UTILIZATION OF OZONE FOR THE IMPROVEMENT OF *MENTHA PIPERITA* L. QUALITY BY REDUCTION OF MICROBIAL LOAD AND IMPACT OF THE PROCESS ON THE HERB PROPERTIES

– Research paper –

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Abstract: Herbs can be contaminated with microorganisms, which affects their quality while increasing the risk of food poisoning. Thus, there is a need for efficient decontamination techniques. In this study, the microbial load was reduced in peppermint herb (*Mentha piperita* L.) exposed to ozone at 10 ppm for 60 minutes. The quality of the ozonated herb was assessed by the determination of microbial load and essential oils content. Total microbial content was reduced by 4 log cfu g⁻¹ of the microorganisms whereas the contamination with molds was reduced from 10.07 log cfu g⁻¹ of the microorganisms to zero. Moreover, sensory evaluation of the herb aroma was conducted. These characteristics were not significantly affected by the treatment. Therefore, ozone can be used to improve the peppermint herb quality by reduction of the microbial contamination of peppermint herb while having no adverse effect on the herb aroma.

Keywords: quality management, ozone, herbs, essential oil content, microbial load

INTRODUCTION

Herbs are often used raw as spices, pharmaceutical or cosmetic products. Due to the limited shelf life of fresh herbs, they are often dried. However, due to the fact that spices in general are prepared from plants grown in tropical and subtropical regions with high humidity and temperatures, one can observe significant bacterial and fungal contamination; microbial loads as high as 10⁸ cfu g⁻¹, (colony forming unit per 1g of the product) (Schweiggert et al., 2005; Akbas and Ozdemir 2008). Despite the fact that the easiest way to inactivate fungal and microbial activity is the reduction of water content, drying procedure is insufficient to deal with viable spores (Brodowska et al., 2014). Assessment of the microbial quality of raw plant resource requires the determination of presence/absence of pathogens e.g. *Escherichia coli*, *Salmonella*, *Shigella* spp, *Listeria monocytogenes*, etc., that cause food poisoning.

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The amount of non-pathogenic microorganisms should also be determined because they can be responsible for the deterioration of sensory attributes of the product (Doğan and Gürler 2018). The microbial contamination is mainly a result of the exposition of different plant parts with external environment during cultivation and the plant susceptibility to infections during drying and storage (Song et al., 2014). Moreover, microbial contamination of herbs depends on which parts of the plant undergo processing.

The microflora of the plant material may also depend on its origin due to the presence of microorganisms naturally inhabiting plants (epiphytic microflora) and the environmental pollution (soil, water or air) (Brodowska et al., 2014). Epiphytic microflora is predominantly Gram-negative bacteria belonging to the genera *Pseudomonas*, *Enterobacter*, *Flavobacterium*, *Alcaligenes*, *Aerobacter*, *Chromobacterium*, *Spirillum*. The group of Gram-positive bacilli include spore of *Bacillus*, whereas the typical

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molds belong to the genera of *Alternaria*, *Fusarium*, *Cladosporium*, *Penicillium* (Kunicka-Styczyńska and Smigielski, 2011). According to the studies reported by (Wójcik-Stopczyńska et al., 2009), the herbal spices that are available on the Polish market, contain a high number of mesophilic bacteria, e.g., up to 6 log cfu g⁻¹, and thermophilic bacteria up to 5.18 log cfu g⁻¹. Bacterial contamination varies greatly, which depends on the type of herb and manufacturer (Wójcik-Stopczyńska et al., 2009).

Decontamination techniques should enable the inactivation of various pathogens, while having none, or only a marginal impact on the product quality. The concentration of essential oils, that is a key element for determination of the quality of herbal resource, can change during the herb treatment with procedures involving water vapor.

Ozone is a powerful oxidant, that is well known for its antimicrobial activity (Akbas and Ozdemir 2008; Alexopoulos et al., 2013; Alwi and Ali, 2014; Khadre et al., 2001; Smilanick, 2003; Sung et al., 2014; Torlak et al., 2013; Wani et al., 2015; Wysok et al., 2006). However, utilization of oxidants such as ozone may pose a potential threat to the terpenes contained in the volatile fraction of spices, such as black pepper (Zhao and Cranston, 1995) and other.

The composition of essential oils and their content is a very important parameter determining the quality of peppermint (*Mentha piperita* L.). The

quality of the herb can be assessed based on the Essential oils content inside the herb, which is around 3% w/w. Some compounds contained in the essential oils fraction in peppermint herb are: α -pinene, β -pinene, menthone, menthol, carane, β -burbonen, β -caryophyllene, germacrene-D (Adaszyńska et al., 2013). Terpenes that are the basic components of essential oils are susceptible to degradation by ozone molecules, e.g., d-limonene, α -terpinene and α -pinene were reported to be degraded by ozone molecules present indoors (Kephalopoulos et al., 2007; Weschler and Shields, 1999). Other properties, as colour of spice can also be affected (Brodowska et al., 2014).

The most sensitive tool for the evaluation of the peppermint quality is smell. Very often, variations of sensory properties occur for food components even though they do not differ in other properties determined by instrumental analysis. The goal of this research was to improve the quality of the pepper mint herb using ozone by reduction in microbial load while preserving standard properties of the plant. To determine the impact of gaseous ozone on the properties of treated herb, the analysis of essential oils content and its composition, moisture content, pH of infusions and the sensory analysis of aroma of the essential oils and infusions prepared from peppermint prior and after ozone treatment were performed.

MATERIALS AND METHODS

Reagents and substrates

Peppermint herb that was acquired from a local producer in a neighborhood of Rzeszów (southern Poland). The microorganisms present in plant material were analyzed in Department of Food Chemistry and Toxicology University of Rzeszów. For that purpose several media e.g., Czapek solution agar and Nutrient Agar (BTL Ltd., Łódź, Poland), were utilized. A 70% solution of ethanol was provided by Chempur (Poland).

Ozone generator

Apparatus for the ozone treatment of plant material was fed with ozone generated by a TS 30 (Ozone Solution, Hull USA) ozone generator, and the ozone concentration was measured by a 106M UV Ozone Solution detector (Ozone Solution, Hull USA), over the range of 0-1000 ppm (Balawejder et al., 2014).

Decontamination procedure

The peppermint herb was placed into the reactor chamber of the device (Figure 1) that has already been successfully utilized for various detoxication procedures (Balawejder et al., 2014).

Figure 1. Scheme of the apparatus used for the ozone treatment of herbs

In our previous studies it was determined that optimal time of living plant ozonation should not

exceed 30 minutes. After that time on the investigated plant organisms necrosis was observed. In the case of dried plant material this time was twofold increased without such consequences. Shorter exposition times (Piechowiak et al., 2019a; 2019b) allowed satisfactory effects during the treatment of raspberry and highbush blueberry fruits. But in a case of dried plants a resistance spores could be observed, therefore we decided to increase ozonation time. Longer exposure than 60 min would be not optimal in terms of technological procedures and economical effectiveness.

Samples (~100 g) of the herb were exposed to ozone at the concentration of 10 ppm for 60 min with the gas-flow rate of 4 m³ h⁻¹ at room temperature. Prior and after the ozone treatment, a number of parameters, including: the microbial load, moisture content, sensory properties, content and composition of essential oils were determined. The experiments were conducted in triplicate.

Preparation of the substrates and cultivation of microorganisms

The microbial cultures were prepared by mixing 1 g peppermint before and after the ozonation and drying procedure with 9 mL of sterile physiological solution. Then, the sample was gradually diluted to achieve a concentration of 10⁻⁷ g/mL.

The microbiological media were prepared as follows: Czapek solution agar (BTL Łódź Poland) 50 g of the powder per 1000 mL of water and Nutrient Agar (BTL Łódź Poland) 38g of the powder per 1000 mL of water. Subsequently, those solutions were boiled and stirred for 15 min. Then, the media were cooled down and transferred into Petrie dishes (approximately 15 mL per dish).

After solidification of the media, a 0.1 mL sample of the solution containing bacteria was transferred into the Petrie dishes using the surface spread plate method. Prior and after each transfer, the spreader was treated by 70% solution of ethanol (Chempur Piekary Śląskie Poland) and heated up over the flame. Incubation was conducted under following conditions: Nutrient Agar 35 °C 48 h⁻¹, Czapek solution agar 25 °C 24 h⁻¹. The latter is suitable for cultivation of molds, which presence is undesired due to production of mycotoxins.

Composition of the essential oils

The analysis of the composition of the essential oils in the dried peppermint was performed using a Deryng type apparatus. For this purpose, a round bottom flask was filled with 50 g of the peppermint

and 400 mL of distilled water. Distillation was conducted for 1 hour (after one hour the volume of essential oil was constant), and as soon as it was accomplished, the mixture was cooled down for 30 minutes. Subsequently, the essential oils that were collected during this procedure, were dried over anhydrous magnesium sulfate and subjected to chromatographic analysis. The analysis was conducted using a Varian GC-450 gas chromatograph coupled with a MS-240 mass spectrometer. The separation of analytes was done on a capillary column Varian, (Palo Alto California USA) L(m) x ID (mm) x OD (mm) – 30 x 0.25 x 0.25. The stationary phase was VF- 5ms, film thickness (μm): 0.25. The settings of the analysis were: the temperature of injector 200 °C, the sample volume 1 μL, split 1:100, the column oven start 50 °C for 1 min, gradient 300 °C 10 min⁻¹, 20 minute isothermally. The gas flow rate was 1 mL min⁻¹ (He purity 5.0).

Essential oils content and composition

In order to verify the impact of ozone on the essential oil content and composition, the essential oils were isolated using Deryng apparatus. The analysis of the essential oils content prior and after the ozonation procedure allowed determination of possible losses of the essential oils. The calculation of their content within the peppermint herb was done according to equation 1:

$$\text{essential oil content} = \frac{a \times 100\%}{b} \quad (1)$$

where:

a – volume of essential oil [cm³],

b – mass of the pepper mint sample [g].

The calculation based on this formula allowed determining the percentage of the essential oils in pepper mint prior and after the ozonation process which was 1.24% ± 0.21 and 1.28% ± 0.07 respectively. The analysis showed no statistically significant differences in the average content of essential oils at the significance level α = 0.05 between the two experiments.

Composition of the volatile fraction

The volatile fraction of peppermint was analyzed using HS-SPME method with a PDMS fiber thickness 100 μm (Supelco Pennsylvania USA). The PDMS fiber was exposed to volatile fraction of peppermint herb for 30 minutes by placing the fiber over the herbal material. Then, the fiber was placed into the injector for 5 minutes. The settings of the chromatographic analysis were: exposition time of the solid phase: start 50 °C 5 min⁻¹, gradient 250 °C in 10 min⁻¹, 10 minutes isothermally, temperature decrease to 30 °C

(20 °C min⁻¹) 5 minute isothermally. The gas flow rate was 1 ml min⁻¹ (He).

Determination of the moisture content

The moisture content in peppermint herb was determined using a moisture analyzer RADWAG MAX 50/1. The maximal temperature during this procedure was 105 °C.

Determination of acidity and pH of the peppermint infusion

The solutions of the peppermint herb were prepared by mixing 1 g of the product and 50 mL of boiled distilled water. After 20 minutes, the infusions were transferred through a filter into a 200 mL flask and filled up with distilled water. Acidity was determined by titration of 25 mL of the prepared solution with 0.01 NaOH versus phenolphthalein as indicator. pH was measured with a CP-315 pH-meter.

Sensory analysis

The impact of the ozonation procedure on the quality of the peppermint sensory quality was determined by analyzing responses to a questionnaire that included following questions: attractiveness and intensity of the infusion smell, attractiveness and intensity of the essential oil smell, before and after ozonation. The quantity of the respondents group was 40 tutored people. The portions of 5 mL of essential oils and infusions prepared from 1 g of product and 50 mL of water were coded and presented to respondents and rated using a five grade scale. The aroma attractiveness and the intensity of aroma of coded samples of the essential oils and the infusions were rated as follows: 1-very weak/bad, 2-weak, 3-average, 4-good, 5-very well. Sensory analysis was conducted according to PN-ISO 4121:1998 – Polish version. The statistical analyzes were conducted using *Statistica 13 (StatSoft Poland)*.

RESULTS AND DISCUSSION

Changes of the microbial load as a result of the ozonation procedure

In our study, Czapek solution agar and Nutrient Agar substrates were utilized. The number of the microorganisms in the peppermint herb was calculated for 1 g of the product and presented as log cfu g⁻¹.

It was observed that the ozone treatment was effective in inactivation of the microorganisms, in particular molds, which can be grown on Czapek solution agar substrate. On that substrate an initial number of 10.07 log cfu g⁻¹ of the microorganisms was detected, whereas after the ozonation procedure no microorganisms were cultivated.

The ozone treatment at the ozone concentration of 10 ppm resulted in decontamination of peppermint herb from molds. This is very important observation due to the fact that absence of molds can stop the generation of mycotoxins that are products of the mold metabolism. The presence of mycotoxins can cause the ban of the utilization of contaminated plant material.

In case of microorganisms growable on the Nutrient Agar medium, it was shown that ozonation allowed reduction in the total microbial load from 12 to 8 log cfu g⁻¹. Prior to the ozonation procedure, a number of colonies of different colours was observed. After the ozonation process the colonies colour was white, they had uniform shape and clearly marked edges of the colony.

These colonies presumably arose from spore forms that had an increased resistance to disinfectants.

Other researchers also observed the positive impact of ozonation procedures on the reduction of microbiacal load of microorganisms belonging to various species. The reduction of the the microorganisms belonging to *Enterobacteriaceae* (2 to 3 log cfu g⁻¹) was observed by (Achen and Yousef, 2001) who observed reduction in *E. coli* for over 3 log cfu/g during utilization of ozonated water with ozone concentration of 25 mg L⁻¹ and 5 minutes long exposure for decontamination of apples. Also, Akbas and Ozdemir in 2008 utilized gaseous ozone at concentration of up to 1 ppm for decontamination of flaked red pepper. Only a marginal effect on the *E. coli* population was observed e.g., reduction of 1 log cfu g⁻¹ of the initial population (Akbas and Ozdemir, 2008). Similar results were also observed during ozonation of spinach at 1 ppm for 10 minutes (1 log cfu/g reduction) (Wani et al., 2015). However, at a higher ozone concentration (10-50 ppm) over 1 log cfu g⁻¹ reduction was observed (Wani et al., 2015). Also, the reduction of the *E. coli* counts for about 3 log cfu/g in lettuce and green bell pepper was achieved using ozonated water at ozone concentration of 0.5 mg/L after 30 min⁻¹ of washing (Alexopoulos et al., 2013). In another research Alwi and Ali in 2014 observed reduction of *E. coli* O157 and *Salmonella enterica* sv. *typhimurium* counts for about 1-2 log cfu g⁻¹ after exposing bell pepper to ozone at concentrations ranged from 0.5 ppm up to 9 ppm (Alwi and Ali., 2014).

Ozonation was also utilized for the reduction of the microbial load on oregano where after 90 minutes of ozonation the counts of *Salmonella typhimurium* were reduced by 1.88 log at 2.8 ppm and by 2.5 log at 5.3 ppm. A longer exposition of oregano to ozone (e.g., for 120 minutes) resulted in higher reduction in the counts of *Salmonella* by 2.8 log cfu g⁻¹ at 2.8 ppm and 3.7 log cfu g⁻¹ at 5.3 ppm (Torlak et al., 2013). It should be noticed that detection of pathogens should result in elimination of contaminated plant material.

Essential oils composition

The composition of the essential oils was determined based on the analysis of chromatograms (see Figure 2), using NIST8 (Mass Spectra Library) and CAS (Chemical Abstracts

Service). All of the detectable compounds were summarized in Table 1. As it can be observed, the analytical procedure allowed identification of the compounds expected to be found within the essential oils fraction in peppermint herb, such as: α -pinene, β -pinene, menthone, menthol, carane, β -burbonen, β -caryophyllene, germacrene-D (Adaszyńska et al., 2013) (Table 1). It could be anticipated that some of those essential oils and in particular those that have a double bond within their chemical structure, may undergo degradation due to ozone oxidative potential. However, the data acquired by the chromatographic analysis showed that the essential oils composition is similar for the control and ozonated peppermint samples.

Figure 2. Chromatograms of the Essentials oil before and after the ozonation procedure

Table 1. Composition of the essential oils of the peppermint herb prior and after exposure to ozone

Retention time [min]	Compound name	Content before ozonation %	Content after ozonation %
5.48	α -Pinene	Trace	trace
6.11	3-Carene	Trace	trace
6.24	β -Pinene	Trace	trace
7.11	Eucalyptol	2.48%	4.81%
9.08	(E)-Menthone	45.62%	39.99%
9.17	(Z)-Menthone	9.23%	9.65%
9.23	Neomenthol	3.15%	3.33%
9.35	Menthol	28.39%	32.34%
9.57	L-Menthol	Trace	Trace
10.27	Pulegone	Trace	Trace
10.54	Piperitone	Trace	0.73%
10.69	m-Menth-3(8)-ene	Trace	Trace
10.88	Carane	8.75%	8.70%
11.16	Cyclohexene, 3-methyl-6-(1-methylethyl)	Trace	Trace
12.30	β -Bourbonene	Trace	Trace
12.80	β -Caryophyllene	0.89%	Trace
13.28	α -Caryophyllene	Trace	Trace
13.37	β -Cububene	Trace	Trace
13.61	(E)-Germacrene-D	0.96%	Trace

Comparison of the volatile fraction

Although no differences in the composition of the essential oils contained in the ozonated peppermint herb were observed, ozone could have still impact on the volatile fraction. The volatile fraction of the peppermint contains compounds that are responsible for smell of the herb. That trait is highly valued by potential consumers. Therefore, the composition of volatile fraction prior and after ozonation was analyzed using the HS-SPME method. That sampling technique involves the utilization of a fiber coated with the extracting phase (polymer or sorbent). It enables extraction of various substances from liquid or gaseous phases. This method allows determination of the presence of volatile organic compounds with high vapor pressure. These compounds can be generated in the process of decomposition of essential oil components. As ozone acts mainly on the surface, these compounds should potentially be also on the surface of ozonated plant material. HS-SPME analysis is expected to reveal their presence. All of the identified compounds are summarized in table 2 (see Table 2). Analogously to the essential oils content, the volatile fraction content was determined based on the analysis of chromatograms (Figure 3) using NIST8 (Mass Spectra Library) and CAS (Chemical Abstracts Service). Just like in the case of the essential oils composition, the analysis of the composition of the volatile fraction showed that the composition of the volatile fraction prior to and after ozonation was similar. The lack of significant differences proves that the applied ozone dose did not affect the composition of the head space of ozonated material. However, sensory analysis can often detect differences in odor that cannot be detected by instrumental methods. Therefore, the next step

was a sensory analysis of the ozonated mint and the control sample.

Moisture content of the peppermint herb

We expected that ozonation could induct the drying process, where peppermint herb was exposed on the gaseous stream for 60 minutes, might cause a mass loss. As we find out, the presence of ozone had negligible impact on the weight and moisture of the peppermint samples e.g., -10% of moisture content after ozonation and 12% without ozonation.

Acidity and the pH value of the peppermint infusion prior and after the ozonation procedure

Acidity was determined using titration of the peppermint infusions with sodium hydroxide and the pH value was measured prior and after 20 minutes of the brew. It was observed that the acidity of the infusion was constant throughout the experiment, however the pH of the infusion was slightly reduced (see Table 3).

Sensory assessment of the organoleptic properties

To assess the sensory quality of the herb prior and after the ozonation, a group of 40 consumers was asked about the perception of aroma attractiveness and intensity of the essential oils and the peppermint infusions. The results of the analysis are reported in Table 4. The statistical analysis based on the test of statistical significance of the difference between two means revealed that at the significance level $\alpha = 0.05$ no difference between the attractiveness and intensity of the assessed oils and infusions was found. This clearly confirms that the ozonation procedure had no negative impact on the sensory quality of the peppermint.

Figure 3. Chromatogram of the volatile fraction of the peppermint herb before and after ozonation procedure

Table 2. Composition of the volatile compounds of the peppermint herb prior and after exposure to ozone

Retention time [min]	Compound	Content before ozonation %	Content after ozonation %
8.12	α -Pinen	Trace	trace
8.42	Camphene	Trace	0.002
8.94	β -terpinene	Trace	0.85
9.06	2-Thujene	1.613	1.799
9.30	Bicyclo [3.1.1]heptane	0.137	1.273
10.15	S-(-)-Limonene	0.4	0.709
10.22	Eucalyptol	7.654	8.987
10.46	3-Carene	0.01	0.04
10.96	Trans-para-2-menthen	0.076	0.116
11.46	Linalool	0.423	0.423
12.37	(-)-Isopulegol	0.387	0.44
12.52	(E)-Menthone	26.932	31,160
12.66	(Z)-Menthone	8.968	10.633
12.86	Menthol	20.081	17.345
13.47	1-n-Propylcyclohexanol	0.883	0.667
13.82	Pulegone	1.308	1.1088
14.07	Piperitone	2.424	2.075
14.51	Menthyl Acetate	7.495	6.201
15.91	β -bourbobene	2.462	1.648
16.42	β -Caryophyllene	4.454	2.715
17.20	Beta-cububene	1.928	1.402
18.50	Caryophyllene oxide	0.544	trace

Table 3. Determination of the acidity and the pH of the peppermint infusions prior and after the exposition of the peppermint drought to ozone enriched atmosphere

	Time of brew	Acidity	pH value
Before ozonation	10 minutes	0.3mL 0.01M NaOH	6.63
	20 minutes	0.3mL 0.01M NaOH	6.64
After ozonation	10 minutes	0.3mL 0.01M NaOH	6.45
	20 minutes	0.3mL 0.01M NaOH	6.47

Table 4. Determination of sensory properties of the essential oils and peppermint drought infusions

Assesed properties	Average	Standard deviation
Attractiveness of the essentials oil aroma	before ozonation	3.800
	after ozonation	3.875
Intensity of the essentials oil aroma	before ozonation	4.025
	after ozonation	3.850
Attractiveness of the infusion aroma	before ozonation	3.750
	after ozonation	3.975
Intensity of the infusaion aroma	before ozonation	3.600
	after ozonation	3.675

CONCLUSIONS

The exposure of the peppermint herb to ozone enriched atmosphere resulted in reduction in various microorganisms including molds and total aerobic microorganisms.

Moreover, it had no effect on the moisture content of the peppermint drought. Also, acidity of the infusions prepared from herb did not change

during ozonation process. pH of the peppermint infusion decreased slightly as a result of ozonation. No difference in the essential oils content prior and after the ozonation procedure was found at the significance of $\alpha = 0.05$.

The chromatographic methods allowed determination of the composition of the essential oils and volatile fraction in the peppermint herb. The chemical analysis was supplemented by a

sensory assay based on the responses of 40 consumers. At the significance level of $\alpha = 0.05$ no differences within the essential oils or the peppermint infusion aroma and its intensity prior

and after the ozonation procedure were found. Therefore, it was possible to improve the quality of peppermint herb by means of ozonation without adverse effects on other parameters of the herb.

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