

MICROBIOLOGICAL AND GC-MS ANALYSIS OF HORSERADISH ROOTS (*ARMORACIA RUSTICANA*)

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ABSTRACT: This paper aims to address the possibility of contamination of the plants used as spices - horseradish (*Armoracia rusticana*) with microorganisms, but also to investigate their composition from the point of view of volatile compounds by GC-MS methods. Their initial use was probably a flavoring agent, which then demonstrated antimicrobial properties by maintaining fresh foods for longer periods and improving the health of those who regularly consume spices. It has been found that horseradish has a low number of bacteria, yeasts and molds of 1-14 CFU / g, in contrast, there are significant amounts of volatile compounds. The most significant values were obtained for isothiocyanates (Isobutyl ITC, sec-butyl ITC, benzyl ITC, 3-butenyl ITC, 3-methylbutyl ITC), which range between 33.1835 $\mu\text{g} / \text{g}$ and 47.45 $\mu\text{g} / \text{g}$ (3-hydroxy-4,5-dimethyl-2 (5H) -furanones) with values ranging from 0.2317 $\mu\text{g} / \text{g}$ to 1.1217 $\mu\text{g} / \text{g}$ and methoxypyrazines, 5103 $\mu\text{g} / \text{g}$ and 1,525 $\mu\text{g} / \text{g}$ (3-isobutyl-2-methoxypyrazine, 3-isopropyl-2-methoxypyrazine). All these elements contribute to the formation of the sensory and aromatic characteristics of horseradish, but also have beneficial effects on the human body.

KEYWORDS: microorganisms, UFC, volatile compounds, GC-MS, horseradish

1. INTRODUCTION (HEADING 1)

Seasonings have been known and used by mankind since ancient times, and are a way of providing organoleptic (taste, smell and flavor) characteristics of superior food quality to stimulate gastric secretions, appetite and digestion (Fuqe et al., 1994). They are in the dry form of different seeds, roots, fruits, bark, milled or whole with special properties conferred by the substances in their composition. Many of these compounds contribute to their microbiological stability, frequently being ethereal oils, aldehydes, esters, ketones, higher alcohols, terpenic hydrocarbons or certain resins, compounds identified in other plants or foodstuffs (Gilbert et al., 1972, Delaquis et al. 1995, Chen et al., 2011, Lengyel 2012, Panaitescu et al., 2017).

Their main use is to add colour or flavor to foods and foods as well as to help them remain preserved for longer. Spices are found all over the world and form an essential part of the culinary practices of certain cultures.

Many traditional remedies have also been linked to the use of spices and have always been sought after. China, India, the Middle East and parts of Africa were regions where spices were commonly used, then expanded to Europe, and their tradition and importance have always been part of our global history.

There are a large number of microorganisms that can contaminate food, but also the raw or auxiliary materials that contribute to their processing (Lengyel, 2014). The presence in food of certain germs and / or species of microorganisms can provide us good information on the hygiene quality of the ingredients used in processing, the process of the technological flow where the contamination has occurred, the inappropriate practices, or in terms of hygiene on the handling, transport, storage and marketing of food products.

There are several main sources of microbiological contamination: soil, water, air, man, plants, animals, insects, rodents. Besides mentioned sources, raw, auxiliary or food can be contaminated during processing, handling, storage, transportation and marketing.

From a microbiological point of view, spices and foods are appreciated by several indicators, set by internal and international standards, so that they are specific, sensitive, resistant and easy to highlight in the laboratory, easy and fast to analyze.

According to the norms, the indicators used are:

- total number of mesophilic aerobic germs (give an overview of microbiological contamination); bacteria: *Salmonella typhi*, *Escherichia coli*, *Bacillus cereus*, *Enterobacter cloacae*, yeasts and molds.

Many of these spices or plants used as spices are rich in biologically active compounds, which give them antibacterial / antifungal activity, however, they are microorganisms that can contaminate them (Ono et al 1998, Soledale et al 1998, Conrad et al., 2006, Wagner et al., 2012).

2. HORSERADISH (*ARMORACIA RUSTICANA*)

Armoracia rusticana has its origins in West Asia and Southeastern Europe, being a perennial plant grown for its spicy, fleshy roots, with food and medicinal uses. It has a high content of vitamins (Bladh et al., 2011), mineral salts, volatile oils (Kroener et al., 2017), especially with sulfur such as glucosylation (Kubler 2010). The plant is processed in the food industry, being used predominantly as a spice (Shehata et al., 2009, Kinai et al., 2000, Kosson et al., 2008).

Horseradish has the ability to reduce inflammation with beneficial effects in treating respiratory diseases, bronchitis, influenza, pneumonia (Goos et al., 2006, Albrecht et al., Fintelmann et al., Schulz 2008, Sampliner et al. Sarli et al., 2012), soothing to the nerves, stimulating digestion and immune system to prevent cancer due to the rich content of antioxidant compounds (Steinmetz et al., 1991, Verhoeven et al., 1996). In addition, horseradish increases the number of red blood cells and helps control heart rate and blood pressure, being a known anti-inflammatory agent (Zao et al., 2005, Cheung et al., Marzocco et al., 2015).

Studies conducted by Sultana et al. 2003 show that horseradish flavor results from the release of volatile isothiocyanates (ITC) by hydrolysis of glucosinolates precursors. Authorized values in New Zealand samples reached 1900.7 mg / kg total isothiocyanate, 1658.1 mg / kg allyl isothiocyanates and 185.2 mg / kg 2-phenylethyl isothiocyanates. Orav et al., 2008 identifies the anise in horseradish extracts, and Marotti et al. (1993) and Piccaglia et al. (2001) p-aldehyde.

New compounds have been identified by Kroener et al. (2017), Imazaki, (2012) such as lactone, 3-isopropyl-2-methoxypyrazine or 3-sec-butyl-2-methoxypyrazine. Wuest (2017) considers that the formation of metoxypyrazines in plants occurs by amidation and condensation of an α -amino acid and an unknown 1,2-dicarbonyl compound to a 2-hydroxy-3-alkylpyrazine which is further methylated with an O-methyltransferase.

In the horseradish extracts sotolone has also been identified, which was reported in Schinduf extract (Blank et al., 1997) and lovage (Blank et al., 1993) and phenylpropanoid (Kundu et al., 2016). Several studies have shown that the content of volatile compounds in horseradish roots ranges from 300 to 3,500 $\mu\text{g} / \text{g}$ and from 30 to 600 $\mu\text{g} / \text{g}$ of fresh weight (Masuda et al., 1996, Sultana et al. , 2003, Horbowicz et al., 2006, Kosson et al., 2008). Kroener et al. 2018 identified values ranging from 0.9-270 $\mu\text{g} / \text{g}$. Ku et al. (2015) recorded the CETP content in horseradish roots at levels ranging from 0.00 to 11.4 pmol / g dry weight, and Scherz et al (2000) at 0.2-25.1 $\mu\text{g} / \text{g}$, This difference may be due to the fact that the horseradish samples analyzed in both studies belong to different varieties, and local pedoclimatic factors are involved.

In the case of sec-butyl ITC Sultana et al., 2003 identified values of $2.77 \pm 0.24 \mu\text{g} / \text{g}$, Kroener et al. 2017 values between 3.7 and 8.7 $\mu\text{g} / \text{g}$ and Masuda et al., 1996) 27 $\mu\text{g} / \text{g}$.

3. MATERIALS AND METHODS

- 6 horseradish samples of native origin / H1 (Hărtibaciului Valley), H2 (Turnișor / Sibiu), H3 (Cut / Sebeș Alba), H4 (Ocna Sibiului), H5 (Gușterița / Sibiu) H6 (Sadu/Cisnădie)

Culture media for microbiological determinations:

-DRBC and VRBGA / selective yeasts and molds, respectively enterobacteria (Merck / Millipore / Germany)

a) Component of DRBC medium (Dicloran-red bengal chloramphenicol agar) - (Yeast and mold selective medium): - Animal / vegetal digest enzyme digest - 5g - D-Glucose - 10g - KH_2PO_4 - 1g - Dicloran - Bengal - 0.025g - Agar - 12-15g - Chloramphenicol - 0.1g - Distilled / deionized water - 1000ml

- Incubation at 25 ° C for 5-7 days - the medium has a pH of 5.6; after sterilization, increasing its value. - Sterilization / autoclaving is performed at a temperature of 121 ° C for 15 minutes.

b) Composition of the VRBGA medium (Red-violet, bile and glucose with Agar) - Enzymatic digest of animal tissues - 7 g Yeast extract - 3 g Bile salts 1,5 g Glucose 10 g - Sodium Chloride - 5g - Neutral Red - 0.03g - Purple Crystal - 0.002g - Agar - 9-18g depending on gelling capacity - Distilled Water - 1000ml

- Incubation at 37 ° C for 1-5 days - the medium has a pH of 7.4 at a temperature of 25 ° C

Microbiological analyzes: determination of the total number of yeasts and molds and enterobacteria

One gram of each sample was weighed into the analytical balance in glass tubes using sterile instrumentation. After weighing each sample, they were placed in the laminar flow hood, where decimal dilutions were made with Ringer's solution.

- In order to count yeasts and molds, sowing was performed by the method of incorporating 1 mL of the dilutions over which the DRBC culture medium was poured, the samples being incubated for 6 days at 25 ° C.

In order to count the enterobacteria from each sample, 1 mL was harvested to be seeded on the DM plates with the medium already plated in the plate followed by plate shaking on the plate surface and on a plaque that developed the growth of enterobacteria (EB) at dilution -1 and -2. In this way, all dilutions were made to a dilution allowing colonization to be visualized and counted. After sowing the plates without the medium, over the samples introduced into them, two layers of medium were poured over a certain period of time. The first layer is the one in which the bacteria will develop, so the casting medium must always be homogenized with the sample by rotating plate movements. The second layer of medium was poured immediately after the first layer solidified, thereby providing microorganisms with anaerobiosis conditions.

After solidification of both media layers, the plates were introduced into the thermostats corresponding to the development temperature of each group of microorganisms. Following incubation at 37 ° C after the time required for development (3 days), the plates were removed from the thermostat and analyzed, and then colonies counted with the colony count (Colony Star / Funke-Gerber / Labortechnik GmbH / Berlin / Germany) and the results reported for the analyzed samples.

Determination of volatile compounds (GC / MS Trace 1300 Thermo Fisher Scientific)

From each of the 6 selected horseradish samples, 1 g of the center of the sample was picked and subjected to extraction with 50 mL of dichloromethane solvent, with stirring for 30 minutes. The extracts were then filtered and distilled at 500 ° C. The obtained distillates were subjected to chromatographic examination.

- For the SPME extraction, divinylbenzene / carboxene / polydimethylsiloxane (DVB / Car / PDMS) fibers (Sigma-Aldrich, Germany) used a 10-minute incubation time at an extraction temperature of $35 \pm 1 \text{ }^\circ\text{C}$ over a period of time 10 min. The volatile compounds were desorbed for 15 minutes at a temperature of 250 ° C, then transferred to the chromatographic column for separation according to the protocol described by Tomsone et al. 2013: "Working conditions: injector - 250 ° C; transfer line to MSD - 260 ° C; oven temperature start - 40 ° C, hold 10 min, programmed from 40 to 60 ° C at 2 ° C min⁻¹, and from 60 to 250 ° C at 20 ° C min⁻¹, hold 5 min; carrier gas (He) - 1 ml min⁻¹; split ratio - 2: 1; ionization - EI + mode".

4. RESULTS AND DISCUSSIONS

The 6 analyzed horseradish samples showed low levels of enterobacteria (between 1 and 3 CFU / g), yeasts and molds (between 2-14 CFU / g), and it is known from the literature that this plant has antibacterial and antifungal effects Kienholz et al., 1960; Tedeschi et al., 2011). The micro-organisms identified on horseradish samples may come from the harvesting environment (the soil) or its handling for processing, the values found being little significant for their hygienic quality.

The following groups of volatile compounds ($\mu\text{g} / \text{g}$) were identified following the GC-MS analyzes performed:

isothiocyanates (ITC): Isobutyl ITC, sec-butyl ITC, benzyl ITC, 3-butenyl ITC, 3-methylbutyl ITC

- sotolone: 3-hydroxy-4,5-dimethyl-2 (5H) -furanone

methoxypyrazine: 3-isobutyl-2-methoxypyrazine, 3-isopropyl-2-methoxypyrazine

As shown in Table 1, the six samples of horseradish studied showed significant values of volatile compounds, the differences between them being explained by the growth and development conditions of this plant. An important aspect may be soil composition and climatic conditions in the area, which

according to the literature may influence the accumulation of these compounds (Scherz et al., 2000). It is noted that Isobutyl ITC is between $9.3545 \mu\text{g} / \text{g}$ for the H4 sample and a maximum of $21.1010 \mu\text{g} / \text{g}$ for the H3 sample, sec-butyl ITC is found to be in the range of $1.2892 \mu\text{g} / \text{g}$ to $7.2213 \mu\text{g} / \text{g}$, (H5 / H2) and benzyl ITC was detected at values between 0 (H4) and $2.6549 \mu\text{g} / \text{g}$ (H3). Two other identified ITC compounds showed values between $5.6673 \mu\text{g} / \text{g}$ - $15.2753 \mu\text{g} / \text{g}$ and $4.6672 \mu\text{g} / \text{g}$ - $17.1437 \mu\text{g} / \text{g}$, namely 3-butenyl ITC, 3-methylbutyl ITC for samples H2 / H4 and H3 / H4. 3-Hydroxy-4,5-dimethyl-2 (5H) -furanone showed subunit maximal values, the peak being detected in sample H3 at a rate of $1.1217 \mu\text{g} / \text{g}$. Methoxypyrazines ranged from $0.11198 \mu\text{g} / \text{g}$ (H5) to $1.17782 \mu\text{g} / \text{g}$ (H1) for 3-isobutyl-2-methoxypyrazine and $0.1111 \mu\text{g} / \text{g}$ (H6) for 3-isopropyl-2-methoxypyrazines.

Table 1. Volatile compounds (isothiocyanates (ITC): Isobutyl ITC, sec-butyl ITC, benzyl ITC, 3-butenyl ITC, 3-methylbutyl ITC, sotolone: 3hydroxy-4,5- , methoxypyrazine: 3-isobutyl-2-methoxypyrazine, 3-isopropyl-2-methoxypyrazines identified in the six horseradish samples (*Armoracia rusticana*)

Compound ($\mu\text{g}/\text{g}$)	H1	H2	H3	H4	H5	H6
Isobutyl ITC	13,2312 $\pm 0,0021$	15,3329 $\pm 0,0011$	21,1010 $\pm 0,0021$	9,3545 \pm 0,0011	11,9879 \pm 0,0015	17,0012 $\pm 0,0015$
sec-butyl ITC	4,8932 \pm 0,0001	7,2213 \pm 0,0005	5,1098 \pm 0,0005	5,6765 \pm 0,0001	1,2892 \pm 0,0001	2,3135 \pm 0,0001
benzyl ITC	1,0093 \pm 0,0001	1,2912 \pm 0,0001	2,6549 \pm 0,0001	0	0,9912 \pm 0,0001	0,7787 \pm 0,0001
3-butenyl ITC	7,8212 \pm 0,0005	5,6673 \pm 0,0006	13,6734 $\pm 0,0023$	15,2753 \pm 0,0025	12,2217 \pm 0,0019	11,1111 $\pm 0,0011$
3-methylbutyl ITC	6,2286 \pm 0,0002	6,9823 \pm 0,0002	4,6672 \pm 0,0001	17,1437 \pm 0,0009	11,1921 \pm 0,0008	12,7878 $\pm 0,0009$
3-hydroxy-4,5-dimethyl-2(5H)-furanone	0,9123 \pm 0,0001	0,1121 \pm 0,0001	1,1217 \pm 0,0001	0,8999 \pm 0,0002	0,2317 \pm 0,0002	0,8989 \pm 0,0005
3-isobutyl-2-methoxypyrazine	1,17782 \pm 0,0001	0,7143 \pm 0,0001	0,9992 \pm 0,0002	0,3992 \pm 0,0002	0,1198 \pm 0,00003	0,2729 \pm 0,0001
3-isopropyl-2-methoxypyrazine	0,3472 \pm 0,0001	0,5121 \pm 0,0002	0,2874 \pm 0,0002	0,1111 \pm 0,0001	0,5487 \pm 0,0031	0,6635 \pm 0,0009

Regarding the total values obtained on groups of volatile compounds, it can be stated that they present significant amounts for each sample, namely: ITC (isothiocyanates, Figure 1) are ranging between 33.1835 (H1) $\mu\text{g} / \text{g}$ and $47,45$ (H4) $\mu\text{g} / \text{g}$, intermediate values being detected for H2 samples at $36,495 \mu\text{g} / \text{g}$, H3 close to maximum by $47,2093 \mu\text{g} / \text{g}$, 11% higher than the minimum for H5 and 8% lower than the H4 sample value for sample H6.

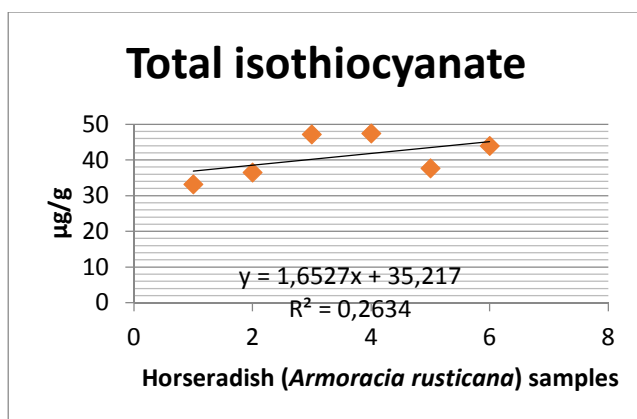


Figure 1. Total isothiocyanate (ITC): Isobutyl ITC, sec-butyl ITC, benzyl ITC, 3-butenyl ITC, 3-methylbutyl ITC in the six horseradish samples (*Armoracia rusticana*)

Following Figure 2 it is found that the samples present a great variety of value with respect to the amount of sotolone. Thus, we have values above $1 \mu\text{g} / \text{g}$ for the H3 sample, lower by 15% for samples H1 and H6, but also to a minimum level, namely for samples H2 and H5 where they are in the amount of $0,1121 \mu\text{g} / \text{g}$ and $0.2317 \mu\text{g} / \text{g}$, respectively.

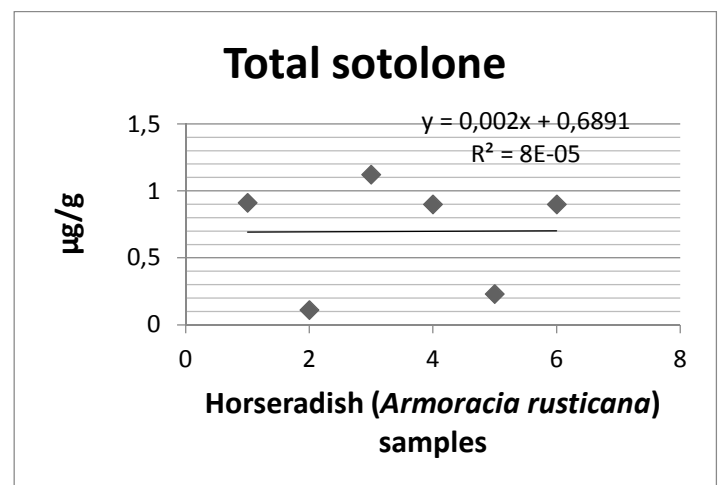


Figure 2. The total sum of sotolone in the six horseradish samples (*Armoracia rusticana*)

Methoxy-pyrazines are compounds identified in many plants, the values determined in the six horseradish samples ranging between a minimum total of 0.5103 µg / g (H4) and a maximum sum of 1.525 µg / g (H1).

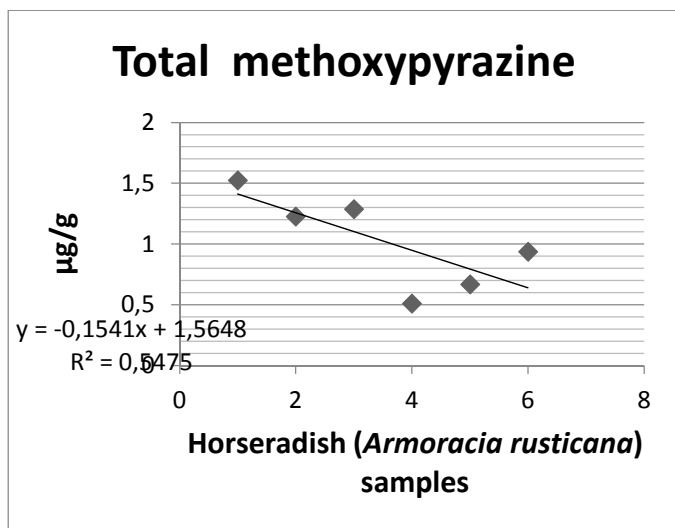


Figure 3. The total amount of 3-isobutyl-2-methoxy-pyrazine, 3-isopropyl-2-methoxy-pyrazine methoxy-pyrazines found in the six horseradish samples (*Armoracia rusticana*)

As noted in Figure 3, close values present the H2 and H3 horseradish samples where the total amount of methoxy-pyrazines is 1.2264 µg / g and 1.2866 µg / g respectively. Subunits of total methoxy-pyrazines are observed for samples H4 and H5 where they only reach 0.5103 µg / g and 0.6685 µg / g respectively.

5. CONCLUSIONS

Based on the microbiological results obtained, it can be stated that both bacteria, yeasts and molds can contaminate plants used as spices such as horseradish, but their number is reduced. At the same time, the development of microorganisms is closely related to the passage of time when the samples are subjected to external environmental conditions, but also to the fact that these plants are handled for food purposes here by interfering with hygiene factors.

As for the composition of the horseradish, it is rich in volatile compounds, predominantly those of isothiocyanate type. The values determined in this study demonstrate that selected samples from different sources (different cultivation areas lead to a different aromatic-volatile profile). Differences are sometimes significant, but overall, each of the selected samples of the study presented specific compounds in quantities that give them the characteristics presented in the literature. Further determinations may be made to detect new compounds or elements so that the horseradish chemical profile will be elucidated.

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