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Formulation and In Vitro Evaluation for Sun Protection Factor of Cosmos Sulphurus Flowers (Asteraceae) Extract Sunscreen Cream

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ABSTRACT

Cosmos Sulphurus(Asteraceae) flowers commonly known as Sonkusum flowers were explored to find flavonoids, so, thin layer chromatography was performed and some of the flavonoids were identified.The reported flavonoids were known to have sunscreen activity and hence SPF factor of the dried flower extract (aqueous) was incorporated in topical formulation and evaluated in vitro with the help of Optometric Model-290 SPF.. The aqueous extract prepared from flower of cosmos sulphurus was used for Thin Layer Chromatography and then into topical formulation. In vitro determination of SPF was done with the help of method specified by The Comité de Liaison de la Parfumerie in Europe (COLIPA). The SPF was found to be 1.93 and Boots Star rating as 3, indicating that Cosmos Sulphurus(Asteraceae) flower extract (aqueous) can be considered as good candidate for Sunscreen or as an additive in any other sunscreen formulation.

Keywords: Sun Protection Factor, in vitro method, flavonoids, Cosmos Sulphurus(Asteraceae) flowers.

INTRODUCTION

As a barrier and immunological organ in the human, the skin especially epidermis, is particularly subjected to external effects. Light is the major environmental component to which skin is exposed daily and this light comprises of UV radiations[1] which have been reported for damaging effects to the skin. There are three types of UV rays; UV-A (320-400 nm), UV-B (280-320 nm), UV-C (200-280nm)[2]. Exposure to UV-A radiation results in premature ageing (photo-ageing) due to dmage to the elastic and collagenic fibers of connective tissue of skin, while UV-B radiation bring about acute[3] inflammation (sun burn) and intensification of photo-ageing. In addition to these changes, UV-B radiations are also reported to induce immune-suppression which reduces normal immunological defense mechanisms of the skin, therefore chances of development of malignant tumor increases [1,3,4]. The application of sunscreens is an efficient method of protecting skin against UV radiations. Thus, it has become a necessity to develop a validated topical sunscreen product which will provide protection against both UV-radiations and hence, topical formulations like sunscreen cream, lotion, spray, gel are prepared.

The efficacy of sunscreens is characterized by the sun protection factor (SPF). The SPF is a numerical rating system calculated by in vitro and in vivo method to indicate the degree of protection provided by a sun care products like sunscreen[5] . SPF is defined as the ratio of the minimal erythema dose (MED) of solar radiation measured in the presence and in the absence of a sunscreen agent[6]. Regulatory agencies like the US-FDA and COLIPA (TheComité de Liaison de la Parfumerie in Europe), TGA has made in vivo testing on human volunteers using an erythemal endpoint to determine the SPF of topical cream mandatory[7]. Although it is a recommended and recognized method by COLIPA, TGA it beneficial to check the effect on formulation onto the skin and it

has several disadvantages like it is a time consuming process, result of the test vary from skin to skin,it also expensive in terms of money, it may be harmful to human because of clinical trials. Having said this, there are still many questions left unanswered about both the scientific accuracy and reproducibility of in vivo measurements of SPF, whereas, an in vitro measurement has the advantage of not exposing human subjects to harmful UV radiation, is cost-effective and provides us with statistically significant data which helps us to develop an effective sunscreen product. Thus, for economical, practical and ethical considerations a suitable method for in vitro determination of SPF is used more often[8].

Sunscreen creams incorporate a wide variety of chemical sunscreen agents like derivatives of 3-benzylidenecamphor, 4-aminobenzoic acid, cinnamic acid, salicylic acid, benzophenone and 2-1 phenylbenzimidazole, Avobenzone and physical sunscreen agents like Zinc oxide, Titanium dioxide which have particular absorbance and are effective over various areas of UV spectrum. To get a broad spectrum sunscreen for getting higher protection over entire UVradiatin more then one sunscreen agents are used in formulaton[8]. The EU has regularly listed 27 different organic and inorganic sunscreen ingredients since two decades, which are approved by Australian Government- Department of Health and Ageing, Therapeutic Goods Administration (TGA) for use in Australia whereas only 16 ingredients are listed in US-FDA monograph, Sunscreen creams incorporate a wide variety of chemicals like derivatives of 3-benzylidenecamphor, 4-aminobenzoic acid, cinnamic acid, salicylic acid, benzophenone and 2-phenylbenzimidazole, Avobenzone and Zinc oxide [9]. The inorganic materials like Titanium dioxide and Zinc Oxide added into sunscreen formulation which provide sunscreen effects by reflecting and scattering the Sun Radiation and which forms an opaque barrier, they are photo stable, do not react with organic sunscreens and due to their light scattering properties there is less variability in the photo-protective effect of inorganic agents as compared to organic agents. However, inorganic sunscreens are cosmetically very les acceptable because its opaque qulaity. The higher refractive index of Titanium dioxide produce white colour after applying on to skin and thus lower cosmetic acceptability.[10] Also, these sunscreen Agents have been increasingly reported for allergic and contact dermatitis, phototoxic and photo-allergic reactions, contact urticaria and even solitary cases of severe anaphylactic reactions.[11] Therefore, the researchers have turned their attention towards developing herbal sunscreen agents which are effective with less or no side effects.

Flavonoids are widely distributed plant pigments. They are water soluble and commonly occur in vacuoles, membrane-enclosed structures within cells. Chemists have identified more than 3,000 naturally occurring flavonoids. Flavonoids are placed into 12 different classes, the best known of which are the anthocyanins, flavonols, and flavones. The basic flavonoid structure is comprised of two benzene rings (A and B) linked through a heterocyclic pyran or pyrone (with a double bond) ring (C) in the middle. The flavonoids are further divided into six subclasses (flavones, flavonols, flavanonols, isoflavones, flavanols, and anthocyanidins) based on the connection of the B ring to the C ring, as well as the oxidation state and functional groups of the C ring. The chemical structures of this class of compounds are based on a C6-C3-C6 skeleton. They differ in the saturation of the heteroatomic ring C, in the placement of the aromatic ring B at the positions C-2 or C-3 of ring C, and in the overall hydroxylation patterns. The flavonoids may be modified by hydroxylation, methoxylation, or *O*-glycosylation of hydroxyl groups as well as *C*- glycosylation directly to carbon atom of the flavonoid skeleton.. Small changes in a 12 flavonoid's structure can cause large changes in its color.

Flavonoids often occur in fruits, where they attract animals which eat the fruits and disperse the seeds. They also occur in flowers, where they attract insect pollinators. In plant, flavonoids play different roles in the ecology. The protective effects of flavonoids in biological systems are ascribed to their capacity to transfer electrons free radicals, chelate metal catalysts, activate antioxidant enzymes , reduce alpha-tocopherol radicals, and inhibit oxidases. Furthermore, flavonoids protect plants from solar UV radiation and scavenge UV generated ROS . Therefore, flavonoids have 3 different photoprotection effects including UV absorption, direct and indirect antioxidant properties, and modulate several signaling pathways. UV-absorbing flavones and flavonols are also present in the leaves of many species, where they protect plants by screening out [13]harmful ultraviolet radiation from the Sun. Literature survey has reported that flavonoids like flavonols [14, 15, 16](quercetin, rutin, kaempferol, myricetin, fisetin, vitexin) flavones (apigenin, luteolin) flavanones (naringenin, hesperitn, naringin, and hesperidin), isoflavones[17,1 18] (genistein) and anthocyanins have shown the potential as sunscreen agents. Flowers and fruits often consist of few or more flavonoids, therefore flowers of ***Cosmos***

Sulphurus. (Asteraceae) were considered as project topic wherein isolation of flavonoids and its characterization was done firstly by determination of total flavonoid content, then characterized by Thin Layer Chromatography technique and subsequently doing its SPF evaluation in a topical sunscreen cream.

Flowers of ***Cosmos Sulphurus***. (Asteraceae) is commonly known as Sonkusum flower and can be found in the local cultivars of Nashik,Niphad,Trimbakeshwar it is[19] originally found at the Sahyadri mountains. Flowers are yellow in color, and have dark orange petals. On drying, the flower color changes to yellow indicating that flavonoids might be present. Literature survey has reported alkaloids but very little investigation has been done in exploring other constituents like Alkaloids, Caretnoids,Fattyacids [20]. As not much of a work is reported in exploring flavonoids, thus, the study was aimed at isolating and identifying the flavonoids followed by developing a validated and effective topical dosage form.

MATERIALS AND METHODS

Plant material: Flowers of ***Cosmos Sulphurus***. (Asteraceae) was collected from rad sides of nashik trimbak road and District- Nashik, Maharashtra state, India. It was authenticated taxonomically with the courtesy from Blatter Herbarium, St.Xaviers Collage, Mumbai.. The herbarium was deposited with Reference No. **16365**.

Extraction of dried flowers of *cosmos sulphurus*. (asteraceae): Dried flowers were ground and extracted in two steps After weighing out a portion of ground material extraction is best carried out in two steps. Firstly with Methanol:Water(9:1) and secondly with Methanol: Water (1:1). At each step sufficient solvent is added to make a liquid slurry and mixture is left for 10 days. Filtration to seperate extract from palnt material can be carried out rapidly by using cotton wool in the neck of filter funnel. The two extracts are then combined and evaporate to about 1/3 the original volume or until the most of the methanol has been removed.

The resultant aqueous extract can then be cleared of low polarity contaminants such as fats, terpenes, chlorophylls, xanthophylls etc. By extraction in 4 seperating funnels with chloroform. 25 ml aq. Extract and 25 ml chloroform taken in each seperating funnel. Thos should be done several times and the extracts combined. The solvent extraced aqueous layer containing the bulk of flavonoids is then evaporated to dryness in water bath at 100º c.after complete evaporation of water semisolid mass of extract is obtained. The solvent-extracted aqueous layer, containing the bulk of flavonoids, was then evaporated to dryness.[22]

The UV spectra of the naturally occurring flavonoids, like flavones and flavonols have been determined as a function of concentration in aqueous solutions. These spectra indicate that the extent of keto–enol phototautomerism in both flavonoids is greatest at high concentrations: a situation which favours molecules aggregation/dimerization. Such behaviors is consistent with phototautomerism being facilitated by a concerted, intermolecular transfer of protons between the partners in the flavonoid dimer. This excited state tautomerism dissipates absorbed energy harmlessly and as such provides a possible mechanism by which these molecules may function in the protection of plants from damaging UV radiation. Along with UV spectroscopy the fluorescence excitation spectra of both flavones and flavonols at high concentrations in aqueous solutions indicate the presence of significant amounts of the enolic tautomeric form in the ground-state. The absorption of the enolic tautomer is at longer wavelengths (510 nm) than that of the keto tautomer (440 nm) and as it extends into the blue spectral region, would account for the yellow appearance of these flavonols in flower petal extracts. Therefore, the wavelengths for flavonoid and flavonol content determination are different. [23]

Determination of total flavonoid content:[24] Aluminium chloride colorimetric method was used for flavonoids determination. 1ml of aqueous extract of flowers of Cosmos Sulphurus(Asteraceae) or 500µ g/ml standard solution of rutin (provided by Yucca enterprises,Wadala, Mumbai) was added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% $NaNO_2$ was added. After 5min, 0.3ml 10% $AlCl_3$ was added. At 6th min, 2ml 1M NaOH was added and the total volume was made up to 10ml with water. The solution was mixed well and the absorbance was measured against reagent blank at 510nm. Total flavonoid content of aqueous extract was expressed as mg rutin/g of extract.

Formula:

$X = A.\ mo.10/\ A_0\ .m$

Where,

X=flavonoid content, mg/g of plant extract, A=the absorption of plant extract solution,

A_0 =the absorption of standard rutin solution, m=the weight of plant extract in g ,mo=the weight of rutin in solution in mg, The total flavonoid content was found to be 0.375 mg/g. (Table1)

Thin layer chromatography [22]: The fact that a spot is visible at all under conditions is a likely indication that it represents phenolic compound. Flavonoids often account for the majority of visible (in UV) spots although blue fluorescent, pink, whitish, orange and brownish spots must be considered as unlikely to represent flavonoids until further investigated (by UV-vis spectroscopy). The typical flavone and flavonol glycoside spot will appear dark purple in the UV. TLC was performed in five different mobile phases like Butanol: Acetic acid: Water (BAW 4:1:5), Forestal (Acetic acid: Water: Hydrochloric acid - 30:10:3), Chloroform: Acetic acid: Water (CAW 3:1.5:0.2), Ethyl acetate: Pyridine: Acetic acid: Water (EPAW 36:36:7:2), Butanol: Benzene: Pyridine: Water (BuBzPW 5:1:3:3). (Table 2) Each of these mobile phases showed dark purple spots under UV at 366nm, the interpretations of probable flavonoid type are as follows; Apigenin, Quercetin, Catechin, Epicatechin, Fisetin, Myricetin, Kaempferol and Daidzein, Pinocembrin and Anthocyanins.

Formulation of sunscreen cream: The Sunscreen cream was prepared by following procedure, the formulation of the cream is specified in (Table no 3).

Step-I: Water phase was prepared by using total 80% water in formulation. firstly (75 %) and then (5 %) water was removed aside from this for final volume makeup. Water was heated to $80^{O}C$ and in it herbal extract of Cosmos Sulphurus, Carbopol 940 , Disodium EDTA, Methyl Paraben was added.

Step-II: Oil phase was prepared by heating propyl paraben, stearic acid, Cetyl alcohol, Cetomacrogal-1000, Cetostearyl Alcohol, along with Teel oil at 80 ^{O}C.

Step-III: Oil phase was added in water phase at 80 ^{O}C with continuous stirring for 20-25 min and then it was homogenized for 15 min. Then cream is cool upto 40^{0} c. Then Triethanolamine is added for adjusting the between 6.4-6.7.

Determination of physical parameters of cream:

Preparation of herbal cream has always been a challenging task and the cream is accepted only if it is tested appropriately for various physical parameters like ease of spreadability, appearance, pH, viscosity and pleasant feeling as specified in (Table No 4)

Determination of in vitro SPF: This study was performed by Transmittance measurement of the flowers of Cosmos Sulphurus (Asteraceae) cream. The Optometrics Model SPF-290 Analyzer measures the sun n protection factor of the cream over a wavelength range from 290nm-400nm. Approximately 110mg of sample was applied and spread on $56cm^2$ area of Transpore tape to obtain a sample film thickness of 2 ml/cm^2 (to get an even film) as suggested in the operational manual of Optometrics LLC for the sample application technique. The samples thus prepared were exposed to Xenon arc lamp for determining the SPF and Boots Star Rating(Table No 5).Graph of Wavelength vs mpf is shown in fig.1,fig.2,fig.3

WIN SPF has used the following equation for calculating SPF value

$$SPF_{SCAN} = \frac{\sum_{290}^{400} E\lambda B\lambda}{\sum_{290}^{400} \frac{E\lambda B\lambda}{MPF\lambda}}$$

Where,

MPF= scan MPF value

$E\lambda$ = spectral irradiance of terrestrial sunlight under controlled conditions

$B\lambda$ = erythemal effectiveness

RESULT

The aqueous extract of dried flowers of Cosmos sulphurus. was subjected to Thin Layer Chromatography using different solvents like Butanol: Acetic acid: Water (4:1:5), Forestal (Acetic acid: Water: Hydrochloric acid - 30:10:3), Chloroform: Acetic acid: Water (3:1.5:0.2), Ethyl acetate: Pyridine: Acetic acid: Water (36:36:7:2), Butanol: Benzene: Pyridine: Water (5:1:3:3). Different number of spots were obtained in different solvents like 5, 1, 4, 3, 1 respectively (Table No.2), all of them appeared as Dark Purple and fluorescent yellow color spots under UV light at 366nm. The compounds were quercetin, kaempferol, fisetin, myricetin, pinocembrin and anthocyanins (yellow color spot). Some of these compounds have reported to have sunscreen activity, therefore, topical formulation was made and evaluated for in vitroSPF determination using Optometrics – 290 SPF model.

The topical cream prepared was Light yellow in color, showed good and uniform spreadability, pH was found to be 6, viscosity and specific gravity was found to be 1690cp and 0.93 respectively (Table No.4), the parameters of cream complies with official acceptance criteria. SPF of this cream is found to be 1.93±0.22 with Boots Star Rating 3 at critical wavelength of 383.33 nm, generally, if the Boots Star rating is more than 2 and when critical wavelength is more than 375 nm (Table No.5), then, the product developed provides good UV-A and UV-B

protection, hence, flowers of Cosmos sulphurus (Asteraceae) topical cream can be considered as good candidate for sunscreen cream or as an additive to any other sunscreen formulation.

DISCUSSION

The Optometrics Model SPF-290 Analyzer is a computer controlled instrument that is designed to measure the sun protection factor of sunscreen preparations. For US-FDA standards the protection factor is calculated over the wavelength range from 290-400nm. To initiate an analysis a reference scan was done with the blank substrate (which consists of data from 23 wavelengths) in the incident beam. The sample was then applied to the substrate and the first sample scan was made. Data was collected in the same manner as the reference data, ratioed to the reference and plotted as a MPF (Monochromatic protection factor). Ratioing the sample signal to the reference signal negates any effect of wavelength dependent variables in the optical system (source, monochromator and detector). Up to 6 sample scans were made to compensate for variables in the substrate and sample application.

The SPF290 software uses Trapezoidal Approx calculation echnique to approximate the integral for SPF and Erythemal UVA protection factor. These include UVA/UVB ratio, critical wavelength and cumulative absorbance. The Average Absorbance method is used for calculating average protection factor; this method averages and computes the standard deviation based on the absorbance scan data. This method of calculation gives a better average value assuming that sample thickness is the largest variable in performing a protection factor measurement.

For the calculation of standard deviation, Diffey's method is [25] used, based on B. L. Diffey's paper on using Transpore Tape® as the substrate for SPF measurements. Diffey's equation applies weighing by recognizing that the MPF measurements for a set of scans have some distribution. Therefore, the standard deviations of the MPF measurements at each wavelength are factored in to the Diffey SPF standard deviation calculation.

Physical Parameters include color, pH, odor, spreadability, specific gravity and viscosity. The color of cream was found to be brown and odor was aromatic, this was done by sensory evaluation, while pH was determined with the help of electronic pH meter by preparing buffer solutions of 4 and 7 pH, calibrating the instrument and followed by measurement of pH, which was found to be 6.7. Specific gravity was determined on the basis of procedure specified in USP, wherein a tared and dry pycnometer was filled first with water boiled till 25°C and calibrated, and then the sample cream was boiled till 25°C and filled in pycnometer. The weight was done only after the temperature was equal to that of balance and then weighed. The tared weight was then subtracted from the filled weight and specific gravity was measured, which was found to be 0.93. The viscosity of Cosmos Sulphurus Cream was determined as per the procedure mentioned in the manual of Brookfield viscometer. The software of Brookfield viscometer (Model no. LV-II + Pro LV) was switched on and he programming of parameters was done. The selection of spindle number (spindle No. LV-IV) for determination of viscosity was done on trial and error basis. The behavior of viscosity of the dosage form was studied at variable RPM (Shear rate) at constant temperature. The viscosity was 26therefore found to be 1690cp[26.]

CONCLUSION

The described in vitro method, though, presents some limits; it has spared the exposure of human subjects to harmful ultraviolet radiations that can pose potential health risks and ethical issues connected with it hence, the results of the present study conclude that the formulated cream has potency to protect against UVA and UVB rays indicating sunscreen activity . it is still preferred and is undoubtedly beneficial as it gives accurate and reproducible results. This method has helped to determine the SPF value of herbal alternatives like flowers of Cosmos Sulphurus (Asteraceae) and stating that it has good sunscreen activity and can be considered as active sunscreen agent or can be incorporated into other sunscreen formulations as an additive to enhance the activity.

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Table 1: Observations for Aluminium chloride assay of Cosmos Sulphurus

Sr. No.	Solution	Concentration µg/ml	Absorbance at 510nm
1	Standard rutin	500	0.2
2	Cosmos Sulphurus. Flower Extract	1000	0.14

Table 2: TLC Profile of Cosmos Sulphurus flowers ' extract

Sr. no.	Solvent system	No. of Spots	Detection	Rf value	Compounds
1	BAW (4:1:5)	5	UV-light	0.90, 0.79, 0.69, 0.60, 0.44	Glycosides, aglycones, sugars, glucose and galactose not separated
2	Forestal (HOAc:H_2 0:HCl) (30:10:3)	1	UV-light	0.84	Flavones,Flavonol, Anthocyanin aglycones
3	CAW (3:1.5:0.2)	4	UV-light	0.93, 0.88, 0.63, 0.54	Iso- rhamnetin, kaempferol, syringitin- laricitrin mixtures
4	EPAW (36:36:7:2)	3	UV-light	0.93,0.89, 0.45	Sugars
5	BBzPW (5:1:3:3)	1	UV-light	0.82	Anthocyanin

B-n-butanol, A-acetic acid, W-water, OHAc-Acetic acid, H 2 0-water, HCl-hydrochloric acid (concentrated), C-Chloroform; E-ethyl acetate, P-pyridine, Bz- Benzene

Table 3: Formula for Sunscreen Cream of Cosmos Sulphurus

SR NO.	INGREDIENTS	Ammount in %
1	cetostearyl alcohol IP	5%
2	cetomacrogol1000 IP	2%
3	cetyl alcohol IP	1%
4	stearic acid IP	2%
5	teel oil	10%
6	carbopol 940 IP	0.5%
7	disodium edta IP	0.2%
8	propyl paraben IP	0.06%
9	methyl paraben IP	0.29%
10	triethanolamine IH	q.s
11	distille water	q.s
12	herbal extract of cosmos sulhurus	0.5%

Table 4: Physical Parameters of Cosmos Sulphrus flower's extract sunscreen cream

Sr.no	Parameters	Observations
1	Color	Light Yellow
2	Odour	Aromatic
3	Spreadability	Good and Uniform
4	pH	6.5
5	Specific Gravity	0.93
6	Viscocity	1690cp

Table 5: Results of SPF and other Parameters of Cosmos Sulphurus flower extract sunscreen cream

Sr no	Parameters	Scan I	Scan II	Scan III	Average
1	SPF	2.11	1.81	1.93	1.95
2	Standard Deviation	0.27	0.29	0.10	0.22
3	UVA/UVB Ratio	0.664	0.639	0.649	0.641
4	Critical Wavelength	384.00	382.67	383.33	383.33
5	Boot Star Rating	***	***	***	***

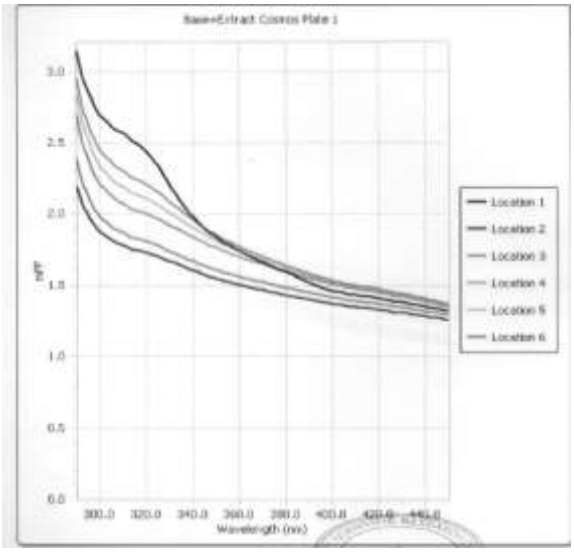

Figure1:Graph of wavelength vs mpf of ScanI

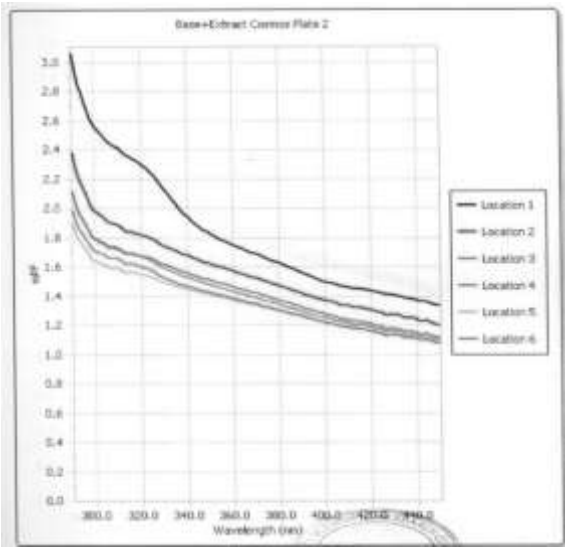

Figure2: Graph of wavelength vs mpf of scanII

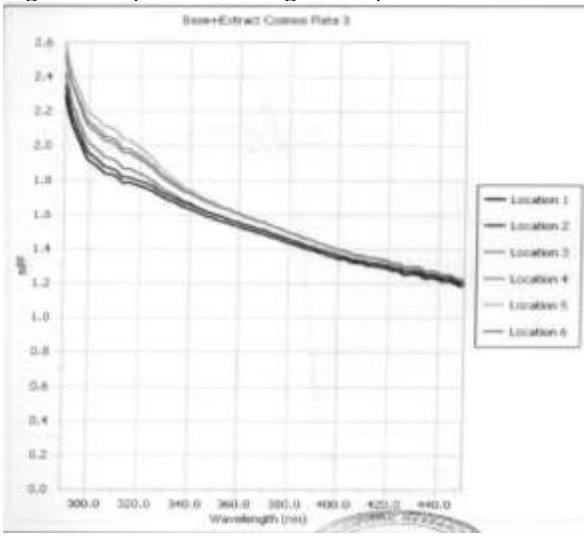

Figure 3: Graph of Wavelength vs mpf of scanIII

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