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SFC and CE—A Comparison of Two Orthogonal Methods for the Analysis of Dihydrochalcones in Apple Leaves

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Abstract: In recent years the analysis of natural products has been carried out using a range of approaches, but mainly utilizing liquid chromatography (LC) or gas chromatography (GC). However, alternative approaches with orthogonal selectivity like capillary electrophoresis (CE) and supercritical fluid chromatography (SFC) have increasingly been employed as well, even though they are often considered niche techniques only. In this study, we intended to confirm and compare their suitability as reliable state-of-the-art methods for the analysis of bioactive compounds by developing CE and SFC for the analysis of dihydrochalcones (DHCs) in apple leaves. The analytes were chosen as they have shown interesting pharmacological effects, such as anti-inflammatory, anti-tumor and immunomodulatory activities, and also present an interesting analytical challenge due to their structural similarity and polarity range. Both methods were well capable to separate the five standard compounds within short separation times and fulfilling the demands for an environmentally friendly "green" technology. CE as well as the SFC assay were validated for linearity, sensitivity, accuracy and precision according to ICH guidelines and met all respective requirements. Using the optimized methods, several *Malus* sp. samples were analyzed whereby a significant difference in the qualitative as well as quantitative DHC profile was revealed, with overall DHC concentrations ranging from 5.47% to 17.24%.

Keywords: supercritical fluid chromatography; capillary electrophoresis; dihydrochalcones; natural product analysis

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1. Introduction

The reviews by Newman and Cragg have shown that drug discovery is still dominated by natural products [1], with plant extracts being the primary source. However, due to the complexity and diversity of this matrix, controlling its consistency and quality always presents a challenging task, especially as even the (qualitative and quantitative) variation in constituents within the same species can be substantial [2]. A selection of analytical techniques is available for the determination of (active) natural products to overcome these obstacles, and because of their robustness, long history of use and broad applicability, techniques like gas chromatography (GC) and high-performance liquid chromatography (HPLC) are often selected. However, due to their orthogonal selectivity, approaches like supercritical fluid chromatography (SFC) and capillary electrophoresis (CE) have steadily grown in popularity, especially as they meet the demand for high separation efficiency in short analysis time [3], whilst at the same time reducing solvent consumption, rendering them as environmentally friendly and "green" analytical methodologies [4–6]. Thus, these approaches are much more than just technical alternatives.

In SFC, separation is carried out using a mostly CO_2-based mobile phase, which is in a supercritical state. It combines the advantageous properties of both a gas and a fluid, namely excellent solvation capacity and high diffusion strength [7]. Together with sub-2 µm stationary phases and a modifier to adjust the polarity of the carbon dioxide, excellent separation efficiencies can be reached in very short analysis times, comparable to UHPLC.

SFC is well established in food and environmental analysis but has also successfully been applied for natural product analysis, especially in the last two decades [5,7–9]. In capillary electrophoresis (CE), separations are performed in small diameter capillaries, typically in the range of 50 μm, filled with buffer solution. Once an electric field is applied the analytes migrate according to their charge state and size; thus, the buffer pH is one of the critical parameters to be considered during method development [10,11]. In contrast to pressure-driven techniques like HPLC (and SFC), in CE the buffer is moved due to the electro-osmotic flow (EOF), resulting in a flat flow profile. This aspect significantly reduces peak broadening [12], and related benefits have been shown in many applications on natural products already [10,11,13,14]. In several monographs of the European pharmacopeia, CE-based methods are presented for quantitative assays [15].

This project intended to highlight and compare the potential of SFC and CE for the analysis of five major DHCs found in apple leaves. They are rich in these natural products with an intriguing pharmacological spectrum that was summarized by Tian et al. [16] and Stompor et al. [17] already: immunomodulatory (trilobatin) [18], anti-inflammatory, antioxidative (phloretin, phloridzin, trilobatin, sieboldin) [19–22], anti-tumor (phloretin) [23–25] and antihyperglycemic effects (phloretin, phloridzin) [26–28]. Furthermore, they have neuro-, hepato-, and cardio-protective potential, which renders them interesting natural compounds for the treatment of various diseases including high blood pressure, diabetes and cancer [16,17].

Previously reported analytical approaches for the analysis of DHCs in apple leaves include several HPLC [29,30] as well as UHPLC [31,32] assays. They covered a maximum of up to three of the DHCs addressed in this publication (Figure 1). CE or SFC were never considered for this separation problem before.

phloretin (**1**) 3-hydroxyphloretin (**2**) phlorizin (**3**)

trilobatin (**4**) sieboldin (**5**)

Figure 1. Structure of the investigated DHCs.

2. Materials and Methods

2.1. Chemicals and Reagents

All organic solvents (methanol, acetonitrile, isopropanol) as well as most of the additives/chemicals used, i.e., formic acid, ammonium formate, ammonia, diethylamine and phosphoric acid, were purchased from VWR International (Vienna, Austria). Oxalic acid was from Alfa Aesar (Karlsruhe, Germany). Sodium tetraborate, sodium hydroxide and phosphate salts for preparing CE buffers came from Merck (Darmstadt, Germany). HPLC-grade water was prepared in-house with an Arium purification system (Sartorius, Göttingen, Germany). Carbon dioxide of the 4.5 grade (purity $\geq$ 99.995%) required for SFC analyses was obtained from Messer (Gumpoldskirchen, Austria).

Standards of the five dihydrochalcones phloretin, 3-hydroxyphloretin, phloridzin, trilobatin and sieboldin were isolated by chromatographic means in-house from *Malus micromalus* leaves during previous projects and had a purity of over 95% (confirmed by

LC-MS and NMR). They are well soluble in methanol and the respective solutions were stable for at least 4 weeks if stored at 4 °C, which was confirmed by repeated analyses.

2.2. Plant Materials and Sample Preparation

The analyzed plant samples were collected in Southern Tyrol (Italy) during a previous research project (EUREGIO EXPoApple2 [33]). The fresh leaves were freeze-dried immediately after harvest and stored protected from light and air as ground powder. An overview of all samples is given in the Supplementary Materials, Table S2.

Prior to extraction, the powder was sieved (45 mesh) to ensure homogeneity. A total of 50 mg of each sample was carefully weighed and extracted four consecutive times with 2.5 mL of methanol in an ultrasonic bath (Bandelin Sonorex, Berlin, Germany) for 10 min at ambient temperature. The extracts were centrifuged (5 min, 1500× *g*) and the clear supernatants were combined and diluted to 10.0 mL of methanol in a volumetric flask. Before analysis, all sample solutions were filtered through a 0.45 µm syringe filter (PFTE; Macherey-Nagel, Düren, Germany). To ensure the comparability of the results, identical sample solutions were analyzed by CE as well as SFC.

To confirm the efficiency of the employed extraction procedure, one sample (**Malus-2**) was submitted to a fifth extraction and this solution was analyzed by CE and SFC for possible remnants of dihydrochalcones. Respective levels were always below 0.5% of the initial concentration, therefore the applied extraction was considered to be exhaustive.

2.3. Analytical Instrumentation

For all SFC experiments, an Acquity UPC^2-system from Waters (Milford, MA, USA) comprising a binary solvent manager, a column oven, convergence and sample manager as well as a PDA-detector was employed, which was operated with Empower 3 software, release 2 (Waters). Optimal separations of the five dihydrochalcone derivatives from *Malus* sp. were achieved on a Torus™ Diol column (3.0 × 100 mm; 1.7 µm particle size) from Waters. The mobile phase comprised CO_2 (A) and 2.5 mM oxalic acid in methanol (B). Elution was performed at a flow rate of 1.10 mL/min in gradient mode from 30 to 45% B in 4.5 min, holding this concentration for 0.5 min (total run time 5 min). After each run, a two-minute re-equilibration step with the initial solvent composition was added. Column temperature, ABPR and injection volume were adjusted to 40 °C, 2000 psi and 1 µL, respectively. The analytes were monitored at 280 nm, which is in agreement with the literature [29].

CE analyses were performed on an Agilent 7100 instrument (Waldbronn, Germany) consisting of an autosampler, diode array detector and thermostated capillary compartment. Fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with an inner diameter of 50 µm and effective length of 62.5 cm were employed. The best results were obtained with a 25 mM borax (sodium tetraborate tetrahydrate) buffer, containing 2.5% isopropanol as an additive and being adjusted to a pH of 8.25 with 2% phosphoric acid. Samples were injected hydrodynamically (50 mbar, 3 s) and during analysis, the applied voltage and temperature were 25 kV and 30 °C, respectively. The detection wavelength was 210 nm. After finishing a run (10 min), a reconditioning step was required, during which the capillary was flushed with 0.1 N NaOH (2 min), buffer solution (2 min), and then applying 25 kV for 1 min. For CE experiments, not only the sample solutions but also buffers and rinsing solutions were membrane filtered (see details under 2.2).

2.4. Method Validation

To assure that the performance characteristics of both developed methods actually meet the requirements for the intended application they were validated according to ICH guidelines [34]. In order to confirm linearity and set up calibration curves, a stock solution of all standard compounds was prepared by dissolving 5 mg of **1–5** in 5.0 mL methanol using a volumetric flask. This solution (1.0 mg/mL) was then serially diluted in a ratio of 1:1 with methanol. Regression parameters were calculated using Microsoft Excel 2019.

The limit of detection (LOD) and limit of quantification (LOQ) were determined in a visual manner, whereby the former value was expressed as three times the baseline noise; the limit of quantification corresponded to three times this value. To do so, the calibration solution was diluted till the respective values were reached. Selectivity (peak purity) of the separation was confirmed by symmetric peak shapes and consistent UV spectra throughout the peaks, which indicated that no co-elution or signal overlap occurred. Precision was evaluated both intra- and inter-day. On each of three consecutive days, five individual extracts of sample **Malus-2** were prepared as described in Section 2.2 and subsequently analyzed by CE and SFC. Intra-day precision was determined by comparing the results of the same day, for inter-day variation those of all three days were evaluated. To establish the assay's accuracy, spiking experiments were conducted. Three concentration levels of all standards **1–5** were prepared and spiked individually to samples of **Malus-2** before extraction: low spike (25 μg/mL), medium spike (50 μg/mL) and high spike (100 μg/mL). By comparing the theoretically calculated analyte concentration with the one determined practically, the percentage recovery rates could be reported. All measurements, regardless of whether CE- or SFC-based, were done in triplicate. Statistical comparison of the methods was accomplished by paired *t*-test as well as Bland-Altman plot, both were calculated using Microsoft Excel 2019. For the *t*-test, the significance level was 5%.

3. Results and Discussion

3.1. Method Development

Our aim was to develop two complementary methods that enable the fast and reproducible separation of five major DHCs found in apple leaves, allowing their straightforward and sensitive quantitation, at the same time fulfilling all validation criteria and being applicable to crude extracts without extensive sample preparation. The optimal results are shown in Figure 2, depicting the separation of the identical standard mixture as well as a sample solution by CE and SFC. The chosen analytes feature a conjugated double-bond system, and a detection wavelength of around 280 nm is reported as optimum in the literature [29,32]. We observed the same for the SFC method developed (acidic conditions); however, for CE separations buffers with pH values in the alkaline range were favorable. This resulted in divergent UV spectra of the compounds so the detection wavelength was changed to 210 nm, at which the sensitive determination of all DHCs was possible (for comparison of UV spectra see Supplementary Materials Figure S12).

To mimic routine conditions and the complex matrix, for most of the method development a surrogate sample (i.e., test solution), a mixture of the extracts of **Malus-2** and **Malus-5** spiked with a standard solution of **2** (3-OH-phloretin), was used. It contained all the DHCs under investigation as well as possibly interfering plant constituents.

3.1.1. Supercritical Fluid Chromatography

As a first step, a selection of six stationary phases was screened. All columns had the same dimension (3 × 100 mm) and were filled with sub-2 μm particles, respectively. They covered both the Waters Viridis (BEH C18, HSS C18SB, BEH 2-EP) and Torus (DIOL, DEA, 1-AA) line (See also Table S1). A pre-filter (Waters KrudCatcher) was always used in order to prevent contamination of the column when analyzing crude extracts. Initial experiments applying a generic CO_2-methanol gradient, i.e., from 0 to 20% modifier in 10 min and holding this concentration for two minutes, already indicated that due to the polarity of the target substances, higher modifier concentrations were obligatory to enable their elution.

Out of the columns tested, the Diol phase seemed to be the most promising candidate, therefore it was selected for further optimization. According to West et al., who evaluated various SFC-specific columns by LSER (linear solvation energy relationship) this stationary phase exhibits polar propanediol moieties bonded to silica, permitting dipole-dipole interactions (positive *e* and *s* values) as well as hydrogen bonding with acidic/basic species [35]. On a 1-AA column, a material with similar properties, the analytes eluted even earlier;

yet, the baseline was less stable and the system pressure was significantly increased so this phase was excluded from further method development. The BEH material, another polar phase but with different chemistry (non-bonded/hybrid silica), did not permit a good separation (Figure 3A). Here most of the analytes coeluted at the beginning of the chromatogram. Other stationary phases were not suitable either (Figure 3B).

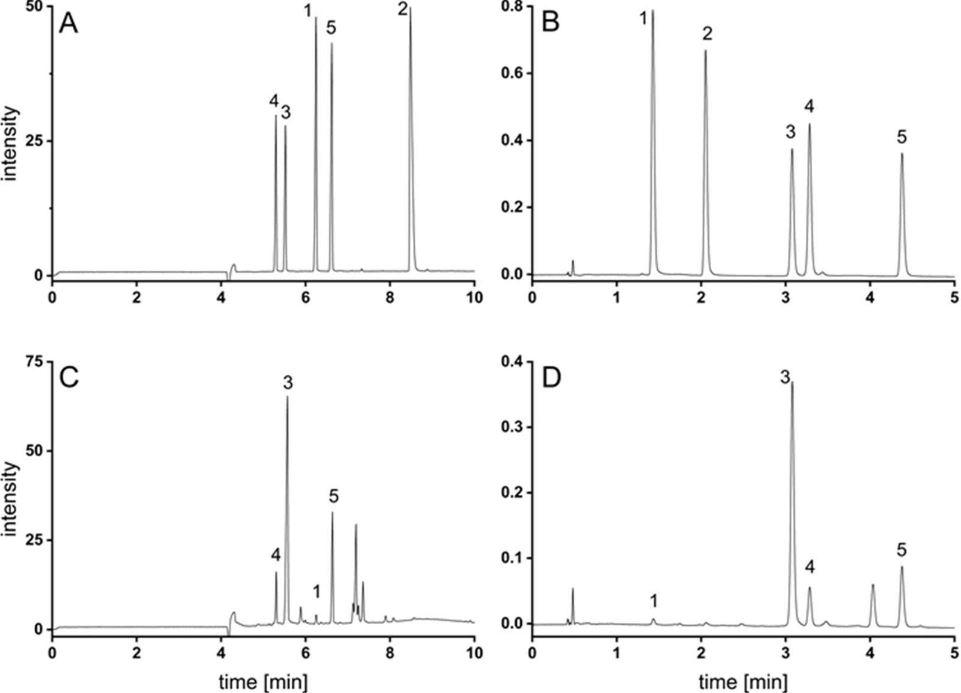

Figure 2. Optimal separation of DHCs by CE ((**A**): standard mixture, (**C**): sample **Malus-2**) and SFC ((**B**): standard mixture, (**D**): sample **Malus-2**). CE: 25 mM borax (sodium tetraborate tetrahydrate) buffer containing 2.5% isopropanol at pH 8.25; injection: hydrodynamic (50 mbar, 3 s); voltage: 25 kV and temperature: 30 °C; detection: 210 nm; SFC: Torus™ Diol (3.0 × 100 mm; 1.7 µm particle size); mobile phase: CO_2 (**A**) and 2.5 mM oxalic acid in methanol (**B**), gradient from 30 to 45% B in 4.5 min, held for 0.5 min; flow rate: 1.10 mL/min; column temperature: 40 °C; ABPR: 2000 psi; injection volume: 1 µL; detection: 280 nm. Peak assignment according to Figure 1.

The second optimization step concerned the mobile phase. In SFC the polarity of pure CO_2 is similar to hexane, so most of the time the polarity of the eluent has to be adjusted by the use of organic solvents, especially when working with rather polar analytes like in this study. Methanol is the most widely used modifier and also was the first choice in the current study; however, the effects of acetonitrile and isopropanol were evaluated as well. The best results were achieved with methanol, as, e.g., even as little as 10% isopropanol led to significant peak broadening (Figure 3C). Although all five standards could already be separated with the modifier methanol, different additives were evaluated to further improve peak shapes as well as to reduce analysis time. In this context, a selection of acidic and basic additives was screened. The addition of bases (NH_3, DEA) led to deteriorated peak shape and resulted in a severe broadening of the signals of **2** and **5** (see Figure 3D). Commonly used volatile acids like formic acid also had no beneficial effects, but with phosphoric acid in a concentration of 0.1%, peak symmetry could be slightly enhanced. However, the resolution between **4** and other coeluting substances decreased. In two papers, the addition of oxalic acid was described as being advantageous for the SFC analysis of flavonoid glycosides [36,37]. Accordingly, we evaluated the effects of different concentrations of this uncommon additive, in combination with or without phosphoric acid, to conclude that 2.5 mM oxalic acid in methanol without any further additive permitted the optimum separation of **1–5**, may it be concerning resolution, peak shape, or analysis

time. Water also has been described as a possible additive for SFC; 0.5% water was added to the modifier, but without any positive effects. On the contrary, pressure fluctuations and reduced reproducibility were the result.

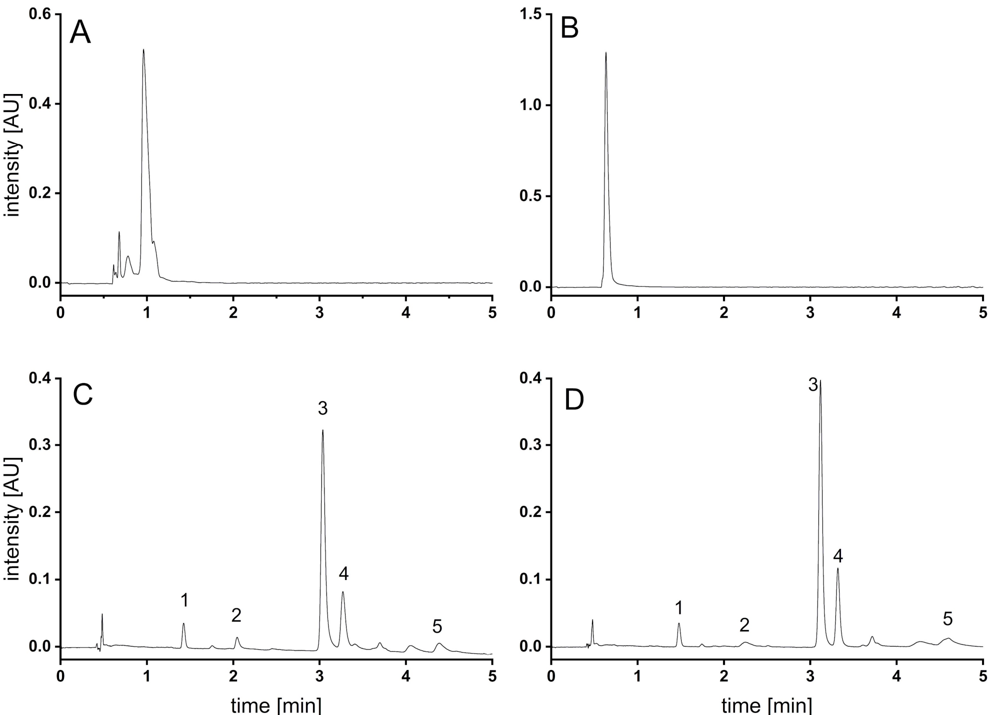

Figure 3. Influence of different parameters on the SFC-separation of the test solution: using a Viridis® BEH (**A**) and Viridis® HSS C18 SB column (**B**), adding 10% isopropanol to the modifier (**C**), using 0.1% of DEA as an additive (**D**). All other parameters were optimal (see Figure 2).

With stationary and mobile phases being defined, the optimal gradient was now searched. As already mentioned, DHCs represent unusual analytes for SFC due to their polarity, so the initial modifier concentration had to be rather high, or else no or only partial elution of the analytes occurred. Thus, beginning the analysis with 30% of 2.5 mM oxalic acid in methanol was a good starting point in terms of a fast separation. The optimal endpoint of the gradient was determined at 45%, resulting in well-resolved peaks in only 5 min. Further increases in the modifier concentration were not possible because of the resulting very high system pressure.

Subsequently, the effects of column temperature were examined. Below 40 °C the required analysis time was prolonged as well as the system pressure substantially increased. Above 40 °C the retention times could be only marginally reduced; therefore, this temperature was also not raised as constantly high temperatures may decrease the column life. In terms of ABPR and flow rate, these parameters were set to 2000 psi and 1.1 mL/min, respectively, being a good compromise between the speed of analysis, resolution and the instrument's pressure limit. The injected sample volume was, typical of SFC, 1 µL, the detection wavelength 280 nm, where all the assayed compounds show a maximum under the selected conditions. For detailed figures on the evaluated parameters, see Supplementary Materials Figures S1–S6.

3.1.2. Capillary Electrophoresis

As the type and pH of the buffer are usually the most relevant parameters in CE, different buffer systems were tested for their ability to resolve **1**–**5**. They included sodium tetraborate as well as sodium phosphate buffers of different molarity (15–35 mM) and pH (pH 6–10). When comparing the results with both buffer types it quickly became clear that borate-based buffers are advantageous, most likely because of their known ability to form

complexes with phenolic compounds, as has already been shown for flavonoids [38]. The pH range of phosphate buffers from 6 to 8 was also not suitable to create sufficient EOF, as this driving force in CE increases with pH. Accordingly, the resolution between peaks was generally higher when using borate buffers, whilst at the same time the analysis time was shorter. Concerning the optimal pH, it was noticed that an insufficient separation of the aglycones **1** and **2** occurred over pH 9.0, at pH values below 8 the resolution of phloridzin and sieboldin declined (Figure 4A,B). Thus, a good compromise was reached at pH 8.25, where the calculated resolution between all target compounds was always higher than 4. It is a known effect in CE that the EOF decreases with increasing buffer molarity. Accordingly, an increasing tetraborate concentration prolonged analysis time, but at the same time resolution between some peak pairs increased due to better peak shape. Above 40 mM of borax, the separation of **1–5** was not possible in less than 10 min anymore, and at the same time, the resulting current was too high, leading to Joule heating and inconsistent results due to air bubble formation in the buffer. A good separation combined with reproducible results was obtained with a 25 mM borax buffer (at pH 8.25), a concentration that, therefore, was selected for all further experiments. Parameters with minor effects on the results were temperature and applied voltage (Figure 4C). A capillary temperature of 30 °C was selected because of a more stable baseline (compared to higher values) and reduced analysis time (compared to 20 °C). An applied voltage of 25 kV permitted fast and, at the same time, well-reproducible results. The reason for selecting 210 nm for detection has already been discussed above.

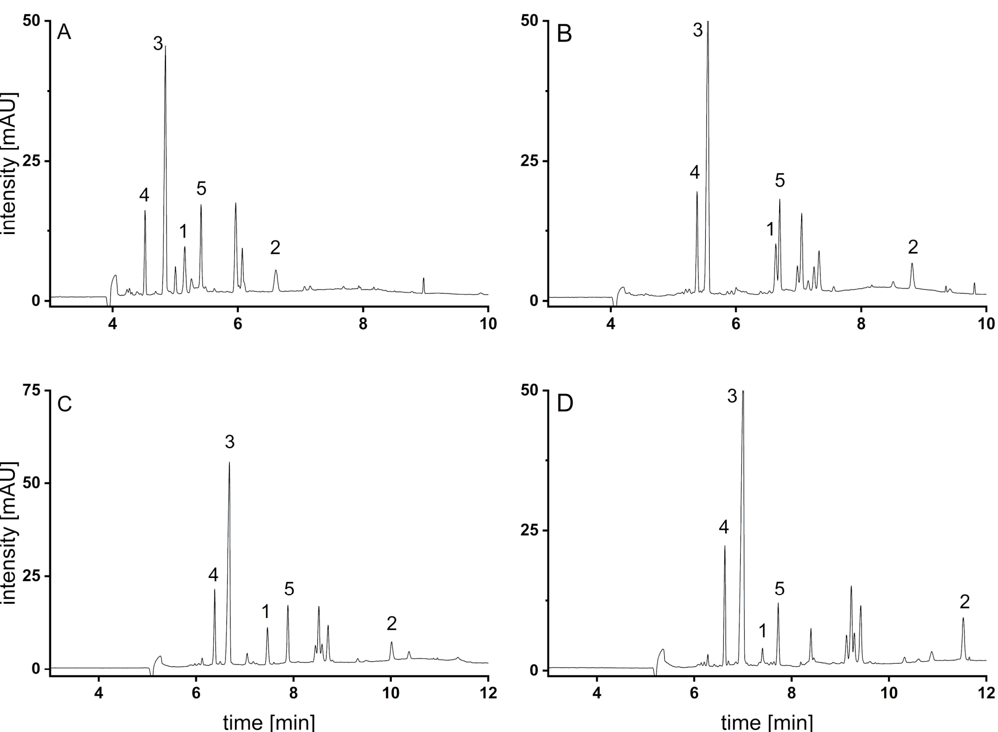

Figure 4. Influence of different parameters on the CE-separation of the test solution: using borate buffers at pH 7.5 (**A**) and 9.0 (**B**), applying 20 kV (**C**), and increasing the isopropanol concentration to 7.5% (**D**). All other parameters were optimal (see Figure 2).

Different organic solvents can be added to a CE buffer to modify analyte solubility and buffer viscosity [12]. Protic (methanol, isopropanol, butanol), as well as aprotic solvents (acetone and acetonitrile), were evaluated in this respect, in a first step, always adding 5% of solvent to the selected electrolyte. Only with isopropanol could the resolution be enhanced so its concentration was varied up to 7.5% to investigate respective effects in detail. With 2.5% isopropanol in the buffer especially the separation of **5**/**1** could be improved, whereas more than 5% significantly prolonged the analysis without any gain

in resolution (Figure 4D). The former was therefore considered beneficiary and selected. As for SFC, detailed figures on the impact of individual parameters can be found in the Supplementary Materials, Figures S7–S11).

3.2. Method Validation

Table 1 summarizes the validation data of both developed methods. The assays had excellent linearity with correlation coefficients higher than 0.999 (CE: ≥0.9993; SFC: ≥0.9994), covering a concentration range from 3.91 to 1000 µg/mL. Concerning SFC, the linear range of phloretin and 3-hydroxyphloretin was even wider, starting from 1.95 µg/mL. The LOD and LOQ values of both techniques were very comparable and were shown to be always less than or equal to 0.98 and 2.93 µg/mL, respectively. Again, lower values could be determined for the two aforementioned analytes if analyzed by SFC (LOD: 0.49 µg/mL; LOQ: 1.62 µg/mL). In terms of precision, the CE assay (maximum intra-day deviation of 5.73% for **1** on day two, maximum inter-day deviation of 5.15% for **4**) was surpassed by SFC (maximum intra-day deviation of 2.59% for **5** on day two, maximum inter-day deviation of 1.65% for **1**), whilst still being within the usually accepted 10% margin for, by nature, inhomogeneous samples like plant extracts. The accuracy of both methods was very acceptable too. The recovery rates for SFC were between −2.97% (**2**, low spike) and +2.45% (**3**, low spike); for the CE the respective range was from −4.71 (**1**, high spike) to +0.57% (**2**, high spike). Concerning selectivity, all examined peaks showed to be pure with no signs of coelution in the respective UV spectra. Further confirmation of this parameter by hyphenation to MS was not possible as for CE analysis a non-volatile borax buffer was used. The same applies to the use of oxalic acid as an additive in SFC; however, this aspect is discussed controversially in the literature [36].

Table 1. Validation results of both techniques: SFC in plain text, CE in italics.

	1	**2**	**3**	**4**	**5**
Regression equation	y = 4517.7x + 923.76 *y = 0.409x − 1.072*	y = 4034.1x − 12932 *y = 0.708x + 2.433*	y = 2402.6x + 4736.6 *y = 0.230x − 0.173*	y = 3057.4x − 9252.6 *y = 0.226x − 0.103*	y = 2662.3x − 10703 *y = 0.387x − 0.556*
R^2	0.9998 *0.9999*	0.9999 *0.9993*	0.9994 *0.9999*	0.9999 *0.9999*	0.9999 *0.9999*
LOD [a]	0.49 *0.98*	0.49 *0.98*	0.98 *0.98*	0.98 *0.98*	0.98 *0.98*
LOQ [a]	1.47 *2.94*	1.47 *2.94*	2.94 *2.94*	2.94 *2.94*	2.94 *2.94*
Precision					
intra-day [b]	1.40 *5.73*	- -	1.65 *3.61*	2.02 *5.15*	2.59 *5.12*
inter-day [c]	1.65 *3.78*	- -	1.09 *2.54*	0.98 *5.15*	0.70 *2.89*
Accuracy [d]					
high spike	98.2 (0.50) *95.3 (0.84)*	97.7 (0.56) *100.6 (0.84)*	101.2 (0.97) *96.2 (1.37)*	101.5 (1.19) *95.4 (1.26)*	101.4 (0.89) *98.7 (1.38)*
medium spike	98.7 (0.66) *98.3(0.91)*	99.4 (1.15) *95.8 (1.12)*	100.6 (0.35) *97.3 (0.77)*	102.0 (0.32) *100.5 (1.36)*	102.3 (1.09) *96.7 (1.14)*
low spike	99.9 (0.50) *95.2 (0.87)*	97.0 (0.56) *98.6 (1.13)*	102.5 (0.40) *96.4 (0.96)*	101.4 (1.18) *96.9 (1.68)*	102.4 (0.89) *97.9 (0.84)*

[a] µg/mL. [b] Maximum deviation within one day in percent based on peak area. [c] Deviation over three days in percent based on peak area. [d] Expressed as recovery rate in percent, $n = 3$, relative standard deviations in parentheses.

3.3. Analysis of Malus sp. Samples

To confirm the practical applicability of the developed methods, sample solutions of seven different *Malus* species (see Table S2) were prepared in an identical manner and analyzed by SFC as well as CE. The target analytes were assigned according to their specific retention/migration time and UV spectra in comparison with standards. The quantitative results presented in Table 2 indicated a rather variable DHC profile in the samples, in qualitative as well as quantitative terms. However, the results obtained by SFC and CE were in good agreement. For example, by both methods compound **2** (3-hydroxyphloretin) was only found in sample **Malus-1**, whereas **4** occurred in six of the seven analyzed specimens.

Conversely, samples **Malus-2** and **-6**, representing *Malus prunifolia* and *Malus sikkimenis*, comprised four of the five standard substances. Overall, the main compounds were either **5** (**Malus-1**, **Malus-4**) or **3**, which is in good agreement with the literature [29]. In terms of total DHC content a pronounced variability ranging from 5.47% to 17.29% was observed (average values of SFC and CE). An interesting observation, known from the literature, is the fact that the synthesis of **4** (trilobatin) and **5** (sieboldin) is encoded on the same genetic allele and thus these analytes never show up individually [29]. This was confirmed in our study as **Malus-3**, *M. huphensis*, lacked both analytes whereas they were found in all other samples. A second literature statement could also be corroborated: as phloretin (**1**) is the known precursor of the other DHCs, its content in plant material is generally low or it occurs in the glycosylated form [29,39]. In our study, phloretin was always the DHC with the lowest concentration, even to the point that it could not be detected in the plant material anymore. Two DHCs seem to be of special pharmacological relevance: trilobatin, as it possesses the highest antioxidative potential amongst the DHCs, and sieboldin, as it is 300 times sweeter than sucrose and has shown antidiabetic potential; it might be an interesting source for a novel sweetener [29]. **Malus-1** and **Malus-7**, representing *M. micromalus* and *M. floribunda*, contained the highest concentrations of both analytes in our sample set.

Table 2. The content of DHCs in different *Malus* species as determined by SFC (plain text) and CE (italics). Mean values are expressed as mg per g of dried biomaterial with corresponding relative standard deviation in parentheses. Assignment of compounds as in Figure 1.

Compound \ Sample	Malus-1	Malus-2	Malus-3	Malus-4	Malus-5	Malus-6	Malus-7
1	-	0.10 (0.78)	-	-	0.20 (0.30)	0.08 (0.30)	-
	-	*0.14 (1.99)*	-	-	*0.24 (2.98)*	*0.13 (1.55)*	-
2	0.41 (0.72)	-	-	-	-	-	-
	0.37 (1.42)	-	-	-	-	-	-
3	-	9.53 (0.73)	15.57 (0.89)	-	3.64 (0.89)	6.44 (1.36)	5.68 (1.50)
	-	*9.50 (0.98)*	*15.45 (2.78)*	-	*3.55 (2.54)*	*6.29 (2.43)*	*5.52 (1.51)*
4	2.54 (0.79)	1.24 (1.21)	-	2.66 (1.39)	1.59 (0.99)	0.64 (1.18)	1.21 (0.85)
	2.62 (1.22)	*1.43 (1.22)*	-	*2.77 (0.93)*	*1.72 (2.93)*	*0.59 (2.81)*	*1.27 (1.57)*
5	14.15 (0.21)	2.41 (0.71)	-	7.30 (1.67)	Det.	0.40 (1.49)	3.35 (0.61)
	14.36 (0.50)	*2.58 (1.70)*	-	*7.44 (2.96)*	*Det.*	*0.38 (2.64)*	*3.37 (1.35)*
Σ of DHCs	17.09	13.28	15.57	9.96	5.43	7.56	10.24
	17.39	*13.65*	*15.45*	*10.21*	*5.51*	*7.39*	*10.16*

When analyzing the different samples, only one contained a compound below LOQ (concordant result of CE and SFC), **5** in **Malus-5**, which therefore was not quantified; its qualitative annotation was still possible, however. In cases where the selected DHCs were not assigned (Table 2), there was no signal at the respective retention/migration time. This confirmed two aspects: (1) suitability of the sample amount used for extraction and the preparation protocol; (2) adequate sensitivity of the assay.

One aspect that proved to be important when analyzing apple leaf extracts by CE was the appropriate treatment of the capillary between runs. One disadvantage of this technique is the sometimes-observed problematic reproducibility especially when complex samples like plant extracts are assessed. The equilibration from run to run is therefore crucial. Washing steps with sodium hydroxide solution (to assure complete deprotonation of the silanol groups) and buffer are common practice. However, for this separation problem, the application of voltage (25 kV for 1 min) prior to injecting the sample proved to be beneficiary in terms of reproducibility. Hasemann already reported a similar observation in his dissertation, assuming a further charge stabilization of the capillary inside through this step [40].

3.4. Comparison of Methods

CE as well as SFC proved to be suitable for the analysis of the five selected dihydrochalcones in apple leaves, and all standards could be well resolved and unambiguously assigned in the extracts by both techniques. The required analysis time was below 10 min regardless of which method was selected. Both approaches fulfilled ICH validation criteria, yet by trend, the method's precision and accuracy were slightly better when using SFC. This is not surprising as the injected sample volumes in CE are in the nano-liter range so even minute variations during injection will have a negative effect on respective parameters. When comparing the relative standard deviation of the quantitative results, again CE proved to be slightly less reproducible; however, overall the values were very consistent between CE and SFC (maximum deviation for compound **5** in sample **Malus-1**: 14.15 mg/g (SFC)/14.36 mg/g (CE)) and the maximal tolerated RSD of ±5% never was exceeded. At the same time, concerning environmental friendliness, CE is definitely advantageous, as the consumption of sample and organic solvents are more or less negligible, and water-based buffers were used.

To compare the developed SFC and CE approaches by statistical means a Bland–Altmann plot [41,42], in addition to a paired *t*-test [43,44], was conducted on the basis of the quantitative results of the extracts. In the Bland–Altmann plot, all calculated differences were in the range of ±1.96 times the standard deviation, and thus the results of both methods were very comparable (see Figure S15). This was also indicated in the *t*-test. The null hypothesis was that there was no significant difference between the values determined by either of the methods. As the determined *p*-value was 0.15 (0.15 > 0.05) this hypothesis could be confirmed within a 95% confidence level.

A comparison to already published (U)HPLC approaches—given that they did not cover all of the analytes mentioned in this paper—shows that the SFC and CE assays presented here demonstrably convince in terms of separation speed (a recently published HPLC-DAD method required 30 min to separate three out of the five compounds [29]) and economic/environmentally friendly operation (superior greenness). The analysis time of the reported UHPLC assays is similar to our SFC approach [31,32]. In the study conducted by Walia et al. [32], the LOQ values (≤0.87 µg/mL) were lower than in the assays presented here; yet, our methods are demonstrably advantageous in terms of resolution, especially concerning the analysis of real samples.

4. Conclusions

This study confirms that viable natural product analysis approaches are not limited to the use of standard techniques like HPLC or GC, but also include less common approaches like CE and SFC. The latter techniques offer separation orthogonality combined with high separation efficiency and speed, which are transferable to the analysis of complex sample matrices as well. The application presented here of such niche techniques for the quantitative determination of dihydrochalcones is hopefully a convincing example. Not only was this interesting group of natural products analyzed by CE and SFC for the first time, but also the aforementioned benefits were impressively confirmed, as, e.g., the quantitative analysis of five DHCs in a complex matrix (apple leaf extracts) in less than 9 min (CE), resp. 5 min (SFC), has never been reported before. The slightly diminished sensitivity compared to UPLC was expected as typical for the methods used and did not limit the practical applicability of both assays.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations10040239/s1, Figure S1: Impact of stationary phase on the separation; Figure S2: Impact of methanol and basified methanol as modifier on the separation, Figure S3: Impact of temperature on the separation, Figure S4: Impact of different flow rates on the separation, Figure S5: Impact of different backpressure on the separation, Figure S6: Impact of the gradient on the separation, Figure S7: Separation of the test solution with a phosphate buffer pH 8.50, Figure S8: Resolution of the target analytes at different pH values, Figure S9: Resolution and peak

symmetry of the target analytes at different voltages, Figure S10: Resolution of the target analytes at different temperatures, Figure S11: Resolution of the target analytes using different organic additives, Figure S12: UV spectra of the five DHCs in comparison, Figure S13: SFC chromatograms of the analyzed samples, Figure S14: CE electropherograms of the analyzed samples, Figure S15: Statistical method comparison using a Bland–Altmann plot, Table S1: Stationary phases evaluated during this study, Table S2: Available information on the samples used in this study.

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