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Assessment of N-Acylethanolamines levels in dry achenes from four cultivars of *cannabis sativa* L.

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SUMMARY: We utilized high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS), on dry achenes from four cultivars of *Cannabis Sativa* L.; *CA*, *Cannabis Sativa* L. *Cultivar Amnesia*; *CB, Cannabis Sativa* L. *Cultivar Beldia*; *CM, Cannabis Sativa* L. *Cultivar Mexicana*; *CK, Cannabis Sativa* L. *Cultivar Khardala*, to detect and quantify N-acylethanolamines (NAEs), which are bioactive compounds involved in lipid and energy metabolism. These plants were grown in Chefchaouen, northern Morocco. All four varieties displayed identical NAE lipid profiles, dominated by those derived from 16C and 18C fatty acids. In general, the NAE species presented the following concentration order: [LEA] > [OEA > POEA] > [SEA] > [PEA]. NAE-MUFA was the most abundant type, followed by NAE-PUFA and NAE-SFA, comprising 44, 37, and 19% of all NAEs, respectively, across the varieties. This research provides first-time quantification of NAEs in Cannabis achenes, thus enriching our understanding of these plants' pharmaceutical and nutritional potential.

***KEYWORDS**: Cannabis achenes;* Cannabis sativa *L; N-acylethanolamines (**NAEs**); NAE-Monounsaturated (**NAE-MUFA**); NAE-Polyunsaturated (**NAE-PUFA**); NAE-Saturated (**NAE-SFA**).*

RESUMEN: ***Evaluación de los niveles de N-aciletanolaminas (NAEs) en aquenios secos de cuatro cultivares de* cannabis sativa *L.*** Empleamos cromatografía líquida de alta resolución-espectrometría de masas (HPLC-MS/MS), en aquenios secos de cuatro cultivares de *Cannabis Sativa* L: *CA*, *Cultivar Amnesia*; *CB, Cultivar Beldia*; *CM, Cultivar Mexicana*; *CK Cultivar Khardala*, para detectar y cuantificar N-aciletanolaminas (NAE), compuestos bioactivos implicados en el metabolismo lipídico y energético. Estas plantas se cultivaron en Chefchaouen, al norte de Marruecos. Las cuatro variedades mostraron perfiles lipídicos de NAE idénticos, predominando los derivados de los ácidos grasos 16C y 18C. En general, las especies de NAE presentaron el siguiente orden de concentración: [LEA] > [OEA > POEA] > [SEA] > [PEA]. NAE-MUFA son los más abundante, seguido de NAE-PUFA y NAE-SFA, que presentan el 44%, el 37% y el 19% de todos los NAE respectivamente, en todas las variedades. Esta investigación proporciona por primera vez una cuantificación de las NAE en los aquenios del *cannabis*, enriqueciendo nuestra comprensión del potencial farmacéutico y nutricional de la planta.

***PALABRAS CLAVE**: Aquenios de Cannabis;* Cannabis sativa *L; NAE-Saturados (NAE-SFA); NAE-Monoinsaturados (NAE-MUFA); NAE-Poliinsaturados (NAE-PUFA); N-aciletanolaminas (NAEs).*

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1. INTRODUCTION

Developing countries have increasingly valued the potential of cannabis as a raw material source for the food industry, along with pharmacopoeia, and medicinal purposes, (Baldini *et al.,* 2018). By contrast, in Morocco the pharmacological and medicinal value of Cannabis is relatively unexplored. As a result, its value often hinges on its content, developed as cannabinoid products, such as THC, CBD, CBC, and CBN, (Ouhtit *et al.,* 2024). These secondary metabolites contribute to the plant's well-known psychotropic effects. However, with the introduction of a new law n°13-21 (Bulletin officiel, 2021) which

legalized the use of Cannabis in Morocco, Cannabis achenes could potentially support the agricultural economy in a meaningful way, through their pharmaceutical, medicinal, and agro-alimentary potential, (Ouhtit *et al.,* 2024).

Cannabis achene oil ranks among the most nutritious vegetable oils due to its high polyunsaturated fatty acid (PUFA) content, which makes up approximately 80%, (Ouhtit *et al.,* 2024). Linoleic acid (C18:2, n6, LA) and α-linolenic acid (C18:3, n3, ALA) emerge as the most dominant FAs with a 3:1 ratio, (Matthäus *et al.,* 2008). As essential lipids in mammals that cannot be synthesized de novo, these FAs need to be incorporated into the diet. LA and ALA serve as precursors for the n6 and n3 FA families, respectively. They can be elongated and desaturated into highly unsaturated forms with ≥ 20 carbon atoms and ≥ 3 double bonds, chiefly arachidonic acid (ARA, 20:4n-6), and docosahexaenoic acid (DHA, 22:6n-3), (Innis *et al.,* 1999). The n3 and n6 PUFAs play a role in various physiological processes and influence human health in multiple ways, (Murru *et al.,* 2013).

Our study evaluates a specific biochemical aspect of Cannabis; the content of N-acylethanolamines (NAEs) in achenes, an area where no data is currently available.

N-Acylethanolamines (NAEs) function as fatty acid (FA) amides which are derived from an N-acylated phoshatidylethanolamine (NAPE) precursor, a minor membrane lipid derivative of the common membrane phospholipid, phosphatidylethanolamine (PE). The NAPE substrate is metabolized by a phosphodiesterase (PLD) to release phosphatidic acid (PA) and N-Acylethanolamines NAE, (Chapman, 2004). The NAE species represent a vital family of endogenous phospholipid mediators. They play a significant role in cellular signal transduction within both animal and plant tissues, (Blancaflor *et al.,* 2006). These molecules are synthesized "in situ" at elevated concentrations when cells are subjected to pathophysiological conditions. Interestingly, NAEs form a crucial part of the endocannabinoidome system (ECS), which controls various physiological functions in multicellular eukaryotes, including neurotransmission, embryonic development, implantation, feeding behavior, and cell proliferation, (Chapman, 2004).

NAEs were first identified during an examination of phosphatide composition in wheat flour, (Bomstein, 1965). Several subsequent studies have investigated, quantified, and elucidated the metabolic pathways of NAE biosynthesis. For instance, de la Roche *et al.,* (1973), demonstrated phospholipid level alterations in dry and germinating wheat seeds due to elevated activity of the enzyme Phospholipase-D (PLD). PLD degrades certain phospholipids in the endosperm, accounting for over 80% of total phospholipids in dry seeds, thus releasing new N-acylated molecules which are crucial for embryo development during germination, (de la Roche *et al.,* 1973).

NAEs are generally classified as a group of endogenous molecules with cannabimimetic activity (endocannabinoids, EC) capable of producing effects akin to cannabinoids in vivo, either mediated or not mediated by cannabinoid receptors. The NAE-endocannabinoid capable of activating the two cannabinoid receptors, CB1 and CB2, and producing effects similar to cannabinoids, primarily THC, is known as Anandamides or N-arachidonoylethanolamine (AEA). This nomenclature derives from the Sanskrit word "Ananda", signifying "happiness" and referring to the psychotropic effects of THC. AEA has garnered increased pharmacopoeial interest due to its identification and isolation in the porcine brain, and in various tissues of mammalian species including humans, (Felder *et al.,* 1996). However, some NAEs, such as N-palmitoylethanolamine (NAE-16:0, PEA) and N-oleoylethanolamine (NAE-18:1, OEA), lack affinity for CB1 and CB2 receptors. These NAEs have been shown to be avid ligands of the nuclear peroxisome proliferator receptor (PPAR)-α, (LoVerme *et al.,* 2005). Multiple studies have highlighted the cytoprotective properties of NAEs, attributing them critical roles in the regulation of hypothalamic functions in mammals, particularly in controlling pituitary hormone secretion, (Weidenfeld *et al.,* 1994), thermoregulation, and the sleep/wake cycle. In the event of tissue damage, NAEs trigger apoptotic and anti-inflammatory mechanisms in damaged cells to prevent necrosis spreading to neighboring cells, (Hansen *et al.,* 2000).

Within plant tissue, NAEs represent a lipid-mediated pathway controlling phytohormone-mediated regulation of plant growth and development, (Blancaflor *et al.,* 2014). NAEs' primary functions encompass scavenging phospholipid bilayer-destabilizing precursors such as free FA and ethanolamine, thereby offering stability and membrane protection against physiological and environmental changes within

cells, (Chapman, 2004). Moreover, NAEs play pivotal roles in activating cell defense genes, (Blancaflor *et al.,* 2006). NAE types identified in seeds typically span 12–18 carbons in length with zero to two double bonds, with NAE abundance in desiccated seeds recorded as micrograms per gram of fresh weight. Predominantly, N-linoleoylethanolamine (NAE-18:2), NAE-16:0, and NAE-18:1 emerge as the most abundant types of NAEs found in seeds. NAE profiles seem to reflect the total FA profiles in acyl lipids from the species of origin, (Chapman *et al.,* 1999).

The aim of this study is to assess the N-acylethanolamines (NAEs) levels from dry achenes using four *Cannabis sativa* L. *cultivars; CA, Cannabis Sativa* L. *Cultivar Amnesia; CB, Cannabis Sativa* L. *Cultivar Beldia; CM, Cannabis Sativa* L. *Cultivar Mexicana; CK, Cannabis Sativa* L. *Cultivar Khardala*. The biochemical aspect of cannabis achenes will be discussed for the first time, highlighting a latent potential for the field of industry.

2. MATERIALS AND METHODS

2.1. Preparation of plant materials and samples

The field trials were set up in El Kalâa, a small village in the region of Chefchaouen (35° 13’ 110’’ N, 5° 14’ 42’’ W), 873(m) in altitude. The village has a mountainous morphology with the famous Jbel el Kalâa summit (1721 m). The climate is typically mountainous, with frequent rainfall, cold in winter and mild or hot in summer. Rainfall is generally between 800 and 1400, but sometimes it can exceed 2000 mm/year. During the summer (July, to mid-August) a dry period occurs with scarce rainfall and high temperatures which sometimes reach or exceed 40 °C, (Benabid, 1982).

Four *Cannabis sativa* L. commercial cultivars namely; *CA, Cannabis Sativa* L. *Cultivar Amnesia; CB, Cannabis Sativa* L. *Cultivar Beldia; CM, Cannabis Sativa* L. *Cultivar Mexicana; CK, Cannabis Sativa* L. *Cultivar Khardala*, were studied. Certified seeds were sown according to the local traditional method of hemp cultivation, then subjected to the same processes of harvesting, drying and production of resin and achenes. Sowing was carried out between March and April (2020) and harvesting between August and September (2020). At the maturity phase, during which more than 90% of brown seeds appeared, the female plants were harvested and then subjected to sun-drying for 4 days. A total of four samples (1 kg for each sample) of hemp seeds were collected from the harvested and dried plants. The collected seed samples were cleaned and excluded from the unripe and the empty seeds, then stored at 4 °C. Herbarium specimens of the *Cannabis Sativa* seeds used in this study were deposited in the herbarium of the Applied Botany Laboratory, Department of Biology, Faculty of Sciences of Tetouan, Abdelmalek Essaâdi University.

The seeds were air-dried beforehand and had an average moisture content of 7.614 ± 1.623 % according to the AOAC Official Method 925.40 (2000), such that 5 grams of each seed variety were placed in a temperature-stabilized oven at 60 ºC until constant mass. Moisture content was calculated as percent (%) of the loss in recorded weight.

2.2. Phytochemical profiling of NAEs using HPLC-MS

Total lipids were extracted from dry Achenes samples according to the method of Folch *et al.* (1957). Deuterated N-acylethanolamine (NAEs) and congeners were added to the samples as internal standards before extraction for quantification by isotope dilution. Aliquots of the lipid fraction were used for their quantification. Internal deuterated standards N- arachidonoylethanolamine $[2H]^8$AEA, N-oleoylethanolamine $[2H]^2$OEA, N-palmitoylethanolamine $[2H]^4$PEA, N-stearoylethanol- amine $[2H]^3$SEA, 2-arachidonoyl-glycerol-d5 $[2H]^5$2AG, were purchased from Cayman Chemicals (MI, USA).

NAE quantification was carried out using an Agilent 1260 UHPLC system (Agilent, Palo Alto) equipped with a mass spectrometry (MS) Agilent Technologies QQQ triple quadrupole 6420 with an electrospray ionization (ESI) source, using positive mode (ESI+).

A Poroshell 120 EC-C-18 column (Agilent, Palo Alto, CA, USA) with 2.7 µm particle size and 3 × 100 mm was used with a mobile phase of CH3OH/H2O/HCOOH (80/20/0.1, v/v/v) at a flow rate of 0.5 mL/min. N_2 was used as a nebulizing gas with a pressure of 50 psi, a drying gas temperature of 300 ºC, a flow of 11 L/min, and 4000 V capillary voltage. For each standard, the precursor ion $[M+ H]^+$ was determined during a full scan (SCAN) in MS, and subse-

quently, the obtained product ion (PI) was monitored for each transition in MRM mode in MS/MS. The parameters of source, such as cone voltage or fragmentor (CV) and collision energy (CE), were optimized for each MRM transition (Table 1), (Murru *et al.,* 2021).

The data was acquired by the MassHunter workstation acquisition software (version B.08.02), analyzed with MassHunter software for qualitative analysis (version B.08.00 SP1) and by the MassHunter workstation software for quantitative analysis (version B.09.00).

2.3. Statistical analysis

All measurements were taken on dry samples, and values were expressed as means ± SD of two replicates from each independent experiment. The differences among the four cultivars were assessed using One-way Anova and Tukey's test. Statistical analyses were made using the SPSS package version 23 (IBM, Armonk, NY, USA). Differences were deemed significant at a probability level of 5%. Principal component analysis (PCA) was done for cultivars based on the major compounds. The XLSTAT software was used for different data processing.

3. RESULTS AND DISCUSSION

3.1. Total NAE analysis

The test for homogeneity of variance revealed a p-value < 0.05 for the variable "total NAE rate" in the four achene varieties: *CA, Cannabis Sativa* L. *Cultivar Amnesia; CB, Cannabis Sativa* L. *Cultivar Beldia; CM, Cannabis Sativa* L. *Cultivar Mexicana; CK, Cannabis Sativa* L. *Cultivar Khardala*. According to Tukey's test, the four cultivars were categorized into two homogeneous subsets. The NAE average levels in our achene samples were 921.38 ± 260.22 pmol/g dry weight. Figure 1 shows significant differences in total NAE levels only between CA and CM (p-value < 0.05); CM achenes had the lowest total NAE levels (751.83 ± 2.15 pmol/g dry weight), while the highest levels were contained in CA achenes, at greater than 1.75-fold (1309.23 ± 132.61 pmol/g dry weight).

We reported the total NAE levels identified in the seeds and tissues of different plant taxa in Table 2, such as Soybean, Peanut, Castor oil, Tomato, Okra, Cotton and Corn, which ranged from 490 ng/g in Peanut to 1608 ng/g in Cotton. NAE levels in the CA achenes of our sample (416.66 ± 41.49 ng/g dry weight), were comparable to Peanut seeds.

3.2. Identified NAE species

The different NAE species of the four cultivars studied are presented in Table 3. LEA, SEA and PEA displayed significant differences (p-value < 0.05) in our achene samples. The NAE profile of the four varieties of achenes was similar to the patterns observed in non-leguminous seeds, (Arias-Gaguancela *et al.,* 2022), in which the most abundant were NAE-18:2,

Table 1. MS/MS source parameters to identify several NAE molecules.

NAEs	P.M	Ione precursor → PI	Fragmentor (CV)	CE	Acceleration (V)
EPEA	345	346 → 187	136	10	4
POEA	297	298 → 62.1	128	14	4
LEA	323	324 → 62.1	140	14	4
AEA	347	348 → 62.1	140	10	4
AEAd8	355	356 → 63	130	12	4
DHEA	371	372 → 62	130	10	4
OEA	325	326 → 62	128	14	4
OEAd2	327	328 → 62.1	130	12	4
DTEA	375	376 → 62	140	14	4
SEA	327	328 → 61.7	128	12	4
PEA	299	300 → 62.1	148	14	4
PEAd4	303	304 → 62.1	130	14	4

NAE-18:1 and NAE-16:1 species followed by NAE-18:0 and NAE-16:0.

In a study conducted on plant species, the variability of NAEs among varieties of species such as cotton, was greater than the variability in our analyzed species, (Chapman *et al.,* 1999). Similar data was obtained from a species of the legume family, *the Medicago Sativa.* L species, (Venables *et al.,* 2005). Furthermore, the chemical composition of NAEs can vary due to several factors, such as genotype (variety), plant section used for extraction analysis, extraction method, and conditions (times and temperature). These differences in NAE composition appear upon seed germination and seedling growth, such that, in young seedlings it is similar to that in

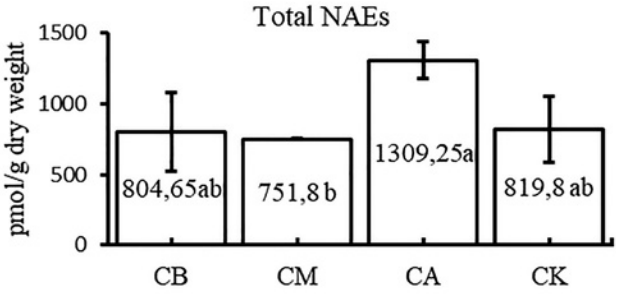

Figure 1. Quantification of total NAE levels in dry achenes from four *cannabis sativa* L cultivars; *CA, Cannabis Sativa* L. *Cultivar Amnesia; CB, Cannabis Sativa* L. *Cultivar Beldia; CM, Cannabis Sativa* L. *Cultivar Mexicana; CK, Cannabis Sativa* L. *Cultivar Khardala*. Bars represent means ± SD of two replicates and expressed by pmol/g dry weight. Values with different superscript letters are significantly different, *p-value < 0.05* according to One-way Anova and Tukey's test.

Table 2. Levels of several NAE molecular species in different plant taxa.

Plant species	Total NAE content	Abundance order of major NAE molecular species *(acyl chain)*	References
Phaseolus vulgaris cv	6.84 ± 0.96	16:0>18:3>18:2>18:1> 8:0>14:0> 2:0	Data summarized from (Venables *et al.,* 2005), total NAE content expressed in µg/g lipid weight.
Medicago truncatula cv. Jemalong	350 ± 26.8	16:0>18:2>18:1>18:3>18:0>14:0	
Medicago sativa cv. 7101	68.0 ± 5.10	18:3>18:2>16:0>18:1>18:0	
Medicago truncatula cv. A17	40.0 ± 4.20	18:3>18:2>18:1>16:0>18:0	
Vigna unguiculata cv. Tohono O'odham	13.4 ± 1.39	16:0>18:2>18:1>18:3>18:0>14:0	
Glycine max cv. Dare	169 ±10.8	18:2>16:0>18:1>18:3>18:0>14:0>12:0	
Bauhinia congesta	3.09 ± 0.52	18:1>18:2>16:0> 2:0>18:3> 4:0>18:0	
Pisum sativum cv. Taos	124 ± 9.74	18:1>18:2>16:0>18:3>18:0>14:0>12:0	
Pisum sativum cv. Early Alaska	144 ± 3.37	18:2>18:1>16:0>18:3>18:0>14:0	
Arachis hypogaea	39.5 ± 1.33	18:1>18:2>16:0>18:0>18:3>14:0	
Lupinus succulentus	28.5 ±1.85	18:2>16:0>18:1>18:0>18:3>14:0>12:0	
Lupinus texensis	73.6 ± 26.4	18:2>18:1>16:0>18:3>18:0>14:0=12:0	
Mimosa borealis	20.5 ± 4.12	18:2>16:0>18:1>18:3> 8:0>12:0>14:0	
Caesalpinia gilliesii	3.70 ± 0.99	18:2>18:3>16:0>18:1>18:0>12:0>14:0	
Cannabis sativa L. CA	416.65 ± 41.48	18:2> 18:1> 16:1> 18:0> 16:0	Data summarized from this present study (2049-Ouhtit *et al.,* 2024). Total NAE content expressed in ng/g dry weight.
Cannabis sativa L. CK	260.82 ± 74.49	18:2> 18:1> 16:1> 18:0> 16:0	
Cannabis sativa L. CB	255.36 ± 87.46	18:2> 18:1> 16:1> 18:0> 16:0	
Cannabis sativa L. CM	238.42 ± 0.71	18:2> 18:1> 16:1> 18:0> 16:0	
Cottonseed	1608 ± 309	18:2> 16:0> 18:1 = 12:0	Data summarized from, (Chapman *et al.,* 1999). Total NAE content expressed in ng/g fresh weight.
Corn	1211 ± 156	18:2 = 18:1> 16:0 = 12:0	
Soybean	1079 ± 172	18:2> 16:0> 18:1 = 12:0	
Peanut	958 ± 78	18:2 = 18:1> 16:0> 12:0	
Okra	792 ± 121	18:2> 16:0 = 18:1 = 12:0	
Tomato	742 ± 156	18:2> 12:0 = 18:1> 16:0	
Castor	627 ± 29	18:2> 12:0> 18:1> 16:0	
Pea	490 ± 89	18:2 = 18:1 = 12:0 > 16:0	

Abbreviations: PEA, N-Palmitoylthanolamine (16: 0-NAE); SEA, N-Stearoylethanolamine (18:0-NAE); OEA, N-Oleoylethanolamine (18:1-NAE); POEA, Palmitoleoylethanolamine (16: 1-NAE); LEA, N-Linoleoylethanolamine (18:2-NAE); Lauroyl-EA, N-lauroylethanolamine (12:0-NAE); Linolenoyl-EA, N-Linolenoylethanolamine (18:3 NAE); Myristoyl-EA, Myristoylethanolamine (14:0-NAE).

Table 3. Contents of several NAE molecular species in different achenes of four *cannabis sativa* L. varieties (pmol/g dry weight).

NAE molecular species	Taxa			
	CB	CM	CA	CK
PEA	44.54 ± 1.96 [b]	49.64 ± 7.96 [b]	153.86 ± 6.21 [a]	64.07 ± 14.71 [b]
SEA	51.84 ± 7.76 [c]	83.61 ± 20.31 [b, c]	144.10 ± 17.68 [a]	105.00 ± 23.19 [a, b]
OEA	256.20 ± 137.65 [a]	206.42 ± 57.63 [a]	355.33 ± 65.20 [a]	202.89 ± 76.96 [a]
PEO	161.45 ± 104.25 [a]	152.84 ± 62.66 [a]	146.78 ± 54.03 [a]	125.25 ± 52.64 [a]
LEA	290.64 ± 42.47 [b]	259.31 ± 89.88 [b]	509.15 ± 10.51 [a]	322.58 ± 67.71 [b]
Total NAEs content	804.65 ± 278.53 [ab]	751.80 ± 2.12 [b]	1309.25 ± 132.58 [a]	819.80 ± 235.18 [ab]

Data represent means ± SED for all NAE species and total content from two replicate treatments on four cannabis achene varieties and are expressed in pmol/g dry weight. Values in the same row with different superscript letters are significantly different ($p < 0.05$) according to One-way Anova and Tukey's test. *CA, Cannabis sativa* L. *Cultivar Amnesia; CB, Cannabis sativa* L. *Cultivar Beldia; CM, Cannabis sativa* L. *Cultivar Mexicana; CK, Cannabis sativa* L. *Cultivar Khardala*; PEA, N-Palmitoylthanolamine (16: 0-NAE); SEA, N-Stearoylethanolamine (18:0-NAE); OEA, N-Oleoylethanolamine (18:1-NAE); POEA, Palmitoleoylethanolamine (16: 1-NAE); LEA, N-Linoleoylethanolamine (18:2-NAE).

the leaves of mature plants, (Wang *et al.,* 2006). In seeds, the proportion of NAE-PUFA decreased more substantially than NAE-SFA species and the oxidative metabolism may contribute significantly to the overall changes during seedling growth, (Chapman *et al.,* 1999).

PEA (NAE-16:0) presented significant differences (p-value < 0.05) among the four achene varieties. The average PEA levels in all four varieties of achenes were 78.03 ± 51.23 pmol/g dry weight; CB achenes showed the lowest levels (44.54 ± 1.96 pmol/g dry weight), while the highest were in the CA achene species (153.86 ± 6.21 pmol/g dry weight), representing 11.75% of the total NAEs.

In addition, SEA (NAE-18:0) showed significant differences (p-value < 0.05). The average SEA of the four species of achenes was higher by 23% than PEA levels. Similar to PEA, CB and CA achenes showed the minimum and maximum levels, 51,84 ± 7,76 pmol/g dry weight and 144,10 ± 17,68 pmol/g dry weight, respectively. In CA achenes, SEA accounted for 10.83 - 11.16% of the total NAEs.

These differences are in accordance with the NAE profile reported for seeds of the legume taxa, (Venables *et al.,* 2005); PEA levels ranged from 0.38 ± 0.03 µg/g lipid weight in the seeds of *Bauhinia congesta* species to 99.7 ± 6.38 µg/g of lipid in the seeds of *Medicago truncatula cv*. Jemalong. SEA levels represented 0.08 ± 0.1 µg/g lipid weight in the seeds of the *Bauhinia congesta* species and 15.9 ± 1.03 µg/g lipid weight in the seeds of *Medicago truncatula cv*. Jemalong.

These two NAE-SFA molecules (PEA, SEA) possess promising therapeutic potential. PEA shows an autocoid negatively modulating mast cell behavior in response to inflammatory noxious stimuli in vivo, (Hesselink *et al.,* 2017). PEA acts as a potent anti-inflammatory and anti-neuroinflammatory agent via activation of the nuclear peroxisome proliferator receptor (PPAR)-α, (Costa *et al.,* 2008), which regulates the activation of genes which are responsible for the synthesis of inflammatory cascades and pro-inflammatory mediators such as cytokines and the tumor necrosis factor alpha (TNF-α), (Hesselink *et al.,* 2017). In addition, the targeting of the transient receptor potential vanilloid-1 (TRPV-1) by PEA is another aspect of its physiotherapeutic importance which confers its anti-allodynic and anti-hyperalgesic effects.

Similarly, SEA is known for its therapeutic effect in modulating immune and inflammatory responses in allergic diseases, in synaptic dysfunction and acute and late neurodegeneration, (Kasatkina *et al.,* 2020).

We found other NAE subtypes biosynthesized by monounsaturated FA (MUFA), such as oleic acid (18:1) for NAE-18:1 (OEA), and palmitoleic acid (16:1) for the NAE-16:1 (POEA) production. We observed no significant changes in NAE-18:1 (OEA) levels in the four achene varieties (p-*value* > *0.05*). The highest levels were in CA achenes (355.33 ± 65.20 pmol/g dry weight), which ranged from 25.44 to 28.61% of the total NAEs. Also, NAE-16:1 (POEA) presented no significant difference (*p-value*

> 0.05), with the highest amount in the CB achenes (161.45 ± 104.25 pmol/g dry weight), ranging from 14.44 to 23.48% of the total NAEs.

Nutritionally, OEA is considered a promising therapeutic agent for weight control, obesity and associated diseases. OEA induces hypophagia and reduces fat mass in rodents and PPAR-α has been shown to be the most widely accepted mediator of the hypophagic action of OEA via signaling homeostatic brain centers, (Brown *et al.,* 2017). Recent studies have revealed that OEA reduces food intake via effects on dopamine and endocannabinoid signaling in the brain, (Sihag *et al.,* 2018). OEA also binds with two other known receptors with moderate potency, namely the G protein-coupled receptor 119 (GPR119), and the capsaicin receptor, transient receptor potential vanilloid-1 (TRPV1) (Im, 2021).

POEA is a palmitoleic acid derivative, and is a well-characterized agonist of GPR119 receptors capable of counteracting the metabolic syndrome associated with complicated obesity. It has pharmacological activity which is similar to that of the oleic acid derivative OEA in the regulation of energy intake through insulin reléase, (Bandres-Meriz *et al.,* 2023). POEA is among the plasma NAEs which act on brain connectivity in homeostatic and reward circuits through hunger and satiety states to maintain homeostasis in humans, (DiPatrizio, 2021). In addition, POEA is a vital and effective nutritional ingredient that can be used against metabolic disorders associated with diet-induced obesity.

Among the polyunsaturated FA (NAE-PUFA), we observed only NAE-18:2, derived by LA which varies significantly (*p-value < 0,05*) among achene varieties, with an average of 345.42 ± 112.17 pmol/g dry weight. CM achenes had the lowest levels (259.31 ± 89.88 pmol/g dry weight), while CA achenes possessed about twice the CM amount (509.15 ± 10.51 pmol/g dry weight). LEA accounted for 35.76 to 42.50% of the total NAEs quantified in CA achenes.

In plant tissue, LEA is a signaling molecule which regulates seed germination and plant growth, (Keereetaweep *et al.,* 2015). According to our data, it is the most abundant molecular species of NAEs identified in the dry seeds of Pea, Soybean, Peanut, Castor, Tomato, Okra, Cotton and Maize, with levels exceeding 800 ng/g fresh seed weight, (Chapman *et al.,* 1999). In another study, LEA ranged from 28.8 ± 5.7 ng/g fresh seed weight in the seeds of the species *Phaseolus vulgaris cv*. Amarillo del Norte to 12740 ± 995 ng/g fresh seed weight in the seeds of the species *Glycine max cv*. Dare, (Venables *et al.,* 2005).

3.3. Relation among NAE species and achene varieties

The NAE relations found in the achenes were studied by principal component analysis (PCA). The first factorial plane, made up of the axes (F1) and (F2), represented 98.12% of the total inertia. The projection of the variables on the first two axes of the PCA (Figure 2), allows us to highlight groupings, oppositions and directional tendencies. The first axis (F1) explains 72.18% of the total variance, while the axis (F2) explains 25.94%. The PEA, LEA and OEA variables are closely linked and evolve in the same direction while positively differentiating on the axis (F2) and on the axis (F1). On the other hand, they are opposed according to the axis F1 to SEA, while the latter is positively correlated with F2. However, POEA shows a strong positive correlation at the F1 axis, and a negative correlation at F2. The projection of the observations (achenes variety) on the two axes of the PCA (F1 and F2) shows a strong separation and a fairly clear differentiation between cultivars. For example, the F2 axis opposes the CA of all the three varieties, while giving it an extreme positive coordinate. Furthermore, the CA is positively correlated to F1, and the achenes corresponding to the CA show a strong affinity for the variables PEA, LEA, OEA, SEA. Conversely, CB and CM are positively correlated to F1 with an extreme positive coordinate for CB, while the latter shows a strong affinity for POEA. CK exhibits a negative correlation at both F1 and F2. The cultivars CA and CB appear to be significant, given their richness in different NAE species which has been identified in this study.

4. CONCLUSIONS

Our research marks the first study that identifies and quantifies various NAE species (PEA, SEA, OEA, POEA, LEA) in Moroccan Cannabis achenes. We demonstrated an abundance of N-Acylethanolamines (NAEs) molecules in the dry achenes of four *Cannabis sativa* L. cultivars; *CA, Cannabis Sativa* L.

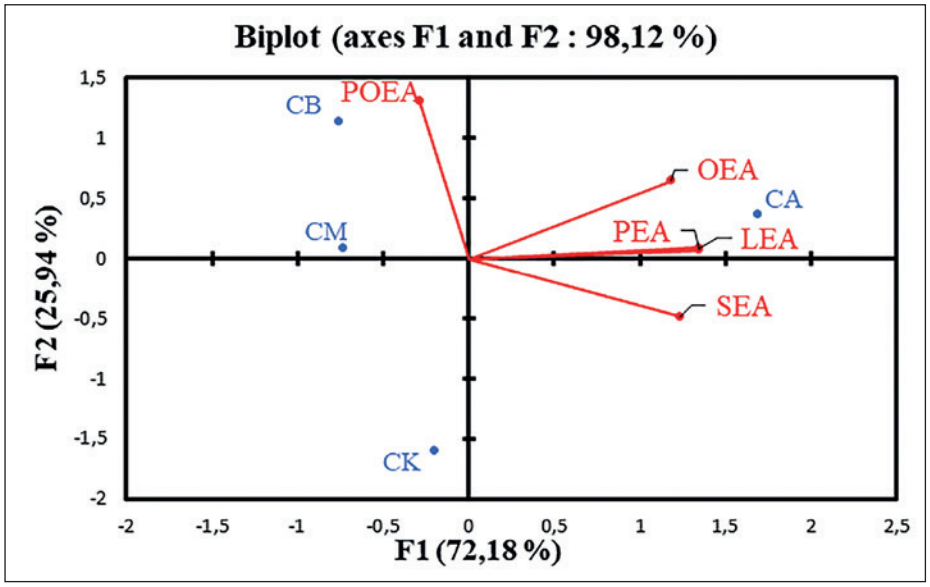

Figure 2. Principal component analysis (PCA) projections on F1 and F2 of variables (NAE species) and cultivars (*Cannabis Sativa* L.); CA, *Cannabis Sativa* L. *Cultivar Amnesia; CB, Cannabis Sativa* L. *Cultivar Beldia; CM, Cannabis Sativa* L. *Cultivar Mexicana; CK, Cannabis Sativa* L. *Cultivar Khardala*. The eigenvalues are symbolized by red segments representing the parameters that most affect each principal component.

Cultivar Amnesia; CB, Cannabis Sativa L. *Cultivar Beldia; CM, Cannabis Sativa* L. *Cultivar Mexicana; CK, Cannabis Sativa* L. *Cultivar Khardala*. The CB, CM and CK presented the same sequence of prevalence: [LEA] > [OEA > POEA] > [SEA] > [PEA], while CA showed a slight dominance of PEA compared to SEA. Furthermore, we detected significant differences in total NAEs and specific NAE specie (PEA, SEA, LEA) levels in the sample of the four achene varieties.

However, all achene varieties showed higher amounts of unsaturated NAEs than saturated NAEs. CB and CM achenes showed higher levels of NAE-MUFAs than NAE-PUFAs and NAE-SFAs; whereas CA and CK achenes showed a slight predominance of NAE-PUFAs over NAE-MUFAs and NAE-SFAs.

In addition to the famous cannabinoids that cannabis is known for, the achenes of the plants present a possible source of active biomolecules with cannabimemetic capacity such as NAEs. The total NAE contents recorded in this study are comparable to those published in studies on the quantification of NAEs in the seeds of different plant species.

This present study is an interesting contribution to the field of the industrial potential for hemp achenes from the Chefchaouen region, northern Morocco.

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AUTHORSHIP CONTRIBUTION STATEMENT

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