Comparison of aroma-impact compound profiles in the Red Moon RM-1 apple fruit cultivar over two successive harvest seasons

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Summary

Fruit aroma, a mixture of volatile organic compounds with odour, as a strong attractant derived from a complex of chemical compounds found in fruits in different amounts and odour-intensities, is distinctively related to their odour-thresholds. Differences in aroma-impact compound profiles in the peel and flesh of red-fleshed Red Moon RM-1 apple cultivar were investigated over two harvest seasons. For this purpose, the laboratory procedure was designed as follows: a sampling method of headspace solid-phase microextraction (HS-SPME) followed by a technique of gas chromatography-olfactometry with flame ionisation detection (GC-FID-O), and with gas chromatography-mass spectrometry (GC-MS) were employed. Apples were harvested in 2020 and 2021 from the same farming orchard. A total of 90 aroma-impact compounds revealed by GC-FID-O were responsible for the overall aroma of apples from 2020 season, and 109 from 2021 season. Aroma-impact compounds comprised esters, alcohols, aldehydes, sesquiterpenes, one ketone and one lactone. The most noticeable odorant compounds common to apples from both growing years were ethyl 2-methyl butanoate, ethyl hexanoate and ethyl butanoate. Other 18 principal odorants found in both apple harvests (with varying odour intensity) comprised namely acetaldehyde, propyl butanoate, butanol, pentanol, 1-octen-3-one, hexanol, (*Z*)-3-hexenol, 5-hexenol, butyl hexanoate, hexyl butanoate, ethyl octanoate, hexyl hexanoate, γ-bisabolene (unknown isomer), 2-phenyl ethanol, 2-hydroxy-γ-butyrolactone, tentatively identified isolongifolene, tetradecanol and farnesol (unknown isomer).

Keywords

red-fleshed apple; aroma-impact compound; headspace solid-phase microextraction; gas chromatography-olfactometry; gas chromatography-mass spectrometry

Flavour as primarily responsible for consumers' enjoyment of fruits, involves integration of sugars, acids and a set of usually tens volatile organic substances comprising aroma. Generally, the odour-producing compounds of fruit aroma are derived from carbohydrates, lipids, phenolic compounds, and mono- and sesquiterpenes, among others, and include a lot of primary and secondary metabolic pathways, many of which have only recently been established [1–3].

Aroma as a complex mixture of many volatile organic compounds (VOCs) has an impact on consumer preference but also serves as an indicator of fruit sensory quality [4]. Volatiles are compounds that apples develop mostly during ripening and in the post-harvest chain [2, 5]. The main precursors for biosynthesis of VOCs in apples are fatty acids and amino acids [4, 6] which are converted by metabolic pathways in the presence of key enzymes (lipoxygenase, alcohol dehydrogenase and alcohol acyltransferase) mostly to aldehydes, ketones, alcohols, esters and terpenes [7, 8].

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The principal chemical structures of apple volatiles are esters and aldehydes [9, 10]. The typical apple juice aroma is mainly determined by them, accounting for 80–90 % of the total volatiles [10]. Aldehydes such as hexanal, (*E*)-2-hexenal or alcohols like (*E*)-2-hexenol with "green" or "grassy" smell are created by chemical or enzymatic oxidation of linoleic and linolenic acid [11] via lipoxygenase and subsequent lyase-mediated cleavage of the intermediate fatty acid hydroperoxides [12]. Recently, CHITARRINI et al. [10] studied volatile profiles of 47 monovarietal cloudy apple juices by the methods of headspace solid-phase microextraction (HS-SPME) gas chromatography coupled with hight-resolution time-of-flight mass spectrometry. The study also mentions a new red-fleshed variety Red Moon (*Malus* × *domestica* Borkh.) cultivar RM-1, in which the percentage composition of the VOCs in apple juice is described: esters accounted for 50 % of the total identified volatiles, with a stronger presence of aldehydes (28 %) and alcohols (22 %). Moreover, red-fleshed apples are consumer-popular for their attractive colour, and especially for their high concentration of polyphenols, primarily the anthocyanins causing red colouration. The precursor of their formation in apples is the phenylalanine [13]. Except the colour, polyphenols contribute together with sugars and acids to aroma, taste, mouthfeel, and particularly astringency of fruits and derived juices [14].

One of the most useful methods for the isolation and preconcentration of volatile fruit fraction is solid phase microextraction (SPME) [15]. Although this technique has been used in fruit aroma analyses for a longer time [16–18], it is still relevant in connection with the newest studies of fruit aromas [19–21], mainly due to its obvious physicochemical advantages regarding the extraction of volatiles, as well as its environmentally friendly characteristics. As a multi-step integrated sample preparation technique, first proposed by ARTHUR and PAWLISZYN [22], it is widely employed by researchers for analysis of volatile and semi-volatile components.

Several works have been published regarding the use of SPME in combination with gas chromatography-mass spectrometry (GC-MS) for isolation and analysis of VOCs in apples. However, most of them focused on volatile profiles of well-known cultivars of white-fleshed apples [6, 10, 11, 15, 16], where differences between cultivars and characteristic volatiles for different harvesting conditions were observed. Due to the rarity of red-fleshed apples, there are just a few published works analysing their volatiles by SPME-GC-MS in fruits or juices. However, the output is usually information regarding the most abundant VOCs in the volatile profiles of several red-fleshed cultivars [10, 13, 17]. As a very effective analytical and quality control technique, gas chromatography-olfactometry (GC-O), coupled with various methods, has been used in several detailed studies on the aroma of fruit species [23–25]. To our knowledge, except our previous publication [26], the GC-O method has not been applied in any other work, published in literature, concerning the analyses of red-fleshed apples.

In this study, we revealed and characterized the aroma-impact compounds in different parts (peel, flesh) of a rare, red-fleshed Red Moon RM-1 apple with an intention to evaluate their qualitative composition and aroma quality. For this purpose, except of GC-MS, we used technique of GC-FID-O that combines instrumental and sensory approach. GC-FID-O can detect volatile compounds with strong aroma perception, even if they are present at low concentration levels, often below the detection limits of commonly used instrumental detectors such as flame ionisation detector (FID) or mass-selective detector (MSD). The aim of this study was to investigate differences in the chemical-sensory profiles of aroma-impact compounds of a relatively new Red Moon RM-1 apple cultivar grown in Slovakia, with respect to the inter-seasonal variability.

MATERIALS AND METHODS

Apple-fruit samples

Two Red Moon RM-1 apple-fruit batches originating from the same farming orchard in the growing location Dunajská Lužná (an altitude of 126 m), Western Slovakia, were used over two successive production years 2020 and 2021. Both batches contained commercialized fruit Red Moon RM-1 harvested at commercial maturity during the 2020 or 2021 season and purchased in early November 2020 or 2021 from the local store Boni Fructi (Dunajská Lužná). Immediately after the purchase, representative apple fruit samples were selected for their uniformity (colour, diameter, lack of damage), and stored at the average temperature 12 ± 2 °C in a storage room with allowed air circulation until the analysis (for a maximum 6 weeks).

Sample processing

Apple-fruit processing was realized according to the procedure detailed in the work of authors ČERVENČÍK et al. [26]. Apple peel/flesh

samples (separately) were always freshly prepared, immediately prior to the HS-SPME GC-analyses. Briefly, two kilograms of apple-fruit samples from each batch were cleaned with tap water, unpeeled to obtain apple peel (< 2 mm in thickness), and deseeded. Top layer of flesh (0.5~1.0 cm) was removed as a waste. Inner flesh was cut to approximately 0.3 × 0.3 × 0.3 cm pieces, whereas the apple peel was cut to 0.3 × 0.5 cm rectangles. With the 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 representative peel or flesh mass for sampling and subsequent analyses. The prepared samples were stored in sealed vials under nitrogen atmosphere in a refrigerator. Subsequently, the isolation of volatiles by SPME and parallel analyses by GC-MS and GC-FID-O were performed soon as possible. All GC-analyses of peel or flesh volatile compounds (separately) were performed in a manual mode (without using an autosampler), always within one day.

Standard reference compounds

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 SPME fibre placed in the headspace (HS) of the sample. Detailed HS-SPME conditions are given in the publication of Červenčík et al. [26]. All HS-SPME extractions were performed as six multiples (n = 6).

Gas chromatography-olfactometry

For determination of retention indices, and to reveal key aroma-impact compounds in apple peel/flesh (each separately), volatiles extracted by HS-SPME were analysed by a combined technique of GC-FID-O, using the concept of detection frequency of posterior assessment of odour intensity (*OI*) [27]. The methodology of sniffing procedure was performed according to Kopuncová et al. [28] with slight modifications. 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. Thus, the percentage of modified nasal impact frequency (*NIF*mod) for each individual odorant was at least 83 %. This corresponds to the minimum percentage from 6 independent GC-O analyses for each sample, during which the odour of each odorant was detected by 3 sniffers. Modified methodology of sniffing procedure did not include recording the individual durations of odorant perceptions. Analyses were realized at the same conditions as given in our study Červenčík et al. [26].

Gas chromatography-mass spectrometry

For identification purposes, in parallel with GC-FID-O technique, 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, Palo Alto, California, USA) coupled to a mass spectrometric detector 5973 inert (Agilent Technologies). Detailed conditions of GC-MS analyses are given in the work of Červenčík et al. [26].

Identification of aroma-active volatiles

Revealed, sensorially recognized aroma-impact compounds were identified based on comparison of their linear retention indices (*LRI*), mass spectra (MS), GC analysis of standard reference compounds (ST), but also by comparison of data on occurrence and aroma description with available literature, and in our in-house database. *LRI* were calculated using the equation of Van den Dool and Kratz [29], and *n*-alkanes C8–C22 were used as reference standards. Obtained *LRI* and MS data were compared and confirmed as detailed in the study of Červenčík et al. [26].

Statistical analysis

Statistical analysis was performed using Unistat, version 6.0 (Unistat, London, United Kingdom) statistical software. Principal component analysis (PCA) was used to define, interpret, and visualize the differences between the compared RM-1 apples coming from different seasons (2020 vs 2021).

RESULTS AND DISCUSSION

GC-FID-O study

The aroma of apples plays an essential role in their consumer acceptance. In order to provide chemically objective data on aroma-impact com-

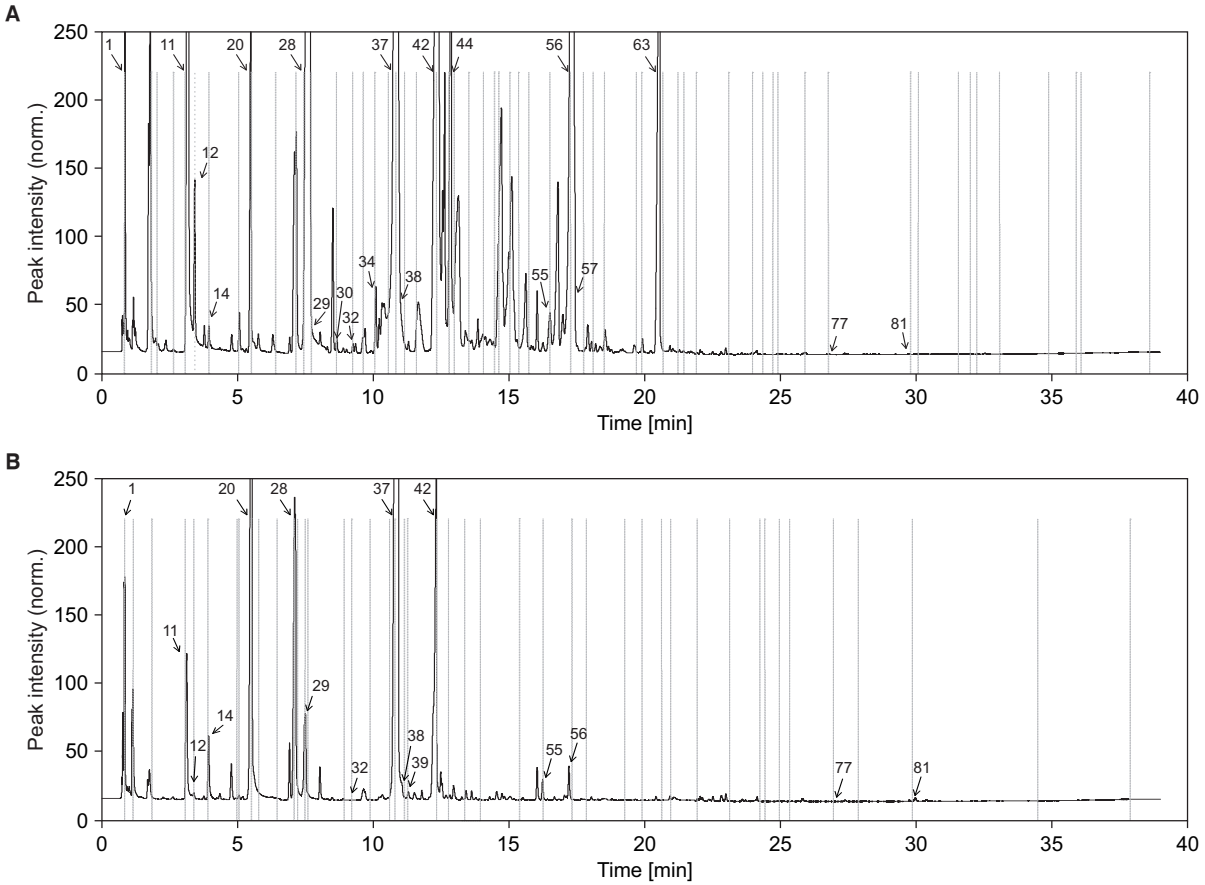


Fig. 1. Illustrative GC-FID-O chromatograms of the apple fruit volatiles overlaid with aromagrams of aroma-impact compounds in Red Moon apple cultivar grown in Slovakia in 2021.

A – peel, B – flesh.

Chromatograms are the records of flame ionisation detector and vertical lines indicate the positions where aroma-active compounds were registered olfactorily. Peak numbers correspond with identification in Tab. 1.

ponents of the aroma complexes of the peel/flesh of apple fruit Red Moon RM-1, analysis of volatile fractions isolated by HS-SPME from the relevant parts of the apple matrix was realized employing GC-FID-O with support of GC-MS. For comparison, samples of apples harvested from the same growing location in Western Slovakia were investigated during production seasons 2020 and 2021. Illustrative GC-FID-O chromatograms overlayed with appropriate aromagrams for apple peel/flesh (season 2021) are depicted in Fig. 1.

The obtained data were processed, and the results are summarized in Tab. 1. Since this study is a continuation of our previous paper by Červenčík et al. [26], Tab. 1 presents data of the same set of apples of Slovak production from 2020 as presented in that paper, but the identity of new odorants (acetaldehyde, isobutanal, 2-methyl-2-butenal, 5-hexenol, β-farnesene, (*E*)-2-hexenyl-hexanoate, 2-hydroxy-γ-butyrolactone, tetradecanol) has been added. In fact, their presence/identification was not sufficiently evident in apples from 2020 [26]. Only the analyses of the richer apple aroma from 2021 contributed significantly to the refinement of the results. Overall, aroma-profiles of apples analysed from both 2020 and 2021 seasons were rich and complex. In total, 83 aroma-zones (odour responses) were revealed by GC-FID-O in the aroma of peel and flesh apples from both years. The aroma-profiles were found to consist of a mixture of volatiles predominantly esters (36), alcohols (18), aldehydes (7) [4, 6], followed by terpenes (14), two ketones, and one lactone, as well as a small group of unknown compounds (5) detected only by GC-O, as their concentrations were below the detection limits of used instrumental detectors FID and MSD, thereby they remained unidentified at this stage. As described in the literature [9], the predominant VOCs in apples and apple juices are esters and

Tab. 1. Aroma-impact compounds in HS-SPME extracts of Red Moon RM-1 apple fruits from the same growing location over two successive production seasons.

No.	*LRI*	Aroma compound	Aroma quality	Odour intensity				*NIF*mod [%]				Identification
				Peel		Flesh		Peel		Flesh		
				2020*	2021	2020*	2021	2020	2021	2020	2021	
1	< 800	Acetaldehyde	Ethereal, solvent, fresh, fruity undertone	1.5	1.5	2	1.5	100	100	100	83	MS, OD, LIT
2	821.0	Isobutanal	Aldehydic, pungent, sharp	1	1.5	1.5	NDO	100	100	100	0	MS, *LRI*, ST, OD, LIT
3	877.4	Butanal	Unpleasant, pungent malty, solvent	NDO	NDO	NDO	1.5	0	0	0	83	MS, *LRI*, ST, OD, LIT
4	893.1	Ethyl acetate	Ethereal, solvent, slight fruity undertone	1.5	NDO	2	NDO	100	0	100	0	MS, *LRI*, ST, OD, LIT
5 + 6	904.8 + NC	Isopropyl acetate + ethanol	Ethereal, alcoholic, solvent	1.5	1	1.5	1.5	100	100	83	100	MS, *LRI*, OD, LIT
7	959.5	Ethyl propanoate	Fruity, sweet, ethereal, slight rum	1.5	1.5	NDO	NDO	100	83	0	0	MS, *LRI*, ST, OD, LIT
8	968.6	Ethyl isobutanoate	Fruity, sweet, apple	1.5	1	NDO	NDO	100	100	0	0	MS, *LRI*, ST, OD, LIT
9	993.0	Methyl butanoate	Fruity, sweet	NDO	1	NDO	NDO	0	83	0	0	MS, *LRI*, ST, OD, LIT
10	1 014.1	Methyl 2-methyl butanoate	Fruity, apple, sweet	NDO	1	NDO	NDO	0	100	0	0	MS, *LRI*, ST, OD, LIT
11	1 040.4	Ethyl butanoate	Intensive ripe apple, banana-pineapple	2.5	3	2.5	2.5	100	100	100	100	MS, *LRI*, ST, OD, LIT
12	1 055.2	Ethyl 2-methyl butanoate	Intensive apple, fresh tropical nuances	3	3	3	2.5	100	100	100	100	MS, *LRI*, ST, OD, LIT
13	1 078.8	Butyl acetate	Fruity, ethereal, solvent, sweet, banana	NDO	1	NDO	NDO	0	83	0	0	MS, *LRI*, ST, OD, LIT
14	1 085.2	Hexanal	Fresh, green, grassy, apple undertone	NDO	1.5	2.5	2.5	0	83	100	100	MS, *LRI*, ST, OD, LIT
15	NC	Isopropyl butanoate	Fruity, apple pits, slightly bitter	2	NDO	NDO	NDO	83	0	0	0	MS, OD, LIT
16	1 096.9	Isobutanol	Ethereal, wine, bitterish, fusel alcohol	2	NDO	NDO	NDO	100	0	0	0	MS, *LRI*, ST, OD, LIT
17	1 100.0	2-Methyl-2-butenal	Fresh cut grass, green	NDO	NDO	1	1.5	0	0	83	100	MS, *LRI*, ST, OD, LIT
18	1 128.9	Propyl butanoate	Fruity, ripe apple, sweet, ethereal	1	2	1.5	1.5	83	100	100	83	MS, *LRI*, ST, OD, LIT
19	1 141.0	Ethyl pentanoate	Apple, sweetish	1	2	NDO	NDO	100	100	0	0	MS, *LRI*, ST, OD, LIT
20	1 151.0	Butanol	Unpleasant, fermented, fusel oil, fruity nuance	1	1	2	2	100	100	100	100	MS, *LRI*, ST, OD, LIT
21	1 164.6	Isobutyl butanoate	Fruity, apple, fresh, ethereal	1.5	NDO	NDO	NDO	83	0	0	0	MS, *LRI*, ST, OD, LIT
22	1 167.2	Ethyl-(*E*)-2-butenoate	Cooked potatoes, ethereal, pungent, alliaceous	NDO	1	NDO	1.5	0	83	0	83	MS, *LRI*, ST, OD, LIT
23	1 193.1	Methyl hexanoate	Fruity, sweet ripe apple, pineapple	2	1	NDO	NDO	100	100	0	0	MS, *LRI*, ST, OD, LIT
24	1 200.0	Limonene	Fresh, citrus, weak sweetish	NDO	NDO	1.5	1	0	0	83	83	MS, *LRI*, ST, OD, LIT
25	1 217.8	2-Methyl-butanol	Ethereal, fatty, leathery, alcoholic undertone	NDO	1.5	1.5	1	0	100	83	83	MS, *LRI*, ST, OD, LIT
26	1 219.4	(*E*)-2-hexenal	Green, vegetable, slight fruity	NDO	NDO	NDO	1	0	0	0	83	MS, *LRI*, ST, OD, LIT
27	1 225.3	Butyl butanoate	Fruity, apple	2	1.5	NDO	1	100	100	0	83	MS, *LRI*, ST, OD, LIT
28	1 241.2	Ethyl hexanoate	Apple, sweet, pineapple, banana, strawberry	3	2.5	2.5	3	100	100	100	100	MS, *LRI*, ST, OD, LIT
29	1 261.8	Pentanol	Balsamic, sweet, fermented smell, fusel oil	1	3	0.5	1	83	100	83	83	MS, *LRI*, ST, OD, LIT
30	1 279.4	Hexyl acetate	Apple, fruity, floral	2	2.5	NDO	NDO	100	100	0	0	MS, *LRI*, ST, OD, LIT

Tab. 1. *continued*

No.	*LRI*	Aroma compound	Aroma quality	Odour intensity				*NIF*mod [%]				Identification
				Peel		Flesh		Peel		Flesh		
				2020*	2021	2020*	2021	2020	2021	2020	2021	
31	NC	Ethyl 5-hexenoate	Fruity, ripe apple	1	NDO	NDO	NDO	83	0	0	0	MS, OD, LIT
32	1306.5	1-Octen-3-one	Earthy, mushroom	1.5	2	2	2	100	100	100	100	*LRI*, ST, OD, LIT
33	NC	Ethyl (*Z*)-3-hexenoate	Fruity, fresh	1	1	NDO	NDO	83	83	0	0	MS, OD, LIT
34	1326.7	Propyl hexanoate	Fruity, pleasant, slight fresh	1	1.5	0.5	NDO	83	83	83	0	MS, *LRI*, ST, OD, LIT
35	1340.8	Ethyl heptanoate	Brandy, slight fruity, fresh smell	1	1.5	NDO	NDO	83	83	0	0	MS, *LRI*, ST, OD, LIT
36	1342.1	6-Methyl-5-hepten-2-one	Pleasant, fragrant, herbaceous, pear, lemon	NDO	NDO	NDO	1	0	0	0	83	MS, *LRI*, ST, OD, LIT
37	1366.0	Hexanol	Fresh, grassy, slight bitter, apple pip	2	2	2	2	100	100	83	100	MS, *LRI*, ST, OD, LIT
38	1396.6	(*Z*)-3-hexenol	Freshly cut grass, green	1.5	2	1.5	1.5	83	100	83	100	MS, *LRI*, ST, OD, LIT
39	NC	3-Octanol	Herbal, earthy, bitter, nutty	NDO	NDO	NDO	2	0	0	0	100	MS, OD, LIT
40	NC	(*E*)-2-hexenol	Herbal mix smell, green, plant cortex	NDO	NDO	1	1	0	0	100	83	MS, OD, LIT
41	NC	5-Hexenol	Green, plant cortex, slight fruity undertones	2	1.5	1	1	100	100	83	100	MS, OD, LIT
42	1420.3	Butyl hexanoate	Pleasant, fruity, apple	1	1	1.5	1.5	83	100	100	83	MS, *LRI*, ST, OD, LIT
43	1422.9	Hexyl butanoate	Fruity, sweet, ripe apple	2	2	2	2	83	100	83	83	MS, *LRI*, ST, OD, LIT
44	1442.3	Ethyl octanoate	Waxy, fruity undertones, soap, brandy, bitter	2	2	1.5	2	100	100	100	83	MS, *LRI*, ST, OD, LIT
45	1469.0	Heptanol	Green, vegetable	NDO	2	NDO	1	0	83	0	83	MS, *LRI*, ST, OD, LIT
46	NC	2-Methylbutyl hexanoate	Ethereal, cooked potato	NDO	2	NDO	1	0	100	0	100	MS, OD, LIT
47	NC	6-Methyl-5-hepten-2-ol	Ripe juicy apple, green, fresh, sweetish	NDO	NDO	NDO	2	0	0	0	83	MS, OD, LIT
48	NC	Ethyl (*Z*)-4-octenoate	Fruit, sweetish	NDO	1	NDO	NDO	0	83	0	0	MS, OD, LIT
49	1483.0	(*E*)-2-hexenyl butanoate	Green, plant cortex, fruity, fatty nuances	1	NDO	1	1.5	83	0	100	100	MS, *LRI*, ST, OD, LIT
50	1503.1	2-Ethyl-hexanol	Pleasant, floral, fragrant, slight rose	NDO	NDO	NDO	1	0	0	0	83	MS, *LRI*, ST, OD, LIT
51	1519.8	Pentyl hexanoate	Sweet, fruity, fatty undertone	1.5	1.5	0.5	NDO	83	100	83	0	MS, *LRI*, ST, OD, LIT
52	1571.7	Octanol	Green, waxy, leaves, grassy, citrus	1.5	1.5	NDO	1	83	100	0	83	MS, *LRI*, ST, OD, LIT
53	NC	Unknown°	Pleasant, fragrant, sweetish, milky	NDO	NDO	NDO	1	0	0	0	83	–
54	NC	Hexyl (*E*)-2-butenoate	Fruity, slight citrus, gingerbread	NDO	1.5	NDO	1	0	83	0	83	MS, OD, LIT
55	1587.4	(*E,Z*)-2,6-Nonadienal	Green, cucumber, slight waxy	NDO	2	NDO	1.5	0	100	0	83	*LRI*, ST, OD, LIT
56	NC	Hexyl hexanoate	Green, herbal, fresh, vegetable, fruity	1.5	1.5	1.5	1	100	100	100	100	MS, OD, LIT
57	NC	Isobutyl octanoate	Fruity, slight floral	1.5	2	NDO	NDO	100	100	0	0	MS, OD, LIT
58	1647.5	Ethyl decanoate	Fragrant, sweetish, milky, slight caramel	NDO	1	NDO	1	0	83	0	100	MS, *LRI*, ST, OD, LIT
59	1652.3	Menthol	Fresh, minty	NDO	1	NDO	1	0	83	0	83	MS, *LRI*, ST, OD, LIT
60 + 61	NC	β-Farnesene + (*E*)-2-hexenyl hexanoate	Fresh, herbal, green	1	1	NDO	NDO	100	83	0	0	MS, OD, LIT

Tab. 1. *continued*

No.	*LRI*	Aroma compound	Aroma quality	Odour intensity				*NIF*mod [%]				Identification
				Peel		Flesh		Peel		Flesh		
				2020*	2021	2020*	2021	2020	2021	2020	2021	
62	NC	α-Bergamotene (unknown isomer)	Pleasant, fragrant, slight balsamic	1.5	1.5	NDO	1	83	83	0	83	MS, OD, LIT
63	NC	α-Farnesene	Herbal, woody, citrus, green paprika	2	1.5	NDO	1	100	100	0	100	MS, OD, LIT
64	NC	Unknown°	Fruity, apple	NDO	NDO	NDO	1.5	0	0	0	83	–
65	NC	Germacrene D	Herbal, woody, planty	NDO	1	NDO	NDO	0	83	0	0	MS, OD, LIT
66	1693.2	Phenyl ethyl acetate	Fruity, sweet, floral	1	1	NDO	NDO	83	100	0	0	MS, *LRI*, ST, OD, LIT
67	NC	Hexyl octanoate	Fruity, apple, sweetish	2	1.5	NDO	NDO	100	100	0	0	MS, OD, LIT
68	1828.5	Anethol	Fragrant, sweet, spicy, anise	1	1	NDO	NDO	83	83	0	0	MS, *LRI*, ST, OD, LIT
69	NC	Bisabolene (unknown isomer)	Planty, fruity	1.5	1.5	NDO	NDO	100	100	0	0	MS, OD, LIT
70	NC	γ-Bisabolene (unknown isomer)	Herbaceous, smoky, balsamic, spicy	1.5	1.5	2	1.5	100	83	83	100	MS, OD, LIT
71	NC	Bisabolene (unknown isomer)	Pleasant, balsamic	1	NDO	NDO	NDO	83	0	0	0	MS, OD, LIT
72	NC	Bisabolene (unknown isomer)	Fruity, fresh	1	NDO	2	NDO	100	0	100	0	MS, OD, LIT
73	NC	Aristolene	Pleasant, flower, sweetish	NDO	1	NDO	NDO	0	83	0	0	MS, OD, LIT
74	1917.9	2-Phenyl ethanol	Floral, sweetish, rose, slight honey	1.5	1.5	1.5	1.5	100	100	100	100	MS, *LRI*, ST, OD, LIT
75	NC	Unknown°	Unpleasant, cellar-like	NDO	2	NDO	NDO	0	100	0	0	–
76	NC	β-Cubebene	Pleasant, fruity	1	1	NDO	NDO	83	100	0	0	MS, OD, LIT
77	2067.0	2-Hydroxy-γ-butyrolactone	Caramel, sweet	2	1.5	3	1.5	100	100	100	100	*LRI*, ST, OD, LIT
78	NC	Unknown°	Fruity, sweet, ripe apple	NDO	NDO	1.5	NDO	0	0	83	0	–
79	NC	1,3-Octanediol	Green, fatty, musty	1	NDO	NDO	1	100	0	0	83	MS, OD, LIT
80	NC	Isolongifolene[t]	Heavy incense-like odour, balsamic, herbal	1	1.5	2	1	100	100	100	83	MS, OD
81	2186.6	Tetradecanol	Fragrant, coconut, sweet, peach undertone	2	3	2	2	100	100	100	100	MS, *LRI*, ST, OD, LIT
82	NC	Farnesol (unknown isomer)	Pleasant, delicate, fresh, floral, linden-like	1	1	1	1	83	83	83	100	MS, OD, LIT
83	NC	Unknown°	Pleasant, fresh, fragrant, slight floral, herbal	1	1	NDO	NDO	100	100	0	0	–

Odour intensity represents the average value of six independent GC-olfactometry analyses ($n = 6$) performed by 3 sniffers for each sample, complying with the requirement of at least 5 citations within each olfactory perception.

*NIF*mod – modified nasal impact frequency, it corresponds to the percentage from six independent GC-olfactometry analyses ($n = 6$) performed by 3 sniffers for each sample, complying with the requirement of at least 5 citations within each olfactory perception.

Compounds were identified on the basis of the following criteria: MS – mass spectrum, *LRI* – linear retention index measured on polar capillary GC column DB-WAX, ST – standard compound, OD – odour quality, LIT – literature reference.

NC – not calculated, NDO – not detected olfactorily, o – aroma substance detected only by GC-olfactometry, t – tentative identification (only on the basis of mass spectrum and aroma character), * – odour intensity data of Slovakian Red Moon RM-1 apple production for 2020 year taken from publication Červenčík et al. [26].

aldehydes, representing summarily 78–92 % of the total volatile composition, followed by alcohols covering 6–16 %. However, only a limited part of them is recognized by the human olfactory system in the apple aroma. Anyway, the aroma-activity of the individual VOCs is determined not only by their concentration, but also their odour threshold concentration, which often varies by several orders of magnitude for different volatiles. This is the main principle of the GC-O technique. Overall, 77 aroma-active compounds were identified in our study by a combination of above-mentioned independent methods. In the case of isolongifolene, only partial information (mass spectrum and odour quality, Tab. 1) was available, and thus only tentative identification was possible. The exact identity of some sesquiterpene stereoisomers such as α-bergamotene, bisabolene, γ-bisabolene and farnesol remains unknown at this stage due to unavailability of relevant reference materials.

RM-1 apples (harvest season 2020)

Apple peel aroma

The relatively abundant aroma of this apple part was created by 55 aroma-impact compounds (Tab. 1). A detailed assessment is provided in the publication Červenčík et al. [26]. Briefly, regarding to the estimated odour intensities, key odour-impact compounds were these 16 substances: ethyl butanoate, ethyl 2-methyl butanoate, isopropyl butanoate, isobutanol, methyl hexanoate, butyl butanoate, ethyl hexanoate, hexyl acetate, hexanol, 5-hexenol, hexyl butanoate, ethyl octanoate, α-farnesene, hexyl octanoate, 2-hydroxy-γ-butyrolactone, and tetradecanol. All mentioned odorants exhibited the highest *OI* levels 2–3, their individual aroma qualities are listed in Tab. 1. Key components of them are esters, important for the apple peel aroma [30]. Esters ethyl 2-methylbutanoate, ethyl hexanoate and ethyl butanoate (*OI*s 3, 3, 2.5) were crucial for the analysed aroma. They are characterised by intensive typical sweet-fruity notes of apples. Most of the revealed aroma-impact esters were in good agreement with the previous studies [4, 6, 8, 10, 17] concerning various apple fruit varieties, such as Pink Lady, Ruixue, Fuji, Golden Delicious, Braeburn, Elstar, Gala, Granny Smith, Jonagold, Pinova, Red Delicious, including Red Moon RM-1, and a rare wild Italian red peel/flesh apple Mela Rossa Val Metauro. In addition to the principal odorants with the highest *OI* scores, odorants with lower *OI*s also have an essential place in the aroma, as complex additive, synergistic and suppressive effects are applied in the overall aroma of the matrix. The final aroma profile of a fruit is the result of a balance between all the emitted volatiles, and any modification in this fine balance would result in changes of the fruit flavour [17].

Apple flesh aroma

As detailed in our previous study Červenčík et al. [26], it was evident that the aroma of the apple flesh from the 2020 season was substantially poorer than the peel aroma (Tab. 1), which is in good agreement with the publication of Li et al. [30]. Overall, it comprised 35 aroma-active compounds. Fifteen of them were perceived olfactorily with high *OI*s 2–3. These key odorants were identified as esters (ethyl acetate, ethyl butanoate, ethyl 2-methylbutanoate, ethyl hexanoate and hexyl butanoate); alcohols (butanol, hexanol and tetradecanol); sesquiterpenes (unknown isomers of γ-bisabolene and bisabolene No. 72, isolongifolene); aldehydes (acetaldehyde and hexanal); one ketone (1-octen-3-one) and one lactone (2-hydroxy-γ-butyrolactone). Concerning the comparison of the apple flesh aroma with the peel one, aroma of flesh involved 6 odorants that were not perceived olfactorily in the peel, such as aldehydes hexanal (*OI* 2.5) and 2-methyl-2-butenal (*OI* 1), terpene limonene (*OI* 1.5), alcohols 2-methyl-butanol (*OI* 1.5) and (*E*)-2-hexenol (*OI* 1), and unknown compound No. 78 (*OI* 1.5). This indicates that C6 aldehyde, but also C6 alcohol together with limonene and 2-methyl-2-butenal, contribute significantly to the fresh, grassy, green aroma of apple flesh. Some aldehydes like hexanal and (*E*)-2-hexenal, which are described as “green” or “grassy” are generated by chemical or enzymatic oxidation of linoleic and linolenic acid [11]. Thus, oxygen plays an important role in the genesis of such aldehydes and alcohols via lipoxygenase and subsequent lyase-mediated cleavage of the intermediate fatty acid hydroperoxides, often being compounds with six carbon atoms [12]

RM-1 apples (harvest season 2021)

Apple peel aroma

The apple peel aroma from fruits harvested in 2021 comprised 61 aroma-impact compounds (Tab. 1). It was 6 more than in 2020. In comparison with the 2020 season, only one overlaid odour response was recorded for two individual aroma-active compounds, i.e. isopropyl acetate + ethanol (Tab. 1., No. 5 + 6). The situation was the same also for aroma compounds β-farnesene + (*E*)-2-hexenyl hexanoate (Tab. 1, No. 60 + 61). Six odour-active compounds reached the highest *OI* levels. There were four esters (with sweet, powerful apple smells), such as ethyl butanoate (*OI* 3); ethyl 2-methyl butanoate (*OI* 3); ethyl hexanoate

(*OI* 2.5); hexyl acetate (*OI* 2.5); and two alcohols (both with *OI* 3), namely pentanol with balsamic, sweet, slight fermented smell, fusel oil-like odour characteristics; tetradecanol with fragrant, coconut, sweet, slight peach undertone. Concerning the comparison with the flesh, 26 apple peel-specific odorants with *OI*s 1–2 were determined (Tab. 1). They belonged to the various chemical groups: 17 of them were esters [30], followed by 6 sesquiterpenes (β-farnesene, germacrene D, anethol, bisabolene unknown isomer No. 69, aristolene, β-cubebene), aldehyde isobutanal, and two unknown compounds No. 75 and 83. Interestingly, menthol was sensory perceived with *OI* 1 in the aroma-profile of apple peel from 2021, but apparently not as a natural constituent of the apple-aroma, but possibly as a residue of peppermint essential oils often used to control post-harvest apple diseases [31].

Apple flesh aroma

The aroma of the flesh produced in 2021 consisted of 48 key aroma-active compounds, 13 less than in the corresponding peel aroma (Tab. 1). This is just another pointing out the fact that the aroma of the fruit is bound primarily in its peel. One of the flesh principial odorants, ester ethyl hexanoate (with powerful sweet aroma of apple, pineapple, with banana and strawberry nuances), was olfactorily perceived at the highest *OI* 3. It was followed by two other esters, ethyl butanoate and ethyl 2-methyl butanoate, and aldehyde hexanal, all of three with a strong *OI* 2.5. Other 8 aroma-active compounds, namely two esters (hexyl butanoate and ethyl octanoate), 5 alcohols (butanol, hexanol, 3-octanol, 6-methyl-5-hepten-2-ol, tetradecanol) and ketone 1-octen-3-one, showed a comparatively high *OI* 2. The large group of remaining odorants (totally 36 compounds) were perceived olfactorily at *OI*s 1–1.5. In summary, 13 aroma-impact volatiles (butanal, 2-methyl-2-butenal, limonene, (*E*)-2-hexenal, 6-methyl-5-hepten-2-one, 3-octanol, (*E*)-2-hexenol, 6-methyl-5-hepten-2-ol, (*E*)-2-hexenyl butanoate, 2-ethyl-hexanol, unknown compounds No. 53 and No. 64, and 1,3-octanediol) – all with *OI*s 1–2, were apple flesh-specific odorants of RM-1 apples harvested in 2021. Finally, as above-mentioned, odorant menthol (*OI* 1) is apparently present as an artefact in the apple flesh aroma profile and probably originates in peppermint essential oils used in the post-harvest treatment of the apples to prolong their shelf-life [31].

Impact of growing season on the aroma of RM-1 apples

Apple peel

As for the comparison of apple peel aromas from fruit grown at the above-mentioned growing location during two seasons, both were complex and rich. Peel aroma from the 2021 was richer by 6 odorants than one from the 2020 (Tab. 1). Three odorants (for apples harvested in 2020: esters ethyl butanoate, ethyl 2-methyl butanoate and ethyl hexanoate) or 6 odorants (for apples harvested in 2021: esters ethyl butanoate, ethyl 2-methyl butanoate, ethyl hexanoate and hexyl acetate; alcohols pentanol and tetradecanol) were perceived olfactorily at the highest *OI*s 2.5–3. Three of these key peel odorants, esters ethyl butanoate, ethyl 2-methyl butanoate and ethyl hexanoate (at mentioned *OI* range), were common to RM-1 apples harvested in both seasons. Nine principal aroma-active volatiles with listed *OI*s 1–2 were peel-specific odorants of apples from 2020. They were predominantly 5 esters (ethyl acetate, isopropyl butanoate, isobutyl butanoate, ethyl 5-hexenoate, (*E*)-2-hexenyl butanoate); followed by 2 alcohols (isobutanol, 1,3-octanediol); and 2 sesquiterpenes (two unknown isomers of bisabolene No. 71 and 72, Tab. 1). Contrarily, 15 odorants characteristic for peel aroma from 2021 were some esters (methyl butanoate, methyl 2-methyl butanoate, butyl acetate, ethyl-(*E*)-2-butenoate; all with *OI* 1), 2-methylbutyl hexanoate (*OI* 2), ethyl-(*Z*)-4-octenoate (*OI* 1), hexyl-(*E*)-2-butenoate (*OI* 1.5), ethyl decanoate (*OI* 1); aldehydes hexanal (*OI* 1.5) and (*E,Z*)-2,6-nonadienal (*OI* 2); alcohols 2-methyl butanol (*OI* 1.5) and heptanol (*OI* 2); sesquiterpenes germacrene D and aristolene (*OI*s 1); and unknown compound No. 75 (*OI* 2).

Apple flesh

Comparing apple flesh aromas of fruit produced on the same growing area in both listed years, these aromas differed quite significantly in the terms of the number of their individual odorants. While in 2020 there were 35, in the following year 48. Twenty-eight of them were common to both seasons: acetaldehyde, isopropyl acetate, ethanol, ethyl butanoate, ethyl 2-methyl butanoate, hexanal, 2-methyl-2-butenal, propyl butanoate, butanol, limonene, 2-methyl-butanol, ethyl hexanoate, pentanol, 1-octen-3-one, hexanol, (*Z*)-3-hexenol, (*E*)-2-hexenol, 5-hexenol, butyl hexanoate, hexyl butanoate, ethyl octanoate, (*E*)-2-hexenyl butanoate, hexyl hexanoate, γ-bisabolene (unknown isomer), 2-phenyl ethanol, isolongifolene, tetradecanol and farnesol (unknown isomer).

Majority of them (15 odorants) were perceived in flesh from fruit of both years at stable *OI*s in the range of 1–2.5 (Tab. 1.). The remaining 13 odorants ranged in overall *OI*s of 0.5–3, but except for isolongifolene, their individual interannual variability was ± 0.5 of estimated *OI* level.

Overall apple aroma

Finally, based on obtained data, aroma-impact volatile compounds such as ethyl acetate and unknown isomer of bisabolene No. 72 (*OI*s from 1 to 2 in the peel and flesh) were specific for the overall aroma of the analysed apples harvested in 2020. On the other hand, the unique aroma-impact volatiles of the overall aroma of apples produced in 2021 were ethyl-(*E*)-2-butenoate, heptanol, 2-methylbutyl hexanoate, hexyl (*E*)-2-butenoate, (*E,Z*)-2,6-nonadienal and ethyl decanoate, likewise with *OI*s 1–2 in the peel and flesh.

Comparing the obtained aroma-related data, it is noticeable that the overall aroma of analysed apples in two different seasons was quite different. Assuming all the agrotechnical conditions remained unchanged during the production periods, the differences in the aroma-related data are mainly attributable to certain environmental factors (number and distribution of days of active sunshine during the vegetation period, temperature characteristics as well as precipitation totals, and their distribution during the growing season). In the season 2020, the average air temperature was 11.9 °C, and the average amount of precipitation reached 723.9 mm·m^{-2} [32], whereas the 2021 season was a little colder (11.2 °C) and with lower precipitation totals (650.9 mm·m^{-2}) [32]. These findings are consistent with study of Wu et al. [32] who investigated 35 apple varieties over two consecutive years and hypothesised that seasonal effects greatly influenced the formation of volatiles. For deeper understanding of the differences between the apple peel and flesh aroma from 2020 and 2021, a PCA was performed. Fig. 2 shows the scores and loadings of the first two principal components (PCs), which describe 62.1 % of the dataset variability. While on PC1 (42.5 %) the samples are separated by the tissue, the driving factor for the sample separation on PC2 (19.6 %) is year. The VOCs with the strongest impact on separation of the samples by tissue were pentyl hexanoate, (*E*)-2-hexenyl hexanoate, isobutyl octanoate, pentanol, ethyl heptanoate and propyl hexanoate. Isobutanol, isopropyl butanoate, 2-methyl butanol, ethyl-5-hexanoate, heptanol, (*E,Z*)-2,6-nonadienal and ethyl decanoate played a dominant role in the separation of the samples by year.

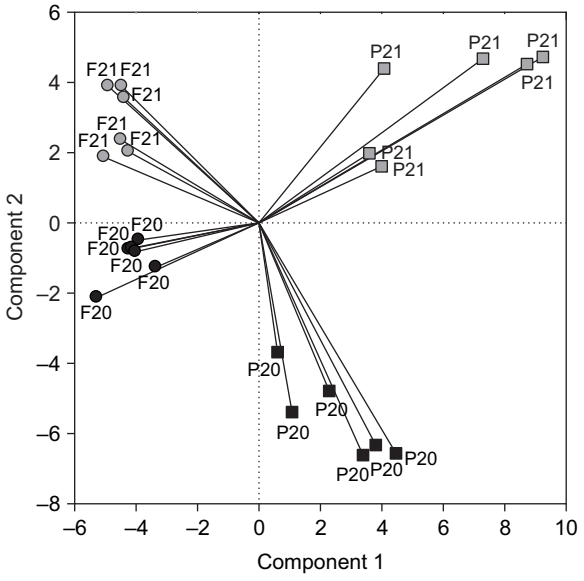


Fig. 2. Plot of principal components demonstrating differentiation of Red Moon RM-1 apples growing in Slovakia according to the harvest years, based on revealed aroma-active compounds and their odour intensities determined by GC-FID-O.

P20 – apple peel from 2020, P21 – apple peel from 2021, F20 – apple flesh from 2020, F21 – apple flesh from 2021.

CONCLUSION

This study provided, for the first time, objective detailed chemical-sensory information on the key aroma-active compounds of peel and flesh of the red-fleshed apple fruit cultivar Red Moon RM-1 grown at one location over two successive harvest seasons. The data were obtained using a unique specific GC-FID-O technique supported by GC-MS. Regarding the results, some differences in the qualitative profiles of the aroma-active compounds are probably due to the variable weather conditions, such as temperature and precipitation, at the time of vegetative growth and ripening of the fruit. An interesting fact related to the 2021 results is, that the odorant menthol appears to be present as an artefact in the aroma of both apple peel and flesh. It is probably derived from the mint essential oils which have come to be used in the recent years in the post-harvest treatment of apples to prolong their shelf-life.

Due to the measured more abundant MS spectra of some RM-1 apple odorants from the 2021 season, it was possible to identify exactly some relevant odorants from the 2020 one. Finally, thanks to the use of a sophisticated analytical method, original, detailed and reliable data

were obtained, which could be useful for quality management of red-fleshed apples production in Slovakia.

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