An easy and green tool for olive oils labelling according to the contents of hydroxytyrosol and tyrosol derivatives: Extraction with a natural deep eutectic solvent and direct spectrophotometric analysis

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Abstract

Natural deep eutectic solvents (NADES) are a green, promising class of solvents with potential applications even in analytical chemistry. Phenolic compounds are among the target compounds of the use of NADES at analytical aims. Hydroxytyrosol, tyrosol and their derivatives (HTD) are a major class of olive oil phenolic compounds, playing a key role in health and sensory properties of olive oils, as well as in their oxidative stability and shelf-life. A health claim is admitted by European Regulations for olive oils containing at least 250 mg of polyphenols per kg of oil, but their determination involves several analytical issues that are being currently debated. In this paper, a NADES based on glucose and lactic acid was used to assess the levels of HTD in extra virgin olive oils.

A set of 163 extra virgin olive oil samples of different origin was submitted to HPLC analysis of HTD and to liquid/liquid extraction with a NADES based of lactic acid and glucose. UV spectra (200-400 nm) of NADES extracts were then acquired and different statistical approaches (both regression and classification) were adopted to relate spectral features to HTD content. The models obtained allowed to assess HTD content for screening purposes ($R^2_{prediction}$ = 0.84, root mean square error of prediction = 35.5 mg kg^{-1}). When applied for labelling purposes, the models obtained allowed to label oils according health claim limits with an error of 0.6%. The proposed method resulted, therefore, a green, cheap and reliable tool for labelling olive oils according to their HTD contents.

Keywords

Olive oil; phenolic compounds; green chemistry; UV spectrophotometry; green solvents

1. Introduction

Natural deep eutectic solvents (NADES) are mixtures, in certain molar ratios, of natural compounds (sugars, organic acids, amino acids, and organic bases) that are abundant in organisms and are individually usually present in food (Vanda, Dai, Wilson, Verpoorte, & Choi, 2018). Due to their high biocompatibility and biodegradability, they are considered as promising green solvents in several kinds of applications: extraction of bioactive compounds and macromolecules, enzymatic processes, removal of undesired components, analytical issues (Fernández, Espino, Gomez, & Silva, 2018; Liu et al., 2018; Lores, Romero, Costas, Bendicho, & Lavilla, 2017; Vanda et al., 2018).

Applications of NADES involve the analytical determination of both inorganic and organic targets (Shishov, Bulatov, Locatelli, Carradori, & Andruch, 2017): procainamide in human saliva (Nugbienyo et al., 2017), gluten in food samples (Lores et al., 2017), ochratoxin A in wheat-derived products (Piemontese, Perna, Logrieco, Capriati, & Solfrizzo, 2017) are some examples of target compounds for which NADES have been proposed as analytical solvents.

In our recent papers (Paradiso, Clemente, Summo, Pasqualone, & Caponio, 2016b, 2016a), we proposed the use of a NADES based on glucose and lactic acid for setting up a screening method of total phenolic compounds (TPC) in olive oils, with a root mean error of prediction of 68.8 mg kg^{-1}. Olive oil phenolic compounds, and particularly hydroxytyrosol, have well-known health benefits, documented for pathologies as diabetes, inflammation, nervous disorders, angiogenesis, cancer, oxidative stress, heavy metal toxicity, hemolysis, LDL oxidation, muscle damage, nephrotoxicity (Difonzo et al., 2017; Navarro & Morales, 2017; Peng, Zhang, Yao, Duan, & Fang, 2015; Wani et al., 2018). On the basis of scientific evidence, the EC Regulation 432/2012, establishing a list of permitted health claims made on foods, provides the following health claim for olive oil: “Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress”. The use of this claim is limited to olive oils which contain “at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil” (European Commission, 2012). While ongoing discussion is trying to reach an unequivocal interpretation of which compounds should be considered to give the sum of 5 mg per 20 g of olive oil (Nenadis et al., 2018; Tsimidou & Boskou, 2015; Tsimidou, Nenadis, Servili, Luis, & Gonzáles, 2018), several attempts are being made to provide reliable

analytical tools to assess the contents of these compounds in olive oils (Celano et al., 2018; Mastralexi, Nenadis, & Tsimidou, 2014; Purcaro, Codony, Pizzale, Mariani, & Conte, 2014; Reboredo-Rodríguez et al., 2016; Romero & Brenes, 2012). The methods proposed are mainly based on liquid-liquid extraction with methanol/water and subsequent chromatographic analysis (using either gas- or liquid chromatography). Regardless of their performances, the analytical methods proposed require in many cases expensive equipment, and always involve the use of toxic or polluting solvents. In one case (Reboredo-Rodríguez et al., 2016) the Folin-Ciocalteu spectrophotometric assay was proposed after acid hydrolysis. Though, considering the entire sample set, differences between the contents of Htyr and tyr determined by HPLC and by Folin-Ciocalteu assay were reported as not significant, the levels observed for individual samples were in some cases relevant and could be misleading for their correct labelling.

In this framework, the use of NADES could provide feasible alternatives. The availability of a fast, cheap and green method to assess the content of specific phenolic classes could bring clear benefits to olive oil producers, retailers and consumers, bringing out of labs this analytical determination, and allowing to perform this determination on site, along the olive oil chain.

The present work was aimed at setting up an easy and green method for the determination of HTD in olive oils by means of a liquid/liquid extraction with a natural deep eutectic solvent and direct spectrophotometric analysis of the extract.

2. Materials and methods

2.1. Reagents and oil samples

Glucose (≥ 99.5%) and lactic acid (90%) were purchased from Sigma-Aldrich (Sigma-Aldrich Co. LLC, St. Louis, USA). Methanol (≥ 99.9%) and acetonitrile (≥ 99.9%) were purchased from Honeywell (Honeywell International, Inc., Morristown, NJ, USA) while acetic acid glacial (≥ 99.8%) from Carlo Erba (CARLO ERBA Reagents S.r.l., Cornaredo, Italy). All solvents were HPLC grade. Ultrapure water from an Elga Purelab Option R system (Veolia Environnement S.A., Paris, France) was used for preparing all solutions. Hydroxytyrosol (CAS 10597-60-1), tyrosol (CAS 501-94-0) and gallic acid (CAS 149-91-7) were purchased from Sigma Aldrich (Sigma-Aldrich Co. LLC, St. Louis, USA). Extra virgin olive oil (EVOO) samples (n = 163)

were obtained from producers and research laboratories. They differed for geographical origin, cultivar, olive maturity and extraction technology. Purified olive oil was obtained according to our previous works (Paradiso, Pasqualone, Summo, & Caponio, 2018).

2.2. NADES preparation

The NADES was obtained by mixing lactic acid, glucose and water (7:1:50 molar ratio), according to our previous work (Paradiso et al., 2016b), with a change in molar ratios to reduce solvent viscosity, by means of magnetic stirrer at 50 °C for about 90 min, until obtaining a clear solution.

2.3. Extraction with NADES

One g of oil was added with 1 mL of hexane and 5 mL of NADES. 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. Afterward, the lower layer (NADES plus phenolics) was recovered, centrifuged again at 9000 rpm for 5 min and finally filtered at 0.45 μm using nylon filters (VWR International, Center Valley, PA, USA).

2.4. Spectrophotometric analysis of NADES extracts

The NADES extracts were analysed in the wavelength range 200-400 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 NADES was used for zero correction.

2.5. HPLC analysis of HTD

Phenolic compounds were extracted according to (Caponio, Summo, Paradiso, & Pasqualone, 2014). The HPLC analysis of the phenolic extracts was performed using an UHPLC binary system (Dionex Ultimate 3000 RSLC,Waltham, MA, USA) equipped with a binary pump and a diode array detector (3000 RS). The stationary phase was an Acclaim 120 C18 analytical column (150 x 4.6 mm i.d.) with a particle size of 3 μm (Thermo Scientific,Waltham, MA, USA). The mobile phases were (A) water/ acetic acid (99:1, v/v) and (B) methanol/acetonitrile/acetic acid (50:49:1 v/v/v) at a constant flow rate of 1 mL min^{-1}. The column temperature was set at 30 °C. The gradient program was as follows: 1 min, 95% A; 10 min, 80% A; 22 min, 56% A; 32 min, 41% A; 46 min, 10% A. Diode array detection was monitored at 280 nm, and spectra were recorded at wavelength range 200–380 nm. The identification of phenolic compounds was performed by

comparing the peak retention times with those obtained by the injection of pure standards and, in absence of these, with data in literature. The quantification was achieved using gallic acid as internal standard and the response factor ratios (RFR) of Htyr and Tyr for free phenolic alcohols and their respective derivatives. The results are expressed as mg kg^{-1} of oil.

2.6. Statistical analysis

Spectra were preprocessed using Solo 8.6.2 (Eigenvector Research, Inc., Manson, WA USA), by smoothing with Savitzky-Golay algorithm (polynomial order: 2, window: 15 pt) and subtraction of background signal using the solvent spectrum. Before building regression and classification models, the sample set was divided into calibration and prediction sets (retaining 66% of the samples in the calibration set) using the Kennard-Stone algorithm. Least squares regression was performed with OriginLab2018. Support vector machine regression (SVM-R) and the SIMCA class-modelling were obtained with Solo 8.6.2. SVM-R was performed after autoscaling of spectra and dependent variable. Cross-validation of models was performed with venetian blinds with 10 splits and 1 sample per split. To assess the prediction quality of the regression models, the root mean square error of prediction (RMSEP) was considered.

3. Results and discussion

The contents of Htyr, Tyr and their derivatives in the EVOO samples, determined by HPLC, are plotted in Figure 1. Htyr was found in the range 0.0-34.0 mg kg^{-1}; the mean content of the 176 samples was 6.4 mg kg^{-1}, while median content was 4.9 mg kg^{-1}. Tyr ranged from 0.7 to 52.0 mg kg^{-1}, with mean and median contents respectively of 3.5 and 1.9 mg kg^{-1}. HTD were measured ranging from 24.2 to 614.5 mg kg^{-1}, with mean and median values of 195.7 and 164.9 mg kg^{-1} respectively.

Figure 2 reports the mean spectrum of the NADES extracts of the 163 EVOO samples, with the range of standard deviation. The most intense absorption was in the range 240-300 nm. Maximum absorption was recorded at 257 nm, with a shoulder at about 277 nm, and a minor peak at about 315 nm. Absorption at 257 nm was found in our previous work as the most correlated to total phenolic content in EVOO assessed by the Folin-Ciocalteu assay, while 277 nm was the wavelength of the peak absorption of phenyl-ethyl-alcohols (Paradiso et al., 2016b).

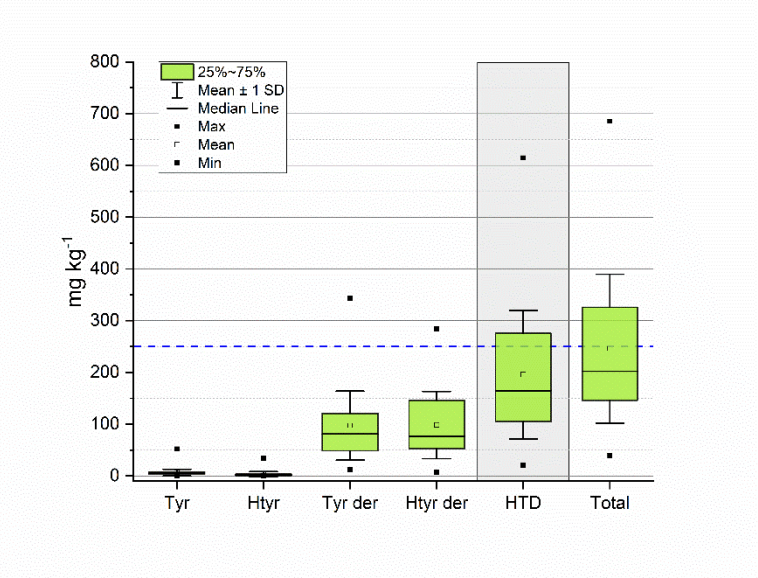


Figure 1 – Plot of contents of Htyr, Tyr and their derivatives in the EVOO samples, determined by HPLC (n = 163).

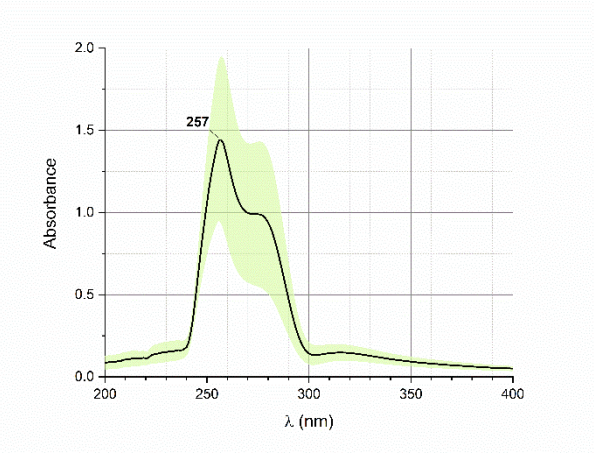


Figure 2 – Mean spectrum of the 163 EVOO samples NADES extracts. Green area corresponds to ± standard deviation of the absorbance at each wavelength.

Correlation analysis with HTD contents of the oil samples gave the results reported in Figure 3. Absorbance in the range 240-300 nm was significantly correlated with HTD in oils, but the highest correlation, with a r value of 0.89, was observed for the absorbance of the extracts at 283 nm, a wavelength near to that of maximum absorption of phenyl-ethyl-alcohols and to that of detection in the HPLC reference method.

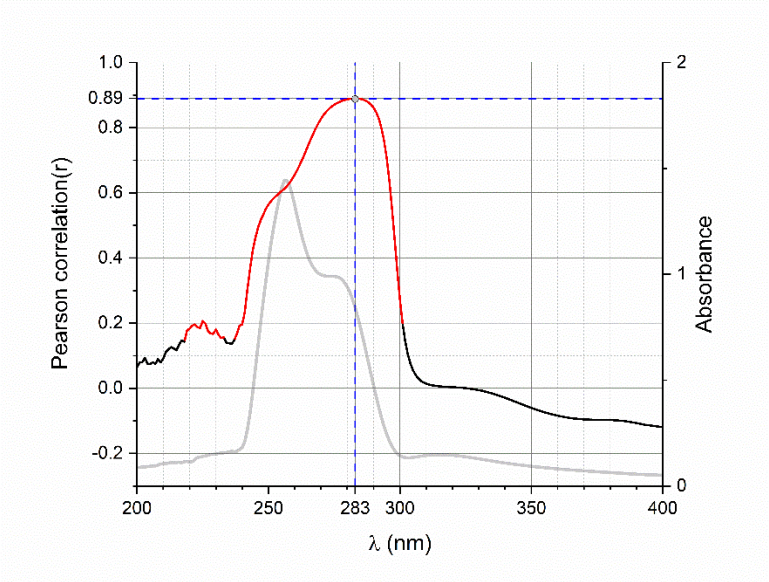


Figure 3 - Correlation (r) of the absorbance of the NADES extracts (n = 163) with HTD content in EVOO. Red line corresponds to significant correlation at p *< 0.05. The mean absorbance spectrum is reported in grey.*

Linear regression analysis provided a regression model with satisfactory screening effectiveness. The model equation was the following.

$$HTD\ (mg\ kg^{1}) = -47.2 + 285.7 * A_{283nm}$$

Adjusted R^2 in calibration, cross-validation and prediction was 0.82, 0.81 and 0.64 respectively, while root mean square error in calibration (RMSEC), cross-validation (RMSECV) and prediction (RMSEP) was 62.4, 63.4 and 58.7 respectively. Measured amounts are plotted versus predicted amounts in Figure 4.

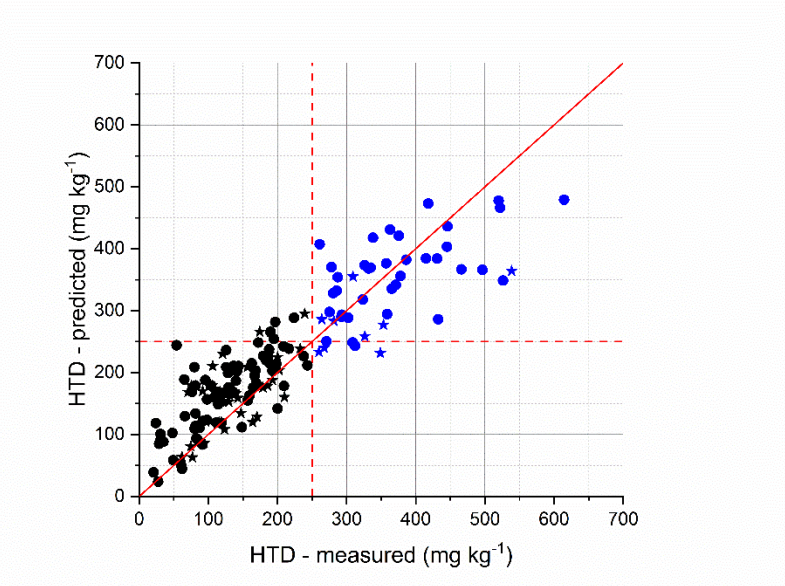


Figure 4 - Results of the linear regression fitting of HTD content as a function of absorbance of NADES extracts at 283 nm. Blue symbols correspond to samples containing > 250 mg kg^{-1} HTD. Circles correspond to calibration set (n = 109), stars correspond to prediction set (n = 54).

RMSEC, RMSECV and RMSEP showed comparable values, pointing out the robustness of the model and confirming in prediction the same fitting performances of calibration. According to the predicted HTD contents, 6 samples out of 109 (5.5%) in the calibration set and 5 out of 54 (9.2%) in the prediction set would be erroneously considered as eligible/not eligible for the health claim. These 11 samples showed HTD contents in the range 174-349 mg kg^{-1}.

Support vector machines (SVM), developed by Vapnik (Vapnik, 1999), are gaining popularity due to empirical performances that are more satisfactory respect to other approaches more prone to overfitting, like artificial neural networks. SVM regression was applied to the whole spectral dataset in order to obtain a model with better predictive capacity. The obtained model, indeed, presented the following values for adjusted R^2 in calibration, cross-validation and prediction: 0.98, 0.85 and 0.84 respectively. RMSEC, RMSECV and RMSEP were substantially reduced at 19.0, 51.6 and 35.5 respectively. Measured amounts are plotted versus predicted amounts in Figure 5. With the SVM regression model, no sample in the calibration set and 2 out of 54 (3.7 %) in the prediction set would be erroneously considered as eligible/not eligible for the health claim. Both samples presented HTD contents near the claim threshold (233 and 260 mg kg^{-1}).

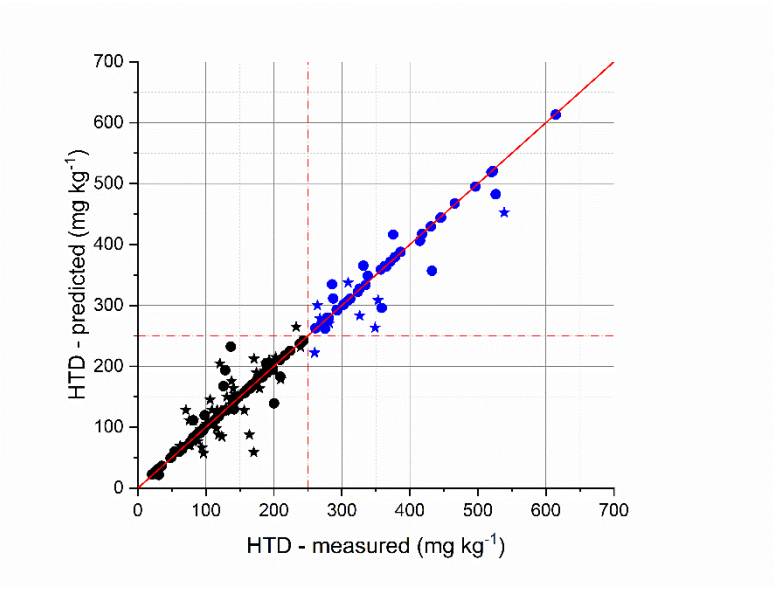


Figure 5 - Results of the SVM regression fitting of HTD content as a function of absorbance spectrum of NADES extracts (200-400 nm). Blue symbols correspond to samples containing > 250 mg kg^{-1} HTD. Circles correspond to calibration set (n = 109), stars correspond to prediction set (n = 54).

As an alternative approach, a class-modelling technique by means of Soft Independent Modelling of Class Analogy (SIMCA), was tested. The SIMCA model was built to classify oil samples according to two classes

respectively characterized by high and low HTD contents (i.e. above and below the threshold of 250 mg kg^{-1}). The obtained model assigned to each sample of both calibration and prediction sets a classification prediction probability for both classes. Figure 6 plots the calibration and prediction sample sets according to the assigned classification probabilities.

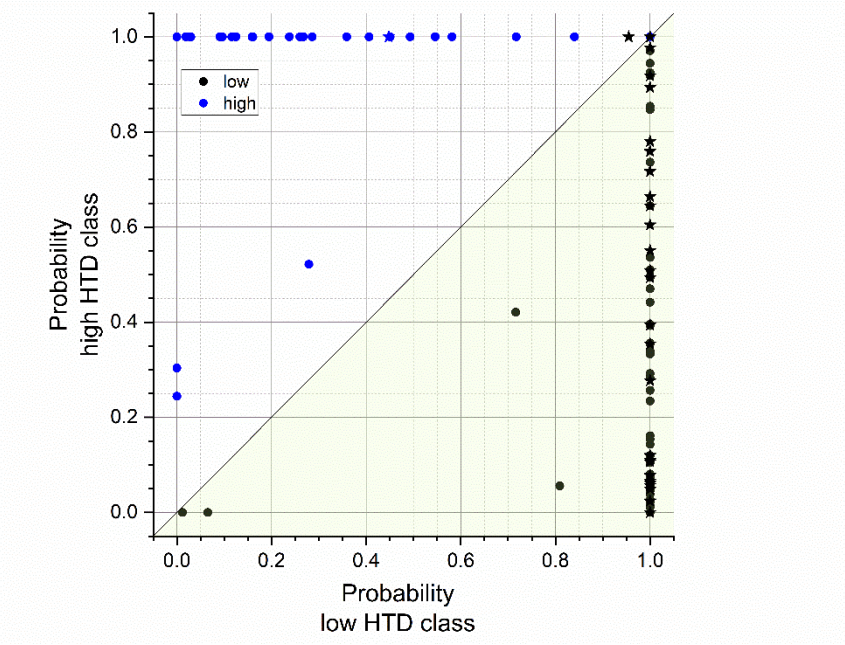


Figure 6 – Results of SIMCA modelling. Oil samples are plotted according to the assigned classification probabilities. Blue symbols correspond to samples containing > 250 mg kg^{-1} HTD. Circles correspond to calibration set (n = 109), stars correspond to prediction set (n = 54).

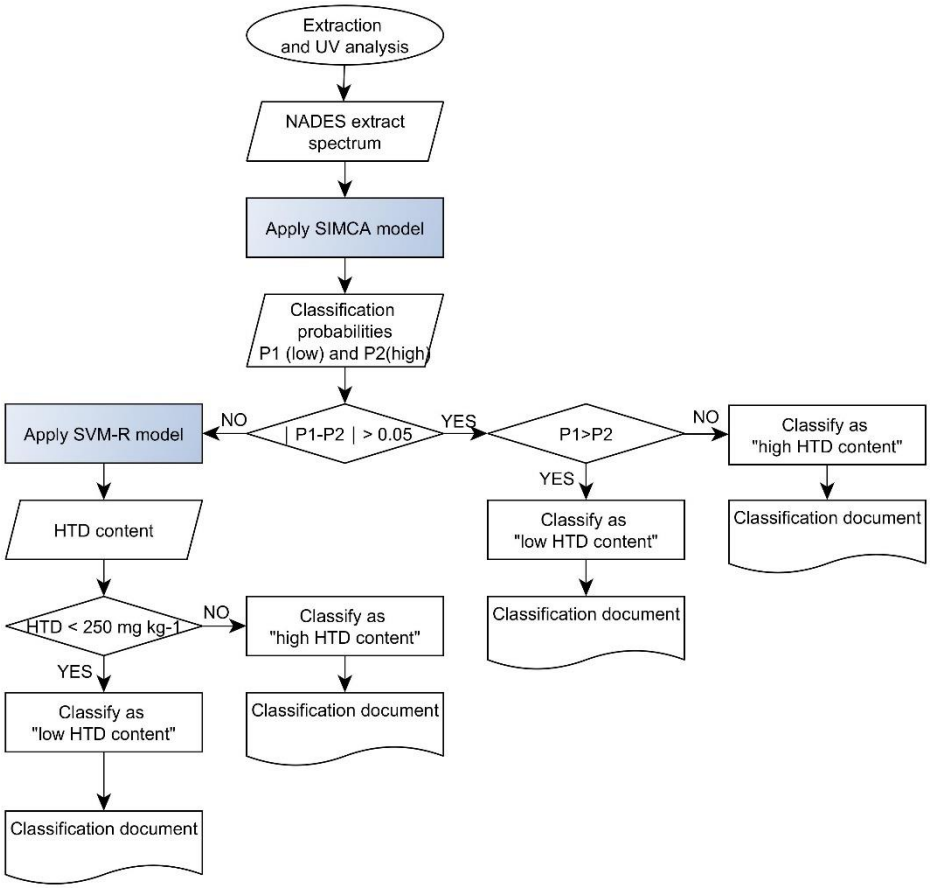
Samples were classified using the most probable classification approach, with a tolerance threshold of $|\Delta p| > 0.05$: samples were assigned to the class with the highest classification probability when the difference of probabilities was higher than 0.05 as absolute value. Using this approach, no sample was incorrectly classified; nevertheless, a large percentage of classification uncertainty was observed (22.9 % in the calibration set and 33.3 % in the prediction set).

As a final approach, a hierarchical model (HM) was developed with labelling aims (Figure 7).

According to the HM, the spectra of NADES extracts were processed by means of the SIMCA model, which assigned the classification probabilities to the oil sample. If the condition $|\Delta p| > 0.05$ was complied, the oil was assigned to the class with the highest classification probability and accordingly labelled. If the condition

206 $|\Delta p| > 0.05$ was not complied, the spectrum was processed using the SVM regression model. The oil was

207 then classified and labelled according to the HTD content predicted by the regression model.



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209 *Figure 7 - Hierarchical model for extra virgin olive oils classification and labelling according to hydroxytyrosol and tyrosol derivatives*
210 *(HTD) contents.*

211 The HM gave the best results in labelling the oil samples. In fact, all the oils belonging to the calibration set

212 were correctly labelled, while only one sample of the prediction set (containing 260 mg kg^{-1} HTD, an

213 amount quite near to the threshold) was erroneously labelled.

214 Table 1 reports a synopsis of the models developed and of their performances.

215 The present method shows an appreciable combination of features, being a fast and green method.

216 Recently, Mora-Ruiz et al. (Mora-Ruiz et al., 2017) proposed a method based on NIR spectroscopy to

217 directly assess the content of Htyr, Tyr and their secoiridoid derivatives. The obtained models for free Htyr

218 and Tyr and their derivatives (test set, n = 75; validation set, n = 18) presented R^2 of prediction in the range

219 0.55-0.84 and RMSEP around 5 mg kg^{-1} for free forms and above 40 mg kg^{-1} for derivatives.

220

Table 1. Synopsis of statistical models' performances [a]

		Linear regression (A_{283})	SVM regression ($A_{200-400}$)	SIMCA ($A_{200-400}$)	HM
Regression parameters					
R^2 cal		0.82	0.98	-	-
R^2 cv		0.81	0.85	-	-
R^2 pred		0.64	0.84	-	-
RMSEC (mg kg^{-1})		62.4	19.0	-	-
RMSECV (mg kg^{-1})		63.4	51.6	-	-
RMSEP (mg kg^{-1})		58.7	35.5	-	-
Labelling results					
Calibration	Accuracy (%)	94.5	100	77.1	100
	Error (%)	5.5	0	0	0
	Uncertainty (%)	-	-	22.9	-
Prediction	Accuracy (%)	90.8	96.3	66.6	98.2
	Error (%)	9.2	3.7	0	1.8
	Uncertainty (%)	-	-	33.3	-

[a] $n_{calibration}$ = 109; $n_{prediction}$ = 54; RMSEC, root mean square error of calibration; RMSECV, root mean square error of cross-validation; RMSEP, root mean square error of prediction.

Inarejos-García et al. (Inarejos-García, Gómez-Alonso, Fregapane, & Salvador, 2013) used NIR spectroscopy to determine individual phenolic compounds in olive oils. On a sample set of 97 oils (divided into calibration ad prediction sets), Htyr derivatives could be determined in the prediction set with a RMSEP of 25.5 mg kg^{-1}. Similarly, Uncu and Ozen (Uncu & Ozen, 2015) used FTIR spectroscopy to obtain PLSR models (n = 64). Model parameters for cross-validation (no prediction on a test set was reported) were the following for Htyr and total phenolic compounds, respectively: R^2 = 0.68 and 0.74, RMSECV = 4.66 and 45.26 mg kg^{-1}. Mid- and near-infrared spectroscopy have the advantage to be directly performed on oil samples, without involving any preparation step. On the other hand, they require expensive equipment. The present approach, though requiring a minimum extraction step, requires cheaper equipment and provides comparable predictive performances for HTD contents and an effective labelling capacity.

4. Conclusions

A fast and green tool for olive oils labelling according to the contents of hydroxytyrosol and tyrosol derivatives. A simple liquid-liquid extraction, using a natural deep eutectic solvent based on glucose and lactic acid, followed by direct spectrophotometric analysis allowed to determine with a mean error of 35.5

mg kg^{-1} the content of these bioactive compounds and allowed to correctly label the 98.2 % of olive oils according to the legal requirements for the health claim. The method proposed is fast and easy and requires cheaper equipment in comparison with other methods.

Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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