

Extraction of bioactive coumarins from lime peel as sample pretreatment before chromatographic analysis

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Abstract: Analysis of lime peel was applied to assess their suitability for various intended purposes, e.g., application in perfumery, cosmetics, and cleaning products, or as a source of bioactive or other value-added compounds. Targeted analysis allows wider usability of the waste part of these natural materials. In the present study, a novel, efficient, lab-simple, time-saving analytical method for coumarins determination in lime peel, including sample pretreatment by extraction and quantification by HPLC with fluorescence detection (FLD), is introduced. Optimal conditions of ultrasound assisted extraction included water as extraction solvent, temperature of 40 °C, and extraction time of 10 min. A magnetic molecularly imprinted polymer was employed as solid phase extraction adsorbent for primary extract cleaning, isolation, and enrichment of coumarins before chromatographic analysis. Recovery of herniarin and umbelliferone was more than 86 % ((RSD ≤ 4.8 %). Linear range of 50–1000 ng/mL with correlation coefficient above 0.998 was obtained for the proposed HPLC-FLD method. The limit of quantification was 8.2 and 44 ng/mL for herniarin and umbelliferone, respectively. These results show that the proposed sample pretreatment procedure is suitable for analytical purposes and is perspective also for the analysis of other citrus samples, as well as for future scale-up preparative isolation of bioactive coumarins from citrus peel.

Keywords: lime peel, coumarins, ultrasound assisted extraction, selective molecularly imprinted extraction, high performance liquid chromatography

Introduction

Natural materials are sources of many bioactive compounds useful in the treatment of a wide spectrum of diseases. They can be components of food, food supplements and cosmetics, as well as cleaning products (fragrance ingredients).

Coumarins, secondary plant metabolites, have an important function in the biochemistry and physiology of plants (Önder, 2020). From a chemical point of view, coumarins are organic heterocyclic compounds and their chemical structure is formed by 1,2-benzopyrone, which consists of a benzene ring connected to a pyrone ring (Annunziata et al., 2020). Citrus fruits are considered very important for the medical, pharmaceutical, perfumery and cosmetic industries due to their nutritional, medicinal, and fragrance properties (Raghavan and Gurunathan, 2021). Natural lemon peel powder was also applied as a heterogeneous catalyst for the synthesis of coumarin based heterocyclic compounds (Ganesh et al., 2020). Citrus peels can be also used in “zero waste” management at home, to cleaning various surfaces or as air freshener; however, grated fine “crumbs” from the peels (mainly lemon and lime) are also used to flavour dishes. Approximately 40–50 % of the total mass of citrus fruit is peel, which is generally considered as

waste. This part of fruits is also a source of bioactive compounds, including coumarins, but some may also have undesirable effects (e.g., coumarin is regulated for the use in food and cosmetics products (Regulation (EC) No. 1334/2008; Regulation (EC) No. 1223/2009); herniarin is prohibited to be used in fragrance compounds (Safety Assessment of Citrus Peel-Derived Ingredients as Used in Cosmetics, 2015)). Citrus waste parts are also used as a source of other high value-added compounds with beneficial properties (e.g., pectin, ascorbic acid, phenolic acids). Several studies reported bioactive simple coumarins, such as umbelliferone and herniarin (Fig. 1), in citrus (Ramírez-Pelayo et al., 2019; Jungen et al., 2021). Higher amounts of these compounds have been determined in peels compared with the edible parts of the fruit (Singh et al., 2020). Antituberculosic, antiinflammatory, antidiabetic, antibacterial, antifungal, and antioxidant properties have been indicated for umbelliferone (Hussain et al., 2019; Mazimba, 2017), while herniarin has demonstrated antiinflammatory, antioxidant, anti-proliferative, and antinociceptive effects (Bose and Pattanayak, 2019). Despite their beneficial effects, they can cause skin, eye or respiratory irritation (at high concentrations), which needs further pharmacological research (Denisow-Pietrzyk et al., 2019; Lin et al., 2023).

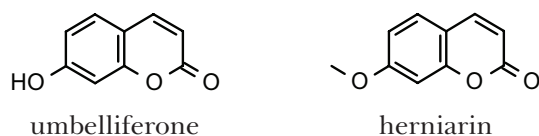


Fig. 1. Chemical structure of simple coumarins investigated in this study.

Thus, the utilisation of waste parts of citrus requires a simple, reproducible, and time-consuming analytical method for targeted analysis and quality evaluation. Sample pretreatment is an important step for subsequent analytes determination, e.g. chromatographic methods. Several extraction techniques, traditional and advanced, have been used for citrus sample pretreatment. Traditional methods, including Soxhlet extraction with methanol (Abosharaf et al., 2020), maceration with ethanol or ethylacetate (Munawaroh, 2017), as well as distillation (Li et al., 2021), were applied for the extraction of bioactive compounds including coumarins. Advanced extraction techniques, such as ultrasound assisted extraction (UAE) with water, methanol/water (80/20, v/v) or methanol (Ramírez-Pelayo et al., 2019; Zhao et al., 2020; Jung et al., 2021; Aznar et al. 2022), microwave assisted extraction (MAE) with water or ethanol (Ciriminna et al., 2017), supercritical fluid extraction (SFE) with CO₂ or CO₂/ethanol, were also used for the isolation of coumarins from different citrus fruits. Solid phase extraction is a frequently used technique for primary extract purification and analyte preconcentration (Aznar et al. 2022; Li et al., 2019; Fan et al., 2015). In response to the requirement of rapid, efficient, and selective extraction, molecularly imprinted polymers (MIPs) have received attention as extraction materials. MIP based extraction is an innovative separation technique with many advantages, which include high selectivity, chemical and mechanical resistance, as well as material reusability. MIP production process is controlled (components of reaction mixture, type of polymerisation procedure, conditions) so as to obtain many complementary cavities with the memory of size, shape, and functional groups of template molecules. The cavities, generated after template removal from the polymeric matrix, have specific recognition and selectivity for the target molecule and sometimes also for its analogues. MIP selectivity is very advantageous in determining compounds in a complex sample, such as natural materials (Chen et al., 2016; Jablonský et al., 2020). In citrus analysis, a hybrid organic-inorganic monolithic MIP allowed the determination of flavone hesperetin (Wang et al., 2018) and of benzimidazole residues (Liang et al., 2019).

This article is devoted to the analysis of lime peel for the purpose of qualitative and quantitative

evaluation of selected coumarins. Sample pretreatment procedures for the extraction of coumarins from lime have been developed. Solvent extraction and solid phase extraction with a selective molecularly imprinted adsorbent were incorporated in the analytical method for the determination of selected coumarins: umbelliferone and herniarin.

Material and Methods

Materials and Chemicals

Methanol (HPLC grade) and acetic acid (for analysis grade) were purchased from Centralchem (Slovakia). Umbelliferone (7-hydroxycoumarin) and herniarin (7-methoxycoumarin) (both 98 %) were purchased from Sigma Aldrich (USA). Lime samples (10 pieces of acid-fruit type, country of origin Mexico) were purchased from a local supermarket. Citrus peels were removed manually, cut into small pieces, and stored in a freezer before analysis.

Solvent extraction

Stirring-assisted solvent extraction: Finely chopped lime peel (1 g) was mixed with extraction solvent (3 mL), deionised water or methanol. The mixture was stirred for 30 min at the constant temperature of 23 or 40 °C. The extract was centrifuged for 1 min at 3000 rpm. The supernatant was collected and stored at -18 °C until HPLC-FLD analysis.

Ultrasound-assisted solvent extraction: An extraction solvent (3 mL) was added to finely chopped lime peel (1 g). The extraction solvent, deionised water or methanol, was added and the mixture was placed in an ultrasonic bath (45 KHz) with constant temperature (23 or 40 °C) for a certain time (10, 20 or 30 min). The extract was centrifuged (1 min, 3000 rpm) and supernatant was stored at -18 °C until HPLC-FLD analysis.

The tested extraction conditions are summarised in Table 1. Extractions were performed in triplicate.

Solid Phase Extraction

Solid Phase Extraction (SPE) was performed with lab-made Fe₃O₄@MIP-umbelliferone (Machyňáková and Hroboňová, 2017). For batch extraction, an aliquot of UAE sample extract (1.5 mL), or coumarins standard solution, or primary aqueous UAE sample extract (obtained at optimal conditions) were mixed with Fe₃O₄@MIP adsorbent (0.1 g, preconditioned with 1.5 mL of methanol/acetic acid (9/1 v/v) and water) and stirred for 30 min (180 rpm) at 23 °C. The sorbent was separated using an external magnet, washed with water (1 mL, 5 min, 180 rpm), and dried at 90 °C. Fe₃O₄@MIP was mixed with methanol/acetic acid (1.5 mL, 9/1 v/v) and stirred for 30 min (180 rpm). The sorbent was separated

Tab. 1. Experimental conditions for the extraction of coumarins from lime peel.

Experiment number	Extraction method	Temperature (°C)	Time (min)	Solvent
1	UAE	23	30	deionised water
2	UAE	40	10	deionised water
3	UAE	40	20	deionised water
4	UAE	40	30	deionised water
5	UAE	40	10	methanol
6	UAE	40	20	methanol
7	UAE	40	30	methanol
8	stirring	23	30	deionised water
9	stirring	40	30	deionised water
10	stirring	23	30	methanol
11	stirring	40	30	methanol

using an external magnet and stored at $-18\text{ }^{\circ}\text{C}$ until the HPLC-FLD analysis.

HPLC analysis

Liquid chromatograph (series 1200, Agilent Technologies, Germany) consisting of a binary pump, automatic injector, column oven, and fluorescence detector (FLD) was used. Separations were performed in reverse phase mode using a Kinetex C18 column (100 mm $\times$ 4.6 mm I.D., 5 μm particle size, Phenomenex, USA). Mobile phase consisted of 1 % aqueous solution of acetic acid (A) and 1 % solution of acetic acid in methanol (B). The gradient elution process was as follows: 0–12 min linear gradient for B component from 30 to 45 %, then to 100 % of B over 0.5 min, and held at 100 % of B for 2 min. Flow rate of mobile phase was 1.0 mL/min, injection volume was 20 μL , and column temperature was maintained at $23\text{ }^{\circ}\text{C}$. Fluorescence spectra were taken in the wavelength range of 250–550 nm, and chromatograms were recorded at excitation wavelength of 320 nm and emission wavelength of 450 nm. The HPLC-FLD method (Hroboňová and Špačková, 2020) was modified and verified using the system suitability test (umbelliferone: $t_{\text{R}} = 6.49 \pm 0.03\text{ min}$, $k = 3.33 \pm 0.01$, $H = 0.013\text{ mm}$; herniarin: $t_{\text{R}} = 12.17 \pm 0.03\text{ min}$, $k = 7.11 \pm 0.02$, $H = 0.004\text{ mm}$; $R_{\text{s}}(\text{umbelliferone/herniarin}) = 16.6$; analyses were realised in triplicate at the concentration level of 0.5 $\mu\text{g/mL}$), and validated which resulted in the limit of detection (LOD = 3.0/12.5 ng/mL), limit of quantification (LOQ = 8.2/44.0 ng/mL) for umbelliferone and herniarin, respectively, and linearity ($R^2 = 0.9998/0.9981$ in the concentration range of 50–1000 ng/mL for umbelliferone and herniarin, respectively).

Statistical analysis

Microsoft Excel 2018 (Microsoft Office, USA) and OriginPro 2018 (OriginLab Corporation, USA)

was used for data processing (linear regression to determine calibration curve coefficients and parameters for LOD, LOQ, and RSD % calculation) and visualisation.

Results and discussion

The first step in the separation of desired natural coumarins from citrus peel is solvent extraction. Solid phase extraction with an adsorbent selective for coumarins, molecularly imprinted polymeric adsorbent, was performed for primary extract purification or analytes preconcentration. These sample pretreatment procedures were optimised to obtain extract suitable for the HPLC-FLD analysis.

Optimisation of the solvent extraction procedure

Solvent extraction is the most common sample pretreatment technique for solid matrices to separate/isolate compounds; solvent selection is crucial for obtaining high extraction efficiency. Solubility of analytes, selectivity, and safety should also be considered in solvent selection. Organic solvents are mostly used in extraction of coumarins from different matrices; however, extraction methods operating at higher temperatures, pressures, or with energy field assistance offer some advantages such as lower organic solvent consumption and shorter extraction time (Rodríguez-García and Raghavan, 2022).

In this work, optimisation of solvent extraction parameters (type of solvent, extraction technique, temperature, time) was performed by one-variable-at-a-time method for a sample of lime peel in which coumarins were detected. Solvents, methanol and deionised water, and conventional stirring assisted and advanced ultrasound assisted extraction procedures were used for the extraction. The yield of the targeted coumarins obtained under different extraction conditions (Table 1) is presented in Fig. 2. Higher temperature ($40\text{ }^{\circ}\text{C}$) increases the solubility

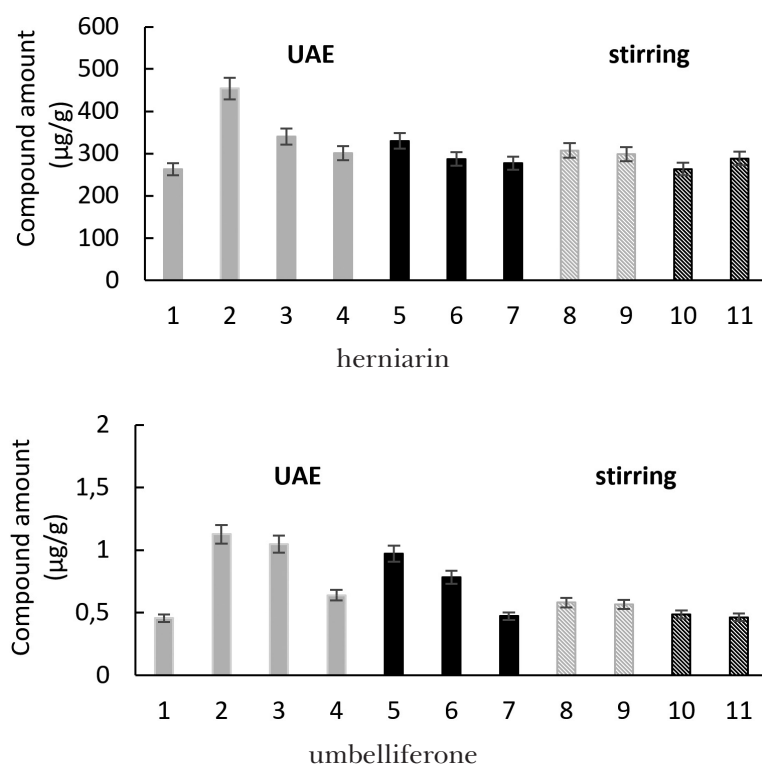


Fig. 2. Amount of coumarins extracted from lime peel at different extraction conditions.
Legend: ■ – deionised water, ■ – methanol; n = 3.

of the compounds and diffusion of solvent into the sample matrix resulting in higher or comparable extraction efficiency for both extraction solvents and type of extraction procedures compared to laboratory temperature (23 °C) extractions (extraction time of 30 min, experimental conditions 1 and 4, 8 and 9, 10 and 11 listed in Table 1). However, such high temperatures may cause the decomposition of thermolabile components and extraction of undesirable impurities (e.g., umbelliferone is chemically and thermally unstable, which may result in many compounds formed during its reflux extraction by methanol, ethanol, buffers, and buffered or non-buffered alcohol/water mixtures (Dawidowicz et al., 2018)). Extraction efficiency depends also on the extraction time and mostly increases with the increased extraction time in a certain time interval. However, increasing time does not significantly affect the extraction efficiency after the equilibrium of the solute is reached inside and outside the sample matrix (Zhang et al., 2018). In addition, long exposure times at high temperatures can lead to lower yield, e.g., due to the decomposition of compounds (Che Sulaiman et al., 2017). The conventional, stirring-assisted, extraction is inexpensive compared to advanced (e.g., UAE) techniques, but has varied efficiency and can be time consuming. In this study, the extraction time for conventional extraction of coumarins was set to 30 min for both investigated solvents. This extraction time was chosen due to

sufficient penetration of solvent in matrix. Results in Fig. 2 show no significant difference between the amount of extracted compounds obtained by either technique at the same extraction time (30 min, solvent/sample ratio: 3 mL/1 g, 40 °C). UAE is efficient also within a short extraction time due to the employment of ultrasonic waves; however, this method is costly (Carreira-Casais et al., 2021). Using the UAE procedure in the tested time interval of 10–30 min and at the temperature of 40 °C (based on the previous experiment; this temperature is well maintained, as the solution heats up during UAE.), higher yields of coumarins were achieved in 10 min. A comparison of the results of coumarins extraction from lime peel using both techniques showed that UAE is more effective. In addition, the highest yields of coumarins were obtained in a shorter extraction time (10 min) using the UAE procedure compared to the stirring-assisted extraction realised at longer time (30 min), which can be translated into economic benefits. Thus, UAE can be proposed as a more suitable sample pretreatment technique for the extraction of coumarins from citrus peel. Optimal conditions for the extraction of coumarin derivatives, umbelliferone and herniarin, were set as: UAE with deionised water as the extraction solvent at 40 °C and extraction time of 10 min. These conditions are suitable for obtaining the primary extract from lime peel and could eventually be applicable also for other citrus fruits (e.g., lemon, orange,

Tab. 2. Summary of selected extraction and purification techniques for coumarins from citrus fruits.

Sample	Coumarins ^a	Technique	Solvent system	Conditions	Ref.
Traditional extraction techniques					
lemon rind	umbelliferone (kvalitative analysis)	Soxhlet extraction	petrolether reextraction with acetone	solvent/sample ratio: 5 mL/1 g time: 12 h (reextraction)	Jose et al., 2014
lemon peel	herniarin (0.01 mg/L of extract)		ethanol	time: 30 days	Arigo et al., 2021
lime peel	coumarins ^b	Maceration	ethylacetate	solvent/sample ratio: 11 mL/1 g (dry peel), 5 mL/1 g (fresh peel) time: 24 h (3×)	Munawaroh, 2017
lemon peel	herniarin (1.3 mg/L of oil)	Distillation	water	ratio solvent/sample: 2 mL/1 g	Li et al., 2021
Advanced extraction techniques					
peel and waste parts of lemon, orange, grapefruit	coumarins ^b	MAE	distilled water	solvent/sample ratio: 0.8 mL/1 g time: 60–80 min	Ciriminna et al., 2017
lemon, lime peel	herniarin (185 mg/kg of lime flavedo; 3.6 mg/kg of lemon flavedo)		deionized water	solvent/sample ratio: 8 mL/1 g time: 30 s	Jungen et al., 2021
citrus peel (sweet orange, tangerine, lime)	coumarins ^b	UAE	methanol	solvent/sample ratio: 3 mL (3×)/1 g time: 30 min	Ramírez-Pelayo et al., 2019
citrus peel	scoparone (0.1–0.5 %) and other coumarins ^b	SFE	CO ₂	temperature: 40 °C pressure: 10 MPa, CO ₂ flow = 1.76 kg/h	Jerkovic et al., 2015
Citrus aurantium amara peel	coumarins ^b		CO ₂ + ethanol	pressure: 17 MPa CO ₂ flow = 2.7 kg/h extraction time = 120 min; static time = 50 min	Trabelsi et al., 2016

MAE – microwave assisted extraction, UAE – ultrasound assisted extraction, SFE – supercritical fluid extraction.

^asamples also contain other coumarins/furanocoumarins but only results for the target analytes are shown.

^bother derivatives of coumarin/furanocoumarins.

pomelo, grapefruit). HPLC-FLD chromatogram of the lime peel extract is shown in Fig. 3B. Table 2 provides an overview of the traditional and advanced extraction techniques used extraction of bioactive substances, including coumarin derivatives, from citrus fruits. Traditional techniques usually require long extraction time (several hours) and the use of organic solvents (ethylacetate, petrolether, ethanol). Advanced techniques involved in coumarins extraction, such as UAE, SFE, MAE, are mostly “greener” and they allow for efficient extraction in shorter extraction time (several minutes). The proposed UAE procedure is an example of efficient and fast extraction suitable as a sample preparation method for targeted HPLC-FLD analysis.

Selective MIP based solid phase extraction

Solvent extraction is considered a good technique for organic compound extraction from different

complex matrices; however, complex extract is mostly unsuitable for HPLC analysis in terms of possible interferences and/or concentration of analytes below LOD. Thus, the combination of UAE and subsequent SPE is useful for primary extract cleaning or analytes preconcentration with a high preconcentration factor. In addition, the use of a selective adsorbent enables the targeted extraction and isolation of analytes.

In the present work, aqueous extract of citrus peel (prepared by proposed optimal UAE conditions), was treated by batch SPE using a group selective magnetic molecularly imprinted polymeric adsorbent (Fe₃O₄@MIP imprinted with the umbelliferone molecule) produced by surface imprinting technology (Machyňáková and Hroboňová, 2017). The core-shell structure of the adsorbent (MIP coated on the surface of magnetic particles, Fe₃O₄) contain specific recognition sites for template molecules on

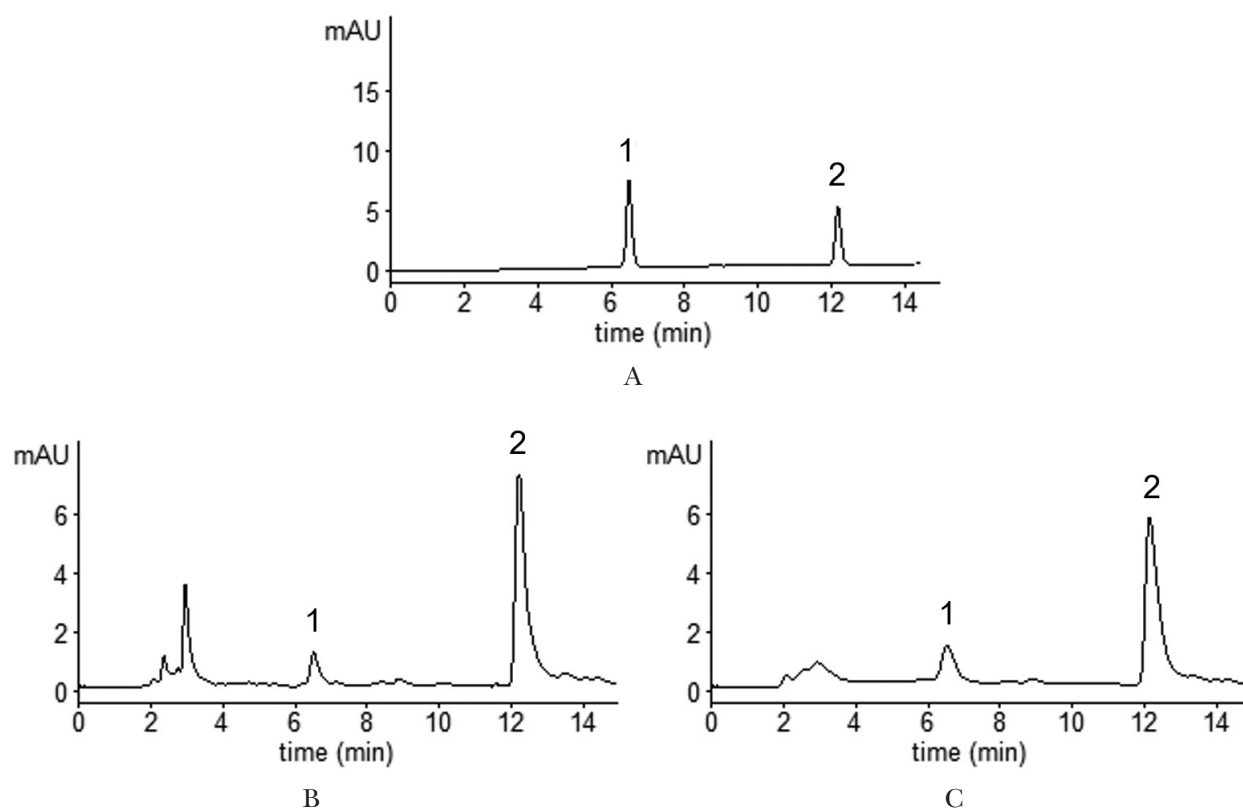


Fig. 3. HPLC-FLD chromatograms of standard solution of umbelliferone and herniarin (A), UAE aqueous lime peel extracts (B) and extract obtained by combined UAE and $\text{Fe}_3\text{O}_4\text{@MIP}$ batch solid phase extraction sample pretreatment procedure (C). Legend: 1 – umbelliferone, 2 – herniarin.

the surface of carrier particles so that the adsorbent can easily rebind and remove the templates. The advantage of $\text{Fe}_3\text{O}_4\text{@MIP}$ extraction is lower solvent consumption and the possibility of similar performance in the separation of solid and liquid phases with external magnetic field, as well as undemanding method development. The $\text{Fe}_3\text{O}_4\text{@MIP}$ -SPE procedure used in this study includes standard steps: adsorbent conditioning, analyte sorption from sample solution, adsorbent washing with water, analyte elution with methanol/acetic acid mixture (9/1, v/v) (extraction conditions were optimised in a previous work, Machyňáková and Hroboňová, 2017). SPE was verified by a recovery study with reference solution of coumarins at the concentration level of 1 $\mu\text{g/mL}$ providing satisfactory recovery values of 93.2 % and 86.9 % ($\text{RSD} \leq 4.8 \%$, $n = 3$) for umbelliferone and herniarin, respectively.

Using the proposed extraction procedure (UAE combined with $\text{Fe}_3\text{O}_4\text{@MIP}$) and HPLC-FLD quantification method, lime peel was analysed. Umbelliferone and herniarin were detected in the lime peel sample (comparing the elution characteristics with standards (Fig. 3A) and their fluorescence spectra) and quantified by the calibration curve method, providing values of 1.1 $\mu\text{g/g}$ and 454 $\mu\text{g/g}$, respectively ($\text{RSD} \leq 5 \%$, $n = 3$). A chromatogram of the

lime peel extract treated by batch extraction on $\text{Fe}_3\text{O}_4\text{@MIP}$ is shown in Fig. 3C. Chromatograms before and after MIP extraction indicated that the extract was purified and batch extraction on the $\text{Fe}_3\text{O}_4\text{@MIP}$ adsorbent can be successfully applied to selective separation of coumarins from lime peel. The peels of citrus fruits (e.g., orange, lemon, grapefruit, pomelo, or different lime species, e.g., Persian lime, Mexican lime) are different both in terms of structural diversity and composition, and thus the combination of the proposed procedures (UAE and $\text{Fe}_3\text{O}_4\text{@MIP}$ -SPE) for sample pretreatment does not always have to be applied. UAE is suitable as the first step in obtaining primary extract. $\text{Fe}_3\text{O}_4\text{@MIP}$ -SPE is convenient to use as an additional purification as well as preconcentration step before the HPLC-FLD analysis. This work shows the possibility of applying both single-step and combined sample preparation procedures for chromatographic analysis.

Research on different citrus peel (sweet orange, lemon, grapefruit, bergamot, pomelo, and clementine) has shown that orange and clementine contain lower amounts of coumarins in comparison with bergamot peel, while much higher amount was determined in flavedo. In lemon, grapefruit, and bergamot peel, trace concentrations of um-

belliferon were determined by the UHPLC-MS method (Lončar et al., 2019; Dugrand et al., 2013; Jungen et al., 2021). The content of coumarins docum, ented in previous works are comparable to the results of this work and they vary in a wide interval depending on the citrus type, e.g. from 0.8 µg/g (orange) to 140 µg/g (grapefruit) (Lončar et al., 2019). Dosoky et al. (2022) observed differences in the coumarins and furanocoumarins content in citrus oils of different crop varieties and origins within the same species. In a sample of lime oil (*Citrus aurantifolia* (Christm., Swingle, Brazil)), herniarin was not detected by the UHPLC-MS/MS method. On the contrary, in samples of lime essential oils (Mexican lime, Key lime, Persian lime), herniarin was determined in the wide concentration interval of 7.4–3880 ppm.

Conclusion

Analysis of citrus peel was applied to assess their suitability for the intended purpose, i.e., application in perfumery, cosmetic and cleaning products, or as a source of bioactive or other value-added compounds. Efficient, lab-simple, and time-saving analytical methods able to reach the requirement of high-throughput screening and ecology can contribute to wider applicability of waste natural materials. This study demonstrates that UAE and selective MIP extraction are an effective tool for sample pretreatment and isolation of coumarins from lime peel for subsequent quantification by the HPLC-FLD method. The proposed extraction conditions for selected type of lime (UAE extraction with water at 40 °C, extraction time of 10 min; Fe₃O₄@MIP extract purification and analytes preconcentration) could become routine sample preparation methods for analytical purposes and they are a perspective tool for other citrus samples analyses, as well as for future scale-up extractions of bioactive coumarins from citrus peel.

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