

DOSINACO™ : Fully automated LC-MS/MS analysis of anticoagulants using a novel reagent kit

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1. Introduction

Novel oral anticoagulants (NOACs) are, as an alternative therapy to vitamin K antagonists (VKAs), used frequently to treat and prevent thromboembolism. Their precise quantitation is necessary to identify the presence/absence of an anticoagulant effect or to determine the concentration of drug that may be helpful for patient management.

Such analysis is mainly done by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). To streamline the workflow, Alsachim has developed a complete reagent kit including stable isotope labeled standards for better precision. Furthermore, we demonstrate here the use of a fully automated sample preparation system "CLAM-2000" (Shimadzu Corporation) coupled online with the LC-MS/MS.

2. Method development

To demonstrate that this multi-analyte approach, with a fully automated system LC-MS/MS, can be used as a walk-away unit, we have used a novel kit for anticoagulants analysis called DOSINACO™ (Alsachim SAS). The kit includes 9 analytes (Acenocoumarol, Apixaban, Argatroban, Betrixaban, Dabigatran, Edoxaban, Fluindione, Rivaroxaban, Warfarin) and their corresponding Stable Isotope Labeled Standards used as internal standard. All parameters, mobile phases, column, Labeled Stable Isotopes Standards, and Controls are delivered together with the kit. The CLAM-2000 (Shimadzu Corporation) was coupled to a tandem MS model LCMS-8045 (Shimadzu Corporation). The CLAM-2000 was programmed to perform protein precipitation followed by filtration and sample collection. The sample is then transported using an arm from the CLAM-2000 to the HPLC without human intervention for LC-MS/MS analysis. The extracted samples were analyzed using a C18 column in 5 min.

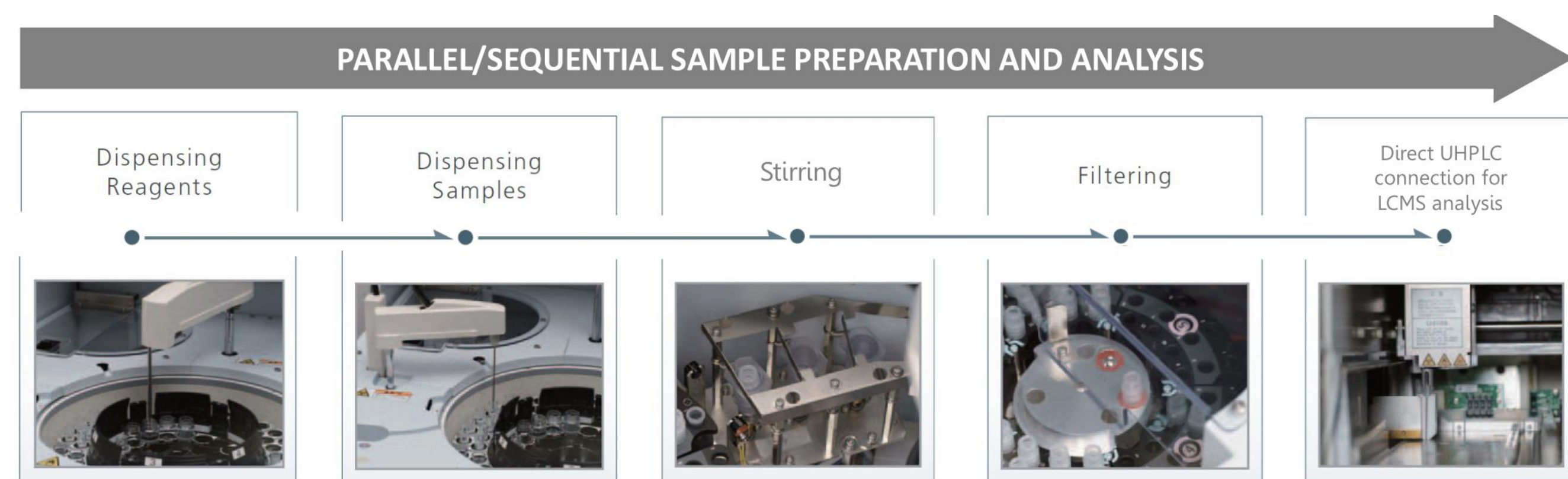


Figure 1: CLAM-2000 sample preparation

The manual sample preparation is time consuming and could be affected by bias caused by the operator due to the liquid transfer steps that are required (Figure 2), moreover it is difficult to maintain the traceability of each step for all the processed samples. Using the CLAM-2000 it was possible to obtain a complete integration of sample preparation steps with the LC-MS/MS quantification.

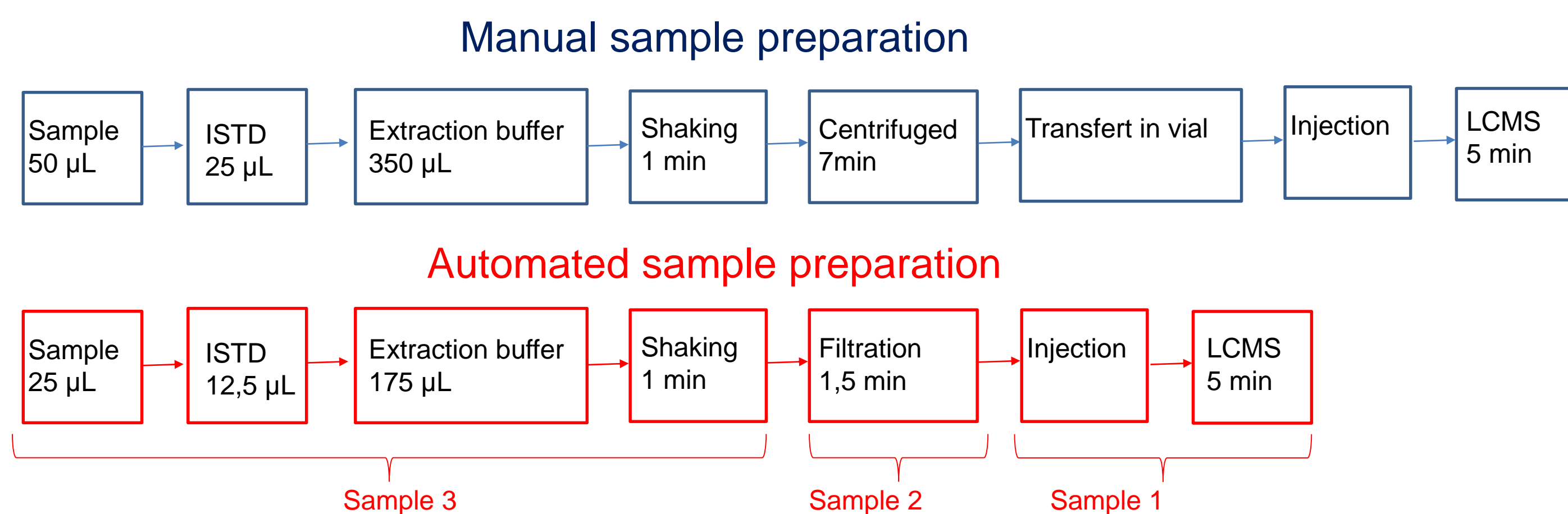


Figure 2: CLAM-2000 fully automated sample preparation and analysis - Due to the overlapped sample preparation, the throughput of the instrument was 1 result each 5 minutes for quantification of all Compounds.

LC	NEXERA X2 system
Column	DOSINACO™ Column C18 2,1x50 mm, 5 µm
Column Temperature	50°C
Mobile Phases	A: H2O 5mM Ammonium formate 0,1% Formic Acid B: MeOH 5mM Ammonium formate 0,1% Formic Acid
Flow rate	0.6 mL/min
Time Program	0-0,5 min: 5%B; 0,5-0,51 min: 20%B; 0,51-2,20 min: 40%B; 2,20-3 min: 100%B; 3-4 min: 100%B; 4-4,01 min: 5%B; 4,01-5 min: 5%B
Injection volume	2 µL
MS	LCMS-8045
Ionization	ESI positive
Nebulizer gas	3 L/min
Interface temperature	400°C
Desolvation line	250°C
Heat Block temperature	500°C
Drying Gas	5 L/min
Heating Gas	15 L/min
Analysis mode	MRM

Table 1: Analytical conditions and source parameters.

Compounds	Quantifier MRM	Qualifier MRM	ISTD	Quantifier MRM	Qualifier MRM
Acenocoumarol	353,90 → 163,20	353,90 → 296,25	[² H ₄]-Acenocoumarol	357,90 → 167,30	357,90 → 300,30
Apixaban	459,90 → 443,30	459,90 → 199,25	[¹³ C, ² H ₂]-Apixaban	468,10 → 451,40	468,10 → 199,20
Argatroban	509,20 → 384,10	509,20 → 70,00	[¹³ C ₆]-Argatroban	515,00 → 390,35	515,00 → 112,30
Betrixaban	452,20 → 324,10	452,20 → 279,00	[¹³ C ₆]-Betrixaban	457,90 → 330,25	457,90 → 285,30
Dabigatran	471,90 → 289,30	471,90 → 144,30	[¹³ C ₆]-Dabigatran	478,10 → 295,25	478,10 → 312,35
Edoxaban	547,90 → 366,10	547,90 → 152,15	[² H ₆]-Edoxaban	554,00 → 372,40	554,00 → 158,30
Fluindione	241,00 → 194,05	241,00 → 175,15	[¹³ C ₆]-Fluindione	247,00 → 220,20	247,00 → 181,15
Rivaroxaban	435,80 → 144,90	435,80 → 231,10	[¹³ C ₆]-Rivaroxaban	441,90 → 145,10	441,90 → 237,35
Warfarin	308,90 → 163,25	308,90 → 251,25	[² H ₆]-Warfarin	315,00 → 163,20	315,00 → 257,30

Table 2: MRM transitions for Anticoagulants (left) and Stable Isotope Labeled Standard (right)

3. Results

The linearity and accuracy of the method was evaluated using 6 reference plasma calibrator levels DOSINACO™. For all the analytes linearity and accuracy were within the analytical acceptable range (90.6%-108.6%). Furthermore in order to estimate the precision of the method, reference plasma control DOSINACO™ were analyzed several times (6 replicates per day during 3 days). For all analytes the CV% values were within acceptable analytical ranges.

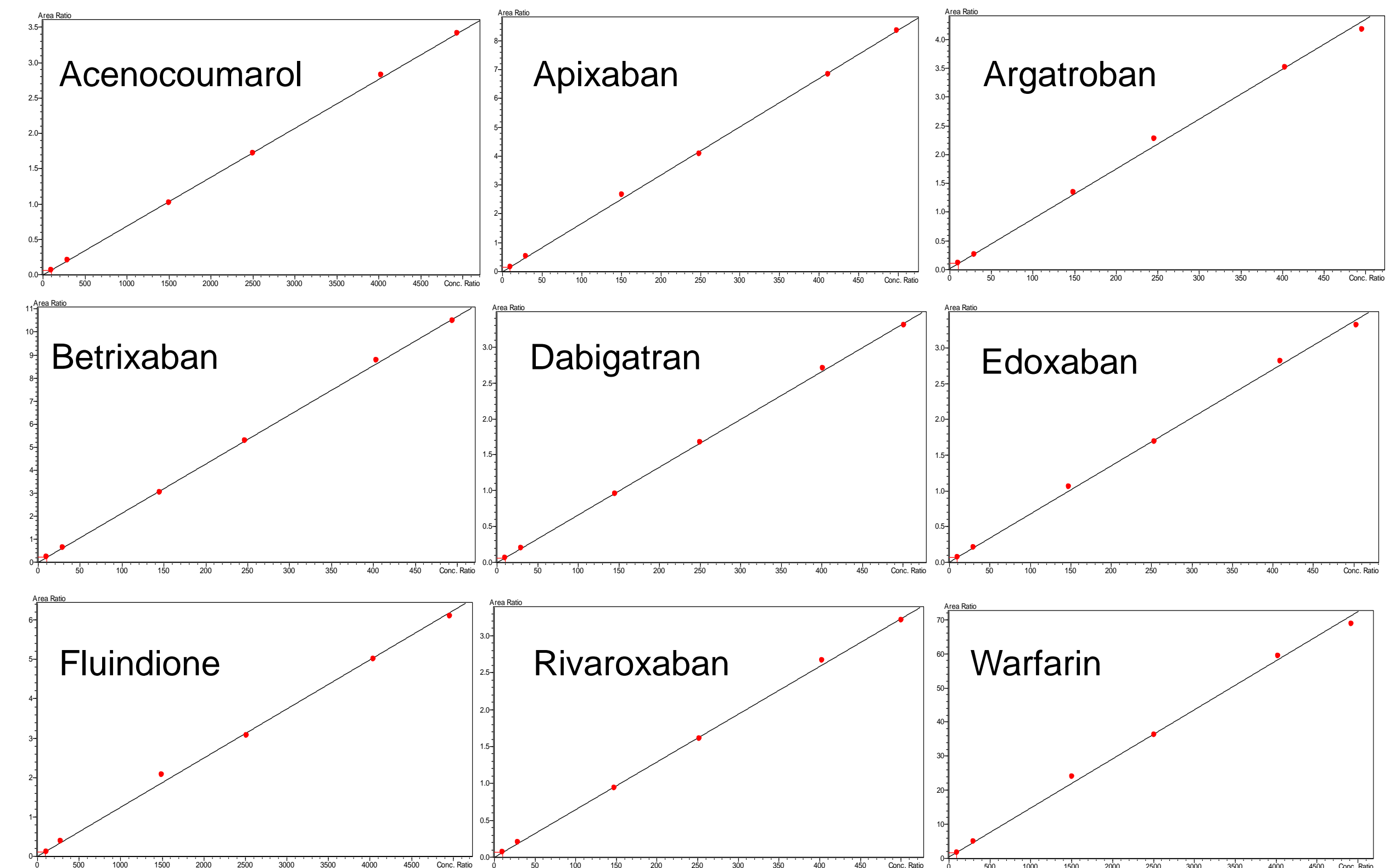


Figure 3: Calibration curves of the Anticoagulants

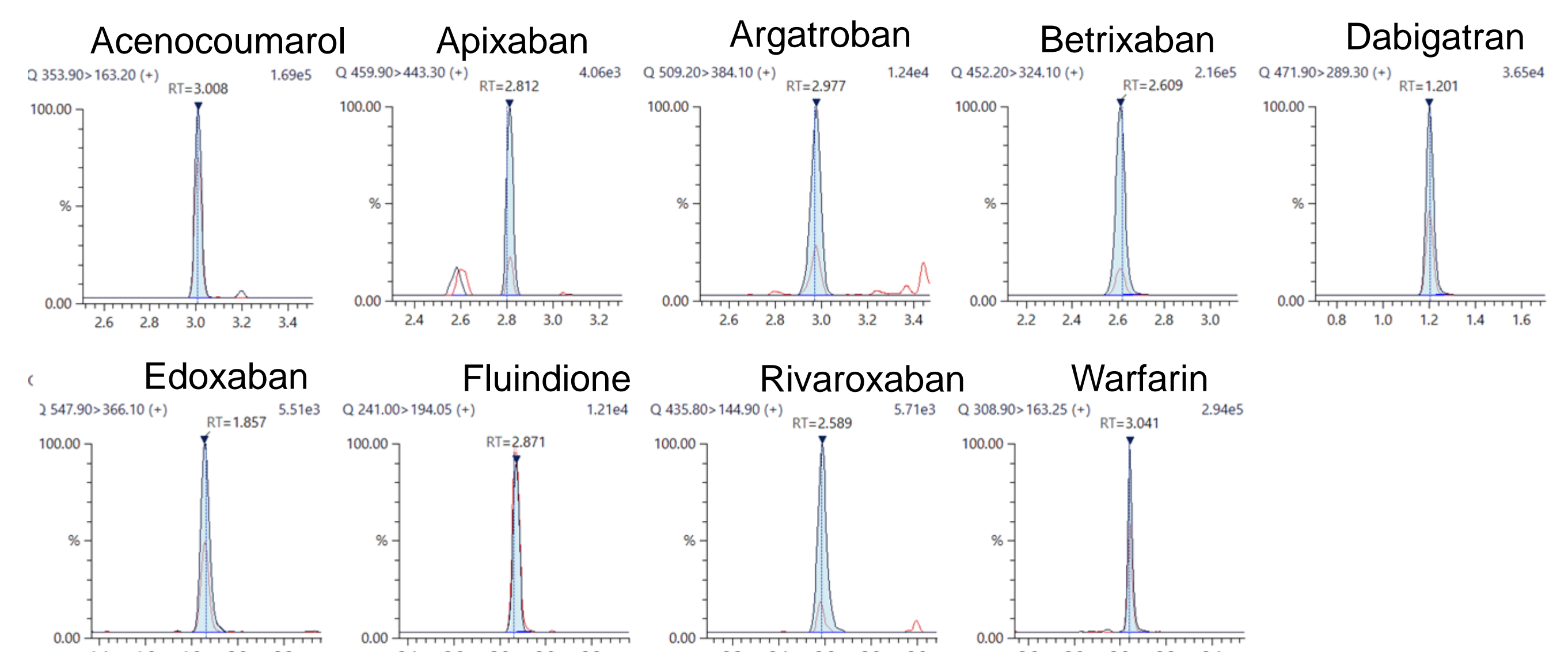


Figure 4: Chromatograms of the Anticoagulants at the Level 1

Compounds	Range (µg/L)	R2	Accuracy (%)			
			QC 1	QC 2	QC 3	QC 4
Acenocoumarol	100-5000	0,999	104,2	99,2	101,3	104,1
Apixaban	10-500	0,999	107,1	106,0	104,3	109,6
Argatroban	10-500	0,999	96,1	95,5	93,7	95,8
Betrixaban	10-500	0,999	98,3	94,1	94,2	96,1
Dabigatran	10-500	0,999	106,6	104,4	103,6	107,8
Edoxaban	10-500	0,999	101,2	103,8	103,1	104,3
Fluindione	100-5000	0,998	116,9	113,9	108,7	108,0
Rivaroxaban	10-500	0,999	99,3	99,9	100,2	102,6
Warfarin	100-5000	0,998	99,5	103,2	96,6	96,1

Table 3: Linearity and Accuracy evaluated using DOSINACO™ plasma controls

Compounds	CV (%)							
	QC 1		QC 2		QC 3		QC 4	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
Acenocoumarol	1,8	2,6	2,0	2,0	2,6	3,0	3,2	2,1
Apixaban	8,0	7,5	6,5	6,7	8,1	6,6	4,2	3,3
Argatroban	5,6	5,3	4,8	4,3	3,3	3,2	3,6	3,6
Betrixaban	1,3	2,3	3,0	2,8	2,2	2,7	1,7	2,2
Dabigatran	2,2	2,7	2,8	2,4	3,4	3,1	1,3	1,5
Edoxaban	3,8	4,7	2,6	2,3	2,2	3,0	1,9	1,7
Fluindione	7,0	7,3	5,1	5,4	3,1	4,1	2,5	2,1
Rivaroxaban	6,7	8,5	4,3	4,4	4,8	4,5	2,1	3,5
Warfarin	4,4	5,6	3,7	7,5	8,3	6,7	4,2	4,2

Table 4: Repeatability evaluated using DOSINACO™ plasma controls: intra-day n=6; inter-day n=3

4. Conclusion

Fully Automated sample preparation procedure led to suitable results for the quantitation of anticoagulants thus eliminating all manual preparation steps. The novel system workflow results in easier and safer operation for users even without Chromatography and Mass Spectrometry experience, thus reducing risk of exposure. It allows to access and analyse hundreds of analytes on the same system without any modification thus improving the quality of service delivered to doctors for quick decision.

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