BPF-003

Valorization of peptide and lipid fractions of hemp (Cannabis *Sativa* L.) seed cake: combined action of pressing and enzymatic hydrolysis for the formulation of a liposomal vector

Maureen Gerlei [1], Cyril J.F. Kahn [1], Cédric Paris [1], Arnaud Bianchi [2], Emilie Velot [2], Michel Linder [1]

[1]Université de Lorraine, LIBio, F-54000 Nancy, France;
[2] Université de Lorraine, CNRS, IMoPA, F-54000 Nancy, France

Abstract

Recent reports from FAO show that between 1990 and 2000, France multiplied its production by 50, becoming the European and world leader in hemp seeds (Cannabis *sativa L.*) production (Xu *et al*., 2020). Currently in this country, the 2015 Energy Transition Act accentuates the priority of producing sustainably within a green economy. This law establishes the circular economy for limiting environmental impact and resource wastage, by exploiting and valorizing waste, now known as co-products. To date, the recovery of co-products (seeds, flowers and aggregates) from hemp cultivation remains limited. First, this study consists in optimizing the yield of hemp seed oil extraction with a screw press with the response surface methodology (temperature, nozzle diameter and frequency). The latter process generates hempseed cakes, in which fraction of lipids and proteins still remains. Thus, a study from a physicochemical point of view allowed us to characterize and quantify all products obtained from this process (hemp seed vs oil and cakes). Secondly, enzymatic hydrolysis of the hemp seed cakes yields residual peptide fractions devided into several molecular weight groups and a heavy phase. Lipid extraction experiments were studied to obtain and characterize polar lipids (phospholipids). This extraction will enable the formulation of lipid nanovectors which will encapsulate a peptide fraction (<10 kDa) and active biomolecules intrinsic (cannabinoids).

Materials & Methods

Protocols

Physicochemical characterization were carried out using the methods described by AOAC.

Lipids were extracted *by* Folch extraction with chloroform/methanol 2:1 (v/v) and Soxhlet extraction with n-hexane (6h).

Fatty acid profiles *were* determined by GC-FID. Fatty acids were esterified according to the Ackman method (1998).

Phospoholipid profiles were obtained by Iatroscan MK5 and LC-MS/MS.

Cannabinoids profiles were quantified by UHPLC-MS/MS.

Liposomes were formulated according to the film hydration method (Bengham, 1964).

Liposome size*:* samples are diluted at 1/400th dilution and analyzed by DLS.

Cell test of cytotoxicity were realized : LDH %, MTT activity and DNA amount in human mesenchymal cell.

Optimization of hemp seed oil mechanical extraction by RSM

Equation of quadratic model :

$$Y = \beta_0 + \sum_{i=j}^{k} \beta i\, Xi + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j$$

Where Y is the response, X_i and X_j are the parameters, and β are the regression coefficients.

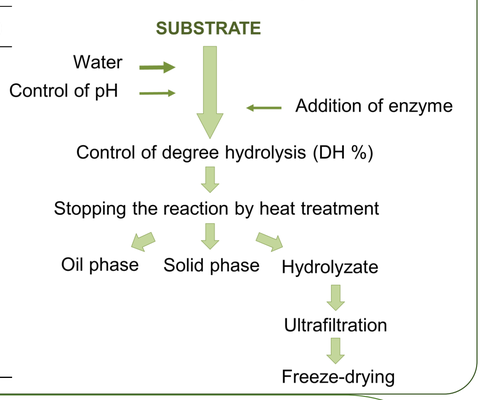
Experimental domain and levels of distribution of variables

Factors	Levels
Temperature (°C)	40 ; 50 ; 60 ; 70 ; 80
Frequency (Hz)	15 ; 20 ; 25 ; 30 ; 35 ; 40 ; 45
Nozzle (mm)	8 ; 10 ; 12

Doehlert design for 3 variables and responses

Experiment	Temperature (°C)	Frequency (Hz)	Nozzle (mm)	Oil mass (g)	Cake mass (g)
1	80	30	10	241.20	751.85
2	40	30	10	134.36	856.18
3	70	45	10	192.71	796.44
4	50	15	10	242.57	739.60
5	70	15	10	251.55	717.63
6	50	45	10	125.09	879.47
7	70	35	10	189.69	803.28
8	50	25	8	209.49	657.00
9	70	25	8	238.88	731.36
10	60	40	8	210.74	766.22
11	50	35	12	133.98	865.55
12	60	20	12	235.79	748.12
13	60	30	10	219.05	762.80
14	60	30	10	222.64	739.80
15	60	30	10	220.75	764.18

Enzymatic hydrolysis



Results

Physicochemical characterization

Physicochemical characterization of hemp seed and cake (g/100g)

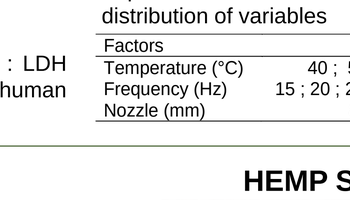
Sample	Hemp seed		Hemp cake	
Reference	Results	Mattila *et al.*, 2018	Results	Potin *et al.*, 2019
Protein	15.7 ± 0.7	25.6 ± 0.6	27.9 ± 1.3	30
Fat	**32.5** ± 1.5	34.6 ± 1.2	**10.4** ± 0.2	8.7
Fiber	30.3 ± 3.9	34.4 ± 1.5	56.4 ± 1.7	54
Ash	8.2 ± 0.5	5.4 ± 0.3	7.3 ± 0.5	6.9
Moisture	8.2 ± 0.1	6.7 ± 0.5	6.8 ± 0.1	5.3

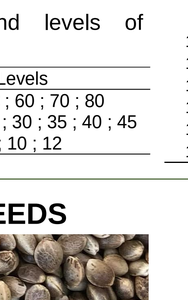
Hemp seed oil cannabinoid profile

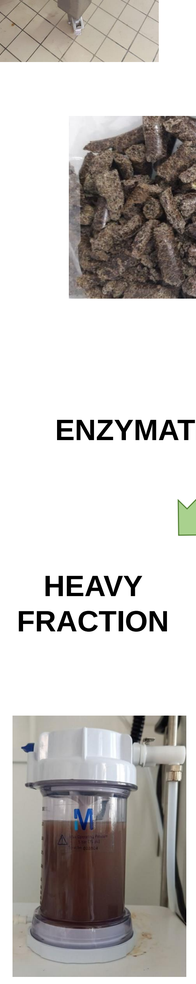
Cannabinoid (mg/g oil)	
Cannabidiolic acid (CBDA)	0.003
Cannabidiol (CBD)	0.085
Cannabigerolic acid (CBGA)	0.630
Cannabigerol (CBG)	0.004
Tetrahydrocannabinolic acid (Δ9-THCA)	0.000
Tetrahydrocannabinol (Δ9-THC)	0.000

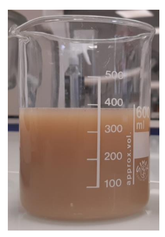
Fatty acids profiles of lipid fraction of seed and cake (%)

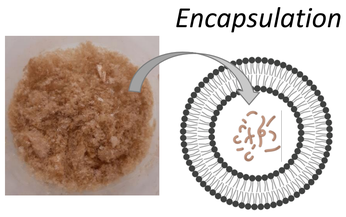
Sample		Seed		Cake
Extraction	n-Hexane	Press	Ethanol	Chloroform/methanol
C12:0				0.19 ± 0.04
C13:0				0.15 ± 0.04
C14:0	0.04 ± 0.00		0.07 ± 0.01	0.23 ± 0.16
C15:0 ISO				0.15 ± 0.01
C15:0			0.04 ± 0.00	0.54 ± 0.02
C16:0	6.79 ± 0.01	6.67 ± 0.01	7.73 ± 0.08	16.18 ± 0.60
C18:0 ISO				0.17 ± 0.03
C18:0	2.91 ± 0.01	2.83 ± 0.00	3.02 ± 0.03	6.60 ± 1.33
C20:0	2.95 ± 0.01	2.90 ± 0.00	3.69 ± 0.05	1.46 ± 0.05
C22:0	0.36 ± 0.00	0.35 ± 0.00	0.67 ± 0.65	0.32 ± 0.04
C24:0	0.15 ± 0.00	0.14 ± 0.00		0.11 ± 0.02
∑ SFA	**13.16**	**12.89**	**15.22**	**26.26**
C13:1 n-4				0.16 ± 0.01
C14:1 n-5				0.05 ± 0.00
C16:1 n-7	0.12 ± 0.00	0.12 ± 0.00	0.13 ± 0.00	0.35 ± 0.02
C18:1 n-9	13.57 ± 0.03	14.34 ± 0.01	13.12 ± 0.26	1.70 ± 0.06
∑ MUFA	**13.69**	**14.46**	**13.25**	**2.26**
C16:2 n-4				0.17 ± 0.01
C16:2 n-6				0.75 ± 0.29
C16:3 n-3				4.74 ± 0.11
C18:2 n-6	55.08 ± 0.02	54.72 ± 0.01	54.40 ± 0.48	51.67 ± 2.50
C18:3 n-3	16.24 ± 0.03	16.15 ± 0.01	16.11 ± 0.25	13.60 ± 2.15
C18:3 n-6	**0.88 ± 0.01**	**0.85 ± 0.00**	**0.85 ± 0.01**	**0.29 ± 0.02**
C18:4 n-3				0.27 ± 0.00
C20:2 n-6				0.12 ± 0.00
∑ PUFA	**72.20**	**71.72**	**71.36**	**71.61**
Ratio n-6/n-3	3.44	3.44	3.22	2.84
PUFA/SFA	5.56	5.56	4.69	2.73







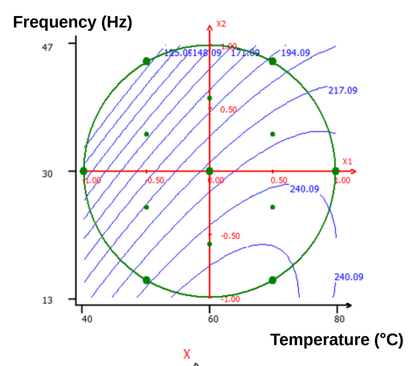


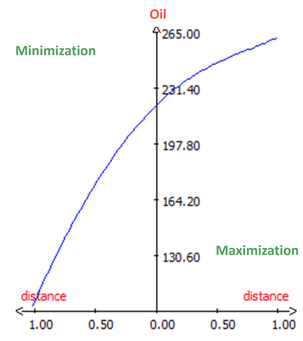


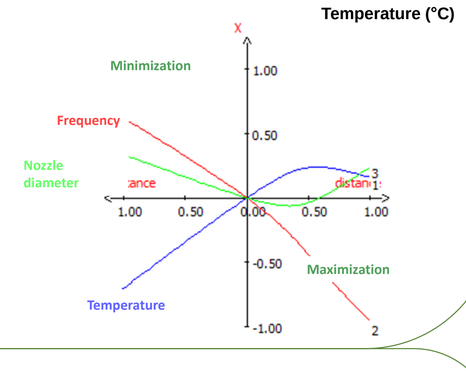
Optimization of yield of hemp oil by press extraction

Effects and interactions

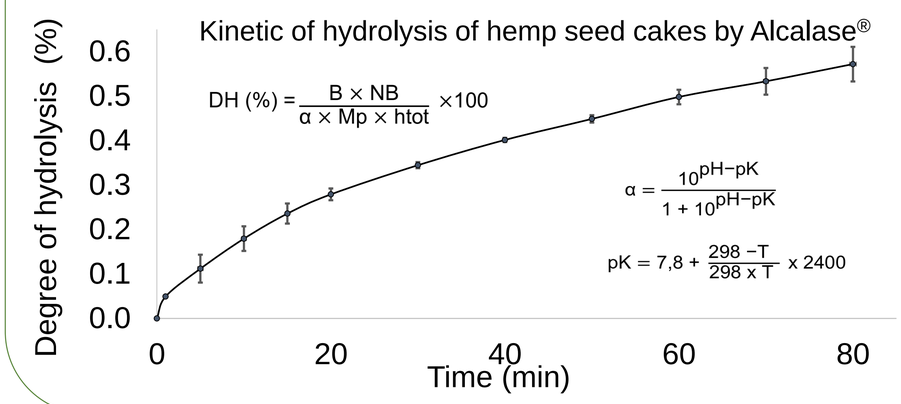
Terms	Coefficient	Significant (%)
β0	220.813	<0.01 ***
β1	46.922	<0.01 ***
β2	-50.790	<0.01 ***
β3	-20.341	0.112 **
β1-1	-33.033	0.191 **
β2-2	-12.767	7.0
β3-3	-15.127	3.48*
β1-2	33.857	0.477 **
β1-3	4.146	62.0
β2-3	-35.620	0.616 **







Control of hydrolysis degree (DH %) of hemp seed cakes

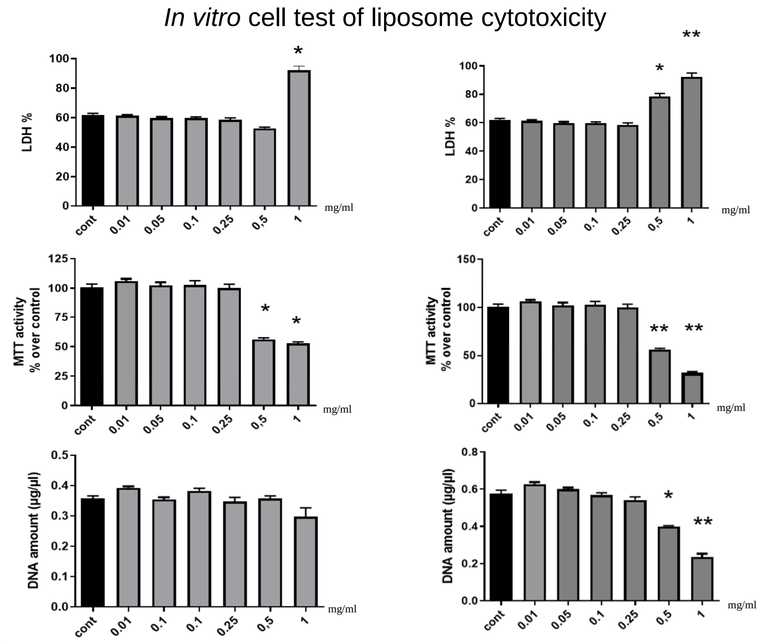


Characterization of empty liposomal vector

Characterization of liposome size

Pdi	Diameter	Zeta potential
0,204	110 nm	-20,4 mV

In vitro cell test of liposome cytotoxicity

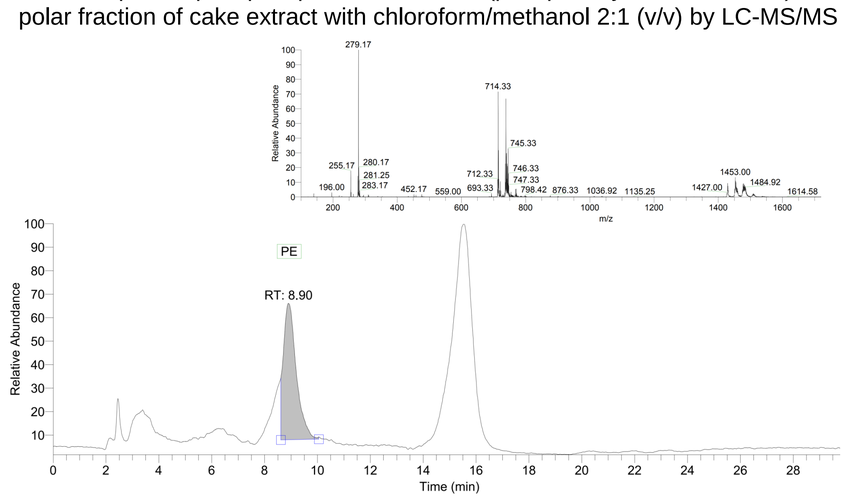


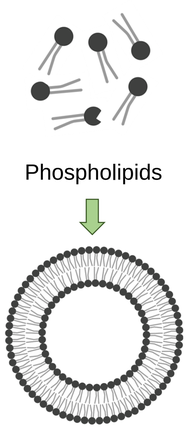
Characterization of polar lipids fraction

Characterization of class of polar lipids in polar fraction (PL) of cake extracted with chloroform/methanol 2:1 (v/v) (Folch) and ethanol (EtOH) by TLC-FID

Class of phospholipids (%)	Fraction PL Folch	Fraction PL EtOH
Phosphatidylcholine (PC)	49.09	36.27
Phosphatidylinositol (PI)	33.26	34.96
Phosphatidylethanolamine (PE)	13.94	12.27
Phosphatidic acid (PA)	0.00	11.29
Others	3.70	5.21

An example of phospholipid identification (phosphatidyletahanolamine) from polar fraction of cake extract with chloroform/methanol 2:1 (v/v) by LC-MS/MS





Conclusion

This study allowed:

- Optimization of hemps seed oil yield obtained by mechanical press.
- The oil and lipid fractions from hemp seeds and cakes are of interesting nutritional quality.
- Interesting characterization in terms of fatty acids and trace of bioactive molecules.
- Valorization of co-products.

- Liposomal-like carriers contain a pool of bioactive molecules:
  - Vectorization capabilities
  - Encapsulation of peptides and their bioactivities
- Presence of natural lipophilic biomolecules in low proportions → Value-added co-product
- Green solvent extraction study is in progress → Development of future formulations in Cosmetics and Nutraceuticals

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