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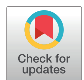
Simultaneous detection of nitrosamines and other sartan-related impurities in active pharmaceutical ingredients by supercritical fluid chromatography



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ABSTRACT

Since July 2018, the pharmacological class of “sartans” has been the subject of considerable media and analytical interest, as it became known that they are contaminated with nitrosamines such as N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA) and N-nitrosodiisopropylamine (NDiPA). Previous compendial methods are not able to detect these new contaminants. Using the latest and innovative Quality-by-Design (QbD) approach, it has now been possible to develop an analytical method that enables to investigate sartans, such as valsartan and losartan. Also a large class of different nitrosamines in the ppb range and sartan-related impurities can thus be determined simultaneously in a single analysis using supercritical fluid chromatography (SFC). By using SFC, a broad spectrum of nonpolar and very polar impurities can be separated and analyzed in under 20 min. The analytical method developed is validated for limit testing according to ICH Q2(R1) and fulfills default thresholds of EMA and FDA for testing of drug substances and genotoxic impurities. Additionally, it can also be adapted to other pharmaceuticals that may be contaminated with nitrosamines, since tetrazole synthesis as the underlying cause of nitrosamine contamination is important for a set of other non-sartan drug substances.

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1. Introduction

Since it became known in July 2018 that the drug substance (DS) valsartan from a major Chinese supplier is contaminated with N-nitrosodimethylamine (NDMA), numerous recalls have been carried out all over the world. Due to change in tetrazole synthesis of the DS, in order to increase the synthesis yield and to avoid highly toxic catalysts, nitrosamines (NAs) were formed and their occurrence not considered by the supplier and the regulatory authorities [1,2]. Valsartan is a member of the group of angiotensin II receptor antagonists, also known as šartans, for the treatment of hypertension and heart failure. Previous compendial analytical methods for the release of the DS are not able to detect NDMA and so NAs emerged in the DS. Nitrosamines, which are not structurally related to sartans, can be formed during synthesis by the reaction of secondary amine-containing solvents (e.g. triethylamine or dimethylformamide, which can contain diethylamine or dimethylamine in trace amounts) and nitrite in an acidic environment [3]. Therefore, depending on the synthesis, different NAs can be formed. As a result, the FDA and EMA carried out further investigations in cooperation with other local organizations, which revealed that other NAs may also be present (e.g. N-nitrosodiethylamine - NDEA) and that other sartans, such as losartan and irbesartan, are also partially affected [1,4–6]. Abdel-Tawab, et al. [5] from the Central Laboratory of German Pharmacists found NDMA contents between 3.7 μg and 22.0 μg per tablet in random examinations of valsartan products.

Nitrosamines are potent carcinogens that can lead to tumors in nearly all organs. For NDMA and almost all other NAs analyzed in this article, the carcinogenic effect has already been shown in a number of animal studies in rats, mice, hamsters, guinea pigs and rabbits, irrespective of the way of exposure [7–9]. Although most the NDMA levels found in the studied valsartan products were low (in the double-digit ppm range) [2], the naturally daily intake of NAs was not negligible and the FDA and EMA assume that chronic exposure to contaminated valsartan products may lead to one additional cancer case per 5,000–8,000 threatened patients [1,10].

Based on this unsustainable situation, EMA and FDA published test methods (GC/MS) capable of detecting only NDMA in sartans

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and an additional method for the simultaneous detection of NDMA and NDEA by UHPLC-MS/MS [11,12].

Disadvantage of these methods is that they add an additional analytical test to the official pharmacopoeial purity monographs. The aim of this work is therefore to develop a new analytical method under Quality-by-Design (QbD) principles, which is capable of replacing conventional purity methods for the determination of related substances in sartans and simultaneously detecting different classes of NAs in trace amounts, so that the release of the drug substance can be implemented using a single and fast technique.

Despite continued use of the well-established liquid and gas chromatography techniques, supercritical fluid chromatography (SFC) adds a complementary strategy in the toolbox of analytical scientists and covers the application domains of different chromatographic modes as normal phase, reversed phase and hydrophobic interaction chromatography [13].

The unique physical properties of supercritical fluids such as low viscosity and high diffusivity, concurrent with a high solvating power make them very interesting for usage as mobile phases in chromatography and are leading to several advantages of SFC compared with traditional HPLC. The most common and preferred fluid used in SFC is CO_2, as it offers excellent properties. This includes a dipole moment of being zero and thus highly lipophilic properties similar to hexane or heptane and the ability to bring it easily into a supercritical state [13,14]. Analysis of non-polar compounds, such as lipids, fat-soluble vitamins and steroids are easily possible. However, for the elution of polar components, the addition of a polar organic solvent, also called modifier, is possible to prompt elution and allow substantial expansion of the polarity range [13,15].

In our study we investigated the applicability of the wide polarity range of SFC technology for the analysis of NAs and other sartan related impurities in active pharmaceutical ingredients (API) in a single run.

2. Materials and methods

2.1. Quality-by-Design development strategy and risk assessment

The term "QbD" originates from the pharmaceutical development guideline Q8(R2)-2009 of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) [16]. The guideline describes the process as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" - also known as QbD. These predefined objectives and targets were also called "Analytical Target Profile" (ATP) [17]. The ATP of the method to be developed should be that (a) all impurities are separated so that they can be detected individually and (b) the method is sufficiently sensitive for all impurities that internationally recognized pharmaceutical regulations on validation and API release are complied with. Furthermore, (c) profound knowledge of the underlying mechanisms and parameters should be acquired in order to understand and control the chromatographic and spectrometric influencing factors in depth.

The harmonized tripartite guideline of the ICH for impurities in drug substances Q3A(R2)-2006 [16] requires a reporting threshold of $\leq$ 0.05% for process- and drug-related impurities "*for qualifying those impurities that were not present, or were present at substantially lower levels, in batches of a new drug substance used in safety*", if the daily administred dose is equal or below 2 g of the API. This defined threshold should be the level, where precise quantification is possible. Therefore, the limit of detection (LOD) for the sartan impurities should be at least 0.03% in our study, to achieve the second predefined objective of the ATP (sensitivity for sartan impurities). In addition, the ICH Q3A guideline also insists that "*lower thresholds can be appropriate if the impurity is unusually toxic*", which is the case for the genotoxic NAs [7]. For this reason, the ICH Guideline M7(R1)-2017 [16], which deals with assessment and control of mutagenic impurities in pharmaceuticals, will be applied to NAs. This guideline M7 uses the concept of the "Threshold of Toxicological Concern" (TTC), to estimate tolerable amounts with negligible risk when administered. With this TTC "*for marketed products, acceptable increased cancer risk is set at a theoretically calculated level of approximately one in one hundred thousand. These risk levels represent a small theoretical increase in risk when compared to human overall lifetime incidence of developing any type of cancer*". Therefore, the LOD for the NAs should be equal or lower than the TTC. The U.S. Environmental Protection Agency (EPA) considers "consuming up to 96 ng NDMA/day is [...] reasonably safe for human ingestion. It is estimated that over the course of a person's lifetime, consuming this amount of NDMA would result in less than one additional case of cancer for every 100,000 people" [1]. Related to an intake of 100 mg losartan or 320 mg valsartan, which corresponds to a usual dosage for high blood pressure and stroke prophylaxis, this amount of NDMA would be approximately equal to a TTC of 1 ppm for losartan and 0.3 ppm for valsartan. The LOD threshold of ATP point (b) for the NAs (sensitivity for nitrosamines) has to be significantly lower than for sartan related impurities and is thus set to the above mentioned values in our study. In order to demonstrate that the objective of point (b) of the ATP is achieved, the method will be validated by limit testing according to the ICH Q2(R1)-2005 guideline for impurities in DS [16].

As method development according to the QbD concept also requires process understanding & control and risk management, which is expressed by point (c) of the ATP, additional control mechanisms and structures must be established to ensure compliance. The ICH guideline Q8 therefore recommends defining "critical quality attributes" (CQAs) for the development and "critical process parameters" (CPPs), "whose variability [have] an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality" [16]. The following CQAs can be defined, which can be used as core parameters for development and optimization: peak resolution (Rs), peak height and symmetry, signal-to-noise and retention. In order to maximize these CQAs, for the SFC-UV-MS/MS method, eight main CPPs will be evaluated. These are the stationary phase, modifier and additives to mobile phase. Additionally, column temperature and gradient slope (and steps) of the mobile phase, type and flow rate of the make-up solvent (for MS/MS coupling) and the mass spectrometric instrument parameters were evaluated. In order to be capable of controlling and understanding all these parameters and their interaction in a multidimensional perspective, the results are evaluated and consolidated using a software-based and statistical approach. The so-called design-of-experiments (DoE) form the centerpiece of the development.

2.2. Chemicals and reagents

Certified reference standards of the analytes valsartan, losartan potassium, losartan impurity C, E, F, G & H and the EPA 8270/Appendix IX Nitrosamines Mix were supplied by Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). Additionally, a 5000 μg/ml reference stock solution of NDMA and a 200 μg/ml reference stock solution of NDiPA in methanol (MeOH) were purchased from LGC GmbH (Luckenwalde, Germany) – see Table 1 for the structures of the included NAs. The pharmacopeial reference substances losartan impurity D, "losartan for system suitability" (contains beside losartan also losartan impurity J, K, L and M) and "valsartan for system suitability" (contains beside valsartan also

Table 1
Nitrosamines included in the method development for impurity screening with their corresponding structures and calculated logP values.

Name	Synonym / Abbreviation	Structure	CAS-Nr.	Rel. molecular Mass [M_R]	logP
N-nitrosodimethylamine	NDMA		62-75-9	74	0.10
N-nitrosodiethylamine	NDEA		55-18-5	102	0.80
N-nitrosomethylethylamine	NMEA		10595-95-6	88	0.40
N-nitrosodi-*n*-propylamine	NDnPA		621-64-7	130	1.7
N-nitrosodiisopropylamine	NDiPA		601-77-4	130	1.4
N-nitrosodi-*n*-butylamine	NDBA		924-16-3	158	2.6
N-nitrosodiphenylamine	NDPhA		86-30-6	198	3.4
N-nitrosopyrrolidine	NPyr		930-55-2	100	0.40
N-nitrosopiperidine	NPip		100-75-4	114	0.80
N-nitrosomorpholine	NMor		59-89-2	116	−0.30

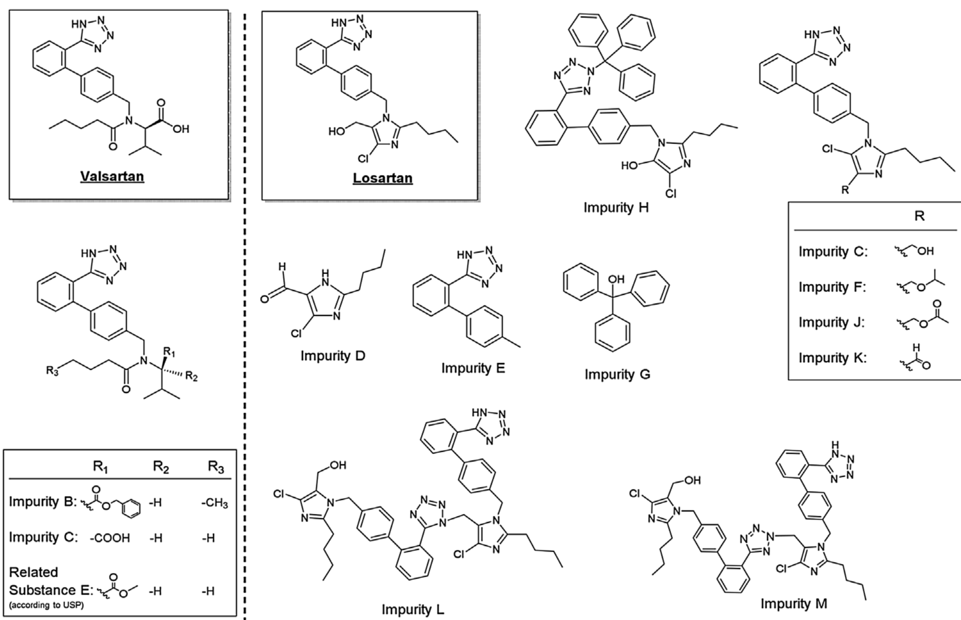


Fig. 1. Valsartan and losartan with their impurities according to Ph. Eur. (USP related Substance E for valsartan was also added).

valsartan impurity C) were acquired from the EDQM (Strasbourg, France). Valsartan impurity B and valsartan related substance E were bought from Toronto Research Chemicals (North York, ON, Canada) and losartan impurity L and M from Phast - Gesellschaft für Pharmazeutische Qualitätsstandards mbH (Homburg, Germany). The structures of all sartans and their impurities from the official Ph. Eur. monographs [18] are displayed in Fig. 1.

For screening experiments HPLC-grade solvents and reagents were used, for method optimization and performance verification these were upgraded to MS-grade purity. All were purchased from VWR International GmbH (Darmstadt, Germany). Carbon dioxide N45 (99.995%) and nitrogen N50 (99.999%) were obtained from Air Liquide Deutschland GmbH (Düsseldorf, Germany) and Argon 5.3 (99.9993%) from Linde AG (Munich, Germany).

Table 2
: Optimized SRMs with corresponding cone voltages and collision energies in ESI + mode for all NAs.

Synonym / Abbreviation	SRM 1 (Quantifier) / SRM 2 (Qualifier)			Cone voltage	Collision energy	Dwell time (sec)
NDMA	75	→	43	36	14	0.05
	75	→	58	36	10	0.05
NDEA	103	→	47	34	8	0.05
	103	→	75	34	10	0.05
NMEA	89	→	61	36	10	0.05
	89	→	47	36	8	0.05
NDPA	131	→	89	26	10	0.05
	131	→	43	26	12	0.05
NDBA	159	→	103	28	10	0.05
	159	→	57	28	14	0.05
NDPhA	199	→	66	26	26	0.05
	199	→	169	26	12	0.05
NPyr	101	→	55	34	14	0.05
	101	→	59	34	16	0.05
NPip	115	→	69	34	14	0.05
	115	→	55	34	24	0.05
NMor	117	→	45	30	14	0.05
	117	→	57	30	14	0.05

2.3. Instrumentation and software

Chromatographic analysis was performed using an Acquity UPC^2 SFC system (Waters GmbH, Eschborn, Germany) equipped with an Acquity UPC^2 column manager with active eluent pre-heaters for up to 4 simultaneous columns, an Acquity UPC^2 PDA detector and an Acquity TQD (Triple Quadrupole Mass Spectrometer). To interface the SFC to the MS a post-column pre-convergence manager splitter (fixed leak) was coupled with a Waters 515 HPLC pump, which was used as a make-up pump to enhance mass transfer to the MS and ionization in the source. For system control, data acquisition and data processing the Empower 3 software (Feature Release 4, Service Release 2, Hotfix 2) from Waters GmbH (Eschborn, Germany)was used.

Instrumentation was operated fully qualified according to the 4Q model of the USP <1058> [19] and under GMP-regulated laboratory environment together with the qualified and validated Empower software.

For column screening, the following columns were chosen: Viridis BEH, Viridis BEH 2-EP, Torus DIOL, Torus 2-PIC, Torus DEA, Torus 1-AA, Viridis CSH Fluorophenyl and Viridis HSS C18 SB (all 100 x 3.0 mm; 1.8 μm – Waters GmbH, Eschborn, Germany).

Fusion QbD software (Version 9.8.1.199 - S-Matrix Corporation, Eureka, California, USA) was utilized for multivariate data analysis and method screening, by full automatized DoE planning and construction. Chemical structures and logP values were generated by ChemDraw Professional (Version 16.0 - PerkinElmer Informatics, Inc., Waltham, Massachusetts, USA).

2.4. Chromatographic conditions and screening procedure

In a first attempt all columns were screened with a generic gradient of 1%B to 40%B in 20 min, followed by a 5 min reequilibration step to the initial conditions (A: CO_2; B: MeOH) at a flow rate of 1.5 ml/min at 25 °C and 50 °C column temperature. Valsartan and losartan were injected as a standard solution containing 0.5 mg/ml each and NDMA as a 10 μg/ml standard solution. Peak shape, retention behavior and peak resolution was analyzed and the four best columns were chosen. Then the nitrosamine mixture was injected as a 10 μg/ml standard solution - peak shape, resolution and retention behavior on the four chosen columns were screened again.

For modifier screening two HSS C18 SB columns were connected in series and the flow rate was adjusted to 1.2 ml/min at 1800 psi back pressure. An initial isocratic step of 2 min was added to the gradient and the gradient was reduced to 30%B in 7 min to prevent excessive back pressure. Four modifiers (B) were screened: MeOH, ethanol (EtOH), isopropanol (IpOH) and acetonitrile (ACN). These were systematically screened at 30 °C and 50 °C column temperature and 0.0, 0.5 and 1.0 initial %B of the isocratic step.

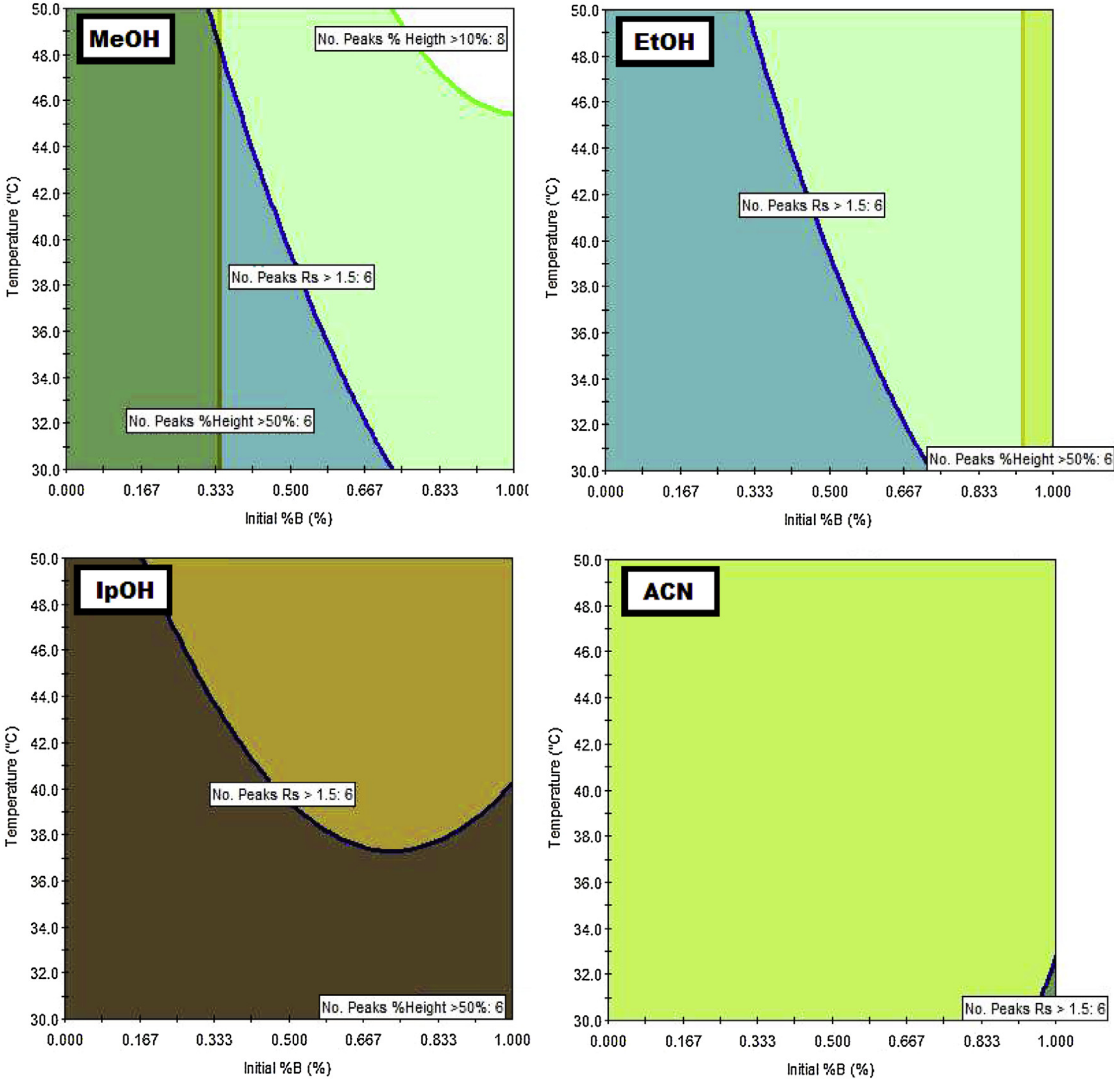
Subsequently, the influence of additives in the mobile phase and make-up solvent were investigated. The following additives were added to MeOH: formic acid (FA), acetic acid (AcOH), trifluoroacetic acid (TFA); also 30% ammonia solution (NH_3) and triethylamine (TEA) for mobile phase screening and 10 mM ammonium formate (NH_4FA) for make-up solvent screening, each 0.1% (v/v). Valsartan and losartan were spiked at the 0.5% level with all their impurities and injected each as 10 mg/ml solutions in MeOH. Samples were prepared by weighting into volumetric flasks and stirring for 5 min.

The final chromatographic method is: CO_2 as eluent A and methanol with 0.1% TFA as eluent B, starting at 2% B for 2 min, then rapidly increased linearly to 10% B within one minute with a 3 min isocratic step, followed by a slow increase to 30% B in further 4.5 min with a 1.5 min hold time and a reequilibration to 2% B for 5 min, resulting in a total run time of 17 min on two HSS C18 SB columns (each 100 x 3.0 mm; 1.8 μm) at 55 °C column temperature and a flow rate of 1.2 ml/min. The injection volume is 1 μl of a 10.0 mg/ml API sample directly dissolved in MeOH by weighting in a corresponding volumetric flask and stirring for 5 min.

2.5. Spectroscopic and spectrometric conditions

Chromatograms were recorded from 190 to 400 nm at a scan rate of 10 Hz with multiwavelength photodiode array detector and evaluated at 230 nm. For peak tracking and high sensitive detection of the NAs, the MS operated in positive electrospray ionization (ESI+) mode with selective reaction monitoring (SRM) for targeted quantitative mass analysis.

MS parameters were tuned by direct infusion of a 1 μg/ml standard solution of all NAs in MeOH (containing also 0.1% formic acid). Optimized parameters are: capillary voltage 3.80 kV, extractor voltage 3 V, source temperature 120 °C, desolvation temperature 250 °C, desolvation gas flow 500 L/h, collision gas flow 0.50 ml/min and span width 0.000. SRMs and optimal cone voltages and collision energies were displayed in Table 2.



Response Variable Goals

Name	Goal	Lower Bound	Upper Bound	Color
Peaks detected	Maximize	9.00	---	Red
No. Peaks Rs > 1.5	Maximize	6.00	---	Blue
No. Peaks %Height >10%	Maximize	8.00	---	Green
No. Peaks %Height >50%	Maximize	6.00	---	Orange

Fig. 2. Ďesign Spaceöf modifier screening for NAs generated by Fusion QbD software – white areas display a positive result for defined goals. Colored areas indicate that at least one response is not achieved – mixed colors are possible, when more than one goal is not achieved. Data generated on two connected HSS C18 SB columns with four modifiers: MeOH (top left), EtOH (top right), IpOH (bottom left) and ACN (bottom right) systematically screened at 30 °C–50 °C column temperature and 0.0, to 1.0%B (%modifier in CO_2).

3. Results and discussion

3.1. Screening

Of the eight screened SFC columns, covering a broad chemical spectrum from normal-phase (NP) to reversed-phase (RP) chromatography, only the Fluorophenyl, C18, Diol and BEH (pure silica) columns showed sufficient separation and retention behavior for losartan, valsartan and NDMA. These columns were selected and tested in order to evaluate, which column has the highest retention and separation power for the NAs, since NDMA showed only weak interactions with the stationary phase in previous screening experiments. Only the HSS C18 SB showed satisfying retention behavior for the sartans, all nine NAs (NDiPA not included at this point – see chapter 3.3 for more details) and proved to be significantly superior to all other columns, so these were immediately discarded for further experiments. This result is congruent to the LC methods usually used for separation and detection of NAs in cosmetics [20], water and biosolids [21,22], rubber [23] or for urine analysis [24], where convention RP-columns (C8 or C18) were used.

In order to further increase the separation performance for the subsequent modifier screening, two columns were connected in series. Modifiers were systemically screened with the DoE support of Fusion QbD software. This approach allows collecting knowledge about the influencing chromatographic parameters and their effects. The data was used to generate a visual “Design Space”

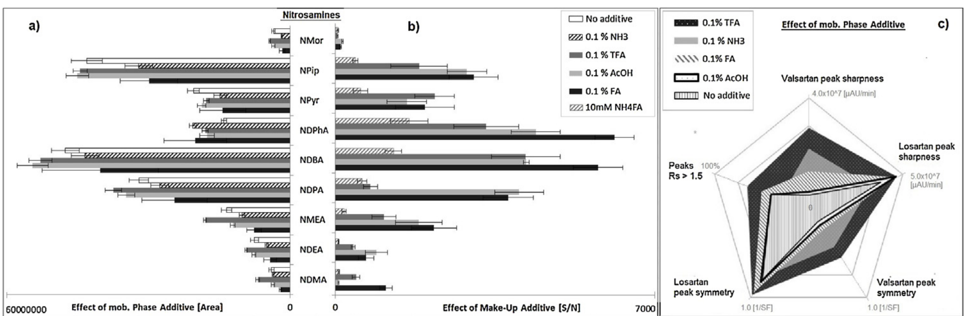


Fig. 3. [a] Effect of mobile phase additives on NA peak areas (left), [b] effect of make-up solvent additives on NA peak S/N (right) and [c] effect of mobile phase additives on sartans (peak symmetry displayed as the reciprocal of the symmetry factor and peak sharpness as peak height/peak width).

("multidimensional combination and interaction of input variables [. . .] and process parameters that have been demonstrated to provide assurance of quality" – ICH Q8), where optimal separation and peak shape is displayed. Fig. 2 shows the result of the modifier screening. The visualized design space is displayed by a white area, were the predefined CQAs are used to discriminate values of each CPP to maximize outcome and demonstrate the space with the highest efficiency for each CQA. By overlaying each CQA discrimination space (displayed by a colored area), only the design space persists uncolored.

Fusion QbD software calculates statistically, without the use of chromatographic terms and laws, the effect on the response goals in a multi-dimensional space. For modifier screening, the first step was therefore to examine only whether all NAs were detectable (response goal #1 - peaks detected), whether a good separation had already been achieved for some of the substances (response goal #2 - resolution) and whether most peaks showed a certain relative minimum intensity of their signals (response goal #3 and #4 - %Height). Response goal #3 and #4 were necessary because the weak modifiers (especially IpOH and ACN) tend to make peaks very broad and flat, so that a relative discriminating factor was necessary from the outset in order not to decrease signal intensity and loose sensitivity. The absolute peak height would not have been a suitable indicator, since all signals clearly differ in their peak height. Therefore, a relative factor was introduced to normalize all NAs.

It can be seen that only MeOH as a modifier at elevated column temperatures is able to elute all NAs from the column (peaks detected), to form them into sharp peaks (%peak height) and thus to ensure sufficient separation (Rs > 1.5). Nevertheless, the white corner section in the MeOH knowledge space of the DoE, which indicates that all response goals have been achieved, shows that the initial conditions were not ideal. Even higher temperatures and more %B (modifier) are needed, to achieve maximum separation and signal intensity. In contrast, EtOH led to a significant peak broadening and with IpOH and ACN it was partially no longer possible to detect them at all. IpOH shows an entire brown design region (mixed color of green, orange and red), which indicates that not every NA was detectable and that the detected signals were unintensive. The same is true for ACN, where all NAs were detected but highly decreased in signal intensity.

Since almost all NAs eluted during the initial isocratic step of the gradient, a very high degree of flexibility remained for the screening of the sartans. For this purpose different mobile phase additives were tested to evaluate their influence on NA separation and ionization (in order not to lose sensitivity) and sartan peak shape and retention. Figure 3a shows the influence on the peak areas for all NAs. Especially the low-responders NDMA, NDEA and NMor a major impact of the eluent additive on their sensitivity was observed.TFA in the eluent showed the best overall outcome for NAs. In parallel, only TFA was also able to decrease tailing and enhance separation for the sartans, without changes in selectivity (see Fig. 3c). AcOH showed no positive influence and formic acid only slightly on sartan separation und peak shape. Ammonia lead to an increase in peak sharpness and retention, but also caused peaks to coelute, since selectivity changed.

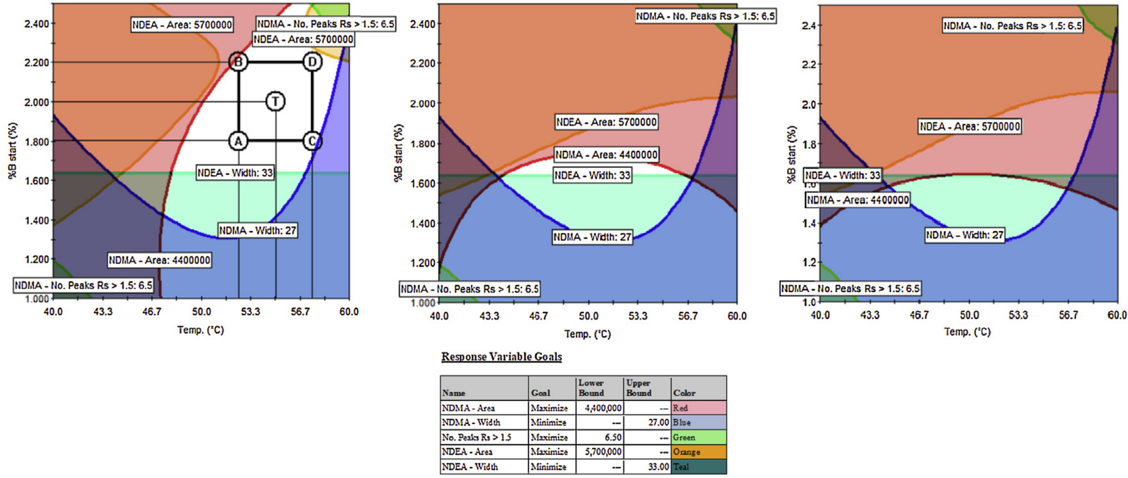
In contrast, the addition of TFA to the make-up solvent, as well as ammonium formate, was found to worsen ionization significantly. FA and AcOH showed comparable properties, but FA proved to be slightly superior, especially for NDMA, where the background noise increased drastically (see Fig. 3b) with AcOH.

Ammonia and TFA are known to be a strong ionization suppressor in mass spectrometry, which explains, why addition in the make-up solvent decrease the S/N values, since the entire flow enters the MS source. Addition of small amounts of TFA to the modifier of the eluent does not have a negative effect on ionization, since only small amounts of the flow are splitted to the MS, but substantially higher volumes also had a negative effect (displayed for the two low-responders NDMA and NDEA in Fig. 4). The mechanisms, which cause TFA to improve the chromatographic separation and peak sharpening, are still unclear, but it is common in SFC to add acidic additives when organic acids are present in the analyte structure - which is the case for the sartans (tetrazole and carboxylic acid derivatives). Suspected mechanisms are ion pairing, suppression of analyte charge and covering of adsorption sites on the surface of the stationary phase [13].

3.2. Optimization

The method screening showed that only MeOH as eluent with an addition of TFA was able to provide sufficient separation and of the NAs and sartans. FA showed the best results as make-up additive. Furthermore, it could be derived from the QbD screening that high temperatures are necessary for separation. Nevertheless, it turned out that the percentage of the modifier in the start gradient was not yet optimized, since only a small corner section of the knowledge space provided successful results (see Fig. 2). On this basis, the amount of the modifier in the start gradient (%B, from 1.0% to 2.5%), the column temperature (from 40 °C to 60 °C) and the concentration of TFA in the modifier (from 0.1% to 0.4% TFA) were further optimized to generate a robust and highly efficient working point for the NAs. An optimization with the goal of sufficient separation and detectability for the sartans and their impurities has not yet been carried out, because the NAs represented the major challenge. Their separation and detection in trace amounts was therefore the first step.

On the basis of Fig. 4, it can be deduced that a higher concentration of TFA does not lead to an improvement of the separation, but has a strongly negative effect on the peak height and thus on the sensitivity. Optimum separation and sensitivity was only achieved at 55 °C column temperature and 2.0%B in the start gradient with 0.1% TFA as additive. Slightly smaller concentrations of TFA prob-



Name	Goal	Lower Bound	Upper Bound	Color
NDMA - Area	Maximize	4,400,000	---	Red
NDMA - Width	Minimize	---	27.00	Blue
No. Peaks Rs > 1.5	Maximize	6.50	---	Green
NDEA - Area	Maximize	5,700,000	---	Orange
NDEA - Width	Minimize	---	33.00	Teal

Fig. 4. Analytical method "Design Space" after QbD eluent optimization for NAs - a robust and satisfying separation is only achieved at 0.1% TFA (left); 0.25% (middle) and 0.4% (right) TFA in the mobile phase negatively impacted the peak areas of NDMA and NDEA due to ion suppression.

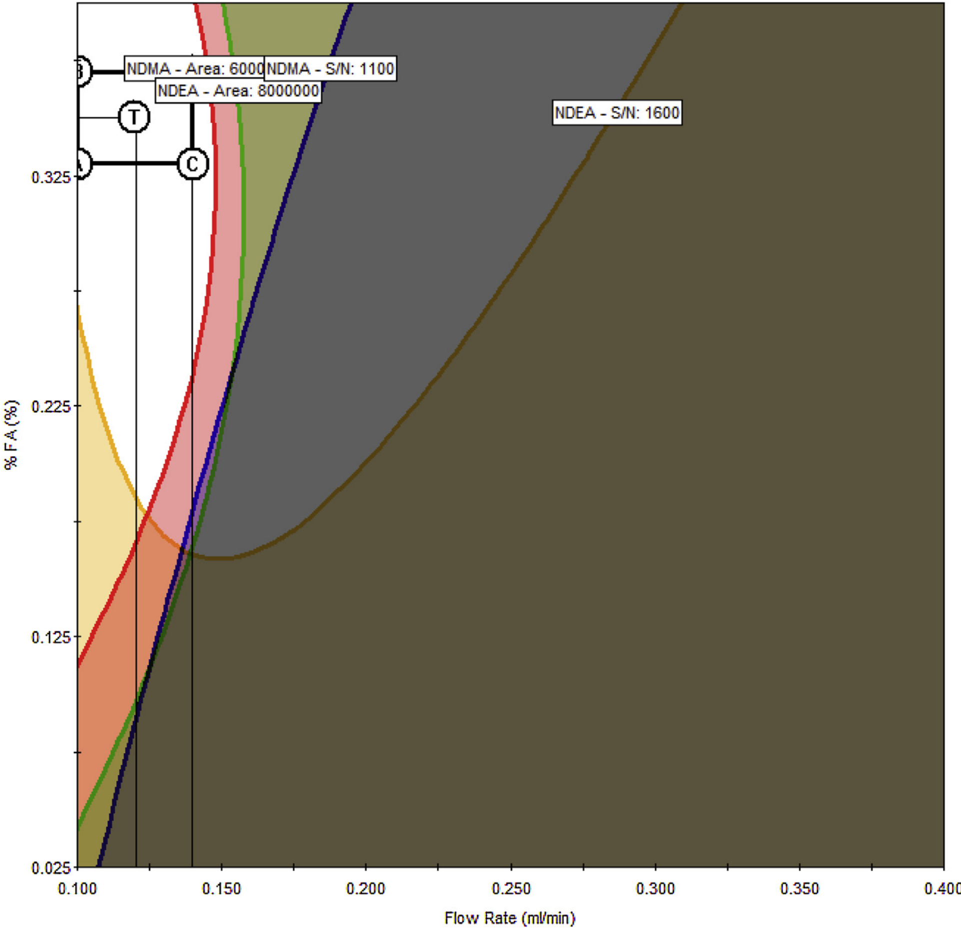


Fig. 5. Optimization of make-up flow rate and percentage of formic acid in MeOH – the best results were obtained at 0.12 ml/min and 0.35% formic acid.

ably would have improved ionization, but higher amounts were necessary to separate the sartans.

The concentration of formic acid (from 0.025% to 0.4%) in the make-up solvent and the flow rate (from 0.1 ml/min to 0.4 ml/min) of the make-up pump were also optimized. The best overall results were obtained at 0.35% formic acid at 0.12 ml/min (see Fig. 5).

After the NAs had been separated with the highest possible sensitivity and selectivity, the gradient for the elution was optimized for the separation of all sartans and their impurities and a step gradient was generated from the linear gradient. Optimum resolution was achieved at a flow of 1.2 ml/min with CO_2 as mobile phase eluent A and MeOH with 0.1% TFA as eluent B (2% B for 2 min, linear to 10% B in 1 min, 3 min hold, linear to 30% B in 4.5 min, 1.5 min hold) on two HSS C18 SB columns (2 × 100 x 3.0 mm; 1.8 μm) at 55 °C. Fig. 6 shows the resulting chromatograms for all NAs and sartans (the final method is described in detail in method chap-

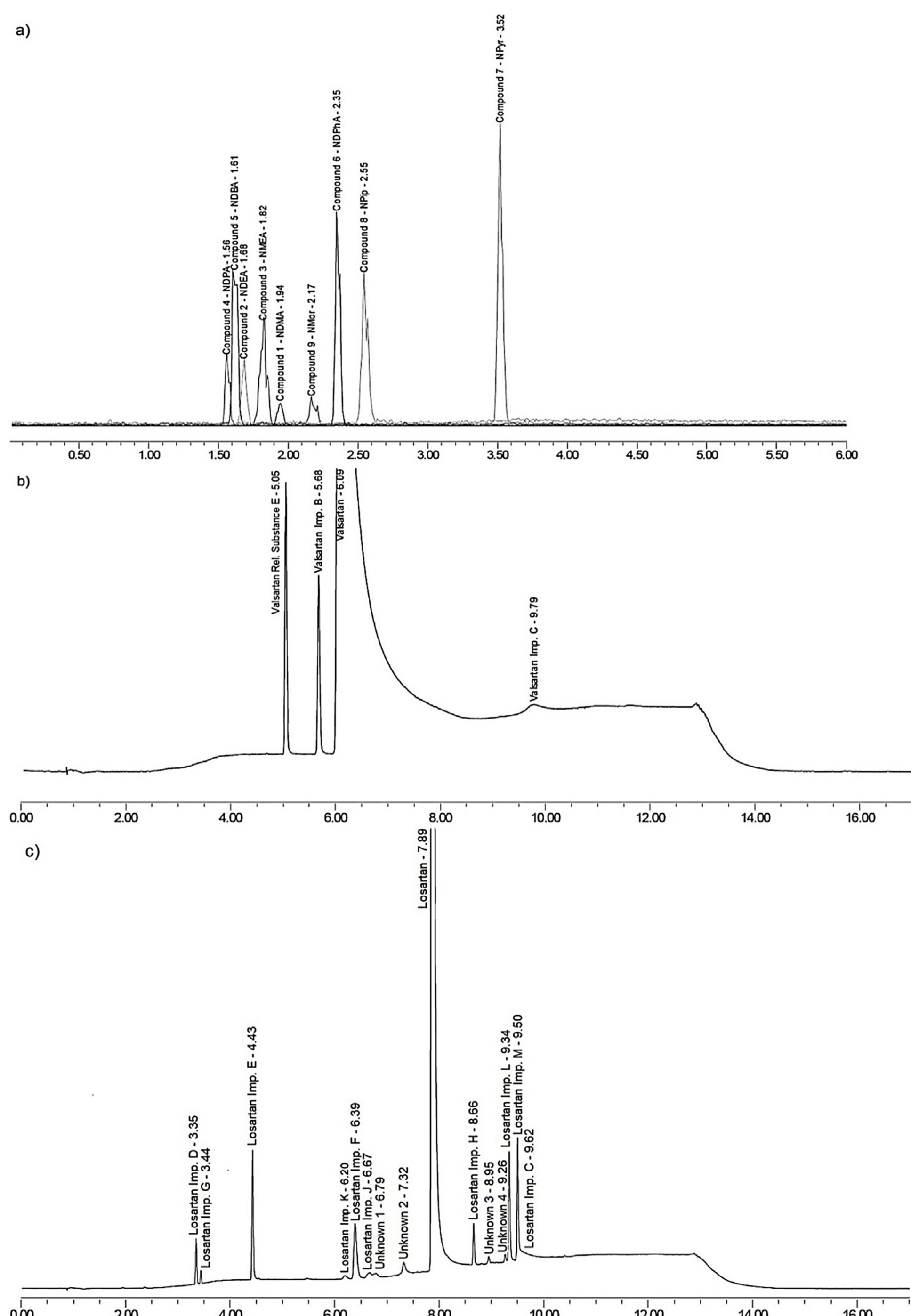


Fig. 6. Representative chromatograms of (a) the nitrosamine mixture (overlay of all 8 SRM quantifier channels – note: NDiPA and NDnPA only displayed as “NDPA”, due to same retention time), (b) valsartan & (c) losartan APIs (at 230 nm detection wavelength), each spiked with their specified and unspecified sartan impurities at the 0.5% level (except for valsartan impurity C and losartan impurity C, J & K, which were spiked at the 0.05% level) - sample concentration: 10 mg/ml.

ter under section 2.4). NAs eluted in their reverse logP order (from non-polar to polar), since the elution strength is orthogonal in SFC, compared to RP liquid chromatography. This effect is inverted above four covalently bonded carbon atoms (which is the case for NDBA, NDPhA, NPip and NPyr), as from this chain length the Van der Waals force increases interaction relevance with the C18 phase of the column, resulting in a logP dependent elution order from polar to non-polar, as common in RP chromatography [25].

3.3. Method validation and application

After the successful method development, where point (a) selectivity and (c) profound knowledge of the ATP were applied, the procedure has been validated according to the ICH Q2(R1) guideline for impurities in DS [16] by limit testing in order to also demonstrate point (b) of the ATP, the sensitivity. LODs were compared with the previously established targets and evaluated with respect to ICH Q3A and M7.

The detection limits were determined by deriving the signal-to-noise ratios from the measured linearity of a six-fold injection at

Table 3
Validation results of limit testing according to ICH Q2(R1) – note that the ICH Q3A(R2) demands a reporting threshold for impurities in drug substances to be ≤ 0.05% (for a daily intake of ≤2 g drug substance) and the ICH M7(R1) for genotoxic impurities (by adoption of the TTC concept) levels of ≤ 1 ppm.

Name	Limit of detection			Selectivity
	[ng/ml]	Per 320 mg valsartan	Per 100 mg losartan	(Separation)
NDMA	4.55	0.14 ppm	0.46 ppm	√
NDEA	1.58	0.05 ppm	0.16 ppm	√
NMEA	1.81	0.06 ppm	0.18 ppm	√
NDPA	0.24	0.01 ppm	0.02 ppm	[√]
NDBA	0.34	0.01 ppm	0.03 ppm	√
NDPhA	0.22	0.01 ppm	0.02 ppm	√
NPyr	3.71	0.12 ppm	0.37 ppm	√
NPip	2.26	0.07 ppm	0.23 ppm	√
NMor	4.20	0.13 ppm	0.42 ppm	√
valsartan impurities	≤ 0.03 %			√
losartan impurities	≤ 0.03 %			√

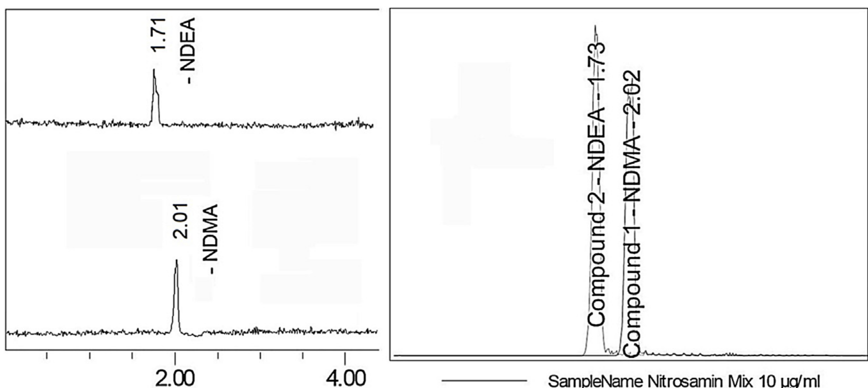


Fig. 7. Detected nitrosamines in losartan API (left) and nitrosamine standard mix for peak verification.

ten levels for each substance. The selectivity could also be shown by this experiment. Table 3 shows the LODs for all substances and displays that all criteria for successful limit testing are met and the ATP is fulfilled. The method developed far exceeds the ATP for all the studied NAs and thus achieved sufficient sensitivity, so that critical quantities can be detected in due time.

Only NDPA and NDBA are slightly coeluting, which does not interfere with its mass spectrometric determination, since this is performed by SRM acquisition, and therefore selectivity is still achieved. Since all NAs are slightly UV-active and can also be determined using PDA detection in higher quantities, this was also tested. It turned out that detection was only possible down to 100 ppm, which is clearly inferior to detection limits achieved by targeted MS/MS.

After finishing the method development it was published that another nitrosamine was detected in a API of a mexican manufacturer, which was not part of the original screening: Nnitrosodiisopropylamine (NDiPA) [26,27]. NDiPA was also subsequently integrated and validated. It turned out that a separation of NDiPA and N-nitrosodi-*n*-propylamine (NDnPA) was not possible, due to complete coelution. Individually evaluated, comparable values for the LODs were obtained for NDiPA and NDnPA. Therefore, both substances are combined in Fig. 6, Tables 2 and 3 as “NDPA” (Nnitrosodipropylamine). Based on the synthesis [3], it appears unlikely, that NDiPA and NDnPA will occur simultaneously in an API batch and the toxicological risk assessment for humans should be the same for both NAs. Therefore, and due to the fact that NDiPA was subsequently incorporated and not part of the original ATP, the coelution is acceptable.

After validation of the method for limit testing, three batches of valsartan and losartan were tested. It turned out that both NDMA and NDEA could in fact be detected in one batch of losartan. Fig. 7 clearly shows traces of the two NAs. NDEA was detected approximately at the LOD and NDMA twice above the LOD. By extrapolation of the detected amounts of NDMA and NDEA in the investigated losartan batch, a corresponding intake of about 90 ng NDMA and 15 ng NDEA per day can be estimated, but it should be noted that the developed SFC-UV-MS/MS method is not validated for quantification. Nevertheless, according to the most recent FDA interim limits, these values can be considered uncritical. FDA and EMA consider NDMA acceptable up to 96 ng per day and NDEA acceptable up to 26.5 ng per day [1]. These limits, which are slightly below the predefined acceptance criteria for LODs in the ATP, can also be achieved by the SFC method.

The values found in our study are therefore below the TTC and have to be considered uncritical. In contrast, in a random sample investigation, values of up to 20 µg NDMA per tablet (thus 200x more) were found in valsartan products by the FDA [28]. This illustrates, why there is an urgent need to develop new sensitive and effective methods, to which we want to contribute with this work.

4. Conclusion and outlook

Through our study, we were able to show that it is possible to incorporate state-of-the-art analytical techniques for the purity analysis of sartans. By using a systematic QbD development approach, contaminants from related API compounds and potentially carcinogenic NAs can be separated and detected simultaneously. This results in a highly sensitive method, which can detect NAs in the picogram to femtogram range on column. The sensitivity of the developed method is comparable to the published LC- and GC–MS/MS methods by FDA and EMA [11,12,29], but outperforms them in terms of speed and is still able to analyze the API related impurities in a single run at the same time.

Only GC headspace methods are superior due to their almost unlimited sample concentration, but are only able to detect the volatile nitrosamines and are also not able to examine sartans.

The developed method is the first approach utilizing SFC to analyze NAs and shows the high potential of this technique. New investigations by the FDA have shown that in addition to the aforementioned NAs, other previously unexpected NAs such as N-Nitroso-*n*-methyl-4-aminobutyric acid (NMBA) could also be detected in the API [1]. This shows that the current tetrazole synthesis process still involves major risks and should be closely monitored analytically. Parr and Joseph [30] have shown in their overview that not only tetrazole-containing sartans can be contaminated with NAs, but also a variety of other tetrazole drugs that might be formed by the same synthesis process than the sartans. Since the developed SFC method separates the NAs during the isocratic step at the beginning, it is also possible to adjust the gradient so that the method can be applied to other DSs, which is another great advantage.

In a next step, we will now try to investigate other non-sartans for NAs and modify another detector that far exceeds the selectivity of the mass spectrometer, to detect NAs in even smaller amounts and prove the advantages of SFC technology for pharmaceutical quality control. Additionally, further NAs will be integrated to generate a complex and comprehensive group monograph for nitrosamines.

Acknowledgments

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