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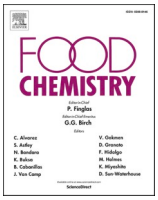

Using fluorescence excitation-emission matrices to predict bitterness and pungency of virgin olive oil: A feasibility study

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ABSTRACT

Unlike other food products, virgin olive oil must undergo an organoleptic assessment that is currently based on a trained human panel, which presents drawbacks that might affect the efficiency and robustness. Therefore, disposing of instrumental methods that could serve as screening tools to support sensory panels is of paramount importance. The present work aimed to explore excitation-emission fluorescence spectroscopy (EEFS) to predict bitterness and pungency, since both attributes are related with fluorophore compounds, such as polar phenols. Bitterness and pungency intensities of 250 samples were provided by an official sensory panel and used to build and compare partial least squares regressions (PLSR) with the excitation-emission matrix. Both PARAFAC scores and two-way unfolded data led to successful PLSR. The most relevant PARAFAC scores agreed with virgin olive oil phenolic spectra, evidencing that EEFS would be the fit-for-purpose screening tool to support the sensory panel.

1. Introduction

Virgin olive oil is defined as the product obtained from the fruit of the olive tree (*Olea europaea* L.) exclusively by mechanical or physical means (IOC/T.15/NC No 3/Rev. 16, 2021). Besides the corresponding chemical-physical parameters (Commission Regulation (EEC) No 2568/91 and its amendments), unlike other food products, it must undergo a sensory assessment in order to be classified within a commercial category: extra virgin olive oil (EVOO), virgin olive oil (VOO) and lampante olive oil (LOO, not edible). Such organoleptic evaluation is conducted by a trained panel following the International Olive Council (IOC) official method (IOC/T.20/Doc. No 15/Rev. 10, 2018), where positive and negative sensory attributes of this valuable food product are rated. Fruity, bitter and pungent are the main positive features, while the list of sensory defects is larger, including fusty/muddy sediment, winey/vinegar or rancid. It should be noted that, among these attributes, just the median intensity of fruitiness (Mf) and of the main perceived defect (Md) are considered for quality grading: Mf should be greater than zero for both EVOO and VOO; Md must be equal to zero for EVOO and ≤ 3.5 for VOO, while for higher Md values, the oil is classified as LOO. Many authors agreed on the controversies associated to the panel test, conducted by a human panel, especially in terms of efficiency and robustness, pointing out the need of setting up a supporting instrumental tool for sensory evaluation (Aparicio-Ruiz et al., 2019; Barbieri, Bubola, et al., 2020; Conte et al., 2020). Since Mf and Md are olfactory perceived attributes, the methods aiming sensory quality grading have been generally based on the analysis of volatile organic compounds (VOC) by different techniques, providing satisfactory results (Barbieri et al., 2020; Contreras, Jurado-campos, & Arce, 2019; Quintanilla-Casas et al., 2020; Sales, Portolés, Johnsen, Danielsen, & Beltran, 2019; Valli et al., 2020; Vega-Márquez, Nepomuceno-Chamorro, Jurado-Campos, & Rubio-Escudero, 2020).

Even though bitter and pungent attributes are not considered for virgin olive oil commercial classification, the regulation allows to state

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their intensity in the label with specific terminology e.g., robust, medium, delicate, well balanced and mild oil (Commission Regulation (EEC) No 2568/91 and its amendments). The above-mentioned instrumental tools based on the aroma profiling are not the best choice to measure bitterness and pungency because these attributes are not perceived through olfactive but tasting receptors, which are stimulated by non-volatile molecules such as polar phenolic compounds (Andrewes et al., 2003; Bendini et al., 2007). Most research regarding olive oil polar phenols, commonly called polyphenols when referred to virgin olive oil, pursued to improve their detection and quantification in view of their health benefits as well as antioxidant properties. Some studies also aimed at the objective measurement of bitter and pungent attributes in virgin olive oils. One of the first studies facing this issue proposed a method based on the absorbance at 225 nm of the olive oil polar extract (Gutiérrez Rosales et al., 1992). Apart from some analytical drawbacks that other authors intended to overcome (Beltrán et al., 2007; Favati et al., 2013; Gutiérrez-Rosales et al., 2003), the main issue of this method was the error due to the fact that some polar compounds not linked to bitter nor pungent notes, such as elenolic acid, also contributed to the absorbance at this wavelength (Inarejos-Garcia et al., 2009). As Inarejos-Garcia et al. (2009) pointed out, this source of error was eliminated when bitterness was evaluated by fluorimetry because the interfering compound does not fluoresce. Based on these results, fluorescence detectors have been used coupled to High Performance Liquid Chromatography (HPLC) for the analysis of polar phenols and the subsequent bitterness prediction (Favati et al., 2013; Inarejos-Garcia et al., 2009). These methods involve sample preparation as well as time-investing data extraction since they are based on a targeted approach. Bitter and pungent notes are elicited by different secoiridoid derivatives known as oleuropein, related with bitterness, and oleocanthal, perceived as pungent (Servili et al., 2009). For this reason, in this context, following a targeted analysis usually requires large sample preparation to extract the mentioned compounds of interest (Demopoulos et al., 2015; Gutiérrez-Rosales et al., 2003; Mateos et al., 2004). Moreover, further identification and quantification is needed.

Disposing of a fast and efficient analytical method to measure bitter and pungent attributes in virgin olive oil becomes a need to support the sensory panel. We hypothesize that fluorescence spectroscopy as such could be the fit-for-purpose tool, given that it is selective, fast and solvent free. This technique has been applied to olive oil for other purposes, such as characterization (Guimet, Ferre, et al., 2005; Lia et al., 2020), authentication (Al Riza et al., 2019; Durán Merás et al., 2018; Guimet et al., 2004; Guimet, Ferre, et al., 2005), discrimination from other edible oils (Poulli et al., 2005; Sikorska et al., 2004; Sikorska et al., 2005) and even to assess deterioration (Tena et al., 2012), but it has not been explored yet for bitterness and pungency measurement. Some of the previous studies applied excitation-emission fluorescence spectroscopy (EEFS) instead of selecting a single excitation wavelength, without a previous separation step (Durán Merás et al., 2018; Guimet, Ferre et al., 2005; Lia et al., 2020). The EEFS allows the acquisition of emission spectra at several excitation wavelengths, obtaining the so-called excitation-emission matrix (EEM), providing comprehensive information regarding the multiple fluorophores present in olive oil (Sikorska et al., 2012), but it requires multivariate, and/or even multiway, methods to be analysed.

Apart from fluorescence, other techniques have been explored to obtain an objective measure of bitterness and/or pungency, such as biosensors (Busch et al., 2006) or electrochemical methods based on amperometric detection (Morozova et al., 2016). However, these techniques seemed to be more suitable for total phenols measurement than for the prediction of the related sensory attributes. On the other hand, Borràs et al. (2016) assayed three instrumental techniques (headspace-MS, FT-MIR and UV–Vis spectroscopy) aiming at developing an electronic panel test. Even if this was suitable for a global organoleptic assessment, more than one technique was still needed to achieve a suitable prediction for bitterness or pungency.

Therefore, the current research aims to explore the feasibility of employing EEFS to develop prediction models for bitter and pungent attributes of virgin olive oil samples, in order to dispose of an efficient screening tool that can support the sensory panel.

2. Material and methods

2.1. Virgin olive oil samples

A sample set of 255 virgin olive oils (152 EVOO, 98 VOO and 5 LOO), produced along the 2019–2020 crop season, were provided by the Institute of Agrifood Research and Technology (IRTA). The oils were produced in Spain, the majority of them in Catalonia; hence, the sample set included different olive cultivars typical of that geographical area. Until analysis, oil samples were stored at −18 ℃ in glass vials with N_2 atmosphere filling the headspace.

2.2. Virgin olive oils organoleptic assessment

The sensory profile of all samples was assessed and graded into the corresponding commercial categories (EVOO, VOO and LOO) according to the IOC official procedure (IOC/T.20/Doc. No 15/Rev. 10, 2018) by the *Panell de Tast Oficial d'olis Verges d'oliva de Catalunya*. Median intensities for bitter and pungent attributes were determined, ranging from 1.7 to 6 and from 2.6 to 5.9, respectively, using a 10 cm open scale. Bitter and pungent intensities of each sample, as well as their commercial categories, are available in Table S1 (Supplementary information). Besides, as any other kind of official control method, quality control was performed in order to prove that results were reliable. In this specific case, quality control of tasters' performance was carried out according to the IOC guidelines (IOC/T.20/Doc. No 14/Rev. 7, 2021). Furthermore, the panel was certified by ISO 17025 and was officially recognized by IOC and EU. Inter-day repeatability results, consisting of 16 duplicate analysis performed along the year 2019, were provided by the panel for the sensory properties of interest.

2.3. Sample analysis by excitation-emission fluorescence spectroscopy (EEFS)

Undiluted virgin olive oils were directly measured in a 10 mm quartz cuvette by front-face EEFS with an angle of 45° and an inclination of 35.9°. The measurements were performed on an FS920 Edinburgh Instruments fluorescence spectrophotometer (Livingston, Scotland, UK) equipped with a Xenon arc lamp as light source (Xe900), a single photon counting detector (Red sensitive photomultiplier, S900), and two Czerny-Turner monochromators (TMS300). The instrument was connected to a temperature control unit (FL300 Recirculating Cooler, Julabo GmbH, Seelbach, Germany) that was set to 20 °C for all measurements.

In order to optimize the EEM acquisition time, two blocks where fluorescence signal was obtained were defined: the low region comprised a measurement range of λ_{ex} = 260–380 nm and λ_{em} = 290–570 nm, while the range for the high region was λ_{ex} = 340–460 nm and λ_{em} = 640–700 nm; with intervals of 5 nm and 1 nm for excitation and emission, respectively. The dwell time was set to 0.01 s/nm, and the slits in both directions were set to 5 nm.

2.4. EEM pre-processing

Artifacts in the signal typically related to EEFS data were handled with Matlab software R2020b®: EEM landscapes included random signal spikes caused by cosmic rays, Rayleigh scattering (2nd order, $\lambda_{em} = 2*\lambda_{ex}$) and a region ($\lambda_{em} \leq \lambda_{ex}$) filled with missing values (Fig. 1a). Signal spikes were automatically detected by calculating the first derivative of emission spectra (Fig. 1b), values above thresholds ($2e^4$ and $7e^4$ for low and high regions, respectively) were replaced by the mean of

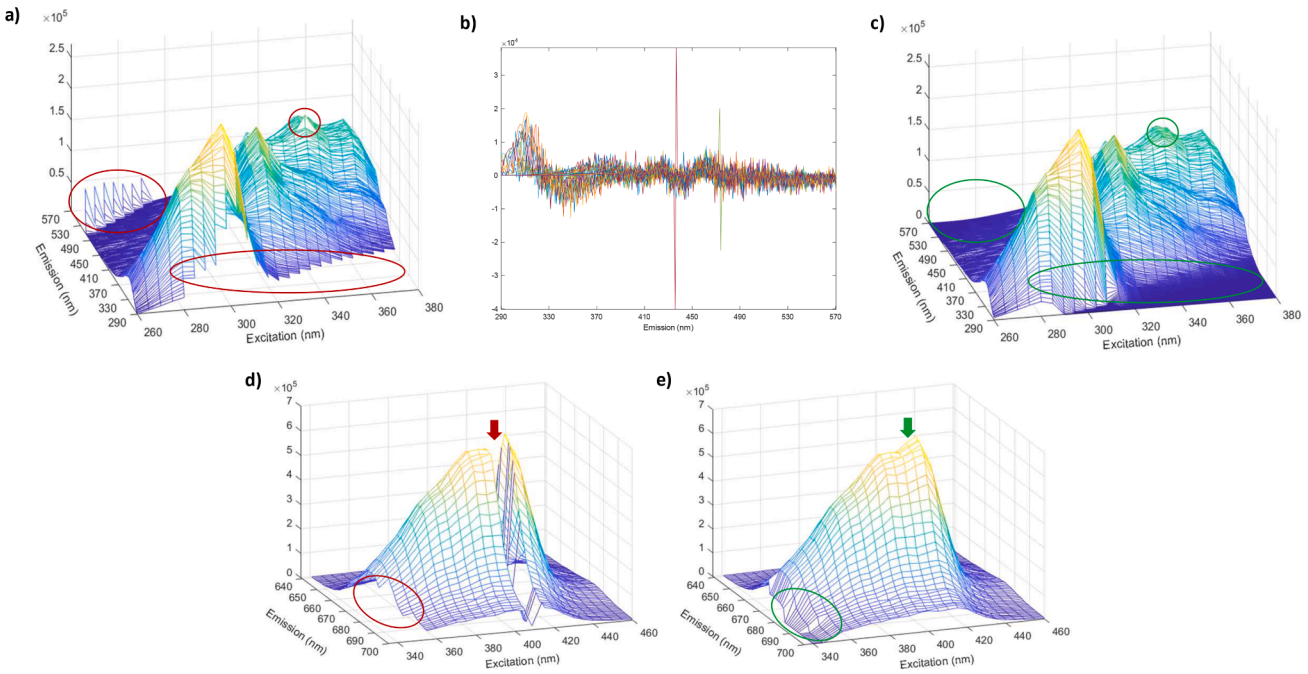


Fig. 1. EEM pre-processing. a) Raw EEM landscape (low region) presenting signal spikes, Rayleigh scattering and missing values for $\lambda_{em} \leq \lambda_{ex}$, b) 1st derivative of emission spectra to detect and remove spikes, c) Pre-processed EEM landscape (low-region) without signal issues, d) Raw EEM landscape (high region) with wrong emissions for a certain λ_{ex} and missing values for $\lambda_{em} = 2^{*}\lambda_{ex}$, and e) Pre-processed EEM landscape (high region) without signal issues.

the emission wavelength on both sides of the spike (Fig. 1c). The *flucut* function from PLS_Toolbox for Matlab (Eigenvector Research Inc, 2019) was applied to solve the remaining issues by interpolation (Fig. 1c). It is important to highlight that, even though a fluorophore cannot physically emit light of higher energy than the excitation source, replacing the area close to the diagonal ($\lambda_{em} \leq \lambda_{ex}$) with interpolated values instead of zeros is necessary to later develop three-way models. Finally, emission signals at certain λ_{ex} were not correctly acquired for

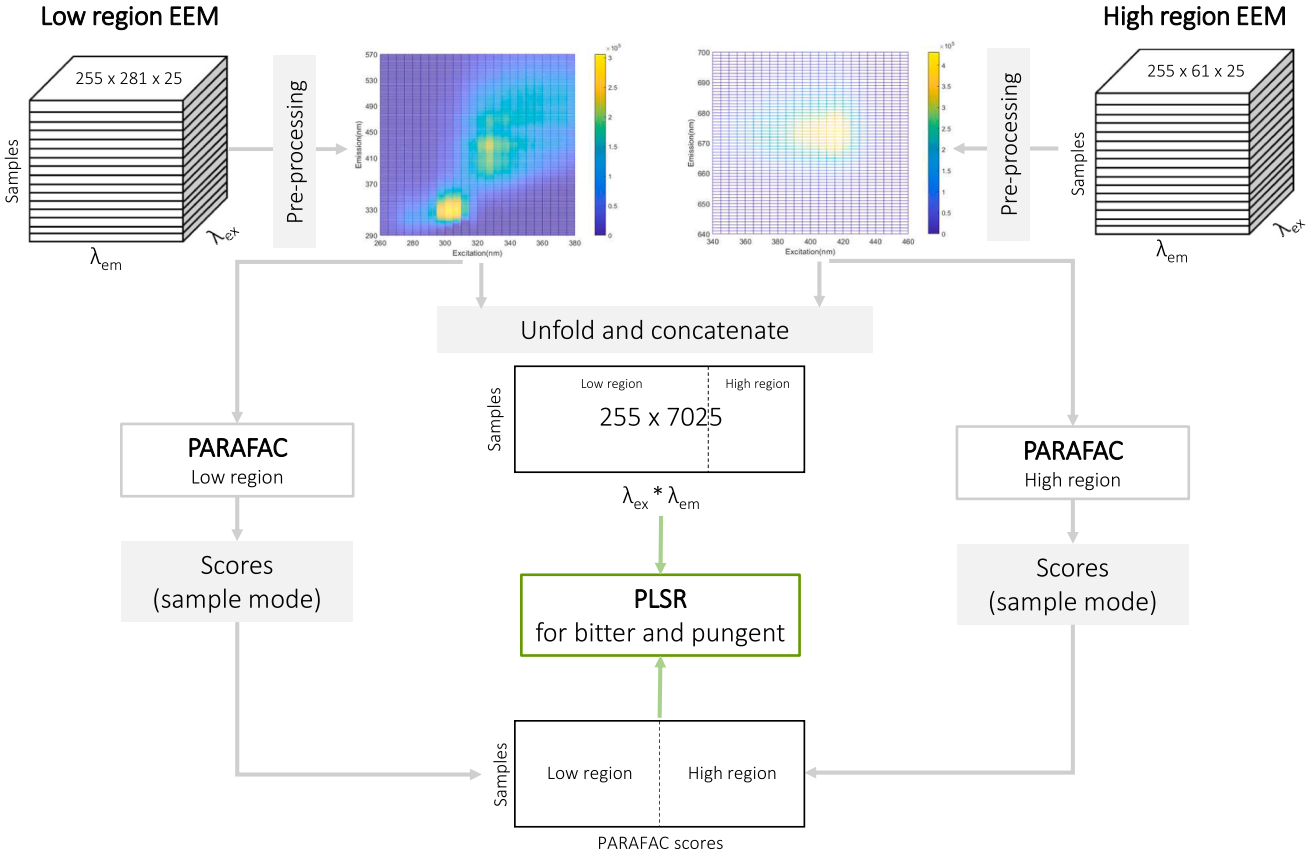


Fig. 2. Data treatment workflow.

some samples, because of shutter problems (Fig. 1d); these values were set to missing and subsequently replaced by interpolation by means of the already mentioned *flucut* function (Fig. 1e). This step also allowed to correct the area where $\lambda_{em} = 2^*\lambda_{ex}$ with interpolation values.

2.5. Regression models

Even though EEM forms a three-way array (samples × emission × excitation), bilinear PLSR was carried out using PLS_Toolbox for Matlab from Eigenvector Research Inc. (Manson, WA, USA) starting from two different scenarios (Fig. 2): i) PLSR on the extracted scores of parallel factor analysis (PARAFAC) applied on the three-way array (EEM), and ii) PLSR on two-way data obtained by unfolding the EEM. The workflow of the data treatment explained in the following sub-sections (2.5.1 and 2.5.2) is shown in Fig. 2. PLSR was selected over other regression tools to develop prediction models for bitter and pungent attributes of virgin olive oils, because it deals with a large number of variables that are likely to be correlated (Wold et al., 2001). PARAFAC models were investigated because a successful prediction model based on scores from PARAFAC could be more easily interpretable chemically and this would make the model more transparent and explainable on a chemical level (Andersen & Bro, 2003). On the other hand, the unfolding model was investigated as it allows using additional information e.g., arising from deviations from Beers law which PARAFAC would often struggle to capture.

2.5.1. PLSR from three-way data: PARAFAC scores

The PARAFAC model has been widely employed for analysing and interpreting fluorescence data. It is particularly useful for EEM decompositions because a successful model provides emission and excitation loadings that can be considered actual estimates of the emission and excitation spectra of each fluorophore (Andersen & Bro, 2003). Further, the scores will then be estimates of the relative concentrations of these fluorophores. PARAFAC models from one to ten components were fitted independently for low and high region EEMs to investigate which was the appropriate number of components. Non-negativity constraint was applied to all modes to avoid unrealistic results that would lead e.g. to negative concentrations.

Scores for the sample mode extracted from the selected PARAFAC models, from both the high and low regions, were used to calibrate PLSR to predict bitterness and pungency. Regression models were cross-validated by venetian blinds with 10 splits, selecting the optimum number of latent variables (LV) according to the lowest root mean square error in cross-validation (RMSEcv). Mean centering was set as pre-processing. Samples with high sum of squared residuals (Q) and/or Hotelling T^2 were considered outliers and, therefore, were removed from the dataset. Finally, PARAFAC loadings for emission and excitation were explored to investigate which compounds were more relevant in regards of bitter and pungent prediction.

2.5.2. PLSR from two-way data: Unfolded EEM

EEM (excitation × emission × samples) for low (25 × 281 × 255) and high (25 × 61 × 255) regions were individually unfolded, resulting in two-way matrices of 7025 and 1525 variables, respectively (Note that measurements below the 1st order Rayleigh scatter line, and above the 2nd order Rayleigh scatter line were removed during the unfolding procedure). The two matrices were subsequently concatenated resulting in a final unfolded matrix **X** (255 samples × 8550 variables) and a response vector **y** for each attribute (bitter or pungent intensities for 255 samples). PLSR models for each sensory attribute were calibrated and cross-validated (venetian blinds with 10 splits). Mean centering was applied as pre-processing. The lowest RMSEcv provided by the PLSR served to select the optimum number of LVs. Outliers were identified by high sum of squared residuals (Q) and/or Hotelling T^2 and were removed from the dataset. Regression vectors of each PLSR model were examined after re-folding them back to EEMs in order to ease their interpretation.

3. Results and discussion

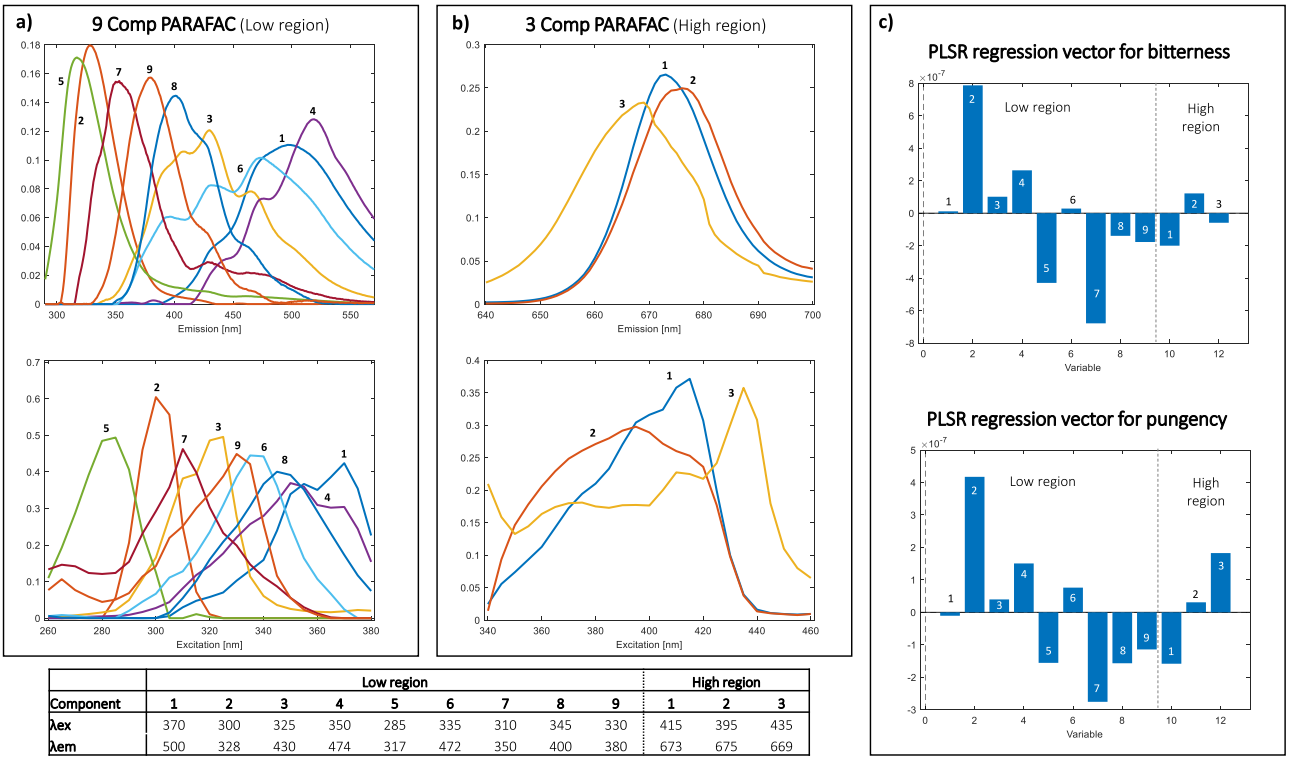
PARAFAC models from one to ten components were fitted for both low and high regions, independently (Supplementary information, Table S2). Since the present study focused on achieving optimal predictions for bitter and pungent attributes in virgin olive oils, the number of PARAFAC components was selected according to the best prediction results. Therefore, PLSR was applied on scores of several combinations of PARAFAC models as follows: components of high region were added to the low region PARAFAC models (starting from 2 components) until the prediction did not improve (Supplementary information, Table S3). As a result, nine components from the low region and three components from the high region were selected as optimal for developing the corresponding regression models. Thus, PARAFAC scores PLSR for bitterness (7 LVs) provided a RMSEcv of 0.6, while for pungency (5 LVs) the RMSEcv was 0.4 (Table 1). Conversely, the PLSR built with the unfolded EEM provided better prediction results for bitterness (RMSEcv for bitter = 0.5, 9 LVs) while pungent prediction did not improve (RMSEcv for pungent = 0.4, 9 LVs) (Table 1). The regression vectors of the PLSR based on the PARAFAC scores provided a straightforward way of assessing the most important fluorophores in prediction, compared to those obtained from the unfolded matrix (Supplementary information, Figure S1). Loadings for emission and excitation spectra of the selected PARAFAC components for regression are shown in Fig. 3. According to the available literature (Baltazar et al., 2020; Eitenmiller et al., 2008; Lia et al., 2020; Sikorska et al., 2012), compounds presenting fluorescence at low region would mainly correspond to polar (polyphenols) and non-polar (tocopherols and tocotrienols) phenols, as well as to some oxidation products (Fig. 3a), while in the high region, chlorophylls were found as the main contributors to the excitation and emission spectra (Fig. 3b). The PLSR regression vectors (Fig. 3c) indicated that fluorophores corresponding to components 2, 5 and 7 were particularly predictive in both bitterness and pungency regression models and, according to their early emission maximum, they could correspond to polar phenolic compounds (Sikorska et al., 2012). Chlorophylls (component 1 and 3, high region) also seemed to carry some information in the case of pungency prediction. There is no evidence in literature that chlorophylls play a role in the pungency tasting mechanism, so we assume they provide information regarding agronomic conditions such as ripening degree of olive fruit or hydric state of olive tree, that are generally correlated with the total polar phenols content (Bengana et al., 2013).

Given that polyphenols are the only compounds whose relationship with bitterness and pungency of virgin olive oil has been proven (Andrewes et al., 2003; Inarejos-Garcia et al., 2009; Servili et al., 2009), regression models were built based on the region where these compounds fluoresce, following the same strategy explained in section 2.5 for full spectra. Since fluorescence spectra of polar and non-polar phenols are sometimes overlapped, the whole block corresponding to the low region has been considered. As found when the whole fluorescence spectrum was considered, prediction results for bitterness were slightly better in case of PLSR models based on the unfolded EEM (RMSEcv = 0.5, 8 LVs) than in those based on the PARAFAC scores (RMSEcv = 0.6, 7 LVs), while prediction of pungent attribute remained equal (RMSEcv = 0.4, PARAFAC scores PLSR with 6 LVs and unfolded EEM PLSR with 8 LVs) (Table 1). Given the obtained results, the measurement of the low region fluorescence spectra seems to provide enough information to predict both bitterness and pungency in virgin olive oils, leading to a decrease of the total analysis time. Concerning the chemometric approach, regression models built from the unfolded EEM as well as the PARAFAC scores provided satisfactory results. In this case, bitterness prediction was enhanced by the unfolded EEM PLSR, however, the fact that regression models based on the PARAFAC scores are rather interpretable would make them the wiser choice.

Table 1
Cross-validation results (venetian blind, 10 splits) for bitterness and pungency prediction in virgin olive oils. PLSR models built with the whole fluorescence spectra (low and high region) and only with the low region, from the PARAFAC scores as well as the unfolded EEM. Results from quality control of sensory panel are also provided.

	Whole fluorescence spectra						Low region fluorescence spectra						Sensory panel
	PARAFAC scores PLSR			Unfolded EEM PLSR			PARAFAC scores PLSR			Unfolded EEM PLSR			
	n[a]	LV	RMSEcv	n[a]	LV	RMSEcv	n[a]	LV	RMSEcv	n[a]	LV	RMSEcv	RMSE[b]
Bitter	225	7	0.6	226	9	0.5	224	7	0.6	222	8	0.5	0.5
Pungent	222	5	0.4	224	9	0.4	221	6	0.4	220	8	0.4	0.4

[a] Dataset without outliers.
[b] Results from duplicate analyses performed along the year 2019 (n = 16).



	Low region									High region		
Component	1	2	3	4	5	6	7	8	9	1	2	3
λex	370	300	325	350	285	335	310	345	330	415	395	435
λem	500	328	430	474	317	472	350	400	380	673	675	669

Fig. 3. a) PARAFAC model for low region (9 components), b) PARAFAC model for high region (3 components), and c) Regression vectors for bitterness and pungency from PLSR built from PARAFAC scores.

Generally, reference values (**y** response) used for developing regression models are obtained through the corresponding official method of analysis. In this case, **y** responses (bitter and pungent intensities) came from the sensory panel test, which is currently the official method. As previously mentioned, this method may present some drawbacks. Some authors highlighted the importance of considering quality control results when developing the corresponding supporting tools based on panel's data (Borràs et al., 2016); even so, this information is rarely taken into account. In the present study, performance results from the panel were considered for both sensory attributes. The precision of the panels as judged from inter-day replicates, expressed as RMSE, was 0.5 for bitterness and 0.4 for pungency (Table 1). Since the reference values from the panels carried these known errors, they would be the lower limits for the observed error in the PLS model. That is, if the PLSR model was perfect, we would still observe a RMSEcv of approximately 0.5 for bitterness as this is the error of the reference that we cannot avoid. Even considering that cross-validation outcomes can be more optimistic than external validation results, this finding indicated the panel test as the main source of deviation (Table 1), especially regarding pungency prediction. In fact, previous studies pointed out the influence of the dynamic perception on the sensory assessment of bitter and pungent attributes in virgin olive oils. Due to different chemoreceptors involved, the time-intensity curves for these attributes show different behaviour, entailing 10 s delay between the maximum perception of bitterness and pungency. Thus, some underestimation may occur when the tasting time is insufficient to reach the maximum intensity of pungency (Esti et al., 2009). Nevertheless, as this is a feasibility study, further data from EEFS measurements as well as sensory panel quality control would be needed in order to prove the abovementioned findings.

In regard to alternative tested methods, biosensors based on tyrosinase or peroxidase showed significant correlation with total polar phenolic composition (Busch et al., 2006). Nevertheless, even if measurements with the peroxidase-based biosensor seemed to be somehow correlated with pungency, the overall correlation with virgin olive oil sensory attributes was not satisfactory enough. On the other hand, the electrochemical method developed by Morozova et al. (2016) found the sensors poised at + 0.4 V to show the highest correlation with bitterness intensity. Still, regression models for bitterness based on PLS did not achieve good prediction results even when several variables (two variables from amperometric sensors at + 0.4 V and + 0.9 V and total phenol values) were used. In view of the results, these analytical tools could be

promising as alternative methods for total phenols measurement, but not really suitable for bitter and pungent prediction. Finally, the electronic panel test suggested by Borràs et al. (2016) showed satisfactory prediction results for both bitter and pungent attributes, when low-level data fusion on e-nose (headspace – MS) and e-tongue (FT-MIR) data was applied, but not when they were independently assessed. Therefore, due to the analytical efforts needed, this method would be more appropriate for a comprehensive sensory description than for predicting just bitterness or pungency.

4. Conclusions

In view of the results, EEFS has proven to be a suitable tool for bitterness and pungency prediction of virgin olive oils and could become the fit-for-purpose screening tool to support the panel. The spectra corresponding to the phenolic fraction (low region) seemed to provide enough information for the sensory attributes' prediction. Hence, the analysis time will be reduced from ten minutes needed to acquire the whole EEM to barely seven minutes. Besides, olive oil samples are directly measured, meaning that no time nor solvent is invested in sample processing or extraction. PLSR from both scenarios, the unfolded EEM and the PARAFAC scores, provided similar prediction results. However, building regression models from the PARAFAC scores seemed to be more convenient given the chemical interpretability of the outcome, than using the unfolded EEMs. In spite of the satisfactory results, also considering the error of panel's performance, further research is needed in order to obtain robust regression models. Besides, a greater number of virgin olive oils samples with a wider range of the attributes of interest would be worth to be included, as well as to carry out an external validation of future regression models.

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CRediT authorship contribution statement

Beatriz Quintanilla-Casas: Formal analysis, Investigation, Methodology, Validation, Data curation, Visualization, Writing – original draft. **Åsmund Rinnan:** Investigation, Validation, Writing – review & editing. **Agustí Romero:** Resources, Writing – review & editing. **Francesc Guardiola:** Supervision, Writing – review & editing. **Alba Tres:** Methodology, Conceptualization, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Stefania Vichi:** Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Rasmus Bro:** Conceptualization, Methodology, Validation, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133602.

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