Exploring Citrus Diversity to Create the Next Generation of Citrus Flavors[a]

The diversity of citrus is not well understood, allowing room for new, compelling discoveries in tonalities.

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Citrus is a key tonality for the flavor and fragrance industry. Citrus notes are widely used in perfumery to provide freshness and lift. They are much appreciated in fine fragrances and home care products, as consumers often associate citrus with cleanliness. According to recent market research on food and beverages (*www.mintel.com*), orange and lemon are in the top five fruit flavors for existing products as well as new product developments. Lime and grapefruit are also important citrus tonalities.

Lately, other citrus varieties are enjoying increased popularity. Indeed, there is a new trend of using less familiar citrus tonalities to provide new sensory experiences to consumers. It is now relatively common to see new products on the market with an original citrus profile: kaffir lime in dry gin (*James Burrough Limited Edition*[b], Spain), finger lime in vinegar (*Causley Fresh*, Australia), dalandan in soda (*Zest-O*[c], Philippines) and yuzu in ice cream (*Mövenpick*[d], Switzerland), among many others.

Thanks to a comparative GC-MS analysis, the molecules responsible for the unique flavor of blood orange were identified, which helped flavorists to mimic the flavor.

A Long History in Citrus Research

Citrus has been an area of active research worldwide for many decades, as reviewed by Rouseff et al.[1] Analysis of citrus oils was markedly accelerated with the development of gas chromatography (GC) in the 1950s. At that time, the major constituents could be separated by using packed GC columns. In the 1960s, the coupling of GC with an olfactometry port (GC-O) helped scientists understand the contribution of these volatile molecules to the overall aroma. In the early 1980s, the general implementation of GC-mass spectrometry (GC-MS) instruments equipped with capillary GC columns allowed the analysis of minor constituents. At that time, analyses were mainly carried out on the most cultivated citrus, such as orange, lemon, mandarin and grapefruit, which are now well documented.[2]

The volatile composition of citrus peel oils is, in general, a mixture of the dominant limonene accompanied by other monoterpenes (γ-terpinene, myrcene, α-pinene, β-pinene, etc.) and sesquiterpenes (germacrene D, *trans*-β-caryophyllene, bicyclogermacrene, etc.), as well as many oxygenated derivatives. Despite this common general profile, each *Citrus* species has a unique organoleptic signature due to a to a well-balanced mixture of major constituents and to the presence of minor components such as neral and geranial in lemon, nootkatone in grapefruit, methyl N-methyl anthranilate in mandarin or linalyl acetate in bergamot, to cite only a few examples.

Citrus has been an important topic for Firmenich's research division for many years. In 1982, Edouard Demole and coworkers detected a strong sulfury note in a grapefruit juice extract during a GC-O experiment. In the corresponding zone of the chromatogram, the instrument did not show any peak, illustrating the higher sensitivity of the human nose for this compound. In order to amplify the potent unknown note, the extraction was repeated, starting with 100 L of juice, and combined with fractionations until the GC-MS instrument was able to detect a peak. On the basis of its MS fragmentation, a chemical structure was proposed that was confirmed by synthesis, leading to the discovery of p-menth-1-en-8-thiol.[3] It was at that time the most powerful flavor compound ever found in nature and a key molecule for grapefruit flavors.[4]

Passionate for citrus tonalities, Regula Naef and coworkers analyzed the volatile composition of various common citrus, from which they identified many new molecules contributing to their overall aroma. One illustration of their work was the comparative analysis of blond and blood orange. Whereas the cultivation of blond orange is widespread, blood oranges require special growing conditions (e.g., cool night temperatures) to develop their fruity and spicy flavor and typical red pulp color. The most important area of production for this fruit is Sicily, with some production also in North Africa and Spain. The limited growing regions mean that the oils are available only in small quantity. Thanks to a comparative GC-MS analysis detailed by Naef et al., the molecules responsible for the unique

[a]This paper represents an expanded version of a talk given as part of the 2013 Flavorcon, which took place November 18–19 in Atlantic City, New Jersey; *www.flavorcon.com*.

[b]*James Burrough Limited Edition* is a trademark of Allied Domecq Spirits & Wine Ltd.

[c]*Zest-O* is a trademark of Schweppes International Ltd.

[d]*Mövenpick* is a trademark of Nestlé.

flavor of blood orange were identified, which helped flavorists to mimic the flavor.[5] Blond orange was characterized by many short aldehydes, while blood orange contained many short esters. For instance, S,S'-ethylidene dithioacetate, identified for the first time in nature, contributes to the juicy and pulpy notes and increases the tropical fruity character of blood orange.[6] Another example of the authors' work was the identification of (2E,4E,7Z)-2,4,7-decatrienal in mandarin and tangerine, which was found to boost mandarin and tangerine type flavors.[7,8] More recently, new branched aldehydes and related alcohols were identified in lemon peel, which contribute positively to the flavor of freshly grated lemon peel.[9]

The Genus *Citrus*: High in Diversity, Yet Not Well Explored

Today, citrus essential oils that dominate the market are mainly obtained from the peel of conventional species such as orange, lemon, lime, mandarin and bergamot. However, citrus diversity has not yet been fully exploited. The genus *Citrus* contains several hundred different species and varieties. This includes the well-known citrus mentioned earlier but also many other less common ones such as kumquat (*Fortunella*), *Microcitrus* and *Poncirus*, which are classified in the genus *Citrus* according to Mabberley's classification (**F-1**).[10] Many are found in specific regions of the world, having different sizes or shapes, peels or pulp colors, as well as unique tastes and aromas.

Room for Discovery!

During the last decade, the number of analytical works on less common citrus varieties has increased.[11] Studies have shown that such varietals are often the source of new molecules (**F-2**), in particular when an enrichment procedure is combined with the use of advanced analytical techniques. For instance, the analysis of yuzu, a Japanese citrus whose pungent aroma differentiates it from other citrus fruits, has led to the discovery of several new odorant molecules. In 2001, 4-mercapto-4-methyl-2-pentanone was identified for the first time by Escher and Mayenzet as an essential constituent for the sulfury note of yuzu.[12] Extraction techniques that could selectively enrich a certain class of molecules (e.g., acids, bases, aldehydes) allowed the identification of a new odor-active acid ((E)-4-methyl-3-hexenoic acid) and three new branched aldehydes of low odor threshold (6-methyloctanal, 8-methylnonanal, and 8-methyldecanal) by Miyazato et al. and Tajima et al., repectively.[13,14]

(2E,4E,7Z)-2,4,7-Decatrienal, found naturally in mandarin (pictured) and tangerine, boosts mandarin- and tangerine-type flavors.

The odor of finger lime's peel is green, floral and woody, with a distinctive fresh herbaceous note.

In 2012, fractionation by silica-column chromatography with subsequent preparative high-performance liquid chromatography (HPLC) on a reverse-phase column was used to enrich an unknown odor-impact compound detected during aroma extract dilution analysis (AEDA). After confirmation by synthesis of the reference compound, it was unambiguously identified as *trans*-4,5-epoxy-(2E,7Z)-2,7-decadienal.[15]

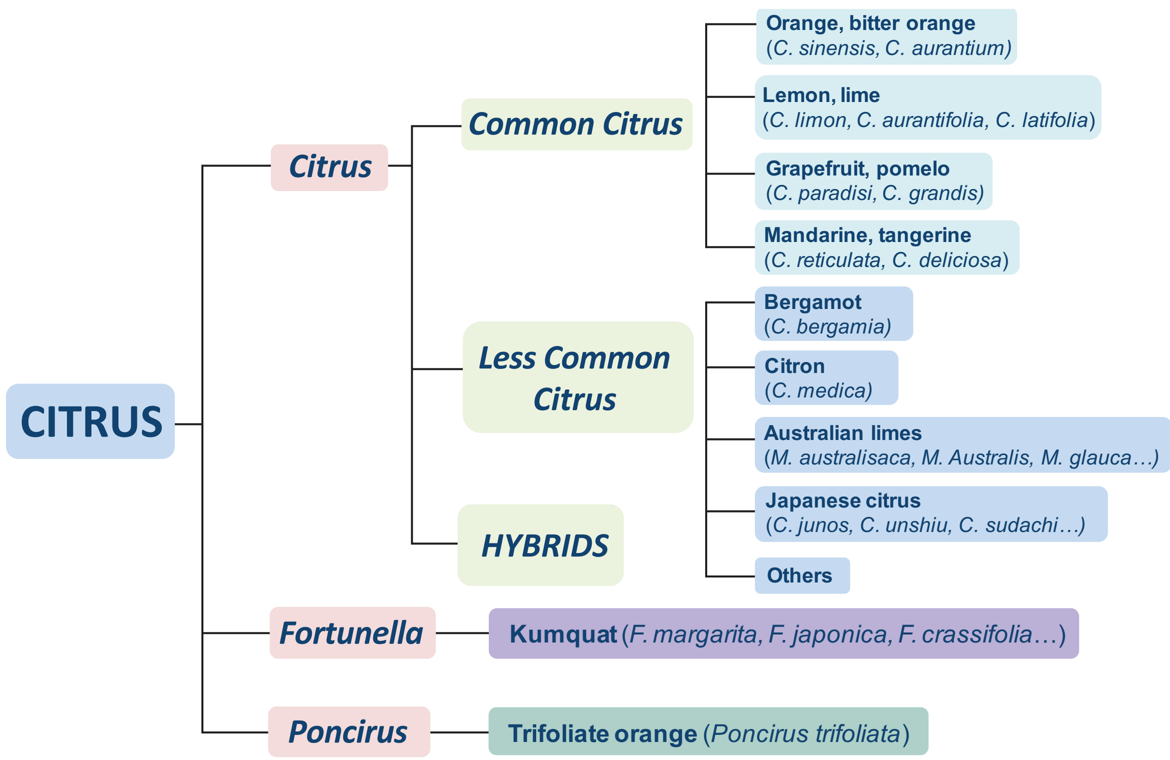
For very potent molecules such as sulfur compounds, the still limited sensitivity of today's instruments can be alleviated by combining a selective extraction technique with a selective detection technique. For example, using affinity chromatography on Affi-Gel 501, an efficient extraction method for thiols, and a GC equipped with an atomic emission detector, Starkenmann et al. could identify new sulfur compounds (3-mercapto-3-methyl-1-butanol, 3-mercapto-1-hexanol and their esters) in the peel of *Poncitrus trifoliata*, known for its unique and strong tropical fruity and sulfury notes.[16,17]

The use of multidimensional (MD) techniques is another useful way of finding new trace constituents that were not detectable with previous techniques. Heart-cut MDGC has been recognized for many years as a powerful tool for increasing chromatographic resolution.[18] In such an instrument, two columns are arranged in a series (e.g., nonpolar stationary phase in the first dimension, followed by a polar stationary phase in the second dimension). The principle is to "heart-cut" the peak of interest in the first dimension, before sending it in the second—ideally orthogonal—dimension to obtain further separation.

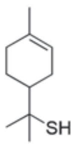
Thanks to repetitive accumulations in a cold trap through two-dimensional GC, Fischer et al. obtained the mass spectrum of an unknown potent sulfur compound in Pontianak (*Citrus nobilis* var. Lour. *microcarpa* Hassk.) and identified it as 1-phenylethanethiol.[19] Its contribution to the sulfurous aroma of this Indonesian citrus was confirmed by aroma recombination experiments. Recently, by combining AEDA with multidimensional techniques (MD-GC-MS-O), Miyazawa et al. identified two new odor-impact compounds in yuzu—(6Z,8E)-undeca-6,8,10-trien-3-one (Yuzunone)[e] and (6Z,8E)-undeca-6,8,10-trien-4-ol (Yuzuol).[f,20] Yuzunone was found to contribute greatly to the unique yuzu aroma and to be useful in perfume and flavor compositions.[21]

[e]Yuzunone is a trade name of T Hasegawa
[f]Yuzuol is a trade name of T Hasegawa

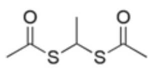
F-1. Illustration of *Citrus* diversity



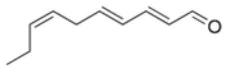
F-2. Examples of odor-impact molecules found in less common citrus



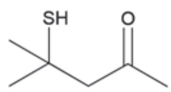
p-menth-1-ene-8-thiol
(grapefruit)



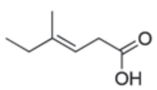
S,S'-ethylidene dithioacetate
(blood orange)



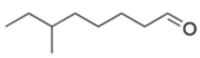
(2E,4E,7Z)-2,4,7-decatrienal
(mandarin and tangerine)



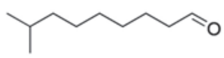
4-mercapto-4-methyl-2-pentanone
(yuzu)



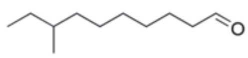
(E)-4-methyl-3-hexenoic acid
(yuzu)



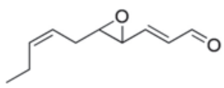
6-methyloctanal
(yuzu)



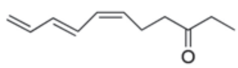
8-methylnonanal
(yuzu)



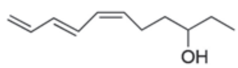
8-methyldecanal
(yuzu)



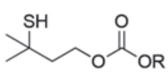
trans-4,5-epoxy-(2E,7Z)-2,7-decadienal
(yuzu)



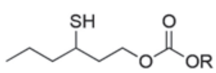
(6Z,8E)-undeca-6,8,10-trien-3-one
(yuzu)



(6Z,8E)-undeca-6,8,10-trien-3-ol
(yuzu)



3-mercapto-3-methyl-1-butanyl alkanoates
R = n-C_3H_7 to n-$C_{15}H_{31}$ (odd C number)
(*Poncirus trifoliata*)



3-mercapto-1-hexyl alkanoates
R = n-C_3H_7 to n-$C_{15}H_{31}$ (odd C number)
(*Poncirus trifoliata*)

Citrus Hybrids Are Opening New Avenues for Flavors

All citrus types hybridize quite easily by cross-pollination. Today, numerous breeding programs are dedicated to the fresh fruit and juice markets. The major objectives are typically to obtain seedless fruits, more resistant fruits, higher crop productivity, higher juice content, longer harvesting periods and so forth. However, the essential oil of citrus hybrids may also benefit perfumery and flavor compositions, for example in the form of higher oil yields, unique organoleptic profiles, improved stability (e.g., low terpene content) or compliance with regulatory requirements (e.g., absence of furocoumarins or low allergen content for fragrance applications). The analysis of the volatile composition of citrus hybrids (leaf, peel, juice) is recent. Several studies have shown that their volatile composition does not result from the exact addition of both parents as some volatile molecules can be over- or under-expressed in the hybrid. [22]

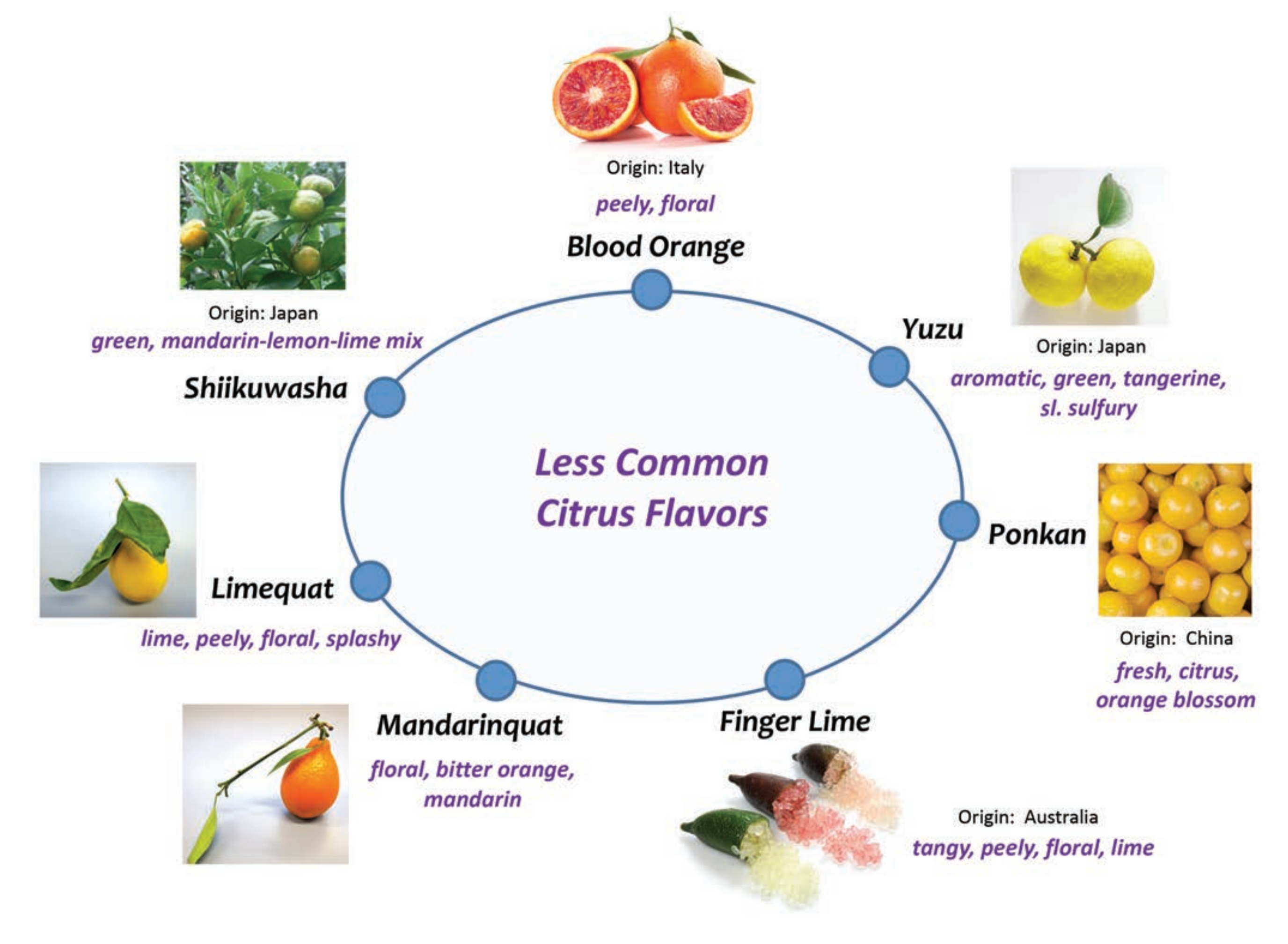
A Need for More Sophisticated Analytical Techniques

Recently, a GC-MS analysis was performed to understand the volatile composition of the peel of two natural citrus hybrids (**F-3**): mandarinquat (a cross between a mandarin and a kumquat) and limequat (a cross between a lime and a kumquat). The aroma of mandarinquat peel was found to be close to kumquat peel with a subtle methyl N-methyl

Despite this common general profile, each *Citrus* species has a unique organoleptic signature due to a to a well-balanced mixture of major constituents and the presence of minor components such as neral and geranial in lemon, nootkatone in grapefruit, methyl N-methyl anthranilate in mandarin or linalyl acetate in bergamot, to cite only a few examples.

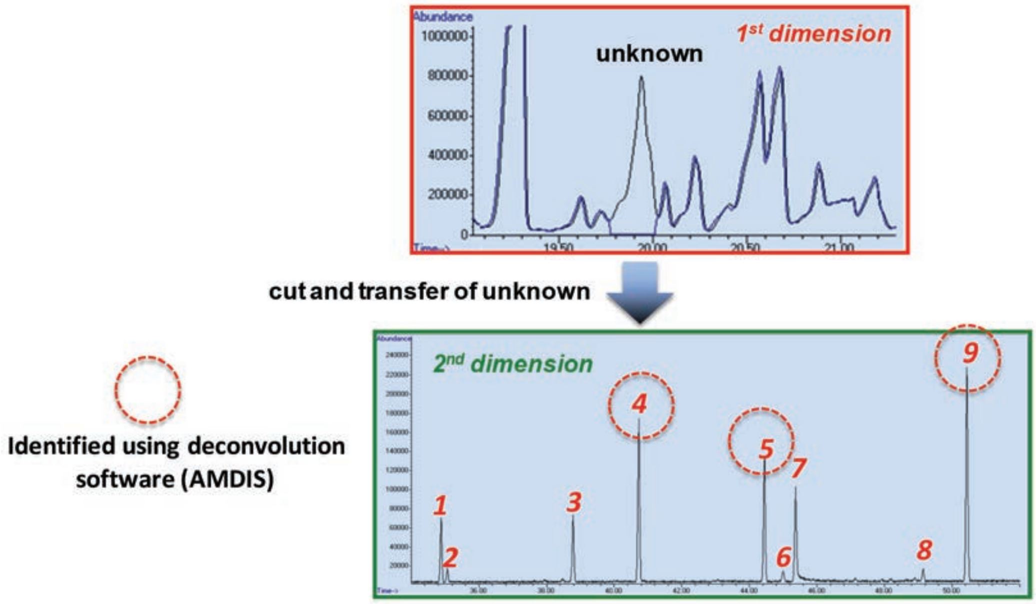
anthranilate note reminiscent of mandarin. The aroma of limequat was close to lime peel but more floral, splashy and slightly spicy. Analysis revealed a high complexity because of the possible addition of the traits of both parents, requiring the use of more advanced analytical tools to solve co-elutions. Thus, further experiments were performed with deconvolution software and heart-cut MDGC.[23] **F-4** gives an example of an unknown GC peak found in mandarinquat, which was cut from the first nonpolar column and sent in a second polar column. MDGC demonstrated that the unknown peak was in fact a mixture of up to nine compounds. All of them were well

F-3. Examples of commercial Firmenich flavors created from the recent analysis of less common citrus varietals



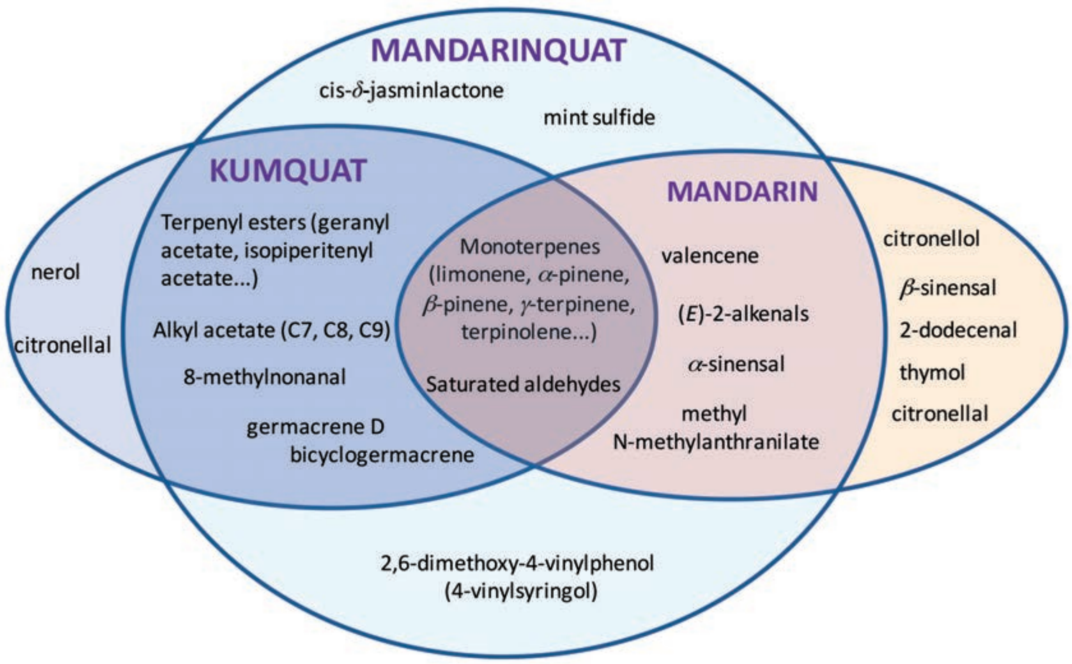
separated in the second dimension and identified by MS, while deconvolution software could identify only three of them. **F-5** illustrates how the volatile composition of the mandarinquat peel extract compares with its parents. Molecules typical of both citrus were identified, along with many molecules typical of mandarin or kumquat only. However, some molecules were less expressed (e.g., methyl N-methylanthranilate) or even absent (e.g., citronellol, citronellal, β-sinensal). To the best of the authors' knowledge, a few compounds that have never been reported in any of the parents were identified, such as mint sulfide, 4-vinylsyringol and *cis*-δ-jasmin lactone ((Z)-7-decen-5-olide). This is of particular interest from a

F-4. Example of use of MDGC for unknown identification in mandarinquat peel extract (modified from[23])



1: (Z)-7-tetradecene; **2:** (*E*)-7-tetradecene; **3:** β-copaene; **4:** β-elemene; **5:** decylacetate; **6:** 7-methyl-3-methylidene-4-(2-propanyl)octahydro-1H-cyclopenta[1,3]cyclopropa[1,2]benzene; **7:** dodecanal; **8:** *1-p*-menthen-9-yl acetate (from peak before unknown 6); **9:** 1,8(10)-p-menthadien-9-yl acetate.

F-5. Simplified figure on mandarinquat composition



Advances in analytical techniques and technology have increased researchers' investigations into the key compounds of citrus aroma.

plant biology perspective. This could be due to the metabolic complementation that activates biosynthetic pathways that may be present but inactive in the parent genome. Moreover, when two genomes fuse to form a hybrid, interactions may occur in which one genome activates or silences genes in the other genome that would otherwise be silent or active in the parent genomes.[24] The identification of new molecules in hybrids may result from these interactions, which may open the door to further discovery.

Creation of New Citrus Flavors

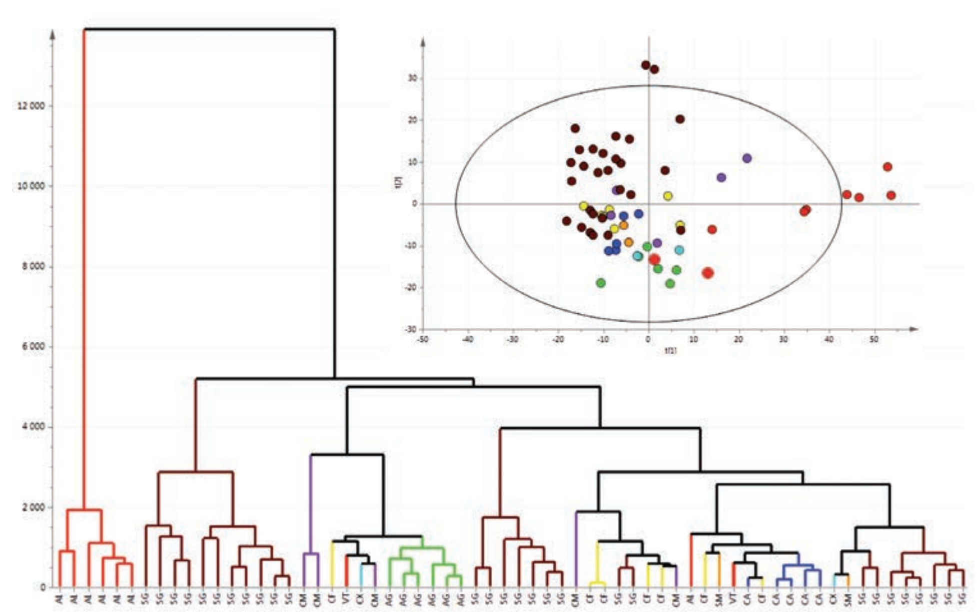
Rare citrus varieties can provide new molecules but also new profiles—i.e., unusual balance of known compounds—leading to a unique organoleptic character. One illustration is the Australian finger lime, which is one of the seven native citrus species endemic to Australia. Finger lime differs from common lime species such as Key lime (*C. aurantifolia*) and Tahiti lime (*C. latifolia*) in terms of its shape, pulp color, aroma and its surprising caviar-like pulp (**F-3**). The odor of its peel is more green, floral and woody, with a distinctive fresh herbaceous note. GC-MS analysis highlighted a unique molecular composition, with isomenthone and citronellal as major constituents after limonene.[25] Isomenthone is a key constituent in mint, but rare in citrus and never in such high amounts. Such a combination of major constituents has never been reported in any other citrus species and likely contributes, at least in part, to the fresh herbaceous and floral notes of the peel. In total, 170 volatile molecules, including six new terpenyl esters, were identified and quantified. The data were provided to a flavorist, who combined his expertise and creativity to convert them into a flavor that tastes like the species studied, and whose profile can be described as juicy, terpenic, green/fresh and floral, with light woody and piney notes.

From a flavorist's point of view, creating the flavor of new citrus varietals can be challenging. The flavor and aroma of fruits other than citrus are often mainly confined to oxygenated components (with notable exceptions such as mango). As mentioned above, the flavor of citrus fruits is considerably influenced by their terpene and sesquiterpene content, as well as by many potent minor or trace constituents that have a strong impact on the overall aroma.[26]

F-3 captures examples of creative interpretations from the analysis of unique citrus varietals, which led to commercial flavors. For instance, for mandarinquat, the flavorist's challenge was to recreate a kumquat character and to combine it with some subtle mandarin notes and slightly floral lactonic sweetness. For limequat, some spicy notes were associated by the flavorist with some floral notes and rounded with a residual sweet finish of coumarins and lactone-like notes.

To create a natural flavor that mimics a new citrus varietal, flavorists must often use as a starting point some extracts (e.g., peel oil, essence oil or water phase) from common citrus that are available on a large scale (mainly byproducts from the juice industry). If needed, these can even be further concentrated or fractionated to reach the individual molecules required for creation. When this approach is coupled with the use of natural chemicals and extracts from non-citrus, the target can be reached quite accurately.

For nature-identical or artificial flavors, a much wider range of raw materials is available, which makes the formulation slightly less challenging. However, when it comes to those same complex terpene and sesquiterpene structures, perfumers and flavorists must still rely on Mother Nature to provide the necessary molecules.



The access to data mining tools (pictured) will help scientists to compare large data sets of citrus samples and to understand their characteristics more rapidly.

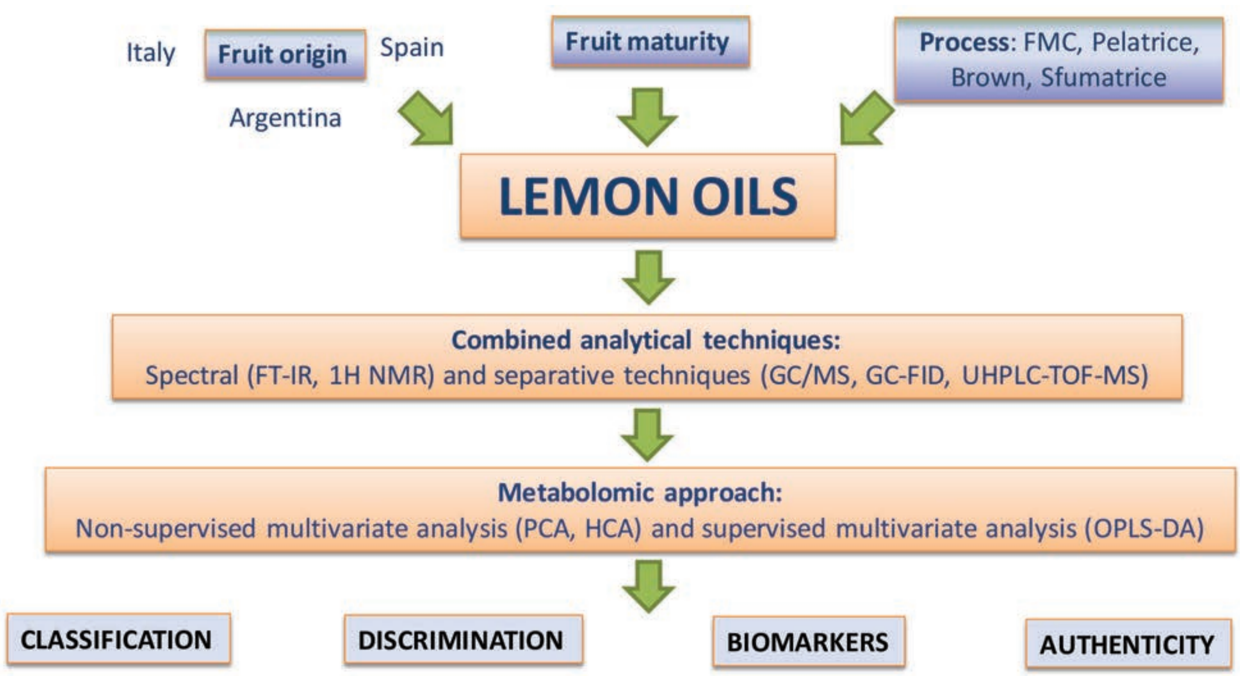
Recently, some complex molecules that are important to citrus, such as valencene and nootkatone, have begun originating from a biotechnology source. The method is based on yeast fermentation and the resulting molecules are natural according to U.S. and E.U. definitions.[27] The availability of such molecules on the flavorist's palette at a low price in the near future is challenging, but this would allow for many new creations that were not previously affordable, while introducing a more sustainable way of obtaining key molecules.

"Citrolomics": A New Tool for Analysis of Large Data Sets of Citrus Oils

The understanding of the performance of raw materials is key to support the creation of unique flavors, too. Recently, chemometric tools were evaluated in collaboration with professors Jean-Luc Wolfender and Serge Rudaz from the University of Geneva (*www.unige.ch*) for the analysis of large data sets of commercial lemon oils. Lemon oils of different fruit maturity obtained from various countries by using different extraction techniques were analyzed with various non-separative (FT-IR, ^{1}H-NMR) and separative analytical methods (GC-MS, GC-FID and UHPLC-TOF-MS). The data obtained were investigated with various chemometric tools. Data sets were first explored with non-supervised multivariate analysis (principal component analysis) and hierarchical cluster analysis to gain an overview and to detect possible outliers. Orthogonal partial least squares discriminant analysis was then carried out to differentiate classes of samples and to highlight biomarkers. The aim of this study was to evaluate whether a metabolomic approach could help discriminate and/or classify lemon essential oils according to their geographical origin, extraction process or fruit maturity (**F-6**). Results showed that the volatile and nonvolatile parts of the oils contained significant

New branched aldehydes and related alcohols have been identified in lemon peel, contributing positively to the flavor of freshly grated lemon peel.

F-6. "Citrolomics" approach



information for classifying the samples according to their origin.[28] Similar results were obtained with the extraction process. The variety of spectral and separation techniques allowed the identification of biomarkers from a wide range of volatile and non-volatile compounds of various chemical families. Although the study focused on lemon oils, the authors are confident that this approach, which can be called "citrolomics," could help in the understanding of the molecular diversity found in the genus *Citrus*, as well as to assess the quality of citrus oils, control their authenticity and better master the cost versus quality ratio, therefore optimizing our sourcing.

Future Opportunities

The creation of citrus flavors requires a good understanding of Mother Nature. Robust analytical data and sensory information are prerequisites for flavorists to create authentic flavors. Taking advantage of technological advancements opens new opportunities. As illustrated above, the combination of selective extraction methods with analytical techniques of improved sensitivity aids in the discovery of new aroma-impact molecules, even in well-studied species. New potent aldehydes have been found recently in common citrus such as orange (e.g., (Z)-8-tetradecenal) and lemon (e.g., 4-methyl nonanal).[9,29] The access to data mining tools will help scientists to compare large data sets of samples and to understand their characteristics more rapidly. The recent successful production of complex high-value molecules via biotechnology offers new perspectives as well.

Most of the exotic citrus and hybrids have not been analyzed in depth yet, and studies have shown that these fruits are often promising sources of unusual molecules or unusual combinations of known molecules. For these reasons, the exploration of citrus diversity is crucial for innovation. The discovery of new molecules can lead to future key ingredients, which will bring more authenticity or a modern twist to classical flavors. Unusual ratios of compounds will inspire new citrus accords, which will bring differentiation to the citrus palette.

Studying the exceptional diversity found in the genus *Citrus* will help provide new taste profiles that better mimic local varieties, offering new taste experiences to consumers. At the same time, it may address many other citrus challenges, such as stability or resistance to diseases. Today, citrus essential oils, which are predominant in the market, are mainly obtained from orange peel (mainly *Citrus sinensis* var. Valencia and Pera) and lemon peel (mainly *Citrus lemon* var. Eureka and Santa Teresa). Lack of genetic diversity is a significant risk in citrus oil production, and hence availability. These low-genetic-diversity species may be more fragile and vulnerable to plant diseases, such as greening, which is currently devastating most of the major cultivated citruses. Some less common citrus varieties, or some citrus hybrids, may be more resistant to the pathogen. Others may contain more stable molecules that would perform better in final applications. For these reasons, there is still a lot to learn from Mother Nature in unraveling her secrets, as well as many opportunities to transform them into winning flavors.

Acknowledgements

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In 2001, 4-mercapto-4-methyl-2-pentanone was identified for the first time by Escher and Mayenzet as an essential constituent for the sulfury note of yuzu.

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