Monika Sitarek*, Paweł Orliński, Jarosław L. Przybył

Warsaw University of Life Sciences – SGGW
Department of Vegetable and Medicinal Plants
Nowoursynowska 159, Warsaw, Poland

* ✉ monika_sitarek@sggw.pl

Fast, efficient extraction and quantification of chlorophylls and carotenoids from fresh parsley leaves (*Petroselinum sativum* hoffm. *ssp. Neapolitanum*)

INTRODUCTION

An efficient, precise chlorophylls and carotenoids extraction method from raw plant material is necessary for research and industrial purposes. Extraction procedures can be diverse in terms of the solvent and conditions (time, temperature, pressure, continuity of the process). The presence of water in the plant material and lipophilic nature of chlorophyll and carotenoid compounds require the usage of organic solvents soluble in water such as acetone, methanol, ethanol, dimethyl sulfoxide. Organic solvents break non-covalent protein-pigment bonds in membranes causing their elution from the tissue and to some extent they reduce the activity of pigment degrading enzymes. Acetone is the most widely used solvent and the common extraction methods are based on simple extraction by shaking, sonication, simultaneous homogenization and extraction of the sample by grinding together with the solvent under conditions of low temperature and light. Heat reflux extraction (e.g. Soxhlet apparatus) is used as well. The plant pigments extraction may be a very time-consuming process, especially in case determination of total content of this compounds in the plant material. On the other hand, the progress of analytical techniques often forces researchers to analyse a large number of samples in a short time. Modern automatic method, such as *pressurized liquid extraction* (PLE) also known as *accelerated solvent extraction* (ASE), solves this problem. Mentioned technique involves elevated temperature and pressure (solvent is heated above boiling points under normal conditions) and requires less solvent. Despite the possibilities offered by this method, only a scant information about the isolation of chlorophylls and carotenoids by this procedure can be found.

The aim of this study was to optimize chlorophylls and two main leaf carotenoids (lutein and β-carotene) PLE (ASE) procedure by varying the solvent (acetone, methanol), the temperature (22-150 °C) and the extraction time (1-50 min.) taking into account the main chlorophyll a degradation product which can be formed during inappropriate extraction conditions. Results were compared to the yield obtained by other simple methods of extraction. Fresh leaves of plain-leafed parsley (*Petroselinum sativum* Hoffm. *ssp. neapolitanum*) were used as a model substrate.

MATERIALS AND METHODS

Sample Preparation

Parsley leaves were purchased from the local shop in February 2016 and prepared for the analysis in the laboratory of Department of Vegetable and Medicinal Plants. Lamina were separated from the petioles, cut into even pieces and immediately frozen under –80 °C in 1 ± 0,001 g aliquots.

Pigment extraction

Accelerated Solvent Extractor – ASE™ 350 (Thermo Scientific™) was used for PLE (Picture 1.). Samples were placed in a 10 mL stainless-steel extraction cell with cellulose or glass fibre filter in the cell outlet. All extractions were performed at 1500 psi, with a 5-min. heat-up time, 50% flush volume and a 30-s purge time. Extraction solvent, temperature, static time and number of static cycles were varied depending on the combination. The extracts were collected in 60 mL glass sample vials fitted with PTFE-coated rubber caps.

Conventional extraction procedures was carried out by shaking (45 min., dark, 22 °C) the samples with three types of solvents (80% aqueous acetone, 96% acetone, 99.8% methanol) or incubation with dimethyl sulfoxide (DMSO) for 2 h at 55 °C in a water bath. Each extract was transferred to a 50 mL volumetric flask and filtered through 0.20 µm PTFE syringe filter prior to HPLC analysis. Triplicate extractions were carried out for each combination.

The experiment no. 1

The influence of the solvent, temperature and time on the extraction yield

Solvents efficiency (methanol and acetone) in different extraction temperature (50, 100, 150 °C) at 1 cycle with 3 different static time (1, 3, 5 min.) was established.

The experiment no. 2

The effect of temperature on the extraction yield

Ten temperature points (22, 40, 50, 60, 70, 80, 90, 100, 125 and 150 °C) were tested at 1 cycle with 5 min. static time.

The experiment no. 3

The influence of the number of cycles and it's static time on the extraction yield

1, 2 and 3 number of cycles were performed with four different static time (1, 2, 5 and 10 min.) at 100 °C.

The experiment no. 4

Comparison of different extraction methods on the extraction yield

Four conventional methods was compared to two temperature (100 and 125 °C) methanol ASE procedure (1 cycle with 15 min. static time).

Picture 1. Accelerated Solvent Extractor – ASE™ 350 (Thermo Scientific™).

HPLC Analysis

Commercially available standards – solutions of chlorophyll a, b, lutein and β-carotene (ChromaDex®) was used as standard stock solutions. Further calibration levels were prepared by diluting these solutions with methanol in 10 mL volumetric flasks (injected volumes ranges: 10, 50, 100, 200, 500 and 1000 µL). The working solutions were injected (1 µL) on a column in six replicates (n=6) using Shimadzu SIL-20AC HT to generate a six-point calibration curve for the each standard compounds separately, using LC Solution chromatography software. The peak table and UV-VIS spectra library (190-800 nm) of individual compounds were created. Standard curve parameters were calculated with MS Excel. Signal-to-noise ratio approach were used to determined LOD (S/N of 3:1) and LOQ (S/N of 10:1). The analyses were performed using a Shimadzu chromatograph equipped with auto sampler SIL-20AC HT, photodiode array detector SPD-M20A PDA and LC Solution chromatography software. A modern C-18 reversed-phase column with core-shell technology (Phenomenex Kinetex® 2.6 µm, C18, 100 Å, 100×4.60 mm i.d.) was used as solid phase. As a mobile phase pure methanol was used. The following conditions were applied: isocratic elution, flow rate 2.0 ml×min^{-1}, oven temperature 30 °C, injection volume 10 µl, total time of analysis 5 min. Peak identification was conducted by comparison of retention time and UV-VIS spectra of standards (190–800 nm). The content of the determined compounds was calculated in mg per 100 g of fresh matter. The results were analysed with multi-way ANOVA and Tukey's HSD test at α = 0.05 significance level using SATISTICA 12 (StatSoft®) software.

RESULTS

The experiment no. 1

All tested factors had a significant impact on the efficiency of pigments extraction. Both, in the case of carotenoids and chlorophylls methanol proved to be a better solvent than acetone. Extending the extraction time resulted in the greatest increase in extraction efficiency at 100 °C. In the case of acetone the efficiency was increased by 58-66% but in methanol it was over 90% for carotenoids and about 64-72% in the case of chlorophyll a, and b. The temperature of 50 °C was too low to carry out the complete extraction in such a short time, while the temperature of 150 °C provided the best results in the extraction of all the compounds tested but it strongly affected the formation of chlorophyll a derived compound. Regardless of the solvent used, in the extracts obtained at 150 °C, 50× higher content of chlorophyll a derivative was detected than in the extracts obtained at 50 °C for the same static time. This compound was also formed with the methanol extraction for 5 min. at 100 °C (4.45 mg × 100 mg^{-1} of fresh matter) and at 1 and 2 minutes, its content was 0.38 mg and 0.47 mg × 100 mg^{-1} of fresh matter respectively. After acetone extraction at the same temperature, the content of this compound was 0.37-0.51 mg × 100 mg^{-1} of fresh matter. The extraction yield of each of the compounds according to the temperature, time and solvent are shown in Tables 1-5.

Table 1. Lutein

	Acetone				Methanol			
Temperature	1 min.	2 min.	5 min.	Mean	1 min.	2 min.	5 min.	Mean
50 °C	1.31 f	1.27 f	1.76 f	**1.45**	1.44 f	1.33 f	1.70 f	**1.49**
100 °C	2.97 e	3.34 e	4.94 d	**3.75**	4.51 d	5.24 cd	8.73 a	**6.16**
150 °C	5.96 c	7.07 b	7.47 b	**6.83**	8.91 a	9.40 a	9.34 a	**9.21**
Mean	**3.41**	**3.90**	**4.72**	**4.01**	**4.95**	**5.32**	**6.59**	**5.62**

Table 2. β carotene

	Acetone				Methanol			
Temperature	1 min.	2 min.	5 min.	Mean	1 min.	2 min.	5 min.	Mean
50 °C	1.24 i	1.30 i	1.74 i	**1.43**	0.96 i	0.87 i	1.72 i	**1.18**
100 °C	3.84 h	3.80 h	6.08 g	**4.57**	5.57 g	6.77 fg	10.61 bc	**7.65**
150 °C	7.51 ef	8.75 de	9.44 cd	**8.57**	11.62 ab	12.69 a	12.43 a	**12.24**
Mean	**4.20**	**4.62**	**5.75**	**4.86**	**6.05**	**6.78**	**8.25**	**7.02**

Table 3. Chlorophyll b

	Acetone				Methanol			
Temperature	1 min.	2 min.	5 min.	Mean	1 min.	2 min.	5 min.	Mean
50 °C	4.60 g	4.35 g	5.51 g	**4.82**	4.25 g	3.83 g	5.61 g	**4.56**
100 °C	8.94 f	9.60 ef	14.20 cd	**10.91**	14.05 cd	16.20 c	23.08 b	**17.77**
150 °C	13.64 cd	13.73 cd	12.01 de	**13.13**	23.82 ab	25.59 ab	26.18 a	**25.20**
Mean	**9.06**	**9.23**	**10.58**	**9.62**	**14.04**	**15.21**	**18.29**	**15.84**

Table 4. Chlorophyll a

	Acetone				Methanol			
Temperature	1 min.	2 min.	5 min.	Mean	1 min.	2 min.	5 min.	Mean
50 °C	13.20 f	11.02 f	16.54 ef	**13.58**	12.00 f	10.33 f	16.32 ef	**12.88**
100 °C	26.21 de	27.73 de	42.20 bc	**32.05**	42.08 bc	48.78 b	72.58 a	**54.48**
150 °C	39.69 bc	39.77 bc	36.50 cd	**38.65**	68.94 a	78.14 a	74.67 a	**73.92**
Mean	**26.37**	**26.17**	**31.74**	**28.09**	**41.00**	**45.75**	**54.52**	**47.09**

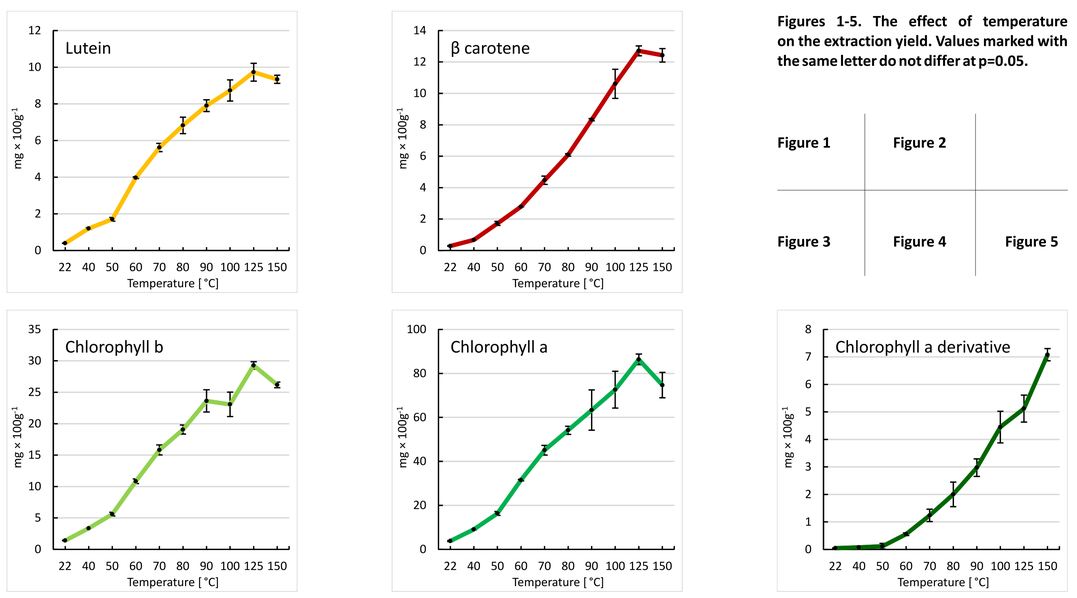
Table 5. Chlorophyll a derivative

	Acetone				Methanol			
Temperature	1 min.	2 min.	5 min.	Mean	1 min.	2 min.	5 min.	Mean
50 °C	0.10 a	0.08 a	0.11 a	**0.10**	0.17 a	0.14 a	0.12 a	**0.14**
100 °C	0.37 a	0.44 a	0.51 a	**0.44**	0.38 a	0.47 a	4.45 b	**1.77**
150 °C	4.44 b	6.64 c	12.00 d	**7.69**	7.97 c	7.27 c	7.08 c	**7.44**
Mean	**1.64**	**2.39**	**4.21**	**2.74**	**2.84**	**2.62**	**3.88**	**3.12**

Tables 1-5. The influence of the solvent, temperature and time on the extraction yield. Values marked with the same letter do not differ at p=0.05.

The experiment no. 2

A temperature increase up to 125 °C resulted in the increase in extraction efficiency for all tested compounds. Increasing the temperature above this limit probably caused the degradation of compounds. Extraction of carotenoids (Figure 1 and 2) at 150 °C gave a little lower scores than extraction at 125 °C, although these differences were not statistically significant. Pronounced trend exists for chlorophylls, for which a reduction in the yield of extraction between 125 and 150 °C was significant and ranged from 29.28 to 26.18 mg × 100 mg^{-1} of fresh matter for chlorophyll b (Figure 3) and from 86.37 to 74.67 mg × 100 mg^{-1} of fresh matter for chlorophyll a (Figure 4). At the same time more rapid increase in the extraction of chlorophyll a derivative by almost 40% is noticeable (Figure 5).



Figures 1-5. The effect of temperature on the extraction yield. Values marked with the same letter do not differ at p=0.05.

Figure 1 Figure 2
Figure 3 Figure 4 Figure 5

The experiment no. 3

Both factors had a significant influence on the extraction of plant pigments. The study shows that the optimum parameters of studied factors was mostly using 3 cycles of 5 minutes each. Both the increase in the number of cycles as well as the extension of the extraction time, not only caused a decrease in the extracted compounds but also caused an increase in the content of the derivative of chlorophyll a in the extracts.

Table 6. Lutein

Number	Time				
of cycles	1 min.	2 min.	5 min.	10 min.	Mean
1	4.76 d	5.21 d	7.39 abc	6.45 c	5.95
3	6.45 c	7.02 bc	8.15 a	7.24 abc	7.22
5	7.20 abc	6.95 bc	8.11 a	7.85 ab	7.53
Mean	6.14	6.39	7.88	7.18	6.90

Table 7. β carotene

Number	Time				
of cycles	1 min.	2 min.	5 min.	10 min.	Mean
1	4.86 d	5.42 bcd	9.85 a	7.63 abc	6.94
3	8.16 abcd	9.00 abc	11.34 a	9.92 a	9.61
5	9.33 ab	9.06 abc	11.18 a	10.74 a	10.08
Mean	7.45	7.83	10.79	8.60	8.67

Table 8. Chlorophyll b

Number	Time				
of cycles	1 min.	2 min.	5 min.	10 min.	Mean
1	16.23 e	17.41 de	26.78 ab	20.85 cd	20.32
3	21.98 c	23.39 bc	28.84 a	22.45 c	24.16
5	23.54 bc	22.07 c	28.55 a	26.85 ab	25.25
Mean	20.58	20.95	28.06	23.38	23.24

Table 9. Chlorophyll a

Number	Time				
of cycles	1 min.	2 min.	5 min.	10 min.	Mean
1	47.57 d	49.79 cd	85.16 a	62.81 bc	61.33
3	66.25 b	68.63 b	94.32 a	66.92 b	74.03
5	70.14 b	63.13 bc	91.70 a	86.87 a	77.96
Mean	61.32	60.51	90.39	72.20	71.11

Table 10. Chlorophyll a derivative

Number	Time				
of cycles	1 min.	2 min.	5 min.	10 min.	Mean
1	2.86 f	3.41 f	4.67 e	6.29 bc	4.31
3	4.91 de	5.67 cde	5.87 bcd	7.92 a	6.09
5	6.35 bc	5.87 bcd	5.69 cde	6.93 ab	6.21
Mean	4.71	4.98	5.41	7.04	5.54

Tables 6-10. The influence of the number of cycles and it's static time on the extraction yield. Values marked with the same letter do not differ at p=0.05.

The experiment no. 4

Incubation of plant tissue in DMSO caused great formation of chlorophyll a derivative (21.00 mg × 100 mg^{-1}) in comparison to other combinations (0.30-5.87 mg × 100 mg^{-1}) and was the worst procedure for the chlorophyll b extraction (Figures 9 and 11). Conventional methanol extraction resulted in the highest yield of chlorophyll a and b (103,83 and 36,50 mg × 100 mg^{-1} respectively) whereas conventional 80% acetone method was the worst in case of chlorophyll a. ASE methods was slightly worse but relatively closer to the best combination than two conventional acetone procedures for chlorophyll a and b extraction (Figures 9 and 10). ASE (at 125 °C) extracts showed the highest content of tested carotenoids while methanol conventional method was the worst procedure for the β carotene extraction (Figures 6 and 7).

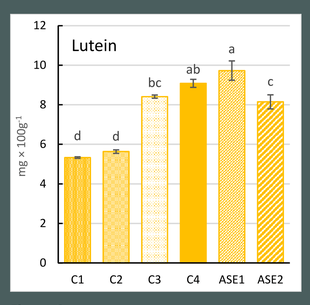


Figure 6

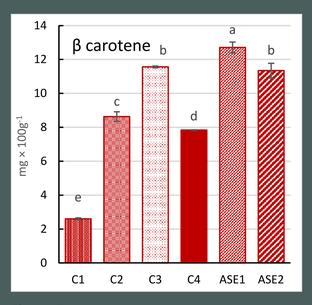


Figure 7

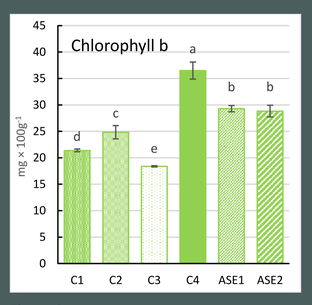


Figure 8

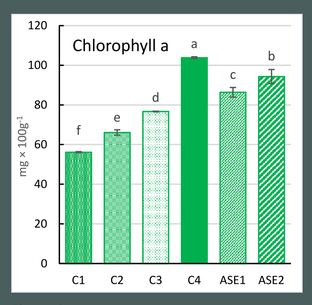


Figure 9

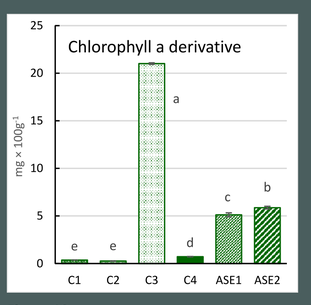


Figure 10

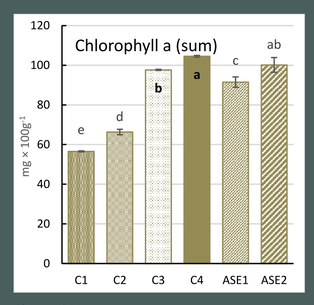


Figure 11

Figures 6-11. Comparison of different extraction methods on the extraction yield. C1 - shaking with 80% aqueous acetone, C2 - shaking with 96% acetone, C3 - incubation with dimethyl sulfoxide (DMSO), C4 - shaking with 99.8% methanol, ASE1 - PLE at 100 °C, ASE2 - PLE at 125 °C. Values marked with the same letter do not differ at p=0.05.