Scientific Research Publishing

American Journal of Analytical Chemistry, 2022, 13, 476-493
https://www.scirp.org/journal/ajac
ISSN Online: 2156-8278
ISSN Print: 2156-8251

Critical Examination of the Suitability of the Folin-Ciocalteu Reagent Assay for Quantitative Analysis of Polyphenols—The Case of Olive-Mill Wastewater

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How to cite this paper: Samara, M., Nasser, A. and Mingelgrin, U. (2022) Critical Examination of the Suitability of the Folin-Ciocalteu Reagent Assay for Quantitative Analysis of Polyphenols—The Case of Olive-Mill Wastewater. *American Journal of Analytical Chemistry*, **13**, 476-493.
https://doi.org/10.4236/ajac.2022.1311032

Received: August 4, 2022
Accepted: November 25, 2022
Published: November 28, 2022

Abstract

The suitability of the Folin-Ciocalteu reagent (FCR) and of HPLC for analyzing polyphenols is evaluated. FCR assay is commonly used although its flows, such as overestimating polyphenol content due to interference by oxidizable species, were previously reported. The wide range of oxidizable species present in biological systems seriously compromises this assay's reliability. Adding small amounts of protein to olive-mill wastewater (OMWW) significantly increased the apparent polyphenol content indicated by the FCR assay. The commonly used "reference" polyphenol as a standard for "total polyphenols" quantification is problematic since each polyphenol responds differently to the FCR. Conversely, HPLC may underestimate polyphenol content. No single HPLC protocol is likely to detect the whole myriad of polyphenols which may be present in a polyphenol-containing system. In analyzing 5 OMWW samples both by FCR assay and HPLC, the polyphenol content indicated by the FCR assay was up to six-fold higher than that determined by HPLC.

Keywords

Folin-Ciocalteu Reagent Assay, HPLC Polyphenol Analysis, Olive-Mill Wastewater, Polyphenols

1. Introduction

About 8000 different polyphenols are produced by plants, some of which help protect the plant from insects, microorganisms or the damaging effects of ultra-

violet radiation [1]. Certain polyphenols produced by plants are effective in preventing animal and human diseases, acting as antioxidants and playing a role in alleviating disorders of the nervous system, fighting infections, slowing ageing, and inhibiting cancer, asthma and heart diseases [2]. The therapeutic activity of polyphenols is derived, for example, from the presence in those compounds of functional groups which can interact with free radicals and heavy metals.

The large family of polyphenols is traditionally arranged in 3 groups [3]: Flavonoids, a group of substances the molecule of which consists of a 15-carbon skeleton containing two phenyl rings and a heterocyclic ring. The molecules belonging to this group are derivatives of flavone; Stilbenoids are compounds composed of two rings bridged by two carbon atoms connected by a double bond and multiple hydroxide groups. The most common compound in this group is resveratrol; Phenolic acids (and aromatic acids in general) are compounds derived from benzoic acid and contain a benzene ring and a carboxylic group. The most common member of this group is cinnamic acid.

The concentration of polyphenols in ripe olives can surpass 8000 $\mu g \cdot g^{-1}$ [4]. Some of the polyphenols originating in olives find their way to the olive-mill wastewater (OMWW), the liquid waste of the olive oil production process. Polyphenols concentration in OMWW differs widely, depending on olive varieties and oil production methods. Thus, according to [4] the average polyphenol concentration in OMWW produced in Portugal was 440 $\mu g \cdot g^{-1}$, in France 25 $\mu g \cdot g^{-1}$, in Italy 4017 $\mu g \cdot g^{-1}$ and in Spain 124 $\mu g \cdot g^{-1}$.

OMWW is often disposed on agricultural land. Loading the soil with OMWW may cause leaching of polyphenols down to the groundwater, thus potentially raising the polyphenol concentration in the groundwater to a harmful level. Although, as stated above, many polyphenols serve beneficial functions, some polyphenols are toxic to plants and their accumulation in soils treated with OMWW may adversely affect the vegetation and reduce crop yield and quality [5].

Two main analytical approaches to quantify polyphenols were applied to date: a spectrophotometric approach based on a color reaction, the Folin-Ciocalteu reagent (FCR) assay, and a chromatographic approach employing various GCMS or HPLC-based procedures, e.g. [6] [7]. The Folin-Ciocalteu reagent is composed of an aqueous solution of sodium tungstate ($NaWO_4 \cdot 2H_2O$), sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), HCl and phosphoric acid to which lithium sulfate ($Li_2SO_4 \cdot 4H_2O$) is added. FCR is an oxidizing agent which reacts with reducing agents under basic conditions to produce a soluble, intensely blue, reduced product [6]. The details of the reaction mechanism of the FCR with polyphenols is not well understood but overall, when the reagent comes into contact with the polyphenol, the molybdenum (Mo(VI)) in the reaction mixture accepts an electron to become Mo(V), while a phenolic group loses an electron to become a phenolate radical [8].

While the FCR method is used extensively to determine the content of polyphenols in biological materials [9] [10] [11] [12] etc., it has some important

shortcomings: 1) It cannot differentiate between individual polyphenolic compounds and hence does not enable the identification of the specific polyphenols present; 2) Polyphenols differ considerably in their quantitative response to the FCR (as shown, for example, in section 3.5 and **Figure 1**) and hence the polyphenol chosen as the standard for quantification may strongly affect the determined apparent polyphenol content; 3) Most importantly, although the FCR is used for detecting and quantifying polyphenols, this reagent is actually responding to an overall reducing capacity of the reaction mixture and hence is subject to considerable interferences, for example due to the presence of sugars, proteins or amino acids [11] [13]. This considerable interference of oxidizable species limits the FCR assay's reliability even for the comparison between the relative (as opposed to absolute) content of polyphenols in different samples because both the composition and the quantity of oxidizable species (including polyphenols) are likely to vary between samples. Although the critical flows in the FCR assay are well documented e.g. [12] [13], references in the literature attribute to the FCR analysis high-precision and accuracy and this reagent was used by numerous research groups (and still is), often as the sole or primary (as opposed to a preliminary) analytical procedure for polyphenol determination [10] [11] [12] [14], etc. The frequent use of the FCR assay to determine polyphenols content persists despite of the availability of alternative (e.g., chromatographic) assays, e.g. [7] [15].

Most of the above shortcomings are eliminated when chromatographic methods for polyphenol analysis, such as HPLC, are used. For example, the latter procedures enable quantitative identification of individual polyphenols by running the unknown samples against known concentrations of specific polyphenol standards. Yet, since complex biological systems such as OMWW may contain numerous components that can interfere with the chromatographic separation, the stationary and mobile phases as well as the running conditions adopted in the chromatographic procedure must all be system-specific so as to suit both the polyphenols present and the matrix in which they are found. Also, there is a high likelihood that any chromatographic procedure applied to a given biological system may not be capable of detecting all polyphenolic species present and thus underestimate the total polyphenol content.

In the present study, a much needed critical evaluation of the commonly used FCR procedure is presented. For the sake of comparison, an HPLC-based procedure for the quantification and identification of some polyphenols commonly found in OMWW is reported and its results contrasted with those obtained by the standard spectroscopic (FCR) procedure. The hypothesis behind the work reported in this paper is that although chromatographic procedures, such as HPLC, are preferred for polyphenol analysis in biological systems to the FCR assay, mainly due to a major interference with the FCR assay by the multitude of oxidizable species present in biological systems, chromatographic assays have a serious limitation as well: In using a chromatographic procedure for polyphenol

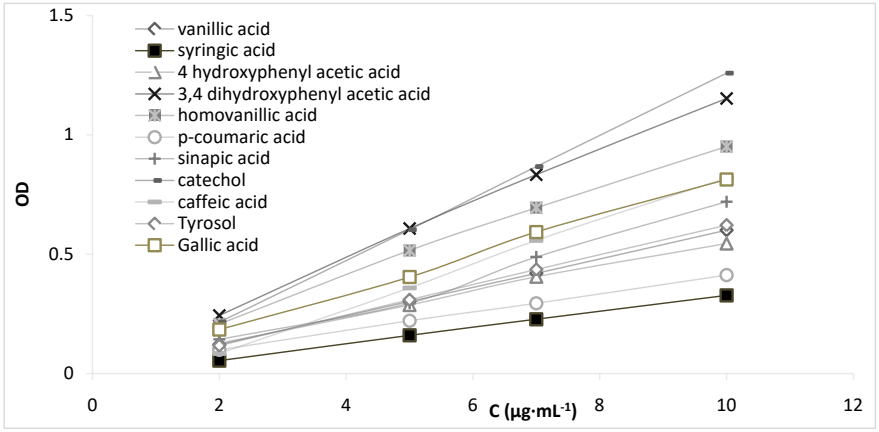


Figure 1. Calibration curves (absorption at 725 nm) for the polyphenols analyzed by the FCR procedure.

analysis, a specific group of polyphenols of interest must be defined, due to the aforementioned likelihood that any chromatographic procedure may not be capable of detecting all of the many polyphenolic species that might possibly be present in a given system. It is posited that due to the great diversity in the properties of polyphenols, determining the "total polyphenols content" in a sample may be of a rather limited utility as compared to the usefulness of the determination of specific groups of polyphenols to be defined by the objectives of the performed analysis.

Although the shortcomings of the FCR procedure in analyzing polyphenol are already well known, this problematic procedure is still often used as the sole analytical method for determining polyphenols (or "total phenolic compounds") in biological systems, e.g. [9] [11] [14] [16] [17]. For this reason and contrary to previous works, this paper quantifies and highlights the problematic nature of the FCR assay and the reasons for the better suitability of HPLC for the determination of polyphenols (while also pointing out the limitations of the latter and similar chromatographic procedures). The quantification of polyphenols in OMWW and in soils amended with OMWW is used as a case study.

2. Materials and Methods

2.1. Materials

FCR was purchased from Sigma-Aldrich (St. Louis, MO, USA) as were all the polyphenol standards employed (**Table 1**) as well as gallic acid ($C_6H_2(OH)_3COOH$), which was used as one universal standard for the FCR analysis, the bovine serum albumin (BSA) and trifluoroacetic acid. All solvents (HPLC grade), $NaHCO_3$ and HPLC grade water were purchased from Bio Lab (Jerusalem, Israel) and all other chemicals from Merck (Darmstadt, Germany). The Gibco Bacto peptone was purchased from Thermo Fisher Scientific (USA). **Table 2** lists the soils to which OMWW was applied and from which extraction of polyphenols was carried out. Soil samples were collected from the surface layer (0 - 3 cm) of plots on which OMWW was disposed. While the first 3 soils listed in **Table 2** are from

the south of Israel, the last two are from the Palestinian Authority [18]. The amount of OMWW disposed on the Revivim soil was approximately 120 m^3 per hectare annually over 4 years and on the Negba and Gilat soils 70 m^3 per hectare on each for one year only [19]. The amount per year and duration of OMWW application to the two Palestinian soils, Rahal and Kamrah (**Table 2**) is not precisely known but is estimated to be as high as 1000 - 2000 m^3 per hectare per annum.

Table 1. The polyphenols investigated in the present study-structure and extraction efficiency from Beit Dagan soil by the two-stage extraction procedure (Section 2.3).

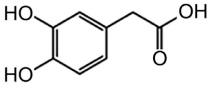
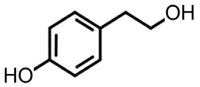
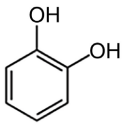
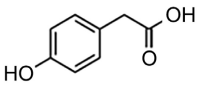
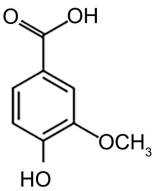
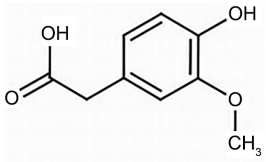
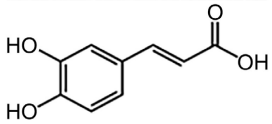
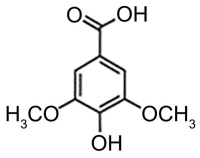
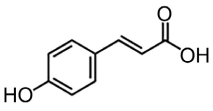
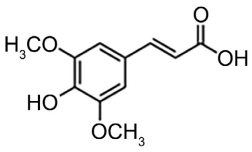
Polyphenol	Structure	Recovery (%)
3,4-Dihydroxyphenylacetic acid		32.3
Tyrosol		99.5
Catechol		118.0
4-Hydroxyphenylacetic acid		95.9
Vanillic acid		79.4
Homovanillic acid		117.9
Caffeic acid		74.0
Syringic acid		41.6
p-Coumaric acid		20.2
Sinapic acid		7.6

Table 2. Texture, organic matter content [18] and protein content in the investigated soils.

Soil	Texture	Organic Matter (%)		Proteins Content ($\mu g \cdot g^{-1}$)[a]	
		Control	Loaded with OMWW	Control	Loaded with OMWW
Revivim	Sandy clay loam	0.2	1.1	104.7	183.4
Negba	Silt loam	0.6	0.8	20.1	15.1
Gilat	Sandy clay loam	1.5	3.0	10.6	81.6
Rahal	Loam	1.2	3.1	9.0	502.5
Kamrah	Loam	1.0	2.2	11.0	51.0

[a]Proteins content measured by the Lowry protein assay.

Beit Dagan soil (a loamy fine sand) served for the polyphenol recovery tests and for comparison between the FCR and the HPLC procedures after it was loaded in the laboratory with OMWW as described below. The OMWW used for the comparison of these procedures was taken from an olive mill in Gilat, Israel. The locations of the olive mills from which OMWW was collected are listed in **Table 3**.

2.2. Loading Soil with OMWW

A portion of 5 g soil was placed in a Petri dish and 1mL of OMWW was slowly added to the soil. The loaded soil was then dried overnight in a fume hood. This process was repeated 5 times so that the soil sample was finally loaded with 5 mL of OMWW, giving a 1:1 (w/v) soil to OMWW ratio.

2.3. Sample Extraction

Two extraction procedures were applied and both types of extract were tested for their suitability to be analyzed for polyphenols, both by the FCR and the HPLC procedures, as described below. Since the objective of the analyses was to determine the efficacy of the FCR procedure in comparison to the HPLC procedure rather than to ascertain that the adopted extraction procedures yielded the total polyphenol content in the investigated systems, no exhaustive polyphenol extraction (e.g., hydrolytic procedures for detecting bound polyphenols) was attempted.

1) *Aqueous extraction*. The soil samples were extracted by shaking 1 g soil with 10 mL of distilled water in the dark, overnight.

The efficiency of the aqueous extraction was determined by extracting a Beit Dagan soil sample loaded as described above with OMWW (section 2.2), and comparing the amount of polyphenols in the extract as determined both by HPLC and by the FCR procedure to the amount of polyphenols in the added OMWW.

2) *Organic solvent extraction*. Extraction of the soils was executed in two stages as follows: First, 1 g soil was shaken in the dark for 5 hrs with 5 mL of a

Table 3. Total concentrations of polyphenols in OMWW samples derived from various olive mills as measured by the HPLC and the FCR procedures and content of the dominant polyphenols as measured by HPLC (μg·mL^{-1}).

Olive Mill	FCR	HPLC		
	Total	Total	Tyrosol	3, 4-dihydroxyphenyl-acetic acid
Kafr Lakff [a]	1647	288	26	222
Kafr Zibad [a]	1715	295	32	219
Kafr Mutalath [a]	2716	416	79	303
Kafr Azzun [a]	1523	294	36	233
Gilat [b]	1697	207	52	143

[a] Palestinian Authority, [b] Israel.

methanol:water (1:1) mixture and then the soil sample was extracted again, following the same procedure, with 5 mL of an acetonitrile:water mixture (1:1). The two extracts were then united. The efficiency of this extraction procedure was determined as follows: Samples of Beit Dagan soil were loaded by the procedure described above (Section 2.2) with a mixture of 10 polyphenols (Table 1) dissolved in acetonitrile, at a concentration of 10 μg·mL^{-1} each and then extracted as described above. The extraction efficiency for each individual polyphenol was calculated by comparing the amount extracted as determined by HPLC to the added amount of that polyphenol.

2.4. Polyphenols Analysis

2.4.1. FCR Assay

The OMWW samples were diluted fivefold with distilled water before being analyzed and the soil extracts as well as the diluted OMWW samples were filtered through a 0.45 μm Teflon filter. Samples of 6.5 mL of the soil extracts or the diluted OMWW were then placed in a test tube to which 3 mL of 20% (w/v) $NaHCO_3$ solution were added. A volume of 0.5 mL of 1:1 diluted FCR reagent was then added and the tubes were incubated for 30 min at room temperature. Absorption at 725 nm wavelength was finally measured with a Genesys 10 spectrophotometer. Both caffeic acid and gallic acid were used for calibration at the concentration range of 0 - 10 μg·mL^{-1}.

2.4.2. HPLC Analysis

Chromatographic separations were performed on an HPLC instrument (Ultimate 3000, Dionex, Germering, Germany) equipped with a PDA detector. The running conditions for the quantitative analysis of the polyphenols were: Column, Kinetex C_{18}, 250 × 2.9 mm, 5 μm particle size (Phenomenex, Torrance, CA, USA); flow velocity, 1 mL·min^{-1}; column temperature, 30˚C; detection wavelength, 240 nm; and injection volume, 10 μL. The mobile phase was composed of acetonitrile and of a 0.04% trifluoroacetic acid solution in water at proportions varied with time. The mobile phase profile was as follows: 5 min isocratic solu-

tion of 10% acetonitrile; 16 Min increasing content of acetonitrile at a gradient of 5% min^{-1}; 1 min isocratic solution of 90% acetonitrile; and finally 1 min decreasing content of acetonitrile at a gradient of 80% min^{-1}. Identification of individual polyphenols by the HPLC procedure was based both on retention time and the UV-Visible spectrum of the analyzed species.

2.5. Protein Analysis

Protein content in OMWW and in extracts of the soils was determined by the Lowry protein assay method – this method is based on color change of the protein-containing solution after reaction with the FCR according to the following procedures: A 0.4 mL sample of OMWW or soil extract was added to 2 mL of a mixture of the solutions, Na_2CO_3 (2% w/v), $CuSO_4$ (1% w/v) and $NaKC_4H_4O_6$ (2% w/v) at a ratio of 1:1: 100 respectively. After keeping the mixture at room temperature for 10 min, 0.2 mL of the FCR diluted with water to half its original concentration was added. The resulting preparation was kept again for 10 min at room temperature. Finally, absorption was measured by a Genesys 10 spectrophotometer (Thermo Scientific, Madison, WI, USA) at both wavelengths 550 nm and 750 nm. Quantitative calibration was performed against solutions of the protein bovine serum albumin at the concentration range of 100 - 1000 $\mu g \cdot mL^{-1}$.

2.6. Demonstration of the Interference by Non-Polyphenolic Species in the FCR Assay

In order to demonstrate the extent of interference by non-polyphenolic species expected in the FCR assay of biological systems, two procedures were employed: Preparation of an artificial "OMWW like" mixture, the composition of which is exactly known, followed by the determination of its apparent polyphenols content by the FCR assay; and, addition of polyphenol-free protein sources to an OMWW sample and analysis by the FCR assay of that sample before and after the addition of the protein source.

1) *Preparation and analysis of the synthetic "OMWW* like" mixture. A mixture of 3 polyphenols which are often encountered in OMWW was prepared by dissolving a 100 $\mu g \cdot mL^{-1}$ each of caffeic acid, 3,4-Dihydroxyphenylacetic acid and tyrosol in distilled water. The mixture was divided into 5 portions and while one portion was left as, to each of the other portions one of the following was added: 0.6% NaCl; 1.7% peptone; 0.6% sucrose; 1.7% peptone + 0.6% NaCl. Each of the above 5 systems was analyzed by the FCR assay (section 2.4 *Polyphenols analysis*).

2) *Preparation and analysis of the protein-enriched OMWW.* A sample of OMWW derived from an olive mill at Kafr Mutalath was divided into three sub-samples. To one sub-sample 1.7% of the peptone was added, to another sub-sample a similar quantity of the BSA was applied and the last sub-sample was kept as is. All there systems were analyzed by the FCR assay. As both the protein additives (the peptone and the BSA) are animal extracts, the probability of the presence of polyphenols in them is very low.

3. Results and Discussion

3.1. Quantification of Proteins in OMWW and Soils

Since the presence of proteins or amino acids interferes with the quantification of polyphenols by the FCR (which is used with some modifications to analyze both proteins and polyphenols), protein content in the OMWW and in the soils loaded with it was determined. A considerable quantity of proteins and amino acids as measured by the Lowry protein assay was detected in the OMWW (60,000 $\mu g \cdot mL^{-1}$) and accordingly, the soils to which OMWW was applied were by and large enriched with proteins (Table 2). Yet, proteins are biodegradable and hence, weather conditions, soil characteristics and agronomic practices, all of which strongly affect microbial activity, also affect the actual content of proteins at any given time in soils treated with OMWW (Table 2).

3.2. Obstacles Encountered in the Determination by the FCR Assay of the Content of Polyphenols in Soils Following Application of OMWW

Only the aqueous extracts of the soils (Section 2.3) could be analyzed by the FCR assay despite the limited efficiency of this extraction, for the following reason: because of the lack of specificity and high reactivity of the reagents employed in the FCR assay, this assay actually detects a wide array of electron donating species, e.g. [11] [13] and accordingly, a wide range of organic solvents cannot be used to extract polyphenols due to a possible interaction between the solvent itself and the FCR. Also, when a more efficient extracting procedure than the aqueous one is used, an extraction of a considerable amount of organic reducing species from the soil is likely to occur and the presence of any such oxidizable species in the extract may contribute to the color formation and thus to a false positive outcome in the FCR procedure. Thus, although the 2-stage extraction with organic solvents was much more efficient in recovering polyphenols than the aqueous extraction (e.g., Section 3.4), the liquid phase in the 2-stage extraction procedure was incompatible with the FCR assay, yielding various interfering side reactions.

It is thus apparent that the use of the FCR assay for polyphenol analysis in biological or other multi-component systems necessitates a rather complex (or alternatively rather inefficient) extraction procedure in order to obtain an even approximately reliable result. Although such interferences were strongly reduced by employing the less efficient aqueous extraction, even in the latter extract, non-polyphenolic components were present and evidently affected the readings of the FCR assay.

The HPLC analysis yielded a polyphenol content considerably lower than the apparent polyphenol content measure by the FCR procedure (125 and 657 $\mu g \cdot g^{-1}$ respectively were measured in the aqueous extract of the OMWW-loaded Beit Dagan soil). Overestimation by a similar magnitude was also reported in the literature [20] when results of the FCR assay were compared to the results ob-

tained by the more phenol-specific chromatographic procedures. It should, however, be reemphasized that any chromatographic procedure such as HPLC, may underestimate the polyphenols content due to its failure to identify part of the many polyphenols that may be present in systems such as OMWW or any other biological system.

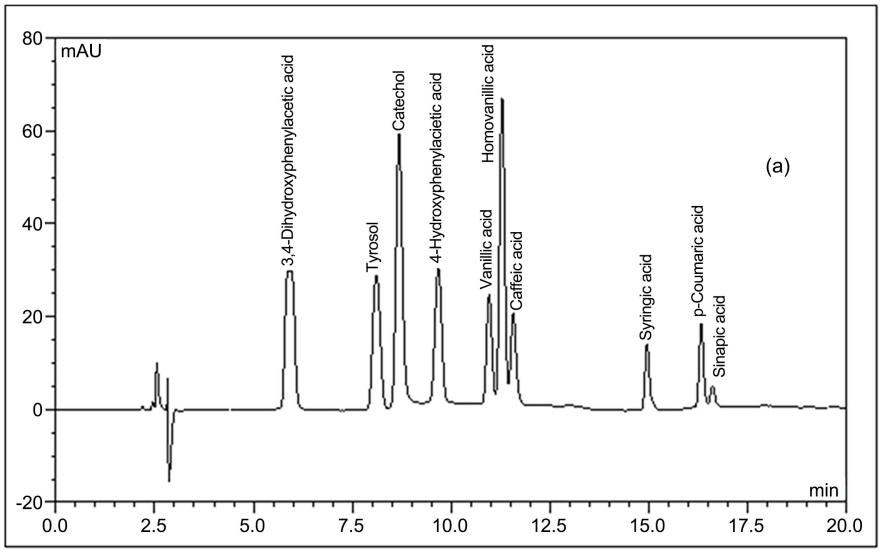
The apparent content of polyphenols in the OMWW-treated and untreated soils as determined by the FCR procedure reveals an interesting pattern. While in the Revivim and Gilat soils (**Table 2**), OMWW addition increased the polyphenol content as measured by the FCR assay by 23 and 29 $\mu g \cdot g^{-1}$ respectively, in the Negba soil the apparent polyphenol content increased by only 5 $\mu g \cdot g^{-1}$. The apparent increase in polyphenol content as determined by the FCR procedure did not correlate with the amount of OMWW added to the soil but it did correlated well with the measured difference in the content of protein between the unamended and OMWW-amended soils (**Table 2**). This observation is in agreement with the assertion that the FCR response assigned to polyphenols is actually due in part to other components, including proteins.

3.3. Limitations of HPLC as a Procedure for the Identification and Quantitative Determination of Polyphenols in Systems Containing a Mixture of Polyphenols

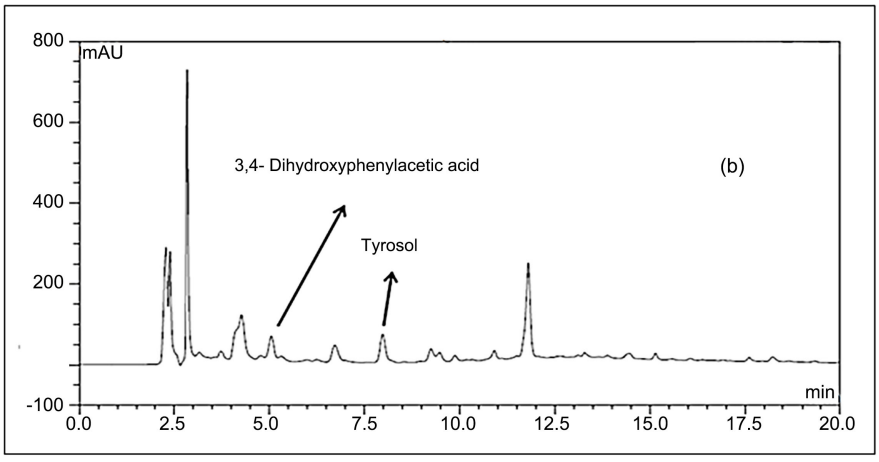
A considerable number of chromatography-based protocols for the separation and quantification of phenolic substances in biological systems was reported, e.g. [21] [22] [23] [24]. The difficulty in developing an efficient HPLC procedure for both identifying and quantifying polyphenols in complex mixtures such as OMWW is compounded by the dissimilarity in structure and polarity between many of the polyphenols found in these olive-originating materials. Thus, it is likely that HPLC protocols which were designed for the quantitative analysis of polyphenols in systems other than OMWW from a given source will not be useful for analyzing that OMWW sample. Each OMWW-based material may requires the adaptation or formulation of a new HPLC procedure. The procedure employed in the present study enabled the separation and quantitative determination of a mixture of 10 polyphenols that are likely to be found in OMWW. A typical chromatogram of such a mixture of polyphenol is depicted in **Figure 2(a)**. Yet, as discussed above and below (section 3.6), like any other specific chromatographic procedure, the protocol adopted in the present study may fail to detect the presence of some other polyphenols that could be found in the investigated, polyphenol-rich systems. For example, although the polyphenol hydroxytyrosol was reported to constitute in some cases nearly (or even over) half of the polyphenol content of OMWW, e.g. [25] [26] [27] [28], this compound was not detected by the procedure adopted in the present study.

3.4. Interference with the FCR Procedure as Indicated by the Recovery Efficiencies of Polyphenols

The apparent recovery efficiency of the aqueous extraction of polyphenols from



(a)



(b)

Figure 2. (a) HPLC chromatogram of a mixture of 10 polyphenols (10 μg·mL^{-1} in acetonitrile each); (b) HPLC chromatogram of OMWW from the Gilat olive mill.

the Beit Dagan soil loaded with OMWW (Section 2.3) as determined by the FCR procedure was ~57%. However, a blank extraction of the same soil to which no polyphenols were added yielded a response equivalent to ~35% of the total content of the polyphenols that were added to that soil with the OWMM. This indicates that the actual efficiency of the aqueous extraction for the polyphenols added with the OMWW was much lower, around 22%, and demonstrates the magnitude of the potential interference to the FCR analysis from various species existing in the soil. This interference is just one example of an error that is built into the FCR assay for polyphenols analysis, an error arising from the above discussed broad reactivity of the FCR.

When the efficiency of the aqueous extraction of the OMWW-loaded Beit Dagan soil was measured by the HPLC procedure, a recovery rate of ~60% was calculated by comparing the measured amount of polyphenols in the OMWW

added to the soil (207 $\mu g \cdot g^{-1}$) to the amount recovered by the water extract (125 $\mu g \cdot g^{-1}$). As expected, both the content of the polyphenols in the OMWW and the amount recovered by the water extraction are lower by a factor of approximately 7 (e.g., Table 3) and 5 respectively than those determined by the FCR procedure. The fact that the HPLC analysis indicated a recovery rate of ~60% as compared to the only ~20% recovery indicated by the FCR procedure suggests that the rate of recovery from the soil of those polyphenols which were added with the OMWW and detected by the employed HPLC procedure was higher than the overall recovery rate of all species which were added to the soil with the OMWW and interact with the FCR but not detected by the HPLC procedure.

The recovery efficiency from the Beit Dagan soil of various polyphenols in the two-stage extraction procedure as determined by HPLC is given in Table 1. An extraction of a blank soil before loading it with the polyphenols did not yield any response at the retention times assigned to any of the polyphenols, indicating the reliability of the recovery values given in that Table.

3.5. Evaluation of the Suitability of a Single Species as a Universal Standard for Quantifying Polyphenols

Caffeic acid and gallic acid are both commonly used as a universal standard for quantifying the concentration of polyphenols when the FCR or similar spectroscopic procedures are used for polyphenols quantification, e.g. [29] [30]. Figure 1 displays calibration curves for different polyphenols analyzed by the FCR procedure. It is apparent that every polyphenol produces a distinct calibration curve and that no individual curve, including that of either caffeic or gallic acid, can represent all polyphenols. Hence, polyphenol concentrations calculated on the basis of a calibration curve of a single reference material, be it caffeic acid or gallic acid and without regard to the types of polyphenols actually present in the system, will be inaccurate, possibly, as demonstrated by Figure 1, by a factor of more than two.

3.6. Critical Comparison between the FCR Assay and Chromatographic Procedures for Quantifying Polyphenols

A comparison between the polyphenol content as measured by the FCR procedure and by the HPLC procedure was conducted using OMWW samples from 5 different sources (Table 3). Very large differences, often six fold or more, are apparent between the concentrations of polyphenols measured by the two methods.

It is most likely that the FCR procedure grossly overestimates the total polyphenol content. The root cause for this is the lack of specificity of the Folin-Ciocalteu Reagent which interacts with many oxidizable species, including proteins, while the HPLC procedure is far more specific to the compounds investigated and it encounters considerably less interference from other components of the analyzed system. However, while the above fact makes chromatographic methods more accurate and reliable, one has to bear in mind that chro-

matographic procedures detect individual polyphenols and there is always a high likelihood that the investigated medium contains polyphenols that the HPLC (or any other chromatographic) protocol being employed fails to detect. Thus, the up to six-fold difference observed between the total polyphenol content as determined by the FCR assay and by the HPLC procedure as reported in the present study (as well as by others, e.g. [20]), is caused, most likely, both by the overestimation by the FCR assay and by the underestimation (due to undetected polyphenolic species), of the HPLC analysis. Since users can always adopt an HPLC procedure which is suitable for the polyphenols most likely to be found in the investigated biological system, it is less likely that a difference of five to six-fold between the total polyphenol content as determined by the FCR and the HPLC analyses will be due just to the underestimation of the latter procedure.

The HPLC procedure used in the present study revealed that among the 10 investigated polyphenols, 2 compounds dominated the polyphenol content of the OMWW samples investigated (**Table 3**): 3,4-dihydroxyphenyl acetic acid and tyrosol. A sample chromatogram of the OMWW from the Gilat mill is given in **Figure 2(b)**. Yet, other polyphenols, not detected by this particular HPLC procedure might, of course, have also been present.

3.7. Demonstration of the of Interference by Non-Polyphenolic Species in the FCR Assay

3.7.1. Analysis of the Synthetic "OMWW Like" Mixture

Addition of any of the additives: peptone, NaCl or sucrose to the mixture of 3 polyphenols at the rates detailed in section 2.6 increased to some degree the apparent polyphenol content as determined by the FCR procedure. Not surprisingly, the most significant response was upon the addition of peptone (an increase of 25%), despite the fact that the peptone contains only 50% proteins and free amino acids [31]. Addition of all 3 additives to the polyphenol mixture yielded an increase of 27% in the mixture's response to the FCR. Obviously, actual OMWW systems vary substantially from the artificial mixture studied here (as well as from each other), both in the nature of their components and in the quantity of any component. The reported results, therefore, only demonstrate the non-specificity of the FCR and not the magnitude of the error in determining total polyphenols content in biological systems by the FCR assay.

3.7.2. Analysis of the Protein-Enriched OMWW

Figure 3 depicts the results of the FCR assay for the OMWW and the OMWW enriched with the protein sources. The addition of 1.7% protein source (a protein content common in OMWW, e.g. [32]), increased the apparent content of polyphenols as determined by the FCR assay by up to 50%, depending on the source of the protein. It should be emphasized that proteins are not the only oxidizable species present in the OMWW which interfere with the FCR assay, e.g. [13] and the content of some such components may be considerably higher than that of proteins (e.g., reducing sugars [33]). And, as mentioned above, the peptone used in this study contains only 50% proteins and free amino acids [31].

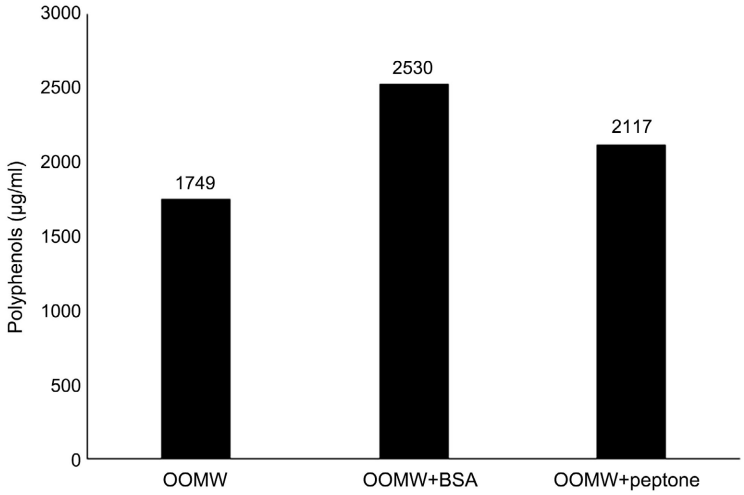


Figure 3. The effect of addition of 1.7% protein source on the apparent content of polyphenols in olive mill waste water (OOMW) as determined by the FCR assay.

4. Conclusion

The Folin-Ciocalteu Reagent assay, a procedure commonly used to detect and quantify polyphenols, may be ill-fitted for total polyphenol analysis in complex biological systems (e.g., OMWW), due to a strong interference by a host of reducing species. The efficacy of the FCR assay is also harmed by the limited suitability of any one polyphenol (e.g., caffeic acid or gallic acid) to serve as a universal standard for calibration in quantitative analysis of total polyphenol content. Finally, the assay lacks specificity and cannot be used to quantify or even identify individual components of mixtures of polyphenols. On the other hand, chromatographic procedures (such as HPLC), enable quantitative analysis of individual polyphenols with considerably less interference from other components of the analyzed system. Thus, the HPLC procedure adopted in the present study revealed a rather high content of two polyphenols in the OMWW samples investigated: 3,4-dihydroxyphenylacetic acid and tyrosol. At the same time, any particular chromatographic procedure is limited by the scope of components it can identify and quantify. The intricacy of the chemical composition of many biological systems and the resulting complexity of the chromatograms of such systems, including OMWW, (e.g. [34] and **Figure 2(b)**) limits further the range of polyphenols any one chromatographic protocol can identify in a given biological sample. Thus, there is a high likelihood that the presence of some polyphenols will not be detected by any specific HPLC or other chromatographic procedure adopted. Just as the FCR assay overestimates the total polyphenol content, chromatographic assays are likely to underestimate it. Hence, the apparent polyphenol content as determined by the FCR assay is as a rule considerably higher than that determined by HPLC. While some polyphenols may be harmful to biological systems and are thus potential polluters of soils and water bodies or a nuisance due to their pungent odor, other polyphenols are valued as antioxidants.

The numerous polyphenolic species that may be present in any OMWW sample (or in many other biological systems) and the large difference in properties between the many polyphenols, e.g. [35], lead to the conclusion that there is little value in assaying the "total polyphenolic content" in a biological system. One should rather define the group or groups of polyphenols which are of interest and quantify these groups by chromatographic procedures suitable to that end.

Acknowledgements

The authors gratefully acknowledge the financial support of the Deutsche Forschungsgemeinschaft (the trilateral OLIVEOIL SCHA849/13 research project).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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