



Extracts of olive polyphenols improve lipid stability in cooked beef and pork: Contribution of individual phenolics to the antioxidant activity of the extract

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ARTICLE INFO

Article history:

Received 11 December 2008

Received in revised form 10 March 2009

Accepted 11 March 2009

Keywords:

Lipid oxidation

Warmed-over-flavour

Antioxidants

Olive

Polyphenol

Beef

Pork

ABSTRACT

Crude polyphenol extracts (50 or 100 mg gallic acid equivalents (GAE)/kg meat) from the waste waters of olive oil pomace reduced the formation of 2-thiobarbituric reactive substances (TBARS) in pre-cooked beef (63–83%) and pork (47–66%). When compared with other antioxidants, the ranking of activities were: tea >> olive > wine. The olive extract contained hydroxy-tyrosol (70.6%), tyrosol (17.5%), caffeic acid (9.5%), *p*-coumaric acid (1.9%) and vanillic acid (0.3%). Relationship between polyphenol composition and antioxidant activity of a blend containing oleuropein, tyrosol, hydroxy-tyrosol, quercetin, rutin and caffeic, vanillic and coumaric acids was described by a first order polynomial model. Quercetin, hydroxy-tyrosol, caffeic acid and oleuropein had the highest contributions to the linear term followed by rutin and tyrosol. We detected the strongest positive synergism between tyrosol and quercetin, hydroxy-tyrosol, oleuropein and to a lesser degree with caffeic acid whilst the effect of vanillic and coumaric acids were not significant.

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1. Introduction

Lipid oxidation plays an important role in the development of undesirable flavours commonly known as “warmed-over-flavour” (WOF) and in the formation of toxic carcinogenic compounds in cooked meat products. For many years, meat processors used synthetic antioxidants like butyl hydroxyanisole (BHA) or butyl hydroxytoluene (BHT) to prevent, or reduce, flavour deterioration. However, concerns about their safety and consumer’s preference for more natural foods has resulted in a high demand for “natural” additives, that can extend the shelf life of both processed and unprocessed meat products.

Olives (*Olea europaea* L.) and olive oil contains polyphenols such as oleuropein, hydroxy-tyrosol and tyrosol (Briante et al., 2002), rutin (Boitia et al., 2001), quercetin (Obied, Bedgood, Prenzler, & Robards, 2007) as well as caffeic (Papadopoulos & Boskou, 1991), vanillic and *o*- and *p*-coumaric acids (Brenes, Garcia, Garcia, Rios, & Garrido, 1999) all with excellent antioxidant properties. These compounds were effective radical scavengers with the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) test (Morello, Vuorela, Romero, Morilva, & Heinonen, 2005). Chimi, Cilliard, Cilliard, and Rahmani (1991) demonstrated that the antioxidant activity of phenolic compounds in linoleic acid was: tyrosol < caffeic < oleurop-

ein < hydroxy-tyrosol. Hydroxy-tyrosol significantly inhibited the lipid oxidation of olive oil (Aparicio, Roda, Albi, & Gutierrez, 1999) and had a positive effect on human health (Manna, Galletti, Cucciolla, Montedoro, & Zappia, 1999). Paiva-Martins and Gordon (2002) reported that oleuropein and hydroxy-tyrosol enhanced the oxidative stability of oil-in-water emulsions whilst quercetin inhibited lipid oxidation in raw fish (Ramanathan & Das, 1992) and beef (Bekhit, Geesink, Ilian, Morton, & Bikkerstaffe, 2003; Shahidi, Zheng, & Saleemi, 1993).

Several publications concluded that the remarkable olive oil resistance to oxidation was closely linked to its total polyphenol content (Franconi et al., 2006; Hrncirik & Fritsche, 2005; Matos, Pereira, Andrade, Scabra, & Oliveira, 2007). Medina, Sacchi, Biondi, Aubourg, and Paolillo (1998) suggested that the improvement in oxidative stability observed when extra virgin olive oil was used as a filling medium in canned fish instead of refined oils was due to the presence of natural polyphenols, normally eliminated during the refinery process. Persson, Graziani, Ferracane, Fogliano, and Skog (2003) reported that frying beef patties in virgin olive oil reduced the content of heterocyclic amines, a well known carcinogenic compound.

From the existing information we could infer that crude extracts of olive polyphenols may provide good protection against lipid oxidation and off-flavour development in pre-cooked meat. However, to our knowledge, the works on this issue were done only in fish (Medina, Satue-Garcia, Bruce German, & Frankel,

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1999; Medina et al., 1998). Since the type of substrate strongly influences antioxidant activity, analysing the efficacy of crude extracts of olive polyphenols for preventing or inhibiting lipid oxidation and off-flavour development in cooked beef and pork is warranted.

Although, olive fruits are rich in polyphenols, only 2% of the total phenolic content passes to the oil phase whilst the remaining 98% is lost in the waste waters (black water, alpechin) and in the solid phase (pomace, alperujo) (Rodin, Karathanos, & Mantravino, 2001). In Australia, olive oil production is done mainly with two-phase mills which use little or no water so the waste is mostly solid in nature. An analysis of the polyphenol composition of Australian mills' pomace showed significant amounts of hydroxy-tyrosol, tyrosol, rutin, quercetin, oleuropein and caffeic acid (Obied et al., 2005, 2007). Lesage-Meessen et al. (2001) reported that hydroxy-tyrosol and *p*-tyrosol were the major compounds detected in Italian mills black waters, but they also identified *p*-coumaric, caffeic, ferulic and vanillic acids.

Developing crude polyphenols extracts from olive oil waste could be a means of adding value to a by product and reducing the environmental impact of waste. This process has significant economic benefits, since it will allow similar, or higher, levels of activity with lower production costs than with a blend of purified polyphenols.

In order to obtain an extract with optimum antioxidant capacity we need to thoroughly establish the relationship between the bioactive composition and the activity of the extract. Several studies demonstrated the positive relationship between antioxidant activity and total polyphenol content (Baldioli, Servili, Perreti, & Montedoro, 1996; Gutfinger, 1981). However, most of the work was done with olive oil and the contribution of the polyphenol composition examined only the effect of individual polyphenols or, at most, binary mixtures, without considering the possible synergies between the mixture's components or the effect of other substrates.

Holistic studies will help to improve the nutritional and sensorial quality of meat products and satisfy consumer's demand for food free of synthetic additives. Extension of shelf life by preventing off-flavour development will provide significant economic gains to retailers, wholesalers and the catering industry. Developing crude extracts from by-products of the olive industry as natural antioxidants in food will also allow the production of new value-added ingredients from low-value raw materials and waste streams.

The project investigates the potential ability of crude polyphenol extracts from the waste waters of olive oil pomace to reduce lipid oxidation in pre-cooked beef and pork. In order to obtain extract of consistent activity, we also determined the relationship between the composition of a mixture of selected polyphenol commonly found in olive products and total antioxidant activity in ground beef.

2. Materials and methods

2.1. Reagents

Oleuropein and tyrosol were from Extrasynthese (Genay, France); rutin, quercetin, caffeic, vanillic and *p*-coumaric acids were supplied by Sigma Aldrich Chemical (St. Louis MO, USA). Hydroxy-tyrosol was prepared by the University of Melbourne (Melbourne, VIC Australia).

2.2. Antioxidant extracts

The olive extract (OE) used in the meat tests was obtained from the waste water of the pomace from olive oil processed with a two-

phase mill. The black water phase, rich in polyphenols, was separated from the pomace by centrifugation, freeze-dried and stored at -20°C until used as an antioxidant.

The commercial wine extract was a concentrated extract prepared by aqueous extraction of red grape (*Vitis vinifera*) skin and sold in Australia to modulate colour and sensory qualities of wine. This product has high levels of antioxidants such as proanthocyanidins, catechin, epicatechin and epicatechin gallate (De Freitas & Glories, 1999). The green tea (*Camellia sinensis*) extract (Polyphenon 30, Mitsui Norin Ltd., Tokyo, Japan) contained about 30% catechins.

2.3. Antioxidant activity of olive extracts in beef and pork. Comparison with other natural antioxidants present in the Australian market

The beef samples were obtained from the semimembranosus (SM) muscle of ten steers from a local abattoir. At 48 h post mortem, each muscle was cut into sections, vacuum-packaged and stored at -20°C in the dark until required for analysis (within 30 days). After removing the subcutaneous fat, sections of each muscle were ground with an Oskar mincer (Brisbane, Australia), using 4 cycles each of 5 s, and divided into 20 g patties. The olive, tea and wine extracts were dissolved in 2 ml of distilled water and added to each patty to a final concentration of 50 (O50, T50, W50) or 100 mg GAE/kg meat (O100, T100, W100). The control samples had 2 ml of water with no antioxidant. Samples were thoroughly mixed and cooked in a water bath at 76°C for 40 min. After cooking the samples were stored at 4°C for 0, 3 or 6 days. In the case of pork, the SM muscles from eight animals were processed similarly. At each storage period, lipid oxidation was determined using the 2-thiobarbituric reactive substances (TBARS) method.

2.4. Relationship between polyphenol composition and antioxidant activity of the extract in ground beef

SM samples from four of the steers used in the previous experiment were ground and treated with different combinations of oleuropein, tyrosol, hydroxy-tyrosol, rutin, quercetin and caffeic, vanillic and *p*-coumaric acids. Within each treatment combination, the concentrations of each polyphenol were similar. The different polyphenol blends were dissolved in 2 ml of ethanol and added to each patty to a final total concentration of 3.9 mmol/kg meat. The control samples had 2 ml of ethanol with no antioxidant. Meat samples were cooked in similar conditions as those described in the previous experiment and stored at 4°C for 0 and 6 days. Lipid oxidation was determined at each storage time with the TBARS method of Nielsen, Sorensen, Skibsted, and Bertelsen (1997).

2.5. Chemical analysis

The total phenolic content of the extracts was determined by the Folin-Ciocalteu method (Fogliano, Verde, Randazzo, & Ritieni, 1999) and expressed as mg of gallic acid equivalents (GAE)/g extract.

Major phenols present in the olive extract were identified by HPLC according to Brenes, Garcia, Duran, and Garrido (1993).

Lipid stability was monitored with the TBARS method (Nielsen et al., 1997). Tests were performed in duplicate samples and the results were expressed as mg malondialdehyde/kg meat (mg MDA/kg meat).

Antioxidant capacity depends on the instrumental method utilised for measuring it. The meat industry prefers results based on sensory tests, however, this technique is expensive and time consuming. Selection of the TBARS test reported by Nielsen et al. (1997) was based on results from a previous work done in the Food Science Australia Meat Science laboratory (Yang, Lanari, Brewster,

& Tume, 2000) that reported a highly significant correlation between both techniques.

Antioxidant activity (AOA_t) of each treatment was calculated using the TBARS values of control and treated meat samples at each time period according to the following equation:

$$AOA_t = 100 * ((TBARS_{Sc_t} - TBARS_{Str_t}) / TBARS_{Sc_t}) \quad (1)$$

$TBARS_{Sc_t}$ and $TBARS_{Str_t}$ are the TBARS values of control and treated samples at time t , respectively.

2.6. Statistical analysis

The antioxidant effect of the olive extract on beef and pork was analysed using the Proc Mixed procedure from SAS (1998). Significant differences amongst least square means were determined with the Student 't' test.

The experimental design adopted to determine the relationship between the polyphenol composition and antioxidant activity at day 6 consisted of a simplex centroid mixture design (Cornell, 1990), replicated twice, run in four blocks corresponding to four animals. The design, generated by the SAS software (1998), included 14 one component blends (3 blends triplicated; 1 was duplicated and 3 had no replications) and 30 binary blends (3 triplicated; 9 duplicated and 3 with no replications) with a total of 128 runs per time point.

The analysis was performed with the following model:

$$AOA_{t\text{pred}} = \sum_{i=1}^n \beta_{ixi} + \sum_{i<j=1}^n \sum \beta_{ijxixj} \quad (2)$$

where β_i and β_{ij} are the coefficients for the linear and interaction terms, respectively, and x_i and x_j are the molar fractions of the individual polyphenols (oleuropein, tyrosol, hydroxy-tyrosol, quercetin, rutin and caffeic, vanillic and *p*-coumaric acids). Treatment effects and estimates of the model parameters were determined using the General Linear Model and Regression Procedures from SAS (1998). Model validation was carried out using combinations of the variables at different levels within the experimental range.

3. Results and discussion

3.1. Total polyphenol concentration of the extracts

The polyphenol composition of the olive extract depends on several factors including fruit variety and ripening, origin, climate and processing conditions (Larrauri, Ruperez, & Saura-Calixto, 1996). Therefore, to reduce variability, the antioxidant extract used in the beef and pork samples from the same batch. The total polyphenol content of the olive extracts was 26.83 mg GAE/g ext. whilst the tea and wine commercial products contained 283.21 and 13.34 mg GAE/g ext., respectively.

3.2. Phenolic composition of the olive extract

The major phenolic compounds identified in the olive extract were hydroxy-tyrosol (70.6%), tyrosol (17.5%), caffeic acid (9.5%), *p*-coumaric acid (1.9%) and vanillic acid (0.3%).

3.3. Antioxidant activity of olive extracts in beef and pork. Comparison with natural products present in the Australian market

Tea and grapes are excellent sources of phenolic antioxidants capable of inhibiting lipid and cholesterol oxidation in meat and fish products (Brannan & Mah, 2007; He & Shahidi, 1997). Currently, there is a wide range of commercial extracts obtained from tea and wine by-products sold as antioxidants for the food and

pharmaceutical industry. Comparing the activity of the olive extract against other natural or synthetic products available in the Australian market will help to promote its application in the food industry.

Figs. 1 and 2 present the influence of the antioxidants dose (50 or 100 mg GAE/kg meat) on the TBARS values of pre-cooked beef and pork, respectively. Control pork was more susceptible to lipid oxidation than control beef ($P < 0.001$), this result agrees with recent reports by Jayathilakan, Sharma, Radhakrishna, and Bawa (2007). The three extracts significantly inhibited ($P < 0.0001$) lipid oxidation, the ranking of effectiveness was: tea \gg olive > wine. Extracts activities were greater in beef than in pork; after 6 days storage, the antioxidant activity of the tea (T50, T100), olive (O50, O100) and wine treated (W50, W100) beef at both doses were 97%, 63–83% and 55–80%, respectively. In the case of pork, the AOA_6 values were 65–75% for the tea, 47–66% for olive and 30–61% for wine extracts.

During the whole storage period, no significant changes in the TBARS levels of tea-treated beef were detected ($P > 0.05$) with polyphenol dose or storage time, antioxidant efficacy was so high that

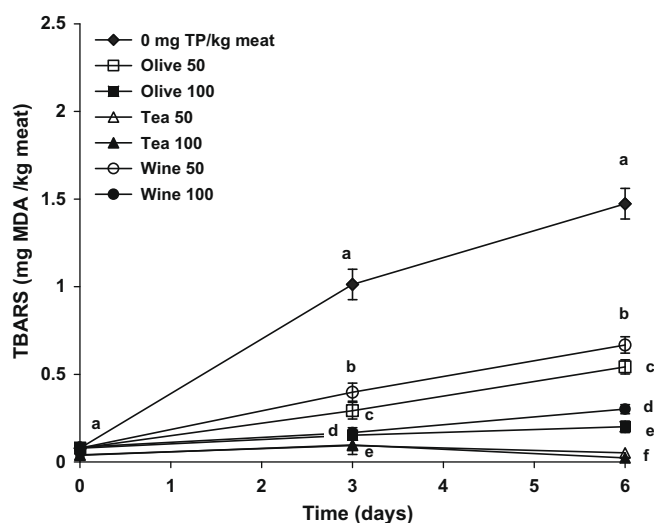


Fig. 1. Effect of different concentrations of olive, tea and wine extracts on the TBARS formation of cooked beef. ^{a–f}Means within each storage period having the same letter are not significantly different ($P > 0.05$).

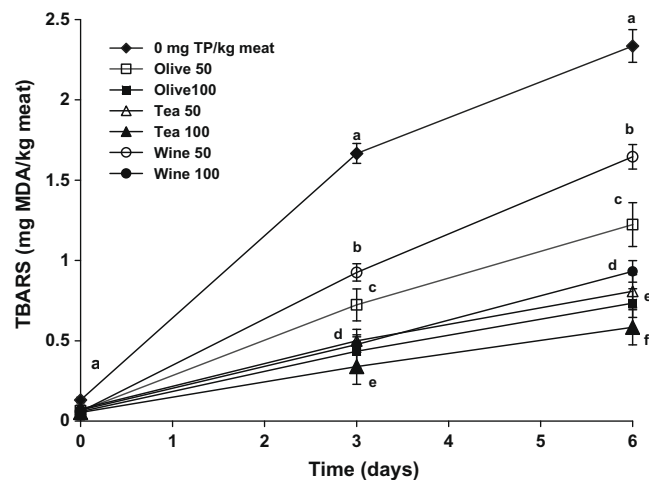


Fig. 2. Effect of different concentrations of olive, tea and wine extracts on the TBARS formation of cooked pork. ^{a–f}Means within each storage period having the same letter are not significantly different ($P > 0.05$).

50 mg GAE/kg meat totally suppressed lipid oxidation. However, at day 6 lipid oxidation in T50 and T100 pork increased 10.5–14.8× and was strongly dependent on the polyphenol level, changing the extract content from 50 to 100 mg GAE/kg meat improved lipid stability 28%. TBARS numbers of the olive and wine treatments increased between 2.6 and 12× in beef and 12.7–28× in the pork samples during storage. Dose effect was also very strong ($P < 0.0001$), lipid stability increased 63–67% in beef and 40–43% in pork when the extracts concentration was enhanced from 50 to 100 mg GAE/kg meat.

The disparity in response to the antioxidant treatments between species could be attributed to differences in the phospholipids, polyunsaturated fatty acids (PUFA) and/or free iron contents (Rhee, Anderson, & Sams, 1996). Increasing the level of these factors enhances the muscle's susceptibility to oxidation and rancidity development (Channon & Trout, 2002; Tay, Aberle, & Judge, 1983) resulting in an enhancement in muscle's antioxidant requirements.

Pork muscle has a greater PUFA (Enser, Hallett, Hewitt, Forsey, & Wood, 1996) and phospholipids (Channon & Trout, 2002) contents and lower free iron concentration than beef. However, results from the current experiment showed that PUFA and phospholipids effects have over compensated the metal ions influence.

3.4. Relationship between antioxidant activity and polyphenol composition

The phenolics selected for this assay included tyrosol, hydroxytyrosol, caffeic, vanillic and *p*-coumaric acids as well as oleuropein, quercetin and rutin. Oleuropein is a major polyphenol in olive oil, fruit and leaves (Briante et al., 2002), quercetin and rutin are very potent antioxidants identified in Australian olive mills waste waters (Obied et al., 2005, 2007).

3.4.1. Antioxidant activity of the individual polyphenols in cooked beef

Fig. 3 shows the effect of tyrosol, hydroxytyrosol, oleuropein, quercetin and rutin as well as caffeic, vanillic and *p*-coumaric acids in the TBARS values of cooked beef patties stored for 0 and 6 days. The most potent antioxidants were quercetin, hydroxytyrosol, caffeic acid and oleuropein with antioxidant activities at day 6 ranging from 80% to 92%. Rutin and tyrosol were less active, their AOA₆ values were 60% and 40%, respectively; *p*-coumaric and vanillic acids were ineffective or slightly prooxidative.

Fig. 4 presents the chemical structures of the polyphenols used in this assay. Results showed that in accordance with previous work done with radical quenching tests in model systems (Rice-Evans, Miller, & Paganga, 1997; Shahidi & Wanasundara, 1992), the antioxidant activity of the polyphenols in cooked patties was

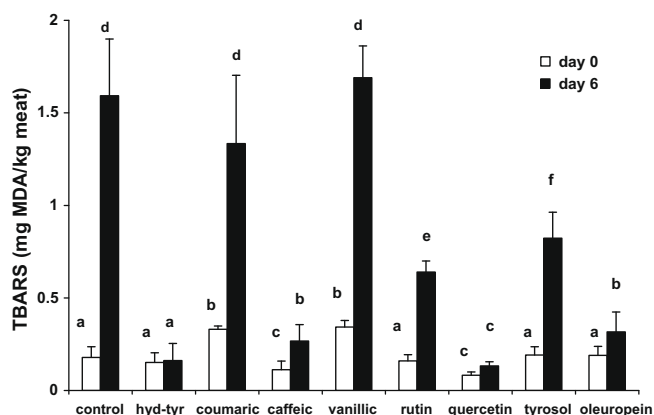


Fig. 3. Effect of individual polyphenols on the TBARS values of pre-cooked beef. ^{a–f}Bars with the same letter are not significantly different ($P > 0.05$).

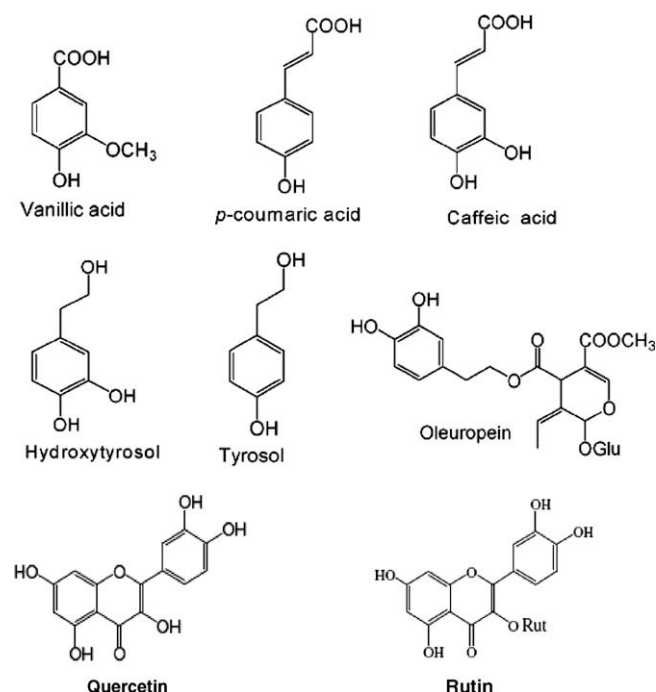


Fig. 4. Chemical structures of the olive polyphenols used in the model.

strongly dependent on the number and position of free hydroxyl groups attached to the aromatic ring. Hydroxytyrosol, caffeic acid and oleuropein, three *o*-diphenols, were more effective antioxidants than tyrosol, vanillic and *p*-coumaric acids with only one OH available. The introduction of a second *o*-hydroxyl group in the tyrosol or the *p*-coumaric acid molecules to form hydroxytyrosol or caffeic acid increased the AOA₆ 85% and 420%, respectively.

Glycosylation of the 3-OH group in ring C of quercetin to form rutin decreased AOA₆ by 50%. Ramanathan and Das (1992) reported that quercetin was 40% more active than rutin in raw fish; the differences in activity compared with our results could be due to a substrate effect, as lipid stability in beef is higher than in fish. The esterification of hydroxytyrosol's -CH₂-CH₂OH group by elenolic acid glycoside to produce oleuropein diminished the effectiveness of the antioxidant from 89% to 80%.

Substitution of the -CH₂-CH₂OH group in tyrosol or hydroxytyrosol by a -CH=CH-COOH as in *p*-coumaric or caffeic acids reduced AOA₆. This effect was particularly strong in monophenols; the antioxidant activity of tyrosol was 3× higher than that shown by *p*-coumaric acid. In contrast, the difference in AOA₆ between hydroxytyrosol and caffeic acid, two biphenols, was 8%. Our results suggested that in polyphenolic compounds with a catechol structure, like hydroxytyrosol or caffeic acid, the biggest structural contributor to their antioxidant capacity in cooked meat are the *o*-OH groups.

3.4.2. Relationship between polyphenol composition of the olive extract and antioxidant activity

The results of the statistical analysis indicated that the contribution of vanillic and *p*-coumaric acids per se, or through their interactions with the other components, to the model was not significant ($P > 0.05$). Consequently, the model was reduced to the effects of oleuropein (OL), tyrosol (T), hydroxytyrosol (OHT), rutin (R), quercetin (Q) and caffeic acid (C).

Analysis of variance showed that all the main effects were highly significant ($P < 0.001$) as well as the interactions between tyrosol and quercetin, hydroxytyrosol, oleuropein and caffeic ($P < 0.001$). Quercetin also interacted with rutin ($P < 0.001$), oleu-

Table 1
Estimated regression coefficients of the polynomial model.

Coefficient	Std. err.		P
Tyrosol (T)	11.42	5.96	0.058
Rutin (R)	64.15	7.07	0.001
Oleuropein (OL)	85.44	5.5	0.001
Caffeic acid (C)	88.15	5.93	0.001
Hydroxytyrosol (OHT)	92.38	5.5	0.001
Quercetin (Q)	92.56	5.5	0.001
Q * T	184.1	15.3	0.001
OHT * T	116.4	15.3	0.001
Q * R	98.5	15.3	0.001
OL * T	94.5	16.4	0.001
C * T	70.4	16.4	0.001
Q * C	45.2	15	0.01
Q * OL	42.7	16.1	0.01
OHT * R	40.2	16.4	0.016
OL * R	33.1	15.3	0.046

ropein ($P < 0.01$), caffeic ($P < 0.023$), whilst hydroxy-tyrosol had a positive synergism with rutin ($P < 0.044$).

Fitness of the polynomial model was highly significant; the coefficient of determination (R^2) was 0.995. Table 1 presents the estimates of the regression coefficients (β_i) of the linear and interaction terms of the significant effects as well as their standard error and degree of significance. From the β_i values we can observe that quercetin, hydroxy-tyrosol, caffeic acid and oleuropein were the highest contributors to the linear term followed by rutin. Although, tyrosol's individual input to the total antioxidant capacity was not significant ($P > 0.05$), the interactions between this compound and quercetin, hydroxy-tyrosol, oleuropein and to a lesser degree with caffeic acid were the strongest (Table 1). The estimates of the interaction parameters also indicated that the contributions of the $Q * OL$, $R * OHT$ and $R * OL$ to the total antioxidant activity were the lowest.

The final model was:

$$\begin{aligned}
 AOA = & 85.44x_{OL} + 92.38x_{OHT} + 92.56x_Q + 64.15x_R + 88.15x_C \\
 & + 11.42x_T + 94.5^*x_{OL}x_T + 42.7x_{OL}x_Q + 116.4x_{OHT}x_T \\
 & + 45.2x_Qx_C + 184.1x_Qx_T + 70.4x_{OHT}x_C + 33.1x_{OL}x_R \\
 & + 40.2x_{OHT}x_R \quad (3)
 \end{aligned}$$

The main effects contribution to the antioxidant activity was much higher than the interactions components. In a six component system with similar polyphenol concentrations ($x_i = 0.167$) the predicted activity was 89%, the main and interactions effects accounted for 81% and 19% of the total AOA, respectively. Increasing tyrosol or rutin to 40% and assuming that the rest of the components were at equal concentrations reduced AOA to 78% and 83%, respectively. In contrast, enriching the polyphenol blend with quercetin (40%) improved antioxidant activity by 10% whilst no effect was detected ($AOA_6 = 88\text{--}90\%$), by adding caffeic acid, hydroxy-tyrosol or oleuropein to similar levels.

The validation tests showed that the experimental and predicted values were in good agreement the correlation coefficient was 0.94.

4. Conclusions

The polyphenol extract from the waste water of olive oil's pomace significantly inhibited lipid oxidation in pre-cooked ground beef and pork. The antioxidant effect increased with the dose and was higher in beef than in pork. In comparison with commercial antioxidants made from tea or wine, the ranking of efficacy was: tea \gg olive > wine.

The relationship between polyphenol composition and antioxidant activity of a mixture of tyrosol, hydroxy-tyrosol, caffeic, vanil-

lic and *p*-coumaric acids and oleuropein, quercetin and rutin was satisfactorily predicted with a polynomial model. Results showed that quercetin, hydroxy-tyrosol, caffeic acid and oleuropein were the highest contributors to the linear term followed by rutin and tyrosol. We detected the strongest positive synergism between tyrosol and quercetin, hydroxy-tyrosol or oleuropein and to a lesser degree with caffeic acid whilst the contributions of the interactions between oleuropein and quercetin or rutin and between hydroxy-tyrosol and rutin were the lowest. *p*-Coumaric and vanillic acids effects were not significant.

Acknowledgements

We thank Ron Kuypers (CSIRO, Australia) and Dr. Robin Shorthose (CSIRO, Australia) for their helpful suggestions and Dr. Alicia Califano (CIDCA, Argentina) and Peter Jones (CSIRO, Australia) for assistance with the statistical design.

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