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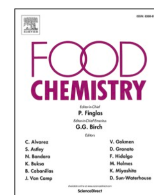

Analytical Methods

Accurate determination of total biophenols in unfractionated extra-virgin olive oil with the fast blue BB assay

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ABSTRACT

The phenolic compounds of extra-virgin olive oil (EVOO) are key contributors of nutritional and sensory quality as well as chemical stability. The reference method for their determination is the HPLC-UV, which is cost-/time-expensive.

In this work, total phenolic compounds were evaluated in unfractionated EVOO adapting the Fast Blue BB (FBBB) assay, which involves the spectrophotometric (absorbance at 420 nm) determination of azo derivatives resulting from the coupling of phenolic compounds with FBBB diazonium salt in alkali pH. When tested on 26 EVOO samples, the FBBB assay and HPLC-determinations were strikingly correlated ($R^2 = 0.9653$), differently from FBBB and Folin-Ciocalteu assays, which showed poor correlation.

The assay is simple, repeatable, robust, rapid and cheap, and results might be evaluated on a printed colorimetric scale. This protocol of the FBBB assay could be routinely used to categorize EVOO according to the health claim allowed by EFSA concerning the content of phenolic compounds.

1. Introduction

The current differentiation of extra-virgin olive oil (EVOO) from lower quality olive oil categories is substantially based on the determination of free acidity, peroxide value and spectrophotometric data (i. e. K_{232}, K_{270}, ΔK), as well as on the evaluation of sensory traits by trained taste panels (European Commission Regulation (ECC) No. 2568/91). These parameters alone are largely insufficient to define "quality" and to rank the virtually unlimited variety of EVOO globally traded and marketed. The European Food Safety Agency (EFSA) has approved four health claims to label EVOO, thereby emphasizing the human health benefits associated with olive oil consumption. Three of these health claims are not specific to EVOO and could be allowed also for other edible oils including non-virgin or refined olive oils, as they concern: i) the high content of oleic acid; ii) the oil as a source of vitamin E; and iii) the relatively high amounts of mono- and/or poly-unsaturated fatty acids (Regulation, (EU), and No. (EU) No. 432/(2012)).

The phenolic compounds of EVOO, also referred to as "biophenols", are responsible of many among the health-promoting effects attributed to EVOO, mainly related to their antioxidant and radical scavenger properties (Gorzynik-Debicka et al., 2018; Lockyer & Rowland, 2014; Martín-Peláez, Covas, Fitó, Kušar, & Pravst, 2013). Phenolic compounds, which vary within a considerably wide range of concentrations (50–1000 mg kg^{-1}) in EVOO depending on olive genotype, elaiotechnical practices, process of oil extraction and storage conditions (Jimenez-Lopez et al., 2020), contribute in a decisive way to the characteristic fruitiness, bitterness, pungency and astringency of EVOO, as well as to oil stability against oxidation (Pedan, Popp, Rohn, Nyfeler, & Bongartz, 2019). Therefore, the concentration of phenolic compounds is a key parameter to sub-segment high-quality oils within the EVOO category because it provides simultaneously a valuable index of nutritional, chemical and sensory quality.

In agreement with the EFSA indications, the Commission Regulation (EU) No. 432/2012 has authorized the EVOO specific health claims "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress", which can be used to label EVOO containing at least 5 mg of hydroxytyrosol and its derivatives (*e.g.*, oleuropein complexes, ligstroside and tyrosol) per 20 g of oil (polyphenols $\geq$ 250 mg kg^{-1}) (EFSA, 2012).

Nevertheless, after several years since its introduction, this health claim is heavily underutilized even for that low percentage of oil brands on the market that have a suitable content of phenolic compounds and

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comply with the EFSA requirements (Bellumori et al., 2019; Caporaso et al., 2015). One of the main drawbacks limiting the commercial exploitation of the claim is undoubtedly the lack of an easily applicable method to quantify phenolic compounds and to assess conformity during EVOO shelf-life (Mastralexi, Nenadis, & Tsimidou, 2014; Tsimidou & Boskou, 2015). Phenolic compounds of olive oil constitute a heterogeneous group of metabolites, also belonging to structurally unrelated classes (Tripoli et al., 2005). The two most abundant olive secoiridoids, namely oleuropein and ligstroside, tend to isomerize or transform into a variety of compounds especially during oil extraction, as a consequence of the rapid deglycosylation mediated by olive fruit β-glycosidase, thus further increasing the multiplicity of the phenolic congeners in EVOO (Velázquez-Palmero et al., 2017 and references therein).

The determination of phenolic compounds in plant extracts has been traditionally carried out with the Folin-Ciocalteu assay (Folin & Ciocalteu, 1927) and its subsequent improvements (Sánchez-Rangel, Benavides, Heredia, Cisneros-Zevallos, & Jacobo-Velázquez, 2013; Singleton & Rossi, 1965). However, this assay suffers from low accuracy and specificity since it is sensitive to a large number of interfering compounds. An array of analytical methods based, among others, on spectrophotometric, spectrometric, colorimetric, enzymatic, electrochemical, and chromatographic techniques, has been proposed for the simultaneous quantification of EVOO phenolic compounds. All these methods have their own analytical or practical limitations and are not routinely used due to lack of consensus (Bartella, Mazzotti, Sindona, Napoli, & Di Donna, 2020; Bellumori et al., 2019; Prior, Wu, & Schaich, 2005). Very recently, an alternative strategy has been proposed by our group to categorize EVOO based on the coulometrically determined antioxidant capacity (Siano, Picariello, & Vasca, 2021). The method recommended by the International Olive Council (IOC) for the accurate quantification of EVOO phenolic compounds is based on the HPLC-UV analysis of hydroalcoholic extracts (80% aqueous methanol, v/v), using syringic acid as the internal standard (IOC, 2009). This protocol requires relatively expensive instrumentation, operational skills, and time of analysis incompatible with the necessity of categorizing EVOO rapidly during bottling. For these reasons, even the HPLC-based protocol is not applied for the routine assessment of EVOO phenolic compounds. To reduce the time of analysis, two groups independently have proposed the determination only of hydroxytyrosol and tyrosol by HPLC, following their release by acidic hydrolysis (Bellumori et al., 2019; Tsimidou et al., 2019). This method still requires the availability of a HPLC and skilled personnel, as well as the hydrolysis with strong acids.

Medina developed a novel spectrophotometric assay for the global determination of phenolic compounds in hydrophilic solutions (Medina, 2011a, 2011b). This assay relies on the coupling reaction between the Fast Blue BB diazonium salt (FBBB), namely 4-benzoylamino-2,5-dimethoxybenzenediazonium-chloride-hemi-[zinc chloride] containing the electrophilic diazonium group ($–N_2^+$), with –OH activated aromatic rings (*i. e.,* phenols), under alkaline conditions. The resulting stable azo complexes are measured by UV–Vis spectrophotometry at the wavelength of 420 nm (Fig. 1). The FBBB assay has been applied to quantify total phenolic compounds in a range of food matrices, including fruits, beverages, cereals (Lester, Lewers, Medina, & Saftner, 2012; Medina, 2011b), and recently legumes, plant seeds and nuts (Pico, Pismag, Laudouze, & Martinez, 2020). The FBBB assay has adequate sensitivity and reliability to monitor the excretion of phenolic compounds into urine of children who had assumed phenols-containing foods (Hinojosa-Nogueira, Muros, Rufián-Henares, & Pastoriza, 2017). Relevantly, the FBBB assay is not significantly biased by the interference of non-phenolic compounds (Hinojosa-Nogueira et al., 2017; Pico et al., 2020).

To the best of our knowledge, the FBBB assay has never been applied to determine phenolic compounds in EVOO or in other non-aqueous media, so far. This research explores the possibility to apply the FBBB assay as a simple, reliable, and cost-/time-effective method to quantify phenolic compounds in EVOO, alternative to the HPLC-based determination.

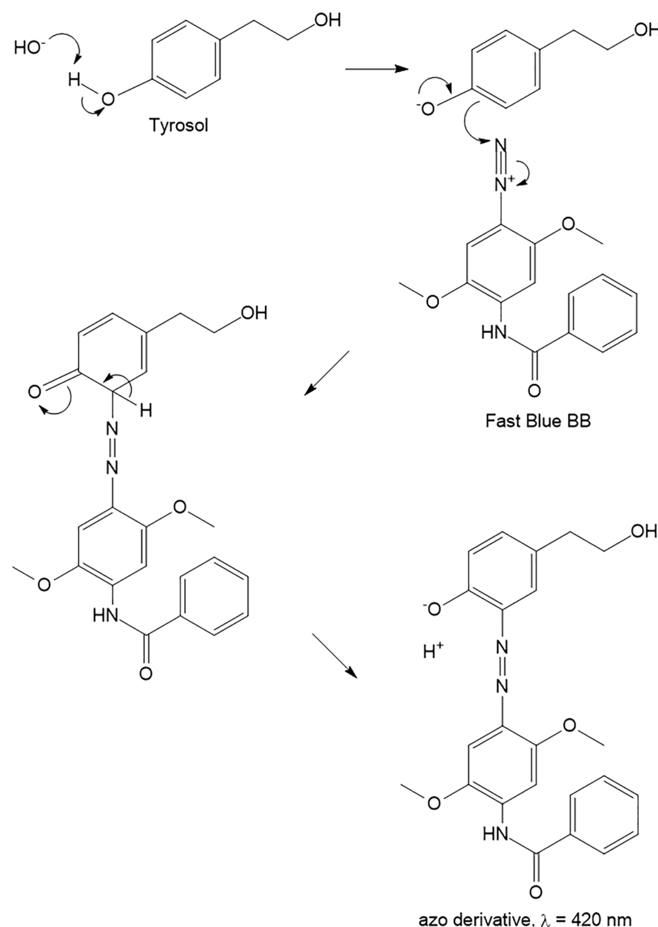

Fig. 1. Coupling of FBBB diazonium salt with tyrosol in alkali, and formation of an azo derivative with absorption band centered at 420 nm.

2. Materials and methods

2.1. Chemicals, reagents, and olive oil samples

High purity chemicals, analytical standards and HPLC-grade solvents were purchased from Sigma-Aldrich (St. Louis, MI, USA). A total of 26 olive oil samples, all commercialized as EVOO, were obtained soon after extraction by cold pressing from local oil mills or from local markets, during oil campaigns 2019 and 2020. EVOO samples were stored in screw-cap dark glass bottles and analysed not later than two weeks after sampling.

2.2. Extraction of phenolic compounds

Preliminary tests of the FBBB assay were carried out with polar extracts from EVOO, which were prepared according to the IOC (2009) protocol. Successively, polar extracts were obtained using a modified protocol, in order to maximize the recovery of phenolic compounds from EVOO samples and to improve accuracy of HPLC determinations (Romero & Brenes, 2012). In this case, polar extracts were obtained by vortexing EVOO with a mixture of methanol/water 80/20 (v/v) using a 1-to-10 (w/w) oil-to-solvent ratio and sonicating for 30 min at room temperature. Afterwards, the mixture was centrifugated for 15 min at 3500 *g* and the upper layer was filtered on disposable 0.22 μm nylon syringe filters (Merck-Millipore, Darmstadt, Germany). The filtrate was stored in glass vials at −26 °C until further analysis.

2.3. Determination of total phenolic compounds with the Folin-Ciocalteu assay

Total phenolic compounds were determined with the Folin-Ciocalteu method (Sánchez-Rangel et al., 2013). Briefly, in a 3 mL plastic cuvette were added consecutively: i) 2300 μL of distilled water; ii) 50 μL of the Folin-Ciocalteu reagent diluted 1:2 with water; iii) 50 μL of oil extract, and iv) after 3 min, 100 μL of a saturated sodium carbonate solution. Following 90 min of incubation in the dark, the absorbance was measured at 765 nm (GE156 Heathcare Ultrospec 2100 UV–Vis; Uppsala, Sweden). Total phenolic compounds were quantified against a calibration curve ($R^2 > 0.99$) built with standard tyrosol (≥99% purity). Results were expressed as mg of tyrosol equivalent (TE) per kg of oil (mg_{TE} kg^{-1} oil).

2.4. HPLC analysis

Phenolic compounds were quantified by reversed phase-high performance liquid chromatography (RP-HPLC), adapting the International Olive Council (IOC) method for the determination of olive oil biophenols (Rev 1, 2017 based on IOC, 2009).

For the HPLC quantification, 1.00 g of EVOO was spiked with a known amount of syringic acid as the internal standard and polar compounds were extracted in methanol/water 80/20 with a 1-to-10 (w/w) oil-to-solvent ratio as described. After centrifugation, the polar fraction (upper layer) was collected and concentrated in a Savant speed-vac up to about 0.5 mL and then diluted to 1 mL with 0.1% trifluoroacetic acid (TFA). One-tenth of the polar extract was separated by RP-HPLC using a modular HP 1100 chromatographer (Agilent, Palo Alto, CA, USA) equipped with a diode array detector (DAD). The stationary phase was a C18 reversed-phase column 250 × 2.1 mm i.d., 4 μm particle diameter (Jupiter Phenomenex, Torrance, CA, USA), kept at a 37 °C using a thermostatic oven. Separations were carried out at a 0.2 mL min^{-1} constant flow rate, applying a 5–65% gradient of the organic modifier (solvent B: acetonitrile/TFA 0.1%) in 5–65 min, following 5 min of isocratic elution at 5% B. After 65 min the% B was increased up to 100%. Solvent A was 0.1% TFA in HPLC-grade water. Effluents were monitored at $\lambda = 280$ nm wavelengths and peaks were integrated using the HPLC ChemStation software vers. A.07.01 (Agilent). The tyrosol-to-syringic acid (internal standard) was determined as described in the IOC (2009) protocol and total phenolic compounds were expressed as mg kg^{-1} TE.

2.5. Fast Blue BB (FBBB) assay

Preliminarily, the FBBB assay was carried out according to the method of Medina (2011b) on a set of n = 25 EVOO samples collected during the olive oil campaigns 2017 and 2018. This assay performed with 100 μL of EVOO polar extracts prepared as recommended by the IOC protocol, *i. e.*, with methanol/water 80/20 (v/v) with a 2:6 (w/v) oil-to-solvent ratio, omitting the internal standard.

Subsequently, the FBBB assay was performed directly on n = 26 EVOO samples, skipping the step for the extraction of polar compounds. For the FBBB assay on unfractionated EVOO, accurately weighed 2.00 g of oil samples were sequentially added with 2 mL of freshly prepared FBBB diazonium salt (0.1% w/v in ethanol) and 2 mL of 5% (1.25 M) NaOH in disposable 15 mL plastic tubes. After incubation for 20 min under agitation and centrifugation for 10 min at 3500*g*, the hydro-alcoholic phase (lower layer) was collected and the absorbance was measured at the wavelength of 420 nm in disposable semi-micro 1.5 plastic cuvettes (Kartell Labware, Noviglio, Milan, Italy) using a GE156 Heathcare Ultrospec 2100 UV–Vis spectrophotometer (Uppsala, Sweden). Photographs were taken with an EOS 7D Mark II (Canon Inc., Tokio, Japan) camera and converted to RGB data images for colour sampling and correction using Adobe Photoshop CC 20.0 (Adobe Co., Mountain View, CA, USA).

2.6. Intra-laboratory validation and statistical analysis

For all analyses, values are averages of three replicates. The intra-day repeatability of the FBBB assay was evaluated performing ten replicate analyses (n = 10) of 2.00 g of one EVOO sample (*i.e.*, sample n. 19). To evaluate the intermediate precision, two operators performed the FBBB assay independently on the same EVOO sample (*i.e.*, sample n. 2) once-a-day over 5 separate days (n = 10). For the intra-day repeatability and intermediate precision, maximum allowed relative standard deviation (% RSD) was 5%. To assess robustness of the FBBB assay, the entire set of n. 26 EVOO samples was analyzed with 1.00 and 2.00 g of oil and results were compared. Pearson correlation analyses were performed using Excel 365 (Microsoft, Redmond, WA, USA) for Windows 10 and XLSTAT v. 5.1 (Addinsoft Co., New York, NY, USA).

3. Results and discussion

3.1. FAST Blue BB assay of EVOO extracts

The FBBB assay was carried out preliminarily with the protocol published by Medina (2011a and 2011b) using the methanol/water 80/20 (v/v) extracts from n = 25 EVOO samples collected during the oil campaigns 2017 and 2018. Polar compounds were extracted from EVOO according to the protocol proposed by the IOC (2009) for the HPLC-based determination, omitting the addition of syringic acid as the internal standard. A calibration curve of ABS (420 nm) *vs* concentration (μg mL^{-1}) was built with seven standard solutions of gallic acid in the concentration range 0–250 μg mL^{-1} ($y = 0.0068x + 0.0646$, $R^2 = 0.9970$). Total phenolic compounds in EVOO extracts as determined with the "classical" FBBB assay varied in the 30–146 mg kg^{-1} range, which were too low figures if compared to those obtained with HPLC for the same extracts (213–790 mg kg^{-1}). The sets of values obtained with the two methods were substantially uncorrelated, exhibiting $R^2 = 0.4530$. Most likely, the inconsistency of the FBBB assay results should be ascribed to the limited repartition of EVOO phenolic compounds in the hydrophilic solution used to produce azo compounds through reaction with the FBBB salt. Since the coupling of FBBB salt with phenolic compounds could involve a different stoichiometry than the coupling with gallic acid, additional calibration curves were built with other standard phenols, including gentisic acid (2,5-dihydroxybenzoic acid) and tyrosol, without improving appreciably the FBBB assay-HPLC correlation. For this reason, we did not pursue this workflow any longer.

3.2. FAST Blue BB assay of unfractionated EVOO

The possibility of carrying out the FBBB assay directly on crude EVOO, omitting the extraction step, was explored. Such a workflow would prevent the issue related to the complete extraction of phenolic compounds, considerably reducing the time of analysis. The rationale of this attempt was also justified by the fact that, in principle, aside from phenolic compounds no other major compounds of EVOO were expected to be reactive with aryl diazonium salts. From a structural standpoint, tocopherols are phenols, and they fluctuate in EVOO within the 100–300 mg kg^{-1} range, with average values of 169 ± 38 mg kg^{-1} (Różańska et al., 2020), which is comparable with the concentration of phenolic compounds in most EVOO samples (Georgiadou et al., 2019). Thus, tocopherols might seriously affect the determination of phenolic compounds with the FBBB assay. On the other hand, more than 90% of EVOO tocopherols is represented by α-tocopherol (Jimenez-Lopez et al., 2020), which does not possess any of the aromatic positions available for coupling and, hence, it is inert toward aryl diazonium salts. β-, γ- and δ-tochopherols, potentially reactive to FBBB, should be considered minor potential interfering compounds, with fluctuations within very narrow ranges of concentration.

Based on these considerations, a modified assay was conceived, making use of the FBBB salt with unfractionated EVOO. For this assay,

accurately weighed EVOO samples (2.00 g) were sequentially added with freshly prepared 0.1% (w/v) FBBB salt in pure ethanol (2 mL) and 5% (w/v) NaOH (2 mL) in disposable plastic centrifuge tubes. After reaction and subsequent centrifugation, the absorbance of the polar fraction (lower layer) was measured at 420 nm. The steps for performing the FBBB assay on EVOO are schematized in Fig. 2. The HPLC analysis of both the ethanol/water layer and the methanol/water 80/20 (v/v) extracts from the oil layer after reaction proved the absence of unmodified phenolic compounds at detectable amounts (Supplementary information, Fig. S1, panel A). The HPLC chromatogram of the water/ethanol layer recorded at 360 and 420 nm contained a broad unresolved peak eluting with 100% of the organic modifier (acetonitrile/0.1% TFA), most likely due to the azo compounds resulting from the coupling of FBBB salt with phenolic compounds (Supplementary information, Fig. S1, panel B). These findings supported the complete conversion of phenolic compounds into the corresponding azo derivatives and their distribution in the hydroalcoholic layer.

The hydroalcoholic phase would be more easily collected if it were the upper layer of the resulting biphasic system. Several other tests were carried out to reduce the density of the hydroalcoholic phase, dissolving the FBBB salt in pure methanol, ethanol or acetonitrile, incorporating up to 10–20% (v/v) of aqueous NaOH. Overall, these adjustments were detrimental to the quality of the analysis in agreement with the observation by Lester et al. (2012), who found that alcohol concentrations higher than 70% affected the FBBB reaction with phenolic compounds.

Notably, after 20 min of coupling reaction between FBBB reagent and EVOO phenolic compounds, the absorbance at 420 nm of the resulting solutions did not change and was stable for several hours. However, the coupling seems rapid, especially if promoted by immerging the reacting system in an ultrasonic bath. Thus, probably the reaction time might be further reduced.

One of the main challenges to develop an assay for the global determination of phenolic compounds in EVOO is the unavailability of an adequate analytical standard representative for the natural complexity of the components. A standard calibration curve was built with a series of blends of an EVOO sample with refined high oleic sunflower seed oil (SSO), which was assumed to contain negligible amounts of phenolic compounds. In the EVOO sample selected for the test, total phenolic compounds were quantified with the HPLC method and were 604 ± 7 mg kg^{-1} (approximated to 600 mg kg^{-1}). The calibration curve was built with eleven varying percentages (v/v) of EVOO in SSO (in triplicate) from 0 to 100% with increments of 10%. In this way, for each standard point, both the amount of EVOO phenolic compounds and their concentration in terms of mg kg^{-1} were easily calculated. Density of EVOO and SSO was assumed the same, that is 0.918 g mL^{-1} (at 20 °C).

A plot of the absorbance 420 nm *vs* concentration of phenolic compounds showed excellent linearity in the range of concentration investigated ($R^2 = 0.9970$), with equation $y = 0.0018x + 0.1504$ (Fig. 3). Thus, the progressive increment of 100 mg kg^{-1} phenolic compounds in EVOO induced a corresponding increase of 0.18 absorbance units, which is easily appreciated visually for light path of 1 cm. In general, colorimetric data on printed paper are or can be made very well correlated with spectrophotometric absorbance (Soda & Bakker, 2019).

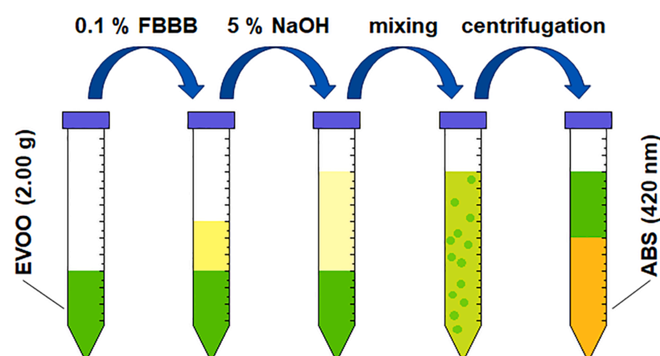

Fig. 2. Schematic workflow of the FBBB assay performed on unfractionated EVOO.

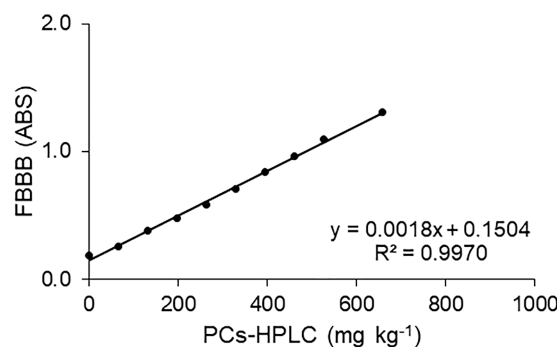

Fig. 3. FBBB assay of sunflower seed oil - EVOO (sample n.19) blends prepared at progressive increments of 10%. of FBBB absorbance (420 nm) *vs* total concentration of PCs in EVOO determined with HPLC-UV (PCs-HPLC). Values on the x-axis are inferred from the HPLC determination of PCs in pure EVOO. Values are means of three replicate analyses.% RDS was < 5% in all cases and error bars have been omitted. PCs-HPLC = phenolic compounds determined with the HPLC method.

Fig. 4 shows a picture of the hydroalcoholic solutions obtained from the standard oil mixtures sequentially disposed. The colours of the test solutions were sampled and reproduced in the lower panel of the figure as well, along with a graduate scale of the concentration of phenolic compounds. The progressive increase of colour intensity, which varied from pale to golden yellow and then to amber, can be easily appreciated by visual inspection.

3.3. FBBB-HPLC and FBBB-Folin-Ciocalteu assay correlations

For the HPLC analyses, the IOC (2009) protocol was modified to maximize the recovery of phenolic compounds. In particular, EVOO samples were extracted with a 1-to-10 (w/v) ratio of oil-to-solvent mixture (Romero & Brenes, 2012) and higher amount of phenolic compounds were injected on a narrower HPLC column compared to the IOC (2009) protocol, which improved resolution and, hence, accuracy of the peak integration.

Total phenolic compounds were determined for 26 EVVO samples with FBBB according to the described procedure, and results were compared with those from both Folin-Ciocalteu assays and HPLC-UV protocol. Values of total phenolic compounds resulting from the FBBB and Folin-Ciocalteu assays in comparison to the HPLC are reported in Supplementary information, Table S1.

The concentration of phenolic compounds in EVOO samples varied within an ample range, between 101 and 626 mg kg^{-1}, as determined by HPLC. The FBBB (ABS 420 nm) *vs* HPLC (mg kg^{-1}) and FBBB *vs* Folin-Ciocalteu (mg$_{TE}$ kg^{-1}) correlation plots are displayed in Fig. 5. The absorbance values obtained with the FBBB assay were strikingly correlated with the concentration of phenolic compounds obtained by HPLC, with $R^2 = 0.9653$ (Fig. 5A). In other terms, the FBBB assay provided values of phenolic compounds highly correlated with those obtained with the HPLC-UV method. It would be difficult to achieve a higher correlation, considering that the two methods rely on different principles, the stoichiometry of the FBBB salt- phenolic compounds coupling might vary, and the HPLC-based determinations are approximate since all the classes of phenolic compounds are evaluated at 280 nm. The linear regression line had equation: $y = 0.0019x + 0.2627$, exhibiting a slope fully consistent with the one obtained from the standard curve with EVOO - SSO mixtures. Once opportunely validated, this or a similar equation could be a reference for inferring the concentration of phenolic compounds in any EVOO sample, without the need of analyzing any

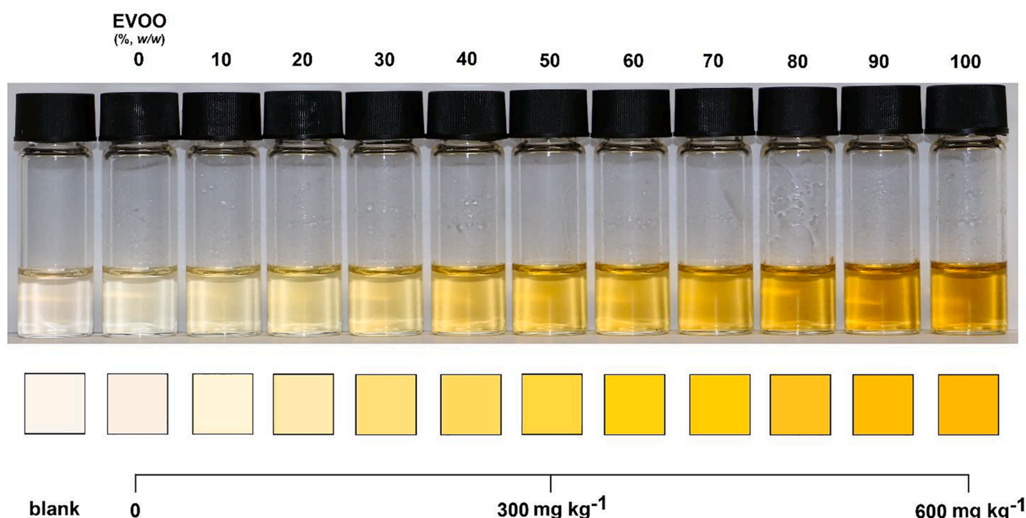

Fig. 4. Photograph image of the hydroalcoholic layer resulting from the FBBB assay of sunflower seed oil – EVOO blends (upper panel). Colours were sampled, corrected and reproduced on a colorimetric scale (lower panel).

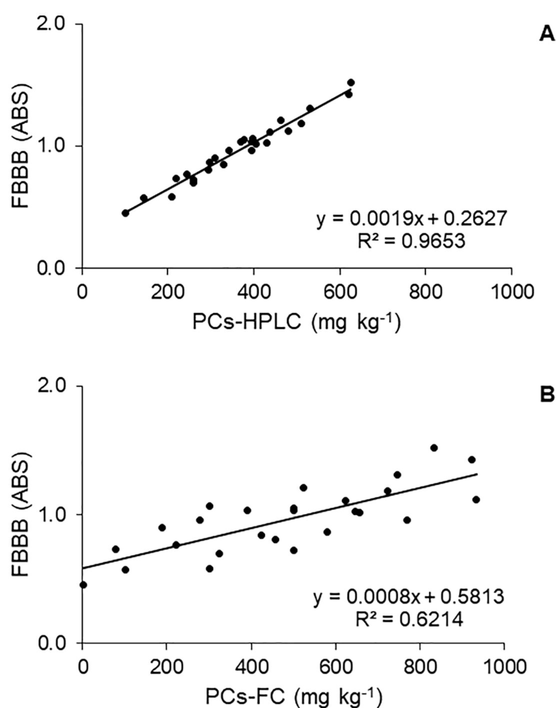

Fig. 5. Pearson correlation of FBBB absorbance (420 nm) *vs* PCs-HPLC (A) and FBBB absorbance (420 nm) *vs* PCs-FC for n. 26 EVOO samples. Values are means of three replicate analyses.% RDS was < 5% in all cases and error bars have been omitted. PCs-HPLC and PCs-FC = phenolic compounds determined with the HPLC and Folin-Ciocalteu methods, respectively.

standard.

In contrast, the results of FBBB and Folin-Ciocalteu assays were poorly correlated with $R^2 = 0.6214$ (Fig. 5B), clearly reflecting the non-specific response of the Folin-Ciocalteu assay and confirming the its scarce consistency for the determinations of phenolic compounds in EVOO compared to the HPLC-based method (Alessandri, Ieri, & Romani, 2014; Ricciutelli et al., 2017).

3.4. *Intra-day repeatability, intermediate precision and robustness*

The FBBB assay for the analysis of phenolic compounds in unfractionated EVOO has been intra-lab validated following the guidelines developed by the Eurachem guide (Magnusson & Ornemark, 2014).

To check for repeatability, an intra-day study was performed with ten replicate analyses of an EVOO sample (EVOO n. 19), obtaining average ABS ± SD (standard deviation) = 1.302 ± 0.035 (% RDS = 2.69).

To test for intermediate precision, two operators analyzed independently one EVOO sample (sample n. 2) once-a-day over 5 separate days (overall 10 determinations), obtaining average ABS ± SD = 1.120 ± 0.043 (% RDS = 3.83). In terms of % RDS, both repeatability and intermediate precision were < 5%, which is the threshold generally accepted for the validation guidelines (AOAC, 2011).

To assess for robustness of the method the entire set of 26 EVOO was assayed with two different sample amounts, namely 1.00 and 2.00 g, maintaining the same proportions of FBBB and NaOH solutions. The ABS values resulting from the two analyses, indicated with ABS_1 (1.00 g EVOO) and ABS_2 (2.00 g EVOO), were highly correlated, as shown in Fig. 6, with $R^2 = 0.9927$.

4. Conclusions

The urgent demand of a simple and cost-/time-effective strategy to assess the content of phenolic compounds in EVOO has prompted the development of numerous dedicated analytical methods over the years. However, due to intrinsic drawbacks or to practical limitations, none among these methods has met the consensus of the scientific community or the approval by EVOO producers and traders. Therefore, the EFSA

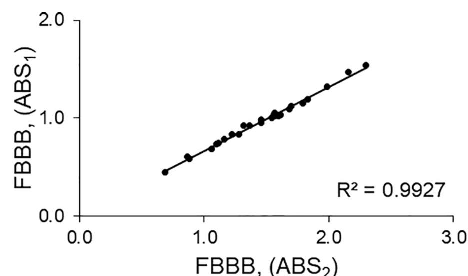

Fig. 6. Robustness test for the FBBB assay. Pearson correlation of FBBB absorbance obtained with 1.00 g and 2.00 g of EVOO samples (n = 26), maintaining unmodified the proportions with volumes of 0.1% FBBB in ethanol and 5% (1.25 M) NaOH solution.

health claim concerning phenolic compounds that has been introduced to classify EVOO on the market is still largely underutilized. Clearly, the failure to use the health claim penalizes the high-quality productions and limits the nutritional exploitation of EVOO benefits by consumers. In this study, for the first time to our knowledge, the FBBB assay has been tested to quantify hydrophobic phenolics in non-aqueous media. The assay is performed directly on EVOO, thereby reducing both sample handling and possible inaccuracy related with the incomplete extraction of phenolic compounds. The FBBB assay applied directly on the unfractionated oil appears a valuable candidate for becoming the elective method to determine total phenolic compounds in EVOO. The assay is rapid, cheap, simple to execute, safe, robust, and can be easily multiplexed. The FBBB assay targets selectively phenolic compounds, thereby minimizing the bias from interfering components, differently from many indirect methods that rely on the assessment of total phenolic compounds through their reducing, antioxidant, or radical scavenging properties.

Interestingly, the FBBB assay could be used also as an indicative colorimetric method to classify EVOO. In this sense, the colour of the resulting solutions could be compared with a printed colour scale, limiting the need of a spectrophotometer for accurate determinations to those EVOO samples with concentration of phenolic compounds close to the threshold value (250 mg kg^{-1}). In perspective, the quantification based on the colour scale could be performed using a smartphone camera and the image processing software of a properly designed app.

In general, once opportunely validated, the FBBB assay can be proposed for a routine screening of phenolic compounds in EVOO aimed at their classification according to the EFSA requirements. Since it does not require particular skills, the assay can be executed *on-site* at the olive mill to classify EVOO at the moment of oil bottling.

Further work is ensured to perform ring trials and inter-laboratory validation of the FBBB assay, in the perspective of proposing it as an official method for the analysis of phenolic compounds in EVOO.

CRediT authorship contribution statement

Francesco Siano: Conceptualization, Formal analysis, Data curation, Writing – original draft. **Ermanno Vasca:** Conceptualization, Data curation, Writing – original draft. **Gianluca Picariello:** Conceptualization, Formal analysis, Data curation, Writing – original draft, Funding acquisition.

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.2021.130990.

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