Research Article

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Quantitation of benzoic and sorbic acid levels from green olives by high-performance liquid chromatography

Abstract

Olive fruit is utilized in two forms: table olives and oil. In our country, food additives that are added consciously and deliberately are used in table olive production to bring the appearance and flavour of olives to the state desired by the consumer, to prevent the deterioration of olives and to extend their shelf life. In Turkey, the amount of preservatives to be added to foods should be compatible with the Turkish Food Codex. This study was carried out to determine the amounts of benzoic ands orbic acids added to extend the shelf life in pickled green olives provided from Bursa market using the HPLC method and to find out whether the findings obtained are within the legal limits envisaged in the Regulation on Food Additives. Among 100 green olive samples supplied, while sorbic acid and benzoic acid were not detected in 79 green olive samples. Sorbic acid was observed in 10 of the samples and benzoic acid was in 1 of the samples, however both acids were detected in 11 of the samples. The detected amounts of sorbic acid ranged between 49,103 and 204,989mg kg-1, while benzoic acid values were within 142,352-153,453 mg kg-1. The preservatives were not detected in the 4 green olive samples which were above 7% of the salt content, whilst they were observed in 20 of the samples with salt ratio below 7%. However their detected amounts were within the levels allowed by Turkish Food Codex.

Keywords: green olive, preservatives, salt, benzoic acid, sorbic acid

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Abbreviations: IOOC, international olive oil council; WHO, world health organisation; FDA, food and drug administration of USA; TFC, turkish food codex; JECFA, The Joint FAO/WHO expert committee on food additives; FAO, food and agriculture organisation; ADI, allowed daily intake; HPLC, high-pressure liquid chromatography; NMKL, nordic committee on food analysis; PVDF, polyvinylidene fluoride; DAD, diode array detector; ODS, *octadecyl* silane; RT, retention times; BA, benzoic acid; SA, sorbic acid.

Introduction

According to the International Olive Oil Council,[1] table olives are defined as "the product prepared from the sound fruits of varieties of the cultivated olive tree (*Olea europaea* L.) having reached appropriate degree of development for processing that are chosen for their production of olives whose volume, shape, flesh-to-stone ratio, fine flesh, taste, firmness and ease of detachment from the stone make them particularly suitable for processing and treated to remove its bitterness and preserved by natural fermentation, and/or by heat treatment, and/or by other means so as to prevent spoilage and to ensure product stability in appropriate storage conditions with or without the addition of preservatives".

Olive fruit can only be consumed after de-bittering procedures which are carried out for removal or degradation of oleuropein, the bitter phenolic glucoside found in all olives in varying amounts, into non-bitter compounds to obtain an acceptable palatable product with improved and ensured sensory characteristics and safety.[1–7] The main methods used for de-bittering are direct immersion of the olives in acidified brine for the diffusion of the oleuropein from the olive flesh into the surrounding brine or treatment with dilute NaOH solution with alkali hydrolysis removal of oleuropein can be achieved rapidly in a matter of hours, however, keeping in brine takes a long time. In recent years, various de-bittering techniques with promising results have been investigated, such as the involvement of fermentation starter culture (lactic acid bacteria and yeasts) having the ability to excrete endogenous enzymes such as esterases, hydrolases and β-glucosidases,[8–16] power ultrasound treatment at different sodium hydroxide concentrations and temperatures,[17] high hydrostatic pressure treatments singly or in combination with natural antimicrobials,[18] or oven/hot/semi-drying.[6,19–22]

The olive fruit can be picked at any stage from the beginning of ripening, when it is green, until it is black and fully mature.[1] The fruits obtained during the period of maturation prior to colouring and in normal sizes are called "green olives." Natural green olives can either directly submerged in brine without any NaOH treatment[23] or treated with lye,[24,25] after which spontaneous fermentation takes place due to the activity of the microbiota present on the olive surface and in the processing plant environment or starter added fermentation. The acidic conditions of the brine result in diffusion or chemical hydrolysis of oleuropein.[26–29]

NaCl plays an important role to improve flavour and increase microbiological stability and safety of the final table olive.[4,30,31] The current practise for the salt ratio used for brining is 10-12% (w/v). Since the excessive consumption of salt has been related to health concerns, such as hypertension and cardiovascular diseases,[32] the reduction or partial substitution of NaCl in fermentation brines of table olives is advisable. Thus, the Turkish Food Codex (TFC) Communiqué on Table Olives have stated that the maximum salt ratio in natural green table olives should be 7% (w/v).

A "food additive" is a natural or artificially synthetic chemical used to *improve* the *sensory properties* (colour, smell, taste) of food to extend shelf life and to reduce nutritional and quality losses, which is involved in production, processing, packaging and/or storage without being a major ingredient.[33–35] All the chemical substances added in order to extend the shelf life of the foods by preventing or retarding deterioration of microbial activity which also result in loss of nutritive value, but does not include salt, sugar, vinegar, spices, herbicides and insecticides are known as "preservatives" or "chemical preservatives".[36,37] The effectiveness of preservatives depends on the pH of the medium, buffering properties, the composition of the medium and the water activity value, the spectrum of activity of the antimicrobial substance, the amount and duration of activity and the number and characteristics of microorganisms present in the medium. The targets of antimicrobials are the walls and membranes of the cells they affect, the protein synthesis system, genetic systems and enzyme systems.[38,39]

Among these substances, benzoic and sorbic acids and their salts are commonly used in foods, pharmaceuticals and cosmetics[40–44] as antifungal and antibacterial agents, however, their usage vary within certain levels. In Turkey, the use of preservatives determined and advised by JECFA (The Joint FAO/WHO Expert Committee on Food Additives) are followed and regulated by maximum permitted levels for different foodstuffs. According to the FAO/WHO recommendations, the daily intake of benzoic acid is specified as 0-5 mg $kg^{-1}$and sorbic acid as 0-25 mg kg^{-1} body weight.[45–47] Benzoic acid (C_6H_5COOH) and its salts are colourless crystalline solid, simplest aromatic carboxylic acid with a benzene ring that are naturally found in blackcurrant, plum and clove. It is not accumulated in the tissues and is rapidly metabolised. The microbial inhibition is dependent on the inactivation of the cell wall and some enzymes in the cell, and the efficient pH is between2.5-4.0.[48] Sorbic acid is a straight-chain monocarboxylic acid which is naturally found in lactone form in the unripe berries of the *Sorbus aucuparia* L. (rowan tree).[49,50] It is a colourless solid that has been used for many years due to its good stability and excellent solubility in water.[51,52] It is mostly preferred in acid and medium acid foodstuffs with a pH range of 3-6[53] to inhibit the growth of fungi and bacteria during storage by inactivating their intracellular enzymes, besides providing easy application.[54,55]

Despite their important function, numerous studies have pointed out that *excessive* consumption of preservatives could lead to a number of serious *health* issues, such as allergies, urticaria,[56] metabolic acidosis, convulsions and hyperpnoea in humans,[57–59] behavioural disorders (i.e. hyperactivity and attention deficit/hyperactivity disorder),[60,61] cancer,[62] cardiovascular heart disease, aside with being toxic and genotoxic when consumed above allowed daily intake (ADI) values.[63,64]

In Table 1 olive processing fruits softening could occur during fermentation by oxidative yeasts and fungi, fermentative moulds and low salt concentrations.[4] Benzoic acid and sorbic acid and their salts are used either alone or in combination at levels 0.5 to 1 g kg^{-1}, particularly for naturally fermented olives packed without heat treatment[65] to control microbial growth. In addition the maximum salt content in table olives is specified as 7% (w/v) in Turkish Food Communiqué on Table 1 Olives,[66] and this could not be evaluated as the only factor for preventing spoilage. Therefore, this study was carried out i) to determine the levels of sorbic acid and benzoic acid and their salts in pickled green olives presented for consumption in Bursa using the HPLC method, ii) to understand whether the findings were within the legal limits envisaged by Turkish Food Communiqué,[66] and iii) to monitor uncontrolled preservative usage that could compensate for low-NaCl content in brine while maintaining safety margin.

Table 1 Occurrence and concentration levels of benzoic and sorbic acids in pickled green olives

Sample	Number of samples preservatives were detected			Concentration of BA (mg kg^{-1})			Concentration of SA (mg kg^{-1})			Concentration of BA+SA (mg kg^{-1})		
	BA	SA	BA+SA	Min	Max	X+SD	Min	Max	X+SD	Min	Max	X+SD
Delicatessens(21)	0	4	5	0	0	0	66,159	203,219	115,286± 9,179	46,445	197,131	137,0556± 27,050
Marketplaces(17)	0	1	3	0	0	0	74,172	92,478	85,011± 8,524	123,029	258,157	180,650± 33,7100
Supermarkets(62)	1	6	2	142,352	153,453	148,568± 4,655	49,103	204,989	148,868± 8,734	53,537	368,809	198,277± 29,524

Materials and methods

Collection of samples

A total of 100 samples were obtained from pickled green olives sold either in various delicatessens (21%), marketplaces (17%) and supermarkets (62%) in Bursa, Turkey, were analysed. The sampling was performed on three consecutive days and the triplicate samples were analysed in triplicate.

Chemicals used

Benzoic and sorbic acids, certified reference material with >99.9% purity; and all the other reagents (i.e. methanol, silver nitrate ($AgNO_3$), potassium bi cromate (K_2CrO_4), glacial acetic acid, sodium hydroxide (NaOH) and Carrez clarification reagents were of analytical purity or for chromatographic use) have been purchased from Sigma-Aldrich, Dorset, UK. The stock solutions and the corresponding dilutions were made in ultra-pure water and were stored in dark places between the experiments at +4°C.

HPLC analysis

The determination of benzoic and sorbic acids were performed according to the NMKL124 method.[67] The acetate buffer was prepared by dilution of 5.7mL glacial acetic acid in 900mL ultra-pure water, the pH was adjusted to 4.7 with 5 N NaOH, made up to 1 L and filtered through 0.45µm membrane filter. For mobile phase 300mL methanol and700 mL acetate buffer were mixed in a 1 L volumetric flask and filtered through a filtration unit (0.45µm).

The olives were de-pitted and homogenised. To the weighed 5±0.0001 g of homogenised olive in a 50mL volumetric flask 2mL of Carrez reagent was added. Following purification the volumetric flask was filled up with 30% water: methanol (v/v) solution. The extracted sample was filtered through Whatman™ Grade 1 qualitative cellulose filter paper, and thereafter passed through a PVDF 0.45µm membrane filter and degassed. All extracts and solutions were freshly prepared prior to HPLC analysis.

Evaluation of benzoic and sorbic acids was performed on a high-performance liquid chromatography (Flexar HPLC System; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) equipped with a diode array detector (DAD) and SPHERI-5 ODS 5UM column (PerkinElmer, MA, USA; 4.6mm×250mm i.d., 5µm particle size). The mobile phase used for chromatographic separation of benzoic and sorbic acids was mixture of acetate buffer and methanol (70:30, v/v). The analysis was carried out isocratically at a flow rate of 1mL min^{-1}, and effective separation and quantification were completed in 6 minutes. The temperature of the column oven was adjusted to 30°C and an automatic injection system was used. The injection volume was 20µL. The detection of benzoic and sorbic acids were carried out at the wavelengths of maximum absorption of the compounds, 235 and 254 nm, respectively. Six parallel analyses were performed for each sample. From the standard stock solutions (1000 mgL^{-1}), prepared by dissolving benzoic and sorbic acid standard substances in methanol: water mixture (40:60, v/v), standard calibration solutions in various concentrations were used for determination of retention times (RT) and quantitation. The following formula was used to calculate the levels of benzoic and/or sorbic acids in the pickled green olive samples as mg kg^{-1}.

$$E = \left(F / B\right) x\, S\, x\, Z \quad (1)$$

Where E, Amount of the preservative in the sample (mg kg^{-1}); F, Peak area of the sample; B, Peak area of the standard; Z, Standard concentration (mg kg^{-1}); S, Dilution coefficient (M/K); M, Completed volumetric water content (mL); K, Amount of the sample weighed at the start (g).

In addition to the determination of the benzoic and sorbic acids in pickled green olives pH and salt contents were determined to compare with the Turkish Food Communiqué on Table 1 Olives.[66]

Results and discussion

In modern food technology with the increased production of processed and convenience foods, among various protection methods chemical protection is becoming a common application for retention of food quality and prevention of deterioration to ensure the consumption of safe food with high nutritional value and extended shelf life. Preservatives could reduce and/or prevent nutritional losses due to microbiological, enzymatic or chemical changes that may occur in foods, in particular by lowering down the pH value and settling the redox potential of the food.[39,68–70] Due to reduction and limitation of NaCl content in table olive processing one could say that chemical preservatives are efficient alternative to prevent spoilage. However, since their excessive use could lead to long term health hazards for consumers their levels in foods and use should strictly be controlled. The occurrence and concentration level of benzoic acid and sorbic acid in the analysed 100 pickled green olive samples from 21 various delicatessens, 17 marketplaces and 62 supermarkets in Bursa province samples were given Table 1.

As shown in Table 1, in the 21 green olive samples obtained from the delicatessens, the average amount of sorbic acid detected was 115,286±9,179mg kg^{-1}, whereas in samples from the marketplaces it was 85,011±8,524mg kg^{-1} and 148,868±8,734mg kg^{-1} in samples from supermarkets. As a result of the HPLC analysis benzoic acid was not detected in samples of delicatessens and marketplaces, nevertheless, of the 62 green olive samples from the supermarkets the average amount of benzoic acid was 148,568±4,655mg kg^{-1}.

In 79 green olive samples neither sorbic acid nor benzoic acid was observed, whereas in 10 of the samples only sorbic acid in 1 sample only benzoic acid and in 11 of the samples both sorbic and benzoic acids were detected. The levels of sorbic acid ranged between49,103 and 204,989 mg kg^{-1}, while the benzoic acid levels were found as 142,352-153,453 mg kg^{-1}. In samples that were detected to contain both sorbic and benzoic acids the levels ranged within 46,445-368,809 mg kg^{-1}. According to TFC Regulations on Food Additives, the allowed maximum level of benzoic acid and sorbic acid that can be added to green olives is 1000 mg kg^{-1}.The levels of benzoic and sorbic acids detected in pickled green olives, either used alone or in combination, were found to be lower than the maximum permitted limits.

Koyuncu[71] reported that benzoic acid was detected neither in black nor green olives, whilst the amount of sorbic acid was 199,00 mg kg^{-1} for black olives and 47,00 mg kg^{-1} for green olives. In another study conducted on 25 black olive samples obtained from different markets in İzmir, Turkey, in 3 of the samples sum of potassium sorbate and sodium benzoate content was detected above the permitted limits of Turkish Food Communiqué on Table Olives [66] whilst was below in the rest of 22 samples.[72] Tokat[73] stated that in 81 black olive samples benzoic acid and in 58 of the samples sorbic acid was not detected. Neither sorbic nor benzoic acid was not detected in 58 samples; however, in 23 of the samples sorbic acid, in 19 of the samples both benzoic and sorbic acids were detected. The amount of benzoic acid in the samples varied between 55,52±10,23 and 452,20±31,80 mg kg^{-1}, whilst sorbic acid ranged between 22,19 2,09 and 451,22±23,87 mg kg^{-1}. The researcher mentioned that the amounts of benzoic acid, sorbic acid and benzoic acid+sorbic acid did not exceed the legal limits specified in the Turkish Food Communiqué on Table Olives.[66]

Sorbic acid and benzoic acids are generally more effective at low pH since they both are in an undissociated form, and thus, penetrate through cell membranes more easily.[64] The pH of samples was between 2.61 and 6.53 (Table 2). In the 100 green olive samples, the salt ratios obtained as a result of salt determination were in the range of 1,285-10,337g 100 g^{-1}, and the average salt content was 4,113±1,444 g100g^{-1}. In 4 green olive samples with salt content was found to be higher than the limits permitted in Turkish Food Communiqué on Table Olives[66] and the average salt content was 8,935±1,409g 100g^{-1}. In the 96 samples, the salt ratio was lower than the limits permitted in Turkish Food Communiqué on Table Olives,[66] with an average of 3,913±1,044g 100g^{-1}. In 21 sorbic acid containing green olive samples the average salt ratio was 4,338±1,099g100 g^{-1}. In the 11 benzoic acid containing samples the average salt ratio was 4,385±1,376g 100g^{-1}.

Table 2 pH and salt contents of pickled green Olives

pH				Salt Content		
	Min	Max	X + SD	Min	Max	X + SD
Delicatessens	2,86	4,62	3,662±0,493	2,774	7,475	4,195±1,379
Marketplaces	2,61	5,36	3,574±0,606	2,161	5,489	4,088±1,099
Supermarkets	2,68	6,53	3,764±0,796	1,285	10,337	4,093±1,572

Tokat[73] expressed that only eight black olive samples were found to have salt content higher than the maximum allowed level of 7% given in the Turkish Food Communiqué on Table Olives.[66] The amount of salt in olive samples were found between 2.04% and 13.02%. The differences between the salt amounts in the samples and the purchasing place were not significant at $p < 0.05$.[75–77]

Conclusion

In conclusion, sorbic and benzoic acid were not detected in the 4 green olive samples which were above 7% of the salt content among the green olive samples studied. Sorbic and benzoic acids were detected in 20 of the samples which were determined to contain a salt ratio below 7%. In accordance with these data, it was determined that green olives with reduced salt ratios contained sorbic and benzoic acid as preservatives at the levels allowed by the codex. However, it is necessary to take necessary measures to ensure the continuous monitoring of the use of food additives at a level that is not threatening public health.

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Conflicts of interest

All authors declare that they have no conflict of interest.

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