



## Differential accumulation of flavonoids by tomato (*Solanum lycopersicum*) fruits tissues during maturation and ripening

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### ABSTRACT

*Objective:* Little is known about physiological functions of flavonoids, specifically in the course of maturation and ripening of fruits. Spatiotemporal changes in the levels of flavonoids were investigated in the present study with focus on possible functional differentiation of individual compounds as related to the maturation or ripening of tomato fruits.

*Methodology and results:* The contents of flavonoids in different tissues of tomato fruits at increasing maturation and ripening stages were determined using high performance liquid chromatography – mass spectrometry. The levels of eriodictyol, kaempferol-glc-rhamnose, naringenin, naringenin-chalcone-hexose and quercetin-glc-rhamnose remained almost constant in the mesocarp and endocarp. The contents of eriodictyol, dicaffeoylquinic acid, naringenin and naringenin-chalcone-hexose significantly ( $P < 0.05$ ) increased in the epicarp from ripening stage 1 onwards. The concentration of dicaffeoylquinic acid increased significantly ( $P < 0.05$ ) in both the mesocarp and endocarp of tomato fruits from ripening stage 1 onwards. Gradual increases in the levels of caffeic-acid-hexose and caffeoylquinic acid in the epicarp and endocarp of tomatoes were observed. The level of kaempferol-glc-rhamnose decreased gradually in the epicarp. The content of quercetin-glc-rhamnose was always higher in the epicarp than in the mesocarp and endocarp. The results obtained indicated increases in the endogenous levels of some flavonoids in the epicarp (especially naringenin) with the onset of the ripening. There was also a gradual decrease and increase in the levels of respectively kaempferol-glc-rhamnose in the epicarp and caffeic-acid-hexose in the endocarp. Thus, an increase in the level of naringenin in the epicarp could be considered as physiological index for the ripening whereas high levels of kaempferol-glc-rhamnose in the epicarp and caffeic-acid-hexose in the endocarp could serve as characteristic traits for respectively immature and red-ripe state of tomato fruits. On the whole, the results point to specific roles of individual flavonoids as some might be involved in the regulation of either the maturation or ripening of tomato fruits whereas others might functionally be needed throughout both processes, and that there would be a specialization of tissues in the synthesis of specific types of flavonoids.

*Conclusions and application findings:* The degree of accumulation of flavonoids in tomato varied according to the nature of the tissue, and the maturation and ripening stages. It is hypothesized that an increase in

the contents of naringenin and caffeic-acid-hexose may be part of natural mechanisms by which ripe tomato fruits prevent the over ripening when they are still attached to the mother plant. Consequently, the enhancement of the levels of these compounds by genetic engineering, conventional breeding or cultural practices could be a novel strategy for extending the shelf-life of tomato fruits.

**Key words:** Naringenin, caffeic-acid-hexose, kaempferol-glc-rhamnose, fruit maturation, ripening, *Solanum lycopersicum* L. cv Balkonstar

## INTRODUCTION

Flavonoids are plant secondary metabolites, which belong to the vast and diversified group of phenylpropanoids having phenylalanine as their common biosynthetic precursors (Verviridis *et al.*, 2007). They play important roles in the control of plant responses to biotic and abiotic stresses (Takahama, 1982; Hahlbrock and Scheel, 1989; Nicholson, 1992; Feucht *et al.*, 1996; Taiz and Zeiger, 2000; Peter *et al.*, 2001; Ryan *et al.*, 2001; Broeckling *et al.*, 2005; Lattanzio *et al.*, 2006; Chennupati *et al.*, 2012; Dasgupta *et al.*, 2013; Liu *et al.*, 2014; Martínez-Lüscher *et al.*, 2014; Nakabayashi *et al.*, 2014). In some plant species flavonoids are required for the growth of the pollen tube (Yistra *et al.*, 1992; Eldik *et al.*, 1997; Guyon *et al.*, 2000; Antognoni *et al.*, 2004) and seed development (Jiang *et al.*, 2013; Lee *et al.*, 2013; Vogt *et al.*, 1994). They also act in plants as allelochemicals (Taylor and Grotewold, 2005; Li *et al.*, 2010; Filho *et al.*, 2013; Liu *et al.*, 2013; Weston and Mathesius, 2013), signalling molecules in symbiotic associations (Zhang *et al.*, 2007; Maj *et al.*, 2010; Mandal *et al.*, 2010; Moscatiello *et al.*, 2010; Zhang and Franken, 2014) and growth inhibitors (Phillips, 1962; Thimann, 1963; Brown *et al.*, 2001; Yoshioda *et al.*, 2004; Besseau *et al.*, 2007; Brunetti *et al.*, 2013). In human and animal nutrition flavonoids are considered as potentially health-promoting substances due to their anti-oxidative, anti-cancer, anti-diabetes and cardiovascular protective effects (Knekt *et al.*, 1996; Knekt *et al.*, 1997; Böhm *et al.*, 1998; Knekt *et al.*, 2002; Ross and Kasum, 2002; Lin *et al.*, 2007; Naruszewicz *et al.*, 2007; Peñarrieta *et al.*, 2008; Sato *et al.*, 2008). They also have anti-microbial, anti-inflammatory, anti-aging, neuroprotective effects (Rauha *et al.*, 2000; Shahidul Islam *et al.*, 2003; Cushnie and Lamb, 2005; Ilija and Hollman, 2005; Neacsu *et al.*, 2007;

Punithavathi *et al.*, 2010; Atanassova *et al.*, 2011; Brunetti *et al.*, 2013). Tomato is one the most consumed fruits worldwide and its global production was estimated in 2012 at about 162 million tons (FAOSTAT, 2012). This consumer's attraction to the tomato fruit is justified by its high content in potentially health-promoting substances such as flavonoids, vitamins and carotenoids (Abushita *et al.*, 2000; Bramley, 2002; Chassy *et al.*, 2006; Fanasca *et al.*, 2006; Moco *et al.*, 2006; Torres and Andrews, 2006). Thus, most of the previous research works focused on the determination of the qualitative and quantitative flavonoid composition of tomato fruits (Tronchet, 1971; Herrman, 1979; Galensa and Herrman, 1980; Hunt and Baker, 1980; Krause and Galensa, 1992; Beecher *et al.*, 1998; Slimestad and Verheul, 2005; Moco *et al.*, 2006; Mitchell *et al.*, 2007; Schiavon *et al.*, 2013). Flavonoids are synthesized by tomatoes because they play an important physiological role in the growth, development and ripening of fruits. Although changes of the endogenous levels of different metabolites in tomato fruits tissues with increasing age have been investigated (Moco *et al.*, 2007) no study exists, to the best of our knowledge, which reports on the spatial and temporal variations in the concentrations of flavonoids in tomato fruits. Furthermore, Moco *et al.* (2007) distinguished five types of tissues in tomato fruits (epicarp, mesocarp, vascular attachment region, columella and placenta, and jelly parenchyma including seeds), some of which according to our point of view are not easily detachable at all fruit maturation stages, what could lead to wrong interpretation of the results. The present study was undertaken to investigate the differential capacity of three easily identifiable and separable tomato fruit tissues (epicarp, mesocarp and endocarp, the

latter comprising vascular attachment region, columella and placenta, and jelly parenchyma including seeds) to accumulate precise type of

flavonoids with emphasis on eventual physiological specialization of these tissues in the course of growth and development.

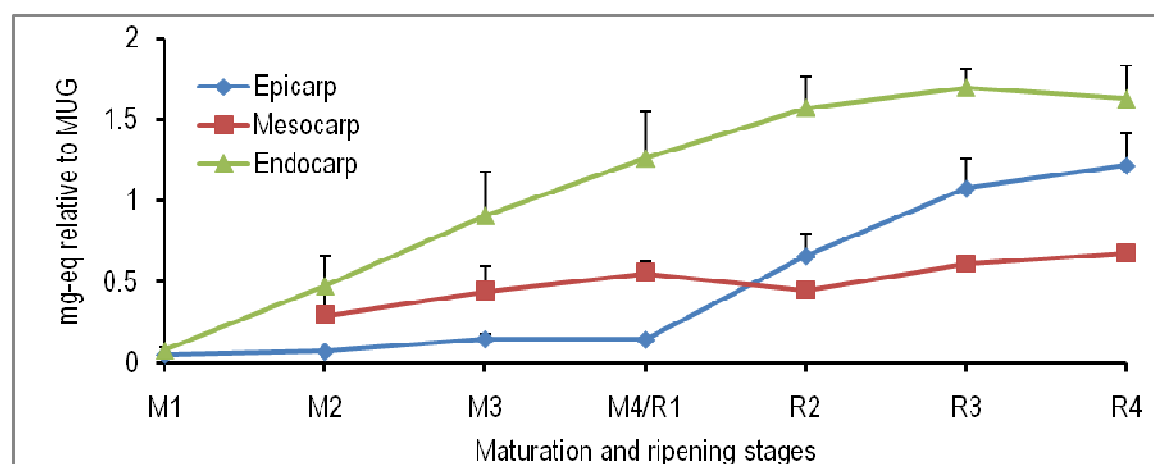


**Figure 1:** Maturation (M) and ripening (R) stages used. M1,  $\phi$  (longitudinal diameter) = 0.7 cm; M2,  $\phi$  = 2.0 cm; M3,  $\phi$  = 3.0 cm; M4/R1, mature green ( $\phi$  = 4.5 cm); R2, turning; R3, pinkish; R4, red-ripe.

## MATERIALS AND METHODS

**Plant material:** Tomato (*Solanum lycopersicum* L. cv Balkonstar) plants were grown in a growth chamber at the Centre for Life and Food Sciences Weihenstephan of the Technical University of Munich (Germany) under a 16h/8h-photoperiod with a light intensity of  $70 \pm 10 \mu\text{mol}/\text{m}^2/\text{s}$  and constant temperature of  $23 \pm 1 \text{ }^\circ\text{C}$ . Tomato fruits were harvested at different maturation stages (Fig. 1), their pericarp, mesocarp and endocarp separated and stored at  $-20 \text{ }^\circ\text{C}$  for the study of spatiotemporal variations of the endogenous level of flavonoids. The tomato fruit tissues were then ground in a mortar under liquid nitrogen. One (1) g each was

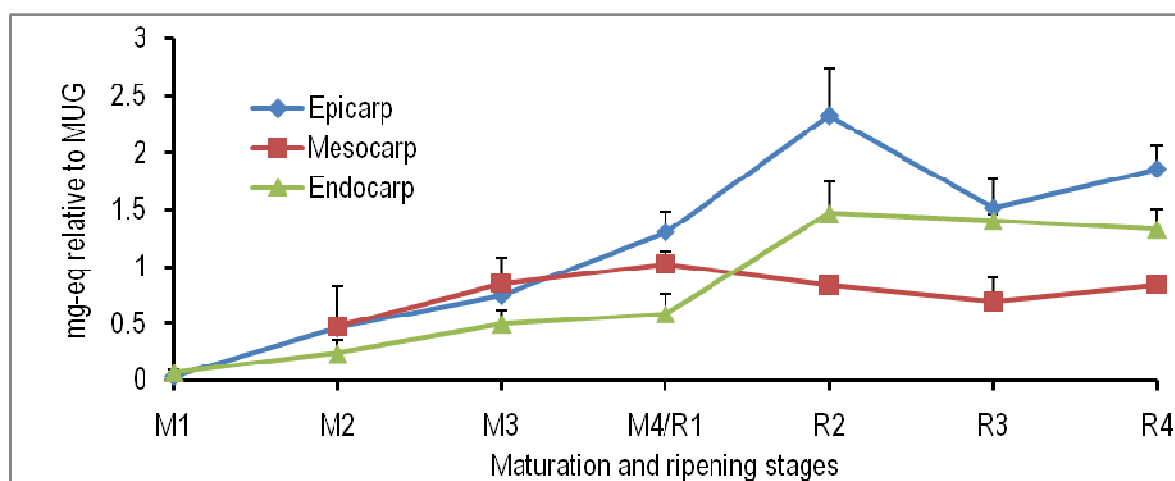
homogenized with a mixture containing 1 ml of methanol and 20  $\mu\text{l}$  of 4-methylumbelliferyl- $\beta$ -D-glucuronide dihydrate solution (9 mg/ml methanol) as internal standard. The mixture was centrifuged (5300 g, 30 min), 2 ml of supernatant then removed and concentrated by speed vacuum. The residue was re-suspended in 150  $\mu\text{l}$  methanol and centrifuged again for 10 min at 13400 g. The supernatant was removed, stored at  $4 \text{ }^\circ\text{C}$  and subsequently analyzed high performance liquid chromatography – mass spectrometry.



**Figure 2:** Spatiotemporal changes in the levels of caffeic-acid-hexose in the epicarp, mesocarp and endocarp of tomato fruits at different maturation (M) and ripening (R) stages. M1,  $\phi$  (longitudinal diameter) = 0.7 cm; M2,  $\phi$  = 2.0 cm; M3,  $\phi$  = 3.0 cm; M4/R1, mature green ( $\phi$  = 4.5 cm); R2, turning; R3, pinkish; R4, red-ripe; eq, equivalent; MUG, 4-methylumbelliferyl- $\beta$ -D-glucuronide dihydrate.

**High performance liquid chromatography – mass spectrometry (LC-MS):** An Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump and a variable wavelength detector, and connected to a Bruker Daltonics Esquire 3000<sup>Plus</sup> ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) was utilized. A C<sub>18</sub> column (150 mm x 2.0 m, particle size 5 µm) held at 25 °C was used. The mobile phase was a mixture of water (A) and methanol (B) both containing 0.1% formic acid. The flow rate was 0.2 ml min<sup>-1</sup>. A gradient of 100% A for 5 min, 100% A to 60% A within 20 min, 60% A to 0% A in 5 min, 100% B for 7 min, 100% B to 0% B within 3 min and 100% A for 10 min was applied. The detection wavelength was 280 nm. The electron spray ionization voltage of the mass spectrometer ranged from -4000 V

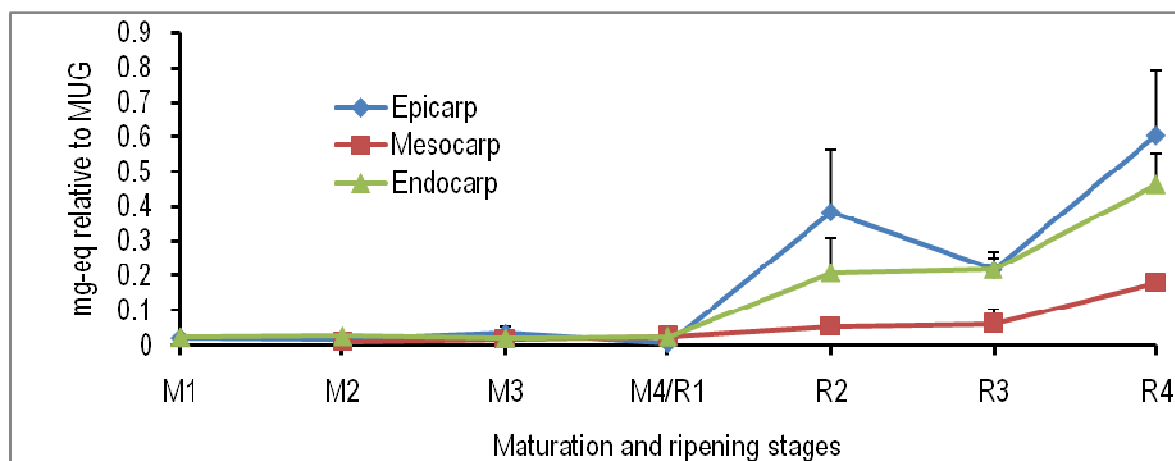
to - 500 V. Nitrogen was used as dry gas at temperature of 330 °C and a flow rate of 10.01 min<sup>-1</sup>. The full scan mass spectra were measured in the range from *m/z* 50 to 800 with a scan resolution of 13000 *m/z* s<sup>-1</sup>. The collision gas for the mass spectrometry was helium with a collision voltage of 1 V. Mass spectra were acquired in both positive and negative ionization modes. Analyses of LC-MS data were performed using the Bruker Daltonics software. Automated comparison of [M-H]<sup>-</sup> and [M-H]<sup>+</sup> was performed using the bioinformatics software R.2.13.1 (R Project for Statistical Computing, version 2.13.1). Compounds were identified using the in-house MS database and the data from Sudjaroen *et al.* (2005), Moco *et al.* (2006) and Mintz-Oron *et al.* (2008).



**Figure 3:** Spatiotemporal changes in the levels of caffeoylquinic acid in the epicarp, mesocarp and endocarp of tomato fruits at different maturation (M) and ripening (R) stages. M1,  $\phi$  (longitudinal diameter) = 0.7 cm; M2,  $\phi$  = 2.0 cm; M3,  $\phi$  = 3.0 cm; M4/R1, mature green ( $\phi$  = 4.5 cm); R2, turning; R3, pinkish; R4, red-ripe; eq, equivalent; MUG, 4-methylumbelliferyl- $\beta$ -D-glucuronide dihydrate.

**Statistical analyses:** Group comparisons were made using One – Way Analysis of Variance (ANOVA) to determine if the variations among means were significantly greater than expected by chance. The Student – Newman – Keuls Test was used to compare means differences. In some few cases where

differences between standard deviations of means were significant, the GraphPad Instat – software automatically proposed the use of rather the Nonparametric ANOVA and Kruskal-Wallis (as post-test).

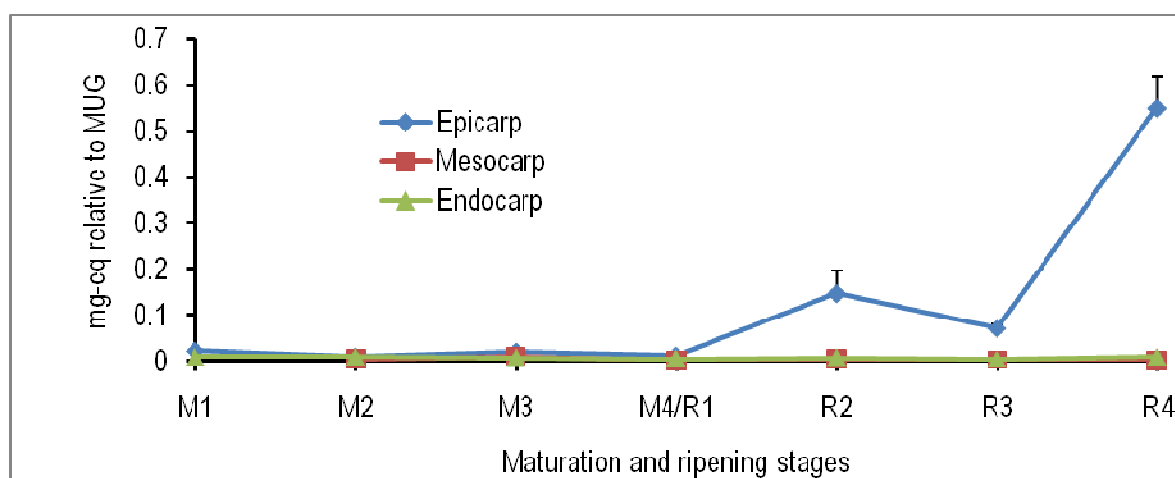


**Figure 4:** Spatiotemporal changes in the levels of dicaffeoylquinic acid in the epicarp, mesocarp and endocarp of tomato fruits at different maturation (M) and ripening (R) stages. M1,  $\phi$  (longitudinal diameter) = 0.7 cm; M2,  $\phi$  = 2.0 cm; M3,  $\phi$  = 3.0 cm; M4/R1, mature green ( $\phi$  = 4.5 cm); R2, turning; R3, pinkish; R4, red-ripe; eq, equivalent; MUG, 4-methylumbelliferyl- $\beta$ -D-glucuronide dihydrate.

## RESULTS

Flavonoids and phenylpropanoids were analyzed in different tissues of tomato fruit at various maturation and ripening stages by LC-MS. Levels were calculated as mg-equivalents relative to the internal standard 4-

methylumbelliferyl- $\beta$ -D-glucuronide dihydrate. The endogenous level of caffeic-acid-hexose increased in all the investigated tissues during the growth and development of tomato fruits.



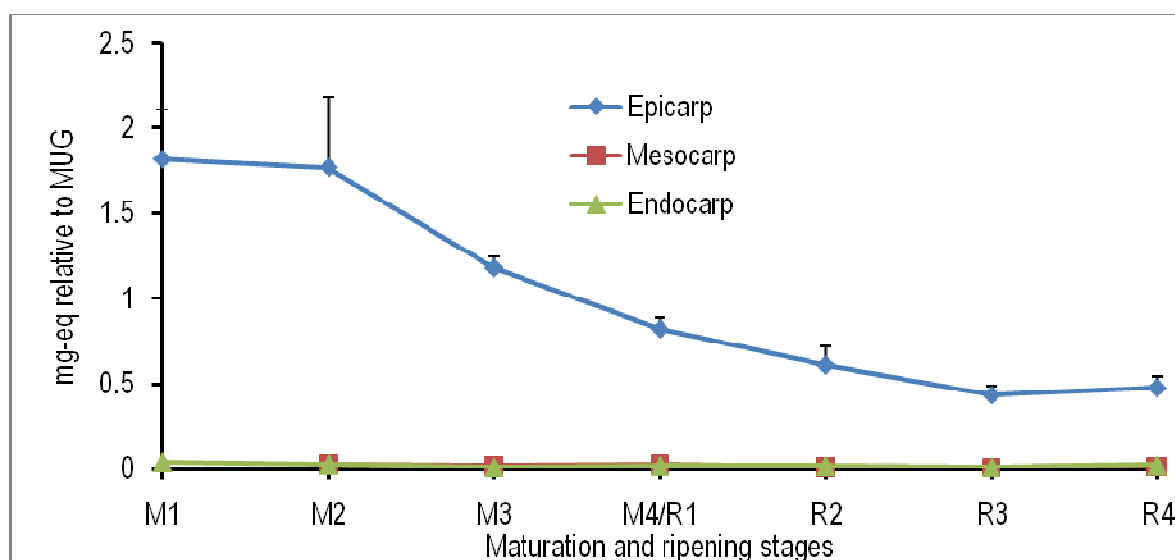
**Figure 5:** Spatiotemporal changes in the levels of eriodictyol in the epicarp, mesocarp and endocarp of tomato fruits at different maturation (M) and ripening (R) stages. M1,  $\phi$  (longitudinal diameter) = 0.7 cm; M2,  $\phi$  = 2.0 cm; M3,  $\phi$  = 3.0 cm; M4/R1, mature green ( $\phi$  = 4.5 cm); R2, turning; R3, pinkish; R4, red-ripe; eq, equivalent; MUG, 4-methylumbelliferyl- $\beta$ -D-glucuronide dihydrate.

From the maturation stage 3 onwards, the content of caffeic-acid-hexose was significantly higher in the endocarp than in the epicarp and mesocarp. At the ripening stage 4, the quantity of this compound found in the epicarp was significantly greater than in the mesocarp (Fig. 2). The concentration of caffeoylquinic acid slightly increased in all the three tissues of tomato

fruits during the experimental period. From ripening stage 2 to ripening stage 4, the level of this compound was significantly higher in the epicarp than in the mesocarp (Fig. 3). The endogenous level of dicaffeoylquinic acid did not change in all the three tissues analyzed from the maturation stage 1 to the maturation stage 4. Thereafter, there were significant

increases in the level of this metabolite in all the three tissues investigated. At the ripening stage 4, the endogenous concentration of dicaffeoylquinic acid was significantly higher in the epicarp and endocarp than in the mesocarp (Fig. 4). The eriodictyol content in the mesocarp and endocarp remained unchanged throughout the maturation and ripening of tomato fruits.

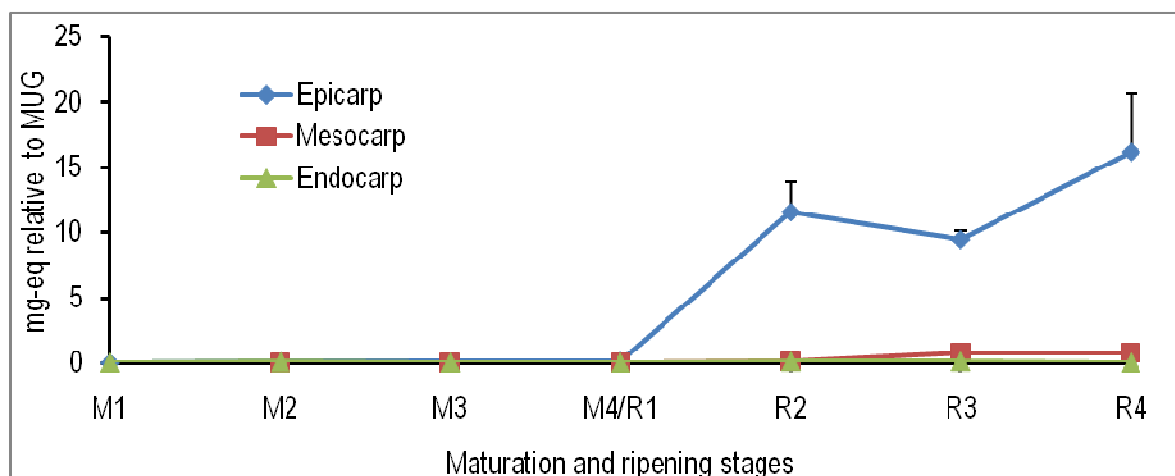
However, there was a significant increase in the concentration of this compound in the epicarp from ripening stage 3 to the ripening stage 4 (Fig. 5). A gradual decrease was observed in the level of kaempferol-glc-rhamnose in the epicarp during the growth and development of tomato fruits.



**Figure 6:** Spatiotemporal changes in the levels of kaempferol-glc-rhamnose in the epicarp, mesocarp and endocarp of tomato fruits at different maturation (M) and ripening (R) stages. M1,  $\phi$  (longitudinal diameter) = 0.7 cm; M2,  $\phi$  = 2.0 cm; M3,  $\phi$  = 3.0 cm; M4/R1, mature green ( $\phi$  = 4.5 cm); R2, turning; R3, pinkish; R4, red-ripe; eq, equivalent; MUG, 4-methylumbelliferyl- $\beta$ -D-glucuronide dihydrate.

No remarkable change could be observed in the concentration of this metabolite in the mesocarp and endocarp during the experimental period (Fig. 6). The endogenous level of naringenin remained nearly constant at low concentration in the epicarp from maturation stage 1 to stage 4, and then increased significantly from the latter stage onwards. Thus, the naringenin content in the epicarp at the ripening 4 was about ninetyfold higher than at the ripening stage 1. No increase in the concentration of naringenin in the mesocarp and endocarp could be observed during the growth and development of tomatoes (Fig. 7). The

content of naringenin-chalcone-hexose remained nearly constant in the mesocarp and endocarp during the maturation and ripening of tomato fruits. However, this metabolite accumulated in the epicarp of tomato fruits increasingly from the maturation stage 4 onwards (Fig. 8). The level of quercetin-glc-rhamnose in all the three tissues studied remained almost unchanged throughout the experimental period. However, during the same period the quantity of quercetin-glc-rhamnose found in the epicarp was significantly higher than that found in other tomato fruit tissues analyzed (Fig. 9).

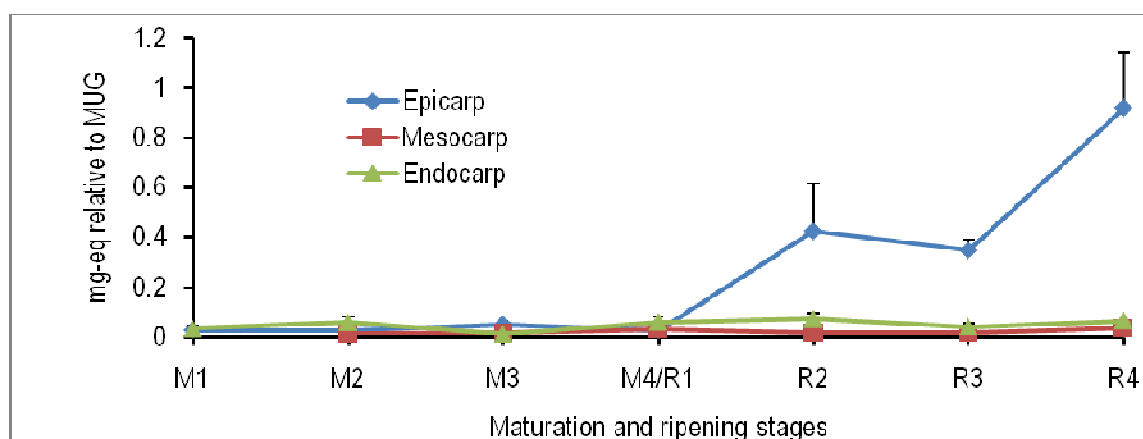


**Figure 7:** Spatiotemporal changes in the levels of naringenin in the epicarp, mesocarp and endocarp of tomato fruits at different maturation (M) and ripening (R) stages. M1,  $\phi$  (longitudinal diameter) = 0.7 cm; M2,  $\phi$  = 2.0 cm; M3,  $\phi$  = 3.0 cm; M4/R1, mature green ( $\phi$  = 4.5 cm); R2, turning; R3, pinkish; R4, red-ripe; eq, equivalent; MUG, 4-methylumbelliferyl- $\beta$ -D-glucuronide dihydrate.

## DISCUSSION

The levels of caffeic-acid-hexose, eriodictyol, dicaffeoylquinic acid, naringenin and naringenin-chalcone-hexose in the epicarp of tomato fruits increased from ripening stage 1 onwards and this trend was more pronounced in the variation of naringenin content. On the contrary the level of kaempferol-glc-rhamnose decreased continuously during the maturation (or growth and development) and ripening phases of tomatoes. Like in other fruits, the peel (epicarp) of tomato fruits is a tissue that undergoes one of the most perceptible changes (e.g. colour change) during transition from development to ripening (Gillapsy

*et al.*, 1993; Carrari and Fernie, 2006; Moco *et al.*, 2007). Obviously, high levels of kaempferol-glc-rhamnose are characteristic for green chloroplast-containing tissues whereas high levels of naringenin, naringenin-chalcone-hexose, eriodictyol, caffeic-acid-hexose and dicaffeoylquinic acid rather characterize coloured chromoplast-containing tomato fruit epicarps although these metabolite do not contribute to the colour. Kaempferol-glc-rhamnose may thus compete with other flavonoids, especially naringenin, for potential substrates.

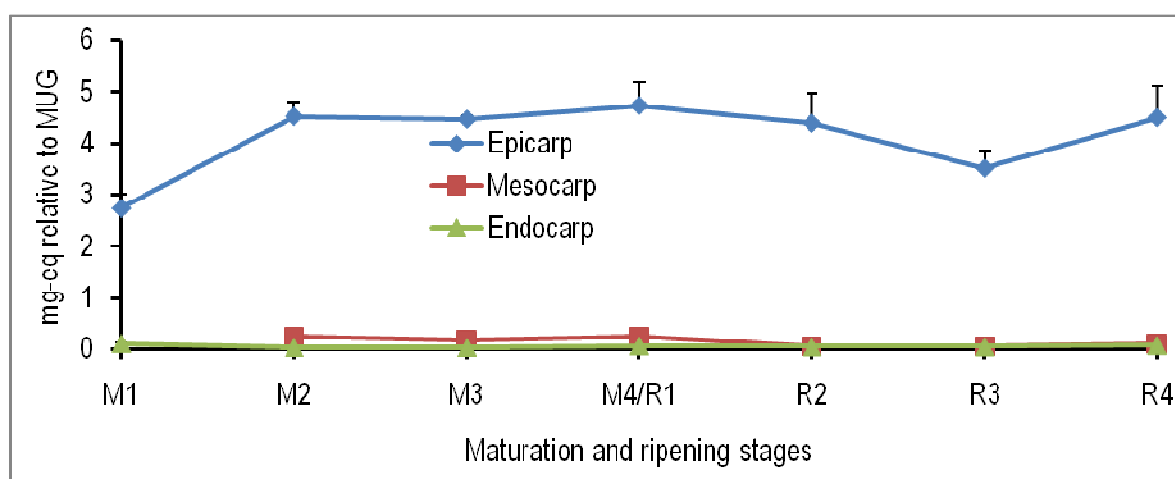


**Figure 8:** Spatiotemporal changes in the levels of naringenin-chalcone-hexose in the epicarp, mesocarp and endocarp of tomato fruits at different maturation (M) and ripening (R) stages. M1,  $\phi$  (longitudinal diameter) = 0.7 cm; M2,  $\phi$  = 2.0 cm; M3,  $\phi$  = 3.0 cm; M4/R1, mature green ( $\phi$  = 4.5 cm); R2, turning; R3, pinkish; R4, red-ripe; eq, equivalent; MUG, 4-methylumbelliferyl- $\beta$ -D-glucuronide dihydrate.



The physiological roles of kaempferol-glc-rhamnose may be confined to the early stage of growth and development of tomato fruits and those of naringenin and other flavonoids restricted to the ripening stages. In this context, kaempferol-glc-rhamnose may stimulate the biosynthesis of growth promoting hormones such as auxins and gibberellins that are massively produced at the cell division and expansion phases of tomato fruit development (Gillapsy *et al.*, 1993; Osorio *et al.*, 2013). The pathway of the biosynthesis of naringenin, naringenin-chalcone-hexose, eriodictyol, caffeic-acid-hexose and dicaffeoylquinic acid may be diverted towards kaempferol-glc-rhamnose in the epicarp of tomato fruits during the development phase. Furthermore, since naringenin has been shown to

competitively antagonize gibberellins (Phillips, 1962), its level and that of other flavonoids which function as growth inhibitors should be maintained at minimum values during the maturation of tomato fruits. Moreover, it is well known that the endogenous ethylene concentration peaks at the transition from breaker (mature green) to turning ripening stages of tomato fruits (Gillapsy *et al.*, 1993; Carrari and Fernie, 2006), a period where the levels of naringenin and some other flavonoids started to increase. Thus, these specific flavonoids may play important roles in the regulation of climacteric ethylene biosynthesis in tomato fruits. In return, ethylene may stimulate their biosynthesis in the epicarp of tomato fruits during the ripening stages.



**Figure 9:** Spatiotemporal changes in the levels of quercetin-glc-rhamnose in the epicarp, mesocarp and endocarp of tomato fruits at different maturation (M) and ripening (R) stages. M1,  $\phi$  (longitudinal diameter) = 0.7 cm; M2,  $\phi$  = 2.0 cm; M3,  $\phi$  = 3.0 cm; M4/R1, mature green ( $\phi$  = 4.5 cm); R2, turning; R3, pinkish; R4, red-ripe; eq, equivalent; MUG, 4-methylumbelliferyl- $\beta$ -D-glucuronide dihydrate.

In fact, it is well established that ethylene, abscissic acid, jasmonic acid and methyljasmonate enhance the biosynthesis of flavonoids (Pirie and Mullins, 1976; Berhow, 1998; Buer *et al.*, 2006; He *et al.*, 2010). Those flavonoids whose levels increased gradually in the epicarp from ripening stage 1 onwards may be implicated in the prevention of the carotenoid photobleaching (Takanama, 1982). Naringenin could also be implicated in physiological mechanisms of the senescence prevention. In fact, Zhang *et al.* (2013) reported that enrichment of the anthocyanins (flavonoids) in tomato fruits led to a significant extension of their shelf-life by delaying over ripening and reducing susceptibility to *Botrytis cinerea*. However, in spite of the above discussed physiological

and defensive roles of kaempferol-glc-rhamnose, naringenin, naringenin-chalcone-hexose, eriodictyol, caffeic-acid-hexose and dicaffeoylquinic acid during the development and ripening of tomato fruits, the exact mechanisms of their involvement in the control and regulation of some cardinal events that occur in the epicarp such as the greening and degreening are not yet exactly known. Apart from dicaffeoylquinic acid, there was no significant change in the levels of all flavonoids analyzed in the mesocarp during the experimental period. The levels of naringenin, naringenin-chalcone-hexose, eriodictyol, quercetin-glc-rhamnose and kaempferol-glc-rhamnose in the endocarp remained nearly constant during the development and ripening of tomato fruits. However,



significant increases in the contents of caffeic-acid-hexose, caffeoylquinic acid and dicaffeoylquinic acid were observed in the endocarp. Contrarily to the mesocarp that seemingly does not undergo important morphological and physiological modifications, noticeable morphological and biochemical changes occurred in the endocarp as spatially delimited in the present study (Moco *et al.*, 2007). This could explain why no remarkable changes in the levels of flavonoids could be observed in the mesocarp. Interestingly, caffeic acid derivatives were the only flavonoids whose

contents significantly increased in the endocarp during the ripening of tomato fruits. This was a sign that the caffeic acid derivatives concerned may play special roles in the control and regulation of metabolic processes occurring in the endocarp of tomato fruits during the ripening stages. Like anthocyanins, caffeic-acid-hexose may be involved in the mechanisms that control the senescence of tomato fruits (Zhang *et al.*, 2013). However, the precise physiological roles of caffeic acid derivatives in the endocarp of tomato fruits need still to be explained.

## CONCLUSION

There were spatial and temporal variations in the accumulation of flavonoids in tomato fruits. Caffeic-acid-hexose, kaempferol-glc-rhamnose and naringenin were the most concerned by these variations. Caffeic-

acid-hexose and naringenin may play important physiological roles in the retardation of the over ripening.

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