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*Full Length Research Paper*

# Effect of *Saccharomyces cerevisiae* fermentation on the colorants of heated red beetroot extracts

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**The aim of the present study was to evaluate the effect of the inoculation of the heated aqueous red beetroot extracts (30, 50 and 60°C) by different concentrations of *Saccharomyces cerevisiae* (0.50, 0.75 and 1.00%). The optimum experimental temperature value and *S. cerevisiae* concentration were found to be 50°C and 0.50% for both sugar consumption and pigment preservation. The antioxidant capacity measured by $ABTS^{\bullet+}$ assay (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation) shows that this extract present an appreciable activity ($EC_{50}$= 10.77 µg.mL$^{-1}$) above the tested samples. The betalains compounds were analyzed and identified using UPLC LTQ Orbitrap MS method. Betalains (vulgaxanthin I, betanin, isobetanin, betanidin and neobetanin) were determined in the fermented extract.**

**Key words:** Red beetroot, fermentation, *Saccharomyces cerevisiae*, betalain compounds.

## INTRODUCTION

In recent years, interest in natural food colorants has considerably increased. Natural colors are found to be nutritional antioxidants and their presence in diet can reduce the risk of cardiovascular diseases, cancer and diseases associated with ageing (Chethana et al., 2007). Red beet color is gaining importance as an alternative to synthetic red colorants for food and pharmaceutical applications. The red coloration in vegetable beet is due to red and yellow pigments namely betacyanins and betaxanthins, together generally know as betalains (Thimmaraju et al., 2003). All betalain pigments are water soluble, a property exploited to extract the pigment. The beet extracts contains 80% of the fermentable carbohydrates and nitrogenous compounds. These free sugars cause spontaneous fermentation of beet extract and also caramelization during and also caramelization during food processing at high temperatures (Delgado-Vargas et al., 2000). Hence, removal of sugars from the extract is very desirable in order to facilitate the application of betalains in food processing. Fermentation is a simple and useful operation.

*Saccharomyces* sp. is the safest and most effective microorganism for fermenting sugars to ethanol and is used in the industry for bioalcohol production (Yoon et al., 2003). Besides, several alcoholic beverages such as wine or liqueurs are obtained from fruit juices fermented by *Saccharomyces cerevisiae* strains (González et al., 2001). Moreover, *S. cerevisiae* has been employed to remove undesired compounds by fermentation, such as monosaccharide and disaccharide by-products in carbohydrate preparations (Yoon et al., 2003) and benzoic acid in concentrated lingo berry juices (Visti et al., 2003).

The aim of this work was to study the fermentation of the aqueous extracts from Tunisian red beetroot with *S. cerevisiae* to obtain concentrated betalains to be used as a red-purple food colorant. Extraction temperature before

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yeast addition and inoculums size were optimized. Sugar consumption, colorant stability and antiradical activity of the extract were studied after fermentation.

## MATERIALS AND METHODS

### Plant material

Red beets (*Beta vulgaris var. conditiva*) were purchased from a local market in Tunisia during November 2008. Roots were washed, peeled, sliced, ground in a blender and stored at -4°C until processing.

### Preparation of samples

Beetroots (50 g) were homogenized with distilled water (250 mL) for 4 h at three different temperatures: 30, 50 and 60°C. The obtained extracts were inoculated with 0.50, 0.75 and 1.00% of *S. cerevisiae* ($10^7$ $CFUmL^{-1}$). The fermentation was run for 20 h at 30°C. At the end of fermentation, the extracts were centrifuged and filtered to remove both cell debris from starter cultures and coarse beet matrix compounds. Uninoculated extracts prepared under the same operating conditions were kept as controls.

### Determination of pH and dry matter

The pH was measured using a Consort P902 pH-meter in order to determine a possible effect of fermentation on the pH of the beetroot extract. Dry matter was determined by drying 1 g of the extract for 24 h at 103±2°C and weighing it after reaching a constant weight.

### HPLC of saccharides

The chromatographic analyses of saccharides were carried out in a series Agilent 1100 (Germany), high performance liquid chromatograph equipped with a RI detector. The chromatographic separation was achieved with a SUPELCO C610H chromatographic column, 30 cm × 7.8 mm. The mobile phase used was $H_3PO_4$ (0.1%). The analyses were performed isocratically at a flow rate of 0.5 $mL.min^{-1}$ and at room temperature. Glucose was used as a standard for the quantitative determination of the sugars.

### Photometric quantification of betalains

Betacyanins and betaxanthins content of the extracts were determined spectrophotometrically (UVIKON XS; Biotech Instruments equipped with LabPower Junior program) following the Nilsson's method. The pigment concentration of liquid samples was determined as absorbance units of 1% relative to the dry extract at 538 and 480 nm, respectively, for betacyanins and betaxanthins. The betalains content (BLC) was calculated as:

$$BLC\ [mg.L^{-1}] = (A \times DF \times MW \times 1000) / (\varepsilon \times 1),$$

Where, *A* is the absorption value, DF the dilution factor and 1 the path lengh (1 cm) of the cuvette. For quantification of betacyanins (Bc) and betaxanthins (Bx), the molecular weights (MW) and molar extinction coefficients (ε) was respectively, 550 $g.mol^{-1}$ and 60000 $L.mol^{-1}.cm^{-1}$ in $H_2O$: λ = 538 nm for betanin, 339 $g.mol^{-1}$ and 48000 $L.mol^{-1}.cm^{-1}$ in $H_2O$: λ = 480 nm for vulgaxanthin I.

### Color measurement

The extract color parameters were measured with a Lovibond PFX195 colorimeter based on the CIE $L^*a^*b^*$ coordinates. $L^*$ describes lightness of the color, going from black ($L^* = 0$) to white ($L^* = 100$); $a^*$ takes a positive value for reddish colors and a negative value for the greenish ones, whereas $b^*$ takes a positive value for yellowish colors and a negative value for the bluish ones. Chrome ($C^*$) and hue angles (h°) were calculated from $a^*$ and $b^*$ Cartesian coordinates by means of the following expressions:

$$C^* = (a^{*2} + b^{*2})^{1/2}$$

$$h° = \text{atan}\ (b^*/a^*)$$

C* is a measure of the chroma (saturation) and represents the "purity" of a color, with lower chroma being less pure. The parameter h° (0–360°) is a measure of Hue and takes values ranging from 0° to 90° for reds, oranges, and yellows; 90 to 180° for yellows, yellow–greens, greens; 180 to 270° for green–cyans (blue–greens) and blues and from 270 to 360° for blues, purples, magentas, and returns again to reds (Barreiro et al., 1997).

### ABTS radical cation decolorization assay

$ABTS^{\bullet+}$ a blue-green chromophore with characteristic absorption at 734 nm; the addition of antioxidants to the performed radical cation reduces it to ABTS, determined by a decolorization (Shirwaikar et al., 2006). Spectrophotometric analysis of $ABTS^{\bullet+}$ scavenging activity was done according to a previously described method (Re et al., 1999). ABTS radical cations were produced by reacting 2 mM ABTS in distilled water with 70 mM potassium persulfate ($K_2S_2O_8$) stored in the dark at room temperature for 24 h. Then, 1 mL of ABTS radical cation solution was added to 1 mL of fermented extracts at different concentrations.

The absorbance was measured at 734 nm, 30 min after mixing the prepared solution. The percentage of radical scavenging was calculated for each concentration relative to a blank containing no scavenger using the following formula:

$$\text{Inhibition}\ (\%) = [(A_0 - A_1)/A_0] \times 100$$

Where, $A_0$ is the absorbance of the control, and $A_1$ is the absorbance of the sample. The sample concentration providing 50% of the inhibition ($EC_{50}$) was calculated by plotting inhibition percentages against concentrations of the samples.

### UPLC LTQ-Orbitrap analyses

The identification of betalain compounds was based on their molecular masses determined by Ultra-performance liquid chromatography heated electrospray ionization-mass spectrometry (UPLC-HESI-MS). The sample was analyzed using an Accela 600 UPLC system (Thermo Scientific, Germany) coupled to LTQ Orbitrap XL Hybrid FTMS operating in Heated Electrospray Ionization mode. Chromatographic separations of sample components were performed on Hypersil GOLD C18 column (Thermo Fisher Scientific, Germany, 50 × 2.1 mm, 1.9 µm, particle size) at a flow rate of 0.40

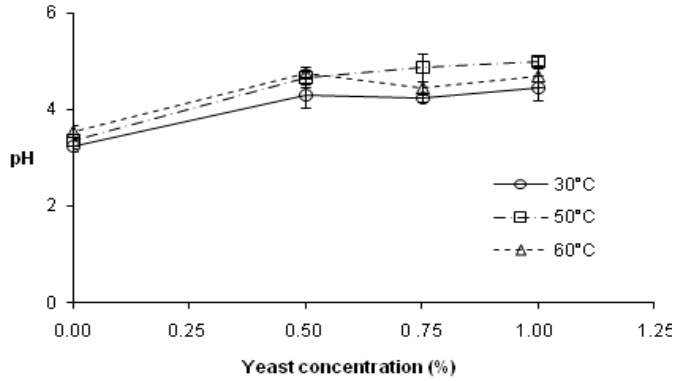


**Figure 1.** pH variation according to the yeast added concentrations of the heated aqueous extract at 30, 50 and 60°C.

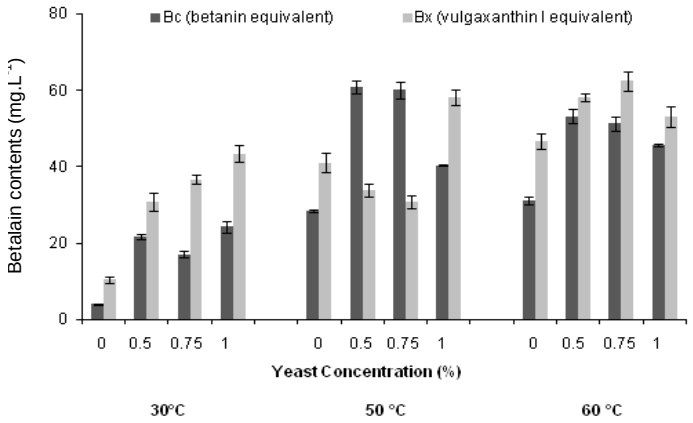


**Figure 2.** Betacyanins (Bc) and Betaxanthins (Bx) (relative to 1% of dry extract) content variation according to the heating temperatures and the yeast added concentrations.

$mL.min^{-1}$. The column was eluted with 0.10% formic acid in water (A) and 0.10% formic acid in acetonitrile 98% (B). A stepwise gradient from 5 to 95% B was applied for 5 min, followed by 2 min isocratically at 95% B.

The Orbitrap MS was operated in both negative and positive mode with masses scanned from 120 to 1000. MS settings were as follows: capillary temperature (270°C), vaporizer temperature (350°C), sheath and auxiliary gas pressures (35 and 10 arbitrary units). For negative ion measurements, the ion source voltage was set to –4000 V, the capillary voltage to –37 V and the tube lens voltage to –204.11 V. For positive ion experiments, the ion spray voltage was 3500 V, the capillary voltage was 13 V and the tube lens voltage was 110 V. Exact mass data were used to assign potential structural formulas using the resident Xcalibur QualBrowser software.

#### Statistical analysis

All data are presented as mean ± standard deviation of triplicate analyzes. For comparisons between samples, data was analyzed by ANOVA and Tukey's multiple comparison test (Statgraphics Centurion XVI). A probability of 5% or less was accepted as statistically significant.

## RESULTS AND DISCUSSION

### pH

The pH control of extracts is an important insurance for the stability of the colorant. Indeed, the degradation of betalains pigments is minimal at pH 4 to 6 (Stintzing and Carle, 2004). Results presented in Figure 1 shows that the pH in *S. cerevisiae* inoculated extracts did not suffer any significant variation in comparison with fresh red beetroot extract (pH= 4.04). Therefore, pH does not affect the stability of pigments. These results are consistent with those of Drdàk et al. (1992) and Castellar et al. (2008). At the same conditions of extraction, the uninoculated extracts (0% of yeast) show a decrease of pH values.

### Sugar concentration

In comparison with the initial extract before fermentation (23.70 $g.L^{-1}$), a drastic decrease of sugar content expressed as glucose concentration was observed for all fermented extracts (Table 1). This result is in agreement with those obtained by Castellar et al. (2008) who reported that *S. cerevisiae* shows a high sugar conversion during fermentation. Nevertheless, the glucose concentration of the inoculated extracts heated at 30°C before yeast addition was higher than those obtained at 50 and 60°C. The sugar content of some of these extracts was detected at the state of trace.

### Betalain content

Both red and yellow pigments were influenced during *S. cerevisiae* fermentation for each heated extract (30, 50 and 60°C), as shown in Figure 2. Indeed, the betalain content increased by the addition of yeast. Otherwise, pigments concentration showed an increase with the temperature. The extracts obtained at 50 and 60°C showed the highest betalain contents. Nevertheless, the heated extract at 50°C were inoculated with 0.50 and 0.75% of yeast which showed the highest betacyanin concentrations of about 61.05 and 60.14 mg betanin equivalent.$L^{-1}$ relative to 1% of dry extract, respectively.

A negative correlation was observed between betalains and sugar content (r = -0.86). This suggests that the decrease of the sugar content lead to a better stability of betalains compounds during processing. Nilsson's method is designed to measure the pigment content of beetroot extract spectrophotometrically without separation of pigments. This technique is not intended to measure

**Table 1.** Glucose concentration ($g.L^{-1}$) in red beetroot extracts.

| Temperature before yeast addition | 30°C | | | | 50°C | | | | 60°C | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % Yeast concentration | 0.00 | 0.50 | 0.75 | 1.00 | 0.00 | 0.50 | 0.75 | 1.00 | 0.00 | 0.50 | 0.75 | 1.00 |
| Glucose | 7.03 | 3.31 | 2.37 | 1.58 | 4.18 | Tr | 0.15 | Tr | 4.11 | 0.80 | 0.19 | Tr |

Tr, Trace.

**Table 2.** Color parameters values determined for red beetroot extracts.

| Yeast concentration (%) | L* | a* | b* | C* | h° |
|---|---|---|---|---|---|
| **30°C** | | | | | |
| 0.00 | 78.14±4.38 | 9.84±0.76 | 38.96±2.15 | 40.18±1.80 | 74.69±0.47 |
| 0.50 | 61.18±3.34 | 15.37±1.83 | 32.89±2.22 | 33.31±2.78 | 64.95±1.14 |
| 0.75 | 64.74±3.29 | 20.14±1.16 | 32.52±2.89 | 38.25±3.53 | 58.22±0,65 |
| 1.00 | 65.90±3.17 | 20.35±1.09 | 30.91±2.19 | 37.00±2.43 | 56.64±0.46 |
| **50°C** | | | | | |
| 0.00 | 56.08±3.39 | 23.96±0.93 | 30.30±1.43 | 38.63±1.08 | 51.66±0.72 |
| 0.50 | 44.93±2.77 | 40.46±2.78 | 17.36±1.11 | 44.02±3.56 | 23.22±0.03 |
| 0.75 | 37.44±2.26 | 37.17±1.67 | 21.23±1.09 | 42.82±2.46 | 29.73±1.36 |
| 1.00 | 32.41±1.32 | 39.46±2.18 | 20.39±1.53 | 44.41±2.44 | 27.32±0.03 |
| **60°C** | | | | | |
| 0.00 | 56.90±3.70 | 19.35±1.01 | 31.84±3.12 | 36.26±3.19 | 58.71±1.17 |
| 0.50 | 47.01±2.33 | 42.67±2.55 | 23.75±1.59 | 48.83±3.00 | 29.10±0.17 |
| 0.75 | 40.34±2.18 | 41.00±2.98 | 21.24±1.23 | 46.17±3.21 | 27.38±0.34 |
| 1.00 | 40.79±2.56 | 41.66±2.71 | 22.59±1.64 | 47.39±3.16 | 28.46±0.18 |

content in heat-treated, partially degraded, or stored colorant mixtures. Large amounts of degradation products will interfere with this method. Therefore, LC analyses were performed to gain a perspective on the respective pigment pattern evolution upon fermentation.

## Color measurement

Color parameters, chroma and hue angle variation are shown in Table 2. $L^*$ values decreased with yeast addition for all samples with higher values observed for inoculated extracts heated at 30°C. The observed decrease of lightness is associated with increased concentrations of betalains in root extracts (r = -0.89). Similarly, $a^*$ and $b^*$ values showed changes with temperature and yeast added concentration, but no significant changes ($p > 0.05$) in chroma value ($C^*$) was observed between samples. Hue angle (h°) of the inoculated extracts heated at 50 and 60°C before yeast added concentrations showed values in the red area of the spectrum.

However, h° values of the inoculated extracts heated at 30°C and controls indicate a yellowish tint, which is in agreement with the decrease of betalains contents (r = -0.91). Thus, betalains are partly decolorized. This decolorization has been reported due to the effect of peroxidase enzyme associated with the beetroot cell wall, which after slicing gets into contact with substrate (Czyżowska et al., 2006) and sugar content (r = 0.89) leading to the caramelization of extracts during incubation (Delgado-Vargas et al., 2000).

## $ABTS^{\bullet+}$ scavenging activity

Generation of the ABTS cation forms the basis of one of the spectrophotometric methods of measuring radical scavenging. The assay involves a decolorization technique in which the radical is generated directly in a stable form prior to reaction with putative antioxidants (Gülçin, 2006). As seen in Figure 3, the studied samples scavenged ABTS radicals in a concentration dependent manner. There was a decrease in the colorization of $ABTS^{+\bullet}$ due to the scavenging capacity of the extracts. At the maximal concentration (200 µg/mL), the scavenging effects of the extract inoculated with 0.50 and 0.75% of yeast

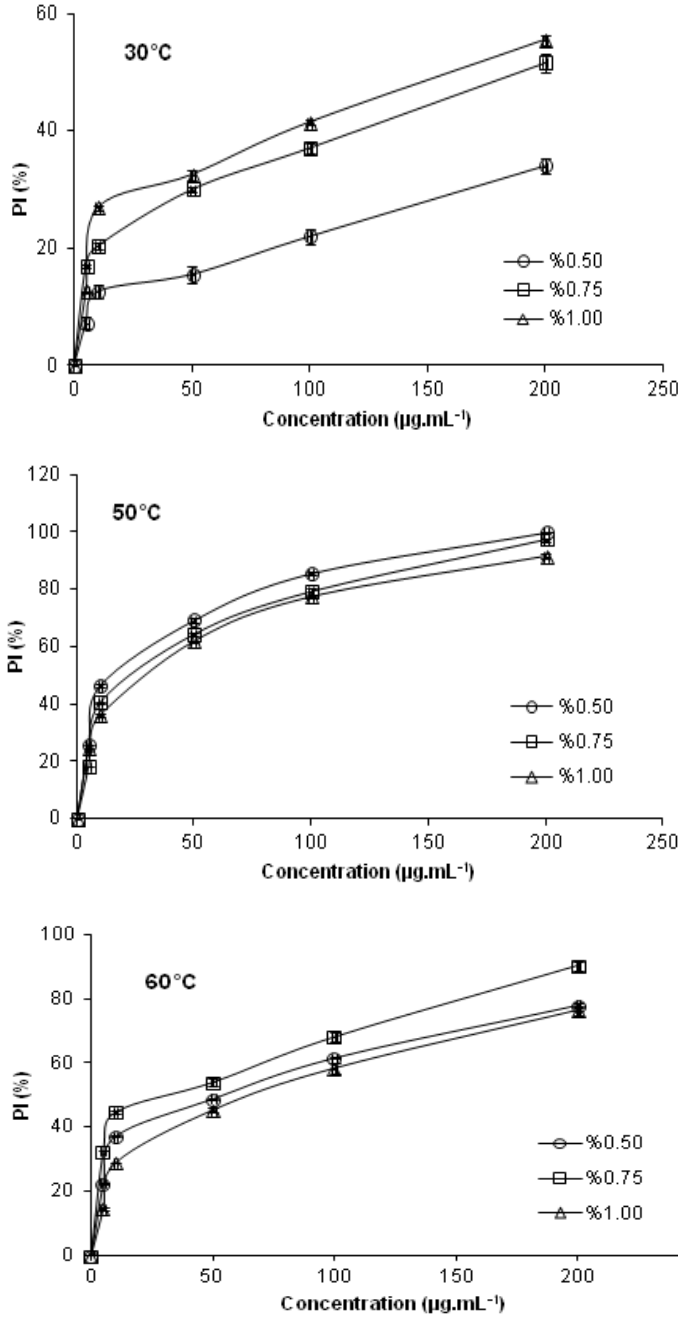


**Figure 3.** Free radical scavenging effect of inoculated extracts by $ABTS^{+\bullet}$ assay.

before heating at 50°C were 99.71 and 97.28%, respectively, which was significantly ($p<0.05$) higher than the other samples.

The determination of $EC_{50}$ of the different samples (Table 3) showed that these extracts presented the lowest concentrations which means the highest $ABTS^{\bullet+}$ scavenging activity ($p<0.05$). The difference in the scavenging activity of the extracts could be attributed to the yeast metabolic activity. During alcoholic fermenta-tion, yeast may change physical (texture and color) and chemical (polyphenols, vitamins, flavours) composition of extracts. Thus, simple sugars, nitrogen compounds and other bioactive molecules are transformed into alcohol production leading to an appreciable antioxidant activity of the extracts (Pérez-Gregorio et al., 2011).

## Betalains pattern

The individual betalains patterns were thoroughly assessed in this work by using UPLC-HESI-MS analyses, a relatively new liquid chromatography technique effectively applied in the analyses of plant and food products (Yu et al., 2006). It was used for the analysis of phenolic acids in beverages such as white wine, grape fruit, and green tea (Zhao et al., 2011; Zuk et al., 2011; Gruz et al., 2008). The major advantages of UPLC over conventional HPLC are enhanced resolution and selectivity, which result in decreased analysis time and solvent consumption (Yu et al., 2006). Coupling UPLC with electrospray ionisation tandem mass spectrometry, thus offers a superior alternative to conventional $HPLC–MS^n$.

Identification of betalains concerned the fermented red beetroot extract presenting simultaneously, the highest colorant stability, lowest sugar content and an appreciable ABTS radical cation scavenging activity. Thus, the heating of the extract at 50°C before yeast addition and the inoculation with 0.50% of *S. cerevisiae* was considered as the suitable parameters for pigments extraction. Representative UPLC chromatogram of the extract is shown in Figure 4. Identified betalains compounds in the extract and their MS data are presented in Table 4. Fresh red beetroot extract is reported to contain one yellow pigment detected at 480 nm, which was the vulgaxanthin I and two main red pigments: betanin and isobetanin detected at 538 nm (Herbach et al., 2004; Kujala et al., 2002; Drdàk et al., 1989). After fermentation, these pigments were also detected in the extract with the appearance of other betalains components identified as betanidin and neobetanin. These results are in accordance with Drdàk et al. (1989), who focused on the effect of fermentation on red beetroot juice by Sephadex G25 column separation. A lactic fermentation also shows quantitative and qualitative variation on the composition of betalain compounds between fresh and fermented red beet juice (Czyżowska et al., 2006). This composition difference is attributed to chemical reactions produced during processing such as isomerization, decarboxylation, or cleavage of betacyanins by heat. *In vitro* isomerization may be induced by increasing temperature and acidic pH conditions (Stintzing and Carle, 2004; Schliemann and Strack, 1998). Neobetanin may also be generated during processing bringing about a betaxanthin-like yellow shift

**Table 3.** $EC_{50}$ values of samples.

| Yeast concentration (%) | | $EC_{50}$ (µg.mL$^{-1}$) [a] | | |
|---|---|---|---|---|
| | | 0.50 | 0.75 | 1.00 |
| Temperature before yeast addition | 30°C | >200 | 193.74±2.93 | 180.22±3.15 |
| | 50°C | 10,77±0.10 | 12,32±0.08 | 40,41±0.22 |
| | 60°C | 51.17±0.30 | 46.30±0.84 | 55,21±0.93 |

Results expressed as means values ± SD of three measurements (level of significance $p<0.05$). [a]$EC_{50}$, ABTS radical cation scavenging activity expressed as Efficient Concentration (EC) values required to decrease the initial ABTS concentration by 50%.

**Table 4.** Identified betalains components in red beetroot extract heated at 50°C before yeast addition and inoculated with 0.50% of *S. cerevisiae.*

| Peaks[a] | Rt (min)[b] | $\lambda_{max}$ (nm) | Molecular weight (g.mol$^{-1}$) | $[M–H]^+/[M–H]^-$(m/z)[c] | Chemical formula | Identification |
|---|---|---|---|---|---|---|
| 1 | 0.42 | 474 | 339.30 | 340/338 | $C_{14}H_{17}N_3O_7$ | Vulgaxanthin I |
| 2 | 0.62 | 538 | 550.46 | 551/549 | $C_{24}H_{27}N_2O_{13}$ | Betanin |
| 3 | 0.95 | 538 | 550.46 | 551/549 | $C_{24}H_{27}N_2O_{13}$ | Isobetanin |
| 4 | 4.29 | 544 | 386.32 | 387/385 | $C_{18}H_{14}N_2O_8$ | Betanidin |
| 5 | 5.80 | 477 | 549.11 | 550/548 | $C_{24}H_{24}N_2O_{13}$ | Neobétanin |

[a] Peak numbers refer to UPLC analysis in Figure 4; [b] Rt, retention time refers to UPLC analysis; [c] $[M–H]^+$, molecular ion in the positive ionization; mode/$[M–H]^-$, molecular ion in the negative ionization mode.

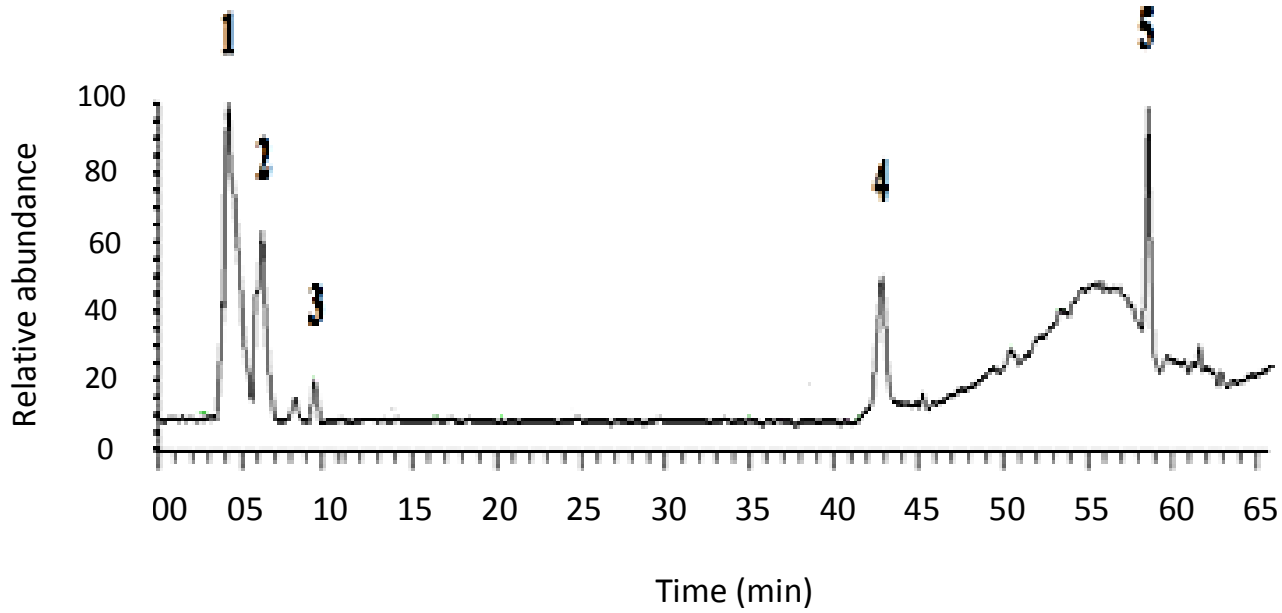


**Figure 4.** Representative UPLC chromatogram of fermented red beetroot extract (temperature before yeast addition = 50°C; yeast concentration = 0.5%).

(Stintzing and Carle, 2004). It is also suspected to result from betanin or isobetanin precursors by a dehydrogenase activity (Stintzing et al., 2005). This dehydrogenase activity might be of plant origin or a result of microbiological activity.

## Conclusion

The effect of the inoculation of the heated aqueous red beetroot extracts (30, 50 and 60°C) by different concentrations of *S. cerevisiae* on pH, sugar content, betalains

content, color parameters ($L^*$, $a^*$, $b^*$) and $ABTS^{+\bullet}$ scavenging activity were studied. A temperature of 50°C and yeast concentration of 0.50% were found to be the most suitable parameters for high sugar consumption, pigment preservation and important antioxidant activity. Betalains compounds of this extract analyzed using UPLC LTQ-Orbitrap method were: vulgaxanthin I, betanin, isobetanin, betanidin and neobetanin.

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