



## Association between the concentration of *n*-alkanes and tolerance to cracking in commercial varieties of sweet cherry fruits



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### ABSTRACT

Fruit cracking is one of the major economic losses in cherry production worldwide. Factors involved in differential tolerance among varieties are unknown. This phenomenon is mainly caused by rainfall during the harvest period and is related to osmotic factors and fruit water permeability. The cuticle is a hydrophobic extracellular membrane and soluble waxes represent a mixture of aliphatic very long chain fatty acids, its derivatives and cyclic compounds like triterpenes. Analysis of waxes from enzymatic isolated sweet cherry fruit cuticles showed that the main components are triterpenes (76%), alkanes (19%) and alcohols (1%).

Cracking *in vitro* was investigated in five sweet cherry varieties. After removal of cuticular wax fruit cracking was significantly increased. Nuclear magnetic resonance analysis (<sup>1</sup>H and <sup>13</sup>C NMR), revealed that fruits of different sweet cherry varieties contain primarily *n*-alkane with 29 carbons and no iso-alkane. Gas chromatography–mass spectrometry (GC–MS) enabled identification and quantification of *n*-alkanes. Varieties with significantly higher concentrations of nonacosane (Kordia, Regina and Lapins) were more tolerant to cracking compared to varieties with lower amounts (Bing and Rainier).

We hypothesized that there is an association between the amount of C 29 *n*-alkane and cracking tolerance in sweet cherry fruits. This difference could be an important factor to explain the different tolerances to cracking in fruits of sweet cherry varieties.

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## 1. Introduction

Cracking is a critical economic problem in sweet cherry (*Prunus avium*) industry, because it can seriously compromise fruit quality and profits. In some years, cracking can affect 90% of the harvested fruits; therefore these fruits lose their commercial value and can only be sold locally or have to be directed to the processing food industry (Simon, 2006; Balbontin et al., 2013; Carrasco et al., 2013). Cuticular cracking in sweet cherry fruits occurs predominantly during fruit ripening and is caused by rainfall and high relative humidity, and over-watering can also cause cracking (Balbontin et al., 2013). External symptoms are cuticular fissures that are visi-

ble on the surface of the fruit mainly in the pedicel area (Beyer et al., 2002).

Cherry varieties have different tolerances to cracking (Blacklock and Jaworski, 2006; Cline et al., 1995). It has been reported that histological properties such as the size of epidermal cells (Yamaguchi et al., 2002), the epidermal layers (Christensen, 1996), as well as size and number of stomata (Peschel et al., 2003) contribute to this disorder. Osmotic concentrations generated by the high sugar content of fruits, are directly related to cracking. Rainwater penetrates the external surface layers of the epidermis (Tyree et al., 1990), increasing the volume of the fruit, triggering cracking (Christensen, 1996). Two paths have been proposed for water uptake in cherries, one through the epidermis and the second through the pedicel. The second, is mainly responsible for an increase in the volume of the fruit (Sekse, 1998). Some of the major problems associated with water uptake are the disruption of the cell wall, leaching of

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vacuolar contents and loss of structure including the dermal layers (Sekse, 1995). Recently, a new hypothesis that attributes cracking to fruit skin shrinking after its rapid cooling caused by a rainfall or by sharp drop of the ambient temperature has been proposed. However, this needs further examination (Koumanov, 2015).

The cuticle is a hydrophobic extracellular polymer membrane, which covers the aerial surfaces of plants. It provides a barrier against pathogens (Stark et al., 2000) and also has a role in reducing uncontrolled transpiration water loss (Riederer and Schreiber, 2001; Buda et al., 2009). The cuticle consists of two fractions: cutin and associated cuticular waxes. Cutin is a polymeric network made principally of oxygenated C<sub>16</sub> and C<sub>18</sub> fatty acids, cross-linked by ester bonds (Heredia, 2003; Riederer and Schreiber, 2001). Studies on isolated cherry fruit cuticles demonstrated that the waxes play an important role against uncontrolled water loss or uptake (Alkio et al., 2014). When waxes are removed, cuticular permeability increased significantly (Knoche et al., 2000). Qualitative and quantitative analysis of fruit cuticle waxes revealed that they are composed mainly of triterpenes (76%), alkanes (19%) and alcohols (1%). Among them, alkanes are pure hydrocarbons lacking functional groups; thereby, reducing their reactivity. They do not form hydrogen bonds and are insoluble in polar solvents such as water (hydrophobic compound) (Croteau et al., 2000; Peschel et al., 2007). Furthermore, characterization of alkanes in cherry wax has shown that the predominant alkane has a chain length of 29 carbons (Peschel et al., 2007).

Studies in fruits of cherry tomato demonstrated that the silencing of *lecer6* elongase, encoding for  $\beta$ -ketoacyl-coenzyme A synthase, generated cuticles three to eight times more permeable to water. This elongase is involved in the elongation of long-chain fatty acids, the substrates for the synthesis of cuticular waxes such as the alkanes (Leide et al., 2007; Buda et al., 2009). These tomatoes showed a significant decrease in the amount of *n*-alkanes (Leide et al., 2007; Cameron et al., 2006). Additionally, expression profile analysis of the orthologous *lecer6* in sweet cherry, suggested that this gene could be involved in conferring a different cracking tolerance (Balbontín et al., 2014).

Gas chromatography–mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR) spectroscopy are powerful tools for analyzing and characterizing plant metabolites (Kwan et al., 2011; Liseč et al., 2006). GC–MS is a very sensitive technology (only small amounts of samples are needed) that can identify and quantify hundreds of metabolites in plants extracts. In the past GC–MS has been extensively used for metabolite profiling. Therefore protocols and software for chromatogram evaluation and interpretation (Liseč et al., 2006) are available. An advantage of the NMR technique is that it is noninvasive. Therefore, the spectra can be recorded from cell suspensions, tissues, and even whole plants, as well as from extracts and purified metabolites. Additionally, NMR analyses may be tailored to the nature of the sample and the metabolic problem that is being addressed, such that the maximum number of metabolites may be detected (Kwan et al., 2011; Krishnan et al., 2005). In addition, chromatographically coupled NMR technologies have found great utility in unequivocal determination of metabolite structures. The aim of this study was to characterize and quantify different alkanes in the cuticular wax of sweet cherry fruits, using NMR and GC–MS to establish an association between the concentrations of the different alkanes and variations in cracking tolerance in *P. avium* varieties. These two techniques are complementary. GC–MS offers quantitative information about the total amount and percent composition of wax, as well as information about the mass and identity of the alkane involved. In contrast, NMR analyzes compounds that contain singled bonds (e.g. alkanes) and determines the exact location of these bonds as well as the stoichiometry.

## 2. Material and methods

### 2.1. Plant material

Five sweet cherry varieties were selected for analyses, based on the differences in susceptibility to cracking (Simon, 2006; Demirsoy and Demirsoy, 2004; Lane et al., 2000; Moing et al., 2004). Bing and Rainier have been reported to be less tolerant to cracking, whereas Regina, Kordia and Lapins are more tolerant to cracking. Sweet cherry fruits (*P. avium* L., cv. 'Rainier', 'Bing', 'Lapins', 'Kordia', 'Regina') were collected in 2009, 2012 and 2013 seasons from: La Palma Experimental Station, Faculty of Agronomy PUCV, Quilota (latitude 32° 54' S and longitude 71° 12' W), Fundo San Luis, Curicó (latitude 35° 02' S and longitude 71° 15' W); Fundo Mirador, Panguipulli (latitude 39° 34' S and longitude 72° 18' W). The agroclimatic data information (rainfall) for the 2009, 2012 and 2013 seasons was obtained from the Meteorological Station, DGA, Faculty of Agronomy, Pontifical Catholic University from Valparaíso; <http://datos.gob.cl/datasets/ver/2719> and Bulletin N° 428 Pluviometric information, December 2013). Fruits were harvested at two different developmental stages, straw-yellow and over-mature and selected for uniformity based on color, size, weight, soluble solids (SS) and absence of damage in its surface.

### 2.2. In vitro cracking assay

The cracking susceptibility percentage was performed using the method described by Verner and Blodgett (1931) and modified by Christensen (1996), with some changes. A time course for 24 h was performed with healthy fruits and the numbers of cracked fruits were visually identified every 6 h. 30 fruits per variety were used with three replicates for the developmental stage over-mature. Analysis of statistically significant difference was performed by Tukey's test ( $p < 0.05$ ).

### 2.3. In vitro chemical and mechanical removal of fruit waxes

Three different treatments (two mechanicals and one non mechanical) were used to remove cuticular waxes of whole fresh fruits. Chloroform was used for the solvent extraction (non mechanical), where the fruits were submerged for 30 s at room temperature in the organic solvent. For the mechanical treatment 1, fruits were gently rubbed with chloroform impregnated tissue paper. For the mechanical treatment 2, waxes were mechanically removed by rubbing the fruit surface with a tissue paper without any solvent. One set of fruits went only through the water incubation time (untreated). In all treatments as well as in the control, the fruits were weighed before and immediately after each treatment to be sure that the wax was extracted.

### 2.4. Wax extraction and alkane purification for NMR analysis

For the NMR analysis of cuticular waxes of sweet cherry (*P. avium* L., cv.), fruits of Lapins, Bing and Rainier varieties from 2009 and 2012 were used, because they are the most common and abundant in Chile. Waxes were extracted from whole fresh fruits for 30 s using CHCl<sub>3</sub> as described in Mayes et al. (1986). Using the same protocol, alkane's purification from cuticular wax was performed with some modifications. The fruits were placed for 1 min in a beaker containing chloroform at 50 °C. The organic solution was filtered with Whatman paper No. 1 in a 250 mL flask and evaporated on a 70 °C heating block in a hood overnight. 50 mL of 1 M potassium hydroxide (dissolved in ethanol) was added to the samples, and incubated at 90 °C for 16 h. Once cooled down to 65 °C, 40 mL of *n*-heptane (ANEDRA) and 10 mL of distilled water were added. The *n*-heptane phase was collected in a different tube, repeating the

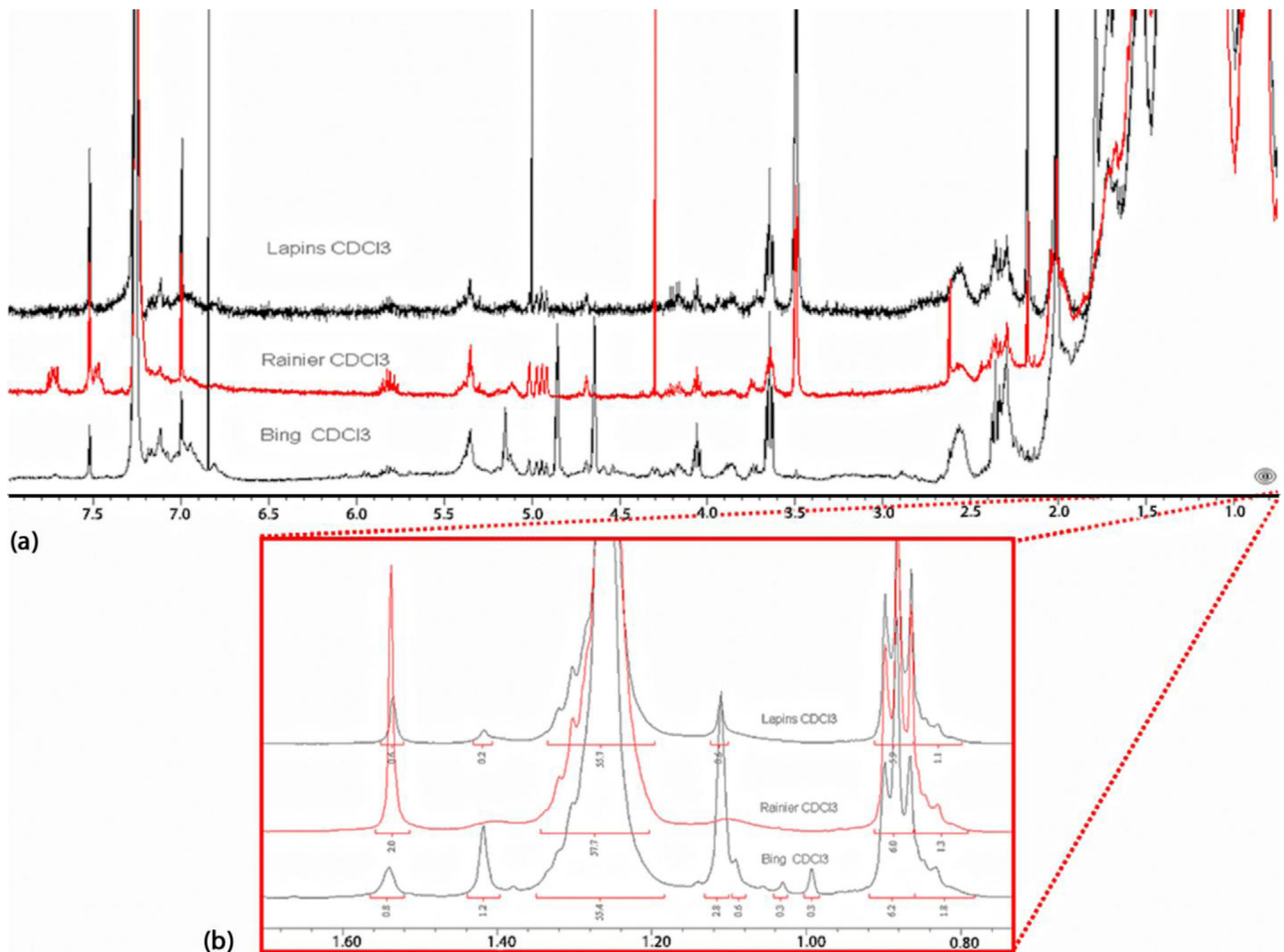
**Table 1**  
*In vitro* fruit cracking assay for five sweet cherry varieties under different chemical treatments.

Varieties	Percentage of cracking <i>in vitro</i>				Fruit treatments
	Time (h)	6	12	18	
Rainier	73 ± 6	93 ± 12	97 ± 6	100	Untreated
Bing	70 ± 10	77 ± 6	80 ± 10	100	
Lapins	27 ± 6 <sup>ab</sup>	53 ± 15 <sup>ab</sup>	70 ± 10	100	
Kordia	40 ± 14 <sup>ab</sup>	87 ± 6 <sup>ab</sup>	93 ± 12	100	
Regina	30 ± 14 <sup>ab</sup>	73 ± 15 <sup>ab</sup>	93 ± 6	97 ± 6	
Rainier	15 ± 7	20 ± 14	20 ± 14	20 ± 14	Solvent extraction
Bing	0	0	7 ± 6	7 ± 6	
Lapins	7 ± 6 <sup>b</sup>	13 ± 6 <sup>b</sup>	13 ± 6	13 ± 6	
Kordia	3 ± 6 <sup>b</sup>	30 ± 10 <sup>b</sup>	57 ± 15 <sup>ab</sup>	70 ± 10 <sup>ab</sup>	
Regina	0 <sup>a</sup>	5 ± 7	40 ± 17 <sup>b</sup>	53 ± 21 <sup>b</sup>	
Rainier	47 ± 7	50 ± 10	53 ± 16	57	Mechanical 1
Bing	47 ± 21	50 ± 10	51 ± 10	53 ± 15	
Lapins	30 ± 17	33 ± 15	40 ± 20	50 ± 10	
Kordia	30 ± 14	50 ± 20	77 ± 6 <sup>ab</sup>	87 ± 6 <sup>ab</sup>	
Regina	10 ± 1 <sup>ab</sup>	53 ± 25	83 ± 21 <sup>ab</sup>	93 ± 12 <sup>ab</sup>	
Rainier	50 ± 10	60 ± 10	66 ± 11	76 ± 6	Mechanical 2
Bing	50 ± 10	53 ± 15	63 ± 11	73 ± 5	
Lapins	26 ± 11 <sup>ab</sup>	33 ± 15	60 ± 10	70 ± 10	
Kordia	10 ± 1 <sup>ab</sup>	65 ± 7	83 ± 12	90 ± 10	
Regina	25 ± 7 <sup>ab</sup>	53 ± 15	90 ± 10 <sup>ab</sup>	93 ± 6 <sup>ab</sup>	

The susceptibility of five different sweet cherry varieties to *in vitro* fruit cracking was determined on fruits that: contained the cuticle wax (untreated); had the cuticle wax removed by chemical method (Solvent extraction); or had the cuticle wax removed using mechanical treatment (mechanical 1 and mechanical 2). *In vitro* fruit cracking was analyzed visually at 6, 12, 18 and 24 h after immersing the fruits in water. Each value represents the average of three replicates of 30 fruits ± standard deviation.

<sup>a</sup> Significant difference with Rainier.

<sup>b</sup> Significant difference with Bing. Tukey's test (*p* < 0.05).



**Fig. 1.** <sup>1</sup>H NMR spectrum for alkanes chemically extracted from fruit cuticular waxes of Lapins, Rainier and Bing sweet cherry varieties. (a) Spectrum of the three varieties between 0.8 and 8.0 ppm. (b) Spectrum of the three varieties between 0.8 and 1.6 ppm showing the aliphatic compounds.

*n*-heptane step once again. Then, the sample was placed in a heat block and allowed to dry at 90 °C for 16 h. The dry residue was resuspended in 10 mL *n*-heptane and purified by passage over a column of ~10 mL of silica gel 60 (MERCK). The purified extract was allowed to dry completely and stored at –20 °C until use in the analysis. Approximately 5 kg of cherries were used to extract 10 mg of alkanes.

### 2.5. NMR spectroscopy

Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker avance spectrometer (Bruker Biospin Corporation, MA, USA) operating at <sup>1</sup>H frequencies of 400 MHz. The experiments were performed at room temperature with 10 mg of cherry fruit alkane samples (two biological replicates and three technical replicates). These samples were dissolved in deuterated chloroform (CDCl<sub>3</sub>) (minimum degree of deuteration for NMR spectroscopy 99.8% stabilized with silver; Cambridge Isotope Laboratories, Inc., Cambridge, UK) to provide a field-frequency lock signal containing 0.03% tetramethylsilane (TMSi) to provide an internal chemical shift standard (Merck, Santiago, Chile). One dimensional (<sup>1</sup>H) spectra were acquired in NMR tubes of 5 mm diameter scans with 1024 points in the time domain and spectral width of 4200 Hz. Carbon-13 (<sup>13</sup>C) NMR were recorded with 32768 points; 24154.59 Hz spectral width operating at 100.61 MHz of frequency. Two-dimensional (2D) NMR and correlation spectroscopy (COSY) measurements were made under the following experimental conditions: 62 scans, pulse delay 8 s and 1024 data points. Data processing and <sup>1</sup>H peak integration were done using XWIN-NMR software ([http://www.bruker-biospin.com/software\\_nmr.html](http://www.bruker-biospin.com/software_nmr.html)). <sup>13</sup>C spectrums for alkanes (octacosane, nonacosane and tricontane) chemical shift predictions were done using ChemDraw Ultra 9.0 CambridgeSoft Corporation.

### 2.6. Cuticle isolation for wax analysis using GC–MS

Exocarp segments were excised from Rainier, Bing, Lapins, Kordia and Regina cherry fruits (twenty biological replicates and three technical replicates) using a cork borer (i. d. 11.5 mm). Only one segment per fruit was excised. Cuticular membranes (CMs) were isolated enzymatically by incubating the fruit segments in 2% pectinase and 2% cellulose (*Aspergillus niger*; Sigma–Aldrich®, Santiago, Chile), dissolved in 50 mM citric acid buffer (Winkler®, Santiago, Chile) at pH 4.0. NaN<sub>3</sub> (Winkler®, Santiago, Chile) was added at a final concentration of 1 mM to prevent microbial growth. The enzyme solution was daily replaced by fresh one. After separation of the CMs from the remaining fruit tissue, CMs were transferred to borate buffer (0.02 M, pH 9.0; Winkler®, Santiago, Chile) for another few days and carefully washed with deionized water (Schönherr and Riederer, 1986). CMs were gently air dried and stored at room temperature in petri-dishes until used in experiments. CM mass was determined gravimetrically. Mass of CM for the whole fruit was calculated by multiplying CM mass per unit area by the surface area of the fruit. The latter was estimated assuming a spherical shape and a density of 1000 kg m<sup>-3</sup>.

### 2.7. Wax extraction and preparation

Wax extraction was performed using the protocol described by Peschel et al. (2007). Samples (2 mg per sample) of CMs were dewaxed with 1 mL CHCl<sub>3</sub> (Fluka, Ulm, Germany) for 16 h at 25 °C in Teflon-sealed reaction vials. After adding 10 µg tetracosane (chloroform solution of 10 mg tetracosane in 50 mL; Fluka, Ulm, Germany) as an internal standard the extracts were reduced under a gentle stream of nitrogen at 60 °C. Hydroxylic and carboxyl groups of alcohols and acids, being important constituents of cherry fruit

wax, were transformed into the corresponding trimethylsilylethers and -esters by derivatization. Derivatization was done using 20 µL *N,O*-bis(trimethylsilyl)-trifluoroacetamid (BSTFA; Machery–Nagel, Düren, Germany) and 20 µL Pyridine (Sigma–Aldrich, Deisenhofen, Germany) for 40 min at 70 °C. Dewaxed CMs (DCMs) were dried and weighed for determining mass per unit area.

### 2.8. Quantitative and qualitative analysis of fruit cuticular waxes

Samples of 1 µL were analyzed by gas chromatography equipped with flame ionization detection (GC–FID; CG–Hewlett–Packard 5890 series H, Hewlett–Packard, Palo Alto, CA, USA) and with on-column injection (30 m DB-1 i.d. 0.32 mm, film 0.2 µm; J&W Scientific, Folsom, CA, USA). Wax compounds were identified by analyzing 1 µL samples by gas chromatography connected to mass spectrometry (GC–MS; quadrupole mass selective detector HP 5971, Hewlett–Packard, Palo Alto, CA, USA). Identification of wax compounds was done comparing obtained mass spectra with mass spectra of known compounds stored in a database.

Mass of wax was calculated based on the amount of the internal standard tetracosane. Content of individual constituents was expressed (i) as percentage of total wax, (ii) on a unit surface area basis by dividing the amount of constituents by the cumulative CM surface area in the respective sample or (iii) on a whole fruit basis by multiplying amounts of constituents per unit fruit surface area by the surface area of the fruit.

### 2.9. Statistics analysis

Analysis of statistically significant difference (Tukey's test) was performed using the STATISTICA software (version 6.0; StatSoft).

## 3. Results

### 3.1. Fruit cracking

The *in vitro* cracking assay (untreated fruits) on five cherry varieties (Rainier, Bing, Lapins, Kordia and Regina) validates the different tolerances of these fruit varieties to cracking. The more susceptible varieties to cracking were Bing and Rainier, both with over 70% of fruits cracked after 6 h incubation in water. On the other hand, Lapins, Kordia and Regina were significantly more tolerant to cracking, with only 27 ± 6%; 40 ± 14% and 30 ± 14% cracking after 6 h of water incubation (Table 1, Untreated). Within 24 h, fruits from almost all varieties were cracked (Table 1, Untreated). To determine if cuticular waxes play a role in fruit cracking or even prevent it, the same cracking assays were performed with fruits where cuticular waxes were removed by solvent extraction and mechanical removal. The solvent extraction treatment significantly reduced the percentage of fruit cracking in the sweet cherry varieties (20% cracking in Rainier, 7% in Bing, 13% in Lapins, 70% in Kordia and 53% cracking in Regina after 24 h of treatment) (Table 1, Solvent extraction). Taking in consideration that chloroform may cause multiple modifications to the fruit surface and physiology, two different mechanical treatments were also used to remove the cuticular wax of the sweet cherry fruits (details in in materials and methods). When partial cuticular wax extraction was performed (mechanical fruit treatments 1 and 2), cracking decreased by 10–50% compared to fully untreated fruits (Table 1). A significant decrease in cracking could be shown after all treatments, suggesting that the cuticular wax plays a major role in determining sweet cherry cracking tolerance.

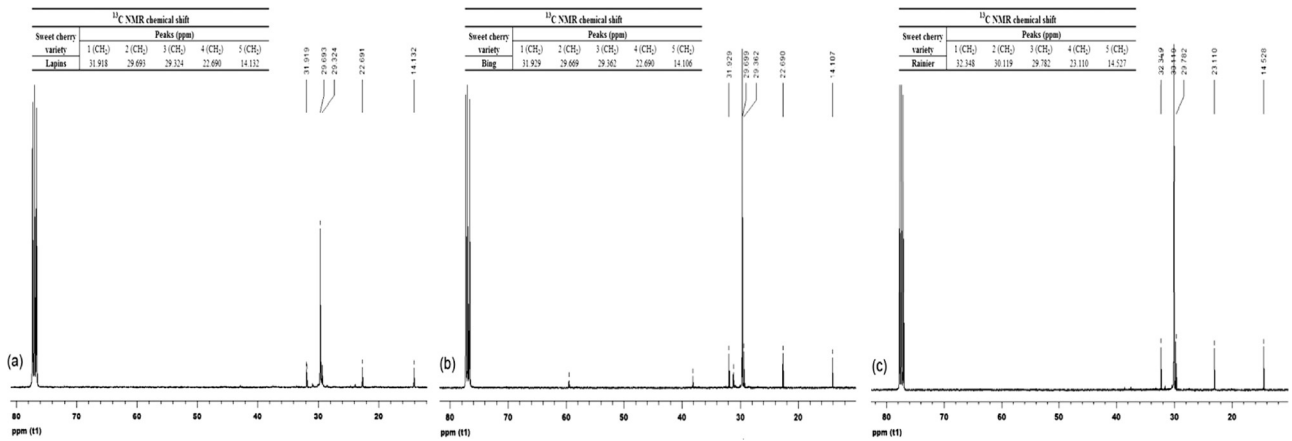


Fig. 2. <sup>13</sup>C NMR spectrum and chemical shifts of alkanes chemically extracted from sweet cherry fruit cuticular waxes. (a) Lapins variety, (b) Bing variety and (c) Rainier variety.

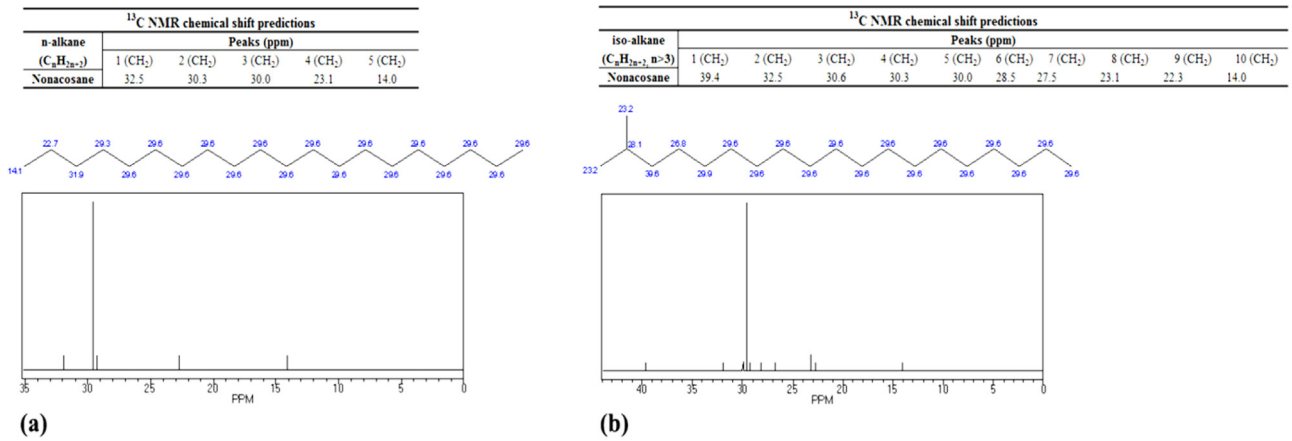


Fig. 3. <sup>13</sup>C NMR chemical shifts prediction of alkanes, (a) skeletal structure and <sup>13</sup>C spectra for n-nonacosane chemical shift predictions, (b) skeletal structure and <sup>13</sup>C spectra for iso-nonacosane chemical shift predictions.

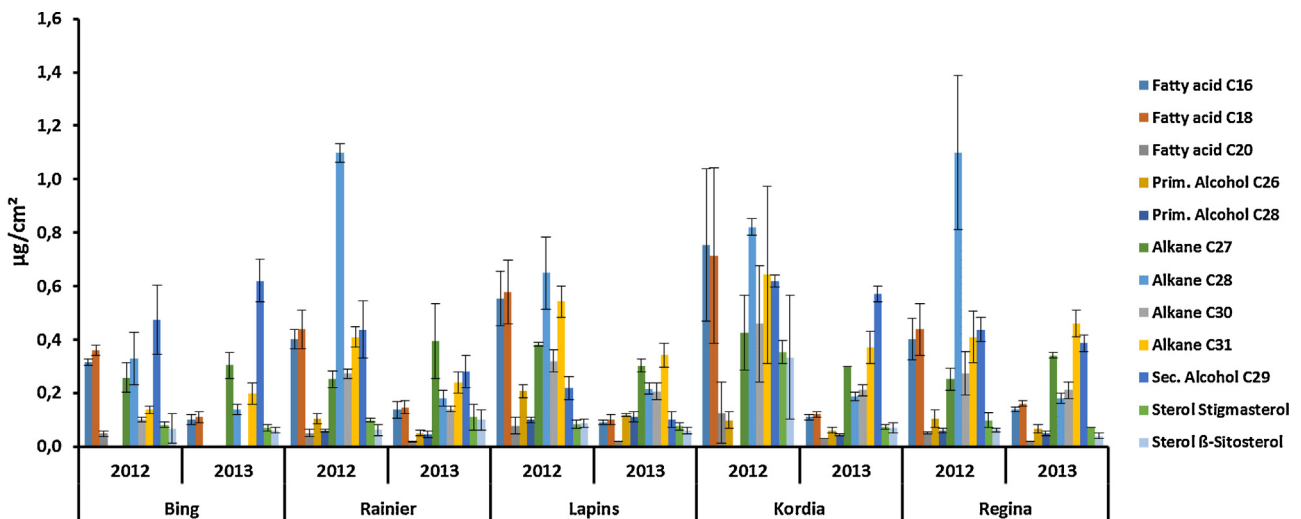
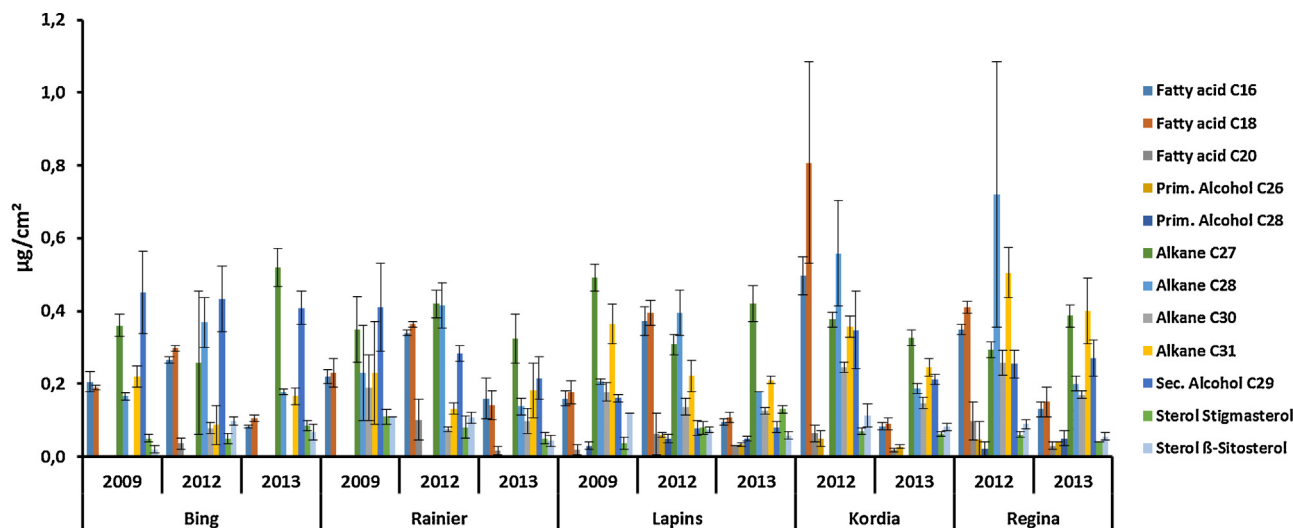


Fig. 4. GC-MS analysis of wax constituents of sweet cherry fruits from Bing, Rainier, Lapins, Kordia and Regina varieties in straw-yellow stage of development. Data are given as means and standard errors of means ( $\pm$ SE). Content of individual constituents are expressed on a whole fruit basis by multiplying amounts of constituents per unit fruit surface area by the surface area of the fruit. Each value represents the average of three replicates technical and twenty biological replicates.



**Fig. 5.** GC–MS analysis of wax constituents of sweet cherry fruits from Bing, Rainier, Lapins, Kordia and Regina varieties in over-mature stage of development. Data are given as means and standard errors of means ( $\pm$ SE). Content of individual constituents are expressed on a whole fruit basis by multiplying amounts of constituents per unit fruit surface area by the surface area of the fruit. Each value represents the average of three replicates technical and twenty biological replicates.

### 3.2. Alkane's analysis

#### 3.2.1. NMR analysis

$^1\text{H}$  NMR spectra of cuticular waxes extracted from fruit of Lapins, Bing and Rainier varieties were analyzed. The spectra from these varieties are very similar, with a major absorbance peak appearing between 0.0 and 2.5 ppm, corresponding to aliphatic compounds like alkanes (Supplementary Figure S1).  $^1\text{H}$  NMR spectra for alkanes extracted from cuticular waxes of fruits show the presence of at least two major components (Fig. 1a) one of them being a saturated fraction between 1.6 and 0.8 ppm (Fig. 1b). The  $^1\text{H}$  NMR spectrum recorded in  $\text{CDCl}_3$  extracts generally show a typical asymmetric triplet centered near 0.84 ppm. That signal corresponds to a terminal methyl group linked to a methylene group. Furthermore a signal with high intensity between 1.18 and 1.38 ppm corresponding to the methylene bulk of alkyl chain (Fig. 1b) could be found. In this range, we can observe a strong absorbance which corresponds to octacosane ( $\text{C}_{28}$ ), nonacosane ( $\text{C}_{29}$ ) and triacontane ( $\text{C}_{30}$ ) alkanes, with nonacosane showing the strongest peak. The stoichiometry was determined considering as reference the integration of the alkane's  $\text{CH}_3$  group, which appears at 0.84 ppm (Fig. 1b). This spectrum also shows a triplet between 4.9 and 5.2 ppm unveiling the presence of an alkane group (between 4.0 and 6.0 ppm), which is present in the three sweet cherry varieties analyzed (Fig. 1a).

#### 3.2.2. 2D COSY and $^{13}\text{C}$ NMR spectrum

In order to confirm the presence of a single signal for this alkane, extracted from cuticular waxes of the fruits Lapins, Bing and Rainier varieties, a 2D COSY NMR experiment was conducted. The analysis showed intermolecular cross peak signals, between the bulk and the methyl groups of these hydrocarbons, present in all three varieties studied (Supplementary Fig. S2, red lines) and no other overlapping signals. These results support the conclusion that cuticular waxes of cherry fruits just contain long chain alkanes.

$^{13}\text{C}$  NMR was performed to reveal what kind of alkane (*n*-alkane or iso-alkane) have the cuticular waxes. The spectrum show a component with high intensity (Fig. 2a–c), position wherein appear the aliphatic compounds. These results confirm the spectrum analysis using  $^1\text{H}$  NMR. In the three varieties studied, these analyses showed the same five chemical shift values (Fig. 2a–c).

The theoretical simulation of  $^{13}\text{C}$  NMR spectrum of *n*-alkane and iso-alkane (nonacosane,  $\text{C}_{29}$ ) was performed to determine the spec-

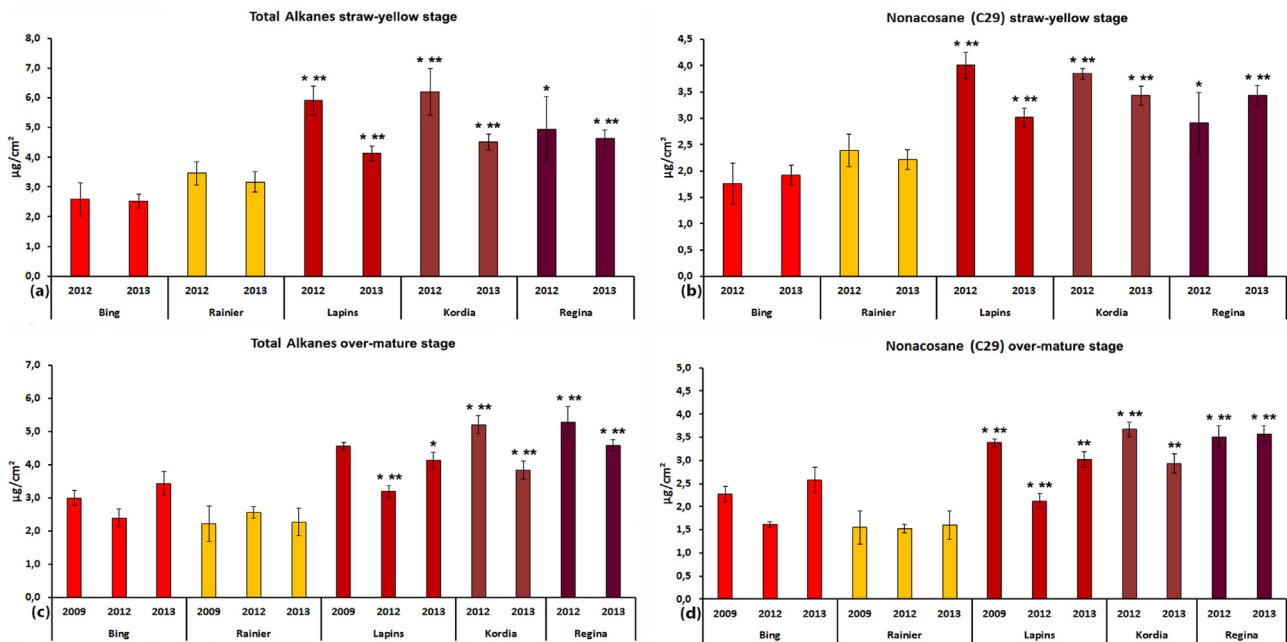
trum, skeletal structure and chemical shifts of these compounds to be able to compare them with the experimental measurements. The theoretical simulation of iso-alkane showed that this compound had ten different peaks and chemical shifts values (Fig. 3b), on the other hand *n*-alkane presented just five peaks with very similar values to experimental data (Fig. 3a). Therefore, the observed chemical shifted signals from the  $^{13}\text{C}$  NMR spectrum obtained from the extractions of the cuticular waxes of fruits, unequivocally indicate the presence of the *n*-alkane.

#### 3.2.3. GC–MS analysis

The GC–MS analysis clearly showed differences between the two different developmental stages (straw-yellow stage and over-mature stage) of the five sweet cherry varieties, with fruits of straw-yellow stage having a higher amount of fatty acids, primary and secondary alcohols, sterols and alkanes (Figs. 4 and 5; Supplementary Figs. S3 and S4). However, the major component of the fruit wax is ursolic acid (60–68%), which is a pentacyclic triterpene (Supplementary Figures S3 and S4). Some recent data published by Belge et al. (2014) show a high concentration of ursolic acid in two varieties of sweet cherry (Celeste and Somerset) after a cold storage. However, the values are lower (47–49% of total waxes) than in the present study. Lapins, Kordia and Regina fruit wax show a higher concentration of C16 and C18 fatty acids than the fruit cuticles of Bing and Rainier. These fatty acids serve as precursors for long chain alkane's biosynthesis (Kunst and Samuels, 2009). It is important to address the fact that compounds such as primary alcohols (C26 and C28) and betulinic acid (triterpene), was not detected in Rainier, Lapins and Kordia at any developmental stage (Supplementary Figs. S3 and S4). The amounts of C29 alkane were left out of these figures to show them in different stages of development/season/varieties as is described in the following paragraph.

#### 3.2.4. Alkane concentrations

GC–MS analysis of cuticular waxes of isolated cuticles from Lapins, Bing, Rainier, Kordia and Regina cherry varieties were conducted from two developmental fruit stages over three seasons (Fig. 6). Bing and Rainier varieties have significant lower concentrations of total alkanes (Fig. 6a and c) and nonacosane (Fig. 6b and d) compared to the varieties with more tolerance towards cracking (Lapins, Kordia and Regina) in both stages of fruit development.



**Fig. 6.** Quantification of nonacosane (C29) and total wax concentrations using GS–MS analysis from sweet cherry fruits (seasons 2009, 2012 and 2013). The fruits were collected in straw-yellow stage and over-mature stage of development. Each value represents the average of three replicates technical and twenty biological replicates  $\pm$  standard deviation. \*: Significant difference with Rainier; \*\*: significant difference with Bing. Tukey's test ( $p < 0.05$ ).

Interestingly, there are seasonal differences in Bing, Rainier and Lapins concerning the total alkanes and especially the C29 alkane amount (Fig. 6c and d). We can observe that these hydrocarbon concentrations are generally higher in 2009 and 2013 samples, compared to 2012 for the same variety. These changes can be due to different agro-climatic conditions presenting the zones where the varieties were grown and harvested.

#### 4. Discussion

An *in vitro* assay revealed differences in the cracking tolerance in sweet cherry fruits from five varieties: Bing, Rainier, Lapins, Kordia and Regina. At six hours Regina, Kordia and Lapins are the most tolerant varieties to the cracking disorder however after prolonged incubation in water almost all fruits from the different varieties cracked (Table 1). Cracking tolerance was significantly altered when the fruit cuticular wax was fully or partially removed. The chloroform removal method (solvent extraction) takes off the epi- and intracuticular waxes which are an effective barrier against water diffusion. Once removed, the water is free to enter or leave under this type of water stress. At the same time, cuticular wax removal leaves many pores exposed in the dermis of the fruit, which increases permeability (Schönherr, 2006; Kerstiens, 2006; Vogt et al., 2004). Increased permeability may cause a reduction in turgor in the epidermal cells, which is reflected in a lower percentage of cracking. Furthermore, chloroform is an organic solvent that damages the cell wall and membranes, solubilizing, precipitating and denaturing lipids (Xiang and Anderson, 1994), polysaccharides (Hussain et al., 2008) and proteins (Asakura et al., 1978). These changes may directly affect the permeability of the epidermis (Merida et al., 1981) and alter the cracking rate. To further understand the role that cuticular wax plays in cracking, two additional mechanical methods were used to remove this layer. The methods using a tissue paper embedded in chloroform and tissue paper without any solvent showed a significant decrease in cracking percentage, but not to the level achieved with solvent extraction. This difference, might be explained because the solvent extraction is able to remove the epi- and intracuticular

waxes (Merida et al., 1981), but when using a method less invasive (mechanical 1 and mechanical 2) epicuticular waxes are mainly extracted. The main function of these waxes is physical protection and becomes a barrier to water movement (Riederer and Schreiber, 2001; Christensen, 1972), however these results show us, that waxes could play a role in the cracking disorder.

The analysis of the NMR spectra and GC–MS data from wax and alkanes extracted from fruit of the different sweet cherry varieties predominantly corroborates the existence of *n*-alkane of 29 carbons (nonacosane). These results are in agreement with qualitative and quantitative alkanes analyzed in different cherries and apple cultivars, where aliphatic hydrocarbons of 29 carbons constitute approximately 75% of total alkanes (Peschel et al., 2007; Verardo et al., 2003). The varieties more tolerant to cracking (Lapins, Kordia and Regina), had a significantly higher concentration of nonacosane compared to the Bing and Lapins varieties. These results could indicate that the varieties with a higher concentration of the C29 alkane are more tolerant to cracking. Probably a cuticle with higher proportion of C29 alkane may be less permeable to water.

Another important association that can be observed is between the alkane concentrations and agro-climatic conditions. A higher concentration of these hydrocarbons could be found in the fruit cuticles harvested in 2009 and 2013 rather than in the 2012 season. The 2009 and 2013 harvest seasons had relatively high temperatures and were almost without rainfall. In fact, those years ended with an annual accumulated rainfall deficit. In contrast, 2012 was a year with a much higher rainfall in the pre and post-harvest season (references in the Plant material section). Some studies have shown that the climatic conditions of seasons, affect the type and cracking percentage in *P. avium* (Measham et al., 2009). This could be linked to the  $\beta$ -Ketoacyl-Coenzyme A Synthase Elongase (*CER6*) pattern expression because this gene is inducible by environmental factor such a light, abscisic acid and osmotic stress like drought and the expression level of *CER6* in the epidermis is one of the factors controlling wax accumulation on Arabidopsis stems (Hooker et al., 2002). Moreover, studies in cherry tomato showed that the silencing of the gene encoding for *CER6* enhances dehydration almost eight fold in the transgenic line compared to the untransformed

plant due to a preferential decrease in the concentration of *n*-alkane with 29 carbon lengths (Leide et al., 2007).

Different concentrations of the main wax components could be seen comparing the five cherry varieties. The C26 and C28 fatty acids could not be found in Bing fruits of both developmental stages. In Rainier, Lapins and Kordia varieties, betulinic acid is not found. These results could suggest the existence of some differences in the wax components between different cherry varieties, similar to what has been shown in a qualitative and quantitative study on cuticles fruits of different tomato varieties (Yeats et al., 2012) as well as recently in sweet cherry under different post-harvest treatment (Belge et al., 2014). In the present work the ursolic acid is the main triterpene (Babalola and Shode, 2013) and apparent in a higher concentration in the cracking tolerant varieties in both seasons investigated. These results might indicate that the cuticular wax must be a dynamic structure (Kosma et al., 2009), that responds to environmental changes and the genetic background of each variety (Yeats et al., 2012).

It is known that C27–C31 alkanes are synthesized from C16–C18 fatty acids (Kunst and Samuels, 2009). Therefore, varieties that synthesize alkanes with a higher chain length, theoretically, should have an increased synthesis or accumulation of precursor molecules. The analysis of fatty acids with 16 and 18 carbon atoms, confirm this statement. Lapins, Kordia and Regina (more tolerant to cracking), had a higher concentration of these fatty acids (16–18 carbons), compared to the Bing and Rainier varieties.

It has been described that C27–C31 alkanes are essential components of epicuticular waxes regulating water permeability in tomato (Vogg et al., 2004) and tobacco leaves (Cameron et al., 2006). This could indicate that higher quantities of this type of alkanes in cuticular wax would decrease the permeability and would allow less entry of water, when the fruit is under water stress such as rain. Kordia, Lapins and Regina, shown higher levels of C29 *n*-alkane presenting more tolerance to cracking when compared to Bing and Rainier. This difference in alkane composition could be an important factor to explain the different tolerance of sweet cherry varieties to cracking.

## 5. Conclusions

The combination of NMR and GC–MS techniques allowed for the determination of the concentration and type of alkane present in the fruit wax of the different sweet cherry varieties used in this study. Moreover, a higher proportion of C29 *n*-alkane in the cuticular waxes, is an important factor for cracking tolerance in sweet cherry varieties fruits. Among the varieties studies, Kordia, Lapins and Regina showed a higher amount of C29 alkane that had an association with a better tolerance to cracking when compared to Bing and Lapins. These results have demonstrated an association between C29 alkane and cracking tolerance. By combining these results with RNA-seq analyses of sweet cherry varieties, as well as reconstruction of the biosynthetic pathways that regulate C29 alkane, molecular markers (e.g. SNPs) may be identified in key regulatory genes and associated with cracking tolerant QTLs in order to incorporate this information and tools in sweet cherry breeding programs to develop new cracking tolerant varieties.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.scienta.2015.10.037>.

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