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Journal:	*Food Additives and Contaminants*
Manuscript ID:	TFAC-2005-393.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	13-Jun-2006
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Methods/Techniques:	Chromatography - HPLC, Chromatography - LC/MS
Additives/Contaminants:	Veterinary drug residues
Food Types:	Drinking water, Milk

Can high-performance liquid chromatography coupled with fluorescence detection under all conditions be regarded as a sufficiently conclusive confirmatory method for B-group substances?

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Abstract

Commission Decision 2002/657/EC requires confirmatory analysis of B-group compounds when detected at levels above the permitted limit. In contrast to banned substances, for B-group substances the use of mass spectrometric techniques is not obligatory and several techniques including LC-UV on two different LC-columns and (single column) HPLC-fluorescence (LC-Flu) are considered to deliver sufficient evidence for the identification of the detected substance. The analysis of sodium salicylate in animal drinking water collected at poultry farms is presented here as an example to show that even in a simple matrix like animal drinking water, fluorescence detection in some cases may provide inadequate specificity. Of 50 samples analysed by LC-Flu 18 tested positive for sodium salicylate. However, only in one sample the presence of the analyte was confirmed with mass spectrometric detection, the others were blank. Consequently, the LC-Flu results obtained were false-non-compliant for sodium salicylate. A second case concerning the analysis of avermectins in milk by

HLPC-fluorescence is briefly described. For a number of samples analysed in the framework of a proficiency test, false non-compliant results for emamectin were reported, due to a background interference sometimes present which practically coeluted with the analyte. The observed retention time difference (1%) was well below the criterion (2.5%) specified in CD 2002/657/EC. Considering the impact of positive findings on individual farmers as well as on trade, product image and food safety perception by the consumer it is concluded that also for B-group substances false-non-compliant results should be avoided whenever possible. This is especially important when the results are treated as and are expected to have the same repercussions as in case of banned A-group substances. In these circumstances only results obtained by mass spectrometry should be considered for confirmatory purposes.

Keywords: Sodium salicylate, B-Group substances, fluorescence detection, confirmatory method, tandem mass spectrometry, avermectines

Introduction

Sodium salicylate (2-hydroxybenzoic acid mono-sodium salt) is a non-steroidal anti-inflammatory drug belonging to the group of salicylates that are commonly used in veterinary practice. It is the sodium salt of salicylic acid and it is used in ointments, creams or solutions for disinfecting wounds. Typically, the active concentration correlates to a dose of 2 to 400 µg kg^{-1} bodyweight/day (EMEA, 1999). For topical applications this substance is authorised in the EU for cattle, horses, sheep, goats and poultry. Sodium salicylate is included in Annex II of Council Regulation 2377/90/EC for topical use on all food-producing animals except fish and hence it has no maximum residue limit (MRL) associated to it (EC, 2000). Recently oral application in porcine and bovine (except milk producing) animals has been included in Annex II (EC, 2004). Sodium salicylate is generally applied via the drinking water at a dose of 40 mg kg^{-1} bodyweight/day (EMEA, 2004). No MRL was deemed necessary as depletion after oral administration was very rapid and residues remaining in edible tissue constituted only 5% of the Acceptable Daily Intake (ADI).

Despite the fact that no MRL has been established for residues of sodium salicylate, the residue control plans of several EU-Member States include the control of sodium salicylate in edible animal products as well as in feed and animal drinking water. Most commonly, sodium salicylate is determined by HPLC with fluorescence detection after aqueous extraction of the sample (Venema et al, 1996; Flagerty et al, 2002). For tissue samples, often a sample clean-up step by means of solid phase extraction is applied (Pirker et al, 2004; RIKILT, 2004).

Although oral administration is allowed for bovine and porcine animals, it is not permitted for poultry. Nonetheless it is suspected that sodium salicylate is sometimes used during the last period of broiler fattening to relieve the animals from pain. Therefore, sodium salicylate was recently targeted in a routine survey in drinking water supplied to broilers. Analysis of the samples was carried out using HPLC with fluorescence detection. According to Commission Decision 2002/657/EC, HPLC-fluorescence (LC-Flu) is a suitable confirmatory method for B-group substances that exhibit native fluorescence or exhibit fluorescence after either transformation or derivatisation and when the performance criteria for chromatographic conditions are fulfilled (EC, 2002). Results of analysis obtained during routine analysis can therefore be regarded as confirmed results in view of CD 2002/657/EC and hence formally those results need no further confirmatory analysis by e.g. mass spectrometric techniques. However, in every-day practice interferences can be observed with LC-Flu despite its alleged specificity.

In this paper we report the observations we recently made with respect to the conclusiveness of LC-Flu results and the possibility of obtaining false non-compliant findings.

Experimental

Chemicals

Reagents and solvents were of analytical grade unless stated otherwise. Acetonitrile, formic acid and sodium hydroxide were purchased from Merck (Darmstadt, Germany); sodium salicylate was purchased from VWR International (Amsterdam, The Netherlands). Calibrant solutions were prepared by diluting aqueous stock

solutions of 10 and 100 mg l^{-1} with mobile phase (water/acetonitrile/formic acid (60/40/0.1 v/v/v) to obtain solutions containing 1, 2, 5, 10, 20, 50, 100, 200 and 500 µg l^{-1}.

Instrumentation

The LC-Flu system comprised of a Gilson 234 auto injector (Gilson, Middleton, WI, USA), a Gilson 307 HPLC pump (Gilson, Middleton, WI, USA), a 10*3 mm PRP-1 precolumn (Hamilton, Bonaduz, Switzerland), a 150*4.1 mm, 5 µm PRP-1 analytical column (Hamilton, Bonaduz, Switzerland), a thermostatic oven (Jones Chromatography, Glamorgan, UK) maintained at a temperature of 25°C and a Jasco 1520 fluorescence detector (Jasco, Easton, MD, USA), set at λ_{ex} 300 nm, λ_{em} 400 nm. The system was run isocratic at a flow rate of 1.0 ml min^{-1} using water/acetonitrile/formic acid (60/40/0.1 v/v/v) as mobile phase. Sodium hydroxide (1M) was added at 0.15 ml min^{-1} to the effluent of the analytical column using a Gilson minipulse 3 peristaltic pump (Gilson, Middleton, WI, USA) and a teflon mixing coil (2 m * 0.8 mm).

For LC-MS/MS confirmatory analysis, a Waters Quattro Ultima tandem mass spectrometer (Waters Corporation, Milford, MA) was used equipped with an ESI ion source operated in negative ionisation mode. A Waters Alliance 2690 LC system was used equipped with a 3*150 mm, 5 µm Water Symmetry® C_{18} analytical column and a thermostatic oven (Spark, Emmen, The Netherlands) maintained at a temperature of 35°C. Chromatography was performed at 0.4 ml min^{-1} using an isocratic system of water/acetonitrile/formic acid (50/50/0.1; v/v/v). The column effluent was split 2:1 before it entered the ESI ion source. The interface was operated at optimised settings

(capillary voltage 2.5 kV, cone voltage 25 V, source temperature 100°C, desolvation temperature 300°C, desolvation gas flow 550 l h^{-1}, cone gas flow 150 l h^{-1}).

Being a small molecule, salicylate (m/z 137) undergoes only few characteristic fragmentations upon collision induced dissociation in negative ESI mode. Only two product ions were observed at a collision energy of 30 eV: a major fragment identified as phenolate anion at m/z 93 (loss of 44, corresponding to the loss of CO_2) and a minor fragment identified as dicyclopentenyl anion at m/z 65 (loss of 72, corresponding to the combined loss of CO_2 and CO) (Figure 1). Hence the precursor ion together with these two product ions were selected for multiple reaction mode (MRM) data acquisition.

(Insert Figure 1 about here)

Samples

Fifty (50) samples of animal drinking water were collected at farms in the Netherlands where broilers were raised and transported to the laboratory for analysis. To mimic the HPLC-mobile phase composition, 6 ml aliquots were mixed with 4 ml acetonitrile and 10 µl formic acid. The samples were subsequently filtered and analysed with LC-fluorescence without further sample preparation. For the LC-Flu analysis every tenth sample was analysed with addition of sodium salicylate at a level of 50 µg l^{-1}.

Samples for which LC-Flu indicated the presence of sodium salicylate at 1 µg l^{-1} or higher were subjected to LC-MS/MS confirmatory analysis. To this end each suspect

sample was analysed in duplicate and with addition of sodium salicylate at approximately the same concentration as determined by LC-Flu.

Results and discussion

Although the therapeutic dose for oral administration of sodium salicylate is around 300 mg l^{-1}, in this survey a low (1.0 µg l^{-1}) cut-off concentration was used. It can be reasoned that in case of illicit use of sodium salicylate via the drinking water, low concentration of residues may persist in the drinking water system for a considerably longer period of time than the actual medication period. Detection of residues of salicylate in the drinking water, even at very low concentrations, may indicate illegal use of this drug in the past. From the water samples taken at the broiler farms analysis by LC-Flu indicated that a substantial number (18 out of 50) contained sodium salicylate in a concentration exceeding 1 µg l^{-1} (Table I). In another 12 samples amounts ranging between 0.3 (the estimated LoD of the method) and 1.0 µg l^{-1} were detected. One sample (entry 15) contained a very high concentration sodium salicylate, estimated (after 1000-fold dilution) at 375 mg l^{-1}. Figure 2 presents typical LC-Flu chromatograms obtained for a standard solution, a blank and two positive samples (entries 15 and 16). No signal was detected in the blank samples while for the samples fortified at 50 µg l^{-1} a mean recovery of 88% was obtained (range 80-105%). For the suspect samples in the low concentration range (1-30 µg l^{-1}), analytical results were very consistent: both the retention time and peak width were very stable and matched with the standard solutions and no other peaks were present in the chromatograms. Furthermore, standard addition of sodium salicylate to the samples did not result in any change of the retention time, nor to detectable peak

broadening. Hence, from an analytical perspective, there is no reason to doubt the validity of the non-compliant findings.

(Insert Table I and Figure 2 about here)

Listed as a B-group substance, strictly speaking using HPLC-fluorescence as detection technique and applying the criteria of CD 2002/657/EC is sufficient to acquire data suitable for confirmation of the analyte's identity. However, the percentage of positive samples (above 1 µg l^{-1}) was surprisingly high (36%) but at the same time the majority of the concentrations found was low; 1000-fold or more below the regular therapeutic dose. Sodium salicylate has been detected in the effluent of waste water treatment plants and in surface water, but always in concentrations well below 1 µg l^{-1} (La Farré et al 2001, Marchese et al, 2003). As these findings could be of considerable significance for further enforcement actions, it was decided to use LC-MS/MS for additional confirmation.

Each suspect sample was re-analysed by LC-MS/MS in duplicate and with addition of sodium salicylate at approximately the same concentration that was determined by LC-Flu. The results are given in Table I. Figure 3 presents the extracted ion chromatograms obtained for the same samples as shown in figure 2. It is clear that with LC-MS/MS no sodium salicylate could be detected in sample 16. In fact not a trace of the analyte could be detected while the limit of detection of the LC-MS/MS method was below 1 µg l^{-1} in drinking water. Sodium salicylate was detected and confirmed in the standard addition sample. The result for sample 16 is representative for all samples in which low concentrations of sodium salicylate were detected by LC-

Flu. The sample extracts used for analysis by LC-Flu were also used in the LC-MS/MS analysis, hence the non-compliant findings by LC-Flu could not have been caused by low-level contamination of the samples, but must have been caused by a fluorescent artefact present in part of the water samples. LC-MS/MS analysis yielded one positive case: in sample 15 a high concentration (400 mg l^{-1}) of sodium salicylate was detected and its identity was confirmed according to the criteria of CD 2002/657/EC.

(Insert Figure 3 about here)

Recently we encountered another case in our laboratory in which a HPLC-fluorescence method produced aberrant results. In the analysis of avermectines in raw milk, derivatisation is used followed by fluorescence detection (λ_{ex} 365 nm, λ_{em} 470 nm) (Schenck, 1999; Danaher, 2001; RIKILT, 2001). Again this is generally considered a highly specific method and suitable for confirmatory analysis. Recently we participated in a proficiency study on avermectins in reconstituted milk. The study comprised 7 samples with unknown concentrations of a number of avermectins, including eprinomectin (EPM), moxidectin (MOX), emamectin (EMA), abamectin (ABA), doramectin (DOR) and ivermectin (IVM). The proficiency test samples were received in a lyophilised state and were reconstituted with water before analysis. However, for the control blank and fortified samples raw milk was used, as no lyophilised control milk was available (nor was it supplied by the proficiency test organiser). As can be seen from Figure 4 the blank raw milk sample produced a very clean chromatogram with only small background interferences and all 6 avermectines could easily be detected and quantified, down to sub-ng/ml concentrations. In

contrast, the reconstituted milk samples produced chromatograms containing significantly more and higher background interferences. In Figure 4 the chromatogram obtained for one of the proficiency test samples is given as an example.

(Insert Figure 4 about here)

We reported this sample (by duplicate analysis) to contain 28, 1.6 and 1.1 µg l^{-1} MOX, EMA and IVM, respectively. We correctly labelled a peak eluting at 5.15 min as not being EPM (retention time 5.25 min, relative difference 2%). However, we learned from the organiser of the proficiency study that we had reported for this sample a deviant (false non-compliant) result for emamectin. It was stated by the organiser that EMA had not been added to any of the samples. Re-examination of the raw analytical data did indeed reveal that the retention time of the peak (8.48 min) was slightly different from that of the EMA standard in the control sample (8.39 min). The retention time difference is only 1% and well below the criterion of 2.5% as specified in CD 2002/657/EC (EC, 2002). This minor difference was found consistently in the duplicate sample as well as in two other samples for which we had reported the presence of EMA (producing two more false positive results). This small retention time difference had escaped attention during the analysis of the raw data and even if it had been noted it would probably not have triggered further action since it complied with the criteria. Furthermore, it should be noted that this peak was prominently present in three samples but virtually absent in the remaining 4 samples. All samples were run in duplicate and produced consistent results. On the basis of the combined information the reported findings of EMA formally remained justifiable. At this moment

it is not yet clear if EMA was detected by any of the other 30 participants (the final report is still pending). However, the conclusion seems justified that the usefulness and conclusiveness of HPLC-Fluorescence strongly depends on the quality of the produced sample extracts and the absence or presence of matrix interferences or other artefacts. When these interferences occur infrequently, it becomes difficult to recognise them as such and to avoid false positives.

Conclusions

From an analytical perspective, animal drinking water could be considered as a simple matrix with little or no risk of interferences. For this reason we used HPLC-fluorescence to analyse sodium salicylate in drinking water samples collected at poultry farms. LC-Flu indicated low levels of sodium salicylate to be present in a substantial number of samples. The identity of sodium salicylate could not be confirmed by LC-MS/MS, however. It was concluded that LC-Flu analysis had generated false-positive results for these samples, probably due to an artefact or an unknown compound that was present in variable amounts. This case and the one described on the analysis of avermectin residues in milk illustrates that despite the fact that LC with fluorescence detection is generally regarded as an acceptable confirmatory method for B-group substances, false-non-compliant results can occur, especially when the analysis is performed at trace levels. The specificity of fluorescence detection can be questioned and consequently so can its conclusiveness.

Considering the possible impact that non-compliant analytical results also for B-group substances may have for the individual farmer as well as on trade, product image

and food safety perception by the consumer, the necessity to use conclusive confirmatory techniques based on mass spectrometry is evident.

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List of Table and Figures

Table I. HPLC-Fluorescence and LC-MS/MS analysis of sodium salicylate in drinking water samples obtained at broiler farms. Concentrations are given in µg l^{-1}. Only those samples are listed that contained more than 1 µg l^{-1} according to LC-Flu analysis.

Figure 1. MS/MS fragmentation pathway of sodium salicylate, negative ESI, collision energy 30 eV.

Figure 2. LC-Flu chromatograms of animal drinking water samples analysed for sodium salicylate. A: standard solution (1 µg l^{-1}) B: blank control sample, C: sample 16 (28 µg l^{-1}) and D: sample 15 (1000x diluted, 375 mg l^{-1})

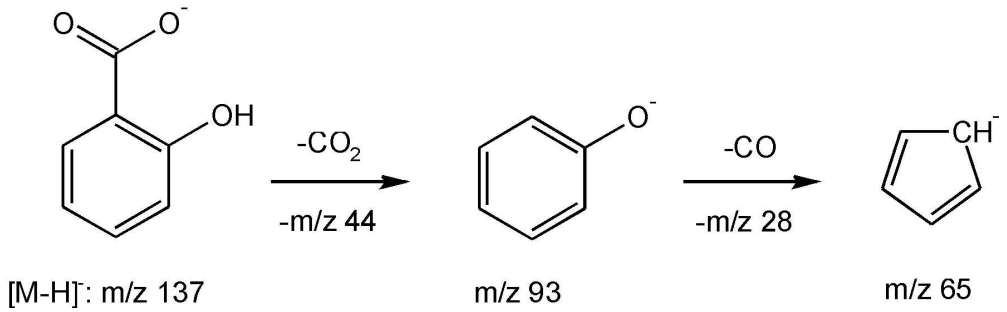
Figure 3. LC-MS/MS chromatograms of animal drinking water samples analysed for sodium salicylate. A. Standard solution (1 µg l^{-1}). B. Sample 16 (compliant), C. Sample 16 spiked with 25 µg l^{-1} sodium salicylate. D. Sample 15 (10,000x diluted, non-compliant, 400 mg l^{-1}).

Figure 4: Analysis of avermectines in milk by HPLC-Fluorescence. A: Blank raw milk sample. B. Raw milk sample spiked with 2.0 µg l^{-1} eprinomectin (EPM), moxidectin (MOX), emamectin (EMA), abamectin (ABA), doramectin (DOR) and ivermectin (IVM). The peak labeled with an asterisk is an impurity sometimes occurring during derivatisation. C: Unknown reconstituted milk sample containing MOX (28 µg l^{-1}) and IVM (1.1 µg l^{-1}). The peak labelled with an asterisk was identified correctly not to be

EPM but an interference on the basis of a slightly different retention time. The peak labelled as EMA (quantified as 1.6 µg l^{-1}) was not recognized being an artifact and was reported as a false positive.

Table I.

Sample code	LC-Flu	LC-MS/MS
1	1.2	<1
2	14	<1
3	2.5	<1
4	6.2	<1
5	2.2	<1
6	2.0	<1
7	1.0	<1
8	2.9	<1
9	5.1	<1
10	2.4	<1
11	1.4	<1
12	1.5	<1
13	5.9	<1
14	1.7	<1
15	375 000	400 000
16	28	<1
17	4.3	<1
18	1.5	<1



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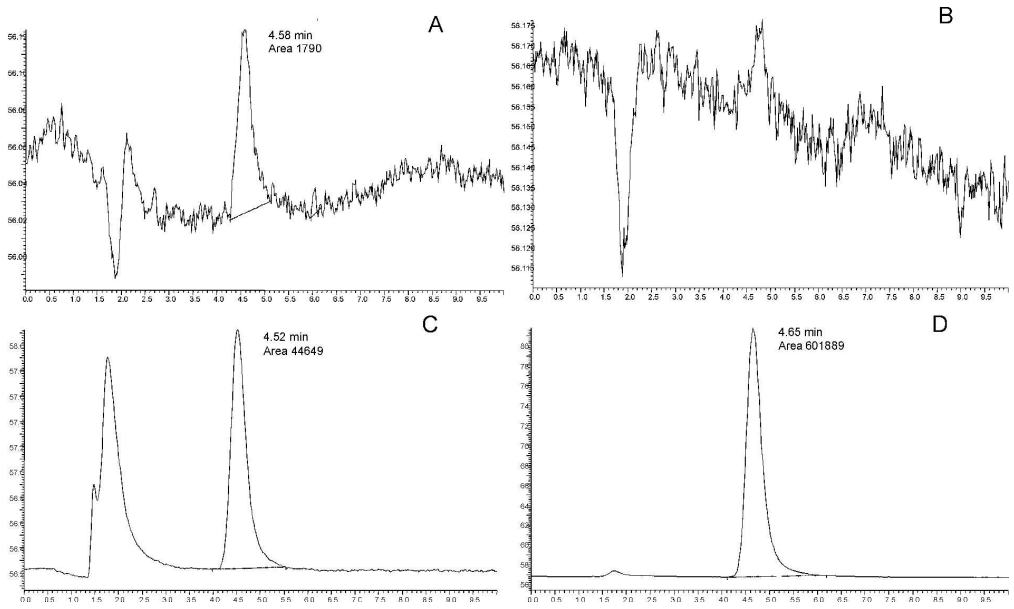

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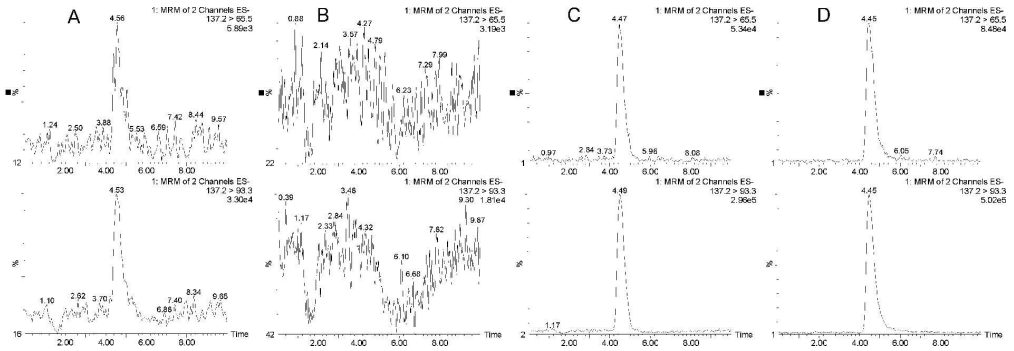

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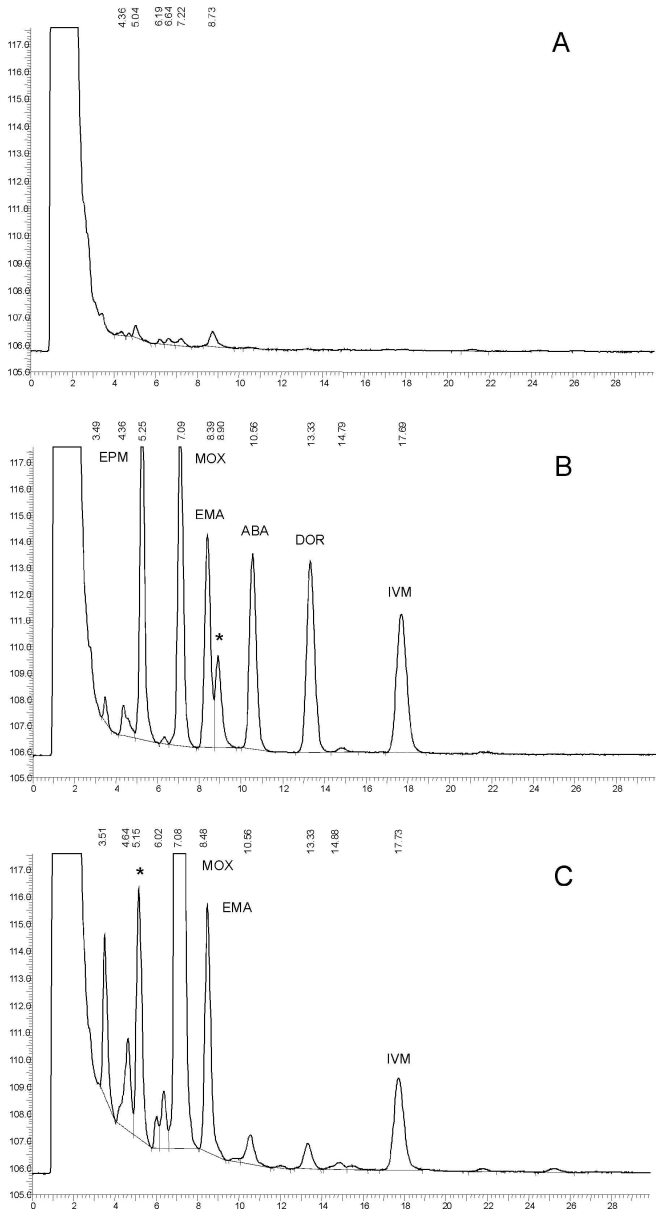

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