Applications

Note 240 | August 2011

Technical Report

Time savings as well as improved reproducibility through centrifugation at 30,000 x *g*

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Abstract

Protocol optimization can contribute substantially to increased laboratory productivity. During sample preparation, centrifugation steps claim a large proportion of the available time. Therefore, these are ideally suited for optimization. During the course of analysis of veterinary drugs, the Centrifuge 5430 R, in combination with Eppendorf Safe-Lock Tubes, achieved a reduction in centrifugation time of up to 75 % at 30,130 x *g*. Furthermore, the measured values showed improved reproducibility compared to standard protocols.

Introduction

This Application Note describes standard sample preparations, which were employed to test the use of the Centrifuge 5430 R (Eppendorf) in comparison with a laboratory centrifuge by a different manufacturer. The analysis protocols performed herein serve the detection of pharmacologically active substances (veterinary drugs) in food of animal origin, per LC/MSMS (liquid chromatography tandem mass spectrometry). Specifically, sample preparation protocols for the detection of metabolites of nitrofurans, sulfonamides and tetracyclines are introduced. These are substances with antibiotic activity. Only one parameter was changed per experiment.

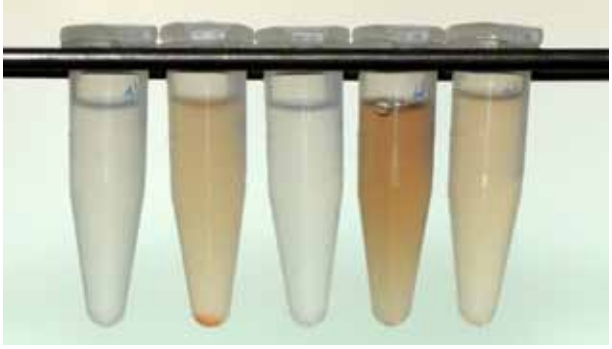

Figure 1: The diversity of matrices used in the analysis of veterinary drugs warrants optimization of sample preparation. Photo: T. Krenz.

Materials and Methods

Experiment I: Sample preparation for the detection of sulfonamides and tetracyclines in shrimp
During this analysis process, antibiotic residuals are extracted from the homogenized sample using an acetonitrile/water/formic acid mix, centrifuged briefly (3,220 x *g*), filtered and concentrated to 0.5 mL.
A water/acetonitrile/formic acid mix is then added to a total weight of 3 g and mixed well, followed by centrifugation at 4 °C in 1.5 mL Eppendorf Safe-Lock Tubes at maximum *g*-force (laboratory centrifuge 1: 16,060 x *g*, Centrifuge 5430 R (Eppendorf): 30,130 x *g*) until a clear solution is obtained.

Experiment IIa: Sample preparation for the detection of nitrofuran metabolites in muscle meat (salted chicken muscle; fortified samples)
For this method, the four nitrofuran metabolites 3-amino-2-oxazolidinone (AOZ), 1-aminohydantoin (AHD), 3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ) and semicarbazide (SEM) are derivatized using 2-nitrobenzaldehyde. Following pH adjustments, the derivatives are extracted with 20 mL ethyl acetate and centrifuged at 3,220 x *g* for 15 min. The supernatant is transferred to a test tube and dried using a parallel evaporator (Syncore, Büchi). The residue is solubilized in 200 µL methanol/water and mixed carefully. This solution is transferred to a 1.5 mL Eppendorf Safe-Lock Tube and centrifuged at maximum g-force (laboratory centrifuge 1: 16,060 x *g*, Centrifuge 5430 R: 30,130 x *g*) at 4 °C until a clear intermediate phase can be removed.

Experiment IIb: Comparison of data reproducibility
After one hour centrifugation at 4 °C, the solvent phase (from experiment IIa) is pipetted into an amber glass vial with micro-insert and measured with LC/MSMS (HP 1100 Agilent Technologies/Quattro Micro, Waters Corporation). Separation was achieved with a Luna C18 column (Phenomenex) (150 x 2 mm, 5 µm particle size) with methanol/ammonium acetate buffer as the mobile phase (flow rate: 0.2 mL/min, injection volume 10 µL). The LC/MSMS was operated in MRM mode (ESI pos.), the following transitions were detected: AOZ: m/z 236 > 104; AMOZ: m/z 335 > 262; AHD: m/z 249 > 134; SEM: m/z 209 > 166. The reproducibility of the concentrations of the nitrofuran metabolites was tested via comparison of the coefficients of variation.

Results and Discussion

Experiment I: Detection of sulfonamides and tetracyclines in shrimps
In this sample preparation the solvent phase is separated from the solid matrix components via centrifugation, in order to be able to analyze it subsequently for sulfonamides and tetracyclines. Figure 2 shows the tubes after 15 and again after 60 minutes of centrifugation (left: laboratory centrifuge 1) and after 15 minutes of centrifugation, respectively (right: Centrifuge 5430 R by Eppendorf).

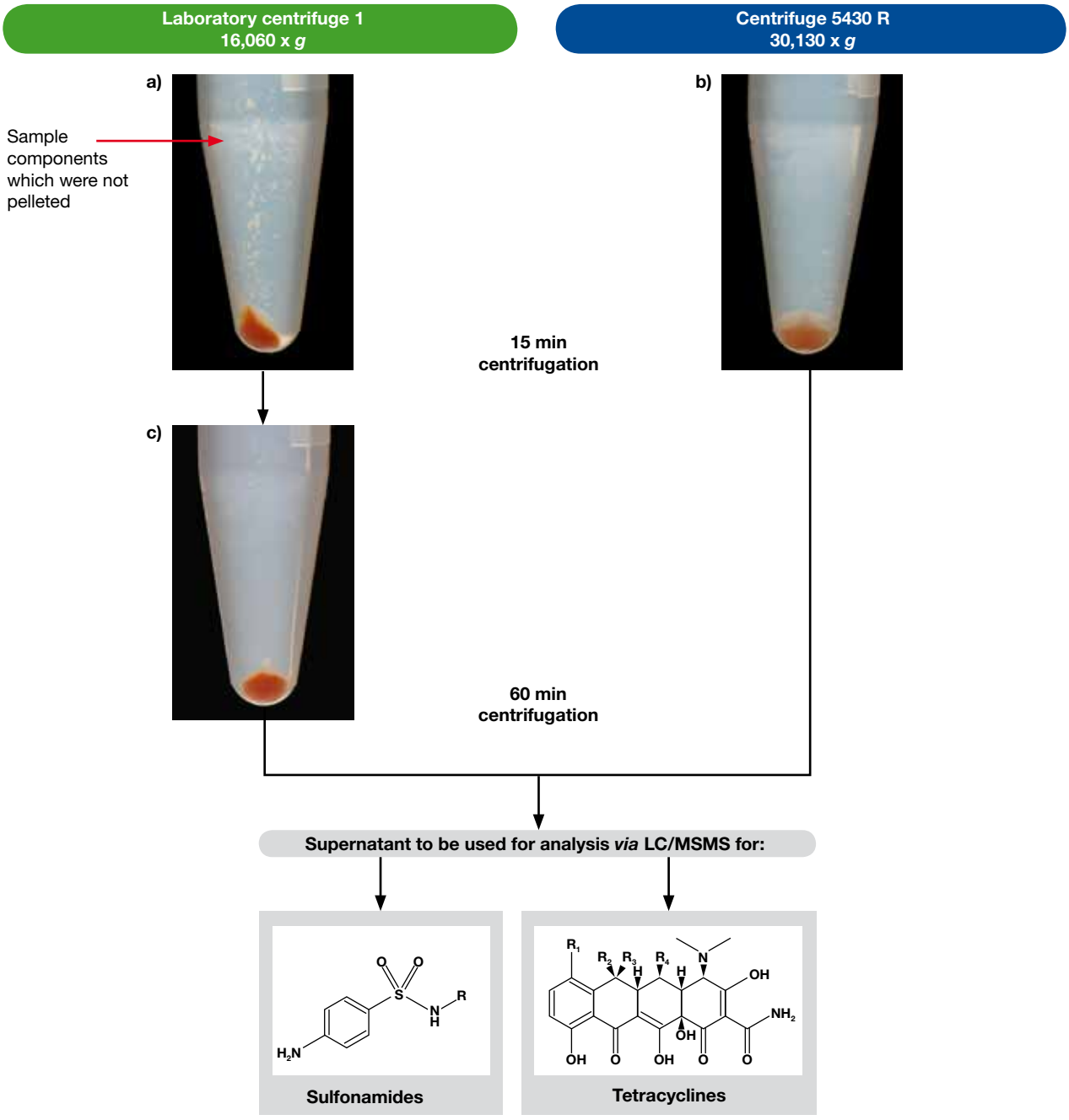

Figure 2: Matrix shrimps extracted following protocol I. After 15 minutes in laboratory centrifuge 1 (a) and Eppendorf Centrifuge 5430 R (b); following 60 min centrifugation in laboratory centrifuge 1 (c). Photos: T. Krenz.

For the matrix shrimps, matrix components were clearly visible inside the tube after 15 min centrifugation time at 16,060 x *g* (fig. 2a). These components partially adhered to the tube wall and would be transferred to the measurement vial due to pipette suction. This could contribute to contamination of subsequently used analysis instruments (e.g. LC/MSMS), thus increasing their maintenance. The Centrifuge 5430 R yielded a satisfactory result within the same time frame (fig. 2b). The supernatant could be used directly for analysis. In contrast, the laboratory centrifuge 1 (16,060 x *g*) took 60 min until the sample supernatant was sufficiently clear for further analysis. Thus, for the sample matrix shrimps, the time required for the centrifugation step could be reduced by up to 75 %.

Experiment IIa: Sample preparation for the detection of nitrofuran metabolites in muscle meat (salted chicken muscle, fortified samples)

The sample preparation for experiment IIa describes the final centrifugation step prior to analysis of nitrofuran metabolites in salted chicken muscle meat. Following extraction and centrifugation, a lipid phase is visible on top. The intermediate layer is the solvent phase to be analyzed. The bottom of the tube holds the solid matrix components which are present in a more or less compact pellet. Figure 3 shows the results after 15 or 60 minutes centrifugation time (laboratory centrifuge 1) and after 15 minutes centrifugation time (Centrifuge 5430 R), respectively.

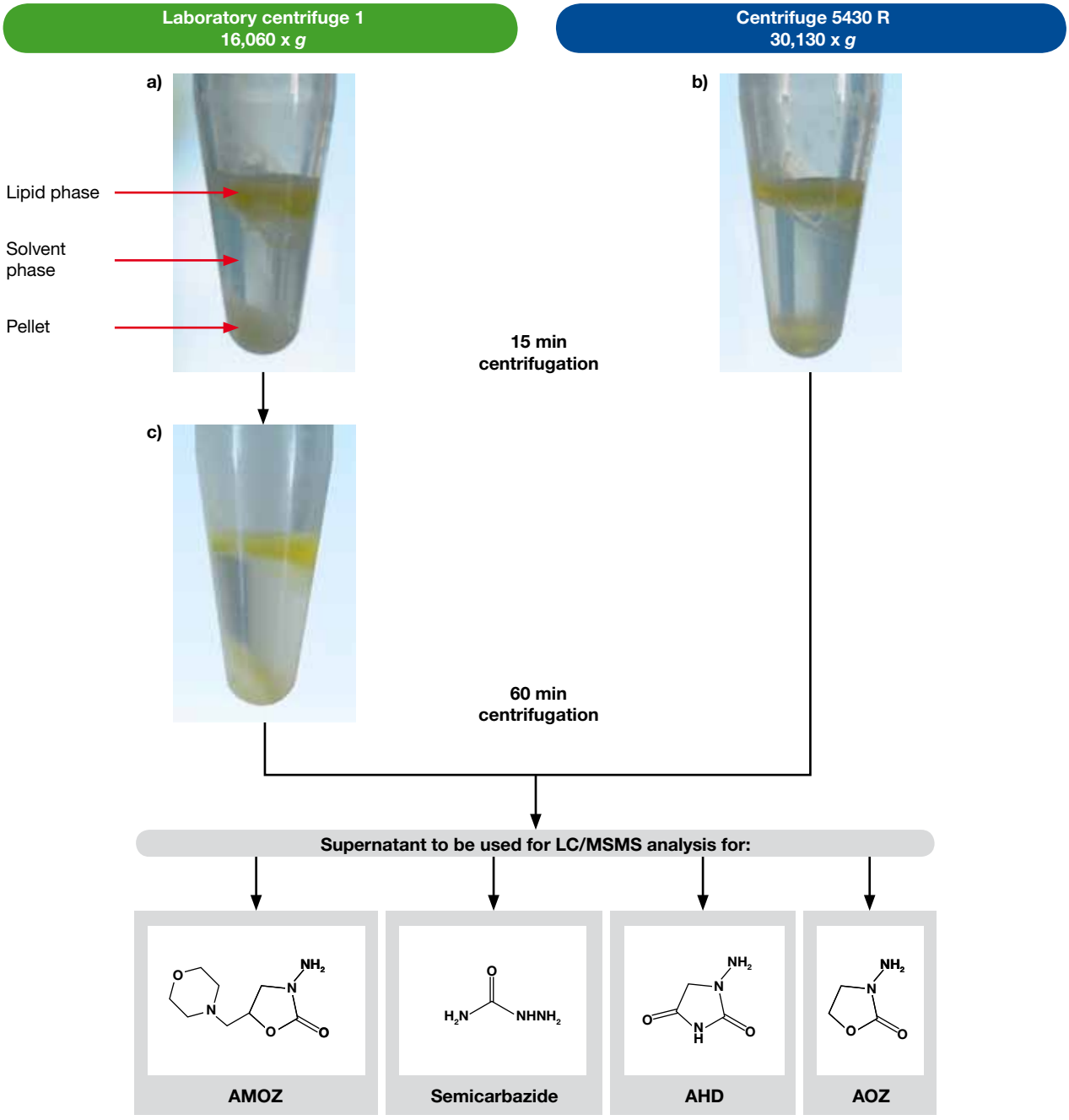

Figure 3: Matrix salted chicken muscle, extracted with experimental protocol IIa. Following 15 min centrifugation in laboratory centrifuge 1 (a) and Eppendorf Centrifuge 5430 R (b); following 60 min centrifugation in laboratory centrifuge 1 (c). Photos: T. Krenz.

In the Centrifuge 5430 R (fig. 3b) the matrix components were enriched in a more compact and tight pellet. After only 15 min, the intermediate solvent phase could be removed without the risk of carrying over pellet components which could potentially contaminate subsequent analysis instruments (e.g. LC/MSMS). For the laboratory centrifuge 1, this step was only possible after 60 min centrifugation (fig. 3c). Thus, the time required for the centrifugation step of the matrix to be analyzed could be reduced by up to 75 % for experiment IIa as well.

Experiment IIb: Comparison of data reproducibility

For sufficient phase separation to be achieved, centrifugation with laboratory centrifuge 1 took one hour in experiment IIa. Therefore, for the purpose of comparison of reproducibility, the measured results from both centrifuges were compared after this maximum centrifugation time (=1 h).

This experimental design was used to test whether the qualitatively visible improved phase separation achieved with the Centrifuge 5430 R at 30,130 x *g* would also lead to improved reproducibility at the data analysis level. Peak areas, as well as parameter concentrations calculated from matrix standard curves, were compared. To this end, quadruple determination of two separate preparations (A and B) of a sample of salted chicken muscle meat were considered, with regards to the following metabolites: 3-amino-2-oxazolidinone (AOZ), 1-aminohydantoin (AHD), 3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ) and semicarbazide (SEM) (attachment 1). The coefficient of variation, which is smaller for more reproducible values, was used as a measure of reproducibility (table 1).

Table 1: Summary of the coefficients of variation for all four metabolites, analyzed by the same method (AOZ, AHD, AMOZ, SEM) following sample preparation II; calculated for laboratory centrifuge 1 and the Centrifuge 5430 R, respectively.

Metabolite	Preparation	Laboratory centrifuge 1	Centrifuge 5430 R
		Coefficient of variation [%]	
AOZ	A	14.4	5.6
	B	12.9	1.2
AMOZ	A	4.4	1.1
	B	6.0	1.9
AHD	A	5.5	3.8
	B	2.2	3.0
SEM	A	4.6	2.0
	B	4.3	3.4

The use of the Centrifuge 5430 R (30,130 x *g*) considerably improves reproducibility of the residuals of the 4 tested antibiotics (only exception: preparation B, measurement of AHD). For laboratory centrifuge 1, the coefficient of variation is between 2.2 and 14.4 %, and for Centrifuge 5430 R it is between 1.1 and 5.6 %. Considering the fact that the varied centrifugation is only one of many steps during sample preparation, while all other preparation steps were performed with identical instruments, the gain in reproducibility is remarkable.

Conclusion

The Eppendorf system of Centrifuge 5430 R and the 1.5 mL Eppendorf Safe-Lock Tubes enables centrifugation at 30,130 x *g*. During the analysis of veterinary drugs, this system led to improved phase separation performance. Thus, centrifugation times could be reduced by up to 75 % for different protocols. Furthermore, data reproducibility could be improved. Further protocol optimizations using the Centrifuge 5430 R and the 1.5 mL Eppendorf Safe-Lock Tubes are planned.

Attachment 1: Comparison of reproducibility of measured values for laboratory centrifuge 1 and the Centrifuge 5430 R following sample preparation IIb. The antibiotic metabolites AOZ, AHD, AMOZ and SEM from two separate sample preparations of salted chicken muscle were taken into consideration. (conc. = concentration)

			Laboratory centrifuge 1		Centrifuge 5430 R	
Substance	**Preparation**	**Injection**	**Peak area**	**Conc. [µg/kg]**	**Peak area**	**Conc. [µg/kg]**
AOZ	A	1	2724	0.24	3591	0.31
	A	2	2851	0.30	3620	0.30
	A	3	2639	0.25	3676	0.34
	A	4	2593	0.33	3559	0.31
		Mean	2702	0.28	3612	0.31
		Standard deviation	113.3	0.04	49.7	0.02
		Coefficient of variation [%]	4.2	**14.4**	1.4	**5.6**
Substance	**Preparation**	**Injection**	**Peak area**	**Conc. [µg/kg]**	**Peak area**	**Conc. [µg/kg]**
AOZ	B	1	2937	0.27	3058	0.23
	B	2	2502	0.20	3060	0.24
	B	3	2898	0.27	3000	0.24
	B	4	2877	0.26	3037	0.23
		Mean	2804	0.25	3039	0.23
		Standard deviation	202.5	0.03	27.8	0.00*
		Coefficient of variation [%]	7.2	**12.9**	0.9	**1.2**
Substance	**Preparation**	**Injection**	**Peak area**	**Conc. [µg/kg]**	**Peak area**	**Conc. [µg/kg]**
AMOZ	A	1	1138	0.52	1191	0.49
	A	2	1152	0.53	1169	0.48
	A	3	1219	0.57	1198	0.49
	A	4	1111	0.54	1140	0.48
		Mean	1155	0.54	1175	0.48
		Standard deviation	45.9	0.02	26.1	0.01
		Coefficient of variation [%]	4.0	**4.4**	2.2	**1.1**
Substance	**Preparation**	**Injection**	**Peak area**	**Conc. [µg/kg]**	**Peak area**	**Conc. [µg/kg]**
AMOZ	B	1	1086	0.48	1038	0.38
	B	2	1122	0.53	937	0.40
	B	3	1112	0.50	881	0.39
	B	4	993	0.47	924	0.39
		Mean	1078	0.49	945	0.39
		Standard deviation	58.8	0.03	66.5	0.01
		Coefficient of variation [%]	5.5	**6.0**	7.0	**1.9**

*because of truncation

			Laboratory centrifuge 1		Centrifuge 5430 R	
Metabolite	**Preparation**	**Injection**	**Peak area**	**Conc. [µg/kg]**	**Peak area**	**Conc. [µg/kg]**
AHD	A	1	1004	0.64	1341	0.67
	A	2	926	0.57	1409	0.67
	A	3	998	0.62	1267	0.62
	A	4	926	0.65	1262	0.64
		Mean	964	0.62	1320	0.65
		Standard deviation	43.4	0.03	69.6	0.02
		Coefficient of variation [%]	4.5	**5.5**	5.3	**3.8**
Metabolite	**Preparation**	**Injection**	**Peak area**	**Conc. [µg/kg]**	**Peak area**	**Conc. [µg/kg]**
AHD	B	1	957	0.58	1184	0.54
	B	2	996	0.60	1136	0.57
	B	3	977	0.60	1144	0.57
	B	4	960	0.61	1135	0.58
		Mean	973	0.60	1150	0.57
		Standard deviation	18.0	0.01	23.2	0.02
		Coefficient of variation [%]	1.8	**2.2**	2.0	**3.0**
Metabolite	**Preparation**	**Injection**	**Peak area**	**Conc. [µg/kg]**	**Peak area**	**Conc. [µg/kg]**
SEM	A	1	594	0.48	732	0.47
	A	2	608	0.47	742	0.49
	A	3	560	0.47	766	0.50
	A	4	531	0.43	766	0.49
		Mean	573	0.46	752	0.49
		Standard deviation	34.6	0.02	17.2	0.01
		Coefficient of variation [%]	6.0	**4.6**	2.3	**2.0**
Metabolite	**Preparation**	**Injection**	**Peak area**	**Conc. [µg/kg]**	**Peak area**	**Conc. [µg/kg]**
SEM	B	1	619	0.47	676	0.41
	B	2	618	0.47	734	0.45
	B	3	591	0.45	708	0.44
	B	4	568	0.43	706	0.42
		Mean	599	0.45	706	0.43
		Standard deviation	24.4	0.02	23.7	0.02
		Coefficient of variation [%]	4.1	**4.3**	3.4	**3.4**

Ordering information

Description	Order no. international	Order no. North America
Eppendorf Safe-Lock Tubes 1.5 mL	0030 120.086	022363204
Eppendorf Safe-Lock Tubes 1.5 mL, PCR clean	0030 123.328	022363212
Eppendorf Safe-Lock Tubes 1.5 mL, Biopur®	0030 121.589	022600028
Eppendorf Safe-Lock Tubes 1.5 mL, DNA LoBind	0030 108.051	022431021
Eppendorf Safe-Lock Tubes 1.5 mL, Protein LoBind	0030 108.116	022431081

Description	Order no. international		Order no. North America	
	Keypad (230 V)	Knob (230 V)	Keypad (120 V)	Knob (120 V)
Centrifuge 5430 with 30 x 1.5/2.0 mL aerosol tight fixed angle rotor FA-45-30-11	5427 000.216	5427 000.410	022620509	022620511
Centrifuge 5430, without rotor	5427 000.011	5427 000.615	022620584	022620596
Centrifuge 5430 R (refrigerated) with 30 x 1.5/2.0 mL aerosol tight fixed angle rotor FA-45-30-11	5428 000.015	5428 000.414	022620601	022620623
Centrifuge 5430 R (refrigerated), without rotor	5428 000.210	5428 000.619	022620667	022620689
Rotor FA-45-24-11-HS, incl. rotor lid, aerosol tight, PTFE-coated	5427 710.000		022654080	

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