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Quantification of Health Claim-Relevant Tyrosol and Hydroxytyrosol after Direct Hydrolysis Improves Customer Understanding and Mitigates Market Distortion

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Abstract: The EFSA-approved claim that olive oil is beneficial for cardiovascular health suffers from ambiguities that lead to a vague and potentially subjective interpretation of the underlying analytical data. Misunderstandings among customers, but also market distortions are possible consequences. In this study, a rapid and simple analytical technique is presented that circumvents the ambiguity by measuring levels of putative health-promoting compounds as the equivalent of tyrosol and hydroxytyrosol, cleaving such moieties from more complex constituents such as oleuropein and oleocanthal. Since the direct hydrolysis of the olive oil is the central element of the process, the reaction temperature, time, reagent concentration and reagent type were optimized. In addition, the influence of co-solvents, which might support the intermittent miscibility of the two phases during hydrolysis, was investigated. The analytical and economic implications are discussed particularly in the context of a commonly used technique.

Keywords: olive oil; hydrolysis; health claim; phenols; bioactive; analysis

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1. Introduction

Extra-virgin olive oil, obtained from the fruit of the olive tree, has been produced, traded and consumed since ancient times. Organic residues, commonly found in ancient Israeli pottery vessels, provide evidence of the storage and possibly the trade of olive oil for at least 7000 years [1]. The victors of the Panathenaic Games (566 BC [2] to the 3rd century AD [3]) received imposing amphorae containing up to almost 40 L of sanctified olive oil, appreciating it not only for the fact of spirituality, but also due to its favorable organoleptic properties. Nowadays, extra-virgin olive oil is valued for its potential human health benefits such as: reduced risk of coronary heart disease, type 2 diabetes, myocardial infarction, stroke and delaying the onset of atherosclerosis [4–10]. The mostly association-based studies attribute the cardioprotective effects to phenolic compounds such as tyrosol, hydroxytyrosol and a number of their derivatives. It could therefore be considered useful to enrich olive oil with phenolic compounds, as suggested by Pedret et al. [6]. The European Commission published a marketing recommendation, i.e., number 432/2012 (commonly abbreviated as health claim) based on a verification of the claim's scientific substantiation by the European Food Safety Authority (EFSA) [11] that olive oil may be labeled as contributing to the protection of blood lipids from oxidative stress. On the contrary, many studies are allegedly flawed, and the beneficial effects are likely overstated [12,13]. Extensive literature examining the beneficial effects of a locally consumed Mediterranean diet, in which extra-virgin olive oil is a key ingredient, has been recently reviewed [14]. However, the effects

of such dietary interventions on inflammatory cytokines were mostly insignificant and showed no effect compared to low-fat diets. The lack of consensus in the literature suggests that while the claimed cardioprotective effects clearly require further research, the thesis of the benefits of extra-virgin olive oil for human health should not be dismissed prematurely.

The EFSA-approved claim *("Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress")* may only be used for marketing purposes of olive oil that contains *"[...] at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil)"*. Regardless of the definition, however, tyrosol and a number of its esters are not polyphenols, so it remains an open question which compounds could be beneficial to health. It is also questioned whether hydroxytyrosol and its esters such as oleuropein, which contains two hydroxy functions attached to a single aromatic ring, should be referred to as polyphenols. Quideau understandably suggests excluding such substances from being labeled as polyphenols [15]. This ambiguity, as well as the wording of the claim, implies that the beneficial effect derives from the phenolic moiety (i.e., hydroxytyrosol or tyrosol) which is also part of the respective esters (e.g., oleuropein and oleocanthal). However, it remains unclear in what form the amount of *"hydroxytyrosol and its derivatives"* is to be determined quantitatively. If an oil meeting the 5 mg weight specification contained only free hydroxytyrosol, this would equate to a daily intake of approximately 32 µmol of the phenol. However, assuming quantitative esterification of hydroxytyrosol, an oil containing only about 9 µmol of the phenolic moiety would already meet the claim (i.e., 5 mg oleuropein). In other words, one would need to consume more than 70 g of such an oil per day to achieve the same claimed effect as from an oil containing 5 mg of released hydroxytyrosol. While a number of studies suggests a significant but clearly vehicle-dependent [8,16] gastrointestinal uptake of hydroxytyrosol and tyrosol [12,17,18], oleuropein was not found on the basal side of either perfused rat intestine [17,19] or Caco-2 monolayers [17]. The hydrolysis of the seco-iridoid to the hydroxytyrosol fraction by simulated [17] or authentic [18] gastric juice is discussed controversially. In the intestine, oleuropein appears to pass through the duodenum, jejunum and ileum [17,18] without being metabolized or absorbed. Experiments on colon microflora suggest that oleuropein [17], but also other phenols [20], are metabolized in the large intestine with the glycosylated seco-iridoid yielding hydroxytyrosol. It is these observations that add to the ambiguities with regard to the health claim, indicating the need for a straightforward quantification method with a clear scope of analysis.

The International Olive Council (IOC) Trade Standards lists a 2017 revised method for quantifying "biophenols" in olive oils using high-performance liquid chromatography (HPLC) with ultraviolet (UV) spectrophotometric detection [21]. After a single extraction using a methanol-water mixture, the phenolic compounds are quantified by a response factor technique employing syringic acid and tyrosol, incorrectly [22] assuming identical absorption-quantity relationships for each of the 31 analytes other than tyrosol. Syringic acid serves as an internal standard and mitigates experimental variance during extraction and general sample handling. However, it may not be assumed a measure of extraction efficiency since syringic acid is added during sample dilution with the extraction solvent. In particular, more hydrophobic derivatives of tyrosol and hydroxytyrosol are likely to be prone to incomplete extraction. The literature offers numerous suggestions for improved extraction methods that also cover more lipophilic moieties. Nevertheless, quantitative determination remains a tedious task given the availability, stability and pricing of many aforementioned compounds other than tyrosol and hydroxytyrosol. The response factor method proposed by the IOC therefore seems to be a reasonable compromise, but the literature suggests a simpler approach, namely the direct hydrolysis of the olive oil, which only requires the quantification of tyrosol and hydroxytyrosol. There is also plenty of literature on the hydrolysis of the polar extract, which was obtained in a similar way to the IOC method [23–26]. In contrast to hydrolysis of the polar fraction, direct hydrolysis of the oil eliminates the ambiguity of the health claim and also simplifies the analytical process by avoiding the error-prone and time-consuming extraction of the polar compounds,

while at the same time cleaving the phenolic moieties of more lipophilic oil components. Bartella et al. [27] propose microwave extraction and simultaneous acidic hydrolysis of the oil, followed by quantification of the two phenols using HPLC hyphenated to mass spectrometry (MS). While extraction and hydrolysis are straightforward, quantification relies on expensive isotopically labeled standards. Furthermore, since only two deuterium atoms were added to each phenol, a restricted upper limit of detection is envisaged since the third isotopic peak of each analyte coincides with the main signal of its respective internal standard, effectively requiring that the analyte's third peak be negligibly small. Otherwise, the ratio of analyte to internal standard would have to be significantly reduced, implying a more costly endeavor. Similarly, Romero and Brenes [28] extracted the olive oil with hydrochloric acid but opted for easier quantification using HPLC with either UV or fluorescence detection. They optimized the solvent ratio and concluded that a 1:20 volume to volume ratio between oil and acid is optimal by achieving quantitative conversion after four to six hours. The authors kept the acid concentration at 2 M, the reaction temperature and the agitation speed at 25 °C and 400 rpm, respectively, while using approximately 50 mL combined volume. Several other methods and protocols for the hydrolysis of olive oil using various acids and solvents in conjunction with HPLC analysis have been described in recent years [29–31].

In this study we propose a rapid and thoroughly streamlined extraction and quantification protocol that circumvents the above-mentioned ambiguities and limitations of both the health claim and the most recent analytical methods. First, a hydrolysis protocol for olive oil is optimized, aiming for maximum yield of both hydroxytyrosol and tyrosol, as they are believed to be the most pharmacologically relevant compounds which are also absorbed intestinally. The molarity of the hydrolytic agent, type of acid, reaction time, temperature and makeup solvent were the parameters under investigation. The scientific and economic implications of the optimized method are followed by a yield comparison of fourteen commercially available olive oil samples that were also treated with a more conventional protocol in which the polar phenols were extracted with a methanol/water mixture prior to hydrolysis.

2. Materials and Methods

Fourteen extra-virgin olive oils from Greece, Italy and Spain were purchased from local supermarkets or directly obtained from local vendors. Hexane (≥97%), methanol (≥99.9%), sodiumdodecylsulfate (10%), acetone (≥99.8%), phosphoric acid (85%), methanesulfonic acid (≥99%), tyrosol (98%), hydroxytyrosol (≥98%), ethanol (≥99.9%) and hydrochloric acid (37%) were obtained from Sigma-Aldrich/Merck. Formic acid (>99%) and citric acid (100%) were purchased from VWR. Acetic acid (100%), nitric acid (69%) and trifluoroacetic acid (≥99.9%) were from Carl Roth. Perchloric acid was obtained from Fluka and acetonitrile from Honeywell. Deionized water was provided by a MilliQ A10 (Millipore, Billerica, MA, USA) dispenser.

2.1. Method Optimization

The optimization was carried out with three individual 50 mg aliquots of an olive oil sample and for each optimization step, implying, for example, 27 individual samples for the optimization of the reaction temperature. Agitation of the 2 mL tubes filled with an aliquot of oil and 1 mL of hydrolysis agent was performed with a laboratory shaker (Eppendorf, Thermomixer comfort, Hamburg, Germany) at 1400 rpm and 80 °C, unless otherwise specified (i.e., during temperature optimization). The reaction time was kept fixed at 90 min except when optimizing this parameter. Centrifugation prior to HPLC-UV analysis was performed using an Eppendorf 5430R centrifuge for 3 min at 5 °C and 21,000 rcf (FA-45-30-11 rotor at 14,000 rpm). The reduced temperature was chosen for repeatability while optimizing the reaction time.

Firstly, the temperature stability of the olive oil used for the optimization was assessed. Accordingly, aliquots were incubated either with plain water or hydrochloric acid and their

tyrosol and hydroxytyrosol yield was monitored over extended periods of up to 20 h. The molarities of sulfuric acid and hydrochloric acid, as two commonly employed acids, were then optimized by investigating the yield upon treatment of the oil with acid concentrations between 5 mM and 2 M. Eleven hydrolytic agents were compared at molarities corresponding to a pH value of 0.3, where achievable. This pH value is similar to the optimized condition of step one, but of course for formic acid, acetic acid, phosphoric acid and citric acid such a pH value could not be reached at the maximum envisaged concentration of 2 M. We thus resorted to 2 M solutions for these acids. After determining the optimal hydrolysis agent, the reaction time was optimized in different steps between 10 and 300 min. The reaction temperature was then studied at 25 °C and in 10 °C increments between 30 °C and 99 °C. Finally, the optimized conditions were tested in conjunction with different dilution solvents for the acid. We tested 0.5 M hydrochloric acid containing various amounts of ethanol (5, 20 and 50 vol%), acetone (5, 20 and 50 vol%) and also 0.05 and 0.1 vol% sodiumdodecylsulfate (SDS), hypothesizing that the addition of a more lipophilic co-solvent would aid the intermittent miscibility during reaction. Higher concentrations of SDS were also tested but dismissed prematurely due to extensive foaming.

The method used for comparison with the optimized conditions involved dissolving the oil in 500 µL of hexane and a three-step extraction with 350 µL each of a 60:40 volume-to-volume mixture of methanol and water. The IOC protocol suggests a single extraction step, but we found this to be insufficient based on previous experiments (data not shown). The three volumes were combined in a 2 mL volumetric flask and adjusted to volume. 200 µL were then transferred to a 2 mL centrifuge tube containing 200 µL of 2 M hydrochloric acid and incubated for 120 min at 1400 rpm and 80 °C. After centrifugation, aliquots of the supernatant were subjected to HPLC analysis.

2.2. Analytical Procedure

Analysis of the samples was performed on a Thermo Scientific UltiMate 3000 HPLC system (Germering, Germany), equipped with an LPG-3400SD pump (0.4 mL/min flow), WPS-3000TSL autosampler (20 µL injection volume, 4 °C), TCC-3000SD column oven (50 °C) and VWD-3100 UV detector (wavelength 275 nm). The following gradient was run on a Thermo Scientific Hypersil Gold 2.1 × 150 mm 1.9 µm column: 3 to 10 vol% B in 7 min, to 100 vol% B within 1 min, kept constant for 2 min, return to 3 vol% B in 1 min and kept constant for 4 min. Solvent A was 0.1 vol% formic acid and solvent B was acetonitrile. Peak integration was performed with the Thermo Scientific Chromeleon software (version 6.80, Waltham, MA, USA). Quantification by the method of external standard (tyrosol and hydroxytyrosol) relied on seven-point linear calibrations (R^2 > 0.999 each).

2.3. Method Validation

The validation was performed using precision, repeatability, accuracy and stability tested on four different olive oil samples and seven calibration points over three consecutive days. Each sample and each calibration point were measured in triplicate and in random order. The analytical method was validated following the high expectations of ICH guidelines and LC-MS evaluation was performed for deeper analyses of the treated samples.

2.3.1. Determination of Linearity, Precision, Repeatability and Accuracy

Target concentrations for tyrosol and hydroxytyrosol were in the range of 3 to 30 ppm, therefore lower and upper limits for the calibration curves were set to 1 to 50 ppm and excellent linearity could be confirmed (see Table 1).

The precision experiments in terms of repeatability and intermediate precision (performed on three consecutive days) were carried out by analyzing four real-life samples of extra-virgin olive oils. Repeatability data shown in Table 2 were generated using numerous injections spread out over a 24 h period (autosampler 4 °C).

Table 1. Interday evaluation of linearity of both compounds 3-Hydroxytyrosol and Tyrosol.

Compound	Day	Linear Equation [1]	R^2
3-Hydroxytyrosol	1	29.999x + 19.217	0.9994
	2	30.610x + 15.649	0.9996
	3	30.660x + 17.712	0.9997
Tyrosol	1	22.984x − 1.3681	0.9994
	2	23.360x − 5.2216	0.9996
	3	23.282x − 2.3758	0.9996

[1] The linear equation was determined on four-fold measurements of every concentration level. Concentration levels were 1, 5, 10, 20, 30, 40 and 50 ppm.

Table 2. Evaluation of repeatability performed on four extra virgin olive oils of both compounds 3-Hydroxytyrosol and Tyrosol.

Compound	Sample	Average Amount (in ppm) [1]	RDS (in %) [2]
3-Hydroxytyrosol	1	4.851	1.25
	2	3.032	1.16
	3	6.168	1.09
	4	4.282	0.71
Tyrosol	1	4.128	0.83
	2	4.903	1.78
	3	5.096	1.55
	4	5.445	0.60

[1] Average was determined on four-fold measurements of each sample. [2] Calculated relative standard deviation of the four injections put in relation to the detected average amount.

The repeatability was assessed on three different days (data of second and third day are shown in the supplementary materials section, see Tables S1 and S2) and the results of the analyzed samples are in good agreement with the measured levels of reference standards in terms of relative standard deviation. The average RSD values of the interested compounds were between 0.60% and 1.78% (for tyrosol) and between 0.71% and 1.25% (for hydroxytyrosol) in olive oil samples. The evaluation of these results confirms the repeatability of the analytical method and shows that no additional matrix effects or influences of the 0.5 M HCl are to be expected.

The data of intermediate precision testing were gathered over three consecutive days by hydrolyzing the same extra-virgin olive oils with the optimized analytical procedure. Each of the four samples was then injected for four times, resulting in a total of twelve measurements per extra-virgin olive oil. The results are presented in Tables 3 and 4 and confirm the robustness of the method in terms of intermediate precision.

Table 3. Intermediate precision experiments of 3-Hydroxytyrosol performed on four extra-virgin olive oils.

Sample	Average Amount (in ppm) [1]			Interday Average (in ppm)	Interday RSD (in %) [2]
	Day 1	Day 2	Day 3		
1	4.851	4.674	4.709	4.745	1.98
2	3.032	2.987	2.947	2.989	1.41
3	6.168	6.067	6.070	6.102	0.94
4	4.282	4.242	4.212	4.245	0.83

[1] Average was determined on four-fold measurements of each sample. [2] Calculated relative standard deviation of the four injections put in relation to the detected average amount.

Accuracy of the analytical method was determined by spiking an olive oil sample which had low tyrosol and hydroxytyrosol levels (5.08 ppm and 6.01 ppm) with reference

standards, resulting in final concentration levels of 100%, 150% and 200% for both compounds (see Table 5). These levels were intended to reflect olive oils with high amounts of the interested compounds, without leaving the linear range of the calibration. The summarized data in Table 6 show high accuracy for tyrosol and hydroxytyrosol within the investigated range.

Table 4. Intermediate precision experiments of Tyrosol performed on four extra-virgin olive oils.

Sample	Average Amount (in ppm) [1]			Interday Average (in ppm)	Interday RSD [2] (in %)
	Day 1	Day 2	Day 3		
1	4.128	4.907	4.132	4.119	0.46
2	4.903	4.909	4.825	4.879	0.96
3	5.096	5.062	5.090	5.083	0.36
4	5.445	5.457	5.487	5.463	0.40

[1] Average was determined on four-fold measurements of each sample on each day. [2] Calculated relative standard deviation of the four injections put in relation to the detected average amount.

Table 5. Investigated concentration levels during accuracy testing.

Nr.	V_{sample} (in mL)	$V_{Standard}$ [1] (in mL)	VH_2O (in mL)	$C_{3\text{-HT/Tyr}}$ (in ppm)	Composition
1	0.2	-	0.2	5	Extract
2	-	0.2	0.2	5	Std_{10ppm}
3	-	0.2	0.2	10	Std_{20ppm}
4	-	0.2	0.2	15	Std_{30ppm}
5	0.2	0.2	-	10	Ex + Std_{10ppm}
6	0.2	0.2	-	15	Ex + Std_{20ppm}
7	0.2	0.2	-	20	Ex + Std_{30ppm}

[1] The added standard mixture consisted of equal concentrations of tyrosol and hydroxytyrosol for each level.

Table 6. Mean recovery (sum of both signals) and relative standard deviation of a spiked and unspiked extra-virgin olive oil sample.

Nr.	$C_{3\text{-HT/Tyr}}$ (in ppm)	Composition	Recovery (in %)	RSD (in %) [1]
5	10	Ex + Std_{10ppm}	99.54	0.89
6	15	Ex + Std_{20ppm}	100.51	0.76
7	20	Ex + Std_{30ppm}	101.02	0.11

[1] Calculated relative standard deviation of three injections put in relation to the detected average amount.

2.3.2. Determination of Solution Stability during Analysis

Stability of compounds during analysis is crucial for method linearity and precision and since every sample and calibration point should be measured at least in triplicate, the effect of set autosampler temperature (4 °C) was monitored over a 24 h period. The stability of both extracts and standards at 4 °C is given and the difference in the quantified amount is in the range of the precision of the chromatographic system. The stability at room temperature of one extract was assessed by leaving it on the laboratory bench for four hours and was then compared to the samples stored at 4 °C. No difference was observed which could be attributed to the extended uncooled storage.

2.3.3. Characterization by LC-MS

The signals of reference standards, unhydrolyzed samples and hydrolyzed samples were additionally analyzed with LC-MS (electrospray ionization positive mode, scan from 50–800 m/z, spectra rate 4 Hz, Maxis Impact qTOF-MS from Bruker, Bremen, Germany). For this purpose, the gradient was slightly changed: 5 to 10 vol% B in 6 min, to 100 vol% B within 2 min, kept constant for 2 min, return to 5 vol% B in 1 min and kept constant for

3 min. By applying these changes, baseline separation of small signals in the MS spectra was achieved.

3. Results

LC-MS was used to analyze reference standard signals from unhydrolyzed and hydrolyzed samples. Figure 1 shows the total ion chromatogram with the superimposed UV chromatogram. The UV-active substances 1 and 4 could be assigned to hydroxytyrosol and tyrosol. Signals 2 and 3 are only present after acid hydrolysis of extra virgin olive oil. Hydrolyzed samples showed additional peaks in the total ion chromatogram between the two main compounds but were mostly inactive under UV. These signals were identified as 283.076 m/z, but no match was found in MetFrag databases (KEGG, HMDB). Further details and MS-spectra are provided as supplementary material (Figures S1–S4).

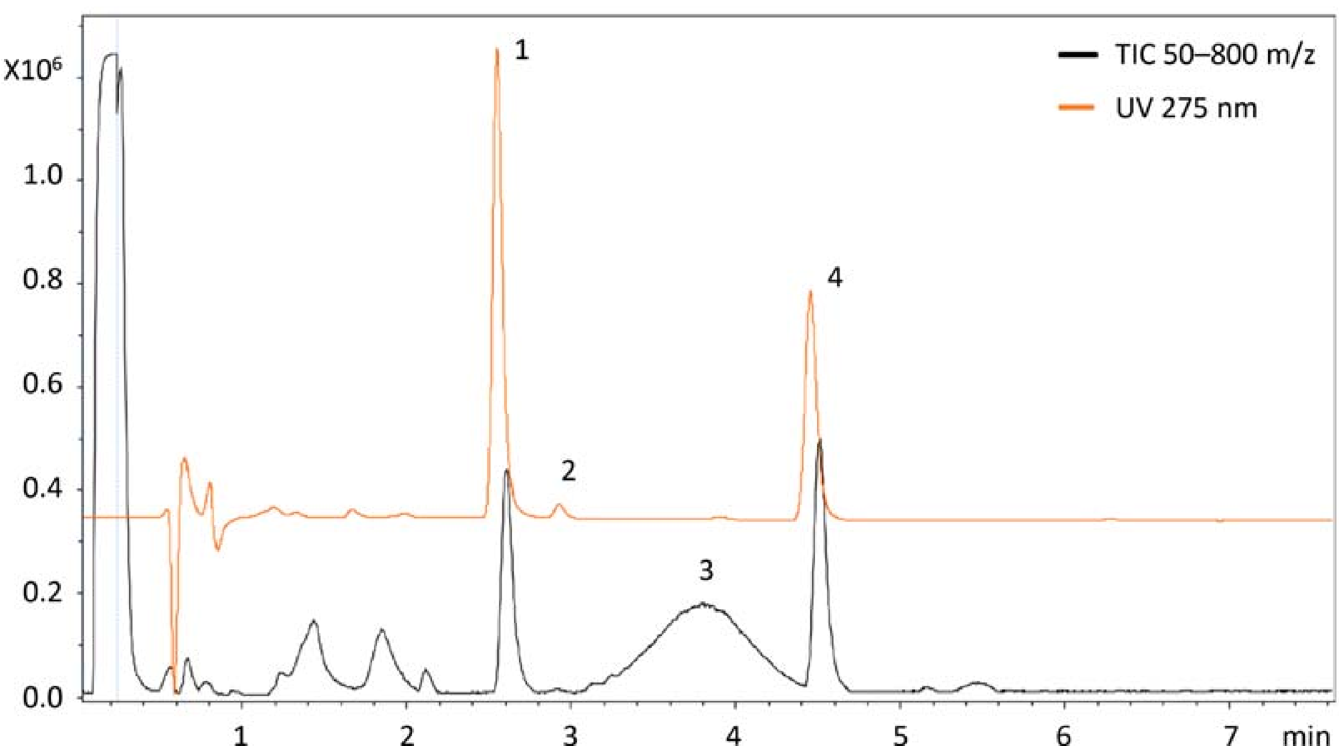

Figure 1. Overlaid UV chromatogram and total ion chromatogram (TIC). UV active substances 1 and 4 are identified as hydroxytyrosol and tyrosol. Signals 2 and 3 are only present after acid hydrolysis of extra-virgin olive oil.

The purity of hydroxytyrosol and tyrosol in standards, non-hydrolyzed samples and hydrolyzed extra virgin olive oils was confirmed (177.051 m/z $[M + Na]^+$ for hydroxytyrosol and 161.056 m/z $[M + Na]^+$ for tyrosol, respectively).

Olive oil samples without hydrolytic agent, which were incubated with water instead, served as a reference for the first assessment of thermal stability. Figure 2 compares the total yield of hydroxytyrosol and tyrosol of the reference samples (bar one) with the yield of five different conditions (bars two to six). Apparently, treatment at 80 °C (bar five) releases only a negligible amount of hydroxytyrosol and tyrosol, and even after 20 h (bar six), the yield remains low compared to even moderately acidic conditions (i.e., 25 °C and 0.5 M hydrochloric acid, bar two). This is also underlined by the chromatogram in Figure 3, which shows the increase in signal upon treatment with acid at constant temperature. It can be appreciated that under such acidic conditions, prolonged incubation at room temperature (bar three) cannot compensate for the benefit of elevated temperature (bar four).

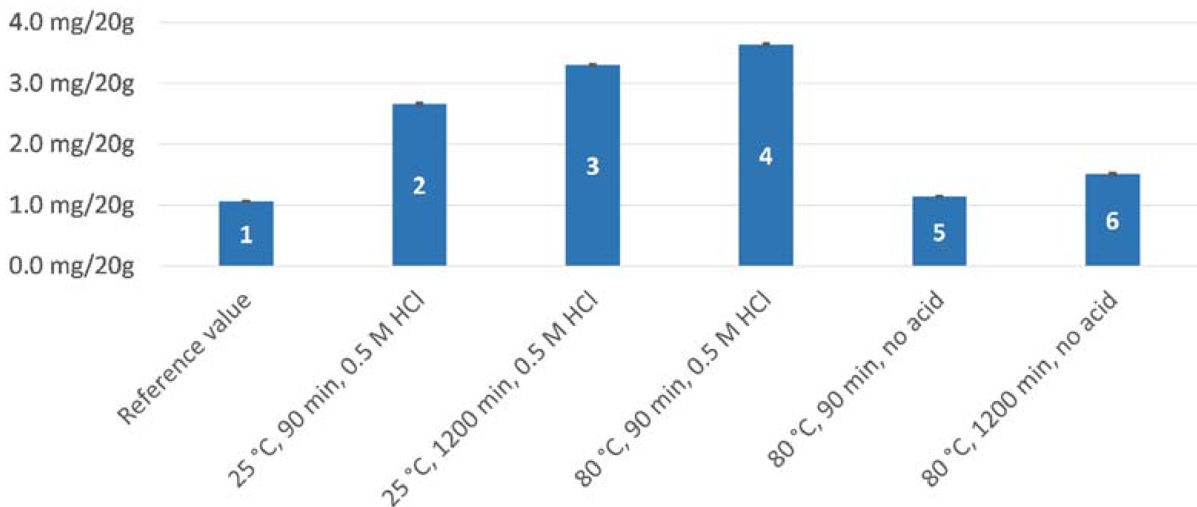

Figure 2. Temperature-induced cleavage in comparison to acidic hydrolysis.

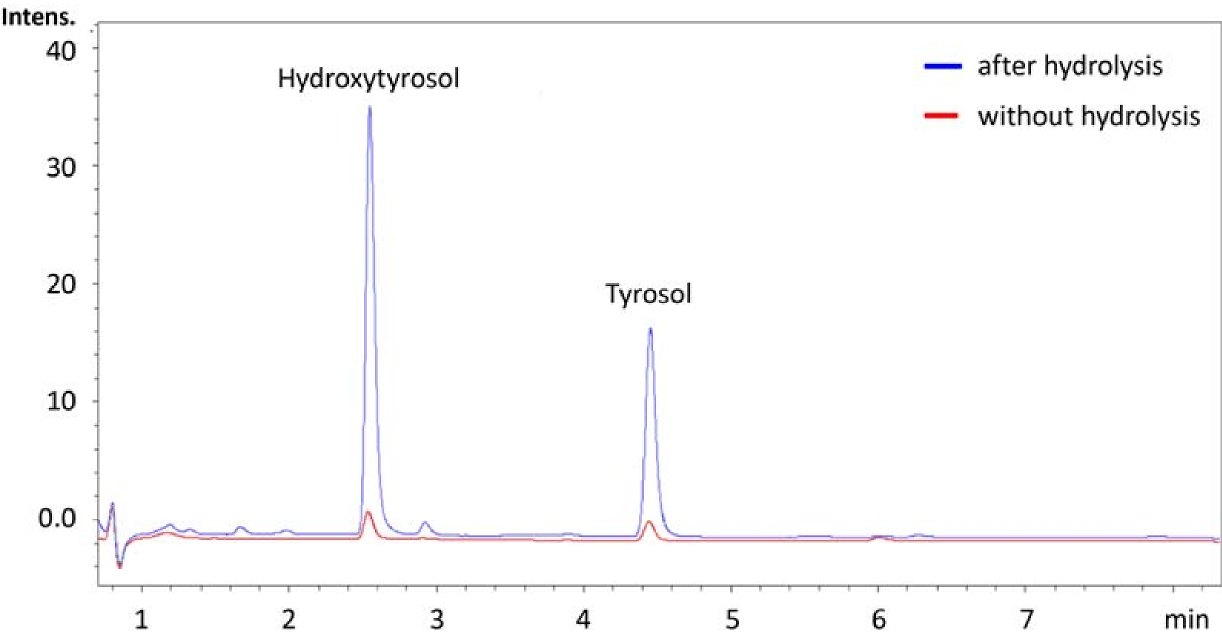

Figure 3. Comparison of a sample treated at 80 °C without acid (red) and a sample treated with 0.5 M hydrochloric acid (blue); the peaks at around 2.5 and 4.5 min represent hydroxytyrosol and tyrosol, respectively.

Considering that hydrochloric acid and sulfuric acid are commonly employed for acidic hydrolysis, a comparison of both acids' hydrolytic capabilities at varying molarities seems warranted. Unsurprisingly, both acids yielded similar values, as can be noted from Figure 4. Hydrochloric acid is responsible for slightly higher amounts of hydroxytyrosol and tyrosol at concentrations of 0.5 or 1.0 M. For the further experiments, and also from an economic perspective, 0.5 M acid concentration was chosen.

Since 0.5 M hydrochloric acid has a pH value of about 0.3, this value was envisaged for the subsequent comparison of hydrolyzing agents. For the weaker acids studied (i.e., formic acid, acetic acid, phosphoric acid and citric acid), 2 M solutions were prepared. pH values of 1.7 (formic acid), 2.7 (acetic acid), 0.9 (phosphoric acid) and 1.4 (citric acid) were observed. The hydrolyses were carried out at 80 °C for 90 min. After preliminary tests, an alkaline hydrolysis with sodium hydroxide, similar to that of Mulinacci et al. [24], was performed. However, due to the saponification of the oil, which probably requires a more complex work-up, further investigations were not considered. While hydrochloric,

sulfuric and nitric acids were responsible for the highest conversion (Figure 5), the weakest acids (i.e., acetic acid and citric acid) showed no hydrolytic activity, apart from the very small increase in phenols attributed to the elevated temperature (Figure 2, bar five). In subsequent experiments with nitric acid, higher than typical standard deviations were observed. Since the yield of hydrochloric, sulfuric, perchloric and nitric acids is very similar, only hydrochloric acid was selected for further optimization.

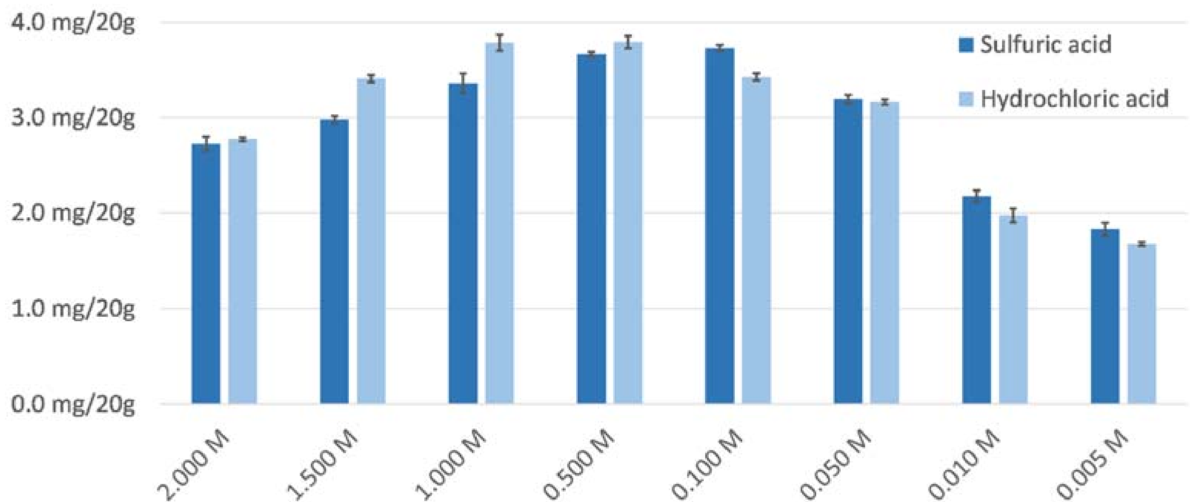

Figure 4. Optimization of hydrolysis using different molarities of hydrochloric and sulfuric acid.

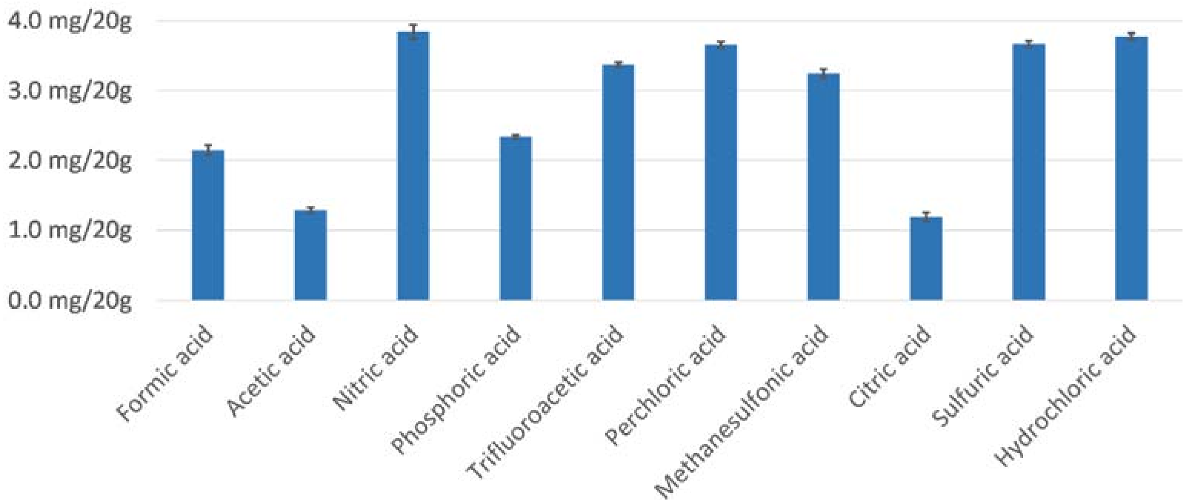

Figure 5. Hydrolytic activity of several acids.

Figure 6(top) reveals that higher temperatures, such as 80 °C, are responsible for increased conversion. While the yield at higher temperatures is arguably quite similar, the observation from Figure 2 justifies that such elevated temperature largely eliminates the need for exhaustive incubation times and makes the use of a laboratory shaker with a heating element reasonable. In this regard, the reader's attention is drawn to Figure 6(middle), which shows the yield at 80 °C for different incubation times. Although the yield does not increase significantly when the incubation time is extended beyond 30 min, longer reaction times can be justified since the remaining time can be efficiently used to prepare a second set of samples. Typically, 24 samples can be incubated in parallel using a commercially available laboratory shaker. What remains to be investigated is the choice of solvent used to dilute the hydrolyzing agent. The use of a more lipophilic co-solvent seems justified by the composition of the oil matrix, which probably improves the miscibility of the two

phases. However, care must be taken to ensure subsequent phase separation. Ethanol and acetone concentrations of up to 50% by volume proved to be unproblematic. For SDS, concentrations above 0.1 vol% were responsible for excessive foaming, which negatively affected the reproducibility of the experiment. The data from Figure 6(below) clearly show that both ethanol and acetone are responsible for deteriorated yields. Probably due to the low concentration, SDS has no effect on hydrolysis and gives results similar to acid diluted with water. More information on optimizing the hydrolysis time is provided as supplemental material (Table S3).

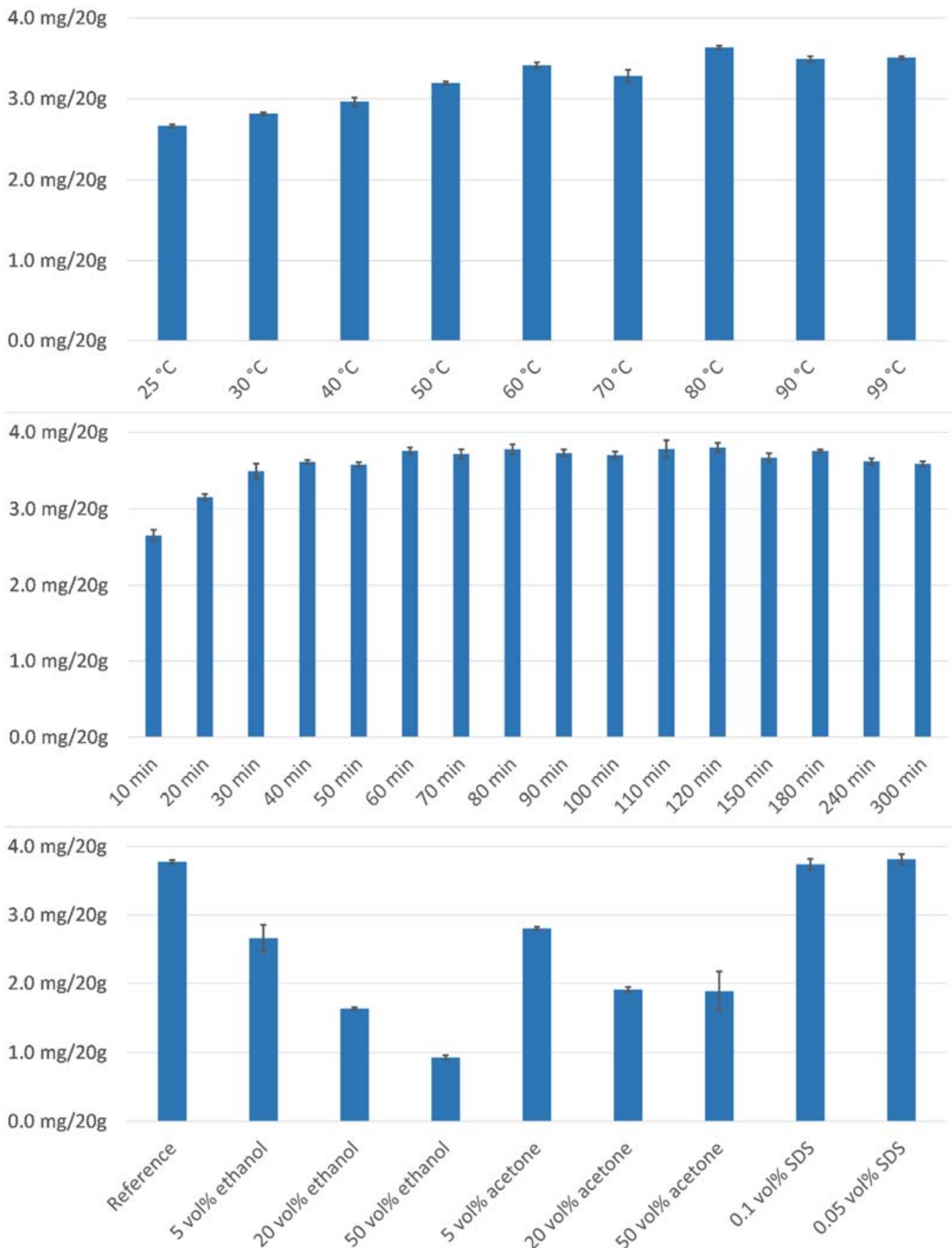

Figure 6. Optimization of hydrolysis temperature (**top**), hydrolysis time (**middle**) and the comparison of several solvents for acid dilution (**bottom**).

Based on these observations, the use of 0.5 M hydrochloric acid at a temperature of 60–80 °C in conjunction with an incubation time of 60–90 min is recommended. Since

no weakening of the signal was observed when known amounts of hydroxytyrosol and tyrosol were incubated in water under the above conditions, the stability of the analytes is assured. However, the yield comparison of fourteen extra virgin olive oils was conducted with and without a small amount of SDS (Figure 7). For most oils, a slight and sometimes even significant increase in yield could be observed when using SDS. This can be attributed to phenols bound to highly lipophilic moieties such as fatty acids. These are likely to be stabilized by SDS in the more hydrophilic hydrolysis environment present. On average, the yield attributed to 0.05% by volume SDS is 11% by weight. Comparing the yield with that of the conventional approach mentioned before, it is clear that the hydrolysis of the polar fraction is not only more labor intensive, but is also responsible for a lackluster yield of tyrosol and hydroxytyrosol. On average, the traditional approach captured only 47 wt% of the two phenols present in the oils tested.

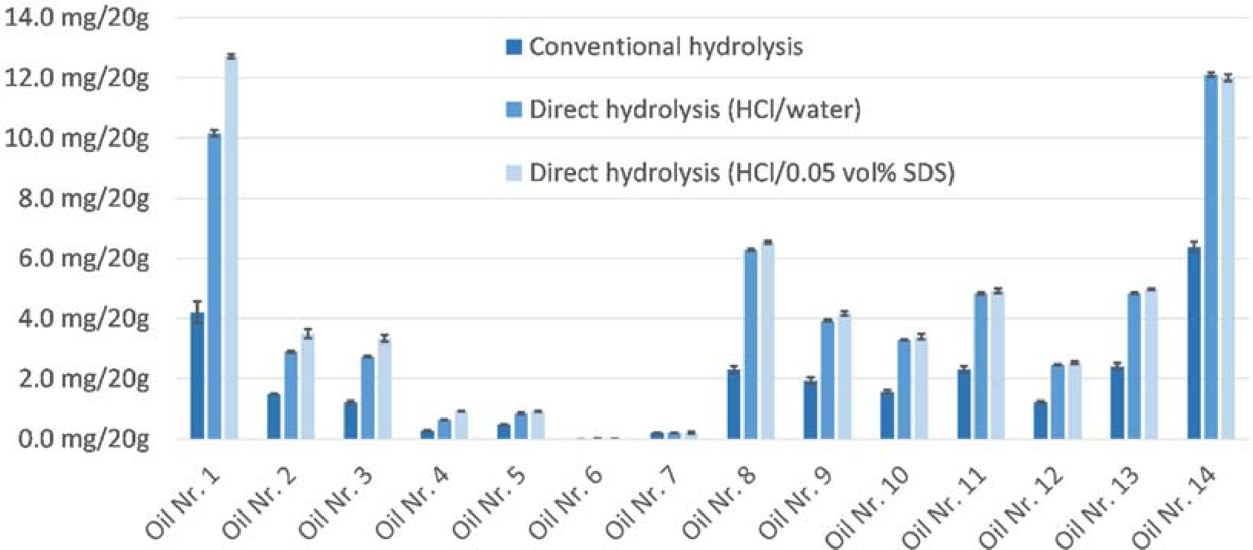

Figure 7. Yield comparison as observed for fourteen extra-virgin olive oils.

The ensuing economic comparison of the direct hydrolysis to the conventional approach relies on the concurrent triplicate determination of eight oils by a single laboratory technician. Since HPLC analysis requires identical resources for both methods, there is no obvious advantage here. However, the cost impact of solvents and reagents is reduced by more than 99.6% in favor of direct hydrolysis since no hexane or methanol is required. Moreover, the direct hydrolysis protocol is performed in about half the time required for traditional extraction and hydrolysis. In the case of laboratory consumables, the monetary impact is reduced by more than 50%. Overall, direct hydrolysis is not only more economical, but also significantly more environmentally friendly since excessive use of solvents and consumables is avoided.

4. Conclusions

In this work a fast, precise, economical, environmentally friendly, reliable and simple method for quantification of tyrosol and hydroxytyrosol in extra-virgin olive oils is proposed. The impact of the investigated parameters is evaluated and maximized, and to ensure selectivity of the analytical procedure and detection of the compounds of interest, a characterization by LC-MS is presented. Furthermore, the analytical method is validated according to stringent ICH guidelines and the combination with modern LC-MS techniques gives a good understanding about acid hydrolysis. The direct hydrolysis approach greatly simplifies the determination of hydroxytyrosol and tyrosol in olive oil, provides reproducible data and circumvents health claim formulation problems. A standardized indication of the total content of hydroxytyrosol and tyrosol in an olive oil would not only

help the consumer but also the producers, as market distortions due to different amounts of high molecular weight esterified phenols are mitigated.

From a scientific point of view, an indication of the content of released phenol makes sense, since in vivo hydrolysis of oleuropein, for example, has been shown in the past, while the thesis of its gastrointestinal absorption has been refuted [17–19]. A critical review of the metabolism of hydroxytyrosol and tyrosol adds to the ambiguity of the issue: the two phenols are minor metabolites of dopamine and tyramine, respectively, but the underlying pathways become more important with increased ethanol intake [8,32–34]. In the past, de la Torre et al. [35] hypothesized that the health benefits of red wine might be due to ethanol interfering with dopaminergic pathways, advocating a re-investigation of the biological effects "*[. . .] on the basis of combined hydroxytyrosol concentrations from red wine and dopamine turnover*". Later studies [32,33] indeed associated the enhanced excretion of tyrosol and hydroxytyrosol with the consumption of red wine and vodka, but also dealcoholized red wine. An ethanol-induced increase in the bioavailability of phenols and, to a lesser extent, the interaction of ethanol with dopamine and tyramine metabolism and other, yet unknown, factors appear to be responsible [32,33].

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10040268/s1, Table S1. Evaluation of repeatability performed on four extra-virgin olive oils of both compounds 3-Hydroxytyrosol and Tyrosol (day 2); Table S2. Evaluation of repeatability performed on four extra-virgin olive oils of both compounds 3-Hydroxytyrosol and Tyrosol (day 3); Table S3. Optimization of hydrolysis time; Figure S1. Recorded mass spectrum of hydroxytyrosol. 177.051 m/z was identified as $[M + Na]^+$, the basepeak at 137.059 corresponds to $[C_8H_9O_2]^+$. Electrospray ionization, positive mode, scan from 50–800 m/z, spectra rate 4 Hz; Figure S2. Recorded mass spectrum of tyrosol. 161.056 m/z was identified as $[M + Na]^+$, the basepeak at 121.064 corresponds to $[C_8H_9O]^+$. Electrospray ionization, positive mode, scan from 50-800 m/z, spectra rate 4 Hz; Figure S3. Recorded mass spectrum of the unknown signal eluting after hydroxytyrosol. Electrospray ionization, positive mode, scan from 50-800 m/z, spectra rate 4 Hz; Figure S4. Recorded mass spectrum of the broad signal eluting between 3.9 min and 4.4 min. Electrospray ionization, positive mode, scan from 50-800 m/z, spectra rate 4 Hz.

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