Article

N-Nitrosamine Impurities in Ethalfluralin: Determination of an Overlooked Deleterious Source in Pesticides

George P. Balayiannis and Helen Karasali

Special Issue
Application Progress of Chromatographic Analytical Methods in Agriculture

Edited by
Dr. Eleftheria Bempelou and Dr. George Balayiannis

https://doi.org/10.3390/agriculture13051104

Article

N-Nitrosamine Impurities in Ethalfluralin: Determination of an Overlooked Deleterious Source in Pesticides

George P. Balayiannis * **and Helen Karasali**

Laboratory of Chemical Control of Pesticides, Scientific Directorate of Pesticides' Control and Phytopharmacy, Benaki Phytopathological Institute, 145 61 Kifisia, Greece; e.karassali@bpi.gr
* Correspondence: g.balayiannis@bpi.gr

Abstract: *N*-nitrosamines are a class of carcinogenic chemical compound. Considering the large-scale application of agrochemicals globally, the elimination of *N*-nitrosamines from pesticides should be a priority for manufacturers and regulators. A set of methods was developed and validated for the determination of the toxicologically relevant *N*-nitrosamine impurity of ethalfluralin (ethyl-*N*-(2-methylallyl) *N*-nitroso amine—EMANA) in 33% $w\ v^{-1}$ emulsifiable concentrate (EC) formulations. Solid Phase Extraction (SPE) was compared with the "dilute and shoot" approach. Gas chromatography (GC) was combined with Flame Ionization Detection (FID) and mass spectrometry (MS). For MS, two mass filtering modes (Selective Ion Monitoring—SIM, tandem mass spectrometry—MS/MS) and two ionization modes (Electron Ionization—EI, Positive chemical ionization—PCI) were applied. It was concluded that, in the case of samples with high nitrosamine concentration (>90 μg g^{-1}), the "dilute and shoot" approach can be applied without compromising the quality of the results. SPE, however, is required to attain the LOQ (0.33 μg g^{-1}) with good recovery (97.4–110.67%), linearity (R > 0.99) and precision (%RSD 0.68–1.74). The LOQ supersedes the limit set by EFSA (1 μg g^{-1}) in the Technical Active Substance—TAS. The concentration range of the methods is 0.05–110 μg g^{-1}. The methods were applied for the official surveillance program of the Greek agrochemicals market.

Keywords: pesticide; impurity; *N*-nitrosamine; GC-MS; chemical ionization; SPE; dilute and shoot

Citation: Balayiannis, G.P.; Karasali, H. *N*-Nitrosamine Impurities in Ethalfluralin: Determination of an Overlooked Deleterious Source in Pesticides. *Agriculture* **2023**, *13*, 1104. https://doi.org/10.3390/agriculture13051104

Academic Editor: Geraldo Andrade Carvalho

Received: 7 April 2023
Revised: 12 May 2023
Accepted: 15 May 2023
Published: 22 May 2023

Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

N-nitrosamines are a class of chemical compounds (Figure 1), a significant number of which are classified by the International Agency for Research on Cancer (IARC) as Groups 1, 2A, 2B and 3 carcinogens [1]. Their mutagenic potential seems to be increased by the number of α-hydrogens available [2]. They can be found in a variety of products, including foods, drugs, cosmetics, toys and pesticides [2].

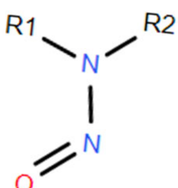

Figure 1. Structure of *N*-nitrosamines.

Although recently there was a significant increase in concern for *N*-nitrosamines in pharmaceutical products [3,4], their presence in pesticides was well-known beforehand. Ross et al., Fishbein and Kearney [5–7] detected them in high concentrations (up to 640 mg·L^{-1}) and studied the mutagenic potential of *N*-nitrosamines in various pesticides, with dinitroanilines among them. These concentration levels are of high toxicological risk since they may lead to human exposures two orders of magnitude greater than exposures from nitrite-preserved

food or from tobacco smoke. Dinitroanilines are one of the most widely used groups of herbicides (ethalfluralin, benfluralin, pendimethalin, trifluralin) [8–11]. During the manufacturing process, depending on reaction conditions, *N*-nitrosamines can be formed as a by-product of the side reaction of the nitrosating agent and secondary amines. (Figure 2). The mutagenic potential of these compounds is significant since they have the maximum possible number of α-hydrogens (four), except from pendimethalin (one), as can be seen in Figure 2. Studies of drug–nitrite reaction products [4] indicate that pesticide–nitrite reaction products could also prove intrinsically more potent as genotoxic agents than several extensively studied *N*-nitroso compounds which are classified by IARC as probably carcinogenic to humans. However, no such relevant study has been carried out for pesticides to confirm or reject this indication.

Ethalfluralin A: $-CF_3$ B: -H, C: -Et, D: -Me-Allyl
Benfluralin A: $-CF_3$ B: -H, C: -Et, D: -Bu
Trifluralin A: $-CF_3$ B: -H, C: $-C_3H_7$, D: $-C_3H_7$
Pendimethalin A: -Me B: -Me, C: -3-pentyl, D: -H

Figure 2. Dinitroanilines and the respective *N*-nitrosamine impurities.

Limits for *N*-nitrosamine levels in various products have been set by regulatory agencies worldwide, while manufacturers have implemented measures to reduce or eliminate these compounds from their products. The Food and Drug Administration in the United States [12] and the European Chemicals Agency in the European Union [13] have set limits on the levels of *N*-nitrosamines in various products (foods, drugs, cosmetics, children's toys, etc.) and require manufacturers to provide information on the presence of *N*-nitrosamines and to test their products before they can be approved for sale so that they are safe for human health and the environment. Similarly, the European Food Safety Authority (EFSA) has set—indirectly—the limits for *N*-nitrosamines in pesticides, with a maximum concentration of 1 $\mu g\ g^{-1}$ in the Technical Active Substance [14].

In recent years, control of relevant impurities such as *N*-nitrosamines has had an increasing importance in the analysis of plant protection products. It is one of the main concerns for illegal, counterfeit or poor-quality pesticides that make up an estimated 7% of the European pesticide market [15]. Faced with this challenge, regulatory authorities are obliged to apply specific, sensitive and robust methods for the fast and efficient monitoring of these compounds [16–21].

In this study, we present a set of methods for the determination of the nitrosamine ethyl-*N*-(2-methylallyl) *N*-nitroso amine (EMANA) in emulsifiable concentrate (EC) formulations of ethalfluralin. EMANA, as shown in Figure 2, is the by-product of the manufacturing process of ethalfluralin. The proposed approach (a) uses the "dilute and shoot" approach with GC-SIM in the case of high concentrations (>90 $\mu g\ g^{-1}$) and low interference samples; (b) combines SPE with the robustness of an FID detector and the sensitivity and selectivity of an MS/MS detector for high interference samples. Solid Phase Extraction (SPE) is required to reach an LOQ lower than the EFSA acceptable maximum concentration (1 $\mu g\ g^{-1}$).

2. Materials and Methods

Analytical standard of *N*-nitrosoethylmethylallyl-amine (99%) was donated by Dow Agrosciences (King's Lynn, UK). HPLC-grade methanol, acetone, 1-chlorobutane and

hexane were obtained from Fischer Scientific (Fisher Scientific, Waltham, MA, USA). Stock and working standard solutions for method validation were prepared in acetone in the corresponding concentrations for each analysis (Table 1). Twenty-five (S1–S25) ethalfluralin commercial emulsifiable concentrate (EC) formulations were collected from the Greek market. The formulations contained 333 g L^{-1} of ethalfluralin.

Table 1. Validation parameters of the methods.

		GC-SIM	GC-EI-MS/MS	GC-PCI-MS/MS	GC-FID
Linearity of response *	Concentration range *	10–300 μg g^{-1}	1–10 μg g^{-1}	0.05–180 μg g^{-1}	1–110 μg g^{-1}
	Correlation coefficient	0.9979	0.9999	0.9998	0.9997
	Slope	7.6943×10^5	1356.4	1.8895×10^5	6653.9
	Intercept	-2.2331×10^4	−5455.36	1.4769×10^4	−2971.52
Sensitivity	Limit of Quantification (LOQ) **	90 μg g^{-1}	1 μg g^{-1} ***	0.33 μg g^{-1} ***	1 μg g^{-1} ***
System Precision *	RSD of Peak Area	8 @ 0.1 μg mL^{-1} (n = 5)	2.9 @0.1 μg mL^{-1} (n = 3)	13.78@0.1 μg mL^{-1} (n = 3)	0.21@10 μg mL^{-1} (n = 5)
	Horwitz RSDr	10.7@ 0.1 μg mL^{-1} (HorRat 0.75)	10.7 @0.1 μg mL^{-1} (HorRat 0.27)	10.7@ μg mL^{-1} (HorRat 1.29)	7.48 @10 μg mL^{-1} (HorRat 0.03)
Method precision	RSD	4.89 @0.3 μg g^{-1} (n = 5)	27.6 @10 μg/g (n = 5)	0.68@0.33 μg g^{-1} (n = 10)	8@20 μg g^{-1} (n = 3)
	Horwitz RSDr	9.086@0.3 μg g^{-1} (HorRat 0.54)	30.321@10 μg/g (HorRat 0.91)	12.66@0.33 μg g^{-1} (HorRat 0.05)	0.14@20 μg g^{-1} (HorRat 0.91)
Accuracy	Low concentration	@90 μg g^{-1} (n = 3) recovery:91.75%	@1 μg g^{-1} (n = 3) recovery:121.2%	@0.33 μg g^{-1} (n = 10) recovery:110.67%	@20μg g^{-1} (n = 3) recovery:101.49%
		RSD 2.88 Horwitz 1.88	RSD 44.56 Horwitz 30.32	RSD 0.68 Horwitz 12.66	RSD 0.14 Horwitz 6.83
	High concentration	@300 μg g^{-1} (n = 3) recovery:103.01%	@10 μg g^{-1} (n = 3) recovery: 95.21%	@180 μg g^{-1} (n = 3) recovery:97.44	@40 μg g^{-1} (n = 3) recovery:100.54%
		RSD 2.35 Horwitz 1.60	RSD 17.21 Horwitz 21.44	RSD 1.74 Horwitz 4.906	RSD 1.13 Horwitz 6.15

All the concentration in the table refer to EMANA concentration $w.w^{-1}$ in the Technical Active Substance (TAS) for facile comparison with the regulatory maximum permitted concentration of 1 μg g^{-1}. * Linearity and system precision were estimated using working standard solutions since blanks of Plant Protection Products are not available. ** LOQ was estimated by standard addition tests on real samples. *** SPE cleanup required to achieve required LOQ.

GC-SIM and GC-MS/MS analyses were performed on a Varian CP-3800 gas chromatograph with electronic flow control and were interfaced to a 1200 L mass selective triple quadrupole mass spectrometer system (Varian, Palo Alto, CA, USA). Chromatographic separation was achieved using a VF-1 MS (30 m × 0.25 mm × 0.25 μm film thickness, Varian, Palo Alto, CA, USA) capillary column along with a programmable temperature vaporization (PTV-1079) injector. The autosampler was a CTC Combi-Pal (CTC Analytics AG, Zwingen, Switzerland). Instrument control and results' processing were carried out using Varian MS Workstation version 9.6 software (Varian, Palo Alto, CA, USA).

GC-FID analysis was carried out on a Thermo Finnigan Trace GC (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a split/splitless injector, operated in splitless mode, a Flame Ionization Detector and an autosampler (Thermo Finnigan AS 2000). The chromatographic column was a DB-1 (30 m × 0.53 mm × 1.5 μm film thickness, J&W Scientific, Folsom, CA, USA). Instrument control and results' processing were carried out using ChromCard v2.10 software (Thermo Fisher Scientific).

"Dilute and shoot" samples for GC-SIM and GC-MS/MS were prepared by weighing the appropriate amount of the plant protection product (PPP) formulation and dilution with acetone to the appropriate volume to attain a final concentration of ~1000 μg mL^{-1}.

The optimized sample preparation process with Solid Phase Extraction (SPE) was performed using Silica gel cartridges (ISOLUTE®, Biotage, Uppsala, Sweden). The cartridge was adapted to the vacuum apparatus (Vac Master, Overland Park, KS, USA). The bed was preconditioned with methanol (MeOH, 5 mL) and hexane (5 mL), and a thin layer of basic

alumina (1 g) and dry sodium sulfate (2 g) was added on top. The SPE column bed was air-dried under vacuum and wetted with 1-chlrorobutane. An accurately weighed amount of the sample (~0.5 mg) was loaded on top of the silica, followed by the addition of 4 mL 1-chlrorobutane in portions (~1 mL). The column was again air-dried under vacuum. The basic alumina and dry sodium sulfate layers together with the cartridge frit were removed and the silica was transferred to an 8 mL glass vial. A mixture of 1-chlorobutane/MeOH (90:10) was added and the resulting slurry was vortexed and finally filtered through a 0.45 µm filter in an amber sample vial. The filtrate was evaporated under a gentle stream of nitrogen to a final volume of 0.5–0.25 mL for further analysis.

"Dilute and shoot" GC-SIM approach was applied (Figure 3A) in the case of ethalfluralin formulations with low interference from the co-formulants and relatively high content of the impurity (>90 µg g^{-1}). The detailed analytical conditions are listed in Table 2 and validation parameters in Table 1.

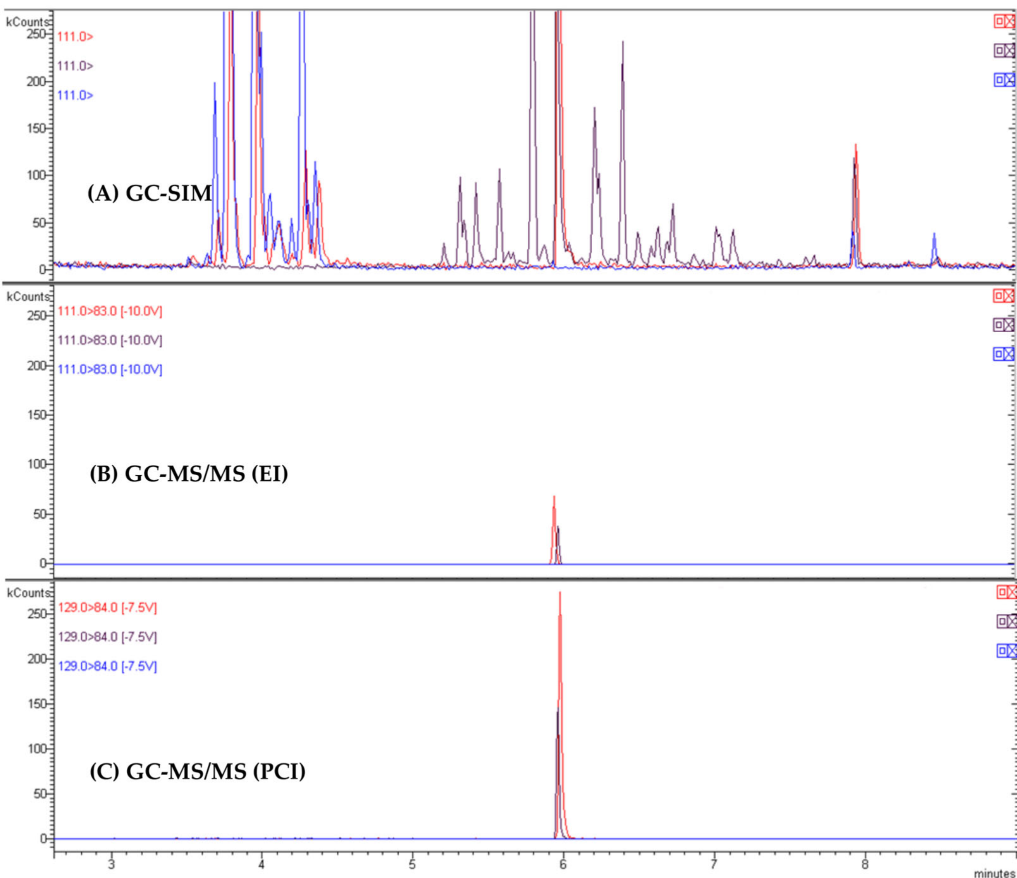

Figure 3. Overlaid chromatograms of samples with low background interference (RED: containing EMANA, BLUE: without EMANA) and high background interference (PURPLE) analyzed by the three mass filtering modes: (**A**): GC-SIM (m/z 111); (**B**): GC-EI-MS/MS (transition 128 > 83); and (**C**): GC-PCI-MS/MS (transition 129 > 84). EMANA retention time: ~6 min. Interfering peaks from co-formulants in SIM (**A**) "disappear" with the MS/MS mode (**B**,**C**). Enhancement of the detection signal achieved with PCI (**C**) is also significant.

"Dilute and shoot" was also tested for lower concentration levels using gas chromatographic separation and tandem mass spectrometry with Electron Impact ionization [GC-MS/MS-(EI), Figure 3B]. The conditions used are presented in Table 2 and the corresponding validation parameters in Table 1.

Table 2. Method instrumental parameters.

	GC-SIM			GC-MS/MS (EI)		GC-MS/MS (PCI)	GC-FID		
Column	VF-5 MS 30 m × 0.25 mm, 0.25 µm film thickness						DB1 30 m × 0.53 mm, 1.5 µm film thick		
Injection Port	250 °C split 100						250 °C, split 30		
Oven Program	Temp (°C)	Rate (°C/min)	Hold (min)	Temp (°C)	Rate (°C/min)	Hold (min)	Temp (°C)	Rate (°C/min)	Hold (min)
	80		0.00	50		0.50	60		5
	220	12	0.00	135	10	0.00	180	5	10
	290	25	30.00	290	70	30.00	270	70	20
Detector Temperatures	Transfer Line/Source: 280 °C/200 °C						Detector (FID): 250 °C		
Mass filtering parameters	* *m*/*z*: 55, 82, 128			** *m*/*z*: 111 > 83, 128 > 111		*** *m*/*z*: 129 > 55, 129 > 70, 129 > 84, 129 > 99			
Q2 pressure (Argon)						1.5 mTorr			
Source pressures	47 mTorr					5 Torr CH_4 (ion source)			
Electron multiplier (V)	1300								

* Proposed fragmentation pattern Supplementary Materials Figure S1. ** Proposed fragmentation pattern Supplementary Materials Figure S2. *** Proposed fragmentation pattern Supplementary Materials Figure S3.

For samples with low content of the impurity (<90 µg g^{-1}) and/or high interference from co-formulants, GC-MS/MS (PCI) was performed after SPE cleanup of small quantities of the sample (0.5 g). The final extract was condensed via evaporation to 0.5 mL. The sample was analyzed under the condition described in Table 2. The LOQ achieved (0.33 µg g^{-1}) was well below the regulatory limit (1 µg g^{-1}), and other validation parameters were also within the acceptable tolerances (Table 1).

The cleaned-up SPE samples also underwent GC-FID analysis using a different column (DB-1) and different chromatographic conditions (Table 2) for confirmation purposes. Samples were concentrated under a gentle stream of nitrogen to a final volume of 0.25 mL. The method was validated (Table 1), and the results confirmed the results from GC-MS/MS (PCI) method. See Figure 4.

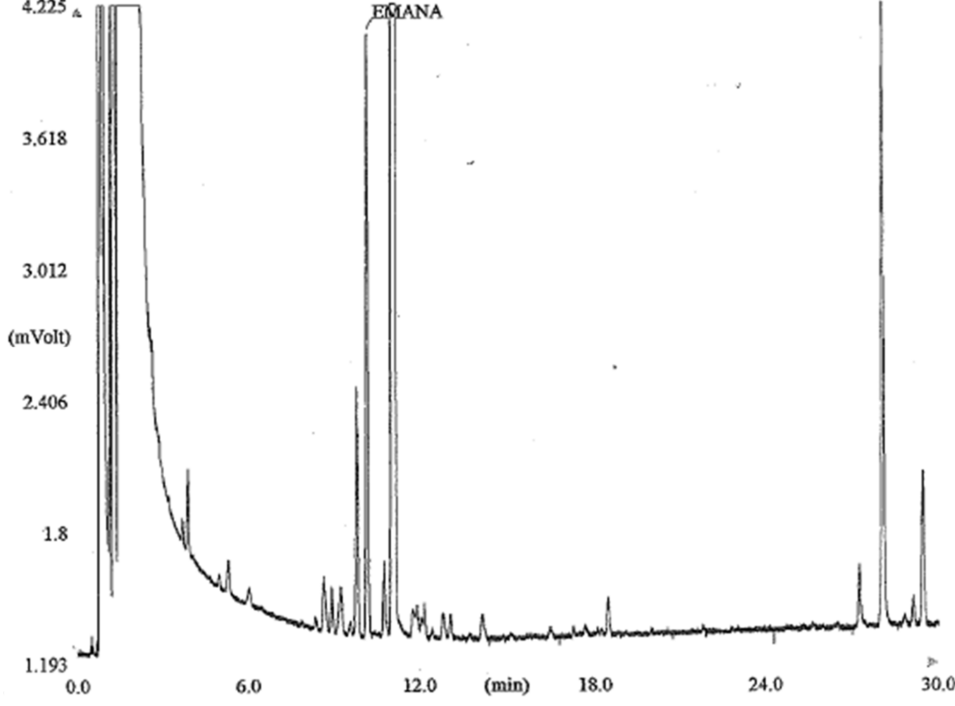

Figure 4. GC-FID chromatogram of real sample with high background interference after SPE cleanup. Retention time of EMANA: 10.15 min.

3. Results

Quality Assurance/Quality Control—Method Validation (Table 1)

Internal validation of analytical methods was carried out via the determination of the analytical quality parameters: precision, linearity, limits of detection and quantification, working range, accuracy, selectivity and sensitivity according to official EU guidelines [22] (Table 1). Linearity was established in all methods using excellent correlation coefficients (R > 0.99). Sensitivity in terms of LOQ was determined in two ways: from recovery experiments and from the standard error of the intercept and slope of the linearity curves. Instrument precision was tested with multiple injections of standard solutions. Method precision was confirmed by testing multiple preparations of the same sample. The modified Horwitz equation was compared with the %RSD of the method using the HorRat ratio. HorRat values for all methods were found below the critical value of two. However, only the GC-PCI-MS/MS and GC-FID methods met the criteria for the HorRat value Hr (Hr < 1) set by EU guidelines [22].

Accuracy was estimated with recovery experiments at two levels. Recovery values and %RSD were satisfactory, ranging from 97.44 to 100.67% for GC-PCI-MS/MS and 100.54 to 101.49% for GC-FID. Selectivity and specificity indicate the degree to which an individual analyte can be determined in a complicate mixture with no interference from the co-formulants or degradation products of the PPP. Lack of interference was demonstrated by a comparison of retention times and mass spectra of the EMANA in the analytical standard and the sample solution, and was confirmed using MS/MS fragmentation and analysis in columns of different polarity. CI-GC-MS/MS and GC-FID analyses were carried out on a DB-1 column, and EI-GC-MS (SIM) and EI-GC-MS/MS analyses were carried out on a DB-5 column. Comparison of the results from all methods indicated that GC-CI-MS/MS is required for the determination of very low concentrations of EMANA. However, in the case of higher concentrations, the application of the less sensitive (higher LOQ) GC-MS and SPE/GC-FID methods can be applied.

The validation results demonstrated the consistency and accuracy of the measurement of EMANA in commercial pesticide formulations and pointed out an absence of systematic error in the developed method.

4. Discussion

The authorization procedure of PPPs includes the identification of impurities and relevant impurities. Specific limits are defined and amended in the Commission Implementing Regulation No. 540/2011 [23] and FAO/WHO specifications [24]. The products are considered safe if these limits are not exceeded. However, relevant impurities are deemed as crucial quality attributes and should be identified when exceeding the threshold defined by the legislation or international specifications.

For nitrosamine analysis, gas chromatography (GC) with a special chemiluminescence detector (also called thermal energy analyzer (TEA)) was the method of choice for about three decades [2]. However, this type of detector is not available in many laboratories since it does not meet wide acceptance for the analysis of other compounds. Today, for many laboratories, the FID detector is a cheap, robust and reliable solution since cost and availability are always weighed when selecting a detector. However, the main criterion for selecting a definitive method is the analytical quality of measurements. For this reason, MS-based techniques are predominantly employed for the identification and quantification of relevant impurities in TAS or formulations. Therefore, most laboratories in research and governmental institutions operate mass selective detectors (MSD) combined with gas chromatographs (GC-MS, GC-MS/MS).

Impurities' determination can be harder in the case of some formulations than in TAS. The presence of co-formulants results in a more complex matrix that can impede detection of an impurity that might be present. In those cases, sample preparation approaches (SPE, LLE) can be optimized to remove co-formulants prior to analysis. In the case that this removal is not feasible, more elaborate separation and/or detection strategies need to be

established. A problem that is often encountered is that sensitive and selective detectors such as MSDs may be susceptible to contamination by the exceptionally high concentrations of the analyzed compounds. Another case is when the increased concentration of the active substance constitutes a chromatographic interference (e.g., by giving a very broad peak, by increased tailing or fronting).

Validation of analytical methods is the only way to verify that a method is fit-for-purpose [25]. Performance characteristics and constraints of a method are established to confirm that it is adequate for resolving a certain analytical problem.

The analysis of real ethalfluralin samples under the frame of the annual market control of plant protection products conducted by the Ministry of Rural Development and Food in cooperation with the Laboratory of Chemical Control of Pesticides of the Benaki Phytopathological Institute was carried out.

As mentioned above in Section 2, the GC-SIM method was used for the analysis of samples with increased content in EMANA and insignificant chromatographic interference from the co-formulants. It was fully validated and applied to readily analyze the first non-compliant samples that were intercepted in the Greek market. As a provision of the national legislation, the method was also inspected during the audit of an independent expert appointed by the manufacturer of the non-compliant PPP.

To achieve an LOQ below the regulatory limit (1 μg g^{-1}), tandem mass spectrometry was tested. With GC-MS/MS-(EI), the LOQ achieved was significantly lower (10 μg g^{-1}), but was still not below the regulatory limit. Performance parameters using standard solutions were very good. However, during the analysis of real samples, method precision at lower concentrations was poor. This is indicative of a strong matrix effect due to interference from the co-formulants. A possible explanation is that Electron Impact ionization (EI), which is considered "hard" and non-selective, provides an inadequate amount of parent ions to break down in the second quadrupole and gives a more intense signal for the corresponding MS/MS transition of EMANA.

Overcoming a strong matrix effect is exceptionally challenging in the case of formulations with strongly interfering co-formulants (Figure 3A—purple-colored chromatogram). To enhance the selectivity and specificity of the MS/MS detector, the application of more "soft" and selective ionization modes than EI were tested. Preliminary Electro Spray Ionization (ESI)–LC-MS/MS experiments indicated very poor ionization of EMANA under ESI conditions. On the other hand, Positive Chemical Ionization (PCI) combined with GC-MS/MS gave very good results (Figure 3C). Chemical Ionization is widely used for various analytes, mostly in the negative mode [26]. However, in the case of nitrosamines and EMANA, the presence of protonation sites on the molecule (nitrogen atoms) favors the use of positive chemical ionization (PCI).

"Dilute and shoot" of high concentration samples (~3300 μg/mL in a.i.) with GC-MS/MS (PCI) was tested to achieve the desired LOQ (<1 μg g^{-1}). However, this approach proved impractical. Sensitivity deteriorated after only a few injections due to overloading of the chromatographic system by the very high concentration of active ingredient and co-formulants. A time-consuming procedure of cleaning the injector port and the ionization source was required. Therefore, it was decided to use SPE cleanup of the sample. In that way, enriched EMANA samples, almost free from the active ingredient and co-formulants, were injected in the chromatographic system and fouling of the system was avoided. The SPE-cleaned samples were further concentrated and analyzed with GC-FID on a column of different polarity for confirmation purposes.

A total number of 25 real samples of PPPs from the Greek market were analyzed to investigate the presence of EMANA and to assess the method's applicability (see Table 3). Most of the samples tested were found to be compliant with the specification of 1 μg/g EMANA in the TAS (0.33 μg/g in formulation). However, in some cases the content of EMANA exceeded the regulatory limits by more than 400%. This was attributed to the omission of a purification step for the removal of EMANA during the manufacturing

procedure. The developed method was successfully utilized for the assessment of EMANA content in ethalfluralin EC formulations.

Table 3. Results from the analysis of commercially available formulation samples from the Greek market.

Sample Code	Concentration of EMANA in the PPP (μg g^{-1})	EMANA in the Technical Active Substance (μg g^{-1})	Sample Code	Concentration of EMANA in the PPP (μg g^{-1})	EMANA in the Technical Active Substance (μg g^{-1})
S1	<LOQ	<LOQ	S14	<LOQ	<LOQ
S2	<LOQ	<LOQ	S15	<LOQ	<LOQ
S3	<LOQ	<LOQ	S16	<LOQ	<LOQ
S4	68.4	203.1	S17	<LOQ	<LOQ
S5	<LOQ	<LOQ	S18	<LOQ	<LOQ
S6	<LOQ	<LOQ	S19	<LOQ	<LOQ
S7	<LOQ	<LOQ	S20	<LOQ	<LOQ
S8	72.8	216.3	S21	<LOQ	<LOQ
S9	<LOQ	<LOQ	S22	<LOQ	<LOQ
S10	72.1	214.2	S23	137.2	406.9
S11	<LOQ	<LOQ	S24	136.1	403.9
S12	<LOQ	<LOQ	S25	<LOQ	<LOQ
S13	<LOQ	<LOQ			

5. Conclusions

As discussed in the introduction, *N*-nitrosamines are associated with carcinogenicity [27]. Since pesticides are applied in bulk quantities, high concentrations of *N*-nitrosamines in pesticide formulations pose a risk mainly for humans and non-target species, but also for the environment. According to the "WHO Guidelines for Water" [28], *N*-nitrosodimethylamine (NDMA), a compound very similar to EMANA (Supplementary Materials Figure S4), has a guideline value of <0.1 μg/L. The guideline also refers to pesticides as one of the sources of contamination of water by NDMA. Therefore, formulations with concentrations of EMANA as high as ~200 μg g^{-1} (200 times above the regulatory limit of 1 μg g^{-1}) in the technical active substance pose a high risk for water contamination and other environmental compartments as well as humans (especially farmers) and other non-target species. No extensive risk assessment of EMANA has been conducted since the registration data for ethalfluralin guaranteed that its concentration would be below 0.05 μg g^{-1}. Today, illegal, low quality and counterfeit pesticides have become increasingly abundant in the international market. This is a new source of impurities, such as EMANA, for the environment.

A set of methods for the determination of the relevant impurity ethyl-*N*-(2-methylallyl) *N*-nitroso amine (EMANA) in a wide range of concentrations in emulsifiable concentrate formulations (EC) of ethalfluralin was developed. "Dilute and Shoot" in combination with GC-SIM was used for samples with exceptionally high concentrations. Solid Phase Extraction (SPE) and Positive Chemical Ionization GC-MS/MS was applied for the determination of formulations with low concentrations of the impurity in matrices with high interference from pesticide co-formulants. GC-FID was used for confirmation. Methods were validated for all the parameters required by the EU Guidelines [22].

The method was applied in routine analysis for post-registration control of plant protection products in the Greek market. The results for non-compliant samples were confirmed following the provisions of national legislation. Re-analyses of the respective counter-samples were carried out under the audit of an independent expert appointed by the manufacturer of the respective PPP.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13051104/s1, Figure S1: EMANA EI-MS (SIM) proposed fragmentation mechanism, Figure S2: EMANA EI-MS/MS proposed fragmentation mechanism, Figure S3: EMANA PCI-MS/MS proposed fragmentation mechanism, Figure S4: Structural resemblance of EMANA, NDMA and NDEA (IARC 2A classification—Probably carcinogenic to humans) and NDPA (IARC 2B classification—Possibly carcinogenic to humans). Figure S5: Target Compound Report from real sample with high interference. Figure S6: GC-PCI-MS/MS chromatograms of solutions of standard EMANA and non-compliant samples.

Author Contributions: Conceptualization, G.P.B. and H.K.; methodology, G.P.B. and H.K.; software, G.P.B.; validation, G.P.B. and H.K.; investigation, G.P.B.; resources, G.P.B. and H.K.; data curation, G.P.B.; writing—original draft preparation, G.P.B.; writing—review and editing, G.P.B.; visualization, G.P.B.; supervision, H.K.; project administration, H.K.; funding acquisition, H.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available on request due to restrictions.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. International Agency for Research on Cancer (IARC). Agents Classified by the IARC Monographs, Volumes 1–132. Available online: https://monographs.iarc.who.int/agents-classified-by-the-iarc (accessed on 5 April 2023).
2. European Medicines Agency (EMA). Nitrosamine Impurities in Human Medicinal Products: CHMP Assessment Report for the Article 5(3) of Regulation EC (No) 726/2004 procedure; EMA: 25 June 2020. Available online: https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-assessment-report_en.pdf (accessed on 5 April 2023).
3. Akkaraju, H.; Tatia, R.; Mane, S.S.; Khade, A.B.; Dengale, S.J. A comprehensive review of sources of nitrosamine contamination of pharmaceutical substances and products. *Regul. Toxicol. Pharm.* **2023**, *139*, 105355. [CrossRef]
4. Brambilla, G.; Martelli, A. Genotoxic and carcinogenic risk to humans of drug–nitrite interaction products. *Mutat. Res./Rev. Mutat. Res.* **2007**, *635*, 17–52. [CrossRef] [PubMed]
5. Ross, R.D.; Morrison, J.; Rounbehler, D.P.; Fan, S.; Fine, D.H. Normal-Nitroso Compound Impurities in Herbicide Formulations. *J. Agric. Food. Chem.* **1977**, *25*, 1416–1418. [CrossRef] [PubMed]
6. Fishbein, L. Overview of Potential Mutagenic Problems Posed by Some Pesticides and Their Trace Impurities. *Environ. Health Perspect.* **1978**, *27*, 125–131. [CrossRef]
7. Kearney, P.C. Nitrosamines and Pesticides: A Special Report on the Occurrence of Nitrosamines as Terminal Residues Resulting from Agricultural Use of Certain Pesticides. *Pure Appl. Chem.* **1980**, *52*, 499–526. [CrossRef]
8. Reportlinker.com, Ethalfluralin: Global Product Intelligence (2020–2025). Available online: https://www.reportlinker.com/p04098753/Ethalfluralin-Global-Product-Intelligence.html (accessed on 5 April 2023).
9. Reportlinker, Trifluralin: Global Product Intelligence (2020–2025). Available online: https://www.reportlinker.com/p03971327/Trifluralin-Global-Product-Intelligence.html (accessed on 5 April 2023).
10. Reportlinker, Pendimethalin: Global Product Intelligence (2020–2025). Available online: https://www.reportlinker.com/p03971303/Pendimethalin-Global-Product-Intelligence.html (accessed on 5 April 2023).
11. Reportlinker, Benfluralin: Global Product Intelligence (2020–2025). Available online: https://www.reportlinker.com/p05245011/Benfluralin-Global-Product-Intelligence.html (accessed on 5 April 2023).
12. Food and Drug Administration (FDA). Control of Nitrosamine Impurities in Human Drugs—GUIDANCE DOCUMENT Rev.1. 2021. Available online: https://www.fda.gov/media/141720/download (accessed on 5 April 2023).
13. European Chemicals Agency (ECHA). Nitrosamines-Substance Infocard. Available online: https://echa.europa.eu/substance-information/-/substanceinfo/100.305.347 (accessed on 5 April 2023).
14. European Commission (EC). Directorate-General for Health and Food Safety (DG SANTE), SANCO/10597/2003—Rev. 10.1: Guidance Document on the Assessment of the Equivalence of Technical Materials of Substances Regulated Under Regulation (EC) No 1107/2009. 2012. Available online: https://food.ec.europa.eu/system/files/2016-10/pesticides_guidance_equivalence-chem-substances_en.pdf (accessed on 5 April 2023).

15. Europol. 122 Tons of Illegal or Counterfeit Pesticides Seized during Operation Silver Axe II. 2017. Available online: https://www.europol.europa.eu/media-press/newsroom/news/record-number-of-1-346-tonnes-of-illegal-pesticides-taken-market-in-2020-global-operation-silver-axe#:~:text=Email-,A%20record%20number%20of%201%20346%20tonnes%20of%20illegal%20pesticides,2020%20global%20operation%20Silver%20Axe&text=Europol%20coordinated%20the%20fifth%20edition,compared%20to%20last%20year\T1\textquoterights%20operation, (accessed on 5 April 2023).
16. Plonka, M.; Walorczyk, S.; Miszczyk, M. Chromatographic methods for the determination of active substances and characterization of their impurities in pesticide formulations. *Trends Anal. Chem.* **2016**, *85*, 67–80. [CrossRef]
17. Plonka, M.; Walorczyk, S.; Miszczyk, M.; Kronenbach-Dylong, D. Simultaneous gas chromatographic determination of chlorpyrifos and its impurity sulfotep in liquid pesticide formulations. *J. Environ. Sci. Health B* **2016**, *51*, 736–741. [CrossRef] [PubMed]
18. Badawy, M.E.I. Development and validation of HPLC methods for analysis of chlorantraniliprole insecticide in technical and commercial formulations. *J. Environ. Sci. Health B* **2018**, *53*, 411–422. [CrossRef] [PubMed]
19. Smith, K.; Prusnick, T. Application of Raman microscopy in pesticide formulation analysis. *Abstr. Pap. Am. Chem. S* **2018**, *256*.
20. Li, Q.Q.; Xie, J.C.; Zhang, J.X.; Yan, H.; Xiong, Y.M.; Liu, W.; Min, S.G. A global model for the determination of prohibited addition in pesticide formulations by near infrared spectroscopy. *Infrared Phys. Technol.* **2020**, *105*, 103191. [CrossRef]
21. Maragou, N.C.; Balayiannis, G. Determination of Ethephon in Pesticide Formulations by Ion Exchange Chromatography with Indirect Spectrophotometric Detection. *Anal. Lett.* **2020**, *53*, 795–806. [CrossRef]
22. European Commission (EC). Directorate-General for Health and Food Safety, SANCO/3030/99 rev.5: Technical Active Substance and Plant Protection Products: Guidance for Generating and Reporting Methods of Analysis in Support of Pre- and Post-Registration Data Requirements for Annex (Section 4) of Regulation (EU) No 283/2013 and Annex (Section 5) of Regulation (EU) No 284/2013. Available online: https://food.ec.europa.eu/system/files/2019-03/pesticides_ppp_app-proc_guide_phys-chem-ana_3030.pdf (accessed on 5 April 2023).
23. European Union (EU). Commission Implementing Regulation (EU) No 540/2011 of 25 May 2011 Implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as Regards the List of Approved Active Substances. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32011R0540 (accessed on 5 April 2023).
24. Food and Agriculture Organization of the United Nations (FAO)—World Health Organization (WHO). *Manual on the Development and Use of FAO and WHO Specifications for Chemical Pesticides*, 2nd ed.; FAO: Rome, Italy; WHO: Geneva, Switzerland, 2022.
25. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). Draft ICH Guideline Q14 Analytical Procedure Development. 2022. Available online: https://database.ich.org/sites/default/files/ICH_Q14_Document_Step2_Guideline_2022_0324.pdf (accessed on 5 April 2023).
26. Anagnostopoulos, C.; Charalampous, A.; Balayiannis, G. EI and NCI GC-MS and GC-MS/MS: Comparative Study of Performance Characteristics for the Determination of Pesticide Residues in Plant Matrix. *Chromatographia* **2015**, *78*, 109–118. [CrossRef]
27. Magee, P.N.; Barnes, J.M. The production of malignant primary hepatic tumors in the rat by feeding dimethylnitrosamine. *Br. J. Cancer* **1956**, *10*, 114–122. [CrossRef] [PubMed]
28. World Health Organization (WHO). Guidelines for Drinking-Water Quality: Fourth Edition Incorporating the First and Second Addenda. Available online: https://www.who.int/publications/i/item/9789240045064 (accessed on 5 April 2023).

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.