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Study of total phenolic composition and antioxidant activity of fresh cheese with red fruits

Fernando Jorge Gonçalves;goncalves7ster@gmail.com
CI&DETS Research Centre/Dep. Food Industry-ESAV, Polytechnic Institute of Viseu, Portugal
Fernando Jorge Gonçalves - goncalves7ster@gmail.com
CI&DETS
Soraia Leitão -
Agrarian School, Polytechnic Institute of Viseu, Portugal
Paula M. R. Correia -
CI&DETS Research Centre/Dep. Food Industry-ESAV, Polytechnic Institute of Viseu, Portugal
Raquel P. F. Guiné - raquelguine@esav.ipv.pt
CI&DETS Research Centre/Dep. Food Industry-ESAV, Polytechnic Institute of Viseu, Portugal

Abstract
The dietary guidelines worldwide recommend the intake of vegetables and fruits like berries, in order to provide their benefits. Moreover dairy products could be beneficial to human health, as source of bioactive compounds. Berry fruits are considered to be a good source of natural phenolic compounds which are known for their high antioxidant activity. The main objective of this work was to quantify the total phenolic compounds and antioxidant activity of new products based on cheese enriched with red fruits. For that, fresh cheeses enriched with red fruits (blueberry or raspberry) were produced. Extracts of methanol and ethanol:water were obtained in order to determine phenolic compounds and antioxidant activity. The results obtained showed that for all cheeses studied the amount of phenolic compounds quantified was higher in the methanolic extracts than in the ethanol:water extracts. However, with regards to the antioxidant activity, it was also higher for the extracts of methanol when the DPPH method was used but lower when the ABTS method was used. Comparing the fruits, the blueberries increased more the phenolic compounds and antioxidant activity when compared with raspberries. It was concluded that the addition of red fruits resulted in cheeses with higher levels of phenolic compounds and antioxidant activity improving their potential health benefits.

Keywords
Cheese; Red fruits; Phenolic compounds; Antioxidant Activity

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1. Introduction

Nowadays it is very common for people to take a greater care with their health, since human diet is considered to be the main factor contributing to a healthy lifestyle. The consumer's exigence has lead the food industry to create novel products, some of them based on traditional ones, by mannaging food ingredients or reducing potential harmful constituents. The dietary guidelines worldwide recommend the intake of vegetables and fruits like berries, in order to provide their health benefits [1]. Cheese has been one of the dairy products that have been boosted by the addition of various ingredients such as food coloring, spices, leaves, and flavoring agents.

All over the world cheese has been present in the human diet since a long time ago. Cheese is a dairy product that can be consumed fresh or maturated. Since then, many technological advances have been applied to its production, allowing obtaining new products. Despite the various differences that distinguish them, cheeses present some common technological aspects including a set of fundamental steps like coagulation (animal or vegetable curds), desorption, molding, pressing and salting. Cheese is mainly composed of proteins, fats, minerals and vitamins. The consumption of dairy products could be beneficial to human health, as milk proteins are an important source of bioactive peptides that possess a number of health benefits, namely antioxidant properties [2].

Berry fruits (e.g. blueberry and raspberry) are considered to be a good source of natural phenolic compounds. Phenolic compounds are secondary plant metabolites; their major role is to protect human organisms against oxidative stress induced by free radical species. A large number of studies on their physiological functions and chemical constituents have been reported [3] and related with their high antioxidant activity [4]. Many studies claim that the intake of berry fruits has a positive and profound impact on the Human health, performance and disease. Because of their remarkable antioxidant capacity, berries have received increasing attention in the last decades. The content of phenolics, micronutrients, phytochemicals and antioxidant capacity in berries is affected by genetic differences, pre-harvest environmental conditions and the degree of maturity at harvest [5] but also by differences in growing locations, processing and storage conditions [6]. Blueberries are flowering plants of the genus *Vaccinium* with dark-purple berries, whose anthocyanins are considered to be nature's most potent antioxidants and have demonstrated properties that extend well beyond suppressing free radicals [7]. Red raspberry (*Rubus idaeus* L.) is a berry crop that contains numerous phenolic compounds, namely flavonoids, ellagic acids and also vitamine C.

The main objective of this work was to quantify the total phenolic compounds and antioxidant activity of new products based on cheese enriched with red fruits.

2. Experimental Procedure

2.1. Cheese preparation

For the preparation of the fresh cheeses the usual processing steps were followed, and they were prepared in a cheese factory following the same workflow. 10 to 12 L of cow's milk, 5 g of salt and 10 g of fruit, in case of cheese with berries (blueberry, raspberry or a mixture of both), were used.
Five different types of cheese were produced in duplicates:

Cheese produced without addition of fruits as control Cheese (CC);
Cheese including fresh raspberry (FRC);
Cheese including a mixture of fresh raspberry and blueberry (5g of each fruit) (FRBC)
Cheese including fresh blueberry (FBC);
Cheese including frozen blueberry (IBC)

Figure 1 shows, as example, a cheese containing blueberry and raspberry.

Figure 1. Cheese with whole raspberries and blueberries.

The cheeses were fresh, hence they did not go through any maturation process, and therefore they were transported to the laboratory for analysis right after production. The transportation step was done under refrigeration at controlled temperature (5ºC).

2.2. Extraction Conditions

In order to quantify the total phenolic compounds and antioxidant activity were obtained extracts using two different extraction solutions: methanol (M) and a mixture (50:50) of ethanol-water (EW) by adaptation of the methodology described by Guiné et al. [8]. For the extraction procedure, each cheese was homogenized and an aliquot of 10 g were taken. Two successive extractions during 60 min under magnetic stirring allowed to obtain a first (E1) and a second (E2) extracts for the different cheeses and extraction solutions.
For each set of experimental conditions used, the process was repeated three times.

2.3. Analysis of total phenolic compounds

The total phenolic compounds (TPC) were analyzed using the Folin-Ciocalteu method [9]. For that, 0.125 mL of each extract were added to 0.75 mL of deionized water and 0.125 mL of the Folin-Ciocalteu reagent. The solution was left to stand for 6 min, and then 2 mL of a 5 % (m/v) solution of Na_2CO_3 were added and the mixture was left to rest in the dark for 90 min at room temperature. For the calibration, standard solutions of gallic acid were prepared and the absorbance was measured in a spectrophotometer at 760 nm. The results were expressed as milligrams of gallic acid equivalents (GAE) per gram of fresh sample, being calculated as a mean of three measurements.

2.4. Analysis of antioxidant activity

The antioxidant activity (AOA) was determined by the $ABTS^{+}$ (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) and DPPH (2,2-Diphenyil-picrylhydrazyl) methods.
For ABTS, 1 mL of $ABTS^{+}$ solution was diluted in 80 mL of ethanol. The initial absorbance was close to 0.700 in all cases. In a tube was placed 2 mL of $ABTS^{+}$ solution with 0.1 mL of the sample extract and after agitation it was left to rest in the dark for 15 minutes [10]. Then the absorbance was measured at 734 nm.
For DPPH, a methanolic solution (6×10^{-5} mol/L) of the radical DPPH• was prepared daily and protected from light. Absorbance was recorded to check the stability of the radical throughout the time of analysis. The initial absorbance was close to 0.700 in all cases. Briefly, 0.1 mL of sample (properly diluted) were added to 2.0 mL of $DPPH^{\bullet}$ solution and stirred. The absorbance at 515 nm was recorded after 30 min of reaction in the dark [11].
For both methods, the percentage of inhibition was calculated according to the equation:

$$\% \text{ Inibhition} = (1-A_f/A_0)\times100 \qquad (1),$$

where A_0 is the value of absorbance of the blank at 0 min and A_f is the absorbance measured of the antioxidant samples at the end of reaction.
A calibration curve was prepared with the standard Trolox and used for quantification. The results were expressed as μmol Trolox equivalents (TE) per gram of fresh sample. The analyses for AOA were performed in triplicate for each of the extracts analysed.

3. Results and Discussion

3.1. Total phenolic compounds

The graph in Figure 1 presents the total phenolic compounds (TPC) quantified by the sum of 1st and 2nd extracts for the five cheeses under study, obtained with methanol (top) and with ethanol:water (bottom).

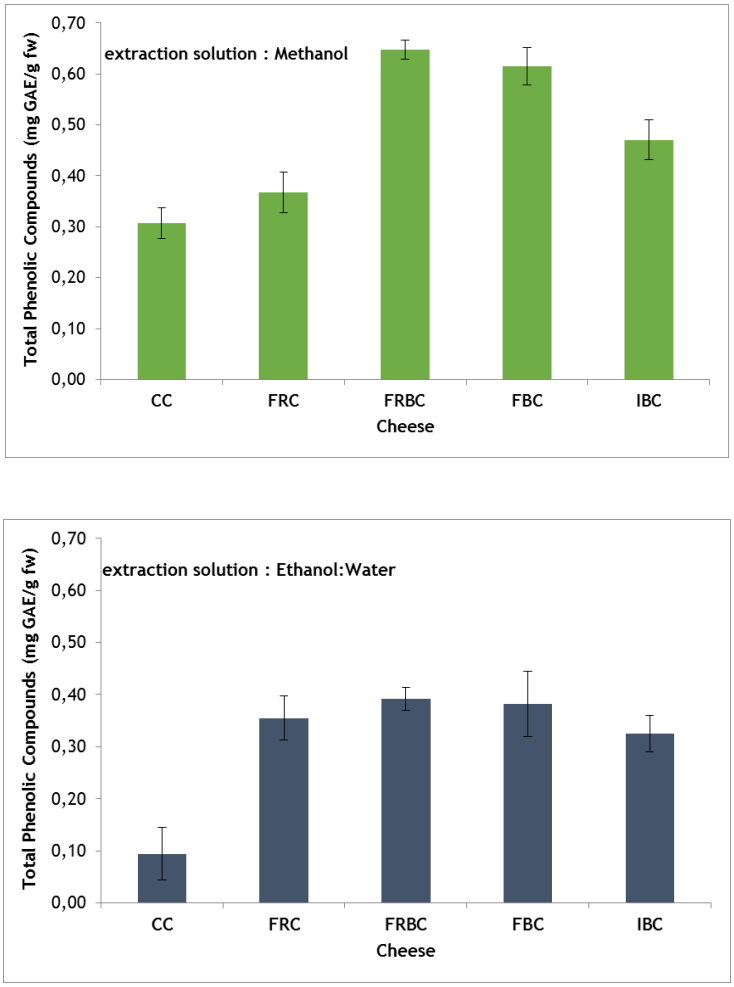

Figure 1 – Total phenolic compounds present in the cheeses (sum of the values obtained for the two extracts).

For methanolic extracts the amount of total phenolic compounds ranged from 0.31 mg GAE/g fw obtained for control cheese (CC) to 0.65 mg GAE/g fw obtained for cheese enriched with the mixture of fresh raspberry and blueberry (FRBC). All the cheeses produced with berry fruits presented higher levels of phenolic compounds comparing with the control cheese.
When the mixture ethanol:water was used to extract phenolic compounds, a similar tendency was observed. The cheese enriched with raspberry and blueberry accounted for 0.39 mg GAE/g fw, slightly higher than the cheese enriched with fresh blueberry (0.38 mg GAE/g fw) and the cheese enriched with fresh raspberry (0.35 mg GAE/g fw). The control cheese accounted only for 0.09 mg GAE/g fw of phenolics. The cheeses containing fruits had approximately four times more phenolic compounds than the control one.
Comparing the effect of the addition of fresh (FBC) and frozen (IBC) blueberry, for both extraction solvents was possible to observe that the use of fresh fruits lead to cheeses with a higher amount of phenolic compounds, around 20% more.

For all cheeses studied the amount of phenolic compounds quantified was higher in methanolic extracts than in the ethanol:water extracts, and in the control cheese that is particularly evident. These results show that methanol was a more efficient solvent when compared to ethanol:water to extract phenolic compounds from this type of cheese.

Figure 2 represents the percentage of recovery of the different extractions in relation to the overall yield. The 1st extraction (E1) ranged from 56% to 76% when methanol was used as solvent and from 53% to 82% in case of ethanol:water. It was not possible to observe any tendency according to the cheese composition or to the use of different extraction solutions. In general the 2nd extraction contributes with percentages higher than 30%, which may suggest that a 3rd extraction could still be performed, to try to extract a higher amount of compounds from the sample.

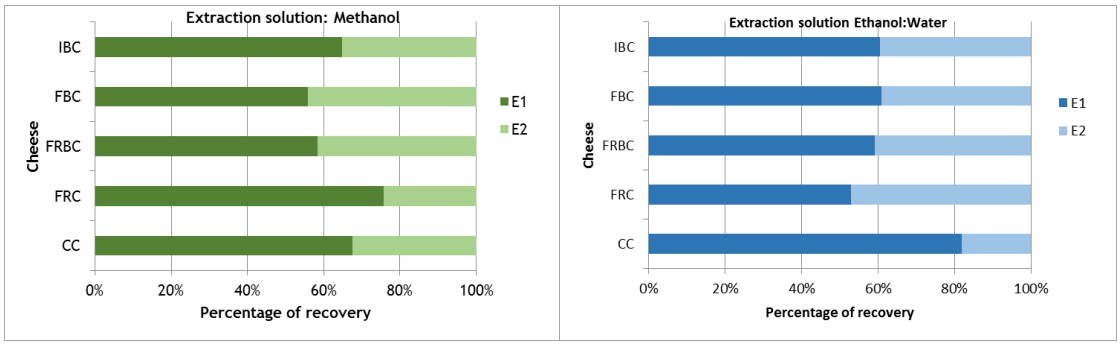

Figure 2 – Percentage of extracted total phenolic compounds (E1- 1st extract, E2- 2nd extract).

3.2. Antioxidant activity

The results in Figure 3 show the antioxidant activity obtained by ABTS method. When methanol was used as extraction solvent, the antioxidant activity for the sum of extracts varied from 1.0 µmol TE/g fw for the control cheese (CC) to 2.0 µmol TE/g fw for the fresh blueberry enriched cheese (FBC). The IBC and FRBC cheeses obtained similar values (1.7 µmol TE/g fw), whereas FRC cheese accounted for 1.4 µmol TE/g fw. In this case, the blueberries induced a higher increase in the antioxidant activity when compared with raspberries. Moreover, the addition of fresh blueberries was 17% more efficient when compared with the addition of frozen blueberries.
In opposition to phenolic compounds, extraction with ethanol:water lead to higher values of antioxidant activity. The control cheese accounted for 1.4 µmol TE/g fw, the lowest value quantified using ethanol:water as extraction solvent, but higher than that obtained with methanol. The antioxidant activity of the control cheese can be atribued to the presence of bioactive peptides [2].The FBC cheese accounted for 2.9 µmol TE/g fw, followed by FRBC (2.4 µmol TE/g fw), and FRC and IBC (both with 2.0 µmol TE/g fw).

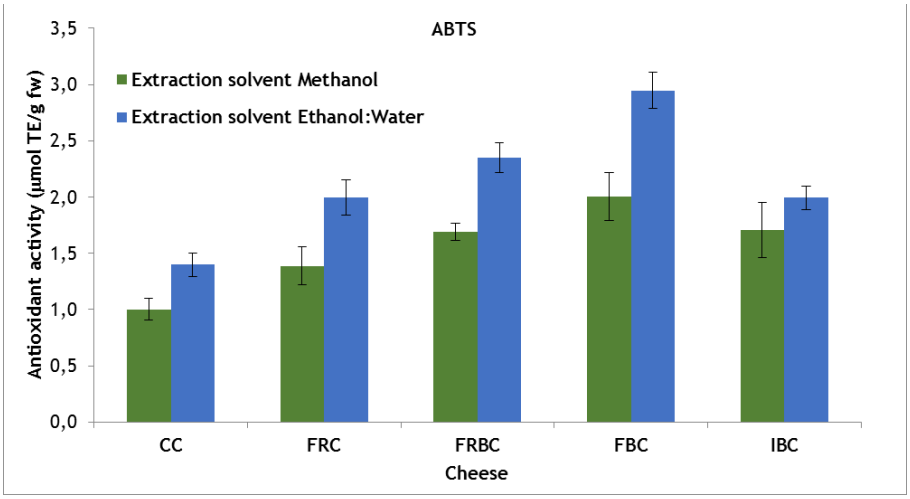

Figure 3 - Antioxidant activity present in the cheeses for ABTS method (sum of the values obtained for the two extracts).

Figure 4 shows the results of antioxidant activity obtained by DPPH method. When methanol was used as extraction solvent, the antioxidant activity for the sum of extracts varied from 0.3 µmol TE/g fw for the control cheese (CC) to 0.6 µmol TE/g fw for the fresh blueberry enriched cheese (FBC). Despite the differences in absolute values, the ranking order was similar to the results obtained by ABTS method. When ethanol:water was used, also FBC cheese exhibited the highest value of antioxidant activity with 0.4 µmol TE/g fw. For both solvents used, the incorporation of fresh blueberries originated cheeses with higher antioxidant activity.

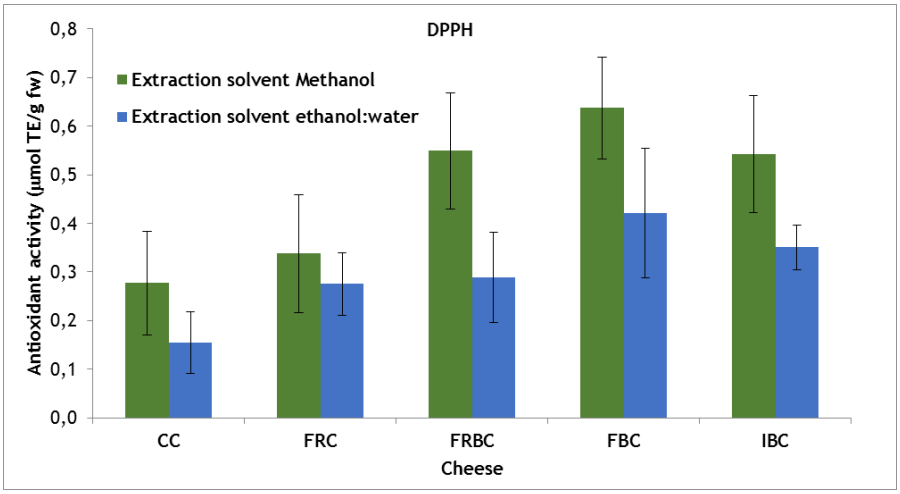

Figure 4 - Antioxidant activity present in the cheeses for DPPH method (sum of the values obtained for the two extracts).

Analyzing the results it's possible to observe that the values quantified by ABTS methodology were superior to those quantified by DPPH. Previous results revealed that bioactive peptides behave differently towards different radicals depending on their medium of solubility. ABTS radical ,being water soluble in nature, is reduced easily by the peptides which are able to donate hydrogen [12].

Figure 5 shows the relative contribution of each extract to the total antioxidant activity determined for all the cheeses, obtained by ABTS and DPPH methodologies using methanol or

ethanol:water as solvent. The results confirm that the antioxidant activity quantified in the 1st extract (E1) corresponded to the highest portion in all cases. For ABTS methodology, that portion ranged from 58 to 80% and from 61 to 68%, when using methanol or ethanol:water, respectively. For DPPH the 1st extract accounted for 59-83% using methanol, and 61-85% using ethanol:water as extraction solvent. Since the 2nd extract ranged from 15 to 42%, considering all extractions, in some cases a third extraction might have been beneficial.

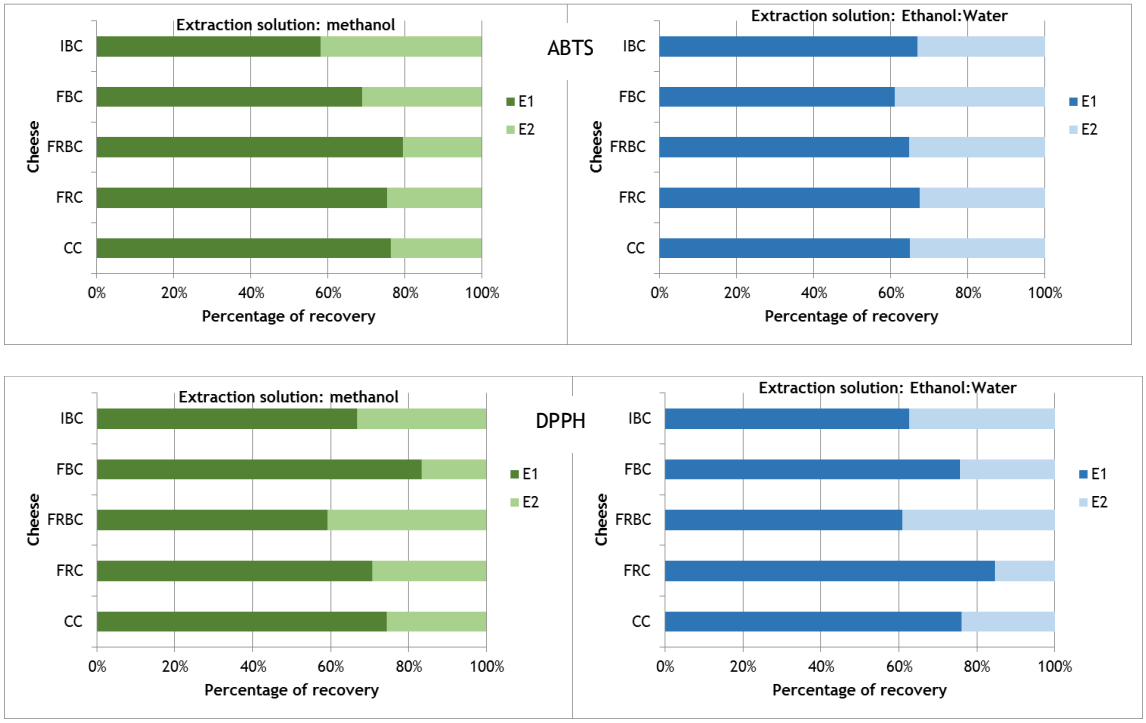

Figure 5 – Percentage of antioxidant activity for ABTS method (Top) and DPPH (bottom) (E1- 1st extract, E2- 2nd extract).

3.3. Correlations

The correlations (R^2) between total phenolic compounds and antioxidant activity were determined. Table 1 shows the correlation values obtained considering all extracts obtained for the two solvents.

Table 1: Correlation values between TPC and AOA for ABTS and DPPH methods considering methanol and ethanol:water extracts.

	Regression coefficient (R^2)	
Extraction solvent	ABTS	DPPH
Methanol	0.5310	0.5955
Ethanol:Water	0.6444	0.5095

The values of the regression coefficient indicate that the relations between total phenolic compounds and antioxidant activity was moderate, allowing to infer the contribution of other molecules than phenolics to the antioxidant activity.

4. Conclusions

The results obtained with the present work showed that the methanol was the solvent that allowed a more effective extraction of phenolic compounds and quantification of antioxidant activity through the DPPH method, whereas the acetone:water allowed a higher quantification through the ABTS method. Regardless of the solvent used, the addition of red fruits, such as raspberry or blueberry, resulted in cheeses with higher levels of phenolic compounds and antioxidant activity.
As to comparing the different fruits added, the blueberries showed to be more effective than raspberries in providing bioactive properties. Also, the addition of fresh blueberries resulted in cheeses with higher antioxidant activity when compared with addition of frozen fruits.
Hence, as a final conclusion, the enrichment of cheese with bioactive compounds from red fruits can be a way to valorize this product since it improves its potential health benefits.

5. Acknowledgement

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6. References

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