Chapter 5

Isolation and GC Analysis of Fatty Acids: Study Case of Stinging Nettle Leaves

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Abstract

Urtica dioica L. is perennial plant with remarkable medical properties and has been widely used in folk medicine and as a food. Fatty acids presented in its leaves were extracted applying three different techniques: classic, automated Soxhlet, and supercritical fluid extraction (SFE). SFE was performed at three different pressures (100, 200, and 300 bar) and two different temperatures (40 and 60°C). Obtained extract was analyzed using GC-FID analytical technique in order to obtain fatty acid profile samples. The highest yield was obtained in the case of automated Soxhlet extraction (218.907 mg/g), while the lowest was observed in the case of classic extraction (15.031 mg/g). Soxhlet approach provided the highest yield of saturated fatty acids (173.348 mg/g), while supercritical fluid extraction gave better results in the case of unsaturated fatty acids (60.062 mg/g). Deeper analysis of SFE extracts revealed relationship between extraction parameters (temperature and pressure) and yields of fatty acids where lower pressure ensured higher yield of unsaturated while higher pressure gave higher yield of saturated acids. On the other hand, increasing in temperature at isobaric conditions negatively influenced the yield of fatty acids except in the case of 300 bar where yield was higher at 60°C.

Keywords: stinging nettle, leaves, extraction, fatty acids, GC-FID analysis

1. Introduction

Urtica dioica L. (stinging nettle) is perennial, wild-growing plant from Urticaceae botanical family [1]. This plant is generally known for biological activity and positive effect which expresses on human health. It has long history of application in folk medicine where it has

been used for treatment of flailing arthritis or paralytic limbs, stimulation of circulation, and warming the joints and extremities [2]. There are wide ranges of studies which have dealt with biological activity of this plant. They have proved antioxidant, antimicrobial, anti-inflammatory, antiulcer, and analgesic properties of plant and its extracts [2–7]. Besides application in medicine, this plant has also been used in human nutrition for a long time. It has been harvested commercially for high content of chlorophylls, which has been used as coloring agent (E140) in food and medical products [8].

Previously conducted studies showed that stinging nettle contains wide diversity of biologically active compounds. There are essential oils and terpenoids as the main components [9, 10], carotenoids [4, 9, 11], fatty acids [9, 11, 12], different phenolic and polyphenolic compounds [1, 3, 9, 13, 14], essential amino acids, chlorophyll, vitamin C, vitamin K, vitamins of B series, tannins, carbohydrates, sterols, polysaccharides, isolectins [4, 9, 11, 15], and minerals [9, 16, 17]. The most important carotenoids are β-carotene, neoxanthin, lycopene, and lutein [11]; fatty acids are palmitic and *cis*-9,12 linoleic and α-linolenic acids [9, 11, 12], while iron has been marked as the most important mineral [9].

Fatty acids or generally fats are presented in many different forms such as saturated, monounsaturated, unsaturated, omega-3, omega-6, trans, or partially hydrogenated fats. These terms are widely presented in media regarding the health subject. Fats represent the most efficient way of storing excess energy. Besides this role, fats represent building blocks of lipid membranes (in the form of phospholipids), play significant roles in maintaining proper function of brain, and participate in process of signal transduction (in the form of diacylglycerols). Certain fats such as linoleic and arachidonic are essential due to inability of human body to synthetize them. Thus, their presence is very important because their involvement in biosynthesis of eicosanoids. This class of biomolecules is further involved in wide range of processes in organism such as platelet aggregation, anti-inflammatory response, allergic reactions, etc. Polyunsaturated fatty acids decrease LDL and cholesterol levels, while saturated acids increase both [18]. It has been found that n-3 and n-6 fatty acids expressed anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic, and vasodilatory properties [19]. The n-3 fatty acids proved to be secondary preventers of several diseases and disorders such as coronary heart disease, hypertension, type 2 diabetes, renal disease, rheumatoid arthritis, ulcerative colitis, Crohn's disease, and chronic obstructive pulmonary disease [19] but also show effects in decreasing the risk of heart disease and cancer [20, 21].

Extraction represents the most common way of isolation of desired compound from the mixture, natural sources, or other matrices. Generally, extraction techniques may be divided into conventional and nonconventional. Usual conventional techniques are classic extraction and Soxhlet extraction. Such techniques usually applied toxic and environmental non-friendly solvents [22]. Due to such drawbacks, nonconventional techniques such as ultrasound-assisted, microwave-assisted, and subcritical water and supercritical fluid extractions have been developed.

Ultrasound (20 KHz–100 MHz) and microwave (300 MHz–300 GHz) penetrate through medium causing characteristic effects which enhance mass transfer. Ultrasound creates compression and expansion, thus producing cavitation [23]. Microwave on the other hand creates uniform heating as a consequence of medium resistance to ion flow. This effect is in close correlation with ionic conduction and dipole rotation mechanisms [23–25]. Supercritical water extraction

relies on application of pressure to maintain water in liquid state during its heating above boiling point. Such state causes decrease in dielectric constant of water together with the polarity. Thus, polarity may, under certain conditions, become close to polarity of methanol [26–28].

Supercritical fluid extraction is a suitable substitution for conventional approaches such as hydrodistillation, steam distillation, and solvent extraction [29]. Techniques have started its commercial application during the 1980s [30]. Industrial scale of this technique found its application in processes such as decaffeination of green coffee beans and black tea leaves, production of hop extracts, isolation of essential oils, oleoresins and flavoring compounds from natural sources, extraction and fractionation of edible oils, and removal the pesticides from plant materials [31, 32]. This technique relies on application of fluids in their supercritical state. Such state can be achieved at pressures and temperatures above critical ones for given fluid. When this state has been achieved, fluid expressed properties between those characteristic for gas and liquid state [33, 34]. Density of fluid is similar to the density of liquids, while viscosity is similar to the values for gas. Behavior of two important factors influences the diffusivity which ranges between values for liquid and gas state. This results in better transport properties [34]. It should be mentioned that most important characteristic of fluids in this state is the ability to modulate their properties throughout modulation of density of fluid itself. This may be achieved by changing the pressure and temperature of system. Such changes in density influence directly the solubility of desired compounds in the fluid [35]. Carbon dioxide is the most commonly used fluid for this technique due to its nontoxic and nonexplosive properties, low price, availability, easy removable from extracts, and moderate critical properties (T_c = 31.1°C; p_c = 73.8 bar) [36].

Due to importance of fatty acids for proper function of human organism, it is also important to establish their occurrence in nature and possibility for utilization. The main goal of this research was to isolate and establish fatty acid profile in stinging nettle leaves. Different extraction techniques were used in order to compare their efficiency for isolation of fatty acids, while GC-FID technique was applied for their quantification. From the presented results, relationship between fatty acid structure and operational conditions was analyzed and established in the case of supercritical fluid extraction.

2. Experimental section

2.1. Plant material

Stinging nettle (*Urtica dioica* L.) leaves were purchased from Institute "Dr Josif Pančić" in 2016. Leaves were dried, grounded in blender, and stored in the paper bags until further processing.

2.2. Extraction procedures

Leaves were extracted using classic, Soxhlet, and supercritical fluid extraction methods. Classic extraction was performed by mixing 5.00 g of leaves with mixture of formaldehyde and ethanol (2:1; v/v) under vacuum at room temperature. Soxhlet extraction was performed using

5.00 g of leaves and 100.00 mL of petrol ether. Process was conducted in automated Soxhlet apparatus Soxtherm S306 (C. Gerhardt, Germany) at 150°C, under constant pressure for 2 h.

Supercritical fluid extraction was conducted using previously described apparatus (HPEP, NOVA-Swiss, Effretikon, Switzerland) [36, 37]. Main parts of this plant are gas cylinder with carbon dioxide, diaphragm-type compressor, extractor vessel with heating jacket, separator, pressure control valve, temperature regulation system, and regulation valves. Extractions were performed at three different pressure (100, 200, and 300 bar) and two different temperature (40 and 60°C) levels, while CO_2 flow rate was maintained constant (0.20 kg/h).

2.3. Sample preparation and fatty acid analysis

Extracts obtained using above-described techniques were further prepared for analysis using method described elsewhere [9]. Basically, fatty acids were extracted from prepared extracts using hexane, then hydrolyzed from their natural forms, and esterified using methanolic solution of KOH.

Analysis was conducted using Agilent 7890A gas chromatograph coupled with FID detector and CP-Sil 88 column (100 m × 0.25 mm × 0.20 μm), with nitrogen flow rate of 1 mL/min, applying previously described analytical method [9]. Nitrogen flow rate in detector was 25 mL/min, and air flow was 400 mL/min, while hydrogen flow rate was 30 mL/min. Oven temperature program was as follows: initial temperature 80°C (0.5 min), then linear increase of 4°C/min up to 220°C (4 min), and linear increase of 4°C/min up to 240°C (10 min). Temperatures of injector and detector were 240°C and 270°C, respectively. Standard solution for calibration was prepared by dissolving standard compounds in hexane in concentration range of 2–120 μg/mL. Limit of detection and recovery was 0.011–0.032% and 83–126%, respectively. Fatty acid contents were expressed as mg of fatty acid per g of extract (mg/g).

3. Results and discussion

3.1. Fatty acid contents in classic and Soxhlet extracts

Fatty acid profile of extracts obtained using classic and Soxhlet approaches is presented in **Table 1**, while chromatograms are given in **Figure 1**. According to their molecular structure, fatty acids (FAs) were divided into saturated (SFAs) and unsaturated (UFAs). UFAs were further divided into monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids.

Presented results indicated that automatic Soxhlet approach was a betted solution for isolation of fatty acids. Fatty acid yield in Soxhlet extract was 14.6-fold higher than yield of classic approach. Classic approach was proved to be better for isolation of UFAs, while SFAs dominated over UFAs in Soxhlet extract. Major FAs in classic extracts were C18:3 (3.368 mg/g), C16:0 (3.022 mg/g), C18:2 (2.775 mg/g), and C20:0 (1.036 mg/g). Domination of those three acids was earlier confirmed by several studies [9, 11, 12]. However, Soxhlet extract showed different profiles where C12:0 (45.639 mg/g) and C16:0 (40.523 mg/g) were major FAs followed by C18:1 (28.773 mg/g), 14:0 (20.656 mg/g), C18:0 (19.045 mg/g), C22:0 (13.385 mg/g), C20:0 (11.512 mg/g), and C24:0 (10.554 mg/g).

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Fatty acid	Content (mg/g)	
	Classic extraction	Soxhlet extraction
C6:0	/	0.667
C8:0	/	4.925
C10:0	/	4.677
C12:0	0.202	45.639
C14:0	0.456	20.656
C14:1	0.085	0.610
C15:0	/	0.722
C15:1	/	0.375
C16:0	3.022	40.523
C16:1	0.158	0.548
C17:0	0.054	0.568
C17:1	0.072	0.288
C18:0	0.493	19.045
C18:1 *cis*-Δ^{9}	0.486	28.773
C18:2 *cis*-$\Delta^{9,12}$	2.775	6.579
C20:0	1.036	11.512
C18:3 *cis*-$\Delta^{9,12,15}$	3.368	2.232
C21:0	0.137	1.075
C20:2 *cis*-$\Delta^{11,14}$	0.423	1.663
C22:0	0.890	13.385
C20:4 *cis*-$\Delta^{5,8,11,14}$	0.087	0.935
C23:0	0.174	0.400
C22:2 *cis*-$\Delta^{13,16}$	0.218	0.775
C24:0	0.644	10.554
C20:5 *cis*-$\Delta^{5,8,11,14,17}$	/	0.685
C24:1	0.251	1.096
SFAs	7.108	173.348
UFAs	7.923	44.559
MUFAs	1.052	31.690
PUFAs	6.871	12.869
Total	15.031	218.907
SFA:UFA ratio	0.90	3.91

Table 1. Fatty acid profile of classic and Soxhlet extracts.

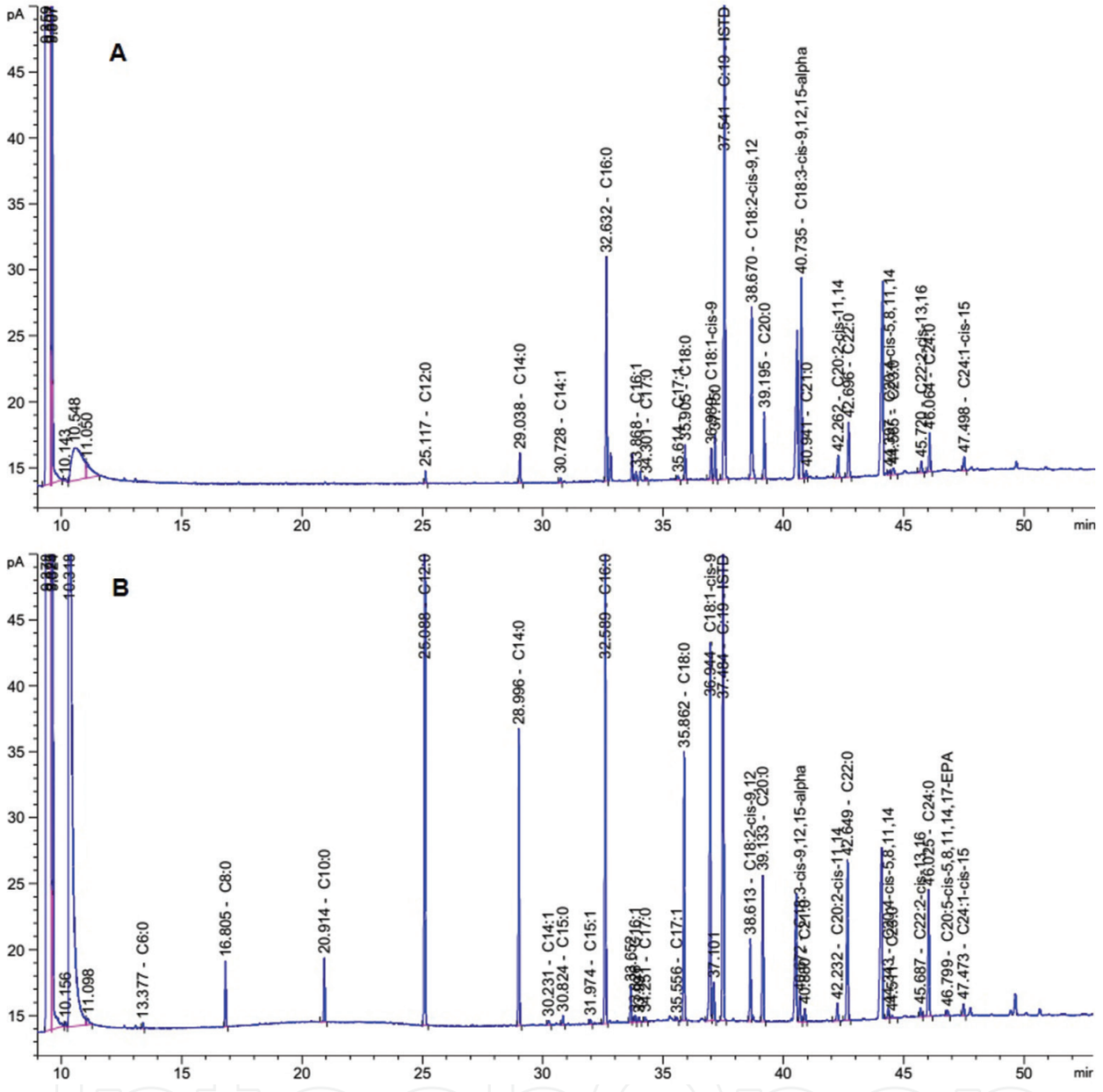

Figure 1. Chromatogram classic extract (A) and Soxhlet extract (B).

Besides the abovementioned differences, it might be also noticed that several fatty acids were detected only in Soxhlet extract (C6:0, C8:0, C10:0, C15:0, C15:1, and C20:5). Also, content of C18:3 PUFA was higher in classic extract. This might be explained with stronger interaction among C18:3 acid and ethanol, which was also previously reported [38].

3.2. Fatty acid profile of SFE extracts

Supercritical fluid extraction was conducted using three different levels of pressure and two different levels of temperature (**Table 2**).

Tuning these two parameters allows higher level of selectivity throughout influence on density of supercritical fluid and vapor pressure of solutes. Results of fatty acids GC analysis are presented in **Table 3**.

Sample	Pressure (bar)	Temperature (°C)
1	100	40
2	100	60
3	200	40
4	200	60
5	300	40
6	300	60

Table 2. Operational parameters of supercritical fluid extraction.

Fatty acid	Sample/content (mg/g)					
	1	**2**	**3**	**4**	**5**	**6**
C6:0	0.376	0.213	0.219	/	0.208	/
C10:0	0.190	/	/	/	/	/
C11:0	0.128	/	/	/	/	/
C12:0	0.209	0.216	0.178	0.158	0.175	0.228
C13:0	0.211	0.181	0.178	/	0.143	/
C14:0	3.859	1.697	2.150	1.573	1.968	1.523
C14:1	1.155	0.860	0.659	0.414	0.611	0.483
C15:0	0.204	0.151	0.173	0.404	/	0.165
C15:1	0.185	0.142	0.612	/	0.376	0.338
C16:0	8.657	6.616	8.010	7.102	6.053	6.331
C16:1	1.075	0.588	0.432	0.702	0.497	0.556
C17:0	0.350	0.294	0.148	0.763	0.192	0.235
C17:1	0.553	0.239	0.329	0.250	0.161	0.171
C18:0	1.996	1.527	1.799	2.279	1.388	1.631
C18:1 *trans*-Δ^9	/	0.126	/	/	/	/
C18:1 *cis*-Δ^9	2.130	1.565	1.640	1.072	1.426	1.297
C18:2 *cis*-$\Delta^{9,12}$	10.900	7.746	9.047	7.347	7.216	7.240
C20:0	3.851	3.986	7.734	13.934	4.286	10.107
C18:3 *cis*-$\Delta^{6,9,12}$	0.431	0.276	/	0.160	0.266	/

Fatty acid	Sample/content (mg/g)					
	1	**2**	**3**	**4**	**5**	**6**
C20:1 *cis*-Δ^{11}	0.159	0.152	/	0.184	0.174	/
C18:3 *cis*-$\Delta^{9,12,15}$	38.564	28.943	24.186	20.504	26.235	22.375
C21:0	0.301	/	17.809	1.483	0.578	0.755
C20:2 *cis*-$\Delta^{11,14}$	3.872	1.364	2.258	1.256	1.794	1.477
C22:0	1.967	1.844	5.945	12.577	3.078	10.638
C20:3 *cis*-$\Delta^{8,11,14}$	0.244	0.292	0.138	0.125	0.447	0.129
C20:3 *cis*-$\Delta^{11,14,17}$	/	0.336	/	/	/	0.719
C20:4 *cis*-$\Delta^{5,8,11,14}$	0.507	0.277	/	0.294	0.338	0.256
C23:0	1.634	0.767	/	0.399	0.821	0.539
C22:2 *cis*-$\Delta^{13,16}$	/	/	0.615	/	/	/
C24:0	1.146	0.950	3.590	8.634	2.923	5.768
C20:5 *cis*-$\Delta^{5,8,11,14,17}$	/	0.603	/	/	0.899	/
C24:1 *cis*-Δ^{15}	/	/	1.263	/	/	/
C22:6 *cis*-$\Delta^{7,4,10,13,16,19}$	0.287	0.165	/	0.261	0.250	/
SFAs	25.079	18.442	47.933	49.306	21.813	37.920
UFAs	60.062	43.647	41.179	32.569	40.690	35.041
MUFAs	5.257	3.672	4.935	2.622	3.245	2.845
PUFAs	54.805	40.002	36.244	29.947	37.445	32.196
Total	85.141	62.116	89.112	81.875	62.503	72.961
SFA/UFA ratio	0.42	0.42	1.16	1.51	0.54	1.08

Table 3. Fatty acid profile of SFE extracts.

Pressure and temperature are considered to be the most influential parameters in SFE. Generally, pressure exhibited positive influence on extraction yield due to its positive influence on density of supercritical fluid. On the other hand, temperature exhibits dual effect as a result of combination of two variables: density and vapor pressure. Density decreases with temperature causing the decreasing in solubility. Vapor pressure increases with temperature, thus increasing the solubility in the same time [30, 39]. Pressure at the point of inversion of those two variables is known as crossover pressure or crossover point [30].

Influence of all abovementioned effects might be noticed in the case of fatty acid profile presented in **Table 3**. The highest yield of FAs was observed at 200 bar and 40°C (sample 3), while the lowest was at 100 bar and 60°C (sample 2). At both temperature levels, similar tendency of FAs yield might be noticed. Yields increased from 100 to 200 bar and then decreased at 300 bar. SFA yields showed same tendency, but the highest was observed in sample 4 (200 bar, 60°C), while the lowest was noticed in sample 2 (100 bar, 60°C). From the presented results, it

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was obvious that higher pressure and temperature levels were better for isolation of SFAs. In the case of UFAs, situation was completely different. The highest yield of UFAs was obtained in sample 1 (100 bar, 40°C), while the lowest was observed at 200 bar and 60°C (sample 4). It might be concluded that lower pressure and temperature levels were better for isolation of UFAs. This was also the same for both MUFAs and PUFAs. Such conclusions might be supported by SFA/UFA ratio, where the highest value was obtained in sample 4 (1.51) and the lowest in samples 1 and 2 (0.42). Chromatogram of sample 3 is presented in **Figure 2**.

Previous investigations of relationship between structure and solubility of organic compounds in supercritical carbon dioxide showed that length of carbon chain and number of double bonds influences their miscibility in supercritical fluids. Thus, increase in carbon number causes decrease in miscibility. Introduction of double bonds showed same effect, but unsaturation expressed favorable effect where alkenes were more miscible than alkanes [40]. Same trends stand for fatty acids. With increasing in number of carbon atoms and molecular weight miscibility decreases, while introduction of double bonds decrease melting point of fatty acids and this increases their miscibility [41, 42]. These parameters together with abovementioned influence of pressure and temperature ensured presented variations in fatty acid profiles (**Table 3**).

C6:0 acid was not quantified in samples 4 and 6. Comparing with its occurrence in samples 3 and 5, it might be concluded that increasing in temperature expressed negative effect on this acid. Same tendency was noticed in the case of C13:0 acid. C10:0 and C11:0 acids were quantified only in sample 1 (100 bar, 40°C). Negative influence of both pressure and temperature on miscibility of this fatty acid was clearly obvious. C15:0 acid was not quantified only in sample 5. C21:0 acid was not observed in sample 2, while C23:0 was not detected in sample 3. All other saturated acids (C12:0, C14:0, C16:0, C17:0 C18:0, C20:0, C22:0, and C24:0) were quantified in all samples.

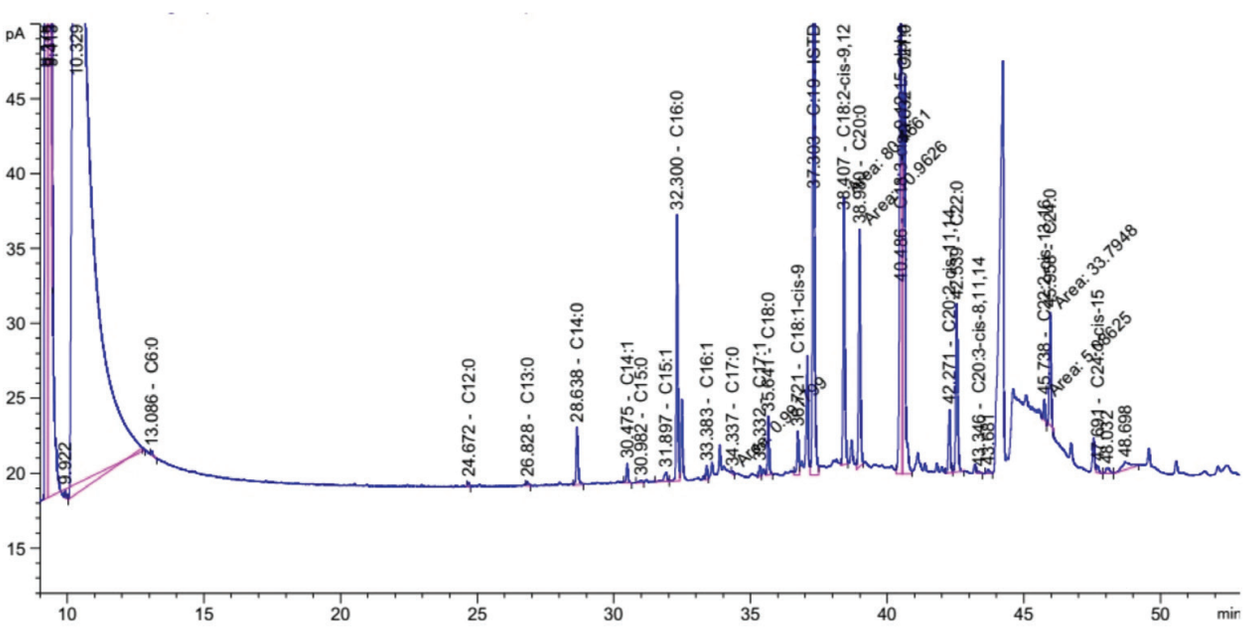

Figure 2. Chromatogram of sample 3 obtained at 200 bar and 40°C.

Comparing the yields for saturated FAs under isothermal conditions revealed existence of two trends: up to C18:0 acid yield decreased with pressure increasing at 40°C. After C18:0 acid, yield increased up to 200 bar and then decreased. On the other hand, at 60°C yield increased at first and then decreased. Changes in temperature under isobaric conditions influenced differently depending on fatty acid. At 100 bar yields decreased with increasing in temperature with the exception of C12:0, C20:0, and C23:0 where yield increased with the temperature. At 200 bar yields dropped except in the case of C15:0, C17:0, C18:0, C20:0, C22:0, C23:0, and C24:0. It might be noticed that more acids reached their maximal yield at higher temperature then in the case of 100 bar. At 300 bar yields decreased only in the case of C6:0, C13:0, C14:0, and C23:0. Such behavior might be explained with previously mentioned dual effect of temperature.

Effects of SFE conditions on yields of unsaturated acids will be considered separately for monounsaturated and polyunsaturated fatty acids. Results showed that *trans*-C18:1 acid was quantified only in sample 2, while 24:1 acid was quantified in sample 3. C15:1 acid was not detected in sample 4, while C20:1 acid was not quantified in samples 3 and 6. C14:1, C17:1, and *cis*-C18:1 acids were quantified in all samples. Isothermal conditions again showed dual effect. At 40°C yield constantly decreased with pressure increasing for C14:1, C17:1, and *cis*-C18:1 acids. In the case of isothermal process, at 60°C, yields reached their maximal values at 200 or 300 bar depending on acid. Isobaric conditions at 100 bar showed that yields dropped with increasing in temperature in all cases except for *trans*-C18:1 acid. At 200 bar trends were the same with the exception of C16:1 and C20:1 acid whose yields increased with temperature. In the case of 300 bar, yields of C16:1 and C17:1 decreased with increasing in temperature.

Polyunsaturated fatty acids were also detected and quantified in all samples. Dominant PUFA was C18:3 *cis*-$\Delta^{9,12,15}$ acid, which was the most abundant acid in all samples. C18:3 *cis*-$\Delta^{6,9,12}$ was not quantified in samples 3 and 6. C20:3 *cis*-$\Delta^{11,14,17}$ acid was detected only in samples 2 and 6, and C22:2 was found only in sample 3, while C20:5 was quantified only in sample 2. C20:4 was not found only in sample 3. Almost all PUFAs reached their maximal yields at 100 bar and 40°C (sample 1) with the exception of 20:3 *cis*-$\Delta^{8,11,14}$, 20:3 *cis*-$\Delta^{11,14,17}$, and C22:2 acids. They achieved their maximal yields in samples 5, 6, and 3, respectively. Both 20:3 acids achieved their maximal yield at 300 bar but at different temperature level. This might be explained with different positions of double bonds in their structures.

Deeper investigation showed that under isothermal conditions at 40°C, yields of most of compounds decreased up to 200 bar and then increased at 300 bar. Exceptions were C18:2 and 20:2 acids whose yields decreased at all three pressure levels. At 60°C, yields of C18:2 and C18:3 *cis*-$\Delta^{6,9,12}$ constantly decreased, while other acids showed different trends (yield increased or decreased up to 200 bar). Presented result showed that generally lower pressure was more beneficial for isolation of PUFAs and MUFAs (100 bar), while higher pressure is better for isolation of SFAs (200 bar). Temperature changes caused decreasing in yield of observed analytes, that is, UFAs, MUFAs, and PUFAs, at all three pressure levels (**Table 3**). They reached maximal levels at 40°C at 100 bar. Therefore, total yield of FAs showed same tendency where the highest yield was at 200 bar and 40°C followed by yield at 100 bat and 40°C. On the other hand, temperature negatively influenced SFA yield at 100 bar, but at 200 and 300 bars, yield of SFAs increased with temperature.

4. Conclusion

Three different extraction techniques were applied for isolation of fatty acids from leaves of stinging nettle. Results showed that automated Soxhlet approach provided the highest yield of fatty acids, while classic extraction achieved the lowest one. Comparison of those two conventional techniques revealed domination of certain saturated and unsaturated fatty acids. In the case of classic extraction, C16:0, C20:0, C18:2, and C18:3 were dominant compounds, while in the case of Soxhlet extraction, main acids were C12:0, C14:0, C16:0, C18:0, C18:1, C20:0, C22:0, and C24:0. It might be noticed that saturated acids absolutely dominated in this sample (SFA/UFA ratio was 3.91). Similar results were obtained in the case of SFE. Dominant acids were C16:0, C18:0, C18:1, C18:2, C20:0, C18:3, C21:0, C20:2, C22:0, and C24:0, but their content was in close connection with operational conditions, that is, pressure and temperature.

Investigation of influence of operational conditions on fatty acid profile revealed complex connection. The highest total yield was obtained in sample 3, followed by sample 1. Both samples achieved high yield at lower temperature level. Under isothermal condition pressure changes influenced differently. At both temperature levels, yield of SFAs increased up to 200 bar and then decreased. Yield of MUFAs decreased with pressure increasing at lower temperature but increase at higher temperature level and 300 bar. Yield of PUFAs decreased up to 200 bar and then increased at both temperature levels. Isobaric processes showed that temperature influenced negatively on yield of SFAs at 100 bar. At higher pressures, temperature exhibits positive effect, which was explained by changes in domination of two variables, that is, density and vapor pressure. On the other hand, temperature increasing negatively influenced yield of UFAs, MUFAs, and PUFAs. Results showed that lower pressure and temperature levels were most beneficial conditions for isolation of unsaturated fats. To conclude, careful choice of operational condition might result in favored isolation of desired group of fatty acids in this particular case.

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