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PII: S0308-8146(16)30762-2
DOI: http://dx.doi.org/10.1016/j.foodchem.2016.05.082
Reference: FOCH 19231

To appear in: *Food Chemistry*

Received Date: 11 February 2016
Revised Date: 10 May 2016
Accepted Date: 12 May 2016

Please cite this article as: Paradiso, V.M., Clemente, A., Summo, C., Pasqualone, A., Caponio, F., Towards green analysis of virgin olive oil phenolic compounds: extraction by a natural deep eutectic solvent and direct spectrophotometric detection, *Food Chemistry* (2016), doi: http://dx.doi.org/10.1016/j.foodchem.2016.05.082

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

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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 = 0.762. 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

1. Introduction

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

Extra virgin olive oil (EVOO) is rich in phenolic compounds, though the concentrations can vary largely depending on several factors such as cultivar, agronomic conditions, extraction technology, storage duration and conditions (Cicerale, Conlan, Sinclair, & Keast, 2009). On the other hand, phenolic compounds play a major role in the overall quality of this highly valuable vegetable oil, affecting its sensory profile as well as its oxidative stability and well-known health properties (Bendini et al., 2007; Cicerale et al., 2009). At present, a widely used method for determining total phenolic compounds is based on the spectrophotometric analysis of water/methanol extracts after colorimetric reaction with the Folin-Ciocalteu reagent (Carrasco-Pancorbo et al., 2005). Research on analytical methods for phenolic compounds in olive oils is ongoing, attempting to 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 were prepared: hydroxybenzoic acid, protocathecuic acid, vanillic acid, tyrosol, *p*-coumaric acid, caffeic acid, apigenin, pinoresinol.

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

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

2.5. Extraction with DES

One g of oil was added with 1 mL of hexane and 5 mL of DES. After intense agitation with vortex, a centrifugation was performed for 10 minutes at 6000 rpm. The supernatant was subjected to further centrifugation for 5 minutes at 9000 rpm. The supernatant was then filtered through a 0.45 µm nylon filter. Two independent extractions were carried out.

2.6. Acquisition of UV spectra of DES extracts

The DES extracts were analysed in the wavelength range 240-400 nm by means of an Agilent Cary 60 spectrophotometer (Agilent Technologies, Santa Clara, USA). The acquisition parameters were the following: 1 cm optical path, 2 nm slit, 60 nm/min scan rate. Pure DES was used for background correction. Mean spectra of the two independent extractions were used for statistical analysis after sample weight normalization.

2.7. Statistical analysis

Correlation analysis, regression analysis, and principal components analysis were carried out using the software XlStat (Addinsoft SARL, New York, NY, USA).

3. Results and discussion

Figure 1 plots the UV spectra of both methanol/water and DES extracts of two different samples of EVOO. Spectra of two independent extracts are represented for each sample.

Methanol/water extracts showed typical spectra with a broad peak at 280 nm (Fuentes et al., 2012) related to phenolic compounds, though not significant correlation has been reported with total phenolic compounds content, probably due to other compounds absorbing at that wavelength (Papadopoulos, Triantis, Yannakopoulou, Nikokavoura, & Dimotikali, 2003). DES extracts did not absorb at the lowest wavelengths, apart a small peak at 248 nm. A bigger, sharp, peak of absorbance was observed at 254±1 nm, followed by another wider peak with maximum at 277±1 nm. A tail in the spectrum, up to about 380 nm was more or less marked in different oils. Repeatability of extraction (n = 8) is represented in Figure 2, reporting the percent variation coefficient of absorbance plotted versus wavelength. Variability was high at short wavelengths but was below 10% in the range of maximum absorbance and kept at about 5% in the range 252-330 nm.

Some reference phenolic antioxidants (belonging to benzoic acid derivatives, cinnamic acid derivatives, phenylethylalcohols, flavonoids, lignans) were solubilized in the DES. The spectra of the solutions were acquired and reported in Figure 3. As can be seen, benzoic acid derivatives showed maximum absorbance at about 260 nm and a further peak at about 296 nm when *o*-diphenolic structure was present. The additional double bond in the cinnamic acid derivatives extended the range of absorption, up to about 360 nm in *o*-diphenolic structures. Phenylethylalcohols and lignans, instead, showed a peak absorption at 277 nm. As regards

flavonoids, apigenin showed a spectrum with a narrow peak at 266 nm and a broad peak at 340 nm. The observed wavelengths of peak absorbance are similar to those typically reported for these compounds also in other solvents (Fuentes et al., 2012; Robbins, 2003).

The spectral properties of the DES extracts of EVOO could be therefore the result of combined absorbance of different phenolic antioxidants contained in the extracts. In order to assess whether information about the total content of phenolic antioxidants in EVOO could be obtained by spectral data of DES extracts, a set of 65 oils was analyzed. Table 1 (upper part) reports the statistical characterization of the sample sets as regards their total phenolic compounds (TPC) content. The whole dataset of UV spectra is reported in a data article (Paradiso, Clemente, Summo, Pasqualone, & Caponio, submitted).

As a first step, a correlation analysis was carried out between absorbance at different wavelengths in the range 252-370 nm and TPC. The Pearson *r* coefficient was plotted versus wavelength in Figure 4. The highest correlation with TPC (r = 0.870) was found for absorbance at 257 nm, corresponding to the observed maximum absorbance of phenolic acids. Also the wavelengths around 280 nm showed high positive correlations, with a local maximum at 275 nm, corresponding to high absorption observed for several reference compounds. On the other hand, a negative correlation of the absorbance at wavelengths higher than 300 nm was observed, with a minimum at 324 nm, an absorption wavelength related to hydroxycinnamic derivatives and flavonoids, both in the present study and in literature when considering standards in methanol/water (Fuentes et al., 2012). The observed correlations appeared to be promising compared to the correlation coefficients between TPC and the absorbance at 280 nm of hexane dilutions and methanolic extracts of oils (r = 0.6924 and 0.3196, respectively; n = 46) observed by Fuentes et al. (2012). We aimed to gain sufficient information about TPC in EVOO from as few spectral variables as possible, in order to hypothesize a rapid, simple screening method for TPC in

EVOO, without the need of chemometric analysis and expensive databases. Therefore, a regression analysis was carried out on the data, after dividing the sample set in two subsets for calibration and validation purposes (n = 45 for calibration and n = 20 for validation, randomly selected): TPC was considered as a function of absorbance at 257 nm, 275 nm and 324 nm of the DES extracts. Backward removal was applied to select the best model, with a removal threshold of 0.1. The obtained regression presented only two absorption wavelengths (257 nm and 324 nm), since absorption at 275 nm was removed from the model. The results of the regression analysis were reported in the lower part of Table 1 and in Figure 5. The regression equation was the following:

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

Adjusted R^2 (0.762) and p-values pointed out the significance of the model. Similar values were obtained for RMSEC and RMSEP, and only two samples of the calibration set showed standardized residuals exceeding the threshold value of ±1.96, confirming the robustness of the obtained model. On the other hand, relative error of both calibration and prediction (24 % and 26 % respectively) were higher than those of the PLS models reported by Fuentes et al. (2012), which ranged from 6.6 % to 14.1 % (relative error of prediction). Nevertheless, the very low amount of data required can justify the choice of this model for screening approaches. Moreover, the high repeatability of spectroscopic properties of independent extracts and the consistency of error indices in both calibration and prediction, together with an examination of the incidence of the different wavelengths in the model could suggest a slightly different selectivity of the DES extraction coupled to direct spectrophotometric analysis respect to water/methanol extraction coupled to Folin-Ciocalteu assay, towards the classes of phenolic compounds. In fact, the model mainly accounted on the absorbance of DES extracts at 257 nm, included with positive coefficient in the model, which was observed in all reference compounds, though being a peak absorption in

phenolic acids. The negative coefficient for the absorbance at 324 nm pointed out that an overestimation of TPC could be reduced by correcting the contribute due to cinnamic acid derivatives and/or flavonoids.

This could be confirmed by literature, since flavonoids were previously reported not to be correlated with the Folin-Ciocalteu spectrophotometric determination of TPC (Alessandri et al., 2014). Moreover, a DES based on glucose (or sucrose) and lactic acid has been reported to be effective in solubilizing cinnamic acids and flavonoids (Dai, van Spronsen, et al., 2013) and extracting them from vegetable matrices (Tang et al., 2015; Wei et al., 2015). Also García et al. (García et al., 2016), while testing several deep eutectic solvents (mainly choline chloride-based) as extraction solvents for phenolic compounds from EVOO, reported different extraction selectivities among the tested solvents.

4. Conclusions

The assessment of the content of phenolic compounds in virgin olive oils is of main importance, due to their role in sensory properties, health effects and storage stability. The DES based on glucose and lactic acid could be used as an extraction medium for phenolic compounds of olive oils. The spectroscopic properties of the extracts was related with the total phenol content of the oils, as assessed by the common Folin-Ciocalteu assay carried out on the methanol-water extracts. Therefore, by simply measuring the absorption of the DES extracts at few wavelengths, a screening of the total phenol content of the oils could be performed, reducing significantly the use of hazardous solvents and reagents.

Direct spectrophotometric analysis of DES extracts could provide a viable approach for green analysis of phenolic compounds in oils, even for on-field analysis (for example in olive mills).

Further investigation is currently being carried out in order to avoid the use of hexane during extraction and to couple this green extraction method to chromatographic analysis.

Acknowledgements

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

Conflict of interest

Authors declare no existing conflict of interest

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Figure captions

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

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

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

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

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

Figure 1

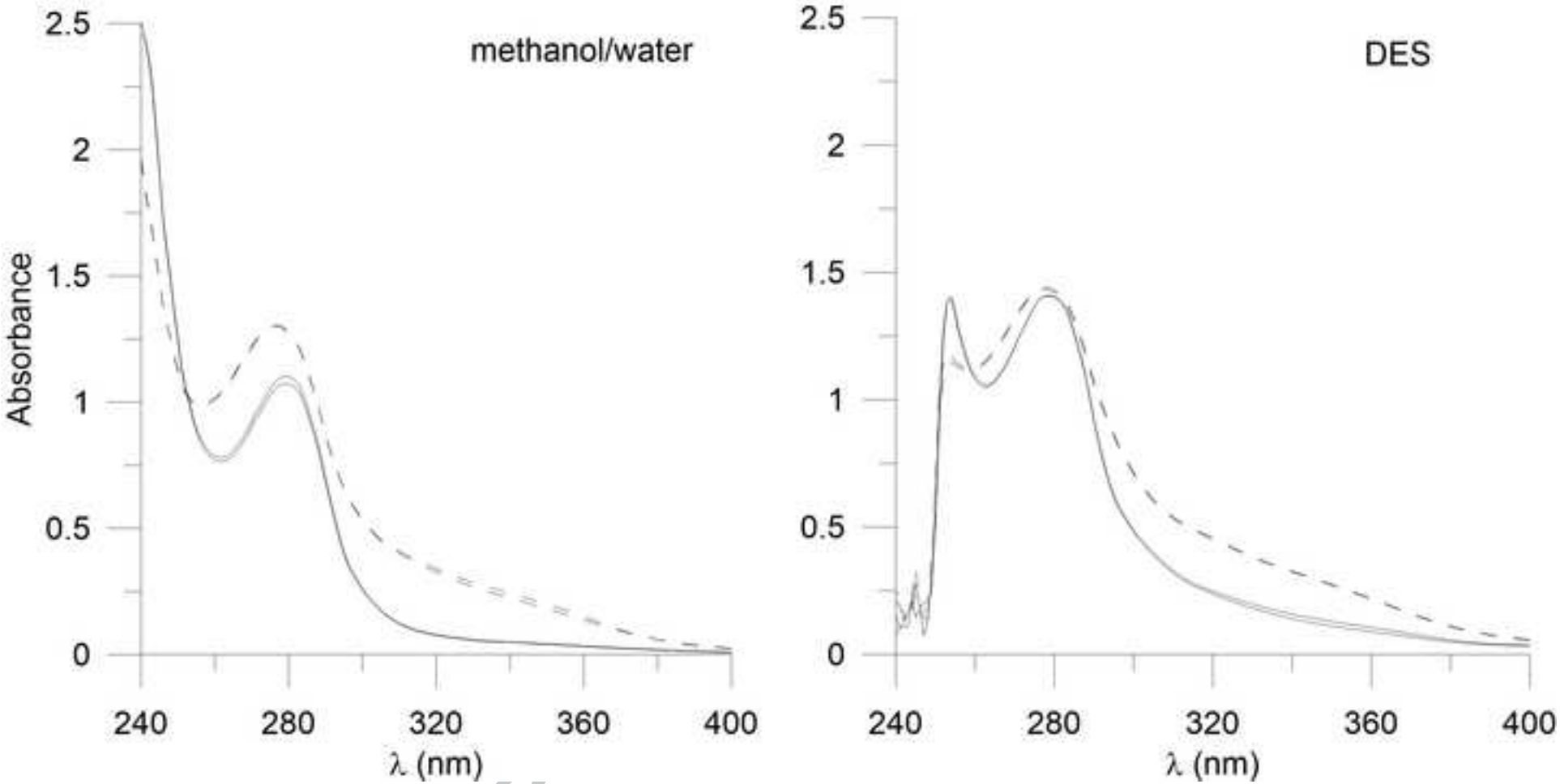


Figure 2

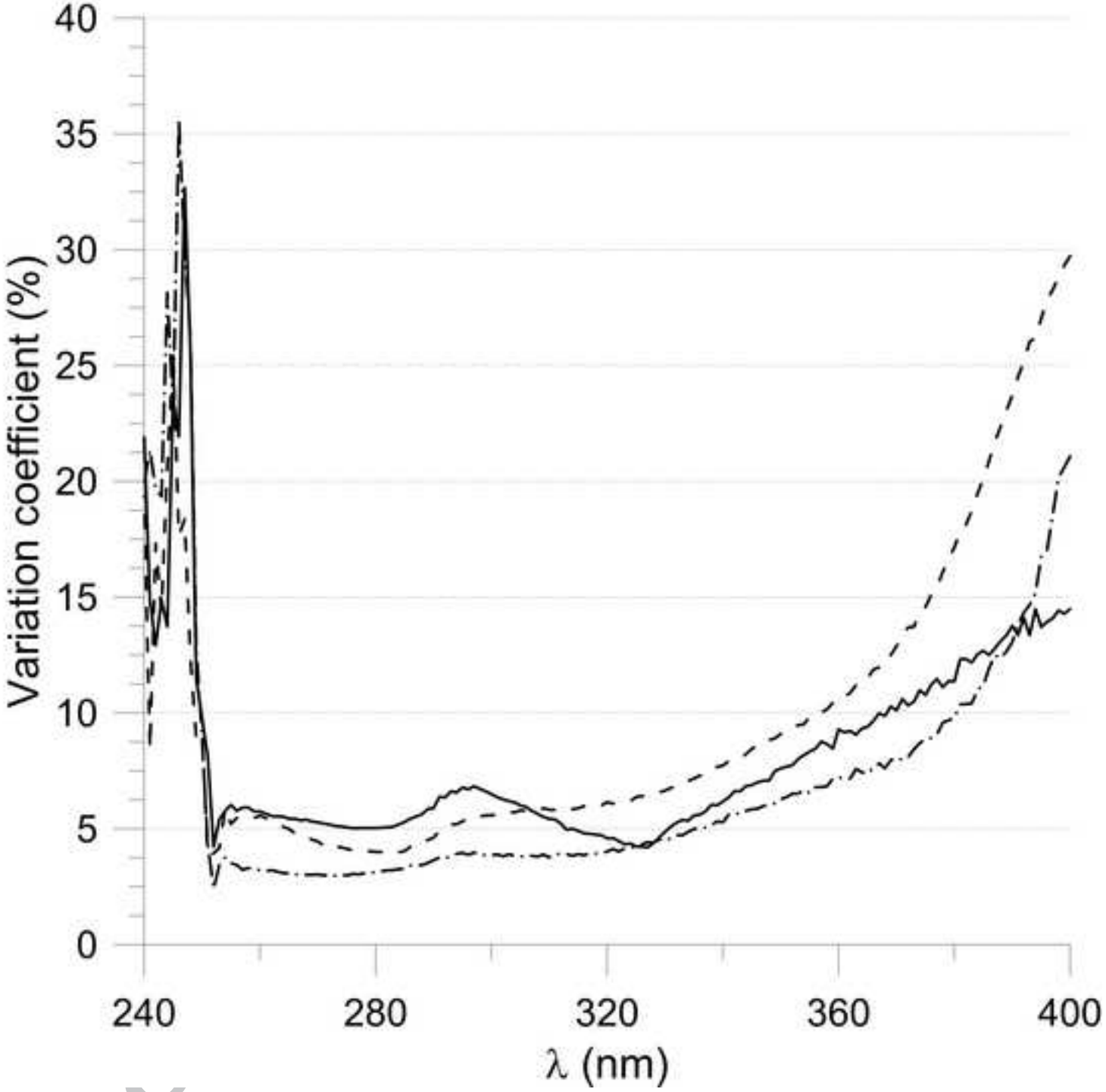


Figure 3

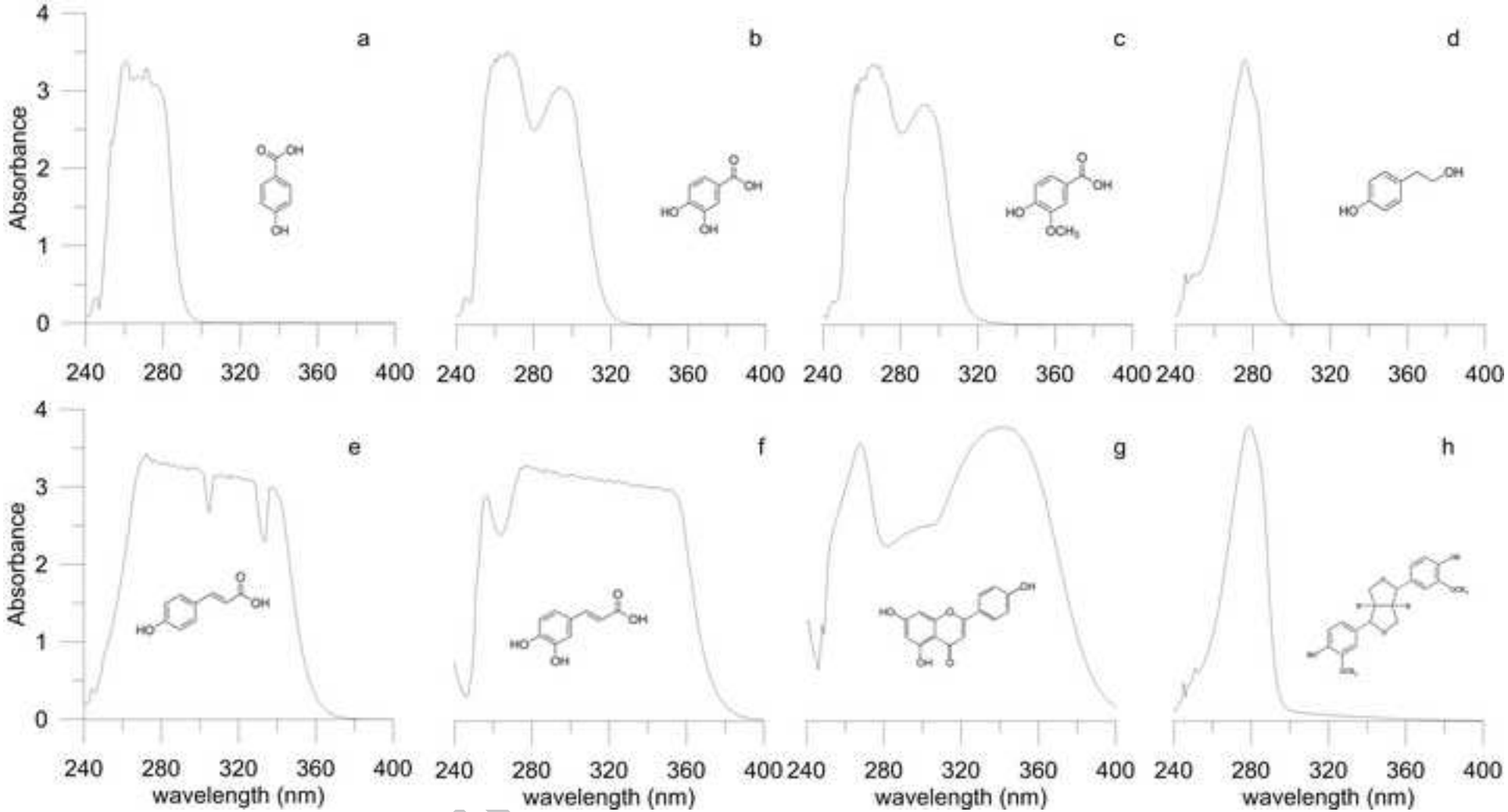


Figure 4

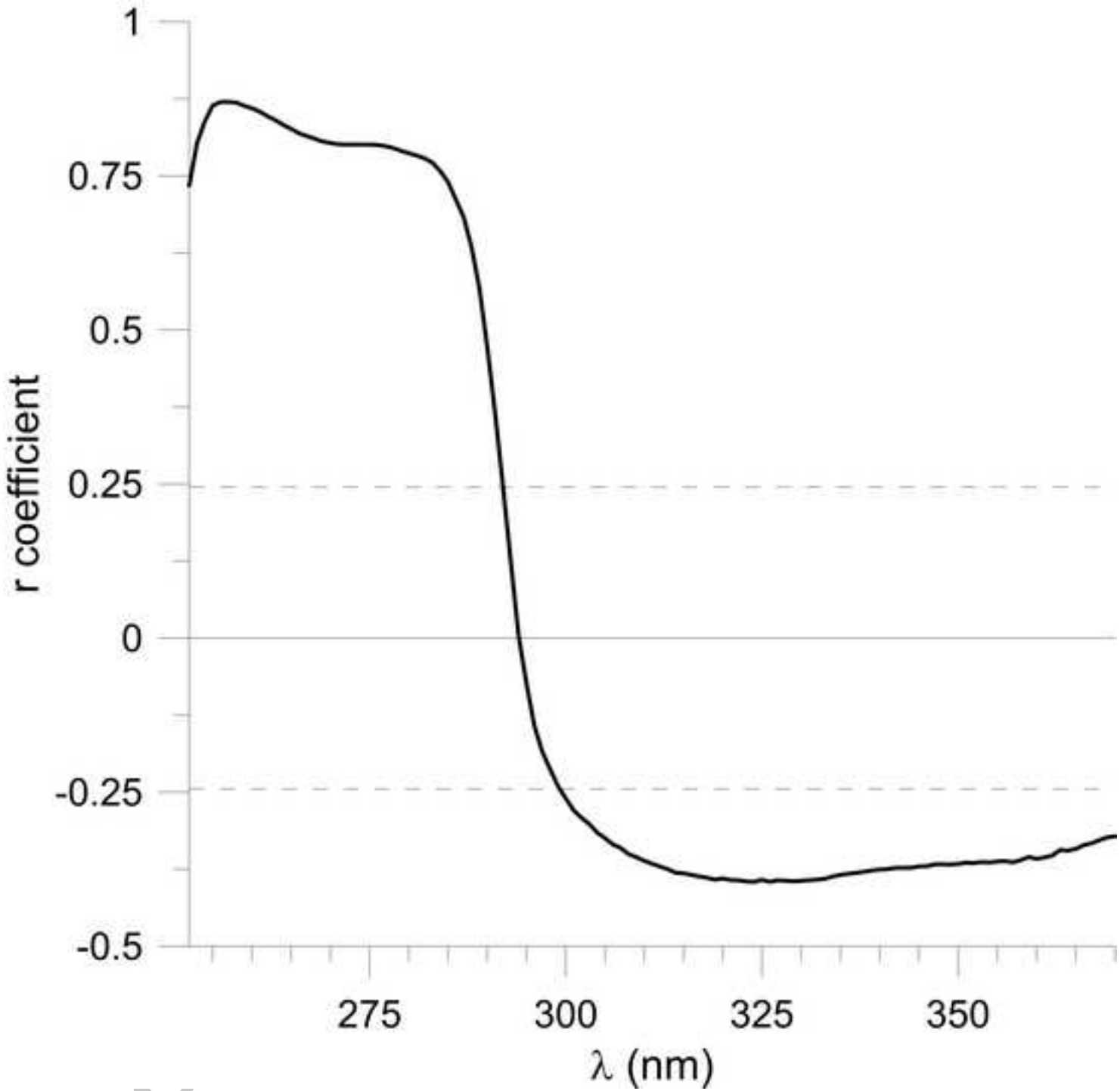
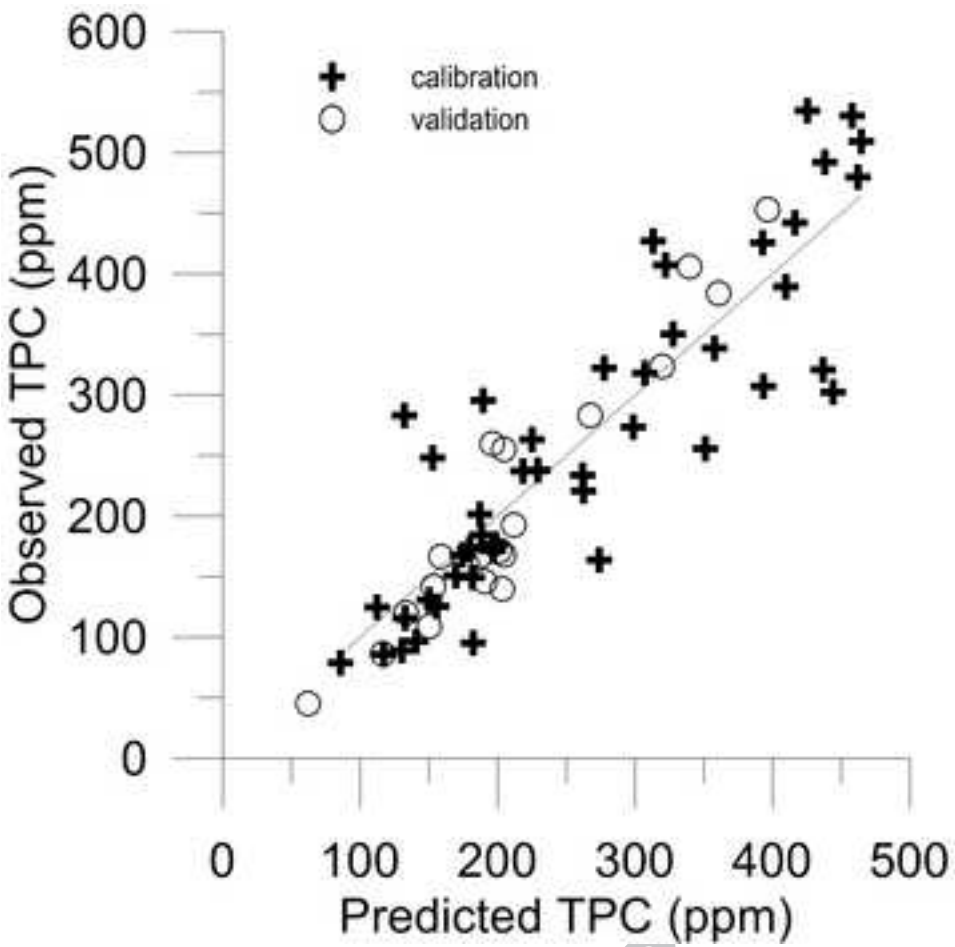


Figure 5



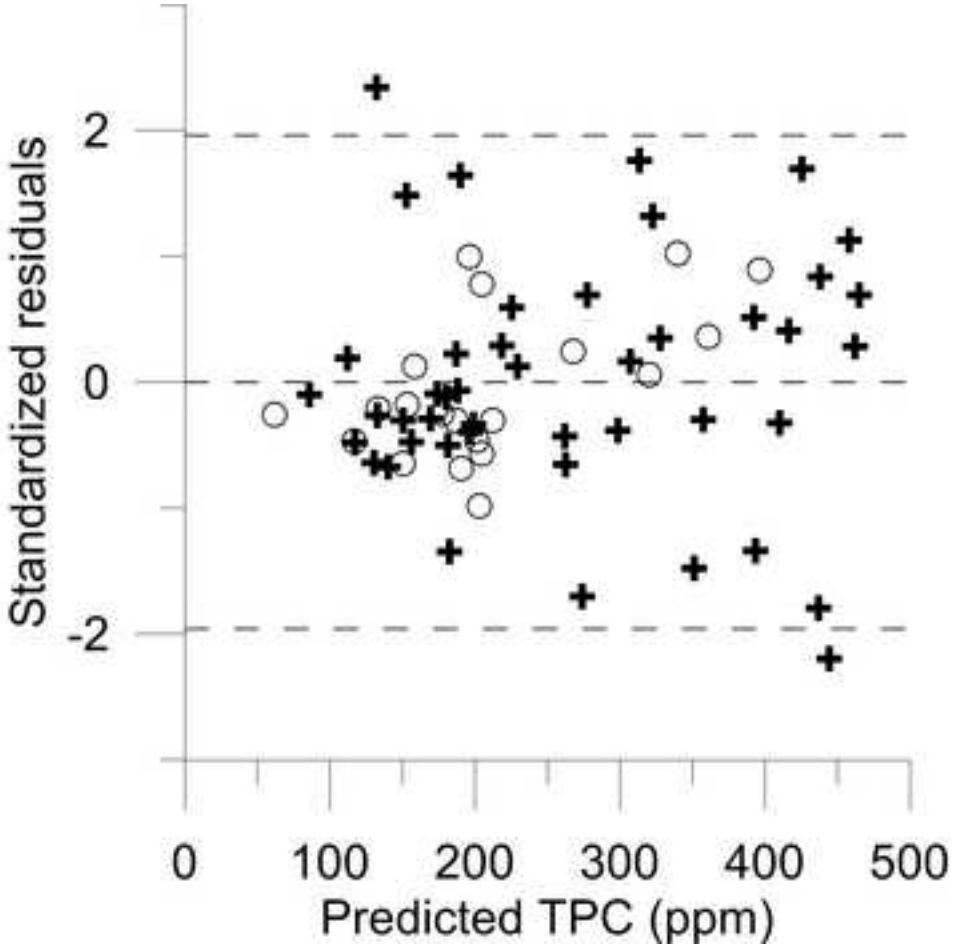


Table 1. Statistical characterization of the sample sets as regards their TPC (ppm) and parameters of the regression of TPC as a function of absorbance at 257 and 324 nm of DES extracts.

	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

TPC = *f*(Abs_{257}, Abs_{324}) – Regression parameters

	R^2 *(adjusted)*	*p-value*	*RMSEC*	*RMSEP*	*REC*	*REP*
Model	0.762	< 0.001	64.5	68.8	24 %	26 %
Abs_{257}		< 0.001				
Abs_{324}		0.005				

TPC, total phenolic compounds; RMSEC, root mean error of calibration; RMSEP, root mean error of prediction; REC, relative error of calibration; REP, relative error of prediction

Highlights – Paradiso et al.

- A deep eutectic solvent (DES) based on glucose and lactic acid was used
- Phenolic compounds were extracted from extra virgin olive oil (EVOO) using the DES
- UV absorption of DES extracts could be related to the content of phenolic compounds
- This is a step towards simple, green analysis of EVOO phenolic compounds