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Advanced Multi-Residue Screening in Veterinary Drug Analysis Using UPLC-ToF MS

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Abstract

This application note demonstrates the feasibility of a truly generic LC-MS method for the analysis of potentially unlimited contaminants with minimal sample preparation. UPLC-ToF MS offers several advantages for such analysis over other techniques. The Waters ACQUITY UPLC System utilizes novel column packing material with a 1.7 μm particle size, operated at elevated pressure to obtain maximum speed and resolution from a chromatographic run. The Waters LCT Premier oa-ToF mass spectrometer with W-Optics and DRE offers unparalleled full spectral sensitivity with <3ppm mass accuracy across a wide dynamic range. The data produced by this combination is rich in information, and has the advantage of being untargeted.

Benefits

- The method presented is suitable for the screening of multiple veterinary drug residues in real matrix samples

- Spectra with high mass accuracy and correct isotope ratios are valuable aids to the identification of contaminants, and may be appropriate for confirmatory analysis

Introduction

A wide variety of veterinary drugs are currently available for antibiotic use in livestock around the world. In many countries, including EU member states and the US, testing for these compounds in animal based food products is mandatory and the permissible concentration levels are strictly regulated. Due to the complexity of matrices (e.g. muscle tissues, urine, milk) and the large number of potential analytes, it is beneficial to use a generic analytical method applicable to a diverse range of samples, which can be reprocessed post-acquisition if 'food scares' arise.

Traditionally, veterinary drug residues in biological matrices are analyzed by microbiological or immunochemical techniques. While these tests can provide a rapid and cost effective screening method for certain classes of compound, they are generally only suitable for one analyte or class, or do not give quantitative results. Currently, therefore, a large number of single residue or group-specific methods of analysis are required in order to analyze a wide array of veterinary drugs.

HPLC with UV or fluorescence detection has been used for quantitation and confirmation of screening results for some time, but in order to obtain sufficient sensitivity and selectivity, extensive cleanup of the samples is often required. More recently, in response to and in anticipation of stricter legislation, many analysts have taken advantage of the increased quantitative and confirmatory capabilities of LC-MS/MS.

However, the increased experimental setup and inherently selective nature of multiple reaction monitoring (MRM) type experiments are restrictive when analytes of interest have not been targeted in the initial sample analysis. This makes post acquisition reprocessing and the discovery of unknown contaminants practically impossible.

This application note demonstrates the feasibility of a truly generic LC-MS method for the analysis of potentially unlimited contaminants with minimal sample preparation. UPLC-ToF MS offers several advantages for such analysis over other techniques. The Waters ACQUITY UPLC System utilizes novel column packing material with a 1.7 µm particle size, operated at elevated pressure to obtain maximum speed and resolution from a chromatographic run.[1] The Waters LCT Premier oa-ToF mass spectrometer with W-Optics and DRE offers unparalleled full spectral sensitivity with <3ppm mass accuracy across a wide dynamic range. The data produced by this combination is rich in information, and has the advantage of being untargeted. Waters ChromaLynx Application Manager, an optional feature of Waters MassLynx Software, quickly and efficiently deconvolutes mass spectra from complex chromatograms to present the user with a readily accessible and easy to interpret dataset. Screening is carried out by comparison to a limitless library of experimental and/or theoretical spectra and further confirmation of identified compounds is presented at-a-glance by accurate mass scoring.
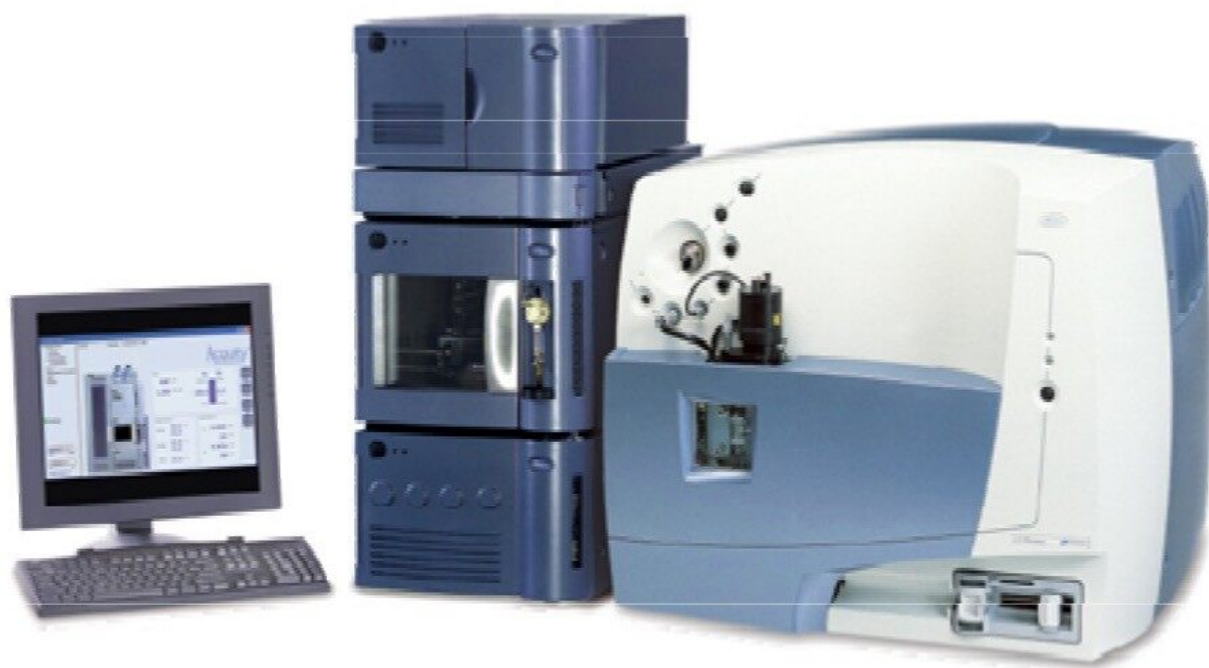
Waters ACQUITY UPLC with LCT Premier Mass Spectrometer.

Experimental

Sample Preparation

Milk (1 mL) was mixed with acetonitrile (1 mL) to effect precipitation of proteins. The supernatant was isolated and diluted five-fold with water and was ultrafiltered over a disposable UF device with a molecular weight cut-off (MWCO) of 30 kDa. An aliquot of 16.6 µL of the ultrafiltrate was injected in the UPLC-ToF system without further sample purification.

Extracted matrix matched standard solutions were prepared with 41 compounds from five classes (tetracyclins, sulfonamides, quinolones, macrolides and benzimidazoles) at 0.5, 1, 2, 5, 10, 25, 50, and 100 µg/L concentrations by adding the appropriate analytes to milk samples prior to processing. These concentrations cover the relevant concentration range with respect to the maximum residue limit (MRL) of the analytes. The spiked samples were allowed to rest for at least 30 minutes before extraction was started to allow adequate equilibration of analytes with the sample matrix.

LC Conditions

LC system:	Waters ACQUITY UPLC
Mobile phase A:	0.1% formic acid
Mobile phase B:	Acetonitrile
Column:	Waters ACQUITY BEH C_{18} 1.7 µm, 2.1 x 50 mm
Column temp.:	40 °C
Flow rate:	350 µL/min
Injection volume:	16.6 µL (calibrated)
Cycle time:	9 minutes

Gradient

Time (min)	%B
0.0	0
1.0	0
6.0	40
7.5	100

MS Conditions

Instrument:	Waters LCT Premier
Ion mode:	Electrospray +
Capillary V:	3200 V
Aperture 1 V:	8 V and 30 V
Source T:	120 °C
Desolvation T:	400 °C
Gas Flow:	600 L/hr
Mass range:	100–1000 Da
Resolution:	10000 FWHM (W-mode)
Acq. time:	0.2 s
Calibration:	$NaCH_2O_2$
LockSpray:	Leucine Enkephalin
Reference:	$[M+H]^+ = 556.2771 Da$

Results and Discussion

Prior to software-assisted processing, the data were interrogated manually to determine the sensitivity on spiked compounds in milk. Using this targeted approach for known analytes also enabled an investigation of the quantitative capability of the UPLC-ToF system. Figure 1 shows good linearity was observed and that the LoD/LoQ are much lower than the MRL concentration.
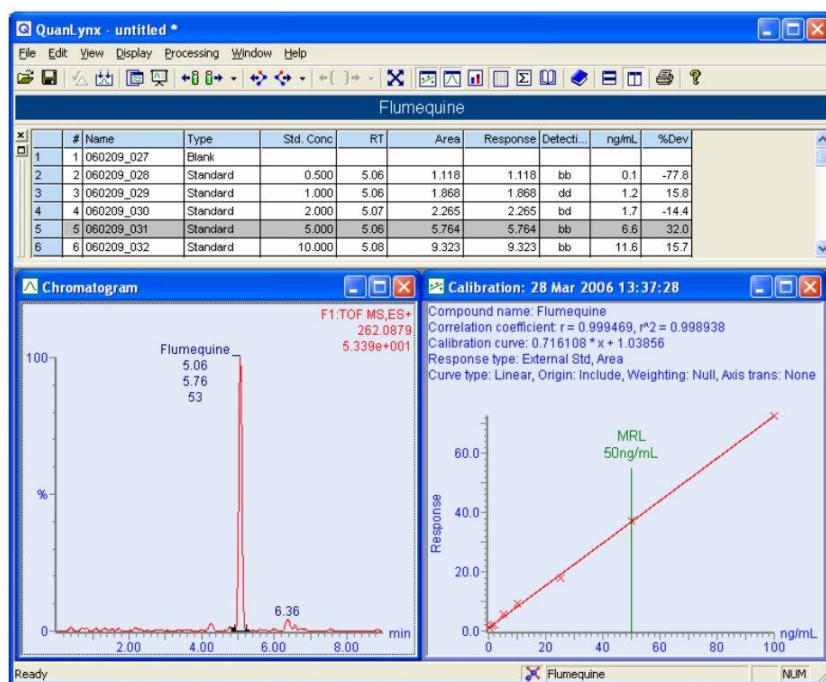

Figure 1. Quantitation of Flumequine. Chromatogram shown is 0.1 x MRL concentration.

Spectra from standard solutions were acquired with an aperture voltage of 8 V and refined in order to create clean library entries with retention time data; and also at 30 V to obtain representative fragments by in-source CID. All spectra were quality checked using i-FIT software, which compares the measured and theoretical isotope ratios to give a score based on the proximity of the match.[2] For the purposes of this study, spectra should have a score of <30. A library was created containing a normal MS spectrum and at least one fragmented spectrum for each compound using the standard library feature in MassLynx. This was then converted to the NIST format with ChromaLynx, which allows filtering of the matched spectra for screening purposes. Figure 2 shows the MassLynx library editor, where compound name, formula and molecular weight are entered as well as optional filtering parameters such as retention time, polarity and cone voltage.
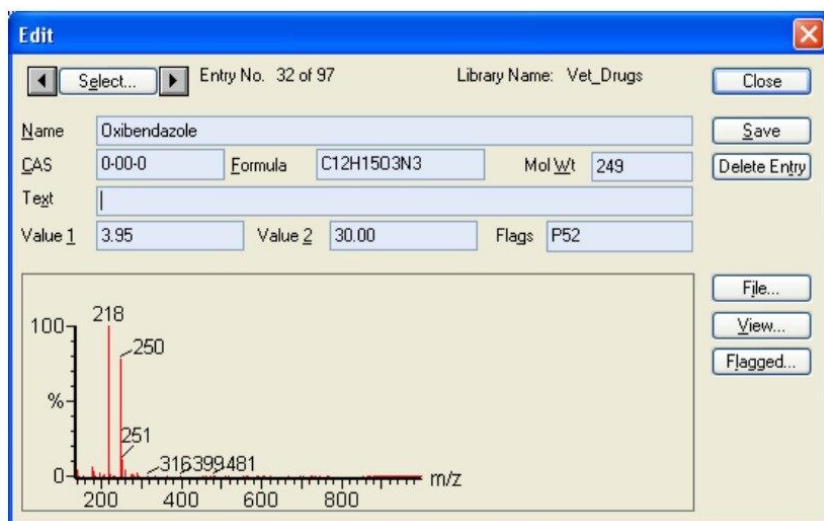
Figure 2. MassLynx library editor.

The ChomaLynx Application Manager has been designed to efficiently deconvolute spectra from complex chromatograms using either automatic or manual peak detection and spectral refinement. Setting up a method is simply a matter of specifying the n most intense ions and a mass window (either in Da or ppm) and the software will deconvolute the data by extracting these masses.

Other parameters, as shown in Figure 3, allow a user to specify peak width and noise limits; rejection factors to avoid multiple occurrences of the same component and internal standard detection parameters if semi-quantitative work is required. A library search method is also created which specifies which libraries to use; how many ions to search against; maximum number of hits and any screening parameters. With these methods in place, a search or a screen can be performed using nominal masses, and is therefore not restrictive as an exactmass library would be. Instead, an extra suite of accurate mass parameters is included which allows the n most intense ions from a deconvoluted mass spectrum to be scored by proximity to the theoretical exact mass of the compound from its library formula. Using this method, not only can a library hit be confirmed at-aglance, but any fragments formed by in-source CID also have an elemental composition based on that of the parent ion assigned to them, which makes structural elucidation much easier.
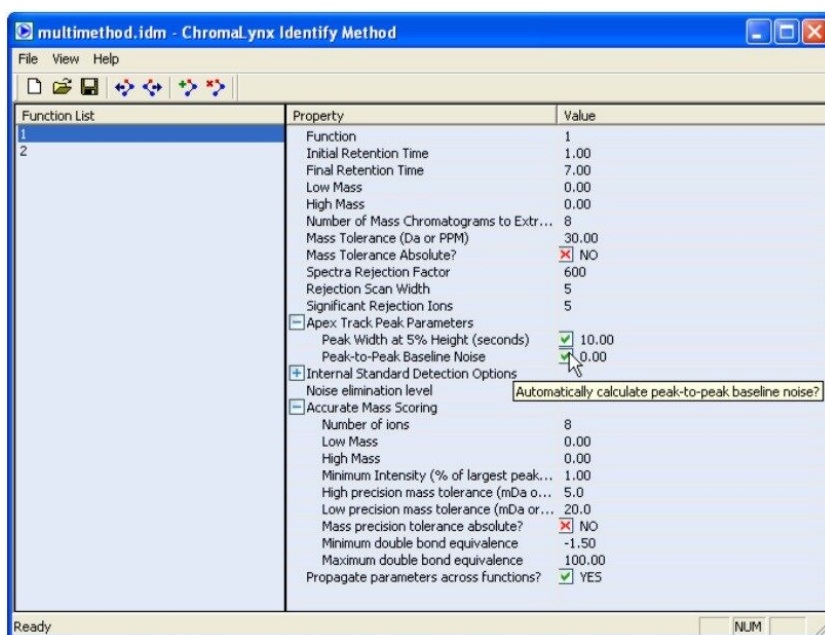
Figure 3. ChromaLynx Identify Method Editor.

Figure 4 overleaf shows the ChromaLynx browser results for a milk sample spiked with several veterinary drugs at a concentration of 10 µg/L - equivalent to the lowest MRL of any of the 41 compounds. The two compounds required to be reported at this level are Flubendazole andOxfendazole, both of which have been correctly assigned by reference to the library created from standards.
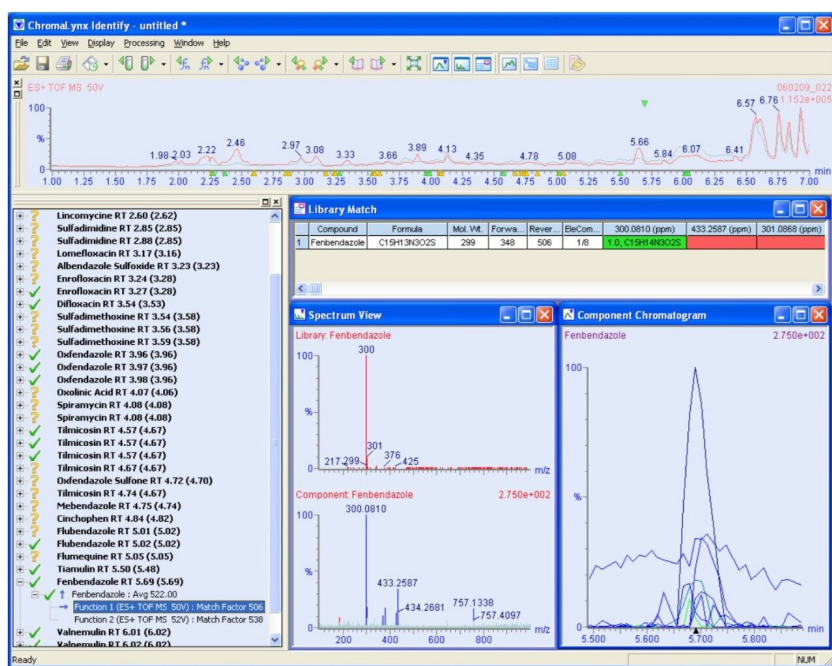
Figure 4. ChromaLynx Application Manager showing Fenbendazole successfully screened from a multi-analyte spiked milk sample at its MRL concentration (10ppb).

Electrospray, being a 'soft' ionization technique, does not produce spectra with the same degree of fragmentation as other techniques, and therefore there are fewer matches to be made than with, for example, GC-MS libraries. Hence, in this study, candidates with a match factor (averaged from the high and low energy functions) over 500 are considered positively identified, and between 250–500 are defined as tentative.

As is clearly displayed, both compounds are positively identified by the green check mark as having a good match to the library entry. However, equally important is the green shaded elemental composition in the match window, showing that the library formula matches the measured mass to <5 ppm (in this case [M+H]$^{+}$ ΔM = 1.0 ppm). A secondary confirmation level of 5–20 ppm is also applied, so matches can still be made if the data is compromised. As an additional quality check, elemental compositions can also be displayed by i-FIT score.

The table in Figure 5 overleaf shows a summary of the MRLs (where applicable), the level at which ChromaLynx screening gives a positive (tentative in brackets) identification and the LoDs for each of the spiked compounds. All library entries are correctly identified at or below their MRL.

		MRL/ µg/L	Screened Level	LoD/ µg/L
Benzimidazoles	Albendazole	100	100 (25)	0.10
	Albendazole Sulfoxide	100	25 (10)	0.20
	Fenbendazole	10	10	0.20
	Oxfendazole	10	10 (5)	0.10
	Oxfendazole Sulfone		100 (25)	0.10
	Mebendazole		100 (25)	0.20
	Levamisole		10 (5)	0.25
	Thiabendazole	100	10	0.20
	Flubendazole		10	0.05
	Oxibendazole		25 (5)	0.05
Macrolides	Spiramycin	200	25	0.30
	Tilmicosin	50	10 (5)	0.50
	Tylosin	50	25	1.00
	Josamycin		25	0.20
	Tiamulin		5 (1)	0.05
	Valnemulin		10	0.10
	Lincomycine	150	10	0.20
Quinolones	Nalidixic Acid		(25)	0.10
	Oxolinic Acid		50 (10)	0.10
	Flumequine	50	100 (10)	0.10
	Norfloxacin		100 (50)	1.00
	Ciprofloxacin		*	1.00
	Lomefloxacin (IS)		50	0.50
	Enrofloxacin	100	10	
	Marbofloxacin	75	10	0.20
	Difloxacin		25 (5)	0.20
	Danofloxacin	30	50 (25)	0.20
	Cinchophen		100 (10)	0.10
	Sarafloxacin		50 (25)	0.20
Sulfonamides	Dapsone	100	(25)	0.10
	Sulfadiazine	100	(100)	0.25
	Sulfamethoxazole	100	*	0.15
	Sulfamethazine	100	*	0.20
	Sulfadimethoxine	100	100 (10)	0.15
	Sulfadimidine	100	25 (10)	0.25
	Sulfadoxine	100	25 (10)	0.05
	Sulfaquinoxaline		100 (25)	0.20
Tetra-cyclines	Doxycycline (-H2O)	100	(25)	0.40
	Tetracycline	100	(100)	1.00
	Oxytetracycline	100	(50)	0.50
	Chlorotetracycline	100	(50)	4.00

* = not in library

Figure 5. Summary table showing analytes' MRL, screened concentration and estimated LoD in milk.

It is important to note at this point that, while in this study a mass accuracy of <5 ppm has been considered confirmatory, there is currently no such consideration in EU legislation. A sample found to contain any of

the analytes should therefore be subjectto further analysis. This is typically performed on a tandem quadrupole type MS/MS instrument, where product ion ratios are used for confirmation. The use of accurate mass spectra for confirmatory purposes is currently under investigation by many groups, and will require validation.

However, at this early stage it is the authors' recommendation that preferably two mass spectral peaks with <5 ppm accuracy, acquired with a mass resolution of at least 10,000 FWHM should be considered. Matching measured and theoretical isotope ratios has proven a reliable and conclusive method for the correct assignment of an analyte's identity and, hence, stringent criteria for this should be compulsory.
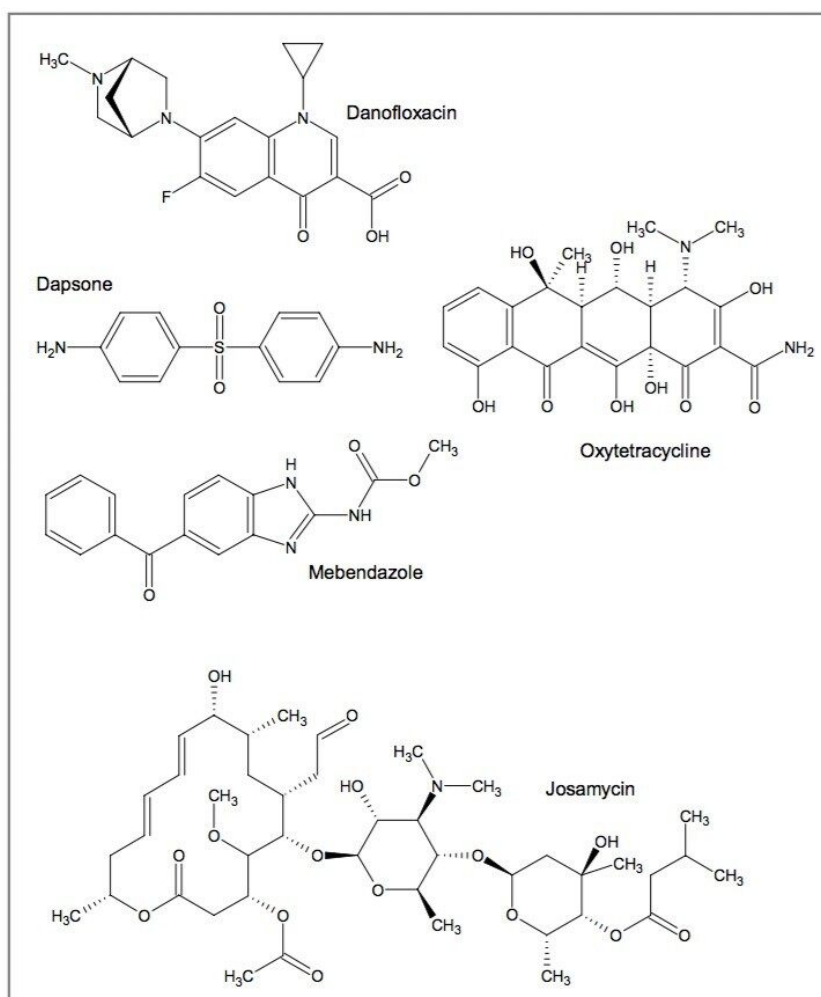

Figure 6. A selection of analytes included in this study. The analytical challenge lies in the diversity of structures and chemistries exhibited.

Conclusion

- The method presented is suitable for the screening of multiple veterinary drug residues in real matrix samples

- A generic, minimal sample preparation for veterinary drug residues in milk samples is sufficient when analysis is carried out by UPLC-ToF MS

- ChromaLynx deconvolution software effectively detects chromatographic peaks, even in complex matrices and when compounds co-elute

- The resulting refined spectra are suitable for library matching against user-compiled reference spectra

- Increased chromatographic resolution from UPLC separation helps to minimize matrix effects and obtain analyte peaks suitable for quantitation in the ToF analyzer

- Spectra with high mass accuracy and correct isotope ratios are valuable aids to the identification of contaminants, and may be appropriate for confirmatory analysis

References

1. Swartz M. E., UPLC, An Introduction and Review, J. Liquid Chromatography and Related Technologies, 2005, 28(7-8), pp 1253–1263.

2. Hobby K., A Novel Method of Isotope Prediction Applied to Elemental Composition Analysis. Poster, presented at BMSS 2005. Waters literature code 720001345EN.

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