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Development of the Automated Solvent-Assisted Flavor Evaporation and its Application to the Identification of the Key Odorants in Walnut Kernels

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The experimental part of the present work was performed between May 2017 and August 2021 at the Leibniz Institute for Food Systems Biology at the Technical University of Munich (before September 15, 2017: German Research Centre for Food Chemistry), Freising, Germany, under the supervision of Priv.-Doz. Dr. rer. nat. habil. Martin Steinhaus.

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1 Summary

Since its introduction in 1999, Solvent-Assisted Flavor Evaporation (SAFE) has become the state-of-the-art method for the artifact-avoiding removal of non-volatiles from solvent extracts obtained from food samples prior to gas chromatography–olfactometry (GC–O). SAFE has almost completely replaced previous high-vacuum transfer approaches for the isolation of volatiles. Despite the outstanding benefits of the SAFE method, a few drawbacks have been identified during its continuous use in the laboratory. These drawbacks were (1) the volumes of the individual extract portions and the length of the time intervals between the portions influenced the yields of the volatiles, (2) unintentionally large portions caused non-volatiles to transfer into the volatile isolate, thus making it unusable for further analysis, and (3) the high level of manpower required. All three drawbacks were associated with the manual operation of the valve. The first part of the present work was thus focused on the development of an automated SAFE (aSAFE) approach, which included the replacement of the manual valve with an electronically controlled pneumatic valve. This finally allowed shorter valve open times and longer closed times. To evaluate the new aSAFE approach, it was applied to model mixtures containing 18 exemplary food odorants and three different levels of fat (non-fat, low-fat, high-fat) as well as to authentic solvent extracts obtained from beer and chocolate. The results indicated higher yields in the aSAFE approach than in the older, manual SAFE (mSAFE) approach. This was particularly the case for high-boiling odorants and for high-fat samples. In addition, the automation substantially reduced the risk of a contamination of the volatile isolate with non-volatiles. To further automate the aSAFE process, a liquid nitrogen refill system and an endpoint recognition and shut-off system were added, which substantially reduced the need for manual intervention during the process.

In the second part of the present work, the newly developed aSAFE method was applied in a study aimed at the identification of the key odorants in fresh walnut kernels. Walnut kernels are appreciated by consumers for their nutritional value, but also for their characteristic aroma, which is clearly distinguishable from the aroma of other tree nuts. Despite 50 years of research, the key odorants responsible for the typical walnut aroma were still unclear. To fill this gap, the volatiles isolated by aSAFE were subjected to odorant screening by aroma extract dilution analysis (AEDA). This resulted in 50 odor-active compounds, among which 37 had not previously been reported as walnut volatiles. Odor descriptions varied widely and included particularly fatty, floral, green, sweaty, and cheesy. Notably, none of the odorants showed a specific walnut-like character, supporting the hypothesis that the aroma of walnuts is formed by a combination of compounds and not a single odorant. The two odorants with the highest flavor dilution (FD) factors were 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one (sotolon) with the smell of fenugreek and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal with the smell of oatmeal. Quantitation by stable isotope dilution assays (SIDAs) and calculation of odor activity values (OAVs) revealed 17 odorants whose concentrations in the walnuts exceeded their compound-specific odor threshold concentrations (OTCs). Aroma reconstitution and omission experiments finally showed that the characteristic aroma of fresh walnuts was best represented by the binary mixture of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal, whose natural concentrations both amounted to ~10 µg/kg. Sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal were thus identified as the key odorant in walnuts. Further sensory studies showed that the walnut character intensifies at higher concentration levels until ~100 µg/kg; the 1:1 ratio, however, needs to be maintained.

These results can guide the breeding of new walnut cultivars with improved aroma. In other tree nuts such as cashew nuts, hazelnuts, and almonds, the concentrations of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal, particularly their ratios, clearly differ from those in walnuts, which explains the lack of a walnut note in these nuts. Aging of fresh walnut kernels did not substantially change the concentrations of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal, however, the concentrations of some fatty acid-derived oxidation products increased. For example, mushroom-like smelling oct-1-en-3-one increased 5-fold during one week of storage at room temperature.

2 Zusammenfassung

Die Solvent-Assisted Flavor Evaporation (SAFE) wurde 1999 als schonende Methode zur artefaktfreien Entfernung nicht flüchtiger Verbindungen aus Lösungsmittelextrakten von Lebensmitten entwickelt. Sie ist heute die Standardmethode zur Isolierung der flüchtigen Fraktion vor dem Screening nach geruchsaktiven Verbindungen mittels Gaschromatographie–Olfaktometrie (GC–O) und hat dabei ältere Hochvakuumtransfer-Verfahren weitgehend ersetzt. Trotz der herausragenden Vorteile der SAFE zeigten sich im Laufe der jahrelangen Verwendung im Labor auch einige Defizite, darunter (1) die Ausbeuten der flüchtigen Verbindungen sind vom Volumen der einzelnen Extraktportionen und auch von den Zeitintervallen zwischen den Portionen abhängig, (2) unbeabsichtigt große Extraktportionen können zu einem Übergang nicht flüchtiger Verbindungen in das Isolat führen, wodurch es für weitere Analysen unbrauchbar wird, und (3) der durch die kontinuierliche Bedienung des manuellen Ventils erforderliche, hohe Personalaufwand. Da alle Nachteile mit der manuellen Bedienung des Ventils in Verbindung standen, konzentrierte sich der erste Teil der vorliegenden Arbeit auf die Entwicklung einer automatisierten SAFE (aSAFE), bei der das manuelle Ventil durch ein elektronisch gesteuertes, pneumatisches Ventil ersetzt wurde. Dadurch konnten die Öffnungszeiten des Ventils verkürzt und die Schließzeiten verlängert werden. Die aSAFE wurde anschließend mit Modellmischungen aus 18 häufig in Lebensmitteln vorkommenden Geruchsstoffen und drei verschiedenen Fettgehalten (null/niedrig/hoch) sowie mit authentischen Lösungsmittelextrakten aus Bier und Schokolade getestet. Die Ergebnisse zeigten, dass die aSAFE im Vergleich zur manuellen SAFE (mSAFE) eine höhere Ausbeute der flüchtigen Verbindungen erzielte. Dies kam insbesondere bei hochsiedenden Geruchsstoffen und fettreichen Proben zum Tragen. Durch die Automatisierung wurde zudem das Risiko der Kontamination des Isolats mit nicht flüchtigen Verbindungen deutlich reduziert. Zur weiteren Automatisierung der aSAFE wurden ein System zum automatischen Nachfüllen des Kühlmittels Flüssigstickstoff und ein System zur Endpunkterkennung mit Abschaltautomatik integriert, wodurch die Notwendigkeit für manuelle Eingriffe während des Prozesses noch einmal erheblich reduziert wurde.

Der zweite Teil der vorliegenden Arbeit befasste sich mit der Aufklärung der Schlüsselgeruchsstoffe frischer Walnusskerne. Hierfür wurde die neu entwickelte aSAFE zur Isolierung der flüchtigen Verbindungen aus den Nüssen eingesetzt. Walnusskerne werden vom Verbraucher nicht nur wegen ihres Nährwerts geschätzt, sondern nicht zuletzt auch wegen ihres charakteristischen Aromas, das sich deutlich vom Aroma anderer Nüsse unterscheidet. Die Erforschung der dafür verantwortlichen Substanzen hatte bereits vor 50 Jahren begonnen, jedoch waren die entscheidenden Schlüsselgeruchsstoffe noch immer unbekannt. Um diese Lücke zu schließen, wurden die mittels aSAFE aus Walnüssen isolierten, flüchtigen Verbindungen einem Screening mittels Aromaextraktverdünnungsanalyse (AEVA) unterzogen. Dies resultierte in 50 geruchsaktiven Verbindungen, von denen 37 bisher noch nicht als flüchtige Verbindungen in Walnüssen bekannt waren. Die Geruchsbeschreibungen waren vielfältig und umfassten hauptsächlich fettige, blumige, grüne, schweißige und käsige Noten. Interessanterweise zeigte kein einziger der 50 Geruchsstoffe einen spezifischen Walnusscharakter. Dies unterstützte die Hypothese, dass das charakteristische Walnussaroma durch die Kombination mehrerer Geruchsstoffe und nicht durch einen einzelnen Geruchsstoff hervorgerufen wird. Die beiden Geruchsstoffe mit den höchsten FD-Faktoren waren

3-Hydroxy-4,5-dimethylfuran-2(5*H*)-on (Sotolon) mit dem Geruch von Bockshornklee und (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal mit dem Geruch von Haferflocken. Die Quantifizierung mittels Stabilisotopenverdünnungsassays (SIVAs) und die Berechnung von Odor Activity Values (OAVs) ergaben 17 Geruchsstoffe, deren Konzentrationen in den Walnüssen ihre spezifischen Geruchsschwellenkonzentrationen überschritten. Durch Aromarekonstitutions- und Weglass-experimente konnte schließlich gezeigt werden, dass das charakteristische Aroma frischer Walnusskerne am besten durch ein binäres Gemisch aus Sotolon und (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal repräsentiert wird, wobei die natürlichen Konzentrationen beider Verbindungen bei ~10 µg/kg lagen. Somit wurden Sotolon und (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal als Schlüssel-geruchsstoffe von Walnüssen identifiziert. Weitere sensorische Untersuchungen zeigten, dass sich der Walnusscharakter bei höheren Konzentrationen bis etwa 100 µg/kg weiter verstärkt, wobei allerdings das 1:1-Verhältnis zwingend erhalten bleiben muss. Diese Ergebnisse können die Züchtung neuer Walnusssorten mit verbessertem Aroma unterstützen. In anderen Nüssen sind die Konzentrationen von Sotolon und (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal, insbesondere ihr Verhältnis zueinander, signifikant unterschiedlich, was das Fehlen einer Walnussnote in diesen Nüssen erklärt. Die Alterung frischer Walnusskerne hatte keinen signifikanten Einfluss auf die Konzentrationen von Sotolon und (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal, jedoch erhöhten sich die Konzentrationen einiger Fettsäureoxidationsprodukte. Beispielsweise stieg die Konzentration des pilzartig riechenden Oct-1-en-3-ons während einwöchiger Lagerung bei Raumtemperatur um das Fünffache an.

3 Abbreviations and Nomenclature

Abbreviations:

AEDA	Aroma extract dilution analysis
ASTM	American Society for Testing and Materials
3-AFC	3-Alternative forced choice
CI	Chemical ionization
EI	Electron ionization
EHMF	2-Ethyl-4-hydroxy-5-methylfuran-3-one
FFAP	Free fatty acid phase
FD	Flavor dilution
FID	Flame ionization detector
HDMF	4-Hydroxy-2,5-dimethylfuran-3(2*H*)-one
HS	Headspace
IS	Internal Standard
GC	Gas chromatography
GC×GC–MS	Comprehensive two-dimensional gas chromatography
GC–GC–MS	Two-dimensional heart-cut gas chromatography−mass spectrometry
GC–MS	Gas chromatography–mass spectrometry
GC–O	Gas chromatography–olfactometry
HVT	High vacuum transfer
LDL	Low-density lipoprotein
LN_2	Liquid Nitrogen
OAV	Odor activity value
OTC	Odor threshold concentration
RM	Reconstitution model
RI	Retention index
SAFE	Solvent-assisted flavor evaporation
aSAFE	Automated solvent-assisted flavor evaporation
mSAFE	Manual solvent-assisted flavor evaporation
SBSE	Stir bar sorptive extraction
SIDA	Stable isotope dilution assay
SPME	Solid phase microextraction

Nomenclature:

2'-Aminoacetophenone	1-(2-Aminophenyl)ethan-1-one
Arachidic acid	Icosanoic acid
Cyclotene	2-Hydroxy-3-methyl-cyclopent-2-en-1-one
(2*Z*,4*Z*)-δ-Deca-2,4-dienolactone	6-Pentylpyran-2-one
γ-Decalactone	5-Hexyloxolan-2-one
(6*Z*)-γ-Dodec-6-enolactone	5-[(2*Z*)-Oct-2-enyl]oxolan-2-one
Eugenol	2-Methoxy-4-(prop-2-en-1-yl)phenol
α-Farnesene	(3*E*,6*E*)-3,7,11-Trimethyldodeca-1,3,6,10-tetraene
γ-Hexalactone	5-Ethyldihydrofuran-2(3*H*)-one
β-Ionone	(3*E*)-4-(2,6,6-Trimethylcyclohex-1-en-1-yl)but-3-en-2-one
3-Isopropyl-2-methoxypyrazine	2-Methoxy-3-(propan-2-yl)pyrazine
Limonene	1-Methyl-4-(prop-1-en-2-yl)cyclohex-1-ene
Linalool	3,7-Dimethylocta-1,6-dien-3-ol
Linoleic acid	(9*Z*,12*Z*)-Octadeca-9,12-dienoic acid
α-Linolenic acid	(9*Z*,12*Z*,15*Z*)-Octadeca-9,12,15-trienoic acid
Maltol	3-Hydroxy-2-methyl-4*H*-pyran-4-one
Methional	3-(Methylsulfanyl)propanal
Myrcene	7-Methyl-3-methylideneocta-1,6-diene
Myristic acid	Tetradecanoic acid
γ-Octalactone	5-Butyldihydrofuran-2(3*H*)-one
δ-Octalactone	6-Butyldihydrofuran-2(3*H*)-one
Oleic acid	(9*Z*)-Octadec-9-enoic acid
Palmitic acid	Hexadecenoic acid
Palmitoleic acid	(9*Z*)-Hexadec-9-enoic acid
α-Pinene	2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene
3-Sec-butyl-2-methoxypyrazine	2-(Butan-2-yl)-3-methoxypyrazine
Sotolon	3-Hydroxy-4,5-dimethylfuran-2(5*H*)-one
Stearic acid	Octadecanoic acid
γ-Terpinene	1-Methyl-4-propan-2-ylcyclohexa-1,4-diene
trans-4,5-Epoxy-(2*E*)-dec-2-enal	(2*E*)-3-[(3-(2*R*,3*R*) and/or (2*S*,3*S*)-Pentyloxiran-2-yl] prop-2-enal
Vanillin	4-Hydroxy-3-methoxybenzaldehyde

4 Introduction

4.1 Molecular Sensory Science

Consumers' food preferences are strongly influenced by the food's sensory properties. Various factors such as the appearance, freshness, nutritional value, and healthiness of food influence the consumer's choice. However, sensory properties such as texture, taste, and especially aroma are most important.[1, 2] Two approaches can be used to investigate the parameters that contribute to the sensory quality of food. In descriptive sensory analysis, a trained sensory panel describes a product in all its sensory attributes, including the smell attributes, appearance attributes, taste attributes, and texture attributes of a food product. Each attribute is assigned a quantitative value.[3] In contrast, molecular sensory science, also known as sensomics, elucidates the molecular background of each individual attribute, that is the sensory-active part of the metabolome. In both cases, descriptive sensory analysis and molecular sensory science, the aim is to use the approach to improve the sensory properties of food products. The sensory-active metabolites include compounds such as odorants, tastants, and chemestetic-active compounds.[4] This study, however, was focused on odorants.

4.1.1 Odor Perception

Odorants reach the olfactory epithelium in two pathways, as shown in Figure 1. One is the orthonasal pathway, in which odorants that evaporate from the food before consumption are inhaled through the nose. The second is the retronasal pathway, in which the odorants enter the nasal cavity mainly through the pharynx during exhalation after swallowing the food.[1]

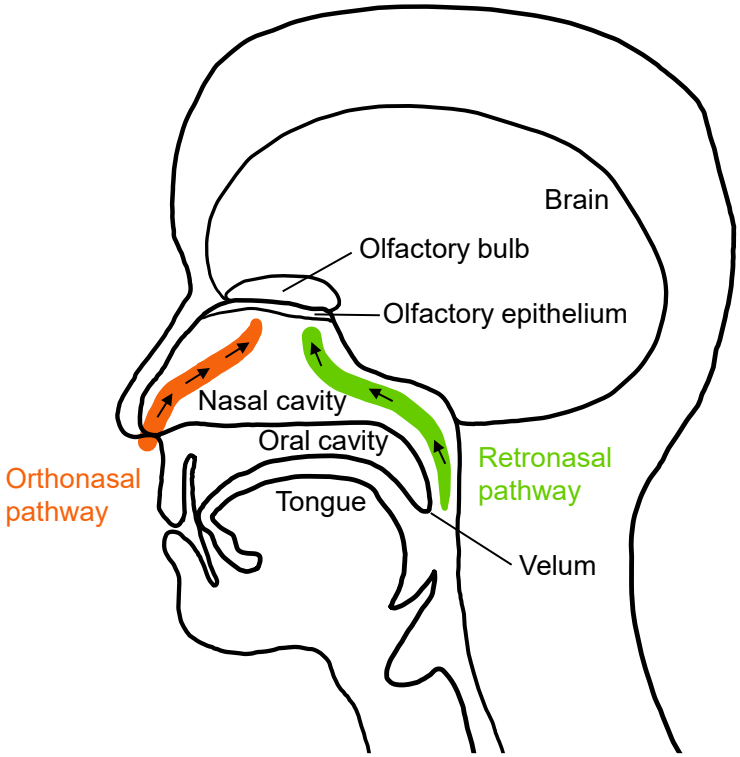


Figure 1: Orthonasal and retronasal pathways (illustration: Christine Stübner)

The olfactory epithelium includes olfactory neurons with ~400 different G protein-coupled olfactory receptors located in the ciliary membranes (Figure 2). Binding of an odorant to a receptor molecule activates a G protein, thus initiating a reaction cascade. Calcium and sodium levels increase in the cytosol of the receptor cell and chloride ions are released from the cell, resulting in depolarization of the cell membrane. The depolarization is transmitted as nerve impulse via the olfactory nerve to the olfactory bulb (bulbus olfactorius) in the brain. There are two olfactory bulbs, and each receives signals from the corresponding nostril, which allows for separate processing and more precise odor perception. Typically, a particular receptor type can be activated by different odorants, and conversely, a specific odorant can activate several receptor types. As a consequence, humans can distinguish approximately 10,000 different odors with ~400 different receptor types. In the olfactory bulb, the axons of receptor cells of the same receptor type converge with the primary dendrite of a mitral cell to form olfactory bulb synapses called glomeruli olfactorii. More than 1000 axons project to a single mitral cell, which reduces the amount of information. The mitral cell transmits the signal to higher brain regions.[1, 5-9] Signaling pathways extend between the two olfactory bulbs and the primary olfactory cortex, the hypothalamus, and the limbic system.[7] The transition from olfaction to odor perception in the brain is smooth. The evaluation of an odor occurs even before its recognition and takes place in the limbic system. In the amygdalae, odors are associated with emotions and memories. In most cases, people first recognize an intuitive liking or disliking of a particular odor before recognizing the specific odor impression. Odor recognition is influenced by previous experience and conditioning. Odors cannot be visualized in the olfactory cortex. However, they can be visualized in the visual cortex, so odors are often associated with a place or an emotion, and phrases such as "it smells like rain" or "it smells like a bakery" are common when people are asked to describe an odor. This unintentional effect is called presemantic implicit memory. The association with a word, and thus the identification of the odor, is achieved by the projection of the olfactory bulb to the thalamus and its transmission to the orbitofrontal cortex. It is known as explicit semantic memory. The permanent storage of olfactory impressions is enabled by the signaling pathway from the olfactory bulb to the hippocampus.[8-11]

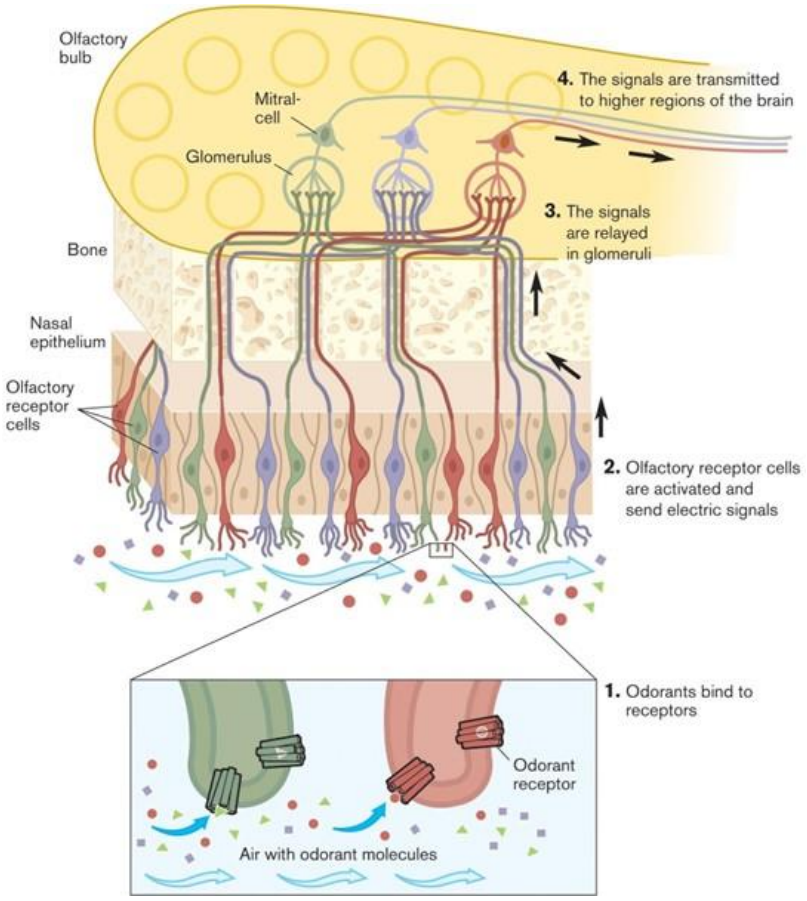


Figure 2: Schematic illustration of the olfactory system[12]

Of the countless volatiles in a food product, only a small number are odor-active.[4] Odorants need to be able to bind to an olfactory receptor.[1, 6] In order to contribute to the overall aroma of a food, it is additionally required that the odorant exceeds its specific odor threshold concentration (OTC) in the food matrix.[1] Ultimately, only a few odorants are crucial for the overall aroma impression. These compounds are referred to as key odorants. The absence of a single key odorant substantially affects the aroma and even can make the food unrecognizable.[1] In a recent meta-analysis, it was shown that of the approximately 10,000 volatiles that have been identified in foods to date, only about 230 were ultimately odor-active in the 227 food samples analyzed. In this study, the odor-active compounds were classified in descending order according to their relative frequency in foods as generalists, intermediates, specialists, and individualists. The meta-analysis identified 16 generalists and 57 intermediates. These were present in 25% and 5–25% of the 227 food samples evaluated, respectively. Specialists are present in a small number of foods. 151 of the nearly 230 odor-active compounds were specialists and found in <5% of the food samples analyzed. Individualists are odor-active compounds in only a single food. Nevertheless, specialists and individualists often contribute significantly to the aroma.[1, 4]

4.1.2 Characterization of Key Food Odorants

The characterization of the key odorants in foods follows a well-established concept developed by Schieberle[13] and Grosch[14]. It can be divided into seven essential steps, as shown in Figure 3.

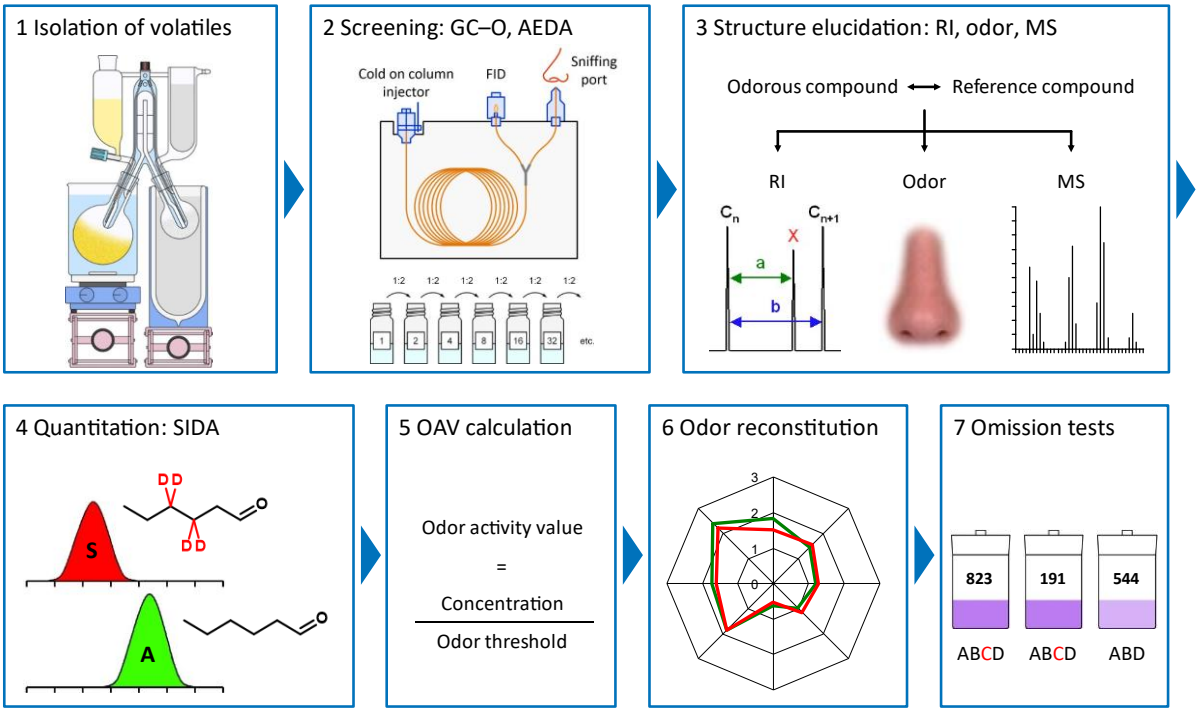


Figure 3: Identification of key food odorants (illustration: Martin Steinhaus)

The first step is the isolation of the volatile fraction, thus the removal of non-volatiles (Figure 3, step 1, and Figure 4). A solvent extract is prepared from homogenized food and a low-boiling, non-polar, organic solvent such as diethyl ether or dichloromethane, followed by drying with an anhydrous salt and filtration. Solvent-assisted flavor evaporation (SAFE)[15], a high-vacuum and low-temperature method, provides a gentle removal of the non-volatiles while the volatiles are obtained with high yields. The application of the high-vacuum allows the temperatures to be maintained at ≤40 °C, thus minimizing the risk of compound degradation and artifact formation.[1] Subsequently, the obtained volatile isolate is concentrated under mild conditions, e.g. using a Vigreux column and a Bemelmans microdistillation device.[16] More details on volatile isolation are discussed in section 4.2.

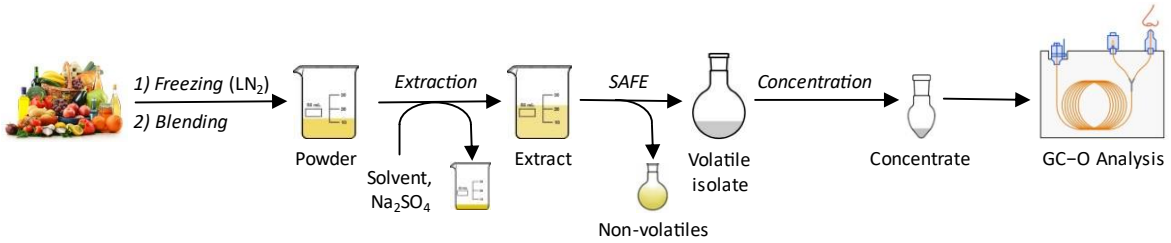


Figure 4: Sample preparation and volatile isolation prior to GC–O (illustration: Martin Steinhaus)

The volatile isolate contains the entire volatiles, including both, odor-active and odorless compounds. Gas chromatography–olfactometry (GC–O)[1] is applied to the volatile isolate as a screening method to detect the odorants by using the human nose as a detector in addition to the flame ionization detector (FID) (Figure 3, step 2, and Figure 5). The volatiles are separated on a GC column. At the end of the GC column, the effluent stream is divided into two equal parts by a Y-shaped splitter. One effluent stream is directed to the FID, while the other is simultaneously directed to the sniffing port. During analysis, the FID signal is recorded by a computer equipped with a GC–O software. At the same time, the assessor's nose is positioned directly above the sniffing port to evaluate the odor. Whenever the assessor detects an odor, an odor descriptor is selected by mouse click from a predefined palette. The software automatically adds the corresponding retention time, establishing a correlation between the FID signal and the odor event. In the past, a recorder was used instead of a computer to record the FID signal, and odor qualities were noted manually.

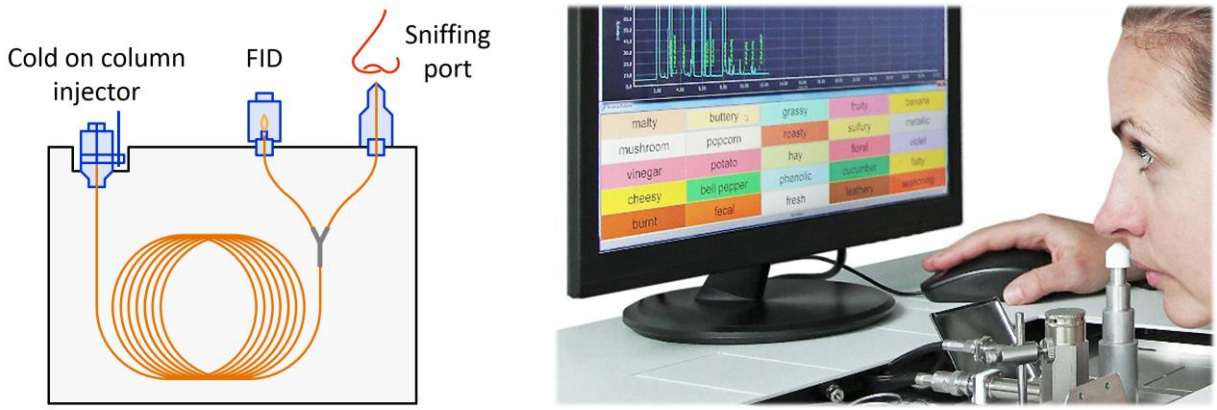
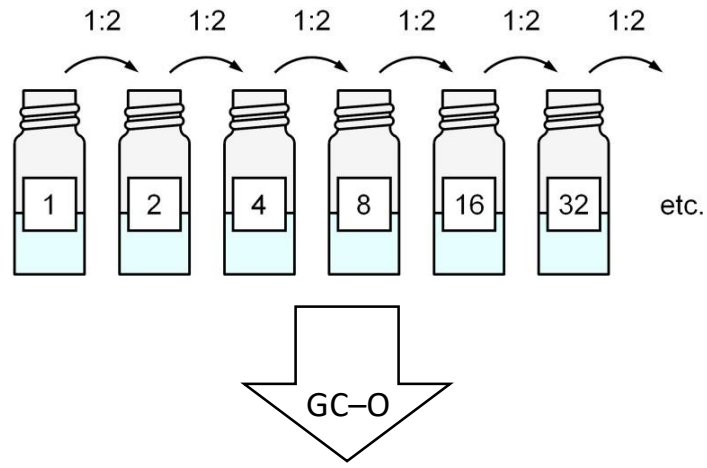


Figure 5: Schematic illustration (left) and application (right) of GC−O (illustration and photo: Martin Steinhaus)

Subsequently, an aroma extract dilution analysis (AEDA)[17] is performed to estimate the odor potency of the individual odorants. The volatile isolate is diluted stepwise by a factor of 2, resulting in dilutions of 1:2, 1:4, 1:8, 1:16, etc. (Figure 6). Starting with the low diluted ones, the samples are subjected to GC–O analysis until at a high dilution level no odorant can be detected during GC–O. A flavor dilution factor (FD) is assigned to each odorant based on the dilution factor of the highest diluted sample in which the odorant was perceived during GC–O. A higher FD factor indicates a greater potential to contribute to the overall aroma. AEDA is a valuable tool for classifying odorants according to their odor potency, but it cannot provide evidence of an odorant's contribution to the overall aroma of the food.



Dilution:	1	2	4	8	…	1024	2048	FD factor
Odorant 1	✓	✓	×	×		×	×	2
Odorant 2	✓	✓	✓	×		×	×	4
Odorant 3	✓	×	×	×		×	×	1
Odorant 4	✓	✓	✓	✓		✓	×	1024
Odorant 5	✓	✓	✓	×		×	×	4
⋮								

Figure 6: Principle of AEDA (illustration: Martin Steinhaus, Christine Stübner)

Structure assignment[1] of odorants detected by GC–O is performed by comparing several parameters of the odorant with authentic reference compounds analyzed under the same conditions (Figure 3, step 3). Parameters such as retention index (RI) on two GC columns of different polarity, odor quality, and mass spectra in electron ionization (EI) and chemical ionization (CI) modes are considered for comparison. Two-dimensional gas chromatography–mass spectrometry (GC–MS) techniques such as GC×GC–MS[18] or heart-cut GC–GC–MS[19] are used to reduce co-elution problems.

The next step is to determine the concentrations of the odorants in the food. Quantitation assays are performed preferably by using stable isotope dilution assays (SIDAs)[13, 20] (Figure 3, step 4, and Figure 7). A stable isotopically substituted analogue of the target analyte, either deuterium- or ^{13}C-substituted,[21] is added as an internal standard at the beginning of the sample preparation. The target analyte and its stable isotopically substituted internal standard can be considered to behave identically in chemical and physical terms under the mild conditions applied. Losses during mild sample preparation are then ideally compensated, provided that the analyte and the internal standard reach equilibrium at the beginning of the sample preparation. The time required to achieve equilibrium depends on the food matrix.[22] The target compound and the internal standard can finally be distinguished by GC–MS analysis due to the different molecular weights resulting from the isotopic substitution. The concentration of the target analyte is calculated from the peak area counts corresponding to the analyte and the standard, the sample amount, and the amount of standard added using a calibration line equation derived from the analysis of analyte/standard mixtures in different concentration ratios.

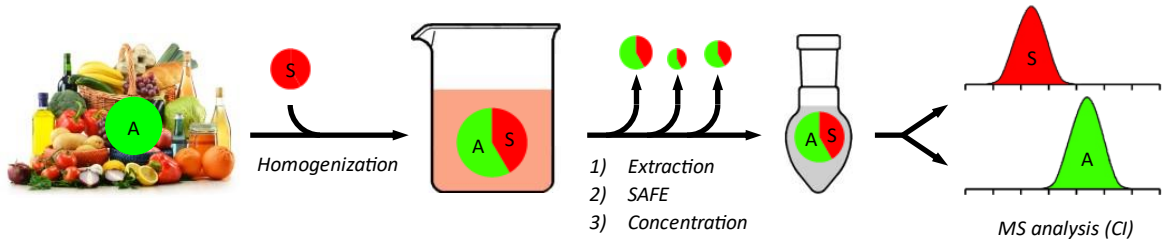


Figure 7: Schematic procedure of SIDA (illustration: Martin Steinhaus)

To identify the key odorants, the next step is to calculate the odor activity value (OAV)[14] of each odor-active compound in the food as the quotient of its concentration in the food and its odor threshold concentration (OTC) in an appropriate matrix (Figure 3, step 5, and Figure 8). The American Society for Testing and Materials (ASTM) standard method for determining odor and taste thresholds by a forced-choice ascending concentration series method of limits is applied.[23] OAVs provide a better approximation of the importance of the odorants to the overall aroma in the food than FD factors because OAVs consider exact concentrations, matrix interactions, and the specific volatility of the compounds. Odorants with an OAV <1 typically do not contribute to the aroma. In contrast, odorants with an OAV ≥1 have the potential to contribute to the aroma, with a higher OAV indicating a higher probability of contribution. However, the actual contribution of a compound to the overall aroma can only be assessed by omission tests after successful aroma reconstitution.

$$\mathrm{OAV} = \frac{\text{odorant concentration } \left(\frac{\mu g}{kg}\right)}{\text{odor threshold concentration } \left(\frac{\mu g}{kg}\right)}$$

Figure 8: OAV calculation

Aroma reconstruction models are created by mixing all odorants with OAVs ≥1 based on the quantitative data (Figure 3, step 6, and Figure 9). Reconstitution models aim to mimic the authentic food matrix as closely as possible in terms of water, sugar, and fat content, as well as pH. A trained sensory panel evaluates the model against the authentic food. If the aroma reconstitution model is in good agreement with the aroma of the authentic food, the reconstitution was successful and provides evidence that all odorants contributing to the aroma are included.[13]

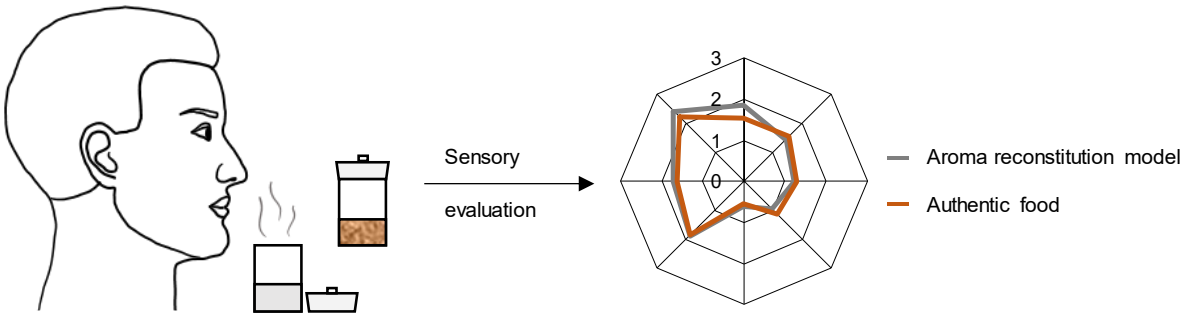


Fig 9: Aroma reconstitution experiments (illustration: Martin Steinhaus, Christine Stübner)

Finally, omission tests are performed to determine the minimum number of compounds required to represent the food's aroma (Figure 3, step 7).[14] In each individual omission test, an incomplete aroma model is created by omitting one or more odorants. The trained panel evaluates this model against the complete aroma reconstitution model using a 3-alternative forced choice (3-AFC) test. If there is no significant difference, the omitted compound(s) do not play a crucial role in the overall aroma of the food and thus are not key odorants. Conversely, if a significant difference is observed after omitting a single odorant, this confirms the importance of that compound, and thus it can finally be declared as a key odorant.

4.2 Approaches for the Isolation of Volatiles

The removal of non-volatiles is crucial prior to GC analyses. Especially for odorant screening, it is in addition essential that the authentic composition of the volatile fraction is preserved, that reactive odorants are not destroyed and no odor-active artifacts are formed. Artifact formation is a particular problem because even minor or trace amounts can be odor-active if the OTCs of the artifacts are at the lower end of the spectrum.[1]

There are methods that do not require time-consuming sample preparation, such as direct injection of solvent extracts or solid phase extraction approaches, such as stir bar sorptive extraction (SBSE)[24] and solid phase microextraction (SPME)[25]. However, these methods require hot injection that promotes artifact formation and thermal degradation.[1, 26-28] Prior to GC–O, a gentle isolation technique should be used to efficiently isolate the volatiles with a high yield, ensuring a consistent composition of the volatile fraction even at trace levels and a complete removal of non-volatiles.

In 1970, Weurman et al. introduced a gentle method for volatile isolation to address the problem of artifact formation, the high vacuum transfer (HVT).[29] The evacuated apparatus consisted of two round bottom flasks connected by a glass tube (Figure 10). Bottle 1, which contained the sample, was at room temperature. Bottle 2, which was initially empty, was cooled to −180 °C. The volatiles in bottle 1 evaporated and recondensed in bottle 2 due to the different vapor pressures caused by the temperature difference in the flasks.

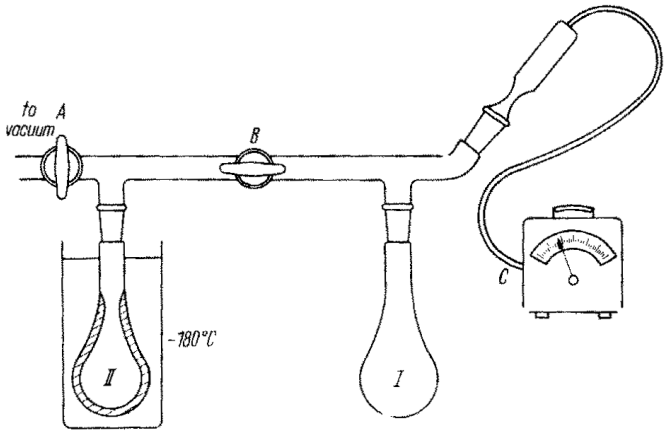


Figure 10: HVT introduced by Weurman et al. in 1970[29]

Schieberle and Grosch improved the HVT approach in 1985 and applied it to a solvent extract obtained from the food sample and a low boiling solvent such as dichloromethane, diethyl ether, or pentane.[30] Their HVT apparatus consisted of a round bottom flask connected by a glass tube leading to a series of gas wash bottles used as cold traps (Figure 11). The solvent extract was placed in the round-bottom flask and cooled with liquid nitrogen. After the apparatus had been evacuated, the cold traps were cooled while the extract cooling was stopped, thus the volatile compounds evaporated from the extract and condensed in the cold traps due to the temperature difference.

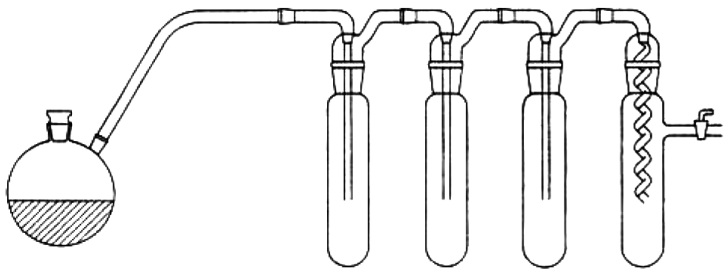


Figure 11: HVT apparatus used by Schieberle and Grosch[30]

A problem in the early HVT setups was that volatiles recondensed in the glass tubes before they entered the cold traps, which resulted in losses, particularly of high-boiling volatiles. To reduce this effect, Sen et al. replaced the glass tube with a double-walled, water-thermostated glass tube that was heated up to 50 °C (Figure 12).[31]

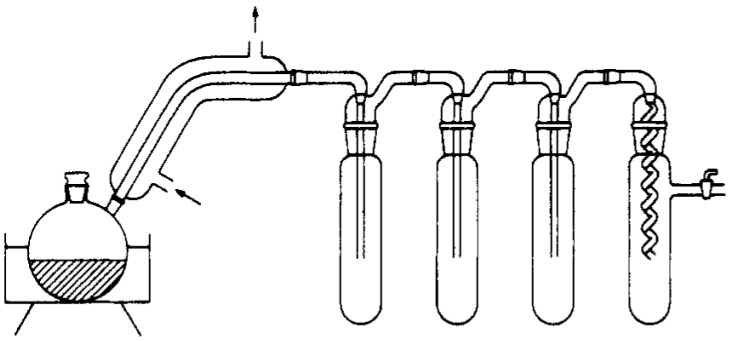


Figure 12: HVT with double-walled glass tube used by Sen et al.[31]

In 1989, further improvement to the HVT approach was presented by Guth and Grosch (Figure 13). The volatile yields were increased by introducing the solvent extract into the round bottom flask in small portions with the aid of a dropping funnel.[32] The extract was added discontinuously at high flow rates over a short time period. This was necessary in order for the extract to be properly transferred from the funnel into the flask, to maintain the vacuum, and to prevent clogging of the funnel outlet by solidified fat when high-fat extracts were applied. This “dynamic HVT” needed no cooling of the round bottom flask. Instead, the flask was thermostated to 34 °C using a water bath. Each portion evaporated in a spray-like manner as soon as it exited the dropping funnel. However, there was some risk that not only volatiles but also non-volatiles in the form of small droplets were transferred to the cold traps.

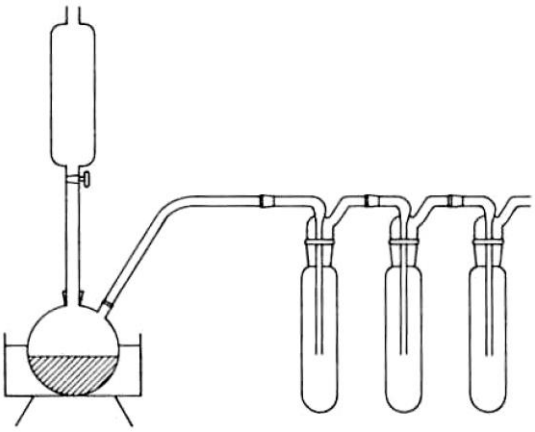


Figure 13: Dynamic HVT used by Guth and Grosch (1989)[32]

To reduce non-volatile transfer, in 1992, Jung et al. extended the HVT apparatus by including an adapter for splash protection between the flask and the thermostated glass tube (Figure 14).[33] Non-volatile droplets were deposited on the adapter walls and did not end up in the volatile isolate in the cold traps.

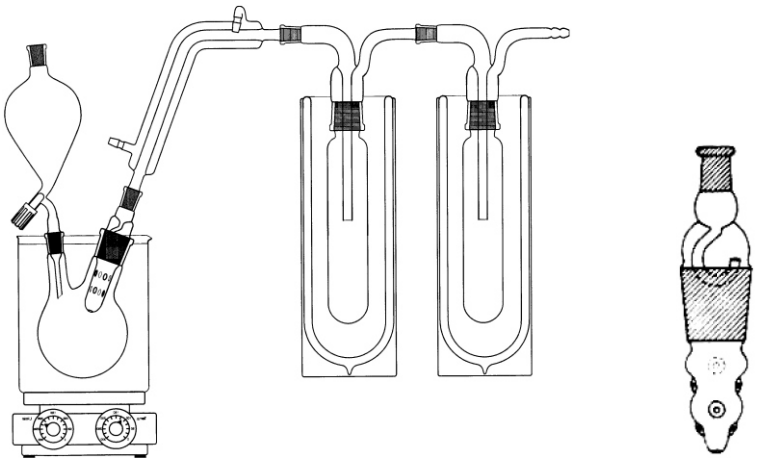


Figure 14: Dynamic HVT used by Jung et al. (left) including a splash protection adapter (right)[33]

In 1999, Engel et al. developed the SAFE by combining the main components of the dynamic HVT setup into a single glass apparatus (Figure 15).[15] The SAFE glassware included a dropping funnel that was equipped with a manual needle valve stopcock to transfer the extract in portions into the evaporation flask. The evaporation flask and the double-walled middle part of the glassware were thermostated at 30–40 °C. The middle part connected the evaporation flask with the recondensation flask. The recondensation flask and a safety cold trap, which protected the vacuum pump in case of a malfunction, were cooled to −196 °C with liquid nitrogen. The temperature of the middle part was kept at 30–40 °C to reduce the recondensation of volatiles and avoid artifact formation. The glass tube between the stopcock and the evaporation flask was also thermostated to prevent clogging of the glass tube by non-volatiles such as solidified fat particles from high-fat extracts. The middle part was equipped with propeller-shaped barriers to effectively retain droplets of non-volatiles.

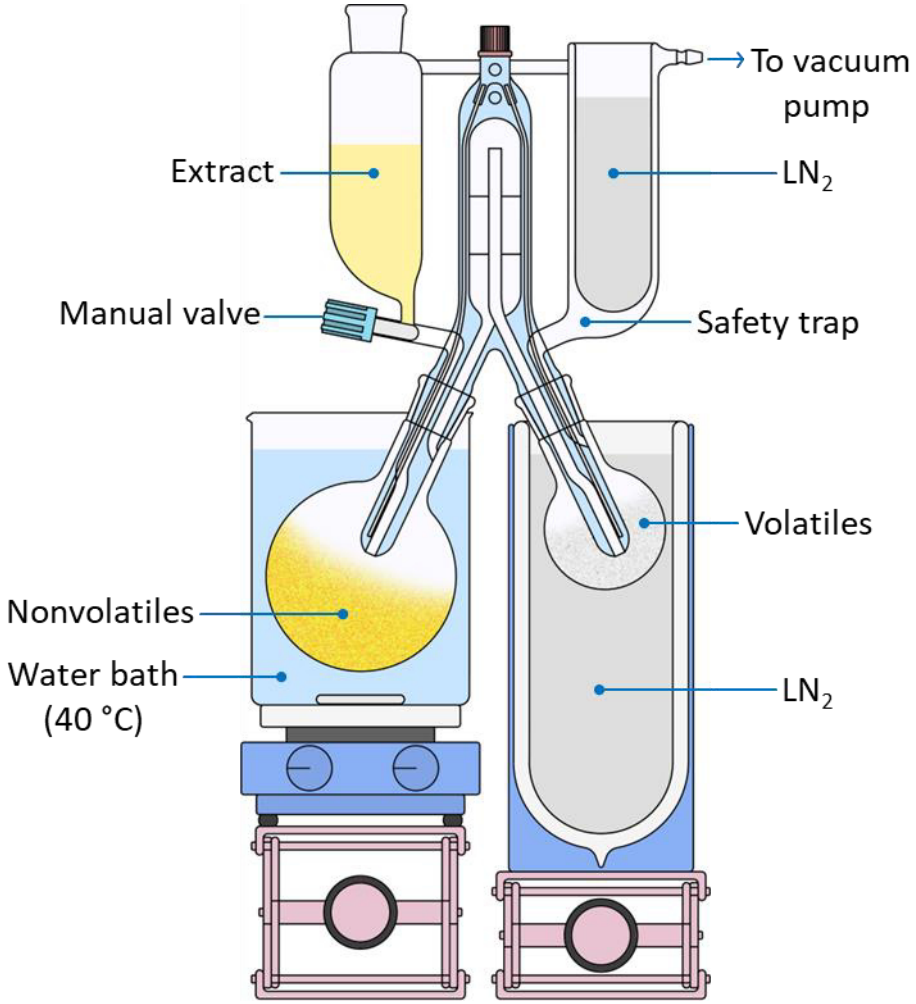


Figure 15: SAFE according to Engel et al. (illustration: Martin Steinhaus according to literature[15])

The SAFE is typically used for solvent extracts obtained from food. To set up the SAFE device, the glass apparatus is completed with the evaporation flask, the recondensation flask, and the valve stopcock. The device is mounted to a lattice lab system and connected to a vacuum pump. The evaporation flask and the middle part are thermostated at 30–40 °C. The recondensation flask and the safety cold trap are cooled with liquid nitrogen. To start the SAFE process, the system is evacuated and the solvent extract is filled into the dropping funnel. The extract is added to the evaporation flask in portions by opening and closing the valve. The volatiles evaporate instantly in a spray-like manner, are transported through the apparatus, and finally trapped in the recondensation flask. Most of the non-volatiles, such as fats and colorants, remain in the evaporation flask or are retained by the propeller-shaped barriers in the middle part. When the dropping funnel is almost empty, the SAFE process is terminated by ventilating the system and the apparatus is disconnected from the vacuum pump. The liquid nitrogen cooling is removed from the recondensation flask. The recondensation flask containing the volatile isolate is then disconnected from the apparatus. The volatile isolate contains only volatiles and is colorless. After thawing, the volatile isolate can be concentrated and used for further analysis.

Compared to previous techniques such as HVT, with the application of SAFE, the yields, in particular of the rather high-boiling volatiles, showed a significant increase. Higher yields were also obtained when SAFE was applied to extracts obtained from high-fat samples. The advantages of the SAFE over the different HVT approaches additionally include an easier and faster setup and reduced bench space requirements.[15] More than 700 citations between 1999 and 2023 evidence that the approach has become established as a state-of-the-art method for artifact-avoiding isolation of volatiles, especially in the field of odorant research prior to GC–O analysis.[34]

4.3 Walnut

4.3.1 The Walnut Tree (*Juglans regia* L.)

The term walnut tree applies to all species of the genus *Juglans* within the family Juglandaceae. The scientific name “*Juglans*” was established in 1737 by Carl von Linné and is based on the Latin words "*Jovis glans*", translated as Jupiter's shine. The genus includes a total of 22 species, including the black walnut (*Juglans nigra* L.), the butternut (*Juglans cinerea* L.), and the most cultivated species *Juglans regia* L. referred to as common walnut or Persian walnut. The German name “Walnuss” is derived from the Old German word wählischnuz, which means “wellsche Nuss” and is synonymous with Gallic or French nut.[35] The present work deals exclusively with the species *Juglans regia* L. Thus, in the following, the term “walnut” always refers to the fruit of this particular species.

Walnut trees are deciduous trees that can reach heights of up to 30 m and are native to a region stretching from southern Europe to southwestern and central Asia.[36] The walnut tree has been cultivated for more than two thousand years.[37] Meanwhile, walnut trees are grown worldwide, but cultivation is limited to regions with mild climatic conditions. On the one hand, the cultivation of walnut trees serves for the production of wood. Particularly the precious heartwood with its dull brown to blackish brown color and streaks is highly valued. Its high dynamic strength and low tendency to splinter make it a durable material for various applications, including the construction of furniture, clocks, and musical instruments.[35] On the other hand, walnut trees are primarily cultivated for the edible seeds of their nut fruits, the walnut kernels.

4.3.2 Walnut Kernels and their Culinary Use

The walnut is not only colloquially, but also botanically classified as a nut. It consists of a brown, woody pericarp, which is divided into two parts. The single seed has wrinkled cotyledons and a light brown seed coat (Figure 16). On the tree, the fruit is enveloped by a green, fleshy husk (Figure 17).[36]

Figure 16: Opened walnut fruit[38]

Figure 17: Walnut fruit with green husk on the tree[39]

The walnut tree produces first fruits at the age of 6–10 years, a full harvest is not possible before 15 years of growth. On average, a 30–60 year old walnut tree yields approximately 50 kg of walnuts per year.[35] However, under optimum conditions, the yield can increase up to 100–150 kg per year.[40]

At maturity, the green husk bursts open. Under natural conditions, the nut detaches from the husk and falls to the ground. In Eurasia, harvesting takes place between September and October and involves shaking the nuts from the trees with mechanical shakers. Husks remaining attached to the nut after harvest should be removed promptly to avoid black spots on the walnut shell. The husk and the walnut tree leaves contain hydrojuglone, a precursor of the natural dye juglone. Therefore, husks can be used in fabric tanning and wood dyeing to shade a dark brown color tone. For the plant, juglone plays a significant role as a wound protectant due to its antibacterial and antifungal properties. When released into the soil, juglone inhibits the root formation of other plants.[35]

The post-harvest processing of walnuts takes place in the country of origin and includes the complete removal of the husk as well as cleaning, hot air drying, and packaging of the nuts.[41] Drying is a crucial step in protecting the nuts from mold and is done within an optimal temperature range between 32 °C and 43 °C. Higher temperatures should be avoided to prevent rancidity.[35] After drying, the water content in the whole nut has decreased to less than 12% and in the kernel to less than 8%, resulting in an overall weight reduction of approximately 50%. Dried nuts can be stored for several months.[40,42] In some cases, walnuts are treated with SO_2 to increase mold resistance and improve appearance, thus increasing their commercial value.[35] Before packing and shipping, unshelled walnuts are classified into three categories. The "Extra" class is reserved for the highest quality, flawless walnuts of a well-defined variety from the previous year’s harvest. Class "I" is intended for walnuts of a well-defined variety of good quality. Class "II" includes all walnuts not qualified for higher classes. The minimum requirements are fulfilled when the shells are intact, dry, clean, and free of husk residues. The kernels must be firm, free from mold, rancidity, and excessive moisture. Walnut size is another grading criterion. Only the lowest class "II" can be used for walnuts with a diameter of 24–26 mm. Fruits between 26 and 28 mm can be classified as "I" or "II" depending on their quality. Walnuts larger than 28 mm, subdivided into 28–30 mm, 30–32 mm, and 32–34 mm,

can be classified into classes “I”, “II” or “Extra”, depending on their quality. The size, grade, and variety, if defined, are indicated on the package.[42] Walnuts are marketed to consumers in two forms: as whole nuts or as walnut kernels. To isolate the kernels from the whole nuts, the shells are cracked and the kernels are separated manually or by machine. Walnut kernels are classified into the same three quality categories as whole walnuts. Instead of the size, the color of the kernels plays a decisive role. The lighter the kernels, the higher the quality. The kernels are sold as “walnut kernel halves”, “quarters”, “large pieces”, or “broken pieces”.[43]

In 2021, the world harvest of walnuts in shells reached 3,500,173 t. The main producing countries China, USA, Iran, Turkey, and Chile together accounted for 75% of the global annual crop. Production of walnuts has increased sevenfold since 1961.[44] Figure 18 shows the walnut-producing countries in 2021 with their crop size indicated by color-coding. Underlying data were taken from the literature.[44]

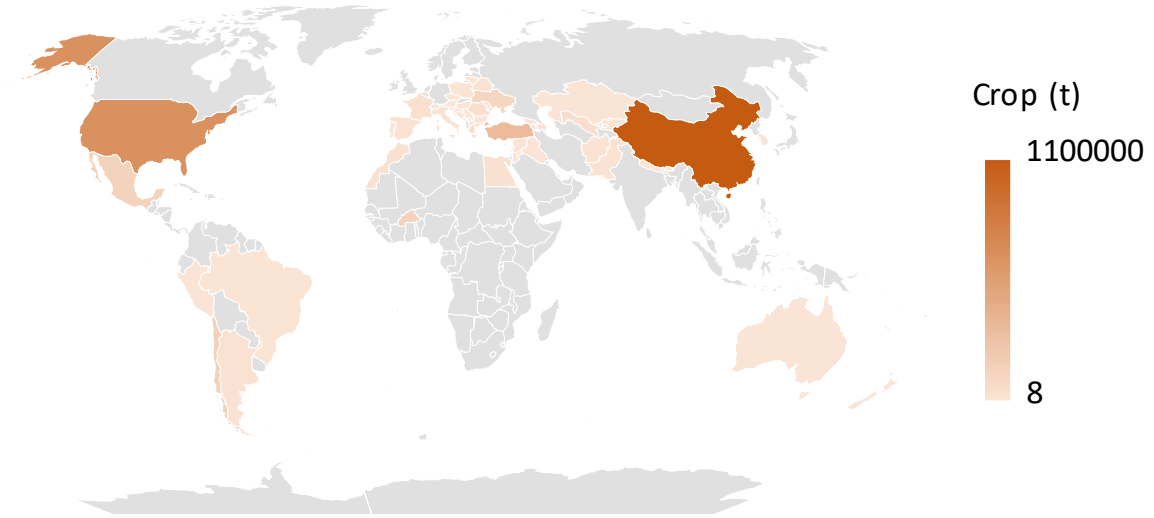


Figure 18: Walnut producing countries in 2021

The substantial growth in walnut production underscores the increasing importance of walnuts in human nutrition. Walnut kernels are appreciated for their overall nutritional value and specific health benefits.[45] They have a high-fat content of 62.5 g/100 g and a favorable fatty acid profile with a high percentage of mono- and polyunsaturated fatty acids. The most abundant fatty acids in walnuts are oleic acid (11.4 g/100 g), the Ω-6-fatty acid linoleic acid (34.3 g/100 g), and the Ω-3-fatty acid α-linolenic acid (7.83 g/100 g), as shown in Table 1.[46] Consumption of Ω-3- and Ω-6-polyunsaturated fatty acids is essential because the human body cannot synthesize them.[45] The optimal balance of linoleic acid and α-linolenic acid in walnuts contributes to the reduction of cardiovascular risks.[47] In numerous studies, walnut consumption has been associated with lower cholesterol and low-density lipoprotein (LDL) levels in human blood.[48, 49] Walnut kernels contain 17% protein and are a rich source of vitamins and minerals (Table 2). Due to their high content of polyphenols, they have remarkable antioxidant and anti-inflammatory properties.[37] Compared to other nuts, the polyphenol content and the associated antioxidant capacity in walnuts are outstanding.[50]

Table 1: Fatty acid composition of 100 g walnut kernels according to Souci et al.[46]

myristic acid	690	mg
palmitic acid	4360	mg
stearic acid	1370	mg
arachidic acid	421	mg
palmitoleic acid	135	mg
oleic acid	11.4	g
linoleic acid	34.3	g
α-linolenic acid	7.83	g

Table 2: Average composition of 100 g walnut kernels according to Souci et al.[46]

energy	685	kcal
water	4.38	g
protein	17	g
fat	62.5	g
available carbohydrates	10.6	g
total fiber	6.14	g
minerals	1.98	g
sodium	2.4	mg
potassium	544	mg
magnesium	129	mg
calcium	87	mg
manganese	2	mg
iron	2.5	mg
cobalt	9.5	µg
copper	880	µg
zinc	2.7	mg
nickel	132	µg
phosphorus	409	mg
chloride	23	mg
fluoride	680	µg
iodide	3	µg
boron	760	µg
selenium	5.5	µg
total sterols	108	mg
total carotenoids	48	µg
vitamin E activity	6	mg
total tocopherols	44	mg
vitamin K	2	µg
vitamin B1	340	µg
vitamin B2	120	µg
nicotinamide	1	mg
pantothenic acid	820	µg
vitamin B6	870	µg

The culinary use of walnuts is diverse. Unripe fruits with husk are used for making nut liqueur and pickled walnuts. Walnuts harvested in-shell when ripe but not dried after harvest are called "fresh walnuts" or "early walnuts" and in German "Schälnüsse". Since the nuts are not dried after harvest, the seed coat remains bitter and must be removed before consumption.[35] These nuts are intended for immediate consumption and have a short shelf life. They are rather found at local farmers' markets than in stores.

Dried walnut kernels are mainly consumed raw or roasted and are used as an ingredient in savory dishes such as salads, pizza, pasta, or appetizers, as well as in sweet desserts and various confectionery products. Popular examples of walnut-based savory dishes include walnut soup, enjoyed in China, Italy, Armenia, and Mexico, as well as US-American Waldorf salad and Greek appetizer Skordalia. Traditional walnut desserts include walnut ice cream, the Georgian confection churchkhela, and the Yewish nunt. The Romanian cozonac and the English walnut cake are well-known walnut pastries.

Walnut kernels can be used to produce cold-pressed walnut oil. The oil is yellow-green and often used in cold cuisine, especially in salad dressings. Walnut oil is not suitable for deep-frying and roasting due to its relatively low smoke point of 160 °C. High temperatures result in an unpleasant aroma caused by fat oxidation.[35]

4.3.3 Odorants and Volatiles in Fresh Walnut Kernels

Walnut kernels can be distinguished from other culinary nuts by their characteristic odor. For nearly 50 years, researchers have been dedicated to deciphering the molecular background of walnut aroma. Clark and Nursten were pioneers in the analysis of walnut odorants and published their scientific results in two subsequent papers in 1976 and 1977. Their studies focused on volatiles in walnut oil and volatiles in the headspace (HS) above walnut kernels, respectively. GC–O and GC–MS allowed to detect 103 and 129 compounds, respectively, of which approximately 30% were identified. Surprisingly, no peak with a pronounced walnut odor was detected in any of the studies, although the researchers observed that the collected eluates from the GC column had a somewhat walnut-like odor. They concluded that the characteristic walnut odor must be due to a combination of different compounds with non-walnut-like odors. Aldehydes and carbonyl compounds were identified as potential contributors, while alcohols and ketones were found to be less influential. Specifically, the authors hypothesized that a mixture of hexanal, pentanal, pentane-2,3-dione, and 2-methylpent-2-enal could mimic walnut odor, but unfortunately, the resulting mixture yielded only a moderate walnut note.[51, 52]

No studies on the odorants in walnuts have been published for a long time. In recent years, however, there has been increasing interest in the volatile composition of walnut kernels, particularly with respect to changes during storage. Overall, the volatile compound fraction is composed of different compound classes, but aldehydes play a prominent role, as previously suggested by Clark and Nursten.[53-55] Hexanal[53-57] and pentanal[53, 54, 56, 57] were frequently detected in the volatile analyses, but nonanal[53, 54] and (2*E*)-non-2-enal[53, 58] were also reported. Hexanal, pentanal, and (2*E*)-non-2-enal are products of the oxidative degradation of linoleic acid, while oleic acid is the precursor of nonanal.[59, 60]

In 2005, Elmore et al. conducted a comprehensive analysis of the volatile composition of walnut kernels from China, Chile, and Ukraine. Using HS–GC–MS, they identified a total of 118 compounds including aliphatic hydrocarbons (20), monoterpenes (5), aromatic hydrocarbons (33), aldehydes (20), ketones (13), alcohols (15), furans (4), esters and lactones (5), and miscellaneous compounds (3). Interestingly, the compounds previously suggested by Clark and Nursten as crucial for walnut odor, namely hexanal, pentanal, 2-methylpenten-2-al, and pentane-2,3-dione, were found at high levels in Chinese walnuts but at low levels in Chilean walnuts.[56]

In 2015, Salcedo and Nazareno studied the effect of the seed coat on the oxidative stability of walnut kernels. The volatiles deca-2,4-dienal, dec-3-en-1-ol, oct-2-enal, hept-2-enal, undec-2-enal, hepta-2,4-dienal, hexanal, nonanal, non-2-enal and pentanal were analyzed as oxidation markers. They found that ground walnuts with seed coats released more volatile compounds during heat treatment than peeled walnut kernels. In particular, the concentration of deca-2,4-dienal increased 34-fold during heating. During storage at room temperature, the seed coat was found to have antioxidant properties.[53]

In 2020, Hao et al. found that vacuum packaging and oxygen-absorbing additives can extend the shelf life of walnuts up to 257 days. A total of 50 volatiles were identified in raw and processed walnuts by HS–SPME–GC–MS. In raw walnuts, 14 volatiles were detected, including the aldehydes hexanal, heptanal, and benzaldehyde, and the alcohols pentan-1-ol, hexan-1-ol, and 2-propylheptan-1-ol. Methyl octanoate, α-phellandrene, and limonene were also found. The concentration of aldehydes, especially the concentration of hexanal, increased with storage time.[55]

In 2021, Grilo et al. analyzed walnut oxidation of two commonly consumed walnut cultivars during a storage period up to 28 weeks. Using SPME–GC–MS, they identified 51 volatile compounds, thereof 26 in each variety. The authors suggested that the Chandler cultivar is less susceptible to oxidation because its volatile metabolome composition changed less during storage than that of the Howard cultivar. The total concentration of volatiles increased 28-fold for the Howard variety, but only 6-fold for the Chandler variety.[57]

Research on walnut odorants resumed in the 2020s. In 2021, Liu et al. analyzed walnut volatiles isolated from raw and roasted kernels using SAFE, GC–MS, and GC–O. By AEDA, 34 odor-active compounds were detected in raw walnut kernels and 10 compounds were proposed as key odorants based on the fact that their OAVs were >1. Fatty and green smelling aldehydes including (2*E*)-non-2-enal, octanal, hexanal, and nonanal were declared as the most important odorants based on the quantitative data.[61] However, their contribution to the walnut odor remained unclear, because no aroma reconstitution experiment was performed.

The most recent study on the key odorants in walnut kernels was published by Peng et al. in 2022. Fresh walnut kernels were used as a reference point to study the changes in walnut odor caused by different heat treatment processes on the molecular level. HS–SPME–GC–MS/O analysis identified a total of 89 odorants in fresh and processed walnut kernels, of which 50 odorants were detected in fresh walnut kernels. Among them, 17 were postulated as key odorants based on OAVs >1. The compounds with the highest OAVs were 3-methylbutan-1-ol, 2-methylbutanal, hexanoic acid, hexanal, and linalool.[62] However, the authors did not substantiate their results with reconstitution experiments.

5 Objectives

As detailed in the introduction, SAFE is a well-established method for volatile isolation. This gentle method allows for the complete removal of non-volatiles while obtaining the volatile fraction with high yields and consistent composition even at trace levels. The use of moderate temperatures prevents artifact formation and compound degradation. However, a critical point of SAFE is the manually operated valve, with the help of which the extract is introduced in small portions into the apparatus. Both, the volume of the individual portions and the time intervals between portions may affect the recovery of the individual volatiles in the volatile isolate. Furthermore, an unintentionally large portion may cause the transfer of non-volatiles, which makes the volatile isolate useless for further analysis. In addition, the operation of the valve requires constant manpower. In order to overcome these drawbacks, the first part of the study aimed at developing a SAFE device with an automated valve while maintaining all the positive features of the original approach. For evaluation, experiments with model solutions and in food matrix were performed. The new SAFE device was then upgraded to fully automatic operation.

The SAFE apparatus with the automated valve was used in the second part of the study to isolate the volatiles of fresh walnut kernels.

As mentioned in the introduction, walnut kernels are appreciated by consumers not only for their nutritional value, but especially for their pleasant aroma. Although walnut volatiles have been investigated in detail, only few studies have addressed walnut odorants. In the last 50 years, the molecular background of the fresh walnut kernel aroma was not unravelled. Thus, the objective of the second part of the study was to identify the key odorants in fresh walnut kernels by applying the following steps: (1) screening for odorants by AEDA, (2) exact quantitation of odorants and calculation of OAVs, (3) aroma reconstitution as proof of success and finally omission tests. Further analyses provided insights into the concentrations of walnut key odorants in other tree nuts and into changes in walnut odorants during aging of fresh walnut kernels.

6 Results and Discussion

This thesis is a publication-based dissertation. The data is summarized in two peer-reviewed articles published in international scientific journals. Copies of the two publications, summaries including the individual authors' contributions, and the publishers' reprint permissions are available in the appendix.

6.1 Automated Solvent-Assisted Flavor Evaporation

6.1.1 Development of the Automated Solvent-Assisted Flavor Evaporation

SAFE was developed by Engel et al. in 1999 and soon became state-of-the-art for the removal of non-volatiles, especially prior to the screening of the volatiles for odorants by GC–O. For this purpose, SAFE is typically applied to solvent extracts obtained from food.[15] SAFE featured better removal of non-volatiles, higher yields, and reduced bench space requirements than previous techniques such as HVT. The setup of the SAFE is described in section 4.2. During continuous use in our laboratory, we have noticed a few limitations of the SAFE, all of which are due to the manual valve used to introduce the extract portions into the apparatus. To improve SAFE, we aimed to preserve all beneficial aspects of the original approach while replacing the manual valve with an automated valve. Hereinafter, the original SAFE approach will be referred to as manual SAFE (mSAFE) because a manually operated valve was used and for the newly developed method the term automated SAFE (aSAFE) will be used because it included an automated valve.

The mSAFE device served as the basis for the development of the new aSAFE device (Figure 19). The middle part of the apparatus including the connection to the evaporation flask (thermostated at 40 °C) and to the recondensation flask (cooled to −196 °C with liquid nitrogen) remained unchanged. The safety cold trap connected to the vacuum pump was also maintained. The manual valve was replaced with a pneumatic valve that was controlled by an electronic control unit. A close-up of the pneumatic valve in its position on the aSAFE device is depicted in Figure 20. The pneumatic valve was originally supplied with a plunger that did not provide the necessary tightness when the apparatus was evacuated. Therefore, it was replaced by a plunger with a tapered tip and an elastomeric O-ring custom-made of PTFE. The glass casing of the plunger was adjusted to the new shape. The plunger position at the pneumatic valve can be adjusted with the adjustment screw at the end of the pneumatic valve. A set screw can be used to lock the adjustment screw in position. An additional modification addressed the glass tube which connects the body of the dropping funnel with the pneumatic valve. Its inner diameter was decreased from ~7 mm to 1 mm. This adjustment allows for the introduction of smaller extract portions into the apparatus. The length of the glass tube was standardized to 4 cm. When the glassware is evacuated and the valve is open, an extract flow rate of 3 mL/s is achieved. To mechanically stabilize the pneumatic valve, which is substantially heavier than the manual valve, an additional glass rod connects the plunger case to the body of the dropping funnel. The pneumatic valve is controlled by an electronic control unit, which is supplied with compressed air and connected to the pneumatic valve with PTFE tubing. The control unit is depicted in Figure 21. With the buttons on the control unit, the open and closed time combination can be set in intervals of 0.1 s and the mode of operation can be switched between manual and automatic.

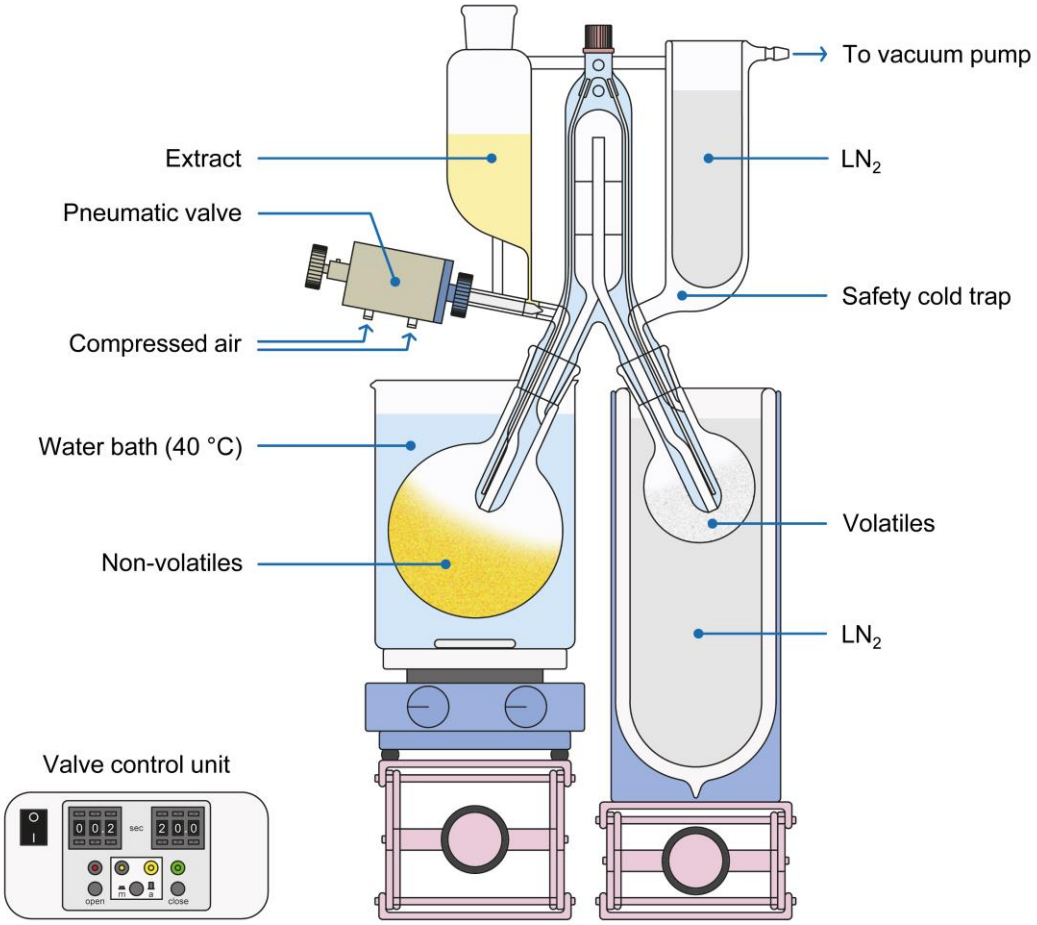


Figure 19: The aSAFE device

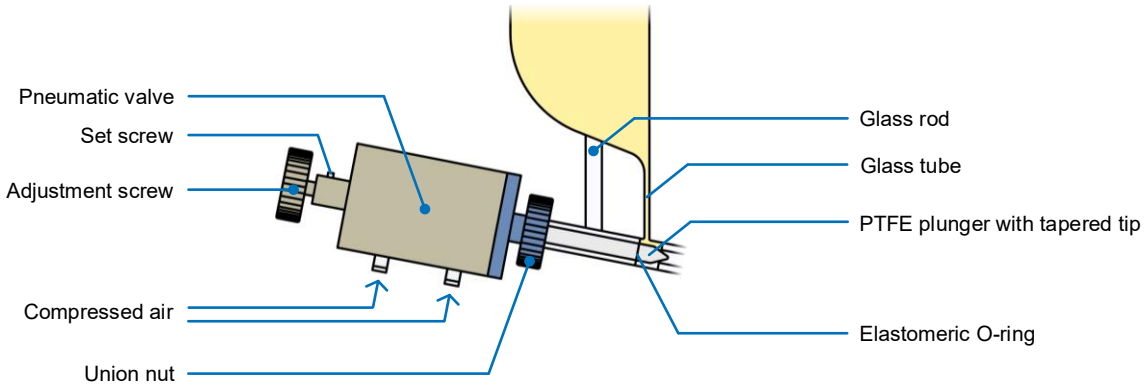


Figure 20: Close-up view of the pneumatic valve on the aSAFE device

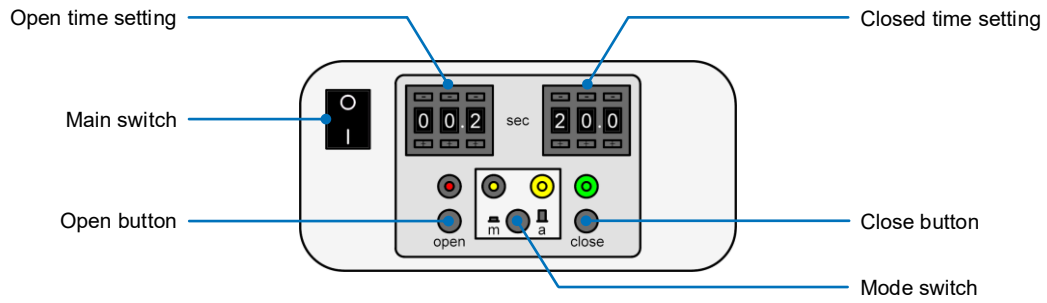


Figure 21: Electronic control unit of the pneumatic valve

To set up the aSAFE in the laboratory, the glass apparatus is mounted to a lattice lab system on the bench, just like the mSAFE. The recondensation flask and the evaporation flask are connected to the apparatus. Thermostatization of the middle part and the evaporation flask is applied and set to 40 °C. Before evacuating the apparatus, the electronic control unit is set to manual operation and the correct operation of the valve is checked with the open and close buttons. Finally, with the valve closed, the apparatus is connected to the vacuum. The Dewar flask around the recondensation flask and the safety cold trap of the glassware are filled with liquid nitrogen. The settings for the valve open and closed times are selected. The closed time should allow for the complete evaporation of the solvent from the previous extract portion. It depends on the lipid content of the extract and the size of the extract portion which in turn depends on the valve open time. To initiate the aSAFE process, the extract is filled into the dropping funnel and the electronic control unit is switched from manual to automatic mode. Now, the valve automatically switches between the open and closed positions according to the settings. Liquid nitrogen needs to be refilled manually whenever necessary. To stop the aSAFE process when the dropping funnel is almost empty, the electronic control unit is switched from automatic mode back to manual mode. After the liquid nitrogen cooling has been removed from the recondensation flask, the apparatus is vented, and the recondensation flask containing the volatile isolate is removed from the apparatus. The entire aSAFE process is documented in a video available on the Internet.[63]

By replacing the manual valve with an automatic valve, the operation of the SAFE was substantially simplified. Extract addition is automated and constant in terms of the volume of the individual extract portions and the total duration of the process. The extract portion volume can be adapted to specific requirements by adjusting the open time. To ensure exhaustive evaporation of the volatile components, the closing time can be adjusted accordingly. Both, open and closed times, can be adjusted in intervals of 0.1 s. The automatization and the selection of small extract portions lead to a reduction of non-volatile carry-overs. Manpower is still required to initiate the aSAFE process, but there is no need to manually open and close the valve anymore. However, it is still necessary to manually refill liquid nitrogen from time to time and to manually stop the aSAFE process before the dropping funnel is totally empty in order to prevent uncontrolled venting.

6.1.2 Evaluation of the Automated Solvent-Assisted Flavor Evaporation

To evaluate the aSAFE approach, we prepared three model mixtures with different fat contents (non-fat, low-fat, high-fat). These fat levels were chosen to represent solvent extracts from non-fat foods, such as fruits, vegetables, and beverages; from low-fat foods, such as milk and meat; and from high-fat foods, such as chocolate and nuts. The model mixtures included 18 characteristic food odorants. All 18 compounds had previously been identified as important contributors to the aroma of various foods and were characterized by their substance classes, boiling points, log P values, and retention indices, all of which covered a typical range. Chemical stability and baseline separation in the GC approach selected for quantitative analysis were also crucial for the feasibility of the experiment. Heptadecane was used as an internal standard. The 18 odorants and the internal standard with their characteristics are depicted in Figure 22. They were present in the model mixtures at concentrations suitable for direct analysis by GC–FID. For evaluation, the odorant yields of the aSAFE approach were compared with those of the mSAFE.

During mSAFE, the operators followed instructions to open the manual valve briefly and close it as fast as possible, ensuring small portion sizes. The process was monitored by observing the evaporation flask and liquid nitrogen around the recondensation flask to ensure complete evaporation and recondensation. The settings for the aSAFE approach were a valve open time of 0.2 s combined with a valve closed time of 5 s, 20 s, or 60 s for the individual experiments. This resulted in 12 model mixture/SAFE approach combinations. Among them, the high-fat mixture was not suitable for the aSAFE with a valve open/closed time combination of 0.2 s/5 s due to incomplete solvent evaporation within 5 s closed time.

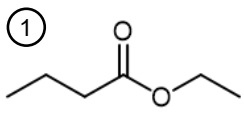
The overall highest yields were obtained with the non-fat model mixture (Figure 23a). For compounds **1**–**13**, (boiling points from 120 to 220 °C), the yields were ~100% in both, mSAFE (Figure 23a, yellow bars) and aSAFE (Figure 23a, blue bars). In contrast, compounds **14**–**18** (boiling points ≥254 °C), exhibited differences in yields between mSAFE and aSAFE. For mSAFE, consistently lower yields were obtained than for aSAFE. This is most probably due to the smaller extract portion volumes in aSAFE. For example, eugenol (**14**) and methyl cinnamate (**15**) showed yields of ~100% with aSAFE, while the yields of mSAFE were only ~80%. Ethyl cinnamate (**17**) and β-ionone (**18**) had yields >90% with aSAFE, but only ~60% with mSAFE. Notably, the lowest yields were not observed for β-ionone (**18**), the compound with the highest boiling point, but for phenylacetic acid (**16**). The yields obtained with mSAFE were only ~20%; with aSAFE, at least yields of 50–60% were obtained. This suggested that SAFE yields not only depend on the SAFE method and the boiling point of the compound but also on the compound class.

The low-fat model mixture (Figure 23b) generally yielded less than the non-fat model mixture (Figure 23a). Until limonene (**8**) with a boiling point of 177 °C, mSAFE achieved yields of ~100%, except for butanoic acid (**5**) which yielded ~90%. Beyond limonene (**8**), the yields obtained with mSAFE decreased from ~80% (**9**) to only ~6% (**18**). The yield of phenylacetic acid (**16**) was only 1%. Notably, the carboxylic acids, namely butanoic acid (**5**), hexanoic acid (**10**), and phenylacetic acid (**16**) had lower yields than expected from their boiling points, a trend observed consistently in both mSAFE and aSAFE experiments.

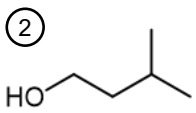
In the low-fat model mixture, aSAFE consistently outperformed mSAFE, especially with longer valve closed times. Compounds **9**–**13**, which yielded 40–80% with mSAFE, showed yields of ~100% using aSAFE (0.2 s/60 s). The yields of eugenol (**14**) and methyl cinnamate (**15**) increased from ~20% (mSAFE) to 80% (aSAFE; 0.2 s/60 s), whereas those of ethyl cinnamate (**17**) and β-ionone (**18**) increased from ~10% to ~60%. For phenylacetic acid (**16**), which yielded only 1% with the mSAFE approach, aSAFE with a closed time of 60 s resulted in a yield of ~40%. However, the differences associated with different closed times were smaller compared to the differences between mSAFE and aSAFE. Thus, reducing the individual extract portion size had a greater impact on the yields than extending the time between two subsequent portions.

The yields obtained from the high-fat model mixture (Figure 23c) using mSAFE were almost identical to the yields of the low-fat model mixture (Figure 23b). However, the aSAFE yields were slightly lower for high-boiling point compounds. In all cases, aSAFE consistently outperformed mSAFE. In terms of reproducibility, there was no distinct difference between mSAFE and aSAFE. Small error bars were typical when the yields were ~100%, while larger error bars were observed with decreasing yields for both, mSAFE and aSAFE.

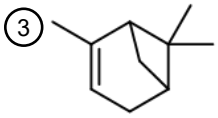
To further evaluate the aSAFE approach, odorant yields were additionally determined from authentic food extracts. Beer was chosen as a non-fat food and chocolate as a high-fat food. We quantitated linalool (**9**), 2-phenylethanol (**13**), and phenylacetic acid (**16**) since they are important odorants in both, beer[64] and chocolate.[65] We compared the mSAFE approach to aSAFE with a valve open/closed time combination of 0.2 s/60 s. The results are depicted in Figure 24 and Figure 25, alongside yields from the corresponding model mixtures for comparison. Comparable yields were obtained from the non-fat model mixture (Figure 24a) and the beer extract (Figure 24b). In both, the model mixture and the food experiment, linalool (**9**) and 2-phenylethanol (**13**) yields were close to 100%. Even higher yields were achieved from the beer extract than from the model mixture for phenylacetic acid (**16**). Comparable yields were also obtained for the high-fat model mixture (Figure 25a) and the chocolate extract (Figure 25b). While aSAFE yields were almost indistinguishable, mSAFE yields for linalool (**9**) and 2-phenylethanol (**13**) were lower from the chocolate extract than from the model mixture, but slightly higher for phenylacetic acid (**16**). However, for all three compounds, aSAFE performed consistently better than mSAFE.



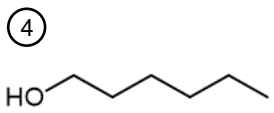
Ethyl butanoate
Odor: fruity
B.p. (°C): 120
Log P: 2.85
RI FFAP: 1027



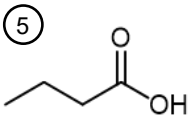
3-Methylbutan-1-ol
Odor: malty
B.p. (°C): 130
Log P: 1.35
RI FFAP: 1206



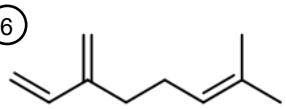
α-Pinene
Odor: resinous
B.p. (°C): 156
Log P: 4.44
RI FFAP: 1009



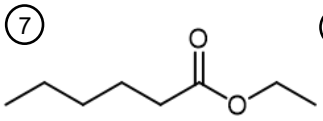
Hexan-1-ol
Odor: grassy
B.p. (°C): 157
Log P: 2.03
RI FFAP: 1350



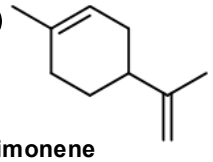
Butanoic acid
Odor: cheesy, sweaty
B.p. (°C): 162
Log P: 1.10
RI FFAP: 1620



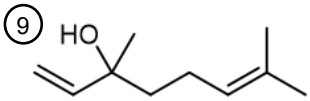
Myrcene
Odor: geranium leaf
B.p. (°C): 167
Log P: 4.82
RI FFAP: 1156



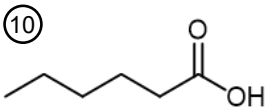
Ethyl hexanoate
Odor: fruity, pineapple
B.p. (°C): 168
Log P: 2.83
RI FFAP: 1226



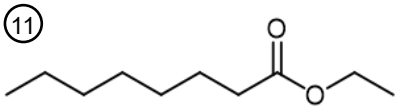
Limonene
Odor: citrusy, lime
B.p. (°C): 177
Log P: 4.38
RI FFAP: 1189



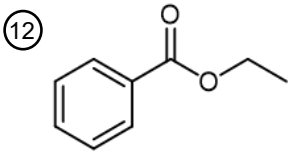
Linalool
Odor: citrusy, bergamot
B.p. (°C): 199
Log P: 2.84
RI FFAP: 1539



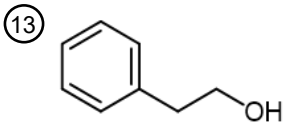
Hexanoic acid
Odor: cheesy, sweaty
B.p. (°C): 203
Log P: 1.75
RI FFAP: 1836



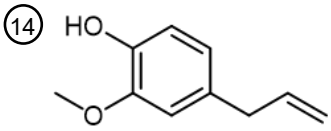
Ethyl octanoate
Odor: fruity
B.p. (°C): 207
Log P: 4.47
RI FFAP: 1441



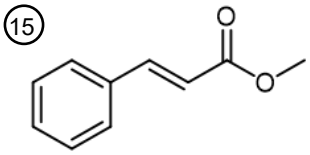
Ethyl benzoate
Odor: fruity, star fruit
B.p. (°C): 212
Log P: 2.59
RI FFAP: 1658



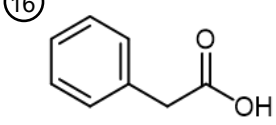
2-Phenylethanol
Odor: floral, honey
B.p. (°C): 220
Log P: 1.30
RI FFAP: 1905



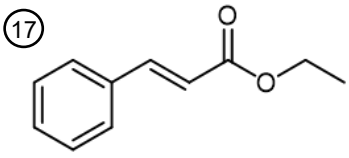
Eugenol
Odor: clove
B.p. (°C): 254
Log P: 1.83
RI FFAP: 2164



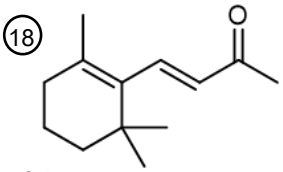
Methyl cinnamate
Odor: sweet, cinnamon
B.p. (°C): 261
Log P: 2,62
RI FFAP: 2056



Phenylacetic acid
Odor: beeswax
B.p. (°C): 265
Log P: 1.40
RI FFAP: 2555



Ethyl cinnamate
Odor: sweet, fruity
B.p. (°C): 271
Log P: 2.90
RI FFAP: 2116



β-Ionone
Odor: floral, violet
B.p. (°C): 271
Log P: 4.00
RI FFAP: 1933

Heptadecane
Odor: odorless
B.p. (°C): 302
Log P: 8.69
RI FFAP: 1700

Figure 22: The 18 food odorants in the model mixtures and the internal standard (IS) sorted by their boiling points

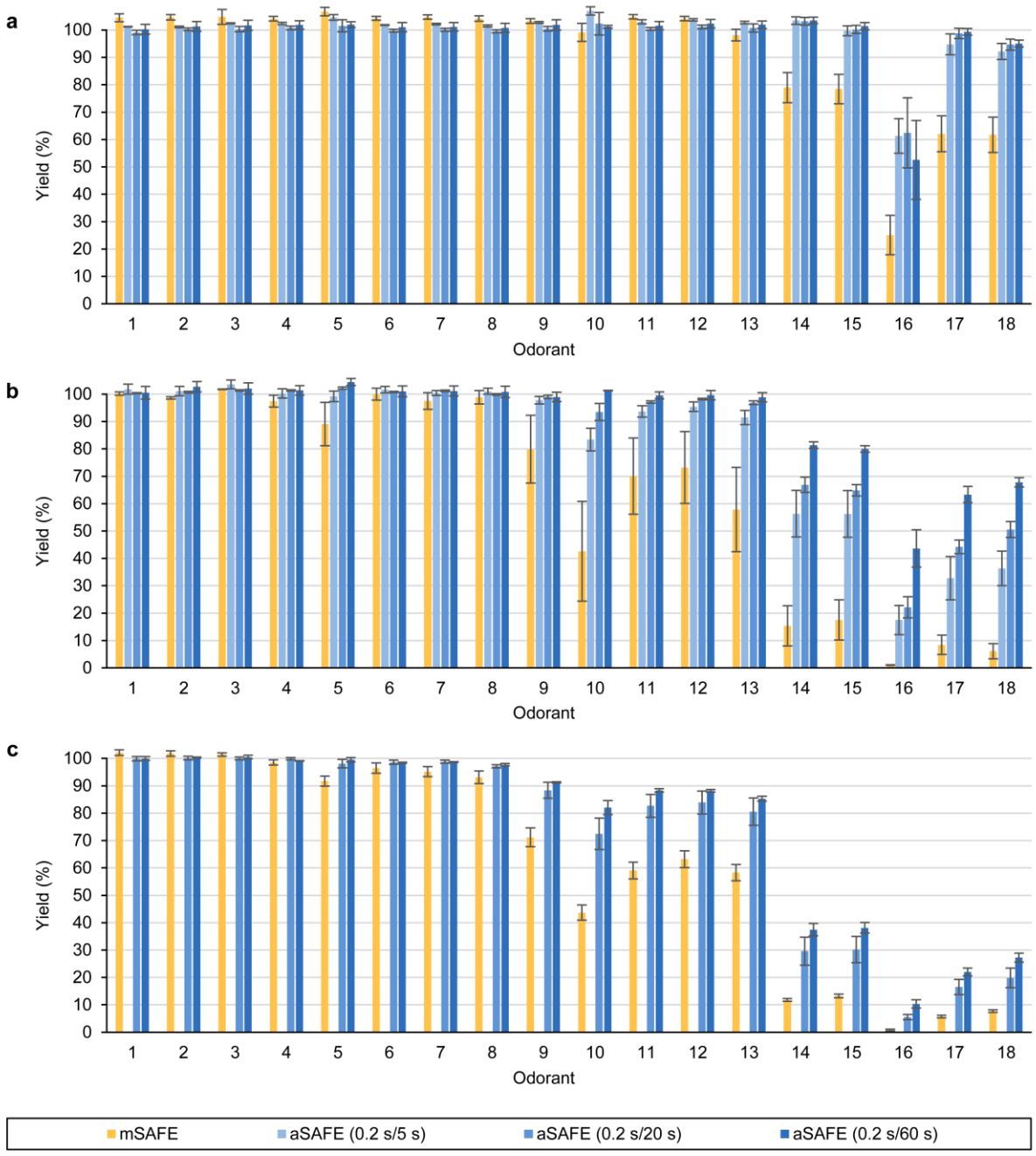


Figure 23: Odorant yields obtained with the aSAFE approach with different valve open (0.2 s)/closed (5 s, 20 s, 60 s) time combinations compared to the odorant yields obtained with the mSAFE approach; the evaluation was carried out using three model mixtures with different fat contents: a) non-fat; b) low-fat (100 mL non-fat mixture + 1 g oil); c) high-fat (100 mL non-fat mixture + 10 g oil); the odorant structures are detailed in Figure 22

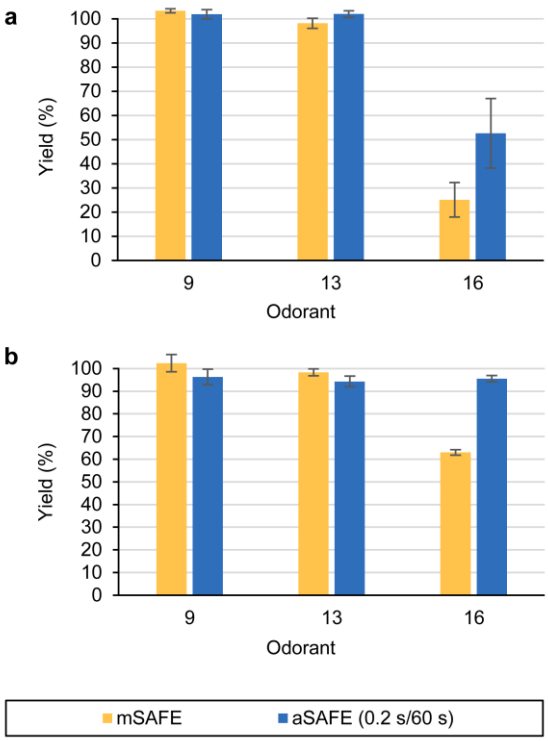


Figure 24: Yields of linalool (**9**), 2-phenylethanol (**13**), and phenylacetic acid (**16**) obtained by aSAFE with a valve open/closed time combination of 0.2 s/60 s applied to a) the non-fat model mixture and b) the beer extract compared to the yields obtained by mSAFE

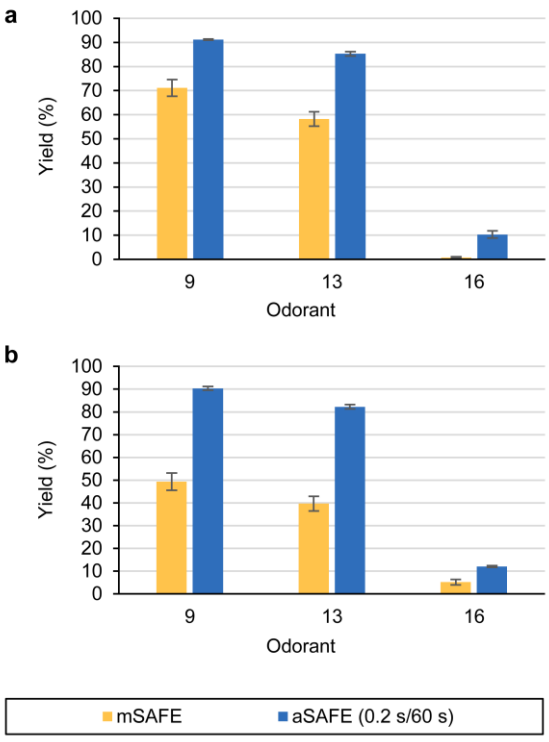


Figure 25: Yields of linalool (**9**), 2-phenylethanol (**13**), and phenylacetic acid (**16**) obtained by aSAFE with a valve open/closed time combination of 0.2 s/60 s applied to a) the high-fat model mixture and b) the chocolate extract compared to the yields obtained by mSAFE

6.1.3 Fully Automated Solvent-Assisted Flavor Evaporation

The new aSAFE approach effectively addressed two significant limitations of the mSAFE approach described in the objectives. It resulted in a significant increase in yields and eliminated the risk of non-volatile carry-overs caused by too large extract portions. However, the reduction in manpower requirements was only partial. An operator is required to refill the cold traps with liquid nitrogen from time to time and to switch from automatic mode to manual mode at the electronic control unit shortly before the dropping funnel is totally empty. If the aSAFE process is not stopped in time, damages to the vacuum system are possible as a result of uncontrolled ventilation of the apparatus.

To overcome the above-mentioned limitations and provide a fully automated SAFE version, we integrated an automated liquid nitrogen refill system and an endpoint recognition and shut-off system (Figure 26). The easiest way to supply both, the safety cold trap between the recondensation flask and the vacuum pump as well as the evaporation flask with liquid nitrogen, was to combine them in a single liquid nitrogen bath. Thus, the safety cold trap was separated from the aSAFE apparatus and placed in a custom-made Dewar vessel filled with liquid nitrogen together with the recondensation flask. The final automated nitrogen refill system consisted of several components including a pressurized liquid nitrogen storage tank equipped with a siphon, a solenoid valve, a transfer hose, a nozzle containing a phase separator, a Dewar vessel with a custom-made lid (Figure 27), and two liquid nitrogen sensors connected to an electronic control unit. At the electronic control unit, manual and automatic mode are selectable. During the automatic mode, the operation of the solenoid valve is controlled by the liquid nitrogen level. For endpoint detection and shutdown of the aSAFE process, a capacitive sensor was integrated into the glass apparatus and controlled by another electronic control unit. Therefore, the glassware needed some adjustment at the glass tube connecting the body of the dropping funnel with the valve. The sensor was cased in a glass cylinder.

To set up the fully automated SAFE, the glass apparatus is mounted to a lattice lab system on a lab bench. Equal to the setup of the aSAFE, the pneumatic valve and the evaporation flask are connected and thermostatization of the evaporation flask and the middle part of the glass device is provided. The Dewar vessel is placed next to the lattice lab system on the lab bench. The recondensation flask is adjusted to the glass apparatus and placed in the appropriate hole on top of the Dewar vessel lid. The pressurized liquid nitrogen storage tank is placed near the Dewar vessel. The liquid nitrogen nozzle of the transfer hose, the liquid nitrogen sensors, and the safety cold trap are placed into the appropriate holes of the Dewar vessel lid. The safety cold trap is connected to the glass apparatus and to the vacuum pump. The liquid nitrogen sensors are connected to the electronic control unit. The further procedure for starting the fully automated SAFE is the same as in the aSAFE process. The liquid nitrogen control unit is switched from manual to automatic mode to start the automatic refill system.

The liquid nitrogen level control unit is designed to maintain a constant cooling environment for the recondensation flask and the safety cold trap. Whenever the liquid nitrogen level in the Dewar vessel falls below the lower liquid nitrogen sensor, the control unit automatically opens the solenoid valve. This continues until the upper sensor is covered in liquid nitrogen, thus, ensuring a continuous supply of liquid nitrogen for effective cooling during the complete aSAFE process and, if required, beyond.

The automated valve operates as known from the aSAFE. For the endpoint recognition, a capacitive sensor is used that senses the change in electrical capacity between solvent and air. When the level of the solvent extract falls below the position of the capacitive endpoint sensor, the electronic valve control unit is disconnected from the power supply by the electronic endpoint control unit. The pneumatic valve remains in the closed position, thus the aSAFE process is stopped. The liquid nitrogen cooling continues until the operator manually stops it before ventilating the aSAFE system. A video showing the fully automated SAFE process is also available on the Internet.[66]

The fully automated SAFE equipment offers additional benefits in terms of handling. All the advantages of aSAFE, especially the quality of the volatile isolate, are unchanged when shifting from aSAFE to fully automated SAFE. However, fully automated SAFE provides the additional benefits of an automated liquid nitrogen supply and an endpoint recognition and shut-off system. The required manpower is further reduced as during the fully automated SAFE process no operator is required except for the start and the finish. These advantages save resources and improve safety in the laboratory. Nevertheless, the crucial aspect for analysis is unquestionably the automatic valve, which improves yields. Therefore, it is up to the user to decide whether to accept the additional costs and space requirements in order to benefit from the further advantages of the fully automated SAFE.

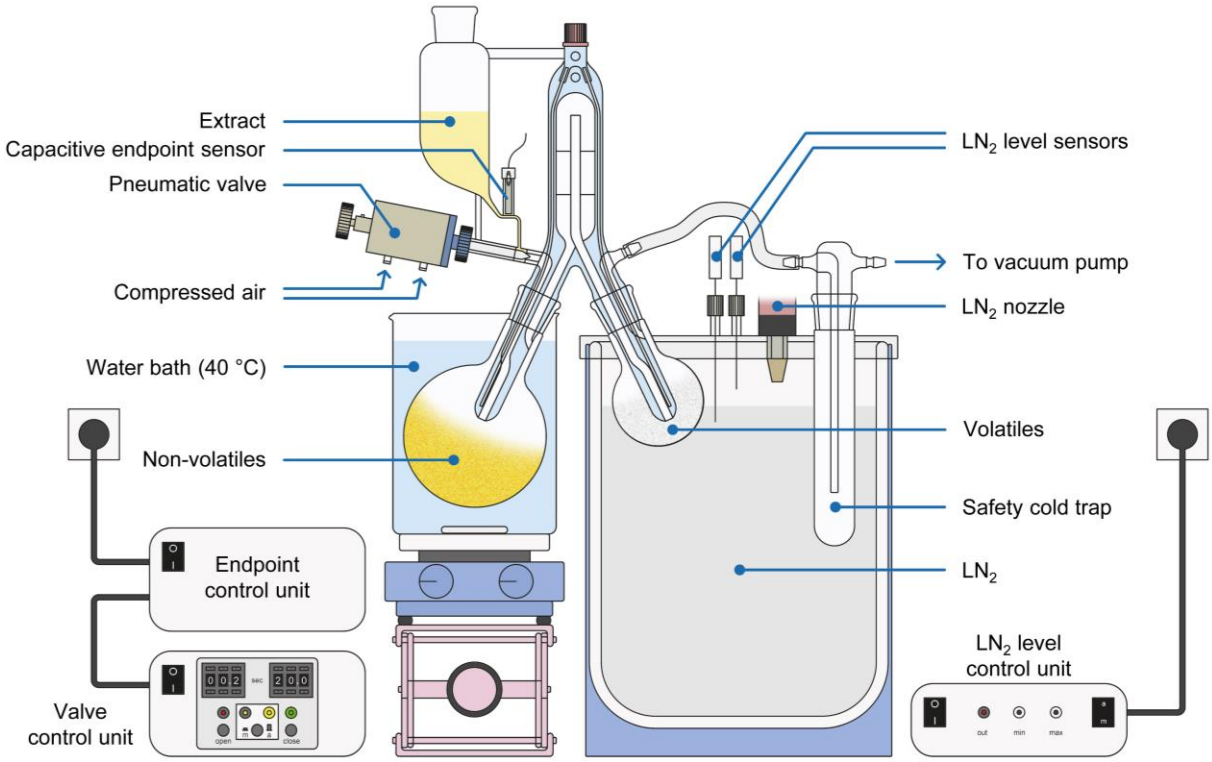


Figure 26: The fully automated SAFE with the liquid nitrogen refill system and the endpoint recognition and shut-off system

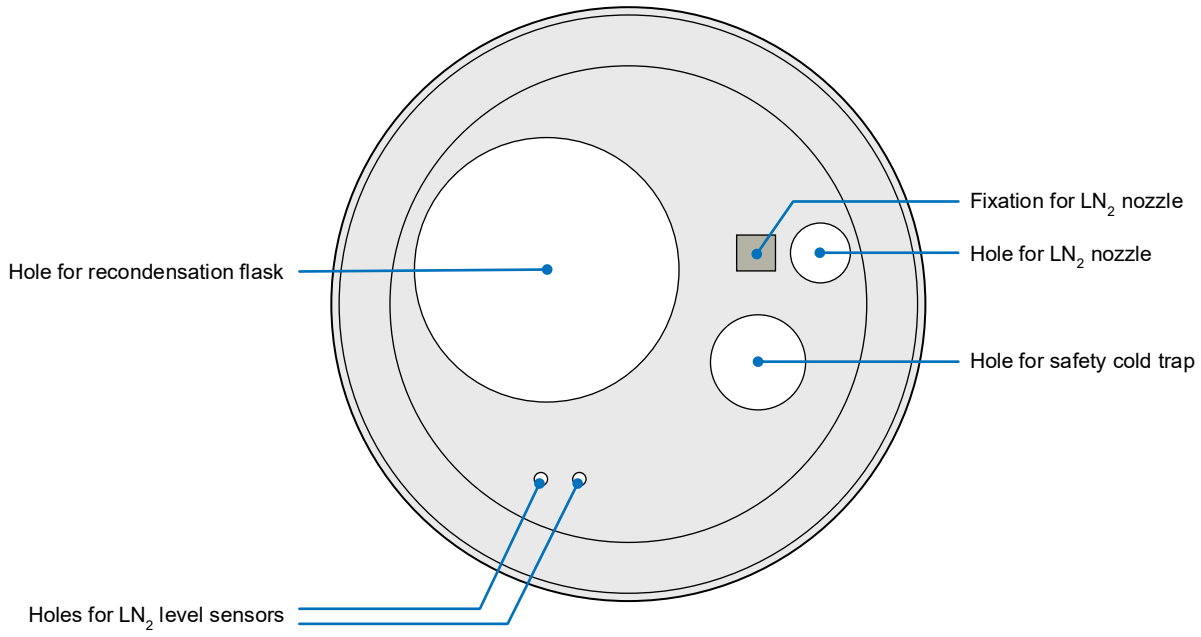


Figure 27: Top view of the Dewar vessel lid

6.2 Key Odorants in Fresh Walnut Kernels

6.2.1 Screening for Odorants

For odorant screening, fresh walnut kernels packaged in an inert gas atmosphere were purchased from a local retail market. The particular brand was selected for the characteristic walnut aroma and the absence of off-flavors.

To obtain a solvent extract, fresh walnut kernels were ground into particles, approximately 1 mm to 3 mm in size, and homogenized with saturated calcium chloride solution to stop further enzymatic reactions,[67] followed by stirring with diethyl ether for 17 h, drying over anhydrous sodium sulfate, and filtration to remove insoluble material.

For AEDA, fresh walnut kernel volatiles were isolated from the solvent extract using aSAFE[68] at 40 °C and a valve open/closed time combination of 0.1 s/10 s. After concentration of the volatile isolate using a Vigreux column and a Bemelmans microdistillation device, the concentrate was analyzed by GC–O in combination with AEDA. The analysis revealed 50 odorants with FD factors ranging from 2 to 1024 (Table 3). During AEDA, no walnut odor was detected at the sniffing-port. This substantiated Clark's and Nursten's hypothesis that walnut odor is caused by a combination of odorants rather than a single odorant.[51, 52]

To achieve preliminary structure assignments for the odorants, the RIs obtained from an FFAP column and the odors perceived at the sniffing port were compared with data compiled in a database.[69] The preliminary assignments were verified by GC–O using authentic reference compounds in appropriate dilutions in parallel with the walnut volatile isolate, first on the FFAP column and second on the DB-5 column. For final confirmation of the structure, the mass spectrum of the compound obtained from the walnut volatile isolate was compared to the mass spectrum of the reference compound obtained under identical conditions. To minimize co-elution problems, the analysis was performed with a GC×GC–MS instrument. Each of the 50 odorants was successfully identified.

The odorant with the highest FD factor of 1024 was the oatmeal-smelling (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**) (Table 3). The second highest FD factor of 512 was obtained for the fenugreek-smelling 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one, better known as sotolon (**46**). The four odorants oct-1-en-3-one (**5**; mushroom-like), 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (HDMF; **40**; caramel), *trans*-4,5-epoxy-(2*E*)-dec-2-enal (**38**; metallic), and 2-methoxyphenol (**31**; smoky) showed FD factors of 256. In decreasing order, 6 odorants respectively had FD factors of 64 (**8**, **9**, **13**, **47**, **49**, **50**) and 32 (**14**, **17**, **24**, **27**, **28**, **34**). FD factors of 16 were determined for 9 odorants (**7**, **10**, **12**, **15**, **19**, **20**, **22**, **23**, **30**) and 23 of the 50 odorants showed FD factors as low as 2–8.

Of the 50 odorants resulting from AEDA, 74% had not previously been reported as walnut volatiles, including (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**) and sotolon (**46**), the odorants with the highest FD factors.[70] That (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal and sotolon have not previously been reported in walnuts may be due to the fact that different sample preparation methods were used compared to the present study. For volatile isolation, headspace techniques were used before GC–O[52, 62] and GC–MS[55, 56]. In the present study, SAFE was used because SAFE is the method of choice for gentle and artifact-free volatile isolation. Liu et al. used SAFE as well for volatile isolation. However, the solvent extract was obtained from a mixture of solvent and

walnut milk, freshly prepared from walnut kernels and water.[61] In the present study, fresh walnuts were coarsely ground and initial enzyme activity was allowed. After inhibition of enzymatic activity, a solvent extract was prepared.

Table 3: Odorants of fresh walnut kernels obtained by AEDA

no.	odorant[a]	odor[b]	RI[c] FFAP	RI[c] DB-5	FD factor[d]
1	butane-2,3-dione	buttery	982	603	2
2	hexanal	green, grassy	1080	802	2
3	γ-terpinene	earthy	1234	1059	4
4	octanal	citrusy	1285	1005	4
5	oct-1-en-3-one	mushroom	1293	979	256
6	2-ethylpyrazine	roasty	1331	916	8
7	(5*Z*)-octa-1,5-dien-3-one	geranium leaf	1364	982	16
8	(2*E*)-oct-2-enal	fatty, citrusy	1419	1061	64
9	3-isopropyl-2-methoxypyrazine	bell pepper	1417	1086	64
10	acetic acid	vinegar	1450	636	16
11	methional	cooked potato	1455	910	4
12	(2*E*,4E)-hepta-2,4-dienal	floral, fatty	1480	1015	16
13	3-*sec*-butyl-2-methoxypyrazine	bell pepper	1496	1167	64
14	(2*Z*)-non-2-enal	fatty, floral	1494	1148	32
15	(2*E*)-non-2-enal	cucumber, green	1532	1163	16
16	2-methylpropanoic acid	sweaty, cheesy	1560	783	8
17	(2*E*,6*Z*)-nona-2,6-dienal	cucumber, green	1584	1154	32
18	undecanal	fatty, floral	1600	1306	8
19	butanoic acid	sweaty, cheesy	1627	827	16
20	(2*E*,4*Z*)-nona-2,4-dienal	fatty	1639	1197	16
21	phenylacetaldehyde	floral, honey	1639	1047	8
22	3-methylbutanoic acid	sweaty, cheesy	1667	863	16
23	2-methylbutanoic acid	sweaty, cheesy	1668	857	16
24	(2*E*,4*E*)-nona-2,4-dienal	fatty	1692	1215	32
25	(2*E*)-undec-2-enal	green, soapy	1747	1362	8
26	α-farnesene	green	1745	1509	8
27	(2*E*,4*Z*)-deca-2,4-dienal	fatty, deep-fried	1752	1296	32
28	(2*E*,4*E*)-deca-2,4-dienal	fatty, deep-fried	1808	1322	32
29	cyclotene	fenugreek	1819	1024	8
30	hexanoic acid	sweaty, cheesy	1838	1015	16
31	2-methoxyphenol	smoky	1862	1087	256
32	(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal	oatmeal	1876	1273	1024
33	(2*E*,4*E*,6*E*)-nona-2,4,6-trienal	oatmeal	1895	1285	2
34	γ-octalactone	coconut	1918	1255	32
35	β-ionone	floral, raspberry	1928	1480	4
36	δ-octalactone	coconut	1967	1292	4
37	maltol	caramel	1974	1114	4
38	*trans*-4,5-epoxy-(2*E*)-dec-2-enal	metallic	2004	1382	256
39	4-methoxybenzaldehyde	aniseed, woodruff	2031	1259	8

Table 3 continued:

no.	odorant[a]	odor[b]	RI[c] FFAP	RI[c] DB-5	FD factor[d]
40	HDMF[e]	caramel	2033	1087	256
41	EHMF[f]	caramel	2077	1139/1148[g]	8
42	4-hydroxy-5-methylfuran-3-one	fruity, caramel	2127	1065	4
43	γ-decalactone	coconut	2133	1496	4
44	eugenol	clove	2169	1354	8
45	(2*Z*,4*Z*)-δ-deca-2,4-dienolactone	sweet, coconut	2170	1459	8
46	sotolon	fenugreek	2205	1111	512
47	2′-aminoacetophenone	foxy	2222	1304	64
48	(6*Z*)-γ-dodec-6-enolactone	sweet, fruity	2389	1658	4
49	2-phenylacetic acid	floral, honey	2553	1267	64
50	vanillin	vanilla	2573	1400	64

[a]Each odorant was identified by comparing its retention indices on two GC columns of different polarity (FFAP, DB-5), its mass spectrum obtained by GC–MS, as well as its odor as perceived at the sniffing port during GC–O to data obtained from authentic reference compounds analyzed in parallel. [b]Odor as perceived at the sniffing port during GC–O. [c]Retention index; calculated from the retention time of the compound and the retention times of adjacent *n*-alkanes by linear interpolation. [d]Flavor dilution factor; dilution factor of the highest diluted walnut volatile isolate in which the odorant was detected during GC–O analysis by any of two assessors. [e]4-Hydroxy-2,5-dimethylfuran-3(2*H*)-one. [f]2-Ethyl-4-hydroxy-5-methylfuran-3-one. [g]EHMF is separated from its tautomer 5-ethyl-4-hydroxy-2-methylfuran-3-one on the DB-5 column, on the DB-FFAP column no separation of the isomers was observed.

6.2.2 Quantitation of Odorants and Calculation of Odor Activity Values

Toward identification of the key odorants in fresh walnut kernels, the major odorants resulting from AEDA were quantitated and their OAVs were calculated.

Fresh walnut kernels were ground to a particle size of approximately 1 mm to 3 mm and then homogenized with saturated calcium chloride solution and finally diethyl ether. To compensate for losses during sample preparation, stable isotopically substituted analogues of the odorants were added to the diethyl ether portion as internal standards. The solvent extract was dried over anhydrous sodium sulfate, freed from insoluble material by filtration, and subjected to aSAFE to remove all non-volatiles.[68] A valve open/closed time combination of 0.1 s/10 s was used. After concentration, the volatile isolate was analyzed by heart-cut GC–GC–MS/(CI) or GC×GC–MS/(EI). A calibration line equation was used to calculate concentrations from the amount of fresh walnut kernels, the amount of added internal standard, and the integrated peak areas of the analyte and internal standard. Isotopologues were available for 23 odorants. Since no isotopologues were available for odorants **20**, **23**, **27**, and **32**, isotopologues of the isomeric compounds **24**, **22**, **28**, and **33** were used instead in the quantitation assays.

Quantitation of the 27 odorants with FD factors ≥16 (Table 3) yielded concentrations in the fresh walnut kernels ranging from 0.0206 g/kg to 44200 g/kg (Table 4). The odorants with the highest concentrations were acetic acid (**10**; 44200 µg/kg) and hexanoic acid (**30**; 2870 µg/kg), followed by (2*E*)-oct-2-enal (**8**; 439 µg/kg), butanoic acid (**19**; 184 µg/kg), (2*E*,4*E*)-deca-2,4-dienal (**28**; 178 µg/kg), (2*E*)-non-2-enal (**15**; 121 µg/kg), 3-methylbutanoic acid (**22**; 118 µg/kg), vanillin (**50**; 105 µg/kg), phenylacetic acid (**49**; 90.2 µg/kg), *trans*-4,5-epoxy-(2*E*)-dec-2-enal

(**38**; 55.7 µg/kg), 2-methylbutanoic acid (**23**; 52.6 µg/kg), (2*E*,4*Z*)-deca-2,4-dienal (**27**; 46.7 µg/kg), (2*E*,4*E*)-nona-2,4-dienal (**24**; 36.6 µg/kg), and (2*E*,4*E*)-hepta-2,4-dienal (**12**; 13.3 µg/kg). The two odorants with the highest FD factors in the AEDA, (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**) and sotolon (**46**), with 10.2 µg/kg and 10.6 µg/kg, respectively, yielded concentrations in the same range. At the low end, 5 odorants were found with a concentration of 0.1–10 µg/kg and 3 odorants with a concentration ≤0.1 µg/kg.

To have the potential to contribute to the overall aroma of fresh walnut kernels, an odorant must normally show a concentration exceeding its odor threshold concentration (OTC) in the food matrix.[1] Therefore, the OAV was calculated for each odorant as the quotient of its concentration in the fresh walnut kernel and its OTC determined in oil. Among the 27 quantitated odorants, 17 odorants exceeded their OTCs in fresh walnut kernels, resulting in OAVs ≥1 (Table 4).

The highest OAVs were calculated for acetic acid (**10**; OAV 130), sotolon (**46**; OAV 46), (2*E*,4*Z*)-deca-2,4-dienal (**27**; OAV 17), 3-methylbutanoic acid (**22**; OAV 13), followed by (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**; OAV 9.3), hexanoic acid (**30**; OAV 6.2), and butanoic acid (**19**; OAV 5.4). The odorants *trans*-4,5-epoxy-(2*E*)-dec-2-enal (**38**), (2*Z*)-non-2-enal (**14**), (2*E*)-oct-2-enal (**8**), 2-phenylacetic acid (**49**), (2*E*,4*E*)-deca-2,4-dienal (**28**), 2-methoxyphenol (**31**), 3-isopropyl-2-methoxypyrazine (**9**), (5*Z*)-octa-1,5-dien-3-one (**7**), (2*E*,4*E*)-nona-2,4-dienal (**24**), and oct-1-en-3-one (**5**) showed OAVs >1 but <5. The concentrations of 10 odorants were found to be below their OTCs, indicating that they are unlikely to play a significant role in the aroma of fresh walnut kernels.

In fact, OAVs are a better approximation for the importance of odorants than FD factors because they are based on exact quantitative data, take into account the specific volatility of each odorant, and consider the individual release behavior as the threshold concentrations are determined in an appropriate matrix.[1] In our study, OTCs were determined in oil because fresh walnut kernels consist of 62.5% fat.[46] However, in this approach, the role of the carboxylic acids may have been overestimated considering that a major part of these odorants would be deprotonated in the natural matrix and therefore not able to contribute to the odor. The minor aqueous phase included in fresh walnut kernels showed a pH of 6.5, which is clearly beyond the pKa values of the carboxylic acids, which are in the range of 4.75–5.0.

Given the overestimation of the carboxylic acids, the importance of the other odorants with high OAVs, in particular, sotolon (**46**; OAV 46), (2*E*,4*Z*)-deca-2,4-dienal (**27**; OAV 17), and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**; OAV 9.3) was emphasized. However, their actual contribution to the walnut aroma remained to be revealed in sensory tests.

Table 4: Concentrations, OTCs, and OAVs of important odorants in fresh walnut kernels

no.[a]	odorant	concentration in walnuts[b] (µg/kg)	odor threshold concentration[c] (µg/kg)	OAV[d]
10	acetic acid	44200	350	130
46	sotolon	10.6	0.23	46
27	(2*E*,4*Z*)-deca-2,4-dienal	46.7	2.8[e]	17
22	3-methylbutanoic acid	118	9.0	13
32	(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal	10.2	1.1	9.3
30	hexanoic acid	2870	460	6.2
19	butanoic acid	184	34	5.4
38	*trans*-4,5-epoxy-(2*E*)-dec-2-enal	55.7	13	4.3
14	(2*Z*)-non-2-enal	13.6	3.6	3.8
8	(2*E*)-oct-2-enal	439	120	3.7
49	2-phenylacetic acid	90.2	26	3.5
28	(2*E*,4*E*)-deca-2,4-dienal	178	66	2.7
31	2-methoxyphenol	3.98	1.8	2.2
9	3-isopropyl-2-methoxypyrazine	0.0206	0.010	2.1
7	(5*Z*)-octa-1,5-dien-3-one	0.0659	0.044	1.5
24	(2*E*,4*E*)-nona-2,4-dienal	36.6	30	1.2
5	oct-1-en-3-one	7.42	6.9	1.1
15	(2*E*)-non-2-enal	121	140	<1
50	vanillin	105	140	<1
40	HDMF[f]	12.8	25	<1
23	2-methylbutanoic acid	52.6	110	<1
47	2′-aminoacetophenone	7.80	21	<1
13	3-*sec*-butyl-2-methoxypyrazine	<0.10	0.46	<1
20	(2*E*,4*Z*)-nona-2,4-dienal	3.48	16[e]	<1
17	(2*E*,6*Z*)-nona-2,6-dienal	8.76	65	<1
34	γ-octalactone	11.5	280	<1
12	(2*E*,4E)-hepta-2,4-dienal	13.3	710	<1

[a]Numbering according to Table 3. [b]Mean of duplicates or triplicates; individual concentrations and standard deviations are available in the Supporting Information file of Publication 2[70]. [c]Odor threshold concentrations determined in low odor sunflower oil. [d]Odor activity value; calculated as ratio of concentration to odor threshold concentration. [e]Approximated from the odor threshold concentration of the (2*E*,4*E*)-isomer in low odor sunflower oil and the ratio of the odor threshold concentrations of the individual isomers in air (Supporting Information file of Publication 2[70]). [f]4-Hydroxy-2,5-dimethylfuran-3(2*H*)-one.

6.2.3 Sensory Tests

To finally identify the key odorants responsible for the aroma of fresh walnut kernels, aroma reconstitution and omission experiments were performed. The walnut matrix was mimicked by a model mixture on the basis of odorless silicone oil; the water content (3.5%) and the pH (6.5) were adjusted with an aqueous buffer solution (H_2PO^{4-}/HPO_4^{2-}). The concentrations of the odorants corresponded to their natural concentrations in fresh walnut kernels, as detailed in Table 4.

The first reconstitution model (RM 1) consisted of all 17 odorants for which OAVs ≥1 had been calculated in the fresh walnut kernels. In the second reconstitution model (RM 2), 12 odorants

with relatively low OAVs (1.1–6.2) were omitted. RM 2 was supposed to include only the 5 odorants with the highest OAVs of 9.3–130. These 5 odorants were acetic acid (**10**; OAV 130), sotolon (**46**; OAV 46), (2*E*,4*Z*)-deca-2,4-dienal (**27**; OAV 17), 3-methylbutanoic acid (**22**; OAV 13), and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**; OAV 9.3). Despite its low OAV, (2*E*,4*E*)-deca-2,4-dienal (**28**; OAV 2.7) had to be included in RM 2 because the reference of (2*E*,4*Z*)-deca-2,4-dienal (**27**; OAV 17) contained the isomer (2*E*,4*E*)-deca-2,4-dienal as an impurity. Thus, (2*E*,4*E*)-deca-2,4-dienal was made up to its natural concentration. The odors of both isomers are virtually identical and have a fatty, deep-fried character. The two aroma reconstitution models RM 1 and RM 2 were evaluated orthonasally by a trained sensory panel in comparison to fresh walnut kernels. The intensity of the “walnut” character was rated by the assessors on a scale from 0 to 3 with 0.5 increments and 0 = not perceptible, 1 = weak, 2 = moderate, and 3 = strong. RM 2, which contained only 6 odorants was rated 2.1. This was significantly higher than the voting of RM 1, which was rated 1.6 (Table 5). Based on this observation, it was assumed that the odorants responsible for the characteristic walnut odor were among the six odorants in RM 2.

Given that the screening by AEDA supported Clark’s and Nursten’s postulation that no single compound alone is able to evoke a walnut aroma, at least 2 odorants would be needed. Therefore, 10 binary mixtures resulting from the 6 compounds of RM 2 were presented to the sensory panel. Odorants **27** and **28** were again treated as a single compound. Reconstitution models RM 3–12 scored “walnut” intensity ratings from 0.1 to 2.3. RM 3–5 and RM 7–10 were rated ≤1, thus were hardly walnut-like. RM 11 contained the fatty, deep-fried smelling deca-2,4-dienal isomers **27** and **28** and the fenugreek smelling sotolon and was rated 1.6, which was comparable to RM 1. RM 12 contained sotolon and the oatmeal smelling (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal and was most clearly perceived as walnut-like with a score as high as 2.3, thus was even higher than the score of RM 2 (2.1). Interestingly, RM 12 contained the odorants sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal in a mass ratio of approximately 1:1 with exact concentrations of 10.6 µg/mL and 10.2 µg/mL, respectively.

Sotolon appeared to have a greater influence on the overall walnut character compared to (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal, as all mixtures containing sotolon (RM 6, RM 9, RM 11, and RM 12) showed more walnut character (0.7–2.3) than the mixtures without sotolon (RM 3–RM 5, RM 7, RM 8, RM 10). The important role of sotolon is further emphasized by the fact that sotolon in combination with the fatty, deep-fried odorants (2*E*,4*Z*)-deca-2,4-dienal (**27**) and (2*E*,4*E*)-deca-2,4-dienal (**28**) in RM 11 also produced a moderate walnut odor. In contrast, the binary mixtures without sotolon only achieved ratings between 0.1 and 0.5. This result is consistent with the fact that the odor of sotolon was described as walnut-like 13 years ago.[71] Nevertheless, it was the simultaneous presence of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal that accounted for the clear walnut character in our experiments.

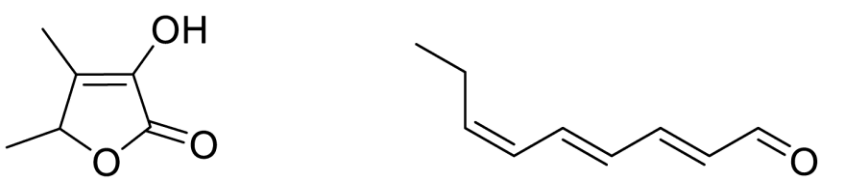
Table 5: Intensity of the characteristic walnut note in binary mixtures and aroma reconstitution models with 6 and 17 odorants at their natural concentration

reconstitution model	odorants[a]	intensity "walnut"[b]
RM 1	all 17 odorants with OAVs >1	1.6
RM 2	**10**, **22**, **27**, **28**, **32**, **46**	2.1
RM 3	**10**, **22**	0.1
RM 4	**10**, **32**	0.3
RM 5	**10**, **27**/**28**	0.4
RM 6	**10**, **46**	1.0
RM 7	**22**, **27**/**28**	0.3
RM 8	**22**, **32**	0.4
RM 9	**22**, **46**	0.7
RM 10	**27**/**28**, **32**	0.5
RM 11	**27**/**28**, **46**	1.6
RM 12	**32**, **46**	2.3

[a]Odorant numbers according to Table 3. [b]Assessors rated the intensity of the odor impression "walnut" on a scale from 0 to 3 with 0.5 increments and 0 = not perceptible, 1 = weak, 2 = moderate, and 3 = strong.

The studies by Peng et al.[62] and Liu et al.[61] suggested walnut key odorants based on the corresponding OAVs calculated from OTCs in water. Liu et al. reported 10 odorants as key odorants of raw walnut kernels, namely (2*E*)-non-2-enal, octanal, hexanal, nonanal, eugenol, 2-(*tert*-butyl)-6-methylphenol, (3*E*)-pent-3-en-2-ol, butane-1,3-diol, 2,5-dimethylbenzaldehyde, and γ-hexalactone. However, the use of OTCs determined in water is to be criticized, given that walnut kernels represent a high-fat food with a fat content of 62.5 g/100 g. Furthermore, all compounds with OAVs ≥1 were declared as key odorants without further sensory evidence. Reconstitution experiments are, however, essential to confirm the postulated key odorants. We prepared a reconstitution model in our laboratory containing the 10 postulated compounds from Liu's study in the reported concentrations. The reconstitution model was prepared on the basis of an odorless medium-chain triglyceride matrix and evaluated by the sensory panel in comparison to fresh walnut kernels. The typical nutty character could not be detected, only a fatty, rancid odor was perceived. This experiment confirmed that sensory reconstitution tests are absolutely necessary to successfully identify key food odorants.

The omission tests following the aroma reconstitution in our study successfully confirmed sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal as key odorants of fresh walnut kernels. The chemical structures of both odorants are depicted in Figure 28.



Sotolon (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal

Figure 28: Key odorants of fresh walnut kernels

Sotolon and (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal had not been previously identified as odorants in walnuts but were known to be odor-active in other foods.[70] Sotolon is classified as a generalist because it is present in odor-active amounts in many different foods.[4] Sotolon can be formed by biochemical processes or by thermal food processing in the course of the Maillard reaction, for example when pan-frying white mushrooms.[72] The odor is described as fenugreek-like, lovage-like, or Maggi Seasoning sauce-like. Sotolon characterizes the aroma of additional herbs and spices, including lovage, blue fenugreek, and Caucasian hogweed,[73, 74] as well as the aroma of other seasoning sauces such as Japanese shoyu.[75, 76] When the aroma of a food is predominantly determined by a single compound, so that its odor is associated with the food, that compound is called a character impact compound of the corresponding food. Thus, sotolon is considered a character impact compound of the mentioned foods. In wine, aroma-active amounts of sotolon have been determined in Sherry,[77] Portwine,[78] and Madeira.[79] In beer it was reported to cause an off-flavor. Notably, in this paper, the odor of sotolon was described as walnut-like.[80] (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal is the character impact compound responsible for the characteristic aroma of oatmeal.[81] It also plays a substantial role in the aroma of black tea[82] and has been identified as an odorant in several other foods, including green tea,[83] hog plum pulp,[84] and prawns.[85] (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal is formed enzymatically or by autoxidation from α-linolenic acid.[81] Surprisingly, the combination of two character impact compounds created a new olfactory impression, in this case the walnut note. Such a case had not been described in the literature before.

To understand the perception of odor mixtures, two main theories are available: the theory of elemental processing and the theory of configural or combinatorial odor processing. According to the elemental processing theory, the components present in an odor mixture are distinct entities that contribute to the overall perception of the mixture.[86, 87] On the other hand, the theory of configural or combinatorial odor processing suggests that the components within an odor mixture interact and combine in ways that create new and unique odors that are distinct from the individual components.[88, 89] For example, research has shown that when the odorants methional, which has a cooked potato-like odor, and (5*Z*)-octa-1,5-dien-3-one, which has a geranium leaf-like odor, are mixed in a 100:1 ratio, they produce a fishy odor.[90] Interestingly, the characteristic walnut odor appears to be produced by a similar effect. Actually, it is even possible to generate the walnut aroma by adding 1 or 2 drops of Maggi Seasoning sauce to a tablespoon of oatmeal, an experiment that can be easily done in the kitchen at home.

Our study continued to focus on binary mixtures containing sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal in different concentrations. The following sensory tests were performed with the same matrix used for the reconstitution and omission tests detailed above. Aqueous solutions of (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal and sotolon were used to define the descriptors "oatmeal" and "fenugreek", respectively. The concentrations were 100 times higher than the OTC. The "walnut" impression was defined by fresh walnut kernels. Seven binary mixtures with different sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal ratios were prepared. To approximate the levels in walnuts, a 1:1 mixture containing 10 µg/kg sotolon and 10 µg/kg (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal was prepared. In subsequent tests, the concentration of one of the two odorants was gradually reduced to 3 µg/kg, 1 µg/kg, and then to zero. The sensory panel was presented with the samples and instructed to orthonasally rate the intensity of the descriptors "walnut", "fenugreek", and "oatmeal". The evaluation was based on the same scale as used in the reconstitution and omission tests, 0 to 3 with 0.5 increments. The scale corresponded to

0 = not perceptible, 1 = weak, 2 = moderate, and 3 = strong. The highest intensity of the walnut note was obtained when sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal were present at concentrations of 10 µg/kg, similar to the levels found in the walnuts (Figure 29). When one of the two odorants was kept at a concentration of 10 µg/kg and the other one was reduced to 3 µg/kg, a moderate intensity of the walnut note was perceptible. However, when one of the two odorants was reduced to 1 µg/kg, the walnut character became weak. The decrease in the walnut note was steeper when the concentration of sotolon was reduced, providing additional evidence that sotolon contributed relatively more to the walnut character than (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal. Interestingly, when the ratio of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal approached 1:1, resulting in the development of the walnut character, the individual odor impressions of both odorants did not completely disappear. Instead, they remained perceptible alongside the walnut note. In other words, the development of the walnut note was not at the expense of the fenugreek note of sotolon or the oatmeal note of (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal, but rather in addition to them.

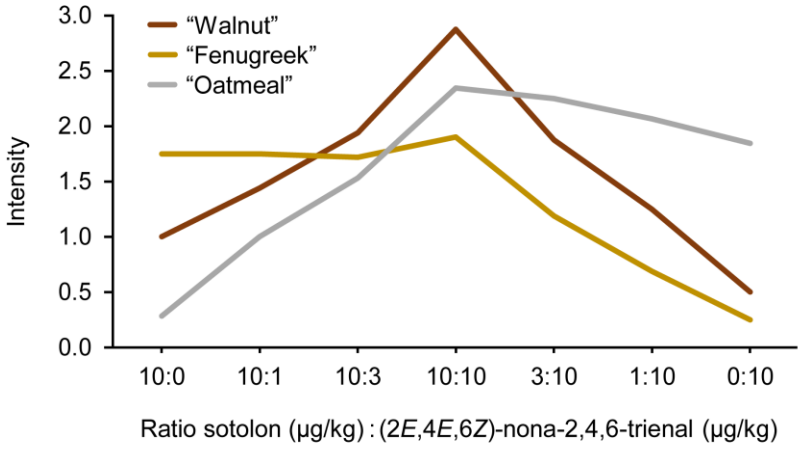


Figure 29: Effect of the sotolon to (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal ratio on the intensity of the "walnut", "fenugreek", and "oatmeal" odor impressions of model mixtures; the evaluation was performed by a trained sensory panel using a scale from 0 to 3 with 0.5 increments and 0 = not perceptible, 1 = weak, 2 = moderate, and 3 = strong

The idea of another sensory test was to investigate whether increasing concentrations of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal would enhance the overall walnut aroma. Each sample was evaluated against the 1:1 mixture containing both odorants at 10 µg/kg. The difference in intensity of the walnut note was rated by the sensory panel on a scale from −3 to +3 with −3 = clearly weaker, −2 = moderately weaker, −1 = slightly weaker, 0 = no difference, +1 = slightly stronger, +2 = moderately stronger, and +3 = clearly stronger. Figure 30 shows that, regardless of the concentration levels, the 1:1 ratio of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal produced the strongest walnut character. When the concentrations of the 1:1 binary mixture were increased from 10 µg/kg to 30 µg/kg and from 30 µg/kg to 100 µg/kg, the intensity of the walnut note also increased. However, when the concentrations increased from 100 µg/kg to 300 µg/kg, a slight decrease in intensity was observed. The data suggests a concentration level of ~100 µg/kg in both odorants to obtain an optimum walnut character.

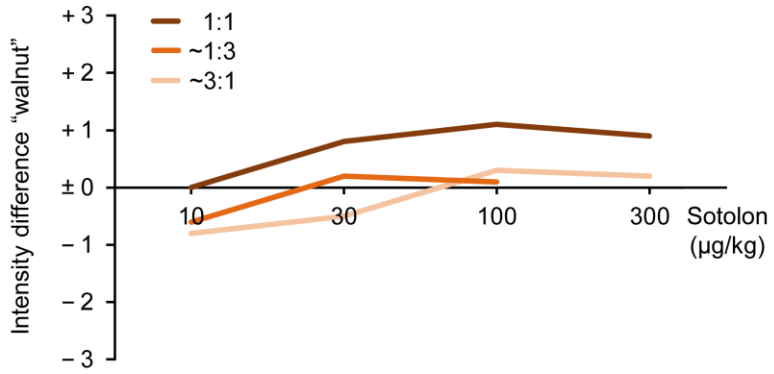


Figure 30: Changes in the characteristic walnut note with increasing odorant concentrations of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal in 1:1, ~1:3, and ~3:1 mixtures; the intensity difference was rated by a sensory panel on a scale from −3 to +3 with −3 = clearly weaker, −2 = moderately weaker, −1 = slightly weaker, 0 = no difference, +1 = slightly stronger, +2 = moderately stronger, and +3 = clearly stronger

In summary, sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal were established as the key odorants of fresh walnut kernels. They occur naturally in a 1:1 ratio with concentrations of ~10 µg/kg. By increasing the concentrations to 100 µg/kg, the intensity of the pleasant walnut character can be increased. Thus, sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal could be considered as markers for the analytical evaluation of the walnut aroma of different walnut varieties. Furthermore, the results may be helpful for future breeding aimed at developing new walnut cultivars with an optimized walnut character. In addition, the results may provide a way to flavor foods with a walnut note without using fresh walnut kernels, which would be beneficial for consumers with walnut allergies.[91]

6.2.4 Quantitation of the Key Odorants of Fresh Walnut Kernels in Other Tree Nuts

Based on the finding that a combination of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal at a 1:1 ratio and concentrations of ~10 µg/kg caused the characteristic walnut note, we were curious to determine the levels of the two odorants in other tree nuts that lack the characteristic walnut aroma. Cashew nuts, hazelnuts, almonds, Brazil nuts, and pecan nuts were selected for analysis.

The results (Table 6) showed that the levels of (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal in cashew nuts, hazelnuts, and almonds were below its OTC of 1.1 µg/kg (Table 4). The concentrations of sotolon in these nuts were lower than in walnuts and ranged from 2.15 µg/kg to 3.55 µg/kg. However, they were beyond the OTC of sotolon of 0.23 µg/kg. Notably, the ratio of sotolon to (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal was greater than 5:1 in these tree nuts. Only in Brazil nuts, the concentration of (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal exceeded the concentration of sotolon. The ratio of sotolon to (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal in this case was 1:2.3. However, both concentrations were significantly lower than those in walnuts. The lower amounts of sotolon

and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal combined with a ratio differing from 1:1 were consistent with the absence of a walnut note in the cashew nuts, hazelnuts, almonds, and Brazil nuts.

Notably in pecan nuts, the concentration of (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal was 7.87 µg/kg, and thus comparable to 10.2 µg/kg determined in walnuts. Furthermore, the concentration of sotolon was 23.6 µg/kg, and thus even higher than in walnuts (10.6 µg/kg). As a result, the ratio of sotolon to (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal was approximately 3:1. In the previous sensory test (Figure 29), the ratio of sotolon to (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal of 3:1 resulted in a moderate walnut note. In agreement with that the pecan aroma showed a moderate walnut character, although clearly weaker than that of walnuts. This was not surprising because pecan nuts (*Carya illinoinensis*) and walnuts (*Juglans regia)* belong to the same botanical family, namely Juglandaceae.

Table 6: (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal and sotolon concentrations in different tree nuts

no.[a]	odorant	concentration (µg/kg)					
		cashew nut[b]	hazelnut[b]	almond[b]	Brazil nut[b]	pecan nut[b]	walnut[c]
32	(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal	<0.20	<0.20	0.560	1.18	7.87	10.2
46	sotolon	3.55	2.15	3.21	0.506	23.6	10.6

[a]Numbering according to Table 3. [b]Mean of duplicates or triplicates; individual concentrations and standard deviations are available in the Supporting Information file of Publication 2.[70] [c]Data taken from Table 4.

6.2.5 Changes during Aging of Fresh Walnut Kernels

The sensory appeal of fresh walnut kernels is highly valued by consumers. However, when stored in ambient air at room temperature or even in the refrigerator, they develop an unpleasant rancid odor within a few days after opening the package. We studied the changes in odorants after one week of storage.

A homogenized batch was prepared from fresh, coarsely ground walnut kernels (particle size approximately 1 mm to 3 mm). One part of this batch was used for the analysis of the fresh material using the approach described in sections 6.2.1 and 6.2.2. The remaining part was then stored in ambient air at room temperature for one week before analysis.

In summary, a comparative AEDA revealed a total of 51 odorants in fresh and aged walnut kernels. Since the previous section 6.2.1 already included a comprehensive analysis of the AEDA results for fresh walnut kernels, the following discussion is focused only on aged walnuts and the differences from fresh walnut kernels. The application of AEDA to aged walnut kernels resulted in 45 odorants (Table 7). Among these, 44 odorants were found to be present in both, aged and fresh walnut kernels, indicating a high degree of similarity. An additional odorant (**51**) was detected exclusively during the analysis of aged walnut kernels. This odorant showed a metallic odor and an FD factor of 32. Unfortunately, the identity of this particular odorant could not be revealed. The odorants with the highest FD factors in the aged walnuts were (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**; OAV 2048), *trans*-4,5-epoxy-(2*E*)-dec-2-enal (**38**; OAV 2048), and oct-1-en-3-one (**5**; OAV 1024). The FD factors of the key odorants sotolon (**46**) and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**) showed only a small difference to the FD factors in the fresh walnuts.

Clear differences were found between the FD factors of fresh and aged walnut kernels for some other compounds. The FD factor of oct-1-en-3-one (**5**) increased from 256 in fresh walnut kernels to 1024 in aged walnut kernels. The FD factor of (5*Z*)-octa-1,5-dien-3-one (**7**) increased from 16 to 256. Those of (2*E*,4*Z*)-nona-2,4-dienal (**20**) and 3- and 2-methylbutanoic acid (**22** and **23**) increased from 16 to 64. In addition, the FD factors of (2*E*,4*E*)-nona-2,4-dienal (**24**), and (2*E*,4*E*)-deca-2,4-dienal (**28**) both increased from 32 to 256. Furthermore, *trans*-4,5-epoxy-(2*E*)-dec-2-enal (**38**) showed an increase in the FD factor from 256 to 2048.

On the other hand, several compounds showed a decrease in the FD factors from fresh walnut kernels to aged walnut kernels. A decrease in FD factors from 64 in fresh walnut kernels to 16 in aged walnut kernels was observed for the odorants (2*E*)-oct-2-enal (**8**), 3-isopropyl-2-methoxypyrazine (**9**), 2'-aminoacetophenone (**47**), and vanillin (**50**). Further decreases in the FD factors were observed for butanoic acid (**19**; from 16 to 4), β-ionone (**35**; from 4 to 1), HDMF (**40**; from 256 to 16), 4-hydroxy-5-methylfuran-3-one (**42**; from 4 to 1), (2*Z*,4*Z*)-δ-deca-2,4-dienolactone (**45**; from 8 to 1). The following odorants were not detected in the aged samples: butane-2,3-dione (**1**), 2-ethylpyrazine (**6**), methional (**11**), (2*E*,4*E*)-hepta-2,4-dienal (**12**), γ-decalactone (**43**), and (6*Z*)-γ-dodec-6-enolactone (**48**), although they had been detected in the fresh material with FD factors of 2, 8, 4, 16, 4, 4 respectively.

Table 7: Odorants of fresh and aged walnut kernels obtained by AEDA

no.	odorant[a]	odor[b]	RI[c] FFAP	RI[c] DB-5	FD factor[e] fresh walnut kernels[f]	FD factor[e] aged walnut kernels
1	butane-2,3-dione	buttery	982	603	2	-
2	hexanal	green, grassy	1080	802	2	1
3	γ-terpinene	earthy	1234	1059	4	1
4	octanal	citrusy	1285	1005	4	4
5	oct-1-en-3-one	mushroom	1293	979	256	1024
6	2-ethylpyrazine	roasty	1331	916	8	-
7	(5*Z*)-octa-1,5-dien-3-one	geranium leaf	1364	982	16	256
8	(2*E*)-oct-2-enal	fatty, citrusy	1419	1061	64	16
9	3-isopropyl-2-methoxypyrazine	bell pepper	1417	1086	64	16
10	acetic acid	vinegar	1450	636	16	16
11	methional	cooked potato	1455	910	4	-
12	(2*E*,4E)-hepta-2,4-dienal	floral, fatty	1480	1015	16	-
13	3-*sec*-butyl-2-methoxypyrazine	bell pepper	1496	1167	64	64
14	(2*Z*)-non-2-enal	fatty, floral	1494	1148	32	8
15	(2*E*)-non-2-enal	cucumber, green	1532	11163	16	64
16	2-methylpropanoic acid	sweaty, cheesy	1560	783	8	4
17	(2*E*,6*Z*)-nona-2,6-dienal	cucumber, green	1584	1154	32	16
18	undecanal	fatty, floral	1600	1306	8	4
19	butanoic acid	sweaty, cheesy	1627	827	16	4
20	(2*E*,4*Z*)-nona-2,4-dienal	fatty	1639	1197	16	64
21	phenylacetaldehyde	floral, honey	1639	1047	8	8
22	3-methylbutanoic acid	sweaty, cheesy	1667	863	16	64
23	2-methylbutanoic acid	sweaty, cheesy	1668	857	16	64

Table 7 continued:

no. [a]	odorant[b]	odor[c]	RI[d] FFAP	RI[d] DB-5	FD factor[e] fresh walnut kernels[f]	FD factor[e] aged walnut kernels
24	(2*E*,4*E*)-nona-2,4-dienal	fatty	1692	1215	32	256
25	(2*E*)-undec-2-enal	green, soapy	1747	1362	8	4
26	α-farnesene	green	1745	1509	8	4
27	(2*E*,4*Z*)-deca-2,4-dienal	fatty, deep-fried	1752	1296	32	16
28	(2*E*,4*E*)-deca-2,4-dienal	fatty, deep-fried	1808	1322	32	256
29	cyclotene	fenugreek	1819	1024	8	4
30	hexanoic acid	sweaty, cheesy	1838	1015	16	16
31	2-methoxyphenol	smoky	1862	1087	256	64
32	(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal	oatmeal	1876	1273	1024	2048
33	(2*E*,4*E*,6*E*)-nona-2,4,6-trienal	oatmeal	1895	1285	2	1
51	unknown	metallic	1906	-	-	32
34	γ-octalactone	coconut	1918	1255	32	16
35	β-ionone	floral, raspberry	1928	1480	4	1
36	δ-octalactone	coconut	1967	1292	4	4
37	maltol	caramel	1974	1114	4	4
38	*trans*-4,5-epoxy-(2*E*)-dec-2-enal	metallic	2004	1382	256	2048
39	4-methoxybenzaldehyde	aniseed, woodruff	2031	1259	8	4
40	HDMF[g]	caramel	2033	1087	256	16
41	EHMF[h]	caramel	2077	1139/1148[i]	8	16
42	4-hydroxy-5-methylfuran-3-one	fruity, caramel	2127	1065	4	1
43	γ-decalactone	coconut	2133	1496	4	-
44	eugenol	clove	2169	1354	8	4
45	(2*Z*,4*Z*)-δ-deca-2,4-dienolactone	sweet, coconut	2170	1459	8	1
46	sotolon	fenugreek	2205	1111	512	256
47	2′-aminoacetophenone	foxy	2222	1304	64	16
48	(6*Z*)-γ-dodec-6-enolactone	sweet, fruity	2389	1658	4	-
49	2-phenylacetic acid	floral, honey	2553	1267	64	64
50	vanillin	vanilla	2573	1400	64	16

[a]Numbering according to Table 3. [b]Each odorant was identified by comparing its retention indices on two GC columns of different polarity (DB-FFAP, DB-5), its mass spectrum obtained by GC–MS, as well as its odor as perceived at the sniffing port during GC–O to data obtained from authentic reference compounds analyzed in parallel. [c]Odor as perceived at the sniffing port during GC–O. [d]Retention index; calculated from the retention time of the compound and the retention times of adjacent *n*-alkanes by linear interpolation. [e]Flavor dilution factor; dilution factor of the highest diluted walnut volatile isolate in which the odorant was detected during GC–O analysis by any of two assessors. [f]Data taken from Table 3. [g]4-Hydroxy-2,5-dimethylfuran-3(2*H*)-one. [h]2-Ethyl-4-hydroxy-5-methylfuran-3-one. [i]EHMF is separated from its tautomer 5-ethyl-4-hydroxy-2-methylfuran-3-one on the DB-5 column, on the DB-FFAP column no separation of the isomers was observed.

A more accurate comparison between fresh and aged walnut kernel odorants was possible on the basis of the quantitative data. The 27 odorants quantitated in fresh walnut kernels were also quantitated in aged walnut kernels. The concentrations found in aged walnut kernels ranged from 0.0228 µg/kg to 75100 µg/kg (Table 8). Similar to fresh walnut kernels, the odorants with the highest concentrations in aged walnut kernels were acetic acid

(**10**; 75100 µg/kg) and hexanoic acid (**30**; 2270 µg/kg). A change was considered significant if the concentration of an odorant increased or decreased by at least ~50%. Compared to fresh walnut kernels, the concentration of (2*E*,4*Z*)-deca-2,4-dienal (**27**) increased by a factor of 1.43, from 46.7 µg/kg to 67.0 µg/kg, while the concentration of acetic acid (**10**) increased by a factor of 1.70, from 44200 µg/kg to 75100 µg/kg. γ-Octalactone (**34**) showed a 57% increase, thus reaching a concentration of 18 µg/kg in the aged walnuts, which still remained below its odor threshold concentration of 280 µg/kg. Odorant **38**, *trans*-4,5-epoxy-(2*E*)-dec-2-enal, showed an increase in concentration of 1.94, from 55.7 µg/kg to 108 µg/kg. Similarly, the concentration of (5*Z*)-octa-1,5-dien-3-one (**7**) almost doubled from 0.0659 µg/kg to 0.144 µg/kg. For oct-1-en-3-one (**5**), however, there was a remarkable increase in concentration by a factor of 5.44, from 7.42 µg/kg to 40.4 µg/kg. A decrease in concentration of approximately 50% was observed for 2-methylbutanoic acid (**23**), which dropped from 52.6 µg/kg to 29.6 µg/kg. Interestingly, the concentrations of sotolon (**46**) and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**), which were the key odorants in fresh walnut kernels, remained virtually unchanged between fresh and aged walnut kernels.

Unlike fresh walnut kernels, aged walnuts showed one compound, (2*E*,4*E*)-nona-2,4-dienal (**24**), below its odor threshold concentrations. Acetic acid (**10**) retained its position as the odorant with the highest OAV, followed by sotolon (**46**), (2*E*,4*Z*)-deca-2,4-dienal (**27**), (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**), 3-methylbutanoic acid (**22**), *trans*-4,5-epoxy-(2*E*)-dec-2-enal (**38**), and oct-1-en-3-one (**5**).

Table 8: Concentrations, OTCs, and OAVs of important odorants in fresh and aged walnut kernels

no.[a]	odorant	OTC (µg/kg)[b]	fresh walnut kernels		aged walnut kernels	
			concentration[c] (µg/kg)	OAV[d]	concentration[e] (µg/kg)	OAV[d]
10	acetic acid	350	44200	130	75100	210
46	sotolon	0.23	10.6	46	9.9	43
27	(2*E*,4*Z*)-deca-2,4-dienal	2.8[f]	46.7	17	67	24
32	(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal	1.1	10.2	9.3	11.8	10.7
22	3-methylbutanoic acid	9.0	118	13	83.8	9.3
38	*trans*-4,5-epoxy-(2*E*)-dec-2-enal	13	55.7	4.3	108	8.3
5	oct-1-en-3-one	6.9	7.42	1.1	40.4	5.9
30	hexanoic acid	460	2870	6.2	2270	4.9
14	(2*Z*)-non-2-enal	3.6	13.6	3.8	15.3	4.3
19	butanoic acid	34	184	5.4	138	4.1
8	(2*E*)-oct-2-enal	120	439	3.7	437	3.6
28	(2*E*,4*E*)-deca-2,4-dienal	66	178	2.7	240	3.6
7	(5*Z*)-octa-1,5-dien-3-one	0.044	0.0659	1.5	0.144	3.3
49	2-phenylacetic acid	26	90.2	3.5	75.6	2.9
31	2-methoxyphenol	1.8	3.98	2.2	4.59	2.6
9	3-isopropyl-2-methoxypyrazine	0.010	0.0206	2.1	0.0228	2.3
15	(2*E*)-non-2-enal	140	121	<1	124	<1
50	vanillin	140	105	<1	122	<1

Table 8 continued:

no.[a]	odorant	OTC (µg/kg)[b]	fresh walnut kernels		aged walnut kernels	
			concentration[c] (µg/kg)	OAV[d]	concentration[e] (µg/kg)	OAV[d]
24	(2*E*,4*E*)-nona-2,4-dienal	30	36.6	1.2	25.4	<1
47	2′-aminoacetophenone	21	7.80	<1	7.48	<1
40	HDMF[g]	25	12.8	<1	7.77	<1
23	2-methylbutanoic acid	110	52.6	<1	29.6	<1
17	(2*E*,6*Z*)-nona-2,6-dienal	65	8.76	<1	11.6	<1
34	γ-octalactone	280	11.5	<1	18.0	<1
12	(2E,4E)-hepta-2,4-dienal	710	13.3	<1	16.1	<1
20	(2*E*,4*Z*)-nona-2,4-dienal	16[f]	3.48	<1	2.31	<1
13	3-*sec*-butyl-2-methoxypyrazine	0.46	<0.10	<1	<0.10	<1

[a]Numbering according to Table 3. [b]Odor threshold concentrations determined in low odor sunflower oil. [c]Data taken from Table 4. [c]Mean of duplicates or triplicates; individual values and standard deviations Supporting Information file of Publication 2[70]. [d]Odor activity value; calculated as ratio of the concentration to the odor threshold concentration. [e]Mean of duplicates or triplicates, standard deviations were <30%. [f]Approximated from the odor threshold concentration of the (2*E*,4*E*)-isomer in low odor sunflower oil and the ratio of the odor threshold concentrations of the individual isomers in air (Supporting Information file of Publication 2[70]). [g]4-Hydroxy-2,5-dimethylfuran-3(2*H*)-one.

The data presented in this study covered only a limited storage period of one week. Other studies investigated the change in the volatile composition of walnut kernels over longer storage periods such as 7 months,[57] 4–12 months,[92] and even 6–15 months.[93] In the following, markers of oxidative degradation in walnut kernels will be discussed, although the studies are not fully comparable to the present study due to the different storage periods. In the study by Grilo et al. on the oxidation of two commonly consumed walnut cultivars, significant amounts of hexanal, pentan-1-ol, pentanal, 1-octen-3-ol, and 2-pentylfuran were observed to accumulate over a storage period of 28 weeks.[57] Caratti et al. reported an increase in the concentrations of hexanal, heptanal, (2*E*)-hept-2-enal, octanal, (2*E*)-oct-2-enal, nonanal, and decanal in walnut kernels over a storage period of 4–12 months.[92] Chakraborty et al. reported an increase in the levels of hexanal, pentan-1-ol, oct-1-en-3-ol, and acetic acid over a storage period of 6–15 months.[93]

Our data also showed that the concentration of acetic acid increased during storage, whereas the concentration of (2*E*)-oct-2-enal remained virtually unchanged between fresh and aged walnut kernels.

It should be stressed that the previous studies focused primarily on volatiles, emphasizing the increase in concentration as a marker of oxidative degradation. In contrast, the current study focused on changes in odorants and their potential contribution to the overall aroma.

Clear suggestions can be made regarding the formation pathways of the odorants that show an increase in concentration in our data. Specifically, *trans*-4,5-epoxy-(2*E*)-dec-2-enal, oct-1-en-3-one, and (2*E*,4*Z*)-deca-2,4-dienal are formed by oxidative degradation of linoleic acid, whereas the formation of (5*Z*)-octa-1,5-dien-3-one starts from linolenic acid.[60] In walnuts, the presence of linoleic acid, oleic acid, and linolenic acid is evident.[46] The oxidative

degradation of linolenic acid is known to occur at a faster rate than that of linoleic and oleic acids.[94]

An interesting observation was that the concentration of the key odorants of fresh walnut kernels, namely sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal remained unchanged after storage of one week storage at room temperature. However, oxidative degradation resulted in the formation of other odorants that may have contributed to the development of the rancid odor.

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8 Appendix

8.1 Publication 1: Development and Evaluation of an Automated Solvent-Assisted Flavour Evaporation (aSAFE)

8.1.1 Bibliographic Data

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8.1.2 Publication Reprint

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ORIGINAL PAPER

Development and evaluation of an automated solvent-assisted flavour evaporation (aSAFE)

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Abstract
Artefact-avoiding isolation of the volatiles from foods is a crucial step before analysis of odour-active compounds by gas chromatography (GC). In the past 20 years, solvent extraction followed by solvent-assisted flavour evaporation (SAFE) has become the standard approach, particularly prior to GC–olfactometry. The manual valve of the SAFE equipment, however, leads to suboptimal yields and the risk of a contamination of the volatile isolate with non-volatiles. We thus developed an automated SAFE (aSAFE) approach by replacing the manual valve with an electronically controlled pneumatic valve. The aSAFE provides clearly higher yields than the manual SAFE (mSAFE), notably from extracts high in lipids and for odorants with comparably high boiling points. Additionally, aSAFE substantially reduces the risk of non-volatiles being transferred to the volatile isolate. Full automatisation is possible by combining the aSAFE approach with an automated liquid nitrogen refill system as well as an endpoint recognition and shut-off system.

Keywords Automated solvent-assisted flavour evaporation · aSAFE · Volatile isolation · Yield · Gas chromatography–olfactometry

Introduction

The isolation of the volatile fraction from foods and beverages is a big challenge, in particular for flavour chemists engaged in the analysis of odour-active compounds with gas chromatographic methods such as gas chromatography–olfactometry (GC–O) [1]. Steam distillation approaches [2] widely used in the early days of GC lead to thermal compound degradation and artefact formation associated with the elevated temperatures [3–6]. Direct injection of solvent extracts without further purification as well as solid phase extraction approaches such as solid phase microextraction (SPME) [7] and stir bar sorptive extraction (SBSE) [8] requires hot injection techniques that also foster thermal degradation and artefact forming reactions (examples in [1, 6, 9, 10]).

The first researchers who clearly addressed the problem of artefact formation in odorant analysis were Weurman et al. [11]. They suggested a new and mild approach for the isolation of food volatiles for which they coined the name "high vacuum transfer" (HVT). In an evacuated system, two round-bottom flasks were connected with a glass tube. Flask 1 contained the food sample and was kept at room temperature, whereas flask 2 was initially empty. When flask 2 was cooled to −180 °C, the food volatiles evaporated in flask 1 and recondensed in flask 2, driven by the different vapour pressures associated with the temperature difference between the flasks. In 1985, Schieberle and Grosch adopted the HVT approach to separate the volatiles and the non-volatiles of extracts obtained from foods with low-boiling solvents such as diethyl ether, dichloromethane, or pentane [12]. A round-bottom flask containing the extract was connected with a glass tube to a series of gas washing bottles serving as cold traps. First, the extract was cooled down with liquid nitrogen. Then, the device was evacuated and cooling was applied to the cold traps, while the cooling of the extract was stopped. As a result, a temperature difference between the frozen extract and the cold traps developed,

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causing the solvent and extracted volatiles to sublime and recondense in the cold traps. Evaporative cooling kept the temperature difference small, thus ensuring a smooth process until the transfer of the solvent was completed, which required ~ 1–2 h/100 mL. To reduce losses associated with a recondensation of volatiles before entering the cold traps, Sen et al. added a water-thermostated double-walled glass tube to connect the round bottom flask and the cold traps and applied temperatures of up to 50 °C [13]. Another HVT variant designed to increase volatile yields was introduced by Guth and Grosch [14]. Instead of placing the entire solvent extract in the round bottom flask before starting the transfer, the extract was introduced in numerous small portions by using a dropping funnel. Discontinuous extract delivery with a high flow over a short period of time was found crucial to properly transfer the extract from the funnel to the flask, maintain the high vacuum, and avoid blockage of the dropping funnel outlet by solidified fat from high-fat extracts. No cooling was applied to the flask; thus, each portion instantly vaporised in a spray-like manner. A major drawback of this "dynamic HVT" approach was the high risk of a transfer of non-volatiles in the form of small droplets. Therefore, in 1992 Jung et al. suggested to insert a splash protection adapter between the round bottom flask and the thermostated glass tube [15]. The adapter forced the vapour stream into hard direction changes; thus, droplets of non-volatiles were deposited at the walls of the adapter and did not reach the cold traps.

In 1999, Engel et al. [16] combined the major parts of the dynamic HVT equipment into a single glass device. For its application, they coined the name "solvent-assisted flavour evaporation" and the acronym SAFE. The equipment is illustrated in Fig. 1. The SAFE device included a dropping funnel with a manual needle valve stopcock used to introduce portions of the solvent extract into the evaporation flask, a double-walled water-thermostated middle part interconnecting the evaporation flask held at 30–40 °C with the recondensation flask held at–196 °C, and a safety cold trap protecting the vacuum pump in case of an operating error. The thermostatisation of the complete middle part significantly increased yields, particularly of volatiles with a high boiling point (b.p.), even though only moderate temperatures of 30–40 °C were applied to avoid the formation of thermal artefacts. Moreover, the tubing between the stopcock of the dropping funnel and the evaporation flask was included into the thermostated part, which prevented its blockage by the solidification of fats when extracts rich in lipids were applied to SAFE. Propeller-shaped glass barriers in the middle part effectively captured droplets of non-volatile material. Furthermore, the SAFE equipment required less bench space and was easier and faster to set up than the HVT equipment, allowing more samples to be processed per working day. Given all these advantages, it was not surprising that SAFE

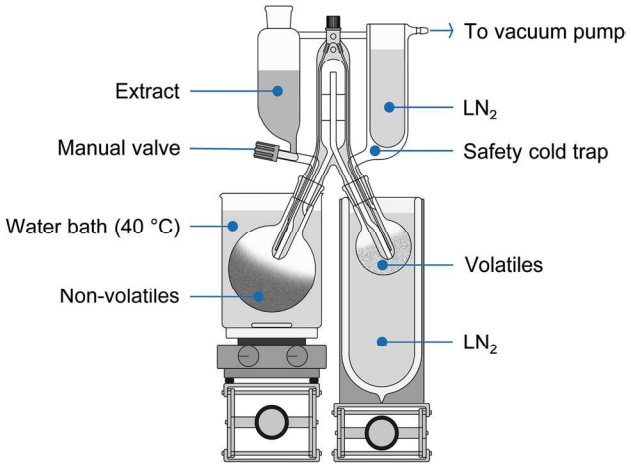

Fig. 1 Equipment for performing SAFE according to Engel et al. [16] (LN_2 = liquid nitrogen)

quickly became the standard approach for the artefact-avoiding isolation of volatiles in academic as well as in industrial food research. It has gained particular importance in the field of food odorants, where it is used to obtain representative volatile isolates suitable for GC–O screening. Between 1999 and 2022, the paper of Engel et al. received more than 600 citations [17].

Nevertheless, while successfully using SAFE for more than two decades in our laboratory, we yet identified some potential for improvement. One drawback of the SAFE approach is the high demand for manpower. During SAFE, the operator needs to be permanently present to open and close the manual valve of the dropping funnel. In addition, the liquid nitrogen level in the cold traps has to be monitored and the traps have to be refilled from time to time. A second drawback refers to yields. Engel et al. showed that SAFE yields of 100% can be achieved for volatiles with low boiling points, but yields decrease for compounds with higher boiling points and when lipids are present in the extract [16]. In addition, we suspected that yields may also differ between different operators and even between two experiments performed by the same operator. This might, for example, lead to problems when two SAFE volatile isolates are subjected to a parallel GC–O screening such as in a comparative aroma extract dilution analysis [1]. We assumed that the SAFE yields depend on the size of the individual extract portions introduced into the apparatus through the dropping funnel as well as on the time span between two portions. A reduction of the individual portion size in combination with an expansion of the time span between two portions was expected to lead to higher yields but would also increase the time required for completing the SAFE process. The third drawback is associated with the fact that large portion sizes might not only decrease the yield but beyond a certain limit they

lead to a significant transfer of non-volatiles to the volatile isolate, particularly when the extracts are rich in fat. In a brief moment of inattentiveness, even an experienced operator may once in a while fail to close the manual valve in appropriate time. Such an operating error eventually means that the volatile isolate is spoiled resulting in a waste of time, material, and manpower.

The drawbacks mentioned above are predominantly associated with the manual operation of the needle valve stopcock at the dropping funnel. The primary aim of the present study was therefore to replace the manual valve at the dropping funnel by an automated valve. The newly designed system was evaluated in terms of yields and finally further developed towards a fully automated SAFE system.

Materials and methods

Chemicals

Odorants **1–3**, **5–18**, and heptadecane were purchased from Merck (Darmstadt, Germany). Odorant **4** was from Thermo Fisher (Waltham, MA, USA). ($^2H_5$)-**13** and ($^2H_5$)-**16** were obtained from CDN Isotopes (Quebec, Canada). ($^2H_2$)-**9** was synthesised as described in the literature [18]. Dichloromethane (DCM) was purchased from CLN (Freising, Germany) and freshly distilled through a column (120 cm × 5 cm) packed with Raschig rings before use.

Food samples

A Bavarian style Pilsner beer, a dark chocolate (70% cocoa), and low odour sunflower oil, brand Thomy (Nestlé, Neuss, Germany) were purchased from a local supermarket.

SAFE equipment

All special glassware was custom-made by Glasbläserei Bahr (Manching, Germany). The pneumatic valve, type PT, the electronic valve control unit, type PAV 90, and the associated tubing were purchased from HWS Labortechnik (Mainz, Germany). The electronic valve control unit was supplied with compressed air at 300 kPa. The plunger of the pneumatic valve was exchanged for a custom-made polytetrafluoroethylene (PTFE) plunger by Glasbläserei Bahr. A PT 50 High Vacuum Pump System (Leybold, Cologne, Germany) ensured a vacuum of ≤ 0.01 Pa in the SAFE glassware. Major parts of the automated nitrogen refill system were purchased from KGW Isotherm (Karlsruhe, Germany). This included the siphon with the solenoid valve, the transfer hose, the nozzle with the phase separator, the Pt100 liquid nitrogen level sensors, the liquid nitrogen level control unit as well as the Dewar vessel for the recondensation flask and the safety cold trap. The latter was a 33 CAL shortened Dewar vessel with a diameter of 30 cm and a custom-made polyethylene top ring (Supplementary file 1, Fig. S1) with tension lock and holes for the sensors, the liquid nitrogen nozzle, a 500-mL recondensation flask (hole diameter 106 mm), and the safety cold trap (hole diameter 52 mm). The storage tank for the cryogenic liquefied nitrogen, type Apollo, volume 100 L, was purchased from Cryotherm (Kirchen/Sieg, Germany) and used pressurised at 70 kPa. The sensor used in the endpoint recognition and shut-off system was a type CFAM 12P1600 and purchased from Baumer (Frauenfeld, Switzerland). The sensor was secured by a 3D-printed polylactic acid (PLA) cap with hooks (Supplementary file 1, Table S1) attached to hooks on the glassware with a pair of elastomeric rings. The sensor was connected to an electronic endpoint control unit purchased from a local electrical engineer. The wiring diagram is available in the Supplementary file 1, Fig. S2.

Determination of SAFE yields with model mixtures

Individual stock solutions (10 mg/mL) were prepared from odorants **1–18** in DCM and checked for purity by GC–flame ionisation detector (FID) analysis of 1:100 dilutions. From the individual stock solutions and DCM, a working solution containing each odorant at a concentration of ~ 100 µg/mL was prepared and divided into 100 mL portions. Portions without further addition served as non-fat model mixture. Low-fat model mixtures and high-fat model mixtures were obtained by adding 1 g and 10 g of sunflower oil, respectively, to 100 mL portions of the working solution.

The model mixtures were subjected to different SAFE approaches. Individual model mixture/SAFE approach combinations were applied in triplicates. To each SAFE volatile isolate as well as to reference portions of the non-fat model mixture without SAFE treatment, 10 mL of a heptadecane solution (10 mg/mL) in DCM was added and the mixtures were analysed by GC–FID. Blank runs between sample injections were employed to demonstrate the absence of carry-over effects. For each GC run, peak areas corresponding to odorants **1–18** were divided by the peak area corresponding to the internal standard heptadecane to obtain the normalised peak areas. Normalised peak areas of three injections were averaged. Yields of the individual SAFE experiments were calculated by dividing the average of the normalised peak areas obtained after SAFE by the corresponding average of the normalised peak areas obtained without SAFE. Finally, the means and standard deviations were calculated from the yields obtained from the three experiments performed for each model mixture/SAFE approach combination as detailed in the Supplementary file 1, Tables S2–S12.

Determination of SAFE yields with beer and chocolate extracts

The beer was degassed by filtration through a folded filter. A portion of the filtrate (200 mL) was shaken with DCM (2 × 300 mL). Phase separation was achieved by centrifugation with a Heraeus Multifuge X3 FR (Thermo Fisher) at 10 °C and 4600 rpm for 20 min. The combined organic phases were washed with brine (2 × 100 mL) and dried over anhydrous sodium sulphate. The supernatant beer extract was used for the yield experiments.

The chocolate was cooled with liquid nitrogen, then coarsely crushed with a laboratory mill Grindomix GM 200 (Retsch, Haan, Germany), and finally ground into a fine powder with a 6875 Freezer Mill (SPEX SamplePrep, Stanmore, UK). The powder (250 g) was stirred with DCM (1000 mL) for 60 min. The mixture was dried over anhydrous sodium sulphate and centrifuged. The supernatant chocolate extract was used for the yield experiments.

Both, the beer and the chocolate extract were divided into ten aliquots, respectively, nine of which were used for further analysis. Three aliquots were used to determine the concentrations of odorants **9**, **13**, and **16** *before* SAFE. For this purpose, defined amounts of the stable isotopically substituted odorants ($^2H_2$)-**9**, ($^2H_5$)-**13**, and ($^2H_5$)-**16** in DCM solution were added as internal standards and the mixtures were subjected to SAFE. Aliquots 4–6 and 7–9 were used to determine the concentrations of odorants **9**, **13**, and **16** after application of different SAFE approaches. For this purpose, the stable isotopically substituted odorants were added to the volatile isolates *after* SAFE. The individual amounts added to the beer extract aliquots were 0.487 µg ($^2H_2$)-**9**, 152 µg ($^2H_5$)-**13**, and 5.19 µg ($^2H_5$)-**16**. The individual amounts added to the chocolate extract aliquots were 4.60 µg ($^2H_2$)-**9**, 27.1 µg ($^2H_5$)-**13**, and 51.9 µg ($^2H_5$)-**16**.

The concentrations of odorants **9**, **13**, and **16** in the beer and chocolate extracts before and after SAFE were finally determined by heart-cut GC–GC–mass spectrometry (GC–GC–MS) analysis of 1:5 dilutions of the volatile isolates (**13** in the beer extracts) or heart-cut GC–GC–MS analysis of concentrates (1 mL) obtained from the volatile isolates by using a Vigreux column (50 × 1 cm) and a Bemelmans microdistillation device [19] (**9** and **16** in the beer extract; **9**, **13**, and **16** in the chocolate extract). Odorant concentrations in the extract aliquots were calculated from the peak area counts of the analyte peak and the internal standard peak in the extracted ion chromatograms of characteristic quantifier ions, the aliquot volumes, and the amount of standard added, by applying a calibration line equation. The calibration line equation was obtained by linear regression after the analysis of analyte/standard mixtures in different concentration ratios. The analyte/standard ratios covered a range of ~ 5:1 to ~ 1:5.

The quantifier ions and the calibration line equations are summarised in the Supplementary file 1, Table S13. Concentration data is available in the Supplementary file 1, Tables S14–S19. Yields of the individual SAFE experiments were calculated by dividing the concentrations after SAFE by the concentrations before SAFE. Finally, the means and standard deviations were calculated from the yields obtained from the three experiments performed for each food extract/SAFE approach combination as detailed in the Supplementary file 1, Tables S20–S23.

GC–FID

A Trace 1310 Series gas chromatograph (Thermo Fisher) was equipped with a TriPlus RSH autosampler, a cold on-column injector, an FID, and a DB-FFAP column, 30 m length × 0.32 mm inner diameter (i.d.), 0.25 µm film thickness (Agilent, Waldbronn, Germany). The carrier gas was helium at 1.9 mL/min constant flow. The injection volume was 2 µL. The oven temperature was 40 °C for 2 min and then increased by 6 °C/min to 230 °C, which was held for 5 min. Data were acquired and evaluated by using the Chromeleon software, version 7.2.8 (Thermo Fisher).

Heart-cut GC–GC–MS

A Trace GC Ultra gas chromatograph (Thermo Fisher) was equipped with a Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland), a cold on-column injector (Thermo Fisher), and a DB-FFAP column, 30 m length × 0.32 mm i.d., 0.25 µm film thickness (Agilent). The carrier gas was helium at a constant pressure of 100 kPa. The injection volume was 2 µL. The oven temperature was 40 °C for 2 min and then increased by 6 °C/min to 230 °C, which was held for 5 min. The end of the column was connected to a moving column stream switching (MCSS) system (Thermo Fisher) supplied with helium as make-up gas at 50 kPa. The MCSS system transferred the column effluent via deactivated fused silica capillaries (0.32 mm i.d.) time-programmed either simultaneously to an FID and a custom-made sniffing port [20] (230 °C base temperature) or via a heated (250 °C) hose to a liquid nitrogen-cooled trap inside the oven of a second gas chromatograph, which was a CP 3800 (Varian, Darmstadt, Germany) equipped with a DB-1701 column, 30 m length × 0.25 mm i.d., 0.25 µm film thickness (Agilent). The oven temperature was 40 °C for 2 min and then increased by 6 °C/min to 230 °C, which was held for 5 min. The end of the second column was connected to a Saturn 2200 ion trap mass spectrometer (Varian) operated in the chemical ionisation mode with methanol as the reagent gas and a scan range of *m/z* 60–250. Data were acquired and evaluated by using the MS Workstation software, version 6.42 (Varian).

Results and discussion

Design and application of the SAFE device with an automated valve

The device was largely based on the original SAFE device introduced by Engel et al. [16] (cf. Figure 1). The middle part including the connections to the thermostated evaporation flask and to the liquid nitrogen-cooled recondensation flask as well as the safety cold trap including the connection to the vacuum remained unchanged. The major modification was the exchange of the manual valve for a pneumatic valve. Minor modifications were required to the plunger casing and the dropping funnel. The new device is depicted in Fig. 2. Details on the modified parts are available in the Supplementary file 1, Fig. S3.

A pneumatic valve and the corresponding electronic control unit were purchased from a laboratory instruments supplier. To combine the pneumatic valve with the SAFE glassware, adjustments were necessary on both sides. The plunger supplied with the pneumatic valve was unsuitable to obtain sufficient tightness when the glassware was evacuated. It was therefore replaced by a custom-made PTFE plunger with a tapered tip. Tightness was achieved by an elastomeric O-ring. The O-ring was buried under the outer PTFE layer of the plunger to make sure that it was not a source of contamination. On the glassware side, a circumferential groove next to the orifice of the plunger casing accommodated a polymeric split washer as abutment for the union nut of the valve. If necessary, the position of the plunger was corrected with the adjustment screw of the pneumatic valve and the position of the adjustment screw was fixed with the set screw. The inner diameter of the glass tube connecting the body of the dropping funnel to the valve was reduced from ~ 7 to 1 mm. This modification was essential to achieve reasonably small extract portion sizes. With glass tube dimensions of 1 mm i.d. and 4 cm length, the flow through the open valve was ~ 3 mL/s when the glassware was under vacuum. The reduction in the size of this tube in combination with the weight of the pneumatic valve made it necessary to stabilise the glassware with an additional glass rod connecting the body of the dropping funnel and the plunger casing. The electronic valve control unit was supplied with compressed air. Depending on the switching state, PTFE tubes delivered the compressed air from the control unit to the pneumatic valve.

To perform a SAFE with the new device, it was mounted to a lattice lab system on the bench. The evaporation flask

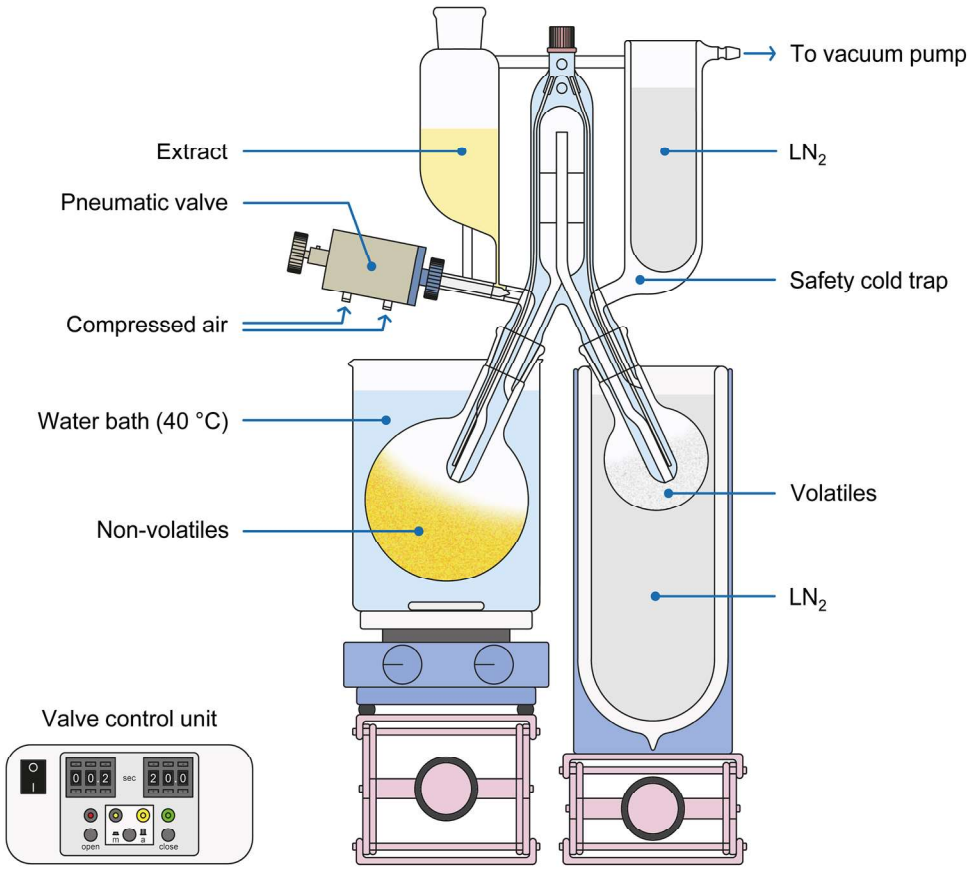

Fig. 2 Equipment for performing an automated SAFE including the pneumatic valve and its electronic control unit

and the recondensation flask were added and fixed with plastic joint clips. The middle part of the device was connected to the circulation thermostat and the water bath was applied to the evaporation flask. In both cases, the temperature was 40 °C. This value was found to be low enough to avoid thermal artefact formation and high enough to keep the lipid fraction of most food extracts in the liquid state.

The mode switch at the electronic valve control unit (Supplementary file 1, Fig. S4) was turned to manual and then the power supply was turned on at the main switch. With the open and close buttons, the correct operation of the valve was checked. With the valve in the closed position, the device was finally connected to the vacuum. After vacuum was established, the recondensation flask was cooled with liquid nitrogen. Then, the settings for the valve open time and the valve closed time were adjusted at the electronic control unit. Valve open times were set to 0.1–0.3 s equivalent to extract volumes of 0.3–0.9 mL. The valve closed times were set to 5–60 s. The selected valve closed time should at least be sufficient to allow for the complete evaporation of the solvent from the previous extract portion. Thus, the minimum setting depended on the valve open time as well as on the lipid content in the extract. High lipid contents clearly delayed the evaporation of the solvent.

As the next step, the extract was filled into the dropping funnel and the automated SAFE process was started by turning the mode switch to auto. Subsequently, the valve automatically switched between open and closed according to the settings. From time to time, liquid nitrogen needed to be added to the cold traps. When the extract level in the dropping funnel eventually reached the capillary at the bottom, the mode switch at the electronic valve control unit was turned to manual. This stopped the automated SAFE process. The cooling of the recondensation flask was removed, the device was ventilated, and the recondensation flask with the isolated volatiles was detached from the device.

The entire process of an automated SAFE is additionally described in a video provided on the internet [21]. To distinguish our new SAFE approach from the older SAFE approach with the manual valve, we suggest referring to the new approach as "automated SAFE" or "autoSAFE" and in writing use the abbreviation aSAFE. Whenever the original SAFE is addressed, we will from now on refer to it as manual SAFE or mSAFE.

Evaluation of the aSAFE approach

To determine the compound yields of the aSAFE approach in comparison to mSAFE, three different model mixtures, namely a non-fat model mixture, a low-fat model mixture, and a high-fat model mixture were prepared to simulate solvent extracts obtained from foods of different lipid content. The mixtures included 18 odorants in concentrations suitable for direct analysis by GC–FID. In their non-volatile lipid contents, the non-fat model mixture represented solvent extracts obtained from foods such as bread, fruit juice, or vegetables, the low-fat model mixture simulated solvent extracts from foods such as biscuits, milk, or meat products, and the high-fat model mixture resembled typical solvent extracts from foods such as chocolate, hard cheese, or nuts.

The 18 odorants used in the models were selected based on their occurrence and odour activity in food, their compound class, their boiling point, their log P value, their stability, and their GC retention behaviour (Table 1). In detail, all 18 compounds had been identified as important odour-active compounds in food [22]. For example, **1**, ethyl butanoate is a major odorant in different kinds of fruit such as strawberry, orange, guava, and kiwifruit [23–26] and **2**, 3-methylbutan-1-ol is a characteristic odorous fermentation by-product in bread [27, 28] as well as in alcoholic beverages such as beer, whisky, and wine [29–31]. The 18 compounds included hydrocarbons (**3**, **6**, **8**), alcohols (**2**, **4**, **9**, **13**), esters (**1**, **7**, **11**, **12**, **15**, **17**), carboxylic acids (**5**, **10**, **16**) as well as a ketone (**18**) and a phenol (**14**). Reactive compounds such as aldehydes and thiols were excluded to avoid interferences by degradation reactions. The 18 compounds covered a boiling point range of 120 to 271 °C, a log P value range of 1.10 to 4.82, and a retention index (RI) range of 1009 to 2555 on the FFAP column used for quantitation by GC–FID. Most importantly, all 18 compounds showed baseline separation during GC analysis.

The three model mixtures were subjected to mSAFE and aSAFE. To perform mSAFE, the operators were instructed to open the manual valve as short as possible to keep the portion sizes small. Before the next addition, complete evaporation and recondensation of the preceding portion was ensured visually. This was achieved by observing the evaporation flask and the liquid nitrogen surrounding the recondensation flask. During a recondensation phase, the liquid nitrogen showed vigorous boiling due to compensation of the heat of condensation, whereas complete recondensation was indicated by smoothening of the liquid nitrogen surface. For aSAFE, the valve open time was set to 0.2 s for all experiments. The valve closed time was varied and set to 5, 20, or 60 s. Of the resulting 12 different model mixture/SAFE approach combinations, however, the combination of the high-fat mixture and the aSAFE with 5 s valve closed time was not applicable, because 5 s was not sufficient to evaporate the solvent completely from the individual high-fat mixture portions.

As expected, the non-fat model mixture resulted in the highest yields (Fig. 3a). Compounds **1–13** with boiling points ranging from 120 to 220 °C showed yields of ~ 100% for both, the mSAFE (Fig. 3a, yellow bars) and the aSAFE (Fig. 3a, blue bars), and the reproducibility of the yields indicated by the error bars was good. Compounds **14–18**

Table 1 Food odorants in the model mixtures used for yield determinations

No.[a]	Odorant	Odour	b.p.[b] (°C)	Log P[c]	RI[d] FFAP
1	Ethyl butanoate	Fruity	120	2.85	1027
2	3-Methylbutan-1-ol	Malty	130	1.35	1206
3	α-Pinene	Resinous	156	4.44	1009
4	Hexan-1-ol	Grassy	157	2.03	1350
5	Butanoic acid	Cheesy, sweaty	162	1.10	1620
6	Myrcene	Geranium leaf	167	4.82	1156
7	Ethyl hexanoate	Fruity, pineapple	168	2.83	1226
8	Limonene	Citrusy, lime	177	4.38	1189
9	Linalool	Citrusy, bergamot	199	2.84	1539
10	Hexanoic acid	Cheesy, sweaty	203	1.75	1836
11	Ethyl octanoate	Fruity	207	4.47	1441
12	Ethyl benzoate	Fruity, star fruit	212	2.59	1658
13	2-Phenylethanol	Floral, honey	220	1.30	1905
14	Eugenol	Clove	254	1.83	2164
15	Methyl cinnamate	Sweet, cinnamon	261	2.62	2056
16	Phenylacetic acid	Beeswax	265	1.40	2555
17	Ethyl cinnamate	Sweet, fruity	271	2.90	2116
18	β-Ionone	Floral, violet	271	4.00	1933

[a]Numbering in the order of increasing boiling points

[b]Boiling point

[c]Common logarithm of the *n*-octanol–water partition coefficient

[d]Retention index

with boiling points of 254 °C and beyond, however, showed clear differences between the mSAFE and the aSAFE. mSAFE yields were consistently lower than aSAFE yields. We ascribed this effect primarily to the reduction of the individual extract portion volumes achieved by the aSAFE approach. Compounds **14**, eugenol and **15**, methyl cinnamate still showed aSAFE yields of ~ 100%, whereas mSAFE yields were ~ 80%. Compounds **17**, ethyl cinnamate and **18**, β-ionone showed aSAFE yields of > 90%, but mSAFE yields of only ~ 60%. Interestingly, the lowest yields were not determined for **18,** β-ionone, the compound with the highest boiling point, but for compound **16**, phenylacetic acid, thus suggesting that SAFE yields were not only influenced by the type of SAFE and the boiling points of the compounds, but also by the compound class.

The low-fat model mixture (Fig. 3b) in many cases showed lower yields than the non-fat model mixture (Fig. 3a). mSAFE yields of ~ 100% were obtained until compound **8**, limonene (b.p. 177 °C), with the exception of compound **5**, butanoic acid, for which the yield was ~ 90%. Beyond compound **8**, limonene, mSAFE yields decreased from ~ 80% (**9**, linalool) to ~ 6% (**18,** β-ionone). The decrease was not continuous. Instead, two compounds, namely **10**, hexanoic acid and **16**, phenylacetic acid, showed clearly lower yields. In summary, the yields of all three carboxylic acids (**9**, **10**, **16**) were lower than expected from their boiling points. This effect was also observed in all further experiments with mSAFE as well as with aSAFE. The aSAFE yields obtained from the low-fat model mixture were again consistently higher than the corresponding mSAFE yields. Moreover, aSAFE yields were higher when the valve closed times were longer. However, these differences were smaller than the differences between mSAFE and aSAFE. Thus, the reduction of the individual extract portion size obviously had a greater effect on the compound yields than the increase of the time span between two portions. For example, for compound **13**, 2-phenylethanol, the aSAFE approaches with 5, 20, and 60 s valve closed time showed yields of 92, 97, and 99%, but the yield of mSAFE was only 58%. The compound with the lowest yields was again compound **16**, phenylacetic acid. This compound showed aSAFE yields of 18, 22, and 44%, and an mSAFE yield of only 1%.

The high-fat model mixture (Fig. 3c) showed virtually the same mSAFE yields as the low-fat model mixture (Fig. 3b), but aSAFE yields were somewhat lower for compounds with high boiling points. Nevertheless, all aSAFE yields were still clearly higher than the corresponding mSAFE yields. Using again compounds **13**, 2-phenylethanol and **16**, phenylacetic acid as examples, the aSAFE approaches resulted in yields of 81–85% (**13**) and 6–10% (**16**), whereas mSAFE yields were only 58% (**13**) and 1% (**16**).

Different from the yields, no clear difference between mSAFE and aSAFE was found in the reproducibility. Error bars were generally small when yields were close to 100%

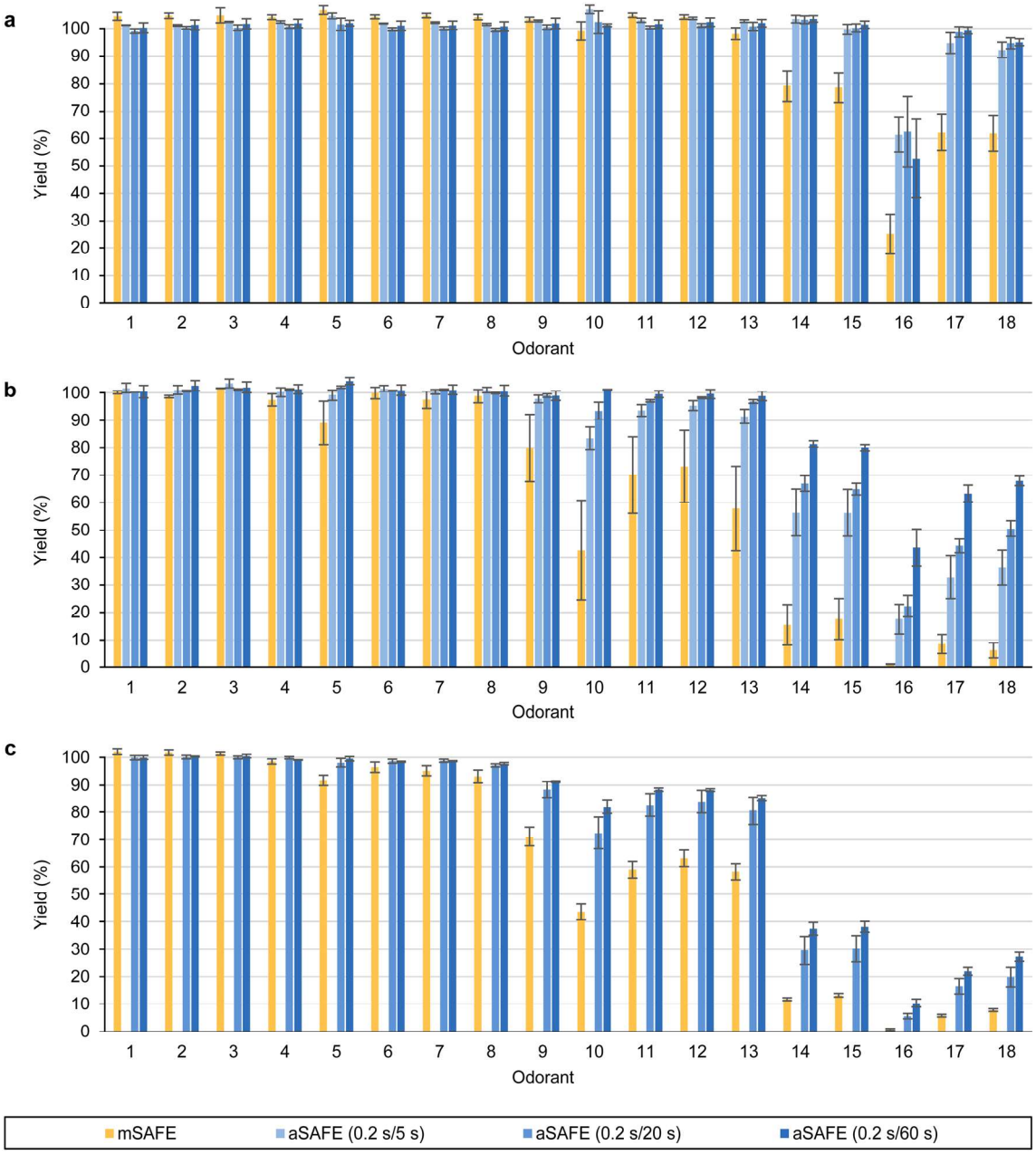

Fig. 3 Odorant yields of the aSAFE approach using valve open/closed time combinations of 0.2 s/5 s, 0.2 s/20 s, and 0.2 s/60 s in comparison to odorant yields of the mSAFE approach applied to three model mixtures of different fat content: **a**, non-fat; **b**, low-fat (100 mL non-fat mixture + 1 g oil); **c**, high-fat (100 mL non-fat mixture + 10 g oil). Odorant numbers refer to Table 1

and tended to increase in size when yields dropped, for both, mSAFE and aSAFE.

To evaluate the aSAFE approach further, we determined odorant yields from real food extracts. Beer and chocolate were chosen as a non-fat and a high-fat material, respectively. Odorants **9**, linalool; **13**, 2-phenylethanol; and **16**, phenylacetic acid were selected for quantitation, because they are present in both, beer [29] and chocolate [32]. The mSAFE approach was compared to aSAFE with a valve open/closed time combination of 0.2 s/60 s. Results are depicted in Figs. 4 and 5 in comparison to the yields obtained with the corresponding model mixtures.

The non-fat model mixture (Fig. 4a) and the beer extract (Fig. 4b) showed comparable yields. In both cases, yields of compounds **9**, linalool and **13**, 2-phenylethanol were close to 100%. The clearly higher absolute amount

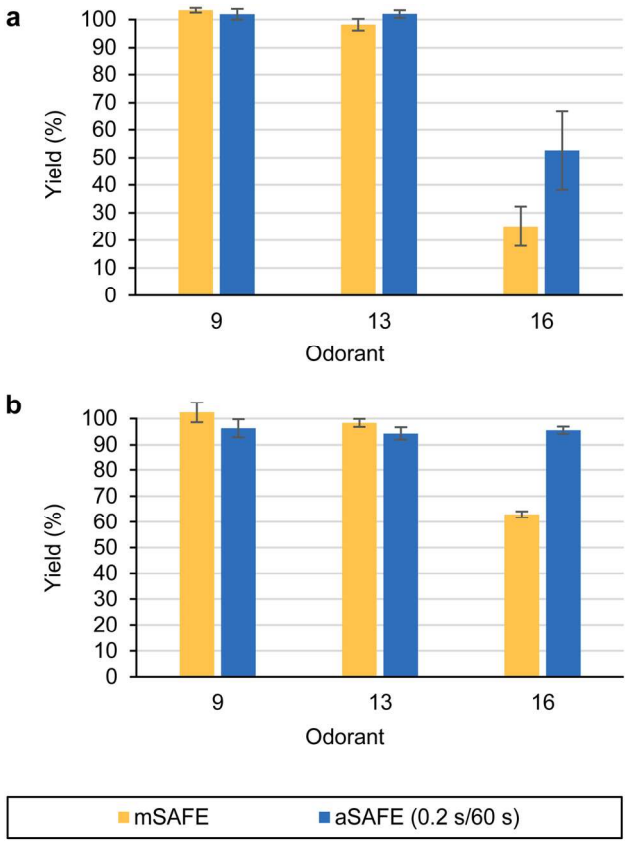

Fig. 4 Yields of compounds **9**, **13**, and **16** after application of the aSAFE approach with a valve open/closed time combination of 0.2 s/60 s, applied to the non-fat model mixture (**a**) and to the beer extract (**b**), in comparison to the yields of the mSAFE approach

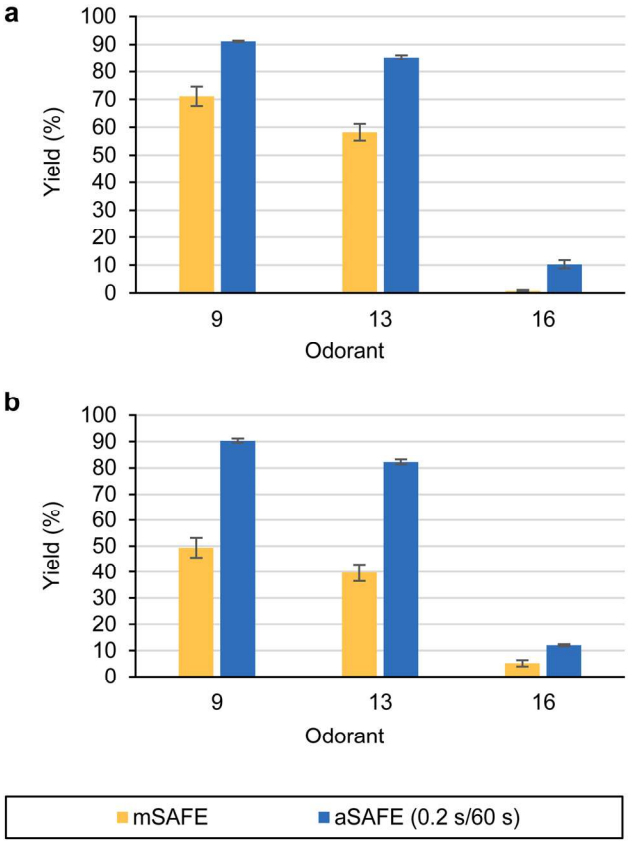

Fig. 5 Yields of compounds **9**, **13**, and **16** after application of the aSAFE approach with a valve open/closed time combination of 0.2 s/60 s, applied to the high-fat model mixture (**a**) and to the chocolate extract (**b**), in comparison to the yields of the mSAFE approach

of **13**, 2-phenylethanol in the beer extract, which was 8000 µg/L (Supplementary file 1, Table S14) compared to ~ 100 µg/L in the model mixture, obviously did not substantially influence the yield. The yields of **16**, phenylacetic acid were even higher from the beer extract than from the model mixture, however, again the aSAFE yield (96%) was clearly higher than the mSAFE yield (63%).

Likewise, the high-fat model mixture (Fig. 5a) and the chocolate extract (Fig. 5b) showed comparable yields. Again, the different absolute amounts of ~ 70 µg/L, ~ 400 µg/L, and ~ 1300 µg/L for compounds **9**, linalool; **13**, 2-phenylethanol; and **16**, phenylacetic acid in the chocolate extract (Supplementary file 1, Table S17) compared to ~ 100 µg/L for all three compounds in the model mixture, seemed to have only little influence on the yields. Particularly, the aSAFE yields were virtually the same. mSAFE yields were somewhat lower from the chocolate extract than from the model mixture for compounds **9**, linalool and **13**, 2-phenylethanol, but higher for compound **16**, phenylacetic acid. For all three compounds, yields from the chocolate extract were clearly higher when aSAFE was used instead of mSAFE.

Further automatisation of the aSAFE approach

With the aSAFE equipment depicted in Fig. 2, two of the major drawbacks of the mSAFE approach detailed in the introduction section were overcome: yields were clearly increased and "accidents" associated with too large extract portion sizes leading to a transfer of non-volatiles could be safely excluded. The manpower requirements, however, were only partly reduced. Although the operator does not need to manually open and close the valve of the dropping funnel anymore, there is still the need to refill the cold traps from time to time. Moreover, the operator is still required to be present towards the end of the aSAFE to stop the process at the electronic valve control unit as soon as there is only a minute amount of the extract left in the dropping funnel. Otherwise, uncontrolled ventilation of the apparatus could lead to damages to the vacuum system. Towards a fully automated SAFE, we therefore added an automated liquid nitrogen refill system as well as an endpoint recognition and shut-off system. The equipment is depicted in Fig. 6.

To supply both, the recondensation flask as well as the safety cold trap with the same automated liquid nitrogen

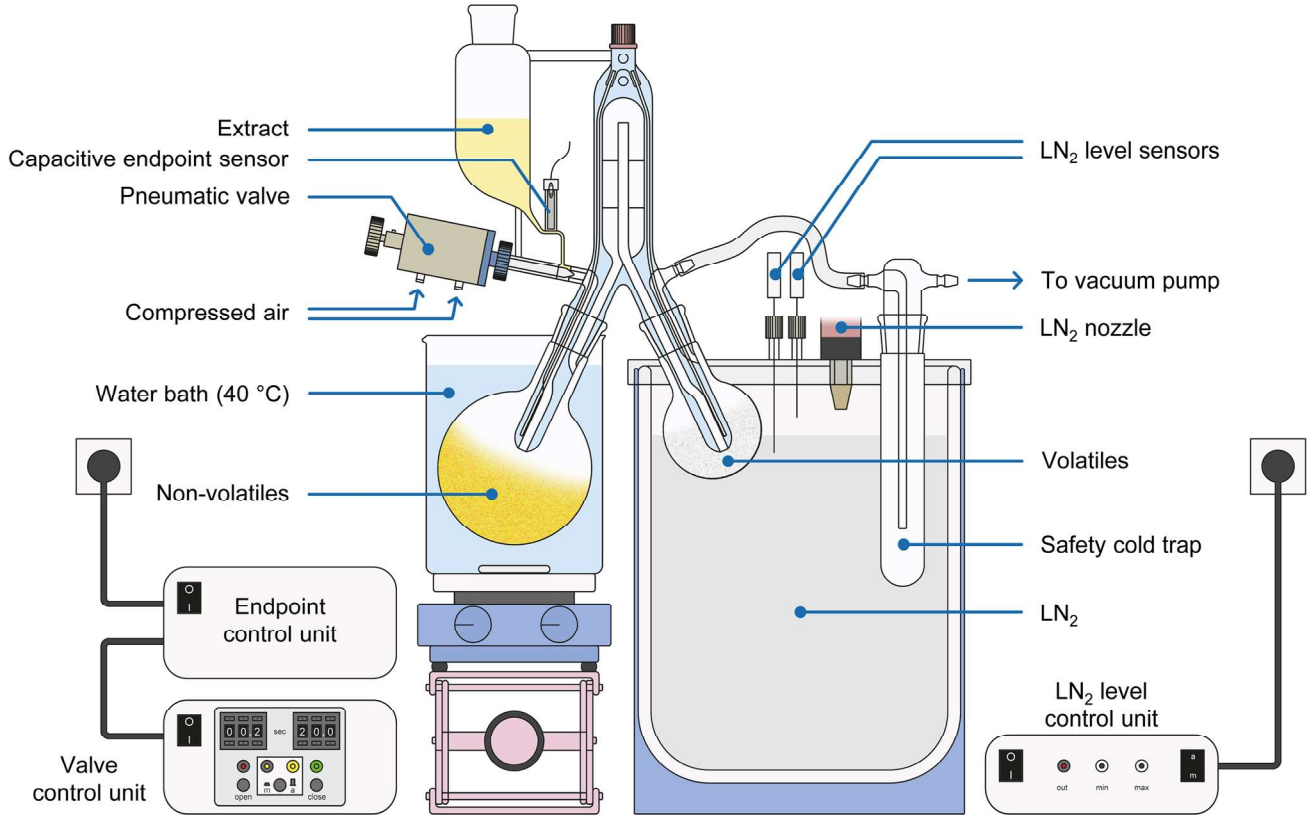

Fig. 6 Equipment for performing a fully automated SAFE including the pneumatic valve, the automated liquid nitrogen refill system as well as the endpoint recognition and shut-off system

refill system, the safety cold trap was separated from the SAFE glassware. The recondensation flask and the safety cold trap were placed in a Dewar vessel filled with liquid nitrogen. The automated nitrogen refill system consisted of a pressurised storage tank for cryogenic liquefied nitrogen with siphon, a solenoid valve, a transfer hose, a nozzle with phase separator, two liquid nitrogen level sensors, and an electronic liquid nitrogen level control unit operating the solenoid valve. The endpoint recognition and shut-off system consisted of a capacitive sensor and an electronic control unit. Adjustments were necessary in the glassware at the tube connecting the body of the dropping funnel with the valve. An additional glass cylinder served as casing for the sensor.

To perform a fully automated SAFE, the system is prepared and started as detailed for the aSAFE. Immediately thereafter, the liquid nitrogen level control unit is switched on. Whenever the liquid nitrogen level in the Dewar vessel drops below the level of the lower liquid nitrogen sensor, the liquid nitrogen level control unit opens the solenoid valve until the upper liquid nitrogen sensor is covered, thus ensuring sufficient cooling of the recondensation flask and the safety cold trap at all times. When finally the solvent extract level reaches the capacitive sensor at the outlet of the dropping funnel, the electronic endpoint control unit disconnects the electronic valve control unit operating the pneumatic valve from the power supply and thus stops the aSAFE process. The pneumatic valve remains closed, whereas the liquid nitrogen cooling is continued until the operator manually stops it before ventilating the system and collecting the isolated volatiles. A demonstration of a fully automated SAFE is available in a video provided on the internet [33].

Conclusion

The new aSAFE approach provides substantial advantages over mSAFE in terms of the yields of the volatiles and the risk of a transfer of non-volatiles. In our labs, aSAFE has meanwhile completely replaced the previous version. The fully automated SAFE equipment provides further advantages in the handling. However, the quality of the volatile isolate is not further improved when moving from the normal aSAFE to the fully automated equipment. Whether the improved handling of the fully automated equipment is worth the additional costs and workspace requirements is thus not a scientific but an economic question that may

be answered differently in an academic and an industrial environment, respectively.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00217-022-04072-1.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human and animal studies This article does not contain any studies with human or animal subjects.

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8.1.3 Summary and Individual Contributions

Isolation of volatile compounds from foods and beverages is a challenge, especially for flavor chemists when using gas chromatography–olfactometry (GC–O) to screen for odorants. Traditional methods such as steam distillation and direct injection have drawbacks due to thermal degradation and artifact formation. The ”high vacuum transfer“ (HVT) technique, which had been continuously improved after its introduction in 1970, for a long time was the preferred approach for the gentle isolation of food volatiles. In 1999, Engel et al. further developed the HVT approach into the solvent-assisted flavor evaporation (SAFE). SAFE used a dropping funnel with a manual needle valve stopcock to introduce solvent extracts into a thermostated evaporation flask, which was connected to a liquid nitrogen-cooled recondensation flask through a double-walled water-thermostated middle part. The recondensation flask was connected to a liquid nitrogen-cooled safety cold trap to protect the vacuum pump. The SAFE approach provided high yields, avoided the thermal formation of artifacts, and in addition was much easier to perform than the previous HVT approach. For this reason, SAFE quickly became the state-of-the-art method for volatile isolation in food aroma research. However, classical SAFE includes certain limitations, including the need for manual intervention and the risk of a transfer of non-volatiles due to unintentionally large extract portions. In addition, there were hints of an influence of the volume of individual portions as well as the time interval between portions on the yields of the volatile compounds. All limitations are associated with the manual valve.

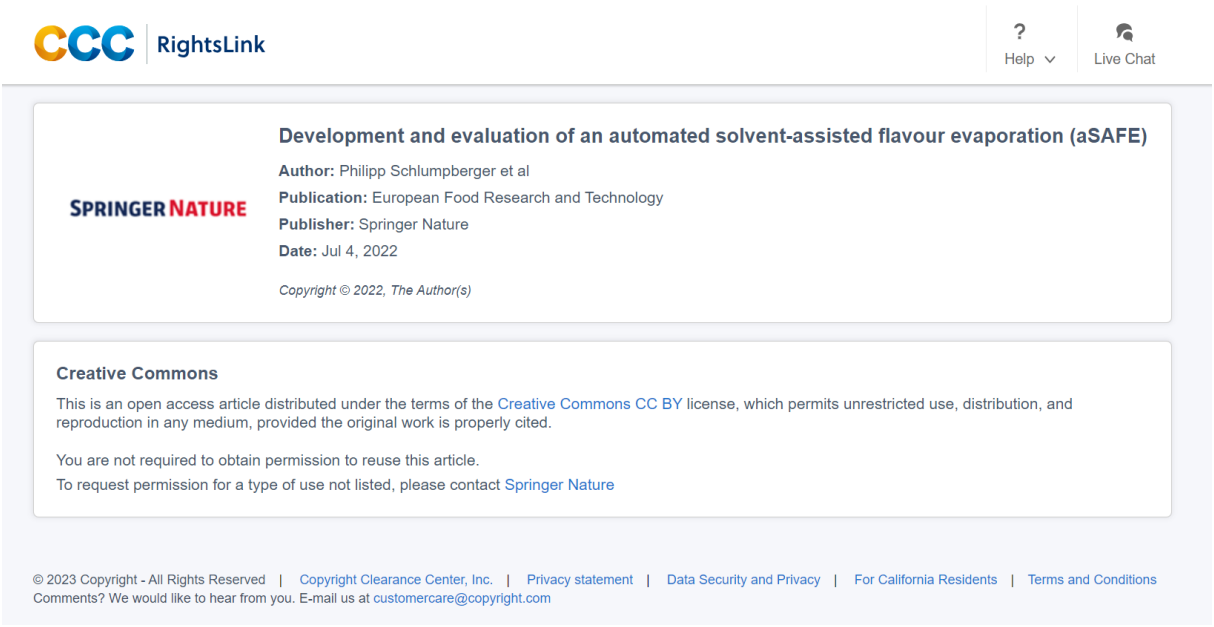
Therefore, the study aimed to improve SAFE by replacing the manual stopcock with an automated valve and evaluating the new approach, particularly in terms of yields. Further development towards a fully automated SAFE system was aimed at reducing manpower and increasing safety in the laboratory. The new version of the safe device was largely based on the original SAFE. The major modification was replacing the manual valve with a pneumatic valve. Accordingly, the original SAFE was referred to as “manual SAFE” (mSAFE), and for the new device, the name “automated SAFE” (aSAFE) was coined.

The aSAFE yields were determined using three different model mixtures (non-fat, low-fat, high-fat) with 18 well-known food odorants as well as two authentic food extracts. The results showed that aSAFE consistently resulted in higher yields than mSAFE. This was particularly the case for compounds with high-boiling points and extracts with high lipid content.

Further automation of the aSAFE was performed by adding an automated liquid nitrogen refill system and an endpoint recognition and shut-off system, which totally eliminated the need for manual operation during the entire volatile isolation process.

Christine Stübner substantially contributed to the development of the aSAFE device and the evaluation experiments with model solutions and food matrices. Christine conceived the idea of the fully automated SAFE approach and primarily accounted for the implementation of the automated liquid nitrogen refill system as well as the endpoint recognition and shut-off system. Philipp Schlumpberger participated in both, the development of the aSAFE as well as the development of the fully automated system. Christine and Philipp jointly evaluated the data and prepared the manuscript. Martin Steinhaus conceived and directed the study, supervised Christine’s and Philipp’s work, and revised the manuscript.

8.1.4 Reprint Permission



8.2 Publication 2: Sotolon and (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal Are the Key Compounds in the Aroma of Walnuts

8.2.1 Bibliographic Data

Title:	Sotolon and (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal Are the Key Compounds in the Aroma of Walnuts
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8.2.2 Publication Reprint

For a reprint of publication 1, please turn to the next page.

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Article

Sotolon and (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal Are the Key Compounds in the Aroma of Walnuts

Christine A. Stübner and Martin Steinhaus*

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ABSTRACT: Fresh kernels of the walnut tree (*Juglans regia* L.) show a characteristic and pleasant aroma, the molecular basis of which was unknown. The application of an aroma extract dilution analysis resulted in 50 odor-active compounds. Among them, 37 had not been reported as fresh walnut kernel volatiles before, including the two odorants with the highest flavor dilution factors, namely, fenugreek-like smelling 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one (sotolon) and oatmeal-like smelling (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal. Quantitations revealed 17 odorants with concentrations in the walnuts that exceeded their odor threshold concentrations. Aroma reconstitution and omission experiments finally showed that the characteristic aroma of fresh walnuts is best represented by a binary mixture of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal. Of both, the natural concentration was ∼10 μg/kg. Further sensory studies showed that the walnut character is intensified when their concentrations are in parallel increased to ∼100 μg/kg. This finding may guide the future breeding of new walnut cultivars with improved aroma.

KEYWORDS: *walnut, Juglans regia L., sotolon, (2E,4E,6Z)-nona-2,4,6-trienal, aroma extract dilution analysis (AEDA), stable isotopically substituted odorants, odor activity value (OAV), aroma reconstitution*

INTRODUCTION

The walnut tree (*Juglans regia* L.) is a huge tree with heights up to 30 m. It is native to a region in Eurasia stretching from southern Europe and the Near East to the Himalayan region and China.[1] Cultivation started more than 2000 years ago.[2] Today, walnut trees are grown worldwide in temperate and subtropical climates, predominantly for nuts. Fruits do not develop before an age of 15−20 years. The fruits are surrounded by a green fleshy husk and consist of a brown, woody, bipartite pericarp and a single edible seed with a light brown seed coat and huge wrinkled cotyledons.[1] The seeds are high in fat and fiber and commonly referred to as walnut kernels. Major exporting countries of whole and shelled walnuts are currently China, the USA, Iran, and Turkey.[3] Raw or toasted, walnut kernels are a popular snack and a common ingredient in bakery products and sweets, and also widely used as a garnish.

Fresh walnut kernels are particularly valued for their characteristic aroma, which is clearly different from that of other tree nuts such as almonds, cashew nuts, and hazelnuts. The first researchers interested in the molecular background of walnut aroma were Clark and Nursten in 1976.[4] They isolated walnut volatiles from the extracted oil in two different ways—one based on Likens-Nickerson extraction, the other one based on a milder, artifact-avoiding high vacuum degassing approach. The isolates were analyzed by gas chromatography−mass spectrometry (GC−MS) and gas chromatography−olfactometry (GC−O) using columns of different polarity. Up to 103 peaks could be separated in the chromatograms, however, none of them showed a specific walnut-like odor. Clark and Nursten concluded that the "odor of walnuts appears to be due to the collective effect of a number of components".[4] This assumption was confirmed in a subsequent study by the same authors.[5] This time, walnut volatiles were directly sampled from the headspace above the kernels. Again, GC−O and GC−MS analyses of the trapped volatiles did not reveal any peak with a specific walnut odor. However, when the entire eluate of the GC column was collected, its odor was clearly walnut-like. Fractionation experiments indicated that carbonyl compounds contributed to the walnut-like odor whereas alcohols did not. Which individual compounds play the key role in walnut aroma, however, remained unclear. Different mixtures of major carbonyl compounds among which were hexanal, pentane-2,3-dione, 2-methylpent-2-enal, and pentanal resulted at best only in a moderately walnut-like aroma.

For many years, the topic of the molecular background of the characteristic walnut aroma was not pursued further. Instead, research on walnut volatiles was focused on differences between origins,[6] differences between varieties,[7] their antioxidant potential,[8] and their suitability to assess the oxidative stability of walnuts after processing and storage.[7,9,10]

More recently, Liu et al.[11] attempted to reinvestigate the compounds responsible for the aroma of walnuts. They isolated the volatiles from raw and roasted walnut kernels by solvent-assisted flavor evaporation (SAFE)[12] and screened them for odorants by GC−O in combination with aroma extract dilution analysis[13] (AEDA). In the raw walnuts, 29 odor-active compounds were detected in a flavor dilution (FD) factor

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range of 1 to 243, among which 11 showing FD factors ≥9 were quantitated and 10 finally resulted in concentrations beyond the odor threshold concentration (OTC) corresponding to odor activity values (OAV = concentration in walnut/OTC) of ≥1. The highest OAVs were obtained for some fat oxidation products such as (2*E*)-non-2-enal (OAV 2217), octanal (OAV 769), hexanal (OAV 753), and nonanal (OAV 500). With this result, Liu et al. declared that they had "provided the integral determination of the key aroma-active compounds" in raw walnuts. However, they did not provide proof of their statement through an aroma reconstitution experiment.[14] When we prepared a solution of the 10 compounds proposed by Liu et al. as key odorants in raw walnuts in the reported concentrations and with an odorless mixture of medium-chain triglycerides as the solvent in our lab, we found that it showed an intense fatty and rancid odor but lacked the specific walnut-like character.

The objectives of the current study were therefore to re-screen the volatiles isolated from raw walnuts for odor-active compounds with a focus on potent odorants that had been overlooked in the previous studies, determine their natural concentrations, and assess their role in the overall raw walnut kernel aroma not only based on OAV calculations but eventually also by aroma reconstitution and omission experiments[14,15] with the aim to unequivocally identify the compounds responsible for the walnut character.

MATERIALS AND METHODS

Nuts. All nut kernels used in this study were purchased at the local retail market in Freising, Germany. In all cases, the kernels were dried but unroasted and the labeling indicated that they had been packaged under an inert gas atmosphere. The walnut sample was selected from numerous brands based on its characteristic and pronounced walnut aroma and the absence of rancid and other off-flavors, which easily develop when walnut kernels are stored in the presence of oxygen. All analyses were performed immediately after opening the package, in most cases within 2 days after purchase or at least before the best-before date.

Reference Odorants. The following compounds were purchased from commercial sources: **1**−**4**, **6**, **8**−**13**, **15**−**19**, **20**−**26**, **28**−**31**, **34**, **39**−**42**, **44**−**50** (Merck; Darmstadt, Germany), **5**, **36**, **37**, **43** (Thermo Fisher Scientific; Waltham, MA, USA), **7**, **32** (Toronto Research Chemicals; Toronto, Canada), **35** (Carl Roth; Karlsruhe, Germany), and **38** (Cayman Chemicals Company; Ann Arbor, MI, USA). Compound **14** was synthesized according to a procedure described in the literature.[16] Compound **21** was freshly distilled before use. Compound **27** was obtained from a commercial sample of **28** as detailed earlier.[17] The same approach was used to prepare compound **20** from a commercial sample of **24**. Compound **33** was synthesized according to Schuh and Schieberle[18] and underwent a first purification step by column chromatography as detailed in their paper. A second and final purification step was performed by preparative HPLC using a system from Knauer (Berlin, Germany) equipped with an Azura sampler AS 6.1 L, an Azura pump P6.1L HPG, an Azura detector MWD 2.1L, and a fraction collector LABOCOL Vario 4000. The column was a Eurosphere II Diol 100-5 (250 × 8 mm). The injection volume was 100 μL and the flow rate was 1.6 mL/min. Solvent A was *n*-hexane/ethanol 90/10 and solvent B was *n*-hexane/ethanol 70/30. The separation program was 0−20 min A/B from 100/0 to 90/10, 20−23 min to 0/100, 23−26 min back to 100/0, and continued 26−30 min. Ultraviolet detection was performed at 220 nm. For data evaluation, the Purity Chrome software, version 5.09.069 was used.

Stable Isotopically Substituted Odorants. The compounds ($^{2}H_3$)-**9**, ($^{13}C_2$)-**10**, and ($^{13}C_2$)-**49** were purchased from Merck. Compound ($^{2}H_3$)-**30** was from Cambridge Isotope Laboratories (Tewksbury, MA, USA). ($^{2}H_2$)-**5**, ($^{2}H_2$)-**7**, ($^{2}H_2$)-**8**, ($^{13}C_2$)-**12**, ($^{2}H_3$)-**13**, ($^{2}H_2$)-**14**, ($^{2}H_2$)-**15**, ($^{2}H_2$)-**17**, ($^{2}H_2$)-**19**, ($^{2}H_2$)-**22**, ($^{13}C_2$)-**24**, ($^{13}C_2$)-**28**, ($^{2}H_3$)-**31**, ($^{13}C_2$)-**33**, ($^{2}H_2$)-**34**, ($^{2}H_2$)-**38**, ($^{13}C_2$)-**40**, ($^{13}C_2$)-**46**, ($^{2}H_3$)-**47**, and ($^{2}H_3$)-**50** were synthesized according to procedures detailed in the literature; individual references are available in the Supporting Information, Table S1. Compound ($^{13}C_2$)-**33** was purified as detailed above for the isotopically unmodified compound **33**.

Miscellaneous Chemicals. Diethyl ether was purchased from CLN (Freising, Germany) and was freshly distilled through a column (120 cm × 5 cm) packed with Raschig rings before use. Odorless silicone oil was from Merck. Medium-chain triglycerides, type Miglyol 812, and silica gel 60 (0.040−0.63 mm) were purchased from VWR (Darmstadt, Germany). The silica gel was purified as detailed previously.[19]

Gas Chromatography. GC−O analyses were performed with a GC−O/FID instrument. For GC−MS analyses, four different instruments were used: a one-dimensional GC−MS instrument with a Paul trap mass analyzer, a two-dimensional heart-cut GC−GC−MS instrument with a Paul trap mass analyzer, a two-dimensional heart-cut GC−GC−HRMS instrument with an orbitrap mass analyzer, and a comprehensive two-dimensional GC×GC−MS instrument with a time-of-flight mass analyzer. Details on the individual instruments are available in the Supporting Information.

Aroma Extract Dilution Analysis. Walnut kernels (150 g) were crushed down to a particle size of ∼1−3 mm using a mortar and pestle. A portion (50 g) of the crushed kernels was placed in a 2 L amber-colored wide-neck Erlenmeyer flask. Under ice-cooling, saturated calcium chloride solution was added (100 mL) to stop enzymatic reactions,[20] before the mixture was homogenized with a stainless-steel blender to facilitate the following extraction step. Diethyl ether (350 mL) was added and the mixture was stirred at ambient temperature in the dark overnight. Under ice cooling, anhydrous sodium sulfate (300 g) was added and the organic phase was decanted through a folded filter paper. The residue was washed with diethyl ether (3 × 100 mL) and the organic phases were combined. Nonvolatiles were removed by automated solvent-assisted flavor evaporation (aSAFE)[21] at 40 °C using an open/closed time combination of the pneumatic valve of 0.1 s/10 s. The distillate was dried over anhydrous sodium sulfate (50 g) and concentrated to a volume of 0.5 mL, first using a Vigreux column (50 × 1 cm) and finally a Bemelmans microdistillation device.[22] When a drop of this volatile isolate was placed on a fragrance test strip and the odor was evaluated directly after evaporation of the solvent, the characteristic walnut aroma was clearly perceivable.

The walnut volatile isolate was subjected to GC−O analysis using the GC−O/FID instrument detailed in the Supporting Information with the FFAP column. Two trained and experienced assessors with complementary olfactory capabilities[14] repeatedly performed GC−O until results were reproducible. By stepwise 1:2 dilution of the volatile isolate with diethyl ether, dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, and 1:2048 of the initial solution were prepared and subjected to GC−O analysis. Each odor-active compound was assigned an FD factor corresponding to the dilution factor of the highest diluted sample in which the odor was perceived by any of the two assessors.[14]

Toward structural identification of the odor-active compounds, odor description and retention index (RI) on the FFAP column were first compared with data compiled in databases.[23,24] Structure proposals were verified by GC−O of authentic reference compounds. If this verification was successful, further confirmation was sought by parallel GC−O analysis of the walnut volatile isolate and the reference compounds using the DB-5 column. Final structure confirmation was achieved by comparing mass spectra of the compounds in the walnut volatile isolate with mass spectra of the reference compounds analyzed under identical conditions. To minimize coelution problems, the GC×GC−MS instrument was employed for this purpose.

Odorant Quantitation. The workup of the walnut kernels (50−150 g) was performed as detailed in the AEDA section. The stable isotopically substituted odorants used as internal standards (cf. Supporting Information, Table S2) were added to the first diethyl ether portion in the extraction step. Depending on the expected target compound concentrations, amounts of the added internal standards ranged from 0.06 to 14.7 μg. The aSAFE distillates were concentrated

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Table 1. Odorants in the Volatile Isolate Obtained from Walnut Kernels

no.	odorant[a]	odor[b]	RI[c] FFAP	RI[c] DB-5	FD factor[d]	previously reported[e]
1	butane-2,3-dione	buttery	982	603	2	5/42
2	hexanal	green, grassy	1080	802	2	5/43
3	γ-terpinene	earthy	1234	1059	4	−/44
4	octanal	citrusy	1285	1005	4	6/45
5	oct-1-en-3-one	mushroom	1293	979	256	6/37
6	2-ethylpyrazine	roasty	1331	916	8	−/38
7	(5*Z*)-octa-1,5-dien-3-one	geranium leaf	1364	982	16	−/−
8	(2*E*)-oct-2-enal	fatty, citrusy	1419	1061	64	6/38
9	3-isopropyl-2-methoxypyrazine	bell pepper	1417	1086	64	−/−
10	acetic acid	vinegar	1450	636	16	46/38
11	methional	cooked potato	1455	910	4	−/−
12	(2*E*,4E)-hepta-2,4-dienal	floral, fatty	1480	1015	16	6/47
13	3-*sec*-butyl-2-methoxypyrazine	bell pepper	1496	1167	64	−/−
14	(2*Z*)-non-2-enal	fatty, floral	1494	1148	32	−/42
15	(2*E*)-non-2-enal	cucumber, green	1532	1163	16	6/38
16	2-methylpropanoic acid	sweaty, cheesy	1560	783	8	−/42
17	(2*E*,6*Z*)-nona-2,6-dienal	cucumber, green	1584	1154	32	−/−
18	undecanal	fatty, floral	1600	1306	8	−/−
19	butanoic acid	sweaty, cheesy	1627	827	16	48/38
20	(2*E*,4*Z*)-nona-2,4-dienal	fatty	1639	1197	16	−/−
21	phenylacetaldehyde	floral, honey	1639	1047	8	48/38
22	3-methylbutanoic acid	sweaty, cheesy	1667	863	16	−/44
23	2-methylbutanoic acid	sweaty, cheesy	1668	857	16	−/−
24	(2*E*,4*E*)-nona-2,4-dienal	fatty	1692	1215	32	−/38
25	(2*E*)-undec-2-enal	green, soapy	1747	1362	8	−/49
26	α-farnesene	green	1745	1509	8	−/−
27	(2*E*,4*Z*)-deca-2,4-dienal	fatty, deep-fried	1752	1296	32	−/42
28	(2*E*,4*E*)-deca-2,4-dienal	fatty, deep-fried	1808	1322	32	11/43
29	cyclotene	fenugreek	1819	1024	8	−/42
30	hexanoic acid	sweaty, cheesy	1838	1015	16	50/38
31	2-methoxyphenol	smoky	1862	1087	256	−/−
32	(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal	oatmeal	1876	1273	1024	−/−
33	(2*E*,4*E*,6*E*)-nona-2,4,6-trienal	oatmeal	1895	1285	2	−/−
34	γ-octalactone	coconut	1918	1255	32	−/−
35	β-ionone	floral, raspberry	1928	1480	4	−/42
36	δ-octalactone	coconut	1967	1292	4	−/−
37	maltol	caramel	1974	1114	4	−/38
38	*trans*-4,5-epoxy-(2*E*)-dec-2-enal	metallic	2004	1382	256	−/−
39	4-methoxybenzaldehyde	aniseed, woodruff	2031	1259	8	−/−
40	HDMF[f]	caramel	2033	1087	256	−/38
41	EHMF[g]	caramel	2077	1139/1148[h]	8	−/−
42	4-hydroxy-5-methylfuran-3-one	fruity, caramel	2127	1065	4	−/−
43	γ-decalactone	coconut	2133	1496	4	−/−
44	eugenol	clove	2169	1354	8	11/−
45	(2*Z*,4*Z*)-δ-deca-2,4-dienolactone	sweet, coconut	2170	1459	8	−/−
46	sotolon	fenugreek	2205	1111	512	−/−
47	2′-aminoacetophenone	foxy	2222	1304	64	−/−
48	(6*Z*)-γ-dodec-6-enolactone	sweet, fruity	2389	1658	4	−/−
49	2-phenylacetic acid	floral, honey	2553	1267	64	−/−
50	vanillin	vanilla	2573	1400	64	−/−

[a]Each odorant was identified by comparing its retention indices on two GC columns of different polarity (DB-FFAP and DB-5), its mass spectrum obtained by GC−MS, as well as its odor as perceived at the sniffing port during GC−O to data obtained from authentic reference compounds analyzed in parallel. [b]Odor as perceived at the sniffing port during GC−O. [c]Retention index; calculated from the retention time of the compound and the retention times of adjacent *n*-alkanes by linear interpolation. [d]Flavor dilution factor; dilution factor of the highest diluted walnut volatile isolate in which the odorant was detected during GC−O analysis by any of two assessors. [e]References that first reported the compound as fresh walnut kernel volatile/walnut oil volatile; the minus sign (−) indicates that there was no report in the literature yet. [f]4-Hydroxy-2,5-dimethylfuran-3(2*H*)-one. [g]2-Ethyl-4-hydroxy-5-methylfuran-3-one. [h]EHMF is separated from its tautomer 5-ethyl-4-hydroxy-2-methylfuran-3-one on the DB-5 column, on the DB-FFAP column no separation of the isomers was observed.

to a volume of 100 μL and subjected to GC−MS analysis using the heart-cut GC−GC−MS instrument (**5**, **8**, **14**, **15**, **19**, **22**, **23**, and **30**), the heart-cut GC−GC−HRMS instrument in the positive CI mode (**17** and **34**), the heart-cut GC−GC−HRMS instrument in the negative CI

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mode (**38**), or the GC×GC−MS instrument (**7**, **9**, **10**, **12**, **13**, **20**, **24**, **27**, **28**, **31**, **32**, **40**, **46**, **47**, **49**, and **50**). All quantitations were performed in duplicates or triplicates.

Peak areas corresponding to the analyte and internal standard were obtained from the extracted ion chromatograms using characteristic quantifier ions. Odorant concentrations in the walnut kernels were calculated from the area counts of the analyte peak, the area counts of the standard peak, the amount of walnut used for the workup, and the amount of standard added, by employing a calibration line equation. The calibration line equation was obtained by linear regression after analysis of analyte/standard mixtures in different concentration ratios. Quantifier ions and calibration line equations are available in the Supporting Information, Table S2. Individual concentration data and standard deviations are available in the Supporting Information, Table S3.

Odor Threshold Concentrations. These were determined orthonasally in low-odor sunflower oil according to the American Society for Testing and Materials standard practice for determination of odor and taste thresholds by a forced-choice ascending concentration series method of limits.[25] Test compounds were checked for purity by AEDA before use and considered suitable for the OTC determination if the FD factor of the target compound was at least 100 times higher than the FD factor of the most potent impurity. Spiked samples were prepared by adding the test substance in ethanolic solution to the oil. To the reference samples, a corresponding amount of pure ethanol was added. The final ethanol concentrations were kept below 300 μL/kg oil. Between two consecutive three-alternative forced choice tests, odorant concentrations differed by a factor of 3. Samples (20 g) were presented to the assessors in cylindrical single-use polystyrene vessels (40 mL nominal volume) with polytetrafluoroethylene lids. The tests were carried out at 22 ± 2 °C room temperature by 12−20 trained assessors in separate booths of a room exclusively dedicated to sensory evaluations.

Sensory Evaluation of Walnut Aroma Model Mixtures. The general matrix used for the aroma reconstitution experiments, omission experiments, and sensory tests with different sotolon/(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal mixtures was an emulsion obtained by mixing 29 g of odorless silicone oil with 1 g of an aqueous phase buffered to a pH of 6.5 ($H_2PO_4^-$/HPO_4^{2-}). The pH corresponded to the pH measured in a homogenate of the walnut kernels with a minimum amount of demineralized water. Aliquots of ethanolic or aqueous stock solutions of the reference odorants were added either to the silicone oil or to the aqueous phase before mixing. Final ethanol concentrations were kept below 300 μL/kg. The model mixtures (30 g) were presented in 100 mL Erlenmeyer flasks with glass stoppers under magnetic stirring to a panel of 14−18 trained assessors. The tests were carried out at 22 ± 2 °C room temperature in the room described before. Assessors were asked to orthonasally rate the intensities of descriptors defined by reference materials. The descriptors "fenugreek" and "oatmeal" were defined by aqueous solutions of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal, respectively. Concentrations were 100 times the OTC. The descriptor "walnut" was defined by freshly crushed walnut kernels. Ratings of all panelists were averaged by calculating the arithmetic mean.

RESULTS AND DISCUSSION

Odorant Screening. GC−O in combination with AEDA applied to the volatile isolate obtained from walnut kernels with a characteristic aroma profile resulted in 50 odor-active compounds, all of which were successfully identified (Table 1). Surprisingly, only 13 of the 50 compounds had previously been reported in walnuts (Table 1, rightmost column). Among the other 37 compounds, 12 were known as walnut oil volatiles, but 25 were unknown in walnuts as well as in walnut oil.

The odor descriptions were highly diverse. Frequently mentioned descriptors included fatty (8×), floral (6×), green (5×), and sweaty, cheesy (5×). None of the odorants was described as specifically walnut-like. This confirmed earlier results[4,5,11] and supported the hypothesis of Clark and Nursten[4,5] that walnut aroma is formed by a combination of compounds and is not caused by a single odorant.

FD factors ranged from 2 to 1024. The compounds with the highest FD factors were oatmeal-like smelling (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**; FD factor 1024) and fenugreek-like smelling 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one, better known as sotolon (**46**; FD factor 512). Both compounds had not been reported as walnut constituents before. (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal is the character impact compound in the aroma of oatmeal,[18] it substantially contributes to the aroma of black tea,[26] and it has been reported as an odor-active compound in a variety of other foods such as green tea,[27] hog plum pulp,[28] and prawns.[29] (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal is formed from linolenic acid.[18]

Sotolon is the character impact compound in many herbs, spices, and seasonings used to flavor savory foods. Herbs and spices include fenugreek seeds, fenugreek leaves, lovage leaves, Transcaucasian hogweed shoots, and blue melilot shoots.[30−32] Ground fenugreek seeds are widely used in commercial curry powders. For this reason, the odor of sotolon is also often described as curry (powder)-like. Fenugreek leaves are used in Indian curry dishes. Fresh lovage leaves and dried Transcaucasian hogweed shoots are used to season soups. Whereas lovage leaves are used all over Europe, Transcaucasian hogweed is specifically used in Armenia to flavor Karshm soup, a local specialty.[31] Dried blue melilot shoots are used in the European alpine region to season local bread and cheese types. Not least, sotolon substantially contributes to the characteristic aroma of soy sauce.[33] Sotolon is not only biochemically formed but also during thermal food processing in the course of the Maillard reaction,[34] for example during pan frying of white mushrooms.[35] Recent metaanalysis has identified sotolon as one of the generalists among the odorants in food, that is, it shows an exceptionally great abundance.[36]

In the order of decreasing FD factors, (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal and sotolon were followed by a group of four compounds, all of which showed an FD factor of 256. These four compounds were mushroom-like smelling oct-1-en-3-one (**5**), caramel-like smelling 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one (HDMF; **40**), also known by its trade name Furaneol, metallic smelling *trans*-4,5-epoxy-(2*E*)-dec-2-enal (**38**), and smoky smelling 2-methoxyphenol (**31**). Oct-1-en-3-one had been detected in walnuts as well as in walnut oil before,[6,37] whereas HDMF had only been known in walnut oil,[38] and *trans*-4,5-epoxy-(2*E*)-dec-2-enal and 2-methoxyphenol had previously been unknown as walnut and walnut oil constituents.

Looking at the compound classes, it became apparent that oxidation products of fatty acids constituted the major group within the 50 compounds listed in Table 1. This group included 16 aldehydes (**2**, **4**, **8**, **12**, **14**, **15**, **17**, **18**, **20**, **24**, **25**, **27**, **28**, **32**, **33**, and **38**), 3 ketones (**1**, **5**, and **7**), and 5 lactones (**34**, **36**, **43**, **45**, and **48**). Further compound classes were amino acid derivatives (**11**, **21**−**23**, **31**, **39**, **44**, **47**, **49**, and **50**), sugar-derived *O*-heterocycles (**29**, **37**, **40**−**42**, and **46**), *N*-heterocyclic pyrazines (**6**, **9**, and **13**), and terpenoids (**3**, **26**, and **35**).

Odorant Quantitation and OAV Calculation. The 27 odorants which showed an FD factor of ≥16 in the screening (cf. Table 1) were selected for quantitation by GC−MS. Stable isotopically substituted odorants were used as internal standards (cf. Supporting Information, Table S2). For 23 compounds, isotopologues were available, allowing for an ideal compensation of potential workup losses. Only for compounds **20**, **23**, **27**, and **32**, no isotopologue was available. These compounds were

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Table 2. Concentrations and OAVs of Important Odorants in Walnut Kernels

no.[a]	odorant	concentration in walnuts[b] (μg/kg)	odor threshold concentration[c] (μg/kg)	OAV[d]
10	acetic acid	44200	350	130
46	sotolon	10.6	0.23	46
27	(2*E*,4*Z*)-deca-2,4-dienal	46.7	2.8[e]	17
22	3-methylbutanoic acid	118	9.0	13
32	(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal	10.2	1.1	9.3
30	hexanoic acid	2870	460	6.2
19	butanoic acid	184	34	5.4
38	*trans*-4,5-epoxy-(2*E*)-dec-2-enal	55.7	13	4.3
14	(2*Z*)-non-2-enal	13.6	3.6	3.8
8	(2*E*)-oct-2-enal	439	120	3.7
49	2-phenylacetic acid	90.2	26	3.5
28	(2*E*,4*E*)-deca-2,4-dienal	178	66	2.7
31	2-methoxyphenol	3.98	1.8	2.2
9	3-isopropyl-2-methoxypyrazine	0.0206	0.010	2.1
7	(5*Z*)-octa-1,5-dien-3-one	0.0659	0.044	1.5
24	(2*E*,4*E*)-nona-2,4-dienal	36.6	30	1.2
5	oct-1-en-3-one	7.42	6.9	1.1
15	(2*E*)-non-2-enal	121	140	<1
50	vanillin	105	140	<1
40	HDMF[f]	12.8	25	<1
23	2-methylbutanoic acid	52.6	110	<1
47	2′-aminoacetophenone	7.80	21	<1
13	3-*sec*-butyl-2-methoxypyrazine	<0.10	0.46	<1
20	(2*E*,4*Z*)-nona-2,4-dienal	3.48	16[e]	<1
17	(2*E*,6*Z*)-nona-2,6-dienal	8.76	65	<1
34	γ-octalactone	11.5	280	<1
12	(2*E*,4E)-hepta-2,4-dienal	13.3	710	<1

[a]Numbering according to Table 1. [b]Mean of duplicates or triplicates; individual values and standard deviations are available in the Supporting Information, Table S3. [c]Odor threshold concentrations determined in low odor sunflower oil. [d]Odor activity value; calculated as a ratio of concentration to odor threshold concentration. [e]Approximated from the odor threshold concentration of the (2*E*,4*E*)-isomer in low odor sunflower oil and the ratio of the odor threshold concentrations of the individual isomers in air (Supporting Information, Table S4). [f]4-Hydroxy-2,5-dimethylfuran-3(2*H*)-one.

quantitated using as internal standards the isotopologues of the isomeric compounds **24**, **22**, **28**, and **33**, respectively.

The results of the odorant quantitations showed concentrations between 0.0206 and 44,200 μg/kg, thus spanning a range of over 6 orders of magnitude (Table 2). High concentrations were determined for acetic acid (**10**; 44,200 μg/kg) and hexanoic acid (**30**; 2870 μg/kg), followed by (2*E*)-oct-2-enal (**8**; 439 μg/kg), butanoic acid (**19**; 184 μg/kg), (2*E*,4*E*)-deca-2,4-dienal (**28**; 178 μg/kg), (2*E*)-non-2-enal (**15**; 121 μg/kg), 3-methylbutanoic acid (**22**; 118 μg/kg), and vanillin (**50**; 105 μg/kg). The concentrations of (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**) and sotolon (**46**), the compounds with the highest FD factors in the screening (cf. Table 1), were interestingly in the same range and amounted to 10.2 and 10.6 μg/kg, respectively. Situated on the low end were the concentrations of 3-*sec*-butyl-2-methoxypyrazine (**13**; <0.10 μg/kg), (5*Z*)-octa-1,5-dien-3-one (**7**; 0.0659 μg/kg), and 3-isopropyl-2-methoxypyrazine (**9**; 0.0206 μg/kg).

By dividing the concentrations in the walnuts by the corresponding OTCs in oil, OAVs were calculated for the 27 odorants (Table 2). Among them, 17 odorants showed an OAV >1. The highest OAVs were calculated for vinegar-like smelling acetic acid (**10**; OAV 130), fenugreek-like smelling sotolon (**46**; OAV 46), fatty, deep-fried smelling (2*E*,4*Z*)-deca-2,4-dienal (**27**; OAV 17), sweaty, cheesy smelling 3-methylbutanoic acid (**22**; OAV 13), oatmeal-like smelling (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**; OAV 9.3), and sweaty, cheesy smelling compounds hexanoic acid (**30**; OAV 6.2) and butanoic acid (**19**; OAV 5.4). Ten further odorants showed OAVs >1 but <5, including *trans*-4,5-epoxy-(2*E*)-dec-2-enal, (2*Z*)-non-2-enal, (2*E*)-oct-2-enal, 2-phenylacetic acid, (2*E*,4*E*)-deca-2,4-dienal, 2-methoxyphenol, 3-isopropyl-2-methoxypyrazine, (5*Z*)-octa-1,5-dien-3-one, (2*E*,4*E*)-nona-2,4-dienal, and oct-1-en-3-one.

OAV data are often used as the basis to discuss the relative contribution of individual odorants to the overall aroma. In fact, OAVs typically provide a much better approximation for the relative importance of odorants than FD factors resulting from AEDA because they are (1) not influenced by workup yields if based on proper quantitations, (2) consider the different volatility of the odorants because threshold data are obtained at room temperature and not at a hot sniffing-port, and (3) consider the different release behavior of the odorants because threshold data are determined in a matrix and not in air.[14] However, the significance of OAV data depends largely on the similarity between the matrix used for the threshold determinations and the real food matrix. For foods high in water, OAVs based on OTCs determined in pure water are considered a good approximation. Considering that walnut kernels are low in water but high in fat, we employed OTCs determined in oil. However, this approach most probably led to an overestimation of the carboxylic acids. The OAVs determined for acetic acid (**10**; OAV 350), 3-methylbutanoic acid (**22**; OAV 13), hexanoic acid (**30**; OAV 6.2), and butanoic acid (**19**; OAV 5.4) were unrealistically high considering that in the natural

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matrix with some aqueous phase and a pH of 6.5, which is clearly beyond the pk_a values of the acids, the major parts would be deprotonated and therefore odor-inactive. Consequently, this would highlight the importance of the other compounds with high OAVs, namely, sotolon (**46**; OAV 46), (2*E*,4*Z*)-deca-2,4-dienal (**27**; OAV 17), and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**; OAV 9.3).

A second point that limits their significance is that OAVs do not consider interactions during the perception of odorant mixtures. Often, the odor of a mixture is dominated by some odorants while the odor of others is totally suppressed even though their OAVs are clearly >1.[14] Sometimes, however, the combination of odorants generates a new synthetic odor that is not perceivable in the individual odorants. For example, it has been shown that the combination of cooked potato-like smelling methional and geranium leaf-like smelling (5*Z*)-octa-1,5-dien-3-one in a ratio of 100:1 results in a fishy smell.[39] A similar effect might generate the characteristic aroma of walnuts. In order to elucidate the compounds being crucial for the characteristic walnut aroma, we proceeded with aroma reconstitution and omission experiments, for which we used a matrix that was closer to walnuts and apart from the predominating oil content additionally considered the water content and the pH of walnuts.

Aroma Reconstitution and Omission Experiments. A first reconstitution model (Table 3, RM 1) included all 17 compounds for which OAVs ≥1 had been determined, dissolved in the natural concentrations (cf. Table 2) in a buffered oil/water emulsion. The second reconstitution model (Table 3, RM 2) was supposed to include only the five odorants with the highest OAVs of 9.3−130, while the other 12 compounds with rather low OAVs (1.1−6.2) should be omitted. However, we faced the problem that the (2*E*,4*Z*)-deca-2,4-dienal (**27**; OAV 17) reference contained some of the (2*E*,4*E*)-isomer (**28**; OAV 2.7). This prompted us—despite its low OAV—to additionally include (2*E*,4*E*)-deca-2,4-dienal in RM 2. When preparing the mixture, the amounts of the two reference compound samples were adjusted in order to result in the exact concentrations previously quantitated, thereby considering the amount of the (2*E*,4*E*)-isomer impurity in the (2*E*,4*Z*)-reference.

Table 3. Intensity of the Characteristic Walnut Note in Aroma Reconstitution Models based on 2 to 17 Odorants in Their Natural Concentrations

reconstitution model	odorants[a]	intensity "walnut"[b]
RM 1	all 17 odorants with OAVs >1	1.6
RM 2	**10**, **22**, **27**, **28**, **32**, **46**	2.1
RM 3	**10**, **22**	0.1
RM 4	**10**, **32**	0.3
RM 5	**10**, **27/28**	0.4
RM 6	**10**, **46**	1.0
RM 7	**22**, **27/28**	0.3
RM 8	**22**, **32**	0.4
RM 9	**22**, **46**	0.7
RM 10	**27/28**, **32**	0.5
RM 11	**27/28**, **46**	1.6
RM 12	**32**, **46**	2.3

[a]Odorant numbers according to Table 1. [b]Assessors rated the intensity of the odor impression "walnut" on a scale from 0 to 3 with 0.5 increments and 0 = not perceptible, 1 = weak, 2 = moderate, and 3 = strong.

A trained sensory panel evaluated the two aroma reconstitution models RM 1 and RM 2 orthonasally in comparison to fresh walnut kernels. Assessors rated the intensity of the odor impression "walnut" on a scale from 0 to 3 with 0.5 increments and 0 = not perceptible, 1 = weak, 2 = moderate, and 3 = strong. To our surprise, model RM 2 with only 6 odorants was rated more walnut-like than model RM 1 with 17 odorants. Obviously, the odorants present in RM 1 but not in RM 2 reduced the typical walnut character in the overall odor profile. This prompted us to hypothesize that the compounds generating the characteristic walnut impression are among the six odorants included in RM 2. In the simplest case, a combination of two of the six would create a walnut aroma. Therefore, we aimed at proceeding with the sensory evaluation of binary mixtures. Given the impurity problem discussed before, the deca-2,4-dienal isomers **27** and **28** were treated as if they were just one compound, which was not considered a problem, because they showed virtually the same fatty, deep-fried odor. Results are displayed in Table 3 (RM 3−12).

A very characteristic walnut note was detected when oatmeal-like smelling (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (**32**) was combined with fenugreek-like smelling sotolon (**46**) (Table 3, RM 12). In this binary mixture, the intensity of the odor impression "walnut" was rated 2.3 out of 3. This score was clearly higher than the scores of all other mixtures including RM 2. Sotolon seems to contribute more to the walnut character than (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal because all mixtures containing sotolon (RM 6, 9, 11, and 12) showed more walnut character (0.7−2.3) than the binary mixtures without sotolon (0.1−0.5). Actually, the term "walnut-like" has been used in some studies to describe the odor of sotolon.[40,41] Nevertheless, in our experiments only the simultaneous presence of (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal was able to push the fenugreek-like odor of sotolon toward a clear walnut character rated with the highest score of 2.3 (RM 12).[43−49]

Sotolon and (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal in Other Tree Nuts. The aroma reconstitution and omission experiments detailed in the previous section suggested that a mixture of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal in a ratio of ∼1:1 and at a concentration level of ∼10 μg/kg is crucial for the characteristic aroma of walnuts. In other tree nuts without a pronounced walnut character, the concentrations would most probably differ from the concentrations in the walnuts. To

Table 4. Concentrations of (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal and Sotolon in Different Tree Nuts

		concentration (μg/kg)					
no.[a]	odorant	cashew nut[b]	hazelnut[b]	almond[b]	Brazil nut[b]	pecan nut[b]	walnut[c]
32	(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal	<0.20	<0.20	0.560	1.18	7.87	10.2
46	sotolon	3.55	2.15	3.21	0.506	23.6	10.6

[a]Numbering according to Table 1. [b]Mean of duplicates or triplicates; individual values and standard deviations are available in the Supporting Information, Table S5. [c]Data taken from Table 2.

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challenge this hypothesis, we additionally quantitated sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal in cashew nuts, hazelnuts, almonds, Brazil nuts, and pecan nuts.

Results of the quantitations (Table 4) revealed levels of (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal below the OTC of 1.1 μg/kg (cf. Table 2) in cashew nuts, hazelnuts, and almonds. The sotolon concentration was also lower than in the walnuts and ranged from 2.15 to 3.55 μg/kg, thus still beyond its OTC. The sotolon/(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal ratio was >5:1 and not ∼1:1 as in the walnuts. The Brazil nut sample was the only one in which the (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal concentration was higher than the sotolon concentration resulting in a sotolon/(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal ratio of 1:2.3, and again both concentrations were clearly lower than those in the walnuts. The lower amounts in combination with a sotolon/(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal ratio clearly differing from 1:1 were in line with the lack of a walnut note in the cashew nut, hazelnut, almond, and Brazil nut samples. By contrast, the pecan nut sample showed some walnut character in the aroma, although not as pronounced as the walnuts. In view of their botany, this was not surprising because the pecan nut tree *Carya illinoinensis* and the walnut tree *J. regia* belong to the same family Juglandaceae. Actually, the (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal concentration in the pecan nuts with 7.87 μg/kg was almost as high as in the walnuts, where 10.2 μg/kg had been determined and the sotolon concentration with 23.6 μg/kg was even higher, resulting in a ratio of sotolon to (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal of 3:1. This raised the question which sotolon/(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal ratio is actually the optimum to achieve the most characteristic walnut aroma. This question was addressed in the following experiments.

Sensory Tests with Different Sotolon/(2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal Mixtures. A first experiment was based on a 1:1 mixture of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal at a concentration level of 10 μg/kg, thus approximating the situation in the walnuts. The matrix was the same oil/buffer mixture used for the reconstitution and omission tests. One of the two components was then reduced in its concentration to 3, 1 μg/kg, and finally omitted totally. This approach resulted in seven samples with different sotolon/(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal ratios. The samples were presented to the trained sensory panel and assessors were asked to orthonasally rate the intensities of the three descriptors "walnut", "fenugreek", and "oatmeal". The same scale previously used for the recombination and omission tests was used, which ranged from 0 to 3 with 0.5 increments and with 0 = not perceptible, 1 = weak, 2 = moderate, and 3 = strong.

The averaged results are depicted in Figure 1. The highest intensity of the walnut note was actually obtained when both compounds were present at 10 μg/kg, which were about the same concentrations as in the walnuts. Moderate intensity of the walnut note was still perceptible when one of the two compounds was present at 10 μg/kg and the other one at 3 μg/kg. However, when one of the two compounds was decreased to 1 μg/kg, the walnut character was only weak. The decrease in the walnut note was steeper when the sotolon concentration decreased, thus further confirming that sotolon contributes somewhat more to the walnut character than (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal. It is noteworthy that, when sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal approached the 1:1 ratio and formed the walnut character, the original odor impressions of the two compounds did not vanish, but were still perceivable in parallel to the walnut note. In other words, the

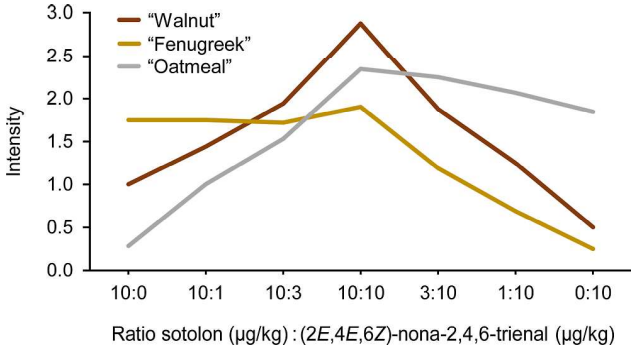

Figure 1. Impact of the ratio of sotolon to (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal on the intensity of the odor impressions "walnut", "fenugreek", and "oatmeal" in model mixtures. Assessors employed a scale from 0 to 3 with 0.5 increments and 0 = not perceptible, 1 = weak, 2 = moderate, and 3 = strong.

walnut note did not develop at the expense of the fenugreek-like note of sotolon and the oatmeal-like note of (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal, but in addition.

In a second experiment, we addressed the question of whether an increase of the sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal concentrations would be beneficial for the overall walnut aroma character or not. The concentration of the 1:1 mixture was increased from 10 μg/kg to 30, 100, and finally 300 μg/kg. To see if at higher overall concentrations the 1:1 mixtures would still represent the optimum ratio, we did the same with the mixtures in which the sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal concentrations differed by one step. All samples were evaluated against the mixture of both compounds at 10 μg/kg and the assessors were asked to rate the difference in the intensity of the walnut note on a scale from −3 to +3 with −3 = clearly weaker, −2 = moderately weaker, −1 = slightly weaker, 0 = no difference, +1 = slightly stronger, +2 = moderately stronger, and +3 = clearly stronger. Averaged results (Figure 2) clearly showed that

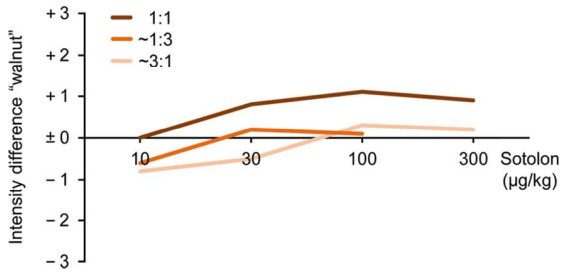

Figure 2. Change in the characteristic walnut note with increasing odorant concentrations (up to 300 μg/kg) shown for sotolon to (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal ratios of 1:1, ∼1:3, and ∼3:1. Assessors rated the intensity difference on a scale from −3 to +3 with −3 = clearly weaker, −2 = moderately weaker, −1 = slightly weaker, 0 = no difference, +1 = slightly stronger, +2 = moderately stronger, and +3 = clearly stronger.

also at higher overall concentrations, the 1:1 ratio of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal resulted in the highest rating for the walnut note. The walnut note of a 1:1 mixture of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal intensified when the concentrations increased from 10 to 30 μg/kg and from 30 to 100 μg/kg but showed a slight decrease when the concentrations further increased from 100 to 300 μg/kg. In conclusion, the sensory tests with the different sotolon/(2*E*,4*E*,6*Z*)-nona-2,4,6-trienal

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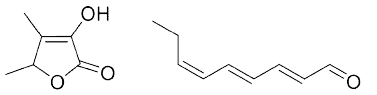
mixtures suggested that a 1:1 mixture of both compounds at a concentration level of 100 μg/kg is desirable to achieve an intense walnut-like aroma character. This result may be helpful to evaluate the aroma of different walnut varieties on an analytical basis and to set targets for the breeding of new walnut cultivars.

In summary, our study showed that the compounds responsible for the characteristic aroma of unprocessed walnuts are fenugreek-like smelling sotolon and oatmeal-like smelling (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (Figure 3). It was surprising that



Sotolon (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal

Figure 3. Key odorants in walnuts.

both compounds had not been detected in walnuts before, although molecular sensory science approaches had been applied in previous studies. It is somewhat speculative to discuss possible reasons for that. However, representativeness of the walnut sample in terms of the aroma properties, sample pretreatment before extraction—particularly the degree of crushing, enzyme inhibition, and water addition, artifact-avoiding isolation of the volatile fraction, and the experience of the assessors performing GC−O may have been critical points. Our results also nicely illustrate that it is not feasible to define key odorants on the basis of OAVs as suggested by Liu et al.[11] OAVs provide a useful tool to select the compounds for the subsequent reconstitution and omission tests but do not allow unequivocally assessing the importance of individual compounds for the overall aroma. An aroma reconstitution experiment is essential to confirm that all key odorants have been captured and only if the reconstitution experiment was successful, omission tests finally allow to identify the key odorants.[14]

In the future, the targeted quantitation of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal may not only be useful for quality control but can also be included in studies aimed at a deeper molecular understanding of variety selection, agricultural parameters, post-harvest handling, and processing on the sensory characteristics of walnuts and walnut products.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c01002.

Information on GC instruments; references on synthetic procedures to isotopically substituted odorants; stable isotopically substituted internal standards, quantifier ions, and calibration lines used in the quantitation assays; individual concentration data used for mean calculations and standard deviations; and odor threshold concentrations of **20**, **24**, **27**, and **28** in air (PDF)

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Notes

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■ ABBREVIATIONS

AEDA, aroma extract dilution analysis; aSAFE, automated solvent-assisted flavor evaporation; EHMF, 2-ethyl-4-hydroxy-5-methylfuran-3-one; FD, flavor dilution; GC, gas chromatography; GC−GC−HRMS, gas chromatography−gas chromatography−high-resolution mass spectrometry; GC−MS, gas chromatography−mass spectrometry; GC−O, gas chromatography−olfactometry; GC−O/FID, gas chromatography−olfactometry/flame ionization detector; HDMF, 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one; OAV, odor activity value; OTC, odor threshold concentration; RM, reconstitution model; RI, retention index; SAFE, solvent-assisted flavor evaporation

■ NOMENCLATURE

2′-aminoacetophenone, 1-(2-aminophenyl)ethan-1-one; cyclotene, 2-hydroxy-3-methyl-2-cyclopenten-1-one; (2*Z*,4*Z*)-δ-deca-2,4-dienolactone, 6-pentylpyran-2-one; γ-decalactone, 5-hexyloxolan-2-one; (6*Z*)-γ-dodec-6-enolactone, 5-[(2*Z*)-oct-2-enyl]oxolan-2-one; eugenol, 2-methoxy-4-(prop-2-en-1-yl)-phenol; α-farnesene, (3*E*,6*E*)-3,7,11-trimethyldodeca-1,3,6,10-tetraene; β-ionone, (3*E*)-4-(2,6,6-trimethylcyclohex-1-en-1-yl)-but-3-en-2-one; 3-isopropyl-2-methoxypyrazine, 2-methoxy-3-(propan-2-yl)pyrazine; maltol, 3-hydroxy-2-methyl-4*H*-pyran-4-one; methional, 3-(methylsulfanyl)propanal; γ-octalactone, 5-butyldihydro-2(3*H*)-furanone; δ-octalactone, 6-butyldihydro-2(3*H*)-furanone; 3-*sec*-butyl-2-methoxypyrazine, 2-(butan-2-yl)-3-methoxypyrazine; γ-terpinene, 1-methyl-4-propan-2-ylcyclohexa-1,4-diene; sotolon, 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one; *trans*-4,5-epoxy-(2*E*)-dec-2-enal, (2*E*)-3-[(3-(2*R*,3*R*) and/or (2*S*,3*S*)-pentyloxiran-2-yl]prop-2-enal; vanillin, 4-hydroxy-3-methoxybenzaldehyde

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8.2.3 Summary and Individual Contributions

The walnut tree, *Juglans regia* L., is native to Eurasia and has been cultivated for over 2000 years. Walnut kernels, the edible seeds of the tree, are rich in fiber and fat and are widely used as a snack or as an ingredient in various foods. The characteristic aroma of fresh walnut kernels has been of interest to researchers since the 1970s. Early studies suggested that the walnut aroma results from more than just a single odorant. However, further investigation of specific odorants responsible for the walnut aroma was limited. The aim of this study was to identify the odorants that are responsible for the characteristic aroma of fresh walnut kernels.

The volatiles were isolated from fresh walnut kernels with the newly developed aSAFE approach. Aroma extract dilution analysis (AEDA) revealed 50 odorants in the volatile isolate. Interestingly, only 13 odorants among them had previously been reported in walnuts. None of the odorants were specifically described as walnut-like, supporting the hypothesis that the walnut aroma results from a combination of odorants rather than a single odorant. The odorants with the highest flavor dilution (FD) factors were (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (1024) and sotolon (512). A group of four odorants additionally showed high FD factors and included oct-1-en-3-one, 4-hydroxy-2,5-dimethylfuran-3(2*H*)-one, *trans*-4,5-epoxy-(2*E*)-dec-2-enal, and 2-methoxyphenol. Quantitation of all 27 odorants with FD factors ≥16 revealed concentrations ranging from 0.0206 µg/kg to 44200 µg/kg. High concentrations were obtained for acetic acid (44200 µg/kg), hexanoic acid (2870 µg/kg), and (2*E*)-oct-2-enal (439 µg/kg). (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal and sotolon showed similar concentrations of 10.2 µg/kg and 10.6 µg/kg, respectively. 17 odorants showed odor activity values (OAVs) ≥1, indicating their potential contribution to the overall aroma. The highest OAVs were calculated for acetic acid (OAV 130), sotolon (OAV 46), (2*E*,4*Z*)-deca-2,4-dienal (OAV 17), 3-methylbutanoic acid (OAV 13), (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal (OAV 9.3), hexanoic acid (OAV 6.2), and butanoic acid (OAV 5.4). Two reconstitution models were evaluated, one including all 17 odorants with OAVs ≥1 and the other including only the six odorants with the highest OAVs (9.3–130). Surprisingly, the reconstitution model with only six odorants was rated more walnut-like than the reconstitution model with 17 odorants. This led to the hypothesis that the characteristic walnut aroma is formed by a combination of two to six odorants. Surprisingly, a 1:1 mixture of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal with concentrations of ~10 µg/kg best resembled the walnut aroma. The walnut note intensified when both compounds were present at higher concentrations, with the 1:1 mixture at 100 µg/kg showing the most intense walnut note.

The concentrations of sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal were determined in further tree nuts. Almonds, cashew nuts, hazelnuts, and Brazil nuts did not show a typical walnut aroma, which was in good agreement with a sotolon to (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal ratio of >5:1 for almonds, cashew nuts, and hazelnuts, and 1:2.3 for Brazil nuts. Interestingly, the analysis of pecan nuts, which had a moderate walnut aroma, resulted in a sotolon to (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal ratio of ~3:1.

Christine Stübner designed and conducted the experiments including the volatile isolations, the GC–O screenings, the structure assignments, the syntheses of odorants and their isotopologues, the quantitation assays, the calculation of OAVs, and the sensory tests. Christine evaluated the data and prepared the manuscript. Martin Steinhaus conceived and directed the study, supervised Christine's work, and revised the manuscript. Martin Steinhaus participated in the GC–O analyses and also in the sensory tests.

8.2.4 Reprint Permission

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Sotolon and (2*E*,4*E*,6*Z*)-Nona-2,4,6-trienal Are the Key Compounds in the Aroma of Walnuts

Christine A. Stübner and Martin Steinhaus*

8.3 List of Publications, Talks, and Poster Presentations

Publications

Publications in peer-reviewed journals:

Stübner, C. A.; Steinhaus, M. Sotolon and (2*E*,4*E*,6*Z*)-nona-2,4,6-trienal are the key compounds in the aroma of walnuts. *J. Agric. Food Chem*. **2023**, *71*, 18, 7099–7108. https://doi.org/10.1021/acs.jafc.3c01002

Schlumpberger, P.; Stübner, C. A.; Steinhaus, M. Development and evaluation of an automated solvent-assisted flavour evaporation (aSAFE). *Eur. Food Res. Technol.* **2022**, *248*, 10, 2591–2602. https://doi.org/10.1007/s00217-022-04072-1

Miscellaneous journal contributions:

Stübner, C. A.; Steinhaus, M. Bockshornklee + Haferflocken = Walnuss. Das Aroma von Walnüssen molekularsensorisch entschlüsselt. *Lebensmittelchemie* **2023**, *77*, S3, S3-024. https://doi.org/10.1002/lemi.202359020

Stübner, C. A.; Schlumpberger, P.; Steinhaus, M. Automated solvent-assisted flavour evaporation. *Lebensmittelchemie* **2023**, *77*, 1, 8–11. https://doi.org/10.1002/lemi.202300103

Stübner, C. A.; Schlumpberger, P.; Steinhaus, M Automated solvent-assisted flavour evaporation: Ein Beitrag zur Geruchstoffforschung 2.0. *Lebensmittelchemie* **2023**, *77*, S1, S1-034. https://doi.org/10.1002/lemi.202352024

Stübner, C.; Schlumpberger, P.; Steinhaus, M Automated solvent-assisted flavour evaporation (aSAFE). *Lebensmittelchemie* **2019**, *73*, S1, S113. https://doi.org/10.1002/lemi.201951113

Talks

Oral presentations at national meetings:

Stübner, C. A.; Steinhaus, M. Bockshornklee + Haferflocken = Walnuss. Das Aroma von Walnüssen molekularsensorisch entschlüsselt. Lebensmittelchemische Gesellschaft (LChG), Fachgruppe in der Gesellschaft Deutscher Chemiker (GDCh), 51. Deutsche Lebensmittelchemietage (German Society of Food Chemistry, a division of the German Chemical Society, 51th National Meeting). Bonn, Germany, August 22, 2023.

Stübner, C. A.; Schlumpberger, P.; Steinhaus, M. Automated Solvent-Assisted Flavour Evaporation. Ein Beitrag zur Geruchstoffforschung 2.0. Lebensmittelchemische Gesellschaft (LChG), Fachgruppe in der Gesellschaft Deutscher Chemiker (GDCh), Regionalverband Bayern, Arbeitstagung 2023 (German Society of Food Chemistry, a division of the German Chemical Society, Bavarian Regional Meeting 2023). Oberschleißheim, Germany, March 7, 2023.

Poster Presentations

Stübner, C.; Schlumpberger, P.; Steinhaus, M. Automated Solvent-Assisted Flavor Evaporation (aSAFE) Lebensmittelchemische Gesellschaft (LChG), Fachgruppe in der Gesellschaft Deutscher Chemiker (GDCh), 48. Deutscher Lebensmittelchemikertag (German Society of Food Chemistry, a division of the German Chemical Society, 48th National Meeting). Dresden, Germany, September 16–18, 2019.