

# **Total (poly)phenol analysis by the Folin-Ciocalteu assay as an anti-inflammatory biomarker in biological samples**

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## ***Abstract:***

The Folin-Ciocalteu method is a well-established and widely used assay for measuring total (poly)phenol content in food/plant products. In recent years, there has been growing interest in applying this method to human samples due to its simplicity and efficacy. However, biological matrices such as blood and urine contain several interference substances that must be eliminated beforehand. This mini-review summarizes the current state of knowledge regarding the use of the Folin-Ciocalteu assay to measure total phenolic content in human urine and blood samples, as well as the preceding cleaning methods to remove interferences. Higher total (poly)phenol levels measured by the Folin-Ciocalteu method have been associated with a decrease in mortality and several risk

variables. We focus on the application of this sustainable assay as a biomarker of poly(phenol) intake and its potential use as an anti-inflammatory biomarker in clinical laboratories. The Folin-Ciocalteu method, with a clean-up extraction step, is a reliable tool for determining total (poly)phenol consumption. Here, we also recommend using the Folin-Ciocalteu assay as means to measure anti-inflammatory activity.

**Keywords:** Antioxidant capacity, biological samples, phenol intake, healthy aging.

**Abbreviations:** F-C, Folin-Ciocalteu; GAE, gallic acid equivalents; SPE, solid-phase extraction.

## 1. Background

The Folin-Ciocalteu (F–C) photometric assay is one of the most common procedures used to determine phenolic compounds, although it was initially developed to detect and quantify tyrosine and tryptophane in proteins, the F–C reagent reacting with the phenolic groups in these two amino acids (Folin and Ciocalteu 1927). The F–C assay was subsequently modified to analyze the (poly)phenol content of red wine (Singleton, Rossi Jr., and Rossi J A Jr. 1965) and is now considered the reference method for phenolic compound quantification and determination in a wide variety of matrices (Wang et al. 2019; Khosravi and Razavi 2020; Jiang et al. 2022). Additionally, it is increasingly being applied in human biological samples (blood and urine) as a way of estimating total (poly)phenol intake (Arenas and Trinidad 2017; Laveriano-Santos et al. 2022).

Although the precise chemical composition of the F–C reagent is undefined, it is known to contain a mixture of phosphomolybdic and phosphotungstic acids, which upon reduction produce a blue chromophore with maximum absorption at 765 nm. The assay is based on an electron-transfer reaction between the F–C reagent (the oxidant) and an antioxidant species (the electron donor) (Figure 1). The extent to which the reagent

changes color after the electron extraction depends on the reducing activity of the antioxidant compounds. It is frequently measured as gallic acid equivalents (GAE), as gallic acid has shown the highest absorbance compared to other compounds as chlorogenic or neochlorogenic acid (Kyoung Chun and Kim 2004). The F–C assay has numerous advantages as a methodology to assess the antioxidant activity of phenolic compounds, including simplicity, reproducibility, and robustness. However, since the test is sensitive to pH, temperature, and reaction time, the reaction conditions need to be carefully selected to obtain reliable results (Singleton, Orthofer, and Lamuela-Raventós 1999). As phenolic compounds only react with the acidic F–C reagent in basic conditions, because a deprotonated OH group in the phenolic ring is required, a sodium carbonate solution is added to the mixture of samples and reagent to increase the pH to approximately 10, avoiding excessive alkalinity. Samples must be incubated with the F–C reagent for 90-120 minutes at room temperature. Up to six hours at room temperature, the blue color remains consistent, and the results obtained are comparable to those obtained after just one hour, although there is an increased standard deviation. Even though color may emerge more rapidly at high temperatures, at > 40 °C the color also disappears more quickly (Singleton, Orthofer, and Lamuela-Raventós 1999). The conventional F–C reagent is only applicable to water-soluble antioxidants (Singleton, Rossi Jr., and Rossi J A Jr. 1965).

Among the most important antioxidants found in plants, (poly)phenols constitute a large and diverse class of organic compounds that contain a phenol functional group consisting of a hydroxyl (–OH) group bonded directly to an aromatic ring. More than 9000 phenolic structures have been described, the most abundant being phenolic acids, flavonoids, stilbenes and lignans; flavonoids and phenolic acids account for 55-60% and 30-40% of dietary (poly)phenols, respectively (Rothwell et al. 2013; Tresserra-Rimbau et al. 2013).

When compared to self-reported information from food frequency questionnaires or dietary recalls, biomarkers of nutritional intake have various advantages for epidemiological and clinical studies (Marshall 2003) as they yield more objective and precise data. Quite recently, the F–C method has been used in blood and urine samples as a biomarker of phenolic intake, although a clean-up procedure is necessary to avoid interferences (Roura et al. 2006). Higher levels of urinary (poly)phenols measured by the F–C assay have been correlated with a decrease in mortality related to cardiovascular disease and lower DNA oxidative damage (Pedret et al. 2012; Zamora-Ros et al. 2013), possibly due to the antioxidant and anti-inflammatory properties of phenolic compounds. Here we provide an updated overview of the use of the F–C assay in urine and plasma as an objective tool for the analysis of total (poly)phenol intake. The limitations and interferences associated with the method, modifications of the extraction procedures used for sample clean-up, and the potential of the assay for use as an anti-inflammatory biomarker in biological samples are also explored.

## **2. Limitations and interferences in the F-C method**

Since 1965, the F–C assay has been widely used to analyze plant-based food, but its application in biological samples started only in the 1990s (Serafini, Maiani, and Ferro-Luzzi 1998; Maskarinec et al. 1999). Overestimation of the total (poly)phenol content is a major concern when applying the F–C test in biological matrices due to the effect of non-phenolic reducing agents in the samples (Blasco et al. 2005), such as aromatic amines, certain amino acids, high sugar concentrations, citric acid, or ascorbic acid (Ainsworth and Gillespie 2007). Tryptophan, indoles, purines, guanine, xanthine, and uric acid are also reported to react with the F-C reagent to yield molybdenum blue (Singleton, Orthofer, and Lamuela-Raventós 1999). As reported by Lamuela-Raventós, urine

contains reductant substances that may potentially interfere with the F–C reagent (see Table 1) (Lamuela-Raventós 2017). Vitamin C, found in urine at a maximum concentration of 100 mg/L, is reactive with the F–C reagent. In contrast, organic acids, such as oxalic, citric, and tartaric acid, as well as folic acid, which has maximum levels in urine equivalent to those of vitamin C, and hippuric acid (10 mg/L) do not react in the F–C assay. Even though the maximum concentration of Fe (II) and some amino acids (Phe, Tyr, Gkut, Arg) in urine is only 1 mg/L, they are detected in the F–C assay. Moreover, the F–C reagent reacts with dopamine (0.4 mg/L), norepinephrine or noradrenaline (0.08 mg/L), and epinephrine or adrenaline (0.02 mg/L).

### **3. Application in biological samples**

Accurate assessment of phenolic intake based on self-reported dietary records and food composition tables can be hindered by the subjectivity of the data and the impact on content by factors such as product variety and degree of ripeness, as well as food processing techniques. Therefore, the development of biomarkers that can be measured in blood and urine is essential for more precise estimates of (poly)phenol intake and to establish the health effects of these dietary constituents (Roura et al. 2006). Most of the studies employed a commercial F-C reagent available from Honeywell (NC, USA).

#### *3.1 Urine samples*

Studies applying the F–C assay to analyze total (poly)phenol content in human urine samples are listed in Table 2. To the best of our knowledge, the first such study was that of Roura *et al* in 2006, in which an initial clean-up step using a solid-phase extraction cartridge ensured that undesired water-soluble compounds were eliminated from the samples. The reliability of the method was validated in a study of 36 volunteers who consumed either a (poly)phenol-rich or a (poly)phenol-free diet (Roura et al. 2006).

However, the most optimized and commonly used methodology in this field was developed by Medina-Remón *et al.*, who increased efficiency and sustainability by using 96-well microtiter plates (Alexander Medina-Remón *et al.* 2009). Thus, interfering substances from spot-urine were removed by solid-phase extraction on Oasis((R)) MAX 96-well plate cartridges before performing the F–C assay, and gallic acid was used as the standard. The phenolic content in urine measured by this quick and easy methodology was demonstrated to be a reliable biomarker of (poly)phenol intake.

In 2011, as part of the InCHIANTI study, Zamora *et al.* corroborated that total (poly)phenols measured in spot morning urine correlated with levels in 24h urine (Zamora-Ros *et al.* 2011). Since then, many studies in elderly or young and healthy populations have applied this procedure either in spot or 24h-urine to assess (poly)phenol intake and associated health outcomes. In elderly cohorts, including those of the PREDIMED and InCHIANTI trials, individuals with higher urinary excretion of (poly)phenols showed improvements in cardiovascular health parameters, such as glucose and triglyceride levels, blood pressure, or body weight (Guo *et al.* 2016; Guo *et al.* 2017; A. Medina-Remón *et al.* 2011). In the InCHIANTI study, Zamora *et al.* also found a negative association between (poly)phenol excretion and mortality in older adults (Zamora-Ros *et al.* 2013). In the same cohort, a lower risk of substantial cognitive decline was observed in individuals with higher urinary (poly)phenols after three years of follow-up, as well as reduced frailty (Rabassa *et al.* 2015; Urpi-Sarda *et al.* 2015). Furthermore, Rabassa *et al.* showed that higher levels of urinary (poly)phenols were associated with a lower risk of physical performance decline (Rabassa *et al.* 2016).

In a cross-sectional study performed in healthy individuals, DNA oxidative stress biomarkers were inversely correlated with the phenolic content in urine, suggesting that phenolic compounds may attenuate oxidative damage (Pedret *et al.* 2012). Haldar *et al.*

showed that the intake of a (poly)phenol-rich curry, assessed using the F-C assay in urine, improved glucose homeostasis in 20 healthy males (Haldar et al. 2019). This approach has also been used in studies of younger populations. A clinical trial with 49 male adolescents demonstrated that urinary (poly)phenols were an accurate biomarker of intake (Hussein et al. 2009). Laveriano *et al.* reported that urinary polyphenols were associated with a better cardiovascular profile in a cohort of Spanish adolescents. Interestingly, they found differences between the sexes, as cardiovascular health in boys was more strongly associated with phenolic excretion than in girls (Laveriano-santos et al. 2020; Laveriano-Santos et al. 2022).

Sample cleaning with solid phase extraction has been modified or alternatives have been proposed. For example, Vinson *et al.* employed a Polyclar solid phase procedure, whereas Kendall *et al.* did not perform any cleaning of the urine samples (Kendall et al. 2009; Vinson et al. 2012). In other studies, samples were only submitted to centrifugation and the supernatant was used to quantify the phenolic compounds (Wruss et al. 2015). However, these approaches do not eliminate the potential interfering substances.

### *Blood samples*

Plasma has also been used as a matrix to measure (poly)phenols, though less frequently (see Table 3). As in urine, a preceding clean-up step is required, which eliminates plasma protein interferences. Hydrolysis has been used to separate the (poly)phenols from the lipids, followed by protein precipitation using metaphosphoric acid to remove plasma proteins (Serafini, Maiani, and Ferro-Luzzi 1998). This methodology was shortened by Arendt *et al.*, who only centrifuged the plasma samples before adding metaphosphoric acid to precipitate the proteins (Arendt et al. 2005). In both studies, circulating levels of (poly)phenols were found to increase after the consumption of alcohol-free wine. The

same procedure was used in plasma samples to examine the reducing capacity of the F–C reagent and (poly)phenol levels after consumption of white tea and a pomace drink, respectively (Müller et al. 2010; Arenas and Trinidad 2017). In a clinical trial with women who consumed fruits and vegetables, Maskarinek *et al.* used ethanol to precipitate plasma proteins before performing the F–C assay; surprisingly, the intervention did not increase total (poly)phenols (Maskarinec et al. 1999). This method was replicated in another study, which observed a decrease in (poly)phenols immediately after exercise (Andersson et al. 2010). Interestingly, Wruss *et al.* did not use any clean-up process to eliminate plasma proteins, submitting the samples only to centrifugation before performing the F–C assay. The participants of the study consumed apple juice and the total phenolic content in plasma was determined after 10 hours to assess (poly)phenol pharmacokinetics (Wruss et al. 2015).

#### **4. Potential future perspectives**

Many pathologies studied in relation to (poly)phenol intake involve inflammatory processes that initiate or worsen the disease. However, the association between total (poly)phenols in biological samples measured by the F–C assay and inflammation has been scarcely studied. A study on healthy men found that dietary (poly)phenols were associated with a better response in vascular and plasmatic inflammatory biomarkers (Hurtado-Barroso et al. 2018; Hurtado-Barroso et al. 2019). Another clinical trial has assessed this relationship, finding that a higher total phenolic content in urine was associated with decreased inflammatory biomarkers in an older Mediterranean population (Alexander Medina-Remón et al. 2017). Numerous clinical trials have observed that phenolic intake is associated with better inflammatory status (Chai et al. 2019; Lockyer et al. 2017; Del Bo' et al. 2021). Moreover, Arancibia *et al.* demonstrated that total

(poly)phenols excreted in urine measured by the F–C assay can serve as a reliable biomarker of anti-inflammatory diets, particularly in women, supporting the inverse relationship between total polyphenol intake and inflammation (Arancibia-Riveros et al. 2023). Altogether, these data suggest that the total phenolic content determined by the F–C assay could be used as a biomarker of inflammation. Considering the simplicity and low cost of the F–C test compared to assays that measure inflammatory molecules, it could be an effective alternative for routine use in any clinical laboratory.

## **5. Conclusions**

The Folin-Ciocalteu assay is a well-known and efficient method for measuring the total phenolic content in plant-based foods and beverages. More recently, due to its speed and simplicity, it has been applied in human urine and blood samples, which are previously submitted to a cleaning procedure to eliminate interferences. The assay is easy to perform, economical, and sustainable, making it ideal for application in routine laboratory analysis as a biomarker of total (poly)phenol intake and potentially of inflammatory status.

The ability to measure total phenolic compounds in biological samples provides valuable information for assessing the impact of dietary interventions and disease prevention strategies. It can also provide valuable insights into the relationship between polyphenol intake, mortality, inflammation, and chronic diseases.

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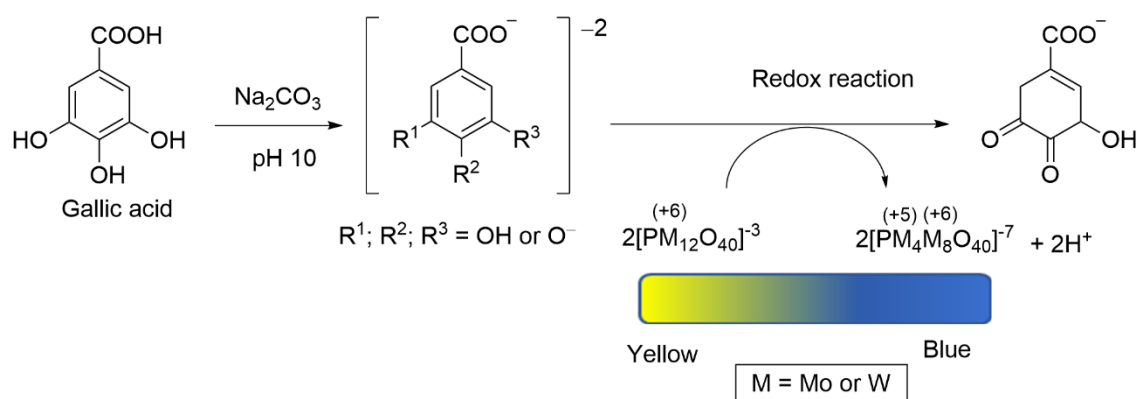
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## Figures

Figure 1. The redox reaction and color variation in the Folin–Ciocalteu assay, together with the metal complex species identified by Munteanu (Munteanu and Apetrei 2021).



## Tables

**Table 1.** Reductant substances that may potentially interfere with the F–C reagent.

<b>Compounds with reducing capacity (maximum levels in urine)</b>	<b>F–C Assay</b>
Vitamin C (100 mg/L)	+
Organic acids: oxalic, citric and tartaric acids (100 mg/L)	-
Folic acid (100 mg/L)	-
Hippuric acid (10 mg/L)	-
Fe (II) (1 mg/L)	+
Amino acids: Phe, Tyr, Glut, Arg (1 mg/L)	Weak
Dopamine (0.4 mg/L)	+
Norepinephrine or noradrenaline (0.08 mg/L)	+
Epinephrine or adrenaline (0.02 mg/L)	+

**Table 2.** Studies performed in human urine samples using the Folin–Ciocalteu assay.

<u>Authors</u>	<u>Cleaning method</u>	<u>Substances removed</u>	<u>Participants</u>	<u>Results expressed as</u>	<u>Validation</u>	<u>Ref.</u>
Roura et al. (2006)	SPE	Water-soluble compounds (sugar, iron, organic acids, amino acids, vitamins and hippuric acid)	36 healthy adults	mg catechin/g of creatinine	Linearity Precision	(Roura et al. 2006)
Medina-Remón et al. (2009)	SPE	Water-soluble compounds (sugar, iron, organic acids, amino acids, vitamins and hippuric acid)	60 adults at high cardiovascular risk	mg GAE/g creatinine	Linearity Sensitivity Accuracy Precision Stability	(Alexander Medina-Remón et al. 2009)
Kendall et al. (2009)	None	None	36 healthy young adults	mg gallic acid/L	Not detailed	(Kendall et al. 2009)
Hussein et al. (2009)	As Medina-Remón et al.	As Medina-Remón et al.	49 adolescents	mg GAE/g creatinine	As Medina-Remón et al.	(Hussein et al. 2009)
Medina-Remón et al. (2011)	As Medina-Remón et al.	As Medina-Remón et al.	589 adults at high cardiovascular risk	mg GAE/g creatinine	As Medina-Remón et al.	(A. Medina-Remón et al. 2011)
Zamora-Ros et al. 2011	As Medina-Remón et al.	As Medina-Remón et al.	928 older adults	mg GAE/g creatinine	As Medina-Remón et al.	(Zamora-Ros et al. 2011)
Pedret et al. 2012	As Medina-Remón et al.	As Medina-Remón et al.	81 healthy adults	mg GAE/g creatinine	As Medina-Remón et al.	(Pedret et al. 2012)

Vinson et al. (2012)	SPE	Non-phenolic interferences	8 healthy adults	μmol/24h urine	Not detailed	(Vinson et al. 2012)
Zamora et al. (2013)	As Medina-Remón et al.	As Medina-Remón et al.	807 older adults	mg GAE/24h urine	As Medina-Remón et al.	(Zamora-Ros et al. 2013)
Rabassa et al. (2015)	As Medina-Remón et al.	As Medina-Remón et al.	652 old adults	mg GAE/24h urine	As Medina-Remón et al.	(Rabassa et al. 2015)
Urpi-Sarda et al. (2015)	As Medina-Remón et al.	As Medina-Remón et al.	811 old adults	mg GAE/24h urine	As Medina-Remón et al.	(Urpi-Sarda et al. 2015)
Wruss et al. (2015)	None	None	35 healthy subjects	(+)-catechin equivalents in mg/L	Not detailed	(Wruss et al. 2015)
Guo et al. (2016)	As Medina-Remón et al.	As Medina-Remón et al.	573 adults at high cardiovascular risk	mg GAE/g creatinine	As Medina-Remón et al.	(Guo et al. 2016)
Rabassa et al. 2016	As Medina-Remón et al.	As Medina-Remón et al.	368 old adults	mg GAE/24h urine	As Medina-Remón et al.	(Rabassa et al. 2016)
Medina-Remón et al. (2017)	As Medina-Remón et al.	As Medina-Remón et al.	1139 adults at high cardiovascular risk	mg GAE/g creatinine	As Medina-Remón et al.	(Alexander Medina-Remón et al. 2017)
Guo et al. (2017)	As Medina-Remón et al.	As Medina-Remón et al.	573 adults at high cardiovascular risk	mg GAE/g creatinine	As Medina-Remón et al.	(Guo et al. 2017)
Hinojosa-Nogueira et al. (2017)	SPE	Water-soluble compounds (sugar, iron, organic acids, amino acids, vitamins and hippuric acid)	228 children	mg GAE/g creatinine	Not detailed	(Hinojosa-Nogueira et al. 2017)
Halder et al. (2017)	As Medina-Remón et al.	As Medina-Remón et al.	20 men	mg GAE/mmol creatinine	As Medina-Remón et al.	(Haldar et al. 2019)
Hurtado-Barroso et al. (2018)	As Medina-Remón et al.	As Medina-Remón et al.	22 healthy men	mg GAE/mmol creatinine	As Medina-Remón et al.	(Hurtado-Barroso et al. 2018)

Hurtado-Barroso et al. (2019)	As Medina-Remón et al.	As Medina-Remón et al.	22 healthy men	mg GAE/mmol creatinine	As Medina-Remón et al.	(Hurtado-Barroso et al. 2019)
Laveriano-Santos et al. (2020)	As Medina-Remón et al.	As Medina-Remón et al.	1194 adolescents	mg GAE/g creatinine	As Medina-Remón et al.	(Laveriano-santos et al. 2020)
Laveriano-Santos et al. (2022)	As Medina-Remón et al.	As Medina-Remón et al.	1151 adolescents	mg GAE/g creatinine	As Medina-Remón et al.	(Laveriano-Santos et al. 2022)

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SPE, solid-phase extraction; GAE, gallic acid equivalents.

**Table 3.** Studies analyzing human blood samples using the F-C assay.

<u>Authors</u>	<u>Cleaning /extraction method</u>	<u>Substances removed</u>	<u>Participants</u>	<u>Results expressed as</u>	<u>Validation</u>	<u>Ref.</u>
Serafini et al (1998)	Protein precipitation and extraction. Hydrolysis with HCl acid Metaphosphoric acid (MPA) 0.75 mol/L	Proteins, lipids, and hydrolyzed conjugates.	10 healthy subjects	quercetin equivalents mg/L	Recovery Limit of detection reproducibility	(Serafini, Maiani, and Ferro-Luzzi 1998)
Maskarinec et al (1999)	50 mL of plasma were mixed with 50 ml of 95% ethanol followed by vortex and centrifugation for 5 min at 11003 g	Protein precipitation with ethanol	33 females	micromole/L quercetin equivalents	Not detailed	(Maskarinec et al. 1999)
Arendt et al. (2005)	As in Serafini et al.	As in Serafini et al.	78 healthy subjects	mg catechin equivalents/L	As Serafini et al.	(Arendt et al. 2005)
Müller et al. (2010)	As in Serafini et al.	As in Serafini et al.	70 healthy subjects	mg catechin equivalents/L	As Serafini et al.	(Müller et al. 2010)
Andersson et al (2010)	Method used by Maskarinec et al (1999)	Ethanol precipitation	22 females	micromole/L quercetin equivalents (+)-catechin equivalents in mg/L	Reproducibility CV<10%	(Andersson et al. 2010)
Wruss et al (2015)	None	None	35 healthy subjects	(+)-catechin equivalents in mg/L	As Serafini et al.	(Wruss et al. 2015)
Arenas and Trinidad (2017)	As in Serafini et al.	As in Serafini et al.	10 healthy subjects	micromole/L GAE	As Serafini et al.	(Arenas and Trinidad 2017)

GAE, gallic acid equivalents.

