ALTRAFLORA
NATURAL EXTRACTS

Date: 30.12.2024

Analysis report – SFE and SWE extracts

Description of sample(s)

Sample name: Extracts of rosemary (*Rosmarinus officinalis*), sage (*Salvia triloba*), and oregano (*Origanum onites*)

Sample type: Supercritical CO_2 and subcritical water extracts

Scientific name: *Rosmarinus officinalis*, *Salvia triloba*, *Origanum onites*

Manufacturing date: -

Volume/mass of sample(s): A total of 176 samples were taken for analysis (10.0–30.0 ± 0.1 mg)

Customer: -

Applied methods of sample preparation and analysis

Analysis date: 26.11.2024 – 19.12.2024

Analyst: Dr. Deniz Can Köseoğlu

Sample preparation:

Rosmarinic acid: Dilution in 20:80 methanol:water at a concentration of 1.0–3.0 ± 0.1 mg mL^{-1}

Carnosic acid and carnosol: Dilution in pure methanol at a concentration of 1.0–3.0 ± 0.1 mg mL^{-1}

Analyses and additional services:

Test supercritical fluid extractions (SFE using CO_2) and determination of extract yield (%)

Test subcritical water extractions (SWE) and determination of extract yield (%) after lyophilization

Construction of the Overall Extraction Curve (OEC) from SFE data

Gravimetric analysis of moisture content (%)

HPLC-DAD analysis of phenolic acids and diterpenes (rosmarinic acid, carnosic acid, and carnosol)

Total Phenolic Content (TPC) analysis using the Folin-Ciocalteu spectrophotometric method

Checked and approved by:

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Professor Abdullah Akdoğan

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Extracts of rosemary *(Rosmarinus officinalis)*, sage (*Salvia triloba*), and oregano (*Origanum onites*), supercritical CO_2 and subcritical water (SFE and SWE, respectively). This report was prepared for -.

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Description of laboratory sample preparation and analysis protocols

Supercritical CO_2 extraction (SFE)

Sample preparation of dried rosemary and sage consisted of grinding on a cutter with a blade speed of up to 28000 rpm to a particle diameter of ≤1 mm. The moisture content of all samples was ≤12% for SFE compatibility.

SFE extractions were carried out on a SuperEx SC-1000 system with an extraction vessel of 1 L able to withstand maximum operating pressure and temperature of 500 bar and 200 °C, respectively. The raw material was placed in the vessel using a steel loading basket equipped with 10 µm mesh filters. After sealing, the vessel was heated to the set temperature and pressurized. Upon reaching the specified temperature and pressure, extraction was carried out in a dynamic (flow-through) mode with a constant flow of CO_2. The outflow pressure after exiting the vessel was released by an automatic backpressure regulator, settling the extract in the separator. After the specified extraction time or the required solvent to raw material ratio was reached, the extract was collected using a built-in needle valve. Any residues stuck to the walls of the separator were washed off with food-grade ethanol, which was subsequently evaporated off in a rotary evaporator.

The objective of SFE was extraction of two phenolic diterpenes with antioxidant properties: carnosic acid (CA) and carnosol (CRN). This was achieved in two stages. In **Stage 1**, pure CO_2 at 100–450 bar was used to remove residual waxes from dried herbs and/or their steam distillation residues (referred to as Post-Distillation or PD samples). Subsequently, the de-waxed material was subjected to SFE with 10–15% ethanol (EtOH) as a co-solvent at CO_2 pressures of 200–350 bar. Three parameters were evaluated in all extracts: the overall extraction yield (% of loaded material), concentration of CA and CRN in the extracts (% extract), and recovery of CA and CRN from the raw material (mg g^{-1} d.w.). No second-stage SFE was carried out on oregano. All extractions with associated parameters are summarized in the table below.

Stage	Raw material	Extraction parameters					
		Pressure (bar)	Temperature (°C)	CO_2 flow (mL min^{-1}*)	EtOH flow (% of CO_2)	Time (min)	S/M**
1	Rosemary (dry)	100	45	100	-	240	92
1	Rosemary (dry)	300	45	100	-	240	82
1	Rosemary (dry)	450	45	100	-	240	96
1	Rosemary (PD residue)	350	45	100	-	240	88
1	Sage (dry)	200	45	100	-	240	90
1	Sage (PD residue)	350	45	100	-	240	191
1	Oregano (dry)	450	45	100	-	240	95
2	Rosemary (dry, de-waxed at100 bar)	350	45	100	10	180	190
2	Rosemary (dry, de-waxed at 300 bar)	350	45	100	15	180	174
2	Rosemary (dry, de-waxed at 450 bar)	350	45	100	15	180	219
2	Rosemary (PD, de-waxed at 350 bar)	350	45	100	10	180	170
2	Rosemary (PD, de-waxed at 350 bar)	200	45	100	10	180	277
2	Sage (dry, de-waxed at 200 bar)	350	45	100	15	180	187
2	Sage (PD, de-waxed at 350 bar)	350	45	100	10	180	918
2	Sage (PD, de-waxed at 350 bar)	200	45	100	10	180	309
2	Sage (PD residue, **not de-waxed**)	350	45	100	15	180	188

*Assuming density of approximately 0.890 g mL^{-1}; **The solvent/material mass ratio (*w/w*)

Subcritical water extraction (SWE)

SWE was carried out on both de-waxed and post-distillation rosemary, sage, and oregano to extract rosmarinic acid (RA), which is a potent phenolic antioxidant. Extractions with water heated past 100 °C under pressure were carried out on the same SC-1000 system used for SFE. Water was either added to a pre-heated

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extraction vessel up to the set pressure, maintained for a certain time, and syphoned via a relief valve (static extraction mode), or continuously pumped through the vessel and a cooling coil into a separator (dynamic extraction mode). Similarly to SFE, the overall yield (% d.w.), RA concentration (% of extract), and recovery (mg g^{-1} d.w.) were evaluated at various temperature and extraction times. Comparison with similar extract obtained via spray-drying were also made. All SWE extractions are summarised in the table below.

Raw material	Extraction parameters					
	Pressure (bar)	Temperature (°C)	Extraction mode	Water flow (mL min^{-1})	Time (min)	S/M
Rosemary (dry, de-waxed at 450 bar)	50	130	Static	30	20	14
	50	150	Dynamic	30	20	32
	50	150	Dynamic	30	60	121
Oregano (dry, de-waxed at 450 bar)	50	125	Static	30	20	14
	50	125	Dynamic	30	20	26
	50	110	Dynamic	30	25	21
Rosemary* (post-distillation residues)	50	140	Dynamic	30	35	7
Oregano* (post-distillation residues)	50	140	Dynamic	30	35	14

*5 replicate extractions carried out

Sample preparation of CA, CRN, and RA for analysis by liquid chromatography (HPLC-DAD)

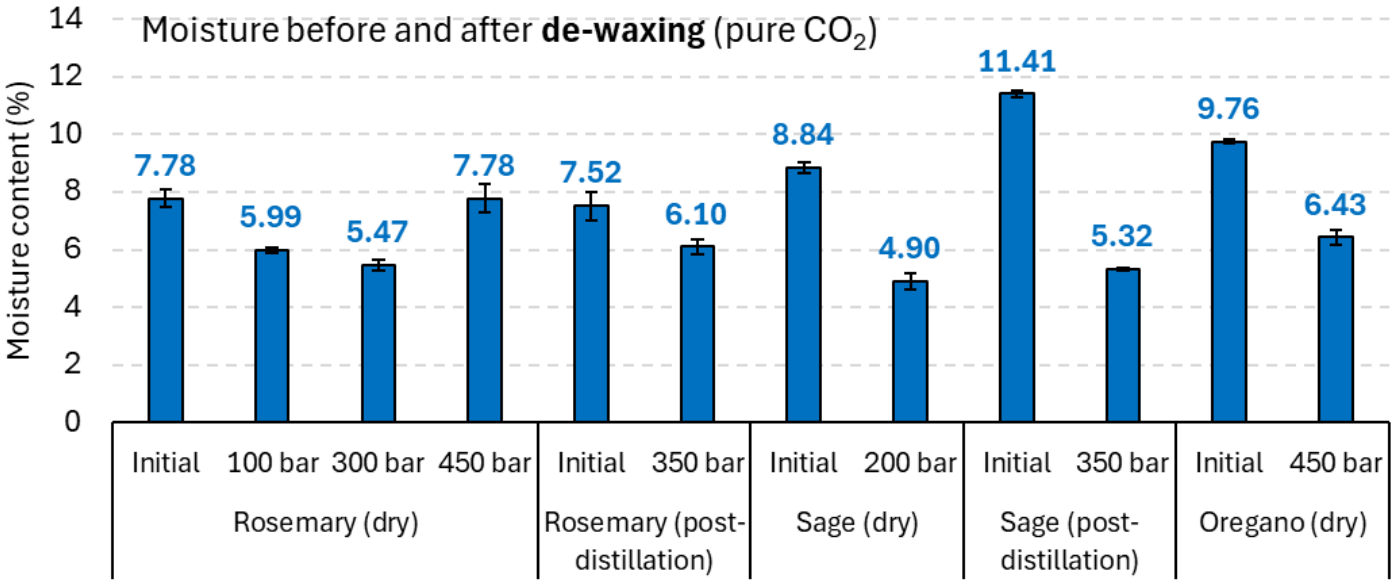
A sample of the extract (10.0–30.0 ± 0.1 mg) was diluted in either 20:80 methanol:water (for rosmarinic acid) or pure methanol (for carnosic acid and carnosol) at a concentration of 1.0–3.0 ± 0.1 mg ml^{-1} in a glass vial, shaken by hand (1 min) and vortexed (1 min). Then, an aliquot of 1 ml was passed through a membrane filter (PVDF, 0.22 µm) into a vial for HPLC-DAD analysis (injection volume 10 µl).

The following equipment and parameters were used to analyze the obtained solution: Shimadzu i-Series LC-2050-3D liquid chromatograph with diode array detector and a C_{18} column (250 × 4.6 mm, 5 µm). For carnosic acid and carnosol, isocratic elution with acetonitrile : water with 0.5% H_3PO_4 (65 : 35 *v/v*, 1.5 mL min^{-1}) for 35 min was carried out at a column temperature set to 25 °C. For rosmarinic acid, gradient elution was carried out with water (0.1% H_3PO_4) : methanol (0.1% H_3PO_4) as mobile phases A and B, respectively. The elution program was as follows: 40% B was increased to 50% in 10 minutes, 60% in 5 minutes (held for 10 min), and returned to 40% in another 5 minutes (held for 5 min for equilibration). The flow rate was 1.0 mL min^{-1} and the column was thermostated at 30 °C. Peak area measurements were performed at wavelengths of 230 nm (carnosic acid and carnosol) and 330 nm (rosmarinic acid). Calibration was performed using a series of standard solutions (20–500 ppm).

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General information – Moisture content in samples

Sample type	Measurements (%)			Mean ± SD
	1	2	3	
Rosemary (dry)	7.64	8.15	7.56	7.78 ± 0.32
Rosemary (dry, de-waxed at 100 bar)	6.07	5.9	6.01	5.99 ± 0.09
Rosemary (dry, de-waxed at 300 bar)	5.51	5.64	5.25	5.47 ± 0.20
Rosemary (dry, de-waxed at 450 bar)	7.23	8.15	7.97	7.78 ± 0.49
Rosemary (PD residue)	8.09	7.23	7.24	7.52 ± 0.49
Rosemary (PD residue, de-waxed at 350 bar)	5.8	6.25	6.25	6.10 ± 0.26
Sage (dry)	8.61	9.00	8.9	8.84 ± 0.20
Sage (dry, de-waxed at 200 bar)	4.93	5.19	4.59	4.90 ± 0.30
Sage (PD residue)	11.54	11.4	11.29	11.41 ± 0.13
Sage (PD residue, de-waxed at 350 bar)	5.35	5.34	5.26	5.32 ± 0.05
Oregano (dry)	9.82	9.68	9.78	9.76 ± 0.07
Oregano (de-waxed at 450 bar)	6.55	6.11	6.62	6.43 ± 0.28



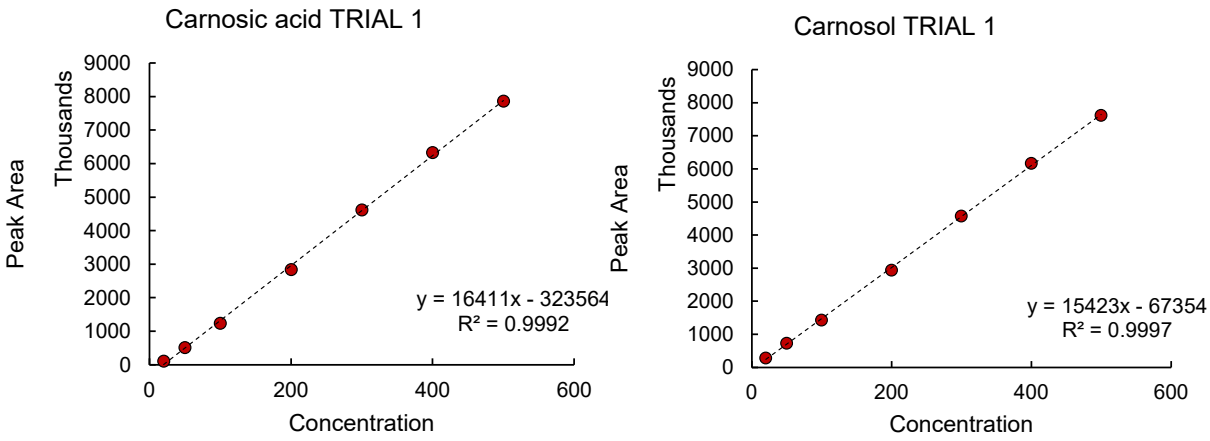
It is apparent that moisture content generally decreases after SFE dewaxing treatment.

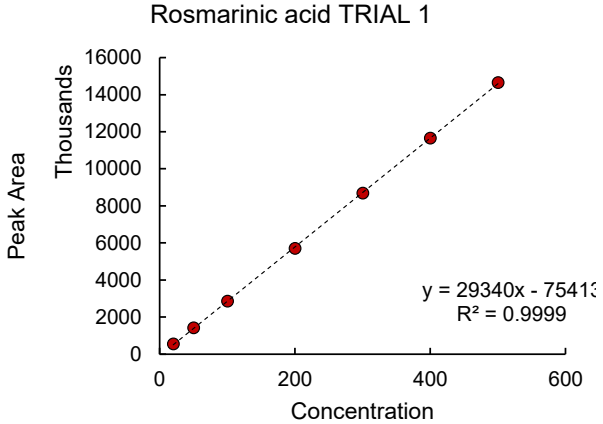
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General information – Calibration data and graphs for carnosic acid, carnosol, and rosmarinic acid

Analyte name	CAS	Concentration (ppm)	RT* (min)	Peak area
Carnosic acid	3650-09-7	20	27.241	109802
		50	26.534	509193
		100	26.136	1236326
		200	25.790	2838862
		300	25.563	4617797
		400	25.421	6329691
		500	25.327	7858954
Carnosol	5957-80-2	20	14.587	283862
		50	14.597	728595
		100	14.601	1427531
		200	14.612	2942169
		300	14.597	4573910
		400	14.592	6171612
		500	14.583	7615242
Rosmarinic acid	20283-92-5	20	13.861	554877
		50	13.853	1425575
		100	13.856	2851899
		200	13.851	5708528
		300	13.860	8688947
		400	13.857	11654226
		500	13.851	14652620

*Retention time

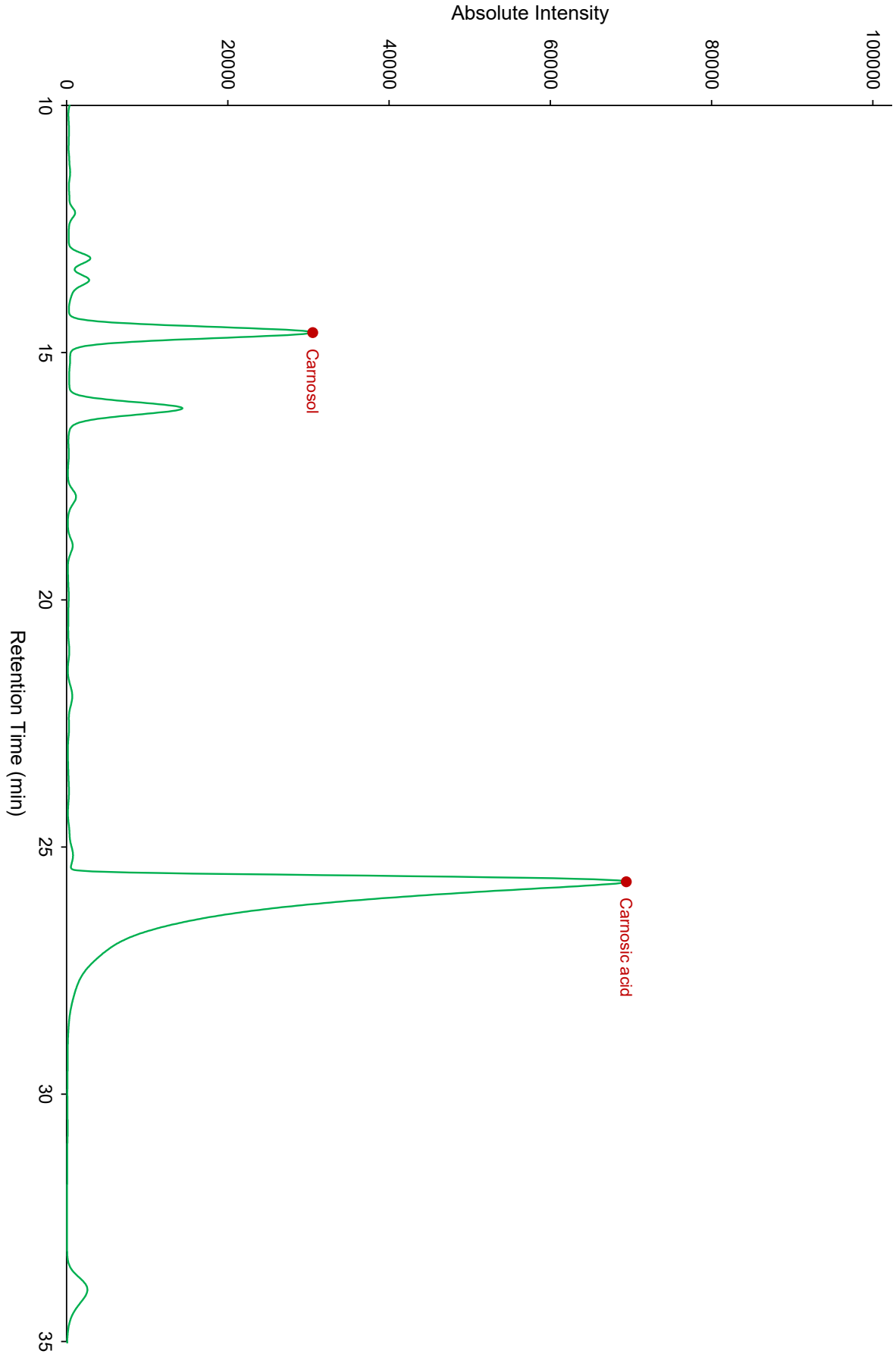




Extracts of rosemary *(Rosmarinus officinalis)*, sage (*Salvia triloba*), and oregano (*Origanum onites*), supercritical CO_2 and subcritical water (SFE and SWE, respectively). This report was prepared for -.

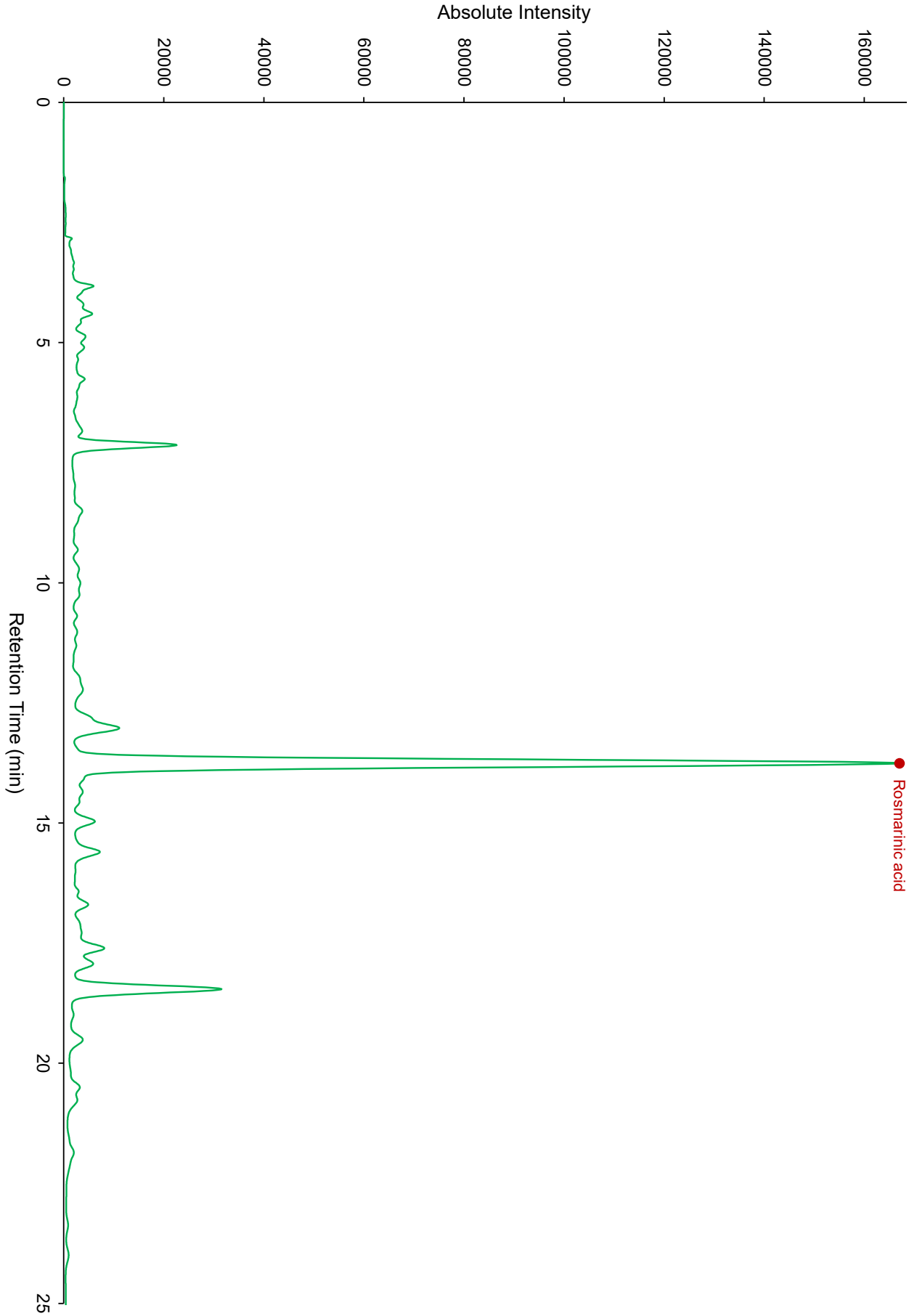
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Results – Example chromatogram of carnosic acid and carnosol (HPLC-DAD)



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Results – Example chromatogram of rosmarinic acid (HPLC-DAD)

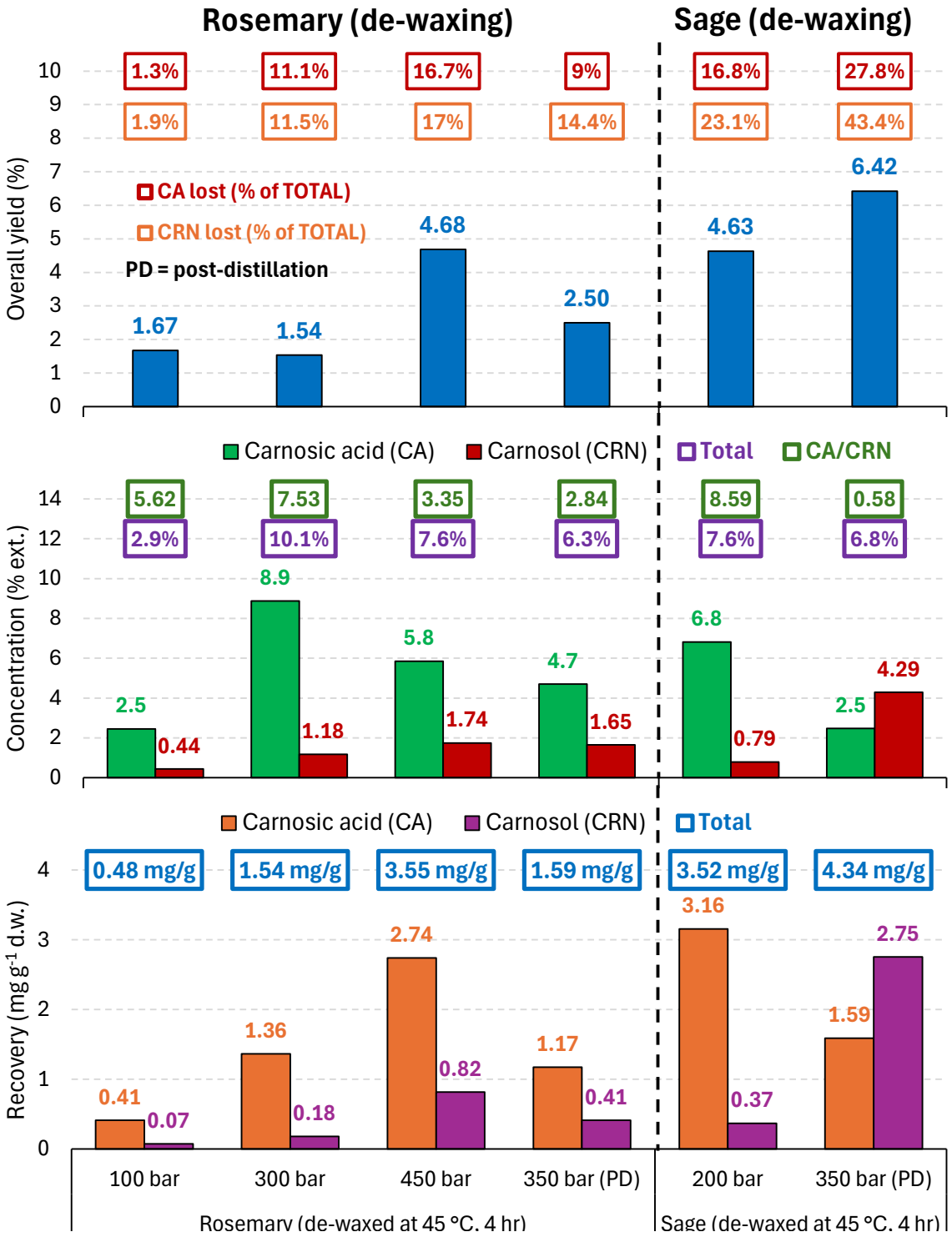


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SFE results – Effect of de-waxing on content of carnosic acid and carnosol in extracts

The potential losses of carnosic acid and carnosol occurring during SFE with pure CO_2 were evaluated by comparing overall yields, concentrations, and recoveries of carnosic acid and carnosol following de-waxing treatments at various pressures ranging from 100–450 bar. The results are summarised in the graph below.



It is apparent extraction at higher pressures causes an increasing proportion of total carnosic acid (CA) and carnosol (CRN) present in the raw material to be extracted despite no co-solvent being used. Here, total CA and CRN is defined as the amount extracted by successive SFE with and without co-solvent. The loss is most evident with rosemary, where raising the pressure from 100 bar to 450 bar increases the loss of CA and CRN from 3.2% to a significant 33.7%, extrating >3.5 mg g^{-1} of the raw material. A similar increase in CA and CRN loss is also observed

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for sage, where 4.34 mg g^{-1} (71.2%) are lost at 350 bar compared to 3.52 mg g^{-1} (39.9%) at 200 bar, despite the fact that the former extraction was carried out on post-distillation residues. Another observation was the generally lower CA/CRN ratio observed in the post-distillation residues. Since CRN is a degradation product of CA, this is attributable to thermal degradation occurring in the material during distillation. A particularly precipitous decrease is seen with sage, where CA/CRN drops from 8.59 to 0.58 (such that CRN > CA) despite a higher total recovery. These observations may point to the unsuitability of distillation residues for diterpene extraction.

SFE results – Extraction of CA and CRN from de-waxed plants using SFE with ethanol co-solvent

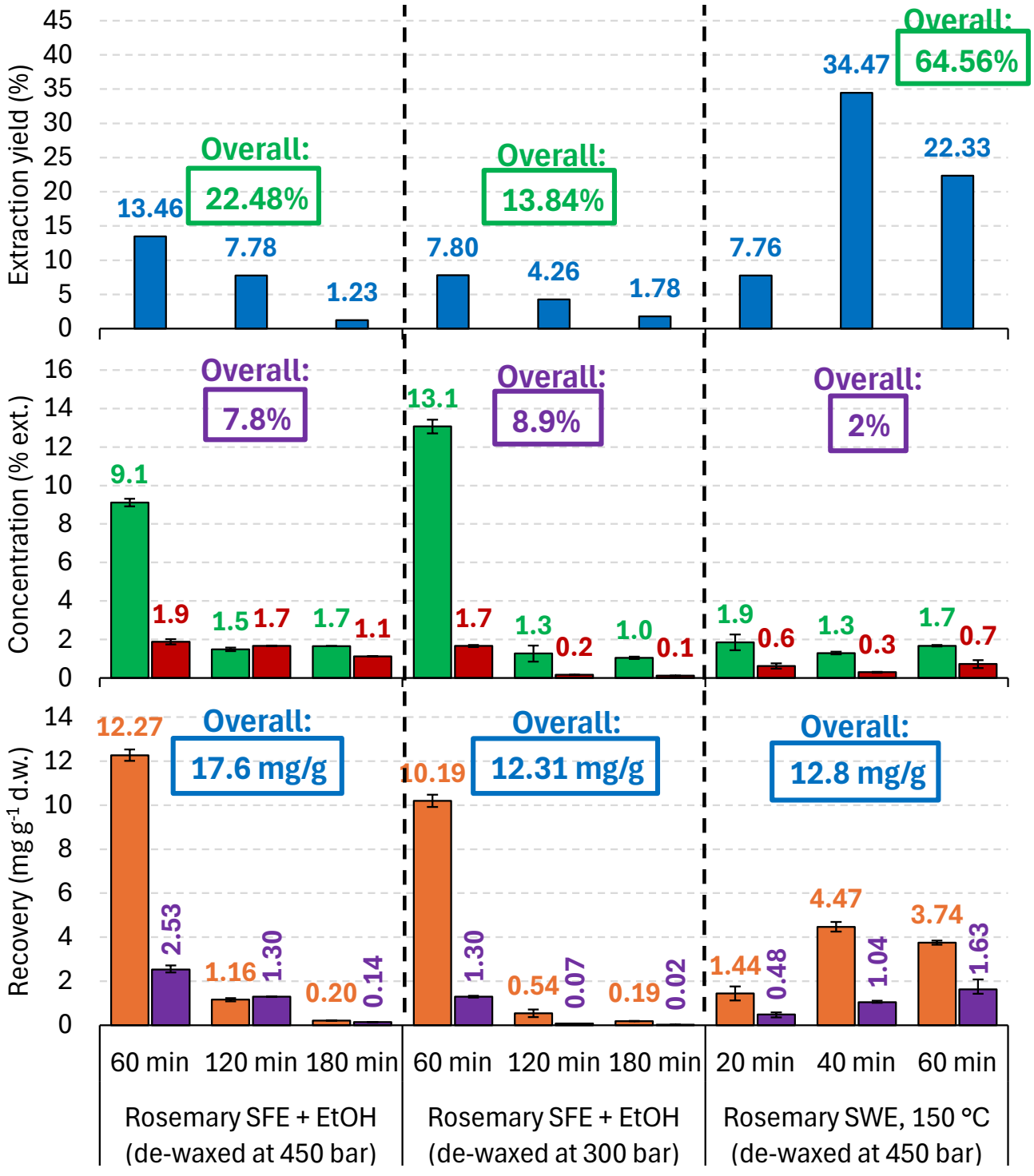


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The graph above summarizes the results of SFE with 10–15% ethanol co-solvent following de-waxing of freshly dried and previously steam-distilled plant material. It is immediately evident that rosemary dewaxed at 100 bar yielded by far the highest total recovery and CA/CRN ratio relative to other rosemary samples, while the lowest recoveries (despite a higher overall extract yield) were obtained from post-distillation residues. Similarly, sage dewaxed at 200 bar yielded an impressive CA/CRN ratio of 12.78 at a total concentration of 17.7% (despite a lower overall yield), while post-distillation residues never exceeded a ratio of 1.15 at a <8% concentration. In fact, without de-waxing, more CRN was recovered relative to CA (a ratio of 0.84). It also appears that extraction with co-solvent at 350 bar is preferable in all respects relative to that at 200 bar for both rosemary and sage.

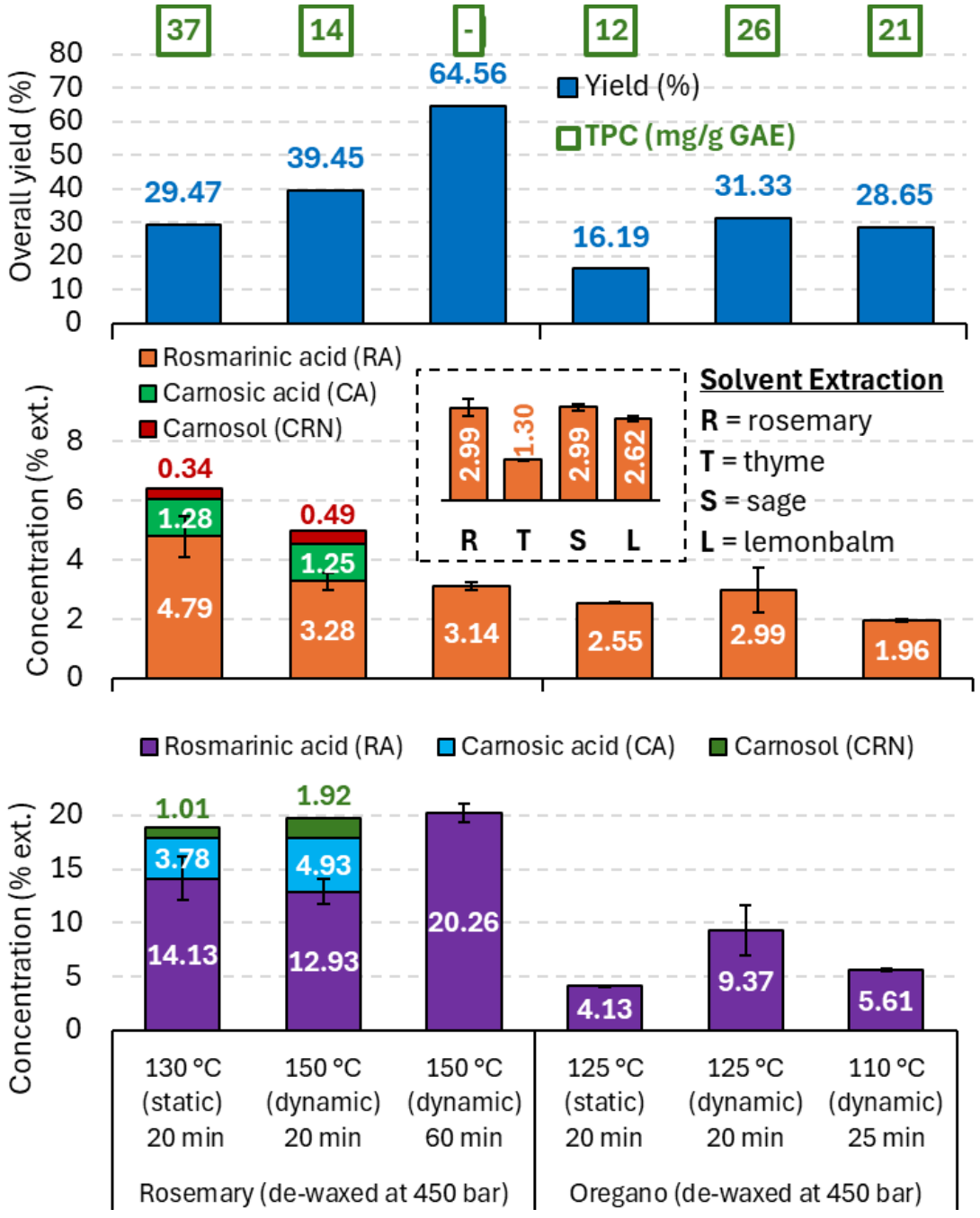
SFE results – Determination of optimal extraction time through periodic extract collection



Extracts of rosemary *(Rosmarinus officinalis)*, sage (*Salvia triloba*), and oregano (*Origanum onites*), supercritical CO_2 and subcritical water (SFE and SWE, respectively). This report was prepared for -.

Overall yields, concentrations, and recoveries of CA and CRN in extract fractions collected every 20–60 minutes are summarized in the graph above for 3 extractions. These include two SFE processes with 10% co-solvent (350 bar) run on rosemary dewaxed at 450 bar and 300 bar and collected every hour. A subcritical water extraction (SWE) at 150 °C was also included for comparison. Evidently, the majority of CA and CRN are recovered in the first hour of extraction (at a CO_2/material ratio of **ca. 23 g g^{-1}** and an EtOH/material ratio of **ca. 3.5 mL g^{-1}**), while continuing extraction for another hour increases the overall yield but causes significant dilution of the extract. Extracting for an additional hour is of little benefit as both overall yield and recovery decrease dramatically. Another observation is the significantly reduced concentration of CA and CRN obtained via SWE (2% versus >8% via SFE), despite similar total recovery of ca. 12.8 mg g^{-1} (most of which occurs at 40 and 60 minutes of extraction). Coupled with a dramatically higher overall yield (65% versus 14–22% via SFE), this highlights the reduced selectivity of SWE relative to SFE.

*SWE results – Recovery and concentration of rosmarinic acid (**TRIAL 1**)*



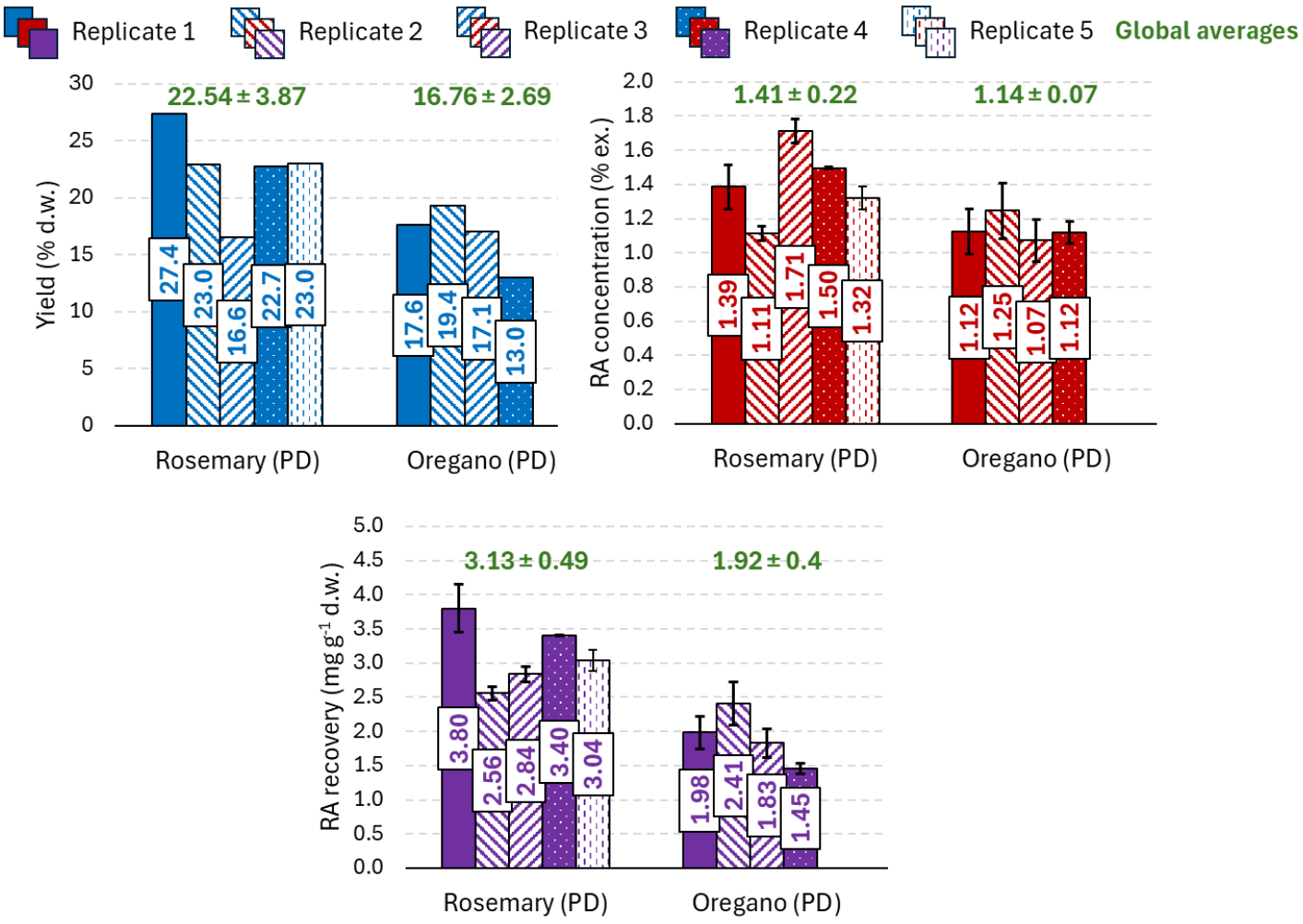
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The figure above highlights the high overall yields (30–65%) and recoveries of the highly polar rosmarinic acid (RA) from both dewaxed rosemary and oregano achievable by SWE in 20–60 minutes. Generally higher recoveries were achieved for rosemary (12–20 mg g^{-1}) compared to oregano (4.13–9.37 mg g^{-1}), although this may partly be explained by a higher extraction temperature. Notably, significantly more RA was recovered from rosemary relative to the less polar (and therefore less water-soluble) CA and CRN, which highlights the different selectivity of SWE compared to SFE. Concentrations of RA (% extract) were similar and often higher than in organic solvent (aqueous ethanol) extracts of various Lamiaceae plants, including rosemary, thyme, sage, and lemonbalm.

For oregano, switching from static to dynamic mode at 125 °C without increasing the extraction time (20 min) significantly boosted the recovery and concentration of RA from 4.13–9.37 mg g^{-1} and 2.55–2.99 %, respectively. Conversely, a similar switch from static to dynamic extraction for rosemary while increasing the extraction temperature from 130 to 150 °C slightly reduced both of these metrics, which may indicate thermal degradation of RA at 150 °C or below. Despite this, increasing the extraction time from 20–60 minutes resulted in a much higher overall yield (40–65 %) and RA recovery (13–20 mg g^{-1}). These observations indicate that extraction at a maximum of 140 °C at up to 30–60 minutes could represent a good balance of high recovery and minimal degradation.

*SWE results – Rosmarinic acid in **post-distillation residues** of rosemary and oregano (**TRIAL 2**)*

Five replicate SWE extractions were carried out on **post-distillation** residues of rosemary and oregano obtained locally in order to compare the overall yield, as well as the concentration and recovery of rosmarinic acid (RA) to those obtained from freshly dewaxed plant material that did not undergo distillation at ca. 100 °C. The results are summarized below.



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It is apparent that while the **overall yield** is comparable to that using freshly-dewaxed material, the **recovery** and **concentration** of rosmarinic acid are both **significantly lower**. This may indicate partial degradation of rosmarinic acid during distillation such that a consistently smaller amount is present in post-distillation residues. Nonetheless, the recovered amount could be sufficient for the use of such extracts as natural antioxidants. Further testing is required.

Main outcomes

SFE conclusions:

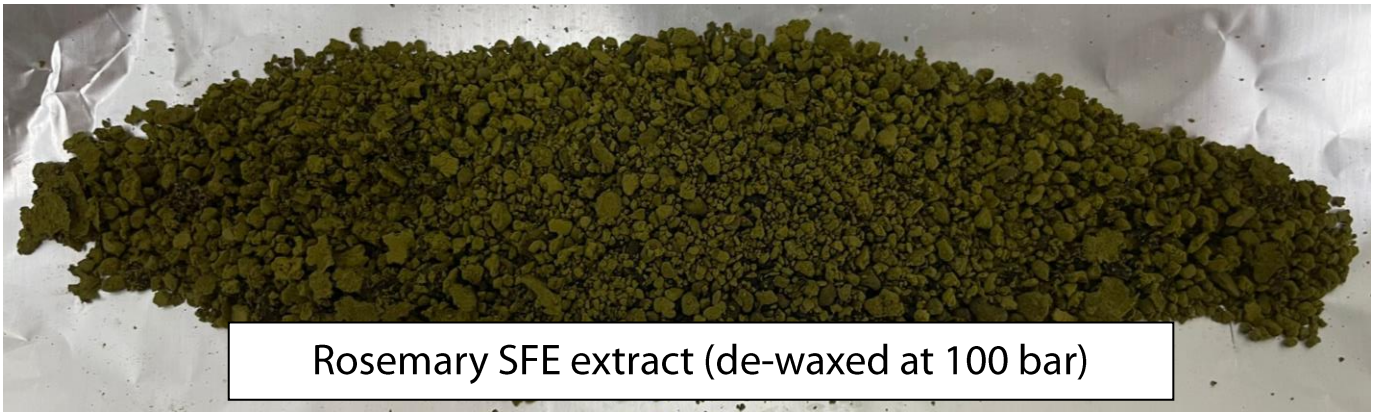
- De-waxing using SFE with pure CO_2 results in **losses of CA and CRN** at pressures **>100 bar**
- Recoveries and concentrations of CA and CRN are **significantly lower in post-distillation residues** compared to the freshly dried and dewaxed plant material
- The ratio of CA/CRN is similarly lower in distillation residues, indicating degradation of CA to yield more CRN
- Overall, combined CA and CRN **concentrations** of **>15–17%** and **recoveries** of **>20–30 mg g^{-1}** are achievable with freshly dried rosemary or sage **dewaxed at <200 bar**. Total yields can reach **>20%** and **>10%** for rosemary and sage, respectively.
- The **majority (>90%)** of total CA and CRN is extracted in the first **60–75 minutes**, with extended extraction times diluting the total concentration by a **factor of 2 or more**.
- A desirable CA/CRN ratio is **>8–12**, such that **CA comprises >85–90% of the assemblage**.

SWE conclusions:

- A similar recovery of CA and CRN to SFE is achievable with SWE (subcritical water), but **significant co-extraction** of water-soluble phenolic acids reduces their concentration to <2%.
- Conversely, extraction of RA (rosmarinic acid) is only possible with SWE. Concentrations of **ca. 5%** at **30% yield** are achievable at **130 °C** with rosemary.
- Concentrations decrease to **ca. 3% at 150 °C**, possibly due either to thermal degradation or reduced selectivity.
- Raising the extraction time from **20 to 40–60 minutes** increases extraction yield approximately two-fold.
- **Dynamic** extraction yields **better** recoveries and concentrations relative to **static** extraction at the same temperature.
- Based on the above, a good combination of parameters for RA extraction are a temperature of **140 °C**, **dynamic** mode, and an extraction time of **40–60 minutes (S/M > 20)**.
- SWE of post-distillation residues appears to yield significantly less rosmarinic acid relative to extraction of freshly dewaxed plant material that did not undergo distillation.

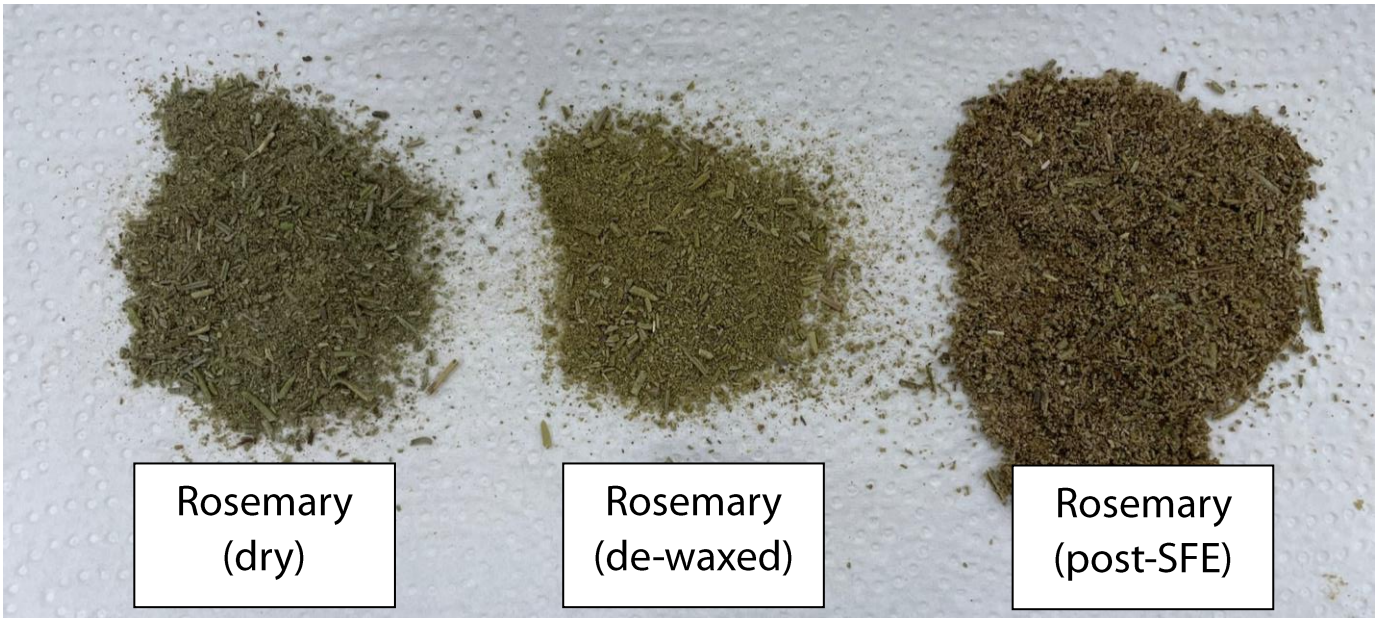
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Extract and plant material photographs









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