Journal of Chromatography A 1718 (2024) 464710

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# Application of chiral stationary phases for the separation of vitamin A acetate isomers

Nicole Schräder [a], Wan Xia Zhu [c], Alexander Jaekel [b], Mo Legelli [a,b], Daniel Meyer [b], Kevin Streckel [a], Michaela Wirtz [a], Stefan Lamotte [b,*]

[a] Department of Natural Sciences, University of Applied Sciences Bonn-Rhein-Sieg, Rheinbach, Germany
[b] Department of Analytical and Material Science, BASF SE, Carl-Bosch-Straße 38, Ludwigshafen am Rhein 67056, Germany
[c] Department of Analytical and Material Science, BASF Advanced Chemicals Co. Ltd., Shanghai, China

ARTICLE INFO

*Keywords:*
Chiral stationary phases
High performance liquid chromatography
Vitamin A acetate isomers
Cellulose stationary phases
Amylose stationary phases

ABSTRACT

The separation of vitamin A acetate isomers is essential for quality assurance of e.g. nutrition supplements, cosmetics, and pharmaceutical ingredients. High performance liquid chromatography (HPLC) is currently the most suitable analytical method for tackling this challenging separation task. However, the existing methods based on normal phase chromatography (NPC) are poorly reproducible due to the typical disadvantages of NPC, such as long equilibration times and fluctuation in retention factors. A new reversed phase method developed in our labs allows the separation of the isomers applying a chiral stationary phase (CSP). This phase consists of an immobilized polysaccharide which can be used in every chromatographic mode. However, they are not typically used in reversed phase mode. Through the screening of various stationary phases with different polysaccharide based chiral selectors, the choice of the ideal stationary phase could be confirmed, allowing to draw conclusions about the retention mechanism. The CSP Chiralpak IG-3 was found to be the most suitable among the examined. Regarding the separation mechanism, the spatial helical structure of the polysaccharide derivatives was confirmed to be of particular significance. In addition to the stationary phase, the mobile phase was tested for optimization regarding composition, gradient parameters as well as temperature using chromatographic method optimization software for the sake of method robustness.

## 1. Introduction

Vitamin A, or retinol, is a compound essential for vital body functions and is used as food additive, in animal feed production, cosmetics, and the pharmaceutical industry. Commercially relevant are the retinol esters, especially vitamin A acetate, due to their increased stability [1]. The global market in 2021 was approx. 600 million euros and is expected to increase to approx. 1 billion euros by 2030 [2].

Vitamin A acetate has several stereoisomeric forms depicted in Fig. 1. The four *cis* isomers and the two *dicis* isomers all exhibit a lower bioactivity than the predominant form, the all-*trans* isomer [3]. Additionally, two vitamin A acetate molecules can undergo a cycloaddition, forming a six-membered ring by Diels-Alder reaction. The resulting molecules are called kitols and are depicted in Fig. 1 as well. The cycloaddition can also occur intramolecularly forming a four-membered ring, in the following designated as “ring closure” isomer.

Since the individual isomers differ in their bioactivity, the accurate and precise analysis of the isomer composition is necessary to assure the quality of the product. For this, high performance liquid chromatography (HPLC) is currently the most frequently applied analytical method. As retinoids show intense absorption in the ultraviolet (UV) range, HPLC is preferably combined with a UV detector [4].

While the separation of the various retinoids such as retinol, retinal or retinoic acid (Fig. 1) from each other is also possible with reversed phase chromatography (RPC), the separation of the individual isomers has so far exclusively been achieved by normal phase chromatography (NPC) [4]. For the separation of the *cis-trans*-isomers of vitamin A acetate, silica gel is the primarily used stationary phase [4]. However, application of NPC comes with the issue of poor reproducibility in retention times due to its dependence on humidity. The water content of the mobile phase heavily affects the activity of the silica gel, thus influencing the retention mechanism and behavior.

Therefore, application of the more robust RPC would be advantageous. During our method development, various classical reversed phase

* Corresponding author.
*E-mail address:* stefan.lamotte@basf.com (S. Lamotte).

https://doi.org/10.1016/j.chroma.2024.464710
Received 30 October 2023; Received in revised form 30 January 2024; Accepted 1 February 2024
Available online 2 February 2024
0021-9673/© 2024 Published by Elsevier B.V.

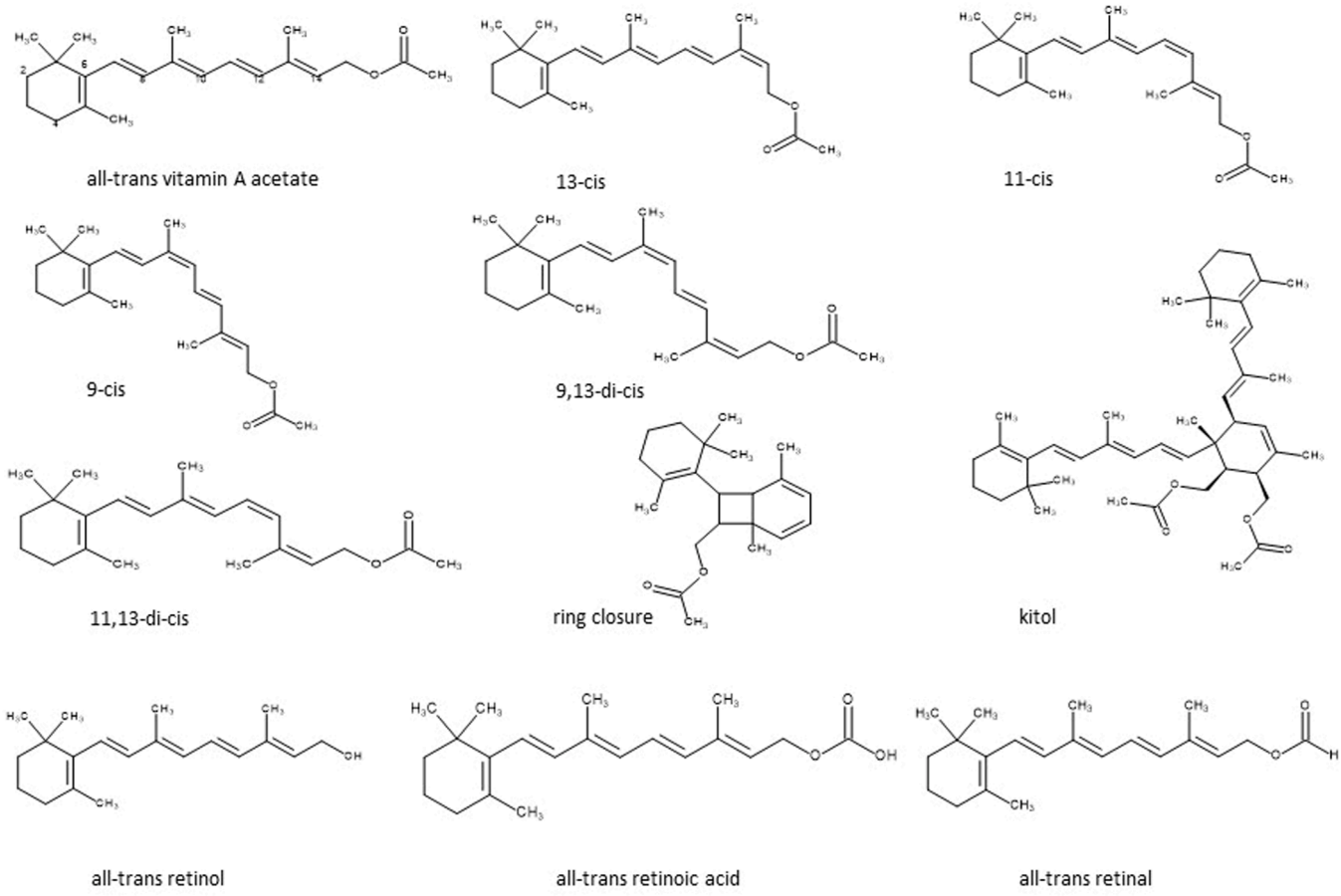


**Fig. 1.** Structural formulas of retinoids including retinol, retinal, retinoic acid and stereoisomers of vitamin A acetate as well as Diels-Alder-products of Vitamin A acetate.

(RP) stationary phases were screened. None of those, even with high carbon load or pronounced shape recognition, were able to provide sufficient selectivity. Promising results were achieved with porous graphite-based stationary phases. However, as carbon by nature has a high adsorptive surface, these columns show large variations in lot-to-lot reproducibility and are consequently not suited for such frequent analyses.

Eventually, a method was developed using a chiral stationary phase (CSP) where the separation mechanism is based on the helical structure of polysaccharides. The column used contains a chiral sorbent which consists of a silica gel base on which a layer of amylose tris(3-chloro-5-methylphenylcarbamate) is immobilized. CSPs are normally chosen for separation of enantiomeric pairs. Among the various existing CSPs, the polysaccharide-based stationary phases are the most common ones due to their excellent separation performances in a wide range of chiral separations [5,6]. With this CSP, it is also possible to separate non-chiral constitutional isomers of vitamin A acetate in RPC which makes this method much more reproducible and robust compared to NPC.

The idea of separating non-enantiomeric positional isomers with the help of chiral stationary phases is not new [7] and has successfully been achieved in a reversed phase mode before [8,9]. However, to our knowledge it has never been applied to the separation of isomers of vitamin A and its derivatives. With the considerable production output and the importance of the everyday substance, the development of a reliable and robust method as it is presented here is highly relevant for continuous quality assurance [2]. Although the separation has been able to prove itself in a routine setting, it is not known whether this particular CSP is the most appropriate for the application, as the separation mechanism has not yet been investigated for these compounds.

This study serves to create a deeper understanding of the underlying retention mechanism by screening different chiral selectors and confirming that selectivity-wise, there is no better suited polysaccharide-based column to separate vitamin A acetate isomers. Furthermore, the separation is optimized concerning mobile phase composition, gradient steepness, and temperature with the help of an optimization software with additional benefit of visualization of the method's robustness.

## 2. Materials and methods

### 2.1. Materials

The chromatographic analyses were performed on a Vanquish Horizon UHPLC system from Thermo Fisher Scientific (Germering, Germany). The system consists of the Vanquish Binary Pump H with built-in degasser, the Autosampler Vanquish Split Sampler HT, two column ovens both equipped with Vanquish Column Thermostat H (one with 6-position/7-port column switching valve), and the Vanquish diode array detector (DAD) HL (190–680 nm).

The columns for screening are the originally used amylose-based CSP Chiralpak IG-3 from Daicel (Osaka, Japan), the amylose-based Chiralpak IA-3, ID-3, IE-3, IF-3 and IH-3, as well as the cellulose-based IBN-3, IC-3, and IJ-3 for comparison. All columns have the same dimensions of 150 × 3.0 mm and a particle size of 3 µm. The differences in chiral selectors of the glucose units are shown in Fig. 2.

The Chromeleon 7.2 SR5 software from Thermo Fisher Scientific, OriginPro 2023 (Learning Edition) and Microsoft Excel were used to evaluate the chromatograms. Mobile phase and temperature optimization of the methods was performed by the method optimization software

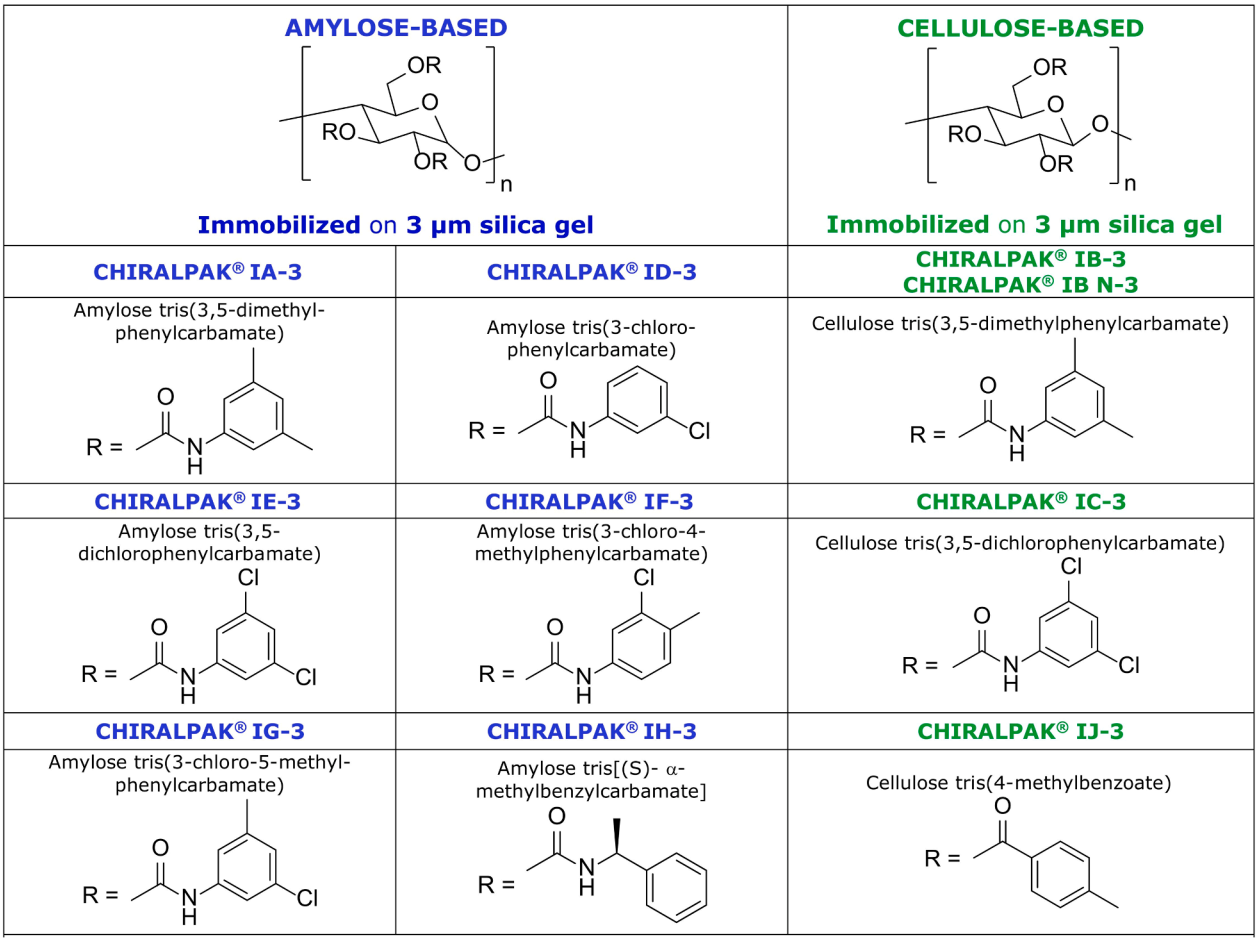


**Fig. 2.** Structure of the used chiral columns (with permission from Daicel Chiral Technologies).

**Table 1**
Gradient of the original method and of the optimized method by Dry Lab model. Isocratic step added to compensate for the dwell volume.

| original method IG-3 ($T$ = 35 °C) | | | optimized method for IG-3 ($T$ = 40 °C) | | |
|---|---|---|---|---|---|
| time (min) | A% | B% | time (min) | A% | B% |
| 0 | 45 | 55 | 0 | 45 | 55 |
| 23 | 30 | 70 | 30 | 25 | 75 |
| 35 | 20 | 80 | 31 | 45 | 55 |
| 50 | 0 | 100 | 41 | next injection | |
| 51 | 55 | 55 | | | |
| 60 | next injection | | | | |

| optimized method for IA-3 ($T$ = 28 °C) | | | optimized method for IF-3 ($T$ = 60 °C) | | |
|---|---|---|---|---|---|
| time (min) | A% | B% | time (min) | A% | B% |
| 0 | 37 | 63 | 0 | 45 | 55 |
| 8 | 33 | 67 | 12 | 37 | 63 |
| 12 | 28 | 72 | 15 | 31 | 69 |
| 23 | 25 | 75 | 30 | 25 | |
| 24 | 37 | 63 | 31 | 45 | 55 |
| 34 | next injection | | 41 | next injection | |

DryLab by the Molnár Institute (Berlin, Germany).

The eluents were HPLC-grade deionized water from a Millipore Milli-Q Integral 3 water purification system (Merck, Darmstadt, Germany) and acetonitrile (ACN) ≥99.9%, CHROMASOLV LC-MS from Honeywell (Morristown, NJ, USA). Formic acid 98–100% from Bernd Kraft (Duisburg, Germany) was used to acidify the ACN. The solvent for the vitamin A acetate sample was propan-2-ol for spectroscopy Uvasol, ≥99.0% from Merck (Darmstadt, Germany). To prevent oxidation of the sample, 2,6-di-tert-butyl-methylphenol (BHT), ≥99.0% (GC) from Sigma Aldrich (St. Louis, MO, USA) was added. The vitamin A acetate samples (2.8 million IU $g^{-1}$) were made available by the production site at BASF SE, Ludwigshafen am Rhein, Germany.

### 2.2. Methods

For sample preparation, approx. 120–150 mg of vitamin A acetate were weighed into an amber 100 mL volumetric flask. Approx. 200 mg $L^{-1}$ BHT was previously added to the propan-2-ol to generate a sample solvent. The weighed sample was dissolved in approx. 50 mL of the solvent, placed in an ultrasonic bath for approx. two minutes and the volumetric flask was filled up to the calibration mark with the solvent.

For HPLC, a column temperature of 35 °C was set, the injection volume was 5 µL, and the flow rate 1 mL $min^{-1}$. The gradient conditions are described in Table 1, where eluent A corresponds to water and eluent B to acetonitrile with 0.1 vol% formic acid. The chromatogram was recorded with the DAD at a wavelength of 295 nm. The parameters described above applied to all columns to be compared.

The method was optimized for three selected stationary phases (IG-3, IA-3, and IF-3) using method optimization software to generate a model for separation on the columns with respect to changes in temperature, eluent composition and run time. Four initial basic experiments were required for each column. This model optimizes these parameters for robustness. In the experiments, two different gradients (50% to 100% ACN in 15 min and 45 min respectively) were measured at two different temperatures (30 and 60 °C). The remaining parameters and sample preparation were kept identical.

**Table 2**
Absorption maxima of the mono- and dimeric isomers in elution order [6].

| Retinoid | Solvent | λ max |
|---|---|---|
| 11,13-*dicis* retinol | Ethanol | 311 |
| 11-*cis* retinol | Ethanol | 319 |
| 9,13-*dicis* retinol | Ethanol | 324 |
| 9-*cis* retinol | Ethanol | 323 |
| 13-*cis* retinol | Ethanol | 328 |
| All-*trans* retinol | Ethanol | 325 |
| All-trans retinyl acetate | Ethanol | 325 |

## 3. Results and discussion

### 3.1. Peak identification

As the UV absorption spectra of predominant isomers are known and specific, these signals can be assigned accordingly. The absorption maxima of the mono- and dimeric isomers are listed in elution order in Table 2 [6]. However, assignment of the kitols is often difficult due to their chirality. The presence of additional chiral centers in the molecule leads not only to diastereomeric isomer separation but also to enantiomeric isomer separation. For this application the enantiomeric separation of the kitols is counterproductive as it results in broad and asymmetric signals and decrease of the limit of quantification.

Fig. 3 shows the assignment of the peaks and the structural formula of the *cis* isomers for the IG-3 CSP [10]. For every separation, the individual elution order indicates the basic retention mechanism. In this case, with increasing retention the planarity of the analytes' molecular structure increases. Therefore, the shape recognition of the stationary phase is expected to be the predominant retention mechanism. This has been proven by Van 't Hoff experiments as the adsorption entropy decreases with increasing retention. The results can be found in the supplementary materials.

Still, there are other underlying interactions responsible for the retention. Most importantly, these are polar interactions between the analytes and the carbamate group in the form of hydrogen bonds, as well as π-π interactions with the phenyl groups of the CSP. The π-π interactions occur between the phenyl ring and the conjugated double bonds of the vitamin A acetate species. Isomers with sterically exposed π-electrons are expected to show increased interactions, thus leading to increased retention. This is especially the case for the 9-*cis* isomer which is, however, not the most retained isomer. Thus, another interaction seems to be more influential. Due to the helical structure of the polysaccharide derivatives, the CSP displays hydrophobic cavities into which the isomers can intercalate differently based on their shape determined by their configurations. In previous studies the influence of the helical structure of the polymer has also been considered the most influential factor for selectivity regarding enantiomers, or in this case diastereomers [5,11]. This observation also corresponds to the assumed shape recognition by increasing linearity of the isomers. The above-mentioned Van 't Hoff experiments indicate that shape selectivity is the predominant retention mechanism as selectivity decreases with increasing temperature. The role of the different substituents on the phenyl ring of the CSP and further clues to the separation mechanism become apparent when comparing the different columns.

### 3.2. Comparison of the CSPs

For almost all compared CSPs the elution order remained unchanged. However, for CSP IJ-3 (cellulose tris(4-methylbenzoate)) the elution order of 9-*cis* and 11-*cis* were inverted. This is not surprising as both CSPs differ in their helical structures (cellulose vs amylose) as well as their ligands (benzoate ester vs carbamate), so their retention mechanisms are not comparable. Van 't Hoff experiments performed using the IJ-3 CSP further support this, as adsorption enthalpies are nearly identical for all visible isomers, unlike for the IG-3, where their differences in adsorption behavior are reflected by their respective adsorption enthalpies. The results can be found in the supplementary material.

Despite this, there are significant differences between the fictional retention factors (k*-values) and more strikingly the selectivity and resolution of all investigated CSPs. The relative retention times and the resolutions in relation to the all-*trans* isomer signal are summarized in Table 3. However, in the case of co-elution, the accurate determination of the resolution is not possible. All chromatograms are shown in Figs. 4 and 5.

Only the IG-3 CSP separates all *cis* isomers with sufficient selectivity ($\alpha > 1$) and resolution ($R_S > 1.5$). Acceptable separations were also achieved using the CSPs IA-3 (amylose tris(3,5-dimethylphenylcarbamate)) and IF-3 (amylose tris(3-chloro-4-methylphenylcarbamate)). However, co-elution of the 9,13-*dicis* isomer and another *cis* isomer was observed on both IA-3 and IF-3. Compared to cellulose based supports, the amylose phenylcarbamates generally show improved separation and thus seem to have a helical structure more suitable for separation by shape selective recognition of analytes, as was also discussed by Okamoto [11]. This becomes particularly evident in the direct comparison of CSPs with the same pendant group as shown in

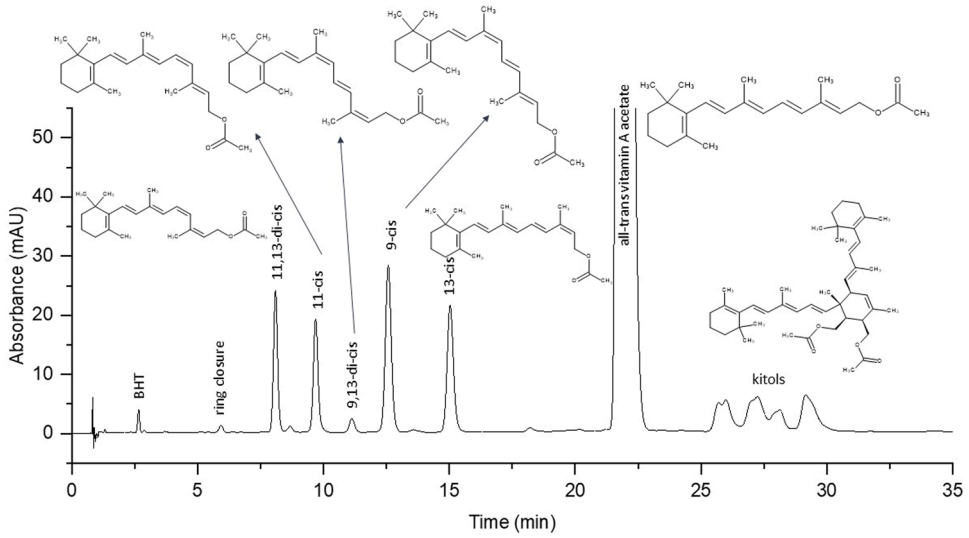


**Fig. 3.** Assignment of the isomers in the chromatogram of Chiralpak IG-3.

**Table 3**
The relative retention time (RRT) and the resolution both regarding the all-*trans* peak.

| Amylose-based: | | | | | | |
|---|---|---|---|---|---|---|
| | Chiralpak IG-3 | | Chiralpak IA-3 | | Chiralpak ID-3 | |
| Analytes | RRT | Resolution $R_s$ | RRT | Resolution $R_s$ | RRT | Resolution $R_s$ |
| BHT | 0.12 | 46.7 | 0.25 | 32.4 | 0.25 | 26.2 |
| ring closure | 0.28 | 31.4 | 0.54 | 17.3 | 0.58 | 12.1 |
| 11,13-*dicis* | 0.38 | 27.7 | 0.67 | 11.9 | 0.71 | 7.9 |
| 11-*cis* | 0.45 | 23.3 | 0.72 | 9.5 | 0.75 | n.a. |
| 9,13-*dicis* | 0.51 | 20 | n.a. | n.a. | n.a. | n.a. |
| 9-*cis* | 0.58 | 16.3 | 0.76 | 8.5 | 0.77 | n.a. |
| 13-*cis* | 0.69 | 11.7 | 0.83 | 5.6 | 0.84 | 4.3 |
| all-*trans* | 1 | 0 | 1 | 0 | 1 | 0 |
| kitol | 1.21 | n.a. | 2.07 | 27.8 | 2.78 | 27.9 |
| kitol | 1.22 | n.a. | 2.11 | 27.4 | 2.85 | 36.5 |
| kitol | 1.28 | 6.6 | 2.2 | 23.3 | 2.93 | n.a. |
| kitol | 1.32 | 8.9 | 2.26 | n.a. | 3.01 | n.a. |
| kitol | 1.38 | 10.3 | 2.29 | n.a. | 3.05 | n.a. |
| kitol | | | 2.33 | n.a. | 3.11 | n.a. |
| kitol | | | | | | |
| | Chiralpak IE-3 | | Chiralpak IF-3 | | Chiralpak IH-3 | |
| Analytes | RRT | Resolution $R_s$ | RRT | Resolution $R_s$ | RRT | Resolution $R_s$ |
| BHT | 0.21 | 36.9 | 0.21 | 31.7 | 0.37 | 25.4 |
| ring closure | 0.64 | 12.9 | 0.51 | 15.5 | 0.66 | 11.2 |
| 11,13-*dicis* | 0.77 | 8 | 0.64 | 12.1 | 0.84 | n.a. |
| 11-*cis* | 0.82 | 6.2 | 0.68 | 10.7 | 0.86 | n.a. |
| 9,13-*dicis* | 0.84 | n.a. | n.a. | n.a. | n.a. | n.a. |
| 9-*cis* | 0.89 | 3.6 | 0.71 | 9.5 | 0.9 | 3 |
| 13-*cis* | 0.89 | 3.6 | 0.81 | 6 | 0.94 | 1.9 |
| all-*trans* | 1 | 0 | 1 | 0 | 1 | 0 |
| kitol | 2.51 | 34.1 | 2.17 | 29.9 | 2.36 | 25.9 |
| kitol | 2.64 | 35.5 | 2.2 | 30 | 2.5 | 35.3 |
| kitol | 2.7 | 34.1 | 2.25 | n.a. | 2.56 | 36 |
| kitol | 2.77 | n.a. | 2.29 | 32.6 | 2.61 | n.a. |
| kitol | 2.81 | 41.5 | 2.34 | 34 | 2.65 | 34.5 |
| kitol | | | 2.38 | n.a. | 2.77 | 37.9 |
| kitol | | | 2.41 | n.a. | 2.82 | n.a. |
| | | | 2.44 | n.a. | | |
| Cellulose-based: | | | | | | |
| | Chiralpak IBN-3 | | Chiralpak IC-3 | | Chiralpak IJ-3 | |
| Analytes | RRT | Resolution $R_s$ | RRT | Resolution $R_s$ | RRT | Resolution $R_s$ |
| BHT | 0.33 | 30.8 | 0.23 | 32.5 | 0.33 | 27 |
| ring closure | 0.66 | 12.9 | 0.65 | 9.1 | 0.48 | n.a. |
| 11,13-*dicis* | 0.85 | 5.3 | 0.84 | 4.9 | 0.69 | 9.9 |
| 11-*cis* | 0.9 | n.a. | 0.89 | 3.3 | 0.73 | n.a. |
| 9-*cis* | 0.92 | n.a. | 0.95 | n.a. | 0.74 | n.a. |
| 13-*cis* | 0.94 | n.a. | n.a. | n.a. | 0.94 | 1.8 |
| all-*trans* | 1 | 0 | 1 | 0 | 1 | 0 |
| kitol | 2.48 | n.a. | 2.75 | 28.1 | 2 | 22.4 |
| kitol | 2.5 | n.a. | 2.83 | n.a. | 2.04 | n.a. |
| kitol | 2.55 | n.a. | 2.89 | 35.8 | 2.13 | 17.4 |
| kitol | 2.58 | 39.1 | 2.94 | n.a. | 2.22 | 21.8 |
| kitol | 2.66 | n.a. | 3 | 36.3 | | |
| kitol | 2.69 | 39.2 | 3.12 | 42.1 | | |

Fig. 6.

Understanding the differences in selectivity requires consideration of the spatial structures of the amylose and cellulose derivatives. Various studies on CSPs have dealt with their structure, particularly, the amylose and cellulose trisphenylcarbamates (ATPC and CTPC) due to their popularity. Each CSP helix consists of a glucose backbone, while the substituents on the glucose units point outwards. The carbamate groups are on the inside, while the phenyl rings face to the outside. ATPC and CTPC differ in their degree of rotation of the helix. According to [11], ATPC form a left-handed 4/3 helix and CTPC form a left-handed 3/2 helix as confirmed by X-ray structural analysis. ATPC and CTPC retain their helical structure and form a lyotropic liquid crystalline phase of high order in solution in the mentioned study. Thus, the shape selectivity is fostered by both the helical structure as well as the highly ordered layer on the silica gel surface [5]. The ATPC show comparably a higher selectivity due to the favorable helix rotation and therefore preferably defined cavities and an even more highly ordered structure, which allows improved shape selective recognition. This preference for amylose-based phases due to their superior steric interactions with positional isomers of longer carbon chains has also been observed in the separation of fatty acids [9].

Changes in the type and the position of the pendant group considerably affect selectivity and k*-values. A methyl group has *a*+*I* effect pushing charge density into the carbamate group via the phenyl ring. This increases the electron density at the carbonyl group while decreasing the polarity of the amine group. In contrast to the methyl group, halogens have a -I effect resulting in opposite polarity changes [5]. In the case of vitamin A acetate derivatives investigated in this study, the above mentioned +*I* effect enhances the formation of hydrogen bonds which increases retention. As shown in the chromatograms of ID-3 and IE-3 (Fig. 4), chlorine as a sole substituent does not provide sufficient retention and selectivity, as the secondary structure of the amylose helix is the decisive factor. Our investigations demonstrate that the combination of -I and +*I* effect forces the IG-3 to higher order, resulting in improved shape selectivity.

Apart from interaction with analytes, the phenylcarbamate groups also interact with each other intramolecularly, further supporting the structure of the helix [12,13]. The different substituents influence the number of hydrogen bonds. Methyl groups increase NH acidity and are thus beneficial for the number of intramolecular bonds which can be confirmed by $^1$H nuclear magnetic resonance (NMR) spectroscopy. Previous studies investigated the influence of different substituents using infrared (IR) spectroscopy to semi-quantitatively determine intramolecular bonds [13,14]. Circular dichroism (CD) spectroscopy also confirms that IG-3 has the most ordered secondary structure of the amylose columns [14] which can explain the high molecular planarity recognition of the CSP for the present application.

### 3.3. Influences on the separation mechanism

Previous studies, although generally focused on enantiomeric separations, have shown that π–π-stacking as well as hydrophobic interactions are the predominant separation mechanisms for polysaccharide-based CSPs. The selectivity of these phases however can be traced back to the shape and interaction with the helix [5,8,15,16]. This has been proven to be true in the case of vitamin A acetate isomers with this study.

Relevant retention mechanisms for separation of vitamin A acetate isomers are polar interactions with the carbamate group like hydrogen bonds, dipole-dipole interactions as well as hydrophobic and π-π interactions. Furthermore, the substituents on the phenyl ring affect retention, as the resulting intramolecular bonds are directly related to the number of available adsorption sites at the amine group. Methyl groups cause a higher electron density at the carbonyl group and a resulting increase in intramolecular bonds stabilize the superstructure, while opposingly reducing the number of adsorption sites. Chlorine has the opposite effect [5,14,17].

Regarding the here investigated vitamin A acetate isomers, we conclude that the dominating effects are the π-π interactions between the phenyl ring and the conjugated double bonds or -CH of the vitamin A acetate and the order of the CSP. The interaction strength depends on how exposed the π-π electrons are, with interactions being particularly strong for the 9-*cis* isomer. The methyl groups also strengthen these interactions through their +*I* effect, whereas chlorine with its -I effect weakens them. The combination of -I and +*I* effect fosters the shape

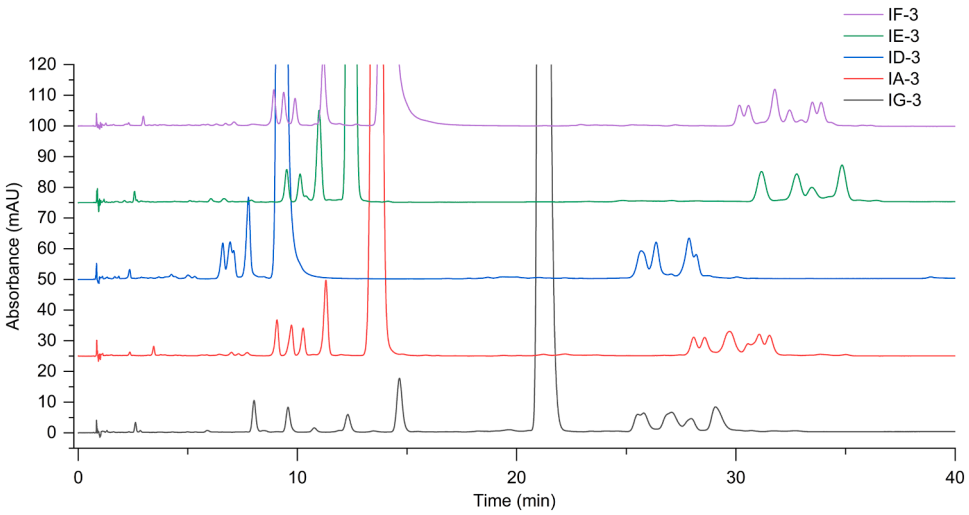


**Fig. 4.** Chromatograms of Chiralpak IG-3, IA-3, ID-3, IE-3 and IF-3 (amylose-based).

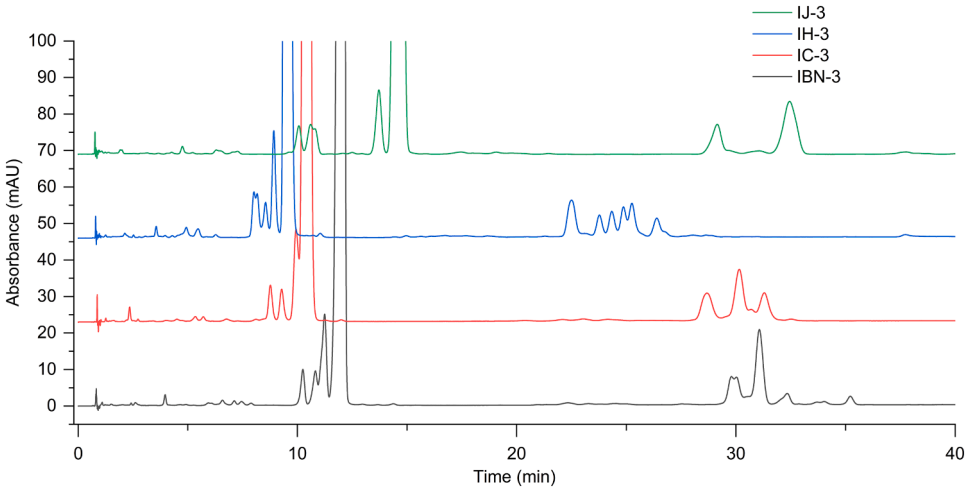


**Fig. 5.** Chromatograms of Chiralpak IBN-3, IC-3, IJ-3 (cellulose-based) and IH-3 (amylose-based).

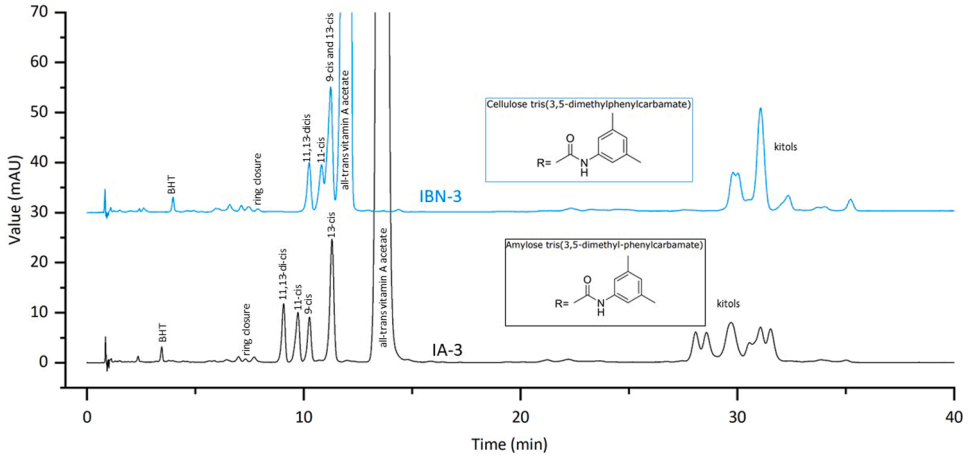


**Fig. 6.** Comparison of IA-3 (amylose-based) and IBN-3 (cellulose-based). Both with the same pendant group tris(3,5-dimethylphenylcarbamate).

selectivity necessary for vitamin A acetate isomer separation.

However, hydrophobic interactions also affect the separation, especially regarding the kitols. The methyl groups on the phenyl ring increase the possibility of hydrophobic interactions. Kitols are more retained since they are dimers and as such significantly more hydrophobic compared to monomers. This is especially true for the comparison of IG-3 and IA-3, indicating that the methyl group is less pronounced in the IG-3 CSP.

To sum up, the most relevant factor for the selectivity of the isomer separation is the secondary structure of the amylose or cellulose derivatives. Thus, the different helical structures of CTPC and ATPC have the most significant effect on the separation, which is due to the intercalation mechanism of the isomers in the cavities formed by the helix. The isomers are able to intercalate in different ways and interact with the various pendant groups located inside the cavity structure. This creates the basis of molecular shape recognition for the isomer separation.

The selectivity seems to be significantly influenced not only by the type but also by the position of the substituents on the phenyl ring. Thus, a chlorine and a methyl substituent in meta-position to each other resulted in improved selectivity compared to the same substituents in ortho-position. This could be caused by different dipole moments and/or by attenuation of the methyl group as well as the π-electron system by the chlorine.

Finally, the differences in size of the substituents play a role. Since the chlorine substituent occupies a considerable amount of space, it is responsible for the shape recognition by the helical structure as it hinders the intercalation of molecules and influences the secondary structure of the amylose or cellulose.

### 3.4. Software aided optimization

The results of four initial runs of two different gradients at two different temperatures each were the basis for a software aided optimization. The resolution of the critical peak pair is a function of temperature and analysis time. The results are illustrated in so-called resolution maps which help to find the best suited gradients for a specific separation requirement. The new method parameters for the three most promising CSPs are described in Table 1 [18]. The obtained methods were compared to the original method.

For the proposed method for the IG-3, the retention times are almost identical to the original method, indicating that the original method had already been optimized. The resolution of the *cis* isomers is according to prediction, but the calculated resolution of the kitols could not be achieved. This is due to the previously mentioned enantiomer separation of the kitols on CSPs, which the software is unable to reflect. Therefore, for the kitols the prediction is not matching perfectly with the experiment. However, this is not important in practice as the kitols are quantified as a group of signals, where no separation is required.

The method for the IA-3 CSP and IF-3 CSP differ not only in analysis time but also temperature (see Table 1). However, selectivity of the IG-3 CSP remains superior.

## 4. Conclusion

Column screening revealed the IG-3 CSP as the only investigated CSP to separate all isomers of vitamin A acetate with sufficient selectivity. The manually developed ideal method was confirmed as such by method development software.

By comparing the chromatograms of the different CSPs and the findings of previous studies on the structure of chiral polysaccharides, conclusions were drawn about the underlying separation mechanisms. Although the polar interaction with the carbamate group is the main reason for the retention of the analytes, the reason for the shape selectivity of the CSP is the helical structure of the polymer chains, which enables molecular shape recognition due to its high degree of order. The pendant groups on the amylose or cellulose units also have a significant impact on the selectivity of the corresponding CSP.

Finding the optimal column for a specific separation task requires a deeper understanding of the underlying retention mechanism. For the separation of vitamin A acetate related substances, we propose the following improvements:

(A) Replacement of the chlorine on the IG-3 CSP with another halogen to tune the size of the cavity. The effect of different halogens with respect to the separation of different enantiomers has already been investigated by Chankvetadze et al. [13].
(B) Modification of the IH-3 CSP with a phenyl substituent analogous to the IG-3.
(C) Adjustment of the mobile phase e.g. by the replacement of ACN with a protic solvent which is expected to have an effect on the elution order especially with respect to the kitols.

Based on these findings the authors would like to invite the chromatographic community to consider the use of CSPs not only for separation of chiral compounds such as longer-chained positional isomers (e.g. carotenoids, polyunsaturated fatty acids (PUFAs)).

## CRediT authorship contribution statement

**Nicole Schräder:** Data curation, Formal analysis, Investigation, Software, Validation, Visualization, Writing – original draft. **Wan Xia Zhu:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation. **Alexander Jaekel:** Writing – review & editing. **Mo Legelli:** Data curation, Formal analysis, Validation, Visualization, Writing – original draft. **Daniel Meyer:** Writing – review & editing. **Kevin Streckel:** Writing – review & editing. **Michaela Wirtz:** Project administration, Supervision, Writing – review & editing. **Stefan Lamotte:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

## Declaration of competing interest

This scientific work was sponsored by the scientific and technology achievement recognition (STAR) initiative of BASF SE. Some of the authors are employees of BASF SE. Besides this no other interests are declared. No AI was used in this scientific writing process even not for language improvement.

## Data availability

The data that has been used is confidential.

## Acknowledgments

The authors gratefully acknowledge Markus Doerr, BASF for the molecular modeling calculations performed for this work. Furthermore, we would like to thank the scientific and technology achievement recognition (STAR) initiative of BASF SE for sponsoring this study.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.464710.

## References

[1] W. Bonrath, B. Gao, P. Houston, T. McClymont, M.A. Müller, C. Schäfer, C. Schweiggert, J. Schütz, J.A. Medlock, 75 years of vitamin A production: a historical and scientific overview of the development of new methodologies in chemistry, formulation, and biotechnology, Org. Process Res. Dev. 27 (2023) 1557–1584, https://doi.org/10.1021/acs.oprd.3c00161.
[2] Food & Beverage, Global vitamin A market – industry trends and forecast to 2029, Data Bridge Market Research (2022).
[3] H. Weiser, G. Somorjai, Bioactivity of *cis* and *dicis* isomers of vitamin A esters, Int. J. Vitam. Nutr. Res. 62 (1992) 201–208.
[4] P.V. Bhat, P.R. Sundaresan, High-performance liquid chromatography of vitamin A compounds, Crit. Rev. Anal. Chem. 20 (1988) 197–219, https://doi.org/10.1080/00078988808048812.

[5] Y. Okamoto, E. Yashima, Polysaccharide derivatives for chromatographic separation of enantiomers, Angew. Chem. Int. Ed. Engl. 37 (1998) 1020–1043, https://doi.org/10.1002/(SICI)1521-3773(19980504)37:8≤1020:AID-ANIE1020≥3.0.CO;2-5.
[6] A.B. Barua, H.C. Furr, Properties of retinoids, Mol. Biotechnol. 10 (1998) 167–182, https://doi.org/10.1007/BF02760863.
[7] J.A. Sellers, B.A. Olsen, P.K. Owens, P.F. Gavin, Determination of the enantiomer and positional isomer impurities in atomoxetine hydrochloride with liquid chromatography using polysaccharide chiral stationary phases, J. Pharm. Biomed. Anal. 41 (2006) 1088–1094, https://doi.org/10.1016/j.jpba.2006.01.063.
[8] F. Ianni, F. Blasi, D. Giusepponi, A. Coletti, F. Galli, B. Chankvetadze, R. Galarini, R. Sardella, Liquid chromatography separation of α- and γ-linolenic acid positional isomers with a stationary phase based on covalently immobilized cellulose tris(3,5-dichlorophenylcarbamate), J. Chromatogr. A 1609 (2020) 460461, https://doi.org/10.1016/j.chroma.2019.460461.
[9] M. Olfert, S. Bräuer, M. Wolter, S. Buckenmaier, E. Brito-de la Fuente, M. Lämmerhofer, Comprehensive profiling of conjugated fatty acid isomers and their lipid oxidation products by two-dimensional chiral RP×RP liquid chromatography hyphenated to UV- and SWATH-MS-detection, Anal. Chim. Acta 1202 (2022) 339667, https://doi.org/10.1016/j.aca.2022.339667.
[10] G. Schlotterbeck, L.H. Tseng, H. Händel, U. Braumann, K. Albert, Direct on-line coupling of capillary HPLC with 1H NMR spectroscopy for the structural determination of retinyl acetate dimers: 2D NMR spectroscopy in the nanoliter scale, Anal. Chem. 69 (1997) 1421–1425, https://doi.org/10.1021/ac960902b.
[11] Y. Okamoto, Chiral polymers for resolution of enantiomers, J. Polym. Sci. A Polym. Chem. 47 (2009) 1731–1739, https://doi.org/10.1002/pola.23215.
[12] E. Yashima, Polysaccharide-based chiral stationary phases for high-performance liquid chromatographic enantioseparation, J. Chromatogr. A 906 (2001) 105–125, https://doi.org/10.1016/S0021-9673(00)00501-X.
[13] B. Chankvetadze, L. Chankvetadze, S. Sidamonidze, E. Kasashima, E. Yashima, Y. Okamoto, 3-Fluoro-, 3-chloro- and 3-bromo-5-methylphenylcarbamates of cellulose and amylose as chiral stationary phases for high-performance liquid chromatographic enantioseparation, J. Chromatogr. A 787 (1997) 67–77, https://doi.org/10.1016/S0021-9673(97)00648-1.
[14] W. Bi, F. Wang, J. Han, B. Liu, J. Shen, L. Zhang, Y. Okamoto, Influence of the substituents on phenyl groups on enantioseparation property of amylose phenylcarbamates, Carbohydr. Polym. 241 (2020) 116372, https://doi.org/10.1016/j.carbpol.2020.116372.
[15] E. Yashima, C. Yamamoto, Y. Okamoto, NMR studies of chiral discrimination relevant to the liquid chromatographic enantioseparation by a cellulose phenylcarbamate derivative, J. Am. Chem. Soc. 118 (1996) 4036–4048, https://doi.org/10.1021/ja960050x.
[16] R.B. Kasat, NH.L. Wang, E.I. Franses, Effects of backbone and side chain on the molecular environments of chiral cavities in polysaccharide-based biopolymers, Biomacromolecules 8 (2007) 1676–1685, https://doi.org/10.1021/bm070006h.
[17] M. Lämmerhofer, Chiral recognition by enantioselective liquid chromatography: mechanisms and modern chiral stationary phases, J. Chromatogr. A 1217 (2010) 814–856, https://doi.org/10.1016/j.chroma.2009.10.022.
[18] Molnár-Institut, Berichte drylab optimierung vitamin A-Acetat BASF, 2022. Unpublished results.