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SHORT COMMUNICATION

HLB determination for nanoemulsions of four essential oils from Northern Peru

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Essential oils, recognised for their diverse biological activities, are of considerable interest for the development of medicinal, cosmetic, and repellent formulations. However, their limited aqueous solubility restricts practical applications. This limitation can be addressed through nanotechnology, particularly by employing nanoemulsions, which not only enhance aqueous dispersion but also improve the bioavailability and stability of active constituents. To obtain stable formulations, it is crucial to determine the hydrophilic–lipophilic balance (HLB), which depends on the proportion and type of surfactants used in the emulsions. The objective of this study was to establish the HLB of nanoemulsions formulated with essential oils from four plant species native to northern Peru: *Minthostachys mollis*, *Bursera graveolens*, *Myrcianthes oreophila*, and *Artemisia absinthium*. For this purpose, 11 nanoemulsions were prepared with each essential oil using the low-energy input method, and their droplet size and polydispersity index were determined. The chemical composition of the oils was analysed by gas chromatography–mass spectrometry (GC–MS). The results indicated that optimal HLB values were 13.08 (F4) for *M. mollis*, 11.16 (F7) for *B. graveolens*, 12.44 (F5) for *M. oreophila*, and 13.72 (F3) for *A. absinthium*. The oil of *M. mollis* showed a high concentration of pulegone (75.35%), whereas *B. graveolens* oil was dominated by limonene (71.16%). In its turn, *M. oreophila* oil contained α-pinene, 1,8-cineole, linalool, and β-pinene as major constituents, while cis-chrysanthenol was the principal component of *A. absinthium* oil. These findings highlight the development of stable nanoemulsions of four active essential oils from Northern Peru with practical application in health and agriculture areas.

Keywords: *Artemisia absinthium*, *Bursera graveolens*, Chemical analysis, *Minthostachys mollis*, *Myrcianthes oreophila*, Nanotechnology

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Introduction

Nanoemulsions are thermodynamically metastable liquid–liquid dispersions with nanometric-sized droplets that provide efficient solubilization of low-polarity compounds in water, enhanced bioavailability of active substances, and improved stability compared to conventional emulsions. They are typically composed of a water phase, an oil phase, and surfactants, which enable the dispersion of immiscible liquids, such as essential oils (EOs), in aqueous systems[1,2]. The dispersion of active oils in water is a common challenge in formulation development, and nanoemulsions represent an effective strategy to overcome this limitation[3].

To achieve suitable stability, each oil requires a well-defined hydrophilic–lipophilic balance (HLB), which depends on the type and proportion of surfactants used in the formulation. In cases where the optimal HLB value for a given oil is unknown, it must be determined to use appropriate tools such as ternary phase diagrams or established protocols, including the method proposed by Ostertag *et al.*[4]. This method involves preparing a series of formulations with varying HLB values, followed by measurements of droplet size and polydispersity index (PDI). The most suitable HLB corresponds to the formulation exhibiting the smallest droplet size and lowest PDI[4].

Among the most promising biologically active essential oils from Northern Peru are *Minthostachys mollis*, *Bursera graveolens*, *Myrcianthes oreophila*, *Artemisia absinthium*, and *M. mollis* demonstrates anti-ulcer, antimicrobial, and anti-inflammatory properties[5,6], while *B. graveolens* EO exhibits strong repellent, fumigant, and acaricidal activities[7,8]. *M. oreophila* is recognised for its antifungal activity[9], and *A. absinthium* possesses anti-inflammatory, antioxidant, and antimicrobial effects, in addition to beneficial actions on the

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gastrointestinal and urinary systems[10]. Despite these findings, the development of formulations, such as nanoemulsions, that enhance their biological potential, as well as chemical studies, is still scarce in the literature. In this context, the chemical difference among the oils and the related physicochemical characteristics lead to the need for customised development of nanoemulsions, so that for each oil studied, an optimal HLB value must be determined to ensure the stability of each formulation. Therefore, the aim of this study was to evaluate the optimal HLB values for each of the four essential oils to develop stable and biologically active nanoemulsions, as well as to determine their chemical compositions.

Material and Methods

Plant material and extraction of the essential oils

At least three specimens of *M. mollis* (leaves), *M. oreophila* (leaves), and *A. absinthium* (leaves) were collected at 2500-3000 m.a.s.l. in cultivation sites of Otuzco (La Libertad, Peru), in May 2022, while the specimens of *B. graveolens* (bark) were collected in the dry woods of Sullana (Piura, Peru), in April 2022. The plant material was identified by the botanist Dr. José Mostacero-León (Universidad Nacional de Trujillo), under the registration codes HUT-091-2026, HUT-102-2026, HUT-098-2026, and HUT-096-2026, respectively. Each mixture of plant material (1000 g) from the four species was submitted to steam distillation for 4 h. In the end, the oils were collected and stored at 4ºC for further analysis.

Gas chromatography/mass spectrometry analysis

The essential oils were analysed using a gas chromatograph Thermo scientific, equipped with a mass spectrometer using electron ionisation. Gas chromatographic (GC) conditions were as follows: injector temperature, 260ºC; flame ionisation detection (FID) temperature, 290ºC; carrier gas, Helium; flow rate, 1 mL/min; and split injection with split ratio 1:40. The oven temperature was initially 60ºC and was increased to 290ºC at a rate of 3ºC/min. Exactly 1 mL of each sample, dissolved in dichloromethane (1:100 mg/µL), was injected into a TG-5MS column (0.25 mm I.D, 30 m in length, and 0.25 µm film thickness). Mass spectrometry (MS) electron ionisation was at 70 eV, and the scan rate was 1 scan/s. Retention indices (RI) were calculated by extrapolating the retention times of a mixture of aliphatic hydrocarbons (C_9-C_{30}) analysed under the same conditions[11,12]. Substances were identified by comparing their retention indices and mass spectra with those reported in literature[13]. The MS fragmentation pattern of compounds was also compared with NIST mass spectra libraries. Quantitative analysis of the chemical constituents was performed by flame ionisation gas chromatography (CG/FID), under the same conditions as GC/MS analysis. Percentages of these compounds were obtained by the FID peak area normalisation method.

Nanoemulsification method and determination of hydrophilic-lipophilic balance (HLB)

Emulsification was performed by modification of the low-energy method described by Ostertag *et al.*[4]. Emulsions comprised 5% (w/w) of essential oil, 5% (w/w) of surfactants, and 90% (w/w) water. Tween 80 and Span 20 were used as surfactants to prepare nanoemulsions. Eleven emulsions of each EO with HLB values ranging from 8.6 to 15.0 were prepared by mixing the surfactants in different proportions. For the preparation of nanoemulsions, oily phases constituted by each essential oil and surfactants were homogenised by magnetic stirring (400 rpm) for 30 minutes at room temperature. After this, the aqueous phase (distilled water) was added to the oily phase under the same continuous magnetic stirring (400 rpm) for 1 h. The formulations were analysed to verify their stability by analysis of droplet size and polydispersity index values. The formulations with the lowest values of droplet size and polydispersity index indicated the adequate HLB for each essential oil.

Nanoemulsion characterization

Droplet size and polydispersity index (PDI) of the nanoemulsions were determined by photon correlation spectroscopy (NICOMP-NANO Z300). Droplet measurements were performed in triplicate, and the average droplet size was expressed as the mean diameter ± standard deviation.

Results and Discussion

Chemical identification of essential oils by GC-MS

Chemical analysis of EOs is an important tool for nanoemulsion development, since a notable difference in the chemical profile can compromise the required HLB value for each oil nanoemulsion. In this sense, the *M. mollis* leaves EO showed a high concentration of pulegone (75.35 %) (Table 1), being the second highest concentration recorded worldwide (behind Padremonti, Argentina, with 76.3%)[14] and the highest amount quantified in Peruvian territory. Hence, the *M. mollis* EO collected for this work belongs to the pulegone/menthone chemotype profile[15]. Pulegone possesses various pharmacological properties, such as antihyperalgesic, antioxidant, antimicrobial, anti-feeding, antifungal, antiviral, and pesticide activities, and has a significant role as an abortifacient and emmenagogue, as well as precursor of synthetic drugs[16-21], so that *M. mollis* leaves EO located in Otuzco can be an important source of this substance. In turn, the *B. graveolens* bark EO presented a significant amount of limonene (71.16%), which is a multifunctional compound with potent therapeutic effects, including gastroprotective, hepatoprotective, antioxidant, anticancer, antidiabetic, anti-inflammatory, cardioprotective, immunomodulatory, anti-fibrotic, and anti-genotoxic activities[22-27]. Although limonene is known to be usually the main substance of *B. graveolens* EOs, the amount quantified in this study was about two-fold higher than those found in some works[7,28-32]. The analysis of *M. oreophila* EO by GC-MS is one of the first chemical reports for this species, which revealed the presence of alpha-pinene, 1,8-cineole, linalool, and beta-pinene as major substances of the oil. Linalool and 1,8-cineole were also the major compounds in oils from *M. rhopaloides* and an unspecified *Myrcianthes*, respectively[33], whereas alpha and beta pinenes were found in concentrations between 5.9 and 7.5 % in *M. fragrans*[34]. Finally, cis-chrysanthenol was the main substance of the *A. absinthium* EO, which presents an associated chemotype already described in a study with samples collected in the Auvergne region, France[35].

Determination of hydrophilic-lipophilic balance (HLB) in EOs nanoemulsions

The formulations which displayed lowest droplet size mean were defined as the most stable nanoemulsions for each EO and consequently indicated the most suitable HLB value for each EO emulsification. The HLB values of 13.08 (F4), 11.16 (F7), 12.44 (F5), and 13.72 (F3) were defined for the *M. mollis*, *B. graveolens*, *M. oreophila,* and *A. absinthium* EO nanoemulsions, respectively (Table 2). These results demonstrate the best surfactant combinations for each nanoemulsified essential oil, in order to optimise the application of these oils for medicinal, cosmetic, and repellent purposes, among others. However, long-term stability and pharmacological biological efficacy studies must be carried out to better understand its technological

Table 1 — Qualitative and quantitative analysis of the four essential oils by GC-MS

	MM				BG				MO				AA			
Substance	Rt	Exp. AI	Theor. AI	%	Rt	Exp. AI	Theor. AI	%	Rt	Exp. AI	Theor. AI	%	Rt	Exp. AI	Theor. AI	%
Isobutyl isobutyrate									2.70	898	908	3.12				
alpha-thujene									2.85	914	924	0.53	2.92	922	924	13.44
alpha-pinene	2.92	921	932	0.19					2.92	921	932	21.60				
Camphene									3.11	942	946	0.36				
Thuja-2,4(10)-diene													3.17	949	953	0.24
Sabinene													3.39	974	969	1.64
beta-pinene	3.44	979	974	0.19					3.45	981	974	14.32	3.44	980	974	0.67
Myrcene	3.59	996	988	0.29												
1,8-dehy drocineole					3.63	1000	988	0.33								
3-p-menthene					3.72	1005	984	0.18								
Isoamyl isobutyrate									3.91	1016	1007	0.74				
alpha-terpinene									3.97	1019	1014	3.69				
1-p-menthene					4.04	1024	1021	0.17								

(*Contd.*)

Table 1 — Qualitative and quantitative analysis of the four essential oils by GC-MS (*Contd.*)

Substance	MM				BG				MO				AA			
	Rt	Exp. AI	Theor. AI	%	Rt	Exp. AI	Theor. AI	%	Rt	Exp. AI	Theor. AI	%	Rt	Exp. AI	Theor. AI	%
Limonene	4.15	1030	1024	0.52	4.18	1032	1024	71.16	4.16	1031	1024	2.51				
1,8-cineole	4.21	1034	1026	0.22					4.23	1035	1026	19.25	4.22	1034	1026	25.83
Sylvestrene					4.23	1035	1025	0.57								
beta-ocimene Z	4.28	1037	1032	0.24												
beta-ocimene E	4.44	1047	1044	0.28												
gamma-terpinene	4.63	1058	1054	0.05					4.64	1058	1054	0.42	4.64	1058	1054	0.29
p-mentha-3,8-diene	4.82	1069	1068	0.05												
p-cymenene					5.20	1090	1089	0.12								
Linalool	5.36	1099	1095	0.22					5.39	1132	1095	15.95				
trans-p-mentha-2,8-dien-1-ol					5.77	1123	1119	0.24								
alpha-camphonelal	5.87	1129	1122	0.06												
cis-beta-terpineol					6.00	1136	1140	0.15								
neoiso-3-thujanol					6.09	1141	1147	0.58								
trans-pinocarveol													6.12	1143	1135	0.61
trans-beta-terpineol					6.23	1149	1159	0.11								
neo-isopulegol	6.23	1149	1144	0.16												
Camphor													6.25	1150	1141	1.72
p-Menth-3-en-8-ol	6.28	1152	1145	0.12												
iso-menthone					6.40	1159	1158	0.43								
iso-isopulegol	6.43	1160	1155	0.11												
Umbellulone	6.57	1169	1167	1.71												
Menthofuran					6.57	1169	1159	1.63								
cis-chrysanthenol													6.64	1173	1160	42.91
Borneol													6.71	1177	1165	1.15
neo-menthol					6.75	1179	1161	0.13								
Lavandulol					6.85	1185	1165	0.20								
Terpinen-4-ol									6.85	1185	1174	0.34				
iso-verbanol	6.86	1185	1176	1.42												
Terpin-4-ol													6.87	1185	1174	0.5
alpha-terpineol					7.13	1201	1186	1.76	7.14	1201	1186	1.99	7.14	1201	1186	0.45
Dihydrocarveol	7.17	1203	1192	0.27	7.23	1206	1192	0.17								
neo-dihydrocarveol					7.29	1209	1193	0.16								
cis-piperitol					7.34	1211	1195	0.10								
Verbenone	7.57	1222	1204	0.29												
Methyoxythiophenol	7.70	1228	1210	0.13												
trans-carveol					7.714	1229	1215	0.35								
Coahuilensol methyl ether	7.86	1236	1219	0.10												
cis-carveol					7.96	1241	1226	0.16								
cis-pulegol	8.08	1245	1226	0.46												

(*Contd.*)

Table 1 — Qualitative and quantitative analysis of the four essential oils by GC-MS (*Contd.*)

	MM				BG				MO				AA			
Substance	Rt	Exp. AI	Theor. AI	%	Rt	Exp. AI	Theor. AI	%	Rt	Exp. AI	Theor. AI	%	Rt	Exp. AI	Theor. AI	
Pulegone	8.19	1253	1233	75.33	8.32	1250	1233	0.13	8.14	1250	1233	0.46				
Carvone					8.43	1256	1239	0.58								
Citronellyl formate	8.82	1283	1271	0.47												
E-anethol													9.09	1297	1282	0.31
Citronellyl formate	8.82	1283	1271	0.47												
Dihydro carveol acetate	9.59	1322	1306	0.47												
delta-elemene					10.01	1342	1335	0.13								
Piperitenone	10.26	1354	1340	0.58												
4aα,7α,7aα-Nepetalactone	10.37	1359	1357	0.06												
beta-elemene					11.14	1397	1389	0.21								
beta-caryophyllene	11.68	1441	1417	0.56									11.68	1442	1417	0.42
beta-longipinene									11.68	1442	1400	0.47				
beta-humulene					12.07	1475	1436	0.12								
trans-cis-Iridolactone	12.17	1483	1443	0.10												
9-epi-E-caryophyllene					12.28	1493	1464	0.10								
Citronellol isobutanoate	12.36	1500	1482	0.06												
gamma-cadinene					12.85	1541	1513	0.19								
trans-beta-guaiene	12.92	1548	1502	0.19												
delta-cadinene													12.92	1548	1522	0.31
Alpha-bulnesene									13.03	1557	1509	0.48				
Premnaspirodiene	13.06	1559	1505	0.08												
Z-nerolidol	13.23	1574	1531	0.10												
E-nerolidol					13.48	1595	1561	1.09	13.48	1596	1561	0.41				
Vetiselineol	14.85	1768	1730	0.38												
8-α-11-elemodiol	14.94	1780	1746	0.61												
beta-vetivone	15.95	1848	1822	0.06												
Total Identified				86.60				81.25				86.64				90.49

MM = *Mintostachys mollis*; BG = *Bursera graveolens*; MO = *Myrcianthes oreophila*; AA = *Artemisia absinthium*; Rt = Retention Time; AI = Arithmetic Index

Table 2 — Nanoemulsion formulations and their respective droplet size and PDI mean according to each HLB value

			MM		BG		MO		AA	
Formulations	Tensoactive composition	HLB	Droplet size mean (nm)	PDI mean	Droplet size mean (nm)	PDI mean	Droplet size mean (nm)	PDI mean	Droplet size mean (nm)	PDI mean
F1	Tween 80	15.00	20.95 ± 1.55	0.41 ± 0.01	120.83 ± 62.73	0.66 ± 0.06	2313.93 ± 1290.954	0.59 ± 0.04	13.57 ± 1.11	0.50 ± 0.02
F2	Tween 80 / Span 20 (9:1)	14.36	19.20 ± 1.10	0.46 ± 0.05	816.97 ± 204.87	0.75 ± 0.09	145.13 ± 40.95	0.56 ± 0.02	12.26 ± 0.71	0.52 ± 0.07

(Contd.)

Table 2 — Nanoemulsion formulations and their respective droplet size and PDI mean according to each HLB value (*Contd.*)

			MM		BG		MO		AA	
Formulations	Tensoactive composition	HLB	Droplet size mean (nm)	PDI mean	Droplet size mean (nm)	PDI mean	Droplet size mean (nm)	PDI mean	Droplet size mean (nm)	PDI mean
F3	Tween 80 / Span 20 (8:2)	13.72	20.15 ± 1.52	0.44 ± 0.04	333.63 ± 126.76	0.64 ± 0.07	50.40 ± 2.72	0.53 ± 0.03	**10.67 ± 1.50**	0.41 ± 0.09
F4	Tween 80 / Span 20 (7:3)	13.08	**16.40 ± 1.28**	0.44 ± 0.04	30.97 ± 7.05	0.54 ± 0.02	65.70 ± 5.00	0.51 ± 0.00	13.90 ± 2.97	0.46 ± 0.01
F5	Tween 80 / Span 20 (6:4)	12.44	25.17 ± 1.87	0.50 ± 0.07	25.90 ± 5.12	0.55 ± 0.03	**14.25 ± 0.21**	0.48 ± 0.00	16.13 ± 4.54	0.52 ± 0.06
F6	Tween 80 / Span 20 (5:5)	11.80	198.8 ± 12.44	0.65 ± 0.06	53.80 ± 21.44	0.74 ± 0.14	42.10 ± 3.90	0.48 ± 0.00	LS	LS
F7	Tween 80 / Span 20 (4:6)	11.16	82.77 ± 6.75	0.59 ± 0.06	**13.67 ± 2.55**	0.51 ± 0.06	27.17 ± 4.95	0.57 ± 0.07	LS	LS
F8	Tween 80 / Span 20 (3:7)	10.52	477.0 ± 18.11	0.51 ± 0.09	32.07 ± 3.48	0.59 ± 0.05	30.97 ± 4.49	0.61 ± 0.06	LS	LS
F9	Tween 80 / Span 20 (2:8)	9.88	52.95 ± 3.15	0.49 ± 0.04	116.00 ± 12.75	0.27 ± 0.08	131.13 ± 65.64	0.58 ± 0.07	LS	LS
F10	Tween 80 / Span 20 (1:9)	9.24	500.05 ± 23.68	0.60 ± 0.13	1534.00 ± 442.80	0.18 ± 0.12	LS	LS	LS	LS
F11	Span 20	8.60	LS	LS	LS	LS	LS	LS	LS	LS

MM = *Mintostachys mollis*; BG = *Bursera graveolens*; MO = *Myrcianthes oreophila*; AA = *Artemisia absinthium*; HLB = Hydrophilic-Lipophilic balance; PDI = Polidispersity index; LS = Nanoemulsion which displayed low stability, such as phase breakage and high creaming.

and biological significance. Furthermore, concerns on medium/large-scale production of nanoemulsions are also a step to be surpassed, since the practical use in agriculture or even as a health product demands an advance in terms of industrial scalability.

Conclusion

This study determined the HLB values between 11.16 and 13.72 for the four nanoemulsions containing bioactive essential oils from the species *Minthostachys mollis*, *Bursera graveolens*, *Myrcianthes oreophila*, and *Artemisia absinthium*, which are native or adapted to northern Peru. These results aimed to determine the best combination of surfactants used in each of the four nanoemulsions to ensure maximum stability. The chemical composition of each essential oil was also determined, highlighting the massive concentration of pulegone in *M. mollis* and of limonene in *B. graveolens*.

Conflict of interest

The authors declare no conflict of interest.

AI use disclosure

There was no data generated by Artificial Intelligence in this work.

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