Musts with an Increased Content of Lignans from Added Spruce Knot Chips

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Abstract

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Red and white musts were enriched with the lignan hydroxymatairesinol, which is the main lignan contained in spruce knots. Chips from the milled spruce knots were then used to enrich grape musts. After enrichment, the musts were stored and samples were taken in 1, 5, 9, and 12 months. The samples were subjected to a variety of analyses and sensory evaluations. Analyses included hydroxymatairesinol and alpha-conidendrin content, antioxidant activity (determined by the FRAP method), content of total polyphenols, sensory evaluation (intensity of woody aroma, intensity of bitterness and astringent taste, and consumer acceptability), and must antimutagenicity. The analysis of variance allowed predicting which factors such as grape type, quantity of added wood chips, sugar addition, method of preservation, and storage time had the most significant influence on the analytical parameters (lignan content, antioxidant activity, and total polyphenol content). In all cases lignan content in the musts was significantly influenced by the addition of spruce wood chips. Total polyphenol content in the musts was significantly affected by the type of musts and by heat treatment (time of thermomaceration). Evaluation of must antimutagenicity showed that all samples, except the sample of white musts after thermomaceration without holding at temperature and without adding chips (10 g/20 kg mash), inhibited mutagenicity.

Keywords: enrichment of must; HMR; CONI; antioxidant activity determined by FRAP; total polyphenol concentration; sensory evaluation

Lignans can be found in significant amounts in plant products such as flax seeds, sesame, and coniferous tree knots (Meagher & Beecher 2000; Holmbom *et al.* 2003; Milder *et al.* 2005). In the last-mentioned material the special type of lignan 7-hydroxymatairesinol (HMR) was found.

After consumption of plants that contain lignans, enteric bacteria, which occur naturally in the human gut, are converting plant lignans to mammalian lignans, called enterolignans.

Enterolignans are absorbed by the intestines and further modified in the liver. The estimated intake of

lignans from un-supplemented food is about 1 mg/day. Intake of lignans should be viewed as supporting a modern healthy lifestyle just like increased fibre, vitamin C, folic acid, vegetarian-based diet, low BMI, increased physical activity, and not smoking. It has been observed that the reduced lignan intake is often associated with an increased intake of saturated and polyunsaturated fatty acids and to a lesser extent, with the consumption of carbohydrates and cholesterol (Peterson *et al.* 2010).

Lignans have also been found to have other properties such as insecticidal, fungicidal, antioxidant, antitumor, antiviral, antibacterial, estrogenic, anti-estrogenic, and last but not least, protective properties with regard to the heart disease (Harmatha 2005; Adlercreutz 2007; Yatkin *et al.* 2014).

Our own experiments have revealed that the knots on Norway spruce (*Picea abies* L.) trees, which are grown in Czech highlands forests (Balík *et al.* 2015), can provide a rich source of HMR.

The main objective of our study was to prepare lignan-enriched grape musts through the addition of lignan-rich spruce knot chips (after resin removal) into the production process. Over a period of 12 months for grape musts we analysed samples to determine the effects of the enrichment process. We performed sensory evaluation and analysis of lignan content, antioxidant activity (using the FRAP method), total polyphenol content, and antimutagenicity of musts after selected storage times.

The analysis of variance helped us predict significant impacts of the preparation process parameters (addition of wood chips, sugar content, preservation method, heat treatment – thermomaceration time, storage time) on analytical parameters (lignan content, antioxidant activity, and total polyphenol content).

MATERIAL AND METHODS

Sample preparations. In red and white musts, spruce knot chips were added during grape thermomaceration in amounts of 0, 10, or 20 g (for white musts) and 0, 20, or 40 g (for red musts) per 20 kg of mash (expected yield was 10 l of must). Musts were prepared by thermomaceration at 80°C without holding time or by thermomaceration at 80°C with holding time of 20 minutes. All musts were placed in 0.5 l bottles and pasteurised at 85°C for 20 minutes.

Sample storage. Prepared samples of musts were stored for 12 months at temperatures between 1°C and 3°C. Samples of musts were analysed after 1, 5, 9, and 12 months of storage.

Lignan content analysis. HMR was identified as the main lignan in spruce knots. Alpha-conidendrin (CONI) was also found in small concentrations. Methods of the analysis of both HMR and CONI were based on extraction of spruce knot mass using 96% ethanol followed by HPLC. However, before HMR extraction, non-polar substances such as terpenes and resins were removed using hexane.

Both 7-hydroxymatairesinol and α-conidendrin concentrations were assessed using an HPLC apparatus (Hewlett Packard 1050; Hewlett Packard, Palo Alto, USA) with diode array detector (DAD Agilent G1315B; Agilent Technology, Prague, Czech Republic), and Phenomenex Luna C18 column (2) (3 μm, 2 × 150 mm). The mobile phase consisted of water–acetonitrile–*o*-phosphoric acid. Mobile phase A consisted of 5% acetonitrile + 0.1% *o*-phosphoric acid and mobile phase B consisted of 80% acetonitrile + 0.1% *o*-phosphoric acid. For separation, the gradient from 20% B to 80% B within 20 min was used, the flow rate was 0.25 ml/minutes. The temperature of the analysis was 25°C. HMR and CONI were detected at 220 nm.

Determination of antioxidant activity using the FRAP method. Determination of antioxidant activity using the FRAP method was carried out at pH 3.6 in acetate buffer (23 mmol/l sodium acetate trihydrate in a solution of 34 mmol/l acetic acid). The reaction mixture contained 12 mmol/l $FeCl_3$ solution, 10 mmol/l 2,4,6-tris(2-pyridyl)-s-triazine in 40 mmol/l HCl solution, and a buffer at a ratio of 1 : 1 : 10. Two ml of the reaction mixture was mixed with 25 μl of the sample (diluted with deionised water) in a disposable plastic cuvette (10 mm) and the solution was measured using a Helios Beta spectrophotometer (after 10 min) at the wavelength of 593 nm. The antioxidant activity was calculated from a Trolox calibration curve.

Total polyphenol determination using the Folin-Ciocalteu reagent. In this method, 0.5 ml of white must (or 0.1 ml of red must) was put into a 50 ml volumetric flask with approximately 20 ml of deionised water and mixed with 1 ml of the Folin-Ciocalteu reagent. The flask was shaken and then, after 3 min, 5 ml of 20% Na_2CO_3 was added to the mixture, shaken thoroughly again and water was added to bring the volume to 50 ml. After standing for 30 min, the colour intensity (the amount of phenol) in the mixture was measured in 10 mm cuvettes at the wavelength

of 765 nm using a Helios Beta spectrophotometer (Unicam UV Spectrometry, Cambridge, UK) [relative to a control (blank) sample]. Total polyphenol content was calculated from a calibration curve using gallic acid.

Sensory assessment. Sensory evaluation of enriched musts was performed by a panel of trained evaluators. A line segment of 100 mm in length (equivalent to 100 points) was used, along which the evaluators marked the value of the parameter being tested. The number of evaluators ranged from 6 to 12. Evaluators assessed the intensity of the woody aroma of the must (0 very weak, 100 very strong), astringent intensity, and bitterness of the must (0 very weak, 100 very strong), and their assessment of consumer acceptability of the must (0 unacceptable, 100 outstanding). Evaluation of all sample parameters was marked on the same straight line (differentiated by sample codes). Parameters valid for individual samples were evaluated by mean values and standard deviations.

Statistical methods. All analytical parameters were measured using 2 trials with the exception of sensory evaluation, where there were 9 to 12 evaluators. Arithmetic mean and standard deviation were calculated for all parameters. These values were plotted in all figures (the column heights are means and whisker or bar segments represent standard deviations). The statistical evaluation of the data was done using analysis of variance and QC Expert 3.1 statistical software (TriloByte Statistical Software, Pardubice, Czech Republic). In musts, the following factors were evaluated: type of must (red, white), quantity of added spruce (S) knot chips (S0 g, S10 g, S20 g, or S40 g), heat treatment (thermomaceration, T0 zero holding time at 80°C, or T20 thermomaceration at 80°C for 20 min) and storage time (1, 5, 9, and 12 months). Lignan content, antioxidant activity, and total polyphenol content were evaluated as variables that could affect the above-mentioned factors of musts.

Examination of sample antimutagenicity. Determination of antimutagenic effects of the must was carried out using the Ames test. Testing was performed using *Salmonella typhimurium* strain TA98, which can grow only on histidine fortified media. This bacterial strain can undergo substitution or gene frameshift mutations that render the bacterial strain histidine prototrophic (i.e. revertant). As a positive control mutagen, 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline (IQ) was used at a dose of 10 ng per Petri dish. This is an indirect mutagen, i.e. it requires metabolic activation by the cytochrome

P450 enzyme complex, which was achieved with rat liver S9 fractions (containing microsomes). The tests were evaluated by counting revertant colonies on Petri dishes using a QCount colony counter (Spiral Biotech, Astor, USA). Each sample was tested in triplicate (100 μl per dish) and the average number of histidine prototrophic revertant colonies of *S. typhimurium* TA98 was calculated.

Antimutagenicity was expressed as % inhibition of mutagenicity (I) according to the formula:

$$\text{Inhibition (\%)} = 100 - (R_t/R_m) \times 100$$

where: R_t – number of revertants per Petri dish in the presence of the mutagen and the test sample; R_m – number of revertants per Petri dish – positive control (mutagen alone)

The result was evaluated according to the following scale in %: 0–20 negative; 20–40 weakly positive; 40–60 positive; > 60 strongly positive.

Tests were also carried out for all the samples without the application of mutagen to eliminate the mutagenic potential of the test samples themselves.

RESULTS AND DISCUSSION

Content of lignans. After one-month storage samples of red musts that were subjected to thermomaceration at 80°C without holding time contained somewhat less lignans compared to samples after thermomaceration held for 20 min at 80°C (Figure 1A). Lignan content was directly related to the quantity of spruce knot chips added. For given quantities of spruce knot chips (20 and 40 g) lignan content in red musts was almost twice that of white musts. After the addition of 40 g of spruce knot chips lignan content increased to more than 50 mg/l (real values ranged about 50–75 mg/l). Storage for five months decreased lignan content in all samples. Lignan content after storage for nine months was comparable to samples stored for five months. After 12 months of storage lignan content was almost the same as after 1 month. Lignan content in samples without addition of spruce knot chips was below the lignan detection limit.

Samples of white must after thermomaceration held at temperature compared to thermomaceration without holding time exhibited higher lignan content (Figure 1B). Increasing the quantity of spruce knot chips from 10 g to 20 g doubled the lignan content. For samples without addition of spruce knot chips
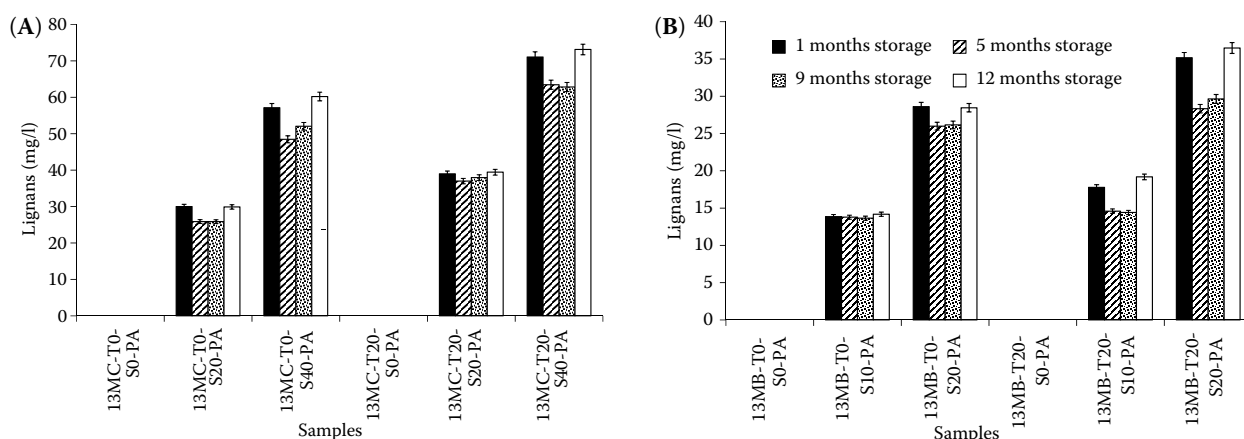
Figure 1. Lignans content in samples of musts of red (**A**) and white grapes (**B**), with the addition of spruce knot chips, during storage

Explanation for Figures 1–4: 13 = year of test; MC = red must, MB = white must; T0 = thermomaceration at 80°C, 0 min holding time; T20 = thermomaceration at 80°C, 20 min holding time; S0, S10, S20, S40 quantity of spruce knot chips (0, 10, 20, 40 g) per ≈ 20 l of grape mash (expected press volume was ≈ 10 l of must); assumption (5% HMR in chips); PA pasteurisation (85°C, 20 min holding time)

lignan content was below the detection limit. Storage for five months produced only a slight decrease in lignan content in white musts containing 20 g of spruce knot chips and subjected to thermomaceration without holding time. In white musts stored for 5 and 9 months that were subjected to thermomaceration with a holding time of 20 min and with the addition of 20 g of spruce knot chips the lignan content was lower compared with musts stored for one month. After nine months of storage there were no further changes in lignan content. After 12-month storage lignan content of must was almost the same as after 1 month of storage.

Sensory evaluation. The woody aroma of red grape musts increased with the quantity of added spruce knot chips; this was true of samples after thermomaceration with holding time or without holding time (Figure 2A). Astringent intensity and bitterness also increase in relation to the quantity of added spruce knot chips.

Parameter values for samples after thermomaceration without holding time did not differ. Consumer acceptability (CA) of enriched musts was lower compared to control samples. However, between the enriched samples there was no change relative to the quantity of spruce knot chips added (i.e. CA 20 g = CA 40 g).
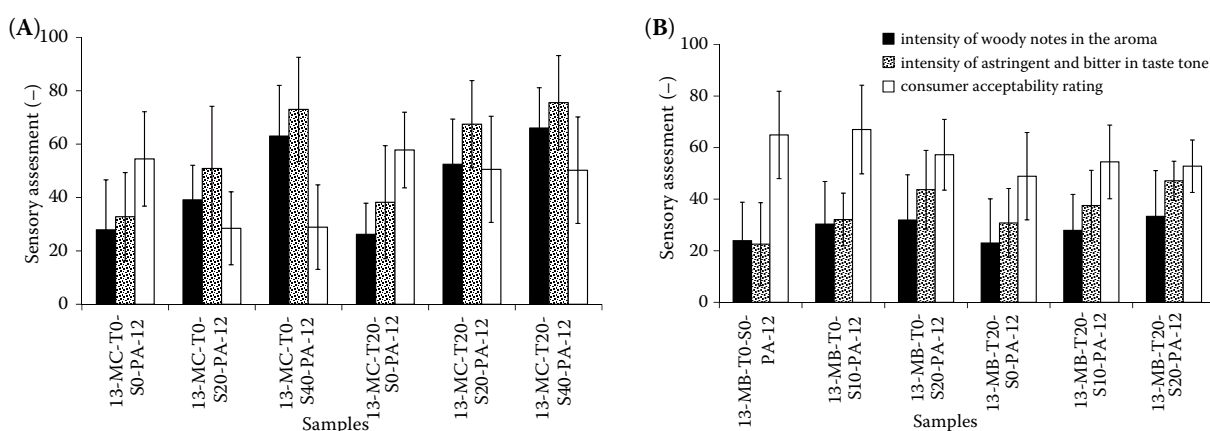

Figure 2. Sensory evaluation of red (A) and white (B) grape musts , with the addition of spruce knot chips, evaluated after 12 months of storage, the number of assessors = 9
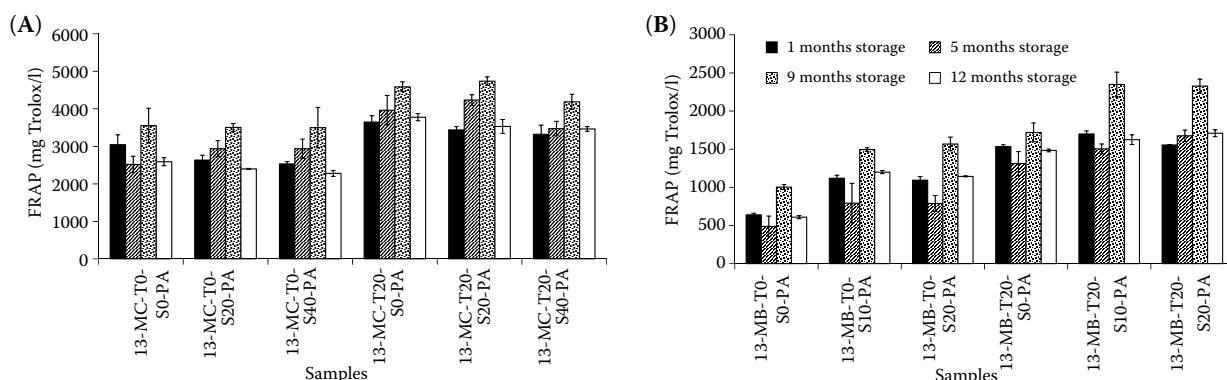
For explanation see Figure 1

Figure 3. Antioxidant activity of red (**A**) and white (**B**) grape musts, with the addition of spruce knot chips, during storage
For explanation see Figure 1

Figure 2B shows that woody aroma in white grape must was slightly more intense in enriched samples after thermomaceration, regardless of the holding time. Astringent intensity and bitterness were increased by the addition of spruce knot chips; these attributes were also slightly increased in samples after thermomaceration with holding time compared to samples without holding time. Consumer acceptability did not change very much with the addition of spruce knot chips; the best quality was found for must samples after thermomaceration without holding time and with 10 g of spruce knot chips.

Antioxidant activity. The antioxidant activity of lignan-enriched red grape must (relative to 1 month) increased in nearly all samples after 5 and 9 months of storage (Figure 3A); however, after 12 months antioxidant activity was similar to values observed after 1 month of storage.

The antioxidant activity in lignan-enriched white grape musts fluctuated during storage (Figure 3B). The highest value was obtained after 9 months of storage. After 5 months of storage, there was a slight decrease. Antioxidant activity after 1 and 12 months was almost the same.

Content of total polyphenols. Total polyphenol content in samples of lignan-enriched red grape musts fluctuated slightly during storage, with the highest values occurring after 1 month of storage (Figure 4A).

After 1 and 12 months of storage the total polyphenol content of lignan-enriched white grape musts reached the highest value and was almost the same for both storage periods; while other storage times tended to fluctuate (Figure 4B). For lignan-enriched red and white musts, samples after thermomaceration with holding time exhibited higher total polyphenol values compared to samples after thermomaceration without holding time.

Statistical data evaluation by analysis of variance. The lignan content in musts was affected statistically significantly by the type of must (i.e. red, white) and the quantity of added spruce knot chips (0, 10, 20, 40 g per 20 kg of mash) (Table 1).
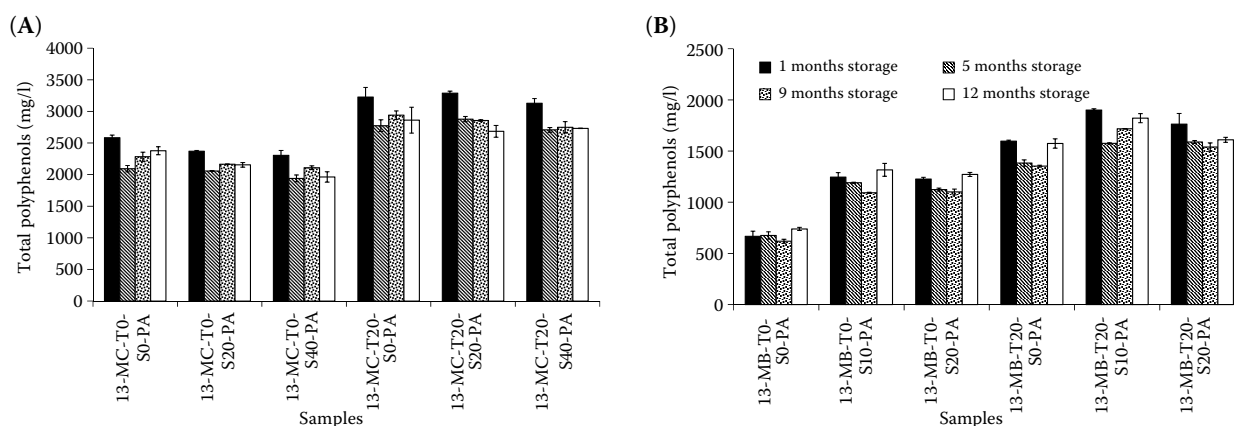

Figure 4. Total polyphenol contents in red (**A**) and white (**B**) grape musts, with added spruce knot chips, during storage
For explanation see Figure 1

Table 1. Analysis of variance for musts with the addition of spruce knot chips, parameter lignans content, antioxidation activity, and total polyphenol content

Predictor (factor)	Sum of squares	*F*-statistics	*P*-value	Significance
Lignans content				
Grape must type	3 065.6	7.882	0.00730	significant
Added knot chips	2 0158.8	370.5	3.27E-32	significant
Heat treatment	284.5	0.633	0.43031	insignificant
Storage time	64.5	0.045	0.9871	insignificant
Antioxidation activity				
Grape must type	780.7	140.0	1.49E-15	significant
Added knot chips	196.0	3.418	2.49E-02	significant
Heat treatment	140.3	7.194	0.010127	significant
Storage time	69.9	1.059	0.375682	insignificant
Total polyphenol content				
Grape must type	18 103 238	113.6	5.13E-14	significant
Added knot chips	3 931 035	2.682	5.77E-02	insignificant
Heat treatment	5 350 647	12.26	0.001042	significant
Storage time	527 561	0.311	0.817532	insignificant

Antioxidant activity was affected statistically significantly by the type of must (i.e. red, white), quantity of added spruce knot chips (0, 10, 20, and 40 g per 20 kg of mash) and by heat treatment (thermomaceration without holding time at 80°C vs. thermomaceration held at 80°C for 20 minutes) (Table 1). Content of total polyphenols was affected statistically significantly by the type of must (i.e. red, white) and heat treatment (thermomaceration without holding time at 80°C vs. thermomaceration held at 80°C for 20 min) (Table 1).

Must antimutagenicity. The highest antimutagenicity (% inhibition of mutagenicity) for red musts was found in a sample after thermomaceration with 20 min holding time (Table 2), but without the addition of spruce knot chips (72%), followed by a sample after thermomaceration with 20 min holding time with added spruce knot chips (40 g per 20 kg of grape mash).

For white musts the highest antimutagenicity was also found in samples after thermomaceration with 20 min holding time, but without the addition of spruce knot chips (64%), followed by samples subjected to thermomaceration with 20 min holding time with added spruce knot chips (20 g per 20 kg grape mash) (Table 2). Red musts exhibited higher antimutagenicity compared to white musts. All four above-mentioned assessments of antimutagenicity were strongly positive according to the scale. All test samples without the application of the mutagen exhibited no mutagenic activity.

Table 2. Determination anti-mutagenicity for red and white musts with the addition of spruce knot chips

Sample	R_t	SD	I (%)	Sample	R_t	SD	I (%)
Red musts				**white musts**			
13 MC T0 S0 PA	90	7.8	56.5	13 MB T0 S0 PA	108	16.6	48.8
13 MC T0 S20 PA	69	2.1	66.7	13 MB T0 S10 PA	132	14.4	37.4
13 MC T0 S40 PA	89	12.4	57.0	13 MB T0 S20 PA	125	3.6	40.7
13 MC T20 S0 PA	58	4.2	72.0	13 MB T20 S0 PA	76	4.2	64.0
13 MC T20 S20 PA	90	9.8	56.5	13 MB T20 S10 PA	119	3.9	43.6
13 MC T20 S40 PA	66	7.3	68.1	13 MB T20 S20 PA	82	8.7	61.1
IQ mutagen	R_m 207	9.3		IQ mutagen	R_m 211	8.2	

R_t – mean number of histidine prototrophic revertants (3 replications) on Petri dish (mutagen and sample); R_m – mean number histidine prototrophic revertants (3 replications) on Petri dish (mutagen alone); SD – standard deviation; I (%) – inhibition of mutagenicity

CONCLUSIONS

Lignan-enriched red and white musts subjected to thermomaceration at 80°C without holding time had the lower lignan content compared to samples after maceration at 80°C for 20 minutes. The intensity of woody aroma, and the bitterness and astringent tastes of lignan-enriched red and white musts were directly related to spruce knot chip quantities, regardless of the thermomaceration holding time.

Antioxidant activity of lignan-enriched red grape musts generally increased for storage times of 5 and 9 months. Antioxidant activity after 1 month of storage was about the same as that after 12 months. In all samples of lignan-enriched white musts, antioxidant activity reached the highest values in 9 months of storage.

Total polyphenol content in the samples of lignan-enriched red grape musts was highest after 1 month of storage. The content of total polyphenols after 1 and 12 months of storage in lignan-enriched white grape musts was almost the same, with fluctuations associated with 5 and 9 months of storage. In both lignan-enriched red and white musts, total polyphenol content was higher in samples after thermomaceration with holding time compared to those without holding time.

The highest antimutagenicity (% inhibition of mutagenicity) was observed in unenriched (i.e. no spruce knot chips) red and white musts after thermomaceration at 80°C with 20 min holding time. Red musts exhibited higher antimutagenicity values compared to white musts.

The obtained analytical data and process parameters were evaluated using the analysis of variance.

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