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Bioactive and volatile organic compounds in Southern Brazilian blackberry (*Rubus fruticosus*) fruit cv. Tupy

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Abstract

Blackberry (*Rubus fruticosus*, cultivar Tupy), an expanding fruit crop in southern Brazil, is greatly appreciated for its flavor and bioactive potential with limited characterization of its metabolite content. The purpose of this study was to characterize the bioactive and volatile organic compound (VOC) content of mature blackberry fruit of cultivar Tupy. Gallic acid, (-)-epicatechin, ferulic acid, and quercetin were the main phenolic compounds found in mature fruit. Among the VOCs identified in 'Tupy' blackberry were important flavor components characteristic of fruit berries, including hydrocarbons, alcohols, aldehydes, ketones, esters, and terpenoids. Some of the VOCs had not been previously found in blackberry, while others have been associated with typical blackberry flavor.

Keywords: flavor; phenolic compounds; blackberry.

1 Introduction

Blackberry (*Rubus* spp) was introduced to Southern Brazil in the 1970s and has adapted well to the temperate climate of the region with high productivity (up to 10,000 kg/ha/year) and low costs of implementation and management (Antunes, 2002; Finn, 2008). The Embrapa Pelotas (RS, Brazil) breeding program has released several blackberry cultivars including the high quality cultivar 'Tupy', which is also currently cultivated in Mexico and exported to the United States. 'Tupy' is a cross between cv. Comanche and an unknown clone called 'Uruguay' originating from that country (Antunes, 2002; Finn, 2008).

Blackberry fruit can be consumed fresh or processed as jam, cake, marmalade, ice-cream, frozen, dried, juice, wine, and liqueur (Antunes, 2002; Finn, 2008). Product acceptance is dependent on fruit flavor, which is composed of volatile compounds, and also on the balance between acids and sugars. Fruit metabolism during ripening, postharvest handling, and storage are determinants for flavor volatile production and also dependent on factors such as maturation stage and plant species (Christensen et al., 2007; Riu-Aumatell et al., 2004). Several methods are available for aroma analysis including solvent extraction and techniques for pre-concentrating volatiles such as solid-phase micro extraction (SPME) (Riu-Aumatell et al., 2004). Although many studies have analyzed volatiles from different *Rubus* species (Kallio & Linko, 1973; Malowicki et al., 2008; Meret et al., 2011; Pyysalo, 1976), and from different blackberry cultivars (Du et al., 2010; Georgilopoulos & Gallois, 1987; Klesk & Qian, 2003), no one has studied the volatiles produced by Brazilian blackberry fruit cv. Tupy.

The contribution of fruit consumption to human health goes beyond supplying essential nutrients for nourishment to include a diverse array of bioactive secondary metabolites from fruit extracts such as phenolic compounds, tocopherols, and ascorbic acid that have been implicated in the prevention of cardiovascular diseases and cancers (Seeram et al., 2006).

As part of an effort to increase consumer acceptance through the increased knowledge of quality attributes and as a support to breeders for improvement of fruit quality of this expanding fruit crop, this study reports the main bioactive and volatile compounds identified in 'Tupy' blackberry.

2 Materials and methods

2.1 Materials

Blackberry (*Rubus fruticosus*) fruits of cultivar Tupy were handpicked on the same day at 9 AM at maturity stage from a local grower in Morro Redondo RS, Brazil, and stored at –80 °C for further analyses. At harvest, fruit had 11.2° brix and titratable acidity of 0.97% of malic acid equivalents. Mature fruit from 10 'Tupy' clones were pooled and then separated into three biological replicates. For each analysis, the appropriate amount (described below) of sample from each replicate was used.

SPME (Supelco, Bellefonte, PA, USA) DVB/CAR/PDMS 50/30 fibers were used for sample volatile collection. The fibers were conditioned prior to use according to supplier's instructions.

All chemicals were GC or HPLC grade and purchased either from Sigma (Saint Louis, MO, USA) or Fluka (Milwaukee, WI, USA), except for β-cryptoxanthin, lycopene, lutein, and zeaxanthin, which were purchased from Chromadex (Irvine, CA, USA).

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2.2 HPLC analyses of bioactive compounds

All bioactive compound analyses were performed using a Shimadzu HPLC, with FL and UV detectors, a CLC-GODS guard column, and a Shimadzu RP-18 CLC-ODS (5 μm × 4.6 mm × 150 mm) column at 25 °C.

Phenolic compounds

Briefly, 5 g of sample were ground in liquid nitrogen, dissolved in methanol (30 mL), and acidified with HCl (1.2 M) (Hakkinen et al., 1998). The extract was incubated in a water bath at 35 °C in the dark for 24 h. The mixture was filtered and concentrated to dryness. The residue was redissolved in methanol (5 mL), centrifuged at 7,000 rpm for 10 min, and 30 μL of the supernatant was injected in the HPLC system. HPLC gradient separation used mobile phase A (water:acetic acid, 99:1) and B (methanol), at a flow rate of 0.8 mL min^{-1}. The gradient started at 100% A and linearly changed to 60% A and 40% B at 25 min, then at 37 min to 95% A and 5% B until 42 min, and back to the initial conditions after 3 min. The UV detector was set at 280 nm. Total run time was 45 min.

Compound identification was based on retention time comparison to the following standards: caffeic acid, ferulic acid, gallic acid, *p*-hydroxybenzoic acid, quercetin, and (-)-epicatechin. Quantification was based on an external standard calibration curve.

Carotenoids

The sample (5 g) was ground with 2 g of celite; cold acetone was then added, and the mixture was stirred for 10 min. (Rodrigues-Amaya, 2001). The sample was filtered under vacuum, and acetone was used to wash the material until the filtrate passing through the funnel became clear. The extract was partitioned using 30 mL of petroleum ether and 100 mL of distilled water. The organic phase was washed three more times and then transferred to a volumetric flask, and the volume was brought to 50 mL with petroleum ether. 25 mL of the sample solution were saponified using potassium hydroxide (1.5 N in ethanol) in the dark for 18 h. Upon phase separation, the extract was concentrated to residue and resuspended in the HPLC initial mobile phase (methanol:acetonitrile, 30:70 v/v). The extract was centrifuged at 9,000 rpm for 6 min, and a 25 μL aliquot was injected in the HPLC.

HPLC gradient separation used mobile phase (A - methanol, B – acetonitrile, and C - ethyl acetate) at a flow rate of 0.9 mL min^{-1}. The gradient started at 30% A and 70% B and linearly changed to 10% A, 80% B, and 10% C at 10 min; then at 35 min the solvent ratio was 5% A, 80% B, and 15% C until 40 min and back to initial conditions after 2 min. Total run time was 42 min. The UV detector was set at 450 nm. Compound identification was based on retention time comparison to the following standards: β-cryptoxanthin, lycopene, lutein and zeaxanthin, and β-carotene. Quantification was based on external standard calibration curves.

Tocopherols

Tocopherol extraction was performed according to the method described earlier for carotenoids (Rodrigues-Amaya, 2001). HPLC gradient separation used methanol (A), acetonitrile (B), and isopropanol (C) at a flow rate of 1 mL min^{-1}. Gradient started at 40% A, 50% B, and 10% C, and it was linearly changed to 65% A, 30% B, and 5% C at 10 min; it was maintained for 2 min, and then back to the initial conditions for a total run time of 15 min. Fluorescence detector was set at an excitation wavelength of 290 nm and emission 330 nm. Compound identification was based on retention time comparison with α-, δ- and γ-tocopherol standards, and quantification was based on an external calibration curve.

L-Ascorbic acid

HPLC gradient separation used mobile phase A (water:acetic acid, 99.9:0.1) and B (methanol) at a flow rate of 0.8 mL min^{-1}. The gradient started at 100% A, at 5 min it was reduced to 98% A and 2% B, held for 2 min, then back to initial conditions for a total run time of 10 min. The UV detector was set at 254 nm (Ayhan et al., 2001). Compound identification was based on retention time comparison to an L-ascorbic acid standard, and quantification was based on an external calibration curve.

2.3 CG-MS analysis of volatile compounds

Chromatographic analyses of volatile compounds were performed with a Shimadzu GCMS-QP5050 equipped with a OV5 column (Ohio Valley Specialty Chemical, USA), (30 m × 0.25 mm × 0.25 μm), using helium as carrier gas (99.99% pure, White Martins, Brazil) at a flow rate of 1 mL/min; and the injection split was set at 1:10. Temperature programming was as follows: oven temperature started at 60 °C and then ramped up 5 °C/min until 250 °C. Both injector and detector were set at 250 °C. Mass spectra were obtained in the range of 40 m/z to 400 m/z, scan speed 20 scans/s and 70 eV. Compound identification was based on the compounds' unique fragmentation pattern confirmed by comparisons to the NIST05 library.

Headspace SPME

Fresh fruit (3 g) were ground and placed in a 15 mL vial with 3 mL water and 1 g of sodium chloride (added to increase extraction recovery). The vial was heated to 60 °C for 15 min, and the SPME fiber was exposed to the headspace for 30 min. Sample injection was performed placing the SPME fiber in the heated chromatograph injection port for 5 min.

Solvent extraction

Twenty grams of ground fruit were dissolved in 100 mL of hexane or acetone. The suspension was sonicated for 1 h and then filtered and concentrated to dryness. The residue was resuspended in 5 mL of the extraction solvent, and an aliquot of 0.5 μL was injected in the GC/MS system under the same conditions used for volatiles with the following temperature differences: injector and detector were maintained at 300 °C and the oven was heated from 70 °C to 280 °C at 10 °C/min.

3 Results and discussion

3.1 Bioactive compounds

Table 1 shows the quantified bioactive compounds found in 'Tupy' blackberry grown in Southern Brazil, which includes phenolic compounds, carotenoids, tocopherols, and ascorbic acid. Gallic acid (145 mg $100g^{-1}$) and (-)-epicatechin (94 mg $100g^{-1}$) were the predominant phenolic compounds. Blackberry species *Rubus glaucus* and *R. adenotrichus* have been shown to contain the phenolic compounds gallic acid, (-)-epicatechin, ferulic, and caffeic acids, in addition to kaempferol, ellagic, and *p*-coumaric acid. 'Marion' (*Rubus* sp. hyb) and 'Evergreen' (*R. laciniatus*) blackberries have ellagic acid, gallic acid, quercetin, and kaempferol (Mertz et al., 2007; Siriwoharn & Wrolstad, 2004). In general, the qualitative and quantitative phenolic profile of 'Tupy' was similar to others of previously reported in studies on blackberry.

Table 1. Content of bioactive compounds in blackberry cv. Tupy.

Compounds	Concentration (mg $100g^{-1}$ f.w. ± std deviation)
hydroxybenzoic acid	1.44 ± 0.20
gallic acid	145.85 ± 3.10
quercetin	20.62 ± 0.90
caffeic acid	1.69 ± 0.30
ferulic acid	22.09 ± 1.20
(-)-epicatechin	94.29 ± 4.10
δ-tocopherol	0.49 ± 0.03
(β+γ)-tocopherol	0.24 ± 0.02
α-tocopherol	0.12 ± 0.01
L-ascorbic acid	0.90 ± 0.02
β-cryptoxanthin	0.24 ± 0.10
lutein + zeaxanthin	0.51 ± 0.30
β-carotene	0.01 ± 0.01
lycopene	0.02 ± 0.01

'Tupy' had a small amount of the bioactive molecules tocopherols, carotenoids, and ascorbic acid (Table 1). The *δ*- and *α*-tocopherol peaks were well resolved; however, *β*- and *γ*-tocopherol did not separate under the chromatographic conditions used and were therefore quantified together. Ascorbic acid, an antioxidant molecule active in oxidative stress reactions, was found in low amounts in 'Tupy' (Table 1) when compared to those of previous reports, from 14.3 to 17.5 mg $100\ g^{-1}$ fw, which were found in other cultivars (Pantelidis et al., 2007). Carotenoids are among the most important pigments accumulated in fruits and may serve as precursors for VOCs. *β*-ionone and geranylacetone, for example, are produced from the degradation of *β*-carotene and lycopene, respectively (Stevens et al., 1979). Although both *β*-carotene and lycopene were found in this study, their concentrations were low (Table 1). The results found in the present study are in agreement with the hypothesis that antioxidant metabolites and defense compounds, such as the quantified bioactive molecules, compensate each other and are not all expressed at the same time.

3.2 Volatile organic compounds

Table 2 shows all 45 volatile compounds identified in the blackberry collected with SPME. The identified compounds represented approximately 77% of the total area of the chromatogram (Figure 1); the vast majority (97.7%) was composed of terpenoids, and limonene was the predominant compound (Table 2). Identified volatiles extracted with hexane were primarily hydrocarbons (Table 3), and those extracted with acetone were furans and pyrans (Table 4). Approximately 60% of the total peak area of the chromatogram (Figure 2) of hexane-extracted volatiles were identified, and most of the compounds were aliphatic, while only 13% were aromatic. In the chromatogram of the acetone extract (Figure 3), the identified compounds represented 82% of the total peak area.

Aroma volatile compound formation is associated with pigment formation during ripening; [3] however, some compounds

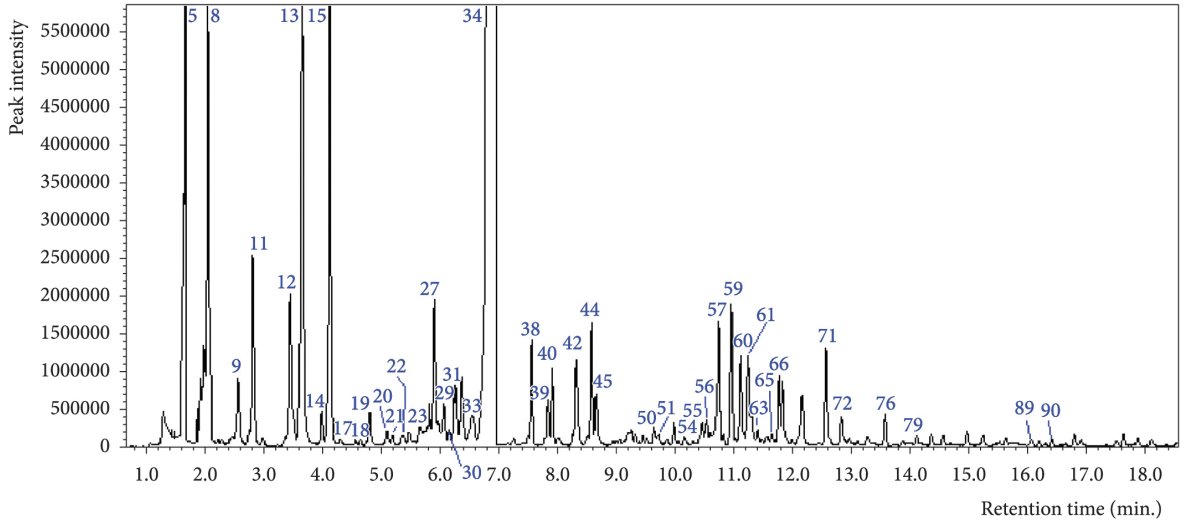


Figure 1. Total ion chromatogram of volatile compounds from blackberry cv. Tupy collected using SPME (*chromatographic conditions described in the materials and methods section*).

Table 2. Volatile compounds identified in blackberry cv. Tupy.

peak	RT (min)	area %	Name	MW	S	class
5	1.68	3.65	methyl ethyl ketone	72	95	ketone
8	2.06	3.58	heptane	100	96	hydrocarbon
9	2.57	0.77	toluene	92	94	hydrocarbon
11	2.81	2.05	hexanal	100	91	aldehyde
12	3.45	1.52	2-hexenal	98	97	aldehyde
13	3.65	4.55	heptanal	114	98	aldehyde
14	3.98	0.17	2-heptanone	114	98	ketone
15	4.13	3.24	2-heptanol	116	98	alcohol
17	4.56	0.17	methyl-hexanoate	130	92	ester
18	4.65	0.18	α-thujene	136	91	terpenoid
19	4.80	0.44	α-pinene	136	97	terpenoid
20	5.11	0.24	camphene	136	97	terpenoid
21	5.19	0.23	heptenal	112	94	aldehyde
22	5.36	0.30	benzaldehyde	106	90	aldehyde
23	5.47	0.22	1-heptanol	116	91	alcohol
27	5.91	1.16	β-myrcene	136	95	terpenoid
29	6.07	0.47	ethyl-hexanoate	144	98	ester
30	6.16	0.22	octanal	128	96	aldehyde
31	6.26	0.59	α-phellandrene	136	96	terpenoid
33	6.56	0.60	terpinolene	136	96	terpenoid
34	6.95	63.26	limonene	136	94	terpenoid
38	7.56	0.73	α-terpinene	136	97	terpenoid
39	7.83	0.60	1-octanol	130	92	alcohol
40	7.91	0.52	linalool oxide	170	98	terpenoid
42	8.32	0.96	o-cimene	136	91	terpenoid
44	8.58	0.79	linalool	154	96	terpenoid
45	8.66	0.47	nonanal	142	95	aldehyde
50	9.64	0.25	trans limonene oxide	152	92	terpenoid
51	9.72	0.24	isopinocarveol	152	93	terpenoid
54	10.16	0.22	nonenal	140	92	aldehyde
55	10.45	0.36	isoborneol	154	90	terpenoid
56	10.53	0.30	ethyl benzoate	150	94	ester
57	10.73	1.12	terpinen-4-ol	154	91	terpenoid
59	10.96	1.13	p-cymen-8-ol	150	93	terpenoid
60	11.11	0.78	α-terpineol	154	95	terpenoid
61	11.24	0.76	methyl salicylate	152	96	ester
63	11.40	0.19	decanal	156	95	aldehyde
65	11.64	0.23	verbenone	150	92	ketone
66	11.77	0.53	p-mentenal	152	95	aldehyde
71	12.56	0.96	(-)-carvone	150	95	terpenoid
72	12.82	0.37	geraniol	154	95	terpenoid
76	13.57	0.39	vitispirane	192	95	terpenoid
79	14.11	0.17	theaspirane	194	92	terpenoid
89	16.19	0.13	α-copaene	204	93	terpenoid
90	16.40	0.18	damascenone	190	92	ketone
area total % identified				77.14		
area % alcohols	4.06			area % hydrocarbons	4.35	
area % aldehydes	0.53			area % ketones	4.23	
area % esters	0.76			area % terpenoids	75.38	

MW = molecular weight; S = similarity with mass spectra; % area = area % related to the total peak area.

such as hexanal and (*E*)-2-hexenal that can be generated from oxidation or lipoxygenase-catalyzed oxidative degradation of fatty acids (Sanz et al., 1997) typically decrease with fruit maturity. Volatile composition also varies both among and within cultivars, thus being genotype specific (Du et al., 2010).

The major chemical groups of VOCs responsible for the flavor of fruit berries are esters, alcohols, ketones, aldehydes, terpenoids, furanones, and sulfur compounds (Christensen et al., 2007). For industrial purposes, 'Black Diamond', a thornless blackberry cultivar with large fruit size and high yield, is considered ideal. 'Marion' is not as large or productive, but it is regarded as having the ideal flavor with predominance of furaneol, linalool, β-ionone, and hexanal (Du et al., 2010). The ideal volatiles found in 'Marion' were also found in 'Tupy'.

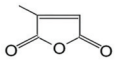
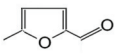
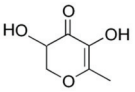
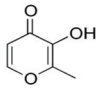
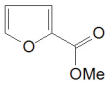
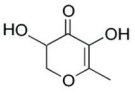
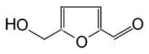
Furaneol, linalool, geraniol, ethyl hexanoate, *trans*-2-hexenol, and β-ionone are responsible for fresh fruit, floral, strawberry, and raspberry aromas, while 1-octen-3-ol, myrtenol, eugenol, and α-terpineol account for vegetal, woody, moldy, and cooked fruit flavors (Du et al., 2010). Heptanol and *p*-cymen-8-ol have also been identified as the most important volatile component of blackberry flavor, and their contribution has been described as fruity-herbaceous and flowery-spicy, respectively (Ibáñez et al., 1998).

Table 3. Semivolatile compounds identified in blackberry cv. Tupy extracted with hexane.

peak	RT (min)	Name	Area %	MW	S
4	3.65	aliphatic hydrocarbon	3.97		
5	3.69	aliphatic hydrocarbon	1.99		
6	3.76	aliphatic hydrocarbon	1.31		
9	3.95	aliphatic hydrocarbon	0.98		
10	4.04	aliphatic hydrocarbon	5.06		
11	4.11	C2-benzene	1.63	106	93
12	4.18	aliphatic hydrocarbon	1.71		
16	4.60	n – decane	8.91	142	93
20	4.95	aliphatic hydrocarbon	2.44		
21	5.04	C3-benzene	2.67	120	92
22	5.15	aliphatic hydrocarbon	1.64		
28	5.46	C4-benzene	1.27	134	92
32	5.87	aliphatic hydrocarbon	1.50		
36	6.15	n – undecane	5.05	156	97
52	7.57	naphthalene	0.66	128	94
53	7.66	n – dodecane	2.46	170	95
55	7.86	aliphatic hydrocarbon	0.71		
64	9.09	n – tridecane	1.81	184	95
65	9.16	C1 naphthalene	0.50	142	91
66	9.44	C1 naphthalene	0.18	142	96
72	10.33	aliphatic hydrocarbon	0.49		
73	10.78	n – tetradecane	1.55	198	96
74	11.08	C2 naphthalene	0.23	156	95
75	11.41	C2 naphthalene	0.23	156	95
76	11.49	C2 naphthalene	0.29	156	94
78	12.17	aliphatic hydrocarbon	0.87		
80	13.24	n – pentadecane	1.81	212	98
81	14.47	C3 naphthalene	0.17	170	94
83	17.07	n – hexadecane	1.90	226	97
85	21.46	n – heptadecane	1.84	240	97
86	21.61	pristine	1.29	268	93
88	23.73	n – octadecane	1.29	254	97
89	23.89	phytane	0.49	282	93
91	25.34	n – nonadecane	0.91	282	95
92	26.64	n – eicosane	0.50	296	95
93	27.76	n – heneicosane	0.26	310	95
		% area identified compounds			60.54
% aliphatic					52.72
% aromatic					7.84

MW = molecular weight; S = similarity with mass spectra; % area = area % related to the total peak area.

Table 4. Semivolatile compounds identified in blackberry cv. Tupy extracted with acetone.

peak	RT	% area	compound	MW	S	Structure
1	3.85	2.69	furandione	112	91	
2	4.15	1.39	furfural	100	93	
3	4.36	0.64	dihydro-dihydroxy-pyranone (two isomers)	144	93	
5	5.19	5.22			96	
6	5.88	2.41	maltol	126	97	
7	5.98	0.84	methyl furoate	126	92	
8	6.93	3.62	dihydro-dihydroxy-pyranone	144	91	
9	7.60	45.50	hydroxy-furfural (two isomers)	126	98	
10	8.35	36.80			93	
18	13.77	0.67	hexanoic acid	116	96	$CH_3(CH_2)_4COOH$
% area identified						82.57

MW = molecular weight; S = similarity with mass spectra; % area = area % related to the total area of identified peak.

The following compounds found in 'Tupy' had been previously found in other *Rubus* spp.: 2-heptanol, 1-hexanol, 1-octanol, hexanal, nonanal, 2-decenal, 2-heptanone, and methyl salicylate in Andean blackberry (*R. glaucus*); 2-butanone, hexenal, and nonanal in arctic bramble (*R. articus*) (Meret et al., 2011); 1-hexanol, 2-heptanol, 1-octanol, *p*-cymen-8-ol, 2-heptanone, *α*-terpineol, pulegone, isoborneol, myrtenol, 4-terpineol, carvone, elemicine, nonanal, benzyl alcohol, benzoic acid, and 3-hydroxy-7,8-dihydro-β-ionol, in *R. laciniata* (Georgilopoulos & Gallois, 1987); acetic and hexanoic acids, *trans* 3-penten-1-ol, 2-heptanol, 3-methyl-2-buten-1-ol, benzyl alcohol, and linalool in hybrids between *R. idaeus* and *R. arcticus*; and *β*-ionone, 2-heptanone, geraniol, limonene, linalol, *α*-pinene and 4-terpineol in *R. idaeus* (Machado et al., 2007; Malowicki et al., 2008).

Blackberry fruit processed as juice alone or in combination with other fruit had furfural, 3-methyl-butanal, 3-methyl-1-butanol, phenylacetaldehyde, *trans*-furan linalool oxide, 6-methyl-5-hepten-2-one, *α*-terpineol, and *E*-nerol as the main volatile aroma compounds (Georgilopoulos & Gallois, 1987; Vazquez-Araujo et al., 2010), indicating that these compounds found in 'Tupy' may be associated with an advanced ripening stage.

Moreover, some of the identified volatile compounds have been associated with plant pathogen control. Hexanal, 1-hexanol, (*E*)-2-hexen-1-ol, (*Z*)-6-nonenal, (*E*)-3-nonen-2-one, methyl salicylate, and methyl benzoate exhibited potential as postharvest fumigants for control of *Botrytis* on strawberry (Archbold et al., 1997).

4 Conclusion

The main flavor active compounds previously reported in *Rubus* spp. are also present in the Southern Brazilian blackberry 'Tupy'. 'Tupy' is a high quality blackberry with the potential to be used in an array of new products based on its bioactive and aroma volatile profile. Further studies investigating optimization of production, postharvest conservation, and processing with the goal of quality improvement and grower and consumer benefits are needed to support the wide utilization of this cultivar.

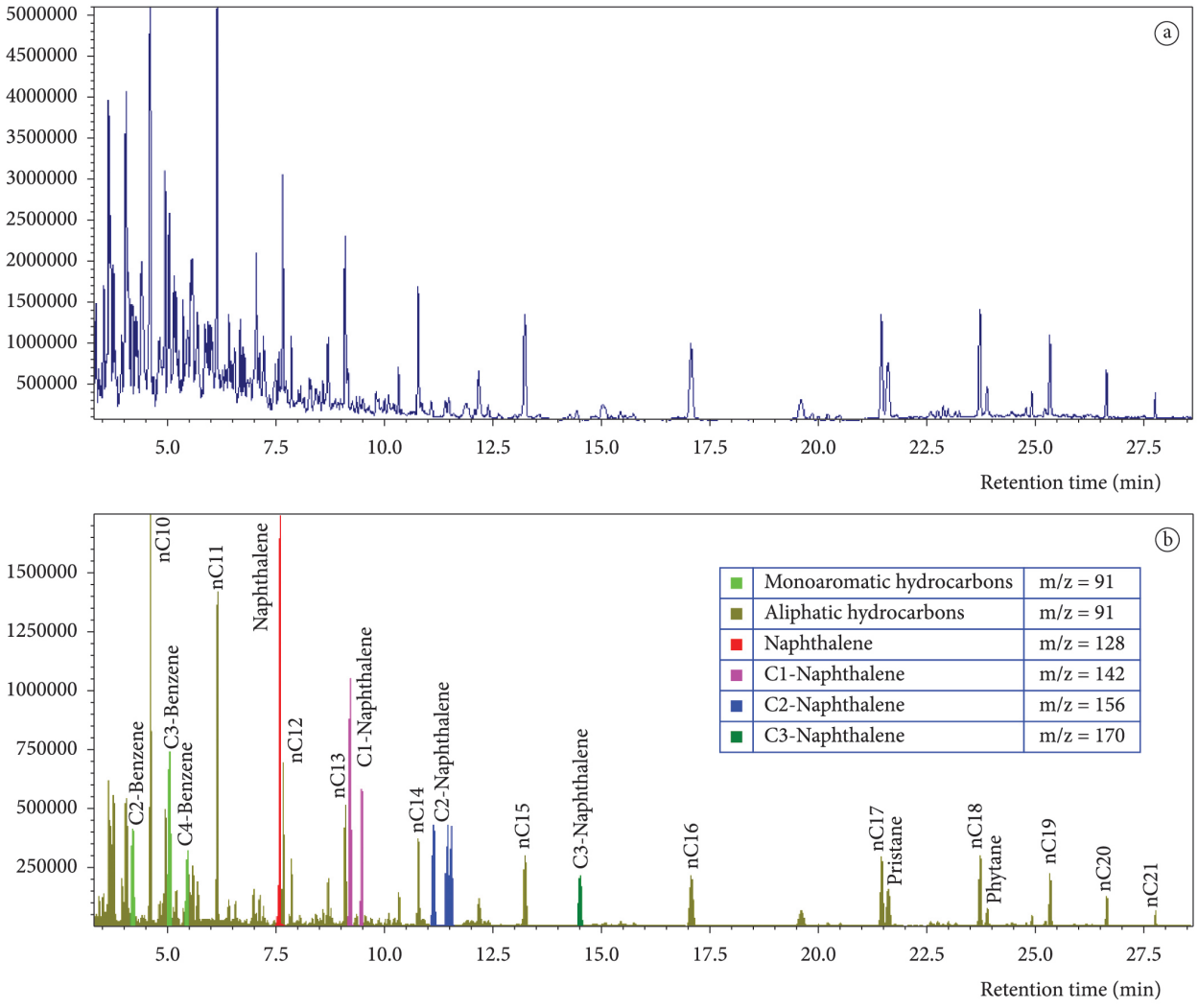


Figure 2. (a)Total ion chromatogram and (b) Monitored ion chromatogram of blackberry cv. Tupy volatiles extracted with hexane (*chromatographic conditions described in the materials and methods section*).

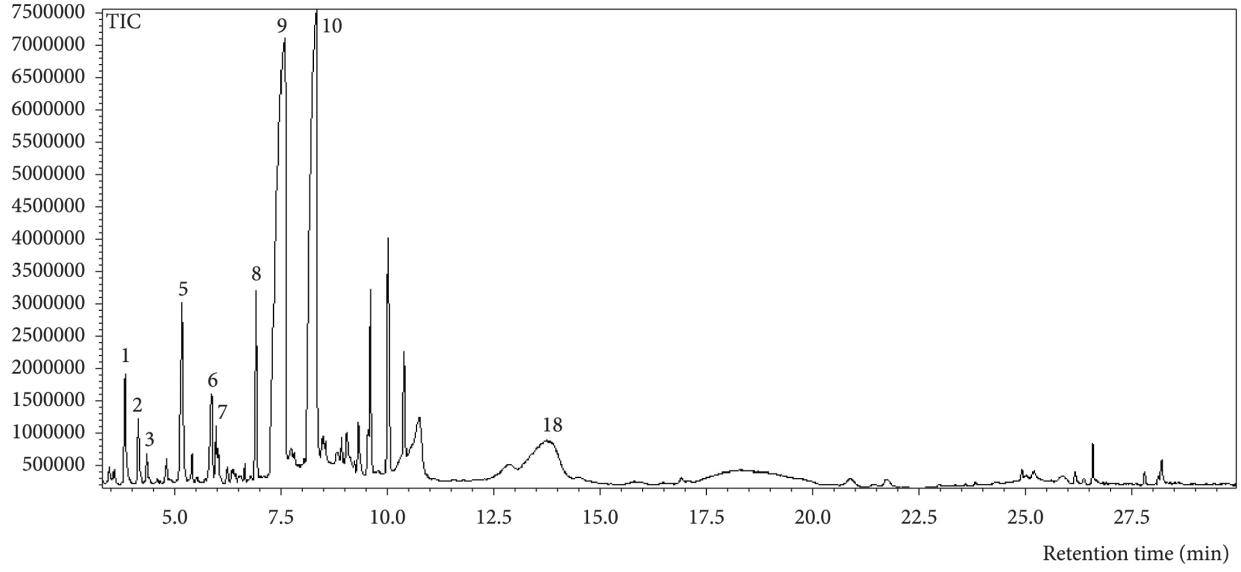


Figure 3. Total ion chromatogram of blackberry cv. Tupy volatiles extracted with acetone (*chromatographic conditions described in the materials and methods section*).

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