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Markus Jungen, Nenad Dragičević, Miriam Rodriguez-Werner, Simone Schmidt, Katy Dinis, et al. A pragmatic authenticity assessment of lemon (*Citrus limon* [L.] Burm.f.) juices by its profile of coumarins, psoralens, and polymethoxyflavones. *Food Control*, 2022, 146, pp.109529. 10.1016/j.foodcont.2022.109529 . hal-03904326

HAL Id: hal-03904326

<https://hal.science/hal-03904326v1>

Submitted on 16 Jul 2024

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A pragmatic authenticity assessment of lemon (*Citrus limon* [L.] Burm. f.) juices by its profile of coumarins, psoralens and polymethoxyflavones

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Abstract

Coumarins, psoralens and polymethoxyflavones have long been described as authenticity markers in lemon (*Citrus limon* [L.] Burm.f.) and lime (*Citrus × aurantifolia* [Christm.] Swingle; *Citrus × latifolia* [Yu.Tanaka]) juices. However, the lack of quantitative concentration limits for these markers hampers the differentiation of natural variability and acceptable technical process variations from unacceptable lacks in good manufacturing practice (GMP) or even deliberate food fraud. In this study, a total of 139 lemon and lime samples, including fruits, juices, juice concentrates and peel oils from eleven countries and covering all usual processing methods on the market, were analysed using three different liquid chromatographic methods of three different labs. First, we confirmed the considered analytical approaches to yield comparable results and analysed the results in detail per product type and processing technology. After carefully eliminating samples prone to authenticity doubts, we used the remaining dataset (125 samples) for establishing maximum concentration limits for coumarins, psoralenes and polymethoxyflavones for the differentiation of authentic lemon juices produced under GMP conditions, i.e., compliant with the AIJN Code of Practice, from adulterated products made with unacceptable procedures such as excessive co-extraction of flavedo or the admixture of foreign *Citrus* species, particularly lime.

Keywords: *Citrus*; food fraud; chromatography; polyphenols

Chemical compounds studied in this article: Herniarin (PubChem CID: 10741), Limettin (PubChem CID: 2775), Isopimpinellin (PubChem CID: 69079), Bergapten (PubChem CID: 2355), Nobiletin (PubChem CID: 72344), Tangeretin (PubChem CID: 68077), Sinensetin (PubChem CID: 145659)

Abbreviations: AIJN, European Fruit Juice Association; AIJN CoP, AIJN Code of Practice; EFSA, European Food Safety Authority; ESI, electrospray ionization; FAO, Food and Agriculture Organization of the United Nations; GMP, Good Manufacturing Practice; HESI, heated electrospray ionization; IFU, International Fruit and Vegetable Juice Association; LoD, limit of detection; LoQ, limit of quantification; *mt*, metric tons; PC, principal component; PCA, principal component analysis; PDA, diode-array detector; SGF, SGF International e.V.; SIM, single ion monitoring; THF, tetrahydrofuran; UHPLC, ultra-high performance liquid chromatography; UV, ultraviolet; v/v, volume by volume

1. Introduction

Lemon juices and concentrates are economically important and growing commodities in the food and beverage industry, but especially in the fruit juice industry. In addition to the use of lemon juice or lemon juice from concentrate as consumer goods, they are often added to naturally acidify fruit nectars and soft drinks, allowing “clean labelling” by replacing acidifying additives. In 2019, the main exporting countries for lemon juice were Brazil, the USA, Mexico, Italy, and Peru (listed in descending order of their market share), while the largest producers of lemon juice concentrate were Argentina, Peru, South Africa, Mexico, and Egypt (listed as above). According to FAO data, exports have increased by about 45% for lemon juice concentrate and by about 28% for lemon juice since 2010 (Food and Agriculture Organization of the United Nations).

Rising demand as well as difficult harvest and processing situations have created incentives for potential fraud and, thereby, have boosted the importance of an accurate authenticity assessment of lemon juice. Within the framework of routine controls of the voluntary control system of the juice industry as conducted by SGF (SGF International e.V.), adulterations such as the addition of sugar and (citric) acid, but also the admixture of foreign species such as lime (*Citrus × aurantifolia* [Christm.] Swingle and *Citrus × latifolia* [Yu.Tanaka] Tanaka) were increasingly detected in lemon juice concentrates from 2014 onwards compared to previous years. For revealing sugar and acid additions, stable isotope analysis is the analytical method of choice (Jahromi et al., 2015; Jamin et al., 2005; Rinke, 2016), but for foreign fruit additions in *Citrus* juices, the analyst is dependent on the use of chromatographic methods. The flavanone glycosides hesperidin, naringin, neohesperidin, eriocitrin and narirutin can be used to detect possible additions of certain foreign species in *Citrus* juices, as shown by Cautela et al. (2008). However, this approach fails to detect the addition of lime to lemon juice. Therefore, the fingerprint of polymethoxylated flavones as measured by HPLC-DAD has played a prominent role in this regard since the 1990s (Hofsommer, 1999; Ooghe, 2001). Initially used mainly to assess the authenticity of orange juices, it soon became clear that other *Citrus* species-specific fingerprint patterns might allow detecting further adulterations. When looking at lemons and limes, the focus has been set on coumarins and psoralens in addition to polymethoxylated flavones. The search for suitable marker parameters for the addition of lime or other *Citrus* species to lemon juice has been occupying the scientific community

for some time. While McHale and Sheridan (1989) examined peel oils of key limes, persian limes, bergamot, grapefruit, bitter orange, sweet orange, mandarins, and tangerines, P. Dugo et al. (2009) and P. Dugo and Russo (2011) directly compared lime and lemon oils, suggesting the coumarin herniarin as well as the psoralens bergapten and isopimpinellin to be characteristic markers for lime. Costa et al. (2014) reported the composition of flavonoid glycosides as well as coumarins and psoralens in Italian lime juice. Lehnert and Ara (2014) established herniarin (referred in their study as 7-methoxycoumarin) as particular lime marker in lemon juices, later being supplemented by the aforementioned psoralens bergapten and isopimpinellin (Lehnert et al., 2017). Jungen et al. (2021) confirmed the usefulness of these previously discussed lime markers bergapten and isopimpinellin, proposing the additional consideration of a further psoralen tentatively assigned to 5-geranyloxy-8-methoxypsoralen. By using modified extraction and chromatographic separation techniques, Li et al. (2021) detected additional coumarins and methoxylated flavones in lemon and lime juice previously reported only in other *Citrus* juices. For instance, sinensetin and heptamethoxyflavone hitherto described in mandarin, orange, grapefruit, and pomelo (except sinensetin) were identified in lemon, but not in lime. The studies of Lehnert and Ara (2014), Dugrand-Judek et al. (2015) and Lehnert et al. (2017) observed elevated levels of total coumarins and psoralens in peel oil and the outer parts of the fruits compared to the juice from the endocarp. By this, apart from fraudulent addition of foreign *Citrus* species, the applied processing technique and its intensity could be traced as well. For instance, prohibited processing methods for lemon juice production according to the European Fruit Juice Directive (European Council, 2012) such as whole-fruit-processing could be detected. Here, Jungen et al. (2021) suggested to combine the targeted analysis of bergapten, isopimpinellin and the tentatively assigned 5-geranyloxy-8-methoxypsoralen together with the phenolic compound phlorin as albedo-marker for assessing potential over-extractions as well as for studying the influence of applied extraction technologies.

Despite the above-mentioned marker parameters from literature, the authentication of market samples is still challenging and final decisions whether a sample is to be claimed or not are often made based on single laboratory-specific (non-public) databases. In the younger past, this situation led to uncertainties in the *Citrus* processing industry, particularly in cases when two laboratories delivered different interpretations, while the measured parameters in both laboratories bore practically the same

results. The situation is aggravated by the fact that the respective reference guideline for lemon juices of the AIJN Code of Practice (CoP) does not have any quantitative ranges describing the contents of the analysed coumarins, psoralens and polymethoxyflavones that are commonly found in lemon juices (AIJN European Fruit Juice Association, 2019).

Fruit juices and fruit juice concentrates contain variable amounts of volatile oils, heavily depending on the extraction technique applied. Based on standing European regulation, it is allowed to restore once removed flavour including volatile peel oil constituents from a juice, potentially lost during processing. However, according to the reference guideline for lemon juice/juice concentrate from the AIJN CoP, a product may not contain more than 0.5 mL/L of volatile oils (AIJN European Fruit Juice Association, 2019). Since the highest amounts of coumarins, psoralens and polymethoxyflavones occur especially in flavedo and albedo of lemon fruits, it can be assumed that the contents of coumarins, psoralens and polymethoxyflavones in lemon juices are to a great extent influenced by the peel oil content of the juice, as noted already by Jungen et al. (2021). For these reasons, we conducted a study aiming at a jointly approved evaluation practice of lemon juices and juice concentrates, in which a total of 138 samples (fruits manually squeezed, industrially processed juices and juice concentrates, and peel oils) were analysed in three independent and accredited laboratories. The study was conducted covering products from harvest seasons 2019, 2020 and 2021. The dataset then should be adjusted by removing potentially non-authentic samples according to current practice and then, should be used to propose an approach for deriving maximum concentration limits for coumarins, psoralens and polymethoxyflavones that would not be exceeded in juices made in compliance with the AIJN CoP. The approach was designed to support the future differentiation of acceptable natural compositional variations and acceptable process-related variations in lemon juices from variations caused by illegitimate processing methods, unacceptable lacks of GMP and even deliberate food fraud.

2. Materials and methods

2.1. Chemicals

Our interlaboratory trial included that the required standards and solvents were purchased from different suppliers by the participating laboratories as follows.

Chelab purchased the standards for 6-methylcoumarin ($\geq 99\%$), herniarin ($\geq 98\%$), bergapten ($\geq 99\%$), limettin ($\geq 98\%$), nobiletin ($\geq 98\%$), and tangeretin ($\geq 98\%$) from Sigma Aldrich (Taufkirchen, Germany), isopimpinellin ($\geq 95\%$) from Phytolab (Vestenbergsgreuth, Germany) and sinensetin ($\geq 98\%$) from Cayman Chemical (Ann Arbor, Michigan, USA). Toluol and methanol were from VWR (Hannover, Germany), acetonitrile from Carl Roth (Karlsruhe, Germany) and tetrahydrofuran (THF) was purchased from Thermo Fisher (Kandel, Germany).

Eurofins applied the standards for bergapten ($\geq 99\%$), herniarin ($\geq 98\%$), and limettin ($\geq 98\%$) from Sigma Aldrich (St. Quentin Fallavier, France), and isopimpinellin ($\geq 99\%$), nobiletin ($\geq 99\%$), sinensetin ($\geq 99\%$), and tangeretin ($\geq 95\%$) from Extrasynthèse (Genay, France). Methanol, formic acid, and water were all LC-MS grade and were purchased from Thermo Fisher (Les Ulis, France).

GfL purchased standards for herniarin ($\geq 98\%$) and limettin ($\geq 98\%$) from Thermo Fisher (Kandel, Germany), phellopterin ($\geq 95\%$) (internal standard), nobiletin ($\geq 98\%$) and sinensetin ($\geq 90\%$) from PhytoLab (Vestenbergsgreuth, Germany) and bergapten ($\geq 99\%$), isopimpinellin ($\geq 95\%$), and tangeretin ($\geq 98\%$) from Sigma Aldrich (Taufkirchen, Germany). Acetonitrile, methanol, and toluol were from Chemsolute (Renningen, Germany) and THF from Merck (Darmstadt, Germany).

All solvents used were at least of analytical or HPLC grade. De-ionized water was used unless stated otherwise.

2.2. Samples

Sample collection aimed at covering a representative market picture, comprising all commonly used extraction technologies, dominant cultivars, cloudy and clear products. Thus, lemon and lime samples from eleven different countries were sampled during routine inspections and sample requests of the SGF as shown in Table 1, comprising a total of 20 lemon fruit samples, 42 lemon juices, 45 lemon juice concentrates and 26 cold-pressed lemon oils as well as 5 cold-pressed lime oils and 1 lime juice. Typically used lemon varieties of Europe (ICI Business (on behalf of CBI), 2020; Klimek-Szczykutowicz, Szopa, & Ekiert, 2020) were: Femminello Siracusa, Femminello Trapani, Eureka, Fino, Primofiore and Verna. Samples from early, middle, and late harvested varieties Primofiore and Verna were additionally obtained. A total of 41 of the samples were sampled during presence audits in step controls at different

points of the extraction process (19 lemon fruit samples from fruit grading, 15 lemon juices after finisher/centrifuge and 7 lemon juice concentrates after the evaporator or from concentrate blending tank) by independent and SGF-trained and -accredited auditors, guaranteeing the authenticity of the samples. Table 1 further provides an overview on the geographical origin and the dominant extraction technology documented during sampling. Noteworthy, “squeezer-type” (e.g., JBT/FMC extractors) and “reamer-type extractors” (e.g., Brown extractors) dominate globally, while “rotary press extractors” (e.g., Flli. Indelicato) are mainly used in Italy and Turkey. Samples either were aseptically packed into sterile bags for shipping and subsequent storage at room temperature or were non-aseptically packed prior to shipping and storing at -18 °C until further analyses, except for peel oils (stored at 5 °C) and lemon fruit (stored at room temperature). Shipment to the three laboratories was made in cooled and padded styrofoam boxes by overnight express delivery.

2.3. Determination of coumarins, psoralens and polymethoxyflavones

2.3.1. HPLC-DAD method A (Chelab, laboratory A)

Fresh fruit were manually extracted into fresh juice using a *Citrus* press prior to analyses. Juice sample processing and HPLC-DAD settings were generally based on a method of Pupin et al. (1998). A gradient elution was implemented to improve HPLC separation of coumarins and psoralens.

Briefly, an aliquot of 10 mL of the respective juice sample was transferred to centrifuge tubes. Then, internal standard (20 µL of 6-methylcoumarin ISTD solution (conc. 5,000 mg/L in MeOH) for lime products and 10 µL of 6-methylcoumarin ISTD solution (conc. 500 mg/L in MeOH) for lemon products) and 5 mL of toluene were added. The tubes were subsequently sealed, shaken manually for 20 s, and centrifuged (10 min, 25 °C, 8,500 × g). The supernatant organic phase was carefully removed at room temperature and collected in a conical flask. The aqueous phase was extracted two more times with 5 mL of toluene each as described. The combined organic phases were concentrated to dryness under reduced pressure at 40 °C. Then, the dried extracts were re-dissolved in 0.5 mL methanol. After centrifugation (5 min, 25 °C, 16,200 × g), the solution obtained was used for HPLC analysis.

Lime and lemon oil samples were diluted with methanol (0.10 to 0.20 g sample in 5 mL), combined with internal standard as described above, and centrifuged (10 min, 25 °C, 16,200 × g) prior to transferral to vials for HPLC analysis.

Quantitative analyses were performed by HPLC-DAD (1260 Infinity II LC system, Agilent Technologies, Santa Clara, USA) equipped with a reversed phase C18 column (Reprosil 100 C18, 250 × 4.6 mm, particle size 5 µm) with a C18 precolumn of the same material (Dr. Maisch, Ammerbuch-Entringen, Germany). Using the ternary eluent A (water/acetonitrile/THF: 88/8/4, [v/v/v]) and the binary eluent B (acetonitrile/THF: 96/4, [v/v]), the gradient program was as follow: isocratic at 30% B (30 min), 30 to 80% B (10 min), 80 to 30% B (5 min), isocratic at 30% B (10 min). Flow rate was 0.7 mL/min, total run time was 55 min and injection volume 20 µL. Column oven temperature was set at 30 °C. UV/Vis spectra were recorded from 190 to 400 nm with a detection wavelength for sinensetin, limettin, nobiletin, tangeretin at 330 nm, while herniarin, isopimpinellin, bergapten were recorded at 320 nm. Compound identification was based on the comparison of retention times and UV/Vis spectra of the target compounds with those of authentic reference standards. External calibration curves of herniarin, sinensetin, limettin, isopimpinellin, bergapten, nobiletin and tangeretin ranging from 0.03 to 45 mg/L were used for quantitation. The internal standard 6-methylcoumarin was merely used for quality control and monitoring of diverging retention times. The limit of detection (LoD) and limit of quantification (LoQ) were determined by injecting a series of reference standards in the range of 0.01 mg/L to 45 mg/L with 14 concentrations levels, a confidence interval ($\alpha=0.05$) of 95% and tolerated error at limit of determination ($k=3$) of 33.33%. Additionally, the linearity graph was plotted (concentration vs. peak area response) using Mandel's test ($P = 99\%$) to check for eventual non-linearity. Based on the sample concentrations, the LoD and LoQ were calculated. The dilution factor of juices was 0.05 and 5 for oils.

2.3.2. HPLC-DAD method B (GfL, laboratory B)

Lemons were extracted with a heavy cast iron lever *Citrus* press to obtain the juice. Concentrates were pre-diluted with double-distilled water in a ratio of 1:5 (w/w), juices were used directly. An aliquot of 5 mL of juice or diluted concentrate was pipetted into a 15 mL centrifuge tube and combined with 0.05 mL of phellopterin solution (100 mg/L) as internal standard and 5 ml of toluene prior to thorough

mixing for 10 s on a vortex-mixer (top speed, Model Heidolph Reax top, Heidolph Instruments, Schwabach, Germany). After phase separation, which could optionally be facilitated by centrifugation, the upper organic phase was transferred to a conical flask. Toluene was removed on a rotary evaporator (40 °C) and any solvent residues were removed under a nitrogen stream. The dried extract was redissolved in 0.5 mL of methanol and filtered (membrane filter, 0,2 µm / 0,45 µm, PTFE, Macherey-Nagel) into an HPLC vial prior to HPLC analysis. *Citrus* oils were diluted 1:10 (v/v) with methanol prior to membrane-filtration into vials for HPLC measurement.

Analysis was performed by HPLC-DAD (1100 series HPLC system, Agilent Technologies), using a reversed phase C18 column (Hypersil ODS, 250 × 4.6 mm, particle size 3 µm, VDS optilab, Berlin, Germany) operated at 50 °C. Eluent A (water) and eluent B (binary mixture of acetonitrile and THF [60:40, v/v]) were used for the gradient program as follows: 0 to 15 min from 75 to 65% A, 15 to 20 min from 65 to 40% A, 20 to 22 min from 40 to 20% A, 22 to 28 min isocratic at 20% A, 28 to 28.1 minutes from 20 to 75% A and 28.1 to 35 minutes isocratic at 75% A. Flow rate was 0.8 mL/min, total run time 35 min, and the injection volume 20 µL. Detection wavelength was 330 nm. UV/Vis spectra were recorded in the range of 200-450 nm. Compound identification was based on the comparison of retention times and UV/Vis spectra of the target compounds with those of authentic references described above. The contents of the individual substances were determined based on external calibrations (linear range 0 to 0.85 mg/L) with the above-mentioned reference compounds, as calculated according to the generally applicable procedure of the internal standard method. Limits of detection (LoD) and quantification (LoQ) under repeatability conditions were determined based on DIN 32645 (DIN-Normenausschuss Materialprüfung (NMP), 2008) .

2.3.3. UHPLC-MSⁿ (Eurofins, laboratory C)

Lemon juices were extracted from the fresh fruit samples using an electric household-type *Citrus* squeezer. Juice concentrates were rediluted with de-ionized water to single juice strength level (8 °Brix). Aliquots of 5 mL of the samples were centrifuged for 5 min at 3,300 × g. The supernatants of juices and rediluted juice concentrates were collected and an aliquot of 250 µL of the supernatant was combined with 750 µL de-ionised water in an HPLC vial before analysis. Aliquots of 100 µL of the supernatant

of centrifuged peel oils were added to a graduated flask of 100 mL and made up with methanol. Subsequently, aliquots of 250 μ L of the diluted methanolic oil were combined with 750 μ L de-ionised water directly in a HPLC vial for analysis.

Analyses were performed on a ThermoFisher Vanquish Flex UHPLC system, composed of a binary pump, a refrigerated sampler set at 7 °C sample temperature, and a column oven, connected to a ThermoFisher high resolution Orbitrap mass spectrometer QExactive Plus with a heated electrospray ionization source (HESI). The UHPLC separation was achieved using a reversed phase C18 column (C18 Hypersil Gold column, 50 \times 2.1 mm, particle size 1.9 μ m, Thermo Fisher) at a flow rate of 0.4 mL/min. The column temperature was set to 30 °C. The mobile phases were water (A) and methanol (B) both acidified with 0.1% formic acid, with the following gradient elution: 0-5 min, B: isocratic at 35%; 5-8 min, B: 35-70%; 8-8.5 min, B: 70-98%; 8.5-11.5 min: B: isocratic at 98%; 11.5-11.6 min, B: 98-35%; 11.6-15 min, B: isocratic at 35%. The injection volume was 1 μ L.

ESI-MS data were acquired in the positive ion mode operating in SIM (selected ion monitoring) with a resolution of 70,000 at scan ranges of m/z 100-1500. Nitrogen at 50, 15 and 0 arbitrary units served as sheath, auxiliary and sweep gas, respectively. Capillary temperature and voltage were 310 °C and 3.20 kV, respectively. The auxiliary gas heater was set to 350 °C. An inclusion list containing the mass-to-charge ratio (m/z) for all the coumarins, psolarens, and polymethoxyflavones analysed was used to perform MS/MS analysis on these precursor ions. Precursor ions were used for quantification, whereas product ions confirmed the compounds' identities. Calibration curves for the above-mentioned standards were constructed at different concentration levels (5, 10, 50, 100, 250 and 500 μ g/L) for compound quantification. HR-MS calibration was performed in positive and negative ion modes using commercial calibration solutions, directly infused into the HESI source.

2.4. Descriptive statistics, data evaluation and visualisation

The differences in analytical methods of the three involved laboratories were analysed by comparing the respective limits of detection (LoDs) and limits of quantification (LoQs). Because of differing LoQs, the obtained data was left-censored (" $<$ LoQ"). To be able to calculate descriptive statistics from continuous, left-censored quantitative data, values denoted with " $<$ LoQ" were substituted with the

medium bound values, i.e. half the LoD or LoQ (LoQ/2 in case of oils and LoD/2 in case of juices and juice concentrates), as described by European Food Safety Authority (2010) and proposed by the work of Antweiler and Taylor (2008), and George et al. (2021) comparing different approximation calculations and mathematical models to represent left-censored data.

Arithmetic means and standard deviations were calculated as based on measurements performed on individual samples. Descriptive statistics were expressed from arithmetic means per sample. Statistical evaluations and graphical presentations were created using Python 3.5 software (Python Software Foundation) and the NumPy (Harris et al., 2020), pandas (Stéfan van der Walt & Jarrod Millman, 2010; Wes McKinney, 2010), Matplotlib (J. D. Hunter, 2007) and Seaborn libraries (Waskom, 2021). For principal component analysis, the measured values were also normalised in Python, the procedure used corresponded to the calculation of a *z*-value by removing the mean and scaling to unit variance with the StandardScaler from the class *sklearn.preprocessing* and *sklearn.decomposition* (Pedregosa et al., 2011). The non-parametrical Kruskal-Wallis Test was used for comparing means of non-normally distributed data (Kruskal & Wallis, 1952).

3. Results and discussion

3.1. Comparability of different analytical approaches

Among our 138 samples (see table 2) available, a total of 65 samples (10 manually squeezed lemons, 7 lemon juices, 17 lemon juice concentrates, 26 lemon peel oils and 5 lime peel oils) were analysed by all three laboratories with their respective methods of analysis as described above, while the residual 73 samples were analysed by only two laboratories. Specifically, 59 of the mentioned latter 73 samples were analysed by labs A and C, the residual 14 by labs A and B.

An evaluation of the 65 samples (manually squeezed lemons, lemon juices, juice concentrates, peel oils and lime peel oils) measured by all three different analytical methods was conducted to study the comparability of the methods, using the non-parametric Kruskal-Wallis test. The detailed results may be found in the Supporting Information (S1, S2, and S3). The comparability of the analytical methods considered was generally limited by different LoDs and LoQs of the previously described methods, requiring an adapted approach of handling the resulting “left-censored” data. Nevertheless, the results

of the analyses fully suffice for the purposes of this work, as illustrated by the example chromatograms of a typical lemon juice and a typical lime juice measured by the three methodologies in Figure 1: Limettin [1] (lemon: 1.34 ± 0.60 mg/L, lime: 11.92 ± 2.47 mg/L) was quantifiable in both samples, while herniarin [3] (lemon: <LoD, lime: 3.99 ± 1.34 mg/L), bergapten [2] (lemon: <LoD, lime: 4.74 ± 1.27 mg/L) and isopimpinellin [4] (lemon: <LoD, lime: 2.89 ± 0.87 mg/L) could only be quantified in the lime juice, while in the lemon juice they were all below the LoD of all methods.

3.2. Coumarins, psoralens, and polymethoxyflavones in lemon products

Juiced products

When considering products to be obtained from the endocarp (juices), we used a multivariate approach by means of a principal component analysis (PCA, Figure 2) to consider the hand-pressed (dark grey circles), industrially processed juices (light grey circles) and juice concentrates (black circles). Based on our analysis of results for coumarins, psoralenes and polymethoxyflavones, 87.54% of the variance was explained by the first two principal components (PCs), with the first PC already explaining 47.48% of the variance. In the scores plot, a clear cluster in the coordinates' origin was distinguished from six juice concentrates, which were marked as atypical (red triangles, Figure 2). Searching for an explanation of these findings, the loadings were considered (Figure 2). In fact, the 5 samples separated in positive direction along PC2 were characterised by higher contents of the polymethoxyflavones sinensetin, nobiletin and tangeretin compared to the other samples, which on average contained only barely detectable levels (cf. also Table 2). The question was now how such higher contents might be explained. Pupin et al. (1998) described 1.84 ± 0.55 mg/L of sinensetin in Brazilian orange juice concentrates, and Russo et al. (2021) reported the three aforementioned polymethoxyflavones to occur in oranges (sinensetin: 0.06 to 0.10 g/kg, tangeretin: 0.42 to 0.92 g/kg, nobiletin: 0.39 to 0.81 g/kg) and mandarins (sinensetin: up to 0.15 g/kg, tangeretin: 1.23 to 5.96 g/kg, nobiletin: 0.66 to 2.19 g/kg) as well as in bergamot (only sinensetin: 0.07 to 0.10 g/kg) and grapefruit (only nobiletin: 0.08 to 0.46 g/kg). According to our results and the mentioned literature, atypically increased levels of these compounds in lemon juices might thus point out the presence of *Citrus* species other than lemon. Noteworthy, nobiletin, sinensetin and tangeretin were qualitatively

detected by Li et al. (2021) in hand-pressed lemon juices of Chinese provenance. Here, however, so-called Volkamer lemons (*Citrus volkameriana*) were also included, and these are natural hybrids of the citron (*Citrus medica* [L.]) and mandarin (*Citrus reticulata* BLANCO), thus reasonably explaining the occurrence of nobiletin, sinensetin and tangeretin. Volkamer lemons must be botanically distinguished from lemons (*Citrus limon* [L.] Burm. f.). Xi et al. (2017) reported nobiletin, sinensetin and tangeretin in peels from 5 different Chinese lemon cultivars but according to the examined cultivars only 3 of them can be considered as *Citrus limon* [L.] Burm. f. (Feiminailao, Pangdelusaningmeng and Beijingningmeng). The other cultivars, however, correspond to rough lemon (Cuningmeng, *Citrus × jambhiri* Lush.) or Rangpur lime (Limeng, *Citrus × limonia* Osbeck).

As a consequence of the above evaluation, we discarded these 5 atypical samples from statistical evaluation of our results, assuming a significant co-processing or carryover issue in their production. Similarly, an additional sample separated in positive direction of PC1 showed higher contents of the psoralens bergapten and isopimpinellin and the coumarin herniarin (Figure 2, red triangle in lower right corner). Lehnert et al. (2017), Russo et al. (2021) and Jungen et al. (2021) identified the psoralens bergapten and isopimpinellin as lime markers, while herniarin contents were described especially as parameter showing an intensive processing of lemons to juice and juice concentrate (Jungen et al., 2021). The sample was discarded as well from further statistical processing.

Upon comparing analytical results of all juices and concentrates remaining after elimination of outliers by PCA with values reported in literature, we found good agreement regarding levels of coumarins, psoralenes and polymethoxyflavones. The levels of the coumarin limettin were 1.04 ± 0.83 mg/L in manually squeezed juices, 0.90 ± 0.66 mg/L in industrially processed juices and 0.84 ± 0.57 mg/L in juice concentrates, representing only insignificant differences. The psoralen bergapten was detected just above the LoQ of 0.02 mg/L with 0.02 ± 0.05 mg/L in only 8 of 42 of the industrially produced juices. These findings on limettin and bergapten confirm an earlier report of Jungen et al. (2021), whereafter limettin had been found not only in flavedo, but in small amounts also in the endocarp and thus also in lemon juices, while bergapten had not been found in the endocarp and therefore not in the juices. Bergapten had been present only in whole-processed and harshly tincture-pressed lemon juices, being absent in hand-pressed or industrially-pressed juices from 100%

lemons, excluding any carry-over, over-extraction or processing of other *Citrus* species due to the nature of the controlled academic experiment (Jungen et al., 2021). In our hand-pressed juices and the juice concentrates, the content of bergapten was on average below the LoQ of 0.02 mg/L and 0.15 mg/L, respectively, but 1 out of 20 manually squeezed lemon juices and 3 out of 45 concentrate samples exhibited quantifiable amounts of this compound. Since the occurrence of bergapten in the flavedo of lemons is well accepted, we assume that the eventual appearance of low amounts of bergapten does neither indicate a lack of manufacturing practices nor an admixture of other *Citrus* species, but rather an entry via the lemons' flavedo. Whether this is to be judged as overprocessing, might be debated in the future.

Herniarin, a previously discussed lime marker (Hofsommer, 1999; Lehnert & Ara, 2014, 2014), was present in quantifiable amounts only in manually squeezed and industrially processed lemon juices with 0.02 ± 0.05 mg/L and 0.03 ± 0.09 mg/L (LoQ = 0.02 mg/L juice), respectively. In lemon juice concentrates, the contents were below the LoQ of 0.15 mg/L (re-constituted single strength juice) on a mean level, and only 3 out of 45 samples contained quantifiable amounts. Besides the fact of the noticeably different LoQs, another possible explanation for these observations is the frequent practice in the industry of blending and fining lemon juice concentrates to meet product specification requirements in terms of citric acid content and turbidity. Such products are clearly different from manually squeezed juices on a laboratory scale or lemon juice “off the *Citrus* press”.

In agreement with Russo et al. (2021), concentrations of the polymethoxyflavones nobiletin, sinensetin and tangeretin were below the LoQ of 0.02 mg/L in juices (hand-pressed and industrially processed) and of 0.15 mg/L in juice concentrates.

Lemon peel oils

Lemon peel oils were considered in this study to provide representative insights into the coumarin, psoralen and polymethoxyflavone levels encountered in peel oils, where they are to be expected to occur in a most concentrated form. Considering already established maximum levels of peel oil allowed to be present in *Citrus* juices (AIJN Code of Practice: 0.5 mL/L) would then allow an estimation of the levels of coumarins, psoralens and polymethoxyflavones to be maximally

expected by (accepted) peel oil intake. For this purpose, we also used a multivariate approach with a PCA to identify deviant peel oil samples and exclude them from the statistical evaluation. Figure 3 shows the scores and loadings plots of the PCA calculated on the levels of the coumarins, psoralens and polymethoxyflavones examined in our lemon peel oils sample set (Tables 1 and S1-2). The first two PCs explained 83.54% of the variance with 54.29% on PC1 and 29.25% on PC2. As with the products produced from the endocarp, atypical products were identified from the score plot. Most peel oils were almost on a straight hypothetical line (black circles, descending from the lower edge of the upper left quadrant to the lower right quadrant), 6 peel oils (red triangles) were off this hypothetical line. In the loadings plot, the different classes of coumarins and psoralens (dark and light green boxes) as well as polymethoxyflavones (orange boxes) were grouped together, except for sinensetin which was not quantifiable ($< \text{LoQ}$ of 5 mg/L) in all analysed lemon peel oils and thus allocated in the coordinate origin of the loadings plot, in agreement with earlier reports of P. Dugo et al. (2009), P. Dugo and Russo (2011), and Russo et al. (2021). The polymethoxyflavones nobiletin and tangeretin were present in quantifiable amounts in 4 (nobiletin) and 2 (tangeretin) out of 26 peel oils (average below LoQ of 5 mg/L, Table 2), exerting high loadings in the positive PC2 direction. In contrast, we found coumarins and psoralens in all studied peel oils at variable levels, i.e., bergapten (5.69 to 145.22 mg/L; avg. 11.18 mg/L, Table 2), isopimpinellin (5.10 to 33.04 mg/L, avg.: 7.02 mg/L), limettin (542.12 to 1325.08 mg/L, avg.: 857.10 mg/L), and herniarin ($< \text{LoQ}$ to 59.28 mg/L, avg. 9.8 mg/L), all having negative loadings on PC2 and strongly positive loadings on PC1. We particularly highlight the presence of isopimpinellin, a compound often thought to be lime-specific and not being detectable when prevailing in an “unconcentrated” form such as in lemon juices or fruit parts including flavedo (Jungen et al., 2021). We now sought to define whether a lemon peel oil would be atypical. Two samples (red triangles in upper right quadrant) showed distinct contents of nobiletin (9.60 and 10.65 mg/L) and tangeretin (8.11 and 6.94 mg/L) greater than those spanned by all other samples (nobiletin: 22 of 26 samples $< \text{LoQ}$ of 5.00 mg/L, 2 samples between 7.15 to 9.60 mg/L; tangeretin: 24 of 26 samples $< \text{LoQ}$ of 5.00 mg/L), pointing to the potential presence of other *Citrus* species like grapefruit, bitter orange, orange, and mandarin, which were reported to contain these compounds in higher concentrations by Russo et al. (2021). Three samples showed

simultaneously increased contents of limettin (1199.84, 1201.99 and 1325.08 mg/L), herniarin (24.66, 40.99 and 59.28 mg/L), bergapten (36.25, 75.35 and 145.22 mg/L), and isopimpinellin (7.13, 13.11 and 33.04 mg/L), pictured as red triangles in lower right quadrant. Compared to the lemon peel oils classified as typical (with contents of limettin of 857.10 ± 176.5 , bergapten of 11.18 ± 9.21 , herniarin of 9.83 ± 7.31 , and isopimpinellin of 7.02 ± 2.15 mg/L), the analytical profile of the 3 aforementioned samples did not allow excluding the absence of lime as described by P. Dugo et al. (2009), P. Dugo and Russo (2011), and Russo et al. (2021). One further sample showed parallelly increased contents of nobiletin (7.80 mg/L) and isopimpinellin (6.23 mg/L) to levels at well above the LoQ (5 mg/L, resp.). Both compounds are commonly not found in quantifiable amounts. These findings raise doubts about the authenticity of the samples, possibly caused by a multiple carryover of orange and/or mandarin (nobiletin) and lime (isopimpinellin). In brief, these 6 samples were not considered for further statistical analysis.

When comparing the composition of juices with peel oils, we highlight that, among the compounds targeted herein, the coumarin limettin was the dominant compound in all juice types and concentrates at 0.05 to 5.42 mg/L (for averages, see Table 2) as well as in the peel oil (542.12 to 1325.08 mg/L), where it was more abundant than in the juice by a factor of about 1,000. The situation was similar for the coumarin herniarin (with a factor of about 600) and the psoralen bergapten (with a factor of 400) as shown in Table 2. The near absence of bergapten and isopimpinellin (Table 2), which have earlier already been discussed as lime markers in adulterated lemon juices (Hofsommer, 1999; Jungen et al., 2021; Lehnert et al., 2017; Lehnert & Ara, 2014), was in agreement with previous results of a study by Jungen et al. (2021). There, both psoralens were not quantifiable in hand-pressed and industrially produced lemon juices, but in dissected flavedo (bergapten) and in whole-processed and tincture-pressed lemons (bergapten and isopimpinellin).

Dugrand et al. (2013) have earlier reported lower contents of bergapten (traces) and isopimpinellin (0.82 ± 0.29 mg/kg) in Eureka lemon peels (flavedo and albedo) than those found in the peel oils of our study (11.18 ± 9.21 and 7.02 ± 2.15 mg/L, resp.). Since the peel oil harbouring the aforementioned compounds originates from the oil cells of the flavedo, it is evident that peel oils contain the aforementioned compounds in more concentrated form than the whole peel or flavedo.

3.3. Comparison of lemon and lime peel oils

For the comparison of the lemon peel oils with lime peel oils adjusted for the atypical samples, scores and loadings plot of the respective PCA (Figure 4) demonstrated a clear differentiation of the lemon oils (black circles) from all lime peel oils (grey triangles), including an atypical lime peel oil (red triangle). Considering the loadings, lime peel oils differed from the lemon peel oils mainly by higher loadings of the coumarins herniarin and limettin as well as the psoralens bergapten and isopimpinellin on both PCs. The deviant apparent lime peel oil was distinguished by the high influence of atypically increased levels of polymethoxyflavones tangeretin, sinensetin and nobiletin (Figure 4), being uncommon also in comparison to earlier results of Russo et al. (2021). For this reason, the presence of orange and/or mandarin material in this sample was suspected. The sample was removed from the sample set for further statistical evaluation. Nevertheless, the compositional variability of lime peel oil (grey triangles) was substantially larger than that in lemon peel oils, despite having had only a few lime peel oil samples ($n=5$). For instance, the high content of limettin in lime peel oil (1582.34 to 4138.86 mg/L, avg. 2179.35 ± 575.29 mg/L) clearly exceeded that found in all lemon peel oil samples (542.12 to 1325.08 mg/L, avg. 857.10 ± 176.35 mg/L). The difference was even more pronounced for herniarin, isopimpinellin and bergapten (Table 2), as expected by studies of Lehnert et al. (2017), Jungen et al. (2021) and Russo et al. (2021).

3.4. Deduction of maximum levels

To date, lemon juice authentication is hampered by a lack of quantitative parameters allowing the differentiation of acceptable biological and processing-related variations from unacceptable lacks in good manufacturing practice (GMP) and deliberate food fraud. The to date open question is beyond which concentration limits for a series of discussed marker compounds a lemon juice would be subject to suspecting an illegitimate lack of GMP or food fraud. In other words, beyond which concentration limits the juices would be considered non-compliant with current European legislation and the AIJN Code of Practice (CoP). Our proposed approach tackles this question by proposing concentration limits for coumarins, psoralens and polymethoxyflavones as based on adding up (i) an “endocarp extraction-based” concentration fraction, representing what is to be maximally expected in lemon juices produced

with still AIJN CoP-compliant good manufacturing practices, with (ii) a plausible certainty fraction, hereafter called the “peel oil-based” fraction, representing what would maximally be brought into the juice by a peel oil input at the maximum allowed peel oil content of 0.5 mL oil per L of juice according to the AIJN CoP. The inclusion of this peel oil-based fraction is of particular importance for assessing juices from concentrates, in which flavour oils potentially rich in peel oil constituents including the marker compounds can be added until reaching 0.5 mL volatile oils per L. The so-derived concentration limits are thought to be unreachable with AIJN CoP-compliant methods, i.e. being unreachable unless extracting unacceptably high amounts of peel oil from the lemon flavedo or unless admixing foreign *Citrus* species. The approach will be described in more detail in the following.

(i) *The “endocarp extraction-based” fraction.* The “endocarp extraction-based” fraction of coumarins, psoralens and polymethoxyflavones refers to the concentrations that would be maximally expected after de-juicing with “high-intake” but AIJN CoP-compliant processing technologies such as reamer-type extractors that had resulted in the highest concentrations in our study (cf. Table 3). For instance, highest limettin concentrations were found in reamer-type made juices from concentrate, while highest bergapten and herniarin concentrations in those of (not-from-concentrate) juices made with reamer-type extractors (Table 3). Consequently, the “endocarp extraction-based” fractions were deduced by adding up the mean concentration of the mentioned processing category where the highest intake of the respective compound had been noted with one corresponding standard deviation. By these means, for instance, the estimated maximum “endocarp extraction-based” fraction of limettin was set at 2.44 mg/L juice (Table 4), derived adding up the mean (1.17 mg/L) and the corresponding standard deviation (1.27 mg/L) as measured in reamer-type made juices from concentrate (Table 3). Following this approach, the “endocarp extraction-based” fraction for bergapten and herniarin was 0.14 mg/L and 0.22 mg/L, respectively (Table 4). The “endocarp extraction-based” fractions for isopimpinellin, nobiletin, sinensetin, and tangeretin, all being unquantifiable in all lemon juices studied, were mathematically set at 0.019 mg/L, right below the LoQ of 0.02 (Table 4).

(ii) *The “peel oil-based” fraction.* This fraction represents a certainty amount to be added later to the “endocarp extraction-based” fraction in order to yield final maximum levels that can only be surpassed when certainly applying manufacturing practices not complying with the AIJN CoP, such as excessive

extraction of non-endocarp parts as well as carry-over and admixture of foreign *Citrus* species. For this purpose, the “peel oil-based” fraction refers to the maximum coumarin, psoralen and polymethoxyflavone concentrations that would be expected upon introducing the today well-accepted maximum content of volatile oils (= peel oil) of the Reference Guideline for Lemon of the AIJN Code of Practice, namely 0.5 mL peel oil per L juice. This would include the addition occurring during reconstitution of concentrates to juices with the respective flavour, which includes peel oil constituents. Noteworthy, surpassing the volatile oil level of 0.5 mL/L is used already today to suspect excessive non-endocarp extraction (AIJN European Fruit Juice Association, 2019). Multiplying this maximum allowed peel oil content (0.5 mL/L) with our maximum found concentrations of the mentioned compounds in lemon peel oils will thus yield an indication about how much would be maximally introduced solely by the aforementioned “peel oil-based” fraction. For instance, the highest level of limettin found in the lemon peel oils studied was 1,096.62 mg/L (Table 4). If 0.5 mL of peel oil with limettin at a concentration of 1096.62 mg/L peel oil (= 1.09662 mg/mL) were present in one litre of juice (Table 4), these 0.5 mL of peel oil would then introduce 0.55 mg of limettin per L of juice ($1096.62 \text{ mg/mL} \times 0.5 \text{ mL} = 0.55 \text{ mg}$). Analogously, bergapten found at maximally 45.75 mg/L peel oil would be introduced by a theoretical maximum “peel oil-based” input of 0.023 mg/L juice, and herniarin and isopimpinellin (max. in peel oil: 26.76 and 12.60 mg/L peel oil) at ca. 0.013 and 0.006 mg/L, respectively. Since nobiletin, sinensetin, and tangeretin were found at levels < LoQ, i.e. <5.00 mg/L, a value of 4.99 was used to derive ($[4.99/1000] \times 0.5 \text{ mg/L}$) a rounded value of 0.002 mg/L (Table 4).

Ultimately, to derive maximum levels not to be exceeded in juices produced in compliance with the AIJN CoP, the maximum “endocarp-based intake” and the maximum “peel oil-based intake” were summed up and rounded up to an accuracy $\pm 0.5 \text{ mg/L}$ (limettin), $\pm 0.05 \text{ mg/L}$ (bergapten, herniarin) and $\pm 0.01 \text{ mg/L}$ (isopimpinellin, nobiletin, sinensetin, tangeretin). The results of this estimation of proposed maximum levels for the mentioned compounds in juices on single strength level are shown in Table 4. According to our own data and the literature on industrially produced lemon juices available to us, none of the previously reported concentrations of these compounds in authentic, AIJN CoP-compliantly produced lemon juices exceed the maximum levels reported herein. For instance, Jungen et al. (2021) reported from 0.3-0.4 mg limettin per L of lemon juices made with squeezer-type industrial

extractors, in agreement with the results of our study (0.67 ± 0.42 mg/L, Table 3). However, the same authors have reported ca. 6.6 ± 1.0 mg limettin per L of whole-processed lemons, righteously exceeding the proposed max. level of 3.00 mg/L. Lime juice, which can be produced from the whole fruit according to the AIJN CoP and European legislation, had a concentration of 180.7 mg limettin per L juice as presented in Jungen et al.'s (2021) study. Such a limettin level would require a dilution by a factor of ca. 60 to fall below the maximum proposed level of 3.00 mg/L or a factor of ca. 27 to fall below the level mentioned above for whole-processed lemons. By these means, also comparably small additions of lime juice to lemon juice might become reasonably traceable and, depending on the found concentration, even distinguishable from overextracting non-endocarp lemon parts. This approach might be particularly worth investigating further when considering more lime-specific parameters such as bergapten, herniarin, and isopimpinellin, which Jungen et al. (2021) found at 59.5, 32.9, and 24.5 mg/L in whole-fruit processed lime juice and, thus, would require dilution factors of ca. 298, 132 and even 817 to fall below the proposed max. levels (0.20, 0.25, and 0.03 mg/L), respectively. Similar considerations could be made with our proposed limits for nobiletin, sinensetin, and tangeretin for detecting the presence of other species like mandarin or orange, being beyond the scope of this paper. Noteworthy, these latter considerations on dilution factors are to be considered preliminary and might require a broader database prior to being established in practice. Nevertheless, they are exemplarily included herein to show the functioning and potential of the whole approach for distinguishing AIJN CoP-compliant lemon juices from juices made under a lack of GMP (e.g., overextracting non-endocarp parts) or the admixture of lime or other species.

4. Conclusions and outlook

First, three different analytical methods were demonstrated to yield comparable results on concentrations of a series of authentication-relevant marker compounds in more than 138 lemon and lime samples. By careful data analyses, we eliminated samples subject due to authenticity doubts. The remaining adjusted database was then used to demonstrate an approach for the derivation of quantitative concentrations limits for selected coumarins, psoralens and polymethoxyflavones that would allow the differentiation of authentic lemon juices produced under GMP conditions, i.e.,

compliant with the AIJN Code of Practice, from adulterated products made with unacceptable procedures such as excessive co-extraction of flavedo or the admixture of foreign *Citrus* species, particularly lime.

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Table 1. Products, predominating *Citrus* extraction technology and geographical origins of analysed lemon (*Citrus limon* [L.] Burm. f.) and lime (*Citrus × aurantifolia* [Christm.] Swingle and *Citrus × latifolia* [Yu.Tanaka] Tanaka) samples.

Origin	Extraction technology	Product	<i>n</i>
Argentina	reamer-type and squeezer-type	lemons	1
		lemon juice	18
		lemon juice concentrate	13
		cold-pressed lemon oil	3
Bolivia	n/a	lemons	1
Brazil, São Paulo	squeezer-type	lemon juice concentrate	8
		lime juice	1
		cold-pressed lemon oil	1
		cold-pressed lime oil	1
China	squeezer-type	lemon juice concentrate	1
Israel	squeezer-type	lemon juice	1
Italy	rotary press extractors	lemons	5
		lemon juice	8
		lemon juice concentrate	6
		cold-pressed lemon oil	3
Mexico	squeezer-type	lemon juice concentrate	1
		cold-pressed lime oil	3
South Africa	reamer-type and squeezer-type	lemons	1
		lemon juice	4
		lemon juice concentrate	4
		cold-pressed lemon oil	6
Spain	squeezer-type	lemons	12
		lemon juice	9
		lemon juice concentrate	10
		cold-pressed lemon oil	13
		cold-pressed lime oil	1
Turkey	rotary press extractors	lemon juice concentrate	1
Uruguay	reamer-type	lemon juice	2
		lemon juice concentrate	1

Table 2. Contents of coumarins, psoralens, and polymethoxylated flavones in lemon (*Citrus limon* [L.] Burm.f.) and in lime (*Citrus × aurantifolia* [Christm.] Swingle and *Citrus × latifolia* [Yu.Tanaka] Tanaka). The whole sample set measured with different analytical techniques (*n*) were reduced by eliminating atypical samples according to PCA analyses (Figures 2 to 4) to yield the adjusted sample set (*n_{adj.}*).

	Lemon				Lime
	Manually squeezed juice	Industrially processed juice	Juice concentrate	Peel oil	Peel oil
<i>n</i>	20	42	45	26	5
discarded	0	0	6	6	1
<i>n_{adj.}</i>	20	42	39	20	4
Limettin [1]	1.04 ± 0.83	0.90 ± 0.66	0.84 ± 0.57	857.1 ± 176.35	2,179.35 ± 575.29
Bergapten [2]	< 0.02	0.02 ± 0.05	< 0.15 ^b	11.18 ± 9.21	900.09 ± 378.56
Herniarin [3]	0.02 ± 0.05	0.03 ± 0.09	< 0.15 ^b	9.83 ± 7.31	2,035.05 ± 1649.55
Isopimpinellin [4]	< 0.02 ^a	< 0.02 ^a	< 0.15 ^b	7.02 ± 2.15	1,103.71 ± 317.3
Nobiletin [5]	< 0.02 ^a	< 0.02 ^a	< 0.15 ^b	< 5.00 ^c	< 5.00 ^c
Sinensetin [6]	< 0.02 ^a	< 0.02 ^a	< 0.15 ^b	< 5.00 ^c	< 5.00 ^c
Tangeretin [7]	< 0.02 ^a	< 0.02 ^a	< 0.15 ^b	< 5.00 ^c	5.06 ± 1.44

Values represent means ± standard deviation of at least two analytical replicates (*n* = 2) per sample. Expressed as mg/L.

^a: LoQ of targeted substances in juice single strength: 0.02 mg/L

^b: LoQ of targeted substances in juice concentrates: 0.15 mg/L

^c: LoQ of targeted substances in peel oils: 5.00 mg/L

Table 3. Contents of coumarins, psoralens, and polymethoxylated flavones in lemon (*Citrus limon* [L.] Burm.f.) in industrially juiced products by processing technology after eliminating atypical samples according to PCA analyses (Figures 2 to 4) to yield the adjusted sample set (*n_{adj.}*).

	Reamer-type		Rotary press		Squeezer-type	
	Juice	Concentrate	Juice	Concentrate	Juice	Concentrate
<i>n_{adj.}</i>	11	4	8	5	23	30
Limettin [1]	1.01 ± 0.83	1.17 ± 1.27	1.4 ± 0.75	0.91 ± 0.29	0.67 ± 0.42	0.79 ± 0.48
Bergapten [2]	0.05 ± 0.09	< 0.15 ^b	< 0.02 ^a	< 0.15 ^b	< 0.02 ^a	< 0.15 ^b
Herniarin [3]	0.07 ± 0.15	< 0.15 ^b	< 0.02 ^a	< 0.15 ^b	0.02 ± 0.05	< 0.15 ^b
Isopimpinellin [4]	< 0.02 ^a	< 0.15 ^b	< 0.02 ^a	< 0.15 ^b	< 0.02 ^a	< 0.15 ^b
Nobiletin [5]	< 0.02 ^a	< 0.15 ^b	< 0.02 ^a	< 0.15 ^b	< 0.02 ^a	< 0.15 ^b
Sinensetin [6]	< 0.02 ^a	< 0.15 ^b	< 0.02 ^a	< 0.15 ^b	< 0.02 ^a	< 0.15 ^b
Tangeretin [7]	< 0.02 ^a	< 0.15 ^b	< 0.02 ^a	< 0.15 ^b	< 0.02 ^a	< 0.15 ^b

Values represent means ± standard deviation of at least two analytical replicates (*n* = 2) per sample. Expressed as mg/L.

^a: LoQ of targeted substances in juice single strength: 0.02 mg/L

^b: LoQ of targeted substances in juice concentrates: 0.15 mg/L

Table 4. Calculation of the proposed maximum levels of coumarins, psoralenes and polymethoxyflavones in lemon (*Citrus limon* [L.] Burm.f.) juices by adding up “endocarp extraction-based” fractions and “peel oil-based” fractions. All values expressed as mg/L.

	Calculated maximum “endocarp extraction-based” fraction ^a	Maximum contents in lemon peel oils ^d	Calculated maximum “peel oil-based” fraction ^b	proposed maximum levels on single strength level ^c
Limettin [1]	2.44	1,096.62	0.548	3.00
Bergapten [2]	0.14	45.75	0.023	0.20
Herniarin [3]	0.22	26.76	0.013	0.25
Isopimpinellin [4]	0.019 ^e	12.60	0.006	0.03
Nobiletin [5]	0.019 ^e	4.99 ^f	0.002	0.02
Sinensetin [6]	0.019 ^e	4.99 ^f	0.002	0.02
Tangeretin [7]	0.019 ^e	4.99 ^f	0.002	0.02

^a: refers to the concentrations maximally expected when using “high-intake” but AIJN CoP-conform processing technologies; calculated by adding up the mean and the standard deviation of the concentrations noted for the processing category in Table 3 that had yielded the highest concentration of the respective compound.

^b: refers to the maximum concentrations that would be expected upon introducing 0.5 mL peel oil with maximum content of the respective compound per L of juice; 0.5 mL/L is the today well-accepted maximum content of volatile oils (= peel oil) of the Reference Guideline for Lemon of the AIJN CoP.

^c: Sum of “endocarp extraction-based” fraction and “peel oil-based” fraction.

^d: Values represent maximum values of three analytical replicates ($n = 3$) per sample with exception of nobiletin (two analytical replicates per sample, $n = 2$).

^e: Maximum content was set at 0.019 mg/L due to the LoQ of the targeted substances in lemon juice at 0.02 mg/L.

^f: Maximum content was set at 4.99 mg/L due to the LoQ of the targeted substances in peel oils at 5.00 mg/L.

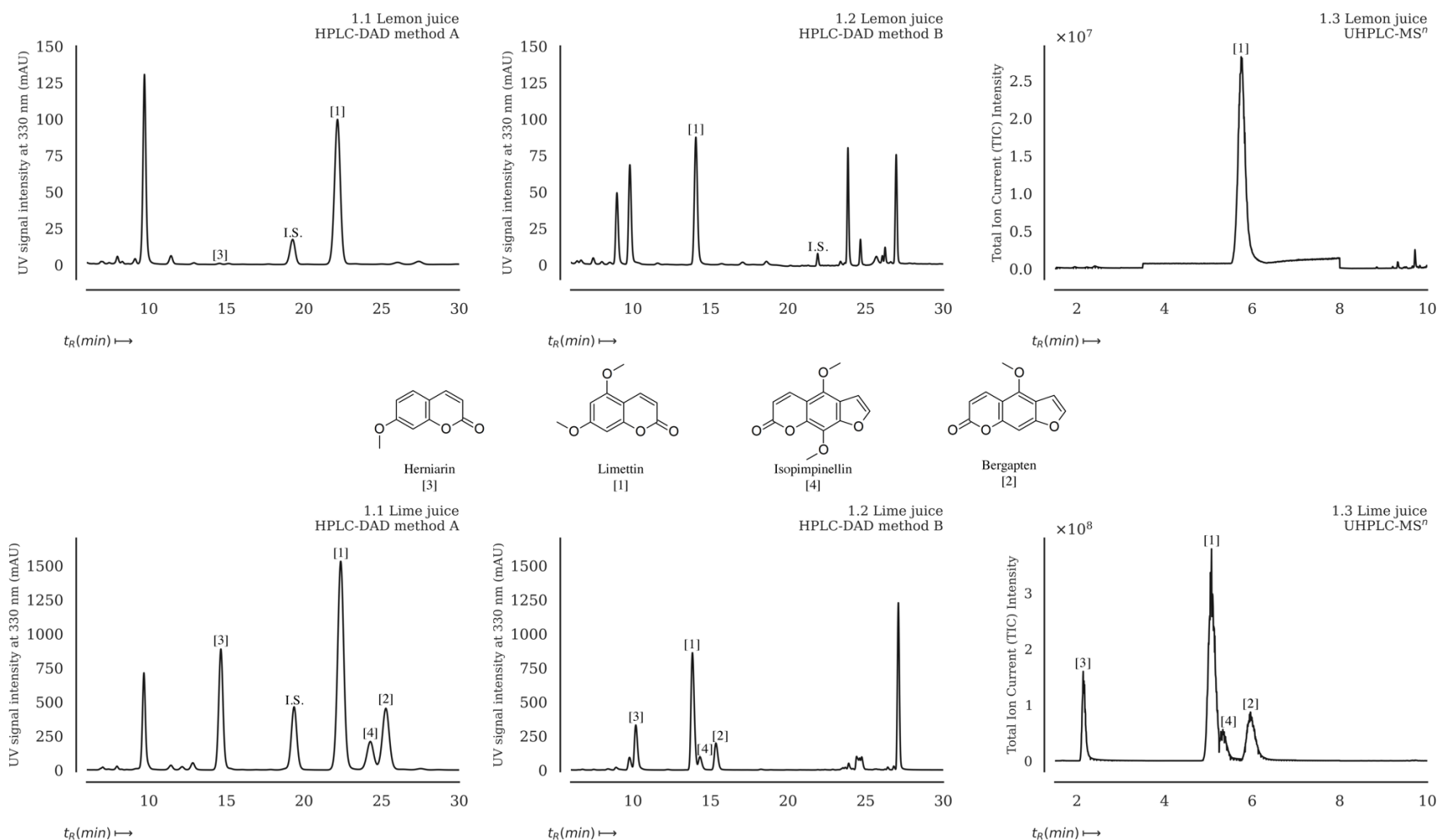


Figure 1. Representative chromatograms of typical lemon (*Citrus limon* [L.] Burm.f.) and lime (*Citrus × aurantifolia* [Christm.] Swingle) juices as analysed by three laboratories (A, B, and C) with HPLC-DAD method A (1.1), HPLC-DAD method B (1.2), and UHPLC-MSⁿ (1.3), respectively. Signals of the coumarins limettin [1] and herniarin [3] as well as the psoralens isopimpinellin [4] and bergapten [2] were found in lime juice, but, among these, only limettin [1] was detectable in lemon juice. Different internal standards [I.S.] were used in each method. t_R : Retention time in min.

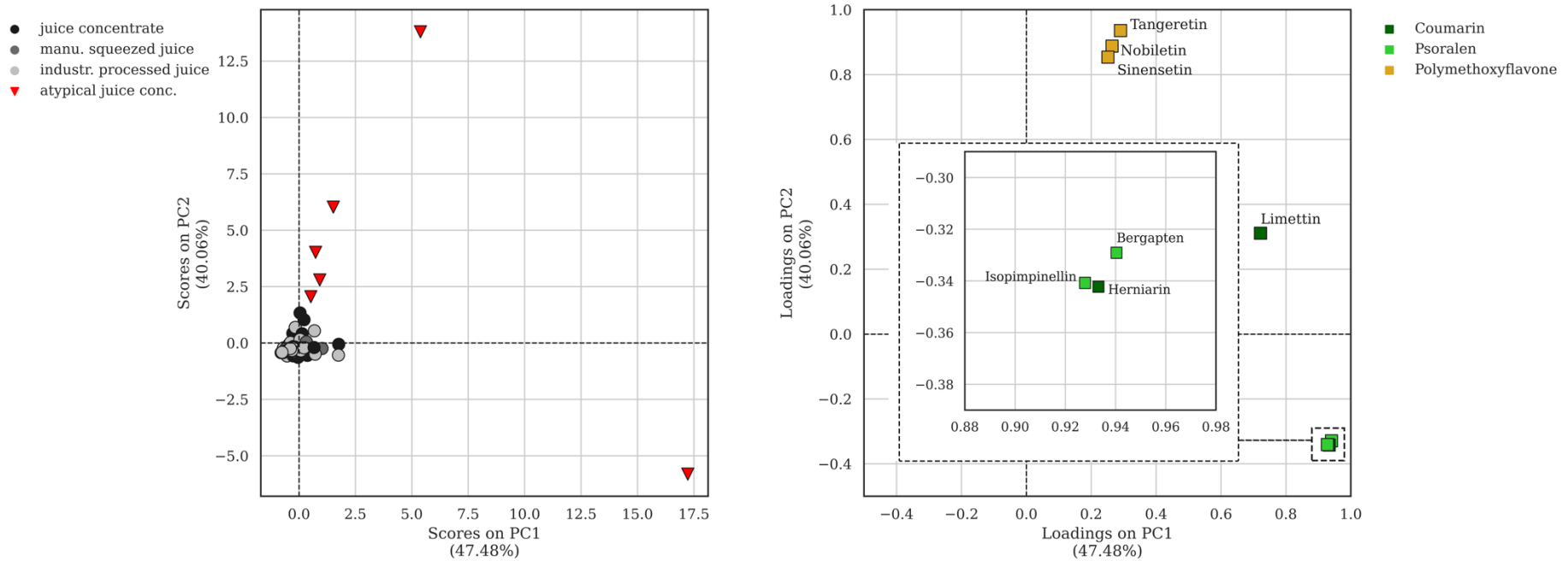


Figure 2. Score and corresponding loading plots of the principal component analyses (PCAs) on lemon (*Citrus limon* [L.] Burm.f.) samples from the endocarp (manually squeezed juice, industrially processed juice and juice concentrate), calculated based on coumarins, psoralens and polymethoxyflavones.

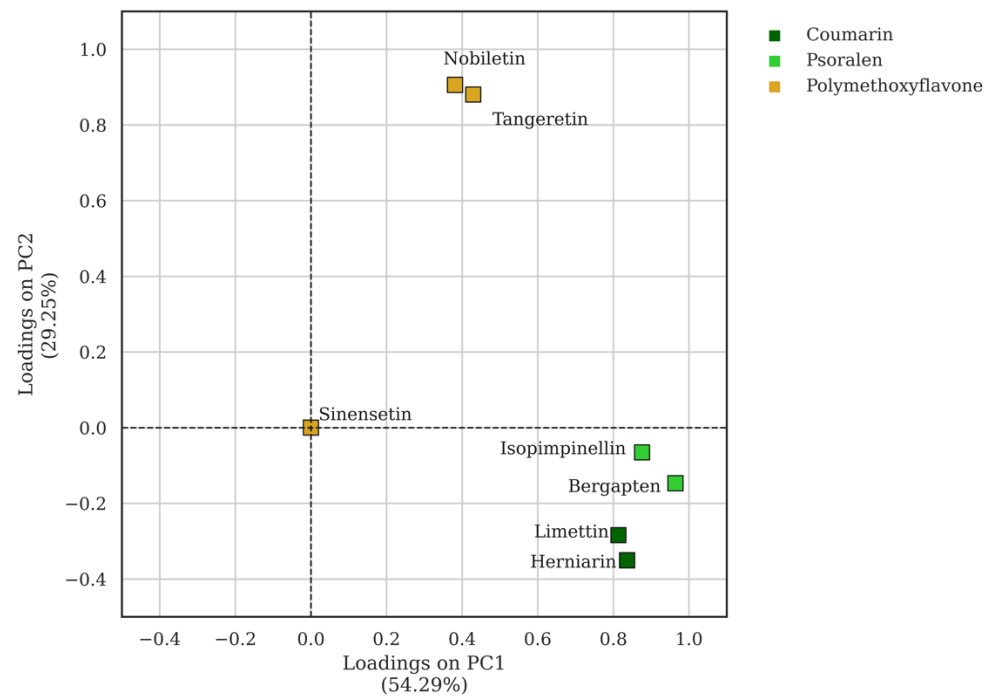
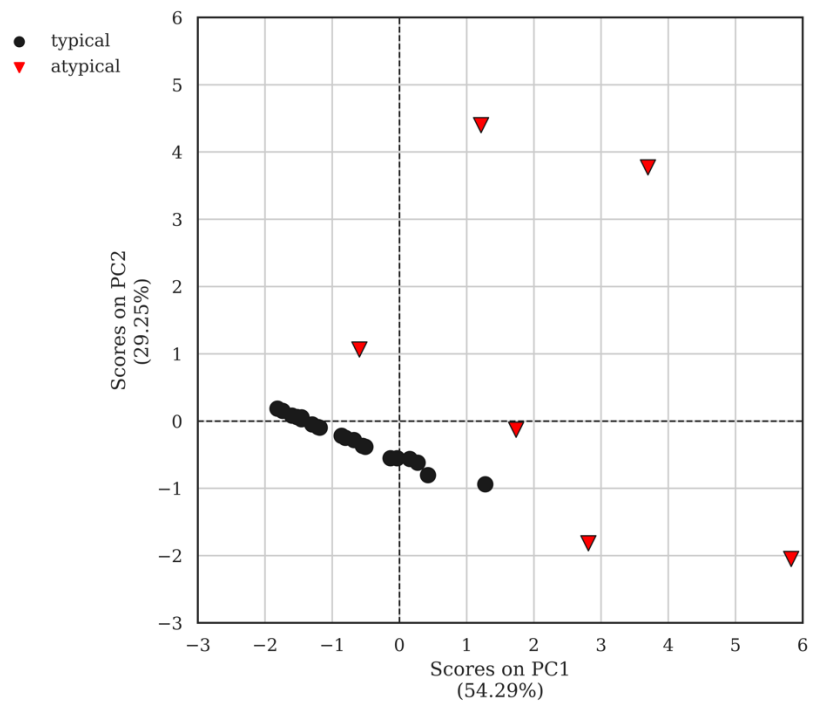


Figure 3. Score and corresponding loading plots of the principal component analyses (PCAs) on lemon (*Citrus limon* [L.] Burm.f.) peel oils, calculated based on coumarins, psoralens and polymethoxyflavones.

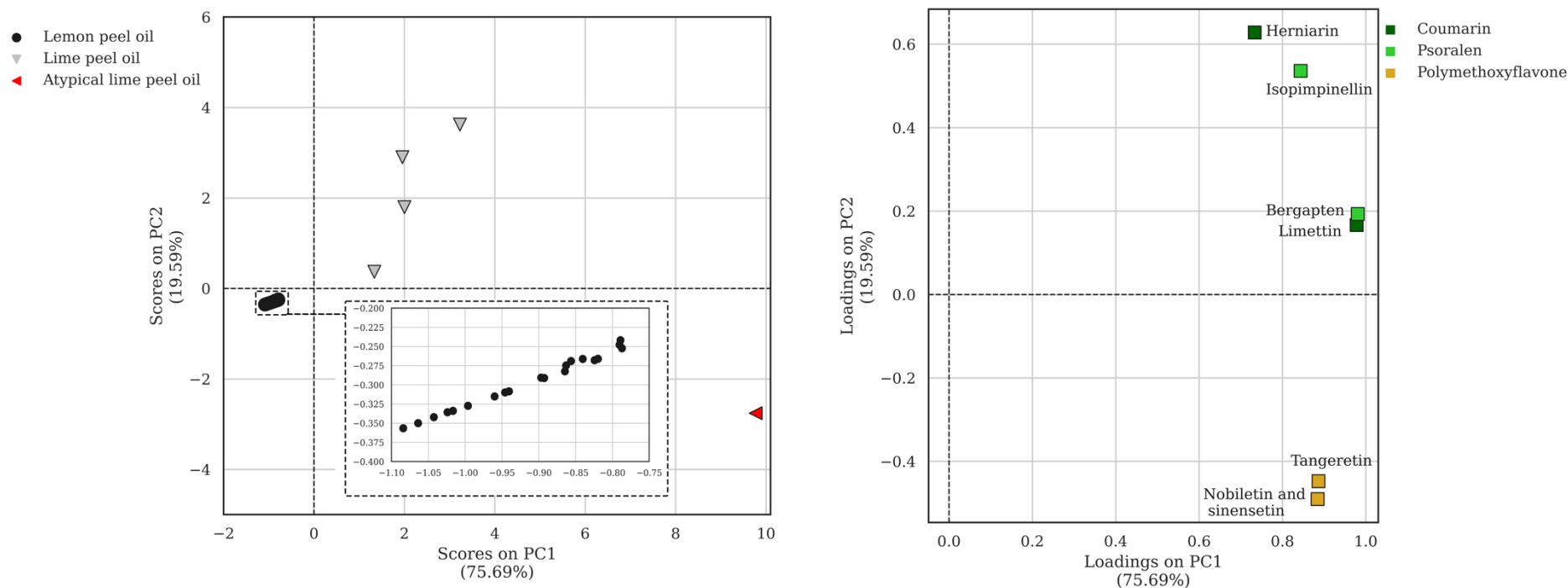


Figure 4. Score and corresponding loading plots of the principal component analyses (PCAs) on lemon (*Citrus limon* [L.] Burm.f.) and lime (*Citrus × aurantifolia* [Christm.] Swingle and *Citrus × latifolia* [Yu.Tanaka] Tanaka) peel oils, calculated based on coumarins, psoralens and polymethoxyflavones.