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Technical Report

Determination of heterocyclic oxygen compounds in *Citrus* essential oils by Supercritical fluid chromatography-tandem mass spectrometry

SFC-MS/MS: sample preparation and measurement

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Abstract:

Oxygen heterocyclic compounds (OHCs) are secondary metabolites mainly present in the non-volatile fraction of cold-pressed *Citrus* essential oils. Under this denomination coumarins, furocoumarins and polymethoxyphlavones are included. These compounds possess numerous beneficial properties for human health; however, even more often the ingestion of large amounts of coumarins or the interaction of furocoumarins with UVA rays could be toxic to human health. Due to their photoactivity, furocoumarins levels are constantly monitored by opinions and regulations issued by the International Fragrance Association (IFRA) and the European Food Safety Authority.

This research has been aimed at the validation of an analytical approach, based on supercritical fluid chromatography coupled to tandem mass spectrometry, for the analysis of OHCs in *Citrus* essential oils. Among eight columns tested, packed with different stationary phases, the pentafluorophenyl allowed the best baseline separation in 8 min and by using less than 10% of methanol. Calibration curves of twenty-eight standards (coumarins, furocoumarins, polymethoxyflavones) were constructed on spiked lemon distilled essential oil and the method was validated according to the EURACHEM guidelines, by calculating linearity, limit of detection (LoD), limit of quantification (LoQ), accuracy, intra-day, and inter-day precision. The quantitative profiles of five cold-pressed *Citrus* essential oils were determined.

Keywords: Supercritical fluid chromatography · Tandem mass spectrometry · Oxygen heterocyclic compounds · *Citrus* essential oils · Green chemistry · Quality control

Introduction

Coumarins (Cs), furocoumarins (FCs), and polymethoxyflavones (PMFs) are secondary metabolites, mainly present in the non-volatile fraction of cold-pressed *Citrus* essential oils [1], commonly named oxygen heterocyclic compounds (OHCs). These compounds possess numerous beneficial properties for human health [2]. However, ingestion of large amounts of Cs or the interaction of FCs with UVA rays could be toxic to human health [2,3]. As a consequence, the European regulation (EC) No 1223/2009 on cosmetic products includes furocoumarins in the list of prohibited substances "except for normal content in natural essences used. In sun protection and in bronzing products, furocoumarins shall be below 1 mg/kg" (EC 1223/2009) [4, 5]. However, the opinions of the International Fragrance Association (IFRA) about the maximum limit of FCs in cosmetics are numerous and have changed over the years [6,7]. Among these suggestions, the scientific articles published by Macmaster and co-workers [8] opened new discussion about the need to dispose of more sensitive method in order to quantify 15 selected FCs in finished products.

For these purpose, the investigation of these compounds has become of great interest not only for cosmetic but also for food and pharmaceutical sectors. According to the bibliographic data, the analytical techniques employed to the analysis of OHCs are mostly based on high-performance liquid chromatography (HPLC) in normal and reverse phase mode.

However, supercritical fluid chromatography (SFC) could represent a valid alternative for the analysis of Cs, Fc, PMFs, due to its benefits, such as the faster analysis time and the low solvent consumption [9].

To the best of the authors' knowledge, there are only two research articles on OHCs in *Citrus* essential oils carried out with SFC technique. The first approach refers to the analysis of PMFs from sweet orange and mandarin essential oils. In this article is reported a baseline separation of six PMFs within 6 min [10]. The second study [11] is focused to the qualitative analysis of 18 compounds among the class of FC, in lemon essential oil. The separation was obtained in 11 min by using a dimethylpentafluorophenylpropyl stationary phase. The authors concluded that this technique is suitable for studying the composition of essential oils thanks to the great advantage of short analysis time and high selectivity [11]. The present research is focused on the development of a rapid analytical method with a low impact on the environment, through the use of SFC coupled to triple quadrupole mass spectrometry detector (QqQ), in order to analyze OHCs in *Citrus* essential oils.

The SFC method has been optimized starting from the screening of different columns, in order to achieve the separation of 28 target compounds. The MRM acquisition ensured the selectivity needed for the correct quali-quantitative evaluation and provided competitive LoQs. To the best of the author's knowledge, this is the first report on the analysis of 28 OHCs in cold-pressed *Citrus* essential oils by using SFC-QqQ-MS technique.

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Experimental

Materials and samples

Byakangelicol, psoralen, byakangelicin, cnidilin, 8-geranyloxypsoralen, bergapten, phellopterin, and epoxyaurapten standards were purchased from Herboreal Ltd. (Edinburgh,UK), while aurapten, bergamottin, citropten, cnidicin, coumarin, epoxybergamottin, isoimperatorin, isopimpinellin, oxypeucedanin, oxypeucedanin hydrate, nobiletin, herniarin, isomeranzin, merazin, merazin hydrate, 5-geranyloxy-7-methoxycoumarin, sinensetin, tangeretin, tetra-O-methylscutellarein, and 8-methoxypsoralen standards were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany). LC–MS grade methanol (MeOH) and 2-propanol purchased from Merck Life Science and 4.8 grade carbon dioxide (CO_2) supplied by Rivoira were used for SFC analyses. HPLC grade ethanol (Merck Life Science) was used to prepare stock solutions and to dilute essential oils prior to the analysis. A total of 5 cold-pressed *Citrus* essential oil samples were analyzed in this study: one lemon, one bergamot, sweet orange, bitter orange, one mandarin. All samples were supplied by Simone Gatto Srl (San Pier Niceto, Messina, Italy), except one bergamot sample purchased at a local store. All standards and stock solutions were kept at – 18 °C prior to be used.

Samples preparation

Five cold-pressed *Citrus* essential oils were analyzed without any pretreatment. Before the SFC-QqQ-MS injection, each oil was diluted 1:1000 (v/v) and 1:20000 (v/v) with ethanol. Each sample was analyzed in triplicate.

SFC-QqQ-MS Instrumentation (Shimadzu)

The SFC-QqQ-MS analysis was performed on a Shimadzu Nexera-UC system (Shimadzu, Kyoto, Japan), consisting of a CBM-20A communication bus module, two LC-20ADXR dual plunger parallel-flow pumps, an LC-30ADSF CO_2 pump, a SFC-30A backpressure regulator, a DGU-20A 5R degasser, a CTO-20AC column oven, a SIL-30AC autosampler, and a LCMS-8050 triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (the entire SFC flow was directed into the MS). The entire system was controlled by the LabSolutions ver. 5.80.

Column screening and SFC-QqQ-MS method optimization

The SFC-QqQ-MS analysis was performed on a Shimadzu Nexera-UC system (Shimadzu, Kyoto, Japan), consisting of a CBM-20A communication bus module, two LC-20ADXR dual plunger parallel-flow pumps, an LC-30ADSF CO_2 pump, a SFC-30A backpressure regulator, a DGU-20A 5R degasser, a CTO-20AC column oven, a SIL-30AC autosampler, and a LCMS-8050 triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (the entire SFC flow was directed into the MS). The entire system was controlled by the LabSolutions ver. 5.80. The mobile phase chosen to evaluate the column efficiency was composed by CO_2 (solvent A) and MeOH (co-solvent); all columns were tested at 1 mL min^{-1} flow rate except F5 column (150 × 4.6 mm, 2.7 µm) which was tested at 2 mL min^{-1}. The standards were analyzed in different linear gradient modes (0–60% B; 0–30% B; 0–10% B, in 10 min). The column which provided the best separations was subjected to the evaluation of further parameters. In particular, two solvents were used as co-solvents, MeOH and 2-propanol. Two different values of back pressure were evaluated: 120 and 150 bar.

Optimized SFC-QqQ-MS method

Separation of OHCs was performed on a Core-shell Column (150 × 2.1 mm, 2.7 µm) column by using CO_2 (solvent A) and MeOH (solvent B), from 0 to 10 min increasing from 2 to 10 % of B. Flow rate: 1.0 mL min^{-1}; make-up pump flow rate: 1.0 mL min^{-1}. The injection volume was 2 µL. The oven temperature and the BPR were set at 40 °C and 120 bar, respectively. The analyses were performed with the following mass parameters: interface temperature, 350 °C; DL temperature, 250°C; heat block temperature 200 °C; nebulizing gas flow (N_2) 3 L min^{-1}; drying gas flow (N_2) 5 L min^{-1}; event time 0.024 s for each event; acquisition mode: MRM; acquisition time: 10 min for all targets. An automatic method, as included function of the software LabSolutions ver. 5.80 and based on set retention times and Q transitions, was used for peak integration.

Method validation

In order to construct OHC calibration curves, a mix of all the standards at concentration of 10 mg L^{-1} was prepared from the stock solutions and used for the following dilutions 3, 2, 1, 0.5, and 0.1 mg L^{-1}; the lower dilutions 0.05, 0.01, 0.005, 0.003, and 0.001 mg L^{-1} were prepared by using the mix of standard at concentration 0.1 mg L^{-1} as starting solution.

The linearity of the method was then evaluated by constructing the matrix-matched calibration curve considering ten concentration levels in the concentration range 0.001–3 mg L^{-1} . Five replicates were carried out for each concentration level. The significance of the y-intercept was assessed for each compound through the regression study and the curve was forced to zero when the P-value was greater than 0.05 [12].

The performance characteristics of the developed SFC procedure were investigated in validation experiments. The limit of detection (LoD) and the LoQ were calculated according to the Eurachem guidelines [22] by the "use of the white sample to create an added material" (Eurachem definition). The un-spiked blank matrix was analyzed to highlight the total bsence of Cs, FCs, and PMFs. To calculate LoDs, LoQs, accuracy (R'%), intra-, and inter-day repeatability, the distilled lemon essential oil was spiked with a concentration of 0.01 mg L^{-1}.

Accuracy (R'%) was assessed by analyzing the spiked blank matrix (n = 5), then the difference between the calculated and the real concentrations was expressed as the average of five measurements multiplied by 100, and divided for the real concentration (0.1 mg L^{-1}).

Repeatability was evaluated for all compounds as CV% of the peak areas of five replicates performed in the same day and after 1 week of storage, for intra- and inter-day repeatability, respectively.

Table 1 MS parameters for the 28 oxygen heterocyclic compounds: $[M + H]^+$, MRM quantifier (Q), qualifier (q′), and second qualifier (q″) ions (collision energy (CE) in V). Retention times, limits of detection and quantification (expressed as mg L^{-1}), linearity ranges (expressed as mg L^{-1}), and intra- and inter-day repeatability (expressed as CV%), values for each compound analyzed. C, coumarin; FC, furocoumarin; PMF, polymethoxyflavon.

N.	Compound (IUPAC name)	Class	Rt	MRM transitions				Linearity range	Repeatability		LoD	LoQ
				$[M+H]^+$	Q (CE)	q′ (CE)	q ″ (CE)		intra-day	inter-day		
1	Coumarin (chromen-2-one)	C	1.11	147	91 (-24)	103 (-19)	65 (-34)	0.05-1	0.8	1.8	0.01	0.0334
2	Isomeranzin (7-methoxy-8-(3-methyl-2-oxobutyl)chromen-2-one)	C	1.55	261	189 (-16)	130 (-30)	103 (-38)	0.01-1	0.8	5.5	0.0011	0.0036
3	Herniarin (7-methoxychromen-2-one)	C	1.6	177	121 (-21)	78 (-39)	77 (-30)	0.005-1	0.3	4.3	0.0008	0.0028
4	Oxypeucedanin (4-[[(2R)-3,3-dimethyloxiran-2-yl]methoxy]furo[3,2-g]chromen-7-one)	FC	1.86	287	203 (-13)	269 (-9)	147 (-34)	0.01-1	1.6	5.4	0.0016	0.0053
5	8-geranyloxypsoralen (9-[(2E)-3,7-dimethylocta-2,6-dienoxy]furo[3,2-g]chromen-7-one)	FC	1.87	339	203 (-17)	138 (-11)	95 (-24)	0.5-10	1.2	4.1	0.047	0.1536
6	Psoralen (furo[3,2-g]chromen-7-one)	FC	2.02	187	131 (-24)	115 (-22)	77 (-42)	0.01-1	1.2	7.3	0.0008	0.0027
7	Aurapten (7-[(2E)-3,7-dimethylocta-2,6-dienoxy]chromen-2-one)	C	2.09	299	163 (-11)	106 (-39)	119 (-27)	0.005-0.5	2.5	3.9	0.0006	0.0022
8	Meranzin (8-[[(2S)-3,3-dimethyloxiran-2-yl]methyl]-7-methoxychromen-2-one)	C	2.12	261	189 (-16)	130 (-30)	77 (-53)	0.003-3	0.4	1.3	0.0006	0.0021
9	8-methoxypsoralen (9-methoxyfuro[3,2-g]chromen-7-one)	FC	2.31	217	202 (-20)	174 (-28)	89 (-53)	0.005-1	0.5	1.1	0.0007	0.0022
10	Epoxyaurapten (7-[(E)-5-(3,3-dimethyloxiran-2-yl)-3-methylpent-2-enoxy]chromen-2-one)	C	2.45	315	163 (-17)	135 (-12)	153 (-8)	0.005-1	1.8	4.4	0.0009	0.0032
11	Cnidicin (4,9-bis(3-methylbut-2-enoxy)furo[3,2-g]chromen-7-one)	FC	2.53	355	173 (-36)	219 (-20)	287 (-9)	0.05-2	1.5	7.1	0.0032	0.0103
12	Phellopterin (4-methoxy-9-(3-methylbut-2-enoxy)furo[3,2-g]chromen-7-one)	FC	2.54	301	233 (-11)	218 (-26)	270 (-8)	0.01-1	1.4	4.8	0.0021	0.0071
13	Byakangelicol (9-[[(2R)-3,3-dimethyloxiran-2-yl]methoxy]-4-methoxyfuro[3,2-g]chromen-7-one)	FC	2.56	317	231 (-17)	233 (-11)	175 (-31)	0.001-1	0.7	1.4	0.0014	0.0046
14	Citropten (5,7-dimethoxychromen-2-one)	C	3.09	207	192 (-19)	121 (-25)	164 (-23)	0.01-1	0.5	1.7	0.003	0.0101
15	Cnidilin (9-methoxy-4-(3-methylbut-2-enoxy)furo[3,2-g]chromen-7-one)	FC	3.1	301	233 (-13)	173 (-29)	218 (-26)	0.005-1	2.4	3.8	0.0008	0.0025
16	Isopimpinellin (4,9-dimethoxyfuro[3,2-g]chromen-7-one)	FC	3.15	247	217 (-24)	232 (-17)	189 (-33)	0.005-1	0.3	3.6	0.0006	0.0019
17	Meranzin hydrate (8-(2,3-dihydroxy-3-methylbutyl)-7-methoxychromen-2-one)	C	3.54	279	149 (-14)	57 (-13)	121 (-34)	0.05-2	1.2	3.9	0.0065	0.0217
18	Bergapten (4-methoxyfuro[3,2-g]chromen-7-one)	FC	3.8	217	202 (-19)	174 (-29)	89 (-48)	0.01-1	2.3	7.3	0.0004	0.0014
19	Isoimperatorin (4-(3-methylbut-2-enoxy)furo[3,2-g]chromen-7-one)	FC	3.84	271	203 (-13)	147 (-29)	131 (-29)	0.01-1	2.1	6.5	0.0004	0.0014
20	Oxypeucedanine hydrate (4-[(2R)-2,3-dihydroxy-3-methylbutoxy]furo[3,2-g]chromen-7-one)	FC	3.99	305	203 (-19)	147 (-35)	91 (-49)	0.005-1	2.6	7.4	0.0004	0.0014
21	5-geranyloxy-7-methoxy-coumarin (5-[(2E)-3,7-dimethylocta-2,6-dienoxy]-7-methoxychromen-2-one)	C	4.01	329	193 (-16)	137 (-37)	148 (-29)	0.01-1	0.7	2.6	0.0007	0.0023
22	Bergamottin (4-[(2E)-3,7-dimethylocta-2,6-dienoxy]furo[3,2-g]chromen-7-one)	FC	4.08	339	203 (-13)	147 (-37)	131 (-35)	0.01-1	0.3	2.7	0.0018	0.0059
23	Epoxybergamottin (4-[(E)-5-(3,3-dimethyloxiran-2-yl)-3-methylpent-2-enoxy]furo[3,2-g]chromen-7-one)	FC	4.46	355	203 (-16)	153 (-8)	135 (-14)	0.01-1	1.7	3.8	0.0009	0.0032
24	Byakangelicin (9-[(2R)-2,3-dihydroxy-3-methylbutoxy]-4-methoxyfuro[3,2-g]chromen-7-one)	FC	4.48	335	317 (-8)	175 (-33)	233 (-13)	0.01-1	1.5	7.6	0.0014	0.0047
25	Tangeretin (5,6,7,8-tetramethoxy-2-(4-methoxyphenyl)chromen-4-one)	PMF	4.84	373	343 (-27)	358 (-20)	325 (-26)	0.005-1	0.4	1.4	0.0004	0.0015
26	Nobiletin (2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxychromen-4-one)	PMF	5.08	403	373 (-29)	388 (-21)	355 (-28)	0.005-2	1.7	5.6	0.0008	0.0027
27	Tetra-O-methylscutellarein (5,6,7-trimethoxy-2-(4-methoxyphenyl)chromen-4-one)	PMF	7.43	343	282 (-25)	313 (-26)	298 (-25)	0.005-1	2.3	5.9	0.0009	0.0032
28	Sinensetin (2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxychromen-4-one)	PMF	7.53	373	343 (-29)	312 (-25)	329 (-28)	0.001-1	0.3	2.2	0.0013	0.0046

Results and discussion

This research was carried out in order to validate a more environmentally-friendly analytical strategy aimed at the separation and quantification of Cs, FCs, and PMFs in cold-pressed *Citrus* essential oils. The best separation of all OCHs was obteined with column F5 (150 × 2.1 mm, 2.7 µm), Fig. 1 reports a SFC-QqQ-MS chromatogram. In addition, another F5 column with an internal diameter of 4.6 mm was tested and provided good results; however, due to the larger internal diameter, it was necessary to increase the flow rate with MeOH from 1 to 2 mL min^{-1}, but this was in contrast with the main advantage of this approach, that is the concept of green chemistry, and consequently the limited use of organic solvents. The separation of all compounds was achieved in less than 8 min, by using a F5 stationary phase and a mobile phase comprising CO_2 and MeOH. In SFC, the use of a modifier is required, to increase the solvent strength of the mobile phase and to improve the elution of polar analytes. The method developed in this work involved the use of MeOH as modifier with a flow rate of 1 mL min^{-1} was selected, despite a column with 2.1 mm internal diameter was employed. This flow rate of mobile phase can be used thanks to the use of CO_2 as main solvent of the SFC analysis conditions. In fact, it is well known that one of the main advantages of this technique is based on the gas-like capability to maintain a low viscosity, typical of gases, and consequently low back pressure. Calibration curves in MRM acquisition mode were constructed on distilled lemon essential oils according to EURACHEM guidelines (Table 1) and previous studies [22,23]. Different linearity ranges were obtained for each compound, depending on the ionization efficiency, which set the LoQ and the highest point of the curve. Good determination coefficients (R^2) were obtained, in the range 0.9952–0.9996. The regression study was performed for all compounds in order to establish the significance of the intercept. Curves were forced to zero when the P-value resulted greater than 0.05. The LoQs resulted in the range between 0.0015 and 0.1536 mg L^{-1}, as reported in Table 1. Intra-day and inter-day repeatability were reported as percentage coefficient of variation (CV%) in terms of peak areas between five replicates of a spiked blank at concentration 0.1 mg L^{-1} performed in the same day and after 1 week, respectively. All values were within a maximum shift of 8%, demonstrating the robustness of the method. R′% resulted in the range between 80.0 and 118.6%, that is within the maximum shift admitted (± 20%), meaning that the method ensures a correct quantitative evaluation. A recurring problem in the analysis of OHCs in cold-pressed *Citrus* essential oils, using a RP-HPLC/PDA technique, is the presence of critical pairs.

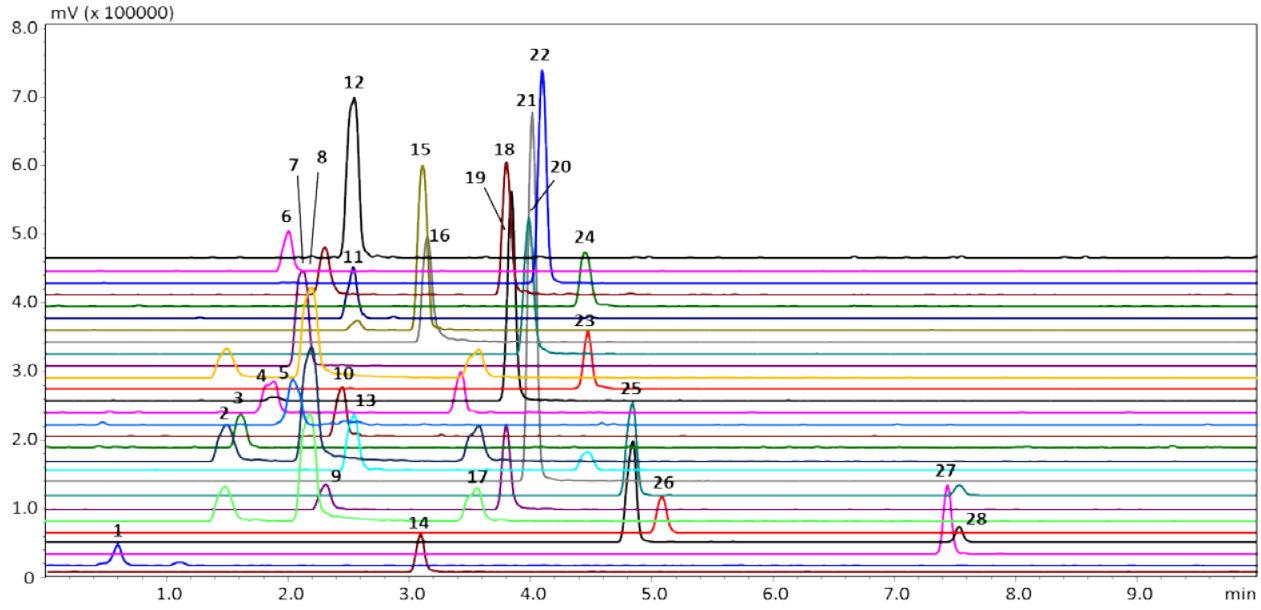

Fig. 1 SFC-QqQ-MS (MRM) chromatogram of the target compounds.

As reported by Russo et al. [15], there are four critical pairs of OHCs: citropten and isopimpinellin in lime; bergamottin and 5-geranyloxy-7-methoxycoumarin in lemon and bergamot; meranzin and isomeranzin in bitter orange and grapefruit; and nobiletin and tetra-O-methylscutellarein in mandarin and sweet orange essential oils. The partial coelution of these components has been overcome by the selectivity of the tandem MS detection in MRM mode. Regarding the SFC-QqQ-MS separation of the critical pairs cited above, citropten/isopimpinellin and bergamottin/5-geranyloxy-7-methoxycoumarin possess very similar retention time but the MRM acquisition ensures the method selectivity, allowing the correct peak integration and consequent quantification. Nobiletin/tetra-O-methylscutellarein and meranzin/isomeranzin are not critical pairs in SFC separation as can be seen from retention times reported in Table 1. On the other hand, SFC-QqQ-MS method showed different coelutions, in particular referring to peaks 11 (cnidicin)-12 (phellopterin), 15 (cnidilin)-16 (isopimpinellin), and 23 (epoxybergamottin)-24 (byakangelicin), as is possible to see in Fig. 1. However, epoxybergamottin and byakangelicin are not naturally occurring in the same *Citrus* oil, then this aspect does not represent a limitation, whereas cnidicin and phellopterin are both present in lemon essential oil and cnidilin and isopimpinellin are both contained in lime essential oil. In these cases, the MRM acquisition allows the selective quali-quantitative determination. Isomer couples are well separated, as can be observed for 8-methoxypsoralen/bergapten, 8-geranyloxypsoralen/ bergamottin, and phellopterin/cnidilin. OHCs that show the presence of an epoxy substituent in the side chain are more retained on the stationary phase surface respect to homologues without the epoxy-group, like aurapten/epoxyaurapten and bergamottin/epoxybergamottin. A similar behavior can be observed for meranzin hydrate, oxypeucedanin hydrate, and byakangelicin, more retained on F5 stationary phase respect to their more polar homologues meranzin, oxypeucedanin, and byakangelicol, respectively. This kind of behavior of SFC separation reflects the NP-HPLC separation mechanism [16].

To summarize, the SFC-QqQ/MS method resulted in a shorter analysis time and less critical pairs, compared to the HPLC–PDA [15] and HPLC–MS/MS approaches. Furthermore, methods can be compared each other from an ecological point of view, in terms of organic solvent employed for the chromatographic separation. The amount of organic solvent consumed for the LC separation, considering the elution gradient and the conditioning step, is around 15.2 mL of MeOH and 0.7 mL of THF for each analysis. The SFC separation requires 0.5 mL of MeOH plus around 0.18 mL for conditioning, and 10 mL of the same solvent used for the make-up pump, which is stopped at the end of the acquisition. To summarize, 16 mL of organic solvent are necessary for the LC method, about 10.7 mL for the SFC one. A difference of 5 mL per analysis could appear insignificant, but not in the context of using the SFC method as QC procedure for routine analysis of numerous samples per day. Five samples of cold-pressed *Citrus* essential oil were analyzed by the new optimized SFC-QqQ-MS method. Quantitative data showed that lime essential oil is the richest in OHCs content; on the other hand, clementine essential oil is the poorest. Bergamottin, 5-geranyloxy-7-methoxy-coumarin, and citropten are the OHCs most representative for lemon and bergamot essential oils. Tangeretin is the most abundant PMF in mandarin essential oils. Bergamot, and bitter orange essential oils showed the presence of all the class of OHCs. Lemon essential oil contained only Cs and FCs, while mandarin, and sweet orange samples were characterized by the presence of PMFs only. Osthol, 5-isopentenyloxy-7-methoxycoumarin, 5-demethyl-nobiletin, 5-demethyl-tangeretin, isosinensetin, hexamethoxyflavone, heptamethoxyflavone, imperatorin, and epoxybergamottin hydrate were only identified in cold-pressed *Citrus* essential oils, and not quantified due to the lack of standard materials. Definitely, the advantages related to the development and validation of the new method are multiple, going to the ecological point of view, which is the main characteristic of the SFC approach, to the sensitivity of the method, with low LoQs. Because of the short analysis time, the method could be considered for QC of FCs, or total OHCs profile, in *Citrus* essential oils.

Table 2 Quantitative results (mg L^{-1} ± standard deviation) of OCHs contained in lemon, bergamot, bitter orange, sweet srange and red mandarin cold-pressed *Citrus* essential oil samples.

Compound	Lemon	Bergamot	Bitter orange	Sweet orange	Mandarin
Bergamottin	6367 ± 85.7	20552 ± 382.6	83 ± 0.3	-	-
Bergapten	-	3248 ± 17.2	-	-	-
Byakangelicin	11 ± 0.8	-	-	-	-
Byakangelicol	1705 ± 8.7	-	-	-	-
Cnidicin	107 ± 3.6	-	-	-	-
Cnidilin	-	-	-	-	-
Epoxybergamottin	-	-	1365 ± 12.8	-	-
Isoimperatorin	140 ± 0.9	-	-	-	-
Isopimpinellin	-	-	-	-	-
Oxypeucedanin	145 ± 0.8	-	-	-	-
Oxypeucedanin hydrate	17 ± 1.6	-	-	-	-
Phellopterin	250 ± 1.9	-	-	-	-
8-geranyloxypsoralen	2155 ± 8.8	-	-	-	-
Epoxybergamottin hydrate	-	-	+	-	-
Imperatorin	+	-	-	-	-
Tot C	10897 ± 112.8	23800 ± 554.8	1365 ± 12.8	-	-
Aurapten	-	-	-	-	-
Citropten	1625 ± 50.6	3148 ± 50.8	-	-	-
Epoxyaurapten	-	-	-	-	-
Herniarin	-	22 ± 0.2	-	-	-
Isomerazin	-	-	356 ± 5.9	-	-
Meranzin	-	-	1612 ± 4.6	-	-
Meranzin hydrate	-	-	254 ± 6.8	-	-
5-geranyloxy-7-methoxycoumarin	2598 ± 29.8	1846 ± 14.2	-	-	-
5-isopentenyloxy-7-methoxycoumarin	+	-	-	-	-
Osthol	-	-	+	-	-
Tot FC	4223 ± 80.4	3356 ± 65.2	2222 ± 17.3	-	-
Nobiletin	-	-	501 ± 14.8	17 1722 ± 8.9±	1721533 ± 15.19
Sinensetin	-	240 ± 0.8	-	413 ± 20.9	54 ± 3.5
Tangeretin	-	-	944 ± 58.3	935 ± 40.2	2477 ± 13.2
Tetra-O-methylscutellarein	-	23 ± 3.6	-	514 ± 6.9	84 ± 0.1
Heptamethoxyflavone	-	-	+	--	--
Tot PMF	-	263 ± 4.4	1445 ± 73.1	3584 ± 76.9	4148 ± 31.9

Conclusions

The developed SFC-QqQ-MS method showed to be a valid and environmentally friendly analytical approach for the analysis of 28 selected OHCs in *Citrus* cold-pressed essential oils. This approach is greener than the HPLC–MS/MS approach previously developed. A fast separation has been achieved with a low consumption of MeOH in less than 8 min. All the validation parameters resulted satisfactory, with low LoQs, that could allow the quantification of these compounds even when they are contained at trace level in finished products. The quali-quantitative profiles of the 5 cold-pressed essential oils analyzed resulted quite coherent with the data previously reported. The SFC-QqQ-MS method is then clearly suitable to be applied for the quality control of OHCs in *Citrus* essential oils.

In addition, thanks to the sensitivity of the method, it could be employed for Cs and FCs determination in finished cosmetics and foods. Moreover, an on-line SFE-SFC- QqQ-MS method could be developed in the next step of this research, in order to evaluate the advantage of a rapid and direct extraction and analysis method, in terms of time saving, reduced environmental impact, and method automation of QC procedure of OHCs.

References

[1] Russo M, Rigano F, Arigò A, Dugo P, Mondello L. Coumarins, psoralens and polymethoxyflavones in cold-pressed *Citrus* essential oils: a review. J Essent Oil Res. 2021. https:// doi. org/ 10. 1080/ 10412 905. 2020. 18578 55.

[2] Hung WL, Suh JH, Wang Y. Chemistry and health effects of furanocoumarins in grapefruit. J Food Drug Anal. 2017. https://doi. org/ 10. 1016/j. jfda. 2016. 11. 008.

[3] Scott BR, Pathak MA, Mohn GR. Molecular and genetic basis of furocoumarin reactions. Mutat Res Genet Toxicol. 1976. https://doi. org/ 10. 1016/ 0165- 1110(76) 90012-9.

[4] Serrano-Pérez JJ, Olaso-Gonzáles G, Merchan M, Serrano-Andrés L. Singlet oxygen generation in PUVA therapy studied using electronic structure calculations. Chem Phys. 2009. https:// doi. org/ 10. 1016/j. chemp hys. 2009. 04. 014.

[5]European Parliament, Official Journal of the European Union, L 342 (2009) 59, 22.12.2009. Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (recast). https:// eur- lex. europa. eu. Accessed 07 Feb 2022.

[6] International Fragrance Association (IFRA). IFRA Standard. 48th Amendment *Citrus* oils and other furo-coumarins containing essential oils. 2015. http:// www. ifrao rg. org. Accessed 07 Feb 2022.

[7] International Fragrance Association (IFRA). IFRA analytical method—quantitative determination of furo-coumarins by HPLC-DAD. 2013. http:// www. ifrao rg. org. Accessed 07 Feb 2022.

[8] Macmaster AP, Owen N, Brussaux S, Brevardc H, Hiserodt R, Leijs H, Bast N, Weber B, Loesinge G, Sherlockf A, Schippa C, Vey M, Fréroti E, Tissoti, Chaintreau A. Quantification of selected furocoumarins by high-performance liquid chromatography and UV-detection Capabilities and limits. J Chromatogr A. 2012. https:// doi. org/ 10. 1016/j. chroma. 2012. 07. 048.

[9] Zoccali M, Donato P, Mondello L. Recent advances in the coupling of carbon dioxide-based extraction and separation techniques. Trends Anal Chem. 2019. https:// doi. org/ 10. 1016/j. trac. 2019. 04. 028.

[10] Dugo P, Mondello L, Dugo G, Heaton DM, Bartle KD, Clifford AA, Myers P. Rapid analysis of polymethoxylated flavones from *Citrus* oils by supercritical fluid chromatography. J Agric Food Chem. 1996. https:// doi. org/ 10. 1021/ jf960 249t.

[11] Desmortreux C, Rothaupta M, Westb C, Lesellier E. Improved separation of furocoumarins of essential oils by supercritical fluid chromatography. J Chromatogr A. 2009. https:// doi. org/ 10. 1016/j. chroma. 2009. 08. 080.

[12] Ellison SLR, Barwick VJ, Duguid TJ. Practical statistics for the analytical scientist: a bench guide. 2nd ed. RSC; 2009. https:// doi. org/ 10. 1039/ 97818 47559 555.

[13] Magnusson B, Örnemark U, editors. The fitness for purpose of analytical methods: a laboratory guide to method validation and related topics. 2nd ed. 2014. https:// www. eurac hem. org/ images/ stori es/ Guides/ pdf/ MV_ guide_ 2nd_ ed_ EN. pdf. Accessed 06 May 2022.

[14]. Arigò A, Rigano F, Micalizzi G, Dugo P, Mondello L. Oxygen heterocyclic compound screening in *Citrus* essential oils by linear retention index approach applied to liquid chromatography coupled to photodiode array detector. Flavour Frag J. 2019. https:// doi. org/ 10. 1002/ ffj. 3515.

[15] Russo M, Bonaccorsi I, Costa R, Trozzi A, Dugo P, Mondello L. Reduced time HPLC analyses for fast quality control of *Citrus* essential oils. J Essent Oil Res. 2015. https:// doi. org/ 10. 1080/ 10412 905. 2015. 10274 19.

[16] Dugo P, Favoino O, Luppino R, Dugo G, Mondello L. Comprehensive two-dimensional normal-phase (adsorption)-reversed-phase liquid chromatography. Anal Chem. 2004. https:// doi. org/10. 1021/ ac035 2981.

First Edition: March, 2023

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3656-02301-PDFIK