Basil as functional and preserving ingredient in "Serra da Estrela" Cheese

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Running title: Basil as functional and preserving ingredient

Abstract

Antitumor, antimicrobial and antioxidant activities of basil were studied, along with its characterization in phenolic compounds, organic acids and soluble sugars. The results placed basil as a valuable candidate for functionalization and conservation of food products, maintaining their nutritional properties, while increasing their shelf life and potential health effects. The basil leaves were then incorporated in "Serra da Estrela Cheese", either in its dehydrated form or as a decoction. The cheeses were then subject to a nutritional evaluation, being characterized for their fatty acids, minerals and CIE color parameters. To assess the combined effects of plant incorporation and storage time, a 2-way ANOVA was used to process the results, further analysed through a linear discriminant analysis. Overall, basil leaves provided antioxidant activity to the cheeses, reduced the moisture, and preserved the unsaturated fatty acids and proteins. Comparing both incorporation types, the decoctions had a higher functionalizing and conservative effect.

Keywords: *Ocimum basilicum* L.; "Serra da Estrela" cheese; functionalization; shelf life increase; natural additives

1. Introduction

Consumer awareness of health implications regarding food is growing considerably, namely due to the increasing literacy of consumers and their concerns towards healthier lifestyles. This has driven the food industries to alter many food ingredients, or reduce the load of harmful constituents. The advertisement of healthier food is present in many labels, and the production of healthier foodstuffs is a growing trend. In many cases, plants, mixtures of plants or even extracted compounds are added to the food, in order to provide their beneficial properties (Jiménez-Colmenero, Carballo, & Cofrades, 2001; Neacsu, Vaughan, Raikos, Multari, Duncan, Duthie & Russell, 2015). Among the aromatic and medicinal plants available to the food industry, basil (*Ocimum basilicum* L.) has promising beneficial effects. This plant, native to the tropical regions of Asia, Africa, Central and South America, is a green herb that can reach about 90 cm of height, displaying lanceolate leaves, which are glossy and fragrant. One of the most known uses of this herb, is as a spice and ingredient among the Italian and Southeast Asian cuisines. Still, many medicinal properties are attributed to this plant, *e.g.*, pain and inflammation soother, nasal douche, cough suppressor, antipyretic, constipation and diarrhoea alleviation, wart removal and kidney malfunction treatment (Javanmardi, Khalight, Kashi, Bais, & Vivanco, 2002; Wiart, 2006; Benzie & Wachtel-Galor, 2011). Some of these ancient claims have been completely or partially confirmed through scientific studies, which revealed additional properties, namely as a diuretic and gastritis treatment (Boskabady, Kiani, & Haghiri. 2005). Its antimicrobial, antifungal, antimutagenic and antioxidant activities have also been confirmed, especially in its essential oils, which are particularly active (Berić, Nikolić, Stanojević, Vuković-Gačić, & Knezević-Vukčević., 2008; Siddiqui,Bhatti, Begum, & Perwaiz 2012; Govindarajan, Sivakumar, Rajeswary, & Yogalashmi, 2013; Flanigan & Niemeyer, 2014; Abassy,

Pathare, Al-Sabahi, & Khan, 2015). Despite all these beneficial effects, very few studies report the incorporation of basil in foodstuffs, although there are some reports. The essential oils have been added to fish gelatine films to maintain the quality of the fish (Tongnuanchan, Benjakul, & Prodpran, 2014). The seed gum of basil has also been incorporated in ice cream and cheese, to maintain their rheological, physical and sensory properties (Hosseini-Parvar, Matia-Merino, & Golding, 2015; Javidi, Razeni, Behrouzian, & Alghooneh, 2015). Still, there are no reports describing the use of the dehydrated leaves as a functional ingredient in foodstuffs.

In Portugal, "Serra da Estrela" cheese is referred as one of the most valued products. It is produced with only three key ingredients, ewe's milk, salt and milk thistle (*Cynara cardunculus* L.) to coagulate the milk proteins. Furthermore, the manufacture process remains very similar to the initial methodology. Previous studies from our research group reported the incorporation of other plant species in this cheese, namely chestnut flowers (*Castanea sativa* Mill.) and lemon balm (*Melissa officinalis* L.), to promote the shelf life, confer functional properties and maintain sensory features, with very promising conclusions (Carocho, Barreira, Antonio, Bento, Morales, & Ferreira, 2015a). In the present study, and due to its important bioactivities, basil was tested for its functional and preserving properties, in order to develop a novel food product with this natural and appreciated ingredient.

2. Materials and methods

2.1. Plant material

The company "Cantinho das Aromáticas, Lda", provided the basil leaves and stems. Decoctions were then prepared following the procedures described by Carocho, Barros, Bento, Santos-Buelga, Morales & Ferreira (2014a), 5 grams of the sample were added

to 1 L of cold water, which, after being heated to the boiling point was left for 5 minutes and finally filtered through a Whatman No.4 filter. After lyophilisation (FreeZone 4.5 Labconco, Kansas, USA), the decoctions were frozen until further analysis.

2.2. Standards and reagents

HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany). Formic and acetic acids were purchased from Prolabo (VWR International, Fontenay-sous-Bois, France). 2,2- Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Phenolic standards were bought from Extrasynthèse (Genay, France). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also other individual fatty acid isomers, and trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid). Mueller-Hinton agar (MH) and malt agar (MA) were obtained from the Institute of Immunology and Virology, Torlak (Belgrade, Serbia). Fetal bovine serum (FBS), l-glutamine, nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and Dulbecco's Modified Eagle Medium (DMEM) media were acquired from Thermo Fischer Scientific (Waltham, MA, USA). Ellipticine and sulforhodamine B were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). Micro (Fe, Cu, Mn and Zn) and macroelements (Ca, Mg, Na and K) standards (> 99% purity), as well as $LaCl_2$ and CsCl (> 99% purity) were purchased from Merck (Darmstadt, Germany). Sulphuric, hydrochloric and nitric acid were obtained from Fisher Scientific (Waltham, MA, USA) and nitric acid was purchased from Sigma (ST. Louis, MO, USA). All other reagents were purchased from specialized retailers. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Chemical characterization and bioactive properties of the natural ingredient based on basil decoctions

2.3.1. Analysis of phenolic compounds

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) as previously described by Carocho et al. (2014a). The detection was carried out with a PDA (photo-diode array detector) detector using 280 and 370 nm as the preferred wavelengths. This equipment was coupled to a mass spectrometer (MS) (API 3200 Applied Biosystems, Dramstadt, Germany) using a triple quadrupole-ion trap analyser, equipped with an ESI (electrospray ionization) source. The spectra were detected in negative ion mode, between m/z 100 and 1700. The compounds were characterized through a comparison with authentic standards (when available), but also through retention times, UV and mass spectra. For the quantification, calibration curves were used, and were injected in known concentrations, between 2.5 and 100 μg/mL. The curves equations were: caffeic acid (y=359x−488.4; R^2=0.999); quercetin-3-*O*-rutinoside (y=222.79x−243.11; R^2=0.999); quercetin-3-*O*-glucoside (y=316.48x−2.9142; R^2=1.000); kaempferol-3-*O*-rutinoside (y=175.02x−43.877; R^2=0.999); and rosmarinic acid (y=312.2x−424.06; R^2=0.999). The results were expressed in mg per g of lyophilized decoctions.

2.3.2. Analysis of organic acids

Organic acids were determined following a procedure previously described by Carocho, Barros, Calhelha, Ćiric, Soković, Santos-Buelga, Morales, & Ferreira (2015b). The detection and identification of these compounds relied on a Shimadzu (Shimadzu Corporation, Kyoto, Japan) ultra-fast liquid chromatograph, coupled to a diode array

detector (DAD) with the wavelengths set at 215 and 245 nm (for ascorbic acid). The used column was a reverse phase C18 SphereClone (Phenomenex, Torrance, CA, USA). The compounds were identified by comparison with calibration curves of commercial standards. The equations were: ($y = 9 \times 106x + 377\ 946$; $R2 = 0.994$); quinic acid ($y = 612\ 327x + 16\ 563$; $R2 = 1$); malic acid ($y = 863\ 548x + 55\ 591$; $R2 = 0.999$); shikimic acid ($y = 8 \times 107x + 55\ 079$; $R2 = 0.999$); citric acid ($y = 1 \times 106x + 16\ 276$; $R2 = 1$); succinic acid ($y = 603\ 298x + 4994.1$; $R2 = 1$); fumaric acid ($y = 148\ 083x + 96\ 092$; $R2 = 1$). The results were expressed in mg per g of lyophilized decoctions.

2.3.3. Analysis of sugars

Soluble sugars were determined by HPLC coupled to a refraction index (RI) detector as described by Carocho et al. (2015b). The detection was carried out using a high performance liquid chromatograph, coupled to a refraction index detector, with a Eurospher 100-5 NH_2 column (5 µm, 250 mm × 4.6 mm i.d., Knauer, Berlin, Germany). The identification of the compounds was achieved through the relative retention times of commercial standards, through the internal standard method. The results are expressed in mg per g of lyophilized decoctions.

2.3.4. Evaluation of the antitumor activity and hepatotoxicity

The antitumor activity was evaluated by the Sulphorhodamine B assay that has been previously described by Carocho et al. (2014b). Four human tumor cell lines were used, namely MCF7 (breast adenocarcinoma), NCI H460 (lung carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma), as well as a positive control (ellipticine). The cells were maintained as adherent cultures. For each cell line, a density of 7.5×10^3 cells/well for MCF7and HCT15, and 1.0×10^4 cells/well for HeLa and

HepG2 were pippeted into in 96-well plates, with different concentrations of the extract being pippeted on them. Hepatotoxicity evaluation was also carried out, using porcine liver, which was acquired from certified abattoirs. The followed methodology was previously described by Abreu, Ferreira, Calhelha, Lima, Vasconcelos, & Adega (2011). A phase-contrast microscope was used to monitor the growth of the cell cultures. They were sub-cultured and plated in 96 well plates (density of 1.0 × 104 cells/well). DMEM medium was used, with 10% of FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin. Ellipticine was used as a positive control, and the results were expressed in in GI_{50} values in µg/mL (sample concentration that inhibited 50% of the net cell growth).

2.3.5. Evaluation of the antibacterial activity

To determine the antibacterial activity, the methodology of Espinel-Ingroff (2001) was followed. The Gram-negative used bacteria: *Escherichia coli* (ATCC (American type culture collection) 35210), *Pseudomonas aeruginosa* (ATCC27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC (National collection of type cultures) 7973). The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined by the microdilution method, previously detailed by Carocho et al., (2015b). Streptomycin and ampicillin were used a positive controls, while 5% dimethyl sulfoxide (DMSO) was used as negative control. The results were expressed as mg/mL of the decoction extract.

2.3.6. Evaluation of the antifungal activity

For the antifungal activity, the procedure previously described by Booth (1971) was followed, using the following microfungi: *Aspergillus fumigatus* (ATCC 1022), *Aspergillus ochraceus* (ATCC12066), *Aspergillus versicolor* (ATCC 11730), *Aspergillus niger* (ATCC6275), *Trichoderma viride* (IAM (Culture Collection, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Japan) 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum* var. *cyclopium* (food isolate). The minimum fungicidal concentrations (MFC) were determined by serial sub-cultivation following the procedures by Carocho et al., (2015b). DMSO at 5% was used as a negative control, while bifonazole and ketoconazole were used as positive controls. The results were expressed in mg/mL of the decoction extract.

2.3.7. Evaluation of the antioxidant activity

The *in vitro* antioxidant activity assays were performed following the previously described methodology by Carocho et al. (2014a). The decoctions were dissolved in water at a 10 mg/mL concentration, and further diluted into sequential concentrations. DPPH radical-scavenging activity was evaluated using an ELX800 microplate reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA) and calculated in percentage after 1 hour of reaction in the dark. The reducing power (RP) assay evaluated the capacity of the extracts to reduce Fe^{3+} to Fe^{2+}, measuring the absorbance at 690nm. Inhibition of *β*-carotene bleaching (β-C) was evaluated through the *β*-carotene/linoleate assay. In this assay, the samples, by neutralizing the linoleate free radicals, avoid their contact with *β*-carotene, which reduces its discoloration. The thiobarbituric acid reactive substances assay (TBARS) used porcine brain homogenates that react with ascorbic acid to create the malondialdehyde-thiobarbituric acid chromogen. The extracts act by inhibiting the

lipid peroxidation of the ascorbate radical, through a colorimetric assay at 532 nm. The used formula was: $[(A-B)/A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively. The results of the antioxidant activity were expressed in EC_{50} value (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay).

2.4. Cheese production

The cheeses were produced in Seia, Portugal, in a certified dairy, under strict supervision of the authors. Initially, the milk, collected from ewes (Breed "Churra Mondegueira) arrived at the facility (5 ºC, pH 6.88) and was immediately placed in a reservoir to maintain the temperature. According to Martins, Belo, Vasconcelos, Fontes, Pereira and Belo (2009), ewe's milk is composed of about 11% of non-fat solids, 8% of fat, and 6% of crude protein, in which 4% is casein. Salt (30 g/L milk) and milk thistle (*Cynara cardunculus* L.) (0.4 g/L) were added to the milk, and thoroughly mixed. Each cheese used about 2.5 L of milk. The coagulation time was approximately 1 hour, after which, an automated machine pumped the cheeses into molds and pressed them to remove the serum. After some seconds of pressing, the same equipment placed the cheeses onto conveyor belts, after which they were collected by the personnel. A second pressing then took place for 2 hours, with 5 KgF of pressure being applied. After this step, the cheeses were embalmed with cloths to avoid deformation and placed in cold chambers. The first chamber varied between 95 to 100% of relative humidity and 7 to 9 degrees Celsius, while the second one varied between 80 to 82%, and 11 to 13 ºC. The cheeses remained in each chamber for approximately 15 days. Every second week they were washed with water to remove or avoid exterior contamination.

2.5. Incorporation of basil leaves and the natural ingredient based on basil decoctions

Three lots of cheese were made, one without any incorporation (control), one with the incorporation of the dried basil, and finally one incorporated with the decoction of basil. The incorporations were carried out between the first and second pressing, by manually mashing the cheese and thoroughly mixing it with the extracts. All the following steps were the same for all three lots.

In order to determine the quantity of dried plants or decoction to be added to the cheese, the EC_{50} value of the DPPH scavenging activity was used. The EC_{50} of basil decoction was 0.141 mg/mL. This value was adjusted to the quantity of milk used for each cheese, resulting in the addition of 352.5 mg of basil decoction per cheese. For the lot incorporated with dried basil, the decoction extraction yield (25.7%) was used. Therefore, 25.7% corresponded to the EC_{50} of the decoction (0.141 mg/mL), and 0.549 mg/mL corresponded to 100%. Once again, by adjusting the amount of milk used per cheese, 1.37 g of dried plant was added to each one.

2.6. Nutritional composition and antioxidant activity of the cheese lots

After approximately 1 month of maturation, the cheeses were brought to the laboratory under controlled temperature. Each lot was further divided into two sub-lots; one was placed in a fridge (5ºC and a relative humidity of 50%) with controlled temperature and humidity for 6 months, while the other sub-lot was immediately prepared for analysis. The cheese was peeled, cut into small cubes, frozen, lyophilized, and milled down. After the 6 months, the other sub-lot was subject to the same treatments, detailed below, except the antioxidant activity, which was not carried out in those lots.

2.6.1. Nutritional composition

Several nutritional parameters (moisture, proteins, fat and ash) as well as the energy value, based on the official AOAC procedures (AOAC, 2012). Moisture was determined by desiccation at a constant temperature of 100 ± 2 °C. Total protein content (N × 5.38) was calculated as nitrogen content by the Kjeldahl method, while crude fat relied on the extraction of dried samples with petroleum ether using a Soxhlet apparatus. Finally, the ash content was determined by incineration at 550 ± 15 °C.

2.6.2. Fatty acids

Fatty acids were determined by gas chromatography, using a DANI 1000 (Contone, Switzerland), coupled to a split/splitless injector and a flame ionization detector (FID), as previously described (Barros, Oliveira, Carvalho, Ferreira, 2010). The identification was carried out by comparing the relative retention times of the fatty acids methyl esters of the samples to commercial standards. The results were expresses in relative percentage of each fatty acid.

2.6.3. Mineral composition: macro and microelements

The procedure for the total mineral content followed the 930.05 AOAC methodology. The samples were incinerated at 550 ± 15 ºC, being the residue dissolved in HCl, HNO_3 and water. Regarding the microelements (Fe, Zn and Cu), these required no additional treatment and were measured directly, while the macroelements (Ca, Mg, Na and K) were diluted at a 1/10 reason to avoid interferences. The followed methodology was previously reported by Fernández-Ruiz, Olives, Cámara, Sánchez-Mata & Torija (2011), using an Analyst 200 Perkin Elmer (Perkin Elmer, Waltham, MA, USA) atomic absorption spectroscope (AAS) with air/acetylene flame. The absorbances were compared with responses of pure analytical standard solutions for AAS, made with

$Fe(NO_3)_3$, $Zn(NO_3)_2$, NaCl, KCl, $CaCO_3$ and Mg bands. The results were expressed in mg/100 g of fresh weight.

2.6.4. Antioxidant activity

The antioxidant activity of the cheese samples was only carried out for the cheeses stored for 1 month, using the same methodologies as the ones previously described in this manuscript. The DPPH assay was not carried out due to high amount of fat present in the cheese, which interferes with the results of this assay.

2.6.5. CIE color parameters

For the detection of external color, a Konica Minolta spectrophotometer (Konica Minolta, Chroma Meter CR-400, Tokyo, Japan) was used. Six readings were performed on each side of the cheese. The used Illuminant was C, at 2º, relying on an 8mm opening of the diaphragm. The CIE colour L* (lightness), a* (redness) and b* (yellowness) values were reported through the Spectra Magic Nx software (version CM-S100W 2.03.0006, Konica Minolta).

2.6.6. Statistical analysis

The three lots of cheese (control, decoction of basil, and dried basil) were labelled and two samples of each lot were subject to immediate analysis (after 1 month of maturation), while the others were stored at constant temperature for 6 months, after which they were also analysed. All the analyses were carried out in triplicate, with the data being expressed as mean±standard deviation, and maintaining the decimal places allowed by the magnitude of the standard deviation. An ANOVA with type III sums of squares was performed using the general linear model (GLM) procedure. The dependent

variables were analysed using a 2-way ANOVA, with the factors "basil leaf" (BL) and "storage time" (ST). When a statistically significant interaction (BL×ST) was detected, the two factors were evaluated simultaneously by the estimated marginal means (EMM) plots for all the levels of each single factor. Alternatively, if no statistical significant interaction was verified, the means were compared using Tukey's honest significant difference (HSD) multiple comparison test to evaluate the BL effect, or by a *t*-student test to assess the effect of ST.

Apart from comparing the effects of different natural additives formulation and storage time for the basil, the results for all parameters were compared simultaneously, aiming to verify which formulation was the most suitable, independently of the storage time. A stepwise technique, linear discriminant analysis (LDA), using the Wilk's λ method with the usual probabilities of F (3.84 to enter and 2.71 to remove), was applied for variable selection. This procedure uses a combination of forward selection and backward elimination procedures, where before selecting a new variable, it is verified whether all variables previously selected remain significant (Palacios-Morillo, Alcázar, Pablos & Jurado, 2013). With this approach, it is also possible to identify the significant variables that most contribute to the possible discrimination of a natural additive (dry material or decoction). To verify which canonical discriminant functions were significant, the Wilks' λ test was applied. A leaving-one-out cross validation procedure was carried out to assess the model performance. All statistical tests were performed at a 5% significance level using IMB's SPSS (Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA)).

3. Results and Discussion

3.1. Basil plant characterization as functional ingredient

3.1.1. Phenolic compounds of basil decoctions

The retention times, λ_{max}, pseudomolecular ion, main fragment ions in MS^2, tentative compound identification and quantification are presented in **Table 1**.

The phenolic composition of the decoction was characterized by the presence of eleven caffeoyl derivatives, as revealed by the observation of their mass spectra at *m/z* 179, 161 and/or 135 typical of caffeic acid. The other six compounds corresponded to flavonoids (quercetin and kaempferol) derivatives.

Caffeic acid (compound 2), quercetin-3-*O*-rutinoside (compound 10), quercetin-3-*O*-glucoside (compound 11), kaempferol-3-*O*-rutinoside (compound 15) and rosmarinic acid (compound 16) were positively identified according to their retention time, mass spectra and UV-vis characteristics, in comparison with commercial standards. The presence of caffeic and rosmarinic acids has been extensively reported in basil (Tada, Murakami, Omoto, Shimomura & Ishimaru, 1996; Jayasinghe, Gotoh, Aoki, & Wada, 2003; Hossain, Rai, Brunton, Martin-Diana, Barry-Ryan, 2010; Kwee & Niemeyer, 2011; Harnafi, Ramchoun, Tits, Wauters, Frederich, Angenot, Aziz, Alem, & Amrani, 2013; Vlase, Benedee, Hanganu, Damian, Csillog, Sevastre, Mot, Silaghi-Dumitrescu, & Tilea 2014; Koca & Karaman, 2015), as quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucoside (Lee & Scagel, 2009; Hossain et al., 2010; Vlase et al. 2014).

Compound 1 ($[M–H]^-$ at *m/z* 311) and 4 ($[M–H]^-$ at *m/z* 473) presented a fragmentation pattern that allowed identification as caftaric and chicoric acid (dicaffeoyltartaric acid). Both these phenolic acids, as compounds 2 and 16 (caffeic acid and rosmarinic acid, respectively) have been described by many authors as being the main phenolic acids in basil (Jayasinghe et al., 2003; Lee & Scagel, 2009; Hossain et al., 2010; Kwee and Niemeyer, 2011; Harnafi et al., 2013; Koca & Karaman, 2015).

Compounds 3, 5, 6, 7, 9, 13 and 17 were also identified as caffeic acid derivatives (trimers and tetramers) according to their UV, mass characteristics and comparison with literature (Guo, Zhan, Zhu, Fan, Wu, Liu, Yang, & Qu, 2008; Chen, Zhang, Wang, Yang, & Qang, 2011; Ruan et al., 2012; Barros, Dueñas, Dias, Sousa, Santos-Buelga, & Ferreira, 2013; Carocho et al., 2015b). The quantification of all these derivatives was made based on caffeic and rosmarinic acids calibration curves. Compound 3 ($[M-H]^-$ at *m/z* 597) was identified as yunnaneic acid F, based on its UV and mass characteristics (Chen et al., 2011; Barros et al., 2013). Similarly, compound 9 ($[M-H]^-$ at *m/z* 571) presented a fragmentation pattern similar to the one described for yunnaneic acid E (Guo et al., 2008; Carocho et al., 2015b), being tentatively associated to this compound. Compound 6 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 537, UV spectrum and fragmentation pattern consistent with the caffeic acid trimer lithospermic acid A. Salvianolic acids H/I, with the same molecular weight as lithospermic acid A, were discarded as possible identities because they present quite a different fragmentation pattern (Chen et al., 2011; Ruan, Li, Li, Luo, & Kong, 2012; Barros et al., 2013). Furthermore, the presence of lithospermic acid A in basil was already reported by Tada et al. (1996) and Lee & Scagel (2009). Compound 5 ($[M-H]^-$ at *m/z* 493) showed a UV spectrum, molecular mass and fragmentation pattern similar to salvianolic acid A, although it could not correspond to this compound, which was expected to elute later than rosmarinic acid (Chen et al., 2011; Ruan et al., 2012; Barros et al., 2013), so compound 5 was assigned as a salvianolic acid A isomer. Compound 7 ($[M-H]^-$ at *m/z* 555) was tentatively assigned as salvianolic acid K, due to a similar fragmentation pattern presented by Hauck, Gallagher, Morris, Leemans, & Winters (2014) in the identification of the phenolic profile of *Dactylis glomerata* L. Compound 13 showed a pseudomolecular ion $[M-H]^-$ at *m/z* 719 and an MS^2 majority fragment at *m/z* 359

corresponding to $[M-2H]^{2-}$; these mass characteristics coincided with those of sagerinic acid, a rosmarinic acid dimer (Barros et al., 2013; Carocho et al., 2015b). Compound 17 ($[M-H]^-$ at *m/z* 717) presented a fragmentation pattern with successive losses of 198 mu (danshensu) or 180 mu (caffeic acid) units, coherent with salvianolic acid B (also known as lithospermic acid B) (Ruan et al., 2012; Barros et al., 2013).

The remaining compounds (peaks 8, 12 and 14) corresponded to flavonol glycosides. Compounds 8 ($[M-H]^-$ at *m/z* 595) and 12 ($[M-H]^-$ at *m/z* 579), releasing a unique MS^2 fragment at *m/z* 301 and 285 ($[M-H-132-162]^-$, loss of a pentosyl and hexosyl moieties), were tentatively identified as quercetin-*O*-pentosyl-hexoside and kaempferol-*O*-pentosyl-hexoside, respectively. Compound 14 ($[M-H]^-$ at *m/z* 505) also gave one MS^2 fragment at *m/z* 301 (quercetin; $[M-H-42-162]^-$, loss of an acetylhexoside moiety), being assigned as quercetin-*O*-acetylhexoside. As far as we know, these compounds have not been described previously in basil samples, either.

Rosmarinic acid followed by chicoric acid were the main phenolic derivatives in basil, as already described by other authors (Kwee & Niemeyer, 2011; Harnafi et al., 2013; Koca & Karaman, 2015). Otherwise, quercetin-3-*O*-rutinoside was the most abundant flavonoid.

3.2. Organic acids and soluble sugars of basil decoctions

Organic acids and sugars are displayed on **Table 2**. Regarding the content of organic acids, analyzed by UFLC-DAD, oxalic, quinic, malic, shikimic and citric acids were detected. The most abundant was quinic acid, followed by oxalic acid, and the less abundant was shikimic acid. Comparatively, the decoctions of basil showed a higher quantity of oxalic, quinic, shikimic, and citric acids than decoctions of lemon balm and chestnut flowers (Carocho et al., 2014a, 2015b). Only three soluble sugars were

detected, two monosaccharides, glucose and fructose, and one disaccharide, sucrose (**Table 2**). The most abundant one was fructose. Compared with other plant decoctions, basil showed much lower concentrations of sugar than lemon balm and chestnut flowers (Carocho et al., 2014a, 2015b).

3.3. Bioactivities of basil decoctions

In **Table 3**, the various bioactivities are detailed, namely the antitumor, antimicrobial and antioxidant activity. In terms of the antitumor activity, after screening the decoctions against various tumor cell lines, the most sensible line was HepG2. Furthermore, no hepatotoxicity was detected for the PLP2 (non-tumor porcine liver cells). Still, these results for HepG2 cell line were better than the ones for the same line using chestnut flower decoction (Carocho, Calhelha, Queiroz, Bento, Morales, Soković, & Ferreira, 2014b).

In terms of antimicrobial activity, the basil decoctions were tested against various Gram negative and Gram positive bacteria, and fungi, all classified as food contaminants. Both the MIC's and MBC (MFC for fungi) were determined. Regarding bacteria, the most sensitive one to basil decoctions was *Listeria monocytogenes*, for which the MIC was below the value obtained in the positive controls. *Staphylococcus aureus* also showed more sensitive to the effects of the decoction (lower MIC value) than to streptomycin, whereas *Salmonella typhimurium* and *Enterobacter cloacae* were more sensitive to the decoctions than to ampicillin. Fungi were generally more susceptible to the decoctions than bacteria. *Trichoderma viridae* and *Penicillium ochlochloron* were more sensitive to the basil than the positive controls, both for the MIC and MFC. *Aspergillus ochraceus*, *Aspergillus niger* and *Penicillium funiculosum* were better

inhibited by basil than by ketoconazole for both MIC and MFC, and bifonazole for the MIC.

Finally, regarding the antioxidant activity, the decoction of basil proved to have high activity, especially for the TBARS assay, with the lowest EC_{50}, which was better than the obtained for lemon balm. Furthermore, the reducing power assay was also better than the one displayed for chestnut flowers infusions. However, the assayed basil decoctions did not score as effectively in the β-carotene bleaching inhibition assay (Carocho et al., 2014b, 2015b).

3.4. Nutritional composition and antioxidant activity of the analyzed cheeses

Table 4 shows the proximate composition, mineral contents and color parameters of the three lots of cheeses (non-incorporated, incorporated with dehydrated basil, and incorporated with basil decoction), as well as for the two storage times; no storage (T0) and six months of storage (T6). Each section of the table is subdivided in two parts; basil leaves (BL) (dehydrated leaf or decoction) and storage time (ST), both including the control cheese. In all cases, the results are presented as the mean of each BL for both storage times, and also the mean value of ST considering all the different incorporation types. This approach was selected in order to help identify the optimal ST independently of the incorporated material, and also the best incorporation type regardless of ST. Thus, the standard deviation should not be interpreted as a measure of accuracy of the applied methodologies, since it encompasses the results obtained from samples prepared in different conditions (variation of the non-fixed factor: ST or BL). Furthermore, the interaction between these two effects was also evaluated. When a significant interaction was found ($p < 0.05$), no multiple comparisons could be

presented. In those cases, the influence of each individual factor was drawn from the estimated marginal means (EMM) plots (**Figure S1**).

Regarding the proximate composition, moisture was the most abundant parameter, followed by fat and proteins. A significant interaction was found for all parameters (BL×ST) ($p < 0.05$), thus, general tendencies were extracted by the EMM plots. As expected, moisture tended to decrease over the course of the 6 months of cheese maturation, while fat and protein content consequently showed a significant increase. Considering the BL effect, moisture and proteins did not undergo significant changes, oppositely to fat, ash and energy values. In the EMM, it could be concluded that the decocted samples, followed by the dehydrated basil incorporated ones, lost more moisture over the course of the 6 months (**Figure S1 A**), and that both the decoctions and the dehydrated plant incorporations preserved the proteins (**Figure S1 B**) and ash, while also rising the energetic value.

Regarding mineral composition, Ca and Na were the most abundant elements, whilst Cu and Fe were the scarcest ones. For all minerals, the interaction among factors was significant, so general tendencies could only be drawn from the EMM plots. In terms of BL, Na and Zn did not show statistically significant differences, in line with the observed for Fe content when evaluating the effect of ST. The remaining elements were trendily higher in samples stored for 6 months, most likely due to a concentration effect induced by the moisture decrease. Likewise, functionalized samples tended to present higher mineral contents than the corresponding controls.

In terms of the color parameters, it is clear that L^* and b^* decreased along the ST, while a^* rose, meaning that the cheese became darker whilst increasing the magenta and blue hues. For L^* and a^*, the interaction of each factor was significant, contrarily to the observed for b^*, allowing to present the multiple comparison test results. Thus, it might

be concluded that the decoctions aided the cheeses to become more yellow, when compared to the other incorporations. Still, regarding BL, the effect was significant for *a** and *b**, but not for *L**. Finally, ST exerted a significant effect in all parameters. Further tendencies were extracted from the EMM plots, and interestingly, all color parameters showed the same behaviour, with the cheeses incorporated with the dried plant showing higher differences than the control and decoction incorporated cheese for *L**, *a**, and *b**. Although expected due to the color of the visible leaf parts in the cheeses, *b** showed these differences at T0 and T6, while the other two parameters showed a higher difference at T6 (**Figure S1 C**).

After analysing the individual molecules and bioactivities of the basil, and determining its suitability for the incorporation, it was manually added to cheeses in predefined concentrations. In terms of the antioxidant activity, the same assays, used for the basil decoctions were used in the cheese, apart from the DPPH (due to the high amount of fat in the samples), and this procedure was only carried out for T0 (data not shown). In all the assays, the incorporated samples showed lower EC_{50} values when compared to the control samples, meaning an increased antioxidant activity. For the lipid peroxidation inhibition assays (β-carotene bleaching inhibition assay and TBARS) the decoction showed lower EC_{50} values (6.6 and 5.8 mg/mL, respectively). While for the reducing power assay, the dried basil incorporation showed the lowest value (EC_{50}: 13.2 mg/mL), much lower than the 47.3 mg/mL recorded for the control cheese.

Table 5 shows the fatty acids profile in relative percentage. The most abundant fatty acids were palmitic acid (C16:0) and oleic acid (C18:1), while C11:0, C13:0, C14:1, C20:3, C22:0, C23:0, C24:0 and C24:1 were detected under 0.2%. As expected in dairy products, the SFA prevail, followed by MUFA. For all fatty acids, the interaction

among factors was significant, so trends were extracted from the EMM plots. Regarding BL, C6:0, C16:0 and C18:3 were the least affected molecules, as indicated by the lack of a significant effect ($p > 0.05$). In the case of ST effect, C12:0, C14:0, C18:1, C20:0 and C20:4 did not vary significantly. The EMM plots allowed to uncover some particular tendencies, namely that the SFA C6:0, C8:0, C10:0, C11:0, C17:0, C24:0, but also C14:1 showed higher amounts for the control cheeses after 6 months of storage, indicating a maintenance of unsaturated ones by the decoctions and dehydrated plants, given that the fatty acids were presented as relative percentage. Inversely, C22:6, a highly unsaturated fatty acid showed higher amounts in the incorporated cheeses for T0, and after the 6 months of storage these values were maintained, proving a prevention of fatty acid peroxidation (**Figure S1 D**).

3.5. Linear discriminant analysis (LDA)

In the previous section, the changes induced by the incorporation type (dehydrated plants or decoctions) and storage time in the cheeses were analysed individually. Despite the different significant variations, the identification of explicit tendencies is easier to determine when all the parameters are evaluated simultaneously. Therefore, two LDA's were applied, where "incorporation type" and "storage time" were sequentially used as grouping factors. The significant independent variables (evaluated parameters) were selected using the stepwise procedure of the LDA, according to the Wilks' λ test. Only the parameters with a statistical significant classification performance ($p < 0.05$) were kept in the analysis.

In the discriminant model designed to verify if the incorporations (dried basil and decoction) had an overall influence in the evaluated parameters, the defined functions (plotted in **Figure 1**) integrated 100% of the observed variance (first: 86.6%; second

13.4%). Among the tested variables, those selected as having significant discriminant ability were proteins, ash, *a**, *b**, Na, K, Zn, C11:0, C14:0, C17:0, which indicates them as the most affected variables in function of the selected incorporation. Function 1, which was better correlated with C11:0 and C14:1 separated the incorporated samples (both types) from the control samples. Function two, more correlated with the color parameters *a** and *b**, was particularly effective in separating the two types of incorporated cheeses, as it can be seen on **Figure 1**.

Regarding the storage time, the single defined function also included 100% of the variance. Among the tested variables, the ones with the highest discriminant ability were fat, *L**, Fe, Na, C13:0, C18:2, C20:4, C23:0, C24:0 and C24:1, being *L** and C18:2 the ones with the highest contribution, which indicates that cheese is prone to suffer a decrease in unsaturated fatty acids and to also undergo an apparent darkening effect.

Conclusions

The antioxidant, antimicrobial and antitumor properties of basil were fully verified. Considering these advantageous results and the large dissemination of basil consumption throughout the world, this plant proved to be a suitable candidate for incorporation in cheese. The addition of the decoctions and dehydrated basil leaves provided a strong antioxidant activity to the cheeses (lower EC_{50} values in all assays). In terms of the nutritional value, the incorporated cheeses lost higher amount of water after the six months of storage with a higher loss for those incorporated with the decoctions. Besides this increased dehydrating effect, the incorporated cheeses did not display greater number (or more profound) cracks on the surface (in fact they were rather low/small). The improved maintenance of the proteins over the 6 months by the

incorporated cheeses might be related to the higher antimicrobial activity, hampering the growth of proteolytic bacteria (the causing agents of undesirable odours and off flavours). Another proof of the antimicrobial capacity of basil was identified through visual assessment (**Figure S2 A**), as indicated by an interior contamination for a control cheese, while the incorporated samples did not show contamination during the course of the 6 months.

Regarding the color parameters, the changes induced by the dehydrated basil were expected, given the green color of the leaves (**Figure S2 B**), which might be attractive to new consumers. For the sceptic consumers, the changes in the samples incorporated with the decoction were barely noticeable (only a brighter yellow).

The minerals, as expected, raised overtime in all samples, due to the loss of moisture. Regarding the fatty acids, SFA showed in general higher values in the control cheeses after 6 months. Since these are given as relative percentages, this would mean higher relative amounts of unsaturated ones in the cheeses incorporated with basil, as it was verified in some particular PUFA.

Ultimately, the incorporation of basil decoctions to the cheese seemed to be better to prevent lipid peroxidation than the dried plant, helping to preserve the proteins and unsaturated fatty acids and accelerating moisture loss. However, dehydrated basil incorporation improved the results regarding the reducing power assay and provided a potentially advantageous exterior appearance. All in all, decoctions could be suitable as natural food additive for preservation and functionalization in this matrix, while the dried plants, apart from these effects could be a desirable ingredient given the color changing capabilities.

Conflict of interest

The authors declare that they have no conflict of interest regarding this manuscript.

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Table 1. Rt, λ_{max}, mass spectral data, tentative identification of phenolic compounds in basil decoctions.

Compound	Rt (min)	λ_{max} (nm)	Molecular ion [M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Content (mg/g lyophilized decoction)
1	5.0	328	311	179(100),149(83),135(72)	Caftaric acid	6.10±0.04
2	11.0	324	179	135(100)	Caffeic acid	1.69±0.04
3	13.5	274,334sh	597	579(8),359(13),295(13),179(21)	Yunnaneic acid F	0.45±0.07
4	14.8	330	473	313(20),293(28),179(86),149(100),135(34)	Chicoric acid	7.77±0.02
5	15.3	282,328sh	493	295(100),267(4),197(8),179(13)	Salvianolic acid A isomer	2.64±0.01
6	15.4	278.328sh	537	493(57),313(20),295(100),179(47),135(22)	Lithospermic acid A	1.97±0.01
7	16.7	276	555	537(3),511(3),493(39),311(10),269(20),197(36),179(29),135(100)	Salvianolic acid K isomer	3.26±0.49
8	17.0	352	595	301(100)	Quercetin-*O*-pentosyl-hexoside	0.27±0.01
9	17.5	268	571	527(100),483(8),439(11),329(9),311(14),285(61),241(72),197(50),179(53),135(60)	Yunnaneic acid E	6.43±0.07
10	18.9	356	609	301(100)	Quercetin-3-*O*-rutinoside	1.73±0.02
11	20.2	356	463	301(100)	Quercetin-3-*O*-glucoside	0.28±0.01
12	20.4	350	579	285(100)	Kaempferol-*O*-pentosyl-hexoside	0.48±0.01
13	20.8	283,332	719	359(100),197(8),179(18),161(50),135(7)	Sangerinic acid	0.97±0.02
14	21.6	350	505	301(100)	Acetyl quercetin-3-*O*-hexoside	0.93±0.03
15	22.3	348	593	285(100)	Kaempferol-3-*O*-rutinoside	0.51±0.03
16	23.6	328	359	197(100),179(94),161(87),135(68)	Rosmarinic acid	26±1
17	27.6	282,338sh	717	537(13),519(100),493(8),339(39),321(92),295(23),279(7),197(3)	Salvianolic acid B	0.85±0.04
					Total phenolic acids	58±1
					Total flavonoids	4.21±0.05
					Total phenolic Compounds	62±1

Table 2. Content in organic acids and soluble sugars in the basil decoctions. Results presented as mean±SD.

Organic acids	Content (mg/g lyophilized decoction)
Oxalic	98±4
Quinic	180±5
Malic	54±3
Shikimic	2.92±0.03
Citric	38±1
Sugars	Content (mg/g lyophilized decoction)
Fructose	12±1
Glucose	5.8±0.6
Sucrose	11.7±0.4

Table 3. Antioxidant, antitumor, hepatotoxic and antimicrobial activity of the basil decoctions. Results presented as mean±SD.

Antitumor activity	**GI_{50} values (μg/mL)**	**Ellipticine**	
MCF7	>400	1.21±0.02	
NCI H460	>400	1.03±0.09	
HeLa	254±5	0.91±0.11	
HepG2	225±5	1.10±0.09	
Hepatotoxicity			
PLP2	>400	2.29±0.18	
Antibacterial activity	**Basil decoction MIC (mg/mL) MBC (mg/mL)**	**Streptomycin MIC MBC**	**Ampicillin MIC MBC**
Staphylococcus	0.125	0.250	0.100
aureus	0.250	0.500	0.150
Bacillus	0.125	0.050	0.100
cereus	0.250	0.100	0.150
Listeria	0.125	0.150	0.150
monocytogenes	0.500	0.300	0.300
Micrococcus	0.187	0.130	0.100
flavus	0.250	0.250	0.150
Pseudomonas	0.250	0.050	0.100
aeruginosa	0.500	0.100	0.200
Escherichia	0.187	0.050	0.300
coli	0.250	0.100	0.500
Salmonella	0.125	0.050	0.150
typhimurium	0.250	0.100	0.200
Enterobacter	0.125	0.050	0.150
cloacae	0.250	0.100	0.200
Antifungal activity	**Basil decoction MIC (mg/mL) MBC (mg/mL)**	**Bifonazole MIC MFC**	**Ketoconazole MIC MFC**
Aspergillus	0.250	0.150	0.200
fumigatus	0.900	0.200	0.500
Aspergillus	0.250	0.100	0.200
versicolor	0.900	0.200	0.500
Aspergillus	0.125	0.150	1.500
ochraceus	0.250	0.200	2.000
Aspergillus	0.125	0.150	0.200
niger	0.250	0.200	0.500
Trichoderma	0.094	0.150	1.000
viride	0.125	0.200	1.000
Penicillium	0.062	0.200	0.200
funiculosum	0.250	0.250	0.500
Penicillium	0.062	0.200	2.500
ochrochloron	0.125	0.250	3.500
Penicillium	0.250	0.100	0.200
verrucosum	0.900	0.200	0.300
Antitoxidant activity	**EC_{50} values (μg/mL)**	**Trolox**	
DPPH	144±5	41±1	
Reducing power	86±1	41.7±0.3	
β-carotene bleaching inhib.	7327±72	18±1	
TBARS	40±1	23±1	

Table 4. Nutritional parameters (g/100 g fw), energetic value (kcal/100g fw), colour parameters and mineral profile of cheese samples added with basil leaves (dry material or decoction as natural additives - BL) at preparation day and after 6 months of storage (ST). The results are presented as mean±SD[1].

		Moisture (g/100 g fw)	Fat (g/100 g fw)	Proteins (g/100 g fw)	Ash (g/100 g fw)	Energy (kcal/100 g fw)
BL	None	43±7	26±2	28±3	4±1	349±27
	Dried plant	37±12	32±6	30±3	4±1	403±66
	Decoction	36±14	32±7	29±5	5±1	402±83
	p-value (n=18)	0.138	0.005	0.415	0.010	0.020
ST	0 months	50±1	25±1	25±1	4±1	328±8
	6 months	28±6	35±1	33±1	5±1	442±48
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	<0.001
BL×ST	*p*-value (n=54)	<0.001	<0.001	<0.001	<0.001	<0.001

		Ca (mg/100g fw)	Mg (mg/100g fw)	Na (mg/100g fw)	K (mg/100g fw)	Fe (mg/100g fw)	Zn (µg/100g fw)	Cu (µg/100g fw)
BL	None	985±155	63±13	1200±393	177±59	0.6±0.3	5.5±0.4	0.4±0.1
	Dried plant	1321±309	112±41	1511±378	222±39	1.0±0.2	5.7±0.5	0.3±0.1
	Decoction	1311±284	100±33	1486±487	262±62	1.2±0.5	5.3±0.5	0.4±0.1
	p-value (n=18)	<0.001	0.001	0.059	<0.001	<0.001	0.245	0.013
ST	0 months	964±95	63±9	992±316	169±36	0.9±0.3	4.8±0.3	0.3±0.1
	6 months	1447±227	120±33	1806±167	272±38	0.9±0.5	6.1±0.3	0.4±0.1
	p-value (n=27)	<0.001	<0.001	<0.001	<0.001	0.755	<0.001	<0.001
BL×ST	*p*-value (n=54)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

		*L**	*a**	*b**
BL	None	58±5	-1.8±0.5	22±4 b
	Dried plant	55±6	-2.5±0.3	19±3 c
	Decoction	54±2	-0.6±0.1	24±3 a
	p-value (n=18)	0.078	<0.001	0.001
ST	0 months	60±3	-2.3±0.5	25±3
	6 months	52±2	-1.2±0.5	18±2
	p-value (n=27)	<0.001	<0.001	<0.001
BL×ST	*p*-value (n=57)	<0.001	<0.001	0.163

[1]Means within a column with different letters differ significantly ($p < 0.05$)

Table 5. Fatty acids profile (relative percentage) of cheese samples added with basil leaves (dry material or decoction as natural additives - BL) at preparation day and after 6 months of storage (ST). The results are presented as mean±SD[1].

	BL				ST			BL×ST
	None	Dried plant	Decoction	*p*-value (n=18)	0 months	6 months	*p*-value (n=36)	*p*-value (n=72)
C4:0	2.3±0.1	3.1±0.3	3.2±0.5	<0.001	2.6±0.2	3.1±0.5	<0.001	<0.001
C6:0	2.6±0.1	2.5±0.2	2.6±0.3	0.789	2.4±0.1	2.7±0.2	<0.001	<0.001
C8:0	2.6±0.2	2.2±0.1	2.2±0.3	<0.001	2.1±0.2	2.5±0.2	<0.001	<0.001
C10:0	7.7±0.5	5.6±0.2	5.7±0.5	<0.001	5.9±0.5	6.8±0.5	0.002	<0.001
C12:0	4.9±0.5	3.5±0.3	3.5±0.2	<0.001	3.8±0.3	4.1±0.5	0.331	<0.001
C14:0	12±1	10±1	10±1	<0.001	11±1	10±1	0.285	<0.001
C15:0	1.5±0.1	1.2±0.2	1.2±0.2	<0.001	1.4±0.1	1.2±0.2	0.006	<0.001
C16:0	25±2	25±2	25±2	0.967	26±2	24±2	0.002	<0.001
C16:1	1.0±0.1	1.0±0.2	0.5±0.2	<0.001	1.0±0.2	0.7±0.3	0.005	<0.001
C17:0	1.0±0.1	0.8±0.2	0.8±0.1	<0.001	0.7±0.1	1.0±0.1	<0.001	<0.001
C18:0	11±1	13±2	14±1	<0.001	12±1	13±2	0.002	<0.001
C18:1	23±4	27±1	26±1	<0.001	26±1	24±4	0.096	<0.001
C18:2	2.2±0.4	1.6±0.5	1.6±0.5	0.046	2.5±0.5	1.0±0.5	<0.001	<0.001
C18:3	1.8±0.1	1.9±0.2	1.8±0.2	0.470	1.7±0.2	2.0±0.2	<0.001	<0.001
C20:0	0.27±0.02	0.32±0.03	0.32±0.02	<0.001	0.30±0.02	0.31±0.04	0.195	<0.001
C20:4	0.20±0.05	0.33±0.05	0.31±0.02	<0.001	0.27±0.02	0.29±0.05	0.373	<0.001
C20:5	0.31±0.05	0.26±0.03	0.26±0.04	0.026	0.25±0.04	0.30±0.05	0.002	<0.001
C22:6	0.18±0.05	0.38±0.05	0.29±0.05	<0.001	0.21±0.05	0.36±0.05	<0.001	<0.001
SFA	71±4	68±1	69±1	<0.001	68±1	71±3	<0.001	<0.001
MUFA	24±4	28±1	27±1	<0.001	27±1	25±3	0.046	<0.001
PUFA	4.8±0.4	4.5±0.5	4.3±0.5	0.024	5.1±0.2	4.0±0.3	<0.001	<0.001

C11:0; C13:0, C14:1, C20:3, C22:0, C23:0, C24:0, C24:1 were also detected but in amounts lower than 0.2%

Figure 1. Mean scores of cheese samples for the two discriminant functions defined from all evaluated parameters.