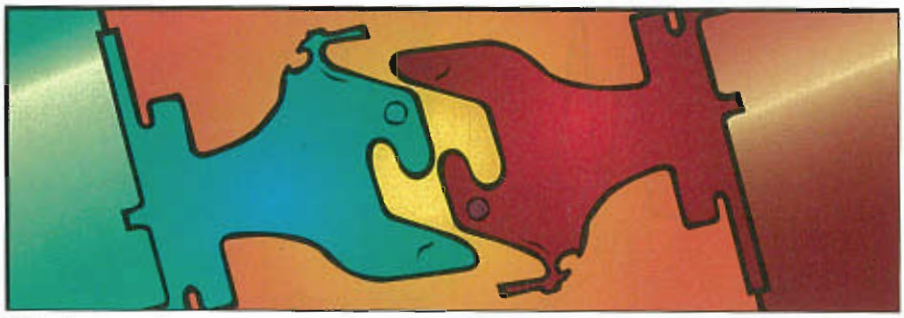
# COUPLING Matters



# Using GC–MS to Identify Residues of Illegal Growth Promoters

**H.F. De Brabander, P. Batjoens, D. Courtheyn, and K. De Wasch**

Gas chromatography–mass spectrometry often is considered a technique free of identification mistakes. This assumption is incorrect — in residue analysis, analysts can obtain both false positive and false negative results caused by interference with matrix components. However, understanding the nature of these interferences and applying some simple rules can prevent most of these mistakes.

In Europe, the word *hormone* has some negative connotations, which are related to the public health danger from the residues of some of these products in foodstuffs of animal origin. Ranchers may use certain hormones as illegal growth promoters when raising livestock. For example, toxicologists have demonstrated that diethylstilbestrol, a synthetic estrogen, is a potential carcinogen (1,2). In human medicine, Melnick and co-workers (3) found analogous experiences with diethylstilbestrol (the so-called diethylstilbestrol daughters). Recently, several cases of poisoning occurred in Spain and France as a result of the consumption of liver from animals treated with clenbuterol (4,5). Moreover, some environmentally persistent alkylphenolic compounds such as nonylphenol and possibly other chemicals show estrogenic activity (6). These environmental estrogens are of public concern in relation to the decreasing quality of human sperm; a British television program called these estrogens an "assault on the male" (7).

A residue can be defined as the trace of a component that is present in a matrix after some kind of administration. A matrix can be anything in which a residue may be present, trapped, or concentrated (for example, meat, urine, feces, or liver). No general agreement exists defining the concentration level of a trace. However, the parts-per-billion level (micrograms per kilogram) in which residues of illegal growth promoters are present may be considered as a trace or even an ultratrace level.

*Chemists often consider GC–MS to be a technique with which they can make no mistakes in identification, except those caused by cross-contamination.*

A residue-analysis procedure consists of three distinct steps:

- First, the analyte must be extracted from the matrix.
- Second, the extract is freed from as many interfering products as possible.
- Third, the analyte is identified and, eventually, the residue is quantified.

Chromatographic techniques are very important in modern multiresidue analyses of illegal growth promoters. Gas chromatography–mass spectrometry (GC–MS) is the technique used most often, with the exception of thin-layer chromatography (TLC) methods (8–10). Low-resolution GC–MS uses two important types of apparatus: quadrupole and ion-trap MS systems. The major difference between the two apparatus types is their method of detecting and recording a chromatogram. Many quadrupole instruments such as the well-known mass-selective detector use selected-ion monitoring for the determination of traces. Relatively high amounts of analyte are required (>10 ng) to record a full-scan spectrum with a quadrupole system. Systems that are based on ion-trap technology can record a full-scan mass spectrum on an analyte at picogram concentrations. With selected-ion monitoring, the ion-trap system monitors a limited number of ions during a selected time interval in the chromatogram. The presence of the analyte is determined by the presence of these diagnostic ions at the correct retention time and in the correct abundance ratio (11).

With systems that are based on ion-trap technology, the whole mass spectrum is stored for each point of the chromatogram. Afterwards, analysts can use a data system to search general or dedicated (homemade) libraries to identify the analyte. The result of this search is expressed as a figure that reflects the fit between the standard and the sample spectrum.

In this "Coupling Matters" column, we will discuss the different methods of residue identification and discuss the possible causes of interferences and ways to avoid them.

## POSSIBLE INTERFERENCES

Chemists often consider GC–MS to be a technique with which they can make no mistakes in identification, except those caused by cross-contamination. This assumption may be true or nearly true in major and minor component analysis, but it certainly is not the case in the residue analysis of illegal growth promoters.

Extracts of biological material such as urine, meat, and feces contain a large variety of components in various concentrations. When using GC–MS, unknown and variable amounts of these matrix components are coextracted with the analyte and introduced into the chromatograph and mass spectrometer. Interference from these matrix components, possibly present at relatively high concentrations (parts-per-million range or higher), and analytes, present at very low concentrations (parts-per-billion range), is possible, and it should be avoided.

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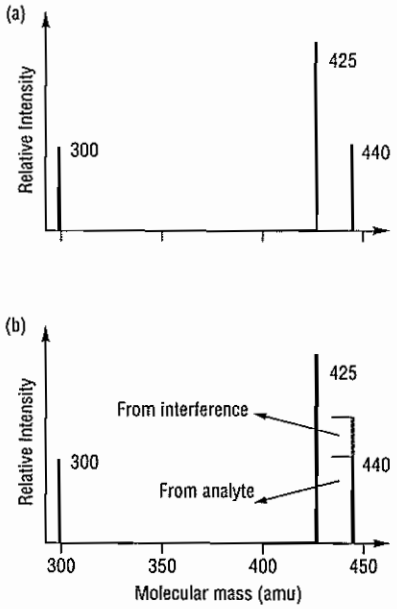


**FIGURE 1:** Selected-ion monitoring mass spectra showing false negative results caused by interferences that disturb the relative ratios of the diagnostic ions. Shown are spectra from (a) a standard and (b) the sample.

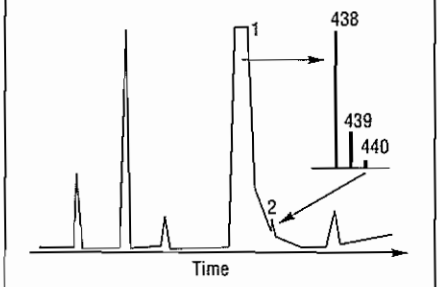


**FIGURE 2:** Isotope interference from samples with several isotope molecules and small amounts of analyte.

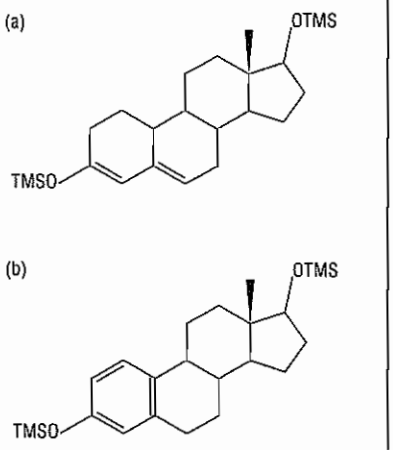


**FIGURE 3:** Formulas of (a) nortestosterone (ditrimethylsilyl, molecular mass = 418) and (b) estradiol (ditrimethylsilyl, molecular mass = 416).

Interferences result mostly from coeluted peaks or background noise. An obtained mass spectrum is a mixture of two mass spectra, which means that analysts can make a false interpretation. Isotope interference is another possible source of interference (12). This phenomenon can occur with any isotope, but $^{13}C$ is a very good example. Carbon has two natural isotopes — $^{12}C$ and $^{13}C$ — in a ratio of 98.9:1.1.

In residue analyses, workers must take three other important parameters into account:

- A very large difference exists between the concentration of the analytes and that of the matrix components.
- The analyte or interference is mostly an organic molecule containing a relatively high level of carbon atoms.
- Interferences may have analogous structures to the analytes.

Anabolic agents such as steroids contain 20–30 carbon atoms, and numerous steroids and metabolites with analogous structures are known.

Using a quadrupole instrument in the selected-ion monitoring mode, analysts fail to observe many interferences because of the highly selective use of the detector. In an ion-trap instrument, high concentrations of coeluted molecules may influence the ionization time of analytes and, therefore, the detection limit. This problem may cause false positive and negative results and incorrect quantification.

## FALSE NEGATIVE RESULTS

Using selected-ion monitoring, the instrument tracks numerous diagnostic ions from the analyte during a time window spanning the expected retention time. These diagnostic ions must be present in the correct relative intensities (±20% for chemical ionization or ±10% for electron impact). Researchers debate about the number of diagnostic ions that must be considered. Two ions certainly are insufficient because false positive results may be generated (see below). Four or more ions would be ideal from a theoretical point of view but impractical at lower concentrations. Using ring tests to compare different testing laboratories, Daeseleire and Van Peteghem (13) observed that the relative intensities of the ions in most laboratories do not remain constant enough when the concentration decreases. The margin on the relative intensities must be increased to 20%, 30%, or higher with an increasing chance of false positives (see later).

The higher the number of ions, the higher the specificity of the methods (fewer false positives) but also the higher the chance of false negative results when the identification criteria are applied strictly. Making a decision based on three ions seems to be an acceptable compromise.

Figure 1 shows that the relative intensity of the ions can be disturbed by background noise and coeluting substances. In this figure, the *m/z* 440 ion results partly from the analyte and partly from interference. In the standard, the relative ratios of the ions are 100 (*m/z* 425 ion), 46 (*m/z* 440 ion), and 41 (*m/z* 300 ion). In the sample, the relative ratios are 100 (*m/z* 425 ion), 68 (*m/z* 440 ion), and 41 (*m/z* 300 ion). The ratio between *m/z* 440 and *m/z* 425 is out of range (the normal range is 41–51), and according to the rules (the quality criteria), the sample must be declared negative, although the analyte is present.

The interference in Figure 1 could have been caused by a molecule with a molecular or fragment ion that has a mass equal to that of one of the analyte's diagnostic ions. However, interference also is possible with molecules containing ions that are one or two atomic mass units less than the diagnostic ions. In the example shown in Figure 1, a molecule will interfere not only when the ions *m/z* 439 and 438 are present but also when the ions *m/z* 424, 423, 299, and 298 are present in a much higher concentration. All these ions will generate isotope peaks that disturb the ratio of the diagnostic ions. This phenomenon is of particular interest on shoulder peaks, as demonstrated in Figure 2. Here, peak 1 contains the *m/z* 438 ion, which has isotope peaks at *m/z* 439 and 440. The *m/z* 440 ion will interfere with the analyte in peak 2 when the diagnostic *m/z* 440 ion is used.

## FALSE POSITIVE RESULTS

To demonstrate the phenomenon of false positive results, consider the following example. Nortestosterone is a well-known anabolic steroid used in cattle fattening. The β form is the active component of this hormone. Estradiol is the female hormone, and the α form is present naturally in relatively high fluctuating concentrations in female animals. It was shown that the disilyl derivatives of α-estradiol and β-nortestosterone were poorly separated under the chromatographic conditions used in most laboratories (12). Figure 3 shows the formulas of the ditrimethylsilyl derivatives of both components. The figure shows that the molecular masses of these two components differ by only two atomic mass units. Because the structures are similar, fragmentation also may be similar.

For the determination of nortestosterone, analysts must monitor three ions: *m/z* 418 (100%), *m/z* 403 (20%), and *m/z* 328 (35%). In the urine of pregnant cows, α-estradiol is present in concentrations $10^4$–$10^5$ times higher than the concentration of nortestos-

terone found after illegal administration. The mass spectrum of estradiol contains the molecular ion *m/z* 416 and the fragment ions *m/z* 402 and 326. De Brabander and colleagues (12) calculated and demonstrated that the isotope peaks of estradiol may generate a selected-ion monitoring signal for nortestosterone. Using by-the-book selected-ion monitoring, analysts will infer the presence of nortestosterone: the three ions are present within the correct retention time windows and with the correct ratios. However, the interfering ions also can be generated by several interferences simultaneously or by stable isotopes of other elements.

*The fact that the interfering endogenous compounds can produce correct ion ratios is not obvious to analysts when using GC–MS in the selected-ion monitoring mode.*

## IMPROVING QUALITATIVE ACCURACY

When using GC–MS for the determination of analyte residues — in particular, illegal growth promoters at the parts-per-billion level — analysts must remember the possibility of interference. Moreover, the consequences for owners of animals that receive false positive results and for the inspection services that obtain false negative results are considerable. However, chemists can invest caution, time, and money in an analysis to prevent a bad decision.

## AVOIDING FALSE NEGATIVES

Analysts should monitor false negatives arising from analyte loss during cleanup, derivatization, or injection by using internal standards. Deuterated analogs of the illegal growth promoters are best suited for that purpose and also can be used for quantification. However, their availability is limited in number as well as quantity. Other internal standards should be used as an alternative. In Belgium, for example, chemists use equilenin (a typical horse hormone) as an internal standard for the analysis of estrogens, androgens, and gestagens in matrices from other animals. Proving that a sample does not contain a certain illegal growth promoter is almost as important. The best way to prove the absence of an illegal growth promoter is using internal standards such as deuterated hormones added to a sample at the normal detection level and monitoring the recovery of these standards.

Chemists should deal differently with false negative results caused by the disturbance of analyte ions' normal peak ratios by one or more interferences. Analysts must be aware that the statistical possibility of this occurrence is high. Instead of immediately declaring a sample negative because the ratio of one of the ions is outside the range proposed by the quality criteria, analysts should add other elements to the analysis. Possibilities include reinjecting the same derivative on another column or using GC–MS–MS, using other derivatization reagents or techniques, and performing a second analysis using a different method.

For example, the GC–MS data could be combined with TLC results. Using this technique the identity of a hormone in a suspect sample may be determined by two values for the ratio to the front in two-dimensional TLC, a characteristic fluorescence after sulfuric acid induction, a retention window in HPLC, a retention time in capillary GC, and mass spectrometric data. Although each technique on its own does not fulfill the quality criteria exactly — they may provide disturbed ratios — the combination may offer sufficient analytical accuracy.

## AVOIDING FALSE POSITIVES

In selected-ion monitoring, analysts may declare false positives when they observe three diagnostic ions at the correct retention time and in the correct ratio windows. However, these ions do not originate from the analyte but are generated by one or more interferences that are present at a high concentration in the final extract. The fact that the interfering endogenous compounds can produce correct ion ratios is not obvious to analysts when using GC–MS in the selected-ion monitoring mode.

With quadrupole instruments, which cannot make a full scan at low concentration, we recommend the following strategy: Perform a second full-scan run on the same sample in the event of a positive result to exclude the presence of isotope-generated peaks that overshadow the low-concentration analytes at the retention time of the analyte. The absence of substantial concentrations of isotope peak generators in the full-scan mass spectrum must be considered a quality criterion.

Analysts can avoid these isotope interferences by using an apparatus that can operate in the full-scan mode at low concentration levels. The quality criteria — three ions — can be extended by using full-scan spectrum matches between the sample spectrum and a homemade library spectrum. One of the problems with systems based on ion-trap technology is the difference between the total spectrum obtained on a trap and that obtained on a quadrupole. This problem has been solved in the most recent ion-trap instruments (for example, the Finnigan GCQ system [Finnigan Corp., San Jose, California]). Moreover, some of these newer instruments offer the power of MS–MS. In the near future, bench-top MS–MS instruments will prove to be powerful tools for obtaining enhanced analytical accuracy.

## CONCLUSIONS

It is a mistake to consider GC–MS as an error-free technique with no identification mistakes. As in any other analytical technique, workers can obtain false positive and false negative results. However, when analysts are aware of the possible causes of these errors, they can apply some simple rules and invest a little more time in analyses to prevent most of these mistakes.

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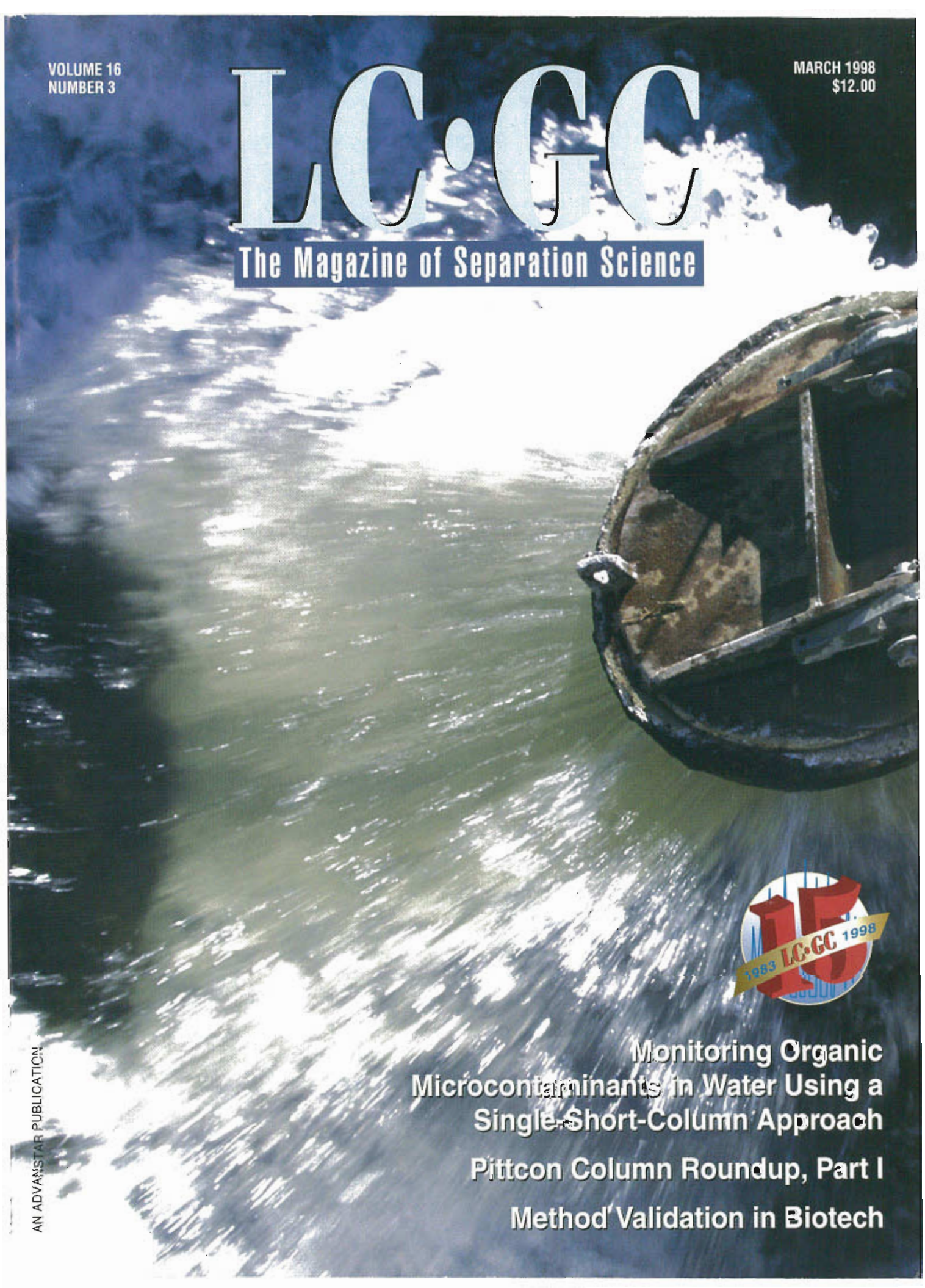




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