separations

Viewpoint

A Critical Appraisal of the Separation Protocols Proposed for the Implementation of the Health Claim on "Olive Oil Polyphenols" (EC Regulation 432/2012)

Maria Z. Tsimidou

Laboratory of Food Chemistry and Technology, School of Chemistry, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece; tsimidou@chem.auth.gr

Abstract: The analysis of the secoiridoid type of phenolic compounds present in virgin olive oil has become a challenging area of research since the first evidence of their presence in the polar fraction of the oil. Separation techniques, mainly liquid chromatographic ones, prevailed over the years of application toward elucidation of their structure, content determination and collection of evidence on cultivar, origin, processing and storage conditions dependence. One of the latest challenges in their analysis was related to the need to address the requirement set by EC Regulation 432/2012 for the implementation of the health claim on 'olive oil polyphenols'. The present work considers in a chronological order the original articles, viewpoints, review articles and other published efforts that appeared in the literature after the issuing of the relevant EFSA scientific opinion in 2011. The EFSA health claim created a lot of expectations among producers of virgin olive oil and boosted research for the development of a 'fit for the purpose' analytical protocol. Emphasis is given to the dedicated separation protocols that have been developed in the last 10 years and to the progress in their validation in comparison to the features of the method that were recently adopted by the International Olive Council.

Keywords: olive oil phenols; EFSA health claim; separation techniques; validated analytical protocols; International Olive Council; European legislation

Citation: Tsimidou, M.Z. A Critical Appraisal of the Separation Protocols Proposed for the Implementation of the Health Claim on "Olive Oil Polyphenols" (EC Regulation 432/2012). *Separations* **2022**, *9*, 351. https://doi.org/10.3390/separations9110351

Academic Editors: Victoria Samanidou, Natasa Kalogiouri and Maria Touraki

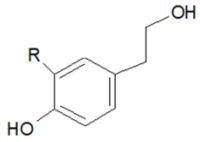
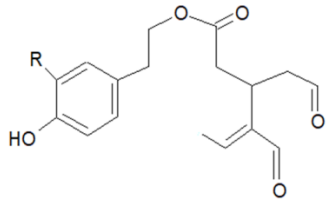
Received: 16 October 2022
Accepted: 3 November 2022
Published: 7 November 2022

1. Olive Oil Polar Phenols

Olive drupes contain an interesting gamut of bioactive compounds that are transferred to virgin olive oil (VOO) during processing by only physical means [1]. Among them, derivatives of secoiridoids such as oleuropein and ligstroside monopolized the interest of researchers, first, for technological reasons, as they were related to the remarkable oxidative stability of the oil [2], its taste [3] and, later on, to a variety of health benefits [4]. Being more polar in nature, and in order to differentiate them from the less polar tocopherols, this group of compounds was named olive oil 'polar phenols', or olive oil 'polyphenols', and it is commonly extracted from the lipid matrix using aqueous methanol solutions [5]. The term 'polyphenols' is still in use even in official documents, though the most abundant compounds present in the oil polar fraction (PF) bear only one phenolic ring, as illustrated in Table 1.

The term 'polar phenols' (PPh) is adopted subsequently for the aim of the present work. The term 'olive oil polyphenols' will be used exclusively when it refers to the EC Regulation 432/2012 [6] and the related European Food Safety Authority (EFSA) opinion [7].

Table 1. Chemical structure, IUPAC/empirical nomenclature and common abbreviation of the most widely known oleuropein and ligstroside derivatives present in virgin olive oil.

Chemical Structure	IUPAc/Empirical Nomenclature	Common Abbreviation
	**1:** (3,4-dihydroxyphenyl) ethanol/hydroxytyrosol, Hytyr **2**: (*p*-hydroxyphenyl) ethanol/tyrosol, Tyr	**1:** 3,4-DHPEA **2**: *p*-HPEA
	**3**: dialdehydic form of decarboxymethyl elenolic acid linked to 3,4-DHPEA/oleacein **4:** dialdehydic form of decarboxymethyl elenolic acid linked to *p* –HPEA/oleocanthal	**3:** 3,4-DHPEA-EDA **4:** *p*-HPEA-EDA

1 and **3**: R = OH; **2** and **4**: R = H.

2. Separation Methods for PPh Speciation and Quantification

Numerous analytical procedures have appeared in the literature since 1980. Most of them involve separation procedures applied to the intact oil or the PF. The latter is obtained using liquid-liquid (LLE) or solid phase (SPE) extraction means. Among published protocols, liquid chromatographic ones coupled with UV, diode array, fluorescence or MS detection systems prevail ([8,9] and references cited therein). Challenging remains the complete resolution of the target compounds and the lack or the cost of appropriate reference compounds. Nevertheless, depending on the aim of the study, e.g., structure elucidation, content determination or collection of evidence on cultivar, origin, processing and storage conditions dependence, researchers develop, adopt or modify separation protocols accordingly. A typical—though not optimum—liquid protocol is that adopted by the International Olive Council (IOC) for 'olive oil biophenols' in 2009 [10]. A characteristic chromatogram, including separation conditions and compound identification, according to the IOC protocol as adopted by Tsimidou and collaborators [11] is shown in Figure 1.

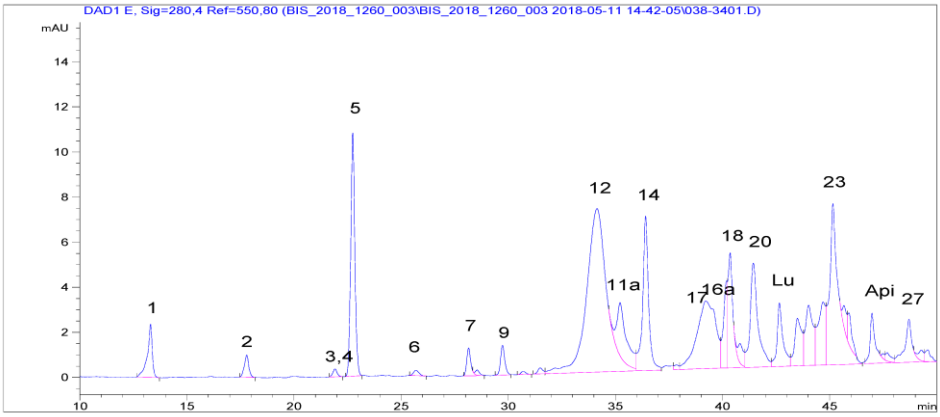


Figure 1. Typical chromatogram of virgin olive phenols present in the polar fraction at 280 nm following the IOC extraction, elution and identification conditions as adopted by ([11], protocol C, with MDPI permission). 1: Htyr; 2: Tyr; 3: vanillic acid; 4: caffeic acid; 5: syringic acid (IS); 6: vanillin; 7: *p*-coumaric acid; 9: ferulic acid; 11a: decarboxymethyl oleuropein aglycone, oxidized dialdehyde form; 12: decarboxymethyl oleuropein aglycone, dialdehyde form; 14: oleuropein aglycone, dialdehyde form; 16a: decarboxymethyl ligstroside aglycone, oxidized dialdehyde form; 17: decarboxymethyl ligstroside aglycone, dialdehyde form; 18: pinoresinol, 1-acetoxy-pinoresinol; 20: ligstroside aglycone, dialdehyde form; Lu: luteolin; 23: oleuropein aglycone, aldehyde and hydroxylic form; Api: apigenin; 27: ligstroside aglycone, aldehyde and hydroxylic form.

One of the latest challenges in their analysis is related to the need to address the requirement set by EC Regulation 432/2012 for the implementation of a health claim on 'olive oil polyphenols'. The health claim refers to their contribution to the 'protection of blood lipids from oxidative stress' and may be used only for 'olive oil, which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil'. It is also noted down that daily intake of this quantity of oil is necessary in order to obtain the particular beneficial effect. To fulfill the strict European legislative rules on the accuracy of any nutritional and health claims present on food labeling [12], dedicated and validated analytical protocols 'fit for the purpose' are mandatory. Such a protocol was not evidenced in the studies cited in the relevant opinion of EFSA ([7] and references cited therein). As a consequence, various separation protocols, as well as spectroscopic or other approaches, were developed over the last decade to address gaps at all stages of PPh analysis [9,11,13–35]. Among them, separation protocols prevail. The next section is focused on the dedicated separation protocols for the implementation of the health claim on 'olive oil polyphenols' that were published from 2011 to June 2022, when the last version of the IOC document, no. 29, was uploaded to its official website [36].

3. Dedicated Separation Protocols for the Implementation of the Health Claim on 'Olive Oil Polyphenols' (EC Regulation 432/2012) 2012–2022

The dedicated efforts using separation techniques for the determination of the target compounds (hydroxytyrosol and tyrosol in free and bound forms) were carried out and supported by different research groups [9,11,13–18,20,22–24,26–30,32,34]. Table 2 presents the institutes involved in each case to point out the rigorousness and collaborative spirit of the various working groups that responded to the market requirement.

Table 2. Dedicated separation protocols on the implementation of the health claim on 'olive oil polyphenols' (EC Regulation 432/2012) in chronological order.

Reference/Publication Year	Research Unit	Participation of Female Scientists (No. Out of Total)	Funding Source
Romero and Brenes [13,16]/2012	Instituto de la Graca/Sevilla/ES	1/2	National funds
Mastralexi et al. [15,17]/2014	Aristotle University of Thessaloniki/GR	2/3	University funds
Purcaro et al. [18]/2014	University of Udine/IT University of Barcelona/ES Stazione Sperimentale per le Industrie degli Oli e dei Grassi/Milano/IT	2/5	University and Institute funds
Tasioula-Margari and Tsabolatidou [20]/2015	University of Ioannina/GR	2/2	University funds
Monasterio et al. [22]/2016	Instituto de Biología Agrícola de Mendoza/AR University of Granada/ES	3/5	National funds
Ricciutelli et al. [23]/2016	University of Camerino/IT	2/7	University funds
Bartella et al. [24]/2018	Università della Calabria/Rende/IT	2/5	University funds
Celano et al. [26]/2018	University of Salerno, Fisciano, IT University of Reggio Calabria, Italy	5/7	National funds
Nenadis et al. [27]/2018	Aristotle University of Thessaloniki, GR University of Perugia/IT Instituto de la Graca/Sevilla/ES Alma Mater Studiorum/University of Bologna/IT	7/11	EU project
Olmo-García et al. [9]/2019	University of Granada/ES CM Europa S.L/Martos/ES	5/7	University funds

Table 2. *Cont.*

Reference/Publication Year	Research Unit	Participation of Female Scientists (No. Out of Total)	Funding Source
Ferro et al. [28]/2019	Instituto Politécnico de Beja/PT University of Aveiro/PT Universidade de Évora/PT	2/4	EU project
Tsimidou et al. [29]/2019	Aristotle University of Thessaloniki/GR Instituto de la Grasa (CSIC)/Seville/ES, Alma Mater Studiorum-University of Bologna/IT	4/6	EU project
Tsimidou et al. [11]/2019	Aristotle University of Thessaloniki/GR University of Perugia/IT Institute for Oliveculture, Science and Research Centre Koper, Zagreb/SI Universitat de Barcelona/ES Eurofins Analytik GmbH/Hamburg/DE Instituto de la Grasa (CSIC)/Seville/ES Alma Mater Studiorum—University of Bologna/IT	4/9	EU project
Bellumori et al. [30]/2019	University of Firenze and Multidisciplinary Centre of Research on Food Sciences (M.C.R.F.S.-Ce.R.A.)/IT University of Bari/IT University Aldo Moro/Bari/IT	5/6	AGER Foundation
Pereira et al. [32]	Universidade de Évora/PT	4/4	EU and national funds
Termopoli et al. [33]/2021	University of Urbino/IT Vancouver Island University/Nanaimo/CA	2/5	University funds
Paradiso et al. [34]/2022	University of Salento/Lecce/IT University of Bari, Italy/ University of Firenze/IT	5/9	AGER Foundation Regione Puglia

Participation of female scientists, seniors and youths is highlighted in a separate column, and it is really considerable (60%). Funding sources are presented in the last column as a proof of the involvement of different stakeholders (universities, institutes, national authorities, the private sector and the EU) to accelerate research progress. Researchers already active in olive oil PPh analysis from the major European olive oil producing countries reacted fast after the authorization of the EFSA health claim [7]. They realized that the analytical gaps and unclear terminology adopted in the official documents would burden the implementation of the health claim in the market [13,15,18,19,22,24,25]. Some efforts were focused on the simplification of the chromatographic profile shown in Figure 1. A prerequisite for this purpose was the introduction of a hydrolysis step for the bound forms in either the intact oil [13] or the polar fraction [15,18,21,24,29]. Optimization of the extraction conditions also became an issue for further examination and validation [5,20,24,26,27,31,34]. Some researchers supported the idea to use simple means to address the legal requirement [13,15,18,21,35]; however, others preferred to avoid—on one hand—hydrolysis of the bound forms and—on the other hand—introduced sophisticated detection means and systems not commonly available in routine analytical laboratories in the public or private sector [24,33]. Some investigators compared HPLC column efficiencies [28] or studied in parallel separation of the target compounds in HPLC and UHPLC systems [29]. Interestingly, some publications presented comparative data produced by different separation (or even NMR) protocols, including that of [10], for the same oil samples in an effort to reach a consensus among all interested parties, i.e., the IOC, the EU, national authorities and the industry [9,11,23,27,29]. Soon after the issuing [6], the IOC advertised an open call for methods 'fit for the purpose' of the health claim, whereas the EU financed projects that incorporated such activities [11,27,29,32]. However, until 2017, the IOC had not made any proposal [37] despite the pressure from the national authorities and the olive oil industry.

It is really impressive that in most analytical protocols data are compared to those obtained using IOC method no. 29 in an effort to make only the necessary modifications for accurate measurement of the target compounds [9,11,23,24,26,27,29,30]. In all of them, validation elements are given regarding the limits of detection and quantification of the target compounds, matrix effects, recovery, etc. In June 2022, the IOC [36] concluded its efforts to address the analytical issue. Thus, in the last version of [10], two methods are proposed: Method 1, COI/T.20/Doc. No 29/Rev.1 2017, for the determination of the biophenols in olive oils using HPLC, and Method 2, for the determination of phenolic compounds in olive oils using SPE-HPLC-DAD. As stated, 'Method 2 can be used to determine the real concentration of phenols to fulfill an EFSA claim as well as the content of individual phenols (e.g., oleocanthal and oleacein (note: oleoscein is wrong!))'. The method is the result of collaborative tests performed by 20 laboratories within the IOC. Unfortunately, there is no recent publication that has conceded a proper peer review process containing data from these tests to explain the choice of the SPE vs. LLE extraction and, in particular, the choice of diol SPE cartridges for the PF isolation. The conditions for PPh extraction are almost identical to those described by Mateos and collaborators 11 years ago [38]. This is clear from the data shown in Table 3. Even in that, publication superiority of SPE on diol cartridge vs. LLE was not supported [38].

Table 3. SPE conditions proposed for the extraction of the olive oil polar fraction according to [36,38].

SPE on Diol Cartridge by Mateos et al. [38]	**SPE on Diol Cartridge using IOC Method No. 29(2) [36]**
❖ A diol-bonded phase cartridge was placed in a vacuum elution apparatus and conditioned by the consecutive passing of 6 mL of methanol and 6 mL of hexane.	❖ Place the SPE diol cartridge) in the SPE equipment. Activate the cartridge by passing 6 mL of methanol and 6 mL of n-hexane without vacuum.
❖ The vacuum was then released to prevent drying of the column.	❖ Make sure the cartridge does not dry during elution.
❖ The oil solution was applied to the column, and the solvent was pulled through, leaving the sample and the standard in the solid phase.	❖ The oil sample is diluted with 6 mL of n-hexane and placed into the activated SPE cartridge. Let the sample enter the cartridge.
❖ The sample container was washed with two 3 mL portions of hexane, which were run out of the cartridge.	❖ Wash the flask with 6 mL of n-hexane and place into the column. Leave it to run out of the cartridge and discard.
❖ The sample container was washed again with 4 mL of hexane/ethyl acetate (90:10, *v*/*v*), which was run out of the cartridge and discarded.	❖ Elute with 4 mL of the eluting mixture n-hexane:ethyl acetate (85:15, *v*/*v*) and discard.
❖ Finally, the column was eluted with 10 mL of methanol, and the solvent was evaporated in a rotary evaporator at room temperature under vacuum until dry.	❖ Elute with 10 mL of methanol and collect the elution in a 25 mL conic flask. Evaporate in a rotary evaporator at room temperature in a vacuum until dry.
❖ The residue was extracted with 500 µL of methanol/water (1:1, *v*/*v*) at 40 °C.	❖ Dissolve the residue in 500 µL of the methanol/water mixture then shake it vigorously with the aid of a vortex.
❖ An aliquot (20 µL) of the final colorless solution was injected into the HPLC system.	❖ Keep this final solution in dark and cool conditions for at least four hours before determination.

Moreover, as shown in Table 4, the proposed RP-HPLC-DAD conditions in [36] are quite similar to those of [38], but it is not clear which compounds should be calculated to fulfill the health claim requirement. Precision data from the interlaboratory study organized by the IOC [36] are given for the total phenol content (mg/kg) after summing up 15 compounds, among which are vanillic acid, vanillin, ferulic acid, pinoresinol, cinnamic acid, 1-acetoxypinoresinol, luteolin and apigenin. Those eight compounds in particular are not secoiridoids and are not considered in the 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil required by the health claim.

Table 4. HPLC conditions proposed for 'olive oil polyphenols' according to [36,38].

HPLC Conditions	by Mateos et al. [38]	IOC Method No. 29 (2) [36]
Column	Lichrospher 100RP-18 column (4.0 mm i.d. × 250 mm; particle size, 5 μm) maintained at 30 °C.	RP18 (4.0 mm i.d. × 250 mm length), 5 μm
Eluting solvents	A, water/acetic acid (97:3, *v*/*v*)	A, water:phosphoric acid (99.5:0.5, *v*/*v*).
	B, methanol/acetonitrile (50:50 *v*/*v*)	B, methanol:acetonitrile (1:1, *v*/*v*)
Gradient	5% B (0 min); 30% B (25 min); 35% B (35 min); 40% B (40min); 70% B (50 min) and 100% B (55 min), followed by 5 min of maintenance.	5% B (0 min); 30% B (15 min); 38% B (30 min); 45% B (40 min); 52.5%B (45 min); 100%B (50 min)
Flow rate	1.0 mL/min	1 mL/min
Detection	240, 280 and 335 nm	280 and 325 nm
Quantification	Internal standard solution, *p*-hydroxyphenylacetic acid, 4.64×10^{-2} mg/mL and *o*-coumaric acid, 9.6×10^{-3} mg/mL in methanol	Internal standard solution, *p*-hydroxyphenylacetic acid, 0.12 mg/mL and *o*-coumaric acid, 0.01 mg/mL in methanol.

The IOC recommendation ignores the endeavors of so many scientists to address the analytical gaps in specific and unambiguous ways over the last decade. The proposed analytical protocol for extraction, separation, identification and quantification seems to be an outdated compromise, whereas there are more recent validated protocols 'fit for the purpose' of the EFSA health claim available.

4. Conclusions

Unequivocally, authorization of the 'olive oil polyphenol' health claim boosted PPh analysis of virgin olive oils from different cultivars and countries, which was a positive activity for the overall interests of SMEs and small producers. Even though the claim could not be used on the labeling for 10 years already, publicity and consumer awareness of the health benefits of olive oil polyphenols was gained in various ways, mainly through social media and scientific publications. However, the bureaucratic procedures of international bodies do not seem to follow scientific progress for the benefit of the olive sector. Researchers from more than 26 institutes who worked hard in this direction should be rather disappointed. The analytical gap cannot be considered addressed on the soundest scientific basis by the recently recommended method of the IOC.

Funding: This research received no external funding.

Data Availability Statement: Not applicable.

Conflicts of Interest: The author declares no conflict of interest.

References

1. Boskou, D. Olive Fruit, Table Olives, and Olive Oil Bioactive Constituents. In *Olive and Olive Oil Bioactive Ingredients*; Boskou, D., Ed.; AOCS Press: Urbana, IL, USA, 2015; pp. 1–30.
2. Tsimidou, M. Polyphenols and Quality of Virgin Olive Oil in Retrospect [*Olea europaea* L.]. *Ital. J. Food Sci.* **1998**, *10*, 99–116.
3. Tsimidou, M.Z. Analytical Methodologies: Phenolic Compounds Related to Olive Oil Taste Issues. In *Handbook of Olive Oil: Analysis and Properties*; Aparicio, R., Harwood, J., Eds.; Springer: Boston, MA, USA, 2013; pp. 311–333.
4. de la Torre, R.; Montserrat Fitó, M.; Covas, M.I. The Bioavailability of Olive Oil Phenolic Compounds and their Bioactive Effects in Humans. In *Olives and Olive Oil in Health and Disease Prevention*, 2nd ed.; Preedy, V.P., Watson, R.R., Eds.; Academic Press: Cambridge, MA, USA, 2021; pp. 193–203.
5. Pizarro, M.L.; Becerra, M.; Sayago, A.; Beltrán, M.; Beltrán, R. Comparison of Different Extraction Methods to Determine Phenolic Compounds in Virgin Olive Oil. *Food Anal. Methods* **2013**, *6*, 123–132. [CrossRef]
6. European Commission. Regulation (EU) No 432/2012 of 16 May 2012 Establishing a List of Permitted Health Claims Made on Foods, Other than Those Referring to the Reduction of Disease Risk and to Children's Development and Health. *Off. J. Eur. Union* **2012**, *L136*, 1–40. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2012:136:0001:0040:en:PDF (accessed on 9 September 2022).

7. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Scientific Opinion on the substantiation of health claims related to polyphenols in olive and protection of LDL particles from oxidative damage (ID 1333, 1638, 1639, 1696, 2865), maintenance of normal blood HDL-cholesterol concentrations (ID 1639), maintenance of normal blood pressure (ID 3781), "anti-inflammatory properties" (ID 1882), "contributes to the upper respiratory tract health" (ID 3468), "can help to maintain a normal function of gastrointestinal tract" (3779), and "contributes to body defenses against external agents" (ID 3467) pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA J.* **2011**, *9*, 2033. [CrossRef]
8. Gómez-Caravaca, A.M.; Lozano-Sánchez, J.; Contreras Gámez, M.d.M.; Segura-Carretero, A.; Taamalli, A. Bioactive Phenolic Compounds from *Olea europaea*: A Challenge for Analytical Chemistry. In *Olive and Olive Oil Bioactive Ingredients*; Boskou, D., Ed.; AOCS Press: Urbana, IL, USA, 2015; pp. 261–298.
9. Olmo-García, L.; Fernández-Fernández, C.; Hidalgo, A.; Vílchez, P.; Fernández-Gutiérrez, A.; Marchal, R.; Carrasco-Pancorbo, A. Evaluating the Reliability of Specific and Global Methods to Assess the Phenolic Content of Virgin Olive Oil: Do they Drive to Equivalent Results? *J. Chromatogr. A* **2019**, *158*, 56–69. [CrossRef]
10. International Olive Council [IOC]. *Determination of Biophenols in Olive Oil by HPLC. COI/T.20/Doc. No. 29*; International Olive Council, [IOC]: Madrid, Spain, 2009. Available online: https://www.oelea.de/downloads/COI-T20-DOC-29-2009-DETERMINATION-OF-BIOPHENOLS-IN-OLIVE-OILS-BY-HPLC.pdf (accessed on 15 October 2022).
11. Tsimidou, M.Z.; Nenadis, N.; Mastralexi, A.; Servili, M.; Butinar, B.; Vichi, S.; Winkelmann, O.; García-González, D.L.; Toschi, T.G. Toward a Harmonized and Standardized Protocol for the Determination of Total Hydroxytyrosol and Tyrosol Content in Virgin Olive Oil (VOO). The Pros of a Fit for the Purpose Ultra High Performance Liquid Chromatography (UHPLC) Procedure. *Molecules* **2019**, *24*, 2429. [CrossRef]
12. European Parliament and the Council of the European Union. Regulation (EC) No. 1924/2006 of the European Parliament and of the Council of 20 December 2006 on Nutrition and Health Claims Made on Foods. *Off. J. Eur. Union* **2006**, *L404*, 9–25. Available online: https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:404:0009:0025:En:PDF (accessed on 9 September 2022).
13. Romero, C.; Brenes, M. Analysis of Total Contents of Hydroxytyrosol and Tyrosol in Olive Oils. *J. Agric. Food Chem.* **2012**, *60*, 9017–9022. [CrossRef]
14. Karkoula, E.; Skantzari, A.; Melliou, E.; Magiatis, P. Quantitative Measurement of Major Secoiridoid Derivatives in Olive Oil using qNMR. Proof of the Artificial Formation of Aldehydic Oleuropein and Ligstroside Aglycon Isomers. *J. Agric. Food Chem.* **2014**, *62*, 600–607. [CrossRef]
15. Mastralexi, A.; Nenadis, N.; Tsimidou, M.Z. Addressing Analytical Requirements to Support Health Claims on "Olive Oil Polyphenols" (EC regulation 432/2012). *J. Agric. Food Chem.* **2014**, *62*, 2459–2461. [CrossRef]
16. Romero, C.; Brenes, M. Comment on Addressing Analytical Requirements to Support Health Claims on "Olive Oil Polyphenols" (EC regulation 432/212). *J. Agric. Food Chem.* **2014**, *62*, 10210–10211. [CrossRef] [PubMed]
17. Mastralexi, A.; Nenadis, N.; Tsimidou, M.Z. Rebuttal to the Comment on Addressing Analytical Requirements to Support Health Claims on "Olive Oil Polyphenols" (EC regulation 432/212). *J. Agric. Food Chem.* **2014**, *62*, 10212–10213. [CrossRef] [PubMed]
18. Purcaro, G.; Codony, R.; Pizzale, L.; Mariani, C.; Conte, L. Evaluation of Total Hydroxytyrosol and Tyrosol in Extra Virgin Olive Oils. *Eur. J. Lipid Sci. Technol.* **2014**, *116*, 805–811. [CrossRef]
19. Tsimidou, M.Z.; Boskou, D. The Health Claim on "Olive Oil Polyphenols" and the Need for Meaningful Terminology and Effective Analytical Protocols. *Eur. J. Lipid Sci. Technol.* **2015**, *117*, 1091–1094. [CrossRef]
20. Tasioula-Margari, M.; Tsabolatidou, E. Extraction, Separation, and Identification of Phenolic Compounds in Virgin Olive Oil by HPLC-DAD and HPLC-MS. *Antioxidants* **2015**, *4*, 548–562. [CrossRef]
21. Reboredo-Rodríguez, P.; Valli, E.; Bendini, A.; Di Lecce, G.; Simal-Gándara, J.; Gallina Toschi, T. A Widely Used Spectrophotometric Assay to Quantify Olive Oil Biophenols according to the Health Claim (EU Reg. 432/2012). *Eur. J. Lipid Sci. Technol.* **2016**, *118*, 1593–1599. [CrossRef]
22. Monasterio, R.P.; Olmo-García, L.; Bajoub, A.; Fernández-Gutiérrez, A.; Alegría Carrasco-Pancorbo, A. Potential of LC Coupled to Fluorescence Detection in Food Metabolomics: Determination of Phenolic Compounds in Virgin Olive Oil. *Int. J. Mol. Sci.* **2016**, *17*, 1627. [CrossRef]
23. Ricciutelli, M.; Marconi, S.; Boarelli, M.C.; Caprioli, G.; Sagratini, G.; Ballini, R.; Fiorini, D. Olive Oil Polyphenols: A Quantitative Method by High-Performance Liquid-Chromatography-Diode-Array Detection for their Determination and the Assessment of the Related Health Claim. *J. Chromatogr. A* **2017**, *1481*, 53–63. [CrossRef]
24. Bartella, L.; Mazzotti, F.; Napoli, A.; Sindona, G.; Di Donna, L. A Comprehensive Evaluation of Tyrosol and Hydroxytyrosol Derivatives in Extra Virgin Olive Oil by Microwave-Assisted Hydrolysis and HPLC-MS/MS. *Anal. Bioanal. Chem.* **2018**, *410*, 2193–2201. [CrossRef]
25. Tsimidou, M.Z.; Nenadis, N.; Servili, M.; García-González, D.L.; Gallina Toschi, T. Why Tyrosol Derivatives Have to be Quantified in the Calculation of "Olive Oil Polyphenols" content to support the health claim provisioned in the EC Reg. 432/2012. *Eur. J. Lipid Sci. Technol.* **2018**, *120*, 1800098. [CrossRef]
26. Celano, R.; Piccinelli, A.L.; Pugliese, A.; Carabetta, S.; Di Sanzo, R.; Rastrelli, L.; Russo, M. Insights into Analysis of Phenolic Secoiridoids in Extra Virgin Olive Oil. *J. Agric. Food Chem.* **2018**, *66*, 6053–6063. [CrossRef]

27. Nenadis, N.; Mastralexi, A.; Tsimidou, M.Z.; Vichi, S.; Quintanilla-Casas, B.; Donarski, J.; Bailey-Horne, V.; Butinar, B.; Miklavčič, M.; García-González, D.L.; et al. Toward a Harmonized and Standardized Protocol for the Determination of Total Hydroxytyrosol and Tyrosol Content in Virgin Olive Oil (VOO). Extraction Solvent. *Eur. J. Lipid Sci. Technol.* **2018**, *120*, 1800099. [CrossRef]
28. Ferro, M.D.; Santos, S.A.O.; Silvestre, A.J.D.; Duarte, M.F. Chromatographic Separation of Phenolic Compounds from Extra Virgin Olive Oil: Development and Validation of a New Method Based on a Biphenyl HPLC Column *Int. J. Mol. Sci.* **2019**, *20*, 201. [CrossRef]
29. Tsimidou, M.Z.; Sotiroglou, M.; Mastralexi, A.; Nenadis, N.; García-González, D.L.; Gallina-Toschi, T. In house Validated UHPLC Protocol for the Determination of the Total Hydroxytyrosol and Tyrosol Content in Virgin Olive Oil Fit for the Purpose of the Health claim Introduced by the EC Regulation 432/2012 for "Olive Oil Polyphenols". *Molecules* **2019**, *24*, 1044. [CrossRef]
30. Bellumori, M.; Cecchi, L.; Innocenti, M.; Clodoveo, M.L.; Corbo, F.; Mulinacci, N. The EFSA Health Claim on Olive Oil Polyphenols: Acid Hydrolysis Validation and Total Hydroxytyrosol and Tyrosol Determination in Italian Virgin Olive Oils. *Molecules* **2019**, *24*, 2179. [CrossRef]
31. Paradiso, V.M.; Squeo, G.; Pasqualone, A.; Caponio, F.; Summo, C. An Easy and Green Tool for Olive Oils Labelling according to the Contents of Hydroxytyrosol and Tyrosol Derivatives: Extraction with a Natural Deep Eutectic Solvent and Direct Spectrophotometric Analysis. *Food Chem.* **2019**, *291*, 1–6. [CrossRef]
32. Pereira, C.; Ana Maria Costa Freitas, A.M.; João Cabrita, M.; Garcia, R. Assessing Tyrosol and Hydroxytyrosol in Portuguese Monovarietal Olive Oils: Revealing the Nutraceutical Potential by a Combined Spectroscopic and Chromatographic Techniques—Based Approach. *LWT—Food Sci. Technol.* **2020**, *118*, 108797. [CrossRef]
33. Termopoli, V.; Piergiovanni, M.; Cappiello, A.; Palma, P.; Famiglini, G. Tyrosol and Hydroxytyrosol Determination in Extra Virgin Olive Oil with Direct Liquid Electron Ionization-Tandem Mass Spectrometry. *Separations* **2021**, *8*, 173. [CrossRef]
34. Paradiso, V.M.; Longobardi, F.; Fortunato, S.; Rotondi, P.; Bellumori, M.; Cecchi, L.; Cosma, P.; Mulinacci, N.; Caponio, F. Paving the Way to Food Grade Analytical Chemistry: Use of a Natural Deep Eutectic Solvent to Determine Total Hydroxytyrosol and Tyrosol in Extra Virgin Olive Oils. *Foods* **2021**, *10*, 677. [CrossRef]
35. Siano, F.; Vasca, E.; Picariello, G. Accurate Determination of Total Biophenols in Unfractionated Extra-Virgin Olive Oil with the Fast Blue BB Assay. *Food Chem.* **2022**, *370*, 130990. [CrossRef]
36. International Olive Council [IOC]. *Determination of Biophenols in Olive Oil by HPLC. COI/T.20/Doc. No. 29 Rev.2*; International Olive Council [IOC]: Madrid, Spain, 2022. Available online: https://www.internationaloliveoil.org/what-we-do/chemistry-standardisation-unit/standards-and-methods (accessed on 9 September 2022).
37. International Olive Council [IOC]. *Determination of Biophenols in Olive Oil by HPLC. COI/T.20/Doc. No. 29 Rev.1*; International Olive Council [IOC]: Madrid, Spain, 2017. Available online: https://www.internationaloliveoil.org/wp-content/uploads/2019/11/COI-T.20-Doc.-No-29-Rev-1-2017.pdf (accessed on 9 September 2022).
38. Mateos, R.; Espartero, J.L.; Trujillo, M.; . Ríos, J.J.; León-Camacho, M.; Alcudia, F.; Cert, A. Determination of Phenols, Flavones, and Lignans in Virgin Olive Oils by Solid-Phase Extraction and High-Performance Liquid Chromatography with Diode Array Ultraviolet Detection. *J. Agric. Food Chem.* **2001**, *49*, 2185–2192. [CrossRef]