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Assessment of Antioxidant and Scavenging Activities of Various Yogurts Using Different Sample Preparation Procedures

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Abstract: Antioxidant activities of various yogurts were assessed using different sample preparation procedures. Specifically, full-fat cow, goat and ewe yogurts as well as cow yogurts with different fat content (4%, 2% and 0%) were employed. Antioxidant activities were determined in two different water-soluble yogurt extracts and also in a total yogurt preparation using the "Clarifying Reagent" for dairy products. Full-fat ewe yogurt preparations exhibited higher antioxidant activities in FRAP and Folin assays as well as higher scavenging capacities against DPPH, hydroxyl and superoxide anion radicals than full-fat cow and goat yogurt preparations. Bradford, Lowry and Ellman's assays confirmed that the strong antioxidant potential of the ewe yogurt was associated with its high protein content. In addition, antioxidant activities appeared to be related with the fat content of cow yogurt. Particularly, in DPPH, FRAP and Folin assays, and also in assays for scavenging of DPPH, hydroxyl and superoxide anion radicals, it was demonstrated that the fat removal led to the increase of the antioxidant/scavenging activities of the cow yogurts due to the increase of their protein/peptide water-soluble content. Moreover, for the first time, results show that the "Clarifying Reagent" for dairy products can be used for the determination of antioxidant and radical scavenging activities of whole yogurt using the FRAP assay as well as the hydroxyl and superoxide anion radicals scavenging assays.

Keywords: yogurt; cow; ewe; goat; antioxidant activity; scavenging capacity

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1. Introduction

Yogurt is a dairy product made by lactic acid fermentation via the action of *Lactobacillus delbrueckii* ssp. *Bulgaricus* and *Streptococcus thermophilus* [1,2]. Cows' milk is most commonly used for the industrial production of yogurt, however, various other commercial yogurts are also generated using milk from goats and/or ewes. Notably, the yogurt produced from cows' milk is the most popular one and is widely consumed throughout the world. Nevertheless, over the past decades, there is a high demand for alternatives to cows' milk due to problems associated with allergenicity, gastrointestinal disorders and the desire for novel dairy products [3,4].

In this direction, various fermented goats' and ewes' milk products such as yogurt are highly produced in Greece and east Mediterranean countries [5,6]. Especially goats' milk has a higher digestibility and lower allergenic properties compared to cows' milk [4–7]. On the other hand, cow yogurt contains at least 3.25% milk fat, but it can also be low-fat (0.5–3% milk fat) or non-fat (less than 0.5% milk fat), which is more preferred due to health concerns.

Yogurt has gained a positive perception by consumers as a functional dairy product and its consumption is increasing worldwide. Particularly, it is considered a healthy food due to the high digestibility and bioavailability of its protein, energy and calcium. In addition, the microbial activities (e.g., proteolysis) that take place during the fermentation process result in the modification of the allergenic properties of milk and the release of a range of bioactive peptides [8,9].

The health-promoting properties of bioactive food components are largely associated with their ability to promote antioxidant and free radical-scavenging mechanisms in the human body. Reactive oxygen species (ROS), including the superoxide radical, hydroxyl radical, hydrogen peroxide, and the peroxyl radical, are known to cause oxidative damage not only to food systems but also to living systems. Hence, there is a great interest in food antioxidants as it is believed that they can protect the human body from the attack of free radicals and delay the progress of many chronic diseases [10,11].

Yogurt antioxidant activities are related to milk constituents and also to those produced during lactic acid fermentation. The total antioxidant capacity of the final product depends on both hydrophilic and lipophilic compounds. Previous data highlighted that the major contributor to the total antioxidant capacity of whole milk was the casein fractions, while albumin was mainly responsible for the total antioxidant capacity of whey protein. In the case of deproteinized milk, other hydrophilic antioxidant compounds, such as vitamin C and uric acid, were the primary source of its total antioxidant capacity. In addition, some lipophilic antioxidants, such as vitamin E and carotenoids, acted as radical scavengers in the lipid phase of the above products [12].

Peptides are the main antioxidants present in yogurt. The latter bioactive compounds are generated during milk fermentation through the action of the lactic acid bacteria starter cultures [11,13,14]. Specifically, many amino acids and small peptides with a molecular weight of less than 1000 Da are released throughout the process, resulting in the strong antioxidant activity of the yogurt [15].

The antioxidant capacity of yogurt has been evaluated using different photometric methods that assess the ferric reducing antioxidant power (FRAP) and the ROS scavenging activities (e.g., DPPH, hydroxyl radicals, superoxide radicals and hydrogen peroxide) of the product [11,15–19]. A significant step in these methodologies is the yogurt sample pre-treatment. Procedures commonly used are the direct application of ultracentrifugation (or centrifugation giving the water-soluble extract) as well as the application of a double pH adjustment at pH 4.0 and 7.0, each followed by centrifugation. In the latter case, an aqueous extract is obtained, and most proteins are removed [11,17,20].

Although the opaque nature of the dairy products renders their energy- and time-consuming pre-treatment necessary for photometric analysis, this bottleneck can be resolved by converting the original sample into a transparent form. To achieve this, the "Clarifying Reagent" for dairy products has been successfully used for the clarification of various milks, creams and cheeses. This commercial reagent is typically a mixture of sodium dodecyl sulfate, sodium hydroxide, Triton X-100 and *n*-butanone. The fact that "Clarifying Reagent" shows low absorbance values in a wide wavelength range, i.e., between 340 nm and 800 nm, provides a significant advantage to its use in a variety of photometric analyses to estimate the quality of dairy products [21,22]. Notably, in contrast with the other dairy products, there are no available data reporting the use of "Clarifying Reagent" in yogurt pre-treatment.

The aim of the present work was to evaluate the antioxidant and scavenging activities of cow, goat and ewe yogurts as well as those of cow yogurts with different fat content. In this effort, two pre-treatment protocols were examined to obtain water-soluble yogurt extracts, while a total yogurt preparation using the "Clarifying Reagent" for dairy products was also tested for the first time. The results obtained were compared in order to assess the effectiveness of "Clarifying Reagent" innovative use in yogurt analysis.

2. Materials and Methods

2.1. Yogurt Samples

Yogurt samples (triplicates) were obtained from various dairy factories located in Greece.

Specifically, full-fat cow yogurt samples were supplied from Dodoni (Ioannina), Mevgal (Thessaloniki) and Olympus (Larissa). Full-fat goat yogurts were provided by EVOL (Volos), Olympus (Larissa) and Inahos (Lamia).

Also, full-fat ewe yogurts were derived from Dodoni (Ioannina), EVOL (Volos) and Kri-Kri (Serres).The gross composition of all of the yogurt samples is given in Table 1.

Table 1. Gross composition of the different full-fat cow, goat and ewe yogurts.

Milk Source	Dairy Factory	Fat (%)	Proteins (%)	Sugars (%)
Cow	Dodoni	3.9	3.6	4.9
Cow	Mevgal	3.8	3.7	5.2
Cow	Olympus	3.9	3.5	5.5
Goat	EVOL	4.0	4.7	5.3
Goat	Olympus	4.0	3.8	4.5
Goat	Inahos	4.0	3.8	4.6
Ewe	Dodoni	6.0	5.3	5.0
Ewe	EVOL	6.0	5.5	5.1
Ewe	Kri-Kri	6.0	5.9	4.7

In addition, cow yogurts with different fat content (4%, 2% and 0%) were obtained from the dairy factories FAGE (Athens), Kri-Kri (Serres) and Neogal (Drama). Their gross composition is shown in Table 2.

Table 2. Gross composition of cow yogurts with different fat content.

Milk Source	Dairy Factory	Fat (%)	Proteins (%)	Sugars (%)
Cow	FAGE	4.0	4.5	6.5
Cow	FAGE	2.0	4.5	7.5
Cow	FAGE	0.0	5.0	7.5
Cow	Kri-Kri	3.9	3.8	4.6
Cow	Kri-Kri	2.0	4.6	5.2
Cow	Kri-Kri	0.1	4.8	5.6
Cow	Neogal	3.8	3.7	5.5
Cow	Neogal	2.0	3.9	5.5
Cow	Neogal	0.0	4.0	5.5

2.2. Reagents

Folin-Ciocalteu's phenol reagent 2N, gallic acid, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) anhydrous, L-glutathione reduced 98%, ferric (III) chloride 97%, albumin from bovine serum (BSA) 96% electrophoresis, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol absolute analytical reagent 99.8% and 1,2,3-trihydroxybenzene (pyrogallol) were purchased from Sigma-Aldrich (Steinheim, Germany). Sodium carbonate anhydrous 99.9%, iron (II) chloride tetrahydrate, 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) and hydrogen peroxide 30% were supplied by Merck (Darmstadt, Germany). Tris(hydroxymethyl)aminomethane 99.5% was from Ferak (Berlin, Germany), while "Clarifying Reagent" for dairy products was from Fluka (Buchs, Switzerland). Copper (II) sulfate pentahydrate puriss, hydrochloric acid min 37% and di-potassium hydrogen phosphate were obtained from Riedel-de Haën (Seelze, Germany). Bradford—Solution for protein determination was from AppliChem GmbH (Darmstadt, Germany), while potassium sodium tartrate (crystals) tetrahydrate was from Mallinckrodt Baker (Phillipsburg, NJ, USA). Ethylenediaminetetraacetic aciddisodium salt (EDTA-Na_2) was purchased from Serva (Heidelberg, Germany).

2.3. Apparatus

The devices used to perform the experiments were a Hettich Zentrifugen Mikro 22R centrifuge (Tuttlingen, Germany), a L7-65 Beckman ultracentrifuge (Indianapolis, IN), a National Labnet Company Microcentrifuge (Iselin, NJ, USA), a Jenway 6505 UV-Vis Spectrophotometer (Vernon Hills, IL, USA), a Raypa Ultrasonic Cleaner (Barcelona, Spain), a Kern ABS balance (Lohmar, Germany) and a Consort C831 Multi-Parameter Analyser pH-meter (Turnhout, Belgium).

2.4. Antioxidant Activities in Yogurt Extracts Obtained by pH Adjustment and Precipitation (pHAP)

2.4.1. Yogurt Extract Preparation

Yogurt sample (10 g) was mixed with 2.5 mL d. water and the pH of the mixture was adjusted to 4.0 using 0.1 M HCl. The mixture was then incubated at 45 °C for 10 min followed by centrifugation (5000× *g*, 10 min, 4 °C). The supernatant was collected, and its pH was adjusted to 7.0 using 1 M NaOH. The neutralized supernatant was recentrifuged (5000× *g*, 10 min, 4 °C) [18,20].

The obtained supernatant was filtered using a 0.45 µm membrane filter D = 33 mm (Millex, Merck Millipore, Burlington, MA, USA) and analyzed according to the methods of Folin–Ciocalteu (Section 2.4.2), DPPH (Section 2.4.4.), hydroxyl radical scavenging activity (Section 2.4.5), superoxide anion scavenging activity (Section 2.4.6), Lowry (Section 2.4.7), Bradford (Section 2.4.8) and Ellman (Section 2.4.9). Prior to FRAP analysis (Section 2.4.3), the above neutralized supernatant was re-adjusted to pH 4.0 using 1 M HCl, recentrifuged (5000× *g*, 10 min, 4 °C) and filtered using a 0.45 µm membrane filter. All supernatants were kept refrigerated and analyzed within 6 h.

2.4.2. Folin–Ciocalteu Assay

In a test tube, 1.9 mL of d. water pH 7.0 were mixed with 100 µL of yogurt extract, followed by the addition of 125 µL of Folin–Ciocalteu reagent and vortexing. After 1 min, 380 µL of 20% sodium carbonate were added and the mixture was vortexed. After incubation in the dark at room temperature for 2 h, the absorbance of the mixture was measured at 750 nm against blank (containing 100 µL of d. water pH 7.0 instead of the yogurt extract). The results were expressed as mg gallic acid or Trolox equivalents per 1 kg of yogurt under the assay conditions.

2.4.3. FRAP Assay

In an Eppendorf tube, 950 µL of yogurt extract were mixed with 50 µL of 30 mM $FeCl_3$ in 0.05 M HCl, and the mixture was incubated in a water bath at 37 °C for 30 min. Then, 200 µL of 9 mM TPTZ in 0.05 M HCl were added. After 10 min, the absorbance of the mixture was measured at 620 nm against blank (containing 50 µL HCl 0.05 M instead of the solution of $FeCl_3$). The results were expressed as mg gallic acid or Trolox equivalents per 1 kg of yogurt under the assay conditions.

2.4.4. DPPH Assay

In a test tube, 1.4 mL of yogurt extract (undiluted as well as diluted at a ratio of 1/1 and 1/4) were mixed with 0.1 mL of 1.25 mM DPPH in ethanol, and the absorbance of the mixture was obtained at 515 nm at various time intervals. Control was made using 1.4 mL of d. water pH 7.0 instead of the sample. For each sample or control, a blank was prepared using 0.1 mL ethanol instead of the DPPH solution.

EC_{50} values of the yogurt extracts were determined at t = 20 min; the amount of sample required to decrease the initial concentration of DPPH by 50%. The exact DPPH concentration was assessed by recording the absorbance at 515 nm of various DPPH solutions.The EC_{50} value of gallic acid at t = 20 min was determined using gallic acid solutions in d. water pH 7.0 instead of the sample (EC_{50} = 308.12 mg/L). The results were expressed as EC_{50} values, g of yogurt per L of reaction mixture.

2.4.5. Hydroxyl Radical Scavenging Assay

In a test tube, 1.5 mL of 2 mM 1,10 phenanthroline, 2 mL of 0.05 M Tris-HCl buffer pH 7.4, 1 mL of 1.5 mM $FeCl_24H_2O$, 1.0 mL of 0.06 M H_2O_2 and 1.5 mL of yogurt extract (sample) were mixed, followed by incubation in a water bath at 37 °C for 1 h. Then, the absorbance of the mixture was measured at 536 nm. For each sample a blank was prepared using 1 mL ofd. water instead of the H_2O_2 solution. The same procedure was followed for the preparation of control except that 4.5 mL of d. water pH 7.0 were used

instead of the sample. The % scavenging activity was estimated according to the formula $[(A_{sample} - A_{control})/(A_{blank} - A_{control})] \times 100\%$ [23]. The results were expressed as mg of Trolox equivalents per 1 kg of yogurt under the assay conditions.

2.4.6. Superoxide Anion Scavenging Assay

In a test tube, 1 mL of 37.5 mM Tris-HCl buffer pH 8.2, 1 mL of 0.75 mM EDTA, 1 mL of 0.3 mM pyrogallol and 0.5 mL of yogurt extract (sample) were mixed. After incubation for 10 min at 25 °C, the absorbance of the mixture was measured at 325 nm. The same procedure was followed for the preparation of control except that 0.5 mL of d. water pH 7.0 were used instead of the sample. The % scavenging activity was estimated according to the formula $[(A_{control} - A_{sample})/A_{control}] \times 100\%$ [24]. The results were expressed as mg of Trolox equivalents per 1 kg of yogurt under the assay conditions.

2.4.7. Lowry Protein Assay

Prior to the analysis, a fresh Lowry solution was prepared by mixing 50 parts of a 2% solution of sodium carbonate with 1 part of a 0.5% solution of $CuSO_45H_2O$ in 1% KNa-tartrate$2H_2O$.Then, 0.25 mL of a 5-fold diluted yogurt extract were mixed with 1.25 mL of the Lowry solution in a test tube. The mixture was kept in the dark at room temperature for 10 min and 0.25 mL of diluted (1 M) Folin–Ciocalteu reagent were added. Then, the mixture was left again in the dark at room temperature for 30 min and afterwards its absorbance was measured at 750 nm against blank (containing 0.25 mL d. water instead of the yogurt extract). The results were expressed as mg of BSA equivalents per 1 kg of yogurt under the assay conditions.

2.4.8. Bradford Protein Assay

In a test tube, 0.5 mL of yogurt extract were mixed with 1.5 mL of Bradford reagent (commercial reagent 4-fold diluted with d. water). After incubation at room temperature for 5 min, the absorbance of the mixture was measured at 595 nm against blank (containing 0.5 mL d. water instead of the yogurt extract). The results were expressed as mg of BSA equivalents per 1 kg of yogurt under the assay conditions.

2.4.9. Ellman's Assay (Total Sulfhydryl Groups)

In a test tube, 0.35 mL of yogurt extract were mixed with 0.65 mL of 0.5 M Tris-HCl buffer pH 7.4, followed by the addition of 0.1 mL of 1 mM DTNB solution in 0.5 M Tris-HCl buffer pH 7.4. After incubation at 20 °C for 1 h, the absorbance of the mixture was measured at 412 nm against blank (containing 0.1 mL of 0.5 M Tris-HCl buffer pH 7.4 instead of the DTNB solution). The results were expressed as mg of BSA equivalents per 1 kg of yogurt under the assay conditions.

2.5. Antioxidant Activities in Yogurt Extracts Obtained by Ultra-Centrifugation (UC)

2.5.1. Yogurt Extract Preparation

Yogurt samples were centrifuged at 30,000× *g* at 4 °C for 30 min. The supernatant was filtered through a 0.45-µm membrane filter and analyzed within 6 h [11].

2.5.2. Folin-Ciocalteu Assay

The procedure described in Section 2.4.2 was followed except that the pH value of the d. water was 4.0 instead of 7.0. The results were expressed as mg gallic acid or Trolox equivalents per 1 kg of yogurt under the assay conditions.

2.5.3. FRAP Assay

The procedure described in Section 2.4.3 was followed. The results were expressed as mg gallic acid or Trolox equivalents per 1 kg of yogurt under the assay conditions.

2.6. Antioxidant Activities in Yogurt Preparation Obtained by the Use of "Clarifying Reagent" (CR)

2.6.1. Yogurt Preparation in "Clarifying Reagent"

In an Eppendorf tube, 0.1 g of yogurt were mixed well with 0.1 mL of d. water. Then, 1.1 mL of "Clarifying Reagent" were added, followed by strong vortexing and sonication for 10 min at 37 °C. Next, centrifugation at 7000× *g* for 10 min was applied and the supernatant (1 mL) was collected and analyzed according to the methods of FRAP (Section 2.6.2), hydroxyl radical scavenging activity (Section 2.6.3) and superoxide anion scavenging activity (Section 2.6.4).

2.6.2. FRAP Assay

In an Eppendorf tube, 100 μL of yogurt preparation were mixed with 100 μL of 3 mM $FeCl_3$ in 0.05 M HCl and the mixture was heated in a water bath at 37 °C for 30 min. Then, 1 mL of 1.8 mM TPTZ in 0.05 M HCl were added. After 10 min, the absorbance of the mixture was measured at 620 nm against blank (containing 100 μL of 0.05 M HCl instead of the solution of $FeCl_3$). The results were expressed as mg gallic acid or Trolox equivalents per 1 kg of yogurt under the assay conditions.

2.6.3. Hydroxyl Radical Scavenging Assay

The procedure described in Section 2.4.5 was followed except that the control was prepared by using 4.5 mL of a mixture of "Clarifying Reagent" and d. water pH 4.0 at the ratio of 1.1/0.2 instead of the sample. The % scavenging activity was estimated using the formula $[(A_{sample} - A_{control})/A_{blank} - A_{control})] \times 100\%$. The results were expressed as mg of Trolox equivalents per 1 kg of yogurt under the assay conditions.

2.6.4. Superoxide Anion Scavenging Assay

In a test tube, 1 mL of 37.5 mM Tris-HCl buffer pH 8.2, 1 mL of 0.75 mM EDTA, 1 mL 0.3 mM pyrogallol, 0.25 mL of d. water and 0.25 mL of yogurt extract (sample) were mixed. After incubation for 10 min at 25 °C, the absorbance of the mixure was measured at 325 nm. The same procedure was followed for the preparation of control except that 0.25 mL of "Clarifying Reagent" and d. water pH 4.0 at the ratio of 1.1/0.2 were used instead of the sample. The % scavenging activity was estimated according to the formula $[(A_{control} - A_{sample})/A_{control}] \times 100\%$. The results were expressed as mg of Trolox equivalents per 1 kg of yogurt under the assay conditions.

2.7. Statistical Analysis

Each dataset was submitted to the general linear model procedure followed by descriptive statistics to test the normal distribution of the residuals, using SPSS v. 17.0 software (Chicago, IL, USA). Statistical comparisons of the mean values were carried out either by one-way ANOVA, followed by Duncan's test, or by Student's *t*-test. Results were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Antioxidant and Radical Scavenging Activities of Full-Fat Cow, Goat and Ewe Yogurts

Antioxidant activities and ROS scavenging activities of full-fat cow, goat and ewe yogurt were studied.

Antioxidant activities as determined by DPPH and Folin assays are presented in Table 3, while those determined by the FRAP assay are presented in Table 4.

Table 3. Antioxidant activities of cow, goat and ewe yogurt preparations obtained by pH adjustment and precipitation (pHAP) or ultra-centrifugation (UC) procedure, as determined by DPPH and Folin assays [1].

Yogurt	DPPH Assay, EC_{50} (g Yogurt/L Reaction Mixture)	Folin Assay (mg Gallic Acid/kg Yogurt)		Folin Assay (mg Trolox/kg Yogurt)	
	pHAP Extract	pHAP Extract	UC Extract	pHAP Extract	UC Extract
Cow	$1151^{\ a} \pm 21$	$68.5^{\ aA} \pm 2.4$	$88.2^{\ aB} \pm 4.5$	$60.3^{\ aA} \pm 2.1$	$77.5^{\ aB} \pm 4.0$
Goat	$1169^{\ a} \pm 18$	$66.4^{\ aA} \pm 1.3$	$83.5^{\ aB} \pm 2.2$	$58.5^{\ aA} \pm 1.1$	$73.4^{\ aB} \pm 1.9$
Ewe	$1071^{\ b} \pm 7$	$76.2^{\ bA} \pm 1.8$	$100.3^{\ bB} \pm 2.2$	$67.1^{\ bA} \pm 1.6$	$88.1^{\ bB} \pm 1.9$

[1] Each value was expressed as mean ± standard deviation of three replicates. Different lowercase letters in the same column represent significant differences in values ($p < 0.05$) between cow, goat and ewe yogurts. Different capital letters in the same row represent significant differences in values ($p < 0.05$) between pHAP and UC preparations of the same yogurt.

Table 4. Antioxidant activities of cow, goat and ewe yogurt preparations obtained by pH adjustment and precipitation (pHAP), ultra-centrifugation (UC), and "Clarifying Reagent" (CR), as determined by FRAP assay [1].

Yogurt	FRAP Assay (mg/L Trolox/kg Yogurt)			FRAP Assay (mg/L Gallic Acid/kg Yogurt)	
	pHAP Extract	UC Extract	CR Preparation	pHAP Extract	UC Extract
Cow	$38.6^{\ aA} \pm 2.3$	$52.9^{\ aB} \pm 1.9$	$150.8^{\ aC} \pm 6.0$	$45.3^{\ aA} \pm 2.7$	$62.0^{\ aB} \pm 2.3$
Goat	$36.9^{\ aA} \pm 1.5$	$51.3^{\ aB} \pm 1.2$	$147.0^{\ aC} \pm 5.0$	$43.3^{\ aA} \pm 1.8$	$60.2^{\ aB} \pm 1.5$
Ewe	$53.7^{\ bA} \pm 1.0$	$66.7^{\ bB} \pm 0.9$	$200.0^{\ bC} \pm 3.7$	$63.0^{\ bA} \pm 1.2$	$78.1^{\ bB} \pm 1.1$

[1] Each value was expressed as mean ± standard deviation of three replicates. Different lowercase letters in the same column represent significant differences in values ($p < 0.05$) between cow, goat and ewe yogurts. Different capital letters in the same row represent significant differences in values ($p < 0.05$) between pHAP, UC and CR preparations of the same yogurt.

According to Table 3, EC_{50} values of pH adjustment and precipitation (pHAP) yogurt extracts followed the order ewe > cow = goat, indicating that ewe yogurt extracts were significantly more active in DPPH scavenging than those of cow and goat yogurts. In the case of standards, gallic acid appeared to be more active than Trolox, exhibiting an EC_{50} value of 0.272 g/L versus 0.293 g/L. Notably, although the yogurt extracts demonstrated a detectable DPPH scavenging activity, their EC_{50} values were 3000-fold higher than those of the standards, i.e., a higher amount of yogurt was required to decrease the initial concentration of DPPH by 50% as compared to the standards. This is logical as the latter correspond to pure antioxidant compounds, while the sample preparations contained only the water-extractable antioxidants that were present in the yogurt.

Antioxidant activities of both pHAP and ultra-centrifugation (UC) yogurt extracts as determined by Folin assay (Table 3) and FRAP assay (Table 4) followed the order ewe > cow = goat, in accordance with the results obtained by the DPPH method. Considering that pHAP and UC yogurt extracts are aqueous systems, it can be deduced that the water-soluble constituents of ewe yogurt (mainly the proteins) have higher antioxidant activities than cow and goat yogurt. Focusing on Folin and FRAP assays, the UC extracts of all the examined yogurts exhibited significantly higher values than the respective ones of the pHAP extracts.This finding is attributable to the fact that antioxidants can be lost or degraded during the various pre-treatment stages for pHAP extract preparation (pH adjustments, thermal incubation and precipitation), while in the case of the UC extracts the water-soluble phase is obtained by applying only centrifugation. In any case, ewe yogurt pHAP and WS extracts exhibited higher antioxidant and scavenging of ROS activities than the respective cow and goat extracts.

Cow, goat and ewe yogurt preparations obtained by the use of "Clarifying Reagent" (CR) were also tested using the FRAP assay (Table 4). The same pattern was followed here

as in the other assays. Specifically, ewe yogurt preparation appeared to be the most active one, while those of cow and goat yogurts exhibited similar activities.

This was also confirmed by the determination of scavenging capacities of cow, goat and ewe yogurt against hydroxyl radicals and superoxide anion, (Table 5). Both ewe yogurt preparations obtained by pHAP and CR exhibited the highest scavenging activity against the ROS. On the other hand, the radical scavenging activities of cow and goat preparations were similar.

Table 5. Hydroxyl and superoxide anion radical scavenging activities of cow, goat and ewe yogurt preparations obtained by pH adjustment and precipitation (pHAP) and "Clarifying Reagent" (CR) [1].

Yogurt	Hydroxyl Radicals (mg Trolox/kg Yogurt)		Superoxide Radicals (mg Trolox/kg Yogurt)	
	pHAP Extract	CR Preparation	pHAP Extract	CR Preparation
Cow	$35.5^{aA} \pm 2.7$	$102.6^{aA} \pm 8.0$	$142.6^{aA} \pm 6.9$	$255.1^{aB} \pm 35.2$
Goat	$33.2^{aA} \pm 1.5$	$96.1^{aA} \pm 4.6$	$137.8^{aA} \pm 5.2$	$215.4^{aB} \pm 15.6$
Ewe	$50.2^{bA} \pm 1.8$	$133.1^{bA} \pm 7.3$	$171.5^{bA} \pm 2.0$	$377.7^{bB} \pm 17.4$

[1] Each value was expressed as mean ± standard deviation of three replicates. Different lowercase letters in the same column represent significant differences in values ($p < 0.05$) between cow, goat and ewe yogurts. Different capital letters in the same row represent significant differences in values ($p < 0.05$) between pHAP and CR preparations of the same yogurt.

It is remarkable to note that each CR yogurt preparation exhibited much higher antioxidant and scavenging of ROS activities than the respective pHAP and UC yogurt extracts (Tables 4 and 5). "Clarifying Reagent" dissolves both proteins and fat [21,22], indicating that these components are potential contributors to the antioxidant and scavenging capacities of yogurt. All the above are consistent with the product composition as ewe yogurt contained higher levels of both protein and fat than cow and goat yogurts (Table 1).

To assess the relation of protein content on the antioxidant potential of the yogurts, cow, goat and ewe pHAP yogurt extracts were analyzed for their protein and total sulfhydryl groups concentration (Table 6). As expected, the protein content of the three extracts examined followed the order ewe > cow = goat in both Lowry and Bradford assays. In addition, the total sulfhydryl groups content followed the same order.

Table 6. Protein and total sulfhydryl groups content of cow, goat and ewe yogurt preparations obtained by pH adjustment and precipitation (pHAP) procedure [1].

Yogurt	Lowry Assay (mg BSA/kg Yogurt)	Bradford Assay (mg BSA/kg Yogurt)	Total Sulfhydryl Groups (mg Glutathione/kg Yogurt)
Cow	$1237^{a} \pm 22$	$2027^{a} \pm 41$	$4.0^{a} \pm 0.3$
Goat	$1214^{a} \pm 17$	$1986^{a} \pm 59$	$3.8^{a} \pm 0.3$
Ewe	$1307^{b} \pm 12$	$2247^{b} \pm 45$	$5.2^{b} \pm 0.2$

[1] Each value was expressed as mean ± standard deviation of three replicates. Different lowercase letters in the same column represent significant differences in values ($p < 0.05$) between cow, goat and ewe yogurts.

During the preparation of yogurt pHAP extract (pH adjustment at 4.0 and centrifugation followed by pH adjustment at pH 7.0 and centrifugation) a large amount of proteins are precipitated and removed. Consequently, the current results indicate that ewe yogurt pHAP extract contains more soluble peptides/proteins and peptides/proteins containing sulfhydryl groups than pHAP cow and goat yogurt extracts.

Protein and total sulfhydryl groups content of the three yogurt pHAP extracts are consistent with their antioxidant and scavenging capacities reported above as it is known that yogurt peptides exhibit antioxidant activities while the –SH group can be oxidized to –S-S-, which also contributes to the antioxidant potential of the product.

Aloğlu & Öner (2011) came to a similar conclusion, by observing that the antioxidant potential of the water-soluble extracts of traditional and commercial yogurts was proportional to their bioactive peptide content [11]. In a study of Citta et al. (2017), the increase of

the antioxidant and scavenging of ROS activities of yogurts during long-term storage was associated with the release of bioactive peptides through protein hydrolysis. The authors characterized the peptides as "sacrificial" antioxidants due to their ability to act quickly against the ROS [25]. Apart from peptides/proteins, the antioxidant activity of yogurts has been also related to the presence of lipids (e.g., linoleic acid), which seem to contribute to the anti-thrombotic and cholesterol-lowering properties of the product [26,27]. The latter strengthens our findings as significantly higher antioxidant activities were recorded via the use of CR (dissolves both proteins and fats) than the water-soluble extracts obtained by pHAP and UC procedures.

3.2. Antioxidant and Radical Scavenging Activities of Cow Yogurts with Different Fat Content

The antioxidant activities and scavenging of ROS capacities of cow yogurt with 4%, 2% or 0% fat were also assessed.

Specifically, the antioxidant activities as determined by DPPH and Folin assays are presented in Table 7 and as determined by the FRAP assay in Table 8.EC_{50} values of yogurt pHAP extracts followed the order 4% > 2% > 0% indicating that, in DPPH scavenging, non-fat yogurt was the most active followed by semi-fat and full-fat yogurt. As in the case of different full-fat cow, goat and ewe yogurts (Section 3.1), the EC_{50} values of cow yogurts with different fat content were 3000-fold higher than those of gallic acid and Trolox standards attributable to the fact that the latter two compounds were pure antioxidants.

Table 7. Antioxidant activities of cow yogurts with different fat content preparation obtained by pH adjustment and precipitation (pHAP) or ultra-centrifugation procedure, as determined by DPPH and Folin assays [1].

% Fat of Yogurt	DPPH Assay EC_{50} (g Yogurt/L Reaction Mixture)	Folin Assay (mg Gallic Acid/kg Yogurt)		Folin Assay (mg Trolox/kg Yogurt)	
	pHAP Extract	pHAP Extract	UC Extract	pHAP Extract	UC Extract
0	$1106^{a} \pm 11$	$86.8^{aA} \pm 2.8$	$93.2^{aB} \pm 1.9$	$76.5^{aA} \pm 2.4$	$81.8^{aB} \pm 1.7$
2	$1155^{b} \pm 11$	$73.7^{bA} \pm 2.6$	$84.3^{bB} \pm 2.4$	$65.0^{bA} \pm 2.3$	$74.1^{bB} \pm 2.1$
4	$1207^{c} \pm 10$	$55.1^{cA} \pm 2.9$	$67.9^{cB} \pm 2.5$	$48.5^{cA} \pm 2.6$	$59.6^{cB} \pm 2.2$

[1] Each value was expressed as mean ± standard deviation of three replicates. Different lowercase letters in the same column represent significant differences in values ($p < 0.05$) between cow yogurts with different fat content. Different capital letters in the same row represent significant differences in values ($p < 0.05$) between pHAP and UC preparations of the same yogurt.

Table 8. Antioxidant activities of cow yogurts with different fat content preparations obtained by pH adjustment and precipitation (pHaP), ultra-centrifugation (UC), and "Clarifying Reagent" (CR), as determined by FRAP assay [1].

% Fat of Yogurt	FRAP Assay (mg/L Trolox per kg Yogurt)			FRAP Assay (mg/L Gallic Acid per kg Yogurt)	
	pHAP Extract	UC Extract	CR Preparation	pHAP Extract	UC Extract
0	$41.0^{aA} \pm 1.1$	$57.0^{aB} \pm 1.3$	$172.8^{aC} \pm 2.9$	$48.1^{aA} \pm 1.3$	$66.9^{aB} \pm 1.6$
2	$36.4^{bA} \pm 1.3$	$51.0^{bB} \pm 1.4$	$152.1^{bC} \pm 3.0$	$42.7^{bA} \pm 1.5$	$59.8^{bB} \pm 1.6$
4	$32.2^{cA} \pm 1.2$	$45.6^{cB} \pm 1.6$	$123.8^{cC} \pm 5.7$	$37.8^{cA} \pm 1.4$	$53.5^{cB} \pm 1.9$

[1] Each value was expressed as mean ± standard deviation of three replicates. Different lowercase letters in the same column represent significant differences in values ($p < 0.05$) between cow yogurts with different fat content. Different capital letters in the same row represent significant differences in values ($p < 0.05$) between pHAP, UC and CR preparations of the same yogurt.

Antioxidant activities of both pHAP and UC extracts as determined by Folin assay and FRAP assay followed the order 0% > 2% > 4%. In both assays, the UC extracts of all the examined yogurts exhibited higher antioxidant activity values than the respective ones in pHAP extracts. This is related to the simplicity of UC procedure (just one centrifugation) against the complex pre-treatment steps of pHAP process (pH adjustment, thermal

incubation and precipitation) that potential lead to antioxidants degradation or loss.CR preparations of cow yogurts with different fat content were also tested using the FRAP assay (Table 8). Again, yogurt with 0% fat appeared to be the most active one, followed by yogurt with 2% fat and that with 4% fat.

Scavenging capacities of cow yogurt with different fat content against hydroxyl radicals and superoxide anion are presented in Table 9. In these assays, pHAP extracts and CR preparations of the yogurts were used. Scavenging capacities of both pHAP and CR yogurt preparations against ROS followed the order 0% > 2% > 4% fat content. Cow yogurt with less fat content in pHAP and UC extracts exhibited higher antioxidant and scavenging of ROS activities than cow yogurt with higher fat content. To sum up, all the antioxidant assays tested indicate that the water-soluble constituents of cow yogurt with less fat content have higher antioxidant and scavenging of ROS activities than cow yogurt with higher fat content. A similar pattern was followed in CR preparations, as the cow yogurt with 0% fat content showed the highest antioxidant and scavenging of ROS potential than the other cow yogurts. This finding indicates that proteinaceous and water-soluble constituents of cow yogurt demonstrate higher antioxidant potential than the lipids.

Table 9. Hydroxyl and superoxide anion radical scavenging activities of cow yogurts with different fat content preparations obtained by pH adjustment and precipitation (pHAP) and "Clarifying Reagent" (CR) [1].

% Fat of Yogurt	Hydroxyl Radicals (mg Trolox/kg Yogurt)		Superoxide Radicals (mg Trolox/kg Yogurt)	
	pHAP Extract	CR Preparation	pHAP Extract	CR Preparation
0	$46.2^{aA} \pm 1.1$	$118.5^{aB} \pm 3.1$	$154.3^{aA} \pm 2.2$	$323.3^{aB} \pm 15.3$
2	$36.7^{bA} \pm 0.8$	$98.9^{bB} \pm 2.2$	$137.8^{bA} \pm 1.7$	$244.1^{bB} \pm 15.2$
4	$27.5^{cA} \pm 0.9$	$76.3^{cB} \pm 4.2$	$120.6^{cA} \pm 1.8$	$174.9^{cB} \pm 15.2$

[1] Each value was expressed as mean ± standard deviation of three replicates. Different lowercase letters in the same column represent significant differences in values ($p < 0.05$) between cow yogurts with different fat content. Different capital letters in the same row represent significant differences in values ($p < 0.05$) between pHAP and CR preparations of the same yogurt.

From another point of view, it was confirmed again (as in Section 3.1) the considerably stronger antioxidant and ROS scavenging activities observed in the CR yogurt preparations as compared with the pHAP and UC extracts. This is attributed to the aqueous nature of the extracts, which do not contain any lipids and have a low protein content. On the other hand, "Clarifying Reagent" dissolves both proteins and fat, indicating that the detected activities might be related to the significant presence of these compound categories. Considering also that the yogurt with less fat content contains higher protein and water-soluble constituents than the respective ones with higher fat content (fat removal leads to the increase of protein and soluble constituents), the yogurt antioxidant activities are mainly attributed to their peptide/protein content.

To confirm the relation of proteins to the antioxidant activities, the pHAP extracts of the cow yogurts with different fat content were analyzed for their protein and total sulfhydryl groups concentration (Table 10). As indicated by both Lowry and Bradford assays, protein content of the three extracts followed the order 0% > 2% > 4% fat. The same order was observed for total sulfhydryl groups. content followed the same order.

During the preparation of yogurt pHAP extract a large amount of proteins are precipitated and removed. Consequently, present results indicate that yogurt pHAP extracts contain more soluble peptides/proteins and also peptides/proteins containing sulfhydryl groups in the order 0% > 2% > 4%.

Table 10. Protein and total sulfhydryl groups content of cow yogurts with different fat content preparations obtained by pH adjustment and precipitation (pHAP) procedure [1].

% Fat of Yogurt	Lowry Assay (mg BSA/kg Yogurt)	Bradford Assay (mg BSA/kg Yogurt)	Total Sulfhydryl Group (mg Glutathione/kg Yogurt)
0	1355 [a] ± 20	2142 [a] ± 41	3.8 [a] ± 0.1
2	1225 [b] ± 19	2005 [b] ± 38	3.3 [b] ± 0.1
4	1134 [c] ± 16	1827 [c] ± 45	2.8 [c] ± 0.2

[1] Each value was expressed as mean ± standard deviation of three replicates. Different lowercase letters in the same column represent significant differences in values ($p < 0.05$) between cow yogurts with different fat content.

Protein and total sulfhydryl groupscontent of three yogurt pHAP extracts are consistent with their antioxidant and scavenging capacities reported above. This is logical as it is known that yogurt peptides exhibit antioxidant activities, while the –SH group can be oxidized to –S-S- exhibiting antioxidant activities.

Analytical results of full-fat cow, goat and ewe yogurts as well as of cow yogurts with different fat content led to some general observations. In both Folin and FRAP assays, UC extracts of all yogurts examined exhibited higher antioxidant activities than the respective pHAP extracts. This can be attributed to materials that are precipitated and removed during the double pH adjustment (pH 4.0 and pH 7.0) applied in the procedure, such as proteinaceous materials [17,20]. Moreover, CR preparations of all the yogurts exhibited much higher antioxidant scavenging capacities than the respective aqueous yogurt extracts. This is associated with the fact that "Clarifying Reagent" for dairy products dissolves both proteins and lipids, indicating that the latter may contribute to antioxidant and radical scavenging capacities of yogurt [21,22]. Finally, DPPH, Folin and FRAP antioxidant assays showed analogous results in the evaluation of all yogurt extracts examined. It is logical as these three assays are based on a single electron transfer mechanism [28].

4. Conclusions

Present results show for the first time that the "Clarifying Reagent" for dairy products can be successfully used for the determination of antioxidant and scavenging activities of whole yogurt using the FRAP assay as well as hydroxyl radicals and superoxide anion scavenging activity assays. Results also show that both water soluble extract and whole full-fat ewe yogurt exhibit higher antioxidant and scavenging activities than the respective full-fat cow and goat yogurt preparations. In addition, it was indicated that both water soluble extract and whole cow yogurt with less fat content exhibit higher antioxidant and scavenging activities than the respective cow yogurt with higher fat. The presence of proteins was found to be the most important factor for the high antioxidant and radical scavenging activities of the yogurts.

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