HPTLC FINGERPRINTING A NOVEL APPROACH FOR CRITICAL DETERMINATION OF SEAWEED EXTRACTED CRUDE PROTEIN AN *ULVA LACTUCA L*

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ABSTRACT

Protein crucial to ensure the future success of marine protein as novel therapeutic that can make a vital contribution to treatment & prevention of various diseases. The advantages of HPTLC in protein analysis, the development of advanced, sufficient to accomplish specific staining protocol leads to improved sensitivity for protein detection on HPTLC plates in comparison to universal protein derivatization of marine crucial peptides. This study aimed at developing a detection methodology for HPTLC separated proteins using mixtures over a broad polarity range, or if necessary allow modifying the separation with only few steps to improve the separation for a specific scope. A staining procedure on HPTLC permits various analytical possibilities. Besides the proof of its applicability for the very first time, parameters such as polarity and different mobile and stationary phases can be utilized these two systems show quite different separation selectivity and their combination into HPTLC process provides excellent separation of protein of the bovine albumin summary. This study adjective focused on the immunological investigation of proteins in marine seaweed extracted ULP following thin-layer chromatographic separation. As well as the proof of its applicability on dissimilar stationary phase materials, the newly developed protein or other substances through their properties as antigen can be used as an approach for semi-quantitative assessment of antigenic proteins. In accumulation to the analysis of intact food allergens, also analysing peptides thereof is worth considering which can be realized using HPTLC-immune staining as well.

Keyword: Protein, Marine, Antigen and Chromatography

INTRODUCTION

Marine seaweeds are well thought-out as a foundation of bioactive compounds as they are gifted to produce an excessive variety of secondary metabolites considered by their biological activities (Baipai 2016). The atmosphere in which seaweeds raise is exacting as they are exposed to a mixture of light and high oxygen concentrations (Kim *et al*., 2008). However, seaweeds occasionally suffer any serious photodynamic injury during metabolism. This point implies that seaweed cells have in defensive mechanisms played by the bioactive compounds (Matsukawa *et al.,* 1997: Zerrifi *et al*., 2018).

Seaweed in asian countries is related to a low incidence of cancers linked to European and North American countries (Kumar *et al*., 2011). Moreover, other assumed positive health effects have been recognized, such as reduced blood sugar and blood pressure, neuroprotective effects, immunomodulatory and, anti-inflammatory among others (Wells *et al*., 2017). A systematic link has been planned due to the presence in marine algae of dissimilar bioactive compounds, containing amino acids, polyphenols, sulfated polysaccharides, carotenoids, lipids and proteins/peptides (Hasim and Rahman 2012: Sathasivam and Ki 2018). Since of their substantial diversity and composition, red seaweeds (*i.e.*, Rhodophyta) have stimulated important interest in the food and the pharmaceutical industry for the search of novel natural nutrients and bioactive compounds (Cian *et al.,* 2015). It is significant that, between seaweeds, red algae contain great amounts of carbohydrates, proteins and minerals (Ruperez 2002). Particular useful properties have been recognized to Rhodophyta proteins/peptides and polysaccharides because of their exclusive composition. Certainly, these polysaccharides have chemical structures and physicochemical properties that differ substantially from folks of land plants (Urbano and Goni 2002; Wang *et al*., 2018).

The analysis of un-denatured proteins, especially proteoforms, is recently stimulating due to their mass of essential and physicochemical characteristics. To overawe some of the complications while using LC-MS or electrophoresis, high-performance thin-layer chromatography (HPTLC) denotes an interesting important technique (Fuchs *et al.,* 2007, Pasilis *et al.,* 2008, Pasilis *et al*., 2008 and Biller *et al*., 2015). In difference to the mostly used electrophoretic methodologies, more degrees of freedom with respect to parting and detection are obtainable. Conventionally, identifying and quantifying proteins on HPTLC plates can be achieved with ninhydrin and fluorescamine were in better for the detection of peptides of staining reagents (Kaiser *et al.,* 1970; Hakanson *et al.,* 1974). The lot of sophisticated method would focus on an extremely exact detection of single proteins

consuming specific antibodies. Showed an antibody based detection on HPTLC plates for evaluating Shiga toxin binding glycolipids holding the amino alcohol sphingosine (Meisen *et al*., 2004). Additionally, herewith already created an anti-body based method to detect a specific protein an sample in this protocol for the investigation of phosphopeptides using trading available antibodies in a HPTLC assay (Morschheuser *et al*., 2016).

MATERIAL METHODS

Extraction seaweed materials

Lyophilized biomass five gram was used for insulin like protein extraction from U. Lactuca L. For this, biomass was homogenised in a solution containing distilled water (DW, 10 mL), 95% ethanol (20 mL) and conc. sulphuric acid (0.72 mL) with uniform shaking for 20 min. Again, 20 mL DW and 95% ethanol (50 mL) were added and the pH adjusted to 1.7 using NaOH and HCL. The suspension was then filtered using Whattman paper (no. 1) and then again the pH adjusted to 3.0. To this suspension, 150 mL of 95% ethanol and 200 mL diethyl ether were added and kept for 12 h at 4°C. After centrifugation at 5,000×g for 20 min, the sediment was washed with acetone and diethyl ether before dissolving in 25% ethanol and the pH adjusted to 8.5 revised (Khanna *et al*., 1976; Krishnamoorthi and Sivakumar 2019).

Protein determination assay

Total protein concentration was determined using commercial kit according the methods of Bradford assays kit Thermo Scientific™ Pierce™ Coomassie (Bradford) Protein Assay Kit Catalogue number: 23200. BSA as the standard.

SDS PAGE

Protein samples were examined by SDS-PAGE on a Mini Protean III cel (Bio-Rad) using a 1x SDS Loading gell, 10% separation gell, 5% stocking gell, respectively with Tris/glycine/SDS buffer. Prior to the SDS-PAGE analysis, protein samples were denatured with loading buffer at 98 °C for 5 min. BSA Protein standard marker (Sigma Aldrich - india) was used as a protein molecular weight marker and protein bands was stained using Coomassie blue G250 dye (Bio-Rad) (Coustets *et al* 2015; Krishnamoorthi and Sivakumar 2019).

HPTLC analysis

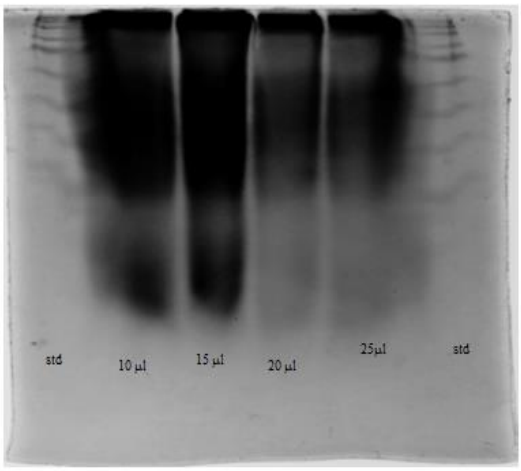
HPTLC of protein extract in two different solvent systems viz. 1. Toluene: Ethyl acetate: formic acid (5: 5: 1) 2. Toluene: Ethyl acetate (9.3: 0.7). Samples were applied on the plate using Camag automatic TLC sampler 4 attached to camag HPTLC system. The samples

(2µl) each were spotted on aluminum backed pre-coated silica gel plate 60F-254 plate (5×10 cm) in the form of bands with width 8 mm by using Hamilton syringe (100µl). Then the plate was developed in the two different solvent systems in a twin trough chamber to a distance of 8 cm. After making dry in air the plates were examined under UV 254 nm and under UV 366 nm. Rf value and the colour of the resolved bands were recorded. Photographs of the plates were captured using camag TLC visualizer. For second solvent system the plate was sprayed with Liebermann-Burchard reagent and heated at 105o till the colour of the spots/ bands appeared without charring. Again the Rf value and the colour of the resolved bands were recorded. It was calculated by using the formula (Distance travelled by solute / Distance travelled by solvent) (Bidhan *et al*., 2015).

RESULTS AND DISCUSSIONS

SDS PAGE

Figure: 1 Seaweed Ulva lactuca l. crude protein using SDS-PAGE profile



The Bradford assay is comparatively free from interference by most generally used biochemical reagents. Conversely, rare chemicals may significantly alter the absorbance of the reagent blank or modify the response of proteins to the dye (Spector 1978; Chial *et al*., 1993).

Obligatory of protein to Coomassie brilliant Blue G250 could shift the absorbance most of the protein peak from 590 nm to 620 nm. Vigour, consequently, appear additional sensible to measure the absorbance at the high wavelength (Compton and jones 1985). Though, as usual pH of the assay, an considerable proportion of the dye is form of (λmax = 650 nm) which inhibits with absorbance measurement of the dye–protein complex at 620 nm.

Measurement at 595 nm represents the finest concession between maximizing the absorbance due to the dye–protein complex while minimizing that due to free dye (Congdon *et al*., 1993).

Table: 1 Bradford assays on ULP extract

S.no	Sample	Absorbance of OD Value
1	Bovine serum albumin	0.305±0.0004
2	Blank D_20	0.037±0
3	Sample	0.077±0.009
4	Extracted protein	0.125±0.005

In our study we separated proteins of marine seaweed ULP samples collected at different stages of ontogenesis. According to SDS-PAGE the presented the same bands of major proteins in studied range from 1 to 210 kDa (Fig. 1). However the amounts of individual proteins differed between different stages of ontogenesis. SDS PAGE is easy and important methods this permit to isolated protein according to their MW and detect of them in assessed to molecular markers so it is an mostly used an biological objects in providing the information. (Krishnamoorthi and Sivakumar 2019).

In addition to the measurement of purity, for a closer examination of the protein isolated and the molecular weight was confirmed using sodium dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Mini-Protean, Bio-Rad Ltd., India) and compared with a bovine albumin standard. SDS-PAGE was performed using a 15% polyacrylamide slab gel and a 4.5% stacking gel and was confirmed after staining with Coomassie brilliant blue R250 and destaining. The molecular weights of the protein subunits were calculated by comparison with a standard ladder (Seo *et al*., 2013).

HPTLC finger printing

Figure: 2 HPTLC fingerprinting on ULP

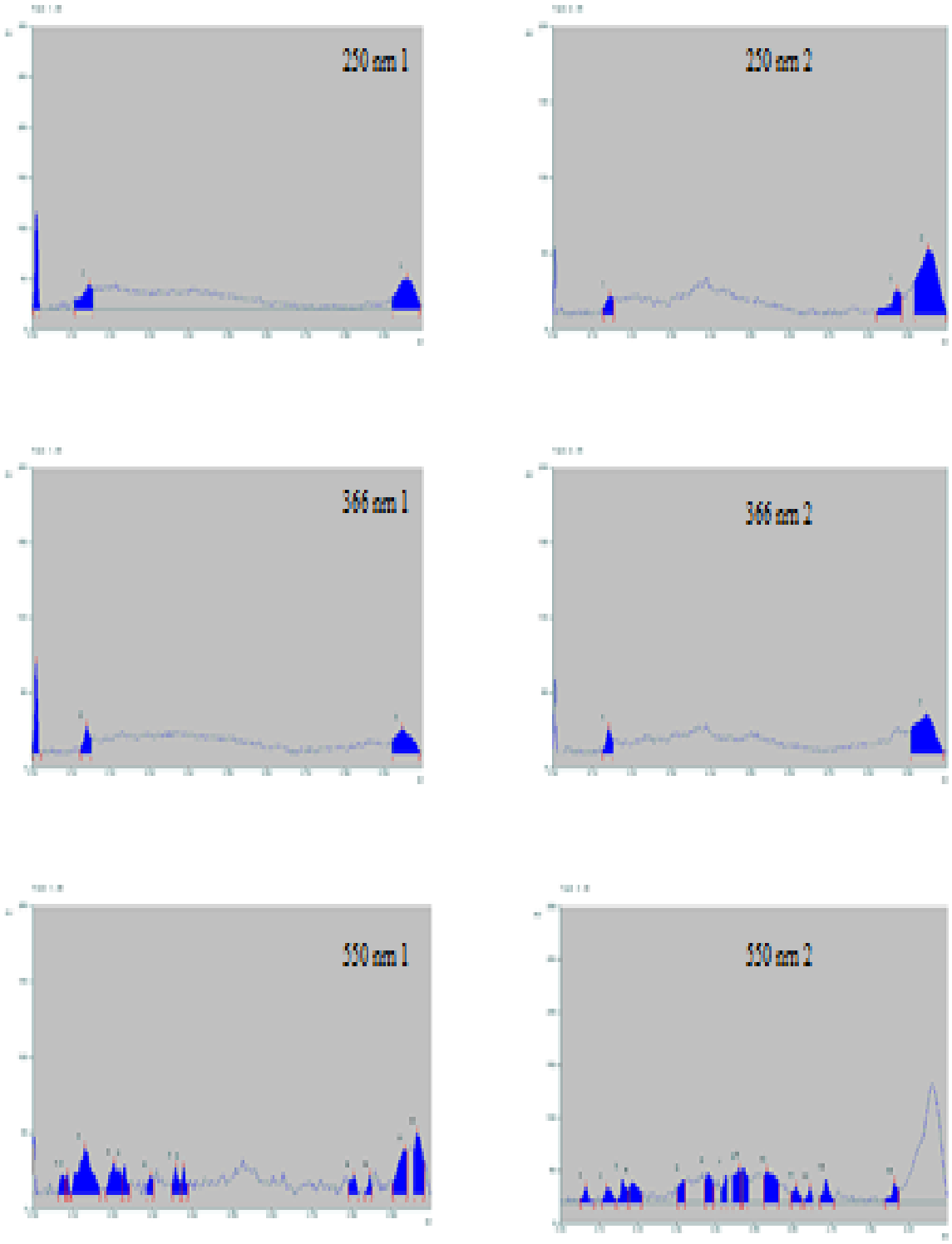


Figure: 3 HPTLC plates on ULP different dots

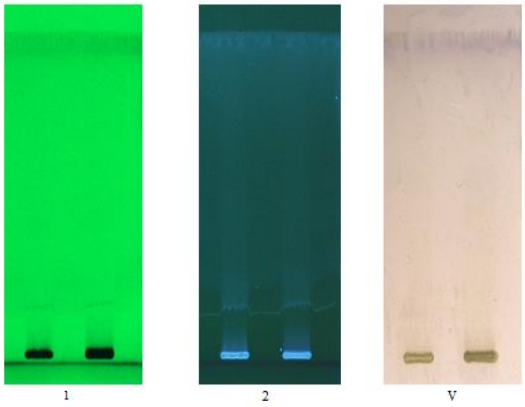
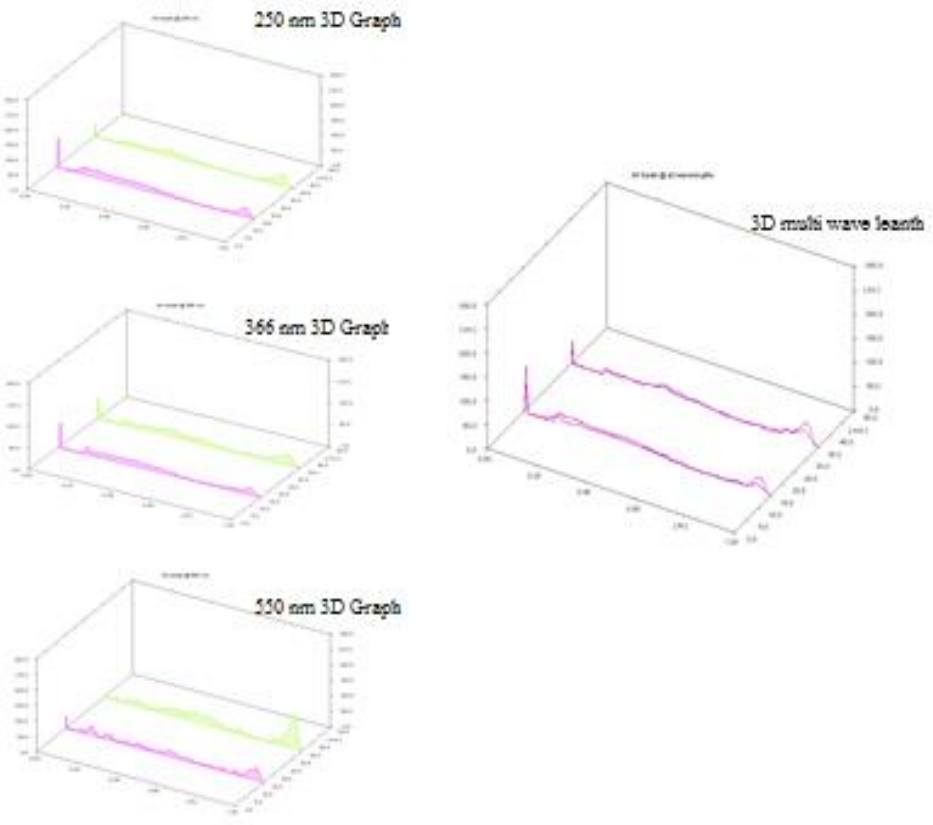


Figure: 4 HPTLC fingerprinting 3D images



HPTLC approach, the evaluation of suitable chromatographic parameters was focused: various solvent systems were applied to achieve an optimal HPTLC separation of the trypsin peptides. The finally established conditions are described in the materials and methods section (Morschheuser *et al*., 2016). Subsequently, suitable parameters for the immunological overlay assay were evaluated based on similar protocols as for the intact proteins (Johnson and Criss 2013). The evaluation was done with regard to composition of the incubation medium in general, time and duration of the incubation steps, antibody and antigen concentrations, and blocking of the surface to inhibit unspecific binding (Frese *et al*., 2013; Cox *et al*., 2014).

The high number of proteins including the incalculable substance varieties and their logical intrigue, HPTLC-based detachment system should be as factor as conceivable to have a few open doors for the various pack of protein diagnostic inquiries. As referenced above, it is hard to locate a one for all technique (Coskun 2016). Particularly with respect to the high fluctuation of proteins (Talley and Alexov 2010). HPTLC appears to give potential outcomes to rapidly react to any partition issue and consolidating the distinctive procedures effectively, in best case even on the web (Attimarad *et al*., 2011). Utilizing HPTLC investigation, partition can be impacted by numerous parameters from which stationary phase, solvents, and utilization of modifiers are the most critical ones (Eric *et al.,* 2007). In a logical working everyday practice, usually practice to perform enhancement of HPTLC-based separation system much dependent on experimentation approaches. Be that as it may, the establishment of a worldwide system dependent on realized connections regarding key streamlining apparatuses ought to be favored (Biller *et al*., 2015; Polak *et al*., 2019).

As a great beginning stage for an enhancement includes test portrayal and determination of an appropriate stationary phase knowing the physic-substance properties of the investigations is of pre-imperative nature (Poole and Dias 2000). Utilitarian substituents, sub-atomic size, just as solvability in various solvents, will influence the determination of the analytical apparatuses (Hayes *et al*., 2015). For example a very low extremity of the analysis isn't appropriate when utilizing silica gel as a stationary phase since it results in low retention, while the inverse would cause retaining that is excessively high (Qin *et al*., 2010). Once investigating the pertinence of various stationary phases in protein partition, a blend of model proteins with abroad scope of properties and in this way expressive to a genuine example may be helpful for assessment (Pedersen *et al*., 2003). For this situation, the accompanying standard proteins are reference article used to be utilized as analyses: cytochrome, insulin,

lacto globulin, myoglobin, lact albumin, lysozyme, bovine serum albumin (BSA), casein, and ovalbumin (Gudiksen *et al*., 2006; Branco *et al*., 2010).

The principles for this decision were to utilize a wide range of polarity and molecular weight, and an isoelectric purpose of the proteins guaranteeing a protein blend with different properties and a subsequent variety in partition conduct. Obvious, the entire fluctuation of proteins can't be secured with this choice. In this underlying methodology, the choice of proteins can be underlined with the accompanying properties (Pergande *et al*., 2017). Myoglobin and insulin are ordinary little model proteins (Strandberg 2009).The expectation of polarity properties of proteins is tougher compared to other analyses, as their structures is more complex and their polar and non-polar functional groups (Wolfenden 1985). Interrelate variably with the sorbent, contingent on their conformation in the mobile phase (Cole and Dorsey 1993). The presence of organic solvents indicates to structural changes, giving rise to diverse conformational states which may stimulus of communicate with the stationary phase (Petrovic *et al*., 2018). Different charge states of a protein could also ensue depending on the pH value causing changes of communicates with the chromatographic media (Di Russo *et al*., 2012; Daniel *et al*., 2015). Furthermore, a protein mixture even obscures the prophecy of a separation condition because of the broad range of adsorption affinities. Therefore, it looks necessary to consider chromatographic systems based on polar as well as less polar stationary phases (Astefanei *et al*., 2017). HPTLC silica gel is the most commonly used adsorbent among the polar materials and the first choice in amino acid and protein analysis (Pasilis *et al*., 2008; Mohammed *et al*., 2012; Tscherch *et al*., 2013). It has been proven to be suitable in most cases and has shown mainly positive results in the analysis of intact proteins (Toby *et al*., 2016).

In case of deeper interest, antigenic peptides can be further analysed by mass spectrometry. At this point, various hyphenation possibilities are worth considering: Coupling to ESI-MS is as well possible as HPTLC-MALDI-MS, but needs to be chosen individually (Heller *et al*., 2003). As the blocking reagent Tween20 tends to interfere with the signals in mass spectrometry, the protocol needs to be complemented in terms of an adequate blocking agent (Gallagher *et al*., 2008). However, in comparison to the use of Tween20 as blocking reagent, the traditionally use of BSA led to an enhanced colouring of the background in HPTLC (Kruger 2009). This might be caused by the high concentration of the antibody and the associated cross-reactivity towards proteins in general. In case of an experimental set-up

where antibodies with affinity towards BSA are used, the blocking agent must be adapted. (Gonzalez-Techera *et al*., 2007).

CONCLUSIONS

General studies of the protein were subsidized to the cohort of vital biologically effective macromolecules compounds. Consequently, they are supposed to be a good resource for food as well as therapeutic and functional food and medicine industries. Commonly users are more familiar currently and conscious about undesirable side effects of drugs and the association between diet and health. This leads to a self medication mind set such as the desire to dodge chemically synthesized drugs and take natural food products or neutraceutical.

The separation of vital systems is the presence of protein and use a suitable modifier in a satisfactory concentration. Circumstances have to be familiar exclusively depending on components and the chromatographic systems.

Dissimilar potentials for follow up detection, especially with regard to protein function, enzyme/peptide activity, and protein identification, special as regard to the hyphenations possible for HPTLC.

Efficacy and determination of separation is glorious and may be slap further improved by optimization of separation conditions as well as procedure of protein consumption.

Conditions must be balanced independently relying upon parts and the chromatographic system. Display is great instruments for precise improvement of the mobile phase. At long last, unique stationary phase science gives modest contrasts in selectivity and offer diverse conceivable outcomes for follow up discovery, particularly as to protein work, compound action, and protein distinguishing proof, the last with exceptional respect to the hyphenations feasible for HPTLC.

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