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Determination of lycopene in tissues and plasma of rats by normal-phase high-performance liquid chromatography with photometric detection

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Abstract

An analytical method for the determination of lycopene in tissues and plasma of rats is described. The method was validated for the determination of lycopene in liver and plasma with respect to selectivity, linearity, accuracy, recovery and precision. Following precipitation of proteins with water–ethanol plasma was extracted with hexane; tissues were extracted with acetone followed by precipitation of proteins with water–ethanol and extraction of lycopene with hexane. Separation and quantification of geometrical isomers of lycopene was achieved by normal-phase HPLC with UV/VIS detection at 471 nm. The method proved to be selective and specific for lycopene in plasma and liver. Detector response was linear in the range from 2 ng/g to 10 μg/g liver and 0.5 ng/ml to 2 μg/ml plasma, respectively. Average recoveries ranged from 96 to 101% in spiked liver samples and from 91 to 94% in spiked plasma samples. Intra-day variability (C.V.) was ≤6% and ≤5% in liver and plasma, respectively. Inter-day precision was ≤9% for liver samples and ≤6% for plasma samples. The procedures were successfully applied to the sample analysis of pharmacokinetic and metabolism studies.

Keywords: Lycopene

1. Introduction

Lycopene, the major carotenoid present in tomatoes, is an acyclic, non-provitamin A carotenoid consisting only of carbon and hydrogen atoms. Tomatoes and tomato products are the main dietary source of lycopene for man. Interest in lycopene grew within the last years, since recent epidemiological studies associated the intake of tomatoes or tomato-based products with decreased risk of disease and various types of cancers [1–6]. There is some evidence that lycopene may account for or at least may contribute to these benefits. Results from Gann et al. and Rao et al. revealed significantly lower lycopene plasma levels in subjects that developed prostate cancer compared to age- and smoking status-matched controls [7,8]. Recently the effects of lycopene-enriched tomato oleoresin on breast cancer were investigated using the 7,12-dimethyl-benz-[*a*]anthracene-induced rat mammary cancer model. In this study the oleoresin-treated rats developed fewer tumors, and the tumor area was smaller than that of unsupplemented rats [9]. It has been sug-

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gested that the beneficial health effects of lycopene are related to its pronounced ability to act as antioxidant [10–12], but other mechanisms like intercellular gap junction communication and metabolic pathways may also be involved [6].

High-performance liquid chromatography with UV-Vis detection is a rapid, robust, sensitive, and selective method to determine lycopene and other carotenoids in a wide variety of matrices. C_{18}-type stationary phases are probably the most employed stationary phases for RP-HPLC separations. However their ability to separate carotenoid stereoisomers is limited. There have been several reports in which separation of *cis*/*trans* lycopene isomers has been achieved on C_{30} columns [13–16]. Also, NP phases generally possess great shape selectivity towards geometric carotenoid isomers. Applications of Hengartner et al. and Schierle et al. included the use of a silica-based stationary phase which provided good separation of lycopene stereoisomers [17,18]. However, these methods required long phase lengths with the combination of up to three 250×4.6-mm columns to give adequate separation.

To support our in-house pharmacokinetic and metabolism studies we developed an analytical method for the determination of lycopene isomers in plasma and different tissues such as liver, spleen, kidney, muscle, or fat. The procedure involves extraction of the analyte from plasma or tissues and quantitative determination by NP-HPLC with UV-Vis detection. Using an alumina-based NP stationary phase, major lycopene isomers present in tissues and plasma could be baseline separated. In the present report we describe the validation of the method for plasma and liver samples of rats.

2. Experimental

2.1. Materials, chemicals and reagents

A lycopene standard (Ro-01-9251/000; Lot No. 12911 B116 RS5) containing (all-*E*)-lycopene as the major component (approx. 97%) and (13*Z*)-, (5*Z*)-, (9*Z*)-, and other (*Z*)-isomers as minor components was from Hoffmann–La Roche (Switzerland). Reference compounds of (all-*E*)- and (5*Z*)-lycopene were also from Hoffmann–La Roche. The antioxidant BHT (2,6-di-*tert*.-butyl-4-methylphenol) was purchased from Sigma (Germany). Basic aluminum oxide (activity I) for chromatography was obtained from Camag (Switzerland). All solvents were p.a. or HPLC grade from Merck (Germany). To remove traces of acids and other impurities from the solvents, hexane, and CH_2Cl_2 were further purified on basic aluminum oxide in an open column.

Blank plasma and liver containing no detectable amount of the analyte was obtained from rats fed with a lycopene-free diet.

2.2. Standard solutions

Stock solutions of lycopene were prepared by dissolving approximately 3 mg of carotenoid together with 250 mg BHT in 20 ml CH_2Cl_2. After equilibration to room temperature the volume was adjusted to 100 ml with hexane. Immediately afterwards the exact concentration was determined by photometric measurement at 471 nm using an $E^{1\%}_{1\ cm}$ value of 3450. Stored in an amber flask at −20°C the stock solutions were stable for at least 3 weeks. Using this storage conditions only minor isomerization was observed. The contribution of (all-*E*)-lycopene to the sum of all isomers declined by 2–3% within 4 weeks. The percentage of (*Z*)-isomers increased by roughly this amount. Working and calibration solutions of lycopene were freshly prepared each day and were obtained by dilution from the stock solution with hexane. Prior to each use the concentrations of stock and calibration solutions were photometrically checked.

For the identification of (all-*E*)- and (5*Z*)-lycopene single reference compounds were available. (9*Z*)-, (13*Z*)-, and other (*Z*)-isomers present in the lycopene standard were identified by comparing the isomer profile with the isomer profile of the standard recorded on a nuclear magnetic resonance (NMR)-controlled HPLC system [17]. Additional information was obtained from photodiode array spectra.

2.3. Chromatography

High-performance liquid chromatography was performed on a Hewlett-Packard Series 1050 HPLC system equipped with an HP autosampler and two independently working HP pumps. Chromatographic

peaks were detected by a Jasco UV-Vis detector (model 970) adjusted to 471 nm or an HP Series 1050 diode-array detector. Hewlett-Packard Chem-Station software was used for peak integration and calculation. Lycopene isomers were separated on a LiChroCART Aluspher Al 100 column (250×4 mm, 5 μm particle size, Merck) protected by a Spherisorb silica guard cartridge (30×4.6 mm, 5 μm particle size, Phase Separations).

Peaks were eluted at a flow-rate of 1.0 ml/min by a gradient specified in Table 1 using the following mobile phases: (A) hexane, (B) hexane–CH_2Cl_2–dioxane (85:12:3, v/v), (C) hexane–CH_2Cl_2–dioxane (37:60:3, v/v). Following each run the column was equilibrated with 100% A) for 15 min to ensure a high reproducibility of retention times.

During the separation of the analytes on the analytical column, the guard column was flushed with hexane–CH_2Cl_2–acetone–2-propanol–*n*-ethyldiisopropylamine (70:20:10:0.5:0.05, v/v) in the backflush mode for 5 min, followed by 5 min equilibration with hexane containing 0.05% 2-propanol. The independent flow of solvent through the guard column was achieved using a switching valve and a second LC-pump.

2.4. Calibration and quantification

Freshly prepared calibration solutions were repeatedly injected into the HPLC system. Peak areas of (all-*E*)-lycopene and relevant (*Z*)-isomers present in the standard were added and their mean value resulting from all injections divided by the spectrometrically measured lycopene concentration of the solutions. The resulting response factor was used to calculate the contents of all geometrical isomers. Results were presented as sum of all isomers. The criterion for (*Z*)-isomers to be used for calibration and quantification were acceptable UV-Vis-spectra recorded by continuous flow diode-array detection. As (all-*E*)-lycopene counts for approximately 97% in the standard, the response factor is mainly dominated by this isomer. As a consequence the amount of (*Z*)-isomers with a lower specific absorption than (all-*E*)-lycopene, e.g. (9*Z*)- and (13*Z*)-lycopene [18], are underestimated by this method.

Table 1
Gradient system used to elute lycopene isomers: following each run the column was equilibrated with 100% A for 15 min

Time (min)	A (%)	B (%)	C (%)
0.00	100		
2.50	100		
2.51	80	20	
4.50	80	20	
14.50	50	50	
17.00	50	50	
17.01			100
25.00			100

2.5. Sample preparation

2.5.1. Liver

Liver was homogenized with a Virtis and a 0.8-g aliquot was placed in a conical glass flask together with 8 ml of the extraction solution (acetone containing 0.25% BHT). After vortex mixing (2 min) the sample was cooled in ice for 25 min and centrifuged at 13 000 *g* at 4°C for 10 min. The supernatant was transferred to a glass tube and the pellet extracted a second time as described before. The combined acetone phases were evaporated to dryness under a stream of N_2 at 30°C, redissolved in 2 ml ethanol–water (1:1, v/v), and extracted twice with hexane (4 and 2 ml). Each extraction step was carried out by vortex mixing for 2 min followed by centrifugation at 13 000 *g* (10 min, 4°C). After evaporating the combined organic phases to dryness under a stream of N_2 the residue was reconstituted in a final volume of 1 ml hexane–CH_2Cl_2 (1:1, v/v). Possible turbidity was removed by centrifugation prior to injection of a 100-μl aliquot into the HPLC system.

2.5.2. Other tissues

Tissues of spleen, small intestine, kidney, heart, lung, brain, fat, muscle, skin, eye, testis, adrenal and thyroid gland were minced to small particles using a pair of scissors. A total of 0.8 g of the material was homogenized together with 6.0 ml of extraction solution (acetone containing 0.25% BHT) in a polytron mixer. After mixing, the polytron was rinsed with 2.0 ml of extraction solution. The homogenate and the rinsing solution were combined, cooled on ice for 25 min and further treated as described for the

liver samples. The final volume of the injection solution varied from 150 to 500 μl depending on the amount of lycopene to be expected in the different tissues. 100-μl aliquots of the solutions were injected for analysis.

2.5.3. Plasma

A total of 0.8 ml water, 1.6 ml ethanol, and 3.2 ml hexane (0.025% BHT) were added to 0.8 ml plasma. The mixture was vortexed for 2 min and then centrifuged at 1800 *g* at 4°C for 10 min. After transferring the hexane layer to a conical glass flask the aqueous suspension was extracted again with 3.2 ml hexane as described above. Following evaporation to dryness of the combined hexane phases under a stream of nitrogen at 30°C the residue was taken up in 3×300 μl hexane–CH_2Cl_2 (1:1, v/v) and quantitatively transferred to an autosampler vial. Again the sample was evaporated to dryness with N_2 and the residue was dissolved in a final volume of 250 μl hexane–CH_2Cl_2 (1:1, v/v). 100 μl of the solution were injected for analysis.

3. Results and discussion

3.1. Selectivity and specificity

The selectivity of the method was determined by evaluating the chromatography of relevant carotene reference substances. None of the carotenes analyzed produced peaks at the retention times of the lycopene isomers.

Blank plasma and liver samples as well as blank samples spiked with lycopene at high and low concentrations (quality control, QC, samples) were extracted and assayed as described before to determine the specificity of the method. The chromatograms of blanks and QC samples were visually inspected for peaks from endogenous substances that might interfere with lycopene isomers. Chromatograms of liver and plasma samples are provided in Figs. 1 and 2, respectively. Neither in plasma nor in liver samples could interference from endogenous compounds be observed.

3.2. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

The relationship between analyte concentration and detector response was evaluated to confirm linearity. Standard solutions of lycopene were prepared over the range of 1.7–11091 ng/ml at eight concentrations. Sets of standard solutions were injected in duplicate on four different days within a period of 4 weeks. Good linearity was observed over the concentration range from 1.7–8319 ng/ml ($y=3.4918x+98.912$, $R^2=0.9991$). At higher concentration levels (data point at 11091 ng/ml) the calibration curve is slightly bent, resulting in a higher *y*-intercept (368.18) and a worse correlation coefficient ($R^2=0.9956$). The coefficient of variance (C.V.) was less than 8% for all concentration levels showing good reproducibility.

At a concentration of 0.8 ng/ml standard solution the signal-to-noise (S/N) ratio was about 3. Using this concentration, LODs were assessed at 1 ng/g liver and at 0.3 ng/ml plasma for the sample preparation procedures described before. Using the lowest concentration of the linear calibration range as LOQ (1.7 ng/ml), back-calculations yielded LOQs of 2.1 ng/g for liver and 0.5 ng/ml for plasma. The upper limit of calibration (ULC) was calculated to be 10.4 μg/g liver and 2.6 μg/ml plasma, respectively. In cases the DAD was utilized for peak detection, the LOQ was at 60 ng/ml standard solution. At this concentration chromatographic peaks showed satisfactory UV-Vis spectra.

3.3. Accuracy

Accuracy was determined using the data sets of the linearity studies. Concentrations of the standards were calculated from the mean peak areas (mean of four data sets) and were compared to the nominal concentrations. Accuracy was calculated as percentage deviation of back-calculated concentrations from nominal concentrations. Table 2 contains a summary of the accuracy data. The deviation from theoretical values is below 11% at all concentration levels studied. Deviations are highest (−10.9%) for the lowest calibration standard (1.7 ng/ml). In the range from 16.6 to 11091 ng/ml differences between

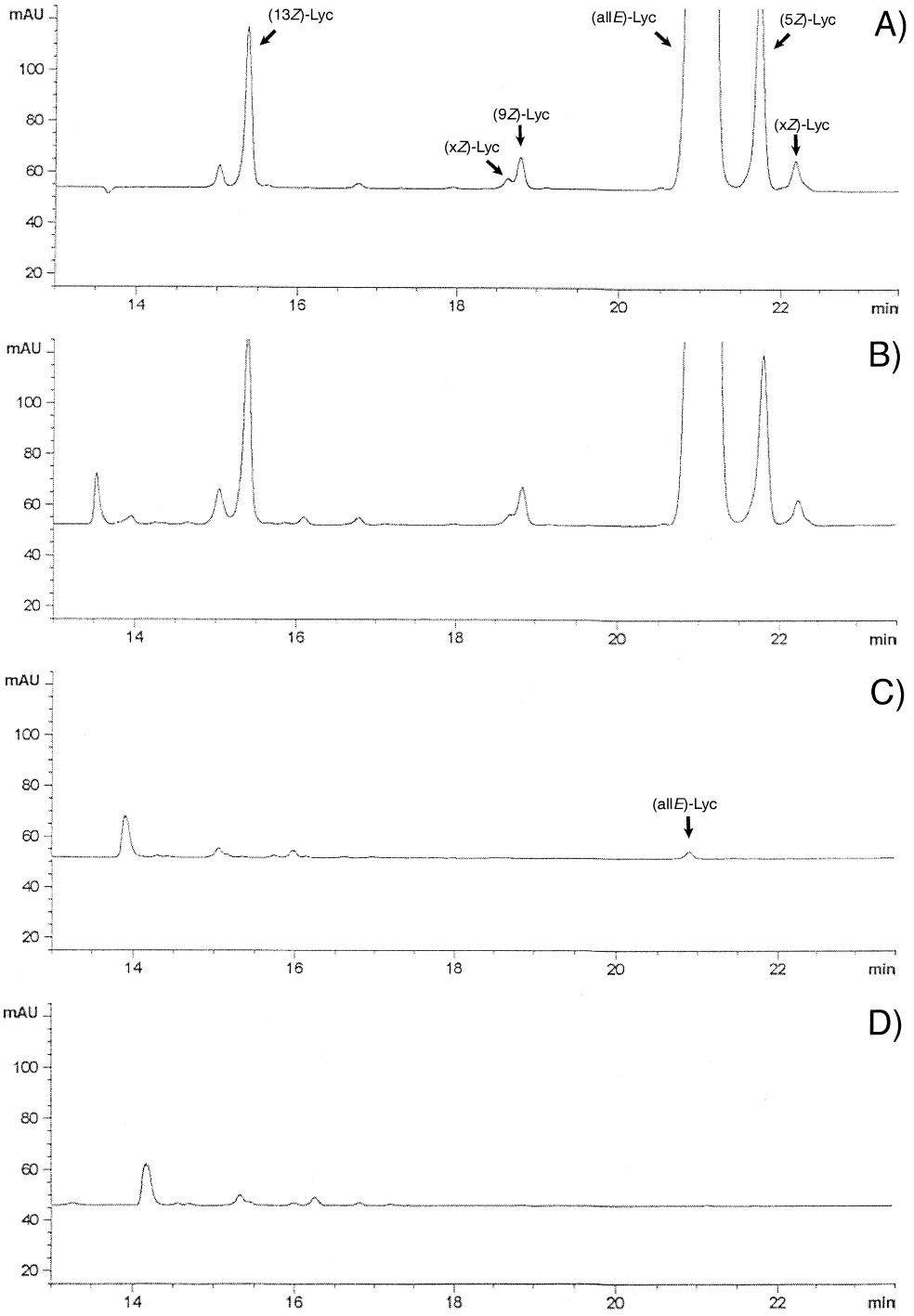

Fig. 1. NP-HPLC chromatograms of a lycopene standard (A), blank liver spiked with lycopene at high (B) and low concentrations (C), and blank liver (D).

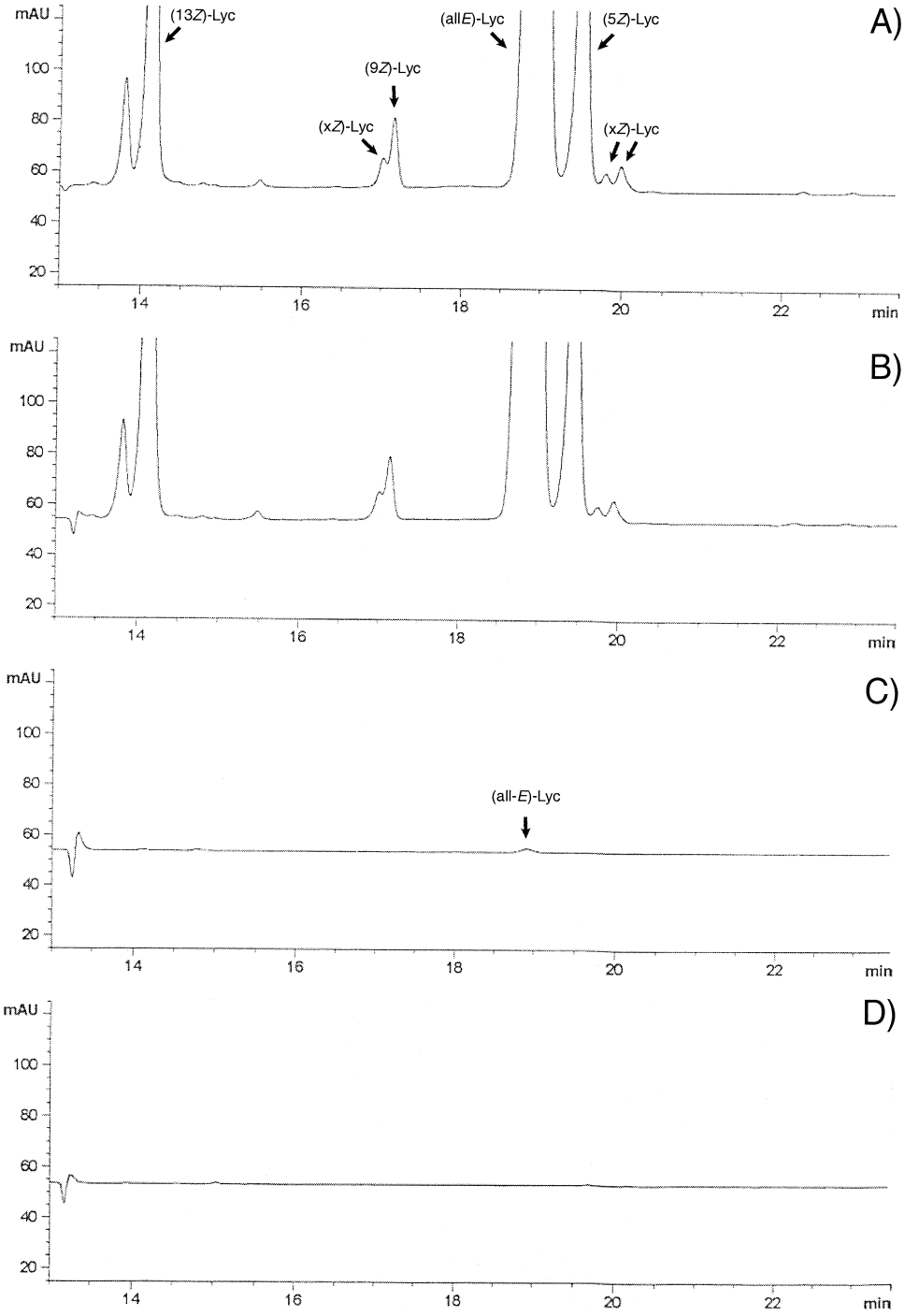

Fig. 2. NP-HPLC chromatograms of a lycopene standard (A), blank plasma spiked with lycopene at high (B) and low concentrations (C), and blank plasma (D).

Table 2
Accuracy of the determination of lycopene over the concentration range of 1.7–11091 ng/ml: accuracy is expressed as percentage deviation of back-calculated concentrations from nominal concentrations

Nominal concentration of standards (ng/ml)	Observed concentration of standards (mean±SD, ng/ml)	C.V. (%)	Accuracy (% deviation)
11091	10249±548	5.3	−7.6
8319	8282±316	3.8	−0.4
6655	6935±84	1.2	4.2
3328	3493±19	0.5	5.0
333	353±3	0.9	6.2
166	175±2	1.0	5.4
16.6	16.8±0.5	3.2	1.0
1.7	1.5±0.1	7.3	−10.9

nominal and observed concentrations are less than 8%.

3.4. Recovery

Recoveries from liver and plasma at two concentrations (high and low calibration range) were studied on five different days. Blank liver was spiked with lycopene to tissue concentrations of approximately 6 and 8000 ng/g, respectively. Plasma concentrations were roughly 1.5 and 1800 ng/ml, respectively. The resulting QC samples were prepared and analyzed as described in Section 2. At each concentration level and for each sample type recoveries were determined in pentuplicate on day 1 and in triplicate on four subsequent days. Percentage recoveries were calculated by comparing the amount of lycopene found in the QC samples to the amount added.

The results of the recovery studies are summarized in Table 3. The mean recovery of lycopene from liver was 96.3% at 8000 ng/g and 101.2% at 6 ng/g. From plasma 90.9% of the spiked lycopene was recovered at high concentrations and 93.6% was found at low concentrations. The coefficient of variance was less than 6% within single study days as well as within the whole study period showing good intra- and inter-day repeatability.

3.5. Precision (intra- and inter-day)

Intra- and inter-day variability was assessed using the data of the recovery studies. Spiked plasma and liver samples (QC samples) at two concentrations each were assayed on five different days. On day 1 five replicate samples and on days 2–5 three replicate samples of each concentration and matrix were prepared and analyzed using the sample preparation and quantitation methods described before. Intra- and inter-day variability was expressed as coefficient of variation. Inter-day precision was calculated from the

Table 3
Recoveries of high and low level lycopene from liver and plasma

			Days					
			1 (n=5)	2 (n=3)	3 (n=3)	4 (n=3)	5 (n=3)	1–5 (n=17)
Liver	High (8000 ng/g)	Mean±SD (%)	96.2±1.9	92.5±2.3	96.6±1.8	98.4±1.8	97.6±2.5	96.3±2.6
		C.V. (%)	2.0	2.5	1.9	1.8	2.6	2.8
	Low (6 ng/g)	Mean±SD (%)	104.9±4.2	95.1±3.2	100.6±2.8	102.1±2.1	100.9±5.8	101.2±4.8
		C.V. (%)	4.0	3.4	2.8	2.1	5.8	4.7
Plasma	High (1800 ng/ml)	Mean±SD (%)	90.8±2.7	87.3±2.7	89.1±1.1	90.5±3.0	96.7±1.3	90.9±3.7
		C.V. (%)	3.0	3.1	1.2	3.3	1.3	4.1
	Low (1.5 ng/ml)	Mean±SD (%)	87.4±3.4	100.0±4.6	96.7±3.8	94.2±1.9	94.0±2.3	93.6±5.5
		C.V. (%)	3.9	4.6	3.9	2.0	2.4	5.9

Table 4
Intra- and inter-day variability of the determination of lycopene in liver

		Intra-day precision					Inter-day precision
	Days:	1 ($n=5$)	2 ($n=3$)	3 ($n=3$)	4 ($n=3$)	5 ($n=3$)	1–5 ($n=5$)
High	Concentration found	8157	8246	7580	7321	7741	8075
	found	7848	8661	7865	7142	8034	8462
	(ng/g liver)	8188	8479	7685	7399	8138	7710
		8211					7287
		7972					7971
	Mean±SD (ng/g)	8075±158	8462±208	7710±144	7287±132	7971±206	7901±437
	C.V. (%)	2.0	2.5	1.9	1.8	2.6	5.5
Low	Concentration found (ng/g liver)	7.8	6.9	6.4	6.2	6.1	7.4
		7.2	6.4	6.4	6.1	5.6	6.6
		7.1	6.6	6.8	5.9	6.2	6.5
		7.3					6.1
		7.5					6.0
	Mean±SD (ng/g)	7.4±0.3	6.6±0.2	6.5±0.2	6.1±0.1	6.0±0.3	6.5±0.6
	C.V. (%)	4.0	3.4	2.8	2.1	5.8	8.6

mean values of the single study days. The results of the precision assay are summarized in Table 4 for liver samples and in Table 5 for plasma samples. Intra-day precision ranged from 1.8 to 2.6% in liver at high concentrations and from 2.1 to 5.8% in liver samples spiked at low concentrations. Day-to-day variability was 5.5 and 8.6%, respectively, in liver at high and low concentrations. In plasma spiked at high concentrations the values for intra-day precision were in the range from 1.2 to 3.3%; at low concentrations coefficients of variation were determined from 2.0 to 4.6%. Inter-day precision for the determination of lycopene in plasma was found to be 4.5 and 5.8%, respectively. The data indicate that the

Table 5
Intra- and inter-day variability of the determination of lycopene in plasma

		Intra-day precision					Inter-day precision
	Days:	1 ($n=5$)	2 ($n=3$)	3 ($n=3$)	4 ($n=3$)	5 ($n=3$)	1–5 ($n=5$)
High	Concentration found	1861	1828	1778	1858	1969	1840
	(ng/ml plasma)	1768	1839	1802	1935	2022	1801
		1866	1737	1758	1812	1990	1779
		1904					1868
		1800					1994
	Mean±SD (ng/ml)	1840±55	1801±56	1779±22	1868±62	1994±26	1856±84
	C.V. (%)	3.0	3.1	1.2	3.3	1.3	4.5
Low	Concentration found	1.3	1.5	1.5	1.5	1.5	1.3
	(ng/ml plasma)	1.4	1.6	1.6	1.6	1.4	1.5
		1.3	1.5	1.6	1.5	1.5	1.6
		1.3					1.5
		1.3					1.5
	Mean±SD (ng/ml)	1.3±0.1	1.5±0.1	1.6±0.1	1.5±0.0	1.5±0.0	1.5±0.1
	C.V. (%)	3.9	4.6	3.9	2.0	2.5	5.8

assay method is reproducible within the same day and within different days; coefficients of variation are less than 10% for all matrices over the concentration range assayed.

3.6. Stability

To check the stability of lycopene during the maximum time of analysis of a run sequence, lycopene standards in a completely filled autosampler tray were measured. As the autosampler used in this study was not equipped with a thermostatting device, samples were kept at room temperature. Stability was calculated as percentage of observed concentrations (sum of all isomers) to the nominal, photometrically measured concentration of standards. Deviations in samples measured 12 h after sequence start were within the precision of the method (Table 6). However, the contribution of single isomers to the total lycopene concentration changed with time. As shown in Table 6, (all-*E*)-lycopene undergoes isomerization to mainly (13*Z*)-lycopene, whereas the percentage of (9*Z*)- and (5*Z*)-lycopene is almost constant. The decline of (all-*E*)-lycopene in favor of (13*Z*)-lycopene is roughly 4–5% within 12 h. These findings concur with results from Schierle et al. who reported an isomerization of the (all-*E*)- to primarily the (13*Z*)-isomer during analytical sample preparation [18].

As a consequence from these results it has to be noted, that the quantitative determination of lycopene isomers is subjected to minor errors if large numbers of samples are analyzed in an unthermostatted autosampler.

Table 6
Stability during HPLC analysis at room temperature

Time in autosampler (h:min)	Change in concentration (%)	Contribution of single isomers to total lycopene (%)			
	Sum of isomers	all-*E*	13*Z*	9*Z*	5*Z*
0:00	0.0	97.4	0.6	0.2	1.8
1:51	−0.1	96.8	1.1	0.2	1.9
3:05	−0.5	96.3	1.5	0.2	1.9
4:19	−1.4	95.9	1.9	0.3	1.9
5:33	−1.6	95.4	2.4	0.3	1.9
6:47	−2.1	95.0	2.8	0.3	2.0
8:01	−3.0	94.4	3.3	0.3	2.0
9:15	−3.1	94.1	3.6	0.3	2.0
10:29	−2.4	93.2	4.4	0.3	2.1
11:43	−3.2	92.6	4.8	0.5	2.1

3.7. Application

The procedures for the determination of lycopene in rat plasma and liver were successfully applied to the analysis of samples from pharmacokinetic and metabolism studies. In a comparative study of lycopene tissue distribution in rat the method was applied to other tissue samples such as spleen, small intestine, kidney, heart, lung, brain, fat, muscle, skin, eye, testis, adrenal and thyroid gland.

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