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QUALITATIVE AND QUANTITATIVE ANALYSIS OF IMPURITIES IN CHLOROCHOLINE CHLORIDE: A REVIEW

BY

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Abstract. This study provides an overview of impurities in an active substance / active ingredient and selective analytical methods of impurities for their identification in chlorocholine chloride with applications and literature references. Moreover, its reviews the importance to understanding what determinates an impurity and identification of potential sources of this impurity is discussed. The analytes include 1,2-dichloroethane and trimethylamine which are relevant impurities acceptable by Food and Agriculture Organization of the United Nations and World Health Organization. After a concise resume introduction regarding the properties of chlorocholine chloride, attention is focused primarily to separation, identification and detection for qualitative and quantitative analysis of these two analytes. The determination method used after identification of impurities are also discussed. In comparation with spectroscopic (UV-Vis, FTIR, NMR), spectrometric (MS), chromatographic (GC, IC) characterization methods, applications in impurities analysis have demonstrated that coupled or tandem techniques (CG-MS, GLC-MS, HPLC-UV-VIS) gain popularity regarding selectivity, high sensitivity, reliability, good accuracy, robustness and precision for monitoring the impurities from chlorocholine chloride production. According

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to European Food Safety Authority, chromatographic techniques are recommended for identification purpose.

Keywords: characterization, detection, separation, 1,2-dichloroethane, trimethylamine.

1. Introduction

Chlorocholine chloride (2-chloroethyl trimethylammonium), known as cycocel or CCC or Chloromequat, is a product with use in agriculture, as well as in horticulture as a plant growth regulator, belongs to the family of pesticides with a content of quaternary ammonium compounds , and fungal diseases are mainly controlled with prochloraz and various conazole fungicides (INCHEM, 1970; Wigfield and Lacroix, 1987; Winek *et al*., 1990; Castro *et al*., 2001; Evans *et al*., 2001; Horak *et al*, 2001; Muthmann and Nadin, 2007; European Food Safety Authority, 2008; Chimcomplex, 2017). Chlorocholine chloride represents one of the most well-known substances that inhibit the growth and development of the vegetative part. It ranks second in the top ten as an active ingredient in the European Union and was first reported for its use in oilseed crops in 2003 (Muthmann and Nadin, 2007).

The physiological action of chlorocholine chloride consists in inhibiting the growth in stem length and increasing the intensity of the plant growth process in width due to the involvement of choline in lipid metabolism (Wigfield and Lacroix, 1987; Wawrzyniak *et al*., 2016; Food and Agriculture Organization, 2018). It also influences the development cycle of vegetative organs, leading, for example, to an increase in flowering. Moreover, it can lead to an increase in chlorophyll formation and root development, producing hardier plants (INCHEM, 1970; Castro *et al*., 2001; Food and Agriculture Organization, 2018).

The impurities identified and standardized in chlorocholine chloride are 1,2-dichloroethane and trimethylamine (vinyl chloride) (INCHEM, 1970).

The experts at the EFSA meeting, (2008) agreed that 1,2-dichloroethane is a relevant but acceptable impurity at the levels proposed in the FAO specification (0.01%). They also agreed that vinyl chloride is a relevant impurity and the highest level proposed in the technical specifications (0.0004 g/L in the technical concentrate, which corresponds to 0.0005 g/kg in the technical material) does not create a significant toxicity concern. As regards crops, the maximum residue levels proposed by the European Union in most types of fruit and vegetables are set at 0.05 mg/kg, while for most cereals it is 2 mg/kg (Alder and Startin, 2005).

It is very important that before starting the development of a method, it is important to define as clearly and in as much detail as possible what the objectives and goals are for the separation intended for the identification of impurities. These questions are:

(1) Should all impurities and degradation products from each other and from the main component be determined?

(2) Is it only necessary to separate impurities and degradation compounds from the main component?

(3) Are all standards available for all impurities, degradation products and solutes identified?

For this question, it may be necessary to separate all impurities and degradants from the main component for a purity test. However, if the objective is to obtain an impurity profile, then all components will need to be separated from each other. In some cases, it is also possible to obtain both impurity profile data and an assessment of the purity of the major component from a single method (Okafo and Roberts, 2003).

Once an impurity has been detected, it becomes necessary to estimate its content. Detectability frequently means that a given component provides a signal at least twice as large as the background noise or baseline. For impurity quantification, the multiple is set much higher. Initial estimates are generally made relative to the parent compound, since in most cases the original impurity sample is not available. When the original sample is available, it is important that it be used for estimations. If estimates indicate that a certain impurity content is greater than 0.1%, it must be characterized according to (Food and Drug Administration) FDA and The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) requirements (Ahuja, 2007).

The development of an active ingredient or active substance requires that meaningful and reliable analytical data be generated at various stages of the development of the active ingredient or active substance. Ensuring the safety of a new chemical compound requires that it meet established purity standards as a chemical entity or when mixed with animal feed for toxicity studies or pharmaceutical excipients for human use. In addition, it should exhibit excellent shelf-life stability.

These requirements require that the analytical methodology used be sensitive enough to measure low levels of impurities. This has led to analytical methods that are suitable for the determination of trace / ultra trace levels, i.e. very small amounts of the order of micrograms or nanograms of various chemical entities. Different methods are available for monitoring impurities. The main criterion is the ability to differentiate the compounds of interest. This requirement reduces the availability of methods primarily to spectroscopic and separation methods or a combination of these methods (Ahuja, 2007). Taking these aspects into consideration, the aim of this study is to identify methods for the quantitative analysis of impurities in chlorocholine chloride.

2. Literature Review

In general, the use of a plant growth regulator can lead to increased yield of a crop, improve quality and increase resistance to stress by regulating cell growth, cell division, rhizogenesis, germination, flowering and ripening (Weng *et al*., 2018).

Chlorocholine chloride was first reported by Tolbert in 1960, and marketed by American Cyanamid and the BASF Corporation in 1966 under the name Cycocel. It is currently available under several trade names, as the pure active ingredient or in mixtures with other pesticides (Castro *et al*., 2001).

In 1970, the Food and Agriculture Organization of the United Nations and the World Health Organization published a report on the regulation and application protocol of chlorocholine chloride treatment per hectare for various crops. This report states that at least 17 countries have regulated the use of chlorocholine chloride as a plant growth regulator specifically to promote more robust growth in wheat, rye and oats and also used to improve grape berry production on bunches and to increase production yield (INCHEM, 1970).

The effectiveness of the substance depends on its concentration in the species, the phase of plant development, the method of applying the substance, as well as external conditions. In horticulture, chlorocholine chloride is used to stimulate the formation of potato tubers, the shortening of pear shoots, the rooting of apple shoots, but also in the culture of tomatoes, beans and some flower species (Wigfield and Lacroix, 1987; Wawrzyniak *et al*., 2016).

In Spain, it is registered for use on pears, grapes, almonds, rye and wheat (Castro *et al*., 2001). It is also widely used in wheat, rice, cotton, tobacco, corn and tomato crops. Taking advantage of the effect by inhibiting cell elongation, chlorocholine chloride improves crop resistance to drought, excess soil water, saline-alkaline soil (Weng *et al*., 2018) and leads to an increase in production yield for wheat, rye crops, oats and triticale. Furthermore, it is used on a wide variety of ornamental, herbaceous and woody plants to promote lateral branching and flowering in plant species such as azaleas, fuchsia, begonia, poinsettias, pelargonium and other species of ornamental plants.

It is also used in cotton crops (United States Environmental Protection Agency, 2007; Food and Agriculture Organization, 2018). More than that can be applied to the plant by spraying (foliar fertilization), ex. Lilium Oriental hybrids 'Sorbonne' – flower varieties with bulbs (Zheng *et al*., 2012); Helianthus tuberosum "Albik" plants (Wawrzyniak *et al*., 2016); cabbage and turnip (Brassica oleracea) (Gholampour *et al*., 2015); natural seed, flowering, fruit development and production yield of tomatoes (Altintas, 2011); coumarin content and crop yield increase (Hou *et al*., 2013).

Regarding quantitative and qualitative investigation of chlorocholine chloride in complex matrices, several studies was made on fruits: apples, pears, grapes, kiwi, peaches, oranges, tomatoes, purees and concentrated fruit juices

(Gabr *et al*., 1985; Startin *et al*., 1999; Evans *et al*., 2000; Mol *et al*., 2000; Castro *et al*., 2001; Careri *et al*., 2002; Reynolds *et al*., 2004; Alder and Startin, 2005; Zheng *et al*., 2012; Chamkasem, 2018), vegetables: carrots, mushrooms (cultivated), mushrooms (wild), sugar pea (Castro *et al*., 2001; Reynolds *et al*., 2004; Alder and Startin, 2005; Chamkasem, 2018); plants-based food: wheat, wheat flour, unprocessed wheat bran, buckwheat, barley, oats, cottonseed and cotton plants, pecan (walnut), tomato plant (Dekhuijzen and Vonk, 1974; Hedin *et al*., 1984; Gabr *et al*., 1985; Allender, 1992; Careri *et al*., 2002; Reynolds *et al*., 2004; Alder and Startin, 2005; Guo *et al*., 2010; Cycon *et al*., 2012; Gu *et al*., 2013; Sytar *et al*., 2014; Mao *et al*, 2015; Weng *et al*., 2018); food of animal origin: milk, eggs, chicken (breast and thigh), pork whey and sow's milk (Nurhayati *et al*., 2003; Reynolds *et al*., 2004; Nurhayati *et al*., 2006; Poulsen *et al*., 2007).

Chlorocholine chloride is a complex chemical compound in terms of identity, technical, physical and chemical properties, chemical analysis methods. It is found as salt with the name of chlormequat chloride (CCC, chlorocholine chloride, Cycocel) and in the form of a cation, Chlormequat. The chemical structures are shown in Fig. 1 (salt) and Fig. 2 (cation).

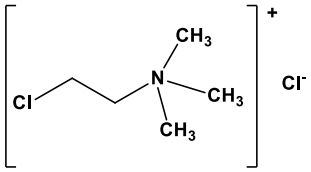

Fig. 1 − Chemical structure of 2-chloroethyltrimethylammonium chloride.

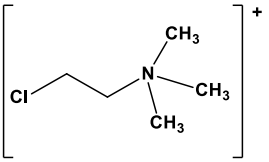

Fig. 2 − Chemical structure of 2-chloroethyl trimethyl ammonium

The material should contain chlormequat chloride together with related manufacturing impurities and shall be a pale yellow to pale yellow liquid with a moderate fishy odor, free of visible foreign matter and added modifying agents (INCHEM, 1970; Evans *et al*., 2001; United States Environmental Protection Agency, 2007; European Food Safety Authority, 2008; European Food Safety Authority, 2014; Chimcomplex, 2017; Food and Agri-culture Organization, 2018; Sigma Aldrich, 2021).

Impurity can be defined as any substance that coexists with the original

active ingredient, such as raw material or reaction intermediates, and produces due to any adverse reactions. The impurity profile is a description of the identified and unidentified impurities present in the products (Dhangar *et al*., 2017).

Technical materials originating from different sources of the same active substance found in plant protection products may have different levels of chemical purity, which means that their differences could be not only in terms of im-purity content, but also in chemical identity.

Impurities in the active substances mainly come from the synthesis process and depend on the type of substrate materials and the type of synthesis technology used, as well as the conditions in which the plant protection product was stored or the type of packaging in which it was added. Without adequate cleaning, impurities can also enter plant protection products on the production line and/or during the packaging process when there is a change in production or packaging from one substance to another (Plonka *et al*., 2016).

Impurities in active ingredients or active substances come from various sources and phases of the process of synthesis and preparation of final products (European Food Safety Authority, 2008). However, most impurities are characteristics of the synthetic route of the manufacturing process. Since there are several possibilities of synthesizing a chemical product, it is possible that the same product from different sources can give rise to different impurities (Kushwaha, 2008), and as a result these paper presents information on the source of impurities, but also specific terms used in the technical field.

According to FAO/WHO Joint Meeting on Pesticide Specifications, 2002; Okafo and Roberts, 2003; Camarasu *et al*., 2006; Ahuja, 2007; Sandra *et al*., 2008; Singh *et al*., 2012; Dhangar *et al*., 2017 the information on the nomenclature and source of impurities are classified as follows:

Frequently used terms: material or materials, intermediate, penultimate intermediate, byproducts, transformation products, products of chemical interactions, similar products.

Terminology used according to the compendial provisions: impure substances, toxic impurities, co-produced components, impurities given by the signal, common impurities, volatile organic impurities, impurities associated with degradation.

Terminology used according to Technical Requirements for Pharmaceuticals for Human Use (ICH): organic impurities, inorganic impurities, other materials, residual solvents, the water, insoluble substances.

Variations in the type and content of impurities can have a significant toxicological effect on the properties of the technical concentrate, as well as on plant protection products. Impurities can have toxic effects, influence phytotoxicity as well as physical properties of products, cause an unwanted increase in the number of residues in food products or contaminate the environment. The risk of toxicological hazards increases with non-original plant protection products, the production of which often takes place without any quality

control of the content of active substances used in the production of a particular plant protection product. Illegal plant protection products can cause: losses for farmers in the form of lost crops, pose a threat to those preparing working solutions, a threat to the environment due to the introduction of unknown (or even banned) substances (Plonka *et al*., 2016).

Determining impurities in commercial plant protection products is a difficult task that cannot be solved simply by a routine analytical approach. Impurity analysis requires efficient separation of components followed by sensitive and selective detection to enable the determination of impurities present at trace or micro-trace levels. On the other hand, sensitive detectors may be prone to contamination, or the active substance, which in plant protection products usually occurs at a high level of concentration, may exhibit chromatographic interference (Plonka *et al*., 2016). Maximum limits for relevant impurities must be <1 g/kg. Maximum limits for these relevant impurities should also be supported with analytical data on each production lot (minimum 5 typical lots) (FAO/WHO Joint Meeting on Pesticide Specifications, 2002).

It is often necessary for impurities to be isolated, as some instrumental methods are not available or further confirmation is required. For example, when coupled or tandem mass spectrometry (LC-MS) techniques are not adequate or do not provide unequivocal characterization, it may be necessary to isolate impurities for further structure confirmation or to perform toxicity studies. Of course, once the structure has been established, these impurities can be synthesized by an appropriate route (Ahuja, 2007).

3. Methods and Analyses

For the isolation, separation and characterization of impurities, the first step must be initiated by simple extraction or partition methods, such as: solid-phase extraction (SPE); liquid-liquid extraction (LLE); accelerated solvent extraction (ASE); supercritical fluid extraction (SFE). It may be possible to extract selective impurities based on acidity, basicity or neutrality. The extraction process usually involves liquid-liquid extraction, in which one phase is an aqueous solution and the other is a nonpolar organic phase. By properly adjusting the pH of the aqueous solution, acidic, basic impurities can be extracted or neutral.

Other separations can be made by chromatographic methods: column chromatography; flash chromatography; thin-layer chromatography (TLC); gas chromatography (GC); high-pressure liquid chromatography (HPLC); capillary electrophoresis (CE); supercritical fluid chromatography (SFC). Frequently, the methods of isolation tend to be the same methods that are used for analysis (Ahuja, 2007). As for impurity characterization, the final step is generally done by matching retention time using spectroscopic (UV, IR, NMR, MS) and chromatographic methods (GC) (Smith and Edwards, 2003; Sandra *et al*., 2008).

With the increasing absolute need for monitoring and controlling impurities, the efficiency of elucidating their chemical structure has become a long-standing problem in industry (Singh *et al*., 2012), which is why coupled or tandem techniques, like gas chromatography-mass spectrometry (GC–MS), liquid chromatography-mass spectrometry (LC–MS), liquid chromatography-nuclear magnetic resonance spectroscopy (LC–NMR), capillary electrophoresis-mass spectrometry (CE-MS) and other tandem techniques are the most selective analytical methods for the identification of impurities (Franke *et al*., 1979; Okafo and Roberts, 2003; Smith and Edwards, 2003; Ahuja, 2007; Sandra *et al*., 2008; Singh *et al*., 2012; Dhangar *et al*., 2017).

Regarding the impurities from Chlorocholine chloride, in Table 1 is presented a synthesis of the quantitative determination of impurities, 1,2 dichloroethane and trimethylamine.

Table 1

Qualitative and quantitative determination of 1, 2-dichloroethane and trimethylamine in chlorocholine chloride

Methods of obtaining	Impurities	Separation method	Qualitative / quantitative analysis	References
Chlormequat chloride technical solution or concentrate (dissolved in water / dimethylacetamide)	EDC*	GC Columns: polyethylene glycol stationary phase and polydimethylsiloxane	Quantitative 0.2 μg/g	FAO, 2018
		settling/phase separation of the reaction product; stripping DCE remaining in the aqueous phase by CCC****; decolourization of the stripped solution with activated carbon; filtering spent active carbon;	Concentration correction of CCC finished product (dilution with demineralized water)	Chimcomplex, 2017
	EDC	Class 1	Quantitative Concentration limit 1870 ppm	Dhangar *et al.*, 2017

Synthesis from EDC and TMA in both aqueous and gas phases	TMA**	Colorimetric sensor range	Qualitative	Li *et al.*, 2016
	EDC	GC-MS, HPLC-UV	Quantitative	Qiao *et al.*, 2010
	EDC		EDC, max. 0.1 g/kg dry CC and chloroethene content: max 0.0004 g/l)	EFSA, 2008
1) Headspace SPME 2) Gas-tight SPME	EDC	GC-MS	Quantitative	Camarasu *et al.*, 2006
The condensation reaction between TMA and EDC	- CC*** - EDC or TMA	GLC, GLC-TEA or MS	Quantitative	Wigfield and Lacroix, 1987
Separation of CCC from CC		Paper chromatography	Qualitative	Koudela and Cleleszky, 1974
Preparation of CC from TMA and Ethylene Chlorhydrin	Trimethylammo-nium salts and trimethylammo-nium hydroxide	Titration prcess	Qualitative	Cundif and Riddick, 1952

Notes: EDC* - 1,2 dichloroethane; TMA** - trimethylamine; CC*** - choline chloride; CCC**** - chlorocholine chloride

4. Conclusions and Future Research

This paper provides an overview of impurities in an active substance / active ingredient and methods for quantitative analysis of impurities in chlorocholine chloride and further provides information on nomenclature, source of impurities, separation techniques and selective analytical methods for their identification. According to the definition in Webster's Dictionary an impurity is something impure or that makes something impure. An impure substance may be defined as follows: a substance of interest mixed or impregnated with a foreign or usually inferior substance. These definitions can help generate a more concise definition of an impurity: any material that affects the purity of the material of interest (Ahuja, 2007).

The identification and quantification of impurities is becoming an increasingly significant part of the control of chemical products, as impurities

affect the quality, stability and safety of these products. Reports in the literature clearly show a definitive shift from the conventional way of elucidating the structure of impurities (involving isolation and spectral analysis) to the use of modern coupled or tandem techniques, and yet liquid chromatography (LC) continues to be a widespread technique, although there are also numerous reports on the use of capillary electrophoresis (CE). In terms of detection, MS systems have the ability to provide, unequivocally, elucidation of the im-purity structure.

Many studies have also appeared in the literature on the use of the LC-NMR system in support of structure characterization, and a few studies on CE-NMR. The use of FTIR as a detector is still limited, due to the problem of sensitivity and the need to avoid salts in the buffer solutions in the mobile phase. However, coupled or tandem techniques are anticipated to remain mainstream and gain increased popularity for impurity characterization as technology im-proves to provide even higher resolution and sensitivity and as instrumentation becomes affordable. A critical review of literature reports reveals the need for correct data collection using justified practical approaches and strategies and the importance of systematic data interpretation (Singh *et al*., 2012; Plonka *et al*., 2016).

Advances in analytical techniques have contributed to the development of new methodologies that reduce the amounts of solvents and reagents required in sample pre-treatment as well as in the instrumental analysis step (eg, fewer mobile phase solvents are used). In this way, the negative impact on human health and the environment is reduced and the techniques approach the principles of green chemistry (Singh *et al*., 2012).

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ANALIZA CALITATIVĂ ȘI CANTITATIVĂ A IMPURITĂȚILOR ÎN CLORURĂ DE CLORCOLINĂ: O SCURTĂ TRECERE ÎN REVISTĂ

(Rezumat)

Acest studiu oferă o imagine de ansamblu asupra impurităților dintr-o substanță activă / ingredient activ și metodele analitice selective ale impurităților pentru identificarea acestora în clorura de clorcolină cu aplicații și referințe din literatură. Mai mult decât atât, se analizează importanța înțelegerii a ceea ce determină o impuritate și se discută identificarea surselor potențiale ale acestei impurități. Analiții includ 1,2-dicloretan și trimetilamină, care sunt impurități relevante acceptabile de Organizația pentru Alimentație și Agricultură a Națiunilor Unite și Organizația Mondială a Sănătății. După o introducere concisă în rezumat cu privire la proprietățile clorurii de clorcolină, atenția se concentrează în primul rând pe separarea, identificarea și detectarea pentru analiza calitativă și cantitativă a acestor doi analiți. Se discută și metoda de determinare utilizată după identificarea impurităților. În comparație cu metodele de caracterizare spectroscopică (UV-Vis, FTIR, RMN), spectrometrice (MS), cromatografice (GC, IC), aplicațiile în analiza impurităților au demonstrat că tehnicile cuplate sau tandem (CG-MS, GLC-MS, HPLC, UV-VIS) câștigă popularitate în ceea ce privește selectivitatea,

sensibilitatea ridicată, fiabilitatea, precizia bună, robustețea și precizia pentru monitorizarea impurităților din procesul de producție pentru clorura de clorcolină. Conform Autorității Europene pentru Siguranța Alimentelor, tehnicile cromatografice sunt recomandate în scopul identificării impurităților.