agriculture

MDPI

Communication

Authenticity Analysis of Cold-Pressed Orange Essential Oils by GC/MS on Polymethoxyflavone Components

Kai-Min Yang [1,†], Chih-Wei Chen [2,†], Min-Hung Chen [3], Hsin-Chun Chen [4] and Li-Yun Lin [5,*]

1 Department of Food Science, National Quemoy University, Kinmen 892, Taiwan
2 Bachelor Degree Program in Food Safety/Hygiene and Laboratory Science, Chang Jung Christian University, Tainan City 711, Taiwan
3 Agriculture and Food Agency, Council of Agriculture, Executive Yuan 8, Guanghua Road, Nantou City 540, Taiwan
4 Department of Cosmeceutics, China Medical University, Taichung 406, Taiwan
5 Department of Food Science and Technology, Hung Kuang University, Taichung 433, Taiwan
* Correspondence: lylin@hk.edu.tw; Tel.: +886-33-432-793
† These authors contributed equally to this work.

Abstract: Citrus essential oil combines numerous components with many qualities and control issues. For example, how to monitor volatile components and nonvolatile substances simultaneously is a major problem. Therefore, the objective of this study was to develop a Gas chromatography/mass spectrometry (GC/MS) method for the compositional study of total constituents in cold-pressed essential oils from major orange species. A polysiloxane GC column (DB-1, nonpolar, low-bleeding) was used in this study; its fingerprint includes terpenoids (Section 1), long-chain hydrocarbons (Section 2), phytosterols, and polymethoxyflavones (Section 3). These markers are more effective in detecting adulteration of essential oil products than volatile components, and more effective than quantitative PMF by 5α-Cholestane for establishing authenticity. The study aims to use the analysis procedure as a routine quality control test for authenticity evaluation in cold-pressed orange essential oils (CP-OrEO).

Keywords: cold-pressed orange essential oils (CP-OrEO); authenticity; polymethoxyflavone; method validation; quality control

Citation: Yang, K.-M.; Chen, C.-W.; Chen, M.-H.; Chen, H.-C.; Lin, L.-Y. Authenticity Analysis of Cold-Pressed Orange Essential Oils by GC/MS on Polymethoxyflavone Components. *Agriculture* **2023**, *13*, 179. https://doi.org/10.3390/agriculture13010179

Received: 10 December 2022
Revised: 4 January 2023
Accepted: 10 January 2023
Published: 11 January 2023

1. Introduction

The orange essential oil has a fresh, sweet, citrus potpourri scent and is traditionally produced by cold pressing the rinds of citrus fruits [1,2]. They are made up of mono-terpene hydrocarbons, oxygenated terpenes, and nonvolatile compounds such as waxes, coumarins, psoralens, and substituted flavones [3,4]. Both volatile and nonvolatile metabolites have anti-oxidative, anti-inflammatory, and anti-cancerogenic properties, and cardiovascular and neuroprotective properties [5–7]. Orange essential oil is simple to obtain and is the least expensive of all essential oils; as for production, consumers favor cold-pressed oils over distilled oils because of the more abundant aroma and better functional properties [2]. Examples include their widespread use in ethnomedicine, juice production, functional food, and extract preparations with application potential in the pharmaceutical and cosmetics industries [1–3].

"Food authenticity is the process of irrefutably proving that a food or food ingredient is in its original, genuine, verifiable, and intended form as declared and represented," according to the International Food Authenticity Assurance Organization (IFAAO) [8]. Food authentication is an interest for (I) regulatory authorities to avoid food adulteration, (II) food processing companies to avoid unfair competition from unscrupulous processors who would gain an advantage from mispresenting the food they are selling, and (III) consumers who expect to purchase and consume genuine, unadulterated, and quality foods for which they typically pay a higher price for [9–11]. As the herb and spice industry grows, aromatic

substances are the most common adulterated food. Economically motivated adulteration from criminals continues to shadow this industry [12,13].

Essential oil is due to the widespread usage of aromatic substances in the food processing industry. Aromatic substances not only improve the flavor and taste of food, but they are also sources of several bioactive molecules that have considerable positive health effects [3,5,14,15]. Aromatherapists and natural health practitioners have used orange oil for centuries due to their function as a mild tranquilizer and antidepressant [16,17]. Food authenticity has grown to be a significant issue for producers, consumers, and legislators, which also is important for food analysis [18–20]. As a result of technological advancements, we can identify unknowns more quickly and precisely. Spectroscopic techniques coupled with chemometrics for detecting adulteration in spices are another example of various food research techniques that have been explored recently [9,21]. In addition, improvements in DNA analysis and mass spectrometry are providing faster, even more precise detection methods. These techniques help protect the herb and spice industry and consumers from fraud by identifying and preventing adulteration [12,22,23].

CP-OrEO mainly uses orange juice by-products as raw materials, and its raw materials mainly come from Bitter Orange (*Citrus aurantium* L.), Sweet Orange (*Citrus sinensis*), blood orange (*Citrus sinensis*), and the processing parameters are different, which makes the CP-OrEO quality standards and authenticity difficult to manage. For the compositional analysis of the total components in cold-pressed essential oils from the main orange species, a gas chromatography/mass spectrometry (GC/MS) approach was devised. It was discovered that the fingerprint made up of terpenoids, phytosterols, and polymethoxyflavones found in CP-OrEO worked better as adulteration indicators than those volatile components. The authors are hopeful that such an analytical approach may be used as a standard quality control test for CP-OrEO's assessment.

2. Materials and Methods

2.1. Materials

Commercial cold-pressed essential oil (samples I, II, and III) and crude orange essential oil (sample V) were obtained from sources in Taichung, Taiwan. Compounding orange blend oil (sample IV) was obtained from Gemfont Corporation (Taipei, Taiwan). The 5α-Cholestane, Tangeritin, sinensetin, and nobiletin were commercially available via Sigma-Aldrich (St. Louis, MO, USA).

2.2. Method Validation

GC/MS experiments are performed with Agilent 6890 GC equipped with DB-1 fused-silica capillary column (60 m, 0.25 mm, 0.25 μm) connected to an Agilent model 5973 N MSD mass spectrometer (MS). The injection (split mode 1:40, 4 mm liner) port temperature was maintained at 250 °C. Helium is a carrier gas with a 1 mL/min flow rate. The electron energy was 70 eV at 230 °C. The oven temperature program was set at 70 °C, followed by an increase of 5 °C/min to 270 °C, and then held for 100 min. The linear RIs were calculated from the retention times of standard n-alkanes under the same chromatographic conditions. The constituents were identified by matching their spectra with those recorded in an MS library (Wiley 7n). The volatile compounds were identified by those in the published literature and performed to determine the relative content of each compound based on the total integrated area. The polymethoxyflavone mass was identified by authentic standards.

This analytical method was developed concerning previous methods [24,25] and then validated by determining the linear regression, including a limit of quantification (LOQ), the limit of detection (LOD), recovery rate, and precision. The calibration curves were established using the external standard for 5α-Cholestane with five different concentrations (1000, 500, 100, 50, and 10 μg/mL), and response linearity was assessed with standards diluted with Isooctane using linear regression analysis of peak area to the concentration of the standards, in triplicate. The variations within a day were determined by comparing the results obtained on three sequential days. Triplicate injections were performed to

test each sample's intraday and intraday variations. LOQ and LOD were calculated via signal-to-noise ratios of 10:1 and 3:1, respectively. We prepared quality control samples: 5 g of CP-OrEO containing 1 mg of internal standard (5α-Cholestane) was dissolved in 10 mL of Isooctane. Recovery for each compound was calculated by the amounts obtained from extractions of known concentrations.

3. Results and Discussion

3.1. Method Development

Cold-pressed orange essential oils (CP-OrEO) are widely used in developing new products of the popular citrus flavor. Because of the relatively simple chemical composition and substantial price differences among citrus flavoring, adulteration has plagued this industry [10,12,13]. Early identification techniques included olfactory sensory reviews, and enhanced methods were soon developed. An obvious downside would be the need for more objective standards, which can fall short when it comes to the assay of said sample oils or adding fillers. Through data obtained from GC analysis [12,13,18], crafted blends can be indistinguishable from authentic essential oils. Agilent J&W DB-1 is nonpolar and low-bleed, and operates under high-temperature limits. It is an excellent choice for general-purpose use in various applications. Precision-engineered DB-1 is bonded, crosslinked, and solvent-rinseable and is equivalent to USP phase G2.

GC techniques are used for adulteration studies of essential oils. Firstly, CP-OrEO volatile fragments are more prone to adulteration than nonvolatile fragments. These components often elute at the upper-temperature limits of the stationary phase, and column bleed can hinder good mass spectral characterization [26–28]. Iso-thermal separations on DB-1 exhibited dramatic temperature dependence, indicating that further optimization was possible with PMF in CP-OrEO. Temperature programming provided the best analysis of the entire volatile fraction of the CP-OrEO. In these analytical conditions, the chromatogram display can be divided into three main sections (Figure 1); terpenes, monoterpenes, sesquiterpenes, terpene oxygenates (Section 1); long-chain hydrocarbons (Section 2); phytosterol, β-sitosterol, and several polymethoxyflavones, tangeritin, sinensetin and nobiletin (Section 3). Content is relatively high, suitable as citrus false or whether the reference is determined based on the harmonic configuration.

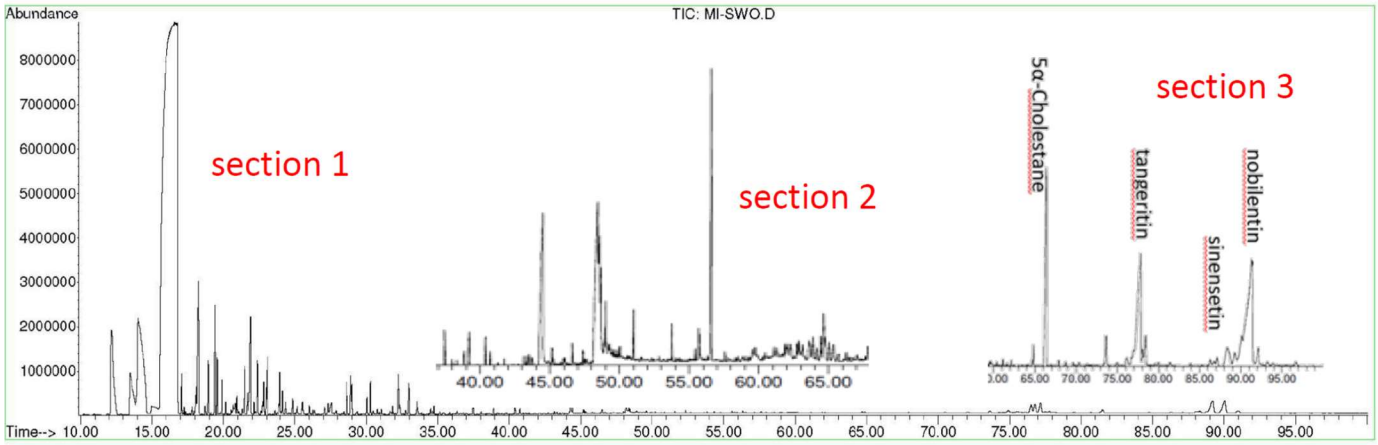

Figure 1. Chromatogram of cold-pressed orange essential oils obtained by GC/MS.

The study of loadings highlighted chemical families or compounds characteristic of each quality of CP-OrEO. The volatile compounds separated from the CP-OrEO are listed in Table 1. The components were further categorized into monoterpene, sesquiterpenes, and oxygenated compounds (alcohols, esters, oxides, and epoxides). The terpene constituted the most dominant volatiles chemical group for 81.80~94.97% of commercial CP-OrEO and 96.87% of crude CP-OrEO, the difference between commercial and crude CP-OrEO, which oxygenated fraction accounted for 5.03~18.20% and 3.13% of the total fraction. In

this study, a total of 21 different volatile compounds were separated from CP-OrEO, mainly characterized by their limonene (79.02~86.52%), β-myrcene (2.96~10.27%), and α-pinene contents (1.64~7.07%). A total of 11 oxygenated compounds were found in CP-OrEO. Oxygenated compounds are mainly characterized by their linalool (0.83~11.30%) and decanal (1.01~4.54%). Oxygenated compounds are generally preferable due to their vibrant aroma and characteristic flavors [7].

Table 1. Volatile compounds of cold-pressed orange essential oils.

Compound	RI
α-pinene	923
sabinene	941
β-myrcene	975
limonene	990
linalool	1048
citronella	1097
α-terpineol	1142
decanal	1193
Z-carveol	1206
E-carveol	1224
carvone	1226
citral	1252
methylbenzoate	1257
α-copaene	1388
β-cubenene	1398
caryophyllene	1433
germacrene D	1490
valencene	1502
δ-cadinene	1528
β-sinensal	1686
α-sinensal	1739

Terpenes can easily lead to polymerization (resulting in the formation of resins) and oxidation by exposure to air and light, which are seen as degradation to the quality of the products. Such deterioration processes result in "off-flavor" [28,29]. The GC profiles of CP-OrEO have long been published and are easily accessible to buyers and producers. Determination is an important aspect of commercially CP-OrEO processing, and it will allow lower terpene fraction with fractionation [1,28]. However, the volatile composition is inefficient in identifying the authenticity of citrus oil. Linalool is usually said to be the most distinctive aroma of citrus oils. Linalool is difficult to separate from terpenes, which is considered to be a sign of the quality of products after deterioration [4].

The citrus fruit contains large amounts of carotenoids, flavonoids, limonoids, tocopherols, phenolic compounds, essential oils, and polysaccharides such as fibers, lignin, and pectin [9]. Analysis regarding quality should be performed under more restrictive parameters concerning its chemical complexity and its differentiation through factors such as genetic causes (variety) or methods used for the production procedures (e.g., pressing and/or solvent extraction) [28–30]. In Figure 1, Section 2 was a long chain of hydrocarbons of CP-OrEO. We identified hexadecanoic, octadecanoal, octadecanoic acid and hexadecenoic acid. These compounds mainly originate from the plant or Carrier Oil and are therefore not biospecific. Section 3 was nonvolatile fractions of CP-OrEO. Due to being less volatile, the nonvolatile fragments can only contribute moderately to the fragrance of citrus oils. It is logical to conclude that adulterators would opt not to invest their time and resources in the nonvolatile fragments, which have little impact on the aroma of CP-OrEO. We analyzed with GC/MS to identify phytosterol and polymethoxyflavones in Section 3 that cannot be removed with determination.

3.2. Validation of the Method

The literature reported that phytochemical characterization and phytosterols occur in many plant species [7,14,17]. However, polymethoxyflavones are exclusively found in citrus plants, particularly sweet orange peels. In this study, the identified polymethoxyflavones were tangeritin, sinensetin, and nobiletin in citrus oils [14]. The analytical separation technique, which identified polymethoxyflavone, was an appropriate marker. To establish a quality control protocol, the results could be used to standardize the chemical composition of the CP-OrEO.

The analytical technique of CP-OrEO uses the standard internal method and uses 5α-Cholestane to Quantification PMF (Table 2). Linearity data were obtained from diluted 5α-Cholestane (10~1000 ug/mL). The slope obtained from least-squares regression analysis indicates high sensitivity (y = 0.5856x − 2.3932; R2:0.996). The relative standard deviation (RSD) of the overall precision for intraday samples and the peak area ratios obtained for 5α-Cholestane was 2.86% (n = 6). The LODs and LOQs for 5α-Cholestane were calculated to be 8.01 ug/mL and 26.75 ug/mL.

Table 2. Validation parameters of the proposed method.

	μg/mL	R^2	LOD	LOQ
5α-Cholestane	10~1000	0.9949	8.01	26.75

The samples were added with 5α-Cholestane to obtain final concentrations of 1 ug/mL, and the nominal analyzed the volume of 5 mL. The mean recovery of 5 α-Cholestane in analyzed samples was within the range of 91.5~99.6% with <3% of mean RSD. Our recovery fell in the acceptable range (mean recovery and RSD range not exceeding 80.00~115.00% and 14.00%, respectively) [25]. In this study, Identification PMF in CP-OrEO showed 24.5 ug/mL of tangeritin, 197.1 ug/mL of sinensetin, and 73.5 ug/mL of nobiletin on crude CP-OrEO; there was 114.2~138.8 ug/mL of tangeritin, 131.4~454.5 ug/mL of sinensetin, and 85.7~420.1 ug/mL of nobiletin on commercial CP-OrEO. Sample IV was compounding orange essential oil and did not detect PMF (Table 3).

Table 3. The analysis of polymethoxyflavones from cold-pressed orange essential oils by GC/MS.

	Cold-Pressed Orange Essential Oils					*m/z*
	I	**II**	**III**	**IV**	**V**	
Recoveries **(%)**	91.5	95.9	94.3	99.6	93.1	
	μg/mL					
Tangeritin	114.2	134.5	138.8	N.D.	24.5	357, 372, 358
Sinensetin	131.4	454.5	329.6	N.D.	197.1	357, 372, 358
Nobiletin	85.7	420.1	201.8	N.D.	73.5	387, 388, 402

Using LC, the analysis of nonvolatile compounds in citrus oils as identifying indicators for adulteration has long been developed. With the GC/MS analysis method in CP-OrEO that we developed, not only can the standards of authenticity be applicable, but volatile-characteristic compounds can also be profiled synchronously. This method can evaluate the overall quality of CP-OrEO while using PMF as the basis for authenticity without being affected by processing parameters.

4. Conclusions

The result could be used to standardize the chemical composition and origin of the CP-OrEO. Compared to prior analysis methods, those reported here represent an improvement in different temperature fraction options that allow for peak confirmation based on retention time. A quantitative PMF by 5-Cholestane may be used for determining adulteration or

aid in identifying the species from which the oil was obtained. The GC/MS experiment is "simple/routine" and provides valuable structural information.

Author Contributions: Methodology, K.-M.Y., C.-W.C. and L.-Y.L.; software, M.-H.C. and H.-C.C.; validation, K.-M.Y. and C.-W.C.; formal analysis, K.-M.Y. and C.-W.C.; investigation, M.-H.C. and H.-C.C.; data curation, K.-M.Y. and C.-W.C.; writing—original draft preparation, K.-M.Y. and C.-W.C.; writing—review and editing, L.-Y.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Haypek, E.; Silva, L.H.M.; Batista, E.; Marques, D.S.; Meireles, M.A.A.; Meirelles, A.J.A. Recovery of aroma compounds from orange essential oil. *Braz. J. Chem. Eng.* **2000**, *17*, 705–712. [CrossRef]
2. Gavahian, M.; Chu, Y.H.; Mousavi Khaneghah, A. Recent advances in orange oil extraction: An opportunity for the valorisation of orange peel waste a review. *Int. J. Food Sci. Technol.* **2019**, *54*, 925–932. [CrossRef]
3. Torres-Alvarez, C.; Núñez González, A.; Rodríguez, J.; Castillo, S.; Leos-Rivas, C.; Báez-González, J.G. Chemical composition, antimicrobial, and antioxidant activities of orange essential oil and its concentrated oils. *CYTA J. Food* **2017**, *15*, 129–135. [CrossRef]
4. Verzera, A.; Trozzi, A.; Dugo, G.; Di Bella, G.; Cotroneo, A. Biological lemon and sweet orange essential oil composition. *Flavour Fragr. J.* **2004**, *19*, 544–548. [CrossRef]
5. Oboh, G.; Olasehinde, T.A.; Ademosun, A.O. Inhibition of enzymes linked to type-2 diabetes and hypertension by essential oils from peels of orange and lemon. *Int. J. Food Prop.* **2017**, *20*, S586–S594. [CrossRef]
6. Lin, L.Y.; Chuang, C.H.; Chen, H.C.; Yang, K.M. Lime (*Citrus aurantifolia* (Christm.) Swingle) essential oils: Volatile compounds, antioxidant capacity, and hypolipidemic effect. *Foods* **2019**, *8*, 398. [CrossRef] [PubMed]
7. Chen, M.H.; Huang, T.C. Volatile and nonvolatile constituents and antioxidant capacity of oleoresins in three Taiwan citrus varieties as determined by supercritical fluid extraction. *Molecules* **2016**, *21*, 1735. [CrossRef]
8. International Food Authenticity Assurance Organization. Available online: https://uia.org/s/or/en/1122281809 (accessed on 15 October 2022).
9. Primrose, S.; Woolfe, M.; Rollinson, S. Food forensics: Methods for determining the authenticity of foodstuffs. *Trends Food Sci. Technol.* **2010**, *21*, 582–590. [CrossRef]
10. Moore, J.C.; Spink, J.; Lipp, M. Development and application of a database of food ingredient fraud and economically motivated adulteration from 1980 to 2010. *J. Food Sci.* **2012**, *77*, R118–R126. [CrossRef]
11. Astill, J.; Dara, R.A.; Campbell, M.; Farber, J.M.; Fraser, E.D.; Sharif, S.; Yada, R.Y. Transparency in food supply chains: A review of enabling technology solutions. *Trends Food Sci. Technol.* **2019**, *91*, 240–247. [CrossRef]
12. Galvin-King, P.; Haughey, S.A.; Elliott, C.T. Herb and spice fraud; the drivers, challenges and detection. *Food Control.* **2018**, *88*, 85–97. [CrossRef]
13. Silvis, I.C.J.; Van Ruth, S.M.; Van Der Fels-klerx, H.J.; Luning, P.A. Assessment of food fraud vulnerability in the spices chain: An explorative study. *Food Control.* **2017**, *81*, 80–87. [CrossRef]
14. Li, S.; Wang, Y.; Wang, Z.; Xiao, H.; Lo, C.Y.; Rawson, N.; Ho, C.T. Quantitative analysis of hydroxylated polymethoxyflavones by high-performance liquid chromatography. *Biomed. Chromatogr.* **2010**, *24*, 838–845. [CrossRef] [PubMed]
15. Ribeiro-Santos, R.; Andrade, M.; Sanches-Silva, A.; de Melo, N.R. Essential oils for food application: Natural substances with established biological activities. *Food Bioprocess Technol.* **2018**, *11*, 43–71. [CrossRef]
16. Ali, B.; Al-Wabel, N.A.; Shams, S.; Ahamad, A.; Khan, S.A.; Anwar, F. Essential oils used in aromatherapy: A systemic review. *Asian Pac. J. Trop. Biomed.* **2015**, *5*, 601–611. [CrossRef]
17. Sowndhararajan, K.; Kim, S. Influence of fragrances on human psychophysiological activity: With special reference to human electroencephalographic response. *Sci. Pharm.* **2016**, *84*, 724. [CrossRef]
18. Do, T.K.T.; Hadji-Minaglou, F.; Antoniotti, S.; Fernandez, X. Authenticity of essential oils. *Trends Anal. Chem* **2015**, *66*, 146–157. [CrossRef]
19. Masson, J.; Liberto, E.; Beolor, J.C.; Brevard, H.; Bicchi, C.; Rubiolo, P. Oxygenated heterocyclic compounds to differentiate Citrus spp. essential oils through metabolomic strategies. *Food Chem.* **2016**, *206*, 223–233. [CrossRef]
20. Fan, H.; Wu, Q.; Simon, J.E.; Lou, S.N.; Ho, C.T. Authenticity analysis of citrus essential oils by HPLC-UV-MS on oxygenated heterocyclic components. *J. Food Drug Anal.* **2015**, *23*, 30–39. [CrossRef] [PubMed]

21. Valand, R.; Tanna, S.; Lawson, G.; Bengtström, L. A review of Fourier Transform Infrared (FTIR) spectroscopy used in food adulteration and authenticity investigations. *Food Addit Contam.* **2020**, *37*, 19–38. [CrossRef] [PubMed]
22. Ch, R.; Chevallier, O.; McCarron, P.; McGrath, T.F.; Wu, D.; Kapil, A.P.; McBride, M.; Elliott, C.T. Metabolomic fingerprinting of volatile organic compounds for the geographical discrimination of rice samples from China, Vietnam and India. *Food Chem.* **2021**, *334*, 127553. [CrossRef]
23. Kim, W.J.; Yang, S.; Choi, G.; Park, I.; Noh, P.; Seo, C.S.; Moon, B.C. Development of conventional PCR and real-time PCR assays to discriminate the origins of Chinese pepper oil and herbal materials from Zanthoxylum. *J. Sci. Food Agric.* **2019**, *99*, 2021–2029. [CrossRef] [PubMed]
24. Food and Drug Administration. International Conference on Harmonisation Guideline on "Validation of Analytical Procedures: Methodology". *Fed. Regist.* **1997**, *62*, 27463–27467.
25. *Department of Health Food and Drug Administration Validation of Analytical Specification in Food Chemistry*; Taiwan Ministry of Health and Welfare: Taipei City, Taiwan, 2012; pp. 1–3.
26. Hansen, F.A.; Pedersen-Bjergaard, S. Emerging Extraction Strategies in Analytical Chemistry. *Anal. Chem.* **2019**, *92*, 2–15. [CrossRef] [PubMed]
27. Danezis, G.P.; Tsagkaris, A.S.; Camin, F.; Brusic, V.; Georgiou, C.A. Food authentication: Techniques, trends emerging approaches. *Trends Anal. Chem.* **2016**, *85*, 123–132. [CrossRef]
28. Silvestre, W.P.; Livinalli, N.F.; Baldasso, C.; Tessaro, I.C. Pervaporation in the separation of essential oil components: A review. *Trends Food Sci. Technol.* **2019**, *93*, 42–52. [CrossRef]
29. Arce, A.; Marchiaro, A.; Martínez-Ageitos, J.M.; Soto, A. Citrus essential oil deterpenation by liquid-liquid extraction. *Can. J. Chem. Eng.* **2005**, *83*, 366–370. [CrossRef]
30. Qin, Z.; Cheng, H.; Song, Z.; Ji, L.; Chen, L.; Qi, Z. Selection of deep eutectic solvents for extractive deterpenation of lemon essential oil. *J. Mol. Liq.* **2022**, *350*, 118524. [CrossRef]