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DESIGN OF EXPERIMENT – OPTIMIZATION OF HPLC ANALYSIS OF VITAMIN A AND E IN MARGARINE AND VEGETABLE OIL

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ABSTRACT

Liquid chromatography is a useful method for selective analysis of vitamin A and E. The native components can be easily tested due to their UV-absorbance and beneficial separation characteristics. To test the food sample, vitamin A and E have to be separated from the fatty part of food. This step is performed by saponification, which is the critical step of procedure due to the heat and light sensitivity of both vitamins. The corresponding standards (MSZ-EN 12822-1, MSZ EN 12823-1) recommend a wide range of saponification temperature and time, giving an opportunity to optimize the recovery of the analyte. Experimental tests were performed from vegetable oil and margarine sample to find the temperature and time settings for the best recovery. Both factors were examined with centered two-level factorial design. Data analysis showed an increasing recovery of analytes towards the corner points of highest possible temperature and shortest possible saponification time. Finally, confirmatory tests were made.

Keywords: HPLC, Vitamin A, Vitamin E, Experimental design, Recovery.

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1. INTRODUCTION

Adequate vitamin and mineral intake is needed for healthy, balanced nutrition. Among fat soluble vitamins, vitamin A has a fundamental role in the vision, and it is the coenzyme of the protein glycosylation (Gropper and Smith, 2013). Vitamin E protects polyunsaturated fatty acids and membrane lipids against oxidation, and has an inflammatory effect. Nowadays, vitamin intake is covered partially by supplementation. To assure the correct dosage of fat soluble vitamins, their concentration in different food matrixes should be measured.

Hungarian standards (MSZ EN 12823-1:2014, MSZ EN 12822), – 'Standards' – describe direct extraction and saponification as sample preparation for fatty samples. Direct extraction requires no heating, assuring the full recovery of the heat sensitive vitamin A and E (Thompson *et al.*, 1980; Nollet, 1996). The method is compatible with normal phase high performance liquid chromatography (NP-HPLC), the sample dissolved in apolar solvent can be directly injected to instrument. Drawbacks of NP-HPLC are:

- Limit of Detection (LOD) is high: sample can not be concentrated due to its high lipid content
- Reversed Phase Liquid Chromatography (RP-HPLC) is commonly used in the laboratories; switching to another analytical task requires washing of HPLC tubing: it is time and solvent consuming. Switching between RP-HPLC eluents would be more simple. Still, RP-HPLC compatible solvents (e.g. methanol, acetonitrile) are not applicable to dissolve fatty samples.

Saponification is also a useful sample preparation method for vitamin A and E (Nollet, 1996). Using this method, sample concentration is possible. If ester forms (e.g. acetate, palmitate) of these vitamins are present, they are splitted as native vitamins and fatty acid. Thanks to this, sample can be tested with the native HPLC standard

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materials. Drawback of saponification is, that The light and heat sensitivity of the analytes should be considered (Gimeno *et al*., 2000; Xu, 2008).

As RP-HPLC systems are more common than NP-HPLC, saponification would be preferred. Test method written in the Standards give good guidelines, excepting two important factors of procedure (temperature, saponification time). They are given in Standard only as interval: this gave the goal of my work to determine the optimal sample preparation conditions.

Recovery of sample preparation of vitamin A and E were optimized with a two factor, two level experimental design, including saponification time and temperature. Sample extracts were examined with reversed phase liquid chromatography. Vitamin A (all-trans retinol) was examined in margarine, vitamin E (α-tocopherol) in vegetable oil.

2. EXPERIMENT

2.1. Materials

Table-1. Reagents

Reagent	Description	Manufacturer
EtOH	Etanol 96% puriss.	Reanal
hexane	Suprasolv n-hexane, GC purity	Merck
KOH	Potassium hydroxide, a.r.	Reanal
Na_2SO_4	Sodium sulphate, Anhydrous, a. r.	Reanal
L-ascorbic acid	Ascorbic acid, a. lt.	Reanal
MeOH	Methanol, HPLC purity	Pancreac

Source: Available chemicals in the laboratory

Vitamin A and E Standards were provided by Sigma-Aldrich. Their purity was verified spectrophotometrically.

2.2. Sample Preparation

Two replicates were prepared in the following manner:

- 5-9 g of vegetable oil (Vénusz) or 3-5 g of margarine (Delma) was weighed into a 250 ml flask
- 300 mg of ascorbic acid was added
- 50 ml of EtOH, then 15 ml of 50 % KOH was added
- Saponification was performed under reflux cooler. Successful saponification was indicated by the disappearing of the oil or fatty drops.
- Sample was rinsed from flask with 50 ml EtOH into a 250 ml funnel
- 120 ml water was added (breaking emulsion), then extraction was made with 3×50 ml hexane
- Hexane phase was washed with water three times
- Hexane phase was rotary evaporated at 50 °C and 260 mbar
- Residue was dissolved in 10 ml of MeOH

2.3. Instrumentation

Reversed phase liquid chromatography was used with:

- HPLC: Agilent 1100 Series, with pump degasser
- Software: Chemstation
- Column: Zorbax Eclipse XDB-C18, 150×4,6 mm, 5 µm
- Eluent: 95:5 methanol:water
- Flow rate: 1 ml/min
- Detector: diode array detector (DAD)

Literal wavelengths were considered as per Table 2.

Table-2. Retention time, detection wavelength of analytes

Analyte	DAD wavelength [nm]	Ret. Time [min]
Vitamin A	325	4
Vitamin E	293	20

Source: MSZ EN 12822:2014, MSZ EN 12823-1:2014

2.4. Experimental

2.4.1. Optimizing Saponification Parameters of vegetable oil

Saponification was examined by the following factors:

- Temperature
- Time

a) Linear experimental design was performed, and result was found adequate.

Table-3. Vegetable oil saponification – linear experimental design

Factor	Time [min]	Temperature [°C]
Low level	35	60
High level	65	82
Center point	50	71

Source: Own experimental design respecting temperature and time ranges given in MSZ EN 12822, MSZ EN 12823:1

b) Corresponding to results, further tests were performed at boiling temperature, below 25 minutes of saponification time.

Table-4. additional test points

Time	Temperature
25 min	82 °C
20 min	82 °C
15 min	82 C

Source: Own experimental design respecting temperature and time ranges given in MSZ EN 12822, MSZ EN 12823:1

2.4.2. Optimizing Saponification Parameters of Delma margarine

Experimental design:

Table-5. Margarine saponification factors - second degree design

	Temperature [°C]	Time [min]
Low level	60	25
Upper level	82	50
Center point	71	35

Source: Own experimental design respecting temperature and time ranges given in MSZ EN 12822, MSZ EN 12823:1

Further experiments:

Table-6. Further test points of margarine saponification

T [°C]	t [min]
82	20
82	18
82	15

Source: Own experimental design respecting temperature and time ranges given in MSZ EN 12822, MSZ EN 12823:1

3. RESULTS

3.1. Vegetable oil: 2 Factors 2 Levels Experimental Design by Statsoft Statistica 7.0

Results were evaluated with a linear model, and was found adequate.

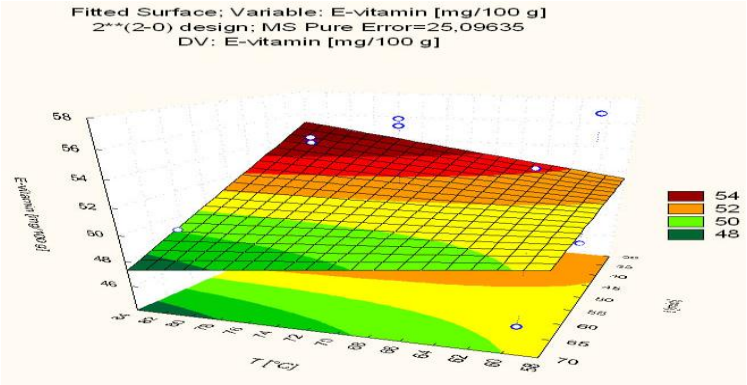

Diagram-1. E vitamin in vegetable oil (linear model)

Vitamin E recovery was found adequate.

3.1.1. Further Tests

Table-7. vitamin E recovery near optimum

T [°C]	t [min]	n	Vitamin E [mg/100g]	RSD%	Recovery
82	15	5	50,3	2,7%	102,7%
82	20	2	47,9	5,2%	97,8%
82	25	2	46,1	11,0%	94,2%

Source: Own experimental design respecting temperature and time ranges given in MSZ EN 12822, MSZ EN 12823:1

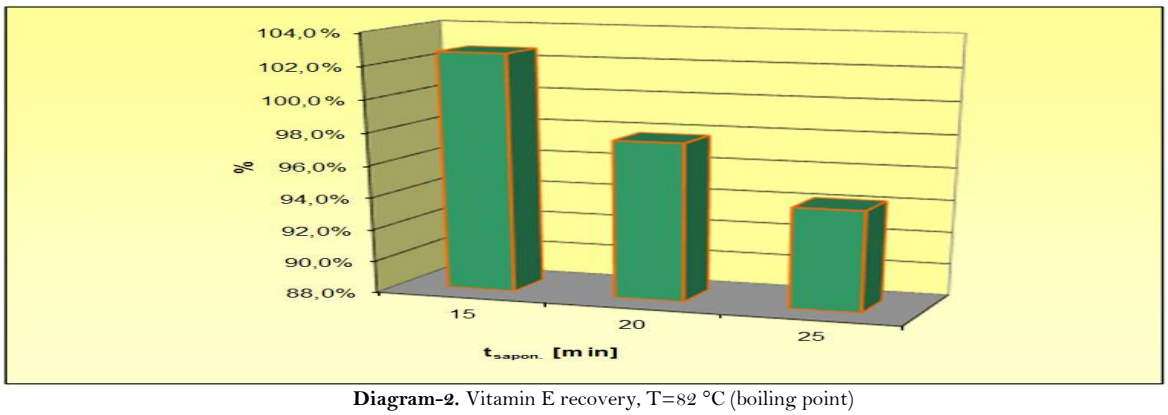

Diagram-2. Vitamin E recovery, T=82 °C (boiling point)

At the sample preparation of Vénusz vegetable oil, optimal recovery of vitamin E can be achieved at the boiling point of the reaction mixture at the minimal time required for saponification.

3.2. Vitamin A in Margarine Delma

Vitamin A and E was examined with a secondary degree 2 factors two levels experimental design (Table 10).

Table-8. Results of margarine saponification

Run	T [°C]	t [min]	Vitamin A	Run	T [°C]	t [min]	Vitamin A
1/1	60	25	0,71	2/5	71	35	0,74
2/1	60	25	0,75	1/6	71	50	0,67
1/2	60	35	0,78	2/6	71	50	0,69
2/2	60	35	0,35	1/7	82	25	0,80
1/3	60	50	0,69	2/7	82	25	0,85
2/3	60	50	0,74	1/8	82	35	0,73
1/4	71	25	0,69	2/8	82	35	0,74
2/4	71	25	0,63	1/9	82	50	0,68
1/5	71	35	0,65	2/9	82	50	0,76

Source: Results of own experiment

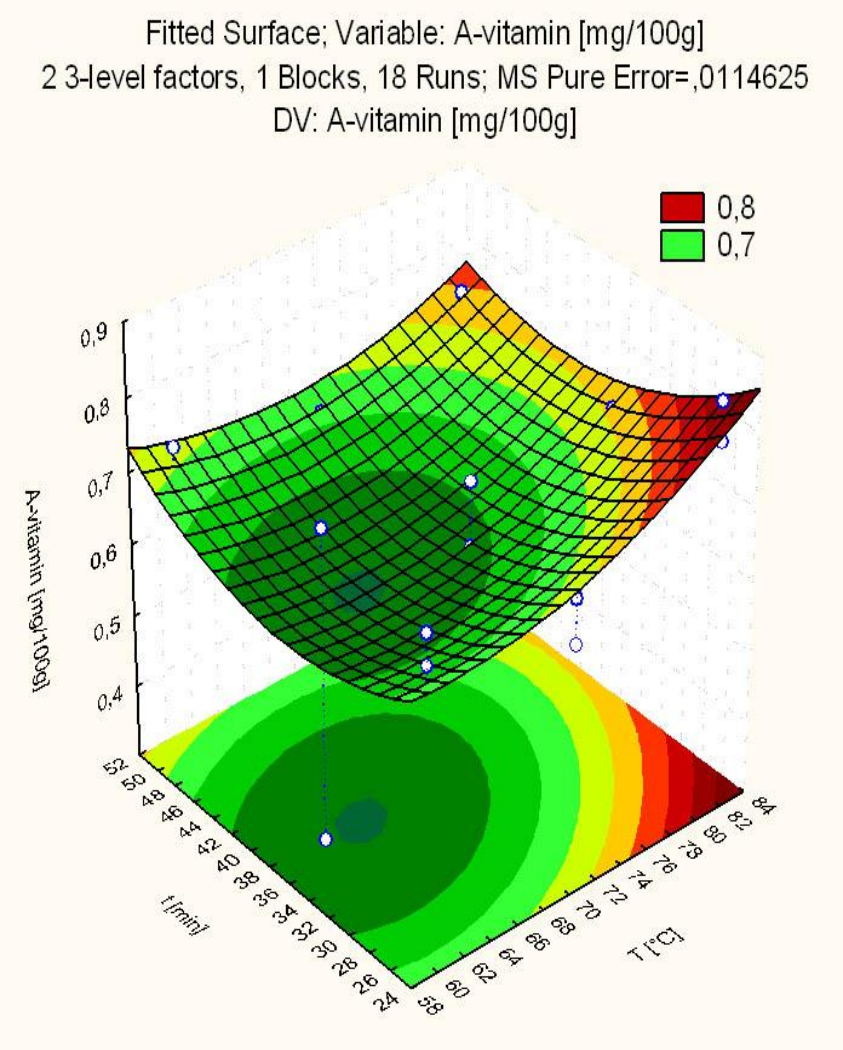

Diagram-3. 3D graph of vitamin A content of Delma margarine

Recovery optimum of vitamin A is at the shortest time at boiling point of the reaction mixture.

Table-9. Gradient results for vitamin A and E saponification

T [°C]	t [min]	n	Vitamin A [mg/100 g]	RSD	Recovery
82	15	2	0,94	6,7%	85,5%
82	18	5	0,97	3,4%	88,7%
82	25	3	0,86	9,0%	78,9%

Source: Results of own experiment

Point 82°C/15 min was found too short, as fatty drops remained before saponification. 18 minutes at boiling point was found optimal.

4. CONCLUSION

Saponification methods in Hungarian Standards MSZ EN 12822:2014 and MSZ EN 12823-1:2014, were optimized for temperature and saponification time factors. Saponification rate and the recovery of analytes can be assured at boiling point temperature with the shortest time needed for saponification. If other settings are used, it is recommended to measure recovery with standard addition.

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STANDARDS

MSZ EN 12822:2014 - Foodstuffs - Determination of vitamin E by high performance liquid chromatography - Measurement of alfa-, beta- , gamma- and delta-tocopherol, 2014, Hungarian Standards Institution.

MSZ EN 12823-1:2014 Foodstuffs – Determination of vitamin A by high performance liquid chromatography – Part 1: Measurement of all-trans-retinol and 13-cis-retinol, 2014, Hungarian Standards Institution.