Principal aroma-active compounds of Red Moon RM-1 apple fruit cultivar as determined by a combined technique of gas chromatography-olfactometry

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Summary

Aroma belongs to crucial natural quality attributes of apples. The profile of aroma-active compounds in the peel and flesh of a rare new red-fleshed Red Moon (RM-1) apple fruit cultivar was investigated. For this purpose, a sampling extraction method of headspace solid-phase microextraction (HS-SPME) followed by a specific technique of gas chromatography-olfactometry (GC-FID-O) and gas chromatography-mass spectrometry (GC-MS) were used. Apples were harvested in 2020 from two different growing areas (Western Slovakia, Northeastern Italy). A total of 58 aroma-active compounds recognized by GC-FID-O were responsible for the overall aroma of the analysed apples from Slovak production, and 52 from the Italian apple production. The aroma-active compounds comprised esters, alcohols, aldehydes, sesquiterpenes, one ketone and one short-chain unsaturated carboxylic acid. Ethyl 2-methylbutanoate was odorously the most noticeable compound of apple peel and flesh, common to apples from both growing localities. Likewise, other 12 principal odorants with various odour intensity levels, such as ethyl butanoate, ethyl acetate, butanol, 1-octene-3-one, isopropyl acetate, hexyl hexanoate, hexanol, bisabolene (unknown isomer), farnesol (unknown isomer), unknown compounds No. 66 and No. 70 and propyl hexanoate were common to all analysed apple samples.

Keywords

red-flesh apples; aroma-active compound; solid-phase microextraction; gas chromatography-olfactometry; gas chromatography-mass spectrometry

Generally, more than 80 million tonnes of apples are produced and consumed annually worldwide as fresh and dried fruit, juices or alcoholic beverages. The EU share on the apple production accounts for almost 12 million tonnes [1].

The genus *Malus* includes 30–35 species of small deciduous trees or shrubs in the family Rosaceae and it is native to the temperate zones of the northern hemisphere, Europe, Asia and North America [2]. The domesticated orchard or table apple *Malus* × *domestica* Borkh. is considered a complex interspecific hybrid. The ancestors are generally known as "wild apples" or "crab apples". Their name is derived from their typically small and tart fruits [3, 4]. 'RM-1' (Red Moon 1) cultivar as a new and distinct apple tree, notable for its upright plant habit, with pink-red skin overcolour and red flesh colour of fruit, has a late season of fruit ripening (late October). It was discovered and selected as a single plant from within the progeny of the open pollination in a controlled environment in Lot, France in 2000. In this original

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area, the tree blooms in the middle of April. The fruit is medium-large, cylindrical with a diameter of 7.5–9.0 cm and with 90 % of overcolour. The red flesh is juicy, firm and moderately aromatic [5, 6].

The volatile aroma-active compounds of apples have been studied for more than 50 years, with more than 300 compounds identified [7, 8]. However, just some of them were shown to contribute significantly to the fruit aroma [7, 9]. Aroma of apples is a crucial criterion to assess fruit quality, exerting a direct influence on their acceptance or refusal by consumers [10, 11].

Biosynthesis of aroma-active compounds in apples involves metabolic pathways in which the main precursors are fatty acids and amino acids. In the presence of key enzymes such as lipoxygenase, alcohol dehydrogenase and alcohol acyltransferase, precursors are transformed to aldehydes, alcohols and esters [12, 13]. In particular, 2-methylbutyl acetate, butyl acetate, hexyl acetate, ethyl 2-methylbutanoate, ethyl acetate, ethyl butanoate, methyl anthranilate and (*E*)-2-hexenal were reported and considered as the most significant volatile compounds contributing to the typical aroma of several apple varieties [12, 14]. It was proven that low concentrations of several aroma-active compounds, such as (*E*)-2-hexenal and ethyl 2-methylbutanoate, may give seemingly an unnecessary impression but they are extremely important in the definition of the overall aroma of apples [15].

A sufficiently selective and sensitive analytical approach is required to determine volatile compounds in apples. A simple solvent-free, equilibrium extraction method of headspace solid-phase microextraction (HS-SPME) for the isolation of volatile compounds [16–18] combined with gas chromatography-mass spectrometry (GC-MS) is frequently used for qualitative and quantitative evaluation of volatile substances in apples and many other fruits [12, 19–21]. For determination of volatiles, which contribute significantly to the overall aroma of apples, a specific technique connecting gas chromatography with olfactometry (GC-O) can be effectively used. Its principle is unveiling and subsequent identification of the volatile organic compounds responsible for a relevant aroma with an additional support of GC-MS. These aroma-active substances are very often present in food matrices in trace or ultra-trace contents, which are usually below the detection limit of commonly used GC detectors such as mass-selective detector (MSD) or flame ionization detector (FID). Individual compounds of the volatile fraction of the sample are separated on the GC column and effluent from is splitted by a cross divider into equally long, inactivated capillaries to an instrumental detector (usually FID or MSD) and a testing outlet (sniffing port). A trained sniffer describes the perceived odour in terms of the intensity of the odour zones perceived according to the training methodology [22]. Several studies used previously gas chromatography in combination with olfactometry to determine the most intensive aroma-active volatile compounds in various apple varieties [23–26].

Recently, several studies focused on the aroma of red-fleshed apples or related juices [27–29]. Zhao et al. [27] studied free and bound forms of aroma of red-fleshed apples during the young fruit period through the expansion period and the con-

Fig. 1. Fruit of the red-fleshed Red Moon (RM-1) apple cultivar.

version period to the ripen period. Lu et al. [28] investigated the effects of post-harvest storage conditions on the fruit quality and relative contents of aroma components of a red-fleshed apple. Chitarrini et al. [29] described cultivar-specific volatile organic compounds in the headspace of cloudy apple juices. Headspace solid-phase microextraction was used to analyse 9 red-fleshed apple juices including RM-1 cultivar. In this context, it is interesting that this new variety with a botanical classification *Malus* × *domestica* Borkh. cultivar 'RM-1' was patented quite recently in August 2017 after several years of research [5, 6].

Although some research has been done on red-fleshed apples, previous studies focused on identification and quantification of the overall profile of individual compounds constituting the volatile fractions. However, the key aroma-active compounds generating principally the aroma were not identified up to now. Therefore, the purpose of this study was determination of principal aroma-active compounds in red-fleshed (RM-1) apple fruits by the specific technique of GC-O. For the first time, the combination of HS-SPME extraction followed by GC-FID-O with support of GC-MS was used to revelation, identification and comparison of the key aroma-impact compounds in peel and flesh of this rare new red-fleshed apple cultivar harvested in Slovakia and Italy.

MATERIALS AND METHODS

Apple fruit samples

Two RM-1 apple fruit (Fig. 1) batches were studied. The first contained the commercialized cultivar grown in Slovakia in Dunajská Lužná at an altitude of 126 m, harvested at commercial maturity during the 2020 season and purchased in early November 2020 from the local store Boni Fructi (Dunajská Lužná, Slovakia). The second batch contained the commercialized cultivar grown in Italy in Eraclea, near Venice at an altitude of 2 m, harvested at commercial maturity during the 2020 season and purchased in early December 2020 from the local store Boni Fructi. Immediately after purchase, representative apple fruit samples were selected for their uniformity (colour, diameter, lack of damage) and stored at a temperature of 12 ± 2 °C in a storage room with allowed air circulation until analysis for a maximum 4–6 weeks.

Sample processing

Two kilograms of apple fruit samples from each batch were washed with tap water, unpeeled to obtain apple peel (< 2 mm in thickness) and deseeded. The top layer of flesh (0.5–1.0 cm) was removed as a waste. The inner flesh was cut to approximately 0.3 cm × 0.3 cm × 0.3 cm pieces and the peel to 0.3 cm × 0.5 cm rectangles. The cutting was done using a ceramic knife to prevent possible metal-induced oxidative degradation of volatile compounds and disposable nitrile gloves were used to prevent the contact of the sample with greasy hands and possible cross-contamination. With a purpose of homogenizing the apple fruit samples (one batch at a time), all apple pieces of peel or flesh (separately) were immediately transferred into a ceramic bowl and stirred gently, to obtain a representative peel mass or flesh mass (separately) for sampling and subsequent analyses.

Reference standards

Chemicals used as reference standards to support identification of volatiles (listed in Tab. 1) were gifts donated by Bedoukian Research (Danbury, Connecticut, USA), Graz University of Technology (Graz, Austria) or French National Institute for Agricultural Research (INRA) laboratories (Dijon, France).

Headspace solid-phase microextraction

Individual samples of either apple peel or apple flesh (always 5 g) were incubated statically in a 40 ml glass vial in a metallic block thermostat (Liebisch, Bielefeld, Germany) at 40 °C for 30 min. The volatile fractions were isolated with a solid-phase microextraction (SPME) fibre placed in the headspace (HS) of the sample. The SPME fibre with divinylbenzene/carboxen/polydimethylsiloxane (DVB/Carboxen/PDMS) coating film (thickness 50/30 μm, length 2 cm), Stable Flex, "For odours" (Cat. No. 57328-U; Supelco, Bellefonte, Pennsylvania, USA) was used. The fibre was initially conditioned by heating in the GC injector block at 250 °C for 1 h. SPME samples were desorbed thermally at 250 °C in the GC injector block during the entire analytical run. All HS-SPME extractions were performed in six replicates (n = 6).

Gas chromatography-olfactometry

In order to reveal principal aroma-active compounds in apple peel and in apple flesh samples (each separately), volatiles extracted by headspace SPME were analysed by a combined technique of gas chromatography with flame ionization detection and olfactometry (GC-FID-O), using the concept of detection frequency of posterior assessment of odour intensity [30]. The methodology of the sniffing procedure was performed

according to KOPUNCOVÁ et al. [31] with slight modifications. The sniffing panel was formed of 3 judges (1 woman, 2 men; aged 28–57 years) who were chosen from 7 assessors trained in sensory evaluation. All assessors were asked to estimate the intensity of each perceived odour using a seven-point scale ranging from 0 to 3 (half values allowed): 0 = none, 0.5 = very weak, 1 = weak, 1.5 = moderate, 2 = strong, 2.5 = very strong, 3 = extremely intensive. Results of GC-FID-O analyses were expressed as average values from 6 independent measurements for each sample, complying with the requirement of at least 5 citations within each olfactory perception. The value of ± 0.5 was considered as a standard error of estimation of odour intensities for the applied intensity scale and engaged well trained sensory panel.

The analyses were performed using a gas chromatograph Agilent 7890A (Agilent Technologies, Palo Alto, California, USA) coupled to a flame ionization detector and an olfactory detector port ODP3 (Gerstel, Mülheim an der Ruhr, Germany). The effluent of the GC column was split to FID and the olfactory detector port at a split ratio of 1 : 1. The column DB-WAX (30 m × 0.32 mm × 0.25 μm; Agilent Technologies) with a polar stationary phase was used for separation of individual volatiles. It operated with a temperature programme 50 °C (1 min), 5 °C·min^{-1} and 250 °C (1 min). Hydrogen was used as a carrier gas at a linear velocity of 45 cm·s^{-1} (measured at 143 °C). Pulse splitless injection (100 kPa for 1 min) was used at an injector temperature of 250 °C. The olfactory detector port (ODP) operated at a temperature of 180 °C, interface temperature was 230 °C and the flow of added nitrogen in the olfactory detector port's humidifier was 12 ml·min^{-1}. The sniffing time of individual judges did not exceed 40 min per one analysis.

Gas chromatography-mass spectrometry

For identification purposes, in parallel with GC-FID-O, fractions of volatile compounds of apple peel or apple flesh obtained via HS-SPME were separated and analysed individually by GC-MS using a gas chromatograph Agilent 6890N (Agilent Technologies) coupled to a mass spectrometric detector 5973 *inert* (Agilent Technologies). HP-INNOWax column (30 m × 0.25 mm × 0.5 μm, Agilent Technologies) with a polar stationary phase operated with a temperature programme 50 °C (1 min), 5 °C·min^{-1} and 250 °C (1 min). An autosampler (Agilent Technologies) was attached. Injector temperature was kept at 250 °C, set to pulsed splitless mode with the pulse pressure set to 100 kPa for 1 min. Helium was used as the carrier gas circulating at 1.2 ml·min^{-1} at a constant flow with a linear velocity of 40 cm·s^{-1}. MS fragmentation was performed under electron ionization of 70 eV. Ion scan range was set to 29–250 *m*/*z*.

Identification of aroma-active volatiles

The revealed sensorically recognized aroma-active volatiles were identified based on comparison of their linear retention indices (*LRI*), mass spectra (MS), GC analysis of standard reference compounds and also by comparison of data on occurrence as well as aroma description with literature [23, 28, 32, 33] and in our in-house database. *LRI* were calculated using the equation of VAN DEN DOOL and KRATZ [34] using *n*-alkanes C_8–C_{20} as reference standards. *LRI* data were compared and confirmed with *LRI* data obtained by the measurement of relevant standard compounds. For this purpose, our in-house database of *LRI* data was used. Identification of compounds by comparison of their mass spectra was performed with NIST21 MS library (National Institute of Standards and Technology, Gaithersburg, Maryland, USA).

Statistical analysis

Statistical evaluation was performed using Unistat v. 6.0 statistical software package (Unistat, London, United Kingdom). Methods of multivariate statistics, primarily principal component analysis (PCA), were used to define, interpret and visualize the differences between the compared Red Moon (RM-1) apple cultivar originating from different countries (Slovakia and Italy).

RESULTS AND DISCUSSION

GC-FID-O study

In the selection of apple fruits, consumers predominantly use visual and organoleptic responses, such as aroma and texture, as quality and preference markers. In order to obtain chemically objective data on principal constituents of the aroma complexes of the peel and the flesh of apple Red Moon (RM-1) cultivar, analysis of volatiles extracted by HS-SPME from the relevant parts of the apple matrix was carried out using GC-FID-O with support of GC-MS. For comparison, samples of apples produced from two different growing locations in Slovakia and Italy were analysed.

Illustrative GC-FID-O chromatograms connected with aromagrams are depicted in Fig. 2. The obtained data were processed and the results are summarized in Tab. 1. Overall, the aromas of the analysed apples from both above-mentioned locations were rich and complex. In total,

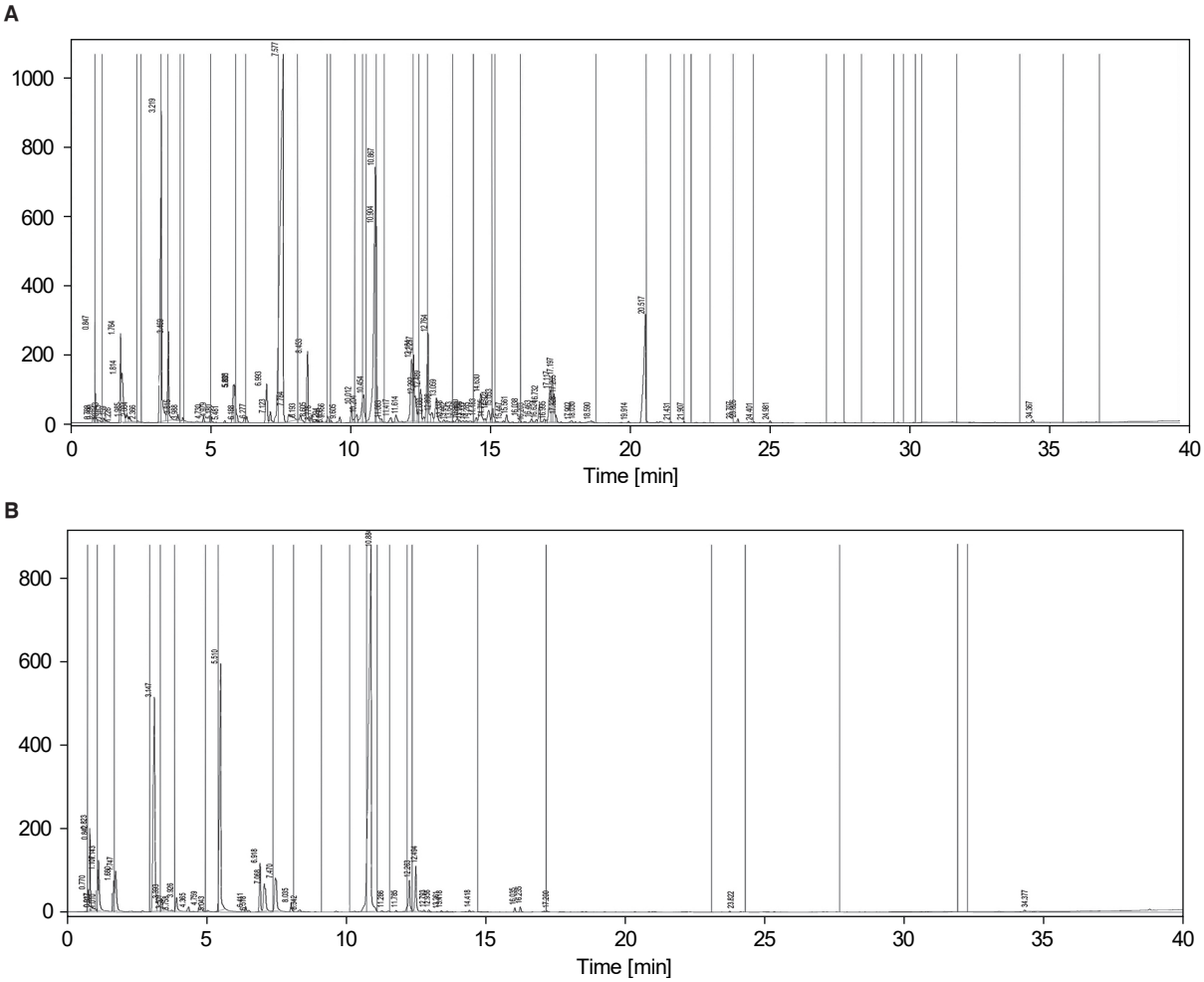

Fig. 2. Illustrative chromatograms of the apple fruit volatiles overlaid with aromagrams, demonstrating GC-FID–O analysis as a tool for revelation of aroma-active compounds in Red Moon apple cultivar grown in Slovakia.

A – peel, B – flesh.
Chromatogram is a record of flame ionization detector and vertical lines indicate the positions where aroma-active compounds were registered olfactorily.

72 aroma-active compounds (odouric responses) were revealed by GC-O technique in the aroma of peel and flesh of apples from both geographic areas. The aroma profiles were found to consist of a mixture of various chemical compounds, mainly esters, aldehydes and alcohols [12, 13], followed by terpenes, one ketone and one short-chain unsaturated carboxylic acid. Fifty-seven aroma-active compounds were identified by a combination of above-mentioned independent methods. In case of 3-octanol, 6-methyl-5-hepten-2-ol and isolongifolene, only partial information was available and thus only tentative identification was possible. Eight compounds were detected only by GC-olfactometry, thereby they remained unidentified at this stage. The exact identity of some sesquiterpene stereoisomers (bisabolene, γ-bisabolene and farnesol) also remains unknown at this stage due to unavailability of relevant reference materials.

Slovakian RM-1 apples

Apple peel

The rich aroma of this apple part was formed by 52 aroma-active compounds (Tab. 1). With respect to the estimated odour intensities, principal odour-active compounds were these 16 substances: ethyl butanoate, ethyl 2-methylbutanoate, isopropyl butanoate, isobutanol, methyl hexanoate, butyl butanoate, ethyl hexanoate, hexyl acetate, hexanol, (*E*)-2-hexenol, hexyl butanoate, ethyl octanoate, α-farnesene, hexyl octanoate, unknown compound No. 66 and unknown compound No. 70. All these compounds showed the highest odour intensity levels from 2 to 3, their individual

Tab. 1. Key aroma-active compounds in headspace SPME extracts of Red Moon (RM-1) apple fruits from two different growing locations.

No.	*LRI*	Aroma compound	Aroma description	Odour intensity				Identification
				Peel		Flesh		
				SK	IT	SK	IT	
1	893.1	Ethyl acetate	Ethereal, solvent-like, slight fruity undertone	1.5	1.5	2	2	LRI, ST, OD, LIT
2	904.8	Isopropyl acetate	Ethereal, slightly fruity	1	1	1.5	1	LRI, ST, OD, LIT
3	NC	Ethanol	Alcoholic, solvent-like	1.5	NDO	1.5	NDO	MS, OD, LIT
4	914.8	2-Methyl butanal	Musty, phenolic-like, malty	NDO	NDO	NDO	1.5	LRI, ST, OD, LIT
5	959.5	Ethyl propanoate	Fruity, sweet, ethereal, fragrant, slight rum-like	1.5	0.5	NDO	1.5	MS, LRI, ST, OD, LIT
6	968.6	Ethyl isobutanoate	Fruity, apple-like, sweet	1.5	1	NDO	NDO	MS, LRI, ST, OD, LIT
7	1 040.4	Ethyl butanoate	Apple-like, sweet	2.5	2	2.5	0.5	MS, LRI, ST, OD, LIT
8	1 055.2	Ethyl 2-methylbutanoate	Typical intensive apple-like	3	2.5	3	1	MS, LRI, ST, OD, LIT
9	1 078.8	Butyl acetate	Fruity, ethereal, solvent, sweet	NDO	2	NDO	2	LRI, ST, OD, LIT
10	1 085.2	Hexanal	Green, grassy, leafy, fresh, apple-like undertone	NDO	NDO	2.5	NDO	MS, LRI, ST, OD, LIT
11	NC	Unknown °	Fresh	NDO	NDO	1	NDO	–
12	NC	Isopropyl butanoate	Fruity, apple pits-like, slightly bitter	2	NDO	NDO	NDO	MS, OD, LIT
13	1 096.9	Isobutanol	Ethereal, wine-like, bitter, fusel alcohol-like	2	1	NDO	1	LRI, ST, OD, LIT
14	NC	Unknown °	Solvent-like, fruity, sweetish	NDO	1.5	NDO	1.5	–
15	1 127.5	2-Methyl butyl acetate	Overripe fruit, sweet	NDO	1.5	NDO	2	LRI, ST, OD, LIT
16	1 128.9	Propyl butanoate	Fruity, apple-like, ethereal	1	1	1.5	NDO	MS, LRI, ST, OD, LIT
17	1 141.0	Ethyl pentanoate	Apple-like, sweetish, fruity	1	1	NDO	1.5	LRI, ST, OD, LIT
18	1 151.0	Butanol	Unpleasant, fermented, alcoholic, fusel alcohol-like, fruity	1	1	2	2	MS, LRI, ST, OD, LIT
19	1 164.6	Isobutyl butanoate	Fruity, apple, fresh, ethereal	1.5	NDO	NDO	NDO	MS, LRI, ST, OD, LIT
20	NC	Pentyl acetate	Ripe fruit, apple, banana	NDO	NDO	NDO	1.5	MS, OD, LIT
21	NC	Butyl 2-methylbutanoate	Fruity, ethereal, solvent, sweetish	NDO	NDO	NDO	1	MS, OD, LIT
22	1 193.1	Methyl hexanoate	Fruity, sweet ripe apple-like, pineapple	2	0.5	NDO	NDO	MS, LRI, ST, OD, LIT
23	1 200.0	Limonene	Fruity, fresh, sweetish, citrus-like	NDO	NDO	1.5	NDO	MS, LRI, ST, OD, LIT
24	1 217.8	2-Methyl butanol	Fruity, sweetish, slight alcoholic undertone	NDO	NDO	1.5	NDO	MS, LRI, ST, OD, LIT
25	1 225.3	Butyl butanoate	Fruity, apple	2	NDO	NDO	NDO	MS, LRI, ST, OD, LIT
26	1 241.2	Ethyl hexanoate	Powerful fruity, ripe apple, sweet, ripe pineapple	3	2	2.5	NDO	MS, LRI, ST, OD, LIT
27	1 261.8	Pentanol	Balsamic, sweetish, fermented, fusel alcohol-like	1	NDO	0.5	NDO	MS, LRI, ST, OD, LIT
28	1 279.4	Hexyl acetate	Apple, fruity, floral, slightly ethereal, sweetish	2	2	NDO	2	MS, LRI, ST, OD, LIT
29	NC	Ethyl 5-hexenoate	Fruity, ripe apple	1	NDO	NDO	NDO	MS, OD, LIT
30	1 306.5	1-Octen-3-one	Earthy, mushroom-like	1.5	1.5	2	2	LRI, ST, OD, LIT
31	NC	Ethyl (*Z*)-3-hexenoate	Fruity, fresh	1	NDO	NDO	NDO	MS, OD, LIT
32	1 324.6	Pentyl butanoate	Fruity, sweet, banana-like, pineapple-like	NDO	1	NDO	NDO	MS, LRI, ST, OD, LIT

Tab. 1. *continued*

No.	*LRI*	Aroma compound	Aroma description	Odour intensity				Identification
				Peel		Flesh		
				SK	IT	SK	IT	
33	1 326.7	Propyl hexanoate	Fresh, fruity, pleasant	1	1	0.5	0.5	LRI, ST, OD, LIT
34	1 340.8	Ethyl heptanoate	Brandy-like, slight fruity, fresh smell	1	1	NDO	NDO	LRI, ST, OD, LIT
35	1 341.6	(*E*)-2-hexenyl acetate	Green, fresh, plant	NDO	NDO	NDO	1	MS, LRI, ST, OD, LIT
36	1 366.0	Hexanol	Fresh, green, apple undertone, slightly bitter, apple pits-like	2	1.5	2	1.5	MS, LRI, ST, OD, LIT
37	1 396.6	(*Z*)-3-hexenol	Freshly cut grass, green	1.5	NDO	1.5	1	MS, LRI, ST, OD, LIT
38	NC	(*E*)-2-hexenol	Herbal mix odour with slight fruity nuance, plant cortex-like	2	1	1	NDO	MS, OD, LIT
39	1 420.3	Butyl hexanoate	Pleasant, fruity, apple	1	2	1.5	NDO	MS, LRI, ST, OD, LIT
40	1 422.9	Hexyl butanoate	Fruity, sweet, ripe apple-like	2	2	2	NDO	MS, LRI, ST, OD, LIT
41	NC	Hexyl 2-methylbutanoate	Green, waxy, apple, spicy, woody undertone	NDO	2	NDO	3	MS, OD, LIT
42	1 442.3	Ethyl octanoate	Brandy, soap-like, waxy with fruity undertones	2	2.5	1.5	NDO	MS, LRI, ST, OD, LIT
43	NC	Unknown [o]	Sour, fresh	NDO	NDO	1	NDO	–
44	1 483.0	(*E*)-2-hexenyl butanoate	Green, plant cortex-like, with slight fruity and fatty nuances	1	1	NDO	NDO	MS, LRI, ST, OD, LIT
45	1 519.8	Pentyl hexanoate	Sweet, fruity, fatty undertone	1.5	1	0.5	NDO	MS, LRI, ST, OD, LIT
46	1 571.7	Octanol	Green, waxy, leaves-like, grassy	1.5	2	NDO	NDO	MS, LRI, ST, OD, LIT
47	NC	3-Octanol [t]	Herbal, woody, earthy	NDO	NDO	NDO	1	MS, OD
48	NC	6-Methyl-5-hepten-2-ol [t]	Green, sweetish	NDO	NDO	NDO	1.5	MS, OD
49	NC	Unknown [o]	Fruity	NDO	NDO	NDO	1	–
50	NC	Hexyl hexanoate	Green, herbal, fresh, vegetable, fruity	1.5	2.5	1.5	1.5	MS, OD, LIT
51	NC	Isobutyl octanoate	Fruity, slight floral	1.5	NDO	NDO	NDO	MS, OD, LIT
52	NC	(*E*)-2-hexenyl hexanoate	Green, herbal	1	1	NDO	NDO	MS, OD, LIT
53	NC	Estragole	Sweetish, slight liquorice-like	NDO	NDO	NDO	1	MS, OD, LIT
54	NC	(*Z*)-α-Bergamotene	Pleasant, fragrant, slight balsamic odour, citrus-like	1.5	1.5	NDO	1	MS, OD, LIT
55	NC	α-Farnesene	Herbal, fresh, citrus, green, woody	2	NDO	NDO	NDO	MS, OD, LIT
56	NC	α-Farnesene + (*E*)-2-Butenoic acid	Fresh, fruity + milky odour	NDO	1	NDO	NDO	MS, OD, LIT
57	1 693.2	Phenyl ethyl acetate	Fruity, sweet, floral	1	NDO	NDO	NDO	MS, LRI, ST, OD, LIT
58	NC	Hexyl octanoate	Fruity, apple-like, sweetish, waxy undertone	2	1.5	NDO	NDO	MS, OD, LIT
59	1 828.5	Anethol	Fragrant, sweet, spicy, anise-like	1	1	NDO	1	MS, LRI, ST, OD, LIT
60	NC	Bisabolene (unknown isomer)	Plant, fruity	1.5	NDO	NDO	NDO	MS, OD, LIT
61	NC	γ-Bisabolene (unknown isomer)	Herbaceous, balsamic, spicy	1.5	1.5	2	NDO	MS, OD, LIT
62	NC	Bisabolene (unknown isomer)	Pleasant, balsamic	1	1	NDO	1	MS, OD, LIT
63	NC	Bisabolene (unknown isomer)	Fruity, fresh	1	1	2	1	MS, OD, LIT
64	1 917.9	2-Phenyl ethanol	Floral, sweetish, rose, slight honey-like	1.5	NDO	1.5	NDO	MS, LRI, ST, OD, LIT

Tab. 1. *continued*

No.	*LRI*	Aroma compound	Aroma description	Odour intensity				Identification
				Peel		Flesh		
				SK	IT	SK	IT	
65	NC	β-Cubebene	Pleasant, fruity	1	NDO	NDO	NDO	MS, OD, LIT
66	NC	Unknown [o]	Caramel-like, sweet	2	1.5	3	1.5	–
67	NC	Unknown [o]	Fruity, sweet, ripe apple-like	NDO	NDO	1.5	NDO	–
68	NC	1,3-Octanediol	Earthy, musty	1	NDO	NDO	NDO	MS, OD, LIT
69	NC	Isolongifolene [t]	Herbal, balsamic, incense-like odour	1	2	2	NDO	MS, OD
70	NC	Unknown [o]	Fragrant, lactone-like, coconut, sweet, dairy, slightly peach-like	2	3	2	1.5	–
71	NC	Farnesol (unknown isomer)	Pleasant, delicate, fresh, floral, linden-like	1	1	1	1	MS, OD, LIT
72	NC	Unknown [o]	Pleasant, fresh, fragrant, slight floral, herbal	1	1	NDO	1	–

Odour intensity represents the average value of six independent measurements ($n = 6$).
SK – Slovakian apple fruit production, IT – Italian apple fruit production, NC – not calculated, NDO – not detected olfactorily, o – compound detected only by GC-olfactometry, t – tentative identification (only on the basis of mass spectrum).
Compounds identified on the basis of the following criteria: MS – mass spectrum, *LRI* – linear retention index measured on GC column DB-WAX, ST – standard compound, OD – odour quality, LIT – literature reference.

aroma descriptions are listed in Tab. 1. Dominant components of them are esters significant for the peel aroma, usually with intensive typical sweet-fruity notes of apples or other fruits such as pine-apple, pear, peach and possibly citrus fruit. Three of the above-mentioned esters were crucial for the analysed aroma, namely, ethyl 2-methylbutanoate, ethyl hexanoate and ethyl butanoate, with odour intensities of 3, 3 and 2.5, respectively. The majority of the detected aroma-active esters was in good compliance with previous studies [12–15, 32, 35] concerning various apple fruit varieties, such as Golden Delicious, Ruixue, Pink Lady or Fuji. The remaining 36 aroma-active compounds reached moderate odour intensity levels of 1–1.5 (Tab. 1). These were mostly other esters predominantly with fresh, ethereal, green, herbal, vegetable, fruity, floral or sweetish notes, but also with slightly fatty nuances. They were followed by several alcohols such as ethanol, butanol, pentanol, (*Z*)-3-hexenol, octanol, 2-phenyl ethanol, 1,3-octanediol, with a wide range of odour qualities such as ethanolic, fermented, yeasty, balsamic, fusel alcohol-like, fresh cut grass-like, waxy, leaves-like, rose, honey-like, earthy or musty undertones. Some terpenes, such as (*Z*)-α-bergamotene, anethol, bisabolenes, β-cubebene or farnesol, with mainly fragrant, balsamic, herbaceous, spicy, green or floral-fruity notes, and one ketone (1-octen-3-one) with earthy, mushroom-like odour, were also present. It was shown that 26 aroma-active compounds were present only in apple peel and they were not detected olfactorily in the flesh. Except for a portion of esters constituting 16 odorants such as ethyl propanoate, ethyl isobutanoate, isopropyl butanoate, ethyl pentanoate, isobutyl butanoate, methyl hexanoate, butyl butanoate, hexyl acetate, ethyl 5-hexenoate, ethyl (*Z*)-3-hexenoate, ethyl heptanoate, (*E*)-2-hexenyl butanoate, isobutyl octanoate, (*E*)-2-hexenyl hexanoate, hexyl octanoate, phenyl ethyl acetate (with odour intensities from 1 to 2), there were some alcohols (isobutanol, octanol and 1,3-octanediol, with odour intensities 2, 1.5 and 1, respectively). They were followed by some sesquiterpenes, such as (*Z*)-α-bergamotene, α-farnesene, anethol, bisabolene (unknown isomers) No. 60 and No. 62, β-cubebene and an unknown compound No. 72, with odour intensity levels from 1 to 2.

Apple flesh

It was obvious sensorially that the aroma of the flesh of apples of Slovakian production was substantially poorer than their peel aroma (Tab. 1). Overall, it comprised 32 aroma-active compounds (by 20 less than the apple peel). Fourteen of them

were perceived olfactorily with odour intensities from 2 to 3. These compounds were identified as esters (ethyl acetate, ethyl butanoate, ethyl 2-methylbutanoate, ethyl hexanoate and hexyl butanoate); alcohols (butanol and hexanol); sesquiterpenes (unknown isomers of γ-bisabolene and bisabolene No. 63, tentatively identified isolongifolene); two unknown compounds No. 66 and No. 70, one aldehyde (hexanal) and one ketone (1-octen-3-one). Whilst two of these apple flesh odourants reached the highest value of odour intensity of 3 (ethyl 2-methylbutanoate and unknown compound No. 66), other three odourants (ethyl butanoate, hexanal and ethyl hexanoate) reached similarly high individual odour intensities of 2.5. Comparing the flesh aroma with the peel one, aroma of flesh included 6 odorants that were not detected olfactorily in the peel, namely, hexanal (odour intensity 2.5), unknown compound No. 11 (odour intensity 1), unknown compound No. 43 (odour intensity 1), unknown compound No. 67 (odour intensity 1.5), limonene (odour intensity 1.5) and 2-methyl butanol (odour intensity 1.5).

Italian RM-1 apples

Apple peel

Aroma of the apple peel from fruits grown in Italy comprised 43 aroma-active compounds. It is worth noting that, due to the overlap between two odours of individual aroma compounds α-farnesene + (*E*)-2-butenoic acid, only one odour response was recorded in this case (Tab. 1, No. 56). Four aroma-active compounds reached high odour intensities of 2.5–3, namely, unknown compound No. 70 (odour intensity 3) with fragrant, lactone-like, coconut, sweet, dairy, slightly peach-like notes, and esters ethyl-2-methyl butanoate, ethyl octanoate, hexyl hexanoate, all having odour intensity of 2.5 and overall a typical intensive apple odour, green, herbal, fresh fruity notes and brandy, soap-like as well as waxy undertones. Regarding the above-mentioned unknown substance No. 70, it is probably a lactone. However, its identity could not be confirmed because it was only detected by GC-O. In comparison with the apple flesh, 18 apple peel-specific odourants were determined. They belonged to various chemical groups, namely, 14 were esters, followed by two alcohols (*E*)-2-hexenol and octanol, one was a sesquiterpene (γ-bisabolene, unknown isomer) and one was a short-chain unsaturated carboxylic acid ((*E*)-2-butenoic acid).

Apple flesh

The aroma of the flesh of apples grown in Italy was composed of 34 aroma-active compounds. It was evident that this was less rich by 9 odourants than the corresponding peel aroma. One of them, the ester hexyl 2-methylbutanoate, was olfactorily perceived at the highest odour intensity of 3. Other 6 aroma-active compounds, namely, 4 esters (ethyl acetate, butyl acetate, 2-methylbutyl acetate and hexyl acetate), one alcohol (butanol) and one ketone (1-octene-3-one), showed a strong odour intensity of 2. The large group of remaining odourants (totally 25 compounds) were perceived at weak to moderate odour intensities of 1–1.5. Two aroma-active esters (ethyl butanoate and propyl hexanoate) were detected in the apple flesh at surprisingly very weak intensity of only 0.5. Summarizing the obtained results, 9 aroma-impact volatiles (2-methyl butanal, pentyl acetate, butyl 2-methylbutanoate, (*E*)-2-hexenyl acetate, (*Z*)-3-hexenol, 3-octanol, 6-methyl-5-hepten-2-ol, unknown compound No. 49 and estragole) were apple flesh-specific odourants of RM-1 apples of Italian provenance (they were not detected olfactorily in the apple-peel).

Aroma of Slovakian vs Italian RM-1 apples

Apple peel

Concerning the peel aroma of apples from the mentioned production localities in Slovakia and Italy, both were rich and complex. The Slovakian apple peel aroma was richer by 9 odorants than the Italian one. Three volatile substances (for Slovakian apples: ethyl butanoate, ethyl 2-methylbutanoate and ethyl hexanoate) or four ones (for Italian apples: ethyl 2-methylbutanoate, ethyl octanoate, hexyl hexanoate, unknown compound No. 70) were perceived at the high odour intensities of 2.5–3. One of these principal peel odorants, the ester ethyl-2-methyl butanoate, was common to RM-1 apples grown in both localities. Fifteen volatiles with listed odour intensities were peel-specific odourants of apples from Slovakian production. They were ethanol (odour intensity 1.5), isopropyl butanoate (odour intensity 2), isobutyl butanoate (odour intensity 1.5), butyl butanoate (odour intensity 2), pentanol (odour intensity 1), ethyl 5-hexenoate (odour intensity 1), ethyl (*Z*)-3-hexenoate (odour intensity 1), (*Z*)-3-hexenol (odour intensity 1.5), isobutyl octanoate (odour intensity 1.5), α-farnesene (odour intensity 2), phenylethyl acetate (odour intensity 1), bisabolene (unknown isomer) No. 60 (odour intensity 1.5), 2-phenyl ethanol (odour intensity 1.5), β-cubebene (odour intensity 1) and 1,3-octanediol (odour intensity 1). On the other hand, characteristic odourants for Italian apple peel aroma were butyl acetate (odour intensity 2), unknown compound No. 14 (odour intensity 1.5), 2-methylbutyl acetate

(odour intensity 1.5), pentyl butanoate (odour intensity 1), hexyl 2-methylbutanoate (odour intensity 2) and (*E*)-2-butenoic acid (odour intensity 1).

Variability between Slovak and Italian samples was obvious also from results of principal component analysis (PCA). The plot of principal components depicted in Fig. 3A indicated the existence of 2 discriminated groups of eigenvectors belonging to the samples from different localities, although the distribution of individual vectors throughout the graph sectors was high. This higher discrimination resulted from 6 independent measurements of 3 judges. As regards the results of PCA for apple peel, the first three principal components (PC) described more than 74.3 % of the total variability of the dataset. For description of maximum variability, 5 PC would be necessary with eigenvalue greater than 1. As followed from the table of eigenvalues (data not presented), descriptors hexanol, octanol and unknown compound No. 70 had the most significant weight for first PC construction. Ethyl 2-methylbutanoate, butyl butanoate, butyl hexanoate, hexyl hexanoate and isobutyl octanoate had a dominant role in the second PC construction. Descriptors ethyl propanoate, 2-methylbutyl acetate, (*E*)-2-hexenol, pentyl hexanoate, hexyl octanoate and isolongifolene played the dominant role in the third PC construction.

Apple flesh

As for the comparison of Slovakian and Italian apple flesh aroma, they were comparable in terms of the number of odourants (32 and 34, respectively). Fourteen of them were common to both growing localities, namely, ethyl acetate, isopropyl acetate, ethyl butanoate, ethyl 2-methylbutanoate, butanol, 1-octen-3-one, propyl hexanoate, hexanol, (*Z*)-3-hexenol, hexyl hexanoate, bisabolene (unknown isomer) No. 63, unknown compound No. 66, unknown compound No. 70 and farnesol (unknown isomer).

Despite the comparable number of odourants in apple flesh, the plot of principal components indicated successful discrimination of Slovakian and Italian samples (Fig. 3B). Three principal components cumulatively described approximately 78 % of the whole dataset variability. Similarly to apple peel, 5 principal components with eigenvalue greater than 1 would be necessary to reach maximum variability. Eigenvalues indicated that, for the construction of the first PC, ethyl butanoate, ethyl 2-methyl butanoate and unknown compound No. 66 had the most significant weight, whereas in the second PC, ethyl 2-methylbutanoate, hexanol, (*Z*)-3-hexenol and bisabolene (unknown isomer) No. 63 played a dominant role. In the third PC, ethyl butanoate, unknown compound No. 66 and unknown compound No. 70 played a dominant role.

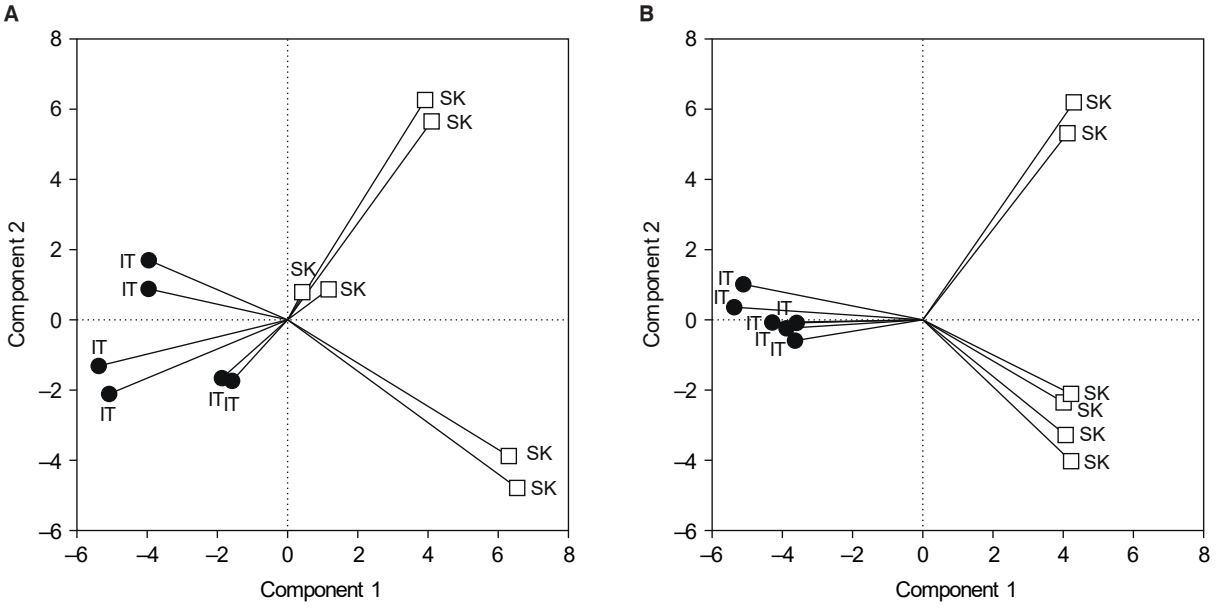

Fig. 3. Plot of principal components demonstrating differentiation of Slovakian vs Italian Red Moon apples based on revealed aroma-active compounds and their odour intensities determined by GC-FID-O.

A – peel, B – flesh.
SK – Slovakian apple fruit production, IT – Italian apple fruit production.

Overall apple aroma

Finally, on the basis of obtained data, odorants such as ethanol, pentanol and 2-phenyl ethanol (with weak to moderate odour intensity levels from 0.5 to 1.5 in the peel and flesh) were specific for the overall aroma of the analysed apple fruit cultivar RM-1 grown in Slovakia. On the other hand, unique odourants of the overall aroma of apples from Italian production were butyl acetate, unknown compound No. 14, hexyl 2-methylbutanoate and 2-methylbutyl acetate (with odour intensities from 1.5 to 3 in the peel and flesh).

Comparing these aroma-related data, it is obvious that the overall aroma of analysed RM-1 apples in two different countries were different to some extent. Differences in them might be attributed to geographical variations such as territory, climate and other environmental factors, e.g. specific soil characteristics, water quality, but also possible different farming conditions. A slightly different timing of the harvest in the monitored year may also have played a role.

CONCLUSION

This is the first study providing objective information on principal aroma-active compounds of the new cultivar of red-fleshed apples Red Moon (RM-1), using the unique specific technique of GC-FID-O. The overall aroma of the analysed fruits from two different countries (Slovakia, Italy) differed. In summary, 72 aroma-active compounds were revealed by GC-FID-O technique in the aroma of peel and flesh of apples grown in both geographic areas. Based on aroma-related data, it can be concluded that aroma of the flesh was substantially poorer than the peel aroma, with no respect to country of the apple production. Ethanol, pentanol and 2-phenyl ethanol were odourants specific for the overall aroma of Slovakian apples, whereas butyl acetate, unknown compound No. 14, hexyl 2-methylbutanoate and 2-methylbutyl acetate were unique odourants of the overall aroma of apples from Italian production. In the future research, the unknown aroma-active compounds (totally 8) or tentatively identified aroma-active compounds (totally 3) should be further investigated. Certainly, further investigation of more samples would be necessary to involve the interseason variability as well as differences between orchards and apple growers.

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