Comparative physiology during ripening in tomato rich-anthocyanins fruits

Borghesi Eva[1], Ferrante Antonio[3] , Gordillo Belén[2], Rodríguez-Pulido Francisco J.[2], Cocetta Giacomo[3], Trivellini Alice*[4], Mensuali-Sodi Anna[4], Malorgio Fernando[1], Heredia Francisco J.[2]

[1] Dipartimento di Scienze Agrarie, Alimentari e Agro-ambientali, Università degli Studi di Pisa, Pisa 56124, Italy

[2] Lab. Colour y Calidad de Alimentos. Universidad de Sevilla. Fac. Farmacia, 41012 Sevilla, Spain

[3] Department of Agricultural and Environmental Sciences, Università degli Studi di Milano, Milano 20133, Italy

[4] Scuola Superiore Sant'Anna, Life Science Institute, Pisa 56100, Italy

*Corresponding author: Alice Trivellini alice.trivellini@gmail.com

Abstract

Solanum lycopersicum (tomato) fruits are important source of nutraceutical compounds with substantial and growing economic and nutritional impacts. Here we assess the physiological diversity affecting the ripening process that yields variation in fruit pigmentation with regard to antocyanins compounds for one nonanthocyanin-accumulating (Ailsa Craig) and two anthocyanin-accumulating tomato genotypes (Anthocyanin fruit type, low pigment accumulation, and Sun Black, high pigment accumulation). Using tomato fruits obtained by traditional breeding we reported a modified hormone equilibrium during the various ripening stages that can be considered a consequence of the anthocyanins accumulation in fruits. Moreover, the fruits of the high pigment accumulation genotype appear more firmness at the comercial stage. Overall, these results showed the considerable potential of exploiting natural genetic diversity to obtain tomatoes with higher levels of secondary metabolites like anthocyanins, and different quality traits such as colour and firmness.

Keywords:, colour; Sun Black tomato; anthocyanins; ripening stage; Ethylene; ABA; firmness;

Introduction

Red colour is a typical trait of commercially grown tomato fruit (*Solanum lycopersicum L.)* and it is mainly due to the presence of the lycopene pigment. Lycopene belongs to the wider group of carotenoids and it can be accumulated both in fruit skin and pulp. Nevertheless, in some wild species of tomato (*S. chilense, S. cheesmaniae*, etc.) skin colour of certain organs, can be also determined by the presence of anthocyanin pigments which provide a typical purple tone. This feature depends on the presence of specific genes, which are activated by sunlight exposure during fruit ripening (Gonzali et al., 2009). Among them genes, the dominant gene "anthocyanin fruit" (*Aft*), is responsible for anthocyanins accumulation in fruit. This gene is present in the *S. chilense* species, and when this species is hybridized with a traditional variety it generates a novel tomato variety '*Aft*' which accumulates low levels of anthocyanins in the skin. Furthermore, from the hybridization of the wild species *S. chilense* and *S. cheesmanie*, both having the recessive gene "atroviolacea" (responsible for the accumulation of anthocyanins in the leaves), derives the novel variety 'Sun Black' (SB), characterized by higher levels of anthocyanins in fruit.

In the last decade growing attention is paid to produce fruit rich in health-related bioactive compounds like anthocyanins considered to offer the best protection against disease either by conventional breeding methods or genetic engineering (Kong et al., 2003; Butelli et al., 2008). The chemical structure of anthocyanins is responsible for their activity as antioxidants and pigments. For these reasons genetic hybridization between traditional tomato species, with low anthocyanins levels and wild ones with a high attitude to accumulate pigments represents a promising approach to be adopted by everyone involved in breeding, growing as well as in the product commercialization to obtain novel tomato varieties with higher nutraceutical value and different appearance features compared to traditional ones.

The mechanisms involved in ripening cover extraordinarily importance in tomato economic sector because, fruits go through a remarkable transformation from something that is uneatable into an attractive, edible fruit. The ripening associated processes include cell wall modifications, fruit softening, the synthesis of pigments, the conversion of starch into simple sugars, and the synthesis of volatile compounds that allow the development of fruit taste and aroma (Leng et al., 2014). All these parameters preponderantly influence

the external appearance of tomato fruits including the skin colour and firmness. From a commercial point of view, colour and firmness are considered key indexes of the global quality of the product, and are strongly affected by the genetic diversity (variety) and by the ripening stage of fruit at harvest.

Undoubtedly, plant hormones have been extensively studied in the various aspects of fruit development, being the ethylene and abscisic acid (ABA) the major contributors to the fruit ripening (McAtee et al., 2013; Kumar et al., 2014). Tomato is a climacteric fruit, and in this type of fruits the ethylene evolution is the key event signaling for the activation of the specific features of fruit ripeness (Bleeker and Kende, 2000; Giovannoni 2001).

Emerging evidence suggest there may be other ethylene-independent factors involved in the control of fruit ripening. A central role for ABA as an intermediate regulator in the perturbation of tomato fruit ripening have been recently proposed (Zhang et al., 2009; Su et al., 2015).

The importance of tomato as an agricultural product have yielded numerous spontaneous and induced mutations, including many that affect fruit development and ripening (Giovannoni 2004). The aim of the present study was to physiologically characterise the ripening of tomato fruits from sunblack genotype, homozygous for both *Aft* and *atv* alleles with altered ability to synthesize anthocyanins, showing an acute black-purple fruit pigmentation. Moreover, using chromametry we assessed the correspondence between hormonal balance, color, firmness and anthocyanin levels.

Materials and Methods

Plant materials

The study was conducted on three tomato (*Solanum lycopersicum*. L.) varieties: Ailsa Craig (AC) characterized by the complete lack in anthocyanins accumulation, and two varieties, obtained by genetic hybridization, Anthocyanin Fruit Type (Aft) and Sun Black (SB), showing a low and a high attitude to accumulate anthocyanins, respectively.

The plants were hydroponically grown in a temperature-controlled glasshouse located in Pisa (latitude 43°430'N; longitude 10°230'E; Italy) during the spring/early summer season. Seedlings were transplanted 50 days after sowing into 1 m long rockwool slabs. The tomato plants were grown vertically with a single stem at a density of three plants m^2. Drip irrigation was carried out using a nutrient solution with an electrical conductivity (EC) of 3.5 dS m^{-1} and pH 6.5. Exhaust nutrient solution was discharged after three weeks

or whenever the EC was higher than 6 dS m^{-1}. The composition of the nutrient solution was as follows (concentrations are expressed in mol m^{-3}): 12 N-NO^{-}_{3}, 1.3 P-POh ,8K+ , 4 Ca2+, 1.2 Mg2+, 9 Na+ , and 1.5 S-SO4 2. Micronutrients were added at Hoagland's concentration (in mmol m^{-3} : B 40, 40 Fe, 1 Cu, 5 Zn, and 10 Mn).

All plants were grown hydroponically in an experimental greenhouse under the same controlled growing conditions. Tomato fruit samples were collected at three ripening stages: mature green (MG) (n=5 for each variety), at turning point (TP) (N=5 for each variety), and mature red (MR) (AC n=13, AFT n=5, SB n=16).

Determination of endogenous ABA

ABA was determined by an indirect enzyme linked immuno-sorbent assay (ELISA) based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA (Vernieri et al., 1989). The ELISA was performed according to the method described by Trivellini et al. (2011), with minor modifications. Flesh and peel samples (100 mg FW) were collected, weighed, frozen in liquid nitrogen, and then stored at -80 °C until analysis. ABA was measured after extraction in distilled water (water:tissue ratio = 10:1 v:w) overnight at 4 °C. Plates were coated with 200 µl per well ABA-4'-BSA conjugate and incubated overnight at 4 °C, then washed three times with 75 mM PBS buffer, pH 7.0, containing 1 g L-1 BSA and 1 ml L-1 Tween 20, keeping the third washing solution for 30 min at 37 °C. Next a 100 µl ABA standard solution or sample and, subsequently, 100 µl DBPA1 solution (lyophilized cell culture medium diluted in PBS buffer containing 10 g L-1 BSA and 0.5 ml L-1 Tween 20, at a final concentration of 50 µg/ml) were added to each well, and competition was allowed to occur at 37 °C for 30 min. Plates were then washed again as described above and 200 µl per well of secondary antibody (Alkaline phosphatase-conjugated rabbit anti-mouse; Sigma, Italy) in PBS buffer containing 10 g L-1 BSA and 0.5 ml L-1 Tween 20, at a final dilution of 1:2,000) was added and incubated for 30 min at 37 °C. Plates were washed again and 200 µl per well p-Nitrophenyl phosphate were added and incubated for 30 min at 37 °C. Absorbance readings at 415 nm were obtained using a MDL 680 Perkin–Elmer microplate reader.

Determination of ethylene production

Ethylene production was measured by enclosing tomato fruits in 1000 ml air-tight containers. Two ml gas samples were taken from the headspace of the containers after 1 h incubation at 22 ± 2°C. The ethylene concentration in the sample was measured by a

gas chromatograph (HP5890, Hewlett-Packard, Menlo Park, CA) using a flame ionization detector (FID), a stainless steel column (150 x 0,4 cm ø packed with Hysep T), column and detector temperatures of 70 and 350°C, respectively, and a nitrogen carrier gas at a flow rate of 30 ml min-1. Quantification was performed against an external standard and results were expressed on a fresh weight basis (pl h-1 g-1 FW).

Anthocyanins composition

Skin anthocyanins levels were measured from fruits at turning point and mature red ripening stages. Around 5 g of tissue were extracted in 10 mL MeOH/4 M HCl, shacked for 1 hour following the method from Mes et al. (2008). The extracts were analyzed by HPLC and the results obtained are presented as $\mu g\ g^{-1}$ of dry weight.

Colour

Colour skin measurement was performed by using a Lutron RGB-1002 (Lutron Electronic Enterprise Co., Taiwan) colourimeter, which is able to detect a dynamic range of 10 bits (1024 values). Determinations were performed on fruit surface (3 measures were taken in the equatorial region of each fruit, every 120 °). The RGB values were then converted to CIELAB parameters (L^*, a^*, b^*, C^*_{ab}, h_{ab}).

Firmness

Fruit firmness was measured by using a penetrometer FT327 (QA Supplies, LLC., USA), data from triplicate measurements are expressed as Kgf.

Statistical analyses

For each experiment, the means from at least five independent biological samples (ABA and ethylene measurements) analyzed with two technical replicates were calculated. Statistical differences among mean values ($p<0.05$) were determined using two-way ANOVA followed by Bonferroni's multiple comparison test to compare the effects of each treatment with the relative hormonal level of the control. hormonal level of the control. The statistical analyses were carried out using GraphPad Prism 5 for Windows v8.0 (GraphPad Software, San Diego, CA, USA) and Statistica® 8.0 (StatSoft Inc., 2007)

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Results

The results were subjected to statistical analysis and showed significant differences ($p<0.05$) in skin colour parameters and anthocyanin levels among the considered varieties at each ripening stage (Table 1).

For all the considered varieties it was observed that at the end of the ripening (MR stage), fruit skin presented the lower values of lightness (L^*) but higher values of chroma (C^*_{ab}) compared to the initial stage (MG). The evolution of the quantitative colour attributes (L^* and C^*_{ab}) indicates that tomato fruit skin tended to get darker and with more intense coloration during the ripening progress. This colour variation was more evident between the TP and the MG stages, when fruits are characterized by a higher rate of *de novo* biosynthesis and accumulation of pigments in the fruit. In case of the variety AC the colour change could be related to a higher accumulation of carotenoids, while, in case of AFT and SB, it could be related to the simultaneous effect of carotenoids and anthocyanins accumulation. With respect to the qualitative attribute of colour (hue, h_{ab}), a progressive decrease of hue values was observed during the ripening, being the effect more marked for SB variety (from 115° to values close to 0° or even negative).

Fig. 1 shows the location of the tomato samples in the CIELAB colour space (a*b*)-plane and lightness (L*) for each variety at different ripening stages. Independently of the tomato variety, a shift of samples from the second to the first and four quadrants was observed during the ripening, that means an evolution of skin tomato color from green to red-orange-blue hues. This colour variation reflects the natural evolution of the pigments in tomato fruits, which is characterised by high levels of chlorophylls in not matured fruits (green colour) and increasing levels of carotenoids and anthocyanins at the end of ripening period (red-orange-blue colour). This is the case of AC and AFT varieties (characterised by low or no anthocyanin accumulation), in which some shades of orange hues due mainly to carotenoids was observed (h_{ab} values around 10°, red-orange region in the (a*b*)-plane, Fig.1a and b). In particular, the SB variety (Fig. 1c) was characterized by an intense red-blue colour (h_{ab} values around -10°, red-blue region in the (a*b*)-plane, Fig.1c), which is consistent with the more intense accumulation of anthocyanin pigments. Based on the dispersion of samples in the (a*b*)-plane, it could be observed that CIELAB colorimetric data allowed a clear differentiation of the different ripening points for AC and AFT varieties (higher dispersion between MG, TP, and MR stages). On the other

hand, SB variety (which was a richer source anthocyanin pigments) showed a minor dispersion of data. This could be due to the high rate of anthocyanin biosynthesis and accumulation from the earlier ripening stages. In this variety, the higher levels of anthocyanin pigments in skin masked the red-orange color of carotenoids, which is typical of the intermediate ripening stages of traditional tomato varieties.

Considering the fruit colour at the commercial ripening stage (MR) (Fig. 2), it was possible to clearly distinguish between the varieties studied. In the genotypes AC and AFT, in which carotenoids were predominant, a more vivid color (higher C^*_{ab} values) and a red/orange hue (h_{ab} between 0 and 10 °) were observed. By contrast, fruits from the variety SB, showing higher predominance of anthocyanins, had less intense but darker bluish colour (samples were closer to the origin of axes into the color diagram and showed lower values of L*).

The firmness of the fruits of three varieties were monitored during development (Fig. 3). The softening pattern of AC and AFT with a similar reduction rate in firmness. The SB fruits had a similar firmness loss until the TP development stage, but at MR stage showed higher values compared with the other varieties, 2.5 Kgf. Along with the progression of ripening it is possible to observe a progressive softening of the fruit and values decrease from 4.0 and 5.5 Kgf of the initial ripening stage, to 1.0 and 3.0 Kgf when the full maturation is reached. Firmness is a phenotypic trait which appears to be genetically determined. In fact, despite the stage of ripening, the two varieties able to synthetize anthocyanins (AFT and SB), showed fruits more firm compared to the traditional variety AC, which completely lack in anthocyanin pigments.

Ethylene biosynthesis was monitored during development. At the MG stage the ethylene was similar and very low (1-2 nL g^{-1} h^{-1}) in all varieties. At the TP stage the higher ethylene production was observed in the AC varieties, while no difference was observed between SB and AFT varieties that showed ethylene levels comprised from 15 to 22 nL g^{-1} h^{-1} (Fig. 4). At the MR stage the ethylene evolution was the same in all varieties and was 20 nL g^{-1} h^{-1} in average.

The ABA content was determined in the flesh and peel in the three development stages (Fig. 5A-B). The higher amount of ABA was found in the peel in all varieties. At MG stage, the higher values were observed in AC variety with 300 ng g^{-1} in the pulp and 1000 ng g^{-1} in the peel. In the pulp the ABA was constant during development and values were similar at TP and MR. In the peel the ABA of AC variety declined at TP and increased at MR stages. In the SB fruits the ABA increased at TP stage and declined in the MR stage.

The highest ABA content was found in the TP stages in both pulp and peel. In the AFT the ABA showed higher ABA values in the MR stage. In the pulp the ABA was constant in the MG and TP stages with values of 200 ng g-1, while in the MR stage the ABA increased up to 300 ng g-1. The ABA content in the AFT peel fruits slightly increased but the differences were not statistical significant.

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Discussion

The anthocyanin content affects the overall tomato quality since influence the visual appearance and hence the consumer's appeal. The accumulation of these bioactive compounds can have direct influence on fruits ripening and postharvest performance. Results obtained confirm the importance of the genotype in influencing the levels of pigments accumulated in the fruit during the ripening process. The genetic background determined significant differences in the external appearance of fruit at commercial ripening stage in terms of both firmness and colour parameters. These evidences were observed in different fruits such as blueberry and strawberry (Cocetta et al., 2015; Kosar et al., 2004). Anthocynins are antioxidant compounds able to counteract or modulate the senescence processes in different plant organs such as leaves, flowers and fruits (Ferrante et al., 2006; Cao et al., 2011; Wen et al., 2015). The delay of senescence is associated with reduction of softening. Texture is an important quality parameter for avoiding mechanical damage during postharvest handling and extend the shelf life of the fruits (Verheul et al., 2015) and it is also an important quality parameter for the consumer.

Ethylene is one of the most important regulator of ripening and senescence of tomato fruits. It is involved in a wide range of network of enzymes activation and leads to many physiological and biochemical changes, including softening and aroma production (Hoeberichts et al., 2002; Alexander and Grierson, 2002). Tomato is a climacteric fruit and shows an ethylene peak during ripening. In our study, it seems that the higher anthocyanins content delayed the ethylene climacteric peak in Aft and SB tomatoes. In AC tomatoes, the ethylene increase was observed at the TP stage, while in AFT and SB the ethylene was still increasing at the MR stage. SB tomatoes seem to produce less amount of ethylene and it may explain the retention of firmness.

The ABA is another important ripening regulators and its levels change according with fruit development stages. The lack of ABA in fruit during ripening alter the normal ripening pathway. In tomatoes fruits, the expression of the key enzyme involved in the

ABA biosynthesis, NCED, increased in the breaker stages and the ABA peak was found at the turning stages (Zhang et al., 2009). These findings were in according with the SB ABA content. The ABA and ethylene are the most important factors of fruit ripening and their interaction is crucial for the fruit development and postharvest senescence (Sun et al., 2010). Moreover, ABA is directly involved in fruit pigmentation and accumulation of nutraceutical compounds (Li et al., 2015).

In conclusion, the data obtained show that SB tomatoes have a modified hormone equilibrium during the various ripening stages that can be considered a consequence of the anthocyanins accumulation in fruits. SB tomatoes beside the higher concentration of bioactive compounds have also a good quality traits for the postharvest distribution chain such as a hardness texture at the commercial stages.

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Figure
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Table 1. Mean values of the colorimetric CIELAB parameters and the Total Anthocyanin content *(*TA, µg g-1 DW*)* in tomato skin for each variety at different ripening stages.

	Ripening stage	AC	AFT	SB
Colour data				
L*	MG	45.6±1.4 $_{a}$	49.5±1.0 $_{b}$	35.9±3.4 $_{ab}$
	TP	53.4±3.6 $_{a}$	57.5±6.2 $_{a}$	34.8±3.9 $_{b}$
	MR	38.7±2.1$_{a}$	40.0±3.1 $_{a}$	32.7±3.7 $_{b}$
C*$_{ab}$	MG	35.9±1.9 $_{a}$	22.2±2.7 $_{b}$	5.2±1.9 $_{c}$
	TP	31.1±11.3 $_{a}$	23.1±2.6 $_{a}$	10.7±12.7 $_{a}$
	MR	54.4±5.9 $_{a}$	62.7±6.7 $_{ab}$	15.6±19.3 $_{c}$
h$_{ab}$	MG	135.0±3.1 $_{a}$	112.6±3.2 $_{b}$	115.4±0.1 $_{b}$
	TP	39.3±17.7 $_{ab}$	58.7 ±36.3 $_{a}$	13.7±4.2 $_{b}$
	MR	6.5±1.9 $_{a}$	7.2±2.6 $_{a}$	-8.3±9.6 $_{b}$
Anthocyanin Content				
TA	MG	-	-	-
	TP	0.00±0.00 $_{a}$	0.13±0.01 $_{a}$	29.78±1.9 $_{b}$
	MR	0.00±0.00 $_{a}$	4.67±0.03 $_{b}$	31.26±0.9 $_{b}$

Different letters in each column indicates significant statistical differences (p<0.05).

Figure legend

Figure 1. Location of the tomato samples in the CIELAB colour space (a*b*)-plane and lightness (*L**) for each variety at different ripening stages: a) Ailsa Craig (AC), b) Anthocyanin fruit type (AFT), and c) Sun Black (SB).

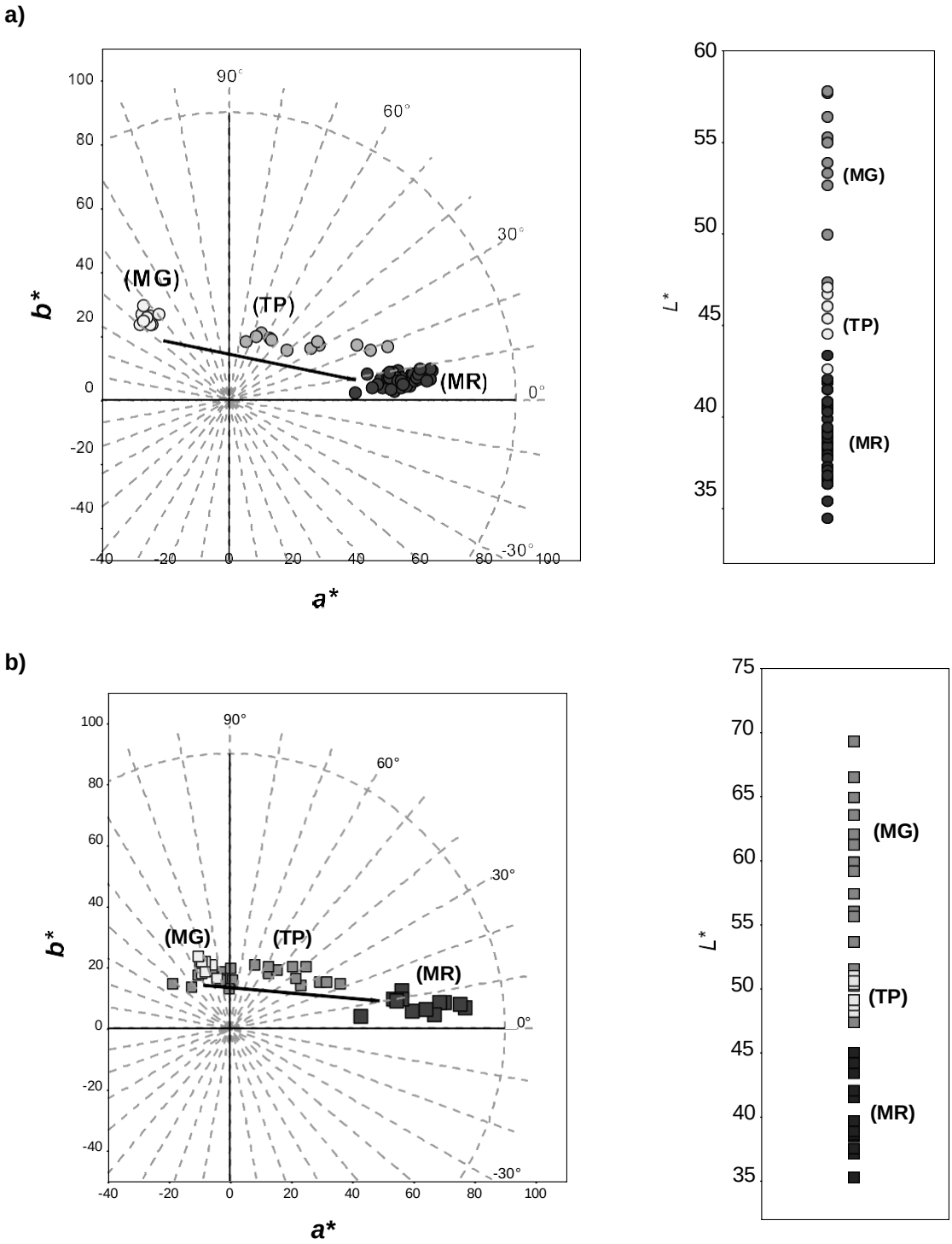
Figure 2. Location of the tomato samples in the CIELAB colour space (a*b*)-plane and lightness (*L**) for each variety at the matured red stage (MR, commercial ripeness grade).

Figure 3. Evolution of fruit firmness (Kgf) during ripening of the three considered tomato varieties. (SB, AFT, AC).

Figure 4. Changes in the rate of ethylene production in AC, AFT and SB fruits. Tomato fruits were harvested at mature green (MG), turning point (TP) and mature red (MR) development stages. Data were subjected to a two-way analysis of variance and differences between developmental stages were analysed by a Bonferroni posttest. Different letters denote significant differences at P < 0.5. Values are means of at least five independent biological samples.

Figure 5. Endogenous ABA content in two fruit tissues (A, flesh tissue; B peel tissue) of tomato at three development stages (mature green MG, turning point TP, and mature red MR). Data were subjected to a two-way analysis of variance. Different letters denote significant differences (Bonferroni post test, P<0.05) of ABA content. Values are means of at least five independent biological samples with two technical replications for each sample

Fig. 1



c)

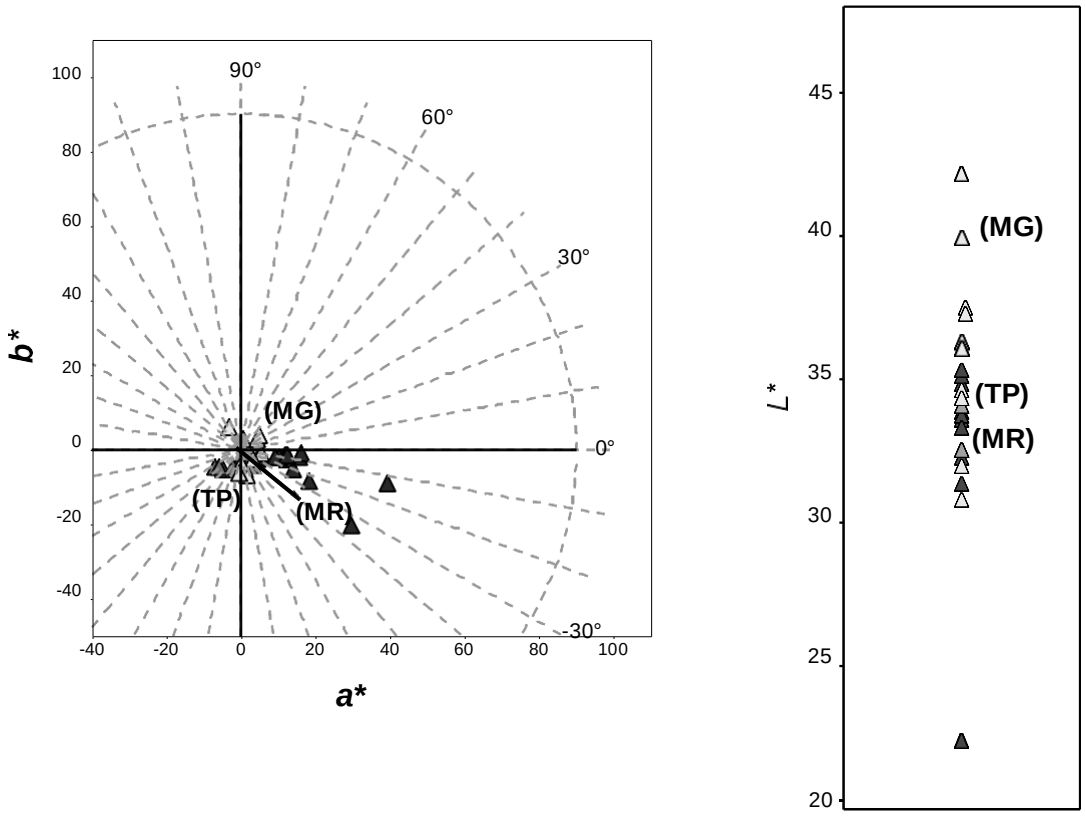
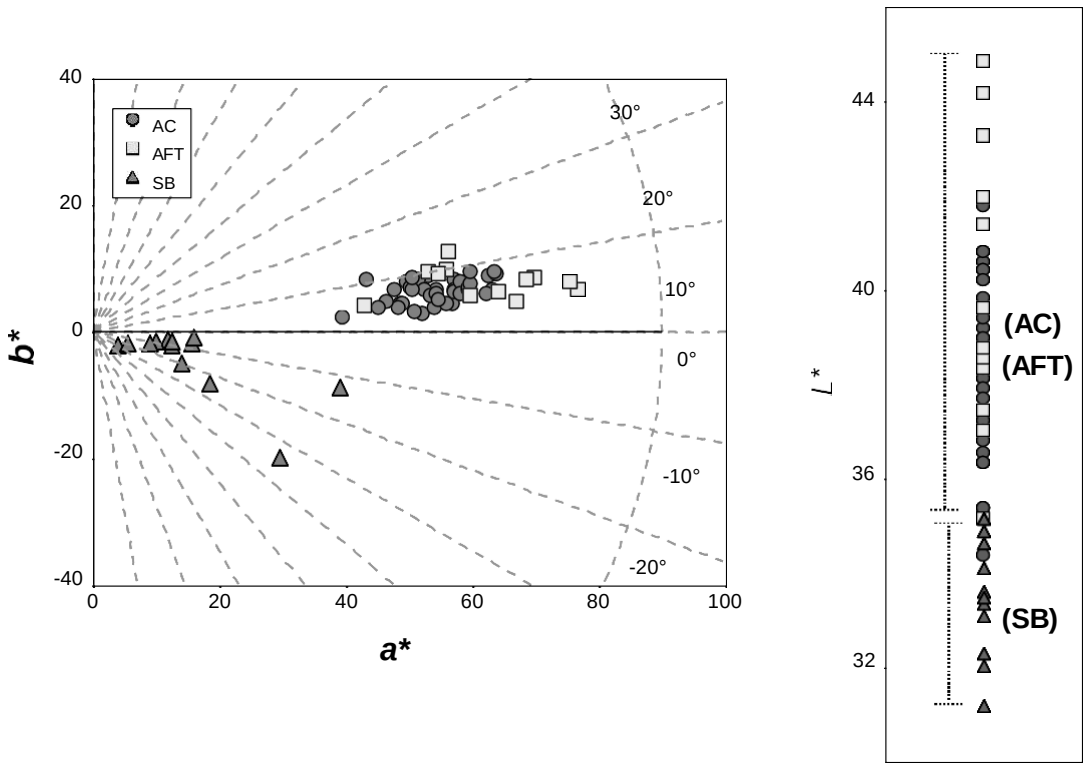


Fig. 2



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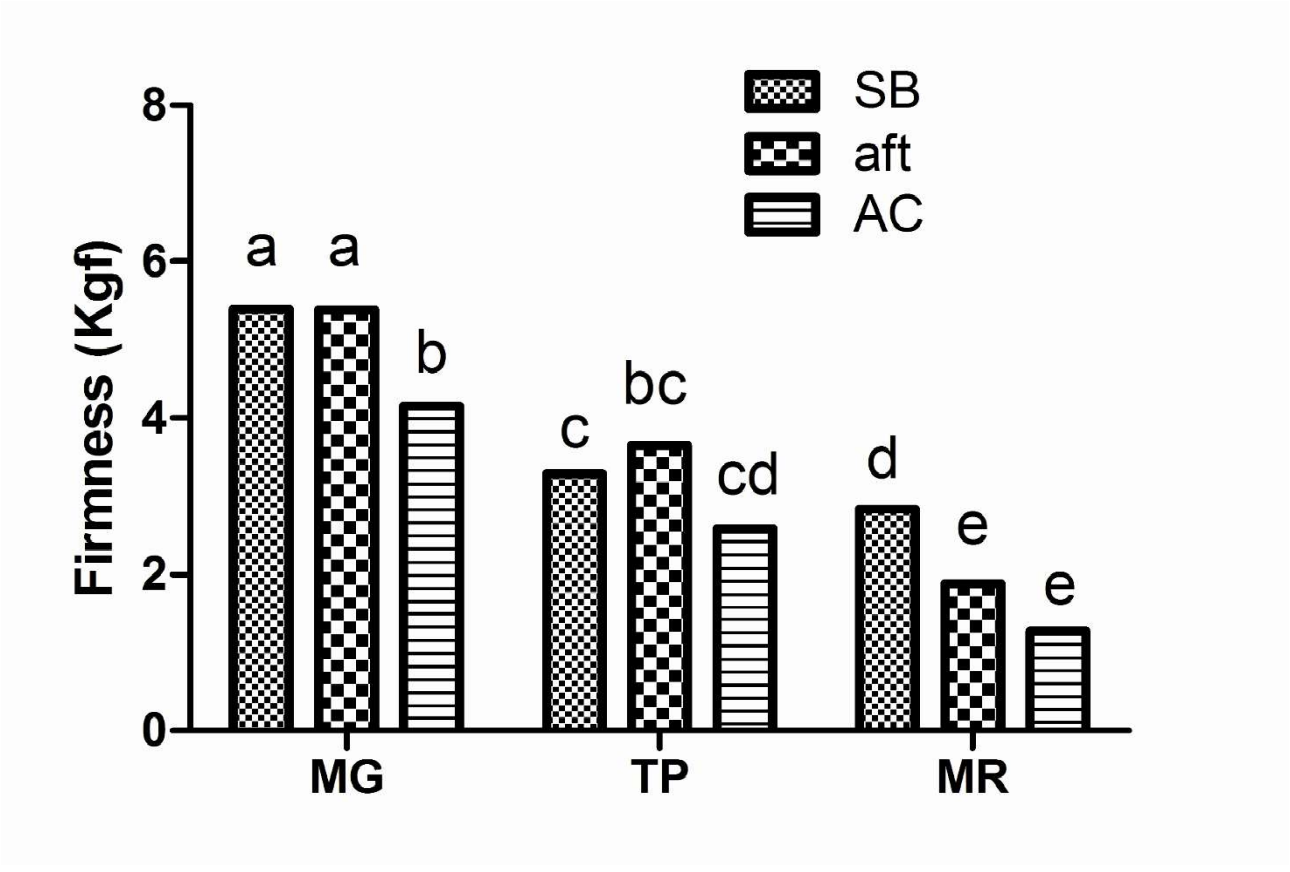
46 **Fig. 3**



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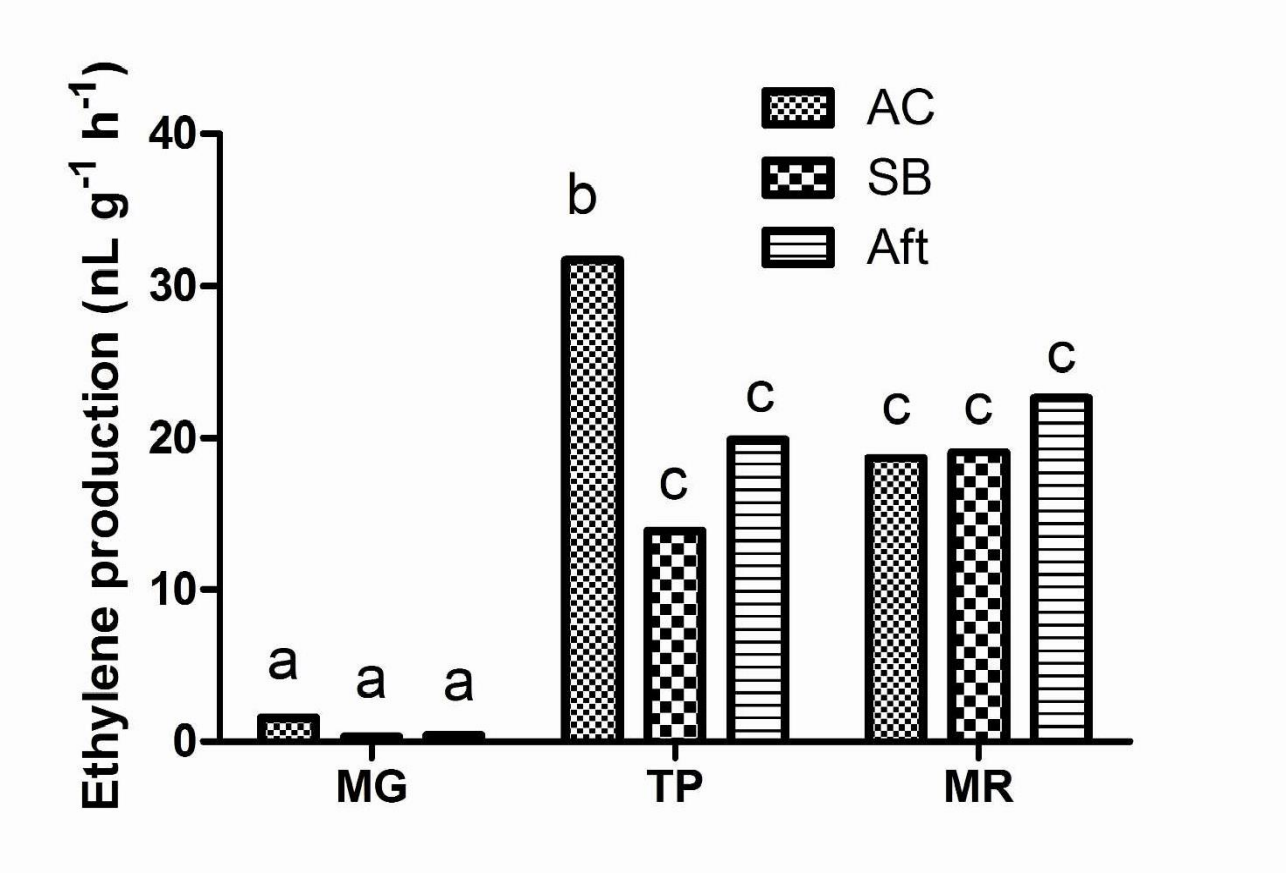
48 **Fig. 4**


Ethylene production (nL g-1 h-1)
40
30
20
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AC
SB
Aft
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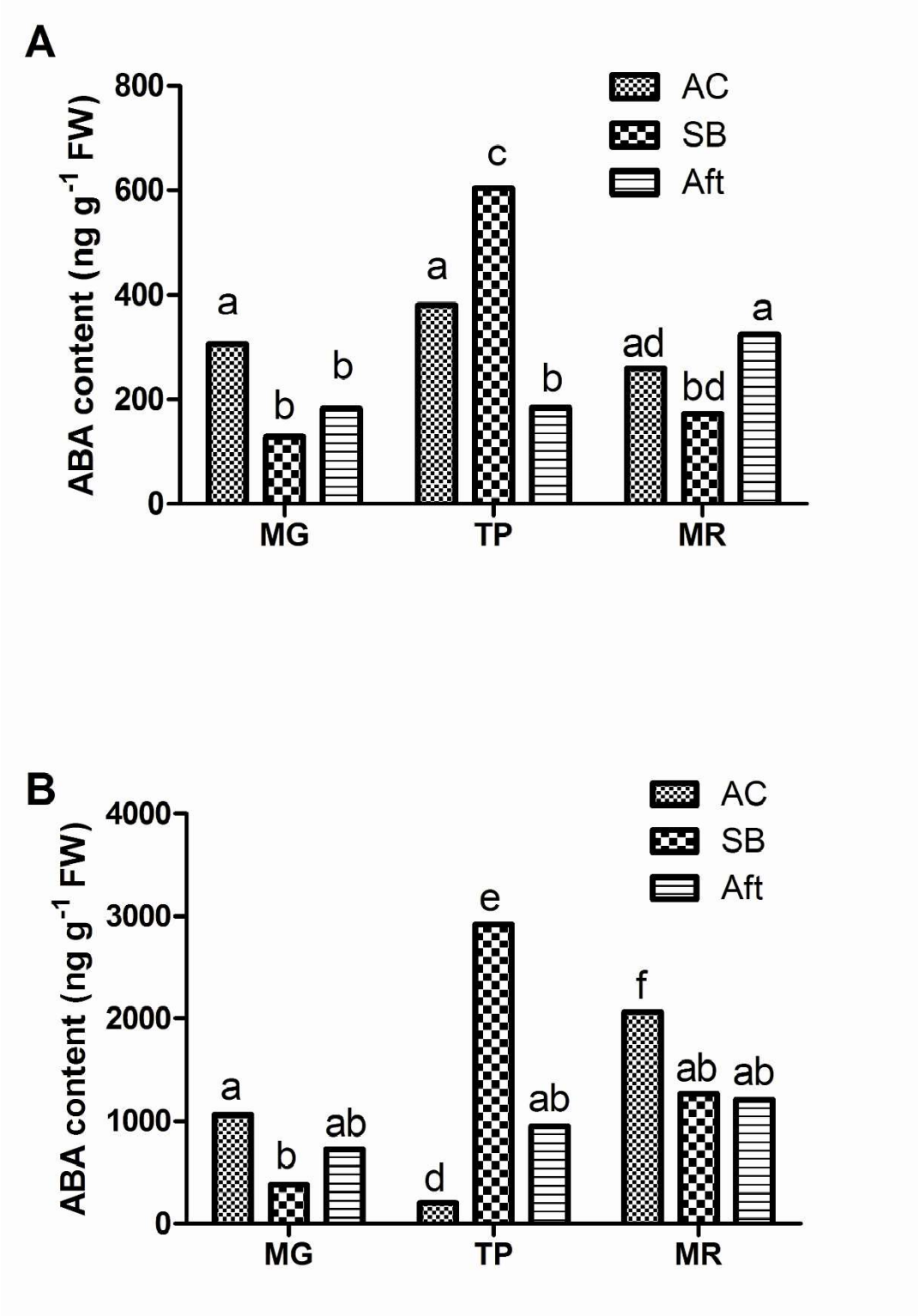
c
MG
TP
MR

Fig. 5


A
ABA content (ng g-1 FW)
800
600
400
200
0
AC
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bd
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ABA content (ng g-1 FW)
4000
3000
2000
1000
0
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