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A widely used spectrophotometric assay to quantify olive oil biophenols according to the health claim (EU Reg. 432/2012)

Patricia Reboredo-Rodríguez[1], Enrico Valli[2], Alessandra Bendini[2]*,

Giuseppe Di Lecce[2], Jesus Simal-Gándara[1], Tullia Gallina Toschi[2]

[1]*Analytical and Food Chemistry Department, Nutrition and Bromatology Group, University of Vigo, Spain*

[2]*Department of Agricultural and Food Sciences, Alma Mater Studiorum- University of Bologna, Italy*

Corresponding author: DiSTAL - Campus of Food Science - Piazza Goidanich 60, 47521 Cesena (FC) Italy

Tel.: +39 0547 338121; Fax: +39 0547 382348. E-mail address: alessandra.bendini@unibo.it

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List of abbreviations

Api, apigenin; **DLA**, dialdehydic form of decarboxymethylelenolic acid linked to tyrosol; **DOA**, dialdehydic form of decarboxymethylelenolic acid linked to hydroxytyrosol; **EVOOs**, extra virgin olive oils; **FC**, Folin-Ciocalteu; **GA**, gallic acid; **HTyr**, hydroxytyrosol; **LA**, ligstroside aglycon; **Lut**, luteolin; **OA**, oleuropein aglycon; **Tyr**, tyrosol; **VOOs**, virgin olive oils.

Abstract

The purpose of this work was to find a simple, cheap and suitable method, among the most widely employed, able to guarantee a proper determination and quantification of the phenolic content of extra virgin olive oils (EVOOs), in order to satisfy the requirements of the specific health claim (EU Reg. 432/2012). Total phenolic content by Folin-Ciocalteu (FC) was used and compared *vs* phenolic profile by HPLC-UV, considering this latter as the most sensitive and specific method for evaluating the phenolic content. Both protocols were performed before and after an acid hydrolysis of the polar phenolic fraction that involves a break of the bound forms of hydroxytyrosol (HTyr) and tyrosol (Tyr), with a simplification of the phenolic profile, and quantification of their total free forms. Results of the phenolic compounds of twelve EVOOs, determined by the different analytical approaches, were statistically compared by means of two-tailed paired *t*-tests: data obtained by the FC assay (expressed as HTyr) before and/or after acid hydrolysis were statistically comparable with results obtained by acid hydrolysis-HPLC (as sum of HTyr and Tyr).

Practical applications:

The promising results obtained in this study show that the simple and cheap colorimetric assay based on the use of the FC reagent, commonly used for the evaluation of phenolic compounds in hydro-alcoholic extracts of EVOO, can be also efficiently applied, without acid hydrolysis of extracts and HPLC analysis, to verify the compliance to the polyphenols health claim introduced by EU Reg. 432/2012. In fact, in order to preserve the positive image of EVOO due to its healthy properties, it is necessary *i)* to share an analytical protocol to determine the amount of hydroxytyrosol and its derivatives having a demonstrated effect of protection of blood lipids from oxidative stress *ii)* to check by this protocol if EVOOs satisfy the EU requirement for including the specific health claim on the oil label.

1. Introduction

The most important phenolic compounds identified in virgin olive oils (VOOs) can be divided into different groups: phenolic acids, phenyl ethyl alcohols as hydroxytyrosol (HTyr) and tyrosol (Tyr), secoiridoids (mainly the dialdehydic form of elenolic acid linked to HTyr or Tyr and oleuropein aglycon isomers), lignans and flavones [1, 2]. Secoiridoids derivatives are the main phenolic compounds in fresh olive oils [3]. The qualitative and quantitative composition of this fraction is affected by genetic and agronomic factors of the olive cultivars (e.g. variety, geographical origin, maturation stage, part of the fruit, storage) as well as technological factors employed during the mechanical extraction of oil (e.g. malaxation operating conditions) [4]. Phenolic fraction is partially responsible for the healthy and sensory properties, together with the higher oxidative stability of the olive oil, in comparison with the rest of edible vegetable oils [5]. Among others, free radical scavenging, anticarcinogenic, cardiopreventive, and antimicrobial properties have been attributed to olive oil phenolics [6]. Most of these biochemical and pharmacological effects have been associated to HTyr and, to a lesser extent, to Tyr. Both substances are absorbed in the intestine and can be detected in urine, plasma, and LDL particles [7-10]. In 2011, the European Food Safety Authority (EFSA) concluded that there is evidence of a cause and effect relationship between the consumption of olive oil phenols and the protection of low-density lipoprotein (LDL) cholesterol particles from oxidative damage [11]. In 2012, the European Commission approved the health claim *"Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress"* for those olive oils which contain at least 5 mg of HTyr and its derivatives (e.g. oleuropein complex and Tyr) per 20 g of product [12]. The enacting of this Regulation requires the development of reliable quantification methods for only *"hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol)"*. As no official method exists so far for the complete separation of all forms of HTyr and Tyr present in oils, it is of utmost importance to find a simple and reproducible protocol to protect consumers and avoid unfair competition [13-14]. The most reliable way to determine the amount required by the European legislation is to hydrolyze all the linked forms and thus determine free forms [11]. Mulinacci et al. (2006) [15] examined both acid and alkaline hydrolysis of the phenolic fraction obtained using a mixture of ethanol and water (7:3, v/v) acidified. Acid hydrolysis was proposed as a quantitative method for the evaluation of the HTyr and Tyr amounts, while alkaline hydrolysis

with the aim of improving the recognition of lignans in the minor polar compounds fraction. In fact, after acid hydrolysis these molecules almost disappeared from the HPLC profile. Afterwards, Purcaro et al. [14] examined also the acid hydrolysis applied to the phenolic fraction (obtained following the IOC method). Despite differentiations applied, they found that acid conditions liberate HTyr and Tyr without destroying them. Then, Romero and Brenes [16], applied a hydrolytic reaction (using HCl) directly to oil to ease the determination of total HTyr and Tyr [13]. More recently, Mastralexi, Nenadis and Tsimidou [13], provided evidences about the superiority of determining total HTyr and Tyr content in the hydrolysate of the phenolic fraction using the conditions proposed by Mulinacci et al. [15]. Recently, the robustness and the expression of such results were under debate in a comment [17] and in a rebuttal [18]. To evaluate the phenolic content of VOOs, the most widely employed methods are spectrophotometric – Folin-Ciocalteu (FC) and molybdate (MoM) assays – and chromatographic. FC assay is a simple and widespread procedure, but it does not permit to distinguish mono- from *o*-diphenols [19-20]. On the other hand, MoM assay is specific for the quantification of molecules having an *o*-diphenolic group [21]. As it is well known, HPLC-DAD determination is characterized by its sensitivity and specificity, but it is time and solvent consuming [22-24] and it is rather cumbersome, due to resolution problems and the lack of standards for a reliable quantification [13-14]. HPLC-MS or HPLC tandem MS (MS/MS) are other more sophisticated chromatographic methods that can be used but they require very expensive analytical tools. A part of its high sensitivity, these detection systems have the capability to both determine the molecular weight and provide structural information [25].

The aim of this work is to assess if the most common colorimetric assay used for the evaluation of phenolic compounds in olive oils, Folin-Ciocalteu (FC), can be also efficiently applied to determine HTyr and Tyr and their derivatives in order to support the health claim introduced by EU Reg. 432/2012. Determinations were performed on VOO extracts previously hydrolyzed by acid media. The acid hydrolysis-HPLC determination was considered as reference method to compare experimental results obtained from the spectrophotometric assays.

2. Material and Methods

2.1 Reagents and chemicals

Commercial phenolic compound standards such as syringic acid (purity ≥98%), caffeic acid (≥99%) and gallic acid (GA, ≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA); HTyr (≥98%) and oleuropein (98%) were bought from Extrasynthèse (Geney, France); Tyr (98%) was from Merck (New Jersey, USA). Folin-Ciocalteu phenol reagent was purchased from Merck (Darmstadt, Germany). The solvents acetonitrile, *n*-hexane, methanol (MeOH), H_2O and formic acid were all HPLC-gradient grade and purchased from Sigma-Aldrich.

2.2 Olive oil samples

Twelve samples of extra virgin olive oils (EVOOs) produced in Italy and Spain from different varieties of olives and obtained by using different technological procedures were analyzed. These samples were selected on the basis of their phenolic content, previously evaluated by the FC assay, in order to cover a quite wide range in terms of phenolic content as can be seen in **Table 1** (from 177.5 mg GA kg^{-1} oil to 687.2 mg GA kg^{-1} oil).

2.3 Phenolic compounds extraction

Phenolic fraction was extracted from EVOOs following the International Olive Council method [26], with some modifications. Briefly, 2.0 g of olive oil were weighed in a 15 mL screw-cap tube and 1 mL of internal standard working solution (IS, containing syringic acid at 0.015 mg mL^{-1}) was added. The tube was shaken for 30 s before adding 5 mL of MeOH/H_2O mixture (80/20, v/v) and shaken again for 1 min. The tube was place in an ultrasonic bath for 15 min at room temperature and then centrifuged at 4000 rpm for 25 min. The MeOH/H_2O phase was collected and was filtered through a 0.2 µm filter with a nylon membrane. Syringic acid was not added in extracts used for the spectrophotometric determinations.

2.4 Acid hydrolysis of the phenolic extracts

Acid hydrolysis of complex phenols of HTyr and Tyr derivatives was carried out by adding 1 mL of 5 M HCl to 1 mL of hydroalcoholic phenolic extract and let react at 100°C in an oven for 1 h (the tube was closed using a screw cap). In order to prepare control samples not subjected to acid hydrolysis, 1 mL of water was

123 added to 1 mL of hydroalcoholic phenolic extract to obtain the same dilution. Preliminarly, a standard of
124 oleuropein (500 mg L^{-1}) and two different phenolic extracts (samples 11 and 12) underwent the above
125 specified analytical protocol to verify the effectiveness of the hydrolytic reactions. On the other hand, the same
126 standard solution and phenolic extracts (samples 11 and 12) were heated at 100°C for 1 h, but without the
127 addition of 5 M HCl, to verify that there was no degradation of phenolic compounds due only to the effect of
128 temperature. The reaction was stopped by freezing the tubes in an ice bath. Then, the hydrolyzed extracts
129 were analyzed by HPLC and/or by spectrophotometric determinations.
130 *2.5 Determination of phenolic content by HPLC analysis*
131 HPLC analysis was performed with an Agilent 1200 Series HPLC system (Hewlett-Packard, Waldbronn,
132 Germany). The column (100 × 3 mm ID, particle size of 2.6 µm) was a C18 Phenomenex Kinetex (Torrance,
133 CA, USA). Gradient elution was carried out with a solvent system of water/formic acid (99.5:0.5 v/v) as mobile
134 phase A and acetonitrile as mobile phase B; the total run time was 13 min (plus 5 min of post-run), and the
135 gradient elution was as follows: 0 min, 95% A; 3 min, 80% A; 4 min, 60% A; 5 min, 55% A; 9 min, 40% A; 10
136 min, 0%; 12 min, 95 % A and 13 min, 95% A. The samples were injected into a 5 µL loop, with a mobile-phase
137 flow rate of 0.7 mL min^{-1} and the column maintained at 40°C. The chromatograms were monitored with MS
138 and UV detectors. The UV wavelengths were suitable for detecting, respectively, derivative of benzoic acid,
139 phenyl ethyl alcohol derivatives and secoiridoids (280 nm) and derivative of cinnamic acid and flavones (330
140 nm). The MS conditions and parameters were set as: ESI interface; nebulizer gas pressure, 50 psi; drying gas
141 flow, 9 L min^{-1} at 350°C; capillary voltage, 3 kV. Nitrogen was used as nebulizer and drying gas. The spectra
142 were scanned within the m/z 50-800 range, both in the positive and negative ion mode. Identification of phenol
143 compounds was achieved by comparing their retention time values with pure standards (in particular, phenyl
144 ethyl alcohols and phenolic acids) and on the basis of the interpretation of their mass spectra; concentrations
145 were then calculated based on the calibration curves of HTyr and Tyr. Calibration curves (r^2=0.999 for both)
146 showed good linearity in the studied range of concentrations.
147 *2.6 Determination of phenolic content by spectrophotometric analysis*

148 Phenolic extracts used for spectrophotometric assays were obtained following the procedure explained
149 in the section 2.3 and analyzed by an UV-Vis 1800 spectrophotometer (Shimadzu Co., Kyoto, Japan).
150 *2.7 Folin-Ciocalteu (FC) assay*
151 The FC assay was performed according to Singleton and Rossi [27] and Mateos et al. [28]. A volume of
152 0.2 mL of the phenolic extract (subjected and not to the acid hydrolysis) was added to 0.5 mL Folin-Ciocalteu
153 reagent and 2 mL of Na_2CO_3 (15% w/v), in a 10 mL volumetric flask reaching the final volume with purified
154 water. Each sample was stored for 2 h at room temperature. Phenolic compounds were detected at 750 nm
155 and quantified using HTyr calibration curve (r^2=0.999). The data were expressed as mg HTyr 20 g^{-1} of oil.
156 *2.9 Statistical Analysis*
157 Two-tailed paired *t*-tests were carried out by the statistical software XLSTAT 7.5.2 version (Addinsoft,
158 USA) to identify significantly differences between the means obtained by the proposed methods (α = 0.05%).
159

160 **3. Results and Discussion**

161 The phenolic content of 12 EVOO samples was evaluated by acid hydrolysis-HPLC and by the FC (before and
162 after acid hydrolysis of the polar phenolic extract). Results allowed to assess if the most common colorimetric
163 assay is an useful tool for the determination of the total content of HTyr and Tyr in VOOs and, consequently, to
164 check if samples satisfied the EU requirement to add the specific health claim on the oil label [12].

165 *3.1 Acid hydrolysis: preliminary experiments*

166 To set up the acid hydrolysis procedure, different acid concentrations (0.5 M, 1 M and 5 M), times of
167 reaction (30 min, 2 h and 6 h) and temperatures (50°C, 100°C) chosen according to studies already reported
168 in the literature [14-16] were preliminarily tested. On the basis of these results performed on two phenolic
169 extracts (samples 11 and 12), after 1 hour of hydrolysis at 100°C by using HCl 5 M (the adopted hydrolytic
170 conditions) more than 90% of OA and LA (and their derivatives) present in the phenolic profiles of samples
171 were hydrolysed to HTyr and Tyr (**Figure 1**). At the same time, flavones such as Lut and Api showed no
172 significant variations after hydrolysis, while the syringic acid showed a slight decrease after the hydrolysis,

making it impossible its use as internal standard for the quantification. Moreover, no degradation of phenolic compounds due only to the high temperature was found by thermostating OA (without acid reagent), following the same hydrolytic condition.

3.2 Comparison between acid hydrolysis-HPLC and the FC assay

FC assay is a widespread method that involves oxidation in alkaline solution of phenols by the yellow molybdotungstophosphoric heteropolyanion reagent and the colorimetric measurement of the resultant molydotungstophosphate blue [27]. In the present experimentation, total phenolic content was determined by acid hydrolysis-HPLC and by the FC method (after and without the hydrolysis of the polar phenolic extract). Taking into consideration the poor contribution of other molecules having reducing activity, in the present investigation the FC assay was used to determine "total phenols" in EVOO extracts, basically containing tyrosol and hydroxytyrosol derivatives. As can be seen in **Table 2**, total phenolics determined by acid hydrolysis-HPLC were expressed as a sum of the concentrations of HTyr and Tyr, identified and quantified each one by using the respective external calibration curves. Concentrations ranged from 1.3 to 8.4 mg HTyr + Tyr 20 g^{-1} oil. On the other hand, FC results were expressed as mg HTyr 20 g^{-1} of oil and ranged from 3.0 to 9.9 mg HTyr 20 g^{-1} of oil (without hydrolysis) and from 2.7 to 8.6 mg HTyr 20 g^{-1} of oil (with hydrolysis). Concentrations obtained by the FC assay have been expressed as HTyr to compare them with the results of the acid hydrolysis-HPLC. In this case, to develop the FC assay with acid hydrolysis extract it was not necessary to introduce modifications because the addition of Na_2CO_3 is enough to reach basic pH. The comparison between the concentrations obtained by acid hydrolysis-HPLC and the FC assay were performed studying test samples (**Table 2**) and looking at the differences between each pair of results using the two-tailed paired *t*-test. The methods studied did not give statistically different values for the mean concentration ($t<t$ (critical value)), but in some EVOOs (5, 6, 11, 12) major differences were detected: considering that a little amount (less of 10%) of secoiridoids did not undergo acid hydrolysis, it is plausible that the results obtained from acid hydrolysis-HPLC (in which only HTyr and Tyr peaks were quantified) can be lower than the ones resulting by FC method without hydrolysis (in which all the molecules detected in the extract were exposed to the reaction). This could explain differences evidenced for samples 11 and 12. Moreover, the higher values

showed by samples 5 and 6 obtained with FC method after hydrolysis, respect to the ones resulting from the same extracts analyzed by acid hydrolysis-HPLC, could be due to hydrolysis of some molecules, as for example lignans or caffeoylquinic acids, with release of guaiacol moieties and caffeic acid respectively, that are efficient as electron donors [29] but not quantifiable as HTyr and Tyr by the HPLC method. It is important to highlight that the same results (data not shown) were found by expressing the results of the FC assay as mg GA 20 g^{-1} of oil (GA it is the most commonly phenolic compound used to quantify the total phenol content) [30,31]. As a conclusion, results obtained by the FC assay (expressed both as HTyr or as GA) do not significantly differ from those obtained following the acid hydrolysis-HPLC (expressed as sum of HTyr + Tyr). In other words, for the verification of the health claim in olive oils object of this study, the simple and rapid FC method, even performed without hydrolysis, could give results proportional to the ones obtained by the more laborious and solvent consuming acid hydrolysis-HPLC. Simple linear regressions has been done between the sum of the concentration of HTyr and Tyr identified by acid hydrolysis-HPLC-DAD and quantified using external calibration curves and the results of the FC assay, expressed as mg GA 20 g^{-1} of oil (without and with the hydrolysis of the polar phenolic fraction). The best correlation coefficient corresponds to the correlation between the acid hydrolysis-HPLC-DAD results and the FC without hydrolysis results ($r = 0.94$).

Conclusions

Considering the analyzed samples no significant differences were found between the total phenolic content determined by acid hydrolysis-HPLC and by the FC method (without and with hydrolysis step). For this reason, the simple and cheap FC method, performed also without hydrolysis, could give results, for the verification of the health claim in olive oils, comparable to the ones obtained by the more laborious and solvent consuming acid hydrolysis-HPLC. Obviously, it has to be considered that this is a preliminary study and a large number of samples would be required to draw any final conclusions from these promising results.

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Conflict of interest

The authors have declared no conflict of interest.

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312

313 **Figure Captions**

314 **Figure 1.** Overlay among three chromatographic profiles of sample 12. Upper trace: not hydrolyzed extract, λ=
315 280 nm; middle trace: hydrolyzed extract, λ= 280 nm; lower trace: hydrolyzed extract, λ= 330 nm for the.
316 Tentative identification of phenolic compounds: I.S., syringic acid; 1, HTyr; 2, Tyr; 3, cinnamic acid derivative I;
317 4, benzoic acid derivative I; 5, cinnamic acid derivative II; 6, benzoic acid derivative II; 7a+b, oxidized form of
318 DOA + pinoresinol; 8a+b, DOA + cinnamic acid; 9, Lut; 10, DLA; 11, 1-acetoxypinoresinol; 12, oxidized form of
319 DLA; 13, Api; 14, OA; 15, LA.

320

321 **Table 1.** Total phenols concentrations in the analyzed EVOOs.

322

EVOO sample	**Total phenols concentration (mg GA kg^{-1} oil)**
1	534.1±1.5
2	687.2±2.9
3	378.8±21.9
4	404.5±23.5
5	219.8±31.0
6	216.7±5.9
7	332.6±18.4
8	348.1±30.5
9	213.5±28.9
10	225.4±16.4
11	177.5±6.9
12	345.3±2.7

323

Table 2. Sum of HTyr and Tyr content (expressed as mg 20 g^{-1} oil) determined in EVOOs by acid hydrolysis-HPLC and by the FC assay.

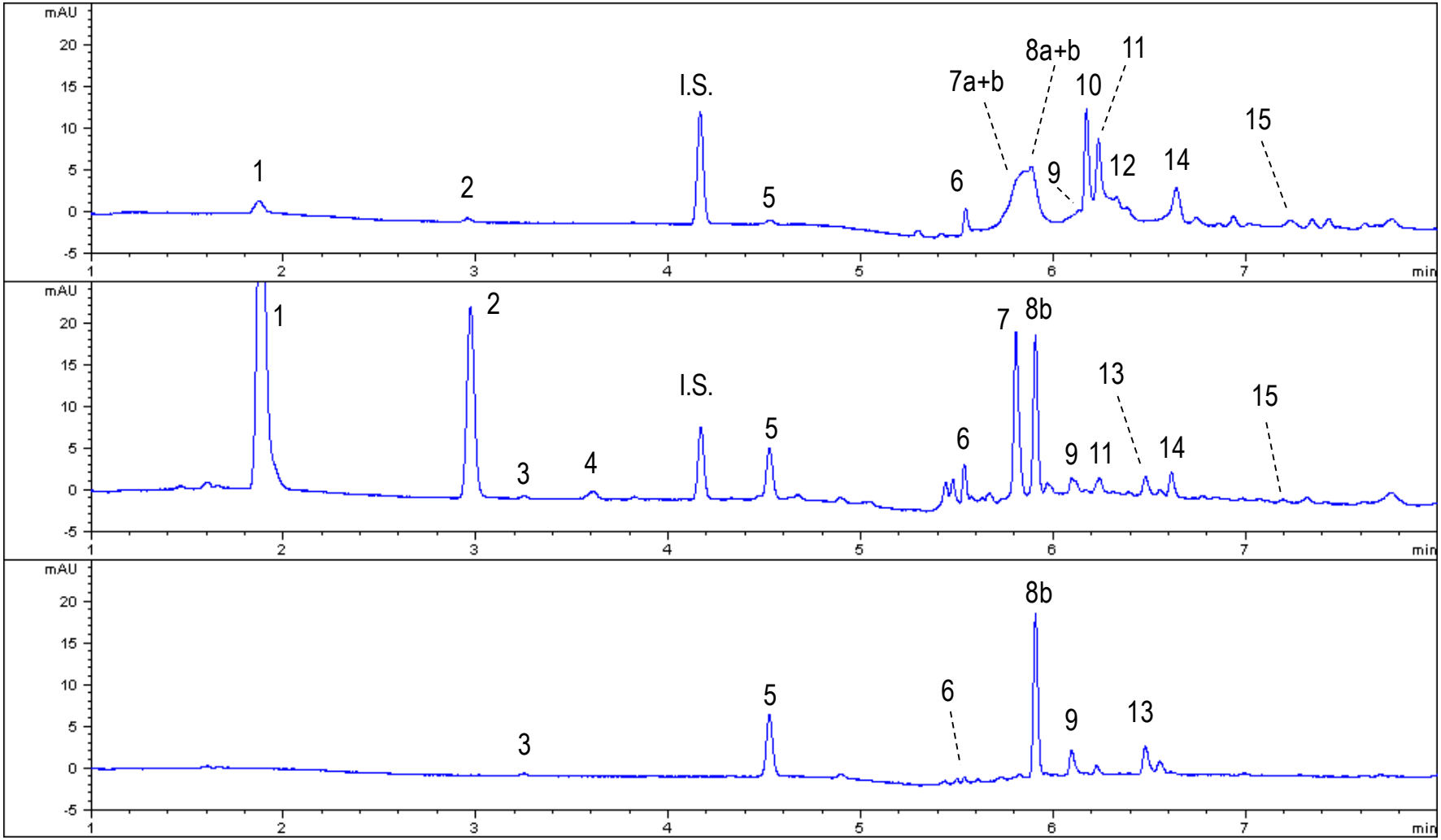
	Concentration (mg 20 g^{-1} oil ± SD)		
EVOO sample	**Acid hydrolysis-HPLC (HTyr+Tyr)**	**Folin-Ciocalteu -without hydrolysis- (HTyr)**	**Folin-Ciocalteu -with hydrolysis- (HTyr)**
1	**5.5±0.2**	**6.8±0.5**	**5.7±0.7**
2	**8.4±0.1**	**9.9±0.4**	**8.6±0.3**
3	**4.9±0.1**	**5.3±0.1**	**4.9±0.9**
4	**5.0±0.2**	**5.3±0.2**	4.4±0.2
5	3.3±0.0	3.0±0.1	**6.2±0.5**
6	2.7±0.1	3.4±0.0	**4.9±0.2**
7	**8.0±0.1**	**7.3±0.4**	**6.8±1.1**
8	**7.7±0.5**	**7.7±0.6**	**7.4±0.9**
9	4.4±0.1	**5.0±0.6**	**5.1±0.4**
10	**6.2±0.2**	**5.2±0.3**	**5.4±0.4**
11	1.3±0.3	4.0±0.4	2.7±0.4
12	**5.1±1.0**	**7.4±0.3**	4.2±0.7
Statistical analysis*			
$\bar{x}_d$		0.629	0.249
S_d**		1.134	1.269
T		1.754	0.621
Conclusion		***t* <*t* (critical value)**	***t* <*t* (critical value)**

*Two-tailed paired *t*-test; $t_{\text{critical value}}$: 2.201.

**Differences are calculated between reference method (HPLC) and the proposed assay.

In bold, concentrations ≥ 5 mg of HTyr and its derivatives (e.g. oleuropein complex and Tyr) per 20 g of product.

Figure 1


mAU
20
15
10
5
0
-5
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3
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5
6
7
min
1
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I.S.
5
6
7a+b
8a+b
9
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11
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6
7
8b
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11
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3
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6
8b
9
13