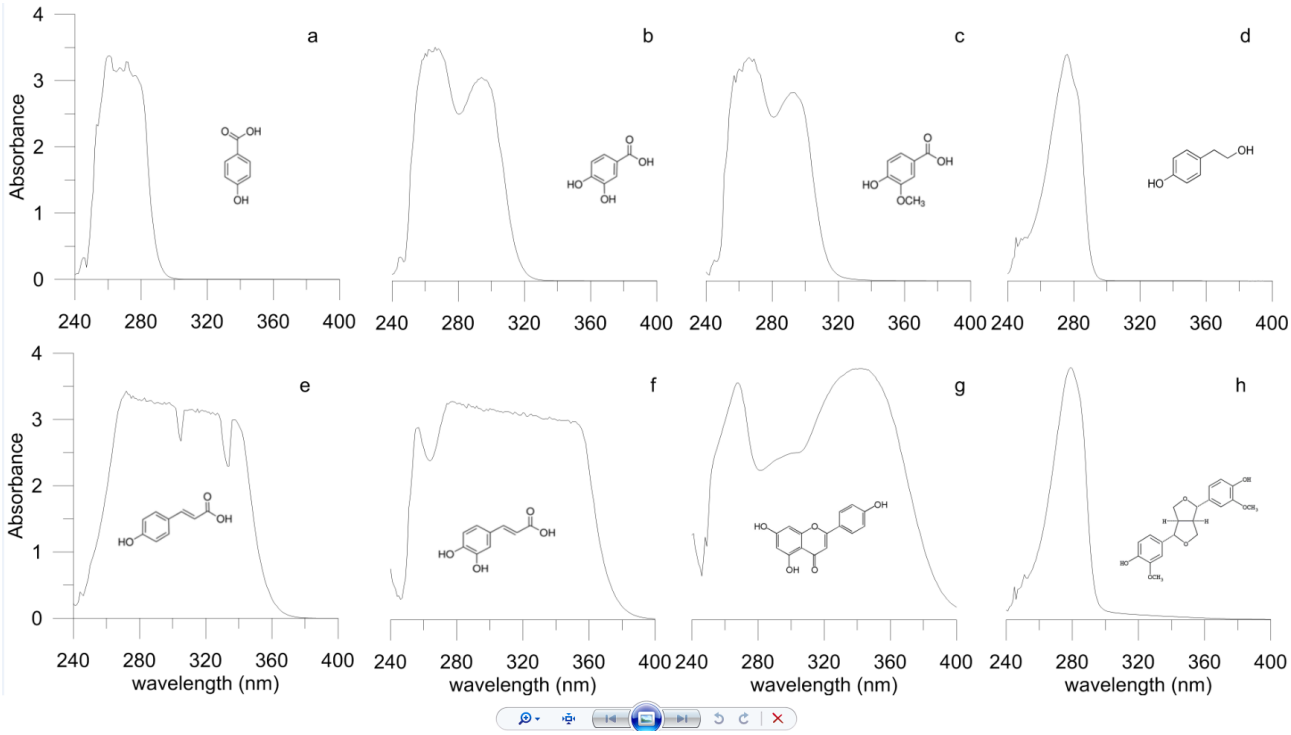

Figure 3 – Paradiso et al.,

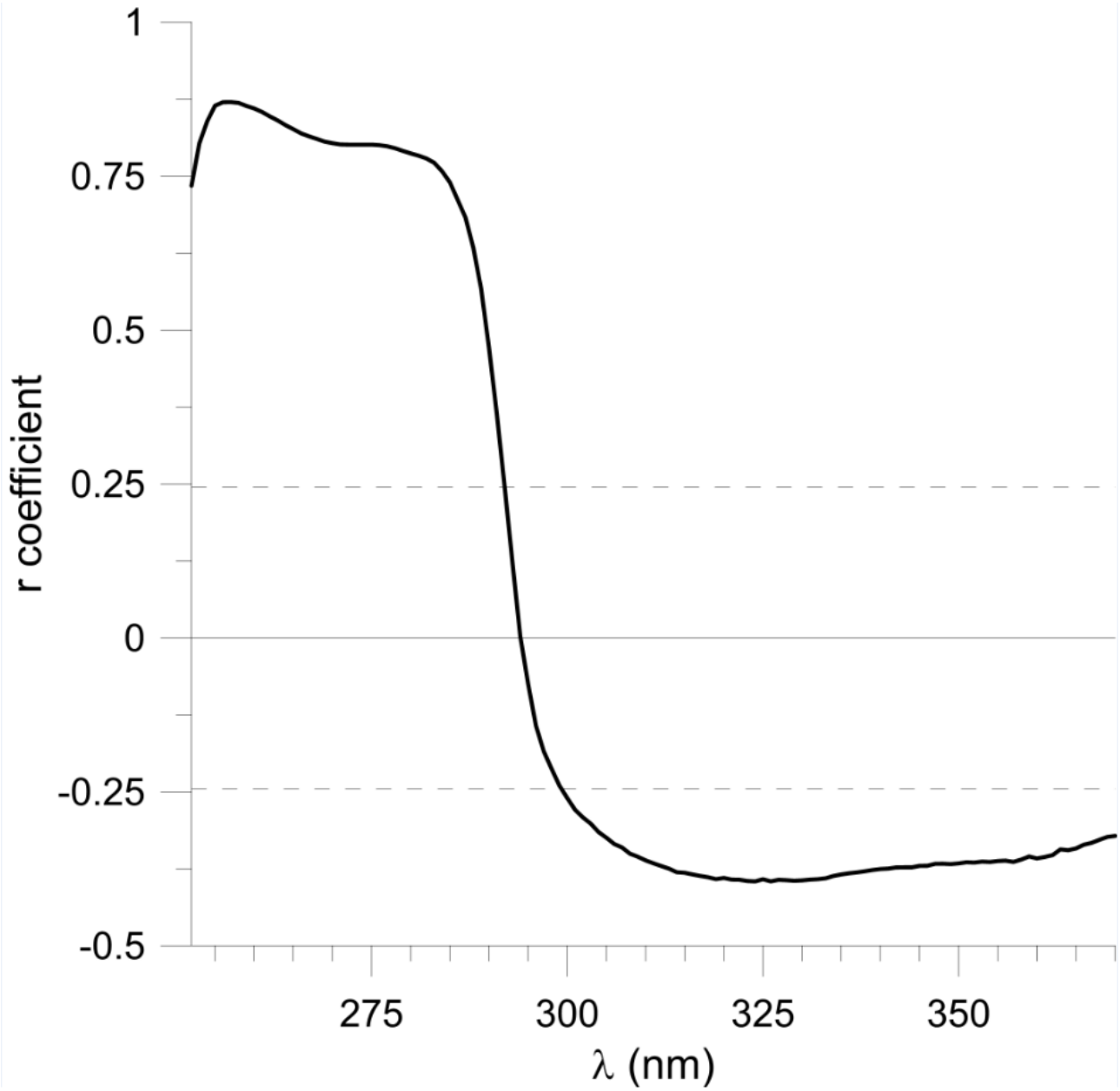

Figure 4 – Paradiso et al.,

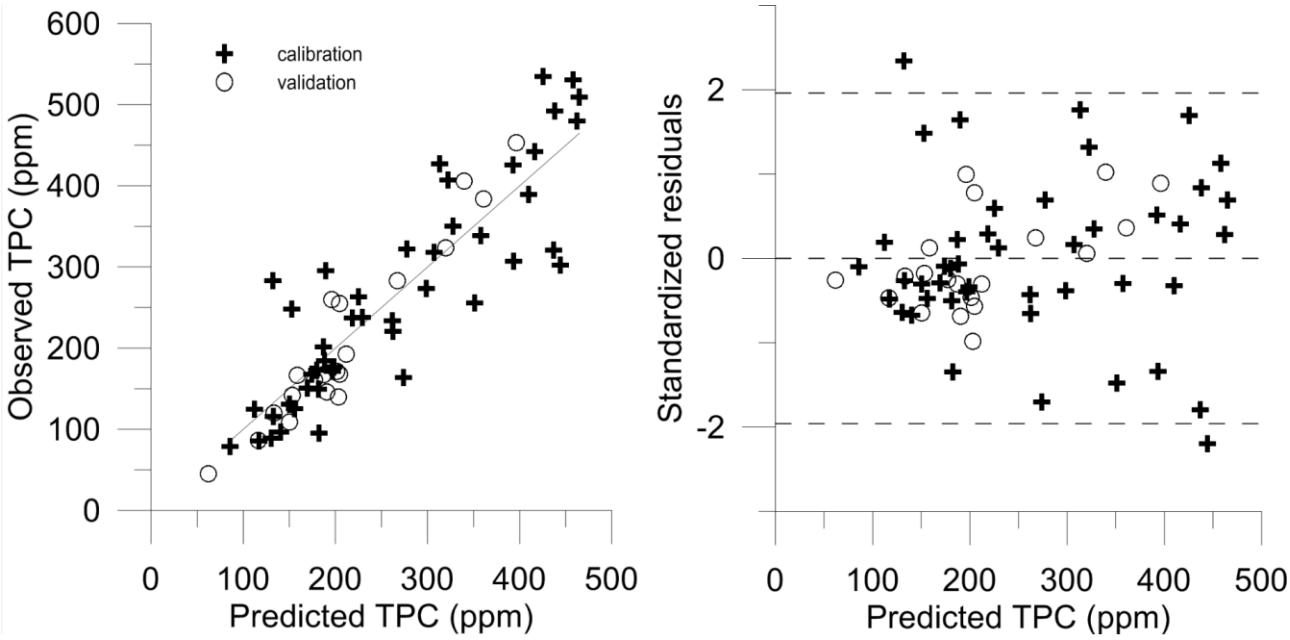

Figure 5 – Paradiso et al.,

294