Prevention of retinal light damage by zinc oxide combined with rosemary extract

Analysis of Actives

Introduction

While not a thorough investigation of bioavailability of the different constituents of rosemary, this analysis provides assessment of the total amount of its primary antioxidant components. The analysis does not distinguish amongst dissolved, solubilized, suspended-microparticulate or carrier-bound forms. The bioresponses reported in the paper establish significance from their total measured amounts. For purposes of comparison across species, data are presented in terms of mg/kg, as are the vitamin / mineral components in the AREDS formulation.

Experimental Methods

Materials

Rosemary extract powder (LRP) and that powder solubilized in omega-3 oil (R/R) were prepared by LycoRed (Beer Sheva, Israel). The omega-3 oil is a commercial product, ROPUFA 75 N-3 EE, manufactured by DSM (Kaiseraugst, Switzerland). Carnosic acid, carnosol, and rosmarinic acid standards were received from Chromadex (Irvine, CA), and the ursolic acid standard was received from Sigma-Aldrich (St. Louis, MO). All solvents were HPLC grade and obtained from VWR (West Chester, PA).

Sample Preparations. Extracts from both LRP and R/R were evaluated and are reported here. LRP is an extract of processed rosemary leaves from which nearly all of the insolubles have been removed. Extraction with alcohol, either ethanol or methanol, was about equally effective, leaving less than 1% of residue. The compositions derived from the following preparations are provided in Table S1.

Rosemary Powder. The composition of the primary actives in the powders were determined by the following procedures.

Ethanol/Aqueous Tween Method (EATM). Following the extraction procedure described in the paper, and analyzing in an alcohol extract either the solution and residue separately, or in suspension.

Alcohol Extraction Method A (AEM-a). LRP (591 mg) was mixed with ~75 mL ethanol and homogenized for 5 mins at 9,500 rpm. This solution was diluted to 100 mL, and analyzed directly by reversed phase HPLC.

Alcohol Extraction Method B (AEM-b). A more concentrated solution was made by mixing 4.25 g of LRP with 75 mL of ethanol. The resulting mixture was homogenized at 9,500 rpm for 5 minutes followed by sonication in an ultrasonic bath (VWR Aquasonic Model 250HT) for 10 minutes. The solution was diluted to 100 mL and analyzed by reversed-phase HPLC.

Rosemary Oil. Approximately 100-150 mg of R/R oil were dissolved in 5 mL of alcohol, and analyzed by reversed-phase HPLC.

Analytical Procedures

Equipment. An Agilent Model1200 series autosampler, pump, diode array detector were used for both HPLC methods.

Procedure A. Ursolic acid was determined by isocratic reversed phase chromatography (Astec C18, 25cmx4.6mm; mobile phase = 90/10/0.1 methanol/water/trifluoroacetic acid) and quantified at 210 nm by external calibration (r2 > 0.99).

Procedure B. Carnosol, carnosic acid and rosmarinic acid were determined by reversed-phase chromatography on a Cyclobond RSP column (25cm×4.6mm) and quantified by external calibration (r^2 > 0.99) at 280 nm1, as described [19].

Calibration Procedure. Linear least squares plots, constrained to pass through the origin, were established for carnosic acid, carnosol, rosmarinic acid, and ursolic acid. The slopes of area (HPLC units) vs. concentration (mg/mL, or ppm) were 1.210, 1.683, 2.440, and 2.223. The corresponding standard deviations were 14, 18, 31, and 97 respectively. These would lead to about a 5% error in the data for all but ursolic acid, which could be about twice this level.

Results

Primary Actives in Rosemary Source Materials

The levels of actives are reported for five samples of rosemary. These included a nominal 10% carnosic acid in the omega-3 oil (R/R – 10%), a representative lot of nominally 5% carnosic acid in omega-3 oil (R/R – 5%), and powder extracts from the three methods described above.

Table S1. Compositions (mass %) of actives in LRP or R/R oil.

Extract solutions	Carnosol	Carnosic acid	Rosmarinic Acid Rosmanol/Epirosmanol	Ursolic acid
R/R-10%	0.95	7.18	0.02	2.76
R/R-5%	0.69	4.67	0.02	4.38
LRP EATM	3.69	23.22	0.15	15.14
LRP AEM-a	3.76	23.25	0.09	15.61
LRP AEM-b	3.92	21.49	0.25	13.99

Analysis of Actives

1. Approximately comparable amounts of the major actives were found using the three powder extraction procedures.
2. The amounts of the rosemary antioxidants in the R/R preparation are approximately one third that in the powder. However, both required dilution and emulsification in order to be presented from an aqueous preparation, as described in the study. The stability of the actives in the R/R was the rationale for some preference of this dosage form [19].

Dosing Levels of Actives

Dose response levels for rosemary powder and R/R oil were determined at the four prepared concentrations from LRP and six from R/R, as described in the study. The amounts of the primary antioxidant active per kg of animal weight are provided in columns 3-6 of Table S2. With the presumption that carnosic acid is the most important antioxidant in the rosemary extracts, its level in the R/R oil required a multiple of about 3X to approximate the effect of the powder extracts.

Table S2. Levels of rosemary-derived actives (mg/kg) as a function of dose and dosage form. (IP injection of rats with an average weight of 0.3 kg.)

Formulation	Dose (LRP or R/R)	Carnosic Acid	Carnosol	Rosmarinic Acid	Ursolic Acid
LRP					
	34.0	7.89	1.25	0.05	5.15
	17.0	3.95	0.63	0.03	2.57
	8.5	1.97	0.31	0.01	1.29
	5.0	1.16	0.18	0.01	0.76
R/R					
	416.7	29.93	3.97	0.08	11.52
	333.3	23.95	3.18	0.06	9.21
	250.0	17.96	2.38	0.05	6.91
	166.7	11.97	1.59	0.03	4.61
	125.0	8.98	1.19	0.02	3.46
	83.3	5.99	0.79	0.02	2.30

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AREDS1: Scaling of Levels of Actives

Table S3 lists the AREDS1 active levels of the formulations [12]. Presuming that for men the mean body mass is about 70 kg, and for women about 60 kg, the approximate dosing levels per kilogram of body mass of each vitamin and mineral are provided. For clarity, the weight of zinc is provided as both the mass of the metal and the [zinc oxide salt].

Table S3. Levels of ingredients (mg/day) and projected amounts per kg of body weight in AREDS1 tablets.

Description →	Actual Formulation			Proposed Formulation		
Ingredient ↓	Amount/day (mg/day)	Amount / kg (women)	Amount / kg (men)	Amount/day (mg/day)	Amount / kg (women)	Amount / kg (men)
Vitamin A (□-carotene)	17.2	0.29	0.25	15	0.25	0.21
Vitamin C	452	7.53	6.46	500	8.33	7.14
Vitamin E	400	6.67	5.71	400	6.67	5.71
Zinc	69.6	1.16 [1.44]	0.99 [1.24]	80	1.33 [1.66]	1.14 [1.42]
Copper	1.6	0.027	0.023	2	0.033	0.029

There are two sections of the table. The first designates the actual amounts (required by DSHEA [Dietary Health and Education Act of 1994] 100% requirement) provided. These levels are guaranteed by the manufacturer, so long as the product has not reached its shelf life. The second, the “proposed” amounts, are the levels indicated in the initial protocol that were anticipated to satisfy the USP requirements operative at the time the protocol was conceived.