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Original article

Risk of de-novo formation of chlormequat and mepiquat in industrial cocoa products assessed by ion chromatography coupled with high-resolution mass spectrometry

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Summary Mepiquat (MQ) and chlormequat (CQ) are two main food contaminants that could be naturally present in various animal and plant foods due to heat treatments. Cocoa and its derivative products seem to be a matrix inclined, for both chemical characteristics and production technologies, to the formation of MQ and CQ. In this study, exploiting the selectivity and sensitivity of IC-HRMS, a new reliable analytical method was developed and validated for MQ and CQ quantification. The LOD of the method was set at 1.9 μg kg^{-1} for both CQ and MQ, and the linearity was defined from 6.25 to 625 μg kg^{-1}. Average recoveries, at 2 μg L^{-1}, were 96.5% and 97.5% for CQ and MQ, respectively, while precision as repeatability (RSDr%) was 2.3% and 5.1% respectively. Subsequently, a laboratory-scale experiment was conducted to assess, for the first time, the actual risk of MQ and CQ formation, confirming in cocoa products the presence of MQ at detectable concentration after 55 min at 180 °C, approximately 8 μg kg^{-1} for cocoa and 7 μg kg^{-1} for chips. However, the investigation of a wide variety of commercial cocoa and nuts products has fortunately ruled out the presence of such contaminants at detectable concentrations.

Keywords Chlormequat, chocolate, cocoa, mepiquat, orbitrap.

Introduction

Chlormequat chloride (2-chloro-N,N,N-trimethylethanaminium chloride, CQ) and mepiquat chloride (N,N-dimethylpiperidinium chloride, MQ) are quaternary ammonium growth regulators (quats) usually used as chloride salt, which work on gibberellin synthesis inhibition. Growth regulators are used in agriculture to reduce vegetative growth including sprout suppression and to keep plants more compact in order to minimise pruning costs and obtain a better vegetative growth and fruit product ratio. MQ, along with CQ, is primarily used as anti-lodging agents in cereal production, including wheat, oats, barley, rye and triticale, in liliaceous, such as onions, leeks and garlic, and in cotton to reduce excessive vegetative growth (Rademacher, 2000).

Toxicology of CQ and MQ has been studied in mice, rats and dogs, and the European Food Safety Authority (EFSA) stated that the chemicals have a low acute toxicity (EFSA Scientific Report, 2008a, 2008b). Currently, both are classified as H302 'Harmful if swallowed', while CQ also classified as H311 'Toxic in contact with skin' and MQ as H412 'Harmful to aquatic life with long-lasting effects'. Maximum residue limits (MRLs) of CQ (sum of CQ and its salts, expressed as CQ-chloride) and MQ (sum of MQ and its salts, expressed as MQ-chloride) in foodstuffs have been established by the European Commission in Annex III of Reg. (UE) 2020/1565 and Annex II Reg. (UE) 2019/50, respectively ('COMMISSION REGULATION EU 2019/50', 2019, 'COMMISSION REGULATION EU 2020/1565', 2020), which in cocoa defines limits of 0.05 and 0.1 mg kg^{-1} for CQ and MQ, respectively, both referring to a generic 'limit of analytical determination'.

Several studies have examined the possibility of MQ neoformation during the roasting/toasting process in coffee and dark barley malts founding that lysine, due to the Maillard-driven degradation, undergoes cyclisation (by decarboxylative deamination) in the presence of a reducing sugar that provides the piperidine intermediate (Hammel *et al.*, 2014). Moreover, the N-methyl groups are transferred from a quaternary nitrogen compound to the nucleophile piperidine generating detectable amounts of MQ. Up to now, trigonelline, choline and glycine betaine have been identified as possible

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methylation agents (Bessaire *et al*., 2014; Yuan *et al*., 2017). Pipecolic acid and pipecolic acid betaine also play an important role in the formation of MQ. The first can form piperidine, albeit with the further requirement of alkylating agents, while the second can form MQ directly through decarboxylation (Yuan *et al*., 2017). The key factor in the amount of MQ produced was found to be temperature: it forms at the typical roasting temperature of coffee (220–240 °C), but in malt roasted at 169 °C, MQ was found to be present. The higher is the temperature, the higher the quantity synthetised; as a confirmation, both dark barley malts and coffee colour showed an excellent positive correlation with MQ content (Esparza *et al*., 2009; Wermann *et al*., 2014; Noestheden, 2015; Bessaire *et al*., 2016; Yuan *et al*., 2017). The possibility of the formation of MQ was also recently confirmed in potatoes, broccoli and meat (Li *et al*., 2021).

The natural formation of CQ was also investigated in dark barley malts (Ekielski *et al*., 2018), and the chemical mechanism was explained by Eriksen *et al*. (2020) evaluating the role of choline in its formation under thermal conditions (200 °C). The formation of CQ was also documented in wheat flour and egg powder after cooking (Yuan *et al*., 2017; Eriksen *et al*., 2020).

No evidence of this risk, to the best of our knowledge, is instead reported in the literature for cocoa-based products. Theobroma cacao contains several alkaloids: mainly theobromine (0.5–2.7%) but also caffeine (about 0.25%) and trigonelline. Endogenous levels of trigonelline in cocoa are lower than those observed in coffee (in dry seeds 27.9 ± 6.1 μmol g^{-1} fresh weight) and vary from 14 to 124 nmol g^{-1} fresh weight in leaves and fruits, in which higher levels have been detected in seeds rather than in the pericarp (Zheng *et al*., 2004). Lysine, which plays a key role, is present in cocoa at approximately 70 μmol g^{-1}. The content is dependent on the action of endogenous proteases; however, it seems to decrease during fermentation down to nearly 50 μmol g^{-1} (De Brito *et al*., 2001). Additionally, a study evidenced that cocoa beans contain 0.3–0.4% of phospholipid of which phosphatidylcholine was considered the major component (Knapp and Others, 1937). Considering that the roasting process of cocoa beans is usually performed between 120 and 150 °C for a time ranging from 5 to 120 min (Sacchetti *et al*., 2016), it seems reasonable to hypothesise the risk of MQ and CQ formation, but to the best of our knowledge, this has not yet been investigated. Moreover, commercial cocoa-based products are often associated with hazelnuts that also present trigonelline (Caligiani *et al*., 2014) and a mean content of lysine of 4.54 mg g^{-1} (Köksal *et al*., 2006).

Due to their extremely polar cationic character and low volatility, extraction and chromatographic separation of CQ and MQ are not trivial. Several approaches have been reported in the literature using gas chromatography–mass spectrometry approaches and, more frequently, liquid chromatography-mass spectrometry (Dos Belmonte *et al*., 2022). Ion-pairing reagents have often been used, resulting in possible mass spectrometry ion source ionisation issues. To avoid this practice, a solution could be the use of an off-line solid-phase extraction (SPE) sample treatment, a technique that is extremely time consuming (Oh *et al*., 2014). As reported by Nardin *et al*. (2017), cation-exchange chromatographic columns represent the best choice to obtain a good chromatographic separation and break down the matrix effect.

The aim of this study was to develop a robust and sensitive analytical method by combining ion chromatography and hybrid quadrupole orbitrap mass spectrometry (IC-HQOMS) to detect MQ and CQ at the legal MRLs. The novelty of the method is the use of cation chromatography combined with high-resolution mass spectrometry. This technique is not very widespread, it is often considered dangerous to combine a very acidic eluent with the mass source, and our work aimed to demonstrate that the technique is safe, robust and above all effective for solving chromatographic problems of polar compound separation and efficient for the precise quantification due to the exact mass.

A laboratory-scale experiment was also performed to investigate the risk of quats formation in cocoa powder and chocolate chip samples subjected to various conditions of elevated temperature and exposure time. In addition, the presence of CQ and MQ in commercial cocoa-based and nuts foods was assessed, assuming the possibility of its neoformation during the roasting processes or industrial baking.

Materials and methods

Reagents and standard solutions

LC–MS-grade methanol (MeOH), LC–MS-grade acetonitrile (ACN) and MS-grade formic acid (FA, 98%) were purchased from Fluka (St. Louis, MO, USA). For mass calibration, a standard mix of n-butylamine, caffeine, methionine–arginine–phenylalanine–alanine–acetate (MRFA) and Ultramark 1621 (Pierce® ESI Positive Ion Calibration Solution, Rockford, IL, USA) was used. Ultrapure water was obtained from Arium® Pro Lab Water System (Sartorius AG, Goettingen, Germany).

CQ chloride (97.2% purity) and MQ chloride (98.6% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mepiquat-d_{16} chloride (d_{16}-MQ, ≥ 97.0%) was purchased from Sigma-Aldrich, and CQ chloride 1,1,2,2-d_4 (d_4-CQ, 100 ng μL^{-1}) was purchased from Dr. Ehrenstorfer (Augsburg, Germany).

Two stock solutions of standards were prepared in polypropylene flasks by dissolving the pure quats in a

50% aqueous MeOH solution: the first containing the two quats in non-labelled form and the second containing deuterated ones. Calibration solutions were prepared at concentrations of 0.15, 0.5, 1, 10 and 50 μg L^{-1} for each native analyte and 20 μg L^{-1} for the isotopically labelled compounds. The calibration solutions were freshly prepared before each analysis, while stock solutions were stored at −4 °C.

Sample preparation

Laboratory-scale experiment

A sample of cocoa powder (about 100% cocoa) and a sample of chocolate chips (>48% cocoa powder), gathered on the Italian market, were submitted to different thermal treatments in a lab oven (TM 120, Argolab, Italy) set at 90, 160, 180 and 230 °C for 15 or 50 min, respectively, in order to test the possible CQ and MQ formation under different thermal and time exposures, as expected for the industrial cocoa bean roasting and baking of cakes and biscuits (Shahapuzi *et al.*, 2015; Domínguez-Pérez *et al.*, 2019). All samples were prepared in triplicate.

Survey of commercial cocoa and nut products

Twenty-four commercial samples of cocoa powder, cocoa creams, shelled almonds and hazelnuts, milk and dark chocolate tablets and chocolate cookies (Table 1) from different national and international producers were collected at food stores in Trentino-Alto Adige (Italy).

Sample analytical extraction

All the samples (two untreated samples, 48 thermally treated ones and 24 commercial cocoa samples) were homogenised and extracted adapting the procedure of QuPPe-PO-methods ('Quick Method for the Analysis of Highly Polar Pesticides in Food Involving Extraction with Acidified Methanol and LC- or IC-MS/MS Measurement I. Food of Plant Origin (QuPPe-PO-Method, Version 12)', 2021). Briefly, 4 g of the sample was placed in a 50 mL falcon (Sartorius, Gottinga, Germania) with 25 mL of a water solution with FA at 0.5% *v*/*v*. Also 20 μg L^{-1} of d_{16}-MQ and d_4-CQ as internal standards was added. The mixture was treated for 60 min in an ultrasonic bath (FALC Instruments, Italy) and adjusted to 50 mL with MeOH. The sample stored at −21 °C overnight was then centrifuged in order to remove precipitated proteins, while supernatant lipids were then filtered through a PTFE membrane (0.45 μm, Sartorius, Gottinga, Germania). The sample was finally transferred into a high-performance liquid chromatography (HPLC) plastic vial before analysis.

Table 1 Commercial samples

Sample n°	Classification	Cocoa and cocoa derivative ingredients	Hazelnut content (%)
Homemade sweet ingredients or ready-to-use products			
1	Roasted cocoa beans	Cocoa beans 100%	–
2	Shelled almonds bio		100
3	Shelled almonds		100
4	Shelled hazelnuts		100
5	Shelled Giffoni's hazelnuts		100
6	Bitter cocoa powder bio	97% of cocoa of which 20% of cocoa butter	–
7	Bitter cocoa powder	Cocoa 99%	–
8	Hazelnut chocolate cream	Dark cocoa 7.4%	13
9	Hazelnut chocolate cream		30
10	Hazelnut chocolate cream	Low fat cocoa 6.5%	18.5
11	Hazelnut chocolate cream	Dark cocoa 9.8%	13.7
12	Milk chocolate with extra cocoa from Ghana	Cocoa 55% (cocoa paste and cocoa butter)	–
13	Milk chocolate with hazelnuts	Cocoa 30% (cocoa paste and cocoa butter)	9
14	Milk chocolate with extra cocoa	Cocoa 45% (cocoa paste and cocoa butter)	–
15	Dark chocolate with whole hazelnuts	Cocoa 50%	25
16	Extra dark chocolate aromatic from Nicaragua	Cocoa 61% (cocoa paste and cocoa butter)	–
17	Extra dark chocolate intense from Peru	Cocoa 74% (cocoa paste and cocoa butter)	–
18	Extra dark chocolate with whole hazelnuts	Cocoa 50% (cocoa paste and cocoa butter)	24
19	Extra dark chocolate	Cocoa >50% (cocoa paste and cocoa butter)	–
20	Chocolate noir intense	86% of cocoa (cocoa paste, cocoa butter, low fat cocoa)	–
21	Chocolate noir extra	70% of cocoa (cocoa paste, cocoa butter, low fat cocoa)	–
Pastries			
22	Chocolate cookies	Extra dark chocolate 14%, cocoa 2%, chocolate 1.8% and low fat cocoa 1.2%	–
23	Chocolate cookies	Cocoa 3.6% and chocolate 3.5%	–
24	Chocolate cookies	Cocoa 3.7% and chocolate 3.1%	–

Chromatographic separation and MS acquisition conditions

An high-performance ion chromatography Dionex™ ICS-6000 HPIC system (Thermo Scientific, Bremen, Germany) equipped with an AS-AP autosampler, an eluent generator, a dual pumps, an automation manager with a six-port automated switching high-pressure valve, an electrolytic Dionex ERS 500 suppressor and a CD conductivity detector was used for the SPE-online concentration and purification (IonPac CS17, 2 × 15 mm, 5.5 µm; with length expressly modified by Thermo Scientific for this study) of the sample and the subsequent chromatographic separation (IonPac CS19, 2 × 250 mm, 5.5 µm; Thermo Scientific). Chromeleon™ 7.2 Chromatography Data System software (Thermo Scientific) automatically managed the system and was used for acquisition control and data processing.

Separation was performed at a flow rate of 0.4 mL min^{-1} with a methanesulfonic acid gradient set at 0.002 mol L^{-1} from 0 to 0.3 min, 0.01 mol L^{-1} from 0.3 to 6 min, 0.04 mol L^{-1} from 6 to 15 min, 0.04 mol L^{-1} held for 1 min (total run time 16 min) and finally returned to 0.002 mol L^{-1} and held for 4 min (20 min) to re-equilibrate the column. Initially, the six-port diverter valve was set to position 1–6 (to waste), 85 µL of the sample was injected, and the gradient started with matrix clean-up with 10% of ACN and 90% of water at 0.4 mL min^{-1} held for 2 min. Sequentially, the diverter valve was set to position 1–2 (to column). After the complete elution of CM and MQ, at 15 min the diverter valve returned to position 1–6 for washing the SPE-online cartridge with formic acid at 1% for 4 min and with 10% of ACN and 90% of water for 1 min. The autosampler was cooled to 5°C and the column to room temperature (24°C). A Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with heated electrospray source (HESI-II) was used for CQ and MQ detection. The tune parameters were set according to Nardin *et al.*, which acquisition conditions were performed with a full MS data-dependent MS^2 analysis (full MS–dd MS^2) (Nardin *et al.*, 2017).

The conductivity detector was used to check the suppressor functionality, and a T-connection to the HESI source has been suitably made to prevent any breakage of the suppressor in the event of pressure changes in the source inlet.

Method validation

Multi-level calibration was performed to evaluate linearity and limit of quantification (LOQ) according to the SANTE guidelines (Guidance SANTE 11312/2021). The concentration levels were from a water/MeOH/FA (69.6:30:0.4, v/v/v) solution followed by 0.15, 0.5, 2, 5, 10 and 50 µg L^{-1}, each one injected four times. Also 20 µg L^{-1} of d_{16}-MQ and d_4-CQ as internal standards was added to compensate for the matrix effect and response drift in the chromatography detection system and accurately compensate for both analyte losses and volumetric variations during the procedure. Recovery was tested by adding 0.5 µg L^{-1} and 2 µg L^{-1} of CM and MQ to samples 2, 12 and 19. Moreover, the standard solution at 0.5 µg L^{-1}, that was injected four times, was used to evaluate the precision as repeatability (relative standard deviation, RSDr%).

Statistical analysis

Statistical analysis was performed using XLSTAT 2023 (Addinsoft, Denver, USA). The data were expressed as mean ± standard deviation (SD). All measurements were carried out in triplicates. The significance of difference was calculated using the Kruskal–Wallis test, and the results with a *P*-value of <0.05 were considered to be statistically significant.

Results and discussion

Method validation

The quantification of CQ and MQ was performed using the masses corresponding to the already charged molecular ions $[M]^+$ with a mass accuracy error ≤5 ppm. The precursor ions (Fig. 1) and confirming fragments of all quats are reported in Table 2. The method allowed the quantification of quats using isotopic dilution-calibration curves obtained by plotting the peak area ratio of the quantifier ions ($A_{standard}/A_{labelled\ standard}$), multiplied by the internal standard concentration vs. the corresponding concentration level.

The limit of detection (LOD) of the method was set at 1.9 µg kg^{-1} for both QC and MQ, thus respecting the European Commission prescriptions for the limits of analytical determination and being this method even more sensitive. Other methods for CQ and MQ analysis in water report LODs lower than ours but require long pre-concentration steps to obtain those results (Dos Belmonte *et al.*, 2022). In our case, similar results are not necessary as our approach already defined LODs extremely lower than the established MRLs for those food matrices. Other authors tried simple preparation like QuEChERS combining than an LC–MS analysis, but finally they failed to get lower LOQs as ours (Pu *et al.*, 2018).

The linearity was confirmed from 6.25 to 625 µg kg^{-1} with calibration curves that showed correlation coefficients (R^2) above 0.996 in all the investigated concentration ranges and residual deviations

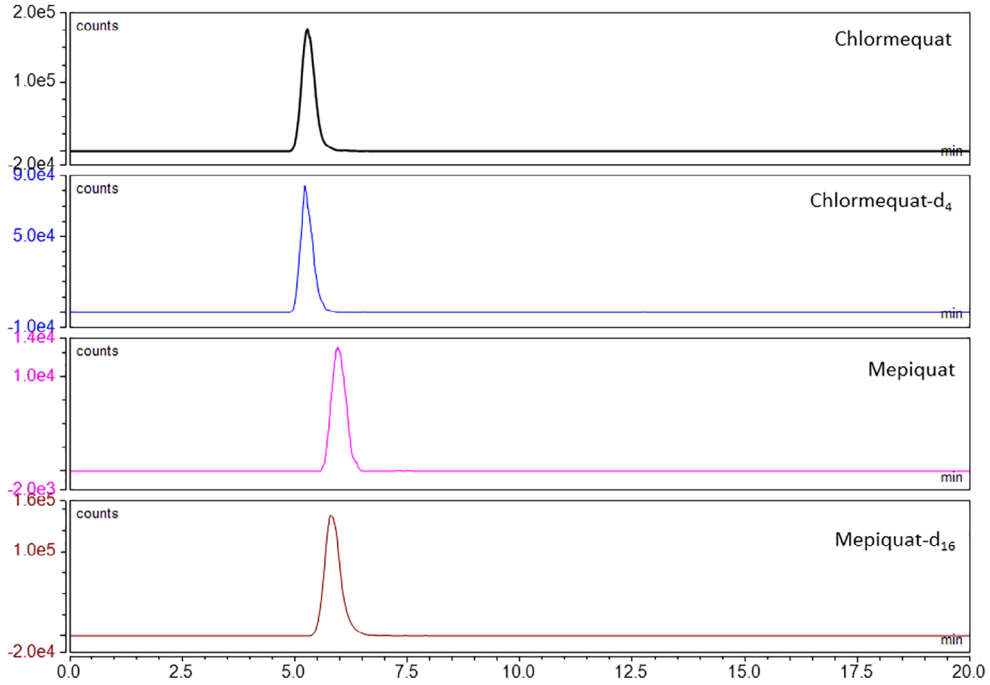

Figure 1 Extracted ion chromatograms of a mixed standard solution at 10 μg/L.

below 10%. Average recoveries, tested at level 2 μg L^{-1}, were 96.5 and 97.5% for CQ and MQ, respectively, while precision as repeatability (RSDr%) was ≤20% (2.3% and 5.1% for CQ and MQ respectively, Table 2).

CQ and MQ neoformation

Due to the chemical composition but also considering the technological processes of derivative preparation, cocoa and above all its commercial derivative products seem to be predisposed for a natural development of MQ and CQ. Initially, the real risk of formation was evaluated in the laboratory by heating cocoa powder and chocolate chips at different temperatures (90, 160, 180 and 230 °C) and for different times (15 and 50 min). This experiment reflected what might happen during an industrial process or during homemade sweet preparation. The starting raw products were free of the two investigated compounds. After the cooking, in none of the samples CQ was detected, while the formation of MQ occurred both in the cocoa and in the chocolate chips, but above 180 °C. At this temperature, the formation appears to be very slow and the MQ concentration was higher than the LOD only after 55 min of cooking (about 2 μg kg^{-1}, Fig. 2), while at 230 °C, the formation was extremely rapid and after 15 min an amount almost equal to that detected after 55 min was formed (approximately 8 μg kg^{-1} for cocoa and 7 μg kg^{-1} for chips). The significance differences calculated using the Kruskal–Wallis test were shown in Fig. 2. The products treated at 230 °C resulted significant different from those treated at 180 °C that in case of chips resulted different from those treated at 160 and 90 °C.

Therefore, it has been shown that the chemical characteristics of cocoa are actually able to favour the development of the MQ but not the CQ. Considering that the roasting process of cocoa beans is usually performed between 120 and 150 °C for a time ranging from 5 to 120 min (Sacchetti *et al.*, 2016), a natural formation during this process is to be considered unlikely. Moreover, the concentrations produced are extremely low compared to those similarly highlighted in coffee, from 1 to 3 mg kg^{-1} (Nardin *et al.*, 2017), where, in fact, the roasting process exceeds 200 °C. This study also confirms what was reported by Li *et al.* (2021), that in different food matrices was able to observe the development of MQ only after cooking for at least 15 min at 220–260 °C. Cocoa-like MQ contents are finally found similarly in pork, and in any case decidedly low compared to the MRL set by law for cocoa.

Commercial cocoa and nut products

Considering the evidence of neoformation during the laboratory experiment, MQ has been researched in a

Table 2 Quat precursor ions, confirming fragments and validation parameters

Compound	RT (min)	$[M]^+$ (m/z)	*m/z* (ppm)	NCE	MS/MS fragments (m/z)	LOD (μg L^{-1})	LOQ (μg L^{-1}) (RSD %; Recovery %)	Recovery at 2 μg L^{-1} (%)	Linearity range (μg L^{-1})	R^2	Precision (RSDr%)
Chlormequat	5.25	122.0729	1.6	80	59.6965 67.5103	0.15	0.5 (4.3; 93)	96.5 ± 0.2	LOQ-50	0.996	2.3
Chlormequat-d_4	5.25	126.0981	0.9	80	58.0657 67.0552						
Mepiquat	5.97	114.1276	1.5	100	113.9636 87.0042	0.15	0.5 (12.4; 89)	97.5 ± 0.3	LOQ-50	0.996	5.1
Mepiquat-d_{16}	5.97	130.2281	0.4	80	71.0294 80.0347						

Note: RT, retention time; m/z (ppm), accurate mass error compared to exact mass; NCE, normalised collision energy; LOD, limit of detection; LOQ, limit of quantitation; RSD%, relative standard deviation.

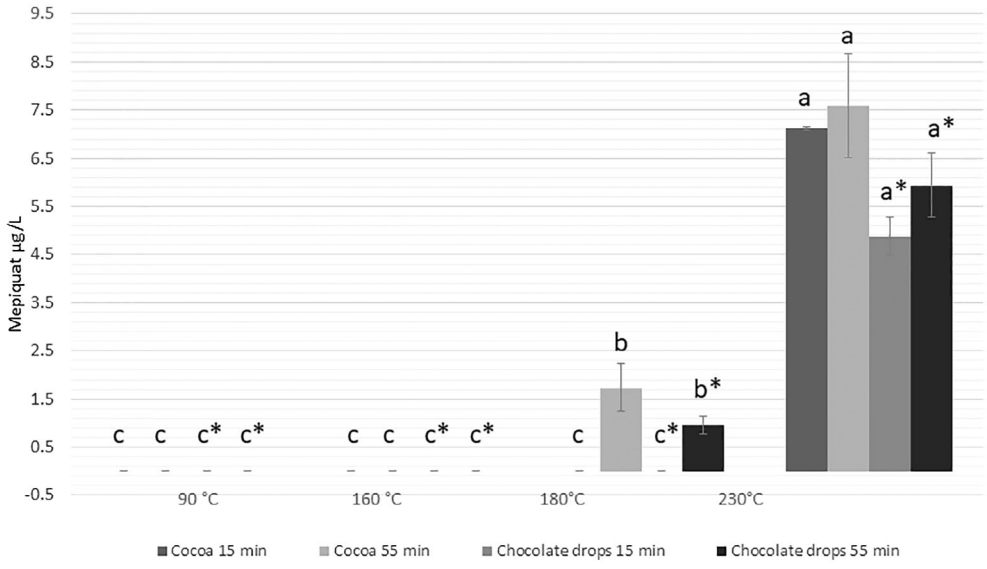

Figure 2 Mepiquat production during treatment at different temperatures and different times. Error bars correspond to standard deviations. a, b, c: significant differences between cocoa treatments; a*, b*, c*: significant differences between chocolate chip treatments; Kruskal–Wallis test (P value < 0.5).

selection of commercial products, including both ready-to-eat products and products used for home preparations. Fortunately, the IC-HQOMS analysis revealed that the quat was always at a concentration below the LOD in all food samples. The presence of hazelnuts and other ingredients that induce the consequent increase in MQ precursors did not promote the trigger of neo-synthesis. Furthermore, neither the roasting of the beans nor the industrial cooking with different heat treatments, reasonably very different in intensity and duration, seem to represent a significant risk factor for the formation of quat. Motivation should stem from the fact that generally the industrial treatments are completely automated and optimised, so never exceed the necessary temperatures or cooking durations. On the contrary, considering the lab experimental evidence, it seems more plausible to suggest a possible risk of MQ formation in the case of home cooking, which, less standardised for control parameters, could more closely reflect what has been observed in laboratory experiments.

Conclusions

Ion chromatography coupled with high-resolution mass spectrometry made it possible the CQ and MQ quantification at low concentrations, in compliance with the EU Regulation for MRLs. In particular, it

allowed us to analyse complex matrices, such as cocoa and derivatives, thanks also to the online-column purification. With a laboratory-scale experiment, we have shown that cocoa and chocolate-based products, when exposed to prolonged heating conditions, can develop MQ. However, its formation was found to be limited and significantly lower than that previously observed in coffee after roasting, and always under the MRL. A survey conducted on a selection of commercial cocoa and nut products collected on the Italian market completely excluded the presence of these compounds, even at very low concentrations. Nevertheless, it is not possible to exclude, in the case of home-baked goods without strict heating conditions, that the neoformation of this contaminant may occur. In spite of this, considering the normal cooking temperature of cakes and biscuits and the actual temperature reached inside a wet food, it is possible to hypothesise only a limited quat formation on the dry and exposed crust.

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Author contributions

Tiziana Nardin: Conceptualization (equal); investigation (equal); writing – original draft (equal). **Riccardo Savastano:** Formal analysis (equal); writing – original draft (equal). **Jakob Franceschini:** Formal analysis (equal). **Franco Abballe:** Validation (equal). **Roberto Larcher:** Investigation (equal).

Ethical approval

Ethics approval was not required for this research.

Conflict of interest

The authors declare no conflict of interest associated with this work.

Peer review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/ijfs.16424.

Data availability statement

The data supporting this study's findings are available from the corresponding author upon reasonable requests.

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