


METHODOLOGY

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Carbon dioxide expanded liquid: an effective solvent for the extraction of quercetin from South African medicinal plants

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Abstract

Background: Quercetin is one of the most important bioflavonoids having positive effects on the biological processes and human health. Typically, it is extracted from plant matrices using conventional methods such as maceration, sonication, infusion, and Soxhlet extraction with high solvent consumption. Our study aimed to optimize the environmentally friendly carbon dioxide-based method for the extraction of quercetin from quince fruit with an emphasis on extraction yield, repeatability, and short extraction time.

Results: A two-step design of experiments was used for the optimization of the key parameters affecting physico-chemical properties, including CO₂/co-solvent ratio, co-solvent type, temperature, and pressure. Finally, gas expanded liquid combining CO₂/ethanol/H₂O in a ratio of 10/81/9 (v/v/v) provided the best extraction yield. Extraction temperature 66 °C and pressure 22.3 MPa were the most suitable conditions after careful optimization, although both parameters did not significantly affect the process. It was confirmed by experiments in various pressure and temperature conditions and statistical comparison of obtained data. The optimized extraction procedure at a flow rate of 3 mL/min took 30 min. The repeatability of the extraction method exhibited an RSD of 20.8%.

Conclusions: The optimized procedure enabled very fast extraction in 30 min using environmentally friendly solvents and it was successfully applied to 16 different plant samples, including 14 bulbs and 2 fruits from South Africa. The quercetin content in extracts was quantified using ultra-high performance liquid chromatography (UHPLC) with tandem mass spectrometry. UHPLC hyphenated with high-resolution mass spectrometry was used to confirm chemical identity of quercetin in the analyzed samples. We quantified quercetin in 11 samples of all 16 tested plants. The quercetin was found in *Agapanthus praecox* from the *Amaryllidaceae* family and its presence in this species was reported for the first time.

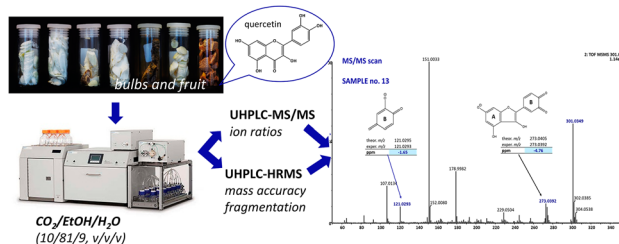
Keywords: Quince fruit, Gas expanded liquid, *Agapanthus praecox*, Targeted analysis, High-resolution mass spectrometry

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Graphical Abstract



Background

Medicinal plants have been used for the isolation of the well-known pharmaceutically active compounds and serve as the source of new compounds having the potential to treat various ailments such as cancer, influenza, malaria, pneumonia, inflammation, diarrhea, tuberculosis, and diabetes. Medicinal plants continue playing an important role in many developing countries in Asia, South America, and Africa, where about 80% of the population uses traditional medicines [1]. In South Africa, more than 30,000 plant species have been recognized, with over 3000 species commonly used in the disease treatment, cure, and management as easily available and low-cost medicine [2, 3]. Moreover, many African plant species demonstrate several promising activities against diseases related to a broad spectrum of biologically active compounds, including mainly secondary metabolites such as saponins, terpenoids, steroids, alkaloids, and phenolic compounds, including tannins, curcumins, lignans, phenolic acids, and flavonoids [4–6]. Flavonoids are the most studied group of polyphenols that have been identified in the fruits, flowers, leaves, bulbs, and other plant parts, where they are responsible for the colors [6]. From another point of view, therapeutic properties of flavonoids include antioxidant, anti-inflammatory, anticancer, and antiproliferative effects due to their free radical scavenging activities and inhibition of the synthesis of the pro-inflammatory prostaglandins and leukotrienes [5–7]. Quercetin (3,5,7,3',4'-pentahydroxyflavone) is one of the most important bioflavonoid compounds having these effects on the biological processes and thus human health. It is considered a compound helping to deal with obesity, diabetes, hypertension, hypercholesterolemia, and atherosclerosis and exhibits a vasodilator effect [4, 8–13]. As a common part of the human diet, quercetin is usually available from many foods, but it is also available as a food supplement [4]. The quince fruit containing quercetin from the deciduous tree or bush *Cydonia oblonga*, family *Rosaceae*, is a beneficial nutritive. Quince

fruits are pear- or apple-shaped with excessive and aggressive astringency. Thus, the consumption of fresh pomes is not typical. On the contrary, quince fruit is commonly used in the food industry as the main part of many products, including jam, jelly, cakes, and liquors [14–17]. The increasing interest in quince fruit is associated with beneficial health properties due to the confirmed phenolics content and antioxidant capacity as well as antimicrobial, anti-inflammatory, and hypoglycemic properties [15, 16].

The efficiency of isolation and extraction of phenolic compounds from a complex matrix is usually achieved using solvents. Conventional laboratory methods include Soxhlet extraction, sonication, infusion, digestion, decoction, percolation, and maceration. They are commonly used despite several drawbacks, such as a long extraction time, high solvent volume consumption, and reduced extraction efficiency. Thus, the methods, which overcome the bottleneck of the traditional approaches, have been introduced in the field of plant extraction. These methods include microwave-assisted extraction and ultrasound-assisted extraction using various organic solvents, which selection is based on the physicochemical properties of the extracted compounds. Nowadays, there is also growing interest in methods applying principles of green sustainable chemistry. Pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) using green solvents are among the most common green solvent extraction techniques in many applications [18–23]. While PLE is based on the application of solvents such as water and ethanol (EtOH) in the temperature over the solvent boiling point and elevated pressure, SFE uses supercritical CO_2 as the main part of the extraction solvent in elevated pressure (>7.38 MPa) and moderate temperatures (>31.1 °C). Due to its low polarity, supercritical CO_2 has limitations in the extraction of polar compounds. Thus, an organic co-solvent is typically added to the CO_2 in a small amount to change its properties and to enable the extraction of more polar compounds [22, 24, 25].

Ethanol, methanol (MeOH), isopropanol, dichloromethane, ethyl acetate, and acetonitrile are commonly used as co-solvents in SFE. Water is sometimes added to improve the extraction of polar compounds [26]. Moreover, CO₂ in its compressed liquid form can be added to organic solvent forming gas expanded liquid (GXL) with unique physicochemical properties. GXL has been shown to be an optimal solvent in many applications [27, 28]. SFE, GXLE, and PLE are commonly used to extract plant material as methods with short extraction time, low consumption of toxic organic solvent, and the possibility of thermolabile compound extraction.

Several methods using CO₂ as a part of the extraction solvent have been developed to extract quercetin from plant materials including *Strachyrtarpheta jamaicensis* leaf [29], St John's wort (*Hyperici herba*) [30], grape (*Vitis vinifera*) skin and bagasse [31, 32], *Rosa damascena* petals [33], *Achyrocline satureioides* flowers [34, 35], *Mimosa pudica* whole plant [36, 37], *Abelmoschus manihot* flowers [38], onion (*Allium cepa*) skin [39], Mango fruit (*Mangifera indica*) [40], leaves of *Morus indica* [41], *Opuntia ficus-indica* leaves [42], *Moringa oleifera* leaves [43], *Taxus chinensis* leaves [44], and sea buckthorn (*Hippophae rhamnoides*) pomace [23]. Ethanol was mostly used as co-solvents in these studies. Acetone [40], methanol [30], and ethyl lactate with water addition [23] were used in other studies, but they do not belong to preferably used co-solvents in SFE. Typically, the co-solvent amount was < 20%, only in few studies, the enhanced co-solvent content varied in the range from 30 to 85% [23, 36, 38, 40, 43, 44]. The temperatures were usually in the range of 40–62 °C, and pressures varied from 20 to 40 MPa. Only two methods did not use any co-solvent [37, 41]. Moreover, Radojković et al. used low temperature, 23 °C, and moderate pressure 15 MPa to extract quercetin from *M. indica* [41]. Most of the methods required long time for extraction, typically from 90 to 420 min. Only one SFE method took less than 1 h including high-performance liquid chromatography (HPLC) determination [32]. HPLC, mostly with ultraviolet and rarely with mass spectrometry (MS) detection, is the method preferably used for plant extract analysis [43].

Our study aimed at optimization of the method enabling selective quercetin isolation from quince fruit and other similar plant parts with an emphasis on the use of green solvents and short extraction times. The composition of extraction solvent was optimized varying conditions from supercritical fluid mostly composed of CO₂ to pressurized liquid with a high percentage of the organic solvent. The pressure and temperature effect on the physicochemical properties of the extraction solvent were tested. The optimized conditions were applied to extract other samples with expected quercetin content, including

fruits and bulbs from South African traditional medicinal herbs and plants, that are traditionally used for the illness treatment. UHPLC with quadrupole-time-of-flight high resolution mass spectrometer (HRMS) was used to confirm the quercetin presence in the extracts. The level of quercetin was quantified using UHPLC hyphenated with triple quadrupole (MS/MS) as a most suitable MS for the quantification and targeted analysis.

Results and discussion

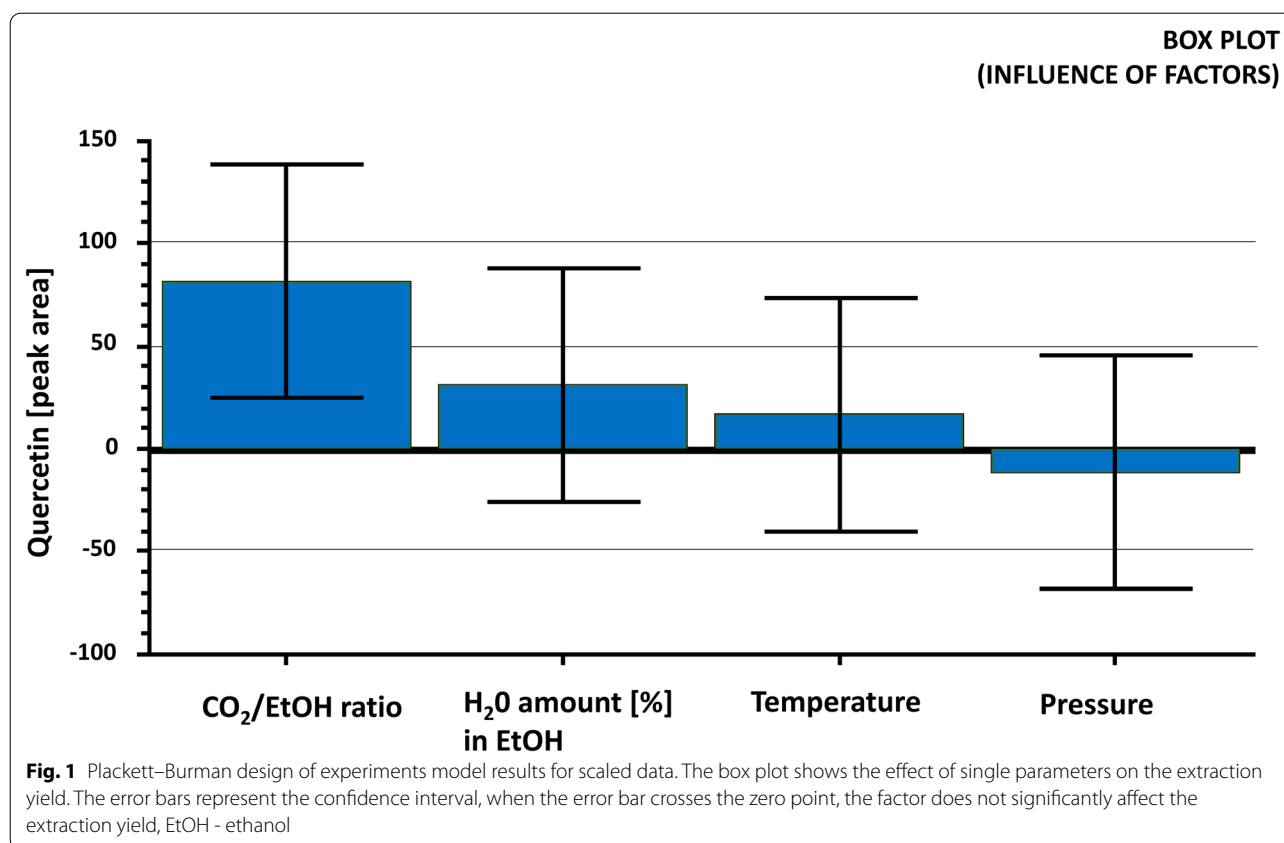
Design of experiments: key parameters optimization

The key parameters, including solvent composition, extraction pressure, and extraction temperature, affect the physicochemical properties of the extractant and thus extraction yield in both, as a single parameter and in interactions. The polarity, density, and viscosity of the extractant are changed based on the temperature, pressure, and solvent composition affecting the solubility of the analyte in the extractant. The optimization of the extraction using CO₂ in the extraction solvent was carried out using MODDE software to design all the experiments.

First, the Plackett–Burman design model was used to identify the effect of single parameters without two-factor interactions on the extraction efficiency. Four factors were tested, including (i) EtOH amount in extractant from 10 to 90%, (ii) water content in co-solvent in the range 0–20%, (iii) the extraction temperature varying in the range 30–80 °C, and (iv) extraction pressure from 10 to 30 MPa. Eleven experiments covering tested ranges were carried out, and they are summarized in Additional file 1: Table S1.

We expected the amount of co-solvent to be the main parameter affecting the extraction yield due to the quercetin structure containing hydroxyl groups and physicochemical properties such as log P 1.99. Indeed, the extraction yields were low at the lowest co-solvent percentage of 10% in the DoE. They increased with the increase in co-solvent volume and water content. Indeed, the highest extraction yields were observed when 90% of EtOH containing 20% water (experiments No. 2 and 3) and 50% EtOH with 10% water (experiment No. 9–11). The results from the preliminary study conferred a valid model with R² and Q² close to 0.8 and 0.6. It is evident that the main parameter affecting the extraction efficiency is the EtOH percentage added to the CO₂, as is shown in the box plot, Fig. 1. This result confirmed the theoretical assumption based on the physicochemical properties of quercetin.

Our study continued with the full factorial design using the Plackett–Burman results to observe single factors and their interactions. Again, the same ratio of co-solvent/CO₂ was tested. In this design, ethanol with 10% water



was used as a co-solvent throughout the study. This water content was chosen since it provided highly repeatable extraction yields in the previous design and was based on the physicochemical properties of quercetin. The temperature in the range 60–80 °C, and pressure from 15 to 30 MPa were tested in 22 experiments with an emphasis on repeatability when different conditions set up were carried out in duplicate and in triplicate for the center point, see Additional file 1: Table S2.

The results showed a valid model for the addition of ethanol with 10% water as the co-solvent. The linearity expressed as R^2 was 0.93 and future prediction precision as Q^2 was 0.79. Good repeatability and reproducibility were confirmed from summaries of fits plot and plot of the replicates. Two experiments, 6 and 17, carried out under the same conditions, had to be eliminated because of the significant difference in extracted yield that had no valid explanation. The observed results confirmed co-solvent amount as the factor most affecting the extraction yield. Indeed, a higher volume of co-solvent led to an increase in the extracted amount of quercetin. Other parameters, as well as their interactions, did not play any significant role.

The MODDE optimizer was used to find the optimal conditions for quercetin extraction. The extraction

solvent composition providing the highest extraction yields included CO₂/EtOH/H₂O in ratio 10/81/9 (v/v/v). This composition was inserted in the ternary diagram published elsewhere [23] to confirm the single-phase composition of the solvent. The effect of temperature and pressure was not so straightforward. We obtained twelve different conditions with pressures and temperatures spread in all tested ranges that should provide similar results, as summarized in Table 1.

As expected, due to the properties of gas expanded liquids, both pressure and extraction temperature did not affect the extraction yield as would be the case of neat CO₂. At high co-solvent amounts, the change in density, compressibility, and thus solubility was not affected significantly by the pressure and temperature change [27].

The extraction yields shown in Table 1 were compared statistically using MODDE and QCExpert (version 3.3.0.7., TriloByte, Czech Republic) software. The tests carried out at a confidence level of 0.95 resulted in normal, homogeneous, and independent data. The Jarque-Berr test for the residues confirmed the normal data distribution as shown in Fig. 2A, where the plotted data are very close to the line showing the ideal case. Figure 2B and C confirm the normal data distribution since the data copy the Gaussian normal distribution.

Table 1 Final conditions obtained from Optimizer, MODDE with the extracted amount of quercetin

Experiment	Co-solvent content (%)	Type of co-solvent	Water content in co-solvent (%)	Temperature (°C)	Pressure (MPa)	Extracted amount (ng/g sample)
N 01	90	EtOH	10	65.9	22.3	125.74
N 02	90	EtOH	10	62.0	16.5	122.62
N 03	90	EtOH	10	79.2	25.9	130.18
N 04	90	EtOH	10	78.0	16.5	141.34
N 05	90	EtOH	10	70.6	29.5	113.80
N 06	90	EtOH	10	60.0	28.5	124.64
N 07	90	EtOH	10	79.6	29.9	174.14
N 08	90	EtOH	10	78.0	28.5	109.12
N 09	90	EtOH	10	70.2	25.6	144.80
N 10	90	EtOH	10	60.0	15.0	130.44
N 11	90	EtOH	10	80.0	15.0	143.14
N 12	90	EtOH	10	80.0	30.0	148.46

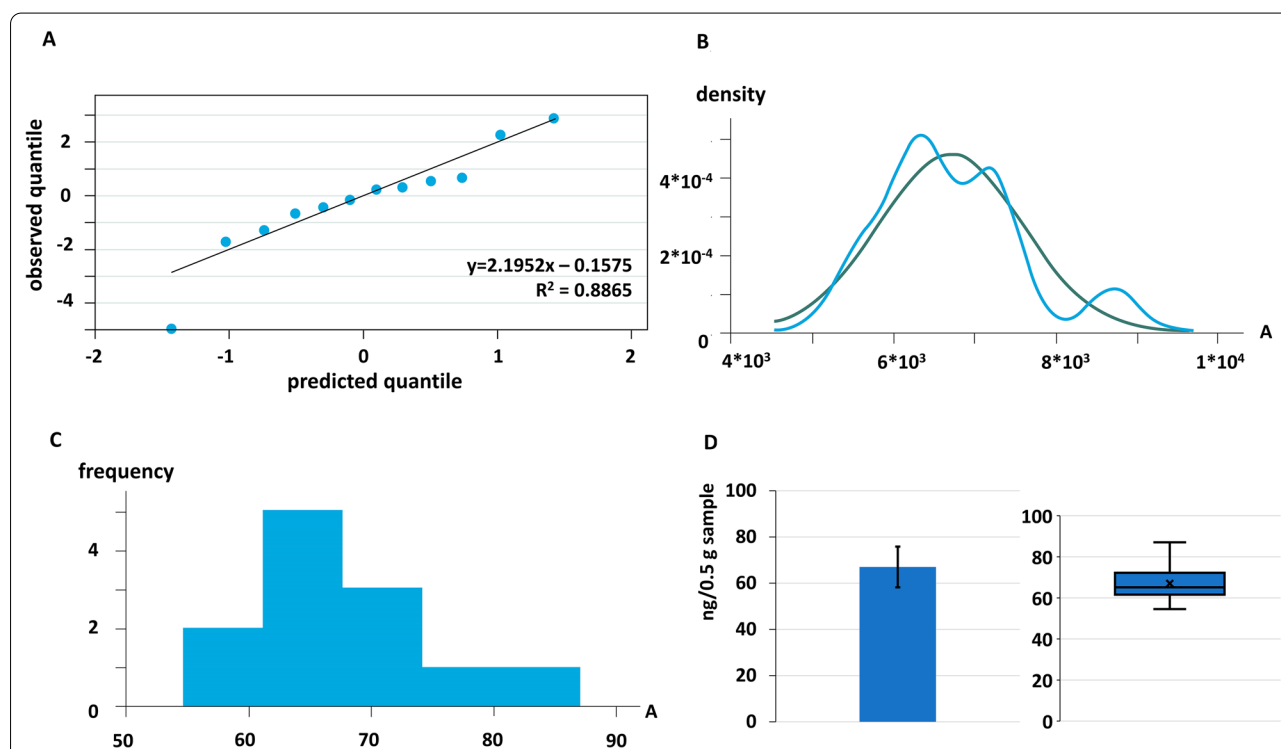


Fig. 2 Tab. Statistical comparison of setpoints for optimal extraction conditions. **A** Jarque-Berr test result showing the normal data distribution. **B** density estimation for obtained data (blue line) and Gaussian normal distribution (green line), **C** data frequency confirming the normal data distribution, **D** average extracted amount of quercetin for all tested conditions and difference between maximal and minimal obtained value

Finally, the first box plot in Fig. 2D illustrates the average extracted amount and error bar the standard deviation. The second plot shows the difference between the maximal and minimal extracted amount that was confirmed as negligible. Based on these results, we

determined final conditions for key extraction parameters, including temperature and pressure. A pressure of 22.3 MPa and a temperature of 66 °C represented mild conditions that could be easily achieved.

Final method tuning

The final step to optimize the extraction conditions was the examination of different flow rates. The flow rates 2, 3, 4, and 5 mL/min were investigated under optimal conditions for extraction solvent composition, temperature, and pressure. The fractions were collected at defined periods at all flow rates to obtain the kinetics profiles shown in Fig. 3 and assume the complete extraction of quercetin from plant material. The kinetic profiles confirm that the extracted amounts were equal in the first 5 min. Then, the curves were very close at flow rates 3, 4, and 5 mL/min, while the extraction yield was lower at 2 mL/min. Thus, the yield did not increase with the increasing flow rate after it achieved 3 mL/min, and the extraction efficiency was not affected by the solubility of the quercetin. The extracted quantities flattened at all tested flow rates after 30 min. This is likely because extraction of all compounds from the material has been completed, and/or the extraction rate is limited by the mass transfer from the matrix. Plots of the extracted amounts versus solvent volume in Fig. 3 overlap during the first part of the extraction process except for 3 mL/min flow rate. According to this result, a flow rate of 3 mL/min was considered optimal since it enabled the

highest extraction yields using the least solvent consumption in 30 min. Compared to the previously published methods summarized in the Background section, the newly optimized protocol enables shorter extraction time and as well as lower solvent consumption.

The repeatability of the optimized method was tested. Three extractions were carried out on three different days, and the relative standard deviation of the extraction yield was calculated. The average extraction yield for nine extracts was 294.37 ng/g sample with RSD 20.8%. The RSD value can be affected by the the packing of the sample and glass beads used as a dispersant in the extraction cell, residual water content in the sample, and thus the drying procedure. As this optimized method was a proof of concept, the RSD value was considered as a satisfactory for the extraction of quercetin from quince plant material using carbon dioxide expanded liquid followed by UHPLC-ESI⁺-MS/MS analysis.

Application of the optimized method in different plant samples

The method was used for the extraction of multiple plant species that were expected to contain quercetin. Various South African lyophilized plant samples from different

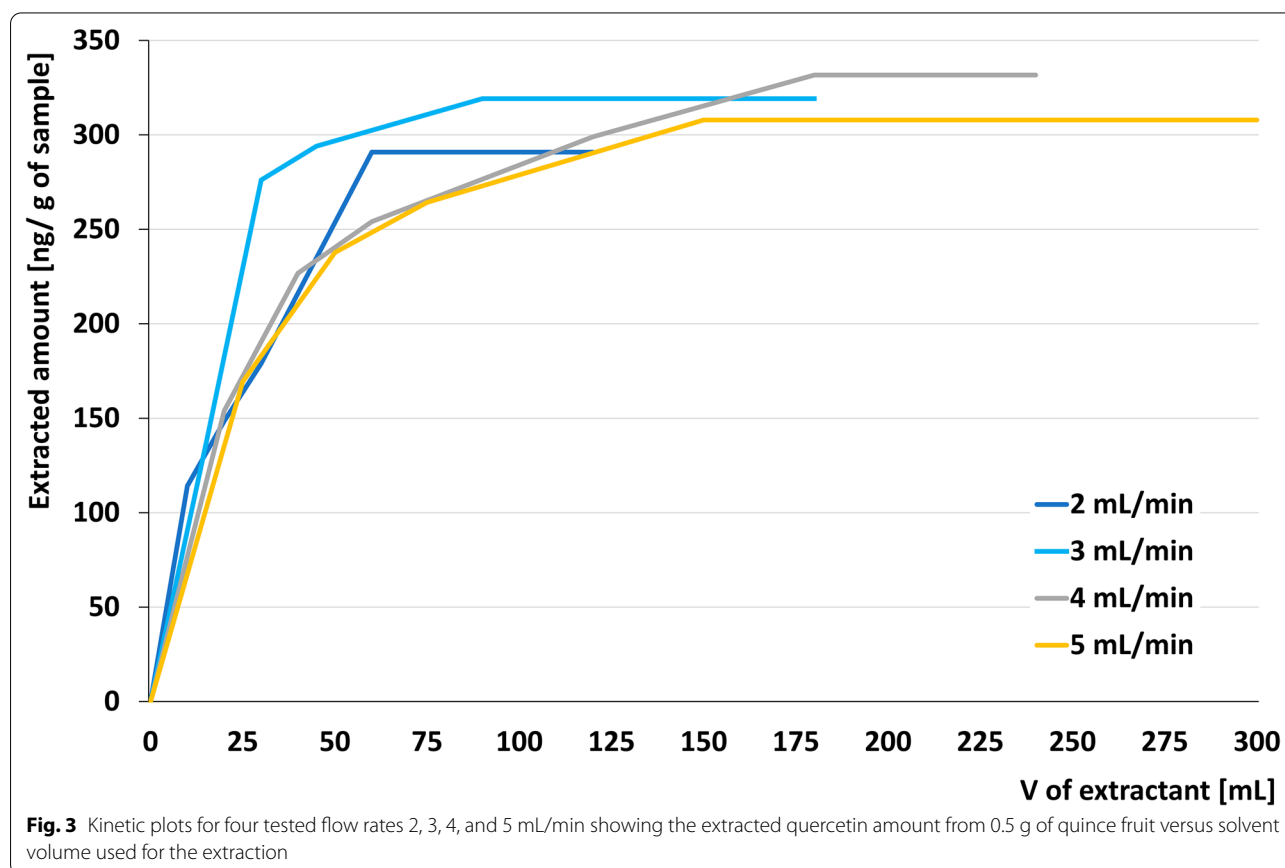


Table 2 The summary of the extracted South African plant samples, their name, extracted part, quantified quercetin amount in a sample, and results used for the quercetin confirmation in the sample.

Sample No	Latin name	Plant part	UHPLC-MS/MS analysis		UHPLC-HRMS analysis		Confirmation	Concentration Per 1 g Sample (ng)
			Concentration (ng/mL)	Deviation of ion ratios (%)	Accuracy of precursor ions (ppm/mDa)	Detection of product ions (m/z 273.0405, 271.0243, 121.0295)		
13	<i>Agapanthus praecox</i> / Amaryllidaceae	bulb	2097.7	2.2	−0.3/−0.09	+	YES	34,402.3
15	<i>Allium cepa</i> / Amaryllidaceae	Bulb	367.4	3.2	−1.3/−0.39	+	YES	5504.9
16	<i>Cydonia oblonga</i> / Rosaceae	Fruit	68.19	−0.3	−0.3/−0.09	+	YES	1118.3
5	<i>Eucomis pole-evansii</i> / Asparagaceae	Bulb	17.23	−0.2	−2.7/−0.81	+	YES	265.6
4	<i>Drimia robusta</i> / Hyacinthaceae	Bulb	16.20	2.2	−3.3/−0.99	+	YES	252.7
3	<i>Scadoxus puniceus</i> / Amaryllidaceae	Bulb	9.34	−2.3	−2.7/−0.81	+	YES	160.6
1	<i>Ornithogalum longibracteatum</i> / Liliaceae	Bulb	2.14	−5.8	−4.7/−1.41	+	YES	33.4
8	<i>Boophone hemanthoides</i> / Amaryllidaceae	Bulb	3.25	−8.3	15.9/4.79	−	MS/MS	52.0
14	<i>Kigelia africana</i> / Bignoniaceae	Fruit	2.01	11.6	−5.7/−1.71	−	MS/MS	32.6
6	<i>Albuca nelsonii</i> / Hyacinthaceae	Bulb	1.39	−0.8	−7.6/−2.29	−	MS/MS	22.8
11	<i>Haemanthus albiflos</i>	Bulb	1.30	14.7	−	−	MS/MS	30.0
7	<i>Tulbaghia violacea</i> / Amaryllidaceae	Bulb	< LOQ	−	−	−	NO	−
9	<i>Bowiea volubilis</i> / Amaryllidaceae	Bulb	< LOQ	−	−	−	NO	−
12	<i>Clivia miniata</i> / Amaryllidaceae	Bulb	< LOQ	−	−	−	NO	−
10	<i>Cyrtanthus obliquus</i> / Amaryllidaceae	Bulb	< LOQ	−	−	−	NO	−
2	<i>Merwillia plumbea</i> / Hyacinthaceae	Bulb	< LOQ	−	−	−	NO	−

ppm parts per million

UHPLC-MS/MS method: LOQ 1 ng/ml,

Designation in column "Confirmation": YES—the quercetin was confirmed by all three criteria (ion ratio, mass accuracy, and typical fragmentation), MS/MS—the quercetin was confirmed only by the UHPLC-MS/MS method based on the deviation of ion ratios, NO—the quercetin was not confirmed

plant families, including fresh quince, were extracted and analyzed to quantify the quercetin amounts. Results are shown in Table 2. While short 5 min separation using UHPLC-ESI⁺-MS/MS method was used for quercetin quantification of extracts carried out during the DoE

providing sufficient selectivity and sensitivity, a different method was employed for analysis of plant samples. Indeed, the short method was sufficient for quince analysis, but not for the analysis of bulbs. Thus, UHPLC-ESI[−]-MS/MS, used for final quantification, was based

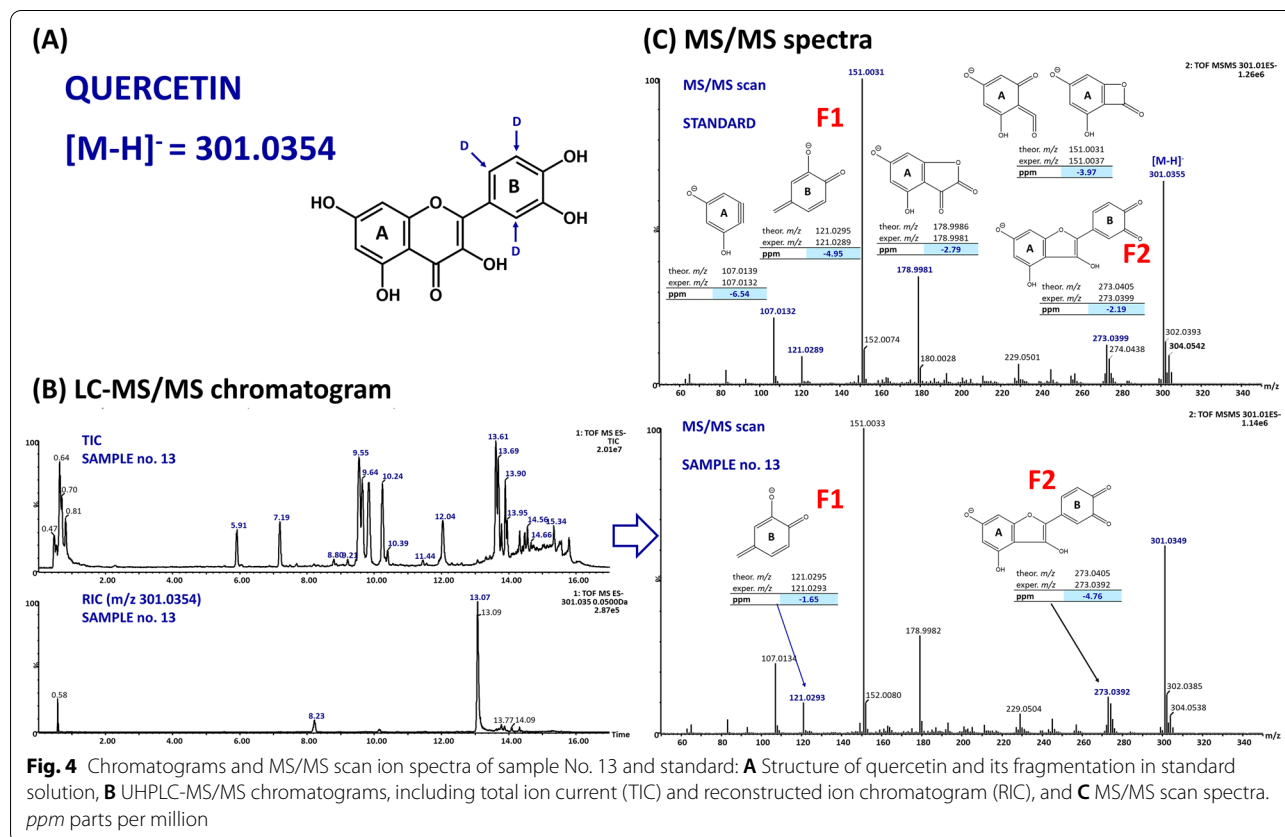
on the separation conditions as well as ionization used in UHPLC-HRMS analysis to get complementary data and easier data evaluation and confirmation. The separation method lasted 17 min and enabled the separation of compounds with a similar and close m/z to quercetin that was crucial, especially at lower quercetin concentration levels. ESI in negative mode was chosen due to better sensitivity and lower background noise in UHPLC-HRMS analysis.

The content of quercetin was calculated from the standard calibration curve in the range 0.5–100 ng/mL analyzed by UHPLC-ESI⁻-MS/MS. The limit of quantification (LOQ) with $S/N \approx 10$ and error -3.8% was determined at 0.5 ng/mL. Consequently, the limit of detection (LOD) was determined at 0.15 ng/mL with $S/N \approx 3$.

Quercetin identity was confirmed via three criteria: (i) ion ratios obtained from UHPLC-ESI⁻-MS/MS analysis, (ii) mass accuracy for quercetin precursor ion with m/z 301.0354 that should be <5 ppm (parts per million) as defined in [45], and/or <1.50 mDa specifically for quercetin, calculated from UHPLC-HRMS data, and (iii) typical fragmentation obtained from measurement of quercetin standard by UHPLC-HRMS method, as shown in MS/MS scan, Fig. 4C. Two quercetin fragments (F), namely F1 with m/z 121.0295 and F2 with m/z 273.0405, were

selected to confirm quercetin in the sample. Other fragments typical of quercetin, including m/z 107.0139, 151.0031, and 178.9986, were observed for quercetin as well as for stable isotopically labeled internal standard (SIL-IS) quercetin- d_3 , thus their use could provide false identification. As shown in Fig. 4A, these fragments were formed by the cleavage of the molecule parts containing cycle B, where the deuterium was bound in the case of SIL-IS. For samples with a concentration lower than LOQ (5 ng/mL), quercetin was confirmed only by the ion ratio calculated from peak areas of quantitative and qualitative SRM measured by the UHPLC-ESI⁻-MS/MS method. The representative chromatograms and MS/MS spectrum of quercetin for the plant extract sample are shown in Fig. 4B,C. Two confirmative fragments F1 and F2 are visible and have mass accuracy lower than 5 ppm.

Analyzed plant extracts were divided into 3 groups based on the detected and quantified quercetin levels, as shown in Table 2. The first group included *O. longibracteatum* (sample No. 1), *S. puniceus* (sample No. 3), *D. robusta* (sample No. 4), *E. pole-evansii* (sample No. 5), *A. praecox* (sample No. 13), *A. cepa* (sample No. 15), and *C. oblonga* (sample No. 16), where the quercetin contents were quantified in the range 2.14–2 097.70 ng/mL, i.e., 33.40–34,000 ng/g sample. The identity of quercetin



was confirmed using all the criteria, ion ratio for the UHPLC-ESI⁻-MS/MS method was less than 5.8%, mass accuracy of the deprotonated molecule of quercetin less than -4.7 ppm, and two typical fragments were observed (Table 2). The highest quantified amount, 34.40 µg/g sample, was observed in the bulb of *A. praecox* (sample No. 13), known as the African lily. To our best knowledge, quercetin has been determined for the first time in this species. A high quercetin amount of 5.80 µg/g sample was also confirmed in the onion bulb (sample No. 15), that is well known as the plant containing quercetin. Quince fruit (sample No. 16) also exhibited a high quercetin amount of 1.12 µg/g sample. The quercetin amount in this extract was different compared with that used during the method development, where the found concentration was lower, 294.37 ng/g sample. This difference might result from two different drying methods used in the study, as is discussed in the Experimental part. Quercetin contents quantified in all the other plant bulbs and fruit were in the range 33.40–265.34 ng/g sample, as summarized in Table 2. The MS and MS/MS spectra of standard and sample No. 13 with the highest concentration of quercetin are shown in Fig. 4.

Quercetin was also analyzed in the second group of plant species, including *A. nelsonii* (sample No. 6), *B. hemanthoides* (sample No. 8), *H. albiflos* (sample No. 11), and *K. africana* (sample No. 14). In this case, the quercetin amounts were very low, close to the LOQ in UHPLC-MS/MS analysis (Table 2). Since the concentration was very low to be seen in UHPLC-HRMS analysis, its presence was not confirmed. Despite that, the UHPLC-ESI⁻-MS/MS method allowed to quantify and confirm quercetin with respect to the deviation of the SRM ion ratio in the range -8.3–14.7% (Table 2) in these 4 plant bulbs.

Finally, quercetin contents were lower than the limit of detection and quantification in both UHPLC-ESI⁻-MS/MS and UHPLC-HRMS methods in *M. natalensis* (sample No. 2), *T. violacea* (sample No. 7), *C. obliquus* (sample No. 10), *B. volubilis* (sample No. 9), and *C. miniata* (sample No. 12), thus its content was not confirmed.

Conclusions

We developed a fast, effective, and environmentally friendly method using CO₂ as a part of solvent for the extraction of quercetin from fruit and bulb samples. The extraction solvent composition, extraction pressure, and temperature, affecting the extraction efficiency were tested. The solvent composition played the most important role, where an increase in extraction yield was observed with an increasing amount of EtOH + 10% water. In contrast, changes in the temperature in the range 60–80 °C and pressure from 15.0 to 30.0 MPa did

not show any significant effect, that was confirmed via 12 experiments under different conditions obtained as optimal from the design of experiments. Finally, the CO₂ expanded ethanol with water in a ratio of 10/81/9 (v/v/v) with 3 mL/min flow rate was used for 30 min quercetin extraction at 66 °C and 22.3 MPa enabling very short and effective extraction of quercetin from different plant material, including bulbs and fruits, compared to previously published methods.

The optimized method was successfully used for quercetin extraction in different bulbs and fruit from South African medicinal plant species. Quercetin was confirmed in 11 extracts using UHPLC-HRMS and UHPLC-ESI⁻-MS/MS. In the extract of *A. praecox* from the *Amaryllidaceae* family, quercetin was confirmed for the first time. This newly optimized method is applicable for the fast green extraction and analysis of plants in which quercetin is expected. It is planned to be applied undescribed plant samples from South Africa to extract and confirm the presence of quercetin.

Methods

Chemicals and reagents

LC-MS grade methanol (>99.9%) and formic acid (98–100%) were provided by Merck KGaA (Prague, Czech Republic). Ethanol (>99.7%) was purchased from VWR International (Prague, Czech Republic). Carbon dioxide (>99.995% purity) was obtained from Messer (Hradec Králové, Czech Republic). Ultrapure water was acquired from the Milli-Q reverse osmosis system (Millipore, Bedford, MA, USA) immediately before use.

Reference standard of quercetin (purity 98.02%) was purchased from MedChemExpress (Sollentuna, Sweden). SIL-IS quercetin-d₃ was purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).

Standard solutions

The quercetin standard stock solution and SIL-IS were prepared by dissolution in MeOH at a 1 mg/mL concentration. The solution was stored at -20 °C and was prepared fresh every week. The diluent mixture, MeOH: water (60: 40, v/v) + 0.1% formic acid, was used for the subsequent dilution of the standard solution. SIL-IS was added to the samples to obtain the final quercetin-d₃ concentration of 20 ng/mL.

Plant samples

Oven-dried quince was used for the optimization of the extraction method that required larger quantities of fruit to optimize method. In contrast, we used the freeze-dried/ lyophilized (VirTis BenchTop Pro with Omnitronics Freeze Dryer—SP Scientific, USA) plant materials for the application of the method since the optimized

method required a much smaller amount of the samples. The commercially sold fruits of *Cydonia oblonga* and the bulb of *Allium cepa* were bought in August 2019 from the Food Lovers Shopping Mall in Pietermaritzburg, South Africa. The other mature plant materials, including *Ornithogalum longibracteatum* Jacq., *Merwillia plumbea* (Lindl.) Speta, *Scadoxus puniceus* (L.) Friis & Nordal, *Drimia robusta* Bak., *Eucomis pallidiflora* Baker *pole-evansii* (N.E. Br.) Reyneke, *Albuca nelsonii* (N.E. Br.), *Tulbaghia violacea* Harv., *Boophone hemanthoides* F.M. Leight., *Bowiea volubilis* Harv. Ex Hook.f., *Cyrtanthus obliquus* (L.f.) Aiton, *Haemanthus albiflos* Jacq., *Clivia miniata* (Lindl.) Regel, *Agapanthus praecox* Willd., and *Kigelia africana* (Lam.) Benth., were collected in the last week of August 2019 from the University of KwaZulu-Natal (UKZN) Pietermaritzburg (PMB) Botanical Garden (29° 37.55' S; 30° 24.13' E) (Table 1). All the plant species were identified by the horticulturist Mrs. Alison Young, after which the voucher specimens were prepared and deposited in the Bews Herbarium at UKZN. The plant samples were grinded using IKA A11 basic analytical mill (IKA-Werke GmnH & Co.KG, Staufen, Germany) to a powder that was stored in closed amber bottle in the dark at room temperature and finally used for the extraction. The quince fruit used for the optimization was grinded in several individual parts of fruit. Consequently, all obtained powders were finally mixed together and used for the sampling. The other plant parts were grinded separately in individual grinding runs.

Analytical conditions

UHPLC-ESI⁺-MS/MS conditions for SFE optimization

Our method for the separation of phenolics and flavonoids published elsewhere [46] was used for the determination of quercetin in the samples obtained during SFE optimization. Briefly, UHPLC-MS/MS system consisted of the Acquity Ultra Performance LCTM (UPLC) system (Waters, Milford, MA, USA) coupled with Micromass Quattro MicroTM API benchtop triple quadrupole mass spectrometer (Waters, Milford, MA, USA). The separation was carried using BEH Shield RP C18 (2.1 × 50 mm, 1.7 μm) and gradient elution with 0.1% formic acid as the mobile phase A and methanol as organic mobile phase B. The gradient started at 5% B in A and was ramped to 95% B in 5 min. Then, the mobile phase composition was switched in 0.1 min to the initial and left for 2 min column equilibration. The flow rate was 0.4 mL/min and the column temperature was 40 °C. The partial loop with needle overfill mode was set up to inject 5 μL. Electrospray in positive ionization mode (ESI⁺) was used for the quercetin ionization. The MS conditions were as follows: capillary voltage + 3.2 kV; ion source temperature 130 °C; extractor 3.0 V; RF lens 0.5 V. Nitrogen was used as a

cone gas at a flow rate of 100 L/h and a desolvation gas at a flow rate 800 L/h at a temperature of 450 °C. Argon served as collision gas. Two selected reaction monitoring (SRM) channels were used for the quercetin analysis with [M + H]⁺ as a precursor ion *m/z* 302.9 to increase the selectivity of the measurement. The observed product ions were 136.92 for SRM 1 and 152.87 for SRM 2. The cone voltage was 40 V, the collision energy 30 eV, and the dwell time 0.05 s. MassLynx 4.1 software was used for MS control and data handling. QuanLynx software was used for data processing and peak integration.

The quercetin amount was calculated from the standard calibration curve analyzed in the range 1–1000 ng/mL with limit of quantification (LOQ) at 10 ng/mL (S/N ≈ 10) and limit of detection (LOD) at 3.3 ng/mL (S/N ≈ 3).

UHPLC-ESI⁻-MS/MS conditions for analysis of South African plant extracts

Quercetin in different plant species was quantified using the UHPLC system ACQUITY UPLC I-Class (Milford, USA) hyphenated with a more sensitive Xevo TQ-XS (Manchester, UK) mass spectrometer. A 2 μL sample was injected in Acquity BEH Shield RP C18 analytical column (2.1 × 100 mm; 1.7 μm). The analytes were separated using gradient elution with 0.1% aqueous formic acid (mobile phase A) and ACN (mobile phase B) at a flow rate of 0.4 mL/min. The gradient started at 2% of eluent B in A and ramped to 30% B in 12 min, followed by a steep increase to 98% B in 3 min. The percentage of B decreased during 0.1 min to the original condition (2%), followed by 2 min equilibration. The total time of chromatographic separation, including column equilibration, was 17 min. Triple quadrupole MS with ESI in negative mode (ESI⁻) was used for the quantification of quercetin. Nitrogen was used as the desolvation and cone gas and argon as the collision gas. The MS conditions were the following: capillary voltage -1.0 kV, ion source temperature 150 °C, desolvation gas flow 800 L/h, desolvation temperature 500 °C, cone gas flow 200 L/h, nebulizer gas pressure 0.6 MPa, and collision gas flow 0.20 mL/min. Two specific qualifier and quantifier SRM transitions were optimized for quercetin [M-H]⁻ precursor ion with *m/z* 300.97 to increase the selectivity of the measurement. The first product ion of *m/z* 150.48 (F1) was used for the quantification, while product ion 178.47 (F2) was used for quercetin confirmation. The ion ratio was calculated from these SRM transitions. The cone voltage was 50 V and the collision energy 20 eV for both product ions. Quercetin-d₃ was used as an internal standard to increase the selectivity of the method when applied to plant extracts. The MassLynx 4.2 Data System was used for MS control and data acquisition. TargetLynx was used

for peak integration and data processing. The method linearity was verified in the range 0.5–100 ng/mL with LOQ ($S/N \approx 10$ and error -3.8%) determined at 0.5 ng/mL and LOD at 0.15 ng/mL ($S/N \approx 3$).

UHPLC-HRMS conditions for quercetin identity confirmation in South African plant samples

The extracts were analyzed using the UPLC I-Class system (Milford, USA) hyphenated with HRMS Synapt-G2-Si quadrupole-time-of-flight mass spectrometer (Milford, USA) to confirm the quercetin content. While the LC conditions were identical as for the UHPLC-Xevo TQ-XS system, the MS conditions were different. The ESI⁻ parameters were capillary voltage -1.3 kV, sampling cone 10 V, source offset 20 V, and ion source temperature 120 °C. The nitrogen desolvation gas flow was 900 L/h and temperature 600 °C. Nitrogen was also used as the cone gas with a flow rate of 50 L/h. Argon was used as collision gas. Nebulization gas pressure was 0.65 MPa. The MS and MS/MS scans in the negative mode were used for the confirmation of quercetin in all plant samples. MS scan was acquired in the range of m/z 50–1200 and MS/MS scan of $[M-H]^-$ precursor ion with m/z 301.0354 was acquired in the range m/z 50–350 at collision energy 20 eV. Leucine enkephalin at a concentration of 200 pg/ μ L was used as the internal calibrant and 0.5 mmol/L aqueous sodium formate solution as an external calibrant. The MassLynx 4.1 Data System was used for MS control and data acquisition. The method linearity was verified in the range 0.5–100 ng/mL with LOQ ($S/N \approx 10$) determined at 5.0 ng/mL and LOD at 1.60 ng/mL ($S/N \approx 3$).

Extraction instrument

An analytical SFE system Waters MV-10 ASFE (Milford, MA, USA) consisted of a fluid delivery module for pumping liquid CO₂ and the co-solvent, an oven for placing the extraction vessels, an automated back pressure regulator for setting the pressure in the system, a make-up pump for pumping make-up solvent to prevent precipitation of the compounds after the expansion following the pressure decrease, and a fraction collector module. The heads of the CO₂ pump were cooled using a chiller operated at 5 °C. The flow was controlled as the volumetric ratio between CO₂ and the co-solvent. After the extraction, the system was flushed with a CO₂/co-solvent mixture for 5 min followed by purging with CO₂. The system was controlled by ChromScope™ software (Waters, Milford, MA, USA).

Milled dried sample (0.50 g) was placed in a 5 mL stainless steel extraction vessel and its volume was filled with glass beads. Dynamic mode of extraction was used in all

experiments. The volume of the collected extract was measured and the extract was stored at -20 °C. Prior to the analysis, 1 mL of the extract was evaporated to dryness using a vacuum concentrator at 30 °C and it was reconstituted in 100 μ L of the diluent mixture containing SIL-IS to achieve a final quercetin-d₃ concentration of 20 ng/mL. The solutions were well shaken and then injected into the UHPLC-MS/MS system.

Design of the experiments and extraction optimization

Linear Plackett Burman designs created in MODDE 12.1 software (Sartorius Stedim Data Analytics AB, Umeå, Sweden) was used in the preliminary study to evaluate the effect of single parameters without two-factor interactions, including extraction temperature, pressure, and extraction solvent composition, i.e., the water content in co-solvent and co-solvent CO₂ ratio. Based on these results, two levels of full factorial design with three center points were created and used to monitor the interaction among the factors affecting the extraction of quercetin from quince fruit. The flow rate and time were kept at 2.0 mL/min and 10 min, respectively. In total, 11 and 22 experiments were run in random order. Multiple linear regression (MLR) was used to calculate the fitting model and response surface. The adequacy of the model was evaluated by R² and Q² values, where R² shows the model fit, and Q² shows an estimate of the future prediction precision. The predicted versus observed plot and coefficient box plots were also used for the model evaluation. The optimum values for tested parameters to gain maximum peak areas and thus the highest concentration was obtained using numerical analysis based on the desirability function. The obtained optimum parameters were compared. Finally, the effects of flow rate and extraction time were studied separately. Flow rates 2, 3, 4, and 5 mL/min were investigated and the extracts were collected at the defined periods representing defined extraction times. The data were plotted as the extracted amount in μ g per g of the sample versus time and solvent amount to understand the extraction kinetics.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13007-022-00919-6>.

Additional file 1: Table S1. Summary of experiments for Plackett–Burman design of experiment model. **Table S2.** Full factorial design and summary of the experiments.

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Author contributions

VP conceived, designed, and supervised the experiments, and wrote the manuscript. LK undertook SFE optimization experiments. HKV designed and undertook UHPLC-MS/MS and UHPLC-HRMS experiments. LN helped with designing of experiments and revised the manuscript. SG, MK collected and pretreated the samples. JVS, FS revised manuscript. KD formulated the idea and revised manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations**Ethics approval and consent to participate**

Not applicable.

Consent for publication

All authors agreed to publish this manuscript.

Competing of interests

The authors declare that they have no competing interests.

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