INNOVHUB
STAZIONI SPERIMENTALI
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innovazione e ricerca

Methods of analysis
Experiences and perspectives

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- Review of analytical methods for the determination of MCPDEs and GE
- Factors characterizing direct and indirect methods
- Innovhub experience: cooperative studies and proficiency tests
- Automatic methods for sample preparation
- Innovhub perspectives

PROCESS CONTAMINANTS

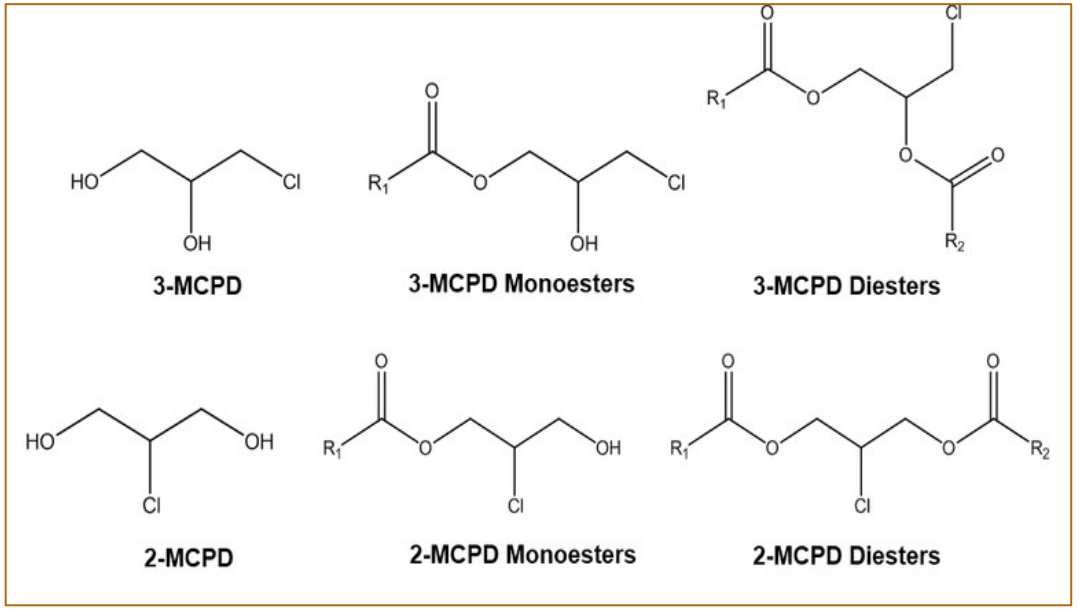
2-monochloropropane-1,3-diol (2-MCPD)
3-monochloropropane-1,2-diol (3-MCPD)
2- and 3-MCPD fatty acid esters (ester-bound MCPDs)

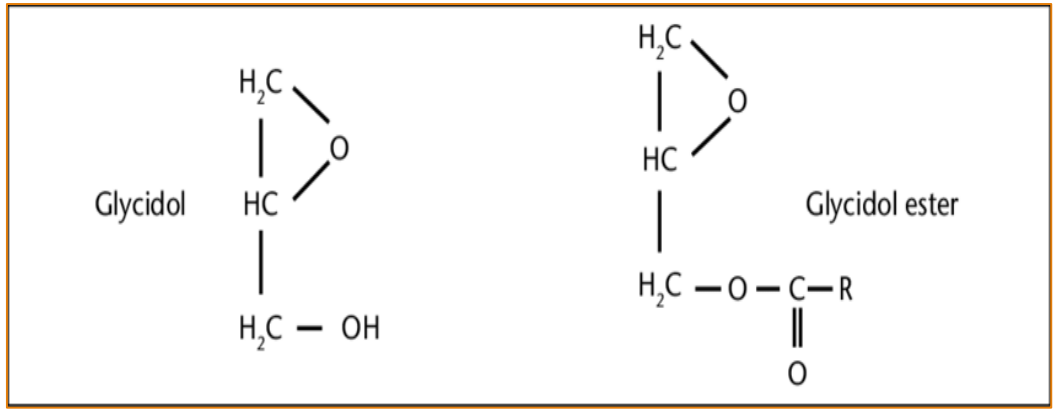
formed from reaction between chlorine containing-compound with acylglycerols, phospholipids and glycerol due to high T° processes in:

- salted foodstuffs temperature-treatment (e.g. soy sauce)
- thermally processed foods (such as bakery products, malt-derived products, cooked/cured fish or meat, chips)
- oils and fats from refining process (deodorisation at T > 230°C).

Glycidol is associated with the formation and decomposition of 3- and 2-MCPD.

Glycidyl fatty acid esters form monoesters with fatty acids (GEs) during the refining of oils and fats from DAGs/MAGs and do not require chlorinated compounds.





HISTORY OF MCPD ESTERS AND GLYCIDIL ESTERS

❑ 1979-1980: First investigations

Chlorinated propanols (as 3-MCPD and Esters 3-MCPD) were identified for the first time in acid-hydrolysed vegetable proteins (ACID-HVP) by Velisek et al. (1979) and Davidek et al (1980).

❑ 2004-2006: The problem started to be considered

High levels of esters of 3-MCPD were identified in some foodstuffs (Svejkovska et al. 2004) and then in refined edible oils (Zelinkova et al.2006).

❑ 2008-2013: Started most of the investigations

Esters of 3-MCPD were found in several foodstuffs as potato chips, crackers, French fries, smoked fish, smoked ham, margarine, vegetable soups, mayonnaise and infant formula (Zelinkova et al. 2009; Hamlet et al. 2011, Weisshaar 2011; Chung et al. 2013).

Then **esters of 2-MCPD** were identified in refined fats and oils (Kuhlmann 2011, Tennant e Gosling 2015).

Fatty acid esters of glycidol (GEs) had been detected as overestimation of 3-MCPD fatty acid esters analysis in refined vegetable oils (Weisshaar & Perz, 2010, Craft et al.2013; Crews et al.2013; MacMahon et al.2013).

ANALYTICAL METHODS DEVELOPED

Indirect methods

Conversion the chloropropanol MCPDs esters and glycidol esters (as MCPD/MBPD) into single compounds quantified as free form.

These methods have in common a series of steps:

- **addition of internal standard** (free or esterified form of isotopically labelled MCPDs/GE)
- **ester cleavage** (alkaline or acidic catalysed transesterification)
- **neutralization and salting out** (with sodium chloride, sulphate salts and sodium bromide)
- **purification and extraction** of free analytes
- **derivatization** with phenylboronic acid (PBA) or heptafluorobutyryl imidazole (HFBI)
- **injection into GC-MS** or optional TripleQuad-MS

Direct methods

Direct determination of all the possible individual MCPDs esters and glycidyl esters.

- ❑ Complexity in the composition of the analytes. A high number of different compounds should be quantified.
- ❑ Several standards are required for a correct quantification. Not always commercially available.
- ❑ Separation of MCPDEs and GEs from the oil matrix applying SPE or gel permeation chromatography (GPC).
- ❑ Provide detailed information on the structure of the individual esters 3-MCPD, 2-MCPD and GE (suitable for research and toxicological studies).
- ❑ Use of sophisticated instrumentation such as: LC-MS, LC-MS/MS, LC-TOF-MS, LC-high resolution-MS.
- ❑ Limitation in the application as routine methods.
- ❑ Official method AOCS-JOCS Cd 28-10 2017– GE in edible oils.

CRITICAL STEPS OF INDIRECT METHODS

- Decomposition of the 3-MCPDs during alkaline hydrolysis resulting in a conversion into glycidol due to strong dependence on the temperature and time of transesterification.

- Necessity of following a strict analytical protocol for alkaline transesterification. Acid transesterification is more robust but requires long time of incubation.

- Formation of additional 3-MCPDs is possible if chloride salts are used in the salting out extraction steps (conversion of glycidol). Strong dependence of the pH value.

- Stability of the mass spectrometer due to strong source contamination.

INDIRECT METHODS

Advantages

- Allow the determination of all ester- bound MCPDs and all glycidol esters through the conversion and the stabilisation of GEs to either a compound structurally similar to MCPD - bromopropandiol (MPBD) or to MCPD itself.
- Validation of three official AOCS methods (2013) by proficiency test
- Commercial availability of standards as 2-3 MCPD, MCPDEs and GE stable isotopes labelled
- Suitable methods in Quality control laboratories for routine analyses
- Simple analytical instruments commonly used in laboratories (GC-MS).

Disadvantages

- Several steps in sample preparation; possibility of errors
- Sample preparation requires different chemical reactions that produce different concomitant reactions (reduction in precision).
- Several critical factors that must be monitored and minimized (i.e. transformation of 3-MCPD into glycidol, formation of additional 3-MCPDs *de novo*)
- Specialized analysts dedicated to analysis
- Long analysis times either by alkaline and acid transesterification (cleavage).

INDIRECT ANALYTICAL METHODS FOR MCPD AND GLYCYDIL ESTERS

Type of transesterification	Analyte	Comments	Reference
H_2SO_4/MeOH	3-MCPD esters	16 h transesterification time -Good robustness – High specificty	Divinova *et al.*(2004); Zelinkova *et al.* (2006)
H_2SO_4/MeOH, pretreatment NaBr/H+ (Unilever)	3- and 2-MCPD esters, glycidyl esters	pretreatment NaBr/H^+ for conversion of glycidyl esters into 3-MBPD esters 16 h transesterification time	Ermacora and Hrncirik (2013)
Alkaline , non specific (DGF C-III 18(09) A) ($NaOCH_3$/MeOH)	Sum of 3-MCPD and glycidyl esters	5 min transesterification time – Salting out with NaCl- Low specificity, not specific to bound 3-MCPD alone (overestimation of 3-MCPD)	DGF (2009)
Alkaline , non specific (DGF C-VI 17(10) B) ($NaOCH_3$/MeOH)	Sum of 3-MCPD and glycidyl esters	5-10 min transesterification time – Modification of (DGF C-III 18(09) A)	DGF (2011 a)
Alkaline with pretreatment (DGF C-III 18(09) B) ($NaOCH_3$/MeOH)	3-MCPD esters	5-10 min transesterification time – Pretreatment by sulphuric acid/propanol mixture to eliminate glycidyl esters – Withdrawn in 2011 as insufficient and not reliable pretreatment step	DGF (2009)
Alkaline chloride free (DGF C-VI 18(10)) ($NaOCH_3$/MeOH)	3-MCPD esters	3-5 min transesterification time - Substitution of sodium chloride with other salts	DGF (2011 b)
Alkaline mild (SGS) (NaOH/MeOH)	3- and 2-MCPD esters, glycidyl esters	16 h transesterification time- Low temperature (-22°C) to eliminate undesirable of MCPD to glycidol. Complex procedure	Kuhlamann (2011)

PROFICIENCY TEST

❑ 2009 -2010 Joint Research Centre (JRC) of the European Commission:

➢ Proficiency Test on the determination of 3-MCPD esters in edible oil

Participants: 34 laboratories

Sample: a virgin olive oil spiked with 3-MCPD dioleate – **assigned value 4,58 ppm**
a refined palm oil - **assigned value 8,77 ppm**

Methods free choice: acid transesterification based methods
alkaline transesterification based methods
unspecified

Satisfactory results % (Z score ≤ 2): for virgin olive oil **85%** (29 out of 34 participants)
for palm oil **56 %** (19 out of 34 participants)

Conclusion: **results were influenced by the method used and instrument calibration.**
Results obtained by acid transesterification were satisfactory
Results by alkaline transesterification were more variables.

❑ 2011 Food Analysis Performance Assessment Scheme (FAPAS):

➢ Proficiency Test on the determination of 3-MCPD esters in edible oil

Participants: 26 laboratories

Sample: a refined palm oil - **assigned value 4,7 ppm**

Methods free choice

Satisfactory results % (Z score ≤ 2): for palm oil **62 %** (16 out of 26 participants)

Conclusion: the results showed only limited improvement.

PROFICIENCY TEST

❑ 2012 American Oil Chemists Society (AOCS)

➢ **Collaborative study for comparison of the three indirect methods in edible oils and fats.**

Determination of 2- and 3-MCPD Fatty Acid Esters and Glycidol Fatty Acid Esters

Participants: 20 laboratories

Sample: seven oils and fats. The same 7 samples were used in each methods.

Methods free choice: one of the three methods proposed **AOCS Cd 29a-13; Cd 29b-13; Cd 29c-13**

Satisfactory results Z score ≤ 2

Reproducibility and repeatability values were calculated according to the AOAC/IUPAC

Conclusion: **The three methods were judged to give the same results with reasonable confidence.**

❑ 2014 Commission Recommendation 2014/661/EU on the monitoring of free MCPD, MCPDEs and GEs in food

The UE recommended **the monitoring in several foods** (such as fine bakery ware, bread and rolls, smoked meat and fish, potato- and cereal-based snacks, fried potato products and vegetable oil containing foods) and **suggested to use the American Oil Chemists' Society standard methods** which have been validated by a collaborative study for vegetable oils and fats.

VALIDATED METHODS

Determination of fatty-acid-bound chloropropanediols (MCPDs) and glycidol esters in fats and oils

AOCS Cd 29a-13 (revised 2017)/ISO 18363-3:2017

Conversion of glycidil esters in 3-MBPD esters. Release of 3-MBPD esters and 2-3-MCPD esters into the free form by slow acid catalysed transesterification at 40°C for 16 h. Derivatization of free analytes with phenylboronic acid (PBA). Simultaneous determination in a single assay. **Known as the *"Unilever method"*.**

AOCS Cd 29b-13 (revised 2017)/ISO 18363-2:2018

Parallel determination of glycidol together with 2-MCPD and 3-MCPD present in bound or free form in oils and fats. Slow alkaline transesterification at - 22°C for 16 h, transformation of the released glycidol into monobromopropanediol (MBPD) and derivatisation of free diols (MCPD and MBPD) with PBA. **Known as the *"3 in 1 method"*.**

AOCS Cd 29c-13 (revised 2017)/ISO 18363-1:2015

Two independent assays (A and B). Fast alkaline transesterification at room temperature for 3-5 min, derivatisation with PBA.
Assay A: sum of 3-MCPD and glycidyl esters (after conversion of glycidol into 3-MCPD). Assay B: specific to 3-MCPD esters only.
Differential measurement for glycidol. Glycidol = (assay A – assay B) * t ; t: transformation factor. **Known as *"DGF C-VI 18 (10)".***

Analysis of 2- and 3-MCPD Fatty Acid Esters and Glycidyl Fatty Acid Esters in Oil-Based Emulsions

AOCS Official Method Cd 30-15 Approved 2017

Extraction procedure for isolating 2-and 3-MCPD and glycidyl esters from oil-based emulsions such as spreads, margarines, dressings, and mayonnaise. After extraction, the process contaminants can be analysed by one of the previously published methods.

INNOVHUB EXPERIENCE

- Survey and careful evaluation of the indirect methods reported in the literature
- Following the indications of EU-Commission Recommendation 2014/661/EU …."...*it is recommended to use the American Oil Chemists' Society standard methods",* the final choice of Innovhub has been for **AOCS Cd 29b-13 method,** although it requires a long sample preparation, but allows to directly quantify the amount of glycidol (as 3-MBPD) and MCPDEs.
- Laboratory experimentation of this method
- First UNI collaborative study on the analysis of GE/2,3-MCPD on various vegetable oils. Each laboratory could choose the preferred method of analysis. 9 oils (refined and virgin) and their mixtures were tested.
  At the end of 2018 the study was repeated on a limited number of samples (N° 3), but, following UNI indications, using **AOCS Cd 29b-13**. In both the collaborative studies our laboratory resulted in line with the other participants, as well as in a proficiency test organized by FAPAS.

FIRST COLLABORATIVE TEST UNI - RESULTS OF INNOVHUB-SSI

Satisfactory results Z score ≤ 2

METHOD USED: AOCS Cd 29b-13

ID Sample	Sum Free 3-MCPD + 3-MCPD esters, expressed as Free 3-MCPD (µg/kg)		Average µg/kg	Assigned Value µg/kg	Z score	Data points n°
	Test A	Test B				
A6	297	306	302	309	-0,33	8
B3	3574	3541	3558	3808	-0,47	8
C2	821	837	829	908	-1,73	8
E1	516	494	505	507	-0,27	8
F4	143	145	144	150	-0,18	8
H2	1172	1182	1177	1230	-0,58	8
H4	20	17	19	2	2,12	8
H5	834	848	841	885	-0,25	8
G5	1795	1763	1779	1791	0,11	8

UNI/CT 003/GL18 «Oli e grassi animali e vegetali e loro sottoprodotti, semi e frutti oleaginosi

FIRST COLLABORATIVE TEST UNI - RESULTS OF INNOVHUB-SSI

Satisfactory results Z score ≤ 2

METHOD USED: AOCS Cd 29b-13

ID Sample	Glycidyl esters expressed as Glycidol free (µg/kg)		Average µg/kg	Assigned Value µg/kg	Z score	Data points n°
	Test A	Test B				
A6	88	91	90	109	-0,09	9
B3	108	112	110	176	-1,12	9
C2	294	291	293	308	-0,71	9
E1	599	535	567	516	1,15	9
F4	316	300	308	321	-0,31	9
H2	479	461	470	526	-1,20	9
H4	< 10	< 10	0	0	nv	9
H5	276	281	279	306	-0,84	9
G5	76	59	68	134	-0,41	9

UNI/CT 003/GL18 «Oli e grassi animali e vegetali e loro sottoprodotti, semi e frutti oleaginosi

FIRST COLLABORATIVE TEST UNI - RESULTS OF INNOVHUB-SSI

Satisfactory results Z score ≤ 2

METHOD USED: AOCS Cd 29b-13

ID Sample	Sum Free 2-MCPD + 2-MCPD esters, expressed as Free 2-MCPD (µg/kg)		Average µg/kg	Assigned Value µg/kg	Z score	Data points n°
	Test A	Test B				
A6	123	123	125	133	0,00	7
B3	1541	1541	1539	1662	-0,59	7
C2	343	343	341	376	-0,39	7
E1	188	188	189	210	-0,93	7
F4	54	54	52	34	0,22	7
H2	497	497	495	543	-0,37	7
H4	< 10	< 10	0	0	nv	7
H5	354	354	358	382	-0,03	7
G5	765	765	757	857	-0,16	7

UNI/CT 003/GL18 «Oli e grassi animali e vegetali e loro sottoprodotti, semi e frutti oleaginosi

SECOND COLLABORATIVE TEST UNI - RESULTS OF INNOVHUB-SSI

Data points n°7

METHOD USED: AOCS Cd 29b-13

ID SAMPLE	Sum Free 2-MCPD + 2-MCPD esters, expressed as Free 2-MCPD (µg/kg)				
	Test A	Test B	Average	Z score	Assigned Value
A3	427	434	431	-0,02	452
B2	654	644	649	-1,04	716
C1	449	454	452	-0,84	516

ID SAMPLE	Sum Free 3-MCPD + 3-MCPD esters, expressed as Free 3-MCPD (µg/kg)				
	Test A	Test B	Average	Z score	Assigned Value
A3	937	907	922	-0,22	945
B2	1411	1433	1422	-0,58	1601
C1	968	961	965	-0,54	996

ID SAMPLE	Glycidyl esters expressed as Glycidol free (µg/kg)				
	Test A	Test B	Average	Z score	Assigned Value
A3	452	455	454	0,00	443
B2	570	581	576	-0,64	603
C1	935	911	923	0,29	919

RESULTS PROFICIENCY TEST FAPAS 2018 - METHOD USED: AOCS Cd 29b-13

	Test A	Test B	Test C	Average	Assigned Value µg/kg	Data points n°	Z score	σ_p
3-MCPD Esters	290,988	304,666	285,38	293,678	298	82	**-0,1**	57,1
2-MCPD Esters	97,063	90,187	90,880	92,710	104	51	**-0,5**	22,9
Glycidyl Esters	99,267	94,073	112,410	101,915	Not set	69	nd	n/a

An assigned value (x_a) was determined for 3-MCPD esters and 2-MCPD esters and in conjunction with the standard deviation for proficiency (σ_p) was used to calculate a z-score for each result. However, is was not possible to determine an assigned value for glycidyl esters due to the bimodality of the distribution of results submitted. Consequently, is has not been possible to calculate z-scores for this analyte.

RESULTS PROFICIENCY TEST FAPAS 2018 - METHOD USED: AOCS Cd 29b-13

Results for this proficiency test are summarised as follows

analyte	assigned value, x_a µg/kg	number of scores, $\|z\| \leq 2$	total number of scores	% $\|z\| \leq 2$
3-MCPD esters	298	73	85	86
glycidyl esters	not set	n/a	n/a	n/a
2-MCPD esters	104	46	53	87

METHODS FOR THE AUTOMATIC SAMPLE PREPARATION

In recent years automation in sample preparation or analysis has become to spread in laboratories and companies. Today there are different solutions for automation in the 2- 3-MCPDEs and GEs analysis, for the steps concerning addition of internal standards, liquid / liquid extraction, evaporation, concentration, derivatization, that connect with the GC-MS instruments used during the analysis.

- *Fully Automated Determination of 3-MCPD and Glycidol in Edible Oils by GC/MS Based on the Commonly Used Methods ISO 18363-1,* ***AOCS Cd 29c-13****, and DGF C-VI 18.* **Dominik Lucas, Andreas Hoffmann, Carlos Gil, GERSTEL Application Note No. 191, 2017**

One analysis is comprised of two assays (A and B)

Manual operation: **weigh in ~ 100 mg oil sample**

Fully automated

- **Addition of internal standard**
- **Ester cleavage(fast alkaline transesterification at room T °C 10 min)**
- **Quenching with NaCl (assay A) and NaBr (assay B)**
- **LLE (purification) 3X**
- **LLE (free analytes extraction) 3X**
- **Derivatization**
- **Evaporation to dryness and recovery with solvent**

Injection into GC-MS

Comparison for 3-MCPD with Methods AOCS Cd 29b-13

	Amount [mg/Kg]		
3-MCPD:	Reference	Cd 29c13	Cd 29b13
Edible Oil 1	0.77	0.68	0.68
Edible Oil 2	0.68	0.63	0.62
Edible Oil 3	0.27	0.29	0.26

Glycidol	Amount [mg/kg]	
	Reference	Automated
Oil 1	0.14	0.18
Oil 2	0.44	0.48
Oil 3	0.11	0.09

	Amount [mg/Kg]
No.:	3-MCPD
1	0.76
2	0.70
3	0.74
4	0.69
5	0.68
Mean	0.71
SD	0.03
RSD %	4.79

METHODS FOR THE AUTOMATIC SAMPLE PREPARATION

References

- *A novel method for the automatic sample preparation and analysis of 3-MCPD-, 2-MCPD-, and glycidylesters in edible oils and fats.* **Ralph Zwagerman and Pierre Overman, Eur. J. Lipid Sci. Technol. 2015, 117, 1-9.**
- *Optimized Analysis of MCPD- and Glycidyl Esters in Edible Oils and Fats using Fast Alkaline Transesterification and ^{13}C-Correction for Glycidol Overestimation: Validation Including Interlaboratory Comparison*. **Ralph Zwagerman and Pierre Overman, Eur. J. Lipid Sci. Technol. 2019, 121, 1800395**

The authors have developed a new automated method for a sample preparation and quantification of 2-3-MCPDEs and GE in oils and fats **using the relatively fast AOCS Official Method Cd 29c-13 with a single step sample preparation**. This method has solved the problem of the possible overestimation of GE, caused by glycidol induced, **using a 3-MCPD-^{13}C3 internal standard**. **The method has been validated against AOCS Official Method Cd 29b-13 using different types of edible oils.**

In **2019** the authors presented the results of an interlaboratory comparison between six laboratories in 4 countries after had made several optimizations and improvements. The interlaboratory comparison showed that the method provides statistically equivalent results for both manual and automated sample preparation.

	LOD ($\mu g\,kg^{-1}$)	LOQ ($\mu g\,kg^{-1}$)
3-MCPDe	10.0	15.0
GE	18.5	41.0
2-MCPDe	2.7	6.9

INNOVHUB PERSPECTIVES

AOCS Cd 29b-13 two assays (A and B)

MANUAL OPERATION
- SAMPLE WEIGHT about 100 mg of oil

FULLY AUTOMATED
- ADD INTERNAL STANDARD
- ADD OF THE SOLVENT AND MIX

MANUAL OPERATION
- RERIGERATION
- SLOW TRANSESTERIFICATION ALKALINE 16h,-22°C
- COLD QUENCHING WITH NaBr

FULLY AUTOMATED
- ORGANIC PHASE EVAPORATION
- LLE (PURIFICATION); 3X
- LLE (FREE ANALYTES EXTRACTION); 3X

FULLY AUTOMATED
- ADD DERIVATIZING
- EVAPORATION TO DRYNESS AND RECOVERY WITH SOLVENT

AOCS Cd 29a-13

MANUAL OPERATION
- SAMPLE WEIGHT about 100 mg of oil

FULLY AUTOMATED
- ADD INTERNAL STANDARD
- ADD OF THE SOLVENT AND MIX
- ADD NaBr (15 min, 50°C)
- QUENCHING NaBr
- EXTRACTION OF ESTERIFIED ANALYTES
- ORGANIC PHASE EVAPORATION
- SLOW TRANSESTERIFICATION ACID 16h, 40°C
- QUENCHING
- LLE (PURIFICATION); 3X
- LLE (FREE ANALYTES EXTRACTION); 3X
- ADD DERIVATIZING
- EVAPORATION TO DRYNESS AND RECOVERY WITH SOLVENT

INJECTION GC-MS

INNOVHUB PERSPECTIVES

- Innovhub-SSI will receive, by the end of the year, a workstation for automated sample preparation for 2-3-MCPDEs and GEs analysis in oils and fats, coupled to our GC-MS.
- The official AOCS methods could be automated
- The automated methods will initially be subjected to validation study for the 2-3-MCPDEs and GEs analysis
- The automatic system will be set to use the **AOCS Cd 29b-13 method** in partially automated mode
- The steps of: addition of Internal standards, ester cleavage, quenching, purification, extraction, derivatization and evaporation **are fully automated**
- Reduction the total analysis time for series of N samples.
- Reduction and/or elimination of human error
- Improved analytical performance.

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Innovare è la nostra tradizione