



Effect of relative humidity on the thin-layer chromatographic separation of E 472 emulsifiers

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Abstract

E 472 emulsifiers are organic acid esters of mono- and diacylglycerides. These emulsifiers are categorized into six subgroups (E 472a-f) based on the specific acid component, *e.g.*, acetic acid, lactic acid, citric acid, tartaric acid, or mixtures of tartaric and acetic acid. The present study investigated the effect of relative humidity (RH) on the thin-layer chromatographic separation of four E 472 emulsifier subgroups, addressing practical concerns due to RH variations between seasons and within laboratories. Three RH-setting techniques affecting plate activity and chamber climate were assessed using saturated salt and aqueous sulfuric acid solutions. Aqueous sulfuric acid solutions for RH adjustment resulted in decreased hR_F values, while no trends in hR_F value changes were observed for saturated salt solutions. Unlike RH itself, the RH setting technique led to substantial changes in the chromatographic fingerprint. Thus, the choice of the RH setting method should be tailored to the specific analyte and analysis goal. Despite variations in chromatographic fingerprints between the three RH setting techniques, the fingerprint remained consistent within the same technique across the tested RH range.

Keywords High-performance thin-layer chromatography (HPTLC) · Food emulsifiers · E 472 · Relative humidity (RH)

1 Introduction

E 472 emulsifiers are organic acid esters of monoacylglycerides (MAG) and diacylglycerides (DAG). These emulsifiers are categorized into six subgroups based on the specific acid component, which can include acetic acid, lactic acid, citric acid, tartaric acid, or mixtures of tartaric and acetic acid (E 472a-f) [1, 2].

Different approaches are used for manufacturing E 472 emulsifiers, the most common two being (1) the transesterification of MAG/DAG with the organic acid or its anhydride and (2) the direct esterification of glycerol with fatty acids and the respective organic acid [3]. The reaction is equilibrium-controlled and, thus, influenced by the reactants' raw material and stoichiometric ratios [4]. By-products like free glycerol, free fatty acids, free organic acid, free glycerides, and polymerized organic acid [3, 5] contribute to the complex composition, also reflected in the chromatographic

fingerprint of the emulsifiers [6, 7]. Recently, two publications on the thin-layer chromatographic (TLC) characterization and differentiation of E 472 subgroups a, b, c, and e [6] and on the quantification of E 472b emulsifiers in foamed food formulations with high-performance thin-layer chromatography coupled to fluorescence detection (HPTLC-FLD) [7] have been published.

During the development of analytical methods, robustness is a crucial parameter. It is referred to as the “capacity [of an analytical method] to remain unaffected by small but deliberate variations in method parameters” [8]. Robustness is related to intrinsic variables contributing to the stability of an analytical method [9]. In HPTLC, method robustness is not only affected by the stationary and mobile phase but also by the gas phase [10] and the relative humidity (RH) affecting plate activity, as well as temperature and several other factors [11]. A few studies investigated the effect of the RH on the chromatographic behavior of different analytes with varying polarity [11–15]. Stationary phases used for evaluation were silica gel [12, 14, 15] and aluminum oxide [11, 13, 14]. RH was set using different techniques depending on the goal of the study. For instance, *Zeeuw* (1968) used activated silica gel plates stored for 24 h at various RH and developed in unsaturated chambers under the same

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RH conditions [15], whereas *Reichel* (1967) kept the plates at ambient temperature in the laboratory for 10 min after application, exposing them to various controlled RH, and carried out the development under the same RH conditions [13]. Generally, RH was tested between 3% and 90%. The retardation factor (R_F), the ratio of the migration distance of a respective substance to that of the mobile phase, or the relative retardation factor (hR_F), was used to evaluate chromatographic separation. For most studies, regardless of whether aluminum oxide or silica gel was used as stationary phase, increasing RH led to increasing hR_F values [12–15], but one study by *Prinzler and Tauchmann* (1967), investigating the separation of sulfoxides on aluminum oxide, described decreasing hR_F values with increasing RH [11]. Increasing hR_F values result from deactivation of the layer caused by adsorbed water with increasing RH [13, 15]. Interestingly, effects were differently pronounced for different analytes [12, 13]. *Suzuki and Matsushita* (1967) compared the separation of polyaromatic hydrocarbons regarding RH on silica gel and aluminum oxide. They observed that effects on hR_F values were more pronounced on aluminum oxide than on silica gel [14].

The effect of the RH on the TLC separation varies for different kinds of analytes and affects method robustness. Thus, the present study aimed to investigate the effect of RH on the TLC separation of four E 472 emulsifier subgroups. The study includes a crucial practical aspect because of the substantial variation of the RH between seasons, even during the day, and that most laboratories have no control over the intralaboratory RH. Therefore, three methods for setting RH influencing plate activity and chamber climate were evaluated.

2 Experimental

2.1 Chemicals

tert-Butyl methyl ether (TBME, CHEMSOLUTE, for high-performance liquid chromatography [HPLC], $\geq 99.8\%$), *n*-heptane (Chemsolute, for HPLC, $\geq 99.2\%$), and methanol (Chemsolute, liquid chromatography–mass spectrometry [LC–MS], $> 99.9\%$) were obtained from Th. Geyer (Renningen, Germany). Chloroform (Chromasolv, for residue analysis, $\geq 99.9\%$) was purchased from Sigma-Aldrich (Steinheim, Germany). Diethyl ether ($\geq 99.5\%$) was provided by Thermo Fisher Scientific (Schwerte, Germany). Ultrapure water ($> 18 \text{ M}\Omega \text{ cm}$) was supplied by a Synergy System (Millipore, Schwalbach, Germany). Formic acid (analytical reagent grade, $> 98\%$) was purchased from Thermo Fisher Scientific, and sulfuric acid (H_2SO_4 , Rotipuran p.a., 96%) was obtained from Carl Roth (Karlsruhe, Germany). Ammonium nitrate (NH_4NO_3 , p.a., $\geq 99\%$) was purchased

from Honeywell (Böblingen, Germany). Magnesium chloride (MgCl_2 , ACS reagent, $\geq 99\%$) and potassium chloride (KCl, for analysis) were obtained from Merck (Darmstadt, Germany). Potassium acetate (CH_3COOK , 98%), primuline (dye content 50%), and molecular sieve (4 Å beads) were provided by Sigma-Aldrich. Potassium carbonate (K_2CO_3 , anhydrous, for analysis, $\geq 99\%$) was obtained from Carl Roth, and sodium chloride (NaCl, 99%) was provided by Häberle Labortechnik (Lonsee-Ettlenschieß, Germany). HPTLC silica gel F₂₅₄s MS-grade plates from Merck were used without pre-washing. E 472a (ACETEM; acetic acid esters of MAG/DAG), E 472b (LACTEM; lactic acid esters of MAG/DAG), E 472c (CITREM; citric acid esters of MAG/DAG), and E 472e (DATEM; diacetyl tartaric esters of MAG/DAG) emulsifier samples of technical grade were provided by different manufacturers and stored in the refrigerator at 4 °C.

2.2 Standard and sample solutions

Stock solutions of each emulsifier were prepared in TBME at a concentration of 500 mg/L and were stored in the refrigerator at 4 °C before HPTLC analysis. Single standard stock solutions of stearic acid (SA), 1- and 2-monostearate (1- and 2-MSt), 1,2- and 1,3-distearate (1,2- and 1,3-DSt), and tristearate (TSt) were prepared in TBME at 1 mg/mL and stored at 4 °C. For comparison, a standard mix solution with a concentration of 0.25 mg lipid class/mL was prepared from the single standard stock solutions and stored at 4 °C.

2.3 Saturated salt solutions

One liter of saturated aqueous solution of each KCl, NaCl, NH_4NO_3 , K_2CO_3 , MgCl_2 , and CH_3COOK was prepared and stored at room temperature (RT) until use. Therefore, according to the solubility of each salt in water at 25 °C, the necessary amount plus 10% accounting for intralaboratory temperature deviation was mixed with ultrapure water.

2.4 Activation of molecular sieve

The molecular sieve was stored at 100 °C for a minimum of 72 h in a drying cabinet. After the activation process, the molecular sieve was cooled at RT and stored in a closed glass vessel until use.

2.5 Sulfuric acid solutions

Aqueous sulfuric acid solutions with different concentrations (10–70% in steps of 10%) were prepared and stored at RT until use.

2.6 High-performance thin-layer chromatography–fluorescence detection

HPTLC–FLD was performed according to *Schuster et al.* [7]. An Automatic TLC Sampler 4 (ATS 4, CAMAG, Muttenz, Switzerland) was used for application. Samples and standards were applied bandwise on 20 cm × 10 cm plates with a distance from the lower edge of 8 mm and the left and right edge of 10 mm. As the rinsing solvent with one rinsing cycle and one filling cycle, TBME was used. After a drying step for 10 min in a fume hood, a twofold development occurred in an Automatic Developing Chamber (ADC 2, CAMAG) equipped with a 20 cm × 10 cm twin-trough chamber (CAMAG). Plate activity was set by different techniques described in Sect. 2.7. For the first development, a mixture of chloroform–methanol–water–formic acid (67:6:1.2:0.2, V/V) was used up to a migration distance of 50 mm, followed by a drying step for 10 min. As the mixture was a two-phase system, it was well shaken until being filled in the developing chamber, which took place briefly before starting the development. The second development was carried out with a mixture of *n*-heptane–diethyl ether–formic acid (55:45:1, V/V) up to a migration distance of 80 mm, including 5 min of drying. After both developments, plate images were captured with the TLC Visualizer (CAMAG) under UV 254 nm and UV 366 nm illumination. Following the second development, the plates were dipped into a solution of primuline (0.05% in acetone–water, 4:1, V/V) with the TLC Chromatogram Immersion Device III (CAMAG, immersion speed 1, immersion time 2) and dried in a stream of cold air for 1 min. Plates were stored in a desiccator at a constant RH of 47%, adjusted by a saturated K₂CO₃ solution for 1 h. After that, plate images at UV 254 nm and UV 366 nm illumination were captured again. Control of the HPTLC instruments and data evaluation was performed with the software winCATS, version 1.4.6.2002 (CAMAG).

2.7 Setting and determination of relative humidity

Three techniques (A, B, and C) to set a constant RH were investigated, and the effect of the RH on the separation of E 472 emulsifiers was evaluated. RH for both developments was adjusted in the same way.

- A. Automatic setting by the humidity control function of the ADC 2 using saturated salt solutions for 5 min. The RH at the end of the humidity control was registered and used for displaying the results. *Note:* This option sets the plate activity before developing the plate rather than setting the relative humidity *during* plate development.
- B. Manual setting of the RH by chamber saturation for 15 min with saturated salt and aqueous sulfuric acid solutions in the ADC 2. Saturated salt and sulfuric

acid solutions were added by the operator, whereas the mobile phase was provided by the ADC 2. Actual RH was measured manually with the help of a hygrometer (TFA, Dostmann, Wertheim, Germany) on the same day as the HPTLC analysis.

- C. Manual setting of the RH by plate preconditioning for 15 min with saturated salt and aqueous sulfuric acid solutions in the ADC 2. Saturated salt and sulfuric acid solutions were added by the operator, whereas the mobile phase was provided by the ADC 2. Actual RH was measured manually with the help of a hygrometer on the same day as the HPTLC analysis.

RH in the laboratory was measured daily with a hygrometer and varied between 30% and 60%. The temperature in the laboratory varied between 25 °C and 30 °C. After the first development, the chamber was cleaned and dried, and the procedure described above was repeated for the second development.

2.8 Evaluation of results

For the evaluation of the results, two approaches were chosen. First, correlations between RH and hR_F values were identified by plotting hR_F values (obtained from the images under UV 366 nm) of selected signals for each E 472 subgroup (Sect. 3.1) against RH values. This evaluation was also done for all RH setting techniques (Sect. 2.7), applying saturated salt and sulfuric acid solutions. Second, the TLC fingerprint alterations were investigated by visual inspection and comparison of the plate images conducted at UV 366 nm. The comparison was performed for all RH levels and all techniques of setting the RH. Examples of observed alterations for the E 472a and E 472b emulsifiers are discussed in Sects. 3.3 and 3.4.

3 Results and discussion

3.1 Experimental design and selection of zones for evaluation

Different saturated salt and aqueous sulfuric acid solutions were utilized to evaluate the effect of RH on the separation of E 472 subgroups. Application of 70% sulfuric acid solution within the humidity control unit of the ADC 2 was not possible due to its high corrosivity, so automatic and manual techniques for setting the RH were evaluated (Sect. 2.7). The first technique (A), the automatic setting of the RH in the humidity control unit of the ADC 2 (which is used for setting the plate activity), was used with saturated salt solutions. Two techniques were investigated for aqueous sulfuric acid solutions: chamber saturation (B), applied for setting the

chamber climate, and plate preconditioning (C) for assessing the plate activity. Thus, in this study, the termini chamber saturation and plate preconditioning refer to the application of saturated salt and aqueous sulfuric acid solutions, not for mobile phase usage. Techniques (B) and (C) were also evaluated with saturated salt solutions for a meaningful comparison of the results.

For better clarity and more straightforward evaluation, selected zones for MAG, DAG, and triacylglycerides (TAG) were evaluated. In the first step, zones were assigned to substance groups, according to *Oellig et al.* 2020 [6], which was possible despite minor changes in the solvent ratios of the mobile phase. For the selection of the zones to be evaluated, differences in the hR_F values were considered, leading to three zones for each emulsifier covering nearly the entire migration distance. For E 472a, selected zones were MAG (a1), monoacetic acid esters (a2), and diacetic acid esters (a3) of MAG. For E 472b, selected zones were MAG (b1), DAG and lactic acid esters of DAG (b2), and TAG (b3). For E 472c, selected zones were MAG and citric acid esters of DAG esterified with an additional glycerol (c1), DAG (c2), and TAG (c3), and for E 472e, MAG (e1), diacetic acid esters of MAG (e2), and TAG (e3) (Fig. 1) [6].

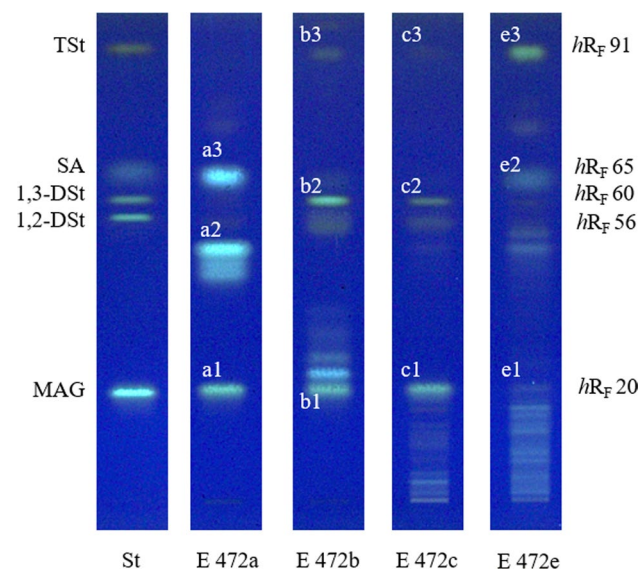


Fig. 1 Separation of E 472 emulsifier subgroups on HPTLC silica gel under UV 366 nm illumination after two-fold development with chloroform–methanol–water–formic acid (67:6:1.2:0.2, V/V) to a migration distance of 50 mm and *n*-heptane–diethyl ether–formic acid (55:45:1, V/V) to a migration distance of 80 mm at a relative humidity (RH) of 33% (set with the ADC 2), and after derivatization with primuline. The standard mix (St) for comparison consists of 1- and 2-monostearate (MSt), 1,2- and 1,2-distearate (DSt), stearic acid (SA), and tristearate (TSt). Zones selected for the evaluation of the influence of the RH on the separation of E 472 emulsifiers are marked with a1 to e3 (a for E 472a, b for E 472b, c for E 472c, and e for E 472e)

The available laboratory for the experiments was not equipped with a humidity control unit or a dehumidifier guaranteeing a constant RH. Hence, during the experiments, RH in the laboratory normally deviated between 30% and 60% and, on some days, even reaching up to 80%. If intralaboratory RH was above 60%, low RH (<30%) could not be achieved with 60% and 70% sulfuric acid solution or molecular sieve within 15 min. This was demonstrated with an experiment with 60% sulfuric acid solution at intralaboratory RH of 68%. RH was measured inside an enclosed twin-trough chamber. One side was filled with 60% sulfuric acid solution, and after 1 h of preconditioning, 34% RH was reached. Compared with the theoretical RH defined as 15% [16], this is a deviation of >100%. Consequently, analyses involving low RH were performed on days when RH was <60% in the laboratory, when theoretical RH values were nearly reachable within 15 min (chamber saturation) or 5 min (automatic humidity control in the ADC 2), respectively (Table 1). To ensure comparable conditions, all salt and sulfuric acid solutions were conditioned for the same duration (15 min) because, for technical reasons, the mobile phase must be added simultaneously with the saturated aqueous salt and sulfuric acid solutions (in the ADC 2).

Table 1 Saturated aqueous salt and sulfuric acid solutions of different concentrations with their theoretical [16, 17] and actual RH measured manually by a hygrometer in a twin-trough chamber after 15 min of saturation (manual procedure of setting the RH) and in the ADC 2 after 5 min of automatic humidity control at a temperature of 25–30 °C and a RH of 30–60% in the laboratory

Substance	Theoretical RH (%) ^c	Actual RH (chamber) (%)	Actual RH (ADC 2) (%)
KCl ^a	84	73	78
NaCl ^a	75	69	70
NH ₄ NO ₃	–	62	63
K ₂ CO ₃ ^a	43	59	48
MgCl ₂ ^a	33	36	34
CH ₃ COOK ^a	23	33	31
Molecular sieve ^b	–	12	9
10% H ₂ SO ₄ ^c	95	82	–
20% H ₂ SO ₄ ^c	88	77	–
30% H ₂ SO ₄ ^d	75	72	–
40% H ₂ SO ₄ ^d	55	55	–
50% H ₂ SO ₄ ^d	35	40	–
60% H ₂ SO ₄ ^d	15	33	–
70% H ₂ SO ₄ ^d	5	10	–

^aAccording to *Greenspan* (1977)

^bHeated at 100 °C for 72 h

^cExtrapolated after *Solomon* (1951)

^dAccording to *Solomon* (1951)

^eAt 25 °C

3.2 Effect of chamber saturation and plate preconditioning with mobile phase on the separation of E 472 emulsifiers

Due to technical reasons (by application of the ADC 2), experiments with the saturated twin-trough chamber involved simultaneous saturation with salt or sulfuric acid solution and the mobile phase. In the original method by *Schuster et al.* [7], saturation or plate conditioning with the mobile phase was omitted. So, the effect of chamber saturation and plate conditioning with the mobile phase was also evaluated. For all E 472 subgroups, no effect on separation was detected, neither for plate conditioning nor for chamber saturation with the mobile phase (data not shown).

3.3 Effect of RH on the chromatographic behavior (hR_F)

For all four emulsifier types, equal trends regarding variation in hR_F values for the selected signals were observed. Exemplarily, data for the E 472b emulsifier is presented in the discussion, while data for E 472a, E 472c, and E 472e are provided in the Supplementary Information. Using aqueous sulfuric acid solutions in different concentrations for the RH setting, a decrease of hR_F values at decreasing RH could be observed for the RH setting technique (B) chamber saturation for 15 min and (C) plate conditioning for 15 min (Fig. 2B and C, blue trend line). Interestingly, the phenomenon was observed only for zones with higher hR_F values (zones a2, a3, b2, b3, c2, c3, and e2 and e3). This result aligns with the literature [13, 15], associating higher hR_F values at increasing RH with stationary phase deactivation due to increasing adsorbed water content. Strikingly, no trends in hR_F value changes were observed across the tested RH range when using saturated salt solutions for all setting techniques (Fig. 2A–C). To estimate and assess the variation of hR_F values, reproducibility data determined in the scope of method validation by *Schuster et al.* (2022) were involved and evaluated [7]. Exemplarily for signal b1, based on the reproducibility, hR_F values ranged at 26 ± 1 . Variations of the hR_F values in the present study were with a few exceptions in this range (hR_F 25–28). When the same technique was used for the adjustment as with sulfuric acid solution, and the same RH were achieved within the same time using saturated salt solutions, the results diverged, which could not be explained. One suggestion is that sulfuric acid vapors are partially responsible for these findings. To investigate this matter, 10 mL of 70% aqueous sulfuric acid were placed in one trough of a twin-trough chamber, and the change of pH was monitored through the color of a pH paper placed on the other trough every 5 min. After 15 min, pH was approximately at 3, and after a further 10 min at 1, confirming acidic vapors in the chamber that could lead to an acidification of

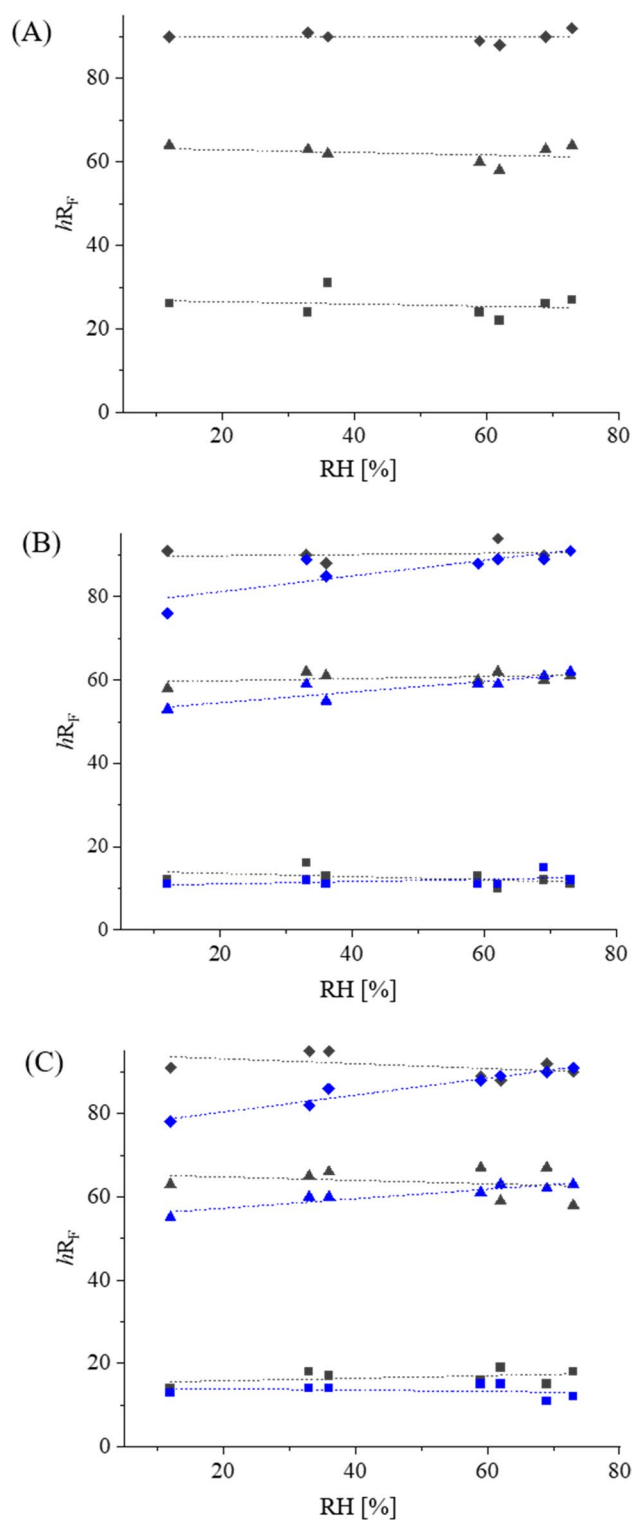


Fig. 2 hR_F values of selected signals (b1 (monoacylglycerides)–square, b2 (diacylglycerides and lactic acid esters of diacylglycerides)–triangle, b3 (triacylglycerides)–diamond) of an E 472b emulsifiers plotted against the relative humidity. Setting of the relative humidity (RH) (A) automatically by the humidity control function of the ADC 2, (B) manually by chamber saturation for 15 min, and (C) manually by plate preconditioning for 15 min by using saturated salt solution (black) and aqueous sulfuric acid solutions (blue)

the stationary phase. Another suggestion is that the highly hygroscopic sulfuric acid can lead to desorption of water from the stationary phase and, thus, influences plate activity. Both possible explanations are not confirmed yet and need further investigation. *Berezkin et al.* (2006) further described changes in the pH of the mobile phase during elution due to its contact with either an acidic (CO_2) or basic (NH_3) gas phase, influencing the acid–base speciation of the tested benzoic acid and aromatic amine mixtures [18].

For zones with low hR_F values (a1, b1, c1, a2, Fig. 2, and SI), differences between the different techniques of RH setting were seen. For example, for signal b1 of the E 472b emulsifier, mean hR_F values increased from 12 [(B) chamber saturation] to 17 [(C) plate preconditioning] to 26 [(A) ADC 2], showing a variance of > 100% between techniques (A) and (B). This indicates that the two developments (Sect. 2.6) might be affected to different extents, possibly due to varying polarity of the mobile phase. Considering these results, the developed method by *Schuster et al.* (2023) proves to be robust against changes in RH.

3.4 Effect of the technique for setting the RH on the chromatographic behavior

Unlike the RH itself, the RH setting technique resulted in a considerable change in the chromatographic fingerprint. This was exemplarily shown and discussed for the RH of 75% (Fig. 3) and additionally visualized for the RH of 33% in the SI. This was most evident in zones with low hR_F values, where the hR_F values exhibited variations for these zones between the different techniques of setting the RH. A more

detailed signal fine structure was detected for technique (A) ADC 2 in comparison with technique (B) chamber saturation and (C) plate conditioning. Examples of alterations in the chromatographic fingerprint are described in detail for the E 472a and E 472b emulsifiers, but changes were also observed for the E 472c and E 472e emulsifiers. Viewing the E 472b fingerprint for technique (C), an initial separation of zone b1, as indicated by the blurred zones above b1, was observed (Fig. 3, E 472b). Prolonging the conditioning time would probably lead to a more detailed signal fine structure. *Zeeuw* (1968) performed plate conditioning for 24 h [15], and *Suzuki and Matsushita* (1967) equilibrated until RH reached a steady state without mentioning a specific time [14].

For the E 472a emulsifier fingerprint, considerable variations were detected for signal a2 applying the different techniques (Fig. 3, E 472a). In contrast to the observations for the signal b2 of E 472b, the signal fine structure was more detailed for the techniques (B) and (C) than for (A). Consequently, the different techniques of setting the RH need to be evaluated for every analyte regarding the best result for a respective goal of the analysis.

Despite variations in the TLC fingerprint between the three techniques of setting the RH, the chromatographic fingerprint within the same technique did not vary over the range of tested RH. However, using the sulfuric acid with technique (C) plate preconditioning delivered changes, as depicted in Fig. 4 for the E 472b emulsifier. With decreasing RH (applying increasing sulfuric acid concentration), the signal fine structure became more precise and detailed, resembling the fingerprint obtained by setting of the RH

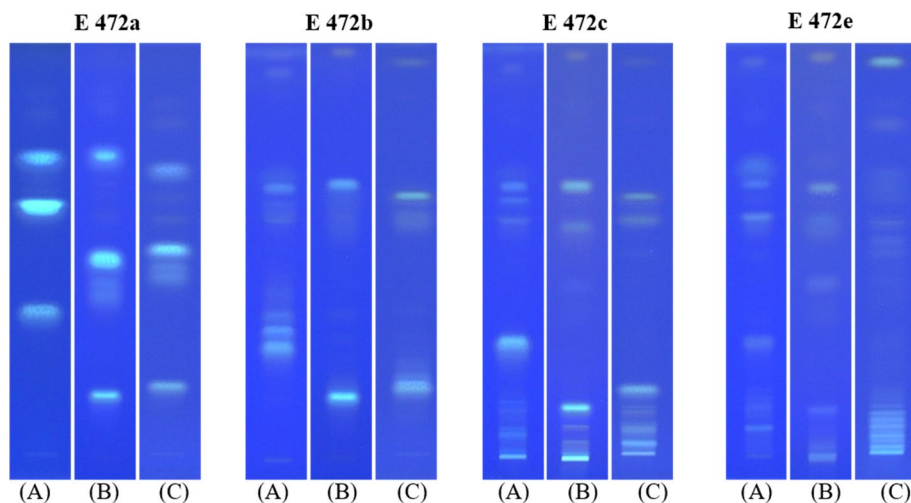


Fig. 3 Separation of E 472 emulsifier subgroups on HPTLC silica gel under UV 366 nm illumination after two-fold development with chloroform–methanol–water–formic acid (67:06:1.2:0.2, V/V) to a migration distance of 50 mm and *n*-heptane–diethyl ether–formic acid (55:45:1, V/V) to a migration distance of 80 mm at a relative humidity

(RH) of 75%, and after derivatization with primuline. Setting of the RH (A) automatically by the humidity control function of the ADC 2, (B) manually by chamber saturation for 15 min, and (C) manually by plate preconditioning for 15 min

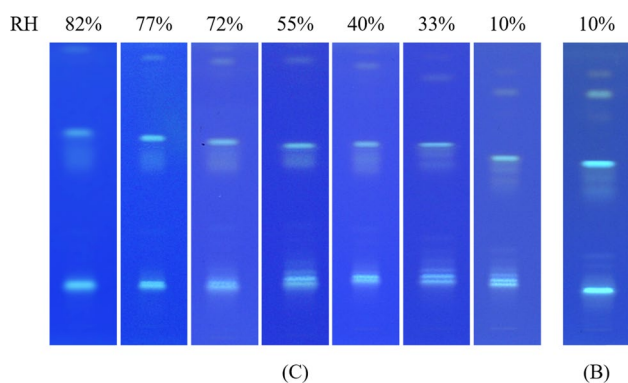


Fig. 4 Separation of an E 472b emulsifier on HPTLC silica gel under UV 366 nm illumination after two-fold development with chloroform–methanol–water–formic acid (67:06:1.2:0.2, *V/V*) to a migration distance of 50 mm and *n*-heptane–diethyl ether–formic acid (55:45:1, *V/V*) to a migration distance of 80 mm at relative humidities (RH) between 10 and 82% set with aqueous sulfuric acid solutions, and after derivatization with primuline. The RH (**B**) was set manually by chamber saturation for 15 min, and (**C**) manually by plate preconditioning for 15 min

with the ADC 2. Surprisingly, this effect was not obtained for technique (B), the chamber saturation. Respective zones were examined by mass spectrometry to investigate a possible cleavage of the ester bonds of the lactylated glycerides that could be related to sulfuric acid vapors in the chamber. No change in mass-to-charge (m/z) ratios was detected in comparison with RH setting with saturated salt solutions. Nevertheless, potential partial hydrolysis at least to a minor degree of the esters in the presence of the acidic vapors could not be ruled out. The results clearly show that the influence of RH varies depending on analytes and chromatographic systems and needs to be evaluated for every method.

4 Conclusion

The present study investigated the effect of RH on the HPTLC separation of four E 472 emulsifier subgroups (a, b, c, e). The study includes a crucial practical aspect because of the substantial variation of the RH between seasons, even during the day, and that most laboratories have no control over the intralaboratory RH. Therefore, three techniques for setting RH influencing plate activity and chamber climate were evaluated. To adjust RH, saturated salt and aqueous sulfuric acid solutions were used. Applying aqueous sulfuric acid solutions for RH adjustment resulted in decreased hR_F values at decreasing RH. Interestingly, this was observed only for zones showing higher hR_F values. It was demonstrated that, after the chamber saturation time of 15 min, the pH in the whole chamber was 1, indicating the presence of acidic vapors. Conversely, no trends in hR_F value

changes were observed using saturated salt solutions for RH adjustment.

Unlike RH itself, the RH setting technique led to substantial changes in the chromatographic fingerprint. Thus, the choice of the RH setting method should be tailored to the specific analyte and analysis goal. Despite variations in chromatographic fingerprints between the three RH setting techniques, the fingerprint remained consistent within the same technique across the tested RH range. However, using sulfuric acid solutions showed different results: with increasing sulfuric acid concentration and, consequently, decreasing RH, the signal fine structure became more detailed, resembling the signal structure obtained with the automatic setting of RH with the ADC 2. Considering these results, the developed method by *Schuster et al.* (2023) proves to be robust against changes in RH.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00764-023-00260-1>.

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Declarations

Conflict of interest The 2nd author, Claudia Oellig is a member of the Editorial Board of the journal. Therefore, the submission was handled by a different member of the editorial board, and s/he did not take part in the review process in any capacity.

Research involving human or animal participants This article does not contain any studies with human or animal subjects.

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