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# The development of a detection method discriminating for mannosylerythritol lipids and acylglycerols

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## Key words

Thin layer chromatography (TLC), Biosurfactants, Mannosylerythritol lipids (MELs), Glycolipids, Pseudozyma, orcinol

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## Introduction

Mannosylerythritol lipids (MELs) are promising biosurfactants produced by *Pseudozyma sp.* when supplied with vegetable oil. Mannosylerythritol lipids are glycolipids, consisting of a mannosylerythritol core esterified with two fatty acid residues and optional acetylation of the mannosyl unit, whereby number and position of acetylation differ which determines behaviour as a surfactant (**Figure 1**) [1].

Enhanced production depends on a careful monitoring of the conversion of vegetable oil (triglycerides) into MELs. Also purification benefits from an improved monitoring of the presence of vegetable oil and



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its degradation products which can easily persist in the purified biosurfactant [3,4]. This can be done by using normal phase thin-layer chromatography (TLC).

Methods described in literature are highly similar and use a solvent mixture of chloroform and methanol in normal-phase chromatography. Sometimes water and/or ammonia is added [2,7,8], in order to change the properties of the mobile phase, such as polarity or hydrogen-binding capacity. Elution is followed by detection with anthrone or orcinol [5]. Although chloroform is a very versatile solvent and dissolves both glycolipids and fat-like substances, the use of this solvent is under concern due to health hazards and its former use as a narcotic. Yet, TLC-methods for the evaluation of MEL-production have not been updated.

This paper reports a new one-pot method to distinguish MELs from vegetable oil and its degradation products, such as mono-acylglycerol (MAG), using acetone and diethyl ether as a mobile phase. The presented method can be used as a cheap and easy method to monitor the appearance of MELs or to evaluate the presence of residual vegetable oil and its degradation products in broth, without using toxic chemicals or advanced equipment. Such an approach allows better control of production and purification conditions.

# Experimental

## Materials and reagents

Silica F254 TLC plates (20x20 cm, Merck) with concentration zone (20x2.5 cm) were used Mobile phases were composed of formic acid (99%, Sigma) and solvent mixture (Acetone and ether: HPLC grade, Sigma; others technical grade or higher, Sigma). A 65/12/2 $CHCl_3$ (HPLC-grade, Sigma)/MeOH (HPLC-grade, Sigma)/saturated aequous $NH_40H$-mixture was used for comparison. 0.1% orcinol solution was prepared

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by dissolving 100 mg orcinol (98%, Acros) in 95 mL distilled water while adding 5 mL 98% sulfuric acid (Acros). 30 g/L stock solutions of MELs and monoacylglycerol (MAG) (analytical grade, Sigma) were made in ethyl acetate. Coconut fat (analytical grade, Sigma) and soybean oil (food grade, Lesieur) were used for triacylglycerols (TAG).

### Instrumentation

100 mL of mobile phase was poured into a tightly closed glass chamber and allowed to equilibrate for at least one hour at room temperature. 6 µL of concentrated sample solutions were spotted on the plate. In the two-chamber method, the plate was firstly developed to a distance of 8 cm (+-30 min), air-dried and transferred into a second chamber and fully developed to a distance of 19 cm (+-2h). After development, plates were removed from the chamber and dried in air after which they were sprayed with 0.1% orcinol solution and heated to 90°C for 5-10 min until purple (glycolipid) or brown (acylglyceroles) spots were observed. Plates were then cooled to room temperature and transferred to a glass chamber with $I_2$ -crystals and allowed to develop overnight, after which relative retention factors ($R_f$) were calculated.

## Results and discussion

Based on Snyders' solvent strength parameters [6], five solvents, namely methanol, tetrahydrofurane (THF), diethyl ether and acetone were selected for screening with MELs, soybean oil, coconut oil (both TAG) and MAG. Formic acid was added as a modifier in concentrations ranging from 0 to 1%. As can be seen in Table 1, a combination of diethyl ether and acetone is expected to separate MELs and fats on one hand, but also to discriminate between MELs, with acetone causing mobility of both groups, while ether separates both groups and between MELs itself.

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Therefore, several mixtures of acetone and ether were tested for their resolving power of a MEL/MAG/TAG-mixture. A 30/70% ether/acetone solvent mixture behaved more as pure acetone and did not bring a clear separation between MELs and acylglycerols. On the contrast, eluentia with at least 70% ether, preferably 80%, were capable of resolving MELs ($R_f$ 0.070.13) and acylglycerols ($R_f$ MAG 0.74; $R_f$ TAG 0.97) as a group, although only two MEL-spots could be distinguished clearly.

However, when samples of fermentation broth extracted with ethyl acetate were spotted, an unexpected, elongated, spot with an $R_f$ between those of MELs and acylglycerols was noticed. Development of the TLC-plate with orcinol en $I_2$ shows a typical colour gradient from purple at the bottom towards brown at the top, indicating a change in ratio between glycolipids and acylglycerols. Presumably, these spots are consisting of an emulsion between MELs and acylglycerols incompletely separated when a higher loading is applied to the plate. No such spots could be observed when samples close to the saturation point, which is more than 34 µL of the used 30 g $L^{-1}$ solution, were sufficiently diluted.

When dealing with fermentation samples, it proved to be difficult to estimate the required dilution to avoid emulsification. In this case a two-step method, which was not prone to emulsification, could be used. Satisfying results were obtained with a first elution in acetone until the solvent front has reached half of the plate, followed by a full elution in diethyl ether. MELs ($R_f$ 0.47-0.41) were separated from triacylglycerols ($R_f$ 0.96) and other fat derivatives, represented by MAG ($R_f$ 0.67). Again, MELs were divided into two spots. Subsequently, it was examined if it was possible to separate these MEL-spots further by an extra elution in ether. This proved to be possible (**Figure 2**), though the extra elution step seemed not feasible due the more laborious character.

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To determine detection and saturation limits of both described methods, several MEL-concentrations were spotted. From these analyses, it can be concluded that 1.67 µl of a 30 g MEL $L^{-1}$ solution, equalling 50 µg MELs, forms a clearly visible spot, while the presence of 0.34 µl of a 30 $gL^{-1}$ MEL solution, equalling 10 µg active substance, can be suspected. Saturation occurred when more than 34 µL MEL-solution, equalling 1000 µg of active substance, was spotted. Therefore, it seems advisable to spot between 10 to 500 µg MELs. When starting from a 30 $gL^{-1}$ solution, between 5 to 17 µL sample could be spotted, an amount which is both easy to handle, reproducible and sufficient for detection.

As last step in the evaluation in this method, the performance of the two-step method was compared to the widely used method of elution in 65:12:2 $CHCl_3$/MeOH/$NH_4OH$, using 2D-TLC [4,9,10]. From this, it can be concluded that spots of the acetone/ether method are equivalent with these as obtained with the chloroform method, namely MAG, and different MEL-types of MELs. The former method discriminates more between the different acylglycerols, while the latter separates MELs better. As described above, this can be amended by a second elution with diethyl ether.

## Conclusion

The acetone/diethyl ether method is a valid alternative for the chloroform method. A one-pot method with 80% of diethylether and 20% acetone is proposed. Detection limit is below 50 µg of glycolipid, or 1.67 µl of a 30 g MEL $L^{-1}$ solution, while the saturation limit is around 1000 µg MELs, or 34 µL of the used 30 g MEL $L^{-1}$ solution. In certain cases, a new spot or smear, consisting of an emulsion of MELs and acylglycerols can appear. The nature of this spot could be revealed by treating the plate with both orcinol and I2. To avoid the occurrence of such spots, tested samples should be diluted sufficiently. In other cases, a two-step method could be used.

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104 Proposed methods are equivalent with the chloroform-based methods and can be used to monitor the
105 conversion of triacylglycerols into MELs, which allows adjusted feeding of an oily substrate, or to check
106 for residual acylglycerols present in a certain MEL-mixture, which is a necessity during purification.

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DRAFT

118 Table 1. Development on full plates (18 cm length) of MEL, MAG and TAG (soybean oil and
119 coconut fat) with an optimal formic acid concentration for each solvent. Soybean oil and
120 coconut fat were separately applied but resulted in identical $R_f$.

| Solvent | pH/ % formic acid | $R_f$ MEL | $R_f$ MAG | $R_f$ TAG |
|---|---|---|---|---|
| Methanol | pH 6 | 0.86 | - | 0.0 |
| Acetone | pH 5 | 0.86-0.95 | 0.86 | 0.96 |
| THF | 0.01 % | 0.82 | 0.84 | 0.94-0.92 |
| Diethyl ether | 0.01% | 0.03-0.06-0.11 | 0.71 | 0.98 |
| Diethyl ether | 0.41% | 0.03-0.06-0.11 | 0.71 | 0.98 |

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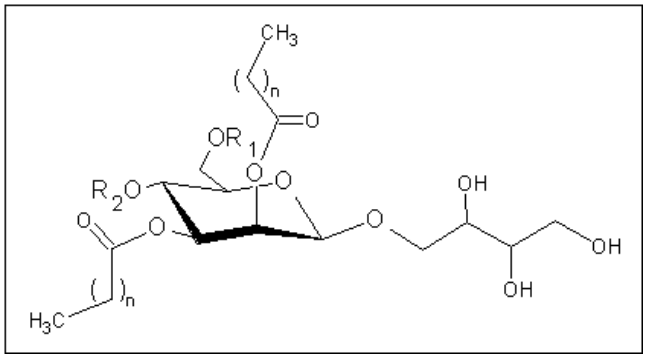
123 


124 Figure 1. Chemical structure of MELs with MEL-A: $R_1$ = $R_2$= -$COCH_3$; MEL-B:$R_1$= -H, $R_2$= - $COCH_3$l; MEL-C:

125 $R_1$= - $COCH_3$, $R_2$= -H; MEL-D: $R_1$ = $R_2$ = -H

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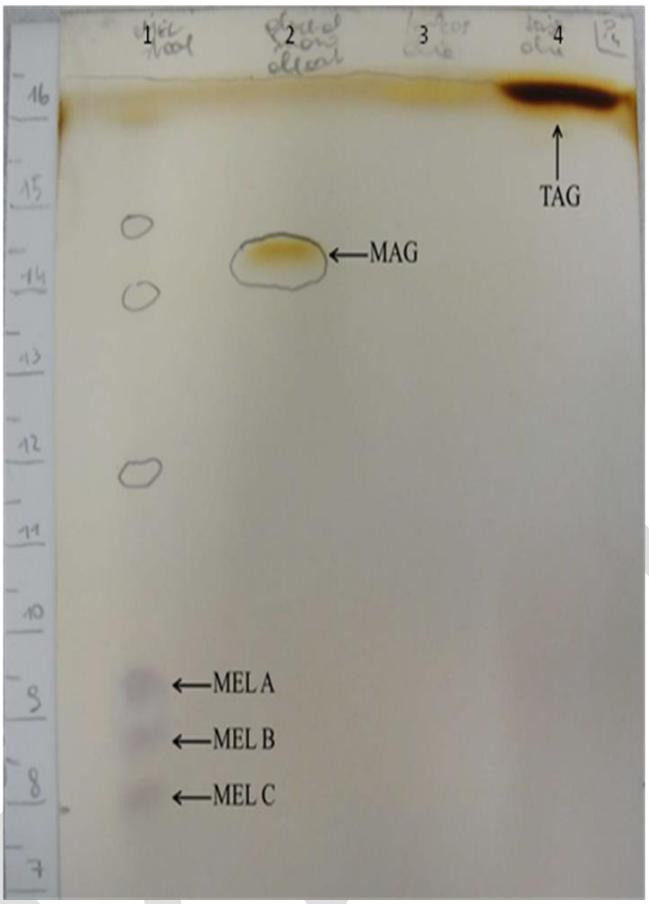


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128 Figure 2. TLC (16 cm) of MEL (lane 1), glycerol monooleate (MAG, lane 2), coconut fat (lane 3) and
129 soybean oil (lane 4) after serial elution ([1/2] Acetone/ [1] $Et_20$ / [1] $Et_20$) and subsequent development
130 with orcinol and $I_2$.