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Authentication of *Citrus* spp. Cold-Pressed Essential Oils by Their Oxygenated Heterocyclic Components

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Abstract: *Citrus* essential oils are routinely adulterated because of the lack of regulations or reliable authentication methods. Unfortunately, the relatively simple chemical makeup and the tremendous price variations among *Citrus* varieties encouraged the interspecies adulteration of citrus oils. In this study, a sensitive UPLC-MS/MS method for the quantitation of 14 coumarins and furanocoumarins is developed and validated. This method was applied to screen the essential oils of 12 different *Citrus* species. This study, to our knowledge, represents the most comprehensive investigation of coumarin and furanocoumarin profiles across commercial-scale *Citrus* oils to date. Results show that the lowest amount was detected in calamansi oil. Expressed oil of Italian bergamot showed the highest furanocoumarin content and the highest level of any individual furanocoumarin (bergamottin). Notable differences were observed in the coumarin and furanocoumarin levels among oils of different crop varieties and origins within the same species. Potential correlations were observed between bergapten and xanthotoxin which matches with known biosynthetic pathways. We found patterns in furanocoumarin profiles that line up with known variations among the *Citrus* ancestral taxa. However, contrary to the literature, we also detected xanthotoxin in sweet orange and members of the mandarin taxon. Using multivariate analysis, we were able to divide the *Citrus* oils into 5 main groups and correlate them to the coumarin compositions.

Keywords: essential oils; *Citrus*; furanocoumarins; coumarins; UPLC-MS/MS

1. Introduction

*Citrus* essential oils (EOs) have several applications in cosmetics, the food industry, and the flavor and fragrance industry. They are also utilized as natural preservatives because of their wide range of biological activities, which include antioxidant and antimicrobial actions [1]. These strong biological activities are attributed to the presence of terpenes, flavonoids, carotenes, and coumarins [2]. Several studies have investigated the volatile composition of various parts of *Citrus* species due to their significant economic importance. All cold-pressed *Citrus* oils contain a portion of non-volatiles fundamentally made of simple coumarins, psoralens, and methoxy-flavones [3]. Coumarins (1,2-benzopyrones) are a huge family of naturally occurring secondary metabolites. Psoralens, also known as furanocoumarins (FCs), are a large family of compounds commonly found in Rutaceae, Apioaceae, and Fabaceae, with Rutaceae containing the highest concentrations [4,5]. FCs contain a furan ring fused to a coumarin core [6]. The fusion helps separate the FCs into linear or angular structural forms. FCs have shown a potential to elicit variable degrees of phototoxic skin reactions. In comparison to angular FCs, linear FCs have often been proven to cause phototoxic responses at lower doses [7].

While studying the volatile composition of *Citrus* oils, the nonvolatile fractions are hard to detect under standard gas chromatography conditions because of their limited volatilities, relatively polar or heat-liable nature. These nonvolatile ingredients may hold the secret to constructing a perfect analytical strategy for interspecies adulteration detection.
This essential fraction of the cold-pressed oil can be used to identify species-specific patterns and establish *Citrus* species fingerprinting. For instance, creating synthetic bergamot oils or adulterating bergamot oils with similar *Citrus* oils like bitter orange are simple strategies to boost profits. Both strategies make it essentially impossible for consumers to detect the difference. The non-volatile fraction contributes very little to the *Citrus* oils’ aroma, but because of its high complexity, commercial unavailability, or extremely high cost in comparison to the *Citrus* oils themselves, it is more difficult to manipulate. Previous studies report the separation and identification of coumarins and FCs in *Citrus* peel extracts and oils using gas chromatography–mass spectroscopy (GC-MS) after derivatization [8], high-performance liquid chromatography (HPLC) [8], enzyme-linked immunosorbent assay (ELISA) [9], reversed-phase (RP)-HPLC [10,11], HPLC-diode array detector (DAD) [12], HPLC-nuclear magnetic resonance (NMR) [13], ultra-performance liquid chromatography coupled with mass spectrometry (UPLC-MS) [14,15], LC-MS [16], and HPLC-UV-MS [17].

The objective of the present study was to develop a sensitive UPLC-MS/MS method to quantify 14 selected coumarins and FCs (Figure 1). This validated method was then applied to the cold-pressed essential oils of bergamot (*Citrus bergamia* Risso & Poit), bitter orange (*C. aurantium* L.), calamansi (*C. × microcarpa* (Bunge) Wijnands), clementine (*C. clementina* Hort. ex Tanaka), grapefruit (*C. × paradisi* Macfady), kumquat (*C. japonica* Thunb.), lemon (*C. limon* Osbeck), lime (*C. aurantifolia* (Christm.) Swingle), mandarin (*C. reticulata* Blanco), sweet orange (*C. sinensis* L.), tangerine (*C. tangerina* Hort. ex Tanaka), and yuzu (*C. junos* Sieb. ex Tanaka) as well as petitgrain EO.

![Chemical structure of key non-volatile components in expressed *Citrus* essential oils](image)

**Figure 1.** Chemical structure of key non-volatile components in expressed *Citrus* essential oils.

### 2. Results and Discussions

#### 2.1. Method Validation

The LC-MS/MS chromatogram of 14 coumarins using the MRM acquisition mode is shown in Figure 2. Specificity, precision, accuracy, linearity, intermediate precision, and LOQ results are summarized in Table 1. The method proved specific to the target compounds since no interferences were found in any of the processed blanks. All com-
pounds met the acceptance criterion of RSD% ≤ 10 based on the precision and intermediate precision results. Compound recovery percentages ranged from 94.07 to 114.53% of the expected value. The linearity of the calibration curve of the 14 compounds was well correlated (r ≥ 0.98) within a range of 0.0001–0.1 ppm. The LOQ values ranged from 0.0001 to 0.005 ppm. These findings demonstrate that the developed method is suitable for analyzing the 14 targeted compounds in EOs.

![LC-MS/MS chromatogram](image)

Figure 2. LC-MS/MS chromatogram (MRM acquisition mode) of 14 targeted coumarins using a Shimadzu LCMS8060.

### Table 1. Linearity of the UPLC-MS Method (Equation and Coefficient of Determination, r²), Limit of Quantitation (LOQ), and Accuracy of the UPLC-MS Method of 14 Coumarins and Furanocoumarins.

| Compound                   | Linear Range (ppm) | Linear Equation | r²   | LOQ (ppm) | Accuracy | Precision | Intermediate Precision |
|----------------------------|--------------------|-----------------|------|-----------|----------|-----------|------------------------|
| **Coumarins**              |                    |                 |      |           |          |           |                        |
| Citroten                  | 0.001–0.1          | Y = 0.9847x + 0.0012 | 0.9991 | 0.001     | 98.92–113.80 | 2.60 | 4.44                      |
| 5-Geranyloxy-7-methoxycoumarin | 0.0001–0.1    | Y = 0.9976x + 0.0002 | 0.9989 | 0.0001    | 94.07–105.44 | 2.23 | 2.05                      |
| Toncarine                  | 0.005–0.1          | Y = 0.9961x + 0.0003 | 0.9991 | 0.005     | 97.50–114.53 | 1.28 | 2.44                      |
| Herniarin                  | 0.001–0.1          | Y = 0.989x + 0.0008  | 0.9996 | 0.001     | 98.25–113.40 | 2.33 | 4.90                      |
| **Linear furanocoumarins** |                    |                 |      |           |          |           |                        |
| 6',7'-Epoxybergamottin     | 0.001–0.1          | Y = 0.9948x + 0.0004 | 0.9990 | 0.001     | 96.83–109.00 | 2.29 | 1.05                      |
| Bergamottin                | 0.001–0.1          | Y = 0.9975x + 0.0002 | 0.9988 | 0.001     | 95.75–104.56 | 2.26 | 3.05                      |
| Bergapten                  | 0.0001–0.1         | Y = 0.9956x + 0.0003 | 0.9994 | 0.0001    | 96.92–109.33 | 2.50 | 0.46                      |
| Biacangelicol             | 0.001–0.1          | Y = 0.9923x + 0.0006 | 0.9992 | 0.001     | 97.17–107.27 | 2.18 | 2.32                      |
| Imperatorin                | 0.001–0.1          | Y = 0.9904x + 0.0007 | 0.9997 | 0.001     | 96.40–106.67 | 2.59 | 2.32                      |
| Isopimpinellin            | 0.0001–0.1         | Y = 1.0002x + 8x10^-6| 0.9989 | 0.0001    | 95.75–112.13 | 2.89 | 2.26                      |
| Oxypeucedanin             | 0.005–0.1          | Y = 0.9946x + 0.0004 | 0.9987 | 0.005     | 96.33–107.73 | 2.62 | 1.17                      |
| Psoralen                  | 0.001–0.1          | Y = 0.9936x + 0.0005 | 0.9985 | 0.001     | 97.00–113.00 | 2.38 | 2.94                      |
| Trioxsalen                | 0.001–0.1          | Y = 0.993x + 0.0005  | 0.9997 | 0.001     | 98.50–106.11 | 2.18 | 2.06                      |
| Xanthotoxin               | 0.001–0.1          | Y = 0.9874x + 0.001  | 0.9997 | 0.001     | 98.50–112.07 | 3.67 | 2.54                      |

### 2.2. Comparison of Citrus EO Coumarin and Furanocoumarin Content

Citrus EOs used in this study were produced by expression in industrial settings. A total of 374 Citrus EOs were screened for coumarins using a 20 min UPLC-MS/MS method targeting 14 coumarins, of which 10 are linear furanocoumarins. The compositions of target compounds greatly differed among the tested Citrus EOs (Table 2). The least quantity of coumarins and FCs was detected in calamansi EO (0.15 ± 0.02 ppm). In comparison, the largest presence of
coumarins and FCs was found in Italian bergamot EO (171,453.11 ± 9227.11 ppm), followed by the Brazilian bergamot EO (52,473.90 ± 1775.63 ppm). Expressed oil of Italian bergamot showed the highest FC content (167,281.60 ± 1017.74 ppm) and the highest level of any individual FC (109,730.67 ± 3150.55 ppm bergamottin). Notable differences were observed in the coumarin and FC levels among EOs of different crop varieties and origins within the same Citrus species. There have been several previous investigations on the non-volatile components of Citrus essential oils reported in the literature (Table 3). The non-volatile components are far more species-specific than the volatile components, which have comparable patterns in different Citrus oils. We found patterns in FC profiles that correspond with published differences among the Citrus ancestral taxa [15,18]. Our findings are in line with previous reports that found a mixture of FCs from the bergapten, xanthotoxin, and isopimpinellin clusters in EOs derived from the citron (C. medica) and papeda (C. micrantha) ancestral taxa [15]. In this study, EOs derived from fruits of the mandarin taxa (mandarin, clementine, and tangerine) showed low total coumarin (2.44–149.45 ppm) and FC levels (2.44–149.45 ppm), not aligning with a previous report that this taxon is nearly devoid of FCs [14,15]. Interestingly, trioxsalen and toncarine were not detected in any of the Citrus EOs. Epoxycoumarottin was absent from calamansi, clementine, mandarin, kaffir lime, and petitgrain oils. The content of psoralen was almost negligible in most of the Citrus EOs but was relatively high in white grapefruit EO (82.65 ± 0.76 ppm). Furthermore, large amounts of xanthotoxin were detected in bergamot and lime EOs. Previous reports indicate that xanthotoxin is absent from sweet orange (C. sinensis, pummelo taxon) and the mandarin taxa [14,15] while we found 4.85 ± 0.32 ppm in sweet orange EO and 0.33–15.24 ppm xanthotoxin in the mandarin taxa EOs. Bergamottin and 5-geranyloxy-7-methoxy coumarin were reported in mandarin, lemon, and lime oils but not in orange oil [17]. The differences between our findings and previous studies could be due to genetic and/or environmental impacts on FC biosynthesis [19]. Our LOQ, however, may be lower than that of other reports because it was based on the weight of EO rather than the weight of fresh fruit peel. Alternative explanations for the differences in our findings include genetic admixture in Citrus varieties or contamination during processing and handling.

Table 2. Total coumarin, total furanocoumarins, and coumarin distribution of the tested Citrus oils.

| Citrus Oil         | Total Coumarin (ppm) | Total FC (ppm) | Coumarin Distribution                                                                 |
|--------------------|----------------------|----------------|--------------------------------------------------------------------------------------|
| Bergamot (Brazil)  | 52,473.90 ± 1775.63  | 48,798.90 ± 174.98 | Bergamottin > imperatorin > bergapten > 5-geranyloxy-7-methoxy coumarin > citropten > xanthotoxin > 6',7'-epoxybergamottin > herniarin > psoralen > oxypeucedanin > isopimpinellin > biacangelicol |
| Bergamot (Italy)   | 171,453.11 ± 9227.11 | 167,281.60 ± 1017.74 | Bergamottin > imperatorin > 6',7'-epoxybergamottin > 5-geranyloxy-7-methoxy coumarin > citropten > xanthotoxin > bergapten > herniarin > oxypeucedanin > isopimpinellin > psoralen > biacangelicol |
| Bitter Orange      | 814.95 ± 9.52        | 809.21 ± 1.30     | 6',7'-Epoxycoumarottin > xanthotoxin > bergapten > imperatorin > bergamottin > citropten > psoralen > 5-geranyloxy-7-methoxy coumarin > herniarin > isopimpinellin |
| Calamansi          | 0.15 ± 0.02          | 0               | 5-Geranyloxy-7-methoxy coumarin                                                   |
| Clementine (Brazil)| 75.26 ± 0.08         | 43.03 ± 0.11     | 5-Geranyloxy-7-methoxy coumarin > bergamottin > imperatorin > citropten > oxypeucedanin > xanthotoxin > bergapten > herniarin > isopimpinellin |
| Clementine (Italy) | 4.69 ± 0.03          | 3.38 ± 0.04      | Oxypeucedanin > bergamottin > citropten > xanthotoxin > biacangelicol > bergapten > 5-geranyloxy-7-methoxy coumarin > psoralen |


| Citrus Oil         | Total Coumarin (ppm) | Total FC (ppm) | Coumarin Distribution                                                                 |
|--------------------|----------------------|----------------|--------------------------------------------------------------------------------------|
| Grapefruit (Red)   | 13,099.29 ± 207.97   | 13,013.55 ± 22.87 | 6',7'-Epoxybergamottin > bergamottin > imperatorin > oxypeucedanin > xanthotoxin > bergapten > 5-geranyloxy-7-methoxycoumarin > citrophan > isopimpinellin > psoralen > xanthotoxin > bergapten |
| Grapefruit (White) | 9163.08 ± 229.85     | 9027.29 ± 25.14 | 6',7'-Epoxybergamottin > imperatorin > bergamottin > oxypeucedanin > xanthotoxin > bergapten > herniarin > 5-geranyloxy-7-methoxy-coumarin > isopimpinellin > citrophan |
| Kaffir Lime        | 75.46 ± 5.13         | 43.15 ± 0.38   | Imperatorin > citrophan > bergamottin > bergapten > isopimpinellin > psoralen          |
| Kumquat            | 169.65 ± 0.72        | 93.19 ± 0.53   | Bergamottin > 5-geranyloxy-7-methoxy-coumarin > imperatorin > citrophan > xanthotoxin > bergapten > oxypeucedanin > herniarin > isopimpinellin > 6',7'-epoxy-b ergamottin > bicangelicol |
| Lemon (Argentina)  | 5404.76 ± 3.60       | 3861.29 ± 3.41 | Imperatorin > bergamottin > citrophan > oxypeucedanin > 5-geranyloxy-7-methoxy-coumarin > xanthotoxin > bergapten > herniarin > isopimpinellin |
| Lemon (Brazil)     | 3321.86 ± 1.84       | 2335.29 ± 1.77 | Imperatorin > bergamottin > citrophan > bergapten > isopimpinellin > psoralen          |
| Lemon (Germany)    | 3107.99 ± 3.27       | 2029.93 ± 2.61 | Imperatorin > bergamottin > bergapten > isopimpinellin > psoralen                      |
| Lemon (Italy)      | 10,874.88 ± 8.28     | 8346.28 ± 9.30 | Bergamottin > imperatorin > oxypeucedanin > 5-geranyloxy-7-methoxy-coumarin > citrophan > xanthotoxin > bergapten > herniarin > isopimpinellin > psoralen |
| Lemon (South Africa)| 4268.48 ± 2.13       | 3185.78 ± 2.81 | Oxypeucedanin > imperatorin > bergamottin > xanthotoxin > bergapten > isopimpinellin > psoralen |
| Lemon (Spain)      | 3343.46 ± 4.76       | 2467.31 ± 4.28 | Imperatorin > bergamottin > bergapten > isopimpinellin > psoralen                      |
| Lemon (USA)        | 2717.40 ± 4.45       | 1985.52 ± 4.93 | Imperatorin > bergamottin > bergapten > isopimpinellin > psoralen                      |
| Lime               | 23,795.43 ± 564.22   | 16,725.07 ± 43.80 | Bergamottin > imperfectin > bergapten > isopimplin > psoralen                         |
Table 2. Cont.

| Citrus Oil            | Total Coumarin (ppm) | Total FC (ppm) | Coumarin Distribution                                                                 |
|-----------------------|----------------------|----------------|--------------------------------------------------------------------------------------|
| Mandarin (Green)      | 32.27 ± 0.35         | 22.77 ± 0.46   | Imperatorin > bergamottin > 5-geranyloxy-7-methoxycoumarin > citropten > xanthotoxin > oxypeucedanin > herniarin > bergapten > isopimpinellin > biacangelicol |
| Mandarin (Red)        | 27.42 ± 0.06         | 19.06 ± 0.08   | Imperatorin > bergamottin > 5-geranyloxy-7-methoxycoumarin > citropten > xanthotoxin > bergapten > herniarin > oxypeucedanin > isopimpinellin > biacangelicol |
| Mandarin (Yellow)     | 52.89 ± 0.04         | 37.96 ± 0.05   | Imperatorin > bergamottin > 5-geranyloxy-7-methoxycoumarin > xanthotoxin > bergapten > herniarin > oxypeucedanin > isopimpinellin > biacangelicol |
| Petitgrain (Lemon)    | 36.22 ± 2.10         | 20.74 ± 0.18   | Herniarin > imperatorin > citropten > bergapten > xanthotoxin > bergamottin > psoralen > 5-geranyloxy-7-methoxycoumarin > oxypeucedanin > isopimpinellin > biacangelicol |
| Petitgrain (Lime)     | 58.93 ± 1.37         | 47.47 ± 0.11   | Imperatorin > xanthotoxin > citropten > bergapten > isopimpinellin > bergamottin > 5-geranyloxy-7-methoxycoumarin > herniarin > oxypeucedanin > psoralen |
| Sweet Orange (Navel)  | 179.26 ± 9.94        | 140.13 ± 0.75  | 6',7'-Epoxybergamottin > bergamottin > oxypeucedanin > imperatorin > 5-geranyloxy-7-methoxycoumarin > citropten > xanthotoxin > bergapten > isopimpinellin > herniarin > oxypeucedanin > psoralen |
| Sweet Orange (Valencia)| 122.27 ± 2.29       | 68.75 ± 0.19   | 5-Geranyloxy-7-methoxycoumarin > imperatorin > bergamottin > citropten > oxypeucedanin > xanthotoxin > bergapten > herniarin > isopimpinellin > 6',7'-epoxybergamottin > biacangelicol |
| Tangerine (Brazil)    | 149.45 ± 1.58        | 94.31 ± 1.45   | Imperatorin > bergamottin > 5-geranyloxy-7-methoxycoumarin > citropten > xanthotoxin > bergapten > 6',7'-epoxybergamottin > oxypeucedanin > herniarin > isopimpinellin > biacangelicol > psoralen |
| Tangerine (Italy)     | 2.44 ± 0.02          | 1.95 ± 0.03    | Bergamottin > bergapten > xanthotoxin > oxypeucedanin > citropten > 6',7'-epoxybergamottin > 5-geranyloxy-7-methoxycoumarin |
| Yuzu                 | 609.06 ± 0.33        | 597.1 ± 0.41   | 6',7'-Epoxybergamottin > biacangelicol > oxypeucedanin > xanthotoxin > bergapten > imperatorin > citropten > bergamottin > herniarin > 5-Geranyloxy-7-methoxycoumarin > isopimpinellin |

Table 3. Non-volatile components of cold-pressed Citrus oils that are reported in the literature.

| Citrus Oil      | Non-Volatile Components | Reported Amount | Reference(s) |
|-----------------|-------------------------|-----------------|--------------|
| Bitter orange   | Bergapten               | 0.035–0.073%    | [3]          |
|                 | Epoxybergamottin        | 0.082%          |              |
|                 | Psoralen                | 0.007%          |              |
Table 3. Cont.

| Citrus Oil | Non-Volatile Components | Reported Amount | Reference(s) |
|------------|--------------------------|-----------------|--------------|
| Bergamot CP | 5-Geranloxy-7-methoxycoumarin | 0.08–0.68% | [3] |
| | 5-Methoxy-7-geranoxycoumarin | 0.04–0.15% | |
| | Bergamottin | 0.68–2.75% | |
| | Bergaptol | 0–0.19% | |
| | Psoralen | 0–0.0026% | |
| | Bergapten | 0.11–0.33% | |
| | Citropten | 0.01–0.35% | |
| Lemon | 5-Geranloxy-7-methoxycoumarin | 0.18–0.28% | [3] |
| | 8-Geranyloxypsoralen | 0.01–0.045% | |
| | Bergamottin | 0.16–0.54% | |
| | Byakangelicol | 0.006–0.16% | |
| | Bergapten | 0.0001–0.035% | |
| | Citropten | 0.05–0.17% | |
| | Isopimpinellin | 0–0.011% | |
| | oxypeucedanin | 0.09–0.82% | |
| Lime | 5-Geranloxy-7-methoxycoumarin | 1.7–3.2% | [3] |
| | 5-Geranoxo-8-methoxypsoralen | 0.2–0.9% | |
| | 8-Geranyloxypsoralen | 0.10–0.14% | |
| | 5-Methoxy-7-geranoxycoumarin | 1.7–5.2% | |
| | Bergamottin | 1.7–3.0% | |
| | Bergapten | 0.17–0.33% | |
| | Citropten | 0.4–2.2% | |
| | Isopimpinellin | 0.1–1.3% | |
| | oxypeucedanin | 0.02–0.3% | |
| Grapefruit | Bergamottin | <0.11% | [3] |
| | Epoxybergamottin | 0.1126% | |
| | Bergapten | 0.012–0.19% | |
| Mandarin | Bergamottin | 0–0.001% | [3] |
| | Bergapten | 0–0.0003% | |
| Mandarin CO₂ | Bergapten | 0.07% | [20] |
| | Citropten | 0.76% | |
| Lemon (coastal) | Bergapten | 0–10 ppm | [21] |
| | Citropten | 700–1300 ppm | |
| | Herniarin | 0–10 ppm | |
| | Isopimpinellin | <5 ppm | |
| Lemon (desert) | Bergapten | 50–350 ppm | [21] |
| | Citropten | 700–1700 ppm | |
| | Herniarin | <10 ppm | |
| | Isopimpinellin | 35–110 ppm | |
| Lemon | 5-Geranloxy-7-methoxycoumarin | 1800–2500 ppm | [21] |
| | 5-Isopent-2'-enyloxyl-8-(2',3'-epoxyisopentylloxypsoralen) | 190–370 ppm | |
| | 5-Isopentenyloxyl-7-methoxycoumarin | 190–360 ppm | |
| | 8-Geranyloxypsoralen | 1600–1910 ppm | |
| | Bergamottin | 1660–1230 ppm | |
| | Byakangelicol | 520–1420 ppm | |
| | Citropten | 350–1420 ppm | |
| | Isoimperatorin | tr | |
| | Oxypeucedanin | 890–1570 ppm | |
| | Oxypeucedanin hydrate | tr | |
Table 3. Cont.

| Citrus Oil | Non-Volatile Components | Reported Amount | Reference(s) |
|------------|-------------------------|-----------------|--------------|
| Lemon      | 5-Geranoxy-7-methoxycoumarin | 2453–2845 ppm  |              |
|            | 5-Isopent-2′-enyloxy-8-(2′,3′-epoxyisopentylloxyxyporalen) | 204–324 ppm |              |
|            | 8-Geranylxyopsoralen | 399–454 ppm |              |
|            | Bergamottin           | 2635–2973 ppm | [22]         |
|            | Byakangelicol        | 555–1640 ppm  |              |
|            | Citropten            | 659–1495 ppm  |              |
|            | Oxypeucedanin        | 863–2200 ppm  |              |
| Lime oil (Mexican type B) | 5-Geranoxy-7-methoxycoumarin | 27,770–45,350 ppm |          |
|            | 5-Isopentenyloxy-7-methoxycoumarin | 2100–2790 ppm |          |
|            | 8-Geranylxyopsoralen | 3800–4540 ppm |          |
|            | Bergamottin          | 25,320–41,590 ppm |          |
|            | Bergapten            | 2160–3920 ppm  |              |
|            | Byakangelicol        | 80–1020 ppm    |              |
|            | Citropten            | 5940–10,950 ppm | [23]        |
|            | Cnidicin             | 70–250 ppm     |              |
|            | Herniarin            | 3350–4670 ppm  |              |
|            | Imperatorin          | 380–660 ppm    |              |
|            | Isoimperatorin       | 70–410 ppm     |              |
|            | Isopimpinellin       | 3010–7300 ppm  |              |
|            | Oxypeucedanin        | 6660–10,720 ppm |              |
|            | Oxypeucedanin hydrate | 1620–1710 ppm |              |
| Lime (type A) | 5-Geranoxy-7-methoxycoumarin | 41,550–63,320 ppm |          |
|            | 5-Isopentenyloxy-7-methoxycoumarin | 4170–4830 ppm |          |
|            | 8-Geranylxyopsoralen | 6520–8100 ppm  |          |
|            | Bergamottin          | 37,300–56,130 ppm |          |
|            | Bergapten            | 2000–3450 ppm  | [23]        |
|            | Byakangelicol        | 0–90 ppm       |              |
|            | Citropten            | 7350–11,740 ppm |              |
|            | Cnidicin             | 90–340 ppm     |              |
|            | Herniarin            | 1460–2970 ppm  |              |
|            | Imperatorin          | 830–900 ppm    |              |
|            | Isoimperatorin       | 5670–10,210 ppm|              |
|            | Isopimpinellin       | 0–260 ppm      |              |
|            | Oxypeucedanin        | 780–1160 ppm   |              |
|            | Oxypeucedanin hydrate | 780–1160 ppm |              |
| Key lime CP | 5-Isopentenyloxy-7-methoxycoumarin | 2790 ± 15 ppm |          |
|            | 8-Geranylxyopsoralen | 4470 ± 28.7 ppm|          |
|            | Bergamottin          | 36,401 ± 150.9 ppm |          |
|            | Bergapten            | 3000 ± 31.1 ppm |          |
|            | Byakangelicol        | 92 ± 9.9 ppm   |              |
|            | Citropten            | 10,950 ± 92.8 ppm |          |
|            | Cnidicin             | 250 ± 62 ppm   |              |
|            | Cnidilin             | 249 ± 7.6 ppm  |              |
|            | Herniarin            | 3880 ± 45.8 ppm|              |
|            | Imperatorin          | 39 ± 10.3 ppm  |              |
|            | Isoimperatorin       | 88 ± 5.9 ppm   |              |
|            | Isopimpinellin       | 7300 ± 46.9 ppm|              |
|            | Oxypeucedanin        | 10,600 ± 85.1 ppm|          |
|            | Oxypeucedanin hydrate | 1690 ± 203 ppm|          |
| Citrus Oil | Non-Volatile Components | Reported Amount | Reference(s) |
|-----------|-------------------------|-----------------|--------------|
| Key Lime (type A) | 5-Geranoxy-7-methoxycoumarin | 306.5–404.5 ppm | [25] |
| | 5-Isopentenylxyloxy-7-methoxycoumarin | <0.1 ppm | |
| | 8-Geranylxyopsoralen | 315.7–328.3 ppm | |
| | Bergamottin | 10–12.4 ppm | |
| | Bergapten | 49.1–63.2 ppm | |
| | Citroten | 2.5–3.5 ppm | |
| | Cnidilin | 8.6–9.6 ppm | |
| | Herniarin | <0.1 ppm | |
| | Isoimperatorin | <0.1 ppm | |
| | Isopimpinellin | 35–36.5 ppm | |
| | Oxypeucedanin hydrate | <0.1 ppm | |
| | Xanthotoxin | <0.1 ppm | |
| Persian Lime | 5-Geranoxy-7-methoxycoumarin | 409.3 ppm | [25] |
| | 5-Isopentenylxyloxy-7-methoxycoumarin | <0.1 ppm | |
| | 8-Geranylxyopsoralen | 315.4 ppm | |
| | Bergamottin | 8.9 ppm | |
| | Bergapten | 48.4 ppm | |
| | Citroten | 2.4 ppm | |
| | Cnidilin | 7.4 ppm | |
| | Herniarin | <0.1 ppm | |
| | Isoimperatorin | 33.1 ppm | |
| | Isopimpinellin | 14.4 ppm | |
| | Oxypeucedanin | <0.1 ppm | |
| | Oxypeucedanin hydrate | <0.1 ppm | |
| | Xanthotoxin | <0.1 ppm | |
| Bergamot oil (Italian) | 5-Geranoxy-7-methoxycoumarin | 194.3–378 ppm | [25] |
| | Bergamottin | <0.1 ppm | |
| | Bergapten | <0.1 ppm | |
| | Citroten | 15.8–25 ppm | |
| | Citroten | 32.6–56.9 ppm | |
| | Cnidilin | 0.5–0.8 ppm | |
| | Herniarin | 33.9–59.4 ppm | |
| | Isoimperatorin | <0.1 ppm | |
| | Isopimpinellin | 16.9–29.3 ppm | |
| | Oxypeucedanin | 21–32.8 ppm | |
| | Oxypeucedanin hydrate | <0.1 ppm | |
| | Xanthotoxin | <0.1 ppm | |
| Bergamot CP | 5-Geranoxy-7-methoxycoumarin | 0.14–0.18% | [25] |
| | Bergamottin | 1.37–1.6% | |
| | Bergapten | 0.18–0.21% | |
| | Citroten | 0.18–0.26% | |
| Bergamot oil (commercial) | 5-Geranoxy-7-methoxycoumarin | 18–37 ppm | [25] |
| | 5-Geranylxyloxy-8-methoxypsoralen | <5 ppm | |
| | 5-Isopentenylxyloxy-8-methoxypsoralen | <5 ppm | |
| | 5-Isopentenylxyloxy-7-methoxycoumarin | <5 ppm | |
| | 8-Geranylxyopsoralen | <5 ppm | |
| | Bergamottin | 68–116 ppm | |
| | Bergapten | 4–10 ppm | |
| | Citroten | 10–13 ppm | |
| | Herniarin | <5 ppm | |
| | Isopimpinellin | <5 ppm | |
| | Oxypeucedanin | <5 ppm | |
Table 3. Cont.

| Citrus Oil       | Non-Volatile Components | Reported Amount       | Reference(s) |
|------------------|-------------------------|-----------------------|--------------|
| Bergamot oil     | Bergamottin             | 96.7 ug/100mg         | [26]         |
|                  | Bergapten               | 152.5 ug/100mg        |              |
|                  | Citropten               | 21.7 ug/100mg         |              |
| Bergamot         | Bergamottin             | 16,312 ppm            | [12]         |
|                  | Bergapten               | 8 ppm                 |              |
|                  | Citropten               | 70.3 ppm              |              |
|                  | Epoxybergamottin        | 53.5 ppm              |              |
|                  | Oxypeucedanin           |                       |              |
| Bergamot         | 5-Geranoxy-7-methoxycoumarin | 0.08–0.104%           | [27]         |
|                  | Bergamottin             | 1.097–1.409%          |              |
|                  | Bergapten               | 0.138–0.209%          |              |
|                  | Citropten               | 0.134–0.212%          |              |
| Bergamot         | 5-Geranoxy-7-methoxycoumarin | 0–2.827 ppm           | [28]         |
|                  | Bergamottin             | 0–39,203 ppm          |              |
|                  | Bergapten               | 0–4.215 ppm           |              |
|                  | Citropten               | 0–6.134 ppm           |              |
|                  | Herniarin               | 0–0.251 ppm           |              |
| Bergamot         | Bergapten               | 1.70%                 | [29]         |
|                  | Citropten               | 0.40%                 |              |
| Bergamot         | 5-Geranoxy-7-methoxycoumarin | 0–3 ppm               | [30]         |
|                  | Bergamottin             | 0–37 ppm              |              |
|                  | Bergapten               | 0–268 ppm             |              |
|                  | Citropten               | 0–14 ppm              |              |
| Bergamot         | 5-Geranoxy-7-methoxycoumarin | 1065 ± 7.5 ppm        | [24]         |
|                  | Bergamottin             | 19.605 ± 73.2 ppm     |              |
|                  | Bergapten               | 2474 ± 28.4 ppm       |              |
|                  | Citropten               | 2232 ± 26.3 ppm       |              |
|                  | Herniarin               | 67 ± 3.2 ppm          |              |
| Bergamot         | Bergapten               | 1.14–2.73%            | [25]         |
|                  | Citropten               | 0.06–0.4%             |              |
|                  |                          | 0.1–0.3%              |              |

2.3. Multivariate Analysis

In order to examine the similarities and relationships between the coumarin compositions and the Citrus essential oils, AHC and PCA were carried out based on a data matrix comprised of 28 Citrus “types” and 12 coumarin components. Based on > 25% similarity, the AHC shows five groups (Figure 3): Group 1 (bergamot from Italy and bergamot from Brazil), Group 2 (lime and lemon from Germany), Group 3 (yuzu, red and white grapefruit), Group 4 (a large group composed of oranges, tangerines, clementines, mandarins calamansi, and petitgrains), and Group 5 (lemons). The PCA analysis (Figure 4) of the Citrus essential oils indicates that F1 and F2 explain 78.43% of the variation in coumarin compositions among the Citrus types. The bergamot group (Group 1) is positively correlated with bergamottin, bergapten, and xanthotoxin; the lemon group (Group 5) positively correlates with biacangelicol and oxypeucedanin as well as citropten and 5-geranyloxy-7-methoxycoumarin. The grapefruit and yuzu group (Group 3) correlate with 6′,7′-epoxybergamottin and psoralen. Group 4 (oranges, mandarins, clementines, etc.) are characterized as having relatively low levels of coumarins. A positive correlation was found between bergapten and xanthotoxin (2 structures related by a common precursor in biosynthesis [15]).
characterized as having relatively low levels of coumarins. A positive correlation was found between bergapten and xanthotoxin (2 structures related by a common precursor in biosynthesis [15]).

**Figure 3.** Dendrogram obtained by cluster analysis of the coumarin composition of *Citrus* essential oils, based on correlation and using the unweighted pair-group method with arithmetic average (UPGMA).
3. Materials and Methods

3.1. Chemicals

Xanthotoxin, herniarin, toncarine, bergamottin, oxypeucedanin, biacangelicol, psoralen, isopimpinellin, bergapten, and imperatorin (purity ≥ 98%) were purchased from Chengdu Alfa Biotechnology (Chengdu, China). 5-Geranyloxy-7-methoxycoumarin (purity ≥ 99%) was bought from Extrasynthese (Genay, France). Trioxsalen and 6′,7′-epoxybergamottin (purity ≥ 98%) were obtained from Cayman Chemical Company (Michigan, USA). Citropten (purity ≥ 99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). LCMS-grade methanol, LCMS-grade water, and HPLC-formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of each standard at a concentration of 10 ppm were prepared by diluting the powder in methanol.
3.2. Essential Oil Samples

Citrus volatile oils from trusted suppliers were obtained from the collection of the Aromatic Plant Research Center (APRC, Lehi, UT, USA). A total of 374 cold-pressed Citrus oil samples from the APRC collection are listed in Table 4. A simple dilute and shoot technique (1 µL oil in 999 µL of methanol) was used for sample preparation. Further dilution was performed whenever needed.

Table 4. Sample information of citrus essential oil samples from the APRC collection.

| Citrus Oil          | Scientific Name | No. of Samples | Origin          |
|---------------------|-----------------|----------------|-----------------|
| Calamansi           | Citrus × microcarpa (Bunge) Wijnands | 5              | Philippines     |
| Tangerine           | Citrus tangerina Hort. Ex Tanaka     | 13             | Brazil          |
| Kumquat             | Citrus japonica Thunb                | 3              | Brazil          |
| Mandarin            | Citrus reticulata Blanco             | 33             | Brazil          |
| Clementine          | Citrus clementina Hort. Ex Tanaka    | 6              | Brazil          |
| Yuzu or Yuja        | Citrus junos Sieb. Ex Tanaka         | 16             | Brazil          |
| Bitter Orange       | Citrus aurantium L.                  | 6              | Japan           |
| Sweet Orange        | Citrus sinensis L.                   | 36             | Brazil          |
| Lime                | Citrus aurantifolia (Christm.) Swingle | 28            | Brazil          |
| Bergamot            | Citrus bergamia Risso & Poit         | 66             | Italy and Brazil|
| Grapefruit          | Citrus × paradisi Macfady            | 45             | South Africa and USA |
| Lemon               | Citrus limon Osbeck                 | 97             | Spain, Argentina, Brazil, Italy, USA, South Africa, and Germany |
| Petitgrain          | Citrus aurantifolia leaf and Citrus limon leaf | 20            | Paraguay        |

3.3. UPLC-MS/MS Analyses

Coumarins were quantified using a NEXERA UPLC system (Shimadzu Corp., Kyoto, Japan) equipped with a mass spectrometer (Triple quadrupole, LCMS8060, Shimadzu, Kyoto, Japan). Target compounds were chromatographed on a Shimadzu Nexcol C\textsubscript{18} column (1.8 µm, 50 × 2.1 mm) with a C\textsubscript{18} guard column (Restek, Bellefonte, PA, USA) at 40 °C. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The compounds were eluted using the following gradient: %10 B at 0 min, %20 B at 0.74 min, %60 B at 5.88 min, %90 B at 10 min, held at %100 B for 4 min, and %10 for 4 min before the next injection. The flow rate was maintained at 0.2 mL/min, and the injection volume was 1 µL. The UPLC system was connected to the MS by electrospray ionization (ESI) operating in positive ion mode. The interface, desolvation line, and heating block temperatures were 350, 250, and 400 °C, respectively. The capillary voltage was 4.5 kV, and CID gas was set at 350 kPa. Nebulizing gas flow was set at 3.0 L/min, and heating and drying gas were set at 10.0 L/min. The detection was completed in multiple reaction monitoring mode (MRM) (Table 5). Samples were run in triplicates with external standards in between. Each run contained a quality control (QC) standard, and at least one QC standard was run at the beginning and the end of the run. The acquired chromatographic results were processed in LabSolutions Insight software version 3.2 (Shimadzu). For each compound, calibration curves (0.005, 0.001, 0.0025, 0.005, 0.01, 0.025, 0.05, and 0.1 ppm) were drawn by linking its peak area and its concentration.
Table 5. Multiple reaction monitoring mode (MRM) parameters.

| Name               | Other Name(s)         | CAS #       | Precursor (m/z) | Product 1 (m/z) | Product 2 (m/z) | Product 3 (m/z) | RT (min) |
|--------------------|-----------------------|-------------|-----------------|-----------------|-----------------|-----------------|----------|
| **Coumarins**      |                       |             |                 |                 |                 |                 |          |
| Citropten or      | 5,7-dimethoxycoumarin | 487-06-9    | 206.90          | 192.10          | 149.10          | 121.15          | 7.61     |
| Limettin 5-Geranyloxy-7- |              | 7380-39-4   | 328.90          | 193.10          | 137.05          | 149.10          | 12.25    |
| Herniarin 7-Methoxycoumarin |            | 531-59-9    | 176.90          | 121.05          | 78.10           | 77.10           | 6.58     |
| Toncarine 6-Methylcoumarin |              | 92-48-8     | 160.90          | 105.05          | 76.95           | 115.05          | 7.18     |
| **Linear furanocoumarins** |                 |             |                 |                 |                 |                 |          |
| Xanthotoxin       | 8-methoxypsoralen     | 298-81-7    | 216.90          | 89.05           | 174.10          | 202.10          | 7.74     |
| Bergamottin 5-geranyloxypsoralen |            | 7380-40-7   | 339.00          | 203.00          | 147.05          | 91.15           | 12.09    |
| Oxypeucedanin      | epoxyisopentynolypso | 26091-73-6  | 286.90          | 202.90          | 147.20          | 91.20           | 8.34     |
| Biacangelicol or  | 5-methoxy-8-(2β,3β- | 26091-79-2  | 317.00          | 233.05          | 231.10          | 218.10          | 8.21     |
| Byakangelicol     | epoxyisopentynolypso |             |                 |                 |                 |                 |          |
| Psoralen           |                       | 66-97-7     | 186.90          | 131.10          | 77.10           | 115.10          | 6.98     |
| Isopimpinellin     |                       | 482-27-9    | 246.90          | 216.95          | 232.05          | 189.05          | 7.57     |
| Bergapten          |                       | 484-20-8    | 216.90          | 202.10          | 174.10          | 89.05           | 7.75     |
| Imperatorin 8-isopentenyloxypsoralen |        | 482-44-0    | 202.90          | 91.15           | 91.15           | 65.10           | 12.09    |
| Trioxsalen 6′,7′-  |                       | 3902-71-4   | 229.00          | 115.15          | 142.20          | 128.10          | 9.62     |
| Epoxybergamottin  |                       | 206978-14-5 | 354.90          | 203.10          | 153.15          | 147.10          | 10.11    |

3.4. Method Validation

Method validation was executed according to the USP<1225> Validation of compendial procedures [31] and ICH harmonized tripartite guideline validation of analytical procedures: text and methodology Q2(R1) [32]. Specificity, precision, accuracy, linearity, intermediate precision, and limit of quantification (LOQ) were determined using standard solutions. Distilled yuzu essential oil was used as a matrix (total coumarins < 0.001 ppm). To prove the specificity of the method, standard solution mixtures and at least three blanks were processed to demonstrate the absence of interferences with the elution of the analytes. Precision and repeatability were determined by injecting six sample preparations spiked to a final concentration of 0.04 ppm and then calculating the RSD% between injections which may reach 10% for each. For the intermediate precision, the repeatability experiment was repeated on a second day and performed by a second analyst with the acceptance criterion of RSD ≤ 10 for each compound and each analyst. To determine the recoveries (accuracy) of the target compounds, three individually prepared samples of yuzu oil were spiked with three concentrations of the standard (LOQ, 0.04, and 0.05 ppm in triplicates). Recoveries were calculated by comparing the absolute peak areas with a reference measurement which must be within 80–120% of the expected value. Five concentrations from 0.001 to 0.1 ppm were used to determine linearity and a coefficient of determination (r) higher than 0.98 was needed. The data obtained during the linearity, precision, and accuracy studies were used to assess the range of the method for the target compounds. The acceptable range was defined as the concentration interval over which linearity, precision, and accuracy are acceptable. To estimate the LOQ, standard mixtures at low concentrations (0.0005 to 0.01 ppm) were analyzed. The calculated LOQ was determined using the signal-to-noise (S/N) ratio (10:1) and then injected 6 times. The acceptance criterion for the LOQ was RSD ≤ 15%. A calibration curve based on the linear range was prepared and injected to estimate the quantity of coumarins in the oil samples. Additionally, QC standards at low (0.05 ppm) and high (0.1 ppm) concentrations were used.
3.5. Multivariate Analysis

The average coumarin concentrations (12 compounds) in the Citrus samples were used as variables in the multivariate analysis. First, the data matrix was standardized by subtracting the mean for each compound concentration and dividing it by the standard deviation. For the agglomerative hierarchical cluster (AHC) analysis, the 24 Citrus samples were treated as operational taxonomic units (OTUs). Pearson correlation was selected as a measure of similarity, and the unweighted pair group method with arithmetic average (UPGMA) was used for cluster definition. Principal component analysis (PCA) was performed for the visual comparison of the coumarin compositions of the different Citrus groups using the 12 coumarin components as variables, with a Pearson correlation matrix. The AHC and PCA analyses were performed using XLSTAT v. 2018.1.1.62926 (Addinsoft, Paris, France).

4. Conclusions

In this study, we developed and validated a simple and sensitive UPLC-MS/MS method for the detection and quantification of 14 selected oxygen heterocyclic compounds (coumarins and furanocoumarins). Targeted screening using this method was successfully completed for the essential oils of 12 different Citrus species. To our knowledge, this is the most comprehensive investigation of coumarin and furanocoumarin profiles across commercial-scale Citrus oils to date. The lowest amount was detected in calamansi oil. Expressed oil of Italian bergamot showed the highest furanocoumarin content and the highest level of any individual furanocoumarin (bergamottin). Remarkable differences were observed in the coumarin and furanocoumarin levels among oils of different crop varieties and origins within the same species. We found potential correlations between bergapten and xanthotoxin which matches with known biosynthetic pathways. Patterns in furanocoumarin profiles lined up with known variations among the Citrus ancestral taxa. Using multivariate analysis, we were able to divide the Citrus oils into 5 main groups (bergamots; lime and German lemon; yuzu and grapefruit; oranges, tangerines, clementines, mandarins, calamansi, and petitgrains; and lemons) and correlate them to the coumarin compositions.

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**Abbreviations**

| Abbreviation | Description                      |
|--------------|----------------------------------|
| AHC          | agglomerative hierarchical cluster analysis |
| DAD          | diode array detector             |
| ELISA        | enzyme-linked immunosorbent assay|
| EO           | essential oil                    |
| FC           | furanocoumarin                   |
| GC-MS        | gas chromatography–mass spectroscopy |
HPLC  high-performance liquid chromatography  
LC-MS  liquid chromatography-mass spectrometry  
LOQ  limit of quantification  
MRM  multiple reaction monitoring mode  
NMR  nuclear magnetic resonance  
OTUs  operational taxonomic units  
PCA  Principal component analysis  
ppm  parts per million  
QC  quality control  
RP-HPLC  reversed-phase-high-performance liquid chromatography  
UPGMA  unweighted pair group method with arithmetic average  
UPLC-MS/MS  ultra-performance liquid chromatography-tandem mass spectrometry

References

1. Mitropoulou, G.; Fitsiou, E.; Spyridopoulou, K.; Tiptiri-Kourpeti, A.; Bardouki, H.; Vamvakias, M.; Panas, P.; Chichilia, K.; Pappa, A.; Kourkoutas, Y. *Citrus medica* essential oil exhibits significant antimicrobial and antiproliferative activity. *IWT-Food Sci. Technol.* 2017, 84, 342–352. [CrossRef]  
2. Viuda-Martos, M.; Ruiz-Navajas, Y.; Fernández-López, J.; Perez-Álvarez, J. Antifungal activity of lemon (*Citrus lemon* L), mandarin (*Citrus reticulata* L.), grapefruit (*Citrus paradisi* L) and orange (*Citrus sinensis* L) essential oils. *Food Control* 2008, 19, 1130–1138. [CrossRef]  
3. Tisserand, R.; Young, R. Essential Oil Safety, 2nd ed.; Elsevier: Amsterdam, The Netherlands, 2014.  
4. Pathak, M.A.; Daniels, F.; Fitzpatrick, T.B. The presently known distribution of furocoumarins (psoralens) in plants. *J. Invest. Dermatol.* 1962, 39, 225–239. [CrossRef]  
5. Murray, R.D.H.; Méndez, J.; Brown, S.A. *The Natural Coumarins: Occurrence, Chemistry, and Biochemistry*; Wiley: Chichester, UK, 1982.  
6. Nitao, J.K.; Berhow, M.; Duval, S.M.; Weisleder, D.; Vaughn, S.F.; Zangerl, A.; Berenbaum, M.R. Characterization of furanocoumarin metabolites in parsnip webworm, *Depressaria pastinacella*. *J. Chem. Ecol.* 2003, 29, 671–682. [CrossRef]  
7. Bourgaud, F.; Hehn, A.; Larbat, R.; Doerper, S.; Gontier, E.; Kellner, S.; Matern, U. Biosynthesis of coumarins in plants: A major pathway still to be unravelled for cytochrome P450 enzymes. *Phytochem. Rev.* 2006, 5, 293–308. [CrossRef]  
8. Ziegler, H.; Spiteller, G. Coumarins and psoralens from Sicilian lemon oil (*Citrus limon* (L.) Burm. f.). *Flavour Fragr. J.* 1992, 7, 129–139. [CrossRef]  
9. Saita, T.; Fujito, H.; Mori, M. Screening of furanocoumarin derivatives in *Citrus* fruits by enzyme-linked immunosorbent assay. *Biol. Pharm. Bull.* 2004, 27, 974–977. [CrossRef]  
10. Thompson, H.J.; Brown, S.A. Separations of some coumarins of higher plants by liquid chromatography. *J. Chromatogr. A* 1984, 314, 323–336. [CrossRef]  
11. Kamiński, M.; Kartanowicz, R.; Kamiński, M.M.; Królicka, A.; Sidwa-Gorycka, M.; Łojkowska, E.; Gorzeń, W. HPLC-DAD in identification and quantification of selected coumarins in crude extracts from plant cultures of *Ammi majus* and *Ruta graveolens*. *J. Sep. Sci.* 2003, 26, 1287–1291. [CrossRef]  
12. Frerot, E.; Decorzant, E. Quantification of total furocoumarins in *Citrus* oils by HPLC coupled with UV, fluorescence and mass detection. *J. Agric. Food Chem.* 2004, 52, 6879–6886. [CrossRef]  
13. Sommer, J.; Bertram, H.J.; Kramer, G.; Kindel, G.; Kühne, T.; Reinders, G.; Reiss, L.; Schmidt, C.O.; Schreiber, K.; Stumpe, W.; et al. HPLC–NMR—a powerful tool for the identification of non-volatiles in lemon peel oils. *Planta Med.* 2004, 70, 217–223. [CrossRef]  
14. Dugo, A.; Olry, A.; Duval, T.; Hehn, A.; Froelicher, Y.; Bourgaud, F. Coumarin and furocoumarin quantitation in *Citrus* peel via ultraperformance liquid chromatography coupled with mass spectrometry (UPLC-MS). *J. Agric. Food Chem.* 2013, 61, 10677–10684. [CrossRef] [PubMed]  
15. Dugo, P.; Mondello, L.; Dugo, L.; Stancanelli, R.; Dugo, G. LC-MS for the identification of oxygen heterocyclic compounds in *Citrus* essential oils. *J. Pharm. Biomed. Anal.* 2000, 24, 147–154. [CrossRef]  
16. 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]  
17. Wu, G.A.; Terao, J.; Ibanez, V.; Llopis-García, A.; Pérez-Román, E.; Borredá, C.; Domingo, C.; Tadeo, F.R.; Carbonell-Caballero, J.; Alonso, R.; et al. Genomics of the origin and evolution of *Citrus*. *Nature* 2018, 554, 311–316. [CrossRef]  
18. Bruni, R.; Barreca, D.; Protti, M.; Brighenti, V.; Righetti, L.; Anceschi, L.; Mercolini, L.; Benvenuti, S.; Gattuso, G.; Pellati, F. Botanical sources, chemistry, analysis, and biological activity of furanocoumarins of pharmaceutical interest. *Molecules* 2019, 24, 2163. [CrossRef]  
19. Lawrence, B.M. Progress in Essential Oils. *Perfum. Flavorist* 2004, 29, 44–59.
21. Dugo, P.; Mondello, L.; Sebastiani, E.; Ottanà, R.; Errante, G.; Dugo, G. Identification of minor oxygen heterocyclic compounds of Citrus essential oils by liquid chromatography-atmospheric pressure chemical ionisation mass spectrometry. J. Liq. Chromatogr. Relat. Technol. 1999, 22, 2991–3005. [CrossRef]

22. Verzera, A.; la Rosa, G.; Zappala, M.; Cotroneo, A. Essential oil composition of different cultivars of bergamot grown in Sicily. Ital J. Food Sci. 1999, 12, 493–501.

23. Bonaccorsi, I.; Sciarrone, D.; Cotroneo, A.; Mondello, L.; Dugo, P.; Dugo, G. Enantiomeric distribution of key volatile components of Citrus essential oils. Rev. Bras. Farmacogn. (Brasil J. Pharmacogn.) 2011, 21, 841–849. [CrossRef]

24. Russo, M.; Torre, G.; Carnovale, C.; Bonaccorsi, I.; Mondello, L.; Dugo, P. A New HPLC method developed for the analysis of oxygen heterocyclic compounds in Citrus essential oils. J. Essent. Oil Res. 2012, 24, 119–129. [CrossRef]

25. Dugo, P.; Mondello, L.; Proteggente, A.R.; Cavazza, A.; Dugo, G. Oxygen heterocyclic compounds of bergamot essential oils. Rivista Italiana EPPOS 1999, 27, 31–41.

26. Kawaii, S.; Tomono, Y.; Katase, E.; Ogawa, K.; Yano, M. Isolation of furocoumarins from bergamot fruits as HL-60 differentiation-inducing compounds. J. Agric. Food Chem 1999, 47, 4073–4078. [CrossRef] [PubMed]

27. Mangiola, C.; Postorino, E.; Gionfriddo, F.; Catalfamo, M.; Manganaro, R. Evaluation of the genuineness of cold-pressed bergamot oil. Perfum. Flav. 2009, 34, 26–32.

28. Costa, R.; Dugo, P.; Navarra, M.; Raymo, V.; Dugo, G.; Mondello, L. Study on the chemical composition variability of some processed bergamot (Citrus bergamia) essential oils. Flav. Fragr. J. 2010, 25, 4–12. [CrossRef]

29. Menichini, F.; Tundis, R.; Loizzo, M.R.; Bonesi, M.; Provenzano, E.; Cindio, B.D.; Menichini, F. In vitro photo-induced cytotoxic activity of Citrus bergamia and C. medica L. cv. diamante peel essential oils and identified active coumarins. Pharm. Biol. 2010, 48, 1059–1065. [CrossRef]

30. Dugo, G.; Bonaccorsi, I.; Sciarrone, D.; Schipilliti, L.; Russo, M.; Cotroneo, A.; Dugo, P.; Mondello, L.; Raymo, V. Characterization of cold-pressed and processed bergamot oils by using GC-FID, GC-MS, GC-C-IRMS, Enantio-GC, MDGC, HPLC and HPLC-MS-IT-TOF. J. Essent. Oil Res. 2012, 24, 93–117. [CrossRef]

31. USP <1225> Validation of Compendial Procedures. 2021. Available online: https://latam-edu.usp.org/wp-content/uploads/2021/08/1225.pdf (accessed on 18 September 2022).

32. ICH Expert Working Group, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use Ich Harmonised Tripartite Guideline Validation of Analytical Procedures: Text and Methodology Q2(R1). 2005. Available online: https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf (accessed on 18 September 2022).