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Research Article

Microwave processing of sunflower achenes and its influence on their quality and enzymes activities

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Abstract: Stricter requirements to environmental compatibility and smaller energy-output ratio highlight the importance of implementing the super high-frequency drying of the crop seeds. The study is aimed at the development of drying optimal parameters and modes of sunflower (*Helianthus annuus* L.) seeds in a settled small-size conveyor microwave unit. In-gel activity of 6 functionally key enzymes (formate dehydrogenase, glutamate dehydrogenase, malate dehydrogenase, diaphorase, leucine aminopeptidase and non-specific esterases) in polyacrylamide gels after disk electrophoresis was analyzed in order to count on an additional assessment of the temperature influence caused by electromagnetic radiation of the tested drying unit on the sunflower achenes metabolism. The correlation analysis showed the existence of the statistically significant ($p < 0.05$) negative dependence between the seed materials heating temperature with germination energy (correlation coefficient −0.783) and achenes germination (−0.797). These two parameters (without processing 88 and 96%, respectively) began to reduce sharply when reaching the heating temperatures of 55°C and more. Enzymes de-activation also started within this range. Considering the collected data about drying of the seed material, the optimal heating conditions were within 26–27 minutes at 800 W and heating temperature 38–40° C. With these parameters the quality of the processed seeds was preserved, and the costs for drying were relatively low (2.61 MJ per 1 kg of the water removed).

Keywords: enzymes histochemical activity; germination energy; germination; microwave drying; sunflower

1. Introduction

The seeds of oil plants are heat-sensitive, and their quality may suffer when they are exposed to excessive heat [1]. Besides, traditional hot air drying requires relatively high energy consumption. Present requirements to environmental compatibility and smaller energy-output ratio require the development of new technologies for the seed drying. Development and use of new equipment, based on high frequency (microwave) processing of the plant material, can be a solution [2]. In Russia, as well as all over the world, the market offers a wide range of domestic and imported apparatus [3]. Unfortunately, the available studies on effects on plant metabolism and optimal processing modes are not yet enough for the successful development of the oil crop seeds microwave-processing technologies in the interests of agriculture.

Sunflower, an essential oil and agricultural food plant, is grown in 72 countries. Russia and Ukraine are the largest producers of the crop in the world [4]. Herewith one of the most vital issues is the necessity of optimal drying procedures for seed material [5]. Due to the peculiarities of the natural and climatic conditions in Russia, the crop collection starts when its humidity is several times higher than the level accepted by the state standards. The agriculture traditionally uses drying units, which allow performing large-scale seed material processing. During this process, the heating is performed inside all the seed mass. That is why the emerging gradients of heat, moisture, and pressure, directed from the inside to the material layer surface, increase the drying efficiency. However, the influence of such physical processes on plants' vital properties has not yet been investigated [6]. Electromagnetic radiation influence on the plants affects all the layers of the living organisms' organization, starting from the gene expression, controlling the metabolism, and ending with the morphological and structural changes [7]. It depends on the frequency, power, and duration of the impact on the plants. The active discussion has been held in the scientific literature on the potential and observed influence of microwave processing on the seeds' metabolites properties [8] in relation with the issues of food safety and extraction recovery of specific components [9]. At the same time, there is not much data on the molecular mechanisms of the thermal and non-thermal microwave radiation influence on a cell [10], affecting the seeds sowing properties. Due to the complex influence of these factors on the plant integrity and to the possible difference of response due to specificity of the biological species [11], particular efforts must be carried out using seeds of different plant crops.

This study has the aim to establish optimal parameters on drying modes of sunflower achenes, that allow the preservation of their essential economic features (germination energy and achenes germination).

2. Materials and methods

2.1. The microwave-unit

The study is conducted using a recently developed small-size conveyor microwave unit, working

at frequency of 2.45 GHz. The temperature and humidity were determined with sensors once it was loaded in the bunker. This information was transferred to the display of the control unit. Heating started after setting the temperature of the set mode. Seeds storing was performed on the second unit's zone, where the moisture is extracted in the inter-seed space. The layer purging with the airflow is performed in the third zone. Further, the dried seed material is put in the required container or a machine through the discharging channel. The unit's output is 50–350 kg/h with the drive drum rotation frequency of 0.1–0.7 min^{-1} and it could be controlled through modulation of the conveyor speed, achenes layer height and electromagnetic radiation power (it can vary within 100–800 W/kg). Material mixing intensity was controlled regulating the fan head and modifuing the section with holes. As a result, we get a product with the quality standards, specified in Russia, and final humidity of 7–9%. When using the developed unit, the aggregate specific energy cost for water evaporation of the biological material was reduced 1.6–1.7 fold in comparison with the existing analogues. The economic balance was of about $6 per 1 ton of the dried seed material and the unit payback period did not exceed 1 year.

2.2. Microwave exposure of seeds

The seed material of the same hybrid used for the tests is from the lots with different initial humidity intended for sowing in farming company Irtyubyak, Kugarchinskiy district, Republic of Bashkortostan. A mathematic model of the humidity dynamics of the seeds during the high frequency drying was used for the design of experiments, where the managing parameters were the power of the microwave radiation P (W) and processing time. Total heating time of this batch of seeds in the process of multiple passages through the drying unit (without taking into account storing and purging) is indicated as *t*. *W(t)* indicates the corresponding humidity value in percent. A mathematical model of the dynamics of changes in humidity is presented in the form of a differential equation:

$$\frac{dW}{dt} = W' = f(P, t_{ПР}, W), \tag{1}$$

with initial condition:

$$W(t_0) = W_0, \tag{2}$$

where, $f(p, t_{ПР}, W) = a_1 + a_2 t_{ПР} + a_3 P + a_4 W + a_5 P t_{ПР} + a_6 W t_{ПР} + a_7 PW$.

An analytical solution to the Cauchy problem (1), (2) can be obtained considering already known $a_i (i = 1...7)$ as a function of parameters P and $t_{ПР}$.

The following notation is introduced:

$$b = a_1 + a_2 t_{ПР} + a_3 P + a_5 P t_{ПР}\ ; \qquad h = a_4 + a_6 t_{ПР} + a_7 P\ . \tag{3}$$

Then Eq (2) takes the form:

$$W' = b + hW\ . \tag{4}$$

After separating the variables and integrating both parts, one gets the following:

$$\int \frac{dW}{b+hW} = \int dt\,, \tag{5}$$

$$\frac{1}{h}\ln\left|b+hW\right| = t + C\,. \tag{6}$$

The constant C is found from the initial condition:

$$C = \frac{1}{h}\ln\left|b+hW_0\right| - t_0 \tag{7}$$

then,

$$\frac{1}{h}\ln\left|\frac{b+hW_0}{b+hW}\right| = t - t_0 \tag{8}$$

Thus, an explicit solution to the tasks (1), (2) is obtained:

$$W = \frac{(b+hW_0)e^{-h(t-t_0)} - b}{h} \tag{9}$$

Unknown parameters a_i $(i = 1,...,7)$ and correspondingly b and h models are determined based on experimental data.

The simulation results were verified by the experimental determination of heating dynamics of the biological material at different powers of the microwave radiation in three replicates (Table 1).

Table 1. Heating conditions of sunflower seeds.

Time of microwave radiation, c	Power, W			
	200	400	600	800
	Germination, %			
30	96	96	96	96
45	96	96	95	95
60	96	94	94	81
120	95	91	53	40
180	95	84	52	25
240	95	60	49	7
300	95	49	47	0

As a result, 15 experimental variants were selected; combining power from 200 to 800 W, processing time from 10 to 308 minutes, changing the final temperature of achenes from 20 to 60 °C

and actual humidity to the level of 7–9%, as established by Russian standards of quality.

The seed material was put in the bunker of the developed drying unit with initial humidity of RH_i = 30%. The heating temperature was determined in 27 points of the microwave drying chamber, using the paraffinic thermometer SP-2P (Russia), which gives relatively negligible errors in the area of the microwave heating. Heating dynamics was obtained by video recording of the thermometer readings.

The germination and germination energy, important indicators of the sunflower seed viability [12], were tested at a constant temperature of 25 °C (50 seeds were used for each treatment option) according to the Russian standard 12038-84 "Agricultural seeds. Methods for determination of germination", which is similar to the requirements of the International Seed Testing Association (ISTA). For this purpose, the boxes filled with 2/3 of wet sand were used. The seeds, pre-heated to 30 ℃ for ten days, were placed into the sand at depth equal to their width. The germination and germination energy were determined on the third and fifth days, respectively. After microwave treatment of seeds, the oil content was determined according to the Russian standard 29033-91 “Grain and derived products. Determination of fat content” at a power of 800 W and a heating temperature of 20, 40 and 60 °C.

2.3. Determination of the enzyme activity

Being the enzymes, the catalysts of most of the chemical processes in the plant cells, we assessed the temperature impact caused by the electromagnetic radiation of the drying unit on the metabolism in the sunflower achenes.

For the enzyme extraction, the seed-lobe mixture variant (200 mg) of each microwave processed lot was ground in the porcelain jar with a two ml cooled extraction buffer (0.1 M Tris-HCI, pH 8.0), containing 17% of sucrose and 0.1% of 2-mercaptoethanol. When analyzing the enzymes variation, the seed-lobes of individual achenes were grinded in the proportional volume of the extraction buffer in the microtubes. The homogenate was centrifuged for 20 min at 12 000 rpm at 4°C. The supernatant liquid was drained with a syringe avoiding intermixing with the floating lipid layer.

The histochemical determination of isozymes was performing using vertical intact disk electrophoresis [13,14]. This has several methodological advantages. It is also relatively simple and allows a high resolving power and sharp isozyme bands due to the gel and buffer discontinuities [15,16], it does not require the use of expensive biochemical equipment and, among other things, it is suitable for performing express-analysis of the electromagnetic radiation effects on the plant metabolism.

For the electrophoresis, we used 0.005 M Tris-glycine electrode buffer (pH 8.3). Polyacrylamide gel concentrations were 7.5% in the separation gel (0.375 M Трис-HCl buffer, pH 8.9) and 2.6% in the concentrating gel (0.0625 M Трис-HCl buffer, pH 6.8).

Six different enzyme activities were screened: non-specific esterases (EST, international classification code EC 3.1.1.), diaphorase (DIA, 1.6.99.1.), formate dehydrogenase (FDH, 1.2.1.2.), glutamate dehydrogenase (GDH, 1.4.1.2.), malate dehydrogenase (MDH, 1.1.1.37.) and leucine aminopeptidase (LAP, 3.4.11.1). Histochemical tests were applied to develop staining of enzyme activities using the standard methods [17] with our minor modifications.

One hundred ml of buffer containing 0.1 M Tris-HCL, pH 8.0 was used for the incubation of EST, DIA, FDH, GDH and MDH. For determining the non-specific esterases activity, 50 mg of 1-naphthyl acetate in 1 ml of acetone and 30 mg of strainer Fast Blue BB were added to the buffer. Isozymes of diaphorase were revealed in the solution, containing 2 mg of 2,6-dichlorophenol-indophenol, 20 mg

of nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), 20 mg of thiazole blue (MTT). Staining of formate dehydrogenase was carried out with an addition to the buffer 50 mg of formic acid (sodium salt), 20 mg of MTT, 20 mg of nicotinamide adenine dinucleotide (NAD) and 2 mg of phenazine metal sulfate (PMS). GDH and MDH were stained using the same reaction mixture, excluding formic acid being substituted with 50 mg glutamic and malic acids (disodium salts), respectively. Leucine aminopeptidase (3.4.11.1, LAP) was determined using 100 ml 0.1 M Tris-maleate buffer with pH 5.4, which contained 20 mg of 1-leucine-2-naphthylamine hydrochloride in 1 ml of dimethylsulfoxide and 10 mg of Fast Black K strainer.

The analysis of the electroforegrams was performed directly on the polyacrylamide gels after the electrophoresis. The staining intensity of isozymes or their absence in some variants of seed's treatment was determined visually after its stabilization as a measure of the activity of the enzymes.

2.4. Statistical analyses

The following software packages were used for the statistical processing of the results: "Excel", "Statu", "Mathcad 15", "Flowvision," and "Statistica 10.0" as well as the correlation analysis. For average values, the relative error was calculated. The statistical dependence between variables was determined by building correlation matrices and assessing the reliability of the correlation coefficients of Pearson. Statistical hypotheses were tested at significance levels of 5, 0.1, and 0.01% by using Student's t-test.

3. Results

3.1. Germination of microwave irradiated seeds

It is determined that the rate of temperature rise of the sunflower seeds at the microwave processing significantly depends on the initial humidity. During the radiation impact, the power is absorbed by the moisture inside the processing product. The quantity of the absorbed power depends on the quantity of this moisture. When the initial humidity (RH_i = 29.5%) was high, its decrease was very sharp compared to the lots of the seed material with the initial humidity of 25.9 and 18%.

Herewith, the microwave sunflower processing affected such practically important seed material parameters as the germination energy and germination (Table 2). The correlation analysis showed the existence of the statistically significant negative dependence between the heating temperature of the seed materials with germination energy (correlation coefficient −0.783) and achenes germination (−0.797). The last two parameters which practically do not differ in processing mode No. 12 (80 and 95% respectively) from the variant without processing (88 and 96%), begin to reduce when reaching the heating temperature of 55° C and more. For example, in modes Nos. 13, 14 and 15, the seeds germination energy decreased to values of 32, 30 and 18%, germination to 49, 40 and 25%, respectively. Enzymes de-activation in gels starts within this range. The mode with the temperature of heating the seeds to 60° C with a microwave power of P = 800 W allows to get maximal yield of oil which increase from the control sample (33,26 %) to 37.51, 41, 44 and 45,85 % at temperatures of 20, 40 and 60 °C, respectively (on average 41.60 ± 2.41%).

Table 2. Germination energy and germination in different microwave sunflower seeds processing modes, %.

No.	Power, W	Temperature, °C	Time, min	Indicators	
				Germination energy*	Germination*
Control				88	96
1	200	20	288	80	96
2	200	20	308	62	74
3	200	20	270	79	95
4	200	35	128	77	95
5	200	36	120	85	94
6	400	20	51	82	96
7	400	22	48	84	96
8	400	28	22	81	94
9	600	30	34	78	95
10	600	44	31	84	94
11	800	35	18	84	96
12	800	30	26	80	95
13	600	58	15	32	49
14	800	56	15	30	40
15	800	59	10	18	25

*: Negative correlations of temperature with germination energy and germination are significant at level $p < 0.001$ (correlation coefficients $r = 0.783$ and $r = 0.797$, respectively).

3.1. The activity of enzymes in irradiated seeds

The histochemical in-gel staining allowed determining that glutamate dehydrogenase and formate dehydrogenase of individual achenes had one, and leucine aminopeptidase and diaphorase - two zones of electrophoretic activity. Malate dehydrogenase happened to have 4 main zones of the histochemical staining. None of the zones showed polymorphism, although malate dehydrogenase was earlier described as having two or triallelic genetic control of the enzyme synthesis [18]. Sometimes heterogeneity by the composition of isoenzymes and samples can be observed. Our study observed this phenomenon for the non-specific esterases. They appeared in gels as a number of the activity zones with different intensity. Some achenes had polymorphic variants (Figure 1, tracks 6–8). A similar spectrum of esterases in sunflower seeds was demonstrated earlier [19].

It is determined that the temperature and exposure time had critical effects, leading to the enzyme de-activation. The activity of all the studied enzymes remained unchanged in the variants at temperatures of 56 °C (Table 2). Differences in the enzymes thermal stability limit varied on a significant level, as it is well-known in science [20]. The isoenzymes of formate dehydrogenase, malate dehydrogenase, and leucine aminopeptidase turned out to be the most thermostable, their histochemical staining did not diminish until the mode of test variant No. 15.

An example of a decrease in the activities of these enzymes under the mode No. 15 (track 5) is shown in the Figure 2 for formate dehydrogenase. The activity of this enzyme is almost identical on tracks 1–4 (test variants Nos. 11–14, respectively).

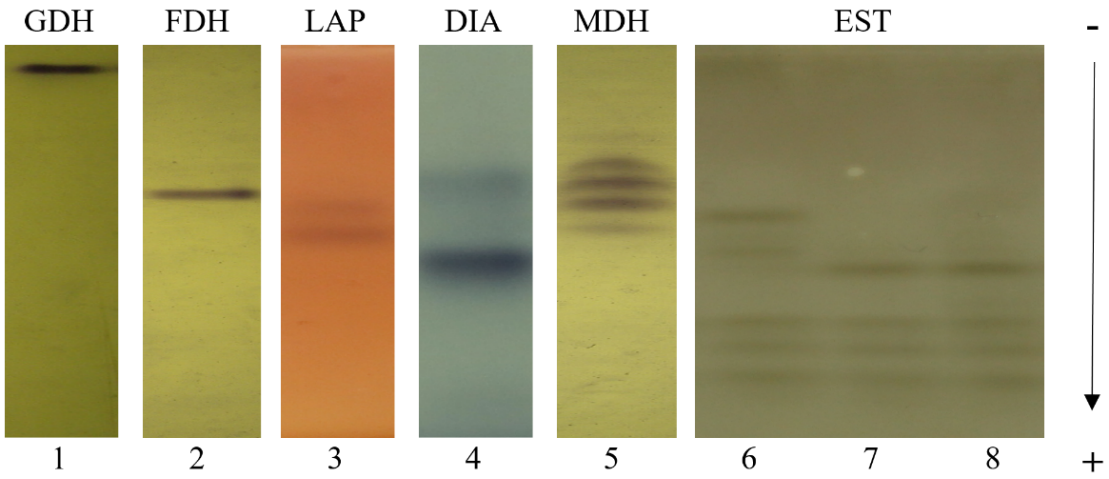

Figure 1. Electrophoregram of the six enzymes studied.

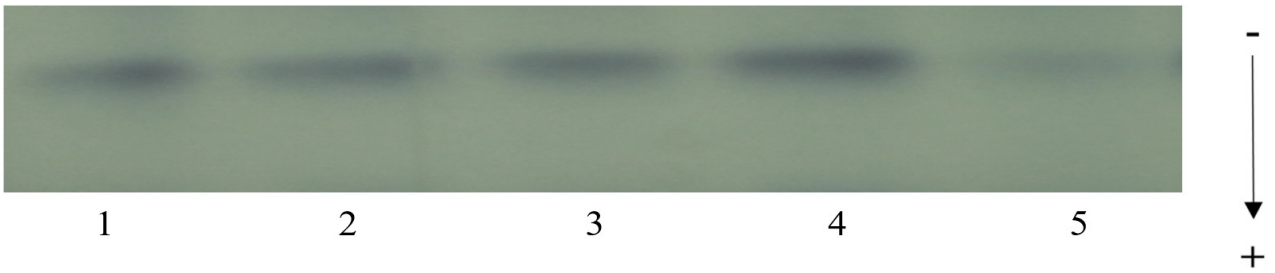

Figure 2. Electrophoregram of the formate dehydrogenase isoenzyme.

GDH - glutamate dehydrogenase, FDH - formate dehydrogenase, LAP - leucine aminopeptidase DIA – diaphorase, MDH - malate dehydrogenase, EST - non-specific esterases. The arrow shows the molecules moving from cathode (-) to anode (+).

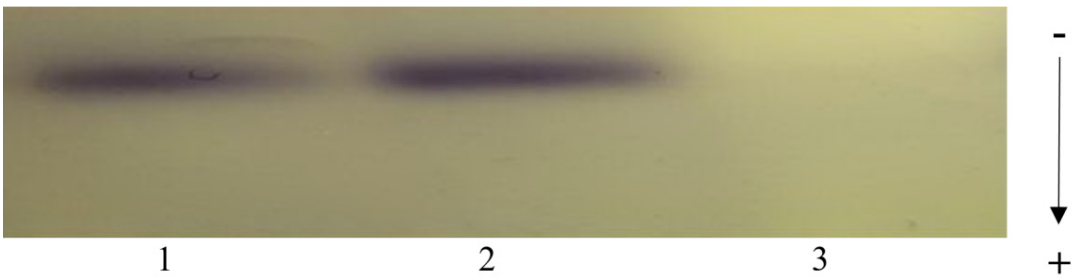

Figure 3. Electrophoregram of the glutamate dehydrogenase isoenzyme.

We observed that the wavelike intensity increased in the histochemical staining at some modes.

The conditions of Mode No. 15 became critical for non-specific esterases and diaphorase, and glutamate dehydrogenase already began to lose activity in mode No. 14. The complete GDH deactivation example is shown in Figure 3. The enzyme activity zone is absent in the sample of track

No. 3 as a result of seed treatment in mode No. 15. The GDH activity in modes No. 1–13 practically did not change (examples are given in tracks 1 and 2).

4. Discussions

Various enzymes keep the catalytic activity within a different and quite wide temperature range [16]. We selected the enzymes with a different functional role in the plants' metabolism for this study [17]. Oxidoreductase, including formate dehydrogenase, glutamate dehydrogenase, malate dehydrogenase, and diaphorase we studied, catalyze the redox reactions. In such a way, they participate in the key metabolites' conjugated flow, ensuring the homeostasis of the main bodies' physiological characteristics and their adaptability. Plants formate dehydrogenase is one of the main enzymes responsible for tolerance development to the influence of stress factors. It is proved by the increase of FDH synthesis under the influence of chemical agents, extreme temperatures, lack of moisture, UV extra amounts, etc. on the plants. This enzyme is responsible for toxical formate oxidation, which is produced in this case [21]. The non-specific esterases and leucine aminopeptidase have the same retardation functions of the stress-modified changes in a cell due to inactivation of toxicants [22].

We observed that the wavelike intensity increased in the histochemical staining at some modes (electropherograms are not presented) - for non-specific esterases (Table 2: test variant 5 and 12), diaphorase (3 and 10) and glutamate dehydrogenase (1, 4 and 10). Variants with 30–44° C were close to the activity temperature optimum of most of the enzymes [23]. Nevertheless, the temperatures of 20°C are significantly lower than the highest catalytic enzymes activities and therefore a different explanation is required to explain the activity bursts for DIA and GDH under these conditions. Recently, it was shown that the enzymes expression could significantly change under the influence of microwave radiation, leading to an increase or decrease of the catalytic activity [24]. This phenomenon was determined using the high-performance liquid chromatography and mass spectrometry for a broad spectrum of the soil bacteria proteins *Bacillus cereus*, processed in the mode with 1800 W at 85°C for 5 minutes. When studying the proteins with the different metabolic role, it turned that less than 39.13% of them were responsible for the synthesis and metabolism of the amino acids or proteins, 21.74% for the carbohydrate metabolism, 8.70% - for the antioxidants and acetyl coenzyme synthesis, 4.35% - for catabolism, detoxification activity, ethanol activity and glyoxylate path, synthesis of a number of the metabolites.

It is known that the stress from high temperatures causes destructive consequences for the metabolism because of the cellular homeostasis destruction and physiologic processes disorder [22], which is associated to the catalytic activity of the enzymes. It comes under notice that the "threshold" temperature, after which the enzymes deactivation begins, coincides with the significant deterioration of the two studied economically important sunflower characteristics - germination energy and germination that are economically important in sunflower seeds. The operating mode of the developed unit to be used under its production conditions will consider this result. It also allows performing other crops' seeds drying. However, a separate analogous "species-specific" study for each of them is necessary in order to determine an optimal microwave processing mode. Inconsistence of the results in this sphere, published in the scientific literature, requires this approach. The following data is shown in the recent review [9]. Germination and germination energy of the lentil (*Lens culinaris*) did not change during the microwave processing, but they decreased for radish (*Raphanus sativus*) and wheat (*Triticum aestivum*) and increased for rice (*Oriza sativa*) and barley (*Hordeum vulgare*).

5. Conclusions

Thus, notwithstanding the participation of the studied enzymes in different metabolism areas, they begin to inactivate in a relatively narrow temperature range during the microwave heating. This threshold level coincides with the temperature at which sunflower achenes germination energy and the germination reduction begin. Based on the received results, which have been tested in the farming company Irtyubyak in an industrial scale, we suggest the following operating modes for practical use of the unit. The seeds with high initial humidity require drying in more soft modes if they are intended for the seed material. The optimal heating for the achenes drying, intended for this purpose, is within 26–27 minutes at 800 W of the microwave radiation and heating temperature of 38–40° C. The quality of the processed seeds following these parameters do not change, and the costs for drying are relatively small (2.61 MJ per 1 kg of the water removed).

Conflict of Interest

The authors declare no conflict of interest.

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