

Adaptation and Validation of Quick, Easy, New, Cheap, and Reproducible (QUENCHER) Antioxidant Capacity Assays in Model Products Obtained from Residual Wine Pomace

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S Supporting Information

ABSTRACT: Evaluation of the total antioxidant capacity of solid matrices without extraction steps is a very interesting alternative for food researchers and also for food industries. These methodologies have been denominated by QUENCHER from quick, easy, new, cheap, and reproducible assays. To demonstrate and highlight the validity of QUENCHER (Q) methods, values of Q-method validation were showed for the first time, and they were tested with products of well-known different chemical properties. Furthermore, new QUENCHER assays to measure scavenging capacity against superoxide, hydroxyl, and lipid peroxyl radicals were developed. Calibration models showed good linearity ($R^2 > 0.995$), proportionality and precision ($CV < 6.5\%$), and acceptable detection limits (<20.4 nmol Trolox equiv). The presence of ethanol in the reaction medium gave antioxidant capacity values significantly different from those obtained with water. The dilution of samples with powdered cellulose was discouraged because possible interferences with some of the matrices analyzed may take place.

KEYWORDS: antioxidant activities, ABTS, Folin–Ciocalteu, free radical scavenging assays, cellulose

INTRODUCTION

Natural antioxidants are increasingly demanded by the food industry to prevent oxidative degradation reactions while satisfying consumers' demands. Furthermore, there is growing interest in knowing the antioxidant value of foods, food supplements, and diets. Therefore, in recent decades, several in vitro and in vivo assays have been developed to determine the total antioxidant capacity (TAC) of foods and biological samples.^{1–3} It is generally recognized that effects of food materials on the redox balance in vivo cannot be merely extrapolated from their activities in vitro, with the possible exception of their antioxidant action in the gastrointestinal tract.^{2,4} However, the determination of the in vitro TAC can be achieved by simple and relatively fast chemical methodologies and may give more relevant information than that obtained from measuring each antioxidant compound separately, as possible synergistic interactions may not be considered.⁴ Therefore, these methods have been largely used to estimate and compare the antioxidant capacity of food items.^{5,6} Because most natural antioxidants are multifunctional, a reliable antioxidant protocol requires the measurement of more than one property relevant to either foods or biological systems.^{7,8} The most commonly applied in vitro TAC methodologies are based on diverse strategies to evaluate (1) the reducing ability of antioxidants, such as the Folin–Ciocalteu (FC) and ferric reducing/antioxidant power (FRAP) assays; (2) the scavenging of stable free radicals by antioxidants, including the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays; and (3) the scavenging of short-lived radicals by competition mechanisms, such as peroxyl radicals in the oxygen radical

absorbance capacity (ORAC) assay. Other less widely used methods assess the free radical scavenging capacity (FRSC) of antioxidants using radicals that are responsible for the oxidative stress damage observed in vivo, such as the superoxide, hydroxyl, and lipid peroxyl radicals. The respective FRSC methods have been named the superoxide radical scavenger capacity (SRSC), hydroxyl radical scavenger capacity (HRSC), and lipid peroxyl scavenger capacity (LPSC) assays.

Most of the TAC and FRSC determinations in foods and antioxidant products have been limited to their soluble compounds, and the extraction procedure has been considered a critical step.^{9,10} This reason motivated the development of the quick, easy, new, cheap, and reproducible (QUENCHER) assays to measure the antioxidant activity of food materials.¹¹ These methods are a very interesting approach that avoids time-consuming solvent extraction steps of the classical protocols. The basis of QUENCHER (Q_c) protocols is to place in direct contact the solid powdered food materials and the reagent solutions. Thus, the soluble antioxidants of the sample quench the radicals present in the reaction medium according to usual liquid–liquid reactions, whereas the antioxidants bound to the insoluble particle matter exert their antioxidant activity by taking advantage of surface reactions occurring at the solid–liquid interface.¹¹

Several of the frequently used TAC methodologies (ABTS, DPPH, ORAC, FRAP, and FC) have recently been adapted to

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76 the QUENCHER approach.^{12–15} Up to now, these assays have
77 been successfully applied to some foods for which the
78 antioxidant activity is largely dependent on the insoluble part,
79 such as cereals and bakery products, seeds, nuts, pulses, and
80 dietary fibers.^{12–19} It must also be noted that a mixture of
81 ethanol/water (50:50, v/v) has been suggested as the most
82 appropriate reaction medium to carry out QUENCHER assays,
83 and “neutral” powdered materials such as cellulose have been
84 proposed as suitable diluting agents when products under study
85 are very rich in antioxidants.^{11,12}

86 Despite the advantages and previous applications of the
87 QUENCHER methods, these assays are not yet extensively
88 used, probably due to a lack of validation studies of these
89 methodologies. In addition, to the best of our knowledge, the
90 QUENCHER concept has still not been adapted to the
91 methodologies that target biologically relevant radicals (SRSC,
92 HRSC, and LPSC), and a statistical validation and correlations
93 between the different Q-TAC and Q-FRSC assays have not yet
94 been performed. These verifications are important to encourage
95 the applicability of QUENCHER protocols as valuable tools for
96 the in vitro antioxidant capacity assessment of different
97 products such as foods, food ingredients, and others.

98 Therefore, to increase the use of the QUENCHER approach,
99 the first aim of the present study was to develop new
100 QUENCHER protocols to evaluate the scavenger capacity of
101 some of the most biologically relevant radicals (superoxide,
102 hydroxyl, and lipid peroxyl); the second aim was to optimize
103 and, for the first time, validate different QUENCHER assays
104 (Q-FC, Q-FRAP, Q-ABTS, Q-DPPH, Q-ORAC, Q-SRSC, Q-
105 HRSC, and Q-LPSC) using three model products of different
106 chemical properties. Secondarily, the best combination of
107 QUENCHER assays for routine analysis was identified, and
108 several factors that might influence the Q-TAC results, such as
109 the presence of ethanol in the reaction medium and the use of
110 cellulose as a diluting agent, were assessed. The study was
111 carried out with three powdered model products, all of them
112 rich in antioxidant compounds, but each containing anti-
113 oxidants of different polarity. Powdered products were made in
114 our pilot plant from wine pomace so that we could work with
115 familiar products for which characteristics and composition
116 were well-known.

117 ■ MATERIALS AND METHODS

118 **Chemicals.** ABTS, cellulose, DPPH, deoxyribose (2-deoxy-D-
119 ribose), 2,2'-diazobis(2-aminodinopropane) dihydrochloride
120 (AAPH), gallic acid (GA), 6-hydroxyl-2,5,7,8-tetramethyl-2-carboxylic
121 acid (Trolox), 4-nitroblue tetrazolium chloride (NBT), phenazin
122 methosulfate (PMS), 2,4,6-tris(2-pyridyl)-S-triazine (TPTZ), and 2-
123 thiobarbituric acid (TBA) were obtained from Sigma-Aldrich Co (St.
124 Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), iron(III)
125 chloride acid (FeCl₃), iron(II) sulfate (FeSO₄), FC reagent, hydrogen
126 peroxide (H₂O₂), L-ascorbic acid (C₆H₈O₆), potassium persulfate
127 (K₂O₈S₂), sodium acetate (NaC₂H₃O₂), sodium carbonate (Na₂CO₃),
128 sodium pyrophosphate anhydrous (Na₄P₂O₇), and trichloroacetic acid
129 (TCA) were purchased from Panreac (Barcelona, Spain).

130 **Samples.** Red wine pomace was kindly supplied by different
131 wineries located at Burgos (Spain). All red wine pomace was mixed
132 and dehydrated until reaching a final water content of <10%. Dried
133 materials were separated, ground, and sieved, thus obtaining three
134 different powdered products,²⁰ which were used as model matrices.
135 One of them was derived from grape seeds (Sd) separated from the
136 wine pomace; this model product represented foods with a significant
137 content of fat and rich mainly in hydrophobic antioxidants. Another
138 product was obtained from the wine pomace free of seeds, which was
139 mainly constituted by grape skins (Sk); this model product

represented food matrices rich in hydrophilic antioxidant and without
or with very low levels of fat. The third powdered product was
obtained from whole (W) wine pomace; this model product
represented food materials with some fat and rich in both hydrophilic
and hydrophobic antioxidants. The particle size of these model
products was <0.250 mm in the cases of Sk and W and <0.355 mm for
Sd.²¹

QUENCHER Antioxidant Capacity Methods. The adapted Q-
TAC and Q-FRSC methodologies were fixed end-point procedures
with the exception of the Q-ORAC method, which was a kinetic assay.
Results are given as mean values ± standard deviation of three
independent samples.

Q-FC Assay. This method was adapted from the FC assay
developed by Singleton and Rossi.²² Briefly, 1 ± 0.005 mg of the
tested products was weighed in a microbalance (MicroPro11,
Sartorius) and mixed with 0.2 mL of Milli-Q (MQ) water and 0.2
mL of FC reagent. After 5 min of reaction, 4 mL of a 0.7 M Na₂CO₃
solution was added, and the final volume was made up to 10 mL with
MQ water. After 1 h of incubation in an orbital shaker, the absorbance
at 750 nm was measured using an UV-vis spectrophotometer (U-
2000 Hitachi). The FC index was expressed as micromoles of gallic
acid equivalents per gram of product (GAE/g) by means of a dose-
response curve for different quantities of the standard.

Q-FRAP Assay. The Q-FRAP procedure was adapted from the
method described by Benzie et al.²³ The reactive mixture was freshly
prepared by mixing 10 mM TPTZ and 20 mM FeCl₃ in 300 mM
NaC₂H₃O₂ buffer (pH 3.6) at a ratio of 1:1:10 (v/v/v) and diluted
10:1 (v/v) in MQ water. Ten milliliters of the FRAP solution was
added to 1 ± 0.005 mg of the samples and incubated at 37 °C for 30
min with continuous stirring. The absorbance at 593 nm was
measured. The results were expressed as micromoles of iron(II)
equivalents per gram of product (Fe(II)E/g) using linear calibration
obtained with different amounts of FeSO₄.

Q-ABTS Assay. The method was adapted from the assay developed
by Re et al.²⁴ and modified by Rivero-Pérez et al.²⁵ The stock solution
of ABTS^{•+} was diluted with MQ water to an absorbance of 0.70 ± 0.02
at 734 nm; 1 ± 0.005 mg of the assessed products was weighed, and
the reaction was started by adding 10 mL of ABTS^{•+} working solution.
After 30 min of incubation in darkness with continuous stirring, the
absorbance of the samples at 734 nm was measured and subtracted
from the absorbance of the ABTS^{•+} working solution. The results are
expressed as micromoles of Trolox equivalents per gram of product
(TE/g), using the dose-response curve described by different
amounts of this standard.

Q-DPPH Assay. This method is based on the scavenging of the
radical DPPH[•].²⁶ A 0.1 mM DPPH[•] working solution in pure
methanol was prepared and diluted until an absorbance at 517 nm of
0.70 ± 0.02.²⁵ Then, 1 ± 0.005 mg of the powdered products was
transferred into a test tube and mixed with 10 mL of DPPH[•] working
solution. The absorbance at 517 nm was measured after 30 min of
continuous stirring at room temperature in the samples and subtracted
from the absorbance of the DPPH[•] working solution. Trolox was used
as standard to perform a calibration curve, and the results were
expressed as micromoles of TE per gram of product.

Q-ORAC Assay. This assay was adapted from the method described
by Ou et al.²⁷ The analysis was conducted in a four-cell fluorometer
(Cary-Eclipse, Varian) with continuous stirring in the cuvette and at
37 °C. Briefly, 0.2 ± 0.005 mg of the assessed products was weighed
and resuspended in 0.8 mL of 75 mM phosphate buffer (pH 7.0,
warmed to 37 °C) just before use. A Trolox quality control (T10,
equivalent to 10 μg of Trolox) and a 300 mM AAPH solution in
phosphate buffer were also freshly prepared. At least one T10 and one
blank were analyzed with every set of samples (same AAPH solution).
In a glass cuvette, 0.4 mL of the samples (the resuspended products,
the T10 control or just phosphate buffer for the blank) was mixed with
2 mL of a freshly prepared 87.5 nM fluorescein solution in phosphate
buffer. The fluorescence reading (λ_{exc} = 493 nm and λ_{em} = 511 nm)
was started, and 0.1 mL of the AAPH solution was added after 2.5 min.
The fluorescence decay curve was monitored for 100 min. The net
area under the curve (net AUC) was calculated for each sample by 209

210 subtracting the blank AUC from the sample AUC. Regression
211 equations between net AUC and different quantities of Trolox were
212 obtained and used for the expression of the Q-ORAC values as
213 micromoles of TE per gram of product.

214 **Q-SRSC Assay.** The Q-SRSC procedure was adapted from the
215 method described by Liu et al.²⁸ and Rivero-Pérez et al.²⁵ Briefly, 1.5 ±
216 0.005 mg of the products was added to the test tube and mixed with a
217 1.5 mL final volume of 78 μM NADH, 50 μM NBT, and 10 μM PMS
218 in 16 mM buffer Tris-HCl (pH 8.0). After the mixture had been
219 shaken for 10 s, the absorbance (A) of the samples (S) at 560 nm was
220 measured. "Oxidized controls" (OC; without products) and "sample
221 controls" (SC; products and buffer) were also prepared. The results
222 are expressed as oxidation inhibition percentages by the equation

$$\text{oxidation inhibition \%} = (A_{OC} - (A_S - A_{SC}))/A_{OC} \times 100$$

223 (1)

224 **Q-HRSC Assay.** The Q-HRSC assay was adapted from the method
225 developed by Halliwell et al.²⁹ and modified by Rivero-Pérez et al.²⁵
226 Briefly, 1 ± 0.005 mg of the powdered products was weighed and
227 transferred into a screw-top test tube. A 1 mL final reaction volume
228 contained a mixture of 1 mM deoxyribose, 0.1 mM C₆H₈O₆, 1 mM
229 H₂O₂, 0.1 mM FeCl₃, and 0.1 mM EDTA in 5 mM phosphate buffer
230 (pH 7.4). OC and SC were also prepared. The tubes were incubated
231 for 60 min at 37 °C with continuous stirring. Then, 1.5 mL of TCA
232 (28% w/v) and 1 mL of TBA (1% w/v) were added, and the capped
233 tubes were shaken and heated at 100 °C for 15 min. The absorbance
234 was recorded at 532 nm, and the results were expressed as oxidation
235 inhibition percent following eq 1.

236 **Q-LPSC Assay.** This assay was adapted from the method described
237 by Rivero-Pérez et al.²⁵ The experiments were carried out in rat liver
238 microsomal preparations,³⁰ and lipid peroxidation was induced using
239 AAPH as oxidant. The total microsomal protein content was
240 determined using the Bradford method.³¹ A microsomal solution
241 containing 10 mg/mL protein in 0.1 M Na₄P₂O₇ buffer (pH 7.4) was
242 prepared and stored at -80 °C until the day of analysis; 0.2 ± 0.005
243 mg of the tested products was placed into a screw-top tube, and 400
244 μL of 50 mM Tris-HCl buffer (pH 7.0), 50 μL of the microsomal
245 solution, and 50 μL of 100 mM AAPH solution in the Tris-HCl buffer
246 were added. OC and SC (replacing the AAPH solution by Tris-HCl
247 buffer) were also prepared. The tubes were incubated at 37 °C for 90
248 min under continuous stirring. Then, 750 μL of TCA (2.8% w/v) and
249 500 μL of TBA (1% w/v) were added, and the capped tubes were
250 shaken and heated at 100 °C for 15 min. Seven hundred and fifty
251 microliters of the reaction mixture was transferred to another tube, and
252 the TBA reactive substances were extracted with the same volume of
253 butanol. The absorbance at 532 nm of the butanol extract was
254 measured, and the results were expressed as oxidation inhibition
255 percent with respect to the OC sample following eq 1.

256 **Statistical Analysis.** The statistical validation of the methods was
257 performed using Statgraphics Centurion XVI version 16.2.04 software
258 (Statpoint Technologies, Inc., Warranton, VA, USA). The statistical
259 analysis of the Q-TAC data was carried out using one-way analysis of
260 variance (ANOVA). Fisher's least significant difference (LSD) test was
261 applied to determine the statistical significance of differences among
262 various groups. A minimum significance level of *p* < 0.05 was
263 considered. Pearson's correlation coefficients were determined to
264 study linear correlations between pairs of the Q-TAC methodologies.

265 ■ RESULTS AND DISCUSSION

266 **Adaptation and Optimization of the Q-TAC and Q-
267 FRSC Methods.** This study shows some modifications with
268 respect to Q-TAC protocols (Q-ABTS,¹² Q-DPPH,¹² Q-
269 FRAP,¹⁴ Q-ORAC,¹⁵ and Q-FC¹⁵) previously published.
270 Moreover, three new QUENCHER methodologies were
271 developed (Q-SRSC, Q-HRSC, and Q-LPSC), which were
272 adapted from validated classical FRSC methods.²⁵

273 The main modification from other Q-TAC assays was the
274 selection of aqueous reaction mediums whenever the method-

ologies allowed for the use of both organic and aqueous 275
solvents. Aqueous medium might better represent the real 276
environment surrounding any food matrix or biological system. 277
Therefore, MQ water or aqueous buffer was initially used in all 278
protocols except in the Q-DPPH assay, which required an 279
organic solvent to dissolve the radical. 280

The particle size of the powdered products is a factor that 281
might influence the results of the Q-TAC and Q-FRSC 282
measurements, and this aspect should be taken into account for 283
comparisons among powders of different particle size. Never- 284
theless, Serpen et al.¹⁷ did not find a remarkable effect of this 285
factor, and Gökmen et al.¹¹ suggested the use of powdered 286
samples of particle size ranging from 0.3 to 0.1 mm in 287
QUENCHER assays. 288

All protocols in this study were optimized for the direct 289
measurement of small quantities of powdered products, 290
considering the final reaction volume and amount of sample 291
weighed. 292

The Q-FC, Q-FRAP, Q-ABTS, and Q-DPPH methods are 293
rather inexpensive and simple methodologies, which allow for 294
the adjustment of the assay volumes (maintaining the 295
proportionality among the reagents) without a relevant increase 296
in the cost of the analysis. Preliminary studies were conducted 297
to establish 1 mg as an advisable amount of product, although 298
satisfactory results can also be obtained with smaller or larger 299
quantities (0.2–5 mg), depending on the TAC of the products 300
and the sensitivity of the assay. 301

In the case of the Q-ORAC method, the reaction volume is 302
restricted by the maximum volume of the cuvette because the 303
fluorescence decay needs to be continuously monitored (kinetic 304
assay). Amigo-Benavent et al.¹³ proposed that the Q-ORAC 305
assay be carried out in alternating stages of incubation at 37 °C 306
in a test tube with centrifugation, transfer to a cuvette, and 307
fluorescence reading. The Q-ORAC method proposed in the 308
present study avoids several centrifugation/transfer steps, is 309
more similar to classical protocols, and reproduces more 310
effectively the advantages of the ORAC assay with respect to 311
other TAC methods.^{2,4,8} However, a fluorometer with a 312
temperature control chamber and constant stirring in the 313
cuvette is required, and the assay might not be suitable for 314
products of very high Q-TAC. 315

With regard to the Q-SRSC, QHRSC, and Q-LPSC methods, 316
the final reaction volume and amount of sample weighed must 317
be adjusted to give results in the range of 0–100% oxidation 318
inhibition. The Q-SRSC assay is a rather simple and quick 319
methodology that can be directly performed in the cuvette or in 320
a test tube. However, the Q-HRSC and the Q-LPSC assays 321
involve more tedious protocols. It is suggested that the amount 322
of sample used be adjusted rather than increasing the final 323
volume, although it must be remarked that the precision of the 324
QUENCHER methods might be compromised when <0.2 mg 325
of the samples is weighed. 326

QUENCHER fixed end-point procedures allow for trans- 327
ferring an aliquot of the reaction medium to a microplate for 328
the absorbance reading step. This alternative increases the 329
repeatability and reduces the time between different measure- 330
ments, so it is especially appropriate for the simultaneous 331
analyses of multiple samples. On the other hand, QUENCHER 332
kinetic assays cannot be carried out directly in a microplate due 333
to the interference of solid products on the absorbance 334
measurement. Furthermore, the extremely small quantity of 335
solid sample required, proportional to the small volume of 336
wells, could put at risk the validity of this procedure. 337

Table 1. Calibration Models of the QUENCHER Total Antioxidant Capacity (Q-TAC) Methods

method	standard ^a	range (μmol)	calibration model ^b	R^2 ^c
Q-FC	GA	0.059–0.588	$A_{750\text{ nm}} = (1.79 \pm 0.009) \mu\text{mol of GA} + (0.005 \pm 0.004)$	0.999
Q-FRAP	Fe(II)	0.036–0.285	$A_{593\text{ nm}} = (2.76 \pm 0.046) \mu\text{mol of Fe(II)} - (0.009 \pm 0.008)$	0.996
Q-ABTS	T	0.083–0.499	$(A_{\text{WS}} - A_{\text{S}})_{734\text{ nm}} = (1.20 \pm 0.015) \mu\text{mol of T} + (0.007 \pm 0.005)$	0.998
Q-DPPH	T	0.040–0.200	$(A_{\text{WS}} - A_{\text{S}})_{517\text{ nm}} = (3.20 \pm 0.033) \mu\text{mol of T} + (0.007 \pm 0.004)$	0.999
Q-ORAC	T	0.010–0.050	net AUC = $(3361 \pm 70.3) \mu\text{mol of T} + (4.78 \pm 2.25)$	0.996

^aGA, gallic acid; Fe(II), iron(II); T, Trolox. ^b $3A_{\text{WS}}$, absorbance of the radical working solution; A_{S} , absorbance of the sample; net AUC, net area under the curve. ^c R^2 , coefficient of determination.

Table 2. Linearity Test, Proportionality, and Detection Limits of the QUENCHER Total Antioxidant Capacity (Q-TAC) Methods

method	linearity test ^a		proportionality test ^b				detection limits ^c			
	CV(Fr) (%)	CV(b) (%)	ANOVA		$a \pm t_{\text{tab}}S(a)$	$t_{\text{cal}}(a)$	$t_{\text{cal}}(b)$	t_{tab}	y_{d}^{d}	x_{d}^{e}
			F_{exp}	F_{tab}						
Q-FC	2.99	0.48	42730	4.38	0.005 ± 0.006	1.45	207	1.73	0.016	12.0 nmol of GAE
Q-FRAP	4.79	1.68	3537	4.67	-0.009 ± 0.015	1.12	59.5	1.77	0.012	15.3 nmol of Fe(II)E
Q-ABTS	3.27	1.25	6328	4.49	0.007 ± 0.009	1.49	80.2	1.75	0.020	20.4 nmol of TE
Q-DPPH	2.57	1.03	9384	4.67	0.007 ± 0.008	1.53	96.1	1.77	0.017	6.68 nmol of TE
Q-ORAC	4.86	2.10	2271	4.97	$4.78 \pm 4.09^*$	2.12^*	47.7	1.81	10.1	3.08 nmol of TE

^aCV(Fr), coefficient of variation of the response factor; CV(b), coefficient of variation of the slope; ANOVA by means of the F test (F_{exp} , experimental F ; F_{tab} , tabulated F ($1, n - 2, 0.05$)). Linearity test acceptance criteria: CV(Fr) < 5%; CV(b) < 2%; ANOVA $F_{\text{exp}} > F_{\text{tab}}$. ^b $a \pm t_{\text{tab}}S(a)$, confidence interval of the ordinate at origin; t_{cal} , calculated Student's t ; t_{tab} , tabulated Student's t ($n - 2, 0.05$). Proportionality acceptance criteria: interval should include 0; $t_{\text{cal}}(a) < t_{\text{tab}}$; $t_{\text{cal}}(b) > t_{\text{tab}}$. ^c y_{d} , results do not meet the acceptance criteria. ^d y_{d} , detection signal; x_{d} , capability of detection. Detection limits calculated for α and $\beta = 0.05$. ^e y_{d} units, absorbance units (Q-FC, Q-FRAP, Q-ABTS, Q-DPPH); net area under the curve (net AUC) (Q-ORAC). ^fGAE, gallic acid equivalents; Fe(II)E, iron(II) equivalents; TE, Trolox equivalents.

Table 3. Precision of the QUENCHER Total Antioxidant Capacity (Q-TAC) and QUENCHER Free Radical Scavenging Capacity (Q-FRSC) Methods

method	units of S_{r} and S_{R}^{a}	repeatability			intermediate precision	
		CV ^b (%)	$S_{\text{r}}(\text{Rp})^{\text{c}}$	$S_{\text{r}}(\text{IP})^{\text{d}}$	CV ^e (%)	S_{R}^{f}
Q-FC	$\mu\text{mol GAE/g product}$	2.06	1.23	1.17	2.37	1.49
Q-FRAP	$\mu\text{mol Fe(II)E/g product}$	4.98	10.8	10.7	6.48	16.0
Q-ABTS	$\mu\text{mol TE/g product}$	2.13	2.24	2.73	3.05	3.26
Q-DPPH	$\mu\text{mol TE/g product}$	3.12	1.24	1.26	3.85	1.59
Q-ORAC	$\mu\text{mol TE/g product}$	5.45*	2.94	2.79	5.48	3.01
Q-SRSC	oxidation inhibition %	3.27	1.05	1.46	6.35	1.62
Q-HRSC	oxidation inhibition %	5.31*	2.79	3.09	6.32	3.28
Q-LPSC	oxidation inhibition %	5.26*	4.01	4.44	6.35	4.87

^aGAE, gallic acid equivalents; Fe(II)E, iron(II) equivalents; TE, Trolox equivalents. ^bRepeatability acceptance criteria: CV < 5%. ^c*, results do not meet the acceptance criteria. ^dEstimate of repeatability (S_{r}) determined by one-variable analysis of seven replicates 1 day (Rp, repeatability experiment). ^eEstimate of repeatability (S_{r}) determined by one-way ANOVA of three replicates on three different days (IP, intermediate precision experiment). ^fIntermediate precision acceptance criteria: CV < 10%. ^gEstimate of intermediate precision (S_{R}) determined by one-way ANOVA of three replicates on three different days (IP, intermediate precision experiment).

338 **Validation of the Q-TAC and Q-FRSC Methods.** The
339 validation study was done by the examination of the following
340 parameters: linearity, proportionality, detection limits, and
341 precision (repeatability and intermediate precision).³² In the
342 case of Q-FRSC assays (Q-SRSC, Q-HRSC, and Q-LPSC),
343 only the precision was evaluated because in these methods a
344 calibration curve is not needed. In the Q-TAC methodologies
345 (Q-FC, Q-FRAP, Q-ABTS, Q-DPPH, and Q-ORAC), a dose–
346 response curve was obtained by testing different amounts of the
347 standard used in each method. The calibration was performed
348 using linear models and the least-squares (LS) method. All of
349 the models presented coefficient of determination (R^2) values
350 >0.995 (Table 1).

351 Once the parameters were estimated, the models were
352 validated by verifying the assumptions related to the residuals

and the functions.³³ The studentized residuals were normally
distributed (Kolmogorov–Smirnov, chi-square, and Shapiro–
Wilk tests), independent (Durbin–Watson test), and homo-
scedastic (Cochran and Bartlett tests) in all cases. The
functional part of each linear model was first validated in a
linearity test. According to the results (Table 2), all of the
models were suitable for describing the linear relationship
between the standard concentration and the response of each
assay. The Q-FC method obtained the best values for all of the
parameters tested, whereas the Q-ORAC assay linearity
response was the least satisfactory, although within the
acceptance criteria. In addition, a proportionality test to
determine whether the mathematical model could be used for
predictive purposes was performed (Table 2). The conditions
of proportionality were corroborated for all methods except Q-

Table 4. QUENCHER Total Antioxidant Capacity (Q-TAC) and QUENCHER Free Radical Scavenging Capacity (Q-FRSC) of the Model Products Obtained from Skins (Sk), Seeds (Sd), and Whole (W) Wine Pomace^a

method	units ^b	Sk		W		Sd	
		value	CV (%)	value	CV (%)	value	CV (%)
Q-FC	$\mu\text{mol GAE/g product}$	74.4 ± 1.07 b	1.43	78.3 ± 2.37 b	3.02	52.6 ± 2.53 a	4.80
Q-FRAP	$\mu\text{mol Fe(II)E/g product}$	275 ± 4.18 c	1.52	224 ± 13.6 b	6.05	140 ± 4.92 a	3.52
Q-ABTS	$\mu\text{mol TE/g product}$	120 ± 5.21 a	4.33	150 ± 2.66 b	1.78	120 ± 0.83 a	0.69
Q-DPPH	$\mu\text{mol TE/g product}$	51.9 ± 1.10 a	2.11	110 ± 3.60 b	3.27	174 ± 4.16 c	2.39
Q-ORAC	$\mu\text{mol TE/g product}$	236 ± 9.92 b	4.19	243 ± 4.17 b	1.72	181 ± 7.96 a	4.39
Q-SRSC	oxidation inhibition %	31.7 ± 1.19 a	3.76	50.5 ± 1.88 b	3.72	30.1 ± 1.20 a	3.98
Q-HRSC	oxidation inhibition %	50.1 ± 2.59 a	5.17	59.0 ± 0.64 c	1.08	53.8 ± 1.08 b	2.01
Q-LPSC	oxidation inhibition %	52.2 ± 2.57 b	4.92	62.5 ± 3.38 c	5.41	33.7 ± 2.02 a	7.27

^aQ-TAC and Q-FRSC values are the mean \pm standard deviation ($n = 3$). Roman letters indicate significant differences among the model products (Sk, W, Sd) within each QUENCHER methodology. ^bGAE, gallic acid equivalents; Fe(II)E, iron(II) equivalents; TE, Trolox equivalents.

ORAC, where the ordinate at origin did not meet any of the criteria. In this assay the Q-TAC is assessed by the net AUC determination and a lag time is observed during oxidation of the fluorescein in the presence of an antioxidant.^{1,27} The unsatisfactory result of the ordinate at origin is explained by the lack of proportionality of this lag time at very small amounts of Trolox. However, a linear response was observed at higher quantities of the standard, as previously observed by Ou et al.²⁷ in the classical approach of this assay.

Having studied the acceptability of the linear models, the detection limits y_d (detection signal) and x_d (capability of detection) of the assessed methodologies were determined as described by Ortiz et al.³³ In general, low limits of detection were observed (Table 2), which confirmed the acceptability of all methodologies to determine the TAC of very small quantities of antioxidants. Among the assays using Trolox as standard, the lowest x_d value corresponded to the Q-ORAC assay, which was corroborated as a highly sensitive methodology, and the highest limits of detection were obtained by the Q-ABTS method. A similar observation was previously reported for a comparison of classical TAC methodologies.²⁵

Finally, two different terms of the precision were evaluated, the repeatability (R_p) and the intermediate precision (IP), determining their estimates (S_r and S_R , respectively) and coefficients of variance (CV).³² The IP refers to the precision under reproducibility conditions when only some of the factors are varied. In this study a single factor (different days) was investigated. The results of the R_p and IP experiments for each methodology are shown in Table 3. It can be seen that similar S_r values were obtained in both R_p and IP experiences for all of the methods. The CV of the assays was the criteria selected to evaluate their acceptability. The R_p of the longest protocols (Q-ORAC, Q-HRSC, and Q-LPSC) exceeded the $CV < 5\%$ cutoff value, whereas those assays with just one or two steps (Q-FC, Q-ABTS, and Q-DPPH) showed a high R_p between measurements. By comparison with the R_p obtained in classical protocols validated using different wines as samples,²⁵ slightly higher CV were observed in the Q-TAC, and especially in the Q-FRSC methods, as all classical assays obtained $CV < 5\%$. The only exception was the Q-ABTS assay, which showed better R_p than the classical approach. In terms of IP (Table 3), all of the methodologies presented satisfactory results, with the lowest CV (%) obtained again by two of the simplest methods (Q-FC and Q-ABTS). Therefore, the differences in the R_p and IP among the methodologies were mainly attributed to the complexity of the protocols. The sensitivity of the assays

could also affect the precision observed, as might be the case for the Q-ORAC method mentioned above.

Q-TAC and Q-FRSC of the Model Products. To provide comprehensive information on the actual TAC of food, it is recommended that at least two TAC assays be used.^{6,8} Similarly, in this study, a broad overview of the antioxidant capacity of the three model products (Sk, W, and Sd) was achieved with the different QUENCHER methodologies under study.

As shown in Table 4, model product W (rich in both hydrophobic and hydrophilic antioxidant compounds) showed the highest Q-TAC and Q-FRSC values in most of the methods, with the exception of the Q-FRAP assay, where Sk (model product especially rich in hydrophilic antioxidant) presented the most elevated antioxidant capacity and the Q-DPPH assay, where Sd (model product especially rich in hydrophobic antioxidant) exhibited the highest Q-TAC values. The difference between the antioxidant capacity of Sk and W was not significant in the Q-FC and Q-ORAC protocols. No significant differences were found between Sk and Sd in the Q-ABTS and Q-SRSC assays. From a comparison of the responses obtained by the three methodologies where Trolox was used as standard, Q-ORAC obtained the highest TAC values, but it must be noted that the sensitivity of this method to Trolox was also more elevated than in the Q-DPPH and Q-ABTS assays.

The obtained results were contrasted with those obtained by classical determinations, and considerable differences were found, except with the Q-DPPH method. Several studies comparing extracts from wine pomace reported a higher antioxidant capacity exhibited by seeds than skins, obtaining the same tendency using different assays (FC,^{34–37} FRAP,^{34,36,37} ABTS,³⁶ DPPH,^{34–37} ORAC,³⁶ SRSC,³⁵ and HRSC³⁴). This fact may be explained because classical protocols involve extraction with solvents, which are capable of extracting both hydrophobic and hydrophilic antioxidants. Therefore, the extracts obtained might not be representative of the antioxidant capacity that solid foods could actually exert, whereas QUENCHER assays may provide more reliable information.¹¹

Correlation Study between the QUENCHER Methodologies. Pearson's correlation coefficients (r) between pairs of the assays were determined using the Q-TAC and Q-FRSC values obtained for Sk, W, and Sd by the different QUENCHER methodologies.

In the literature, due to the wide spectrum of analytical techniques and reaction conditions available, it is difficult to find an agreement on the correlations among different TAC methodologies.^{2,8,38} To the extent of our knowledge, the

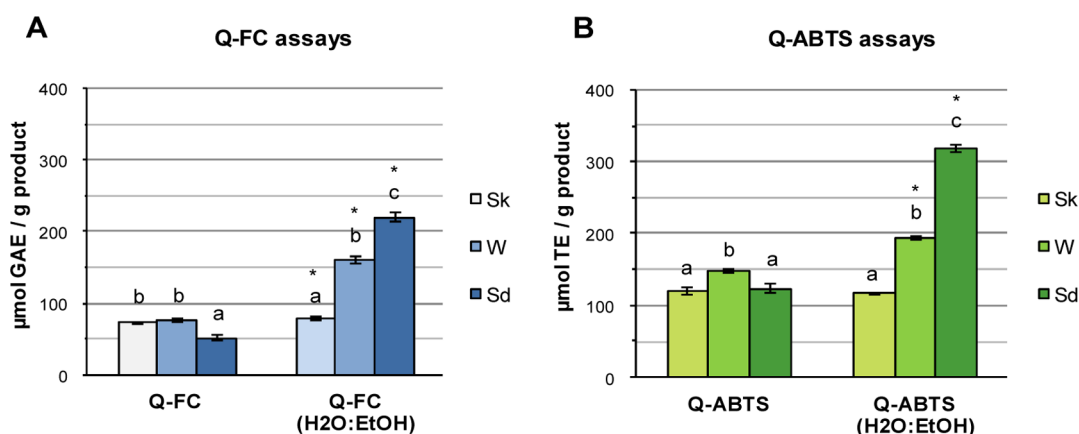


Figure 1. QUENCHER total antioxidant capacity (Q-TAC) of the model products (Sk, skins; W, whole; Sd, seeds): (A) Q-FC assays; (B) Q-ABTS assays. GAE, gallic acid equivalents; TE, Trolox equivalents. Data are expressed as mean values \pm standard deviation ($n = 3$). Roman letters indicate significant differences among the model products (Sk, W, Sd) within each assay. The asterisk (*) refers to significant differences between the Q-TAC and Q-TAC (H₂O:EtOH) approaches for each wine pomace product.

461 correlation between different QUENCHER methodologies has
 462 not been described yet. In this study, an interesting
 463 correspondence among the assays was observed, especially for
 464 the Q-FC and Q-ABTS assays with the rest of methods. A
 465 highly significant ($p < 0.01$) positive correlation was detected
 466 between Q-FC and Q-FRAP ($r = 0.847$), between Q-FC and
 467 Q-ORAC ($r = 0.974$), and between Q-FC and Q-LPSC ($r =$
 468 0.926). The correspondence between Q-FC and Q-FRAP is
 469 due to the fact that both methods measure the reducing
 470 capacity of the sample,³⁸ also detected between the classical
 471 approaches.^{36,37} Furthermore, the significant correlation
 472 between Q-ORAC and Q-LPSC ($r = 0.914$) may be explained
 473 because peroxy radicals are involved in both assays.³⁹ The
 474 positive correlation of the Q-FC with the Q-ORAC and Q-
 475 LPSC assays is very convenient as they are more biologically
 476 relevant methods, but these assays are also more complicated,
 477 and their validation study showed some unsatisfactory results.
 478 However, the obtained results contrast with the weaker
 479 correlation ($R^2 = 0.409$) found between the classical
 480 approaches of the FC and the ORAC assays by Ky et al.³⁶
 481 using also wine pomace as samples. In the present study,
 482 interesting high positive correlations ($p < 0.01$) between Q-
 483 ABTS and Q-SRSC ($r = 0.935$) and between Q-ABTS and Q-
 484 HRSC ($r = 0.903$) were also observed. According to Rivero-
 485 Pérez et al.,²⁵ such strong correlations were not detected in the
 486 classical approaches of these assays using wines as samples. On
 487 the other hand, negative correlations ($p < 0.05$) between Q-
 488 DPPH and Q-FC ($r = -0.781$), between Q-DPPH and Q-
 489 FRAP ($r = -0.986$), and between Q-DPPH and Q-ORAC ($r =$
 490 -0.808) were found in the present study, and the Q-DPPH
 491 method was not significantly correlated with any of the
 492 biologically relevant Q-FRSC assays. In classical methodologies,
 493 the DPPH and HRSC assays were not found to be correlated
 494 either,³⁴ but high positive correlations were usually detected
 495 between the DPPH and other TAC methods (FC,^{35–37}
 496 FRAP,^{34,37} and ABTS²⁵). It must be noted that the Q-DPPH
 497 is the only QUENCHER methodology that was performed in
 498 an organic reaction medium, and its lack of positive correlation
 499 with the rest of the protocols indicates the elevated influence of
 500 the solvent used in these assays.
 501 The different contents of hydrophilic and lipophilic
 502 compounds^{40,41} of the three model products used in this
 503 study allow for assessing the response of each method toward

504 matrices with compounds of high or low water solubility. 504
 505 Anyway, it would be advisable to confirm the correlations found
 506 in the current study by evaluating a wider range of products. 506

507 With the results of both the validation and correlation studies
 508 taken into account, the combination of both Q-FC and Q-
 509 ABTS assays is suggested as a good selection for a general Q-
 510 TAC analysis of powdered foods. The Q-SRSC, Q-HRSC, and
 511 Q-LPSC methods, first adapted to the QUENCHER approach
 512 in this study, might give a more biologically relevant overview
 513 of the antioxidant capacity of the samples. However, the Q-
 514 HRSC and Q-LPSC assays are complicated protocols for
 515 routine analysis, and it has been shown that the combination of
 516 the Q-FC and Q-ABTS methods covers the correspondence
 517 with the results obtained by the three biologically relevant Q-
 518 FRSC assays. 518

**Effects of the Reaction Medium in the Q-FC and Q-
 519 ABTS Assays.** In the QUENCHER methods there is no
 520 extraction step, but the reaction medium is a key factor that
 521 determines the solubility of the compounds present in the solid
 522 powdered product, as well as the interactions between the
 523 antioxidants and the radicals used as probes in the assays. 524

525 Serpen et al.⁴² proposed a water/ethanol (H₂O:EtOH) 50:50
 526 (v/v) ratio as a suitable working solution, providing accurate Q-
 527 TAC results for most of the food matrices studied. Therefore,
 528 the effect of using water or a mixture H₂O:EtOH 1:1 (v/v) as
 529 reaction medium was assessed in the current study using the
 530 two previously selected Q-TAC methods (Q-FC and Q-ABTS). 530

531 The Q-FC (H₂O:EtOH) and Q-ABTS (H₂O:EtOH) assays
 532 were validated as previously described for the rest of the
 533 methodologies and presented satisfactory results for all
 534 parameters evaluated (Supporting Information 1). Slight
 535 formation of white solids was observed in the Q-FC
 536 (H₂O:EtOH) method, probably due to the precipitation of
 537 Na₂CO₃ in the presence of ethanol. Nevertheless, these solids
 538 did not interfere in the measurement. The responses of the Q-
 539 FC and Q-FC (H₂O:EtOH) assays to different standard
 540 concentrations were not significantly different (similar slopes in
 541 the calibration models), whereas a 2.54 times lower response to
 542 Trolox in the Q-ABTS than in the Q-ABTS (H₂O:EtOH)
 543 method was detected. In contrast, Serpen et al.⁴² found similar
 544 slopes of the dose–response lines obtained in the Q-ABTS
 545 assays using different solvent ratios H₂O:EtOH (0:100, 25:75,
 546 50:50, 75:25, 100:0). A possible explanation might be that the

Table 5. Influence of the Amount of Sample Weighed and the Use of Cellulose (C) as a Diluting Agent in the Q-ABTS Assays Evaluated in Model Products Obtained from Skins (Sk) or Seeds (Sd) Separated from Red Wine Pomace^a

method	Q-TAC values ($\mu\text{mol TE/g product}$)				
Q-ABTS	Sk 3 mg	Sk 2 mg	Sk 1 mg	Sk:C (2:1) 3 mg	Sk:C (1:2) 3 mg
	116 \pm 3.97	119 \pm 3.41	122 \pm 3.77	121 \pm 1.88	118 \pm 8.32
Q-ABTS (H ₂ O:EtOH)	Sd 3 mg	Sd 2 mg	Sd 1 mg	Sd:C (2:1) 3 mg	Sd:C (1:2) 3 mg
	126 \pm 5.47 a	139 \pm 5.83 b	148 \pm 3.30 c	153 \pm 3.44 c	167 \pm 5.11 d
Q-ABTS (H ₂ O:EtOH)	Sk 1.5 mg	Sk 1 mg	Sk 0.5 mg	Sk:C (2:1) 1.5 mg	Sk:C (1:2) 1.5 mg
	124 \pm 4.10 cb	131 \pm 4.56 c	116 \pm 8.89 b	117 \pm 4.13 b	93.4 \pm 4.75 a
Q-ABTS (H ₂ O:EtOH)	Sd 0.75 mg	Sd 0.5 mg	Sd 0.25 mg	Sd:C (2:1) 0.75 mg	Sd:C (1:2) 0.75 mg
	314 \pm 5.45	320 \pm 9.30	318 \pm 18.1	323 \pm 10.1	320 \pm 17.4

^aQ-TAC values are the mean \pm standard deviation ($n = 3$). TE, Trolox equivalents. Roman letters indicate significant differences among the different amounts of sample and the dilutions with cellulose tested within each method (Q-ABTS or Q-ABTS (H₂O:EtOH)) for each product (Sk or Sd).

547 calibration curves of Q-TAC methodologies carried out in this
548 study were performed by weighing directly the standards,
549 without their previous dilution in any solvent. Thus, the lower
550 solubility of Trolox in water than in organic solvents played a
551 critical role in the response observed in each method, as
552 previously described by other authors.⁹ This fact may lead to
553 lower Q-TAC values in the Q-ABTS than in the Q-ABTS
554 (H₂O:EtOH) assay due intrinsically to the methods used rather
555 than the samples analyzed. Therefore, as the numeric Q-TAC
556 values obtained by the Q-ABTS assays may not be directly
557 comparable, the tendency observed among the samples (higher
558 or lower Q-TAC values) was the main interest of these
559 analyses.

560 The Q-TAC of Sk, W, and Sd obtained using the Q-FC and
561 Q-ABTS assays can be seen in Figure 1. As previously described
562 for classical methods,^{1,39} the reaction medium greatly
563 influenced the Q-TAC results, with both soluble compounds
564 and insoluble antioxidants attached to the powdered products
565 being affected by the solvent used in the Q-TAC assays. A
566 similar tendency was observed in both Q-FC and Q-ABTS
567 assays when water was replaced by a mixture of H₂O:EtOH
568 (1:1, v/v) as reaction medium. A significant increase in the Q-
569 TAC values of Sd was observed, reaching higher antioxidant
570 capacity than W, for which Q-TAC was also enhanced, but to a
571 lesser extent. The Sk product showed the lowest Q-TAC in
572 both Q-FC (H₂O:EtOH) and Q-ABTS (H₂O:EtOH) assays.
573 Similar results were obtained in previous studies²¹ carried out
574 using conventional methodologies including extraction steps
575 with organic solvents. Indeed, a good correlation between
576 general results obtained using Q-TAC (H₂O:EtOH) and
577 classical methods was observed. In contrast, this fact was only
578 found between Q-TAC (carried out in water) and classical
579 methods when the matrices under study were rich in
580 hydrophilic antioxidants (Supporting Information 2).

581 Differences among results obtained for the three model
582 products using the different Q-TAC methods can be partly
583 explained by their chemical composition, the solubility of their
584 antioxidant compounds in the reaction medium, and their
585 redox reactions with the radical probes or reagents used in each
586 assay. With regard to the chemical composition of the model
587 products tested, it is important to have in mind that skins of
588 grapes are a rich source of anthocyanins and hydroxycinnamic
589 acids, although they also contain some flavanols and flavonol
590 glycosides, whereas gallic acid, flavanols, and proanthocyanidins
591 are mainly present in the seeds of grapes.^{35,37,43} According to

these facts, the Sk model product was rich mainly in
592 anthocyanins and hydroxycinnamic acids, whereas Sd was rich
593 in flavanols from monomers to polymers (proanthocyanins and
594 tannins). A description of the solubility of food antioxidants,
595 including main phenolic classes, can be found in Gökmen et
596 al.¹¹ In general, the water solubility of polyphenols increases
597 with the number of glycosylated hydroxyl groups and with the
598 amount of sugars constituting the carbohydrate moiety. On the
599 other hand, in the case of polymerized polyphenols (such as
600 proanthocyanidins or tannins), the water solubility considerably
601 drops with an increased number of units and with the
602 formation of intramolecular bonds.⁴⁴ Thus, the higher polarity
603 of the polyphenols found in the grape skins allows that they are
604 well solubilized when water is used as reaction medium,⁴⁴
605 whereas the presence of ethanol seems to favor the extraction
606 of hydrophobic antioxidants present in the grape seeds. It must
607 be pointed out that lower H₂O:EtOH ratios are not advised
608 because it may cause the shrinking of the major constituents of
609 the food matrices, such as cellulose and proteins.^{17,42} This
610 could hamper the release of the compounds or the diffusion of
611 the radicals into the solid matrices where they can react with
612 inner bound insoluble antioxidants, leading to lower Q-TAC
613 values.¹¹

The reaction medium also affects the interactions between
615 the antioxidants and reagents involved in the methods by
616 enhancing or decreasing the equilibrium constant of their redox
617 reactions. In the QUENCHER approaches, when the redox
618 equilibrium constant of an antioxidant compound in a
619 determinate medium is higher than its solubility constant, the
620 extraction of this antioxidant from the insoluble matrix and its
621 solubilization in the reaction medium can be thermodynamically
622 enhanced.⁴²

The study of the correlations between the Q-TAC assays
624 carried out in the same reaction medium showed a strong
625 correspondence ($p < 0.01$) between Q-FC (H₂O:EtOH) and
626 Q-ABTS (H₂O:EtOH) ($r = 0.971$), whereas there was no
627 significant correlation between Q-FC and Q-ABTS ($r = 0.496$).
628 This finding shows that, in the Q-TAC (H₂O:EtOH) methods,
629 the factors related to the assay reaction medium (solubilization
630 of the antioxidant compounds) play a more critical role than
631 those related to the method used (reaction with the probes or
632 reagents). Therefore, the mixture H₂O:EtOH 50:50 (v/v)
633 proposed by Serpen et al.⁴² might be adequate to evaluate the
634 global TAC of food materials, but the Q-TAC of hydrophobic
635

636 antioxidants could be overestimated under certain conditions
637 and, then, their real antioxidant role in food matrices.

638 Moreover, the correlations between the biologically relevant
639 Q-FRSC assays and both Q-TAC (H₂O:EtOH) methods were
640 not significant in the case for the Q-SRSC and Q-HRSC
641 methods, and not even a significant ($p < 0.01$) negative
642 correlation between Q-LPSC and Q-ABTS (H₂O:EtOH) ($r =$
643 -0.716) was found. These results further support the selection
644 of the Q-FC and Q-ABTS assays in water as preferred protocols
645 to assess a potentially more biologically relevant Q-TAC of
646 food products and dietary supplements.

647 **Effects of Using Cellulose as a Diluting Agent in the**
648 **Q-ABTS Assays.** Cellulose has been suggested as a diluting
649 agent in previous Q-TAC protocols.^{11,12} However, the affinity
650 of polyphenols to cellulose is well-known,⁴⁵ so it is possible that
651 interactions between the cellulose and these antioxidant
652 compounds interfere in the quantification.

653 The possible influence of cellulose on the Q-TAC measure-
654 ments was evaluated with the Q-ABTS assays (the most widely
655 used Q-TAC assay up to now), and it was compared with the
656 effects of weighing different amounts of sample. The two model
657 products that presented more differences between them (Sk
658 and Sd) were chosen to carry out this study. Two dilutions or
659 product/cellulose ratios (2:1 and 1:2) were used. The
660 quantities of product weighed were adapted to those required
661 by each method to give results within their detection range. As
662 can be seen in Table S5, no influence of the amount of sample or
663 the presence of cellulose on the Q-TAC values was observed
664 for Sk in the Q-ABTS assay and for Sd in the Q-ABTS
665 (H₂O:EtOH) method. However, Q-ABTS results of Sd showed
666 significantly higher Q-TAC values when lower amounts of
667 sample were used, and this effect was more marked when the
668 product was diluted with cellulose. In contrast, the opposite
669 tendency was found for Sk in the Q-ABTS (H₂O:EtOH) assay.
670 In this case, inferior Q-TAC values were obtained when a lower
671 amount of Sk was used, which were only significant in Sk/
672 cellulose (1:2) with respect to the nondiluted Sk sample.

673 The observed effect of weighing different amounts of Sd in
674 the Q-ABTS assay could be due to a better water extraction of
675 its slightly soluble hydrophobic polyphenols^{11,36} when they are
676 at a lesser concentration. Moreover, possible hydrophobic
677 interactions between the antioxidant compounds (favored in
678 the aqueous medium)⁴⁶ could be more relevant when a higher
679 amount of sample is present, thus decreasing their ability to
680 scavenge the soluble ABTS^{•+} radicals present in the reaction
681 medium.

682 With regard to the influence of cellulose in the measure-
683 ments, absorbance values similar to those of the ABTS^{•+}
684 working solution were obtained in both methods (Q-ABTS
685 and Q-ABTS (H₂O:EtOH)) when only cellulose was used as
686 sample (data not shown), corroborating that cellulose alone is
687 inert toward the ABTS^{•+} reagent, as has been previously
688 described.¹² Therefore, the results obtained in this study could
689 be due to the effects of cellulose in the solubility of the
690 hydrophilic or lipophilic compounds present in the products
691 and their capacity to interact with the ABTS^{•+} radicals, both
692 factors influenced by the reaction medium. Hydrogen-bonding
693 and hydrophobic interactions between cellulose and poly-
694 phenols have been described,⁴⁵ which are also dependent on
695 the solvent systems used. Hydrophobic interactions are favored
696 in hydrophilic solvents, whereas hydrogen bonding is favored in
697 the more hydrophobic ones. Thus, the affinity of polyphenols
698 (such as gallotannins and ellagitannins) to cellulose is expected

to correlate with their hydrophobicity, their number of galloyl
699 groups, and their molecular size.^{44,45} This evidence explains
700 that cellulose effects are of particular importance in the
701 hydrophilic solvents when hydrophobic compounds are tested,
702 as happened when Sd was assessed by the Q-ABTS assay. The
703 monomeric and oligomeric proanthocyanidins of the grape
704 seeds might remain insoluble in aqueous reaction mediums.^{40,41}
705 However, their interaction with cellulose through their
706 hydrophobic moieties might raise their water solubility and
707 leave the hydroxyl groups available to interact with the ABTS^{•+}
708 radicals, leading to the increased Q-TAC values observed when
709 the product/cellulose ratio was decreased. On the other hand,
710 in the case of Sk in the Q-ABTS (H₂O:EtOH) assay, the
711 contrary effect may take place. Hydrogen bonding between
712 cellulose and the hydrophilic phenolics present in wine pomace
713 skins could be favored in the presence of organic solvents.
714 Thus, the number of hydroxyl groups available in the molecules
715 to scavenge the radical probes of the assay is reduced.
716

On the basis of the cited results, the use of cellulose as a
717 diluting agent is discouraged in the Q-TAC methodologies,
718 especially if the antioxidant products tested are a source of
719 polyphenols. The main motive is the possible interaction of
720 cellulose with the phenolic compounds involved in the assays,
721 leading to an under- or overestimation of the Q-TAC of the
722 products.
723

In summary, a wide range of QUENCHER analytical
724 techniques to provide a comprehensive initial assessment in
725 vitro of the TAC and FRSC of food materials have been
726 adapted and validated (Q-FC, Q-ABTS, Q-DPPH, Q-FRAP, Q-
727 ORAC, Q-SRSC, Q-HRSC, and Q-LPSC). The Q-TAC and Q-
728 FRSC assays skip the extraction step of the classical antioxidant
729 capacity protocols and are simple, quick, inexpensive, and
730 precise methodological approaches. The QUENCHER meth-
731 odologies validated in this paper working with powdered model
732 products are suitable for application to other foods and food
733 ingredients, both rich in fat (as seeds) and not, as skins, which
734 are rich in fiber and minerals among others. The combination
735 of the Q-FC and Q-ABTS assays using water as reaction
736 medium is proposed as the best choice to analyze a large
737 number of samples. They provide more biologically relevant
738 information than the classical TAC methods using extracts or
739 other Q-TAC approaches using nonaqueous solvents. It is
740 suggested that the same amount of sample be weighed,
741 especially when products rich in hydrophobic antioxidants are
742 assessed. The use of cellulose as a diluting agent is not
743 recommended in the Q-TAC methods. Finally, it must be
744 pointed out that, despite the advantages of the proposed
745 QUENCHER assays over other in vitro methodologies, the Q-
746 TAC or Q-FRSC of food materials and dietary supplements
747 cannot be directly translated into healthful effects provided in
748 vivo.
749

■ ASSOCIATED CONTENT

Supporting Information

Validation of Q-FC (H₂O:EtOH) and Q-ABTS (H₂O:EtOH)
752 methods; correlations between classical TAC, Q-TAC
753 (H₂O:EtOH) and Q-TAC methods. The Supporting Informa-
754 tion is available free of charge on the ACS Publications website
755 at DOI: 10.1021/acs.jafc.5b01644.
756

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767 Notes

768 The authors declare no competing financial interest.

769 ■ ABBREVIATIONS USED

770 ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid);
771 AUC, area under the curve; DPPH, 2,2-diphenyl-1-picrylhy-
772 drazyl; FC, Folin-Ciocalteu; FRAP, ferric reducing/antioxidant
773 power; FRSC, free radical scavenging capacity; GA, gallic acid;
774 HRSC, hydroxyl radical scavenging capacity; LPSC, lipid
775 peroxyl scavenging capacity; Q, QUENCHER; OC, oxidized
776 controls; ORAC, oxygen radical absorbance capacity; SC,
777 sample controls; Sd, product from wine pomace seeds; Sk,
778 product from wine pomace skins; SRSC, superoxide radical
779 scavenging capacity; T, Trolox; TAC, total antioxidant capacity;
780 W, product from whole wine pomace

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