IMPACT OF OZONATION PROCESS ON THE CONTENT OF BIOACTIVE COMPOUNDS WITH ANTIOXIDANT PROPERTIES IN SCOTS PINE (*PINUS SYLVESTRIS* L.) SHOOTS AS WELL AS YIELD AND COMPOSITION OF ESSENTIAL OILS

– Research paper –

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Abstract: This study examined the impact of the ozonation procedure on the content of bioactive compounds in young shoots of *Pinus sylvestris* L. as well as on the yield and the composition of the essential oils and volatile fraction. The ozonation process consisted of exposure of the raw material during 5, 15 and 30 min to the ozone gas with the concentration of 10 and 100 ppm, and then it was stored for 24 hours. In the ozonated pine shoots, the content of vitamin C overall content of polyphenol content and antioxidant potential were determined. It was found that the investigated parameters increased as a result of the utilized procedure. The observed increase in the content of tested bioactive compounds was dependent on exposition time and was found to be highest for ozonated material for 15 min with ozone gas at a concentration of 10 ppm. As a result of the ozonation procedure the content of polyphenolic compounds and vitamin C content increased. Whereas it, did not have a significant effect on composition of the obtained essential oils. It was found that ozone is an effective elicitor, which allows increasing the content of bioactive compounds in pine shoots.

Keywords: *scots pine; ozonation; antioxidant activity; polyphenols; essential oils; HS-SPME;*

INTRODUCTION

Scots Pine (*Pinus sylvestris* L.) is the most popular and widespread conifer in the world. In Europe, it covers a distance of 14 000 km and stretches from Spain to Russia (Mason et al., 2000). *Pinus sylvestris* L. is very important species responsible for wood production, especially in the Scandinavian countries. It is also valued for its healing and aromatic properties and as a source of various plant materials, such as buds (*Pini Gemmae*), young shoots (*Pini Turiones*) and needles (*Pini Folium*) (Strzelecka et al., 2003; Bączek et al., 2017). Young pine shoots are a valuable source of compounds with a high nutritional value including antioxidants, polyphenols and vitamin C (Ulukanli et al., 2014). *Pinus sylvestris* L. like other coniferous species, it is also a valuable source of essential oils (*Pini Oleum*) (Matłok et al., 2020). The world production

of pine essential oils is about 40 t, and their main producers are China and Russia. These oils can be found mainly in needles and non-woody, young shoots, and their yield is about 0.87% of a dry mass (Daugavietis et al., 2002). Pine essential oils contain mainly monoterpenes and sesquiterpenes but also juniperic and sabinic acid derivatives, tannins and vitamin C (Tümenet al., 2010; Thoss et al., 2007; Judžentienė et al. 2008). These compounds, due to their properties, exhibit pharmacological activities, including: expectorant, antispasmodic, antimicrobial, warming, diaphoretic, diuretic and choleretic. Pine oils are also used in the cosmetics and perfumery industries (Motiejūnaitė et al, 2004; Kohlmünzer, 2010; Kędzia et al., 2012).

The market value of aromatic herbal raw materials, including pine shoots, depends on a number of quality parameters. One of the most important is the microbiological indicator (Tanko et al., 2005; European Spice Association, 2013). There is a number of methods that have been developed to reduce the microbial load of aromatic raw materials,

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but it seems that only the use of ozone is the most promising technique because it leaves no residue and is environmentally friendly, compared to other methods (Brodowska et al., 2014a; Torlak et al., 2013; Rice et al., 2017). Nevertheless, essential oils contained in aromatic raw material may be susceptible to reaction with ozone, as reported by Weschler and Shields (1999). Therefore, it is important to verify the optimal doses of ozone that can cause oxidative stress and, consequently, stimulate the production of antioxidants such as

polyphenols and vitamin C while maintaining the efficiency of essential oils that can be obtained from plant resources (Birben et al., 2012).

There is a literature gap on the ozonation process of pine shoots and its impact on the plant material. Therefore, the aim of the presented research was investigation of the impact of ozonation process of young pine shoots (*Pinus sylvestris* L.) on the content of bioactive compounds and the efficacy and composition of the essential oils.

MATERIALS AND METHODS

Plant materials

The plant resource used in the experiment comprised young shoots of Scots pine (*Pinus sylvestris* L.).

The material was as described by Matłok et al. (2020).

The ozone treatment of the plant material

Samples of pine shoots ($500 \text{ g} \pm 5$) were placed into the ozonation chamber described by Antos et al. (2018) and Gorzelany et al. (2016). This device was used in combination with the Ozone Solution TS 30 ozone generator, and the ozone concentration was measured with the 106M UV-Ozone Solution detector, with a measuring range of 0 –1000 ppm. Pine shoots were exposed to gaseous ozone at the concentration of 10 ppm and 100 ppm for 5, 15 and 30 minutes with $4 \text{ m}^3 \text{ h}^{-1}$ flow rate of ozone. The ozonation process was carried out at constant temperature of 20°C.

After the ozonation process the pine shoots samples were placed in container and stored at temperature of 25°C for 24 hours.

The experiments were performed in triplicate. Next, the material was subjected to further analysis.

Total phenolic content assay, antioxidant activity

The total polyphenolic content was determined using the Folin-Ciocalteu reagent method as described by Matłok et al. (2020).

The free radical scavenging activity was determined by ABTS and DPPH according to Matłok et al. (2020).

Ascorbic acid content

The determination of ascorbic acid content was determined using a procedure described by Matłok et al. (2020).

Head Space–Solid Phase Microextraction HS-SPME

The control and the ozonated material were subjected to Head Space - Solid Phase Microextraction Analyses that were performed according to the procedure described by Matłok et al. (2019). The composition of the compounds desorbed from SPME fibre was examined using gas chromatograph (GC-MS, Varian 450GC coupled with 240 MS).

Isolation and chromatographic analysis of the essential oils

Essential oils were isolated from plant material ($200 \text{ g} \pm 1$) with the use of steam distillation method in Deryng apparatus (Farmakopea Polska V, 2002) as described by Matłok et al. (2020).

Composition of the desorbed compounds was examined using gas chromatograph Varian 450 GC with the mass detector (GC-MS) Varian 240 MS as described by Matłok, et al. (2019).

The compounds were identified based on NIST.08 and Willey data base. The composition of the essential oils isolated from the material was examined using a gas chromatograph Varian 450 GC, as described by Matłok et al. (2020).

Statistical analysis

A one-way analysis of variance (ANOVA) and multiple pairwise Tukey test was applied in Statistica 13.

RESULTS AND DISCUSSION

Pine shoots mass reduction

Ozonation process of *Pinus sylvestris* L. young pine shoots resulted in the mass reduction of ozonated plant material (Figure 1). It was observed that the mass reduction of ozonated pine shoots increased with an increasing of the exposure time. The highest reduction was observed for the ozone dose of 10 and 100 ppm and exposition time of 30 minutes. The difference in the observed mass reduction may be attributed to convective drying where the air with ozone surrounds plant material, and thus causes its drying, which in consequence reduces its mass. Mass loss during convection drying was confirmed by several tests (Matłok et al. 2019).

Total phenolic, antioxidant activities and ascorbic acid content

Ozone is highly reactive form of oxygen which mainly decomposes to reactive oxygen species (ROS) such as superoxide anion radical, hydrogen peroxide and hydroxyl radical in contact with herbs surface. The occurrence of oxidative stress in plant cells is one of main causes of reduced quality of plant products including nutritional value. Such changes can be observed by determination of the total antioxidant potential (Zhou et al., 2014). Therefore, in the presented research the impact of different ozonation conditions on the total antioxidant potential of young pine shoots was determined. The antioxidant potential was determined against synthetic radicals, such as ABTS and DPPH. Moreover, the level of polyphenolic compounds and the vitamin C content were determined.

As presented on Figure 2, the ozonation process resulted in the change of antioxidant status of young pine shoots. The exposition of plant material to gaseous ozone at the concentration of 10 ppm for up to 15 minutes of the exposition time resulted in an increase in antioxidant activity and in polyphenol and vitamin C content. During the time of storage 24 h the analysed parameters begun to increase. In the case of a higher ozone dose e.g., 100 ppm, the analysed parameters showed similar tendency but the effect is less spectacular.

Recent reports indicate that antioxidant potential of ozonated plant resources can be correlated with ozonation conditions (Horvitz et al, 2014). A number of research indicate that utilization of mild ozonation conditions results in activation of defensive mechanisms as a consequence of oxidative stress in plant resources. In research by Piechowiak et al. (2019), ozonation of raspberries with an ozone concentration of 8-10 ppm during storage at room temperature for 72 hours resulted in activation of enzymes participating in the neutralization of ROS, e.g., superoxide dismutase and ascorbate peroxidase.

Moreover, it was indicated that an increased level of flavonoids including anthocyanins and the total antioxidant capacity in ozonated raspberries fruits resulted from an increased activity of phenylalanine ammonialyase – an enzyme participating in the biosynthesis of polyphenol compounds. Similar observations were reported in paper by Chen et al. (2019). There, the authors concluded that an increased level of polyphenol compounds in strawberries exposed to ozone at the concentration of 5 ppm for 10 hours resulted from an increased expression of genes encoding phenylalanine ammonialyase.

Roach et al. (2019) concluded that an increased level of activity of the defence mechanisms responsible for reaction against to oxidative stress caused the reduction in accumulation of ROS in ozonated tissue of coriander that was exposed to ozone at the concentration of 0.68 mg L⁻¹ for 10 minutes.

Therefore, an increase of antioxidant activity of young pine shoots after ozonation observed in this study can be attributed to increase of activity of the defence mechanisms responsible for detoxication of ROS, including enzymes responsible for synthesis of antioxidants with a low molecular mass. However, after the maximal antioxidant status was achieved in case of both ozone concentrations, the procedure resulted in gradual decline in levels of compounds that could react with ABTS and DPPH, which was also clearly seen with vitamin C concentration.

Figure 1. Impact of ozonation process on the mass reduction of *Pinus sylvestris* L. shoots.

Figure 2. Impact of ozonation process on the antioxidant status of *Pinus sylvestris* L. On the figure: A - antioxidant activity against DPPH radicals, B- antioxidant activity against ABTS radicals, C- total phenolic content, D - total ascorbic acid content. Mean values with the same lower case are not significantly different to each other according to the T-Tukey test ($\alpha=0.05$).

Figure 3. Impact of ozonation process on the efficiency of essential oils with *Pinus sylvestris* L. shoots

Yield of essential oils

To analyse relations between the ozone dose (ozone concentration and exposition time) and the yield of essential oils expressed as [ml 100 g⁻¹ d.m.] that were isolated from ozonated pine shoots, their yield was determined (Figure 3). It was concluded that the application of a various ozone doses caused an increase of the content of some biologically active compounds in *Pinus sylvestris* L. pine shoots but have only slight impact on yield of isolated essential oils. It was observed that the highest yield of essential oils was 0.586 mL 100 g⁻¹ d.m. and was obtained from ozonated pine shoots that were exposed to ozone gas at the concentration of 100 ppm and an exposure time of 30 minutes. This yield was 11.8% higher than the yield of control sample. An increased yield of essential oils from raw plant materials that were subjected to ozone treatment was also reported by Dąbrowska et al. (2017). Biosynthesis of essential oil compounds, as a secondary metabolites, could be induced with another bioactive compounds synthesis as a result of ozonation process. There it was observed that under the impact of ozone treatment only slight change in the qualitative and quantitative composition of essential oil occurred.

Chemical composition of essential oils

Essential oils contain metabolites generated during vegetation of plants. The composition of the essential oils that can be obtained from a specific plant depends mainly on plant variety, plant localisation and local environmental conditions. In this manuscript we described the composition of essential oils that were analysed after isolation from *Pinus sylvestris* L. from same geographic localization. Essential oils are mainly composed of unsaturated hydrocarbon derivatives that are susceptible to strong oxidizing agents, including ozone. This has been shown in a number of works

in which direct olefin ozonolysis was carried out. It has been shown that direct effect of ozone during the exposure of pine shoots does not affect the level of volatile substances contained in them. For this purpose, isolation and analysis of essential oils were performed. It was found that the main components of the oil isolated from pine shoots are: 3-carene, D-limonene and α -pinene. Judžentienė et al. (2008) stated that these compounds are typical components of oils isolated from *Pinus sylvestris* L. pine shoots. Different ozone doses and variable raw material exposure times were used in our studies. The first stage of assessing the impact of the ozonation process on the quality of the pharmaceutical raw material was the initial analysis of the yield of essential oils. At this stage, it showed no significant differences. The next step was qualitative and quantitative analysis using the GC-MS method (see Figure 4, Table 1). Also this analysis showed no significant differences. Oils obtained from the ozonated raw material did not show the presence of oxygen artifacts or potential oxidation products (see Table 1). This confirms that ozone did not directly oxidize cellular components. This observation is highly important in relation to results of other authors who observed changes in the yield and composition of essential oils in ozonated plant material. Brodowska et al., 2014 observed that during the ozonation procedure the essential oils yield increased. The performed analysis of the essential oils composition showed the presence of new originally absent compounds. It was shown that the presence of those compounds originate from oxidation of unsaturated fatty acids and belong to aldehydes (Brodowska 2014b). The use of ozone in the proposed doses did not show phytotoxic properties, but effectively increased its therapeutic quality by activating metabolic pathways of antioxidant biosynthesis, such as polyphenols and vitamin C.

Chemical composition of HS-SPME

Analysis of the composition of essential oils showed no effect of ozonation on volatile substances isolated from the plant material. This indicates that ozone was mainly surface-active. Pine shoots as well as other green morphological parts of *Pinus sylvestris* L. are covered with resinous substances, which are also rich in volatile substances. These parts are exposed to ozone. Due to potentially generated oxygen derivative components of the raw material, its aroma character may change. An effective method of verifying the presence of this type of artifacts is HS-SPME analysis. Before starting the ozonation process, the volatile fraction typical of fresh raw material was determined.

Similarly to the essential oil, the main components of the volatile fraction of this raw material are: 3-carene, D-limonene and α -pinene, however these compounds are present in different proportions (see Figure 4, Table 1). The analysis of the ozonated raw material showed that the signal intensity of individual components as well as the quantitative composition of the volatile fraction is without significant differences. A similar signal height indicates the lack of mineralization of volatile components located on the surface of the ozonated raw material, and the lack of differences in their composition precludes the ozonolysis of unsaturated terpenes.

Figure 4. Chromatograms for essential oils isolated from the ozonated (10 ppm, 15 min) *Pinus sylvestris* L.

CONCLUSION

The ozone treatment induced a significant metabolic response in pine shoots. It was found that the antioxidant potential, polyphenol content and vitamin C increased after the ozonation procedure. However, this process is limited by ozone dose. The best response to the treatment was observed for the exposure to ozone at the concentration of 10 ppm. Optimal ozonation time with the gaseous ozone at the mentioned concentration was 15

minutes. It should be noted that effects of ozonation under proposed conditions were not phytotoxic. This phenomenon was confirmed by the lack of artifacts in the composition of essential oils, as well as the slight impact of ozonation process on their yield. This observation indicates a lack of direct oxidation of cellular components. It has been found that ozonation is an effective elicitor which increase the content of bioactive compounds in the pine shoots. The pine shoots obtained in this way can be a pharmaceutical material with a composition that is unique in nature

Table 1. Chemical composition of headspace fractions and essential oils isolated from ozonated of pine shoots.

№	RT [min]	Peak share in the chromatogram [%]														Ordinary substance name	No CAS	Indices Referenci Kováčs
		Fresh			10 ppm			100 ppm										
		SPME	OIL	SPME	OIL	SPME	OIL	SPME	OIL	SPME	OIL	SPME	OIL	SPME	OIL			
1	7.448	10.68	9.631	9.16	7.976	8.10	8.097	1.07	9.212	23.08	7.095	17.43	9.598	14.17	8.849	α -pinene	80-56-8	920
2	7.780	-	0.433	-	0.624	-	0.610	-	0.673	-	0.613	-	0.739	-	0.613	camphene	79-92-5	937
3	8.400	-	1.338	-	1.611	-	1.404	-	1.393	-	0.794	-	1.111	-	1.154	β -terpinene	99-84-3	969
4	8.476	6.61	4.369	4.85	3.988	6.46	3.088	0.38	4.111	8.96	4.040	13.85	5.251	4.19	4.175	α -fenchene	471-84-1	973
5	8.831	4.00	4.804	4.01	3.398	4.31	3.720	11.21	4.288	8.35	3.321	4.26	4.138	11.49	4.267	β -pinene	127-91-3	991
6	9.303	62.73	32.715	73.95	40.785	74.07	36.858	65.88	35.105	40.02	27.956	43.79	32.973	53.86	30.667	3- carene	13466-78-9	1016
7	9.660	6.71	10.916	7.42	7.752	0.74	8.524	14.18	6.398	13.92	7.226	15.16	7.890	10.89	8.202	D-limonene	138-86-3	1034
8	10.004	0.87	0.899	trace	0.761	trace	0.685	trace	0.774	0.90	0.899	1.18	0.996	1.04	0.912	(E)- β -ocimene	3779-61-1	1052
9	10.219	0.09	0.776	trace	0.837	trace	0.770	0.79	0.807	0.51	0.731	trace	0.762	trace	0.774	γ -terpinen	99-85-4	1063
10	10.806	8.30	4.152	0.61	5.159	6.34	4.754	6.49	4.098	4.26	4.160	4.32	4.350	4.28	3.961	terpinolen	586-62-9	1093
11	12.062	-	0.191	-	0.238	-	0.189	-	0.238	-	0.198	-	0.264	-	0.230	(S)-cis-verbenol	18881-04-4	1158
12	12.178	-	0.158	-	0.165	-	0.138	-	0.192	-	0.160	-	0.207	-	0.177	(1R)-(-)-myrtenol	19894-97-4	1164
13	12.368	-	0.551	-	0.501	-	0.512	-	0.552	-	0.663	-	0.614	-	0.590	(-)-terpinen-4-ol	20126-76-5	1173
14	12.578	-	0.303	-	0.203	-	0.226	-	0.256	-	0.384	-	0.394	-	0.289	(-)- α -terpineol	10482-56-1	1184
15	13.238	-	0.281	-	0.247	-	0.254	-	0.272	-	0.225	-	0.181	-	0.238	carvacryl methyl ether	6379-73-3	1234
16	14.057	-	0.489	-	0.526	-	0.489	-	0.520	-	0.600	-	0.573	-	0.547	bornyl acetate	76-49-3	1293
17	14.798	-	0.213	-	0.189	-	0.147	-	0.232	-	0.319	-	0.266	-	0.246	ocimen-1-yl acetate	72214-23-4	1342
18	14.940	-	0.416	-	0.342	-	0.085	-	0.441	-	0.665	-	0.584	-	0.479	α -terpinyl acetate	80-26-2	1351
19	15.049	-	0.488	-	0.124	-	0.079	-	0.089	-	0.121	-	0.087	-	0.172	α -longipinene	5989-08-2	1354
20	15.374	-	0.274	-	0.192	-	0.241	-	0.205	-	0.315	-	0.223	-	0.211	α -copaene	3856-25-5	1381

21	15.839	-	0.418	-	0.234	-	0.342	-	0.474	-	0.741	-	0.588	-	0.472	longifolene	475-20-7	1419
22	15.998	-	1.501	-	0.963	-	1.022	-	1.059	-	1.587	-	1.327	-	1.215	β-caryophyllene	87-44-5	1432
23	16.264	-	0.449	-	0.623	-	0.477	-	0.409	-	0.703	-	0.449	-	0.679	α-humulene	6753-98-6	1454
24	16.440	-	0.289	-	0.238	-	0.259	-	0.252	-	0.418	-	0.305	-	0.252	γ-gurjunene	22567-17-5	1469
25	16.666	-	0.211	-	0.232	-	0.280	-	0.218	-	0.477	-	0.212	-	0.187	(E)-germacrene D	23986-74-5	1488
26	16.695	-	0.248	-	0.348	-	0.351	-	0.332	-	0.553	-	0.338	-	0.344	(-)-α-amorphene	23151-88-0	1490
27	16.786	-	0.894	-	0.892	-	0.995	-	0.777	-	1.333	-	0.896	-	0.913	α-murolene	31983-22-9	1497
28	16.853	-	0.361	-	0.521	-	0.456	-	0.397	-	0.693	-	0.402	-	0.521	(+)-valencene	4630-07-3	1503
29	16.983	-	1.309	-	1.710	-	1.712	-	1.624	-	2.516	-	1.637	-	1.757	γ-murolene	30021-74-0	1514
30	17.179	-	0.734	-	1.111	-	1.170	-	0.988	-	1.595	-	1.081	-	0.969	(R)-γ-cadinene	39029-41-9	1530
31	17.272	-	2.008	-	3.007	-	3.409	-	3.066	-	6.060	-	3.272	-	3.096	δ-cadinene	483-76-1	1538
32	17.316	-	trace	-	0.060	-	0.064	-	trace	-	trace	-	trace	-	trace	(-)-isolekene	95910-36-4	1541
33	17.396	-	0.081	-	0.110	-	0.121	-	0.115	-	0.179	-	0.101	-	0.099	cadinadiene	29837-12-5	1548
34	17.458	-	0.092	-	0.209	-	0.205	-	0.220	-	0.323	-	0.172	-	0.174	guaiene	88-84-6	1553
35	17.985	-	0.164	-	0.354	-	0.385	-	0.354	-	0.463	-	0.353	-	0.356	(-)-spathulenol	77171-55-2	1597
36	18.075	-	0.140	-	0.093	-	0.119	-	0.103	-	0.155	-	0.128	-	0.120	β-caryophyllene oxide	1139-30-6	1604
37	18.561	-	0.127	-	0.143	-	0.191	-	0.165	-	0.304	-	0.172	-	0.144	cubenol	21284-22-0	1644
38	18.703	-	0.670	-	1.078	-	1.687	-	1.322	-	2.225	-	1.447	-	1.148	T-cadinol	5937-11-1	1656
39	18.860	-	0.909	-	1.358	-	1.856	-	1.713	-	2.714	-	1.891	-	1.494	α-cadinol	481-34-5	1669
40	22.689	-	0.274	-	0.200	-	0.360	-	0.312	-	0.458	-	0.276	-	0.250	rimuene	1686-67-5	2045
41	23.317	-	0.083	-	0.062	-	0.137	-	0.106	-	0.197	-	0.105	-	trace	cembrene	1898-13-1	2045
42	24.139	-	0.174	-	0.054	-	0.138	-	0.102	-	0.212	-	0.121	-	trace	pimarinal	472-39-9	2115
43	24.722	-	0.350	-	0.398	-	0.431	-	0.364	-	0.559	-	0.315	-	0.221	methandriol	521-10-8	2201
TOTAL		100.0	84.883	100.0	89.616	100.0	87.037	100.0	84.328	100.0	84.95	100.0	86.789	100.0	81.164			

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