CHAPTER

PREPARATION OF FUNCTIONAL YOGURT ENRICHED WITH OLIVE-DERIVED PRODUCTS

11

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11.1 INTRODUCTION

Healthiness is one of the most frequently mentioned reasons behind food choices in EU countries. In addition to traditional healthiness, food may contain specific components with a positive impact on health and because of this foods are considered as functional. Fermented foods constitute an important part of our food. Several potential claims are available on proposed beneficial health effects of fermented dairy foods including yogurt. Milk and yogurt constitute two of the most popular dairy products with high nutritional value, which are proven as successful matrices for the development of various health-promoting functional foods (Rowan et al., 2005). In recent years, a variety of dairy products supplemented with probiotic bacteria and/or bioactive components have been introduced to the market. Because of their health-promoting benefits, there has been an increasing interest in the development and consumption of new functional dairy products.

One category of bioactive components with officially recognized health-protecting properties is olive polyphenols (EFSA, 2011). This category of components appeared as an attractive candidate that could be used for the enrichment of dairy products and increase their health-protecting properties (Zoidou et al., 2014). Oleuropein (Fig. 11.1) comprises the major constituent of *Olea europaea* L. leaves and unprocessed olives (Bianco and Uccella, 2000; Soler-Rivas et al., 2000).

The olive leaf extract has been recognized by the European Medicines Agency (EMA) as an official herbal drug displaying a broad variety of health beneficial properties (EMA monograph). Many in vivo and in vitro studies have indicated that oleuropein exhibits a wide variety of biological activities, including antimicrobial, antioxidant, antiischemic, antihypertasic, anticoagulant, hypolipidemic, and antitumor properties (Hamdi and Castellon, 2005; Obied et al., 2005; Andreadou et al., 2006; Coni et al., 2000; Tassou and Nychas, 1995; Giamarellos-Bourboulis et al., 2006). Thus this natural product has attracted significant scientific and research interest, mainly connected with its potential protective role against infections and diseases, as well as the risk of developing breast, prostate and colon cancers, cardiovascular diseases, and diabetes. Most significantly, according to the scientific opinion of the European Food Safety Authority (EFSA, 2011), oleuropein (as hydroxytyrosol derivative) has been related to the protection of low-density lipoprotein from oxidation. The bioavailability of oleuropein has been studied (de Bock et al., 2013). It is absorbed after oral administration and is metabolized mainly to hydroxytyrosol, which is also a powerful antioxidant.

Although oleuropein is an abundant constituent of unprocessed olives, in most edible olives it is removed during the debittering process, resulting in a significant decrease in its nutritional intake. We

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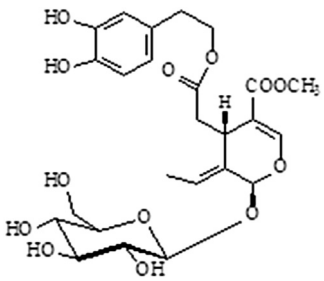

FIGURE 11.1

The structure of oleuropein.

have identified a Greek edible olive variety (*Throuba thassos*), which is particularly rich in oleuropein (Zoidou et al., 2010), and based on this study we determined an acceptable and safe level for the daily nutritional uptake of oleuropein. In this frame, oleuropein appeared as a component that was worth studying for the enrichment of dairy products (Zoidou et al., 2014).

However, pure oleuropein is a relatively expensive ingredient and for this reason we also investigated, and present herein, the use of olive leaf extract as an alternative cheap source with a high concentration of oleuropein. Oleuropein is available as a nutraceutical in the world market mainly as a constituent of olive leaf extract, but until now there are no food preparations with this molecule. The olive leaf extract, besides oleuropein, contains several other bioactive compounds and exhibits antioxidant, antiinflammatory, antiviral, immuno-modulatory, and hypocholesterolemic effects (EMA monograph). Olive leaf has been in the focus of research interest because of its health beneficial effects especially in the treatment and prevention of several chronic diseases. In our study, we used the olive leaf extract in the form of an infusion. It should be noted that the recognized traditional use by the EMA concerns herbal tea in a decoction or infusion form.

Based on these data, we investigated the addition of oleuropein, either in pure form or in the form of dried olive leaf infusion, in milk and yogurt for the production of a new type of functional food.

One of the major challenges that had to be faced during this study was the impact of oleuropein on the taste and texture of yogurt. Since consumers demand products combining increased health benefits with good taste, the bitter taste of oleuropein constituted a serious drawback that had to be investigated. The questionable stability of oleuropein during processing comprised a second barrier that had to be studied. For this purpose, it was necessary to develop new analytical methods for the measurement of oleuropein in leaves, in the infusion, in milk, and in yogurt. Thus the development of functional dairy products based on oleuropein was an intriguing case.

11.2 MATERIALS AND METHODS

11.2.1 CHEMICALS

All solvents and distilled water used throughout the experiments were obtained by Merck (Darmstadt, Germany) and were of high-performance liquid chromatography (HPLC) grade. All mobile phases were vacuum filtered through a 0.2 μm membrane filter (Scientific Resources, Eatontown, NJ, USA) and degassed in an ultrasonic bath prior to HPLC analysis. Pure oleuropein was purchased from Extrasynthese (Genay, France).

11.2.2 OLIVE LEAF SELECTION

The olive leaf selection was based on its oleuropein content. Olive leaves from 10 different varieties and geographic origins from Greece were selected and analyzed for their oleuropein content. The finally selected olive leaves originated from wild trees growing near Lake Volvi (northern Greece). More specifically, dry olive leaves in powder form (100 mg) were extracted with 20 mL of MeOH in a supersonic bath for 45 min. The supernatant was separated by centrifugation at 4000 rpm for 3 min. A part of the supernatant methanol extract (10.0 mL) was mixed with 0.5 mL of internal standard solution (0.5 mg syringaldehyde/mL in acetonitrile) and evaporated under reduced pressure at 40°C.

11.2.3 QUANTITATIVE NUCLEAR MAGNETIC RESONANCE ANALYSIS OF OLEUROPEIN CONTENT IN OLIVE LEAVES

The dry extract was dissolved in 600 μL of CD_3OD. ^1H nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz. Thirty-two scans were collected and the spectra were phased corrected and integrated automatically using TOPSPIN. The quantitation was based on the integration ratio between the aldehydic proton signal of syringaldehyde at 9.75 ppm and the proton of oleuropein appearing at 5.91 ppm. A calibration curve of oleuropein was prepared at seven different concentrations ranging between 70 and 4500 μg in a tube. The solutions for the construction of the calibration curve were prepared by mixing appropriate volumes of a stock solution of pure oleuropein (1 mg/mL in MeOH) with 0.5 mL of the internal standard solution (0.5 mg syringaldehyde/mL in acetonitrile) and evaporation under reduced pressure at 40°C. The equation used for the quantification of oleuropein in a tube was $y = 0.512x + 0.0904$, with $r^2 = 0.995$.

11.2.4 OLIVE LEAF EXTRACT PREPARATION

An aliquot of 24 g of dried olive leaves was added to hot water (450 mL) for 30 min, according to the EMA monograph. The obtained infusion was lyophilized to afford a dry extract (5 g). The oleuropein content of the olive leaf-lyophilized extract was measured by NMR using the previously described method. More specifically, 15 mg of the lyophilized extract were mixed with 0.5 mL of the internal standard solution (0.5 mg syringaldehyde/mL in acetonitrile) and evaporated under reduced pressure at 40°C. The mixture was dissolved in 600 μL of CD_3OD and the ^1H NMR spectrum was recorded at 400 MHz.

11.2.5 SELECTION OF OLEUROPEIN DOSAGE

The determination of oleuropein dosage was based on its quantity that corresponded to the average consumption of 15 olive drupes per day (as measured for *Throuba thassos* olives; Zoidou et al., 2010). For this purpose, oleuropein was added to the milk to achieve three levels: 0.1, 0.2, and 0.4 mg/mL. The organoleptic properties of the novel products were examined by a sensory panel, which evaluated their taste, color, and texture using a 1–10 scale, in accordance with Tamine and Robinson (2000). The respective results suggested to proceed with oleuropein concentrations of 0.1 and 0.2 mg/mL. Based on the measured oleuropein content of the lyophilized olive leaf extract, a respective amount of extract (0.43 mg/mL) affording 0.1 mg/mL of oleuropein was found to be acceptable and was further used.

11.2.6 EXPERIMENTAL PROCEDURE FOR THE PRODUCTION OF MILK AND YOGURT PREPARATIONS

Full-fat raw cow's milk was used for all the experiments. Milk was first spiked with oleuropein and in a next step the content and stability of the contained oleuropein in milk and the produced yogurt were determined during their treatment. A second set of experiments was performed using olive leaf extract in the place of pure oleuropein.

11.2.7 PREPARATION OF MILK ENRICHED WITH PURE OLEUROPEIN: STUDY OF HEAT RESISTANCE AND STORAGE

The resistance of oleuropein during the heating of milk was examined first. In particular, 40 mg of pure oleuropein was added to 200 mL of raw milk (fat content 3.5%) and mixed. The milk was heated at 90°C for 5 min in a water bath under continuous stirring, and then milk was cooled immediately with tap water and put into sterilized glass bottles. Next, the milk was stored at 4°C for 7 days. The oleuropein content was determined immediately after its addition in milk as well as after the heat treatment and every 2 days during storage. The experiment was duplicated.

11.2.8 MANUFACTURE AND STORAGE OF YOGURT ENRICHED WITH PURE OLEUROPEIN

Pure oleuropein was added to 500 mL of cow milk (fat content 3.5%), mixed well, heated at 90°C for 5 min in a water bath, cooled to 43°C, and divided into equal quantities (2 × 200 mL) in two sterilized glass cups. Then, 3% (v/v) of yogurt culture was aseptically inoculated and mixed well, and the milk was incubated at 42°C for 4.5 h until its pH reached 4.45. The prepared yogurts were cooled, stored at 4°C, and their oleuropein content was determined immediately after inoculation and the production of yogurt, as well as every 2 days during their storage. Control yogurt was also manufactured using a similar procedure without the oleuropein addition step. The process was repeated twice using the two dosages selected (0.1 and 0.2 mg/mL).

11.2.9 MANUFACTURE AND STORAGE OF YOGURT ENRICHED WITH OLIVE LEAF EXTRACT

The milk was homogenized and heated. The addition of olive leaf extract to milk was done at 35°C. The amount of added extract was 0.43 mg/mL corresponding to 0.1 mg/mL of oleuropein. Then, the milk was heated at 90°C for 5 min, cooled to 42°C, inoculated with traditional Greek yogurt at 1.5%, poured into 100 g sterilized cups, and incubated to the desired pH (4.6). The yogurts were refrigerated immediately at 4°C for 21 days and analyzed at 7, 14, and 21 days of storage.

11.2.10 PH MEASUREMENT

During the refrigerated storage, the quality of the produced milk and yogurt was monitored by measuring the pH and determining their sensory characteristics. In particular, pH was determined using a Hanna model HI 98240 pH meter (Hanna Instruments, Woonsocket, RI, USA) and titratable acidity was determined by titrating 10 mL of a sample with N/9 NaOH and expressed as Dornic degrees (°D).

11.2.11 SENSORY ANALYSES

Samples were assessed for sensory properties for the following attributes: external aspects (color and dry/humid), flavor, texture, basic tastes (acidic, salty, and bitter), and after-taste (intensity and persistence). The sensory acceptability of the new products was assessed by a panel of five persons of the Dairy Laboratory staff. The products were served at 7–10°C, milk in glasses and yogurt in plastic cups, immediately after their preparation and after 7 and 21 or 35 days of storage at 4°C, respectively. In this respect, their taste, color, and texture were graded using a sensory scale of 1 to 10 as follows: 1–2 bad, 3–4 not satisfying, 5–6 good, 7–8 very good, 9–10 excellent, according to Tamine and Robinson (2000). Their overall acceptability was also determined. Because of the small number of the members of the sensory panel the results should be considered as preliminary.

11.2.12 PHYSICOCHEMICAL ANALYSES

The composition was evaluated by means of Milkoscan (Milkoscan FTIR 120, Foss Electric, Denmark).

11.2.13 RHEOLOGICAL ANALYSES

11.2.13.1 Firmness

Firmness (g) was measured by a Brookfield texture analyzer LFRA 1000 using a 2 cm acrylic cylinder probe. The test speed was fixed at 2 mm/s and the penetration depth was 13 mm. Sample temperature was 20°C. Firmness was defined as the force necessary to reach the maximum depth.

11.2.13.2 Viscosity

Viscosity (cps × 1000) was measured using a Brookfield RV, DV-II viscometer (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA) with a Helipath (Spindle, 94) rotated at 2 rpm for 1 min. Sample temperature was 20°C.

11.2.13.3 Syneresis

Syneresis was measured by syneresis index and water-holding capacity (WHC).

11.2.13.4 Water-Holding Capacity

WHC was determined by a procedure adapted from Akalın et al. (2012). A sample of 20 g yogurt (YO) was centrifuged for 10 min at 5000 *g* at 20°C. The WHC was expressed as percentage (w/w) of yogurt mass after removing the whey expelled (WE) and defined as $WHC\% = 100 \times (YO - WE)/YO$.

11.2.13.5 Syneresis Index

Syneresis index was measured according to the method described by Harwalkar and Kalab (1983) by emptying the yogurt from a cup (100 mL) into a filter, cutting crosswise into four pieces, draining for 24 h at 20°C, and collecting the amount of whey drained off in a graduated cylinder. The relative volumes (V%) of whey drained at 5 min and at 1 min increments thereafter for up to 3 h were plotted against time. The relative volumes at 3 h were estimated as the syneresis index.

11.2.14 QUANTITATION OF OLEUROPEIN IN MILK AND YOGURT

A method for the quantitation of oleuropein in milk and yogurt preparations was developed and validated. The proposed method included an extraction procedure of oleuropein and its quantitation through HPLC analysis.

The extraction procedure for milk was done with the addition of 2 mL of acetonitrile into 1 mL of milk under stirring for 2 min. Then, the mixture was centrifuged at 2000 rpm for 15 min and the supernatant was filtrated through a syringe filter pore 0.45 μm and injected for HPLC analysis.

The extraction procedure for yogurt was as follows: 1 mL of water was added to 1 g of yogurt and stirred for 2 min. Then, the mixture was centrifuged at 2000 rpm for 15 min and the supernatant was filtrated through a syringe filter of pore 0.45 μm. A portion of 20 μL was injected for HPLC analysis.

11.2.15 CHROMATOGRAPHIC INSTRUMENTATION AND METHODOLOGY

HPLC determination of oleuropein was carried out with a system consisting of a Finnigan Spectra system P4000 quaternary pump coupled to a Finnigan Spectra system UV6000LP diode array detector. For the chromatographic separation a Li Chromosorb C18 reversed-phase column (250 × 4.0 mm, ID 5 μm) equipped with a C18 Li Chromosorb precolumn was used.

The gradient elution program was implemented using a system of two solvents. Solvent A was a 2% w/w solution of acetic acid and solvent B was acetonitrile. The flow rate was constant at 1 mL/min and the total chromatographic analysis time was 40 min at ambient temperature. The following gradient program was applied: linear gradient 100%–95% of solvent A for 2 min, 95%–75% of solvent A from 2 to 10 min, 75%–60% of solvent A from 10 to 20 min, and 60%–50% of solvent A from 20 to 30 min. Then, 50% rate of solvent A from 30 to 34 min, which followed a linear gradient to 100% from 34 to 40 min. The injection volume was 20 μL. Oleuropein was determined at 254 nm at a retention time of 17 min. For the quantitation of oleuropein in the extracts, standard solutions of milk and yogurt spiked with oleuropein were prepared. Identification of the eluting peaks was performed by comparing their retention time values (t_R) and the corresponding UV spectra (obtained from the diode array data) with those of the standards. The area of the peak was estimated by Qhromquest V.2.51 software. The oleuropein content of extracts was estimated from the calibration curve of the milk- and yogurt-spiked standards.

11.2.16 STANDARD SOLUTIONS

A stock solution of 1 mg/mL oleuropein in methanol was prepared and diluted in water to produce standard solutions of 0.02, 0.04, 0.1, 0.2, 0.4, and 0.6 mg/mL oleuropein concentrations. Milk and yogurt standards were prepared to provide 0.02, 0.04, 0.1, 0.2, 0.4, and 0.6 mg/mL concentrations of oleuropein by diluting appropriate volumes of the stock standard solutions in milk and yogurt. Stock solution was stored in a refrigerator and used for the preparation of standard solutions of milk and yogurt immediately prior to the analyses.

11.2.17 METHOD VALIDATION

The method was evaluated by measuring the linearity, precision, relative standard deviation percent (RSD%), accuracy, relative percentage error percent (Er%), and determining the limits of detection and

quantitation (LOD and LOQ). Moreover, a reproducibility study (system precision) was also performed. For the linearity study, six milk and yogurt standards containing oleuropein 0.02, 0.04, 0.1, 0.2, 0.4, and 0.6 mg/mL were analyzed four times and the data linearity was verified through linear least-squares regression analysis. In addition, the intraday and interday (for 5 different days) precisions were also determined along with the RSD%. The accuracy was estimated by analyzing three milk and yogurt standards at four concentration levels of 0.02, 0.10, 0.20, and 0.4 mg/mL or g. The results were expressed as Er%, defined as [assayed concentration − true concentration]/[true concentration] × 100. For the recovery calculation, milk and yogurt standards and standard solutions containing 0.02, 0.1, 0.2, 0.4, and 0.6 mg/mL or mg/g of oleuropein were analyzed in accordance with the proposed extraction procedure. The recovery was determined in respect to the experimental response values as the ratio of the peak area for oleuropein in the milk and yogurt standards against that of the standards. The LOD and LOQ were determined by measuring the background response, and running six blank samples of milk and yogurt at maximum sensitivity. The signal-to-noise ratio of 3:1 (peak area ratio of the oleuropein vs. baseline noise) and 10:1 were used for the calculation of the LOD and LOQ, respectively. The reproducibility study was performed by injecting a standard of 0.5 mg/mL in five replicates (n = 5).

11.2.18 LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY QUALITATIVE ANALYSIS

Ultrahigh-pressure liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) monitoring of oleuropein was performed on an Agilent 1290 Infinity UHPLC interfaced to a 6460 triple-quadrupole mass spectrometer with electrospray ionization (ESI) via Jet Stream Technology (Agilent Technologies, Santa Clara, CA, USA). The UHPLC was equipped with a binary pump with an integrated vacuum degasser (G4220A), an autosampler (G4226A) with thermostat (G1330B), and a thermostatted column compartment (G1316C). The samples were analyzed using a Poroshell 120 EC-C18 column (2.1 × 150 mm 2.7 μm, Agilent Technologies). The mobile phase consisted of 0.1% formic acid in water (A) and acetonitrile (B) with the following gradient program: 0–2.5 min with 10% (B), 3–6 min 25% (B), 7.5 min 40% (B), and 8.5–9.5 min 95% (B). The flow rate was 0.4 mL/min, and the injection volume was 1 μL.

Negative ESI mode was used. The drying gas temperatures and flow rate were 250°C and 8 L/min, respectively. The sheath gas temperature and flow rate were 350°C and 11 L/min, respectively. The nebulizer gas pressure, capillary voltage, and dwell time were 45 psi, 3.5 kV, and 200 ms, respectively.

Total ion as well as multiple reaction monitoring mode was utilized to confirm the identity of oleuropein and the peak purity. Precursor and product ions were identified and optimized using a MassHunter Optimizer (Agilent Technologies).

Oleuropein was monitored through the fragmentation of the precursor ion 539.2 to the product ion 275.1 using a fragmentor voltage of 165, collision energy of 20, and retention time of 6.3 min.

11.2.19 MICROBIOLOGICAL ANALYSIS

The total viable microflora in milk and yogurt was enumerated by the pour-plate method using plate count agar (Merck, Darmstadt, Germany). The plates were incubated at 30°C for 72 h (IDF 100B:1991) and microbiological count data were expressed as $\log_{10}$ of colony forming units (cfu) per mL or gram. Yogurt lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*)

counts were estimated according to the IDF standard 117, 2003 and yeasts and molds were counted according to IDF standard 94, 2004. The enumeration was performed on milk or yogurt just after the inoculation with oleuropein or olive leaf extract, and then after 4 and 7 days for milk and after 14 and 21 or 35 days for yogurt at 4°C. All determinations were made in duplicate.

11.2.20 STATISTICAL ANALYSIS

Data were subjected to analysis of variance using Software Statgraphics Plus for Windows v.5.2 (Manugistics, Inc., Rockville, MO, USA) to test the effect of the concentration of oleuropein and storage time on yogurt characteristics.

11.3 RESULTS AND DISCUSSION

11.3.1 HEALTH-PROTECTING PROPERTIES OF OLIVE LEAF EXTRACT AND OLEUROPEIN

According to the EMA monograph, olive leaf herbal tea is a safe traditional herbal medicinal product used to promote the renal elimination of water, in mild cases of water retention after serious conditions have been excluded by a medical doctor. According to the same monograph, herbal tea can be prepared either in the form of decoction or as an infusion.

Besides the officially recognized therapeutic indication, olive leaf extract has been found to possess a significant number of other health-protecting properties based on in vitro, in vivo, and clinical studies. More specifically, the EMA monograph reports several studies supporting that olive leaf can be used to enhance the immune system as an antimicrobial, antiviral, antioxidant, hypoglycemic agent, and also to protect from cardiovascular problems (antihypertensive, antiplatelet, hypolipidemic activity). Although these properties are not supported by sufficient clinical data the beneficial role of olive leaf on human health is well accepted.

Many of these properties have been related to oleuropein. Pure oleuropein has been found to have antimicrobial, antioxidant, antiischemic, antihypertasic, anticoagulant, hypolipidemic, and antitumor properties (Hamdi and Castellon, 2005; Obied et al., 2005; Andreadou et al., 2006; Coni et al., 2000; Tassou and Nychas, 1995; Giamarellos-Bourboulis et al., 2006).

Concerning the available toxicological data, it is known that the LD_{50} in mouse after oral administration is >3000 mg/kg and for oleuropein after intraperitoneal administration it is >1000 mg/kg. Although there are no available data for chronic oral toxicity, the safety profile of olive leaf extracts can be judged as good from the existing clinical data and from their long-term use (more than 30 years) in the European market.

For all these reasons, it was a very attractive proposition to incorporate oleuropein or olive leaf extract in dairy products to create a new type of functional food.

11.3.2 DOSE SELECTION

The selection of the appropriate dose for oleuropein incorporation in novel products was a critical point for this endeavor. Based on the quantity of oleuropein measured for the *Throuba thassos* variety (average 1.2 mg/drupe) (Zoidou et al., 2010) and considering that an intake of 15 olive

drupes per day is quite usual for the Greek population, total oleuropein intake could be estimated as approximately 20 mg per day. In addition, since the average consumption of dairy products corresponds to 200 mL milk or 200 g yogurt per day, we selected three different levels of oleuropein (0.1, 0.2, and 0.4 mg/mL) to incorporate into milk or yogurt (mg/g). These novel products were tested by the sensory panel immediately after their preparation, which found that the first two preparations exhibited acceptable sensory characteristics and were further investigated for their behavior during storage. On the contrary, milk and yogurt containing 0.4 mg/mL or mg/g of oleuropein produced a taste reminiscent of rancid oil and were only marginally acceptable, hence, they were not further studied.

Considering the high cost of pure oleuropein, we also investigated the use of olive leaf extract with standardized oleuropein content as an alternative cheap source of oleuropein. For this purpose it was first necessary to develop a rapid method for measuring oleuropein levels in leaves by quantitative NMR (qNMR). Then, after screening of several different olive varieties, we recognized a source of olive leaves with very high oleuropein content. Olive leaves of wild trees growing near Lake Volvi (northern Greece) were found to contain the highest oleuropein in the lyophilized infusion. Those trees constitute the largest wild olive orchard in Greece with a population of 100,000 trees. The leaves of those trees were used for the preparation of olive leaf infusion according to the EMA monograph. The infusion was lyophilized and the dried extract was analyzed for its oleuropein content by qNMR (Fig. 11.2).

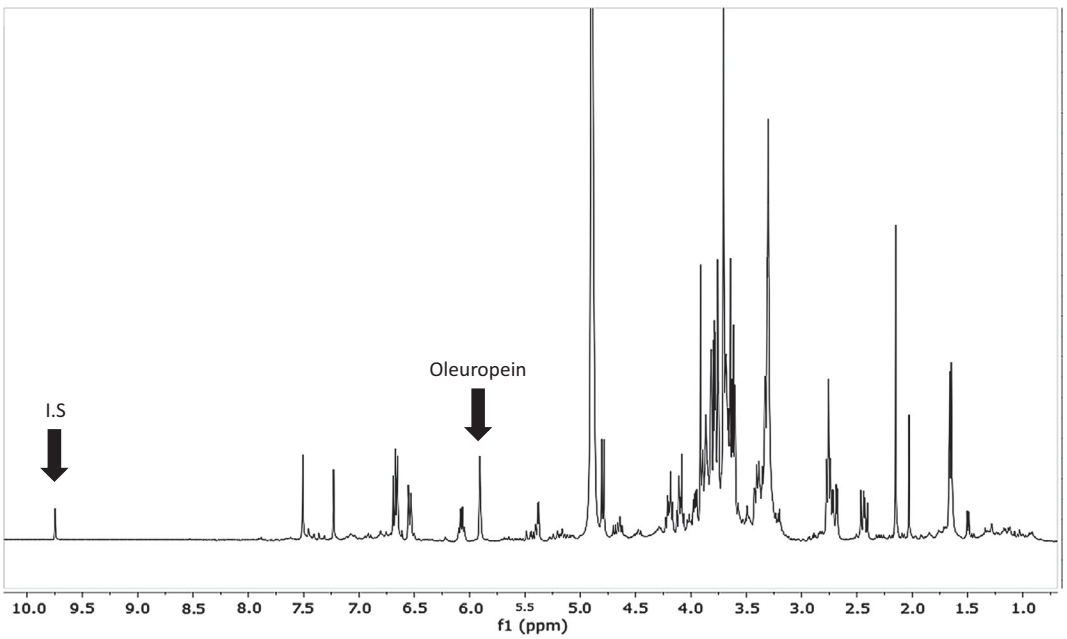

FIGURE 11.2

^{1}H nuclear magnetic resonance (NMR) profile of the olive leaf extract showing the peak of oleuropein used for quantitation. *I.S*, internal standard.

The extract was found to contain 230 mg of oleuropein per gram (23% w/w). Based on this measurement we used a dose of 0.43 mg of extract per mL of milk, corresponding to 0.1 mg of oleuropein. The enriched milk was used next for the preparation of the enriched yogurt. Olive leaf extract standardized in oleuropein content can be easily prepared at a very low cost, much cheaper than pure oleuropein. The concentration used was found to be organoleptically acceptable and the enriched yogurt was further investigated for its physicochemical, rheological, and microbiological behavior during storage.

11.4 MILK ENRICHED WITH PURE OLEUROPEIN

11.4.1 QUANTITATION METHOD VALIDATION

The method was evaluated through the determination of oleuropein content in milk revealing good linearity, precision, accuracy, and reproducibility. All correlation coefficients calculated were higher than 0.990. In addition, the intraday precision, expressed as RSD%, ranged from 3.7% to 7.9% for the three concentration levels and was lower as compared to those of interday precision. The LOD and LOQ were 0.025 mg/mL and 0.074 mg/mL, while the recovery in the milk samples was 73%–93% (average 85%), indicative of a nearly quantitative recovery. The estimated accuracy values were within acceptable levels for oleuropein, while the system reproducibility was low (2.6%). All milk control extracts used in the experiments were oleuropein free, as determined by HPLC analysis.

11.4.2 MILK PROCESSING, HEAT RESISTANCE, AND STABILITY IN COLD STORAGE

According to quantitative HPLC-UV and qualitative LC-MS analysis, oleuropein content in milk was not affected by heat processing, indicating the industrial application potential of this molecule in yogurt manufacture. Oleuropein stability was also tested during the extended storage of milk at 4°C, proving that the molecule remained intact. Furthermore, no metabolites were detected in any screened UV wavelength, indicating that no decomposition of this compound occurred. It must be noted that according to previous reports and patents, the addition of some phenolics to milk prior to heat treatment enhanced its storage stability (Morgan et al., 1971). In addition, according to O'Connell and Fox (2001) the ability of various phenols to improve milk processability is attributed to their interaction with milk proteins. In particular, Sarker et al. (1995) reported that catechin, a green tea polyphenol, increases the volume and improves the foaming properties of β-lactoglobulin, while phenol-rich extracts or purified phenols such as caffeic acid markedly increase the heat stability of milk at 140°C (O'Connell and Fox, 1999).

11.5 YOGURT ENRICHED WITH PURE OLEUROPEIN

11.5.1 QUANTITATION METHOD VALIDATION

The HPLC-UV method was evaluated through the determination of oleuropein in novel yogurt preparation, displaying a linear relationship between oleuropein response (measured as peak area) and the corresponding concentration. The correlation coefficients were higher than 0.990 ranging from 0.997 to 0.9990. The data revealed good precision and accuracy with the lowest values corresponding to the lower quantities of oleuropein (0.02 mg/g). LOD and LOQ were 0.003 and 0.009 mg/g, respectively,

while recovery in the yogurt samples was close to 75%, the lower value obtained at the lower concentration.

All yogurt control extracts used in the experiments were oleuropein free, as determined by HPLC and LC-MS analysis.

11.5.2 YOGURT MANUFACTURE AND STORAGE

The fermentation period of the milk enriched with oleuropein was found to be normal (2.45 h), a value comparable to the control milk. Though oleuropein is considered as an antimicrobial agent, in this study it did not inhibit the growth of lactic acid bacteria when they were incubated together. In previous studies, it has been reported that oleuropein has an antimicrobial activity against brine lactic acid bacteria *Lactobacillus plantarum*, *Lactobacillus brevis*, *Leuconostoc mesenteroides*, and *Pediococcus cerevisiae* (Fleming et al., 1973). However, no data exist regarding the ability of oleuropein to inhibit the growth of yogurt bacteria.

According to HPLC results, the concentration of oleuropein determined by HPLC after 4.5 h incorporation in yogurt was similar to the processed milk. Fermentation for several hours did not cause any degradation to oleuropein, suggesting that this molecule was unaffected by starter bacteria. It must be noted that Giafardini et al. (1994) and Marsilio and Lanza (1998) studied the ability of an oleuropein degrading strain of *L. plantarum* to grow in the presence of oleuropein. They found that in the absence of glucose, oleuropein incorporation in the cultivation medium caused complete degradation of derivative products, whereas in the presence of glucose oleuropein remained almost intact in the cultivation medium. This observation indicates that oleuropein is degraded unless there is another carbon and energy source such as glucose, which may be utilized more readily. McCue and Shetty (2005) also demonstrated that other phenolics were also not affected by yogurt microflora in the presence of kefir culture during yogurt production from soymilk.

Moreover, the acids produced during lactic acid fermentation did not affect oleuropein. After 4.5 h of storage the pH of yogurt containing oleuropein was 4.45, a value comparable to control, indicating that oleuropein neither supported nor impeded lactic acid production.

The stability of oleuropein was also tested during extended storage of yogurt at 4°C. Oleuropein was found to be chemically stable in acidic conditions up to 35 days of storage (a slight reduction after the 27th day was not considered as statistically important). Chemical stability was also confirmed using qualitative LC-MS analysis.

Concerning the bacterial population of the manufactured milk and yogurt during storage, the following data were collected. The average total viable microflora in raw milk was 4.78 log cfu/mL. After heat treatment, it was 2.20 log cfu/mL and after 4 and 7 days storage at 4°C it was 3.45 and 4.64 log cfu/mL, respectively. Viable microflora in the 0-day yogurts was 8.71 log cfu/g. After 14 days, the average counts dropped to 8.33 and at the end of the refrigerated storage they dropped to 7.52 log cfu/g. All yogurt samples, however, contained 10^7–10^8 cfu/g for the entire period of 35 days. In general, there were no significant differences for total microflora among milk or yogurt samples with or without oleuropein.

11.5.3 PH MEASUREMENTS AND SENSORY CHARACTERISTICS

After manufacture and storage, the quality of the two products was evaluated by determining their pH values and sensory characteristics. Concerning the pH, a similar pattern was observed between the test and the control products, since the pH value was 6.45 in milk and was not markedly changed after

storage for 7 days at 4°C. In the case of fresh yogurts, the pH was 4.45 and diminished to 4.29 and 4.24 after 15 and 35 days of storage, respectively.

The sensory acceptability of the novel milk and yogurt was also tested immediately after their preparation and after 7 and 35 days of storage. The two selected concentration levels of oleuropein (0.1 and 0.2 mg/mL or g) gave preparations equally acceptable with the control ones ($P > .05$), since changes in yogurt taste were not observed (Table 11.1).

Many researchers have reported that the addition of phenolic compounds into dairy products alters their organoleptic properties. In some cases, phenols were also responsible for distinct off-flavors caused by protein interaction through Maillard reactions (Parks and Allen, 1973; Walker and Manning, 1976; Luck et al., 1994) or oxidation after the heat treatment of milk (Dumont et al., 1974), or even when they were added as flavoring agents (Maga, 1988). Thus the effect of phenols as functional ingredients on the quality of dairy products has been advocated and attributed to the protein–polysaccharide–phenols interaction. The extent of this interaction depends on the pH, the molecular properties of phenols, or the presence of specific polysaccharides. The enzymatic oxidation to quinones may also play an important role as well (O'Connell and Fox, 2001). The sensory scores of milk and yogurt prepared for the experiment, after 7 and 35 days of storage, respectively, were also determined. It should be noted that the mean scores for taste and color gradually decreased, while the mean scores for texture increased in yogurts as the storage progressed. Generally, the oleuropein-based yogurts were firmer than control yogurts. These changes in sensory characteristics were similar with the control samples. Nevertheless, all the products were acceptable to the sensory panel, characterized as "very good," and none of them had any off-flavor.

Table 11.1 Sensory Analysis of Yogurts Incorporated With Oleuropein During Storage at 4°C (Zoidou et al., 2014)

Criteria	Storage Period (days)	Yogurt Code		
		A	B	C
Texture	1	$8.01^{a, e}$	$8.06^{a, e}$	$7.93^{a, e}$
	7	$8.33^{a, f}$	$8.50^{a, f}$	$8.24^{a, f}$
	35	$8.70^{a, g}$	$9.10^{a, g}$	$8.68^{a, g}$
Taste	1	$7.11^{a, f}$	$6.71^{a, f}$	$7.16^{a, f}$
	7	$7.07^{a, f}$	$6.69^{a, f}$	$7.14^{a, f}$
	35	$6.18^{a, e}$	$5.57^{a, e}$	$6.52^{a, e}$
Color	1	$8.52^{a, f}$	$8.30^{a, f}$	$8.51^{a, f}$
	7	$8.29^{a, f}$	$8.26^{a, f}$	$8.30^{a, f}$
	35	$8.04^{a, e}$	$7.97^{a, e}$	$8.14^{a, e}$
Total	1	$23.64^{a, f}$	$23.07^{a, f}$	$23.60^{a, f}$
	7	$23.69^{a, f}$	$23.45^{a, f}$	$23.68^{a, f}$
	35	$22.92^{a, e}$	$22.82^{a, e}$	$23.34^{a, e}$

A, B: yogurt with 0.1 and 0.2 mg/g oleuropein respectively; C: yogurt without oleuropein.
"a" means in row at the same storage time with a common superscript do not differ significantly (P > .05, LSD test).
"e, f, g" means in column at different storage time with a common superscript do not differ significantly (P > .05, LSD test).

11.6 YOGURT ENRICHED WITH OLIVE LEAF EXTRACT

Having the positive results from the study of yogurt enriched with pure oleuropein we proceeded to the next step where the yogurt was enriched with olive leaf extract. Following a similar method of manufacture and storage as in the first set of experiments we studied the changes in composition, pH, acidity, rheological, and microbiological and sensorial parameters during manufacture and storage. It should be noted that in the case of yogurt enriched with olive leaf extract we investigated more in depth the impact on rheological characteristics. Besides the health benefits related to the nutritional profile and the presence of live microorganisms, rheological characteristics play a very important role in sensory evaluation and in consumer acceptability. The most typical defects of yogurts are low viscosity and reduced firmness or syneresis and liquid consistency (Domagała et al., 2013), and for this reason the impact of the added extract on those specific factors was thoroughly studied.

11.6.1 YOGURT COMPOSITION, PH, AND ACIDITY

The average composition of 0-day yogurts (Table 11.2) revealed that the enrichment with olive leaf extract did not cause any difference in fat. However, total solids and protein content were higher in enriched yogurt compared to the control because of the addition of the extract.

The enriched yogurt also presented higher pH and titratable acidity values (Table 11.3) resulting in a buffering capacity effect. The titratable acidity and pH values had a normal variation during storage and after 21 days the pH was similar and within the acceptance range of 4.30–4.40 in both yogurts.

Table 11.2 Composition of Control Yogurt and Yogurt Enriched With Olive Leaf Extract

Product	Fat (%)	Protein (%)	Total Solids (%)
Enriched yogurt	3.70	3.30	12.50
Control	3.60	3.05	11.65

Table 11.3 pH, Acidity, and Rheological Parameters of Control Yogurt and Yogurt Enriched With Olive Leaf Extract During Storage at 4°C

Product	Control				Enriched Yogurt			
Days of storage	1	7	14	21	1	7	14	21
pH	4.38	4.31	–	4.30	4.46	4.41	4.38	4.40
Acidity (°D)	72	80	–	85	98	78	82	85
Firmness (g)	113	–	–	138	128	151	159	169
Viscosity (cps × 1000)	360			420	380	440	460	460
Water-holding capacity (%)	49		41.88	44.01	49.6		42.83	45.24

11.6.2 RHEOLOGICAL PARAMETERS

Texture is a very important characteristic of yogurts. It is closely linked with the yogurt inner structure, which finally determines the overall quality of the yogurt. The results of the rheological analysis of the yogurts are presented in Table 11.3. The effect of the extract used for the enrichment was significant for all the rheological parameters of yogurt evaluated.

11.6.3 SYNERESIS

Syneresis is the shrinkage of the gel, which then leads to whey separation (Lucey, 2004). Syneresis occurs because of the loss of the ability of the yogurt gel to retain all of the serum phase because of weakening of the gel network.

Syneresis index was estimated by the ability of fresh yogurts to retain the serum. Increased whey expulsion from both yogurts was observed with time (Fig. 11.3).

The separation proceeded in two stages. A rapid rate of whey loss within the first 5–10 min was followed by a slower rate for up to 30 min. During this period the rate of whey drainage was higher for the enriched yogurt. Next, the separated whey increased slowly and after 1 h the relative volume was 44% and 41% for the enriched and the control yogurts, respectively. Approximately 87% and 80% of the whey drained over the 24-h period was drained within 1 h. The syneresis was leveled off at 3 h and was the same (~50%) for both yogurts. The whey drained from the enriched yogurt was very clear while the other was cloudy.

Syneresis was also estimated by the WHC during all storage time. On the 0-day yogurts the WHC was 49%–49.5%. The WHC values decreased during storage. However, the enriched yogurt presented higher WHC than the control yogurt, 42.83% compared to 41.88% after 14 days and 45.24% compared to 44.01% after 21 days.

Casein micelles aggregate through isoelectric precipitation by the action of lactic acid bacteria. During storage the casein strands can be broken and syneresis occurs. Manufacturers attempt to prevent these defects by increasing the total solids content of milk by adding milk ingredients and stabilizers (Sodini et al., 2005; Domagała et al., 2013; Roumanas et al., 2016; Sakkas et al., 2016). Polyphenols have a significant affinity for proteins that lead to the formation of soluble complexes, which can grow in size and even form sediments. Most models suggest that protein–polyphenol complexes are formed

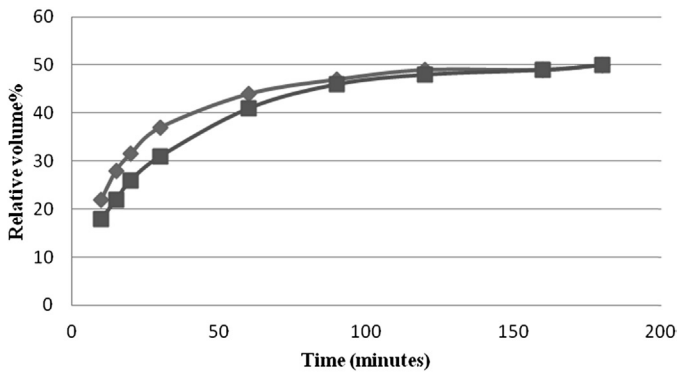

FIGURE 11.3

Separation of whey by draining yogurt: (■) enriched with olive leaf extract and (♦) control.

by multiple weak interactions (mainly hydrophobic) between the amino acid side chains and the polyphenol aromatic rings, indicating that the association of polyphenols with proteins is mainly a surface phenomenon. However, sometimes these interactions could be complemented by hydrogen bonding, which reinforces and stabilizes the complexes (Charlton et al., 2002; Oliveira et al., 2001).

Sastry and Rao (1990) reported that the bonds between polyphenols and proteins are more effective, promoting the formation of a protein network with smaller pores and greater ability to increase water-binding capacities, and at low pH the dissociation of proteins have more binding sites. These polyphenol–casein stable complexes, because of the interaction of protein and polyphenol, may be the reason for the improved syneresis in enriched yogurts with polyphenols, as was estimated by WHC.

11.6.4 FIRMNESS AND VISCOSITY

Firmness and viscosity values increased for both yogurts during cold storage. Yogurts enriched with olive leaf extract developed higher firmness and viscosity throughout the storage period. This positive effect is highly dependent on the higher content of total solids, proteins, and on the type of proteins (Oliveira et al., 2001). A higher protein rearrangement occurred in the enriched yogurt because of the protein–phenolic compound interactions. The effect of these interactions on structural properties has been illustrated by several studies (Ozdal et al., 2013). Sometimes these interactions create stable complexes with stronger internal bonds maximal at the isoelectric point of the proteins (O'Connell and Fox, 2001) and increase also the molecular weight of the proteins (Rawel et al., 2005).

11.6.5 MICROBIOLOGICAL CHARACTERISTICS

Olive leaf extract is a known antimicrobial agent, so it was important to check the survival of yogurt microorganisms in enriched yogurts (Chouchouli et al., 2013; O'Connell and Fox, 2001). It was found that the enrichment of yogurt with olive leaf extract did not cause change in the populations of lactic acid bacteria compared to the control, as also happened in the case of pure oleuropein. The counts of yogurt bacteria increased in the first 7 days, then decreased during cold storage in a similar manner in control and enriched yogurt indicating that the enrichment did not affect their viability. Both yogurts presented a high number of lactic acid bacteria throughout storage above the recommended level of 10^7 cfu/g (Table 11.4) (Codex Alimentarius, 2011). Yeasts and molds were not found.

Table 11.4 Bacteria During Storage of Control Yogurt and Yogurt Enriched With Olive Leaf Extract

Product	Control				Enriched Yogurt			
Days of storage	1	7	14	21	1	7	14	21
Thermophilic cocci[a]	3.28×10^8	7.7×10^8	2.19×10^8	2.13×10^8	3.36×10^8	7.5×10^8	2.83×10^8	2.71×10^8
Thermophilic bacilli[b]	2.05×10^7	2.76×10^7	2.22×10^7	1.39×10^7	9.7×10^6	1.17×10^7	9.54×10^6	1.08×10^7
Yeasts–molds	0	0	0	0	0	0	0	0

[a]Streptococcus thermophilus.
[b]Lactobacillus delbrueckii *ssp.* bulgaricus.

11.6.6 ORGANOLEPTIC PROPERTIES

Regarding the organoleptic properties, both yogurts showed no differences in texture. The addition of the olive leaf extract reflected a bitter taste and a slight sharp after-taste of olive leaf, which, however, did not change the natural yogurt flavor. It should be noted that a light green color was observed in the enriched yogurt.

11.7 CONCLUSION

As presented herein and as shown elsewhere (Zoidou et al., 2014), the addition of oleuropein in milk and yogurt can lead to novel foodstuff preparations. For the first time we demonstrate herein the possibility of using olive leaf extract as an alternative source of oleuropein leading to products with enhanced health-protecting properties. For this purpose it was first necessary to develop and validate two efficient methods for the reliable determination of oleuropein in milk and yogurt preparations. The results indicated that oleuropein is resistant during the heating of milk. During the coagulation of milk, oleuropein was not hydrolyzed by the produced acids or metabolized by lactic acid bacteria, nor was their growth inhibited. Oleuropein was completely soluble in the selected concentrations (0.1 or 0.2 mg/mL) without adding any peculiar taste or flavor, while its stability during milk and yogurt storage at 4°C was unequivocally proven. In the case of yogurt enriched with olive leaf extract, the growth of lactic acid bacteria and pH were similar to the control during cold storage. The enriched yogurt contained more total solids and proteins. The utilization of olive leaf extract in yogurt improved its rheological properties (higher firmness and viscosity and less syneresis). Since from a technological point of view the presence of oleuropein in milk does not have any negative effect in the yogurt manufacturing process and considering the significant biological value of oleuropein was proved repetitively by numerous research reports, it is concluded that this molecule can be added as an active ingredient in milk and yogurt preparations for the production of novel functional foods with significant health benefits. Finally, the olive leaf extract may become a new convenient yogurt ingredient because it appears to improve not only its health-protecting properties but also its rheological properties.

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